respective subgroups. In particular, the Trp in Kabat position 47 in the human- or primate-derived framework region of the Heavy Chain Variable Region as shown in SEQ ID NO: 15 may be replaced by an Leu or by an amino acid residue that has similar properties and the substitution of which leads to alterations that are substantially conformationally or antigenically neutral, producing minimal changes in the tertiary structure of the mutant polypeptides, or producing minimal changes in the antigenic determinants. In particular, the Trp in Kabat position 47 in the human- or primate-derived framework region of the Heavy Chain Variable Region as shown in SEQ ID NO: 15 may further be replaced by an amino acid selected from the group consisting of norleucine, Ile, Val, Met, Ala, and Phe, particularly by Ile. Alternative conservative substitutions may be contemplated which are conformationally and antigenically neutral.

[0340] The Arg in Kabat position 94 in the human- or primate-derived framework region of the Heavy Chain Variable Region as shown in SEQ ID NO: 15 may be replaced by Ser or by an amino acid residue that has similar properties and the substitution of which leads to alterations that are substantially conformationally or antigenically neutral, producing minimal changes in the tertiary structure of the mutant polypeptides, or producing minimal changes in the antigenic determinants. In particular, the Arg in Kabat position 94 in the human- or primate-derived framework region of the Heavy Chain Variable Region as shown in SEQ ID NO: 15 may alternatively be replaced by Thr.

[0341] In another embodiment of the invention, both residues may be replaced in the humanized antibody.

[0342] The Gln in Kabat position 45 in the human- or primate-derived framework region of the Light Chain Variable Region as shown in SEQ ID NO: 12 may be replaced by Lys or by an amino acid residue that has similar properties and the substitution of which leads to alterations that are substantially conformationally or antigenically neutral, producing minimal changes in the tertiary structure of the mutant polypeptides, or producing minimal changes in the antigenic determinants. In particular, the Gln in Kabat position 45 in the human- or primate-derived framework region of the Light Chain Variable Region as shown in SEQ ID NO: 12 may be replaced by an amino acid selected from the group consisting of Arg, Gln, and Asn, particularly by Arg.

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[0343] The Leu in Kabat position 50 in the human- or primate-derived framework region of the Light Chain Variable Region as shown in SEQ ID NO: 12 may be replaced by Lys or by an amino acid residue that has similar properties and the substitution of which leads to alterations that are substantially conformationally or antigenically neutral, producing minimal changes in the tertiary structure of the mutant polypeptides, or producing minimal changes in the antigenic determinants. In particular, the Leu in Kabat position 50 in the human- or primate-derived framework region of the Light Chain Variable Region as shown in SEQ ID NO: 12 may be replaced by an amino acid selected from the group consisting of Arg, Gln, and Asn, particularly by Arg.

[0344] The Asn in Kabat position 53 in the human- or primate-derived framework region of the Light Chain Variable Region as shown in SEQ ID NO: 12 may be replaced by His and Gln or by an amino acid residue that has similar properties and the substitution of which leads to alterations that are substantially conformationally or antigenically neutral, producing minimal changes in the tertiary structure of the mutant polypeptides, or producing minimal changes in the antigenic determinants. In particular, the Asn in Kabat position 53 in the human- or primate-derived framework region of the Light Chain Variable Region as shown in SEQ ID NO: 12 may be replaced by an amino acid selected from the group consisting of Gln, His, Lys and Arg.

[0345] The Thr in Kabat position 87 in the human- or primate-derived framework region of the Light Chain Variable Region as shown in SEQ ID NO: 12 may be replaced by Phe or by an amino acid residue that has similar properties and the substitution of which leads to alterations that are substantially conformationally or antigenically neutral, producing minimal changes in the tertiary structure of the mutant polypeptides, or producing minimal changes in the antigenic determinants. In particular, the Tyr in Kabat position 87 in the human- or primate-derived framework region of the Light Chain Variable Region as shown in SEQ ID NO: 12 may be replaced by an amino acid selected from the group consisting of Leu, Val, Ile, and Ala, particularly by Leu.

[0346] The so obtained variable region comprising at least one CDR of non-human origin embedded in one or more human- or primate-derived framework regions may then be combined with a constant region derived from a human or primate source antibody, particularly with human IgG4 or κ constant regions respectively. The IgG4 constant region

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may be modified by, for example, changing Serine at position 228 in the hinge region to Proline (HuIgG4 Ser-Pro). This mutation stabilizes the interchain disulphide bond and prevents the formation of half molecules that may occur in native human IgG4 preparations. The IgG4 constant region may be further modified by deletion of the terminal Lys in position 439 as shown in SEQ ID NO: 16.

[0347] The modified variable regions may be constructed by method known in the art such as, for example overlapping PCR recombination. The expression cassettes for the chimeric antibody, C2 ChV_H AF and C2 ChV_K, may be used as templates for mutagenesis of the framework regions to the required sequences. Sets of mutagenic primer pairs are synthesized encompassing the regions to be altered. The humanized V_H and V_K expression cassettes produced may be cloned into appropriate cloning vectors know in the art such as, for example, pUC19. After the entire DNA sequence is confirmed to be correct for each V_H and V_K, the modified heavy and light chain V-region genes can be excised from the cloning vector as expression cassettes and transferred to appropriate expression vectors.

MUTATING Fc REGION

[0348] The present invention provides methods for making a polypeptide variant, particularly an antibody comprising a variant region. Such an antibody comprising, for example, a variant Fc region may be used for treating a disease or disorder, such as amyloidosis.

[0349] The "parent," "starting" or "nonvariant" polypeptide is prepared using techniques available in the art for generating polypeptides comprising, for example, an Fc region. In a preferred embodiment of the invention, the parent polypeptide is an antibody and exemplary methods for generating antibodies are described in more detail in the following sections. The parent polypeptide may, however, be any other polypeptide comprising an Fc region, *e.g.* an immunoadhesin. Methods for making immunoadhesins are elaborated in more detail below.

[0350] In an alternative embodiment, a variant Fc region may be generated according to the methods herein disclosed and this "variant Fc region" can be fused to a heterologous polypeptide of choice, such as an antibody variable domain or binding domain of a receptor or ligand.

[0351] The parent polypeptide comprises an Fc region. Generally the Fc region of the parent polypeptide will comprise a native sequence Fc region, and preferably a human native

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sequence Fc region. However, the Fc region of the parent polypeptide may have one or more pre-existing amino acid sequence alterations or modifications from a native sequence Fc region. For example, the C1q binding activity of the Fc region may have been previously altered (other types of Fc region modifications are described in more detail below). In a further embodiment the parent polypeptide Fc region is "conceptual" and, while it does not physically exist, the antibody engineer may decide upon a desired variant Fc region amino acid sequence and generate a polypeptide comprising that sequence or a DNA encoding the desired variant Fc region amino acid sequence.

[0352] In a preferred embodiment of the invention, however, a nucleic acid encoding an Fc region of a parent polypeptide is available and this nucleic acid sequence is altered to generate a variant nucleic acid sequence encoding the Fc region variant D265A.

[0353] DNA encoding an amino acid sequence variant of the starting polypeptide is prepared by a variety of methods known in the art. These methods include, but are not limited to, preparation by site-directed (or oligonucleotide-mediated) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared DNA encoding the polypeptide.

[0354] Site-directed mutagenesis is a preferred method for preparing substitution variants. This technique is well known in the art (see, *e.g.*, Carter et al. Nucleic Acids Res. 13:4431-4443 (1985) and Kunkel et al., Proc. Natl. Acad. Sci. USA 82:488 (1987)). Briefly, in carrying out site-directed mutagenesis of DNA, the starting DNA is altered by first hybridizing an oligonucleotide encoding the desired mutation to a single strand of such starting DNA. After hybridization, a DNA polymerase is used to synthesize an entire second strand, using the hybridized oligonucleotide as a primer, and using the single strand of the starting DNA as a template. Thus, the oligonucleotide encoding the desired mutation is incorporated in the resulting double-stranded DNA.

[0355] PCR mutagenesis is also suitable for making amino acid sequence variants of the starting polypeptide. See Higuchi, in PCR Protocols, pp.177-183 (Academic Press, 1990); and Vallette et al., Nuc. Acids Res. 17:723-733 (1989). Briefly, when small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template.

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[0356] Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al., Gene 34:315-323 (1985). The starting material is the plasmid (or other vector) comprising the starting polypeptide DNA to be mutated. The codon(s) in the starting DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the starting polypeptide DNA. The plasmid DNA is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures, wherein the two strands of the oligonucleotide are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 5' and 3' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated DNA sequence.

[0357] Alternatively, or additionally, the desired amino acid sequence encoding a polypeptide variant can be determined, and a nucleic acid sequence encoding such amino acid sequence variant can be generated synthetically.

[0358] The amino acid sequence of the parent polypeptide is modified in order to generate a variant Fc region with altered Fc receptor binding affinity or activity in vitro and/or in vivo and/or altered antibody-dependent cell-mediated cytotoxicity (ADCC) activity in vitro and/or in vivo and/or altered cell mediated cytoxicity (CDC) activity in vitro and/or in vivo.

[0359] Generally, the modification entails one or more amino acid substitutions. The substitution may, for example, be a "conservative substitution." Substantial modifications in the biological properties of the Fc region may be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

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(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

[0360] Aside from amino acid substitutions, the present invention contemplates other modifications of the parent region amino acid sequence in order to generate, for example, an Fc region variant with altered effector function.

[0361] One may, for example, delete one or more amino acid residues of the Fc region in order to reduce binding to an FcR. Generally, one will delete one or more of the Fc region residues identified herein as effecting FcR binding in order to generate such an Fc region variant. Generally, no more than one to about ten Fc region residues will be deleted according to this embodiment of the invention. The Fc region herein comprising one or more amino acid deletions will preferably retain at least about 80%, and preferably at least about 90%, and most preferably at least about 95%, of the parent Fc region or of a native sequence human Fc region.

[0362] By introducing the appropriate amino acid sequence modifications in a parent Fc region, for example, one can generate a variant Fc region which (a) mediates antibody-dependent cell-mediated cytotoxicity (ADCC) in the presence of human effector cells more or less effectively and/or (b) binds an Fc gamma receptor (Fc.gamma.R) with more or less affinity than the parent polypeptide. Such Fc region variants will generally comprise at least one amino acid modification in the Fc region. Combining amino acid modifications is thought to be particularly desirable. For example, the variant Fc region may include two, three, four, five, etc substitutions therein, e.g. of the specific Fc region positions identified herein.

[0363] For example, in the IgG1 context, an Fc region variant can be generated with reduced binding to the Fc.gamma.R by introducing an amino acid modification at any one or more of amino acid positions 238, 239, 248, 249, 252, 254, 265, 268, 269, 270, 272, 278, 289, 292, 293, 294, 295, 296, 298, 301, 303, 322, 324, 327, 329, 333, 335, 338, 340, 373, 376, 382, 388, 389, 414, 416, 419, 434, 435, 437, 438 or 439 of the Fc region. See, *e.g.* Presta U.S. Pat. No. 6,737,056.

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[0364] IgG1 variants which display reduced binding to Fc.gamma.RI, include those comprising an Fc region amino acid modification at any one or more of amino acid positions 238, 265, 269, 270, 327 or 329. See, *e.g.* Presta U.S. Pat. No. 6,737,056.

[0365] IgG1 variants which display reduced binding to Fc.gamma.RII include those comprising an Fc region amino acid modification at any one or more of amino acid positions 238, 265, 269, 270, 292, 294, 295, 298, 303, 324, 327, 329, 333, 335, 338, 373, 376, 414, 416, 419, 435, 438 or 439. See, *e.g.* Presta U.S. Pat. No. 6,737,056.

[0366] IgG1 Fc region variants which display reduced binding to Fc.gamma.RIII include those comprising an Fc region amino acid modification at any one or more of amino acid positions 238, 239, 248, 249, 252, 254, 265, 268, 269, 270, 272, 278, 289, 293, 294, 295, 296, 301, 303, 322, 327, 329, 338, 340, 373, 376, 382, 388, 389, 416, 434, 435 or 437. See, *e.g.* Presta U.S. Pat. No. 6,737,056.

[0367] It will be understood by one of ordinary skill in the art that similar effects can be obtained by varying specific residues in other Ig Fc regions, though the numbering of the residues may be different. See, *e.g.* Presta U.S. Pat. No. 6,737,056.

[0368] One can design an Fc region with altered effector function, e.g., by modifying C1q binding and/or FcR binding and thereby changing CDC activity and/or ADCC activity. For example, one can generate a variant Fc region with improved C1q binding and improved Fc.gamma.RIII binding; e.g. having both improved ADCC activity and improved CDC activity. Alternatively, where one desires that effector function be reduced or ablated, one may engineer a variant Fc region with reduced CDC activity and/or reduced ADCC activity. In other embodiments, one may increase only one of these activities, and optionally also reduce the other activity, e.g. to generate an Fc region variant with improved ADCC activity, but reduced CDC activity and vice versa. See, *e.g.* Presta U.S. Pat. No. 6,737,056.

[0369] With respect to further amino acid sequence alterations, any cysteine residue not involved in maintaining the proper conformation of the polypeptide variant also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant cross linking. See, *e.g.* Presta U.S. Pat. No. 6,737,056.

[0370] Another type of amino acid substitution serves to alter the glycosylation pattern of the polypeptide. This may be achieved by deleting one or more carbohydrate moieties found in the polypeptide, and/or adding one or more glycosylation sites that are not present in the

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polypeptide. Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used. Addition of glycosylation sites to the polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original polypeptide (for O-linked glycosylation sites). An exemplary glycosylation variant has an amino acid substitution of residue Asn 297 of the heavy chain. See, *e.g.* Presta U.S. Pat. No. 6,737,056.

[0371] Moreover, the class, subclass or allotype of the Fc region may be altered by one or more further amino acid substitutions to generate an Fc region with an amino acid sequence more homologous to a different class, subclass or allotype as desired. For example, a murine Fc region may be altered to generate an amino acid sequence more homologous to a human Fc region; a human non-A allotype IgG1 Fc region may be modified to achieve a human A allotype IgG1 Fc region etc. In one embodiment, the amino modification(s) herein which alter FcR binding and/or ADCC activity are made in the CH2 domain of the Fc region and the CH3 domain is deleted or replaced with another dimerization domain. Preferably, however, the CH3 domain is retained (aside from amino acid modifications therein which alter effector function as herein disclosed). See, *e.g.* Presta U.S. Pat. No. 6,737,056.

BINDING ASSAYS

[0372] The ability of the polypeptide variant to bind an FcR may be evaluated. Where the FcR is a high affinity Fc receptor, such as Fc.gamma.RI, FcRn or Fc.gamma.RIIIA-V158, binding can be measured by titrating monomeric polypeptide variant and measuring bound polypeptide variant using an antibody which specifically binds to the polypeptide variant in a standard ELISA format See, *e.g.* Presta U.S. Pat. No. 6,737,056. FcR binding assays for low

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affinity FcRs are well-known in the art and are described in, *inter alia*, Presta U.S. Pat. No. 6,737,056.

[0373] To assess ADCC activity of the polypeptide variant, an in vitro ADCC assay may be performed using varying effector:target ratios. Useful "effector cells" for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the polypeptide variant may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. PNAS (USA) 95:652-656 (1998).

EXPRESSION VECTORS

[0374] Any suitable expression vector may be used to practice the invention. For example, one of ordinary skill in the art would be able to express IgG1 in, for instance, *pSVgpt*. Expression vector *pSVgpt* is based on pSV_2gpt (Mulligan and Berg, 1980) and includes the ampicillin resistance gene for selection in bacterial cells, the *gpt* gene for selection in mammalian cells, the murine heavy chain immunoglobulin enhancer region, genomic sequence encoding the constant region gene and SV40 poly A sequences. The heavy chain variable region for expression is inserted as a *Hin*dIII to *Bam*HI fragment.

[0375] Expression vector pSVhyg includes the ampicillin resistance gene for selection in bacterial cells, the *hyg* gene for selection in mammalian cells, the murine heavy chain immunoglobulin enhancer region, genomic sequence encoding the kappa constant region gene and including the kappa enhancer and SV40 poly A sequences. The light chain variable region for expression is inserted as a *Hin*dIII to *Bam*HI fragment.

[0376] The DNA sequence is then to be confirmed to be correct for the humanized V_H and V_K in the expression vectors.

[0377] For antibody production the humanized heavy and light chain expression vectors may be introduced into appropriate production cell lines know in the art such as, for example, NS0 cells. Introduction of the expression vectors may be accomplished by co-transfection via electroporation or any other suitable transformation technology available in the art. Antibody producing cell lines can then be selected and expanded and humanized antibodies purified. The purified antibodies can then be analyzed by standard techniques such as SDS-PAGE.

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ANTIBODY WITH IMPROVED AFFINITY, SPECIFICITY, STABILITY

[0378] The CDRL2 sequence ("KVSNRFS") of the mouse C2 antibody may be modified slightly without adversely affecting antibody activity. Conservative substitutions may be made through exchange of R for K at position 50 and S for N at position 53. The two alternative CDRL2 sequences are therefore "RVSNRFS" and "KVSSRFS", respectively. These are incorporated into the murine V_K sequence with no other changes, as C2 VK-R and C2 VK-S, respectively.

[0379] The affinity, specificity and stability of an antibody according to the invention as described herein before or a fragment thereof can be modified by change of its glycosylation profile or pattern resulting in improved therapeutic values.

[0380] To achieve this change in glycosylation pattern, host cells may be engineered such that they are capable of expressing a preferred range of a glycoprotein-modifying glycosyl transferase activity which increases complex N-linked oligosaccharides carrying bisecting GlcNAc. Further, modified glycoforms of glycoproteins may be obtained, for example antibodies, including whole antibody molecules, antibody fragments, or fusion proteins that include a region equivalent to the Fc region of an immunoglobulin, having an enhanced Fc-mediated cellular cytotoxicity.

[0381] Methods of obtaining antibodies with modified glycosylation pattern are known to those skilled in the art and described, for example, in EP1071700, US2005272128, Ferrara et al (2006) J Biol Chem 281(8), 5032-5036); Ferrara et al (2006) Biotechnology and Bioengineering 93(5), 851-861.

PHARMACEUTICAL PREPARATION AND ADMINISTRATION

[0382] The antibodies according to the invention, but particularly a monoclonal antibody according the invention, can be prepared in a physiologically acceptable formulation and may comprise a pharmaceutically acceptable carrier, diluent and/or excipient using known techniques. For example, the antibody according to the invention and as described herein before including any functionally equivalent antibody or functional parts thereof, in particular, the monoclonal antibody including any functionally equivalent antibody or functional parts thereof is combined with a pharmaceutically acceptable carrier, diluent and/or excipient to form a therapeutic composition. Suitable pharmaceutical carriers, diluents and/or excipients are well known in the art and include, for example, phosphate buffered

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saline solutions, water, emulsions such as oil/water emulsions, various types of wetting agents, sterile solutions, etc.

[0383] Formulation of the pharmaceutical composition according to the invention can be accomplished according to standard methodology know to those skilled in the art.

[0384] The compositions of the present invention may be administered to a subject in the form of a solid, liquid or aerosol at a suitable, pharmaceutically effective dose. Examples of solid compositions include pills, creams, and implantable dosage units. Pills may be administered orally. Therapeutic creams may be administered topically. Implantable dosage units may be administered locally, for example, at a tumor site, or may be implanted for systematic release of the therapeutic composition, for example, subcutaneously. Examples of liquid compositions include formulations adapted for injection intramuscularly, subcutaneously, intravenously, intra-arterially, and formulations for topical and intraocular administration. Examples of aerosol formulations include inhaler formulations for administration to the lungs.

[0385] The compositions may be administered by standard routes of administration. In general, the composition may be administered by topical, oral, rectal, nasal, interdermal, intraperitoneal, or parenteral (for example, intravenous, subcutaneous, or intramuscular) routes. In addition, the composition may be incorporated into sustained release matrices such as biodegradable polymers, the polymers being implanted in the vicinity of where delivery is desired, for example, at the site of a tumor. The method includes administration of a single dose, administration of repeated doses at predetermined time intervals, and sustained administration for a predetermined period of time.

[0386] A sustained release matrix, as used herein, is a matrix made of materials, usually polymers which are degradable by enzymatic or acid/base hydrolysis or by dissolution. Once inserted into the body, the matrix is acted upon by enzymes and body fluids. The sustained release matrix desirably is chosen by biocompatible materials such as liposomes, polylactides (polylactide acid), polyglycolide (polymer of glycolic acid), polylactide co-glycolide (copolymers of lactic acid and glycolic acid), polyanhydrides, poly(ortho)esters, polypeptides, hyaluronic acid, collagen, chondroitin sulfate, carboxylic acids, fatty acids, phospholipids, polysaccharides, nucleic acids, polyamino acids, amino acids such phenylalanine, tyrosine, isoleucine, polynucleotides, polyvinyl propylene,

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polyvinylpyrrolidone and silicone. A preferred biodegradable matrix is a matrix of one of either polylactide, polyglycolide, or polylactide co-glycolide (co-polymers of lactic acid and glycolic acid).

[0387] It is well know to those skilled in the pertinent art that the dosage of the composition will depend on various factors such as, for example, the condition of being treated, the particular composition used, and other clinical factors such as weight, size, sex and general health condition of the patient, body surface area, the particular compound or composition to be administered, other drugs being administered concurrently, and the route of administration.

[0388] The composition may be administered in combination with other compositions comprising an biologically active substance or compound, particularly at least one compound selected from the group consisting of compounds against oxidative stress, anti-apoptotic compounds, metal chelators, inhibitors of DNA repair such as pirenzepin and metabolites, 3-amino-1-propanesulfonic acid (3APS), 1,3-propanedisulfonate (1,3PDS), α-secretase activators, β- and γ–secretase inhibitors, tau proteins, neurotransmitter, β-sheet breakers, attractants for amyloid beta clearing / depleting cellular components, inhibitors of N-terminal truncated amyloid beta including pyroglutamated amyloid beta 3-42, anti-inflammatory molecules, "atypical antipsychotics" such as, for example clozapine, ziprasidone, risperidone, aripiprazole or olanzapine or cholinesterase inhibitors (ChEIs) such as tacrine, rivastigmine, donepezil, and/or galantamine, M1 agonists and other drugs including any amyloid or tau modifying drug and nutritive supplements such as, for example, vitamin B12, cysteine, a precursor of acetylcholine, lecithin, choline, Ginkgo biloba, acyetyl-L-carnitine, idebenone, propentofylline, or a xanthine derivative, together with an antibody according to the present invention and, optionally, a pharmaceutically acceptable carrier and/or a diluent and/or an excipient and procedures for the treatment of diseases.

[0389] Proteinaceous pharmaceutically active matter may be present in amounts between 1 ng and 10 mg per dose. Generally, the regime of administration should be in the range of between 0.1 μg and 10 mg of the antibody according to the invention, particularly in a range 1.0 μg to 1.0 mg, and more particularly in a range of between 1.0 μg and 100 μg, with all individual numbers falling within these ranges also being part of the invention. If the administration occurs through continuous infusion a more proper dosage may be in the range

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of between 0.01 μg and 10 mg units per kilogram of body weight per hour with all individual numbers falling within these ranges also being part of the invention.

[0390] Administration will generally be parenterally, eg intravenously. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions and emulsions. Non-aqueous solvents include without being limited to it, propylene glycol, polyethylene glycol, vegetable oil such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous solvents may be chosen from the group consisting of water, alcohol/aqueous solutions, emulsions or suspensions including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose) and others. Preservatives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, inert gases, etc.

[0391] The pharmaceutical composition may further comprise proteinaceous carriers such as, for example, serum albumin or immunoglobulin, particularly of human origin. Further biologically active agents may be present in the pharmaceutical composition of the invention dependent on its the intended use.

[0392] When the binding target is located in the brain, certain embodiments of the invention provide for the antibody or active fragment thereof to traverse the blood-brain barrier. Certain neurodegenerative diseases are associated with an increase in permeability of the blood-brain barrier, such that the antibody or active fragment thereof can be readily introduced to the brain. When the blood-brain barrier remains intact, several art-known approaches exist for transporting molecules across it, including, but not limited to, physical methods, lipid-based methods, and receptor and channel-based methods.

[0393] Physical methods of transporting the antibody or active fragment thereof across the blood-brain barrier include, but are not limited to, circumventing the blood-brain barrier entirely, or by creating openings in the blood-brain barrier. Circumvention methods include, but are not limited to, direct injection into the brain (see, e.g., Papanastassiou et al., Gene Therapy 9: 398-406 (2002)) and implanting a delivery device in the brain (see, e.g., Gill et al., Nature Med. 9: 589-595 (2003); and Gliadel Wafers™, Guildford Pharmaceutical). Methods of creating openings in the barrier include, but are not limited to, ultrasound (see,

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e.g., U.S. Patent Publication No. 2002/0038086), osmotic pressure (e.g., by administration of hypertonic mannitol (Neuwelt, E. A., Implication of the Blood-Brain Barrier and its Manipulation, Vols 1 & 2, Plenum Press, N.Y. (1989))), permeabilization by, e.g., bradykinin or permeabilizer A-7 (see, e.g., U.S. Patent Nos. 5,112,596, 5,268,164, 5,506,206, and 5,686,416), and transfection of neurons that straddle the blood-brain barrier with vectors containing genes encoding the antibody or antigen-binding fragment (see, e.g., U.S. Patent Publication No. 2003/0083299).

[0394] Lipid-based methods of transporting the antibody or active fragment thereof across the blood-brain barrier include, but are not limited to, encapsulating the antibody or active fragment thereof in liposomes that are coupled to antibody binding fragments that bind to receptors on the vascular endothelium of the blood-brain barrier (see, e.g., U.S. Patent Application Publication No. 20020025313), and coating the antibody or active fragment thereof in low-density lipoprotein particles (see, e.g., U.S. Patent Application Publication No. 20040204354) or apolipoprotein E (see, e.g., U.S. Patent Application Publication No. 20040131692).

[0395] Receptor and channel-based methods of transporting the antibody or active fragment thereof across the blood-brain barrier include, but are not limited to, using glucocorticoid blockers to increase permeability of the blood-brain barrier (see, e.g., U.S. Patent Application Publication Nos. 2002/0065259, 2003/0162695, and 2005/0124533); activating potassium channels (see, e.g., U.S. Patent Application Publication No. 2005/0089473), inhibiting ABC drug transporters (see, e.g., U.S. Patent Application Publication No. 2003/0073713); coating antibodies with a transferrin and modulating activity of the one or more transferrin receptors (see, e.g., U.S. Patent Application Publication No. 2003/0129186), and cationizing the antibodies (see, e.g., U.S. Patent No. 5,004,697).

DETECTION/DIAGNOSIS

[0396] In a further embodiment the present invention provides methods and kits for the detection and diagnosis of amyloid-associated diseases or conditions. These methods include known immunological methods commonly used for detecting or quantifying substances in biological samples or in an *in situ* condition.

[0397] Diagnosis of an amyloid-associated disease or condition in a patient may be achieved by detecting the immunospecific binding of a monoclonal antibody or an active

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fragment thereof to an epitope of the amyloid protein in a sample or *in situ,* which includes bringing the sample or a specific body part or body area suspected to contain the amyloid protein into contact with an antibody which binds an epitope of the amyloid protein, allowing the antibody to bind to the amyloid protein to form an immunological complex, detecting the formation of the immunological complex and correlating the presence or absence of the immunological complex with the presence or absence of amyloid protein in the sample or specific body part or area.

[0398] Biological samples that may be used in the diagnosis of an amyloid-associated disease or condition are, for example, fluids such as serum, plasma, saliva, gastric secretions, mucus, cerebrospinal fluid, lymphatic fluid and the like or tissue or cell samples obtained from an organism such as neural, brain, cardiac or vascular tissue. For determining the presence or absence of the amyloid protein in a sample any immunoassay known to those of ordinary skill in the art. (See Harlow and Lane, Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory, New York 1988 555-612) may be used such as, for example, assays which utilize indirect detection methods using secondary reagents for detection, ELISA's and immunoprecipitation and agglutination assays. A detailed description of these assays is, for example, given in WO96/13590 to Maertens and Stuyver, Zrein et al. (1998) and WO96/29605.

[0399] For *in situ* diagnosis, the antibody or any active and functional part thereof may be administered to the organism to be diagnosed by methods known in the art such as, for example, intravenous, intranasal, intraperitoneal, intracerebral, intraarterial injection such that a specific binding between the antibody according to the invention with an eptitopic region on the amyloid protein may occur. The antibody/antigen complex may be detected through a label attached to the antibody or a functional fragment thereof.

[0400] The immunoassays used in diagnostic applications typically rely on labelled antigens, antibodies, or secondary reagents for detection. These proteins or reagents can be labelled with compounds generally known to those skilled in the art including enzymes, radioisotopes, and fluorescent, luminescent and chromogenic substances including colored particles, such as colloidal gold and latex beads. Of these, radioactive labelling can be used for almost all types of assays and with most variations. Enzyme-conjugated labels are particularly useful when radioactivity must be avoided or when quick results are needed.

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Fluorochromes, although requiring expensive equipment for their use, provide a very sensitive method of detection. Antibodies useful in these assays include monoclonal antibodies, polyclonal antibodies, and affinity purified polyclonal antibodies.

[0401] Alternatively, the antibody may be labelled indirectly by reaction with labelled substances that have an affinity for immunoglobulin, such as protein A or G or second antibodies. The antibody may be conjugated with a second substance and detected with a labelled third substance having an affinity for the second substance conjugated to the antibody. For example, the antibody may be conjugated to biotin and the antibody-biotin conjugate detected using labelled avidin or streptavidin. Similarly, the antibody may be conjugated to a hapten and the antibody-hapten conjugate detected using labelled anti-hapten antibody.

[0402] Those of ordinary skill in the art will know of these and other suitable labels which may be employed in accordance with the present invention. The binding of these labels to antibodies or fragments thereof can be accomplished using standard techniques commonly known to those of ordinary skill in the art. Typical techniques are described by Kennedy, J. H., et al.,1976 (Clin. Chim. Acta 70:1-31), and Schurs, A. H. W. M., et al. 1977 (Clin. Chim Acta 81:1-40). Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, and others, all of which are incorporated by reference herein.

[0403] Current immunoassays utilize a double antibody method for detecting the presence of an analyte, wherein. The antibody is labeled indirectly by reactivity with a second antibody that has been labeled with a detectable label. The second antibody is preferably one that binds to antibodies of the animal from which the monoclonal antibody is derived. In other words, if the monoclonal antibody is a mouse antibody, then the labeled, second antibody is an anti-mouse antibody. For the monoclonal antibody to be used in the assay described below, this label is preferably an antibody-coated bead, particularly a magnetic bead. For the polyclonal antibody to be employed in the immunoassay described herein, the label is preferably a detectable molecule such as a radioactive, fluorescent or an electrochemiluminescent substance.

[0404] An alternative double antibody system often referred to as fast format systems because they are adapted to rapid determinations of the presence of an analyte, may also be

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employed within the scope of the present invention. The system requires high affinity between the antibody and the analyte. According to one embodiment of the present invention, the presence of the amyloid protein is determined using a pair of antibodies, each specific for amyloid protein. One of said pairs of antibodies is referred to herein as a "detector antibody" and the other of said pair of antibodies is referred to herein as a "capture antibody". The monoclonal antibody of the present invention can be used as either a capture antibody or a detector antibody. The monoclonal antibody of the present invention can also be used as both capture and detector antibody, together in a single assay. One embodiment of the present invention thus uses the double antibody sandwich method for detecting amyloid protein in a sample of biological fluid. In this method, the analyte (amyloid protein) is sandwiched between the detector antibody and the capture antibody, the capture antibody being irreversibly immobilized onto a solid support. The detector antibody would contain a detectable label, in order to identify the presence of the antibody-analyte sandwich and thus the presence of the analyte.

[0405] Exemplary solid phase substances include, but are not limited to, microtiter plates, test tubes of polystyrene, magnetic, plastic or glass beads and slides which are well known in the field of radioimmunoassay and enzyme immunoassay. Methods for coupling antibodies to solid phases are also well known to those skilled in the art. More recently, a number of porous material such as nylon, nitrocellulose, cellulose acetate, glass fibers and other porous polymers have been employed as solid supports.

[0406] The present invention also relates to a diagnostic kit for detecting amyloid protein in a biological sample comprising a composition as defined above. Moreover, the present invention relates to the latter diagnostic kit which, in addition to a composition as defined above, also comprises a detection reagent as defined above. The term "diagnostic kit" refers in general to any diagnostic kit known in the art. More specifically, the latter term refers to a diagnostic kit as described in Zrein et al. (1998).

[0407] It is still another object of the present invention to provide novel immunoprobes and test kits for detection and diagnosis of amyloid-associated diseases and conditions comprising antibodies according to the present invention. For immunoprobes, the antibodies are directly or indirectly attached to a suitable reporter molecule, e.g., an enzyme or a radionuclide. The test kit includes a container holding one or more antibodies according to the present invention

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and instructions for using the antibodies for the purpose of binding to amyloid protein to form an immunological complex and detecting the formation of the immunological complex such that presence or absence of the immunological complex correlates with presence or absence of amyloid protein.

EXAMPLES

Materials

[0408] The development and preparation of mouse monoclonal antibody ACI-01-Ab7C2 (named "mC2" and hC2 for the humanized C2 antibody, throughout the application) is described in co-pending application EP 05 02 7092.5 filed 12.12.2005, the disclosure of which is incorporated herein by reference.

[0409] Hybridoma cells FP-12H3-C2, producing mouse monoclonal antibody ACI-01-Ab7C2 (named "mC2" and hC2 for the humanized C2 antibody, throughout the application) were deposited 01 December 2005 in co-pending application no EP05027092.5 with the "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, Mascheroder Weg 1 B, 38124 Braunschweig, under the provisions of the Budapest Treaty and given accession no DSM ACC2750.

[0410] Hybridoma cells were cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum and antibiotics (Penicillin/Streptomycin). The isotype of the antibody produced was checked and found to be mouse IgG2b/kappa, as expected.

Assay

[0411] An ELISA for binding to Amyloid Beta provided a reliable measure of the potency of C2 antibodies. Positive control antibodies, murine FP-12H3-C2 antibody (Genovac Lot No: AK379/01), and standard Chemicon antibody 1560 (Lot no: 0508008791).

Choice of human constant regions

[0412] As immune system recruitment is not desirable for the clinical antibody candidate, the selected human constant region for the heavy chain was human IgG4, modified to change Serine at position 228 in the hinge region to Proline (HuIgG4 Ser-Pro). This mutation stabilizes the interchain disulphide bond and prevents the formation of half molecules that may occur in native human IgG4 preparations. The antibody expressed from the production

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cell lines will also have the terminal lysine removed. The sequences of human constant regions HuIgG4 Ser-Pro and human Kappa are given in SEQ ID NO: 17 and 14, respectively.

Example 1 Cloning and Sequencing of Antibody Variable Regions

[0413] Total RNA was prepared from 3 x 10^6 hybridoma cells (one T175 flask) using the Qiagen RNeasy mini kit (Cat No: 74104). RNA was eluted in 50μL water and checked on a 1.2% agarose gel. The conditioned medium from the cells was retained and a sample used for testing in the antibody activity assay.

[0414] V_H and V_K cDNAs were prepared using reverse transcriptase with mouse IgG and κ constant region primers. The first strand cDNAs were amplified by PCR using a large set of signal sequence primers. The amplified DNAs were gel-purified and cloned into the vector pGem® T Easy (Promega). The V_H and V_K clones obtained were screened for inserts of the expected size by PCR and the DNA sequence of selected clones determined by automated DNA sequencing. The locations of the complementarity determining regions (CDRs) in the sequences were determined with reference to other antibody sequences (Kabat EA *et al.*, 1991). The numbering convention of Kabat for antibody variable regions is used throughout this application; hence residue numbers may differ from the strict linear number.

[0415] The DNA sequence and deduced amino acid sequence for mC2 V_K is shown in SEQ ID NO: 29 and 27, respectively. Four clones gave this identical productive sequence. A non-productive aberrant V_K sequence that arises from the hybridoma fusion partner was also found in a number of clones.

[0416] For mC2 V_H, two different productive sequences were isolated. The mC2 V_H AF sequence (see SEQ ID NO: 30) was found in a total of 29 clones, with 14 single base pair changes in individual clones. The mC2 V_H B sequence was found in a total of 8 clones. Five of these represented the majority sequence, with the other 3 clones being variations on this. It is possible that these similar V_H B sequences arose as an artifact of the PCR amplification. A non-productive aberrant V_H was also obtained from the C2 hybridoma and is attributed to defective V-D-J joining.

[0417] In order to determine which is the correct active mC2 V_H, two chimeric antibodies were prepared with the two different V_H sequences, AF and B, combined with the mC2 V_K, to be tested for the correct antibody activity.

Example 2 Construction of Chimeric Antibody Genes

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[0418] A human chimeric antibody in its most common form consists of human constant regions linked to murine (or other non-human) variable regions. A chimeric antibody provides a very useful tool, firstly for confirmation that the correct variable regions have been identified, secondly for use as a control antibody in antigen binding assays with the same effector functions and utilizing the same secondary detection reagents as a humanized or engineered antibody, and also may be used to investigate the pharmacokinetic and other properties of the human constant regions with reference to the particular target for the antibody.

[0419] Two chimeric heavy chain expression vectors were constructed consisting of mC2 V_H AF or mC2 V_H B variable regions linked to HuIgG4 (Ser-Pro) constant region in the expression vector *pSVgpt* (Figure 1). This is based on pSV_2gpt (Mulligan and Berg, 1980) and includes the ampicillin resistance gene for selection in bacterial cells, the *gpt* gene for selection in mammalian cells, the murine heavy chain immunoglobulin enhancer region, genomic sequence encoding the constant region gene and SV40 poly A sequences. The heavy chain variable region for expression is inserted as a *Hin*dIII to *Bam*HI fragment.

[0420] A chimeric light chain vector was constructed consisting of C2 VK linked to human C Kappa constant region in the expression vector pSVhyg (Figure 2). pSVhyg includes the ampicillin resistance gene for selection in bacterial cells, the *hyg* gene for selection in mammalian cells, the murine heavy chain immunoglobulin enhancer region, genomic sequence encoding the kappa constant region gene and including the kappa enhancer and SV40 poly A sequences. The light chain variable region for expression is inserted as a *Hin*dIII to *Bam*HI fragment.

[0421] Expression cassettes for the murine C2 VH and VK sequences were constructed by addition of 5' flanking sequence including the leader signal peptide, leader intron and the murine immunoglobulin promoter, and 3' flanking sequence including the splice site and intron sequence, using the vectors VH-PCR1 and VK-PCR1 as templates (Riechmann et al., 1988). The DNA sequence was confirmed to be correct for the VH and VK in the chimeric expression vectors. The DNA and amino acid sequences of the VH and VK genes in the expression cassettes are shown in Figures 3 and 4.

Example 3 Expression of Chimeric Antibodies

3.1 Expression in stable cell lines

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[0422] The host cell line for antibody expression was NS0, a non-immunoglobulin producing mouse myeloma, obtained from the European Collection of Animal Cell Cultures, Porton UK (ECACC No 85110503). The heavy and light chain expression vectors were co-transfected into NS0 cells by electroporation. Colonies expressing the *gpt* gene were selected in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 0.8 μg/ml mycophenolic acid and 250 μg/ml xanthine. Transfected cell clones were screened for production of human antibody by ELISA for human IgG. Cell lines secreting antibody were expanded and the highest producers selected and frozen down in liquid nitrogen. The best producing cell lines for each antibody were expanded in medium as above but with only 5% FBS. Chimeric antibodies were purified using Prosep®-A (Bioprocessing Ltd). The concentration was determined by ELISA for human IgGκ antibody. The antibodies were also analyzed by SDS-PAGE.

3.2 Transient expression of chimeric antibodies

[0423] To expedite the testing of the different chimeric antibodies, transient expression was used to produce quickly small quantities of cell supernatant containing recombinant antibody for testing. The mC2 V_H and V_K expression cassettes were transferred to vectors based on pcDNA3.1 (Invitrogen) for transient expression. The heavy chain vector included a human IgG constant region. The light chain vector included a human kappa constant region. Both mC2 V_H AF and mC2 V_H B were transfected with mC2 V_K into human embryonic kidney (HEK 298) cells with Lipofectamine 2000 reagent (Invitrogen Cat No: 11668) according to the protocol supplied by the manufacturer. Conditioned medium was harvested from cells 3 days after transfection. The amount of antibody produced was determined by ELISA for human IgGκ antibody.

Example 4 Activity of Chimeric C2 Antibodies

4.1 Activity of chimeric C2 antibodies produced by transient transfection

[0424] Samples of conditioned medium from transient transfection for the two different chimeric antibodies were tested in the ELISA for binding to Amyloid Beta. The results clearly indicate that the C2 VH AF is the correct sequence. The C2 V_H AF/C2 V_K chimeric antibody binds well in the assay, but the C2 V_H B/C2 V_K does not show any binding at all. The Chemicon 1560 murine control antibody showed good binding, but binding by the purified murine C2 antibody supplied was low. It should be noted that a different secondary

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antibody was employed for the murine antibodies with the mouse constant regions compared to the chimeric antibodies with human constant regions, so the results are not directly comparable. Conditioned medium from the C2 hybridoma was later found to give a good result in the assay.

4.2 Activity of purified chimeric C2 antibodies

[0425] The two different C2 chimeric antibodies were purified from stable NS0 cell lines as described and tested using the Amyloid Beta ELISA. The results obtained are in accordance with the results obtained with transiently expressed antibody. The C2 ChVH AF/ChVK antibody binds well in the ELISA and the C2 ChVH B/ChVK antibody does not bind at all.

Example 5 Design of Humanized C2 Antibody Genes

[0426] The mC2 V_H and V_K amino acid sequences were compared to rodent antibody V_H and V_K sequences in the NCBI and Kabat databases.

5.1 Light chain variable region

[0427] The closest match mouse germ line gene to mC2 V_K is bb1, Locus MMU231201, (Schable *et al*, 1999). Only two amino acids differ from this germ line sequence, both located within CDRL1. Mature murine antibodies with similar, but not identical, sequence are found. Several have an identical CDRL2 and identical CDRL3, but the CDRL1 of mC2 seems to be unique. mC2 V_K can be assigned to Kabat subgroup MuV_KII. Position 87 of mC2 V_K is F rather than the Y that is more common in the subgroup, indicating that this framework residue may be important for antibody activity. Comparison with human germ line V_K sequences shows that genes from subgroup V_KII are the best match for mC2 V_K (Cox *et al*, 1994). Sequence DPK15 together with the human J region HuJ_K1 were selected to provide the acceptor framework sequences for the humanized V_K.

[0428] Four humanized V_K sequences were designed. C2HuVK1 consists of mC2 V_K CDRs with frameworks from DPK 15 and human J_K1. In versions 2, 3 and 4 murine residues have been substituted in the framework at positions 45 or 87 or both. Residue 45 may be involved in supporting the conformation of the CDRs. Residue 87 is located at the interface of the V_H and V_K domains. Therefore these residues may be critical for maintenance of antibody binding.

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[0429] The positions and changes that have been made in the light chain framework regions are shown in Table 1. A comparison of the humanized sequences with mC2 V_K sequence, and with DPK15 and human J_K1

5.2 Heavy chain variable region

[0430] The closest match mouse germ line gene to mC2 V_H AF is VH7183, Locus AF120466, (Langdon *et al*, 2000). The comparison is shown in Figure 5. Nine amino acids differ from this germ line sequence, most being located within CDR2. Mature murine antibodies with identical or similar (one residue different) CDR1 or with similar CDR2 (one residue different) are found, but none with all three CDRs identical to mC2 V_H AF. CDR3 of mC2 antibody is unusually short, consisting of only three residues. However, other antibodies are found in the database with CDR3 of this length. mC2 V_H AF can be assigned to Kabat subgroup MuV_HIIID. Residue 47 of mC2 V_H is L rather than the more common W, and residue 94 is S rather than the normal R, indicating that these framework residues may be important for antibody activity. Comparison with human germ line V_H sequences shows that genes from subgroup V_HIII are the best match for mC2 V_H. Sequence DP54 together with the human J region HuJ_H6 was selected to provide the acceptor framework sequences for the humanized V_H.

[0431] Four humanized V_H sequences were designed. C2HuVH1 consists of mC2 V_H AF CDRs with frameworks from DP54 and HuJ_H6. In versions 2, 3 and 4 murine residues have been substituted in the framework at positions 47 or 94 or both. Residue 47 in framework 2 makes contact both with the CDRs and with the V_K domain. Residue 94 may be involved in supporting the conformation of the CDRs. Therefore these residues may be critical for maintenance of antibody binding.

[0432] The positions and changes that have been made in the heavy chain framework regions are shown in Table 2.

Example 6 Construction of Humanized Antibody Genes

[0433] The modified variable regions were constructed by the method of overlapping PCR recombination. The expression cassettes for the chimeric antibody, C2 ChV_H AF and C2 ChV_K, were used as templates for mutagenesis of the framework regions to the required sequences. Sets of mutagenic primer pairs were synthesized encompassing the regions to be altered. The humanized V_H and V_K expression cassettes produced were cloned into pUC19

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and the entire DNA sequence was confirmed to be correct for each V_H and V_K. The modified heavy and light chain V-region genes were excised from pUC19 as *Hin*dIII to *Bam*HI expression cassettes. These were transferred to the expression vectors pSV*gpt* and pSV*hyg* which include human IgG4 Ser-pro or κ constant regions respectively, as for the chimeric antibody vectors. The DNA sequence was confirmed to be correct for the humanized V_H and V_K in the expression vectors.

Example 7 Expression of Humanized Antibodies

7.1 Expression in stable cell lines

[0434] The humanized heavy and light chain expression vectors were co-transfected into NS0 cells by electroporation, as for the expression of chimeric antibodies. Antibody producing cell lines were selected and expanded and humanized antibodies purified, exactly as for the chimeric antibody. The purified antibodies were analyzed by SDS-PAGE.

7.2 Transient expression of humanized antibodies

[0435] To expedite testing of the different humanized V_H and V_K constructs, the C2 humanized V_H and V_K expression cassettes were also transferred to the vectors for transient expression described in section 7.2. The four humanized C2 V_K constructs were co-transfected with the chimeric C2 V_H construct into HEK293 cells. Similarly, the four humanized C2 V_H constructs were co-transfected with the chimeric C2 V_K construct into HEK293 cells. Conditioned medium was harvested from cells three days after transfection. The amount of antibody produced was determined by ELISA for human IgGκ antibody.

Example 8 Activity of Humanized C2 Antibodies

8.1 Activity of humanized C2 antibodies produced by transient transfection

[0436] Samples of conditioned medium from the transient transfection were tested in the Amyloid Beta ELISA. The results obtained clearly indicate that the humanized VH constructs C2 HuVH AF versions 2 and 4 are functional when combined with the chimeric C2 kappa chain, and are comparable to the chimeric C2 antibody in the assay. In contrast, the antibodies containing C2 HuVH AF versions 1 and 3 combined with the chimeric C2 kappa chain show no binding at all in the assay. This indicates that the substitution of the murine residue at position 94 is essential for antibody activity. Antibodies containing the chimeric C2 heavy chain combined with the four humanized C2 kappa chains all showed good binding, comparable to the chimeric antibody, in the ELISA.

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8.2 Activity of purified humanized C2 antibodies

[0437] Eight different humanized C2 antibodies comprising all combinations of two humanized heavy chains and four humanized light chains were purified from stable NS0 cell lines as described and tested using the Amyloid Beta ELISA (figure 6).

[0438] The results obtained clearly indicate that C2 HuVH4 antibodies perform better in the assay than C2 HuVH2 antibodies. Of the C2 HuVH2 antibodies, C2 HuVH2/HuVK3 shows the best binding activity, but this is approximately 2 fold reduced compared to the chimeric control antibody C2 ChVHAF/ChVK. C2 HuVH2/HuVK2 activity is four to five fold reduced compared to the control. The activities of the antibodies comprising C2HuVH4 with the four different humanized light chains are similar. The highest activity is observed for C2HuVH4/HuVK1and all four antibodies are close to the control chimeric antibody in the assay.

Example 9 Modifications to CDRL2

9.1 Design light chain with modified CDR 2

[0439] As noted above, many antibodies share the same CDRL2 sequence ("KVSNRFS") as the C2 antibody. It was decided to test whether CDRL2 could be modified slightly without adversely affecting antibody activity. Two conservative substitutions were selected: R for K at position 50 and S for N at position 53. The two alternative CDRL2 sequences are therefore "RVSNRFS" and "KVSSRFS". These were incorporated into the murine V_K sequence with no other changes, as mC2 VK-R and mC2 VK-S respectively.

9.2 Transient expression of modified CDRL2 antibody

[0440] The two C2 light chain constructs with modified CDRL2 described in Section 11.2.1 were cloned into the light chain vector for transient expression. Each was co-transfected with the chimeric C2 V_H vector into HEK293 cells. Conditioned medium was harvested from cells three days after transfection. The amount of antibody produced was determined by ELISA for human IgGκ antibody.

9.3 Activity of C2 antibody with modified CDRL2

[0441] Samples of conditioned medium from the transient transfection of mC2 V_Ks with modified CDRL2 combined with mC2 V_H were tested in the Amyloid Beta ELISA.(figure 7) Both the VK-R and the VK-S antibodies are comparable to the chimeric C2 antibody,

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indicating that the individual modifications to CDRL2 chosen do not markedly affect the activity of the antibody in the assay.

Example 10 Affinity Determination

[0442] To assess the binding specificity and affinity of mouse (ACI-01-Ab-7-C2) chimeric (AF) and humanized antibodies (H4K1; H4K4), BIACORE.RTM. analysis was performed using amyloid beta 1-42 monomers and fibers as antigen immobilized on a CM5 chip. BIACORE.RTM. technology utilizes changes in the refractive index at the surface layer upon binding of the antibody to the antigen immobilized on the layer. Binding is detected by surface plasmon resonance (SPR) of laser light refracting from the surface. Analysis of the signal kinetics on rate and off rate allows the discrimination between non-specific and specific interaction. The concentration of antibody used was in the range of 0.05 ìM to 1.0 ìM.

	Monomers			**Fibers**		
	k_a(1/Ms)	k_d(1/s)	KD (M)	k_a(1/Ms)	k_d(1/s)	KD (M)
Mouse ACI-01-Ab-7-C2	1,8E+04	2,7E-03	1,5E-07	2,4E+04	9,9E-04	4,1E-08
chimeric AF	4,7E+04	9,5E-04	2E-08	5,1E+04	3,3E-04	6,5E-09
humanized H4K1	5,0E+04	9,5E-04	1,9E-08	4,9E+04	2,3E-04	4,7E-09
humanized H4K4	2,5E+04	4,4E-04	1,8E-08	1,3E+05	3,0E-04	2,3E-09

Example 11 Immunhistochemical Binding Assay

11.1 Human brain sections

[0443] Brains from healthy, non-demented pre-AD and AD patients were obtained from the Universitätsklinik in Bonn after ethical approval. Brains were fixed in formaldehyde and the hippocampus region was dehydrated, embedded in paraffin and 5 μm sections were cut with a microtome. Paraffin sections were stored at RT until use. For fresh material, 5 µm cryosections were cut with a cryostat and sections stored at -80°C until use.

11.2 Immunohistochemistry

[0444] Paraffin sections were deparaffinized and rehydrated by bathing slides in xylene followed by 100% ethanol, 90% ethanol and 70% ethanol. Background was decreased by 30

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minutes incubation in 10%H_20_2, 10% methanol in water. Antigen retrieval was obtained by incubating the slides in 100% formic acid for 3 minutes. After 3 washes in Tris buffered saline (TBS, pH 7.5), non-specific labeling was blocked by a 2 hour incubation of the slides in 10 % BSA, 0.25% Triton X-100 in TBS. After washing (3 washes in TBS) blocking of endogenous antibodies was performed by adding a non-labeled anti-human IgG (Biomeda) and incubating slides in humid chambers overnight at RT. After another 3 washes, the primary human anti amyloid antibody was added to the slides and incubated another 24 hours at RT. Following washing, an alkaline phosphatase labeled secondary anti human IgG (Sigma) was added to the slides and incubated for 2 hours at RT. After washing, slides were developed with Liquid permanent Red (Dakocytomation) washed with water and air-dried before mounting with permanent mounting media (corbitbalsam).

[0445] Cryosection were fixed in methanol for 30 minutes at -80°C and background decreased by adding H_20_2 to the cold methanol to a final concentration of 10% and incubating for 30 minutes at RT. After 3 washes in Tris buffered saline (TBS, pH7.5), non-specific labeling was blocked by a 2 hour incubation of the slides in 10 % BSA, 0.25% Triton X 100 in TBS as above and the same staining procedure as above was carried out.

[0446] Sections were examined with a Leica DMLB microscope and photographed using a Leica DC500 camera and Leica FireCam1.2.0 software.

[0447] Both human antibodies A and C labeled plaques of brains from AD disease patients (figure 8). Both diffuse and cored plaques were labeled. Moreover, diffuse plaques in non - demented pre-AD patients could also be detected by the A and C antibodies. Amyloid in cerebral amyloid angiopathy (CAA) was labeled with both antibodies and some staining of neurons which may correspond to intracellular amyloid was also detected. No labeling was seen on control brains from healthy patient. Plaques could be detected on paraffin sections pretreated with formic acid but no plaques were labeled on paraffin sections without formic acid pretreatment and on cryosections fixed in methanol. The human antibody B did not detect plaques on paraffin sections and the mouse antibody did not stain either paraffin or cryosections of human brains.

Abbreviations:

A = binding chimeric antibody AF (IgG4)

B = non-binding chimeric antibody B (IgG4)

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C = binding humanized antibody H4K1 (IgG4)

Mouse = ACI-01-Ab-C2 mouse antibody (IgG2b)

Example 12 Functionality of mC2 on Amyloid Fibers

12.1 Modification of Conformation of Aâ1-42 Fibers and Initiation of Disaggregation after Binding of the mC2 antibody

[0448] In order to evaluate the mechanism by which the antibody is capable to disaggregate preformed beta-amyloid ($A\beta_{1-42}$) fibers a head-to-head comparison of Thioflavin-T (Th-T) fluorescent assay was performed measuring disaggregation and solid-state Nuclear Magnetic Resonance (NMR) of U-^{13}C Tyrosine10 and Valine12-labeled Aβ1-42 peptide analysing secondary conformation (figure 9A). The mC2 antibody solubilised 35.4 % of the preformed Aβ1-42 fibers and simultaneously induced a shift in secondary conformation from beta sheet to random coiled. The reduction in the population of the beta sheet conformation with respect to the random coil is of the order of 35% and is therefore in close agreement with that measured using fluorescence Th-T assay (figure 9B). These data indicate that the binding of the mC2 antibody initiates a transition of the secondary structure which potentially causes a destabilization of the parallel intermolecular arrangement of the beta sheets affecting a break of elongated fibers into smaller fragments.

12.2 Conformation-dependent Binding Affinity of mC2 antibody

[0449] Since it is well known in the scientific literature that a proportion of the antibody-antigen binding energy can be used for energy-dependent modification of the conformation of an antigen (Blond and Goldberg, 1987), a comparison experiment of the binding affinity of the C2 antibody to the whole $A\beta_{1-42}$ protein and to a smaller, nine amino acid long, peptide comprising the antibody's epitope was performed (figure 10). For this comparison the affinities of the humanized antibody C2 were analyzed by ELISA using biotinylated peptides covering the complete amino-acid sequence of the C2's epitope (produced by Mimotopes and purchased from ANAWA Trading SA) and a biotinylated complete Aβ1-42 peptide (Bachem). The analysis was done according to the manufacturer's (Mimotopes) instructions. As demonstrated in Figure 10, the antibody binds with a 36.0% higher affinity to the peptide comprising its specific epitope (aminoacids 13-21 of the $A\beta_{1-42}$ sequence) than to the whole

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Aβ1-42 protein. It is therefore suggested that the difference in binding affinity energy was used for the energy-consuming transition of the secondary conformation of the amyloid protein to present the antigen in a more acceptable position for the antibody interaction. This explains why the affinity of the antibody is lower for the native (the whole amyloid protein) than for the isolated subunit.

Example 13 Effects of the anti-amyloid hC2 on the aggregation of amyloid beta 1-42 peptide

[0450] To evaluate the ability of the humanized anti-human amyloid beta monoclonal antibody hC2 to mediate anti-aggregating and disaggregating effects on amyloid beta (Aβ) a thioflavin T spectrofluorescence assay was accomplished.

13.1 Inhibition of Aggregation Assay

[0451] Aβ1-42 lyophilized powder was reconstituted in hexafluoroisopropanol (HFIP) to 1 mM. The peptide solution was sonicated for 15 min at room temperature, agitated overnight, and aliquots made into non-siliconized microcentrifuge tubes. The HFIP was then evaporated under a stream of argon. The resulting peptide film was vacuum dried for 10 min and stored at -80°C until used.

[0452] To assay for the antibody-mediated inhibition of Aβ1-42 aggregation the hC2 antibody was pre-diluted in PBS and an assay solution containing the following components was made in a non-siliconized incubation tube: 3.3 or 0.33 mM pre-diluted antibody, 10 mM thioflavin T, 33 mM Aβ1-42, and 8.2% DMSO. Therefore the final molar ratios of antibody to Aβ1-42 were 1:10 and 1:100. Appropriate control solutions were also prepared. The solutions were then incubated for 24 hrs at 37°C, and the spectrofluorescence (relative fluorescence units; RFU) read in six replicates in black 384-well plates (Perkin-Elmer) on a Perkin-Elmer FluoroCount spectrofluorometer. The spectrofluorescence was then measured and % disaggregation calculated as described below.

13.2 Disaggregation Assay

[0453] To assay for antibody-mediated disaggregation of pre-aggregated Aβ1-42, a low-molecular weight Aβ1-42, prepared as described above, was made up as a 110 mM solution in 27% DMSO and 1x PBS. This solution was then allowed to aggregate at 37°C for 24 hrs after which the following were added: 3.3 or 0.33 mM pre-diluted antibody, and 10 mM thioflavin T. This resulted in a molar ratio of 1:10 and 1:100 antibody to Aβ1-42. This

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solution was then incubated for additional 24 hrs at 37°C. The spectrofluorescence was then measured and % disaggregation calculated as described below.

13.3 Calculation

[0454] Inhibition of aggregation or disaggregation is expressed as mean % inhibition or disaggregation, respectively, ± standard error of the mean (SEM) according to the following equation:

$$\% \text{ inhibition} = \frac{(\text{RFU of pos contrl} - \text{RFU of neg contrl}) - (\text{RFU of sample with A}\beta\text{1-42} - \text{RFU of sample without A}\beta\text{1-42})}{(\text{RFU of pos contrl} - \text{RFU of neg contrl})} \times 100\%$$

13.4 Result

13.4.1 Inhibition of Aβ1-42 aggregation

[0455] Inhibition of Aβ1-42 aggregation using the hC2 antibody is shown in Table 1 and Figure 18. At an antibody to Aβ1-42 molar ratio of 1:100 the inhibition averaged 30% (2 independent experiments), whereas at a 1:10 molar ratio the inhibition was 80% (2 independent experiments; see Table 1).

Table 1. hC2-mediated inhibition of Aβ1-42 aggregation at a 1:100 and 1:10 antibody to Aβ1-42 molar ratios.

Antibody	Molar ratio (antibody to Aβ1-42)	
	1:100	1:10
hC2	30.0 ± 4.1%	80.4 ± 6.9%

13.4.2 Disaggregation of pre-aggregated Aβ1-42

[0456] Disaggregation of pre-aggregated Aβ1-42 using the hC2 antibody is shown in Table 2 and Figure 19. At an antibody to Aβ1-42 molar ratio of 1:100 the disaggregation averaged 24%, whereas at a 1:10 molar ratio the disaggregation was 32% (3 independent experiments; see Table 2).

Table 2. hC2-mediated disaggregation of pre-aggregated Ab1-42 at a 1:100 and 1:10 antibody to Aβ1-42 molar ratios.

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Antibody	Molar ratio (antibody to Aβ1-42)	
	1:100	1:10
hC2	23.9 ± 4.4%	31.9 ± 3.5%

[0457] Using the thioflavin T assay, the bi-functional properties of the anti-Aβ humanized antibody hC2 can be demonstrated, namely to inhibit the aggregation of Aβ1-42 into pathogenic protofibrillar conformation and in addition to disaggregate preformed Aβ1-42 protofibrils. hC2 inhibited Aβ1-42 aggregation by 80% at an antibody to Aβ1-42 molar ratio of 1:10. The ability of hC2 to disaggregate pre-aggregated protofibrils of Aβ1-42 at a 1:10 molar ratio was shown to be 32%.

Example 14: Conformation-specific binding of mC2 to different classes of Amyloid Protein

[0458] In order to evaluate the specificity of mC2 to different stages of polymerized amyloid protein, monomeric, polymeric soluble and fibrillic amyloid, an ELISA coated with these different stages of polymeric beta-amyloid was performed (figure 11). Monomers were prepared according to a modified method published by (Klein, 2002), soluble polymeric amyloid beta according to (Barghorn et al., 2005), whereas fibers were performed by incubation of amyloid (Bachem, Switzerland) with a final concentration of 1μg/μl in Tris/HCl pH 7.4 at 37°C for 5 days followed by a centrifugation step (10,000 rpm for 5 minutes). Then amyloid polymers were coated on an ELISA plates with a final concentration of 55μg/ml and binding affinity ELISA by using an anti-mouse IgG monoclonal antibody (Jackson) labelled with alkaline phosphate was performed. As demonstrated in Figure 11 the mC2 antibody binds with higher affinity to soluble polymeric amyloid beta than to fibers and with the lowest to monomers. These data indicate that the antibody's binding is influenced by the amyloid epitope and by the conformation of the different amyloid aggregates.

Example 15: Epitope mapping of AC Immune's monoclonal antibody hC2

[0459] Epitope mapping of the humanized monoclonal antibody hC2 was performed by ELISA using three different peptide libraries. One library comprised a total of 33 biotinylated peptides covering the complete amino acid (aa) sequence of Aβ1-42 (produced by Mimotopes and purchased from ANAWA Trading SA), the second library contains

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biotinylated peptides using peptide 12 (aa12-20 of Aβ) from the first peptide library and substituting each aa in the sequence by an alanine (see table 3 below), and the third library contains biotinylated peptides 13, 14, or 15 (aa 13-21, 14-22 or 15-23 of Aβ) and substituting in each case the last amino acids to an alanine or to a glycine for aa 21 which is already an alanine (see table 4 below). A biotinylated complete Aβ1-42 peptide was used as positive control (Bachem). Epitope mapping was done according to the manufacturer's (Mimotopes) instructions. Briefly, Streptavidin coated plates (NUNC) were blocked with 0.1% BSA in PBS overnight at 4°C. After washing with PBS-0.05% Tween 20, plates were coated for 1 hour at RT with the different peptides from the library, diluted in 0.1% BSA, 0.1% Sodium Azide in PBS to a final concentration of 10 μM. After washing, plates were incubated for 1 hour at RT with the hC2 antibody or a non Aβ binding chimeric IgG4 antibody diluted to 200 ng/ml in 2% BSA, 0.1% Sodium Azide in PBS. Plates were washed again and incubated with alkaline phosphatase conjugated goat anti human IgG for 1h at RT. After final washing, plates were incubated with phosphatase substrate (pNPP) and read at 405 nm using an ELISA plate reader.

[0460] It was shown that the humanized monoclonal antibody hC2 bound specifically to peptides 12,13,14,15 and 16 of the first peptide library. These peptides comprise aa 12-20, 13-21, 14-22, 15-23 and 16-24 respectively of Aβ1-42, suggesting that the epitope lies in region 12-24 of Aβ. A second library with alanine substitutions was used to determine the critical aa for binding to Aβ12-20 (VHHQKLVFF). The binding of the hC2 antibody is lost completely when amino acids 16, 17, 19 or 20 are substituted by an alanine, indicating that these aa are absolutely critical for binding of the antibody to Aβ. The binding of the hC2 antibody is partially lost when aa 15 and 18 are substituted.

[0461] The binding was also almost completely lost when aa 14 was substituted for an alanine, indicating that aa 14 is also very important for binding.

[0462] Finally, a third library was used to determine whether aa 21, 22 or 23 are critical for binding to the epitope. The binding of the antibody to aa 15-23 was reduced when aa 23 was substituted for an alanine, indicating that aa 23 is also important for binding. The binding was partially lost when aa 21 was substituted for a glycine and slightly lost when aa 22 was substituted for an alanine.

Example 16: Neuroprotection by the hC2 Antibody

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[0463] The ability of antibody hC2 to protect neurons from Abeta oligomer-induced degeneration was assessed in an in vitro assay. Embryonic day 16.5-17.5 mouse cortical neurons were isolated, dissociated, and cultured in vitro in N3-F12 media. The cells were grown for nine days in total, and were fed on day 3 and on the day that Abeta oligomer, or Abeta oligomer plus anti-Abeta antibody hC2 was added. At day five ("4 days Abeta") or day six ("3 days Abeta"), certain wells of cells were treated with either 2 μM Abeta oligomer alone, or a combination of 2 μM Abeta oligomer and 50 μg/mL anti-Abeta antibody hC2.

[0464] The Abeta oligomer was prepared by dissolving Abeta 1-42 (rPeptide) in HFIP, from which Abeta peptides were aliquoted into 10μl aliquots at 1mg/ml and then evaporated in a fume hood for 30 minutes and peptide films were stored at -80C until use. Upon use, the peptide film was dissolved in 10μl of DMSO, then 78.6μl of HAMS F12, and the Abeta peptide solution was incubated at 4C for 24-48 hours (25μM final concentration of Abeta).

[0465] For control cells, DMSO-F12 alone was added at the same volume as Abeta-DMSO at day 5, and the cells were cultured for an additional 4 days without any additional treatment. On day 9, neurons from all culture conditions were fixed and stained with Tuj1 (an anti-beta-tubulin antibody), followed by staining with secondary antibodies labeled with FITC to visualize microtubules, and thus neuronal processes in general. The results are shown in Figure 20.Untreated mouse embryonic cortical neurons showed normal morphology after nine days of culture (Figure 20, leftmost panel). Treatment of the cells with Abeta oligomer for three days induced axon degeneration and caused a decrease in the total number of axons (Figure 20, lower center panel), and this effect was even more pronounced at four days of treatment (Figure 20, upper center panel). In contrast, the cells treated with the combination of Abeta oligomer and anti-Abeta antibody hC2 looked similar to control cells (Figure 20, upper and lower right panels). These results indicate that anti-Abeta antibody hC2 was able to protect embryonic mouse cortical neurons from Abeta oligomer-induced degeneration.

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Table 1: Positions and changes made in the humanized C2 light chain framework regions

Position Light chain	45	87	50	53
Mouse C2V_K	K	F	K	N
Humanized C2HuV_K1	Q	Y	K	N
Humanized C2HuV_K2	Q	F	K	N
Humanized C2HuV_K3	K	Y	K	N
Humanized C2HuV_K4	K	F	K	N
Human Germline dpk15	Q	Y	L	N
Mouse C2V_K-R			R	
Mouse C2V_K-S				S

Table 2: Positions and changes made in the humanized C2 heavy chain framework regions

Position Heavy chain	47	94
Mouse C2VHAF	L	S
Humanized C2HuVHAF1	W	R
Humanized C2HuVHAF2	W	S
Humanized C2HuVHAF3	L	R
Humanized C2HuVHAF4	L	S
Human Germline DP-54	W	R

A total of 8 different antibodies were constructed with light chains Humanized C2HuV_K1, C2HuV_K2, C2HuV_K3, C2HuV_K4 and heavy chains C2HuVHAF4 and C2HuVHAF2

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Table 3. Summary of peptides used in the second library

aa that are important for binding are marked in italics and underscore and aa absolutely critical for binding are marked in italics and bold.

p12-20	V	H	H	Q	K	L	V	F	F				
A12	**A**	H	H	Q	K	L	V	F	F				
A13	V	**A**	H	Q	K	L	V	F	F				
A14	V	H	**A**	Q	K	L	V	F	F				
A15	V	H	H	**A**	K	L	V	F	F				
A16	V	H	H	Q	**A**	L	V	F	F				
A17	V	H	H	Q	K	**A**	V	F	F				
A18	V	H	H	Q	K	L	**A**	F	F				
A19	V	H	H	Q	K	L	V	**A**	F				
A20	V	H	H	Q	K	L	V	F	**A**				
aa no.	12	13	***14***	***15***	***16***	***17***	***18***	***19***	***20***				
p13-21			H	H	Q	K	L	V	F	F	A		
p13-21	G21		H	H	Q	K	L	V	F	F	**G**		
p14-22				H	Q	K	L	V	F	F	A	E	
p14-22	A22			H	Q	K	L	V	F	F	A	**A**	
p15-23					Q	K	L	V	F	F	A	E	D
p15-23	A23				Q	K	L	V	F	F	A	E	**A**
aa no.			13	***14***	***15***	***16***	***17***	***18***	***19***	***20***	21	22	***23***

Table 2. Summary of peptides used in the third library.

aa that are important for binding are marked in italics and underscore and aa absolutely critical for binding are marked in italics and bold

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WHAT IS CLAIMED IS:

1. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof, which specifically binds to at least one epitope on the β-amyloid protein wherein the epitope comprises at least two consecutive amino acid residues predominantly involved in binding to the antibody, wherein the at least two consecutive amino acid residues are selected from the group consisting of:

a) –Lys-Leu- embedded within the following core sequence (SEQ ID NO: 10):

Xaa1 – Xaa2 - Lys - Leu – Xaa3 wherein

Xaa1 is an amino acid selected from the group consisting of His, Asn, Gln Lys, and Arg,

Xaa2 is an amino acid selected from the group consisting of Asn and Gln; and

Xaa3 is an amino acid selected from the group consisting of Ala, Val, Leu, norleucine, Met, Phe, and Ile

and

b) Phe-Phe- embedded within the following core sequence (SEQ ID NO: 9):

Xaa3 - Phe – Phe – Xaa4 – Xaa5 – Xaa6, wherein

Xaa3 is an amino acid residue selected from the group consisting of Ala, Val, Leu, norleucine, Met, Phe, and Ile;

Xaa4 is an amino acid residue selected from the group consisting of Ala, Val, Leu, Ser and Ile;

Xaa5 is an amino acid residue selected from the group consisting of Glu and Asp,

and

Xaa6 is an amino acid residue selected from the group consisting of Glu and Asp,

wherein said chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof comprises a variant Fc region, and wherein said variant Fc region comprises at least one amino acid modification relative to a wild type Fc region, such that said molecule has modified effector function than the wild type Fc region.

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2. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1, which specifically binds to at least two epitopes on the β-amyloid protein, wherein said at least two epitopes each comprise at least two consecutive amino acid residues predominantly involved in the binding of the antibody, which are - Phe - Phe –and - Lys - Leu –, respectively, and wherein said at least two distinct binding sites exhibit amino acid sequence –Val - Phe - Phe - Ala - Glu – Asp - shown in SEQ ID NO: 7 and amino acid sequence His - Gln - Lys - Leu – Val - shown in SEQ ID NO: 8, respectively.

3. A humanized antibody or a fragment thereof which comprises in the variable region at least one CDR of non-human origin and one or more human- or primate-derived framework regions and, optionally, a constant region derived from a human or primate source antibody, which humanized antibody or fragment thereof is capable of specifically binding β-amyloid protein, β-amyloid monomeric peptide, polymeric soluble amyloid pepides comprising a plurality of β-amyloid monomeric units, β-amyloid fibers, fibrils or filaments, and a β-amyloid polymeric peptide in isolation or as part of a β-amyloid plaque, at an epitope comprising the following amino acid sequence (SEQ ID NO: 11):

Xaa1 – Xaa2 - Lys - Leu – Xaa3 - Phe – Phe- Xaa4 – Xaa5 – Xaa6, wherein

Xaa1 is an amino acid residue selected from the group consisting of His, Asn, Gln, but particularly His;

Xaa2 is an amino acid residue selected from the group consisting of Asn and Gln, but particularly Gln; and

Xaa3 is an amino acid residue selected from the group consisting of Val, Leu, and Ile, but particularly Val;

Xaa4 is an amino acid residue selected from the group consisting of Ala and Val, but particularly Ala;

Xaa5 is an amino acid residue selected from the group consisting of Glu and Asp, but particularly Glu; and

Xaa6 is an amino acid residue selected from the group consisting of Glu and Asp, but particularly Asp.

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4. A humanized antibody or a fragment thereof according to claim 1, wherein the humanized antibody comprises variable regions with human- or primate-derived framework regions and at least one CDR with an amino acid sequence selected from the group of sequences consisting of SEQ ID NO:1 representing CDR1, SEQ ID NO: 2 representing CDR2 and SEQ ID NO: 3 representing CDR3 of the Heavy Chain Variable Region (HCVR) and SEQ ID NO: 4 representing CDR1, SEQ ID NO: 5 representing CDR2 and SEQ ID NO: 6 representing CDR3 of the Light Chain Variable Region (LCVR).

5. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof, wherein at least one of the amino acids of a light chain and a heavy chain CDR region as given in SEQ ID NOs: 1-6 is changed through a conservative substitution such that the antibody maintains its full functionality.

6. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof, according to claim 5, wherein within the amino acid sequence of CDR2 of the light chain variable region (LCVR) as given in SEQ ID NO: 5, the Lys at Kabat position 50 is replaced by Arg, Gln or Glu, but particularly by Arg, and/or the Ser at Kabat position 53 is replaced by Asn or Thr, but particularly by Asn, and/or the Asn at Kabat position 53 is replaced by Ala, Val, Leu, Ser and Ile; but expecially Ser.

7. A humanized antibody or a fragment thereof according to claim 1, wherein at least one of the amino acids of the human- or primate-derived framework region is changed through a substitution to an amino acid from the corresponding region of murine antibody ACI-01-Ab7C2 or a substitution conservative thereto.

8. A humanized antibody or a fragment thereof according to claim 162, wherein

the Trp in Kabat position 47 in the human- or primate-derived framework region of the Heavy Chain Variable Region as shown in SEQ ID NO: 15 is replaced by an amino acid selected from the group consisting of Leu, norleucine, Ile, Val, Met, Ala, and Phe, particularly Leu and Ile, but especially Leu and/or

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the Arg in Kabat position 94 in the human- or primate-derived framework region of the Heavy Chain Variable Region as shown in SEQ ID NO: 15 is replaced by an amino acid selected from the group consisting of Ser and Thr, but especially by Ser, and/or

the Tyr in Kabat position 87 in the human- or primate-derived framework region of the Light Chain Variable Region as shown in SEQ ID NO: 12 is replaced by an amino acid selected from the group consisting of Phe, Leu, Val, Ile, and Ala, particularly by Leu and Phe, but especially by Phe.

9. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof antibody according to claim 1, wherein the heavy chain variable region (HCVR) has an amino acid sequence that is 90%, particularly 95%, more particularly 98%, even more particularly 100% identical to the sequence set forth in SEQ ID NO: 15 and 16, respectively.

10. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1, wherein the light chain variable region (LCVR) has an amino acid sequence that is 90%, particularly 95%, more particularly 98%, even more particularly 100% identical to the sequence set forth in SEQ ID NO: 12 and 13, respectively.

11. A humanized antibody or a fragment thereof according to claim 1, wherein at least two, but especially three, of the CDR regions of the heavy chain variable region (HCVR) have an amino acid sequence that is 90%, particularly 95%, more particularly 98%, even more particularly 100% identical to the corresponding CDR region set forth in SEQ ID NOs: 1-3.

12. A humanized antibody or a fragment thereof according to claim 1, wherein at least two, but especially three, of the CDR regions of the light chain variable region (LCVR) have an amino acid sequence that is 90%, particularly 95%, more particularly 98%, even more particularly 100% identical to the corresponding CDR region set forth in SEQ ID NOs: 4-6.

13. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1, wherein the antibody is capable of inhibiting

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aggregation of A β monomers and disaggregating polymeric fibrils or filaments, especially polymeric fibrils or filaments paired with a high degree of conformational sensitivity.

14. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1, which antibody, upon co-incubation with a β-amyloid monomeric peptide selected from Aβ monomeric peptides 1-39; 1-40, 1-41, and 1-42, and/or a polymeric soluble β-amyloid peptide comprising a plurality of said Aβ monomeric peptides, and particularly with an Aβ1-42 monomeric peptide and/or an Aβ polymeric soluble amyloid peptide comprising a plurality of Aβ1-42 monomeric peptides, inhibits the aggregation of the Aβ monomers into high molecular polymeric fibrils or filaments, and, upon co-incubation with preformed high molecular polymeric amyloid fibrils or filaments formed by the aggregation of β-amyloid monomeric peptides selected from Aβ monomeric peptides 1-39; 1-40, 1-41, and 1-42, and particularly by the aggregation of Aβ1-42 monomeric peptides, is capable of disaggregating the preformed polymeric fibrils or filaments.

15. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1, which antibody substantially binds to aggregated Aβ in the brain of a subject selected from a mammal and a human.

16. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1, which antibody reduces Aβ plaque burden in the brain of a mammal or a human.

17. A nucleic acid molecule comprising a nucleotide sequence encoding a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1 or a nucleotide sequence selected from a sequence encoding SEQ ID NO: 2 and SEQ ID NO: 3, representing the Complementarity Determining Regions (CDRs) 2 and 3 of the Heavy Chain Variable Region (HCVR), respectively, a sequence encoding SEQ ID NO: 4, representing CDR 1 of the Light Chain Variable Region (LCVR), SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, a sequence encoding the light chain variable region of SEQ ID NO: 22, a sequence encoding the light chain variable region of SEQ ID NO: 22 and the light chain

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constant region of SEQ ID NO: 23, a sequence encoding the heavy chain variable region of SEQ ID NO: 25 and a sequence encoding the heavy chain variable region of SEQ ID NO: 25 and the heavy chain constant region of SEQ ID NO: 26.

18. An expression vector comprising the nucleic acid molecule of claim 17.

19. A cell comprising an expression vector according to claim 18.

20. A composition comprising the antibody according to claim 1 including any functionally equivalent antibody or functional parts thereof in a therapeutically effective amount and optionally further comprising a pharmaceutically acceptable carrier.

21. A composition comprising a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1 including any functionally equivalent antibody or functional parts thereof and, optionally, further comprising a further biologically active substance in a therapeutically effective amount and/or a pharmaceutically acceptable carrier and/or a diluent and/or an excipient, wherein the further biologically active substance is selected from a compound used in the treatment of amyloidosis, compounds against oxidative stress, anti-apoptotic compounds, metal chelators, inhibitors of DNA repair such as pirenzepin and metabolites, 3-amino-1-propanesulfonic acid (3APS), 1,3-propanedisulfonate (1,3PDS), α-secretase activators, β- and γ-secretase inhibitors, tau proteins, neurotransmitters, β-sheet breakers, attractants for amyloid beta clearing/depleting cellular components, inhibitors of N-terminal truncated amyloid beta including pyroglutamated amyloid beta 3-42, anti-inflammatory molecules, and cholinesterase inhibitors (ChEIs), such as tacrine, rivastigmine, donepezil, and/or galantamine, M1 agonists and other drugs including any amyloid or tau modifying drug and nutritive supplements, and, optionally, a pharmaceutically acceptable carrier and/or a diluent and/or an excipient.

22. A method for preventing, treating or alleviating the effects of one or more amyloidosis-related diseases selected from amyloidosis, neurological disorders such as Alzheimer's Disease (AD), Lewy body dementia, Down's syndrome,

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hereditary cerebral hemorrhage with amyloidosis (Dutch type), the Guam Parkinson-Dementia complex; progressive supranuclear palsy, multiple sclerosis; Creutzfeld Jacob disease, Parkinson's disease, HIV-related dementia, ALS (amyotropic lateral sclerosis), Adult Onset Diabetes, senile cardiac amyloidosis, endocrine tumors, and macular degeneration in a subject in need thereof, comprising administering a chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof and/or a functional part thereof according to claim 1 to a subject affected by such a disorder in a therapeutically effective amount.

23. A method according to claim 22, wherein treatment of a subject, particularly a mammal or a human, suffering from an amyloid-associated condition characterized by a loss of cognitive memory capacity leads to an increase in the retention of cognitive memory capacity or complete restoration thereof.

24. A method of diagnosis of an amyloid-associated disease or condition in a subject comprising

(a) bringing a tissue sample or a specific body part or body area of the subject suspected to contain the amyloid protein into contact with an antibody according to claim 1;
(b) allowing the antibody to bind to the amyloid protein;
(c) detecting the antibody bound to the protein; and
(d) correlating the presence or absence of antibody binding with the presence or absence of amyloid protein in the sample or specific body part or area.

25. A method of determining the extent of amyloidogenic plaque burden in a tissue and/or body fluids of a subject comprising

(a) obtaining a sample representative of the tissue and/or body fluids in a subject under investigation;
(b) testing said sample for the presence of amyloid protein with an antibody according to claim 1;
(c) determining the amount of antibody bound to the protein; and

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(d) calculating the plaque burden in the tissue and/or body fluids of the subject.

26. A test kit for the detection and diagnosis of amyloid-associated diseases and conditions comprising an antibody according to claim 1 and optionally further comprising instructions for using the antibodies for the purpose of binding to amyloid protein to form an immunological complex and detecting the formation of the immunological complex such that presence or absence of the immunological complex correlates with the presence or absence of amyloid protein.

27. An isolated light chain variable region (LCVR) comprising human- or primate-derived framework regions and at least one CDR having an amino acid sequence selected from SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6.

28. An isolated heavy chain variable region (HCVR) comprising human- or primate-derived framework regions and at least one CDR having an amino acid sequence selected from the group of sequences consisting of SEQ ID NO: 2 and SEQ ID NO: 3.

29. A light chain variable region (LCVR) of SEQ ID NO: 12.

30. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1, wherein the antibody or fragment thereof substantially binds Aβ monomers and/or Aβ fibers, fibrils or filaments and/or soluble polymeric amyloid beta, and wherein the antibody or fragment thereof does not show any significant cross-reactivity with amyloid precursor protein (APP).

31. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 30, wherein said antibody or fragment binds to an Aβ monomer with a binding affinity represented by a KD in a range selected from a group consisting of from at least about 1 x 10-7 M to at least about 1 x 10-12 M, from at least about 1 x 10-8 M to at least about 1 x 10-11 M, from at least about 1 x 10-8 M to at least about 1 x 10-10 M, and from at least about 1 x 10-8 M to at least about 5 x 10-8. M, and wherein said antibody or fragment binds to an Aβ fiber, fibril or filament with a binding affinity represented by a KD in a range selected from a group consisting of from at least about 1 x 10-7M to at least about 1 x 10-12 M, from

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at least about 1 x 10-8M to at least about 1 x 10-11M, from at least about 1 x 10-9M to at least about 1 x 10-10M, and from at least about 2 x 10-9M to at least about 8 x 10-9M.

32. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 30, wherein the antibody has a higher affinity to soluble polymeric amyloid beta and Aβ fibers, fibrils or filaments, than to Aβ monomers.

33. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 32, wherein said antibody or fragment thereof exhibits a binding affinity to an Aβ fiber, fibril or filament which is at least 10 times, particularly at least 15 times, more particularly at least 20 times, and especially at least 25 times higher than the binding affinity to an Aβ monomer.

34. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1, which antibody substantially binds to aggregated Aβ, Aβ plaques and/or soluble fibers in the mammalian, particularly the human brain but, preferably, does not show any significant cross-reactivity with amyloid precursor protein (APP).

35. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1, which antibody or fragment thereof is capable of shifting the equilibrium between Aβ in its soluble and aggregated state towards its soluble form by disaggregating fibers to soluble poly- and monomeric forms by inducing a shift in conformation and binding and stabilizing the disaggregated and solubilized Aβ forms in the tissue and/or body fluids, particularly the brain.

36. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 35, which antibody or fragment thereof is capable of inducing a transition of the β-sheet conformation towards an α-helix and/or a random coil conformation, but particularly a random coil conformation, even more particularly a random coil conformation at a given location in the molecule, especially in the environment of Tyr 10 and Val12 of the Aβ protein, which leads to an increase

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of the random coil conformation at the expense of the β-sheet conformation and an improved solubilization of the preformed high molecular polymeric amyloid fibrils or filaments.

37. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 36, wherein the decrease of the β-sheet conformation amounts to at least 30%, particularly to at least 35%, and more particularly to at least 40% and more as compared to the respective preformed amyloid polymeric fibrils or filaments incubated in buffer (control).

38. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 35, which antibody or fragment thereof substantially binds to polymeric soluble Aβ protein species, and/or less aggregated soluble Aβ protein species and/or monomeric forms of soluble Aβ protein, thus modifying the peripheral clearing and catabolism of these Aβ protein species and reducing their toxic effects.

39. A method according to claim 23, wherein the humanized antibody or a fragment thereof binds aggregated amyloid beta outside the brain.

40. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1, which antibody or fragment thereof is capable of shifting the equilibrium between Aβ in its soluble and aggregated state towards its soluble form by disaggregating fibers to soluble poly- and monomeric forms.

41. A chimeric antibody or a fragment thereof, or a humanized antibody or a fragment thereof according to claim 1, which antibody or fragment thereof does not substantially bind Aβ in human tissue and/or body fluids, particularly the brain, but is capable of shifting the equilibrium between Aβ in its soluble and aggregated state towards its soluble form by disaggregating fibers to soluble poly- and monomeric forms by inducing a shift in conformation and binding and stabilizing the disaggregated and solubilized Aβ forms in the tissue and/or body fluids, particularly the brain.

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42. A humanized antibody or a fragment thereof according to claim 1, wherein said antibody or fragment thereof exhibits a binding affinity which is at least 5 times, 10 times, particularly at least 15 times, more particularly at least 20 times, but especially at least 100 times higher than the binding affinity of the mouse antibody.

43. A humanized antibody or a fragment thereof according to claim 1, wherein the affinity, specificity and stability of said antibody or fragment thereof has been modified by a change of the glycosylation profile.

44. The antibody of claim 1, wherein the amino acid modification results in a reduced effector function.

45. The antibody or fragment thereof of claim 44, wherein the Fc region amino acid modification comprises a change in one or more of amino acid positions 238, 239, 248, 249, 252, 254, 265, 268, 269, 270, 272, 278, 289, 292, 293, 294, 295, 296, 298, 301, 303, 322, 324, 327, 329, 333, 335, 338, 340, 373, 376, 382, 388, 389, 414, 416, 419, 434, 435, 437, 438 or 439.

46. The antibody or fragment thereof of claim 44, wherein the Fc region amino acid modification comprises a D265A amino acid mutation.

47. An antibody comprising at least one variable region as in SEQ ID NO: 27 or SEQ ID NO: 29, or a variant thereof.

48. A cell line expressing the antibody of claim 47.

49. A method for disaggregating preformed beta-amyloid fibers, comprising interacting an hC2 antibody with preformed beta-amyloid fibers.

50. A humanized antibody or a fragment thereof according to claim 1, wherein said antibody or fragment thereof protects neurons from Abeta-induced degradation.

51. A method of preventing Abeta-induced neuron degradation comprising treating neurons with an effective amount of a humanized antibody or a fragment thereof according to claim 1.

52. The antibody or fragment thereof of claim 1, wherein the variant Fc region is a variant IgG1 Fc region.

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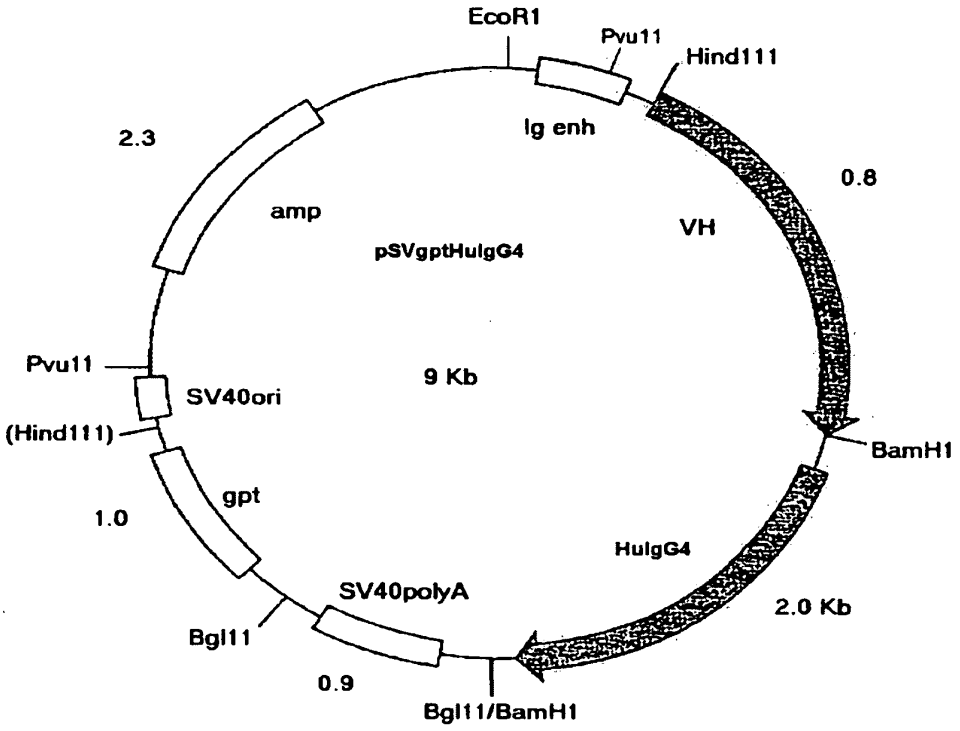


FIG 1
(example 2)

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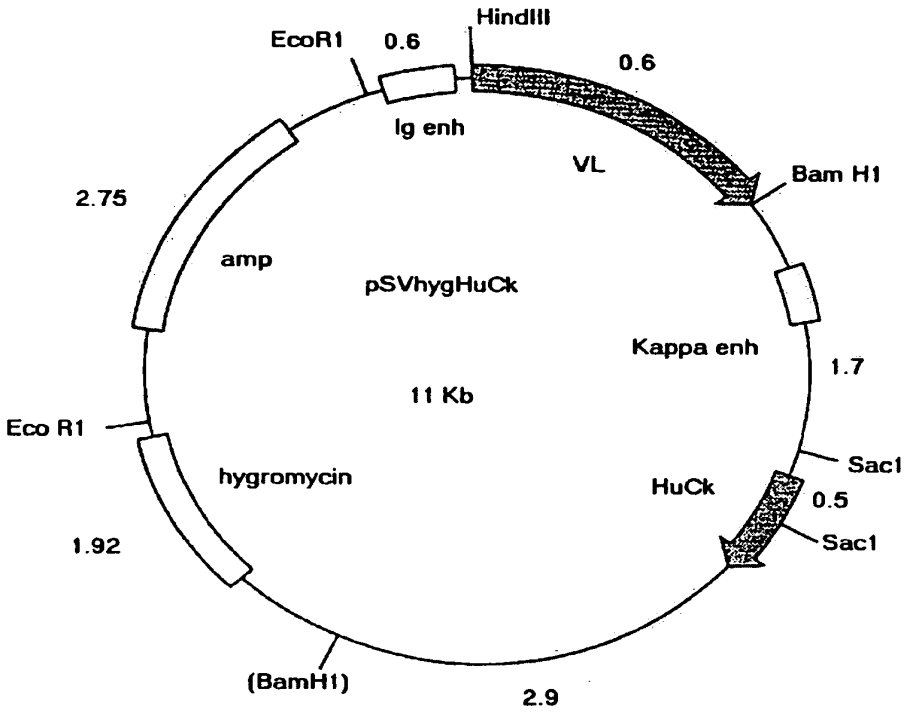


FIG 2(Example 2)

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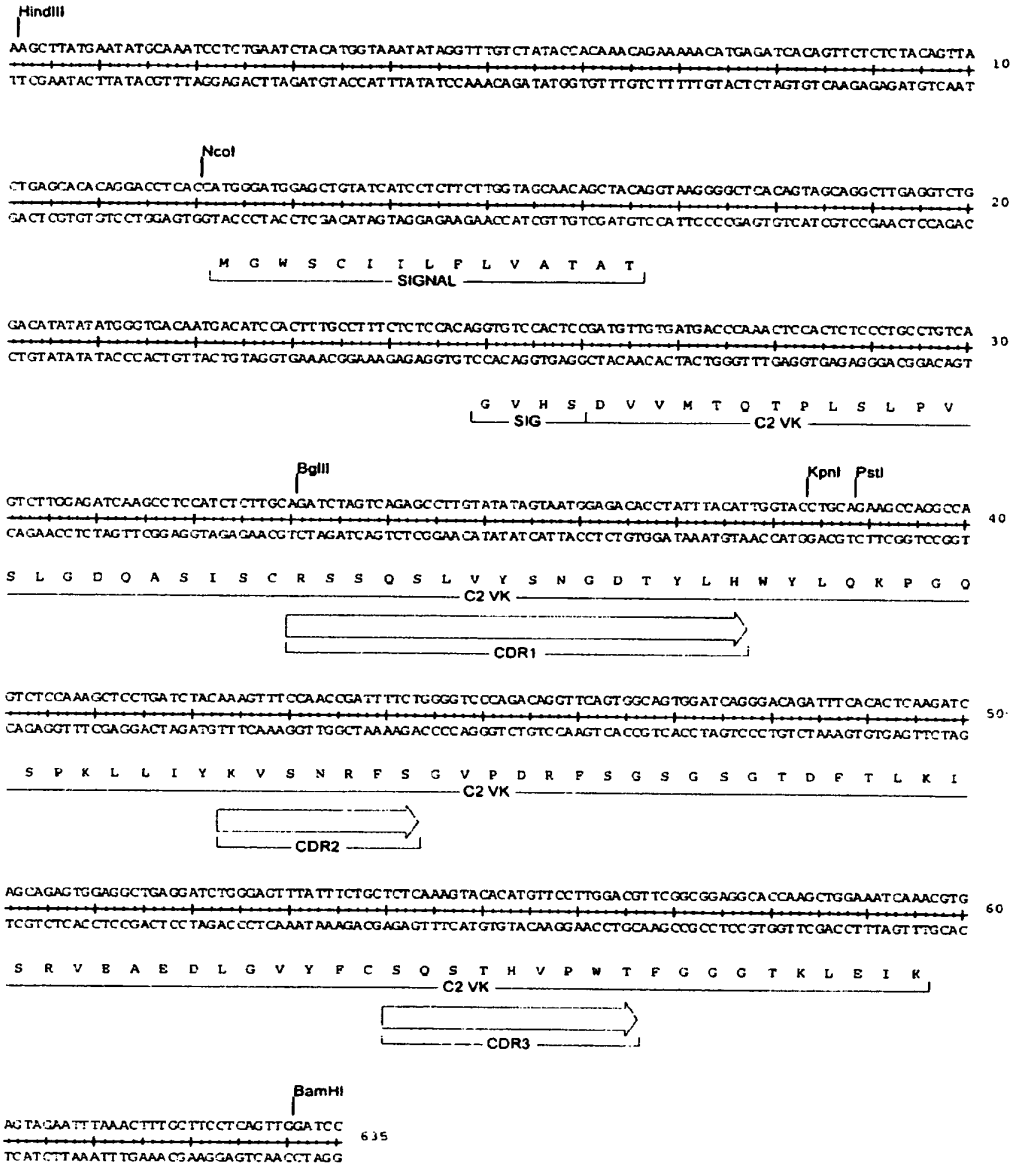


FIG 3
(Example 2)

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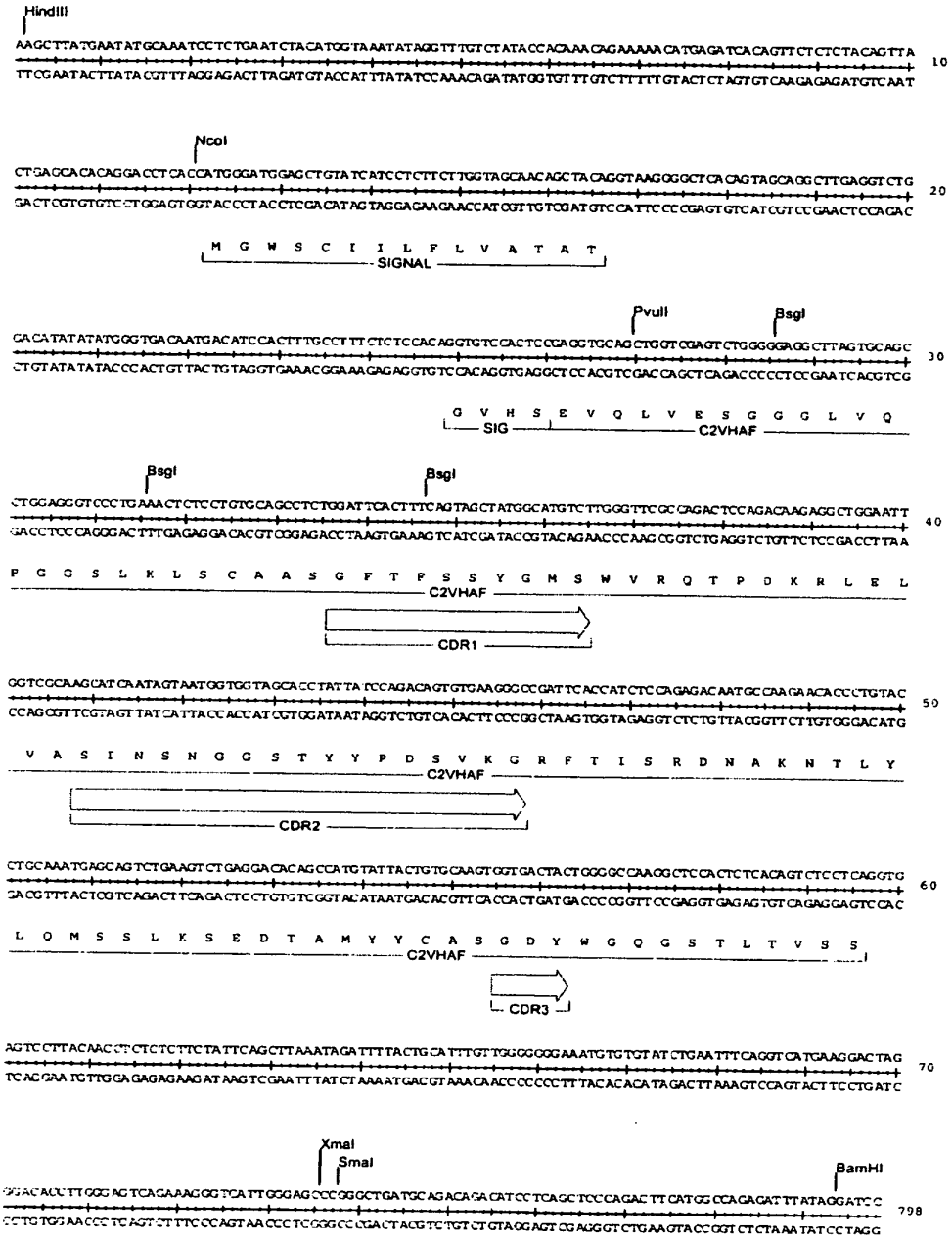


FIG 4

(Example 2)

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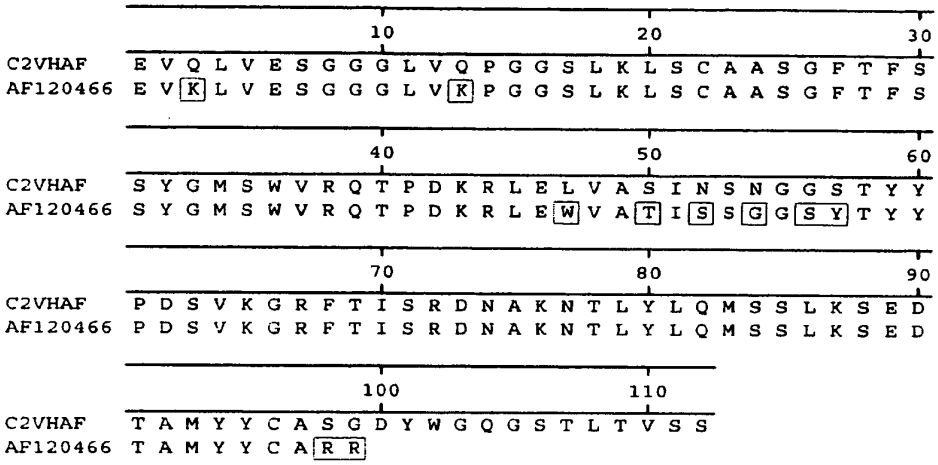


FIG 5
(Example 5.2)

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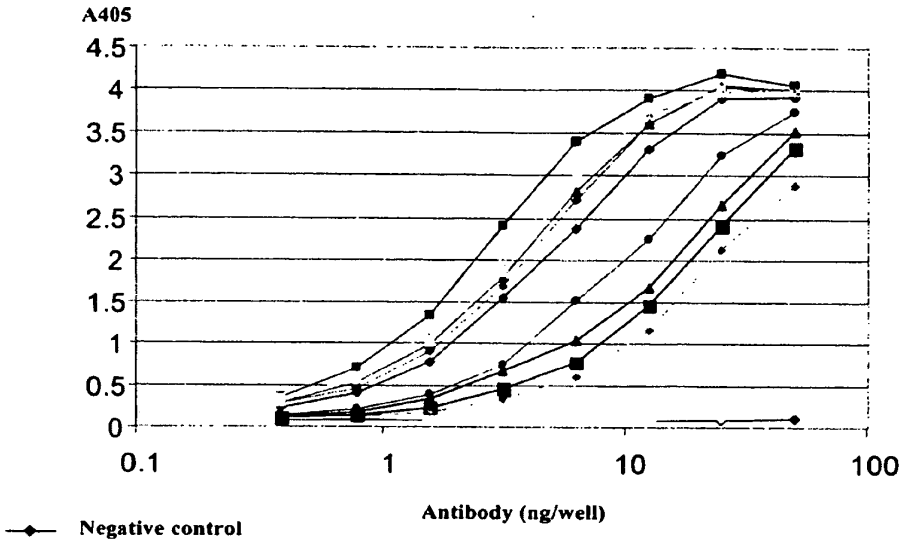


FIG 6
(Example 8)

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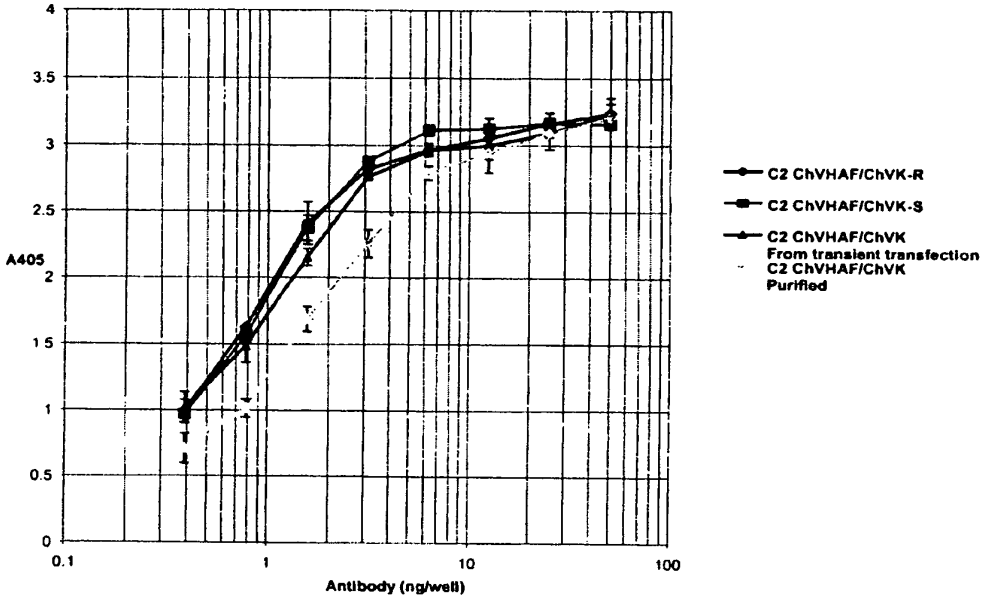


FIG 7
(Example 9)

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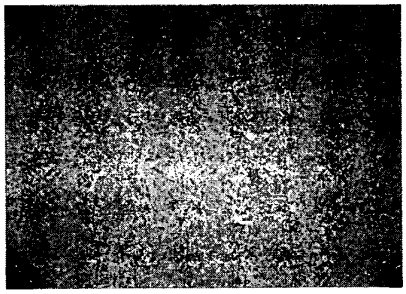
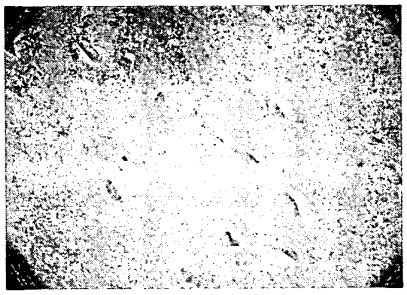
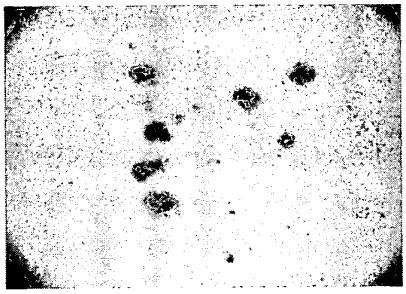
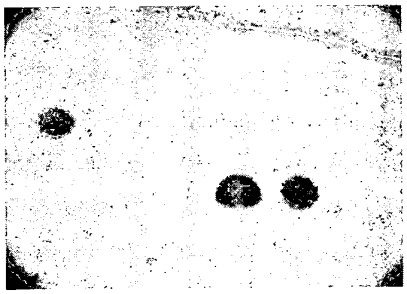
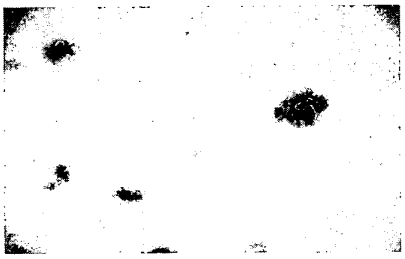
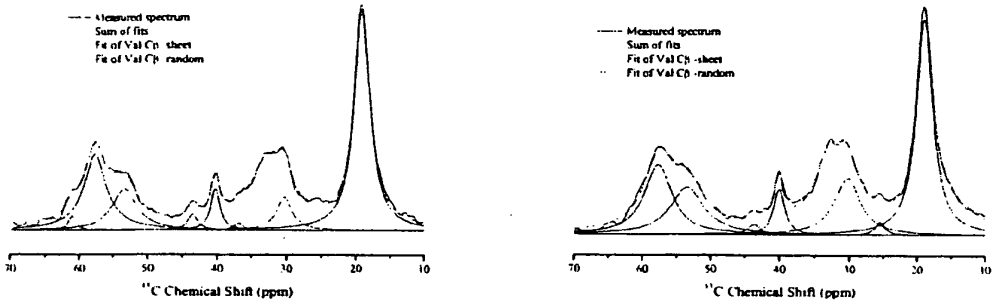
	C: Antibody hC2	A: Chimeric antibody
Control patient		
	Temporal neocortex region I, 40 x	CA4 (Cornu ammonis region 4), 40 x
AD patient		
	Temporal neocortex region I, 40 x	Temporal neocortex region I, 40 x
preAD patient		
	Temporal neocortex region I, 40 x	Temporal neocortex region I, 40 x

FIG. 8
(Example 11)

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A)



B)

Resonance	PBS			Mouse C2		
	δ ISO (ppm)	FWHH (Hz)	% Integral Intensity	δ ISO (ppm)	FWHH (Hz)	% Integral Intensity
Val Cβ -sheet	32.60	479	81.7	33.09	366	53 5
Val Cβ -random	30.27	200	18.3	30.27	340	46.5

A) Comparison of ^{13}C CPMAS spectra and fits for U-^{13}C Tyr10 and Val12 labelled amyloid β1-42 fibres incubated with PBS (left; served as control) or ACI-7-C2 (right) for 24 hrs and then lyophilised. The fits for the two conformations of Val12 Cβ are shown in green (sheet) and blue (random coil). The peak at c33 ppm corresponds to the beta sheet conformation of the fibres whilst that at 30 ppm is a result of random coil conformation.

B): Comparison of the fitted parameters for the two conformations of Val 12 Cβ. The fitted chemical shifts for the two conformations are quite similar but the integral intensities are very different, reflecting a reduction in the original beta sheet conformation by approx 35% (1-(53.5/81.7)). This is in very close agreement with the value we obtained from the fluorescence measurement.

FIG 9
(Example 12)

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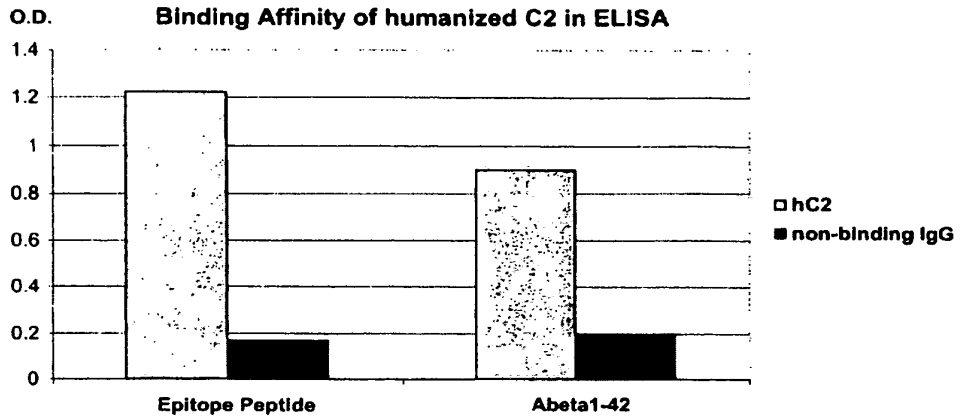


FIG 10
(Example 12)

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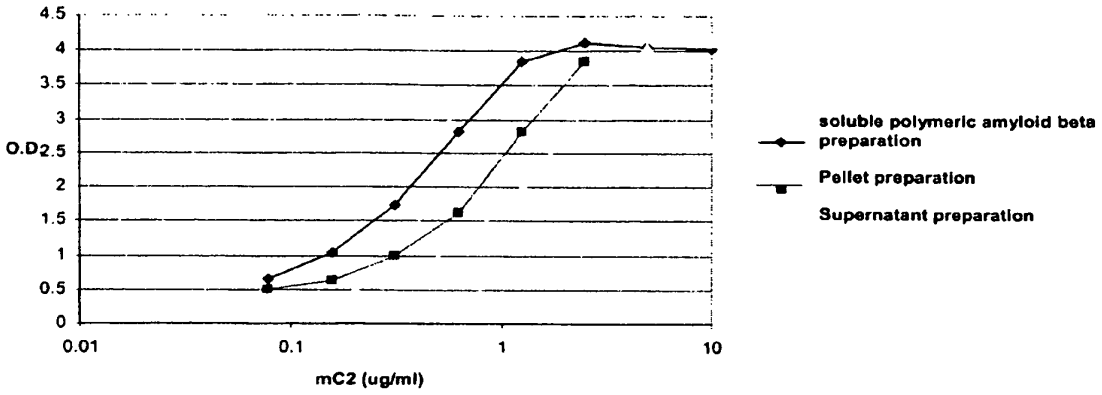


FIG 11
(Example 13)

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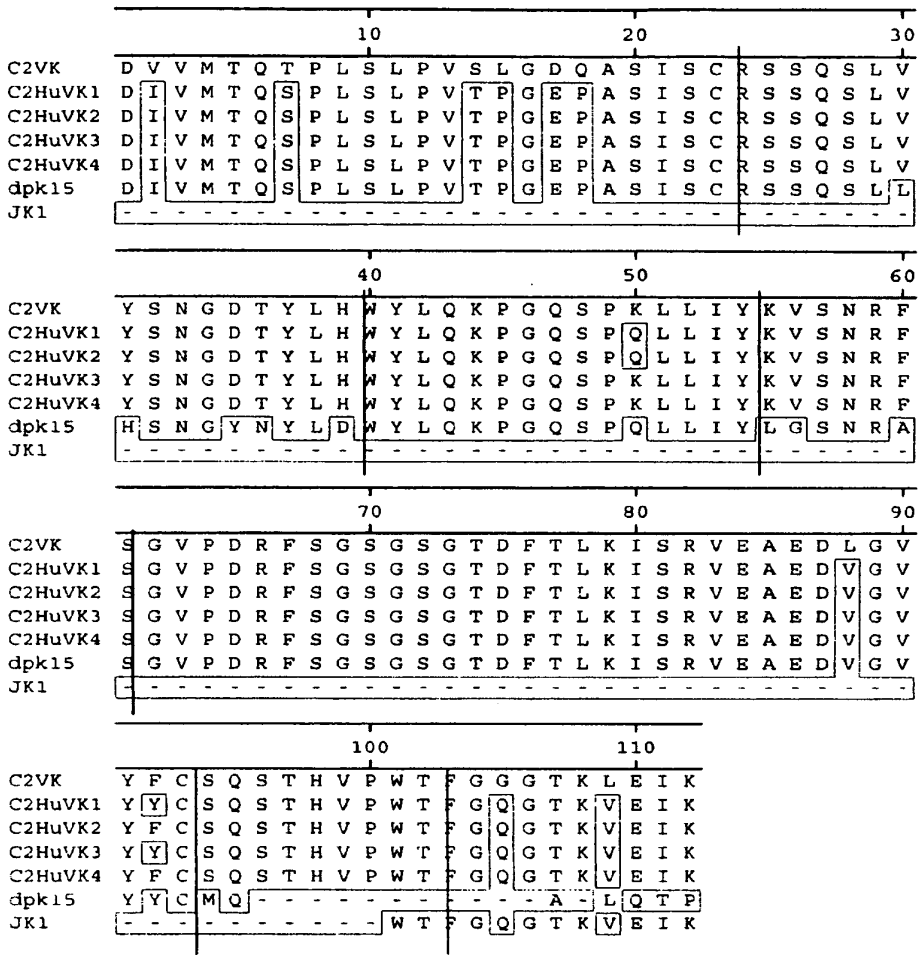


FIG 12.

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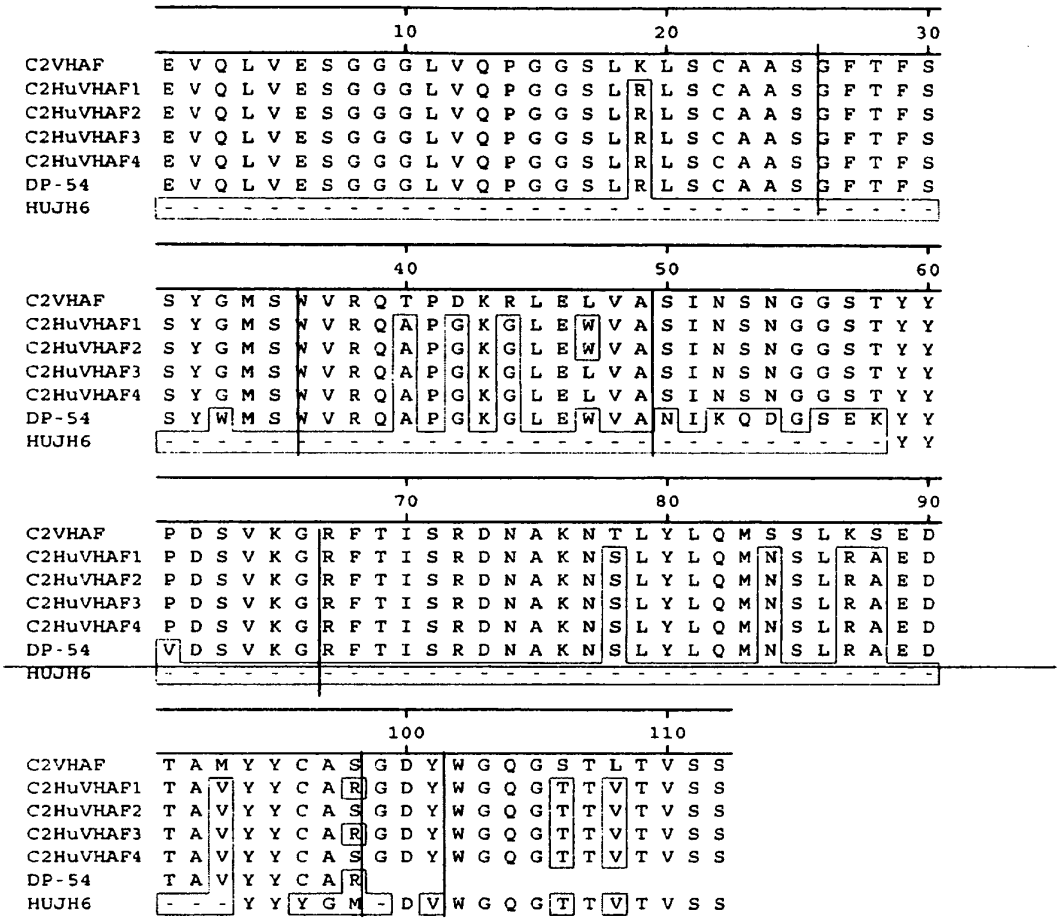


FIG 13.

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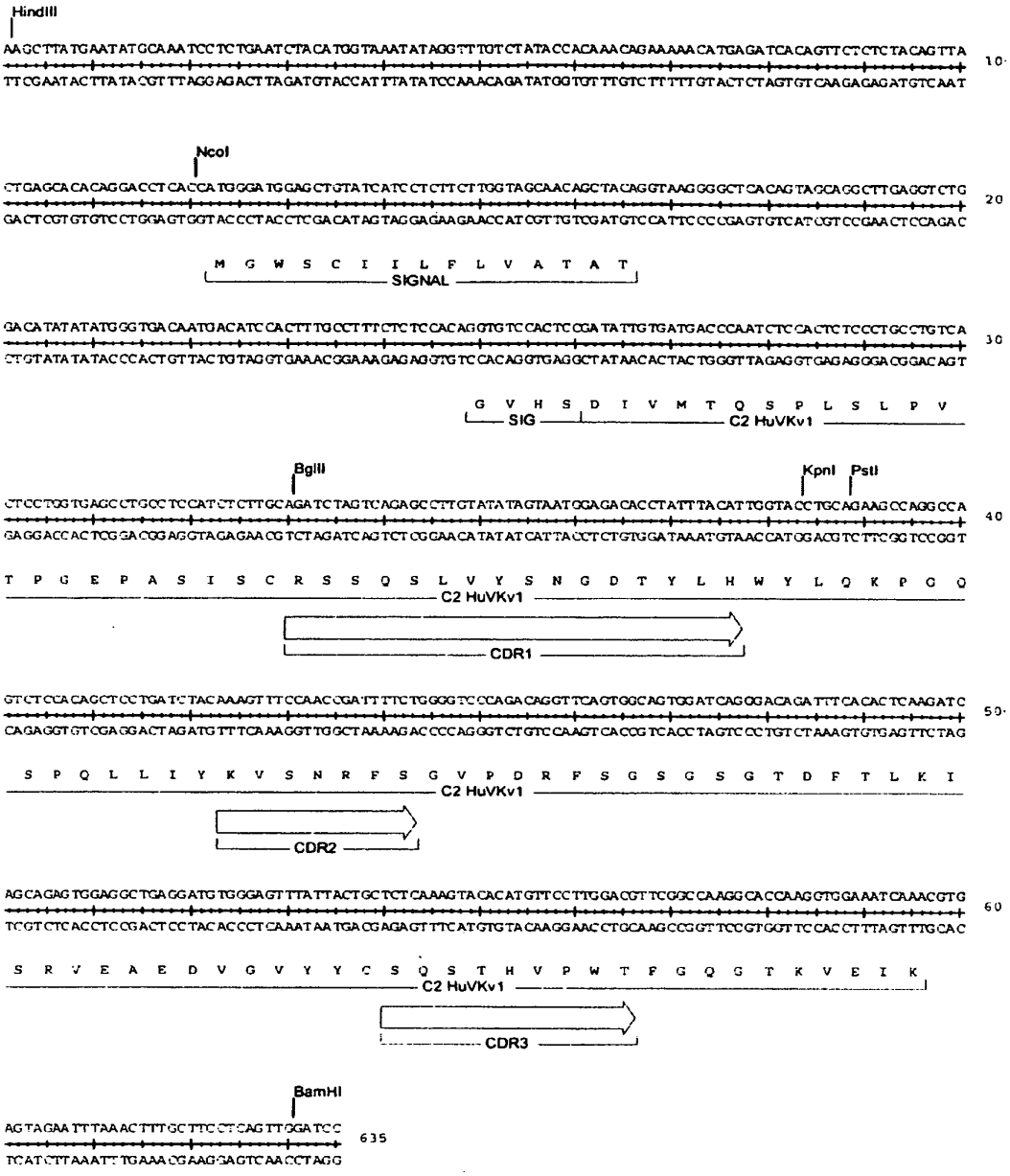


FIG 14

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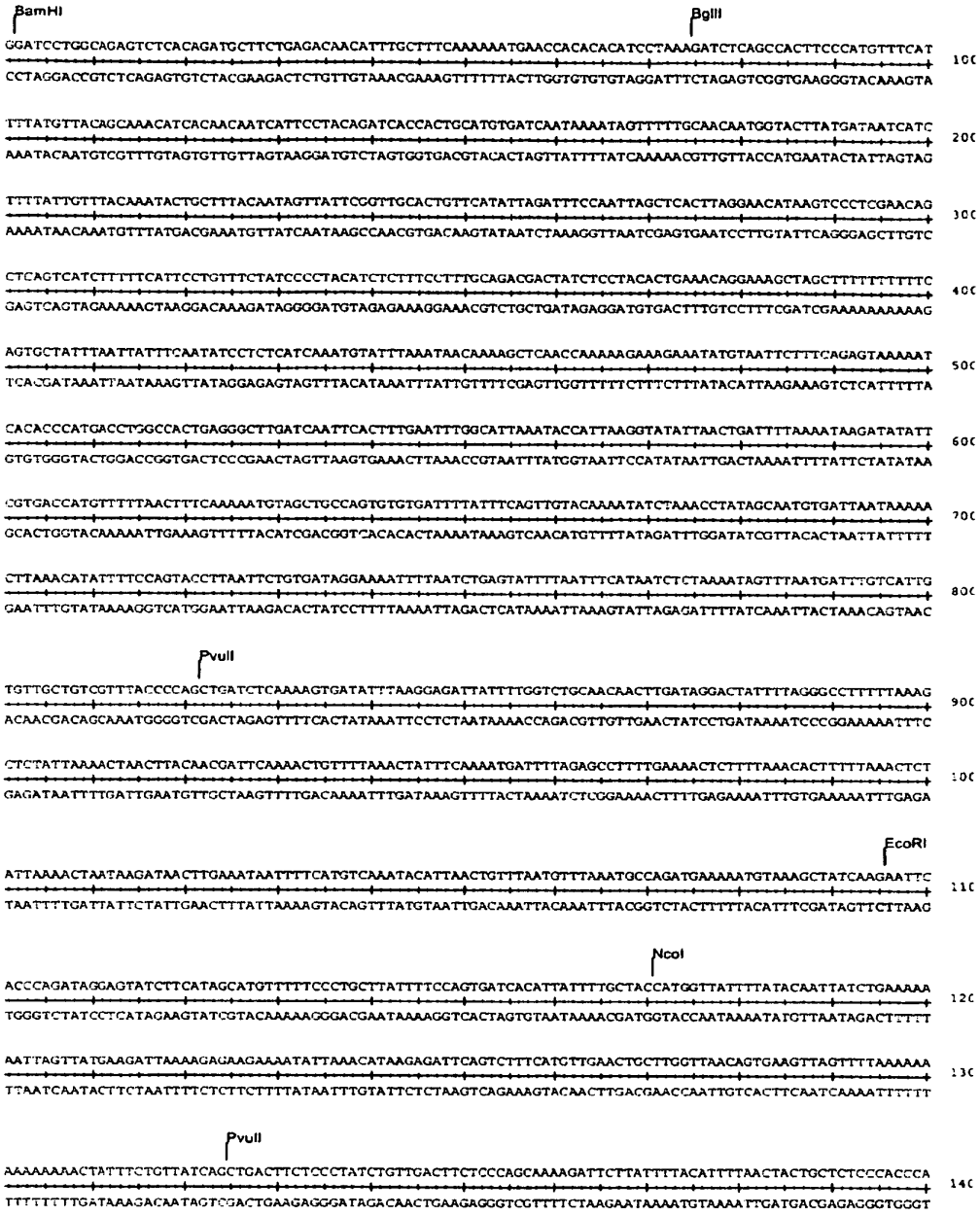


FIG 15-1

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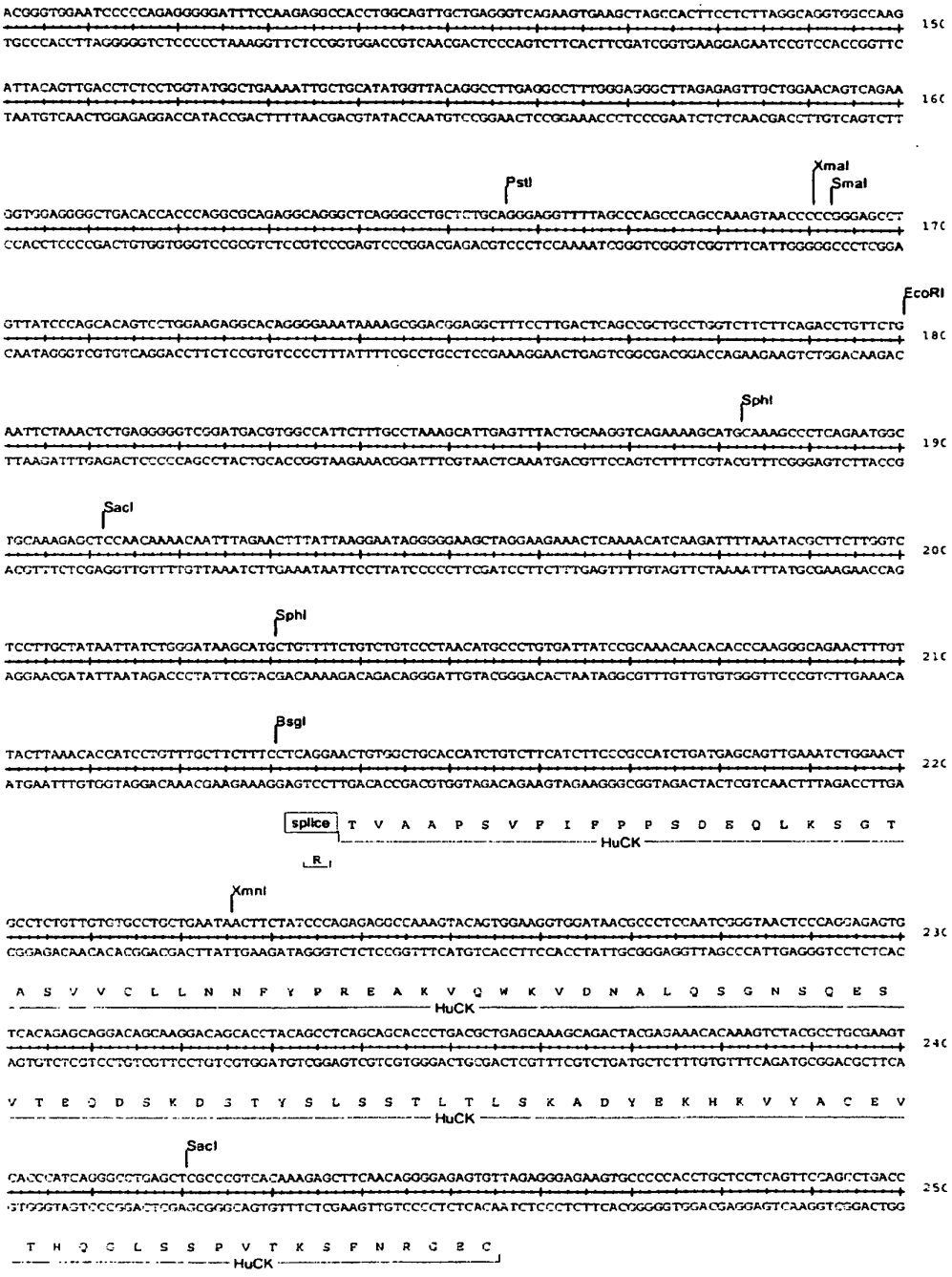


FIG 15-2

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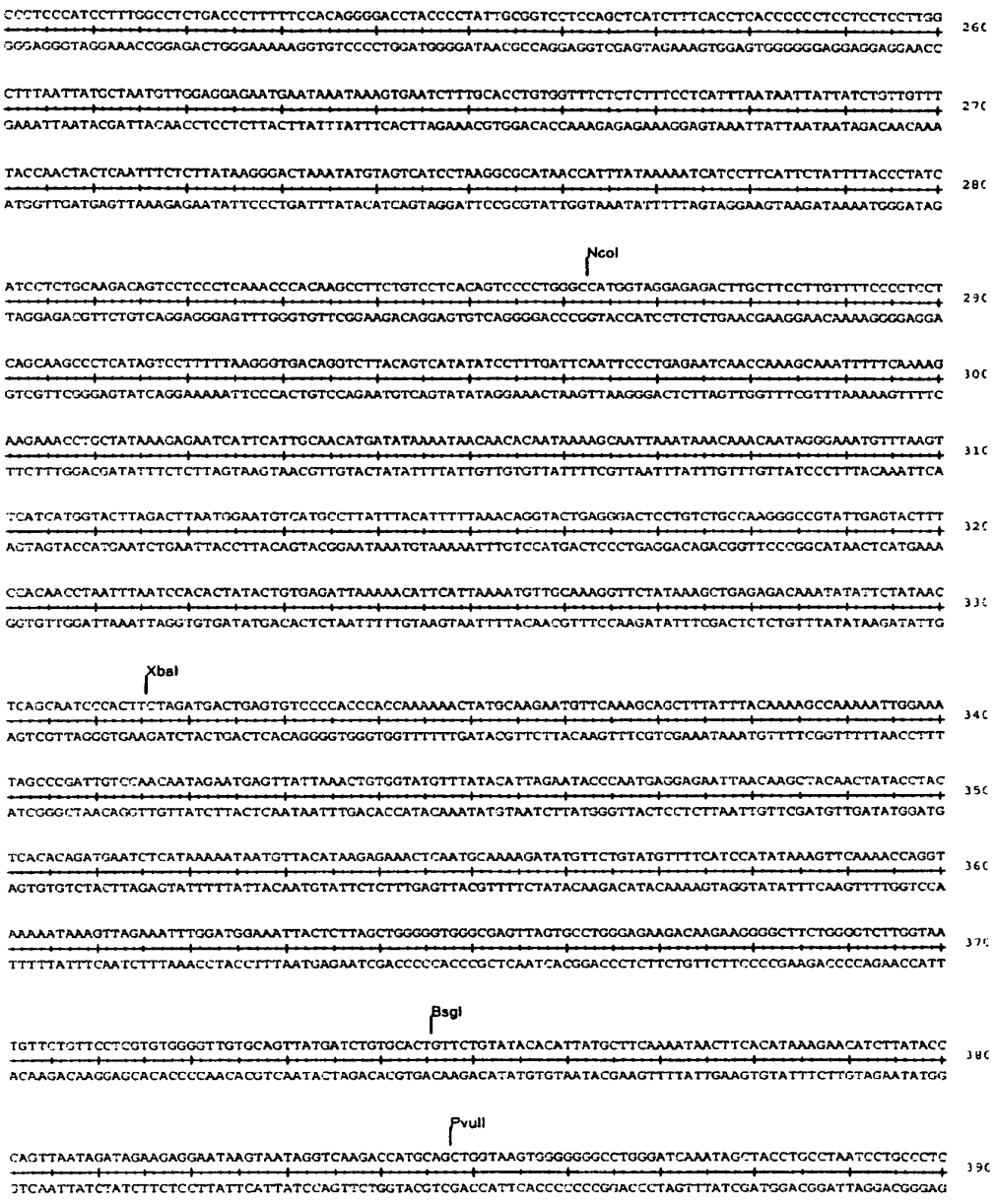


FIG 15-3

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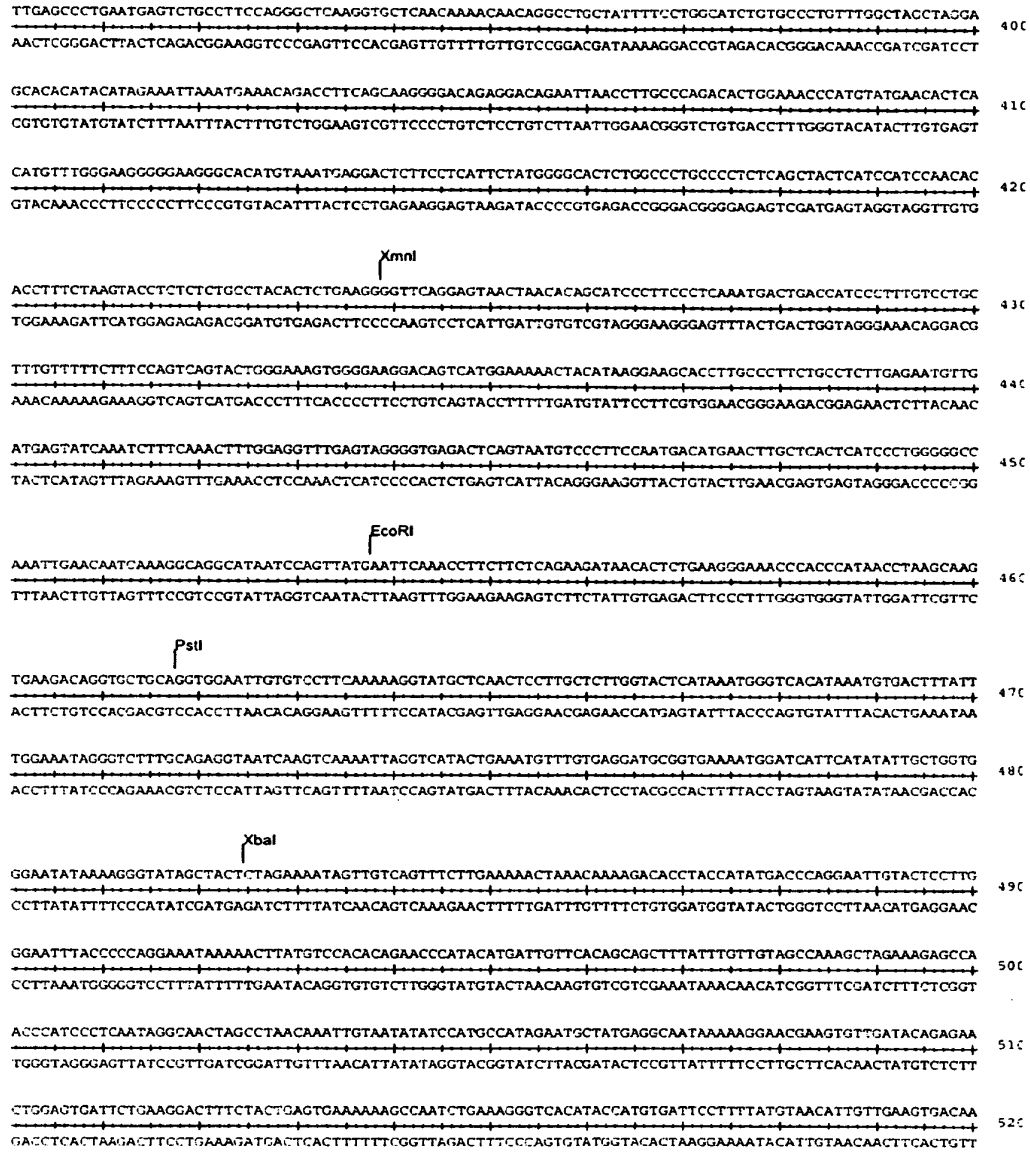


FIG 15-4

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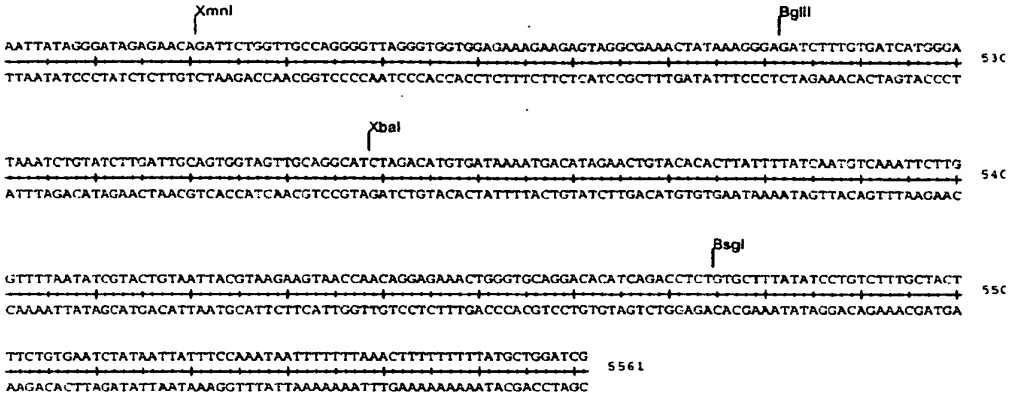


FIG 15-5

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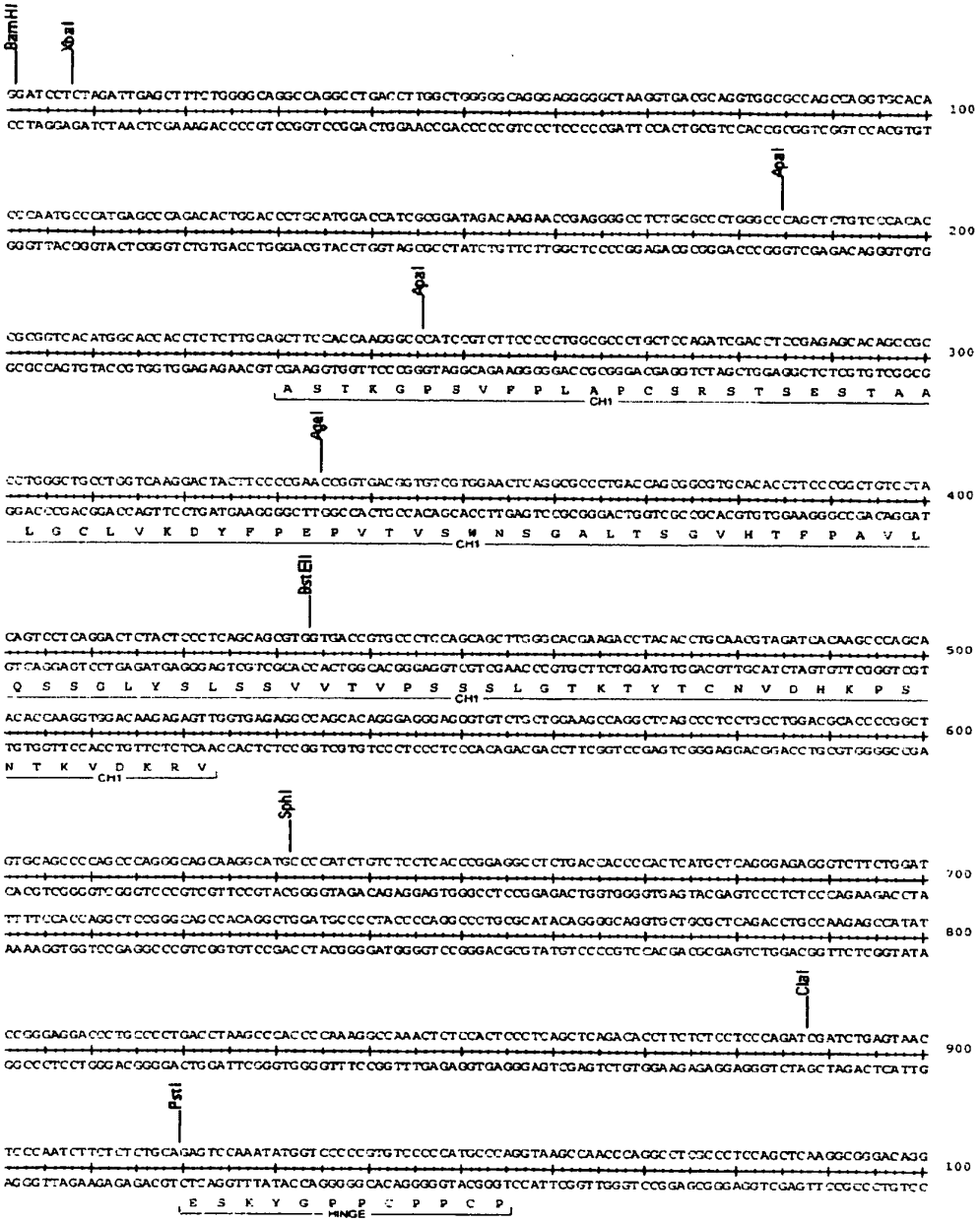


FIG 16 -1

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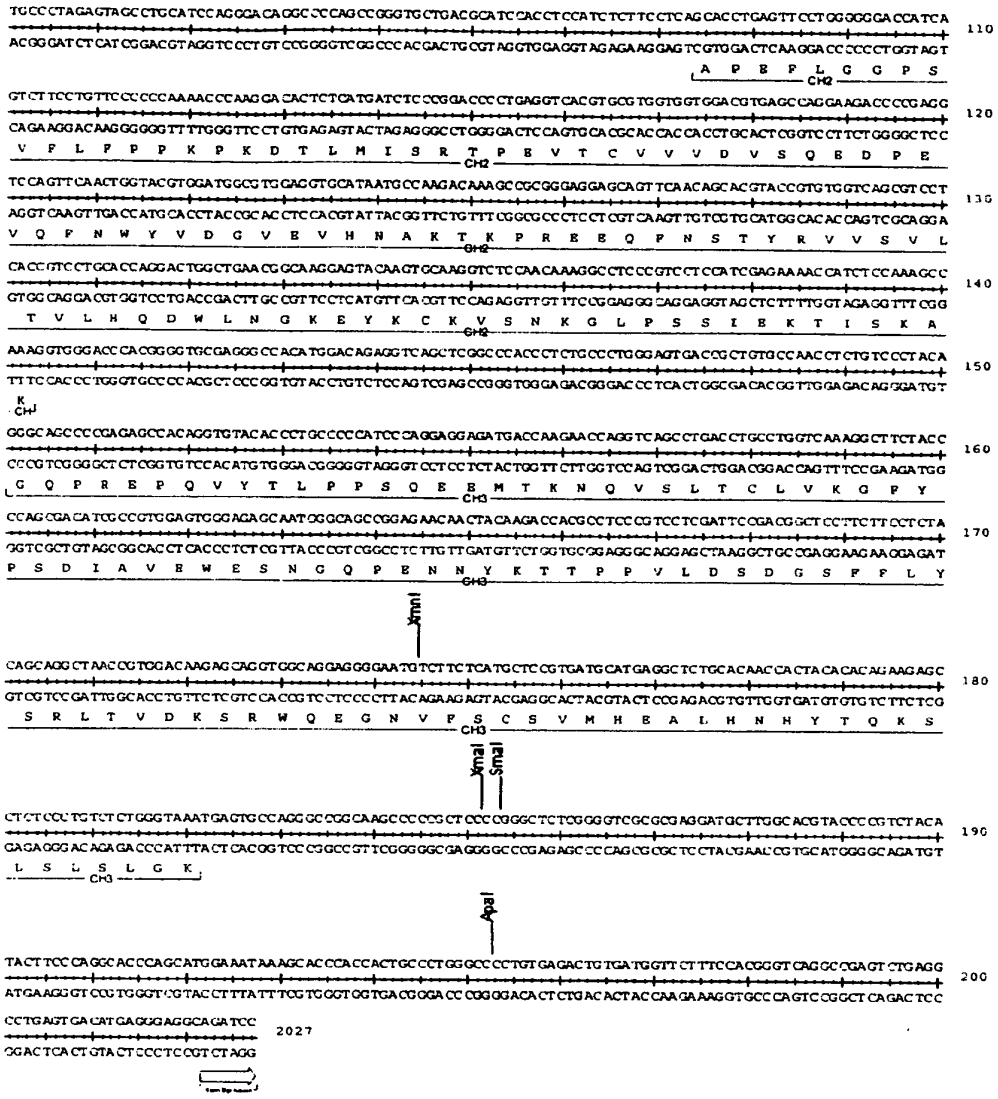


FIG 16 -2

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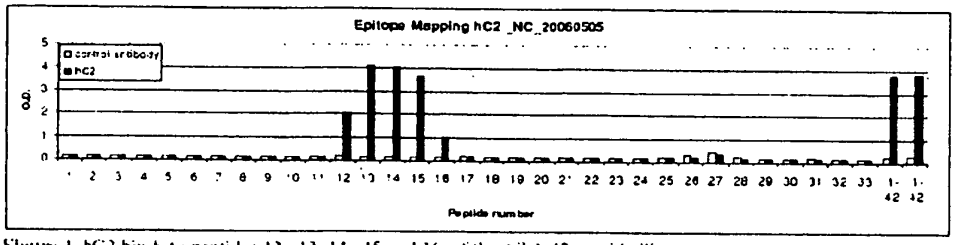


Figure 1. hC2 binds to peptides 12 , 13, 14 , 15 and 16 of the Aβ 1-42 peptide library.
Binding of hC2 to overlapping peptides of Aβ 1-42 was analyzed by ELISA. Binding to the complete Aβ 1-42 and binding of a non-binding chimeric antibody (control antibody) was used as positive and negative controls respectively. The peptide number corresponds to the amino acid in the Aβ 1-42 sequence on which the peptide starts. Results are expressed as OD.

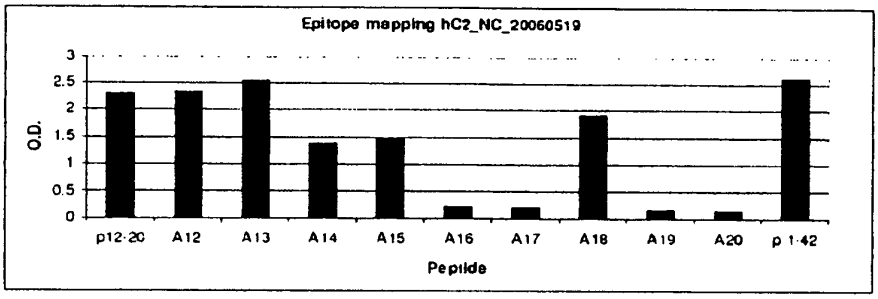


Figure 2. hC2 binding to Aβ 12-20 is completely dependent on aa 16,17,19 and 20 and partially dependent on aa 14,15 and 18.
Binding of hC2 to Aβ 12-20 and alanine substituted Aβ 12-20 was analyzed by ELISA. Binding to the complete Aβ 1-42 was used as positive control. The number corresponds to the aa that is substituted by alanine. Results are expressed as OD.

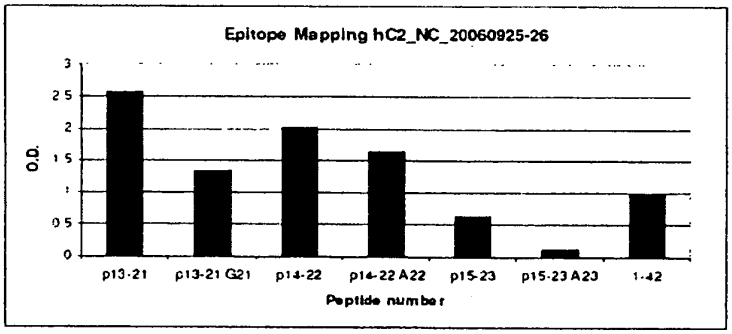


Figure 3. hC2 binding to Aβ 15-23 is dependent on aa 23 and partially on aa 21 and slightly dependent on aa 22
Binding of hC2 to Aβ 13-21, 14-22 or 15-23 and to 13-21G21, 14-22A22 or 15-23A23 was analyzed by ELISA. Binding to the complete Aβ 1-42 was used as positive control. Results are expressed as OD

Fig 17:

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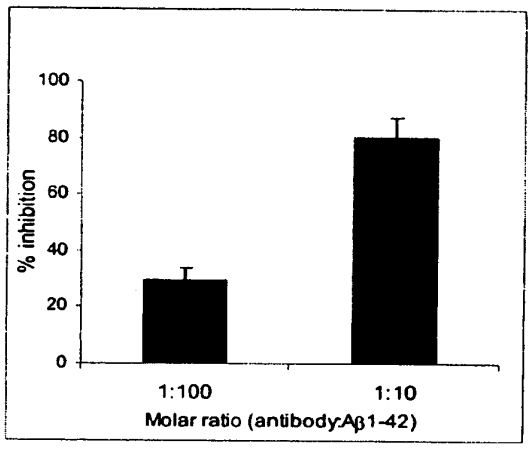


Fig 18

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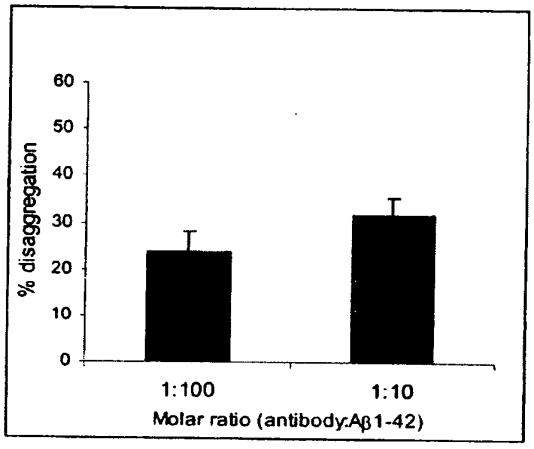


FIG 19

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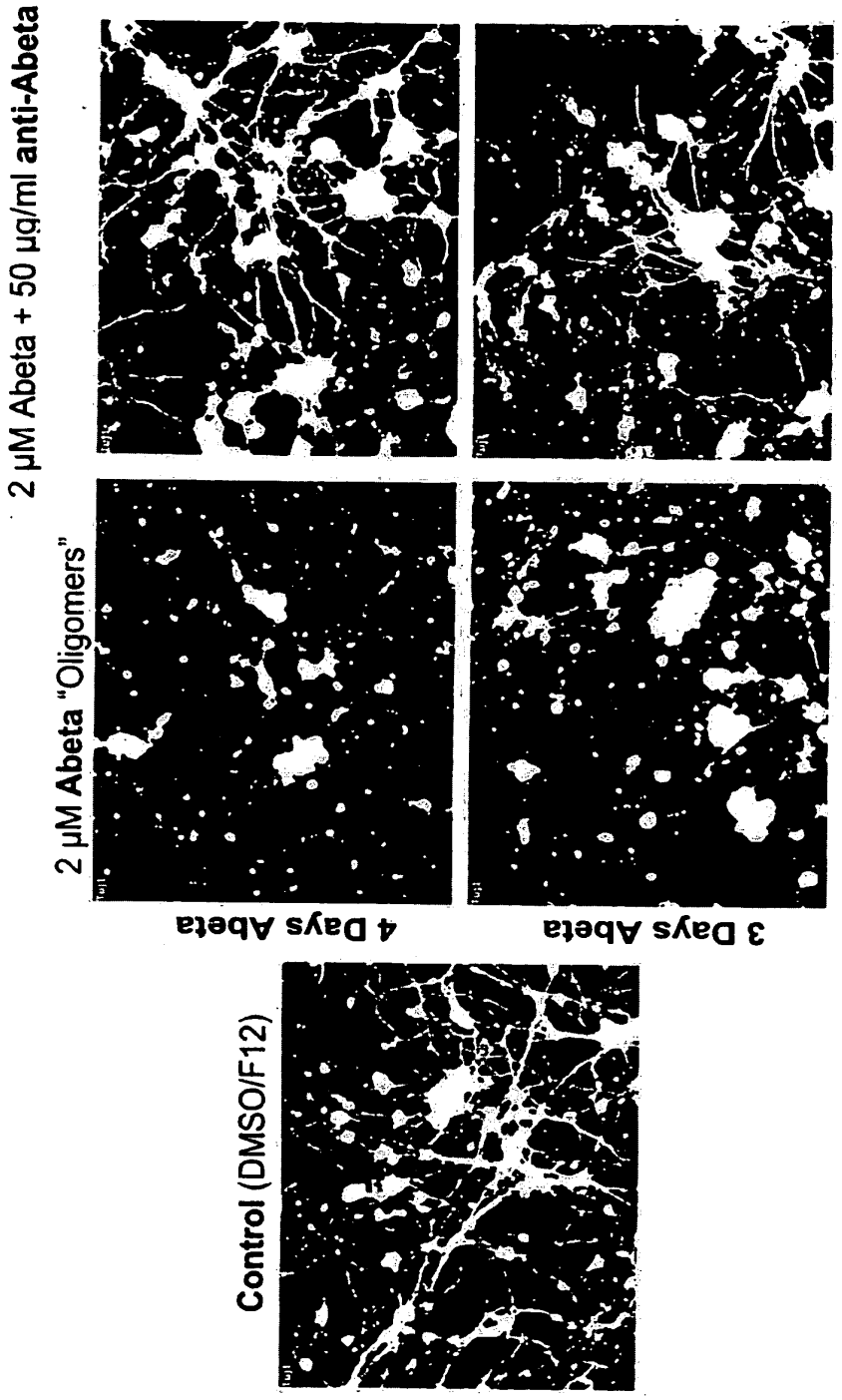


FIG 20

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/007318

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/395 A61P25/28 C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Cilensek, Zoran

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International application No
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International application No
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(54) **Title:** USE OF A CAMELID SINGLE-DOMAIN ANTIBODY FOR DETECTING AN OLIGOMERIC FORM OF AN AMYLOID BETA PEPTIDE AND ITS APPLICATIONS

(57) **Abstract:** Use of camelid single-domain antibodies for detecting an oligomeric form of the amyloid β peptide 42 and their therapeutic and diagnostic applications.

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USE OF A CAMELID SINGLE-DOMAIN ANTIBODY FOR DETECTING AN OLIGOMERIC FORM OF AN AMYLOID β PEPTIDE AND ITS APPLICATIONS

The invention relates to the use of camelid single-domain antibodies for detecting an oligomeric form of the amyloid β peptide 42 and their applications.

Alzheimer's disease (AD) is a progressive, irreversible brain disorder with no known cause or cure. Extracellular fibrillar deposits and senile plaques are prominent and universal AD features. The major component of these aggregates is a water-soluble 40 or 42 amino acid polypeptide called Amyloid β (Aβ). However, the initial focus on the water-insoluble fibrillar amyloid as the central structure in AD pathology has evolved during the last 10 years. This was due to several outstanding discoveries such as the finding of a water-soluble fraction of oligomeric Aβ in the human brain (Kuo *et al.*, 1996). These isolated soluble oligomers were toxic to neurons in culture. The presence and toxicity of oligomeric Aβ was then confirmed and the name of ADDLs (Aβ-derived diffusible ligands) was proposed for these structures (Lambert *et al.*, 1998). Depending on conditions, ADDL compositions can contain predominantly trimers-hexamers, with larger structures of up to 24-mers. ADDLs show important regionally selective neurotoxicity, sparing neurons in the cerebellum while selectively killing neurons in hippocampal CA1 region and entorhinal cortex (Klein *et al.*, 2001). Moreover, oligomers are able to inhibit hippocampal long-term potentiation (LTP) in rats *in vivo* (Walsh *et al.*, 2002) and in hippocampal slices (Wang *et al.*, 2002; Wang *et al.*, 2004). Recently, it has been showed that cognitive deficits are directly attributable to low amounts of soluble oligomeric forms of Amyloid β; trimers and at a lesser extent, dimers and tetramers being particularly active (Cleary *et al.*, 2005; Townsend *et al.*, 2006).

Different models have been proposed for the formation of Aβ fibrils. A simple model proposed by Bitan *et al.* (2003) illustrates how Aβ42 may assemble. Monomers rapidly oligomerize into paranuclei that in turn associate to form large oligomers and protofibrils. Monomers, paranuclei (pentamers, hexamers), and oligomers are predominantly unstructured, but do contain some β-strand and α-helix elements. Protofibril formation involves substantial conformational rearrangements, during which unstructured, α-helix and β-strand elements transform into predomi-

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nantly β sheet/β-turn structures. The final step in the pathway is protofibril maturation into fibrils, a process that appears to be irreversible, at least kinetically (Lomakin *et al.*, 1997).

The need for accurate diagnosis of disorder mediated by amyloid β peptide oligomers such as AD or the Down syndrome is increasingly important as therapeutics become available in some cases. Therefore, a reliable assay for Aβ oligomers would be extremely helpful. However these assays are impaired because monoclonal and polyclonal antibodies, generated by immunization with Aβ oligomers, raised against oligomers also recognize fibrils (Lacor *et al.*, 2004; Lambert *et al.*, 2001; Lambert *et al.*, 2007). These antibodies show, in AD brain sections, the expected AD type immunoreactivity and also a pericellular immunoreactivity (Lambert *et al.*, 2007; Lacor *et al.*, 2004; Gong *et al.*, 2003). A monoclonal antibody raised against oligomers was also obtained by Lee *et al.* (2006). It recognizes oligomers, fibrils and mature senile plaques on AD brain sections. Kayed *et al.* (2003) prepared polyclonal rabbit antibodies that specifically recognize a conformation specific of Aβ high-MW oligomers distinct from that of soluble monomers, low-MW oligomers, and fibrils. A way to discriminate between the different conformations of Aβ is to use alternative "binders" which could recognize non conventional epitopes.

A significant proportion of camelid antibodies are single-domain antibodies, which interact with the antigen via a single heavy-chain binding domain devoid of light chain. This domain is referred to as "VHH" or "VHH antibody". Recombinant VHH is the minimal-sized, intact antigen-binding domain. The absence of VL domain allows the VHHs to attain a higher structural flexibility than that of VH domains associated with VLs. Furthermore, the complementarity determining regions (CDRs) of VHHs, and especially CDR3, are statistically longer than those of conventional VH-VL antibodies (Muyldermans *et al.*, 2001). Small size and increased plasticity appear to endow VHHs with unique potentialities: for instance, several VHHs are capable of inhibiting enzymatic activity by interacting with the active site cavity of enzymes such as α-amylase, carbonic anhydrase and hen egg lysozyme (Desmyter *et al.*, 1996; Desmyter *et al.*, 2002; Transue *et al.*, 1998; Lauwereys *et al.*, 1998). These features may allow camelid VHHs to recognize other unique epitopes that are poorly immunogenic for conventional antibodies.

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International Application No. WO 2004/044204 describes variable fragments of camelid single-chain antibodies (VHH antibodies) capable to specifically bind the amyloid β peptide 42. Among these VHH antibodies, one particular antibody, referred to as VHH V31-1, has been shown to specifically recognize the carboxy terminal end of Aβ42 peptide (Aβ42) in its fibrillar form and intraneuronal Aβ42 deposits.

The Inventors have now found that contrary to what was described in said International Application No. WO 2004/044204, VHH V31-1 antibody does not recognize Aβ42 in its water-insoluble fibrillar form but specifically recognizes water-soluble low-molecular oligomers of Aβ42.

Furthermore the Inventors have also found that unexpectedly said VHH V31-1 antibody inhibits fibrillogenesis, *i.e.* Aβ fibril formation, in spite of the fact that it specifically binds the C-terminus of Aβ42. Indeed, previous studies have indicated that antibodies specific of regions 1-6 (Solomon, 2002; Legleiter *et al.*, 2004) and 17-20 (Liu *et al.*, 2004; Legleiter *et al.*, 2004) of Aβ42 inhibited *in vitro* aggregation and cytotoxicity of Aβ, while antibodies specific of the C-terminus did not inhibit aggregation (Bard *et al.*, 2000; Liu *et al.*, 2004). The regions 17-20 and the C-terminus play critical roles in the aggregation (Ma and Nussinov, 2002; Balbach *et al.*, 2000; Halverson *et al.*, 1990; Jarrett *et al.*, 1993 and Hilbich *et al.*, 1991), which may explain why antibodies specific of the central region of Aβ inhibited aggregation. However there is no clear explanation why previously reported C-terminus specific antibodies had no effect on the aggregation of Aβ.

Therefore, in a first aspect, the present invention relates to the use of a VHH antibody of camelid binding an epitope located at the C-terminal end of the amyloid β peptide 42, for detecting *in vitro* in an appropriate biological sample or tissue, or *in vivo* in an organ (for instance the brain), an oligomeric form of the amyloid β peptide 42.

A VHH antibody of camelid (camel, dromedary, llama, alpaca,...) refers usually to a variable fragment of a camelid single-chain antibody (See Nguyen *et al.*, 2001; Muyldermans, 2001), and also comprises according to the present invention:

- an isolated VHH antibody of camelid,

- a recombinant VHH antibody of camelid, or

- a synthetic VHH antibody of camelid.

In a preferred embodiment, the VHH antibody of the present invention is a variable fragment of a camelid single-chain antibody having the amino acid sequence of SEQ ID NO: 1. This VHH antibody has been described in International Application No. WO 2004/044204. A host cell expressing VHH V31-1 is available at the Collection Nationale de Cultures de Microorganismes (CNCM), 28 rue du Dr. Roux, 75724 Paris Cedex 15, France, under the number I-2936.

As used herein, an "amyloid β peptide" refers to a peptide generated from amyloid precursor protein (APP) by β- and γ-secretase-mediated cleavage. Preferably, the amyloid β peptide is the amyloid β peptide 42 (Aβ42) having the amino acid sequence of SEQ ID NO: 2.

As used herein, the term "oligomer" refers typically to the initially formed, metastable multimer in an amyloid formation reaction (Kodali *et al.*, 2007). Some protofibrils might also be considered oligomers.

In a preferred embodiment of the invention, the oligomeric form (oligomer) of said amyloid β peptide 42 is selected from the group consisting of a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, 7-mer, 8-mer, 9-mer, 10-mer, 11-mer, 12–mer, 13-mer, 14-mer, 15-mer, 16-mer, 17-mer, 18-mer, 19-mer, 20-mer, 21-mer, 22-mer, 23-mer, 24-mer, preferably a dimer, trimer, tetramer or dodecamer (12-mer), and more preferably trimer and tetramer.

The characterization of the binding of a camelid VHH antibody according to the present invention to an epitope located at the C-terminal end of the amyloid β peptide 42 can be performed by an ELISA-based binding assay as described in Example 2 below. Advantageously, the dissociation constant of said VHH antibody is about or less than 2. 10^{-8} M. Dissociation constant measurements may be made using methods known to those skilled in the art, including using the method described in Friguet *et al.* (1985).

The term "C-terminal end of the amyloid β peptide 42" as used herein, refers to the amino acids 25 to 42, particularly to the amino acids 29 to 42 of the amyloid β peptide of SEQ ID NO:2.

In a preferred embodiment of the invention, the epitope consists of a peptide selected from the group consisting of the peptide Aβ 29-40 having the amino acid sequence of SEQ ID NO: 9, the peptide Aβ 33-42 having the amino acid sequence of SEQ ID NO: 10 and the peptide Aβ 35-42 having the amino acid sequence of SEQ ID NO: 11 in the annexed listing of sequences.

The term "detecting" means assessing the presence or absence of an oligomeric form of the amyloid β peptide 42 as defined here above, *in vitro* or *ex vivo* in an appropriate biological sample or tissue (obtained for example by brain biopsy) or *in vivo* in an organ, for instance the brain. This process involves the ability of said VHH antibody (of SEQ ID NO: 1) to bind an oligomeric form of the amyloid β peptide 42.

The detection can be performed by any method known in the art *in vitro* in an appropriate biological sample or tissue or *in vivo* in an organ (for instance the brain). These methods include an appropriate immunochemical technique such as:

- ELISA, EIA, RIA, immunofluorescence, immunocytochemical, immunohistochemical,

- immunoblot analysis performed in a biological sample or tissue (*e.g.*, blood, serum, urine, cerebrospinal fluid) from a subject,

- and also brain imaging.

By way of example, this may be accomplished by linking to said VHH antibody a detectable label that can be visualized or measured, or by using ligands (*e.g.*, an antibody linked to a detectable label) that specifically bind to said VHH antibody.

The level of binding is preferably detected quantitatively.

Advantageously, the detection of the presence of an oligomeric form of the amyloid β peptide 42 as described above is a prognostic marker of appearance of a disorder mediated by amyloid β peptide oligomers, preferably a neurodegenerative disorder such as Alzheimer's disease or the Down syndrome.

In a preferred embodiment of the invention, the detectable label is selected from the group consisting of:

- an enzyme, such as horseradish peroxidise, alkaline phosphatase, glucose-6-phosphatase, beta-galactosidase;

- a fluorophore, such as green fluorescent protein (GFP), blue fluorescent dyes excited at wavelengths in the ltraviolet (UV) part of the spectrum (*e.g.* AMCA (7-amino-4-methylcoumarin-3-acetic acid); Alexa Fluor 350), green fluorescent dyes excited by blue light (*e.g.* FITC, Cy2, Alexa Fluor 488), red fluorescent dyes excited by green light (*e.g.* rhodamines, Texas Red, Cy3, Alexa Fluor dyes 546, 564 and 594), or dyes excited with far-red light (*e.g.* Cy5) to be visualized with electronic detectors (CCD cameras, photomultipliers);

- a heavy metal chelate, such as europium, lanthanum or yttrium;

- a radioisotope, such as [^{18}F]fluorodeoxyglucose or ^{11}C-, ^{125}I-, ^{131}I-, ^{3}H-, ^{14}C-, ^{99}Tc- and ^{35}S- labelled compounds.

- a biotin that can be detected using labeled avidin.

In a second aspect, the present invention provides a method for determining *in vitro* in an appropriate biological sample or a tissue, or *in vivo* in an organ (for instance the brain), an early-stage of a disorder mediated by amyloid β peptide oligomers, preferably by Aβ42 oligomers, (for instance a neurodegenerative disorder such as Alzheimer's disease or the Down Syndrome), comprising a step of detecting, in a subject (a mammal, preferably a human), an oligomeric form of an amyloid β peptide 42 as defined here above, by contacting said appropriate biological sample, tissue or organ with a VHH antibody as defined above, preferably the VHH antibody of SEQ ID NO: 1, preferably labelled with a detectable label as defined above.

According to another aspect, the present invention relates to a method of diagnostic of a disorder mediated by amyloid β peptide oligomers, preferably by Aβ42 oligomers as defined above, in a subject (a mammal, preferably a human) comprising the steps of:

a) contacting an appropriate biological sample, tissue or organ, with a VHH antibody of camelid binding an epitope located at the C-terminal end of an amyloid β peptide 42 as defined above, preferably the VHH antibody having the amino acid sequence of SEQ ID NO: 1, and

b) detecting the binding of said VHH antibody to said biological sample, tissue or organ, a binding constituting a marker of the presence of said disorder (for instance Alzheimer's disease or the Down Syndrome).

According to the invention, said contacting step may be performed *in vitro*, *ex vivo* or *in vivo*.

When the contacting step is performed *in vivo*, the organ is preferably the brain.

The present invention also relates to a method of diagnostic of a disorder mediated by amyloid β peptide oligomers, preferably by Aβ42 oligomers, in a subject (a mammal, preferably a human) comprising the steps of:

a) contacting *in vitro* or *ex vivo* an appropriate biological sample or tissue, or *in vivo* (for instance the brain), with a VHH antibody of camelid binding an epitope located at the C-terminal end of an amyloid β peptide 42 as defined above, preferably the VHH antibody having the amino acid sequence of SEQ ID NO: 1,

b) determining the amount of amyloid β peptide 42 oligomers in said biological sample, tissue or brain, and

c) comparing the amount determined in step (b) with a standard, a difference in amount constituting a marker of the presence of said disorder (for instance Alzheimer's disease or Down Syndrome).

As used herein, the term "standard" refers to the amount of amyloid β peptide 42 oligomers in said appropriate biological or in the brain, which has been determined in a large population of subjects not suffering from a disorder mediated by amyloid β peptide oligomers.

In another aspect, the present invention relates to the use of a VHH antibody of camelid binding an epitope located at the C-terminal end of an amyloid β peptide 42 as defined above, preferably the VHH antibody of camelid having the amino acid sequence of SEQ ID NO: 1 for the preparation of a medicament for treating or preventing a disorder mediated by amyloid β peptide oligomers, preferably by amyloid β peptide 42 oligomers.

The term "treating" includes the administration of said VHH antibody to a patient who has said disorder, a symptom of said disorder or a predisposition toward said disorder, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disorder, the symptoms of the disorder, or the predisposition toward disorder.

The term "preventing" means that the progression of said disorder is reduced and/or eliminated, or that the onset of said disorder is delayed or eliminated.

In a preferred embodiment, said medicament is administered to a subject so as to inhibit the formation of amyloid β peptide fibrils, preferably Aβ42 fibrils, and/or to slow down the progression of said disorder.

In a particular embodiment of the present invention, the medicament can be administered to a subject (a mammal or a human) either directly into the brain or by injection, preferably by intravenous, intraperitoneal, intramuscular or subcutaneous injection. Indeed, the Inventors have shown that the said VHH antibody is capable of transmigrate across a blood-brain barrier model.

In another preferred embodiment, the disorder is a neurodegenerative disorder such as Alzheimer's disease or the Down syndrome.

All amyloid fibrils share common features, including a high content of β-sheet in a classical « cross- β » pattern, a fibrillar morphology in electron microscopy, and the ability to bind and alter the spectroscopic properties of heterochromatic dyes Congo red and Thioflavin T (Sumner Makin and Serpell, 2005; Westermark, 2005).

Then, the present invention also relates to a method of monitoring the therapeutic effect of a VHH antibody of camelid binding an epitope located at the C-terminal end of an amyloid β peptide 42 as defined above, preferably the VHH antibody of camelid having the amino acid sequence of SEQ ID NO: 1 on the regression of a disorder mediated by amyloid β peptide oligomers in a subject comprising the steps of:

a) contacting *in vitro* or *ex vivo* an appropriate biological sample with a compound binding β-sheet amyloid structures such as amyloid fibrils, preferably Congo Red (sodium salt of benzidinediazo-bis-1-naphtylamine-4-sulfonic acid) or thioflavin T (ThT),

b) determining the amount of β-sheet amyloid structures,

c) comparing the amount so determined with amounts previously obtained for the subject, a decrease in amount constituting a marker of the regression of said disorder.

In addition to the preceding features, the invention further comprises other features which will emerge from the following description, which refers to examples illustrating the present invention, as well as to the appended figures.

Figure 1 shows SDS PAGE electrophoresis and immunoblot by V31-1 and mAb 6F/3D. Aβ42 was incubated for several days at 37°C and an aliquot was removed at day 0, 1, 3 and 7. The different fractions were resolved on a 4-12% gel and proteins were transferred onto nitrocellulose membrane. It has to be noted the absence of immunoreactivity of V31-1 for the gel-excluded Aβ reactive material (arrow). Molecular weights are expressed in kDa between the coomassie staining and the immunoblots.

Figure 2 shows the analysis of Aβ42. size by DLS. Aβ42 was resuspended in water (0.20 μM) and incubated at 37°C for several days. Each R_H distribution was normalized to 100 % intensity. Scattering intensity came predominately from polymeric and aggregated Aβ42. Large particles (>1000 nm) were not included in the measurement window. The data are representative of those obtained in each of at least three independent experiments.

Figure 3 shows the binding of VHH V31-1 on Aβ42 fractions. Aβ42 were incubated at 37°C for several days and an aliquot was removed at t=1h, at day 1, day 10 and day 16 and stored at –20°C until further use. The coating of fractions was performed at 4°C overnight and the binding of VHH V31-1 at 4°C for 20 min. Two wells were used for each VHH antibody dilution. Results are expressed as means and standard deviations. This experiment is representative of three independent experiments.

Figure 4 shows the dot-blot immunoassay of Aβ42 by VHH V31-1 and mAb 6F/3D. 2 μl of serial dilutions of Aβ42 (1 μg, 0.5 μg, 0.25 μg, 0.125 μg, 0.062 μg) were dot-blotted.

Figure 5 shows VHH antibody transmigration across *in vitro* blood-brain barrier (BBB). Transport studies were initiated by adding 10-20 μg/ml VHH V31-1 or L1-3 antibody to apical compartment (upper chamber) and the amount of VHH antibodies was determined in the lower chamber at 10 min, 30 min and 60 min.

Figure 6 shows the intraneuronal Aβ42 peptide immunoreactivity of AD patient by various VHHs. Representative photomicrographs of brain tissue stained

with VHH V31-1 (**A** and **B**), VHH L1-3 (**C**) and VHH 61-3 (**D**). When VHH V31-1 is pre-incubated with Aβ42 prior use, only faint neuronal staining can be seen (**E**). The photomicrograph **F** demonstrates both intraneuronal Aβ42 immunoreactivity and the lack of extraneuronal diffuse plaque staining by VHH V31-1 (plaques are indicating by an arrow). The synapses are labelled by VHH V31-1 (**G**).

Figure 7 shows Aβ42 immunoreactivity by dot-blot immunoassay of the formic acid fractions of control and AD patient. 2 µl of serial dilutions (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128) were dot-blotted. VHH V31-1 (**A**) and mAb 6F/3D were used. Aβ42immunoreactivity was determined by densitometry for all of the dilutions. Aβ42 quantities in relative absorbance units (R.A.U.), corresponding to the difference of signal between the AD and control patient, are expressed as a function of sample dilution (**B**).

Figure 8 shows the immunodetection with VHH V31-1 of Aβ42 trimers and tetramers in the human cortical brain formic acid fractions.

The following examples illustrate the invention but in no way limit it.

EXAMPLE 1: Materials and methods

Materials

Aβ42 (SEQ ID NO: 2) and the different Aβ42 fragments (1-11, 10-20, 15-25, 22-35, 29-40 and 33-42 fragments of SEQ ID NO: 2, respectively SEQ ID NO: 5, 6, 7, 8, 9 and 10) used were purchased from Bachem. The monoclonal antibody (mAb) 6F/3D anti-Ab 8-17 (Dako) recognizes synthetic amyloid peptides by dot- and western-blotting. It also specifically stains all type of amyloid deposits in AD brains.

Subjects

Human cortical brain tissue was obtained from the Hôpital Pitié-La Salpetrière, Paris, France. Postmortem brain tissue was examined from representative neurologically normal controls and AD patient (staged Braak-VI, according to Braak and Braak, 1991).

VHH antibodies and expression thereof in a pET system

VHH V31-1 (SEQ ID NO: 1); a host cell expressing VHH V31-1 is available at the Collection Nationale de Cultures de Microorganismes (CNCM), 28

rue du Dr. Roux, 75724 Paris Cedex 15, France, under the number I-2936; it was filed on September 20, 2002;

VHH L1-3 (SEQ ID NO: 3); a host cell expressing VHH L1-3 is available at the CNCM, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France, under the number I-2934; it was filed on September 20, 2002;

VHH L35 (SEQ ID NO: 4); a host cell expressing VHH L35 is available at the CNCM, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France, under the number I-2935; it was filed on September 20, 2002;

VHH 61-3 (SEQ ID NO: 12); a host cell expressing VHH 61-3 is available at the CNCM, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France, under the number I-2933; it was filed on September 20, 2002.

The coding sequences of VHH V31-1, VHH L1-3, VHH L35 and VHH 61-3 antibodies inserted in vector pHEN1, described in International Application No. WO 2004/044204, were subcloned in vector pET22 using the *Nco*I and *Not*I restriction sites according to the manufacturer's instructions (Novagen, Darmstadt, Germany). Transformed *E. coli* BL 21 (DE3) cells expressed VHH antibodies in the periplasm after induction by IPTG 1mM for 3 hours at 20°C. Periplasmic extracts were obtained by spheroplasting cells, suspended in 50 mM sodium phosphate buffer pH 8 containing 20% sucrose and 1 mM EDTA, and hydrolysing the peptidoglycan with 5 mg/ml lysozyme for 20 min at 4°C, in the presence of protease inhibitors (Complete™, Boehringer Mannheim, Germany). The suspension was then centrifuged 2 min at 10,000 rpm. The supernatant corresponding to the periplasmic extract was kept at 4°C. Purified VHH antibodies were obtained by IMAC using a chelating agarose column charged with Ni^{2+} (Superflow Ni-NTA, Qiagen Ltd, UK) according to manufacturer's instructions. The protein content was measured using the Bradford reagent. The purity of the final preparation was evaluated by SDS-PAGE with Coomassie staining and by Western blot.

ELISA

A modified version of a standard ELISA was used to test for the presence of VHH antibodies in culture supernatants. Microtiter plates (Nunc, Denmark) were coated by incubation overnight at 4°C with 1 µg/ml of antigen diluted in PBS. Plates were washed four times with buffer A (0.1% Tween 20 in PBS), and

VHHs were diluted in buffer B (0.5% gelatin in buffer A). The plates were incubated for 2 hours at 37°C and washed again, before adding a horseradish peroxidase-labeled rabbit anti-*c-myc* (A14) (Santa Cruz, Ca, USA) or with a rabbit anti-His tag antibody (Santa Cruz, Ca, USA). Then, the plates were washed with buffer A, and freshly prepared 0.2 % orthophenylenediamine (Dakopatts A/S, Glostrup, Denmark), 0.03% H_2O_2 in 0.1 M citrate buffer, pH 5.2, were added to each well. The peroxidase reaction was stopped by adding 3 M HCl, and the optical density was measured at 490 nm.

Determination of dissociation constants by ELISA

Binding affinity of VHH antibodies was determined as described in Friguet *et al.*, (1985). Various concentrations of Aβ peptides were incubated in solution overnight at 4°C with a known quantity of VHH antibody until equilibrium was reached. The VHH antibody concentration used was determined by preliminary ELISA calibrations. Each mixture (100 µl) was transferred to a well of a microtiter plate previously coated with antigen and was incubated for 20 min at 4°C. The plates were washed with buffer A and free VHH antibodies were detected by adding ß-galactosidase-conjugated goat anti-rabbit Igs (Biosys, Compiègne, France) and 4-methylumbelliferyl ß-D galactoside (Sigma). Fluorescence was read (Fluoroskan, Labsystem, Finland) at 460 nm, after excitation at 355 nm. K_D was estimated from the slope of the regression curve obtained by plotting the reciprocal of the fraction of bound antibody versus the reciprocal of the molar concentration of antigen.

Western Blot

Aβ peptides were suspended in PBS. To an aliquot (5µl), an equal volume of gel loading buffer was added and then treated at 100°C for 5min. Following separation by polyacrylamide gel electrophoresis (PAGE) using NuPAGE Novex 4-12% Bis-tris gel (Invitrogen), semi-dry transfer onto Hybond-C (Amersham) and western blotting were carried out using the Xcell II blot module (Invitrogen). Prior to the immunochemical reaction, membranes were blocked in a 4% skimmed milk solution. Immunoblotting of membranes was accomplished with either VHH antibody or mAb, and respectively revealed by peroxidase-labeled rabbit anti-*c-myc* (A14) antibodies (Santa Cruz, Ca, USA), or rabbit anti-His tag (Santa Cruz, Ca, USA) followed by peroxidase labeled goat anti-rabbit immunoglobulins. Finally, peroxidase activity was visualized using a chemiluminescent kit (Amersham).

Dot-blot

Frozen brain samples were homogenized (1:10 (w/v)) in the Laemmli sample buffer containing 0,25% (W/v) dithiothreitol (DTT) and heat-treated for 10 min. Each sample was then centrifuged at 100,000 g for 60 min. Resulting pellets were resuspended in 500 µl of 100% formic acid and left under agitation at room temperature for 3 hours. Fractions were then centrifuged for 1 hr at 14000xg (Permanne *et al.*, 1995). The supernatants were removed and 2 µl of a serial dilution (from 1/2 to 1/164) were dot-blotted onto Hybond-C nitrocellulose membranes (Amersham). Membranes were blocked and processed for immunorevelation as described for the western blot. Dot-blots were digitized on a Gel-doc (Biorad) at a resolution of 72 dots/inch and saved as 8-bit gray scale PICT files (256 shades of gray). Intensities of immunoreactivity were calculated for each dot with Quantity one software (Biorad).

Preparation of Aβ42 monomers and protofibrils

Samples of 1 mg of Aβ42 powder were dissolved in 500 µl of hexafluoroisopropanol (Sigma), gently stirred at 4°C for 7 days, sonicated for 10min using a Branson ultrasonic bath sonicator and then centrifuged for 10 min at 16000xg. Aliquots containing 50 µg of peptides each were lyophilised and stored at –20°C. Aliquots were dissolved in double distilled water or in PBS pH=7.4 and incubated at 37°C until use.

Dynamic Light Scattering (DLS)

DLS (also termed quasi-elastic light scattering) was used to measure the average diffusion coefficient distribution of Aβ particles of the particles. Each particle present in the sample is characterized by its hydrodynamic radius (R_H), corresponding to the radius of a sphere with a diffusion coefficient equal to that measured. R_H measurements were made at 25°C with a DynaPro MS800 instrument (Protein solution-Wyatt) equipped with a gallium aluminium arsenide 825 nm laser. An aliquot of Aβ42 peptide was diluted to 0.20 µM in double-distilled water and incubated at 37°C. Samples (110 µl) were placed directly into a 3 mm optical pathlength quartz cuvette (Hellma), and the total light scattering intensity at a 90° angle was collected using a 10-s averaging acquisition time. Particle translational diffusion coefficients (D_T) were calculated from autocorrelated light intensity data

(usually 30-40 points) and converted to R_H with the Stokes-Einstein equation. A distribution plot of intensity versus R_H was calculated using the Sedfit 9.3 analysis software (www.analyticalultracentrifugation.com), and intensity-weighted mean R_H values were obtained from each subpeak.

Thioflavin T (ThT) fluorescence assay

Fluorescence emission of ThT is shifted when it binds to β-sheet aggregate structures such as amyloid fibrils (LeVine, 1993). An aliquot of Aβ42 peptide was diluted to 20 μM in double-distilled water or in PBS pH 7.4 and incubated at 37°C. Aβ42 aggregation was measured by periodically removing 30 μl aliquots from the incubation samples and adding them to 2 ml of 5 μM ThT solution (50 mM phosphate buffer, pH=6.5). Fluorescence intensity was monitored at an excitation wavelength of 450 nm and an emission wavelength of 482 nm on a spectrofluorometer using 1 cm light-path quartz cuvettes with both excitation and emission bandwidths of 5 nm. Readings were the results of an average of three values after substracting the fluorescence contribution of free ThT. Each experiment was performed in duplicate.

Statistical analysis

A two-tailed Student's *t*-test was used for comparison of the fibrillogenesis of Aβ42 in the presence or absence of antibody fragments. *P* values < 0.05 were considered as statistically different.

Immunocytochemistry

Immunostaining of brain tissue was performed on 7 μM thick paraffin sections. Sections were de-paraffinized in xylene, rehydrated through ethanol (100%, 96 %, and 90%) and finally brought to water. They were incubated in 90% formic acid, washed again in water, quenched for endogenous peroxidase with 3% hydrogen peroxyde and 20% methanol, and finally washed in water. Non-specific binding was blocked by incubating the sections for 10 minutes in 2% bovine serum albumin in TBS + 0,5% Tween. Appropriate dilutions of primary antibodies were then applied overnight in a humidified chamber at room temperature (typically 1 μg/ml for VHH, and 1:200 for 6F/3D mAb). Slides were washed with TBS-Tween and incubated with secondary antibodies (rabbit anti-His Tag or biotinylated anti-mouse immunoglobulins) in TBS-Tween at room temperature for 2 hours. Slides were then

incubated with either peroxidase goat anti-rabbit immunoglobulins or streptavidine-peroxidase fusions, and developped with diaminobenzidine (DAB) for 2 minutes. After washing with TBS-Tween, slides were counter-stained with hematoxylin and eosin.

Amyloid extraction

Human cortical brain tissue was obtained from the GIE Neuro-CEB of Hôpital de la Pitié- Salpétrière, Paris, France. Amyloid extraction was performed according to Delacourte *et al.*, 2002. Briefly, a total of 50 mg of brain tissue was homogenized in 500 µl of pure formic acid. One hundred µl were evaporated under nitrogen, solubilized in 100 µl of the LDS sample buffer (NuPage, Invitrogen) containing 2% β-mercaptoethanol and boiled 10 minutes before electrophoresis.

EXAMPLE 2: Demonstration of the recognition of oligomeric forms of Aβ42 by VHH V31-1

1. Characterization of the epitopes recognized by VHH V31-1

International Application No. WO 2004/044204 discloses that VHH V31-1 recognizes the carboxy terminal end of Aβ42. To further characterize the epitope recognized by VHH V31-1, the K_D was determined for peptides corresponding to different Aβ fragments (1-11, 10-20, 15-25, 22-35, 29-40 and 33-42). VHH V31-1 did not recognize the fragments 1-10, 10-20, 15-25 and 22-35. The K_D of VHH V31-1 for Aβ 29-40 and Aβ 33-42 was in the same order of magnitude, respectively $2(\pm 0.8).10^{-8}$ M for Aβ 29-40 and 2.10^{-8} M for Aβ 33-42, suggesting that VHH V31-1 recognizes an epitope located at the C-terminal end of Aβ42.

2. VHH V31-1 recognizes preferentially the oligomeric form of Aβ42

To further characterize VHH V31-1, its binding was tested by immunoblotting on Aβ. Aβ42 was incubated for several days at 37°C and an aliquot was removed at days 0, 1, 3 and 7. Standard Aβ42 preparation, *i.e.*, « aging » by incubation of high concentrations of Aβ42 at 37°C for several days, lead to mixed fractions. After SDS denaturation, such preparations showed not only gel-excluded Aβ-reactive material *(i.e.*, SDS-insoluble fibrils), but also Aβ42 monomer and oligomers (Hartley *et al.*, 1999). VHH V31-1 labelled the 4 kDa band corresponding to the monomer, the 8-16 kDa bands corresponding to dimers, trimers and tetramers,

and the 50-80 KDa bands corresponding to 12-mers, but not the additional band at the very top of the gel, suggesting that V31-1 recognizes specifically Aβ42 in its oligomeric form, but not its fibrillar form (Figure 1). In contrast, mAb 6F/3D labelled all the different bands including the gel-excluded fraction.

DLS was then used to study the evolution of the size distribution of Aβ42 particles along the oligomerization process. Aβ42 peptide was dissolved in hexafluoroisopropanol (HFIP), sonicated and then centrifuged to completely remove associated peptides. Lyophilized aliquots were kept at -20°C, resuspended in double-distilled water and analyzed by DLS at different incubation times. At T=1h, the size distribution for Aβ42 showed a single peak with a weight-average hydrodynamic radius (R_H) of approximately 40 nm (Figure 2). At day 1, a single peak centred around 80 nm was observed. At day 10, two peaks were observed: one centred around 40 nm and the other around 400-500 nm. At day 20, two peaks were also observed: one centred around 150 nm and the second around 600-800 nm. It is important to note that because the particle light scattering intensity is proportional to the volume filled by a molecule ($I_D = f(R_H^3, C, 1/\lambda^4)$), the actual abundance of large particles is significantly lower than suggested by the intensity distributions. Bitan et al previously analysed the oligomer size distribution of Aβ42 by DLS (Bitan *et al.*, 2003). Two peaks were observed, one around 10-20 nm, corresponding to pentamer/hexamer units, and the other around 60 nm, corresponding to protofibrils, formed by further self-association of these oligomers. The 40 nm peak that was observed could be a weight-average of the two peaks reported at 10-20 nm and 60 nm

The kinetics of fibril formation was monitored using a ThT assay (Table I below). ThT is a fluorescent dye that specifically binds to fibrillar structures. Aβ42 was resuspended in water and aliquots were periodically removed and added to a ThT solution. A time-dependent increase was observed, suggesting that progressive formation of amyloid fibrils was taking place.

Table I: Kinetics of Aβ42 fibrillogenesis. ThT binding shows an increase at day 1 and 6. Background fluorescence (0.7 10^5 cpm) was subtracted for all measurements.

Incubation time	Fluorescence (10^5 cpm)	Relative response
T=1h	1.19	1.0
T= day 1	4.86	4.1
T= day 6	9.90	8.3

The binding of V31-1 to the different fractions of Aβ42 was analysed in ELISA (Figure 3). The binding of VHH V31-1 to Aβ42 was significantly higher for the 1h-fractions than for the day 16 fractions.

Dot-blot immunoassays were performed on Aβ42 incubated for more than 20 days at 37°C, composed mostly of fibrils. A strong Aβ42 immunoreactivity was obtained with mAb 6F/3D. Conversely, Aβ42 immunoreactivity was weak with VHH V31-1 (Figure 4), thus confirming that it preferentially binds to an oligomeric form of Aβ and not to fibrillar structures.

3. VHH V31-1 recognizes intraneuronal Aβ42 oligomers but not amyloid plaques

The distribution of VHH-specific immunoreactivity in human AD brains was examined. Stained AD brain tissue slices revealed significant intraneuronal immunoreactivity for VHH V31-1, while that of VHH 61-3 was very faint and that of VHH L1-3 undetectable (Figure 6A-D). Normal brain tissues were not labelled with VHHs. The granules were located in the perinuclear region of the cell body. The cells containing the granules were identified as neuronal by their shape. Endothelial cells never contained granules. The specificity of this labelling was confirmed by pre-incubating VHH V31-1 with Aβ42 oligomers (Fig 6E). VHH failed to detect amyloid plaques but detected intracellular granules of neurons surrounding such plaques (Fig 6G). Synapses were also labelled with V31-1 (Fig 6F).

To confirm the immunoreactivity of VHH V31-1 on brain tissues, dot-blot immunoassays were performed on formic acid fractions obtained from an AD patient and from a control. A strong Aβ42 immunoreactivity was obtained in the AD patient fraction. Conversely, Aβ42 immunoreactivity was weak for the control patient

(Figure 7A). To compare the results obtained for VHH V31-1 and mAb 6F/3D, Aβ42 immunoreactivity was determined by densitometry for all the dilutions. Aβ42 quantities corresponding to the difference of signal between the AD and control patients, were expressed as a function of sample dilution. The results obtained with VHH V31-1 and mAb 6F/3D were similar (Figure 7B). Thus the present assay confirmed that VHH V31-1 was able to detect Aβ42 oligomers in brain tissues.

4. VHH V31-1 inhibits the formation of fibrils

To investigate the effects of the VHH antibodies on fibril formation, conditions were selected (lyophilized Aβ42 is resuspended in water then diluted in PBS at the working concentration and sonicated) under which both Aβ42 is able to aggregate *in vitro* and the VHH antibody can bind tightly to Aβ42. Similar experiments were performed with an irrelevant VHH antibody (L35; SEQ ID NO: 4; described in International Application No. WO 2004/044204) as a negative control. A striking feature of the VHH antibodies is their capacity to withstand prolonged incubation at 37°C (Arbabi Ghahroudi *et al.*, 1997).

In the absence of VHH V31-1, the DLS data showed that Aβ42 readily aggregated with the formation of 2 peaks at day 1, one around 30 nm and the other around 100-150 nm. Then as early as day 2, a new peak centred around 300 nm could be detected, which then evolved to 600-700 nm at day 7 (Table II).

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Table II: Effects of VHH on aggregation of Aβ42. Time course of the aggregation of Aβ42 (0.20 μM) in the absence and presence of equimolar concentrations (0.20 μM) of VHH V31-1 and VHH L35 monitored by light scattering. Large particles (>1000 nm) were not included in the measurement window.

	Aβ42			Aβ42 + V31-1			Aβ42+L35		
3h									
R_H (nm)		88			97			109	
5h									
R_H (nm)		95			84			116	
24h									
R_H (nm)	24	125		10	101		14	128	
day 2									
R_H (nm)	35	329		9	122		44	250	
day 3									
R_H (nm)	36	208		25	256		33	232	
day 4									
R_H (nm)	60	498		12	157			135	663
day 7									
R_H (nm)	41	142	665	25	151			107	701

The aggregation of Aβ42 therefore appears to be similar when it is diluted in water (Figure 2) or in PBS (Table II). When Aβ42 is co-incubated with an equimolar concentration of VHH L35, similar pattern of aggregation was observed with the formation of 2 main peaks around 100-150 nm and 600-700nm. Interestingly, the kinetic of fibril formation appeared to be faster when Aβ42 was incubated with VHH L35 than when Aβ42 was alone, as the 600-800 nm peak appeared as early as day 4. When Aβ42 was co-incubated with an equimolar concentration of VHH V31-1, two peaks were observed, one around 10-25 nm and one centred around 150 –200 nm. Noticeably, the 600-800 nm peak could never be detected for this complex, suggesting that high molecular weight aggregates could not form in the presence of VHH V31-1.

The average light scattering intensity of VHH antibodies alone is much lower than the intensity of the VHH antibodies incubated with Aβ42. The absence of formation of fibrils was also confirmed by the ThT assay: fluorescence actually significantly decreased when Aβ42 was co-incubated with an equimolar concentration of VHH V31-1, while it increased when Aβ42 was alone (Table III).

Table III: Time course of the fibrillogenesis of Aβ42 in the absence and presence of antibody fragments. The kinetics of Aβ42 (20 μM) fibril formation was monitored by ThT fluorescence in the absence and presence of equimolar concentrations (20μM) of VHH V31-1 and VHH L35. The samples were incubated at 37°C and 30 μl of the samples were removed periodically and added to 2 ml of 5μM ThT. *P* values resulting from paired t-test analysis : * $P < 0.05$, ** $P<0.01$, *** $P<0.001$ (n=4). This experiment is representative of two independent experiments.

	Fluorescence (10^5 cpm)		
Incubation time	Aβ42	Aβ42 + VHH V31-1	Aβ42 + VHH L35
Day 0	2,3 ± 0.15	1.86 ± 0.15	1.66 ± 0.18
Day 1	4,47 ± 0.63	2.85 ± 0.48*	2.8 ± 0.53*
Day 5	3,33 ± 0,14	1.3 ± 0.14***	3.83 ± 0.36
Day 10	4,4 ± 0.46	1.7 ± 0.14***	3.07 ± 0.33*
Day 15	7.65 ± 2.18	2.06 ± 0.08**	4.21 ± 1. 3

No significant decrease of fluorescence was observed when Aβ42 is co-incubated with an equimolar concentration of VHH L35.

Yan and Wang (2006) have recently shown that the C terminus of Aβ42 is more rigid than that of Aβ40 and is likely preordered for its β-conformation in fibrils and oligomers, therefore acting as an internal seed for aggregation. This notion is supported by recent bioinformatic studies of Aβ42 showing that the C-terminal six-residues of Aβ42 have the best match for the NNQQNY conformation in the crystal structure of fibrils (Thompson *et al.*, 2006; Nelson *et al.*, 2005). It can be postulated that VHH V31-1 may recognize this seed in oligomers and preclude the formation of aggregates.

5. VHH V31-1 recognizes Aβ42 trimers and tetramers in human cortical brain tissue

The distribution of VHH-specific immunoreactivity in human cortical brain tissue was examined. Two main bands at 16 and 20 KDa were immunodetected in the cortical brain formic acid fractions with VHH V31-1 corresponding to Aβ42 trimers and tetramers (Figure 8).

EXAMPLE 3: *In vitro* VHH antibody transmigration across hCMEC/D3

Methods

Immortalized human brain endothelial cells hCMEC/D3 have been previously described in detail in Weksler *et al.* (2005). Cell viability in the presence of VHH antibodies (VHH V31-1 and L1-3) was assessed by MTT assay as described in Weksler *et al.* (2005).

The permeability of hCMEC/D3 cell monolayers to VHH antibodies was measured on transwell polycarbonate insert filters (pore size 3 μm, Corning, Brumath, France) as described in Weksler *et al.* (2005). hCMEC/D3 cells were seeded on the filters at a confluent density of $2x10^5$ cells/cm^2 in EGM-2 medium.

Transport studies were performed 3 days post-seeding as described in Weksler *et al.* (2005). Experiments were initiated by adding VHH antibodies to the upper chamber containing either collagen, coated inserts without cells, hCMEC/D3 cells or hCMEC/D3 cells pre-exposed to various pharmacological modulators for 30 min. Transport studies were conducted at 37°C. The lower chamber was sampled at various time intervals (10, 30 and 60 min) and the presence of VHH antibodies was determined by ELISA and Western Blot (see results).

Results

Figure 5 shows that there is a transcytosis of functional VHH V31-1 while there is no passage of VHH L1-3 across hCMEC/D3. This passage was time-dependant and reached a maximum at 30 min. At 60 min about 1% of VHH V31-1 antibody was present in the lower chamber. This result shows that VHH V31-1 is able to transmigrate across the blood-brain barrier.

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CLAIMS

1°) Use of a VHH antibody of camelid binding an epitope located at the C-terminal end of the amyloid β peptide 42 for detecting *in vitro* in an appropriate biological sample, an oligomeric form of the amyloid β peptide 42 having the amino acid sequence of SEQ ID NO: 2.

2°) The use according to claim 1, characterized in that said epitope consists of a peptide selected from the group consisting of the peptide Aβ 29-40 having the amino acid sequence of SEQ ID NO: 9, the peptide Aβ 33-42 having the amino acid sequence of SEQ ID NO: 10 and the peptide Aβ 35-42 having the amino acid sequence of SEQ ID NO: 11.

3°) The use according to claim 1 or claim 2, characterized in that said VHH antibody has the amino acid sequence of SEQ ID NO: 1.

4°) The use according to any of claims 1 to 3, characterized in that the oligomeric form of said amyloid β peptide 42 is selected from the group consisting of a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, 7-mer, 8-mer, 9-mer, 10-mer, 11-mer, 12–mer, 13-mer, 14-mer, 15-mer, 16-mer, 17-mer, 18-mer, 19-mer, 20-mer, 21-mer, 22-mer, 23-mer, 24-mer, more preferably a dimer, trimer, tetramer or dodecamer.

5°) The use according to any one of claims 1 to 4, characterized in that the presence of an oligomeric form of the amyloid β peptide 42 in said biological sample is a prognostic marker of appearance of a disorder mediated by amyloid β peptide oligomers.

6°) The use according to any of claims 1 to 5, characterized in that said disorder is Alzheimer's disease or the Down syndrome.

7°) An *in vitro* method for determining an early-stage of a disorder mediated by amyloid β peptide oligomers, characterized in that it comprises a step of detecting in a subject an oligomeric form of the amyloid β peptide 42 by contacting an appropriate biological sample or tissue with a VHH antibody as defined in any of claims 1 to 3.

8°) The method according to claim 7 or claim 8, characterized in that said disorder is Alzheimer's disease or the Down syndrome.

9°) A method of diagnostic of a disorder mediated by amyloid β peptide oligomers in a subject comprising the steps of:

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a) contacting *in vitro* or *ex vivo* an appropriate biological sample with a VHH antibody as defined in any of claims 1 to 3, and

b) detecting the binding of said antibody to said biological sample, a binding constituting a marker of the presence of said disorder.

10°) The method according to claim 9, characterized in that said disorder is Alzheimer's disease or the Down syndrome.

11°) A method of diagnostic of a disorder mediated by amyloid β peptide oligomers in a subject comprising the steps of:

a) contacting *in vitro* or *ex vivo* an appropriate biological sample with a VHH antibody as defined in any of claims 1 to 3,

b) determining the amount of amyloid β peptide 42 oligomers in said biological sample, and

c) comparing the amount determined in step (b) with a standard, a difference in amount constituting a marker of the presence of said disorder.

12°) The method according to claim 11, characterized in that said disorder is Alzheimer's disease or the Down syndrome.

13°) The method according to claims 7, 9 or 11 characterized in that the oligomeric form of said amyloid β peptide 42 is selected from the group consisting of a 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, 7-mer, 8-mer, 9-mer, 10-mer, 11-mer, 12–mer, 13-mer, 14-mer, 15-mer, 16-mer, 17-mer, 18-mer, 19-mer, 20-mer, 21-mer, 22-mer, 23-mer, 24-mer, more preferably a dimer, trimer, tetramer or dodecamer.

14°) Use of a VHH antibody as defined in any of claims 1 to 3 for the preparation of a medicament for treating or preventing a disorder mediated by amyloid β peptide oligomers, preferably amyloid β peptide 42 oligomers.

15°) The use according to claim 14, characterized in that said medicament is administered to a subject so as to inhibit the formation of amyloid β peptide fibrils.

16°) The use according to claim 14 or claim 15, characterized in that said medicament is administered to a subject so as to slow down the progression of said disorder.

17°) The use according to any of claims 14 to 16, characterized in that said disorder is a neurodegenerative disorder.

18°) The use according to claim 17, characterized in that said neurodegenerative disorder is Alzheimer's disease or the Down syndrome.

19°) A method of monitoring the therapeutic effect of a VHH antibody as defined in any of claims 1 to 3 on the regression of a disorder mediated by amyloid β peptide oligomers in a subject comprising the steps of:

a) contacting *in vitro* or *ex vivo* an appropriate biological sample with a compound binding β-sheet amyloid structures such as amyloid fibrils,

b) determining the amount of β-sheet amyloid structures,

c) comparing the amount so determined with amounts previously obtained for the subject, a decrease in amount constituting a marker of the regression of said disorder.

20°) The method according to claim 19, characterized in that said disorder is Alzheimer's disease or the Down syndrome.

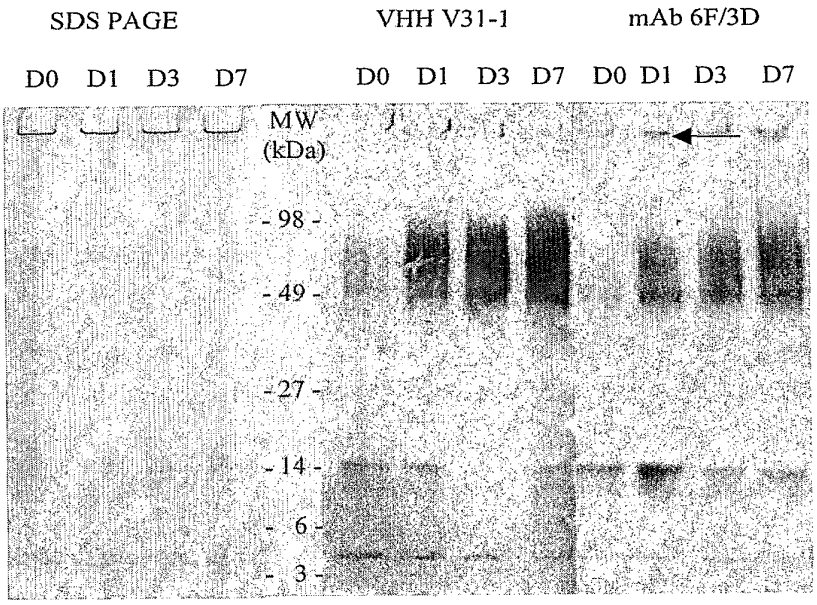

SDS PAGE
VHH V31-1
mAb 6F/3D
D0 D1 D3 D7
D0 D1 D3 D7
D0 D1 D3 D7
MW (kDa)
- 98 -
- 49 -
- 27 -
- 14 -
- 6 -
- 3 -

Figure 1

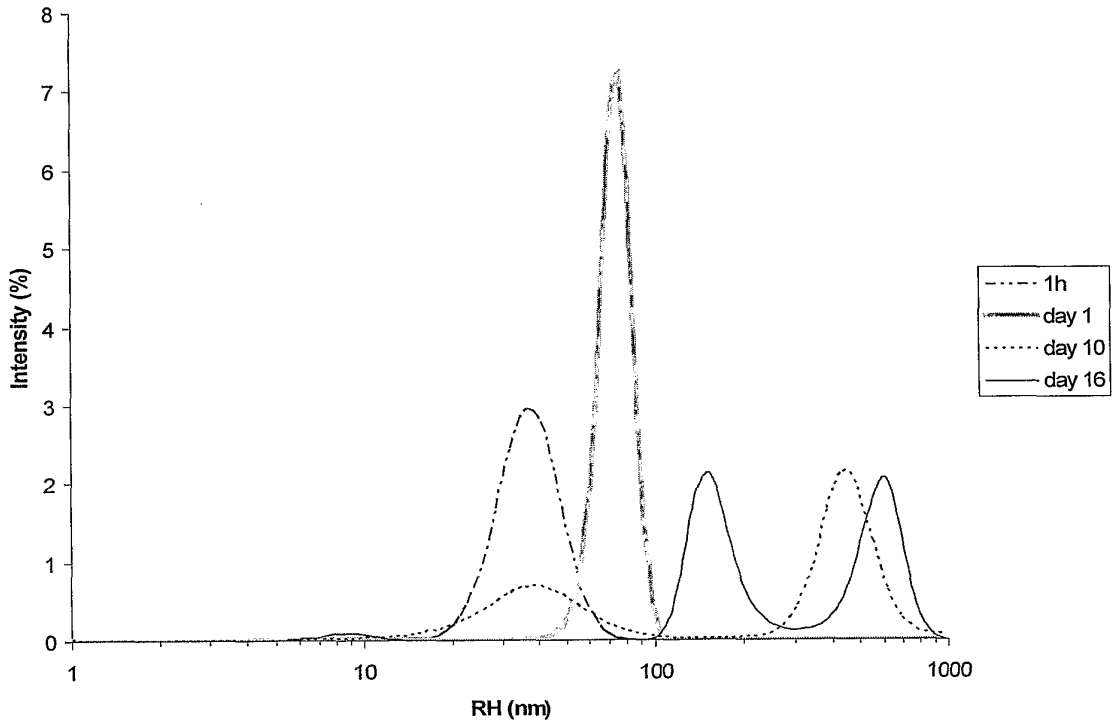


Figure 2

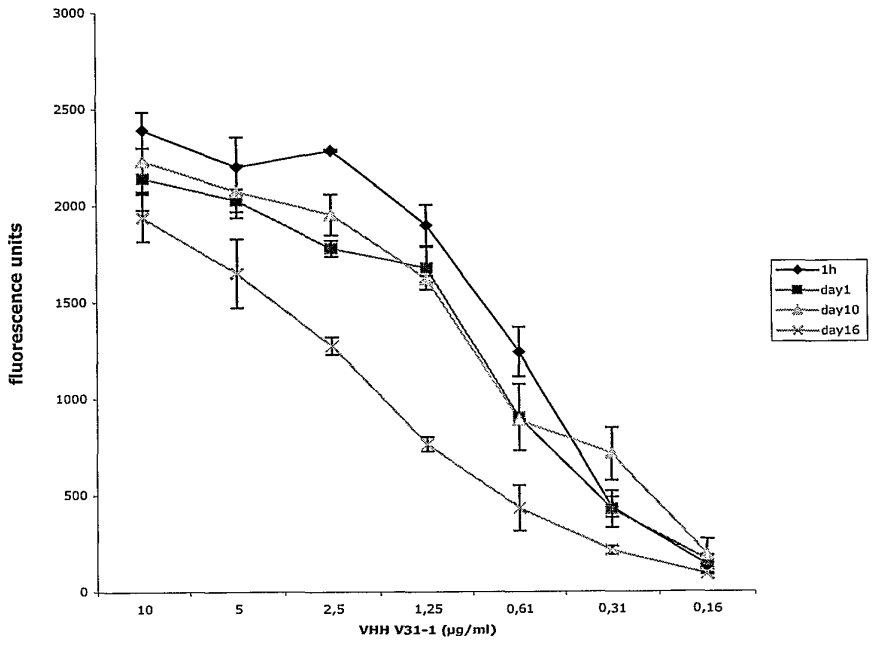

3000
2500
2000
1500
1000
500
0
fluorescence units
10
5
2,5
1,25
0,61
0,31
0,16
VHH V31-1 (µg/ml)
1h
day1
day10
day16

Figure 3

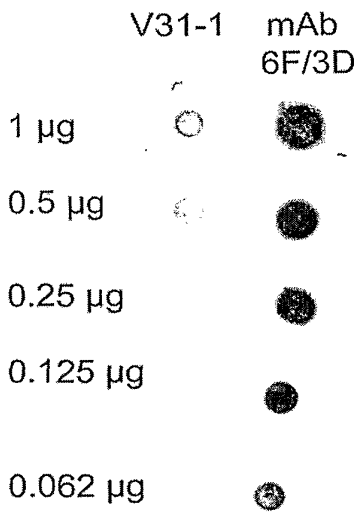


Figure 4

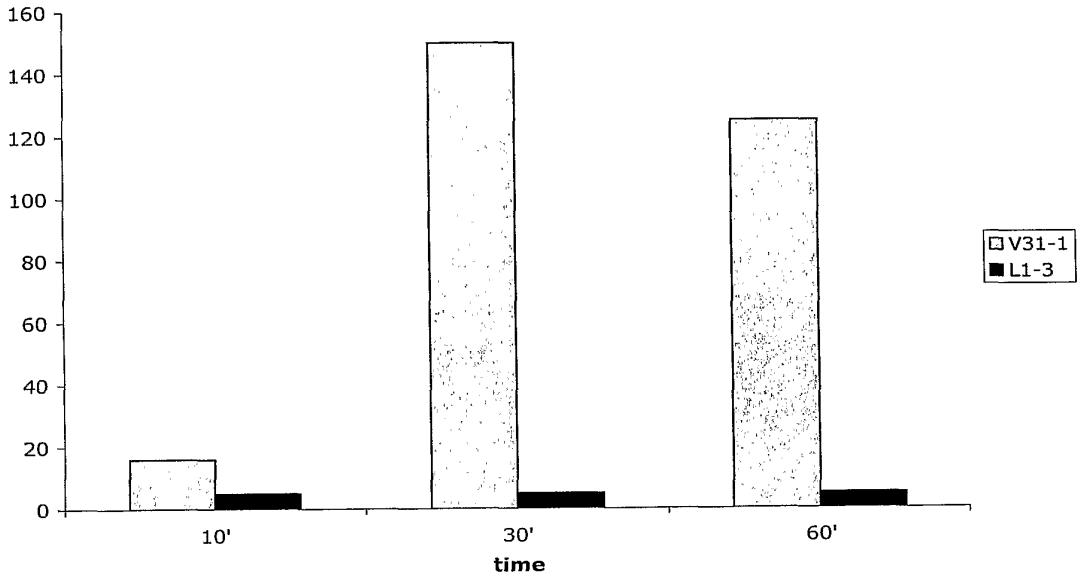


Figure 5

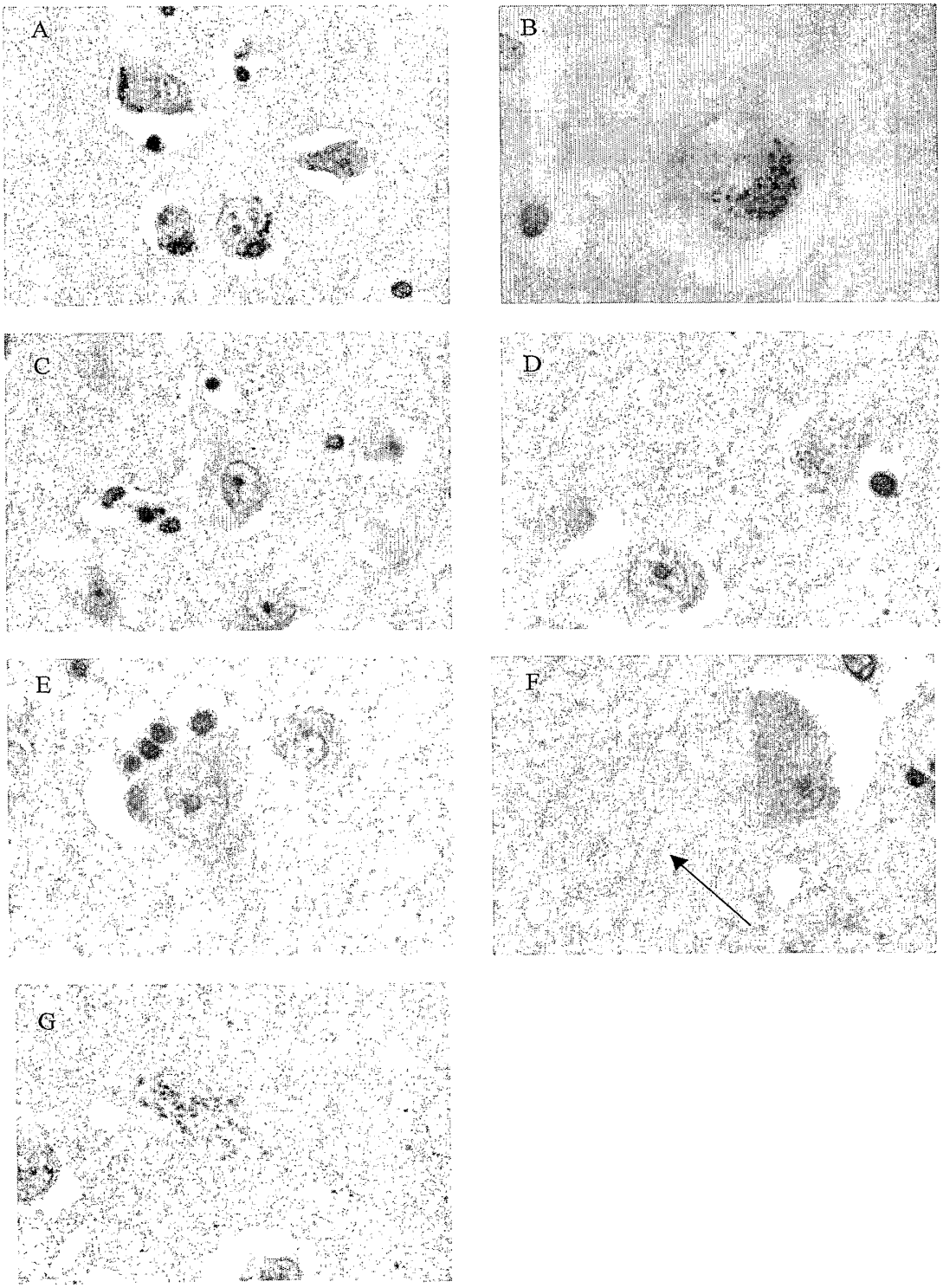
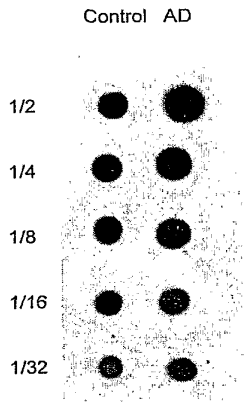
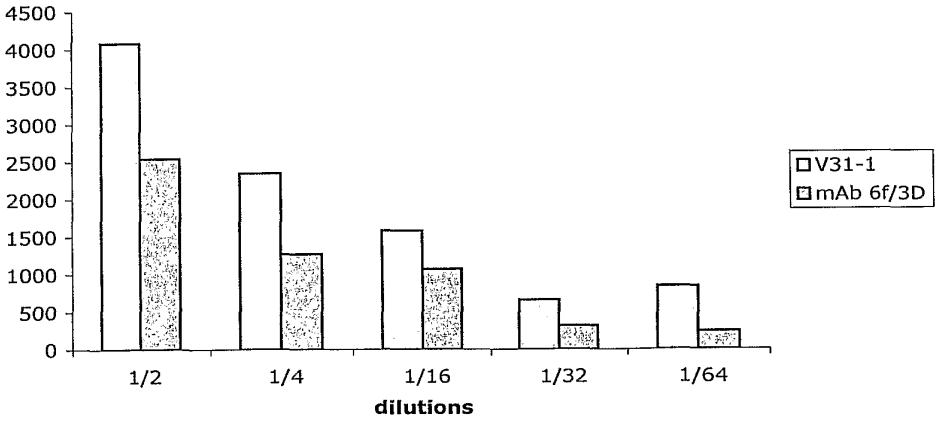


Figure 6

A)


Control AD
1/2
1/4
1/8
1/16
1/32


4500
4000
3500
3000
2500
2000
1500
1000
500
0
1/2
1/4
1/16
1/32
1/64
dilutions
V31-1
mAb 6f/3D

B)

Figure 7

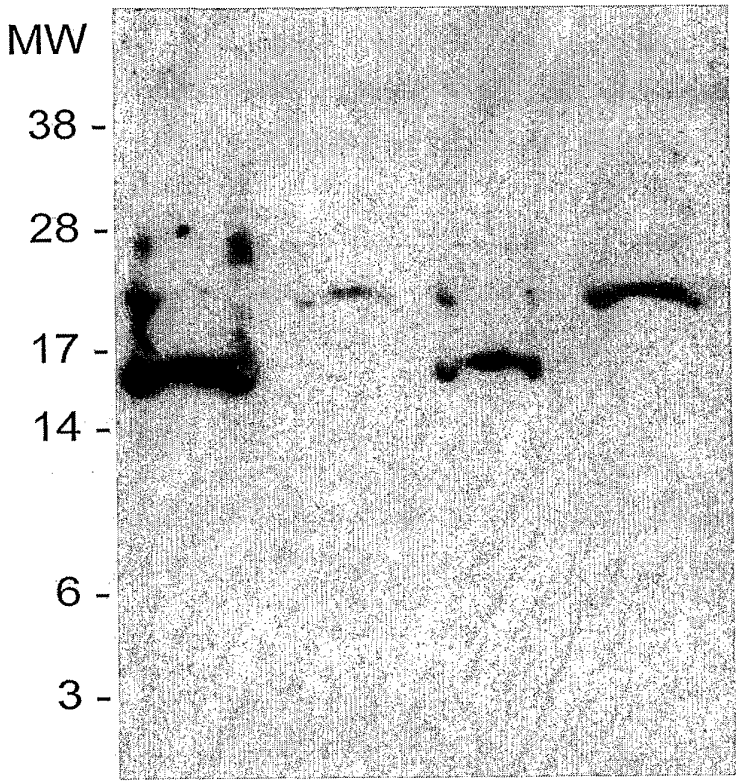


FIGURE 8

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Remarks:
This application was filed on 08 - 06 - 2001 as a divisional application to the application mentioned under INID code 62.

(54) Monoclonal antibody specific for beta-A4 peptide

(57) The instant invention provides for monoclonal antibody which is specific for the βA4 peptide, and in particular the free C-terminus of βA4 "1-42" but not "1-43", and stains diffuse and fibrillar amyloid, vascular amyloid, and neurofibrillary tangles. The instant invention further provides for antibody fragments and constructs thereof which have the same binding specificity. The instant invention also provides for methods of diagnosis, screening and therapeutics for treating unique forms of βA4 peptide, using the antibodies of the instant invention.

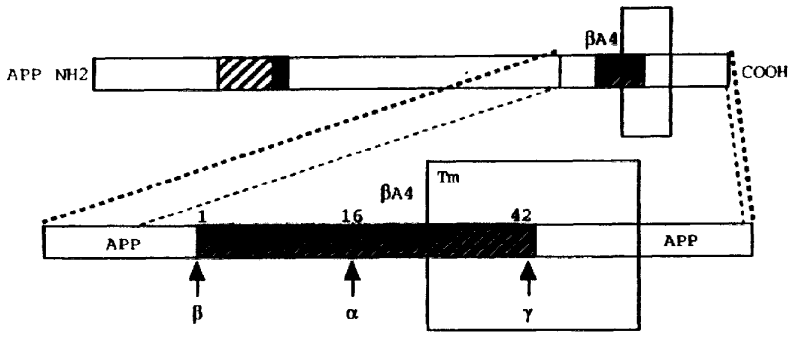


Figure 1

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Description

Field of the Invention

[0001] This application relates to Alzheimer's disease, particularly, to a monoclonal antibody specific for the βA4 peptide derived from Amyloid Precursor Protein, cells which produce such antibody, methods of generaring such monoclonal antibodies, and to methods for using such antibodies in diagnostics and therapy.

Background

[0002] Alzheimer's Disease (AD) is an irreversible progressive neurodegenerative brain disorder. Over the course of several years the progression of AD leads to memory loss, dementia, and finally death. Currently, it is the fourth leading cause of death in the United States, accounting for approximately 100,000 deaths annually. Typically, AD affects primarily the elderly and is therefore, with the aging of modern society, expected to be an increasing health concern in the near future. Soon after the onset of the disease, patients require assistance around the clock. This represents a tremendous psychological as well as financial problem for our society. At present, no proven means for diagnosis, prevention, treatment, or cure of AD exist.

[0003] Neuropathologically, AD is characterized by massive neuronal cell loss in certain brain areas, and by the deposition of proteinaceous material in the brains of AD patients. These deposits are the intracellular neurofibrillary tangles and the extracellular β-amyloid plaques. The major protein component of the β-amyloid plaque is the βA4 peptide. Sequence analysis of purified β-amyloid plaque material and mass spectrometry showed that the maximum length of the βA4 peptide is 43 amino acids. Typically, however, species of the peptide can also end either at position 40 or position 42 (Miller *et al.*, 1993, *Arch. Biochem. Biophys.* 301:41-52). Similarly, at the N-terminus, a certain raggedness can be observed, leading to several different forms of the peptide, starting mainly at position 1, 4 or 11 (Miller *et al.*, 1993).

[0004] Molecular cloning revealed that the βA4 peptide is derived from a much larger precursor protein termed the "Amyloid Precursor Protein" (APP) (Kang *et al.*, 1987, *Nature* 325:733-736) (Figure 1). Figure 1 illustrates the Amyloid Precursor Protein (APP) which is a transmembrane (Tm = membrane region) protein where the N-terminus is located extracellular and the C-terminus is located intracellular (cytoplasmic). βA4 is partially embedded into the membrane. Several alternatively spliced isoforms have been described, which undergo extensive post-translational modifications (Selkoe, 1994, *Ann. Rev. Neurosci.* 17:489-517). The βA4 sequence itself is located partially on the extracellular side and extends partially into the transmembrane region (Figure 2). Figure 2 (SEQ ID NO:3) illustrates the βA4 sequence, shown (encircled area) extending with its C-terminal end into the transmembrane region (Tm, boxed area) and the N-terminal end located in the extracellular part. Asterisks indicate the location of familial mutations in the APP gene; they are either flanking the βA4 sequence, or are centered in the middle portion of the βA4 sequence. The three major cleavage sites (α, β and γ) in APP are indicated. Release of βA4 was therefore postulated to occur through the proteolytic action of one or more proteases on the N-terminus (β-cut) and on the C-terminus (γ-cut) of the peptide (Figure 2) (Selkoe, 1994). The main event during the secretion of APP is at the α-cut (position 16/17 of βA4 "1-42"). This secreted APP molecule (αAPPs) contains the first 16 amino acids of the βA4 sequence at its carboxyl end. The remaining cell-associated APP fragments (so called C-terminal fragments (CTFs)) contain the C-terminal portion of the βA4 sequence and extend to the cytoplasmic region of APP. Therefore, this proteolytic cut results in fragments which may not be processed in such a way that directly or indirectly leads to amyloidogenic fragments (non-amyloidogenic processing) (Selkoe, 1994).

[0005] Recently, it was demonstrated that cell lines which express large amounts of APP through a stably transfected APP cDNA construct produce high picomolar to low nanomolar amounts of βA4 and release it rapidly into the medium (Shoji *et al.*, 1992, *Science* 258:126-129). It has also been found that virtually every primary cell culture and cell line releases βA4 constitutively (Busciglio *et* al., 1993, *PNAS USA* 90:2092-2096). Additionally, healthy controls as well as Alzheimer patients have been shown to have low nanomolar amounts of βA4 in sera and CSF (Seubert *et al.*, 1992, *Nature* 359:325-327). The majority of the detected soluble βA4 species in these body fluids and conditioned media was βA4 "1-40", which is not truly reflective of the overall composition found in β-amyloid plaque depositions. The notion that the production and subsequent release of βA4 is sufficient and therefore responsible for the buildup of β-amyloid plaques in the brains of AD patients could therefore no longer be maintained; other factors must contribute to the deposition of β-amyloid plaques. One straightforward hypothesis is that acute or chronic overproduction of βA4 causes the increased amyloid load observed in AD

[0006] The finding that specific point mutations in and around the βA4 region of the APP gene are linked with certain familial Alzheimer's disease (FAD) cases showed unequivocally that the APP gene is a "disease gene" (Goate *et al.*, 1991, *Nature* 349:704-706; Murtell *et al.*, 1991, *Science* 254:97-99; Levy *et al.*, 1990, *Science* 248:1124-1126; Carter *et al.*, 1992, *Nature Genetics* 2:255-256). In families in which AD is inherited dominantly with a specific age of onset,

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the point mutations in the APP gene are necessary and sufficient to cause AD. Although the vast majority of Alzheimer disease cases are sporadic and probably multifactorial, these familial APP mutations can teach us a great deal about amyloidogenesis, i.e., the generation of the small βA4 peptide from the larger precursor and its subsequent deposition in β-amyloid plaques.

[0007] The double mutation at APP codon 670/671 (the "Swedish variant", at the N-terminus of βA4 in APP) causes a 5 to 8 fold higher release of βA4 in cell cultures stably transfected with that mutated APP cDNA (Figure 2) (Citron *et al.*, 1992; Cai *et al.*, 1993). It is conceivable that this double point mutation leads to an increased turnover of APP due to increased proteolysis at the β-cut, which in turn leads to a higher level of released βA4. Increased amounts of βA4 monomers, as demonstrated by transfection studies with the "Swedish mutation", can explain the faster kinetics of βA4 aggregation to β-amyloid plaques in these families.

[0008] Another FAD mutation lies C-terminal of βA4 at position 717 ("London variant") and does not affect the level of released βA4 in tissue culture (Figure 2). It was recently demonstrated that this 717 mutation changes the "1-40/1-42" βA4 ratio (Suzuki *et al.*, 1994, *Science* 264:1336-1340). Although it is not clear at the moment how the generation of the C-terminus of βA4 occurs, since this part is embedded in the transmembrane region, it is tempting to hypothesize that-the "London mutation" affects the proteolytic breakdown of APP to βA4. Possibly, this point mutation interferes with the cleavage fidelity of the responsible protease at the γ-site. βA4 1-40 exhibits among other things a dramatic difference in its solubility in aqueous solutions when compared to βA4 1-42 (Burdick *et al.*, 1992, *JBC* 267:546-554). The latter is virtually insoluble in water, whereas 1-40 is water soluble up to several mg/ml in vitro. Minor amounts of the longer 1-42 form can enhance precipitation of 1-40 in vitro dramatically. A slightly higher proportion of the longer 1-42 βA4 species would explain the early onset deposition of βA4 to β-amyloid plaques in patients with this "London mutation". The proportion of the 1-42 species to the shorter more soluble 1-40 species may also be one of the critical factors in the sporadic AD cases (i.e. cases where no genetic predisposition was identified). Monoclonal antibodies which specifically bind to the 1-42 species, are therefore useful to investigate the production and presence of βA4 species ending at amino acid position 42, and can be used as a diagnostic indicator of abnormal species present in AD.

[0009] Recent biochemical analyses with one antibody which recognizes βA4 ending at position 40, and one antibody recognizing βA4 species extending to position 42 or further, showed that the contribution of the longer βA4 species might be critical for the onset of the disease (Suzuki *et al.*, 1994). However, the monoclonal antibody of Suzuki does not distinguish between βA4 1-42, 1-43, and longer βA4 species. This is also the case for another reported monoclonal antibody 2G9 (Yang *et al.*, 1994, *Neuro Report* 5:2117-2120). Therefore, in order to avoid this crossreactivity, antibodies which are specific for βA4 species ending at position 42 to the exclusion of the other forms would be very useful in order to avoid crossreactivity with membrane associated C-terminal APP fragments, which are typical cellular products not necessarily associated with the β amyloid plaques.

[0010] One monoclonal antibody recognizing βA4 1-42 has been described (Murphy *et al.*, 1994, *Am. J. Path.* 144: 1082-1088). However, the βA4 1-43 peptide species was not used in these studies, thus it is not known what the exact specificity of this monoclonal antibody would be in response to the 1-43 peptide. Competition studies were only performed with βA4 peptides ending at position 40 ("1-40"), and position 44 ("1-44") and beyond with this antibody. Importantly, the antibody was reported to stain diffuse amyloid, fibrillar amyloid, intraneuronal and extraneuronal neurofibrillary tangles, but *not* vascular amyloid.

[0011] An in vitro biochemical diagnostic test for Alzheimer's disease in its early stages as well as a means of screening for at-risk AD individuals is not available. The current diagnosis of AD requires a detailed clinical evaluation which cannot give clear answers until significant symptoms of dementia and memory loss are manifested. In view of the research referred to above, βA4 1-42 represents a preclinical marker for AD. Thus, identifying the level of or the buildup of βA4 1-42, or other residue 42 terminating species, and how this may progress during the course of the disease, and how it is distributed in the brain, will provide valuable insights into monitoring the course of, as well as for specific diagnosis and possible treatment of AD.

[0012] It would be useful for preparing diagnostic tests, therapeutics and for monitoring assays of AD, to have a monoclonal antibody which, in contrast to the specificity of presently available antibodies (crossreactive with 1-43; reported not to stain vascular amyloid), does stain vascular amyloid and is specific for βA4 peptide ending at residue 42, and therefore extends the diagnostic capabilities of the art, i.e. one that recognizes the free C-terminus of βA4 1-42 and stains diffuse and fibrillar amyloid, neurofibrillary tangles, and vascular amyloid. Such an antibody is the subject of the present application.

Summary of the Invention

[0013] The instant invention provides for monoclonal antibody which is specific for the βA4 peptide, and in particular, the C-terminus of βA4 "1-42" and stains diffuse and fibrillar amyloid, neurofibrillary tangles, and vascular amyloid. In particular, the instant invention provides for monoclonal antibody which is specific for all βA4 peptides in which the C-terminus is residue 42 of the βA4 amino acid sequence. The instant invention further encompasses antibody fragments

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and constructs thereof which have the same binding specificity. The instant invention encompasses in particular the monoclonal antibody known as "Mab 369.2B" and is produced by the cell line "369.2B" which has been deposited under the Budapest Treaty with the American Type Culture Collection (ATCC) on January 26, 1995, and has been assigned the ascension number HB11829. The instant invention encompasses the use of the monoclonal antibody of the instant invention in diagnostic, purification, and therapeutic uses.

[0014] Thus one embodiment of the instant invention encompasses a monoclonal antibody specific for βA4 peptide ending at position 42, wherein said antibody binds to diffuse amyloid, fibrillar amyloid, vascular amyloid, and neurofibrillary tangles. In one particular embodiment, the instant invention provides for a monoclonal antibody which is specific for the C-terminal amino acids of the βA4 1-42 peptide. In the most preferred embodiment the instant invention encompasses a monoclonal antibody that is identified as 369.2B, and is produced by the cell line deposited with the American Type Culture Collection (ATCC) as ascension number HB 11829. A preferred embodiment of the instant invention also encompasses a cell which is identified by the ATCC ascension number HB 11829. In a further embodiment of the instant invention, an immunologically reactive fragment of the monoclonal antibody of the instant invention which is capable of the same binding as the monoclonal antibody of the instant invention, is encompassed.

[0015] The instant invention also provides for methods of generating βA4 specific antibodies which recognize the free C-terminal residue 42. The instant invention also provides for methods for detecting the presence of βA4 peptides ending at position 42, in tissue comprising contacting a tissue sample with monoclonal antibody of the instant invention, by detecting the presence of monoclonal antibody in a selective fashion. The instant invention also provides for methods for selective purification of βA4 peptides ending at position 42, comprising contacting a sample to be purified with monoclonal antibody of the instant invention, separating the βA4 peptide from the sample to be purified, and isolating the βA4 peptide. In a further embodiment, the instant invention provides for methods for detection of βA4 peptide associated with Alzheimer's Disease, comprising contacting a sample to be tested with monoclonal antibody of the instant invention, and detecting the presence of βA4 peptides.

[0016] Thus the instant invention also provides for methods for the prevention of aggregation of βA4 peptide by administering monoclonal antibody of the instant invention. In a preferred embodiment the monoclonal antibody is identicle to 369.2B, or is an immunologically active fragment with equivalent binding specificity thereof. The instant invention thus provides a means for detecting the presence of βA4 peptide comprising an immunologically reactive fragment of the monoclonal antibody of the instant invention. As well as a means for preventing the aggregation of βA4 peptide comprising an immunologically reactive fragment of the monoclonal antibody of the instant invention. The instant invention provides a means for detecting and monitoring the level of βA4 peptide in tissue or fluid samples (e.g. blood, other body fluids, tissue sections, biopsy tissues etc.). In a preferred embodiment, the βA4 peptide being detected, monitored, inhibited or purified is a βA4 peptide with a free carboxy terminal amino acid residue being residue number 42 of the βA4 peptide amino acid sequence.

[0017] All references to publications and patent documents in the prior or subsequent sections are hereby incorporated by reference in the entirety. Specific preferred embodiments of the present invention will become more evident from the following more detailed description of certain preferred embodiments and the claims.

Brief Description of the Drawings

[0018] The invention will be more completely understood from a consideration of the following detailed description, taken in conjunction with the drawings, in which:

Fig. 1 is a schematic showing the βA4 portion of the Amyloid Precursor Protein (APP), its location relative to the cell membrane, and the α, β and γ cleavage sites;

Fig. 2 shows the βA4 portion of APP, its position relative to the transmembrane region of a cell, and the three major cleavage sites (α, β and γ) in APP;

Fig. 3 is a diagram of the clone pGK003 which was used to generate the βA4 1-42 peptide;

Fig. 4A shows SDS-PAGE on a 16 % Tris/Tricine gel, of in vitro translated radioactively labelled βA4 in a wheat germ system;

Fig. 4B shows SDS-PAGE on a 16 % Tris/Tricine gel, of in vitro translated radioactively labelled βA4 from wheat germ system, immunoprecipitated with MAb 286.8A;

Fig. 5 is a graph showing immunoprecipitation of in vitro translated βA4 (IVT βA4) with 286.8A;

Fig. 6 is a diagram of the peptides used to generate the immune response (immunogen) and to screen the sera of mice;

Fig. 7 is a graph showing immunoprecipitation of in vitro translated βA4 vs. antibody concentration; -o- 286.8A, -Δ- 369.2B, -□- 369.6;

Fig. 8 is a graph showing the % of various βA4 sequences immunoprecipitated by MAb 369.2;

Fig. 9 is a graph showing epitope mapping of MAb 369.2 by competitive assay, with -■- being 35-42(OVA) (Oval-

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bumin coupled 35-42 βA4 peptide), -□- being 1-42 βA4 peptide, and -♦- being 1-40 βA4 peptide;
Fig. 10 is a photograph showing the binding of MAb 369.2B to vascular amyloid and other plaques with various morphologies.

Detailed Description of the Invention

[0019] The β-amyloid depositions in Alzheimer's disease brains are made up mainly of βA4 peptides which show both N- as well as C-terminal heterogeneity. The major C-terminal species, identified by peptide sequencing of β-amyloid purified from postmortem brain material, end either at position 40 or 42 of the βA4 peptide which is at most 43 residues long. In vitro experiments with synthetic βA4 peptides ending either at position 40 or 42 indicate profound physico-chemical differences. Previously, the distribution of these two βA4 species in postmortem tissue as well as their generation in vitro could not be addressed due to the lack of specific antibodies against the carboxy end capable of distinguishing between subspecies of βA4 peptide.

[0020] Recent evidence suggests that release of βA4 is a normal event in virtually every cell culture. Typically high picomolar to low nanomolar concentrations of βA4 can be measured in serum and cerebral spinal fluid (Seubert *et al.*, 1993). This finding was surprising because it had been assumed that the production of βA4 is a pathological event since βA4 is massively deposited as β-amyloid plaques in the cortical and hippocampal brain regions of Alzheimer disease patients. Detailed sequence analysis of the released βA4 from cell culture revealed that the major species end at position 40 (Selkoe, 1994). Amyloid plaques purified from postmortem brain show a slightly different picture: amyloid deposits of the congophilic amyloid angiopathy (CAA) are βA4 aggregates surrounding blood vessels and are predominantly βA4 1-40, whereas in contrast amyloid plaques cores (APC) which are present in the brain parenchyma and are not associated with blood vessels exhibit an N-terminal raggedness (starting most commonly at residues 1, 4 and 11) and end mainly either at position 40 or 42 (Glenner and Wong, 1984, *Biochem. Biophy. Res. Comm.* 120:885-890; Masters *et al.*, 1985, *PNAS USA* 82:4245-4249; Miller *et al.*, 1993). Occasionally, longer species ending at 43 or extending even further have been described (Miller *et al.*, 1993). Because the length of the hydrophobic C-terminus is critical for the ability of the peptide to self-aggregate in vitro (Burdick *et al.*, 1992; Jarrett *et al.*, 1993, *Biochem.* 32: 4693-4697), it is entirely possible that the two distinct pathological aggregates, APC and CAA and other vascular β-amyloid plaques, can be explained by the differing properties of the two species 1-40 and 1-42. This could also be the case for the so called "diffuse plaques" (Selkoe, 1994) which are seen frequently in brains of aged humans and are not associated with AD, however, have been proposed to be precursors of fibrillar β-amyloid deposits. A non-fibrillar aggregation of βA4 has been suggested for these structures. It is therefore of primary importance to determine the tissue specific production of these longer βA4 species (i.e., those ending at position 42) and their pathological appearance in brains of AD patients. Recently three reports have been published where antibodies have been described, which distinguish 1-42 and 1-40 species of βA4 (Suzuki *et al.*, 1994; Murphy *et al.*, 1994; Yang *et al.*, 1994). Unlike the antibodies of the instant invention, the antibodies reported by Suzuki *et al.*, and Yang *et al.*, crossreact in a significant degree with both the 1-43 and 1-42 species of βA4 peptide. The antibody of Murphy *et al.*, while not tested for binding with the 1-43 species of βA4 peptide, exhibits a different tissue binding pattern than the antibodies of the instant invention, and thus must recognize a different, or modified epitope from that recognized by the antibodies of the instant invention.

[0021] Positions 29 through 42 of the βA4 peptide lie entirely within the putative transmembrane region of the Amyloid Precursor Protein and are hydrophobic in nature (Miller *et al.*, 1993). Synthetic peptides to the C-terminal sequences in this region must overcome the ability of the 34-42 sequence to form an unusually stable β-structure which is virtually insoluble in water and strong denaturants (Halverson *et al.*, 1990, *Biochem.* 29:2639-2644) if they are to be used to elicit good immune responses against soluble βA4. We designed a hydrophilic spacer five residues long which would overcome those insolubility problems and also extend the presumed epitope away from the proximity of the carrier. To reduce the likelihood of cross-reactivity with the shorter but major βA4 species, 1-40, we chose a minimal peptidyl epitope of 8 residues corresponding to positions 35-42 of the βA4 sequence. The entire synthetic sequence designed in this way was coupled by a free sulfhydryl group on a N-terminal cysteine residue to KLH (keyhole limpet hemocyanin).

[0022] Successful use of spacers and hydrophilic residues in the production of antipeptide antibodies is well documented as is the use of hydrophilic structures to bring insoluble haptens into solution for conjugation (McMillan *et al.*, 1983, *Cell* 35:859-863; Makela and Seppala, 1986, in Handbook of Experimental Immunology, Volume 1: Immunochemistry, Wier, D.M., editor. Blackwell Scientific Publications, Oxford, pp 3.1-3.13). The success of this method in producing specific antibodies may at least in part be attributed to the presence of a free charged carboxyl terminal, especially in context of a hydrophobic sequence, as terminal residues on peptide antigens give rise to significant proportions of antipeptide antibodies (Gras-Masse et al., 1985, in Synthetic Peptides in Biology and Medicine, Alitalo, K. *et al.*, editors, Elsevier, Amsterdam, p 105). This, along with the selective and novel use of a minimal βA4 sequence used as an immunogen maximized the probability of producing an antibody which could distinguish between βA4 species ending at positions 42 with those that do not. Although peptide competition studies did not fine map the antigenic

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determinant, βA4 sequences other than 1-42 were not effective in inhibiting binding. The fact that 1-42 did not totally compete with ^{35}S-methionine-labelled in vitro-translated βA4 may be due either to the particular properties of the molecule itself or to the fact that the 35-42 peptide immunogen was presented in the context of a specific spacer and/or carrier, or that a 200-1000 fold excess of unlabelled peptide is not enough to quench the signal. Non-specific effects of N-terminal residues on antigenic activity are also well documented (Benjamini *et al.*, 1968, *Biochem.* 7:1261-1264).

[0023] The intriguing finding that 25-35 actually enhances the ability of 369.2B and other antibodies to bind to βA4 may be due to a peculiar interaction between the abstracted peptide and the full length βA4 sequence itself. Residues 26-33 are believed to exist as a random coil in aqueous solution (Halverson *et al.*, 1990) and may be able to interact with soluble βA4 in such a way that makes the C- terminus more accessible to the binding sites on antibodies.

[0024] The highly specific antibody of the instant invention, of which 369.2B is a particular example was raised against a synthetic βA4 peptide having residues 35-42, and does not recognize the shorter βA4 species 1-40 in solution or on a solid phase. Furthermore, both 1-40 and 1-43 were unable to absorb out the antibody when used immunohistochemically. A secondary screening method with medium capacity through-put for the screening of hybridoma supernatants using radioactively labelled in vitro translated βA4 was applied so that antibodies culled from the primary screening could be further selected for their ability to immunoprecipitate soluble βA4. This method can be easily adapted to other proteins/antibodies of interest. The resulting MAb 369.2B represents a superior tool to investigate the role of βA4 peptides ending at position 42 in situ, postmortem tissue, transgenic animals, and the in vitro generation of βA4 peptides in established cellular βA4 production models, for diagnostic use, and for therapeutics.

[0025] The monoclonal antibody of the present invention represents an important tool needed to establish a diagnostic test kit. It permits one to measure/quantify the amount of the βA4 1-42 or derivatives thereof (e.g. 4-42 species, and other truncated forms with the "42" carboxy end) in human body fluids (CSF, serum, urine etc.) or tissues. It can also be used to study the distribution pattern of 1-42 or βA4 species ending in residue 42, in AD brains compared to healthy controls. Its exceptional high avidity makes it a superior and novel tool for such testing. The monoclonal antibody here disclosed can also be used in biological model systems such as transfected cell cultures or animal models (transgenic mice), designed to measure and/or influence the presence and/or production of βA4 species ending in amino acid 42. These model systems represent means to identify selective modulators of the production of βA4 ending in amino acid position 42 of the βA4 in biological systems. The antibodies of the instant invention provide for methods of preventing aggregation of βA4 peptide because the specificity of the antibody will allow for the specific interference with the free C-terminal residue, thereby interfering with and disrupting aggregation that may be pathogenic in AD.

[0026] Surprisingly, the antibody of the present invention differs from that of the prior art in that it stains diffuse and fibrillar amyloid, neurofibrillary tangles, and vascular amyloid while being specific for the βA4 peptide free C-terminal residue 42. This unique binding pattern shows that the antibody of the instant invention recognizes a different epitope from that of the prior art, and that the tissue distribution or accessibility of the βA4 peptide recognized by the antibody of the instant invention is also different. Further, the instant invention provides for monoclonal antibody which can precipitate the βA4 peptide out of solution, which was not demonstrated by the antibodies of the prior art. Thus the instant invention provides for unique monoclonal antibodies which recognize a unique subset of βA4 species which has a distinct tissue distribution that is most likely a better diagnostic indicator than what was previously available, and a unique target for therapeutic intervention.

[0027] Thus the instant invention provides for antibodies, antibody fragments and constructs thereof which are specific for the βA4 species of peptide where the C-terminal ends at residue 42. The instant invention also provides for the use of such antibodies, binding fragments and constructs thereof in diagnostic, analytic, therapeutic, and biochemical purification methods which employ the binding specificity of the instant monoclonal antibodies and their use within pharmaceutical formulations.

[0028] The following examples will further explain the instant invention and are shown by way of illustration, and not by way of limitation. The following examples illustrate certain aspects of the above-identified methods and compositions as well as advantageous results.

Example 1: βA4 Peptide Expression System

Preparation of plasmid pGK002

[0029] General cloning and molecular biology procedures are found for example in Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning 2nd edition, Cold Spring Harbor Lab Press. Plasmid pMTI-26, which is a Bluescript KS clone containing 2.415 kb (kilobase pairs) of the APP sequence with a TAG stop codon followed by a BamHI site placed in frame by site-directed mutagenesis after the 42nd amino acid codon of the βA4 region, was modified by excising a 1.8 kb Xba I/Bgl II fragment and religating the plasmid after filling in the ends. The resulting construct, designated pGK002, places the consensus containing initiation codon of the βA4 sequence immediately downstream of the Bluescript T7 promotor.

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Preparation of plasmid pGK003

[0030] Plasmid pGK003 (Fig. 3), used in all of the wheat germ in vitro translations of βA4 to be described below, was made by subcloning a 590 bp (base pair) NotI/Xho I fragment from pGK002 containing the entire human βA4 sequence with the mutagenized stop/Bam HI into a pSP64 polyA vector (Promega Corp.). In preparing this plasmid, pGK002 was digested with Not I and Xho I and the resulting 590 bp fragment was filled in with Klenow, isolated, and ligated with pSP64polyA linearized with Sma I. Figure 3 is a diagram of Clone pGK003. The open reading frame of βA4 1-42 is expressed in vitro from the bacterial SP6 promoter. The 3' untranslated (3'-UT) region of APP is shown in black.

Example 2: In Vitro Transcription and Translation of pGK003

[0031] Plasmid pGK003 was linearized with EcoRV and complete digestion was confirmed by agarose gel electrophoresis. The sample was extracted twice with phenol/chloroform, followed by two chloroform extractions and ethanol precipitation. The resulting pellet was washed once in 70% ethanol, partially dried under vacuum, and resuspended in TE at a concentration of 1 μg/μl.

[0032] In vitro transcripts using linearized templates at 30 μg/ml were prepared in 80 mM HEPES-KOH (pH 7.5) buffer containing 16 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, 3 mM ATP/CTP/GTP/UTP, 800 units/ml RNAsin Ribonuclease Inhibitor (Promega Corp.), 5 units/ml Yeast Inorganic pyrophosphatase (Sigma Corp.), and 1800 units/ml SP6 RNA polymerase (Promega Corp.). The reaction mixture was kept at 37°C for 4 hrs. The resultant transcript was verified by electrophoresis through a 1.2% agarose/TBE/EtBr gel with denatured samples (65°C x 10 min).

[0033] Transcripts were translated using Wheat germ extract (Sigma Corp.). Briefly, transcripts were heated (65°C x 10 min), mixed with the wheat germ extract containing KAc, RNAsin, and a methionine-minus amino acid mixture, and translated at 25°C for 1 hr in the presence of ^{35}S-labelled methionine (Amersham). Translation of a 4 kD (kilo dalton) βA4 protein was verified by SDS-PAGE using 16% Tris/Tricine gel (Novex). Gels were fixed and proteins visualized fluorographically using a commercial system, "Amplify" (Amersham).

[0034] The incorporation of label into in vitro translated βA4, which contains one methionine residue per molecule, was determined by gel slicing. 2 mm slices were solubilized in 1 ml of 30% hydrogen peroxide, 0.75M NH_4OH overnight at 37°C. Next a 10 ml volume of "Ready Value" scintillation cocktail (Beckman) was added and DPMs (Decay per minute) determined using a Beckman LS6000IC scintillation counter in the auto DPM mode. Typical reactions produce -250 ng βA4/ml, or -56 nM.

[0035] In vitro transcription followed by translation of the βA4 clone, pGK003, in a wheat germ system resulted in a single 4 kD protein product when visualized by fluorography on a 16% Tris-Tricine SDS polyacrylamide gel (Figure4A). Figure 4A shows the results of SDS-PAGE on a 16 % Tris/Tricine gel. Lane 1: High MW markers. Lane 2: Low MW markers. Lane 3: In vitro translated βA4 in a wheat germ system. The identity of this 4 kD product was confirmed by immunoprecipitation with βA4 specific antibodies (Figure 4B). Figure 4B shows results of SDS-PAGE on a 16 % Tris/Tricine gel. Lane 1: High MW markers. Lane 2: Low MW markers. Lane 3: In vitro translated βA4 from wheat germ system immunoprecipitated with MAb 286.8A. Transcription and translation of this as well as other βA4 clones in a combined reticulocyte lysate system (TnT) did not result in the same yield or purity of radioactively labelled βA4 (data not shown). This could be due to the short transcript or to the peculiar nature of the βA4 peptide itself.

[0036] The monoclonal antibody 286.8A, which was raised against crude peptide 1-42 and maps to region 3-8 of βA4, was able to precipitate this protein in a concentration-dependent manner (Figure 5). Figure 5 graphs this immunoprecipitation of in vitro translated βA4 (IVT βA4). Increasing amounts of IVT βA4 were immunoprecipitated with a fixed amount of 286.8A (7.4 μg) in 100 μl in RIPA buffer.

Example 3: Immunogen and Screening Peptide Preparation

[0037] Peptides were prepared by standard Fmoc solid-phase procedures. (see for example Gras-Masse *et al.*, 1985).

[0038] Peptide #959, a 14 residue synthetic peptide having an N-terminal cysteine attached to a hydrophilic DGDGD spacer and residues 35-42 of human βA4 (resulting complete sequence: CDGDGDMVGGVVIA (SEQ ID NO: 1)), was coupled to a maleimide-activated KLH (Keyhole Limpet Hemocyanin) carrier using the commercially available "Imject" Activated Immunogen conjugation kit (Pierce). Briefly, 2 mg of peptide were dissolved in 200 μl of conjugation buffer and allowed to react at room temperature for 2 hrs with 2 mg of reconstituted maleimide-activated KLH. The conjugate was purified by gel filtration and used as an immunogen for monoclonal antibody production using standard protocols as described in Example 4.

[0039] Peptide #958, a 14 residue synthetic peptide having an N-terminal cysteine attached to a GGGGG spacer and residues 35-42 of the human βA4, (resulting complete sequence: CGGGGGMVGGVVIA (SEQ ID NO: 2)), was coupled to ovalbumin by dissolving 2 mg of peptide in 200 μl 6M guanidine, 0.01M phosphate pH 7.0 and conjugated

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as above to 2 mg of a reconstituted maleimide activated ovalbumin. The purified conjugate was used in ELISA-screening of monoclonal fusion products. Antibodies screened in this way are specific for the "35-42" determinant rather than the spacer, cysteine bridge or carrier portions of the immunogen.

[0040] Figure 6: Illustrates the peptide used to generate the immunresponse (the immunogen) and the peptide used to screen the sera of mice, as well as fusions, in the enzyme immunoassay plate (EIA) are shown. βA4 sequence 35-42 was synthesized together with a spacer and a C-terminal Cysteine, which was then used to couple it covalently via maleimide bridge to a large carrier molecule. Both, the spacer and the carrier molecule in immunogen and screening peptide are different in order to select for βA4 sequence specific antibodies.

ELISA (Enzyme Linked Immunosorbant Assay)

Biotinylation of MAb

[0041] The N-hydroxysuccinimide ester of biotin is used to biotinylate monoclonal antibody 286.8A. The integrity of the reagent is first verified by watching it's spontaneous hydrolysis in the absence of primary amines: an 0.2 mg/ml solution of NHS-LC-Biotin (Vector Labs, Burlingame, CA) in PBS is monitored at 260nm over time. An OD260 of 1.0 after approximately 2 hrs (rising from and initial OD260 - 0.55) indicates the expected hydrolysis.

[0042] In the biotinylation reaction a 66:1 molar ratio of Biotin to monoclonal 286.8A at neutral pH has been found to give optimal results when the biotinylated 286.8A was tested in an Elisa format. NHS-LC-Biotin 0.6 mg in H_2O at a concentration of 0.1 mg/ml is added (within 5 min of dissolving) to 1 ml (2 mg) of 286.8A in PBS. Nucleophilic attack of the NHS ester is allowed to occur at 25°C for 4 hrs after which 10 mg of glycine in 50 µl H_2O is added to stop the reaction. The reaction is then placed over a 10 ml cross-linked dextran desalting column equilibrated to PBS and 0.5 ml aliquots are collected. The first peak representing the IgG peak is pooled and stored at 4°C until used.

Elisa procedures

[0043] Corning 25801 96-well Elisa plates are coated overnight at 4°C with 100 µl monoclonal 4G8 or other capture antibody at 5 µg/ml typically in H_2O or buffer. The plate is then washed with PBS containing 1% Triton X-100 in a Dynatech Ultrawash plus. Wells are then blocked for 90 min with 300 µl PBS containing 1% Triton X-100 and 1% Elisa grade BSA (Blocking Buffer). After washing antigen or unknown diluted in blocking buffer is added to the wells in triplicates and incubated at room temperature for2 hrs. The plate is washed 2 times and 400 ng biotinylated 286.8A or other detecting antibody is added. After 30 min the plate is more extensively washed (2 times wash, 2 min soak, 2 times wash) and 100 µl preformed Avidin-Biotin-Alkaline Phosphatase Complexes (Vector Labs) are added. The plate is washed (2 times wash, 2 min soak, 2 times wash, 5 min soak, 4 washes) and MUP substrate added at 0.06 mg/ml 1x diethanolamine buffer. Plates are read in a Millipore Cytoflour after 15 min using a 360 nm excitation filter and a 460 nm emission filter.

Example 4: Generation of Monoclonal Antibodies

[0044] Balb/c mice were immunized with multiple I.P. inoculations of KLH conjugated peptide #959. Splenocytes from immunized animals were fused with the mouse myeloma AG8 using standard protocols (Wunderlich et al., 1992, J. Immunol. Methods 147:1-11). Supernatants from resultant hybridomas were screened for immunoreactivity to ovalbumin-coupled peptide #958 using standard Elisa protocols as described above. Hybridomas positive for the expression of immunoreactive MAbs were cloned at least twice by limiting dilution and MAb isotype analysis was performed. Purified MAb IgG was prepared from ascites fluid using protein-A affinity chromatography.

[0045] After fusion screening showed that immunization of mice with peptide #959 conjugated to KLH and screened in a solid-phase ELISA format with peptide #958 coupled to ovalbumin resulted in six positive parental signals (identified as 369.1 through 369.6). Both peptides have amino acids 35-42 of the βA4 region, different N-terminal spacers, and a cysteine for covalent coupling to carrier proteins (Figure 6). The free C-terminus with the charged carboxy group and a limited length of only 8 amino acids favors the generation of antibodies which are specifically directed against longer forms of βA4 peptides; shorter βA4 peptides ending before amino acid 42 would thus not be recognized.

[0046] Figure 6 diagrams the structure of the immunogen (carrier-peptide) and the screening peptide (carrier-screening peptide) used.

[0047] Two of the six parental signals were ultimately not clonable. Of the remaining four, two gave immunoprecipitation/scintillation signals only a few percent above normal non-immune controls; the other two (identified as 369.6 and 369.2) showed signals of 18% and 19% respectively. Production of monoclonal antibodies from ascities fluid and subsequent immunopurification of these clones was done. Table I compares the data obtained with the IPSA for hybridoma supernatants and purified antibodies.

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Table I

[0048]

Table 1.

Comparison of antibody activities in hybridoma cell lines. IPSA data represents the percent of in vitro translated βA4 which could be immunoprecipitated by either hybridoma supernatants or purified antibody.			
Cell Line	Isotype	IPSA (supernatant)	IPSA (purified)
369.1	IgG1/IgG2b	3%	N.D.
369.2	IgG1	19%	25% (with 5 μg)
369.3	IgG1	2%	N.D.
369.6	IgG2b	18%	7% (with 10 μg)

Example 5: Immunoprecipitation/Scintillation Assay for Hybridoma Screening

[0049] To develop and screen for monoclonal antibodies which recognize the βA4 peptide in solution rather than when attached to a solid phase, an assay was developed in which immunoprecipitation of an ^{35}S-methionine-labelled in vitro-translated βA4 (IVT βA4) is measured. A standard amount of in vitro translated βA4 is allowed to form antibody/antigen complexes in a solution which can be optimized for ionic strength, pH, and detergent composition. After the immune complexes are precipitated with Protein G (Omnisorb cells) and washed extensively, bound radioactivity is counted in a liquid scintillation counter, background is subtracted and the efficiency of precipitation calculated. This Immunoprecipitation/Scintillation assay (IPSA) allows for both the rapid identification and characterization of antibodies, and has been used to test a variety of βA4 antibodies. The assay can be applied in general to monoclonal hybridoma supernatants as well as polyclonal sera to identify antibodies which can be used for immunoprecipitations. Typically 18 IPSAs can be performed in one day. This is therefore a fast and informative secondary hybridoma screening method

[0050] Briefly, approximately 1.5 x 10^5 DPMs of ^{35}S-methionine-labeled in vitro-translated βA4 (IVT βA4) were added to 10 μl of a 10x immunoprecipitation buffer (150mM NaCl, 10%NP-40, 5% deoxycholic acid,-1% SDS, 500mM Tris pH8). To this, 90 μl of monoclonal cell supernatant from the monoclonal fusion of interest (our designation # 369) were added and allowed to react for 2 hrs at 4°C. Background was determined using 90 μl of supernatant of a non-reactive clone; the positive control was 90 μl of supernatant containing monoclonal antibody 286.8A which was made previously against a crude synthetic βA4(1-42) preparation. After 2 hrs, 40 μl of a 10% solution of Omnisorb cells (Calbiochem) equilibrated in 1x immunoprecipitation buffer (RIPA buffer, 150mM NaCl, 1%NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50mM Tris pH8) were added and allowed to react for an additional 2 hrs at 4°C with rocking. The cells were pelleted by centrifugation for 5 min at 4500 g and 4°C, and washed 3x with 800 μl cold 1x immunoprecipitation buffer. Pellets were quantitatively transferred to scintillation vials and counted in a Beckman LS6000 scintillation counter in the Auto DPM mode. The percentage of βA4 immunoprecipitated was calculated.

[0051] Immunoprecipitation/Scintillation assays were performed with 1 μg of purified monoclonal antibody 369.2B in a total volume of 100 μl 1x immunoprecipitation buffer to which 5 μg of competing peptide were added. Incubations and precipitations were as described above.

[0052] Figure 7 depicts the percentage of immunoprecipitated IVT βA4 as a function of antibody concentration for MAbs 369.2, 369.6 and MAb 286.8A. Under the conditions of this assay, 369.2 (and further subclone 369.2B) is approximately four times better than 369.6 in immunoprecipitating soluble IVT βA4, but precipitates a little less than half as much as 286.8A. Figure 7 shows the results of Immunoprecipitation of in vitro translated βA4 vs. antibody concentration (μg antibody/100 μl RIPA buffer) where;

$$\text{Percent } \beta A4 \text{ immunoprecipitated} = \frac{(\text{dpms with MAb}) - (\text{dpms with non or preimmune control})}{(\text{total } \beta A4 \text{ dpms/reaction})}$$

Percents with a given MAb concentration varied only a few percentage points between and within experiments.

IPSA for Monoclonal Characterization

[0053] Approximately 1.5 x 10^5 DPMs of ^{35}S-methionine-labeled in vitro-translated βA4 were added to various amounts of purified monoclonal antibody, either 369.2B, 369.6, or 286.8A, in a total volume of 100 μl 1x immunoprecipitation buffer, and allowed to react as described above. Immune complexes were precipitated with Omnisorb, washed, and counted as described above.

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Example 6: Characterization of MAb 369.2B

[0054] To further characterize the best cell line, 369.2B, a competition immunoprecipitation/scintillation assay (Competition IPSA) was performed. In this variation 369.2B was added to an approximate 200 fold molar excess of unlabelled competitor peptide at the same time as labelled in vitro translated βA4 1-42. As expected, peptides to the human βA4 region, 1-40, 1-11, 1-28, 12-28, as well as the reverse peptide 40-1 did not compete with the ^{35}S-methionine-labeled in vitro-translated βA4 for immunoprecipitation, whereas the complete 1-42 peptide did (Figure 8).

[0055] These results were corroborated in a solid-phase ELISA format: the absorbed ovalbumin-coupled screening peptide which contains the βA4 region 35-42, as well as the 1-42 peptide, compete whereas 1-40 did not (Figure 9). The decreased competitiveness of the 1-42 peptide compared to the ovalbumin coupled 35-42 may be due to conformational and/or solubility factors involving the antigenic determinant, or perhaps more simply to the particular stoichiometry of the conjugation (ovalbumin, a carrier with molecular weight of 45 kD compared to 4 kD for the 1-42 peptide, and having somewhere between 5-15 conjugatable maleimide groups per mole of carrier).

[0056] Figure 8 shows Immunoprecipitation Peptide competition/Scintillation Assay for epitope determination of MAb 369.2. Peptide competitor (5 μg) was mixed with in vitro translated βA4 (-1.5 x 10^5 DPMs or -200 pg) then immunoprecipitated with 2 μg 369.2, where;

$$\text{Percent } \beta A4 \text{ immunoprecipitated} = \frac{(\text{dpms with MAb})-(\text{dpms with non or preimmune control})}{(\text{total } \beta A4 \text{ dpms/reaction})}$$

Percents with a given MAb concentration varied typically only a few percentage points between and within experiments.

[0057] Figure 9 shows epitope mapping of MAb 369.2 by competitive assay. C369.2 (50ng IgG/100 μl) was preincubated with or without synthetic competitor peptides (22°C, 1 hr), then subjected to Elisa against plate-bound Ovalbumin-coupled 35 - 42 (200ng/well). Percent competition was calculated relative to MAb binding in the absence of competitor, i.e. where;

$$\%\ \text{competitor} = \frac{(\text{signal w/o competitor}) - [(\text{signal w competitor}) - (\text{background})]}{(\text{signal w/o competitor})}$$

The solid square is 35-42(OVA) peptide conjugate; the open square is 1-42 peptide; and the solid diamond is 1-40 peptide.

[0058] From this data we conclude that monoclonal 369.2B is specific for the C-terminal end of the full length (1-42) βA4 peptide. Although the exact antigenic determinant has not been fine mapped, it clearly involves residues beyond position 40 and, since the antibody was made to a short synthetic peptide the determinant is unlikely to involve other residues of βA4 which may be conformationally juxtaposed. Specifically, 369.2B is a very important tool in recognizing βA4 species ending at position 42.

[0059] One additional and interesting observation from the peptide competition assay is the enhanced immunoprecipitability of in vitro-translated βA4 by the decapeptide 25-35. This phenomena has also been seen in assays using one other monoclonal antibody (i.e., 286.8A) as well as one rabbit polyclonal antisera (data not shown). We also know from other experiments using varying amounts of detergent, specifically SDS, in IPSA assays with MAb 286.8A, that more βA4 can be immunoprecipitated with increasing amounts of detergent (data not shown). SDS, interestingly, has been shown to be ineffective in solubilizing βA4 aggregates, at least as shown by SDS-PAGE (Burdick et al., 1992). However it is not immediately clear why SDS should enhance the immunoprecipitability of βA4.

Example 7: Immunohistochemical Studies

[0060] We have undertaken immunohistochemistry studies with 369.2B. The staining pattern of 369.2B (1/10,000 dilution of a 22 mg/ml ascities purified antibody solution) when compared to the monoclonal antibody 286.8A which we have shown recognizes epitope 3-8 of βA4 and is human specific (data not shown) showed interesting differences. Results obtained from immunohistochemistry demonstrated that 369.2B is an excellent antibody (at 1/10,000 dilution) to specifically label amyloid plaques cores, diffuse as well as fibrillar amyloid deposits and vascular amyloid deposits (Figure 10).

[0061] Figure 10 is a photomicrograph showing β-amyloid plaques, blood vessels, and perivascular deposits of βA4 in a paraffin embedded 10 μm thick section from the brain of a 76 year old female patient with Alzheimer's disease. Tissue sections were pretreated with 88% formic acid (30 min), then labeled using an avidin-biotin-peroxidase kit (Vector Laboratories, Burlington, CA) with nickel-diaminobenzidine as the chromagen. Monoclonal antibody 369.2B labels plaques with a variety of morpologies, including cored, perivascular, and diffuse (non-amyloidotic) plaques. It also labels a subset of amyloidotic blood vessels.

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[0062] Further studies also showed that βA4 1-43 peptide was not able to compete for staining (more than a 1000 fold excess peptide), whereas βA4 1-42 completely abolished the signal (Table 2). Again as expected, 1-40 or 40-1 did not compete for staining. From these studies we can already conclude that this antibody is an excellent tool for immunohistochemistry. As suggested by in vitro studies which show physico-chemical differences between 1-40 and 1-42, it is possible that these two βA4 species show distinct patterns in Alzheimer brains. With the monoclonal antibody of the instant invention, we are now able to begin addressing this question. Thus the monoclonal antibody and methods of the instant invention are useful for diagnostic and therapeutic uses in all immunological and related methodologies which can be applied to the detection, monitoring, extraction, inhibition and modification of unique βA4 species, in the diagnosis and treatment of AD.

Table 2.

Results from competition binding experiments and inhibition of staining, a +++ indicates strong staining, - indicates no detectable staining.		
	Monoclonal Antibody used for Staining	
Competing peptide	N-terminal Mab 286.8A	C-terminal Mab 369,2B
None/buffer	+++	+++
None/DMSO	+++	+++
Human "40-1"	+++	+++
Human "1-16"	-	+++
Mouse "1-16"	+++	+++
Human "1-40"	-	+++
Human "1-42"	-	-
Human "1-43"	-	+++
Human "35-42" with spacer	+++	-

[0063] It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Graham, Paul

(ii) TITLE OF INVENTION: Monoclonal Antibody Specific for BA4 Peptide

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

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(E) COUNTRY: USA

(F) ZIP: 60606

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

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(B) REGISTRATION NUMBER: 26,949

(C) REFERENCE/DOCKET NUMBER: 95,216

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(B) TELEFAX: 312-715-1234

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Asp Gly Asp Gly Asp Met Val Gly Gly Val Val Ile Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Cys Gly Gly Gly Gly Gly Met Val Gly Gly Val Val Ile Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 59 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 4..5

(D) OTHER INFORMATION: /label= Beta
/note= "Beta cleavage site in APP"

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 20..21

(D) OTHER INFORMATION: /label= Alpha
/note= "Alpha cleavage site in APP, residues 16/17
of BA4."

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 46..47

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(D) OTHER INFORMATION: /label= Gamma
/note= "Gamma cleavage site in APP"

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 5..47
(D) OTHER INFORMATION: /label= BA4
/note= "BA4 peptide"

(ix) FEATURE:
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(B) LOCATION: 33..56
(D) OTHER INFORMATION: /label= Tm
/note= "Transmembrane region of APP"

(ix) FEATURE:
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(B) LOCATION: 1..32
(D) OTHER INFORMATION: /label= Ex
/note= "N-terminal extracellular part of APP"

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Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
 1               5                  10                  15
His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
            20                  25                  30
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
        35                  40                  45
Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys
    50                  55
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Claims

1. A monoclonal antibody that binds specifically to βA4(1-42) peptide associated with diffuse amyloid, fibrillar amyloid, neurofibrillary tangles, and vascular amyloid, and not to βA4(1-40) and βA4(1-43) peptides.

2. A monoclonal antibody of claim 1 that recognizes the C-terminal amino acid sequence Val-Val-Ile-Ala of the βA4 (1-42) peptide.

3. A monoclonal antibody of claim 1 or claim 2 that is an IgG class antibody.

4. A monoclonal antibody of claim 3 that is an IgG1 or IgG2b subclass antibody.

5. A monoclonal antibody identified as 369.2B, and is produced by the cell line deposited with the American Type Culture Collection (ATCC) as ATCC number HB 11829.

6. A protein which is an immunologically active fragment of the monoclonal antibody of any one of claims 1 to 5.

7. A cell which is identified by ATCC number HB 11829.

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8. A method of detecting the presence of βA4 peptide ending at amino acid 42 Ala in tissue comprising contacting a tissue sample with protein of any one of claims 1 to 6, and detecting the presence of said protein.

9. A method of selectively purifying βA4 peptide ending at amino acid 42 Ala comprising contacting a sample to be purified with protein of any one of claims 1 to 6, separating the βA4 peptide ending at amino acid 42 Ala from the sample to be purified, and isolating the βA4 peptide ending at amino acid 42 Ala.

10. A method of detecting βA4 peptide ending at amino acid 42 Ala associated with Alzheimer's Disease, comprising contacting a sample to be tested with protein of any one of claims 1 to 6, and detecting the presence of βA4 peptide ending at amino acid 42 Ala.

11. A method of preventing aggregation of βA4 peptide ending at amino acid 42 Ala *in vitro* comprising administering protein of any one of claims 1 to 6.

12. A kit comprising a means for detecting the presence of βA4 peptide ending at amino acid 42 Ala which is a protein of any one of claims 1 to 6, and a suitable detectable signal.

13. A kit comprising a means for preventing the aggregation of βA4 peptide ending at amino acid 42 Ala which is a protein of any one of claims 1 to 6.

14. A method for generating the antibody of claim 1, comprising immunizing a mammal with the peptide Cys-Asp-Gly-Asp-Gly-Asp-Met-Val-Gly-Gly-Val-Val-Ile-Ala (SEQ ID NO:1) conjugated to a suitable immunological carrier.

15. A monoclonal antibody that binds specifically to βA4(1-42) peptide, and not to βA4(1-40) and βA4(1-43) peptides, obtainable by immunizing a mammal with the peptide Cys-Asp-Gly-Asp-Gly-Asp-Met-Val-Gly-Gly-Val-Val-Ile-Ala (SEQ ID NO:1) conjugated to a suitable immunological carrier.

16. A use of a peptide conjugated to a suitable immunological carrier for the preparation of a medicament for the treatment and/or prophylaxis of Alzheimer's Disease.

17. A use of a peptide conjugated to a suitable immunological carrier for the preparation of a medicament for the treatment and/or prophylaxis of Alzheimer's Disease, wherein the conjugated peptide generates an antibody that selectively binds βA4(1-42)peptide and not βA4(1-40) and βA4(1-43)peptide.

18. The use according to any of claims 16 or 17 wherein the conjugated peptide is SEQ ID NO:1.

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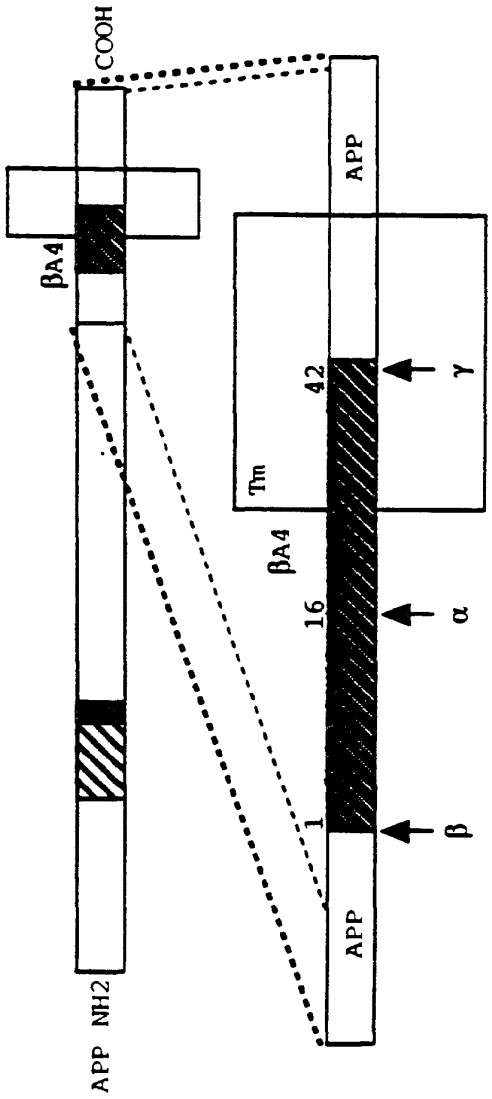


Figure 1

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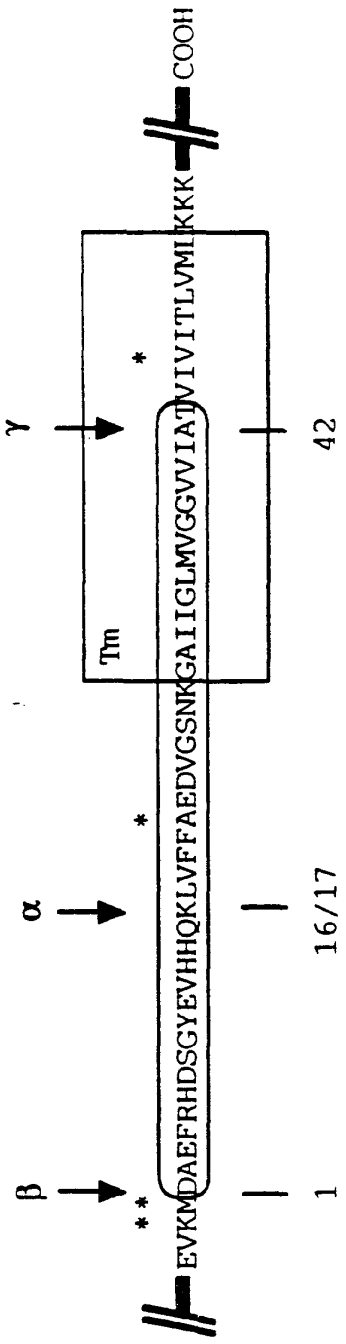


Figure 2

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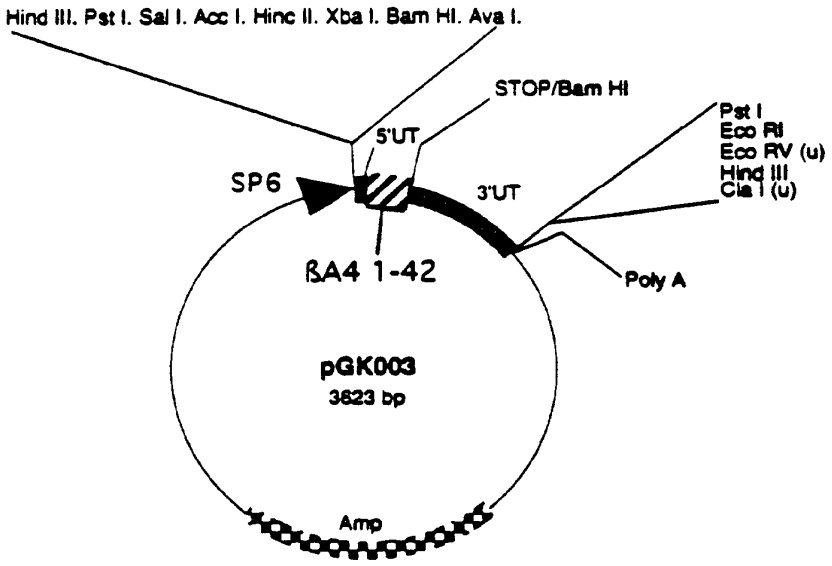


Fig. 3

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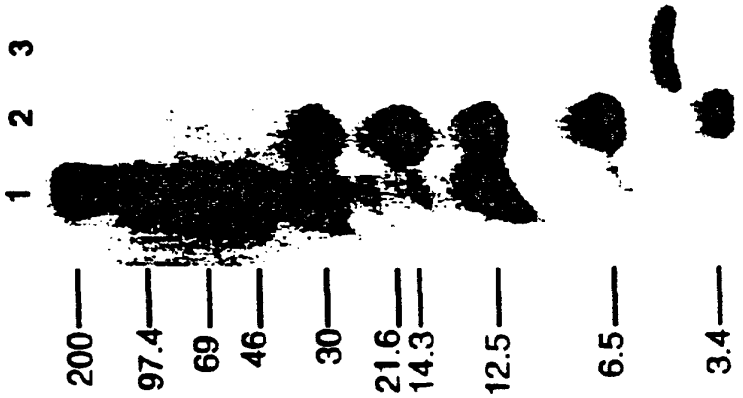


Fig 4B.

e gel.

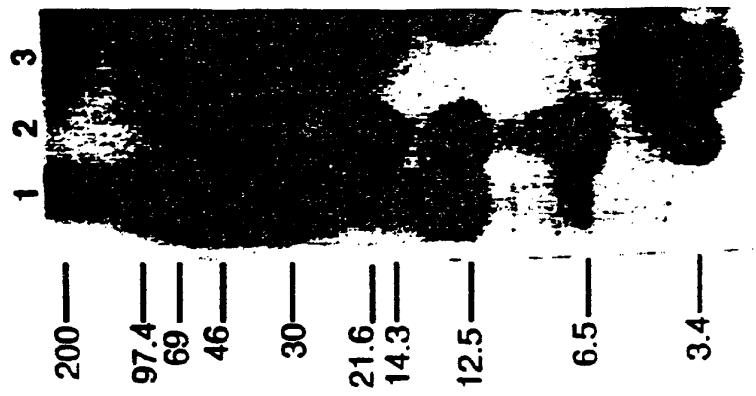


Fig. 4A.

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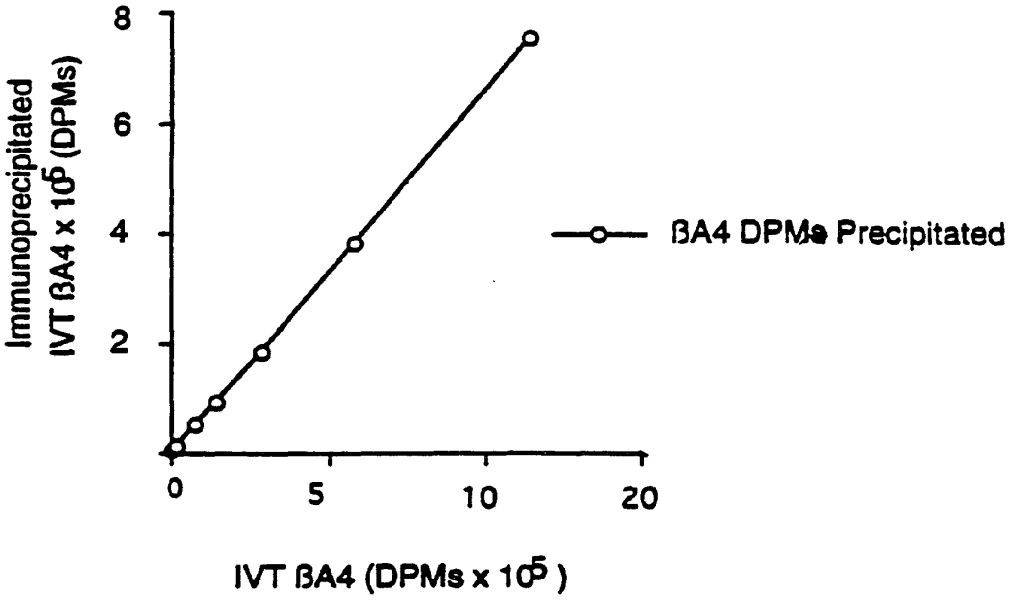
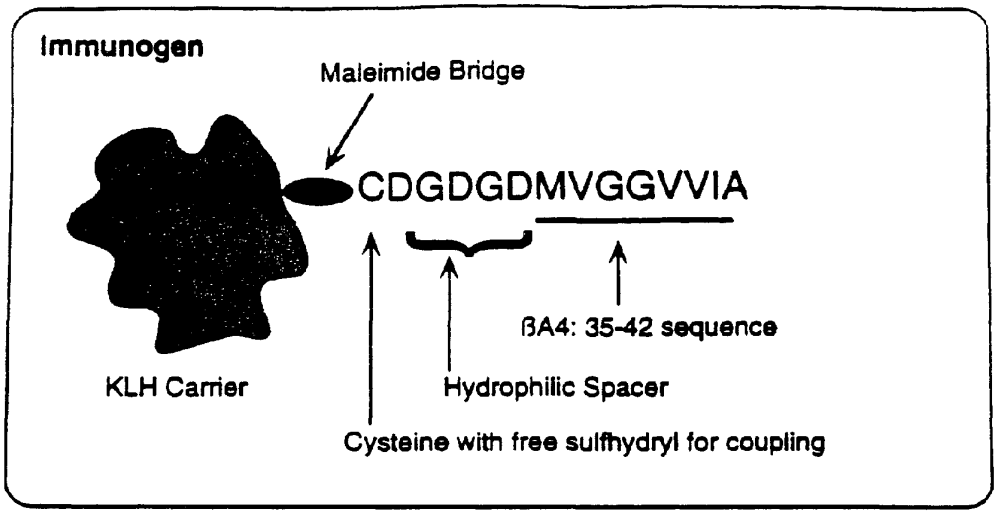


Fig. 5

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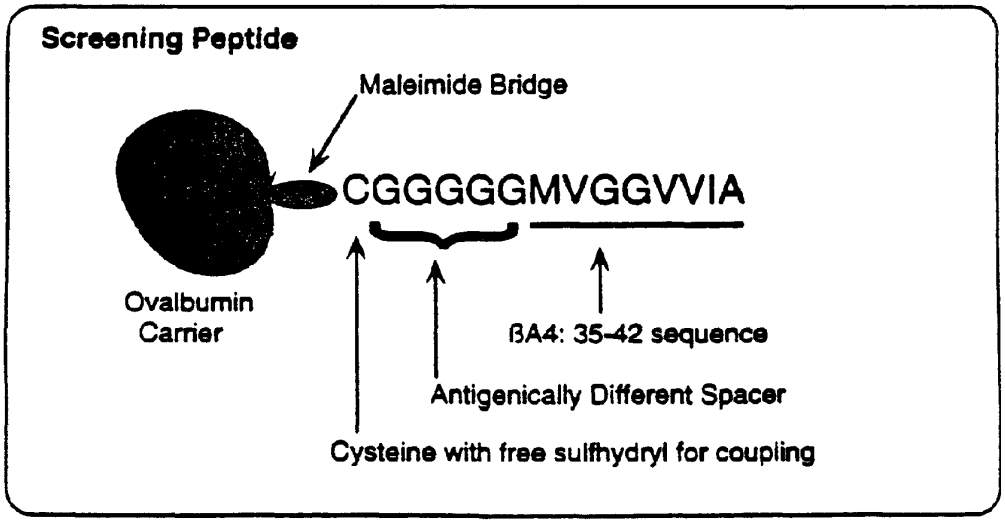


Fig 6

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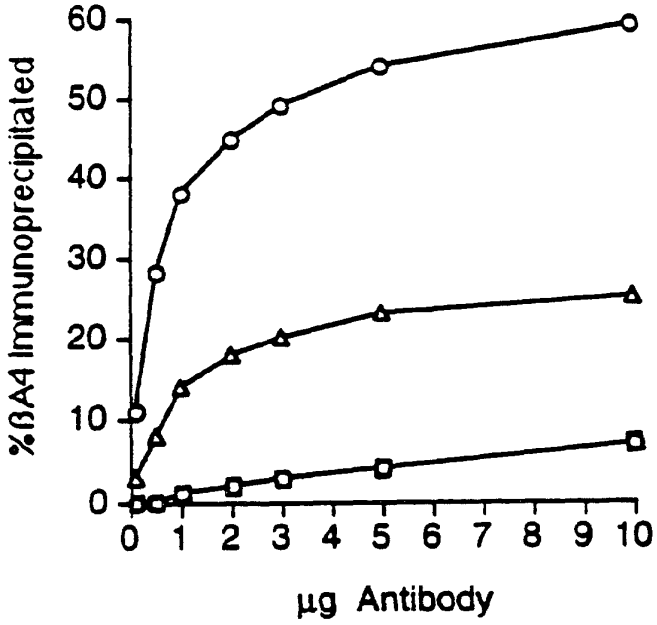


Fig. 7.

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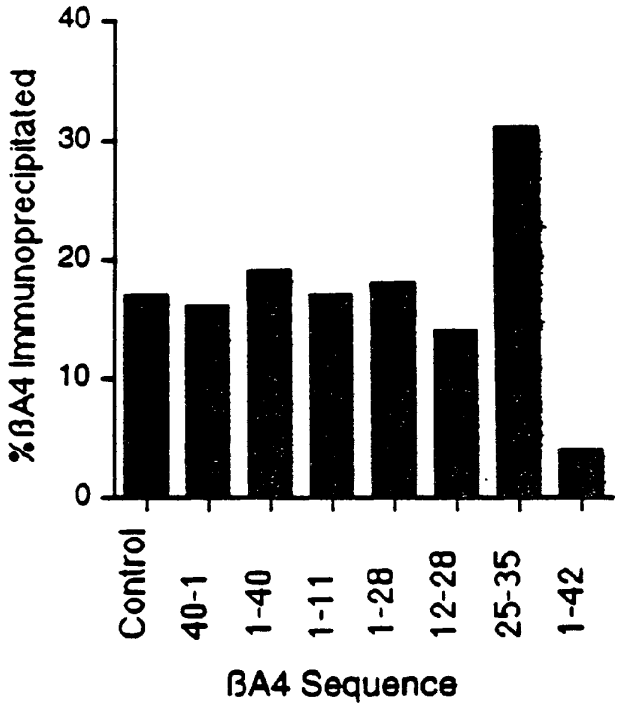


Fig. 8.

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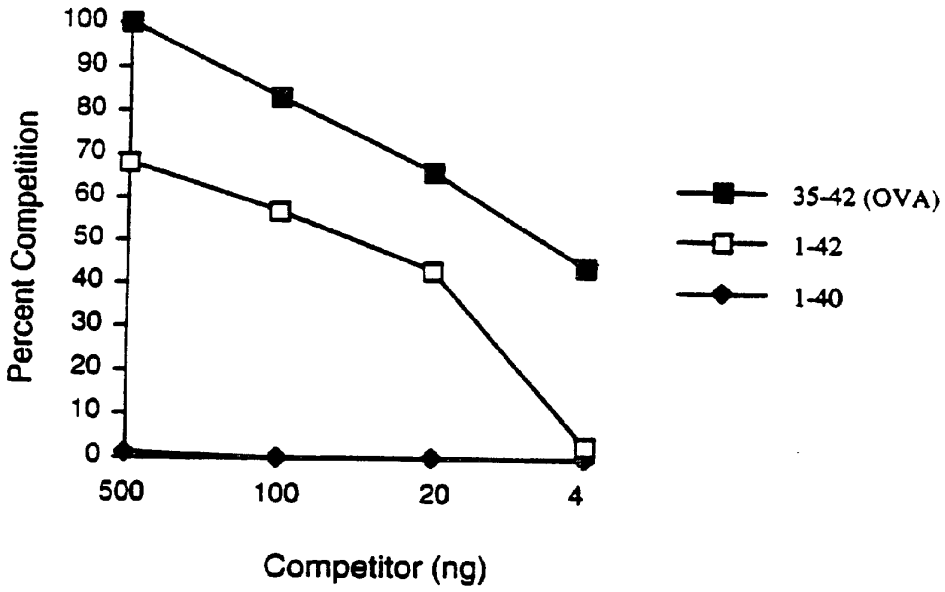


Fig. 9.

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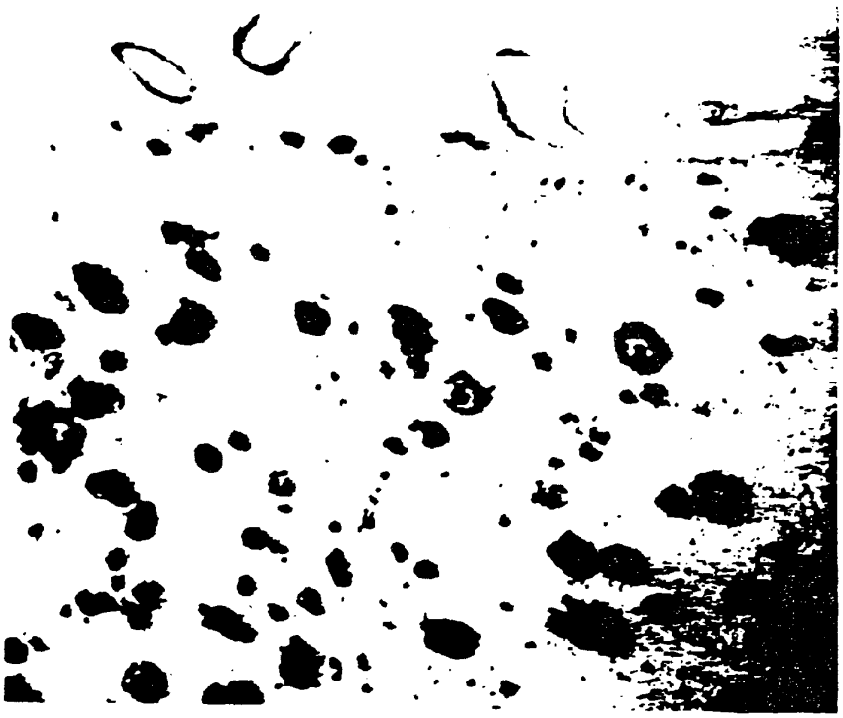


Fig. 10

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European Patent Office
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(11) EP 1 298 436 A2

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Remarks:
This application was filed on 07 - 10 - 2002 as a divisional application to the application mentioned under INID code 62.

(54) Beta-amyloid peptide (BAP) release inhibitor compounds for treating BAP-related diseases and methods for their identification

(57) Elevated levels of βAP in body fluids, such as blood and cerebrospinal fluid, is associated with the presence of a βAP-related condition in a patient, such as Alzheimer's Disease. Soluble β-amyloid peptide (βAP) is measured in biological fluids at very low concentrations, typically in the range from 0.1 ng/ml to 10 ng/ml. The measurement of βAP concentrations in animals or conditioned medium from cultured cells is used for drug screening, where test compounds are administered to the animals or exposed to the cultured cells and the accumulation of βAP in the animal or culture medium observed.

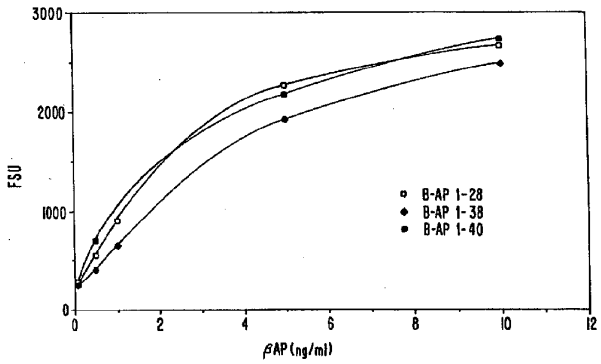


FIG. 1

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Description

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0001] The present invention relates generally to compounds and compositions for treating soluble β-amyloid peptide (βAP) related disorders. The present invention also related to screening methods for the identification of inhibitors of βAP production where βAP is detected *in vitro* or *in vivo* and to diagnostic methods where βAP is detected in patient samples.

[0002] Alzheimer's Disease (AD) is a degenerative brain disorder characterized clinically by progressive loss of memory, cognition, reasoning, judgment and emotional stability that gradually leads to profound mental deterioration and ultimately death. AD is a very common cause of progressive mental failure (dementia) in aged humans and is believed to represent the fourth most common medical cause of death in the United States. AD has been observed in races and ethnic groups worldwide and presents a major present and future public health problem. The disease is currently estimated to affect about two to three million individuals in the United States alone. AD is at present incurable. No treatment that effectively prevents AD or reverses its symptoms and course is currently known.

[0003] The brains of individuals with AD exhibit characteristic lesions termed senile (or amyloid) plaques, amyloid angiopathy (amyloid deposits in blood vessels) and neurofibrillary tangles. Large numbers of these lesions, particularly amyloid plaques and neurofibrillary tangles, are generally found in several areas of the human brain important for memory and cognitive function in patients with AD. Smaller numbers of these lesions in a more restricted anatomical distribution are also found in the brains of most aged humans who do not have clinical AD. Amyloid plaques and amyloid angiopathy also characterize the brains of individuals with Trisomy 21 (Down's Syndrome) and Hereditary Cerebral Hemorrhage with Amyloidosis of the Dutch-Type (HCHWA-D). At present, a definitive diagnosis of AD usually requires observing the aforementioned lesions in the brain tissue of patients who have died with the disease or, rarely, in small biopsied samples of brain tissue taken during an invasive neurosurgical procedure.

[0004] The principal chemical constituent of the amyloid plaques and vascular amyloid deposits (amyloid angiopathy) characteristic of AD and the other disorders mentioned above is an approximately 4.2 kilodalton (kD) protein of about 39-43 amino acids designated the β-amyloid peptide (βAP) or sometimes Aβ, AβP or β/A4. βAP was first purified and a partial amino acid sequence reported in Glenner and Wong (1984) Biochem. Biophys. Res. Commun. 120:885-890. The isolation procedure and the sequence data for the first 28 amino acids are described in U.S. Patent No. 4,666,829.

[0005] Molecular biological and protein chemical analyses conducted during the last six years have shown that βAP is a small fragment of a much larger precursor protein, referred to as the β-amyloid precursor protein (APP), that is normally produced by cells in many tissues of various animals, including numans. Knowledge of the structure of the gene encoding APP has demonstrated that βAP arises as a peptide fragment that is cleaved from APP by as-yet-unknown enzymes (proteases). The precise biochemical mechanism by which the βAP fragment is cleaved from APP and subsequently deposited as amyloid plaques in the cerebral tissue and in the walls of cerebral and meningeal blood vessels is currently unknown.

[0006] Several lines of evidence indicate that progressive cerebral deposition of βAP plays a seminal role in the pathogenesis of AD and can precede cognitive symptoms by years or decades (for review, see Selkoe (1991) Neuron 6:487). The single most important line of evidence is the discovery in 1991 that missense DNA mutations at amino acid 717 of the 770-amino acid isoform of APP can be found in affected members but not unaffected members of several families with a genetically determined (familial) form of AD (Goate et al. (1991) Nature 349:704-706; Chartier Harlan et al. (1991) Nature 353:844-846; and Murrell et al. (1991) Science 254:97-99) and is referred to as the Swedish variant. A double mutation changing lysine^{595}-methionine^{596} to asparagine^{595}-leucine^{596} (with reference to the 695 isoform) found in a Swedish family was reported in 1992 (Mullan et al. (1992) Nature Genet 1:345-347). Genetic linkage analyses have demonstrated that these mutations, as well as certain other mutations in the APP gene, are the specific molecular cause of AD in the affected members of such families. In addition, a mutation at amino acid 693 of the 770-amino acid isoform of APP has been identified as the cause of the βAP deposition disease, HCHWA-D, and a change from alanine to glycine at amino acid 692 appears to cause a phenotype that resembles AD in some patients but HCHWA-D in others. The discovery of these and other mutations in APP in genetically based cases of AD proves that alteration of APP and subsequent deposition of its βAP fragment can cause AD.

[0007] Despite the progress which has been made in understanding the underlying mechanisms of AD and other βAP-related diseases, there remains a need to develop methods and compositions for diagnosis and treatment of the disease(s). Treatment methods could advantageously be based on drugs which are capable of inhibiting the generation of βAP *in vivo*. To identify such drugs, it would be desirable to provide screening assays for potential drugs which can inhibit βAP generation in *in vivo* and *in vitro* models. It would be further desirable to provide methods and compositions for diagnosis of βAP-related conditions, where the diagnosis is based on detection of βAP in patient fluid samples.

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Specific assays for βAP detection should be capable of detecting βAP in fluid samples at very low concentrations as well as distinguishing between βAP and other fragments of APP which may be present in the sample.

2. Description of the Background Art

[0008] Glenner and Wong (1984) Bioahem. Biophys. Res. Commun. 120:885-890 and U.S. Patent No. 4,666,829, are discussed above. The '829 patent suggests the use of an antibody to the 28 amino acid βAP fragment to detect "Alzheimer's Amyloid Polypeptide" in a patient sample and diagnose AD. No data demonstrating detection or diagnosis are presented.

[0009] Numerous biochemical electron microscopic and immunochemical studies have reported that βAP is highly insoluble in physiologic solutions at normal pH. See, for example, Glenner and Wong (1984) Biochem. Biophys. Res. Commun. 122:1131-1135; Masters et al. (1985) Proc. Natl. Acad. Sci. USA 82:4245-4249; Selkoe et al. (1986) J. Neurochem. 46:1820-1834? Joachim et al. (1988) Brain Research 474:100-111; Hilbich et al. (1991) J. Mol. Biol. 218: 149-163; Barrow and Zagorski (1991) Science 253:179-182; and Burdick et al. (1992) J. Biol. Chem. 267:546-554. Furthermore, this insolubility was predicted by and is consistent with the amino acid sequence of βAP which includes a stretch of hydrophobic amino acids that constitutes part of the region that anchors the parent protein (APP) in the lipid membranes of cells. Hydrophobic, lipid-anchoring proteins such as βAP are predicted to remain associated with cellular membranes or membrane fragments and thus not be present in physiologic extracellular fluids. The aforementioned studies and many others have reported the insolubility in physiologic solution of native βAP purified from AD brain amyloid deposits or of synthetic peptides containing the βAP sequence. The extraction of βAP from cerebral amyloid deposits and its subsequent solubilization has required the use of strong, non-physiologic solvents and denaturants. Physiologic, buffered salt solutions that mimic the extracellular fluids of human tissues have uniformly failed to solubilize βAP.

[0010] Separate attempts to detect APP or fragments thereof in plasma or CSF have also been undertaken. A large secreted fragment of APP that does not contain the intact βAP region has been found in human cerebrospinal fluid (Palmert et al. (1989) Proc. Natl. Acad. Sci. USA 86:6338-6342; Weidemann et al. (1989) Cell 57:115-126; Henriksson et al. (1991) J. Neurocbem. 56:1037-1042; and Palmert et al. (1990) Neurology 40:1028-1034); and plasma (Podlisny et al. (1990) Biochem. Biophys. Res. Commun. 167:1094-1101). The detection of fragments of the carboxy-terminal portion of APP in plasma has also been reported (Rumble et al. (1989) N. Engl. J. Med 320:1446-1452) as has the failure to detect such fragments (Schlossmacher et al. (1992) Neurobiol. Aging 13:421-434).

[0011] Despite the apparent insolubility of native and synthetic βAP, it has been speculated that βAP could occur in body fluids, such as cerebrospinal fluid (CSF) or plasma (Wong et al. (1985) Proc. Natl. Acad. Sci. USA 92:8729-8732; Selkoe (1986) Neurobiol. Aging 7:425-432; Pardridge et al. (1987) Biochem. Biophys. Res. Commun. 145:241-248; Joachim et al. (1989) Nature 341:226-230; Selkoe et al. (1989) Neurobiol. Aging 10:387-395).

[0012] Several attempts to measure βAP in CSF and plasma have been reported by both radioimmunoassay methods (Pardridge et al. (1987) Biochem. Biophys. Res. Commun., *supra,* and WO90/12870 published November 1, 1990) and sandwich ELISAs (Wisniewski in *Alzheimer's Disease*, eds. Becker and Giacobini, Taylor and Francas, N.Y. pg. 206, 1990; Kim and Wisniewski in *Techniques in Diagnostic Pathology,* eds. Bullock et al., Academic Press, Boston pg. 106; and WO90/12871 published November 1, 1990). While these reports detected very low levels of βAP immunoreactivity in bodily fluids, attempts to directly purify and characterize this immunoreactivity further and determine whether it represented βAP were not pursued, and the efforts were abandoned. The possibility of βAP production by cultured cells was neither considered nor demonstrated. Retrospectively, the inability to readily detect βAP in bodily fluids was likely due to the presence of amyloid precursor fragments with overlapping regions or fragments of βAP that obscured measurements and to the lack of antibodies completely specific for intact βAP. In fact, the previous findings by both Pardridge et al. and Kim et al. reported levels of βAP four-to-fivefold lower than that shown in the present invention. This is presumably because the antibodies used by both groups would crossreact with other APP fragments containing part of βAP known to be present in CSF thereby interfering with the measurement, if any, of intact βAP. The present invention overcomes these difficulties with the use of monoclonal antibodies specific to an epitope in the central junction region of intact βAP.

[0013] EP 444,856 provides a means of diagnosing Alzheimer's disease using a sandwich immunoassay to "Alzheimer's Disease Associated Protein" (ADAP). ADAP is defined as a material reactive with the monoclonal antibody termed Alz50, originally described by Wolozin et al. (1986) Science 232:648-650. Alz50 has more recently been shown to react specifically with phosphorylated forms of tau (Ksiezak-Reder et al. (1988) J. Biol. Chem. 263:7943-7947; Ueda et al. (1990) J. Neuroscience 10:3295-3304; Lee et al. (1991) Science 251:675-678). Hence, ADAPs represent phosphorylated forms of tau and are unrelated to the amyloid precursor protein of βAP described in this invention.

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SUMMARY OF THE INVENTION

[0014] The present invention provides methods and compositions useful for the identification of β-amyloid peptide (βAP) production inhibitors as well as for the diagnosis and monitoring of βAP-related conditions in patients, where the methods and compositions rely on the specific detection of soluble βAP and/or βAP fragments in fluid samples. For the identification of βAP production inhibitors, a test compound is introduced to an *in vitro* or *in vivo* βAP generation model, and the effect of the test compound on the amount of soluble βAP or βAP fragment generated by the model is observed. Particularly useful as an *in vitro* model are cell lines which express APP variants which overproduce βAP. Test substances which affect the production of βAP and/or βAP fragments, usually by reducing the amount produced, are considered to be likely candidates for further testing for use as therapeutic drugs in the treatment of βAP-related conditions, particularly Alzheiner's Disease. For the diagnosis and monitoring of βAP-related conditions, the amount of soluble βAP and/or βAP fragments in a patient sample,

[0015] The present invention provides a method for detecting a soluble β-amyloid peptide (βAP) in a fluid sample in the presence of β-amyloid precursor protein (APP) or fragments thereof, said method comprising capturing soluble βAP from the sample using a first binding substance under conditions in which the first specific binding substance binds to an epitope at a junction region on the βAP and which is substantially free from cross-reactivity with APP and fragments thereof other than βAP, wherein the junction region is centered at the site between βAP amino acid residues 16 and 17 and is a target for normal proteolytic cleavage; and detecting capture of the soluble βAP using a labelled second binding substance which binds to an epitope on a second region of the soluble βAP other than the epitope on the βAP which is bound by the first binding substance.

[0016] In preferred embodiments the βAP is intact βAP. The soluble βAP may be captured on a solid phase, and the capture is detected by exposing the solid phase to the labelled second binding substance and thereafter detecting the presence of the label on the solid phase.

[0017] The invention also provides a method for detecting a soluble β-amyloid peptide (βAP) in a fluid sample, said method comprising exposing the sample to a first binding substance specific for an epitope at a junction region on soluble βAP disposed between amino acid residues 13 to 28; and detecting binding between the first binding substance and the soluble βAP under conditions where the first binding substance binds to the βAP without substantial cross-reactivity with β-amyloid precursor protein (APP) and fragments thereof other than βAP.

[0018] In preferred embodiments the first binding substance is an antibody, optionally a monoclonal antibody, raised against a peptide consisting of amino acid residues 13 to 28 of βAP.

[0019] The binding of the first binding substance and the soluble βAP may be detected by separating bound complexes of the binding substance and βAP exposing the separated bound complexes to a labelled second binding substance specific for the N-terminal region of βAP, and detecting the presence of label on the bound complexes.

[0020] The second binding substance may be an antibody, optionally a monoclonal antibody, raised against a peptide consisting of amino acid residues 1-16 of βAP.

[0021] The invention also includes a method for detecting a soluble β-amyloid peptide (βAP) in a fluid sample which may contain βAP and βAP fragments of β-amyloid precursor protein (APP) other than βAP, said method comprising exposing the fluid sample to a first binding substance under conditions in which the first binding substance will bind to an epitope on soluble βAP and βAP fragments but will not bind to epitopes on APP fragments which may be present in the sample; and detecting binding between the first binding substance and the soluble βAP and βAP fragments.

[0022] In a preferred embodiment the first binding substance binds specifically to intact βAP or to an approximately 3 kD fragment of βAP. The first binding substance may be immobilized on a solid phase, and binding is detected by exposing the solid phase to a labeled second binding substance and thereafter detecting the presence of the label on the solid phase.

[0023] In other preferred a further embodiments the fluid sample for use in methods of the present invention is selected from the group consisting of culture medium, blood, CSF, urine, and peritoneal fluid.

[0024] The present invention also includes a method for identifying β-amyloid peptide (βAP)production inhibitors said method comprising culturing mammalian cells in a culture medium under conditions which result in production of a soluble βAP peptide which can be detected in the culture medium; exposing the cultured cells to a test compound; and determining whether the test compound affects the amount of soluble βAP peptide present in the culture medium.

[0025] In preferred embodiments the determination of βAP peptide may be specific for soluble βAP present in the culture medium.

[0026] In a further embodiment of the invention, the effect of the test compound is determined by comparing the accumulation of βAP in the culture medium over a time period in the absence of the test compound with the accumulation of βAP in the culture medium in the presence of the test compound over the same time period.

[0027] Also provided by the invention is a method for assaying a test compound for the ability to inhibit β-amyloid peptide (βAP) production by cells, said method comprising culturing a first population of mammalian cells in a culture medium under conditions which result in generation of a soluble βAP peptide which can be detected in the culture

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medium; culturing a second population of the same mammalian cells in a second culture medium under identical conditions to the first population, except that the test compound is present in the second culture medium; measuring the amounts of soluble βAP present in the culture media of the first population and the second population of cells; and comparing the measured amounts of βAP to determine whether the test compound has had an effect on soluble βAP peptide generation by the cultured cells.

[0028] In preferred embodiments the measurement is specific for soluble βAP present in the culture media of the first and second population of cells.

[0029] The cultured mammalian cells used in the methods for identifying and screening for β-amyloid peptide (βAP) production inhibitors, may be from a cell line, optionally human, comprising DNA encoding an amyloid precursor protein (APP) isotype or variant which provides overproduction of βAP in the cultured mammalian cells.

[0030] Test compounds of the invention may be present at a concentration in the range 1 nM to 1mM.

[0031] The invention also includes a method for identifying β-amyloid (βAP) production inhibitors, said method comprising administering a test compound to a mammalian transgenic host comprising DNA encoding an amyloid precursor protein (APP) isotype or variant which provides overproduction of βAP in cultured mammalian cells comprising said DNA; and determining whether the test compound affects the amount of a soluble βAP peptide present in a body fluid.

[0032] The effect of the test compound may be determined by measuring a baseline amount of soluble βAP peptide in a body fluid prior to administering the test compound and remeasuring the amount of soluble βAP in the body fluid after said administering.

[0033] In preferred embodiments the mammalian transgenic host is selected from the group consisting of monkeys, dogs, rabbits, guinea pigs, rats and mice. Optionally, the mammalian transgenic host is a transgenic host having enhanced susceptibility to βAP plaque deposition compared to a mammal of the same species not comprising said DNA.

[0034] In other preferred embodiments the body fluid is selected from the group consisting of blood, CSF, urine and peritoneal fluid. Optionally, the test compound is administered orally, topically, intramuscularly, intravenously, subcutaneously intraventricularly, or intraperitoneally.

[0035] The soluble βAP peptide may be intact βAP or an approximately 3 kD fragment of βAP. The βAP fragment may consist of amino acid residues 11-40 or 17-40 of βAP.

[0036] The DNA may encode an APP having one or several amino acid substitutions directly amino-terminal of the βAP cleavage site. Preferably, the DNA may encode an APP comprising leucine as the residue immediately amino N-terminal to βAP cleavage site. Optionally, said DNA encodes an APP comprising the Swedish mutation (a double mutation $Lys^{595} \rightarrow Asn^{595}$ and $Met^{596} \rightarrow Leu^{596}$).

[0037] The present invention also includes a β-amyloid peptide (βAP) production inhibitor that affects the amount of soluble βAP peptide present in the culture medium of a cell line of mammalian cells that are cultured under conditions which result in soluble βAP which can be detected in the culture medium.

[0038] In preferred embodiments the cultured mammalian cells are from a cell line optionally human comprising DNA encoding an amyloid precursor protein (APP) isotype or variant which provides overproduction of βAP in the cultured mammalian cells.

[0039] The inhibitor of β-amyloid peptide (βAP) production preferably affects the amount of soluble βAP in the culture medium of the mammalian cell line when present at a concentration in the range 1 nM to 1mM.

[0040] Also provided by the present invention is a β-amyloid (βAP) production inhibitor that when administered to a non-human mammalian host affects the amount of a soluble βAP peptide present in a body fluid of that host.

[0041] The mammalian host may be transgenic and has DNA encoding an amyloid precursor protein (APP) isotype or variant which provides overproduction of βAP in cultured mammalian cells comprising said DNA. In a preferred embodiment the non-human mammalian host is selected from the group consisting of monkeys, dogs, rabbits, guinea pigs, rats and mice.

[0042] The non-human mammalian transgenic host may have enhanced susceptibility to βAP plaque deposition compared to a mammal of the same species not comprising said DNA.

[0043] The body fluid may be selected from the group consisting of blood, CSF, urine and peritoneal fluid, optionally wherein the inhibitor is administered orally, topically, intramuscularly, intravenously, subcutaneously intraventricularly, or intraperitoneally.

[0044] In preferred embodiments the soluble βAP peptide, of which production is affected, is intact βAP or an approximately 3 kD fragment of βAP. Optionally, the βAP fragment consists of amino acid residues 11-40 or 17-40 of βAP. In one aspect of the invention, said DNA encodes an APP having one or several amino acid substitutions directly amino-terminal of the βAP cleavage site. In an optional embodiment said DNA encodes an APP comprising leucine as the residue immediately amino N-terminal to βAP cleavage site.

[0045] The present invention also includes a β-amyloid peptide (βAP) production inhibitor as claimed in any preceding claim for use as a pharmaceutical.

[0046] Furthermore, the invention provides for the use of a β-amyloid peptide (βAP) production inhibitor of any of claims 31 to 45 for the manufacture of a medicament for the treatment of Alzheimer's Disease or other βAP-related

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conditions.

[0047] The invention provides a method for identifying β-amyloid peptide (βAP) production inhibitors is provided, said method comprising culturing mammalian cells in a culture medium under conditions which result in generation of a soluble βAP peptide which can be detected in the culture medium; exposing the cultured cells to a test compound; and determining whether the test compound affects the amount of soluble βAP peptide present in the culture medium.

[0048] The culture cells are preferably from a human cell line. The human cell line preferably comprises DNA encoding an amyloid precursor protein (APP) isotype or variant which provides overproduction of βAP in the cultured mammalian cells. Preferably the DNA encodes an APP comprising leucine as the residue immediately amino N-terminal to βAP cleavage site.

[0049] The cultured cells may be exposed to test compounds at concentrations in the range from 1 nM to 1mM.

[0050] The effect of the test compound preferably determined by comparing the accumulation of βAP in the culture medium over a predetermined time period in the absence of the test compound with the accumulation of βAP in the culture medium in the presence of the test compound over the same time period.

[0051] The soluble βAP peptide may be intact βAP. Alternatively, the soluble βAP peptide may be an approximately 3 kD fragment of βAP.

[0052] The invention also provides a method for assaying a test compound for the ability to inhibit β-amyloid peptide (βAP) production by cells, said method comprising: culturing a first population of mammalian cells in a culture medium under conditions which result in generation of a soluble βAP peptide which can be detected in the culture medium; culturing a second population of the same mammalian cells in a second culture medium under identical conditions to the first population, except that the test compound is present in the second culture medium; measuring the amounts of soluble βAP present in the culture media of the first population and the second population of cells; and comparing the measured amounts of βAP to determine whether the test compound has had an effect on soluble βAP peptide generation by the cultured cells.

[0053] The cultured cells are preferably from a human cell line.

[0054] The test compound is preferably present in the second culture medium at a concentration in the range from 1 nM to 1 mM.

[0055] The soluble βAP peptide may be intact βAP. Alternatively the soluble βAP peptide may be an approximately 3 kD fragment of βAP.

[0056] The invention further provides a method for identifying β-amyloid (βAP) production inhibitors, said method comprising administering a test compound to a mammalian host; and determining whether the test compound affects the amount of a soluble βAP peptide present in a body fluid.

[0057] The mammalian host is preferably selected from the group consisting of monkeys, dogs, rabbits, guinea pigs, rats, and mice.

[0058] The mammalian host is preferably a transgenic host having enhanced susceptibility to βAP plaque deposition.

[0059] The transgenic host may comprise DNA encoding an amyloid precursor protein variant.

[0060] The body fluid may be selected from the group consisting of blood, CSF, urine, and peritoneal fluid.

[0061] The test compound may be administered orally, topically, intramuscularly, intravenously, subcutaneously intraventricularly, or intraperitoneally.

[0062] The effect of the test compound may be determined by measuring a baseline amount of soluble βAP peptide in a body fluid prior to administering the test compound and remeasuring the amount of soluble βAP in the body fluid after a predetermined time period has elapsed.

[0063] The soluble βAP peptide may be intact βAP. Alternatively the soluble βAP peptide may be an approximately 3 kD fragment of βAP.

[0064] The invention also provides a method for diagnosing or monitoring a β-amyloid peptide (βAP)-related condition in a patient, said method comprising measuring the amount of soluble βAP or βAP fragments in a patient sample; comparing the measured amount with a predetermined amount of βAP or βAP fragments; and assessing patient status based on a difference between the measured and predetermined βAP or βAP fragment amounts.

[0065] The predetermined amount of βAP may be a normal value and the method provides for diagnosis.

[0066] The patient sample may be blood and the predetermined amount of intact βAP may be in the range from 0.1 ng/ml to 10 ng/ml.

[0067] The patient sample may be CSF and the predetermined amount of intact βAP may be in the range from 0.1 ng/ml to 25 ng/ml.

[0068] The predetermined amount of βAP is a value measured from the same patient at an earlier time and the method provides for monitoring.

[0069] The amount of soluble βAP or βAP fragment is preferably measured by exposing the patient sample to a first binding substance specific for a junction region on soluble βAP disposed between amino acid residues 13 to 28 and detecting binding between the first binding substance and the soluble βAP. The first binding substance may be an antibody raised against a peptide consisting essentially of amino acid residues 13 to 28 of βAP.

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[0070] Binding of the first binding substance and the soluble βAP may be detected by separating bound complexes of the binding substance and βAP, exposing the separated bound complexes to a labeled second binding substance specific for the N-terminal region of βAP, and detecting the presence of label on the bound complexes. The second binding substance may be an antibody raised against a peptide consisting essentially of amino acid residues 1-16 of βAP.

BRIEF DESCRIPTION OF THE DRAWINGS

[0071]

Fig. 1 is a graph illustrating the detection of three synthetic βAP peptides using ELISA assay format with monoclonal antibody 266 as the captured antibody and monoclonal antibody 10D5 as the reporter antibody. Antibody 266 was prepared against a synthetic peptide including amino acid residues 13-28 of βAP. Antibody 10D5 was raised against a synthetic peptide including amino acid residues 1-28 of βAP.

Figs. 2A and 2B are charts comparing the plasma and CSF concentrations of βAP in normal control and AD patients.

Fig. 3 is a chart comparing the CSF concentration of βAP in normal controls (C), Alzheimer's Disease patients (AD), cerebral vascular accident (CVA) patients (stroke), and Parkinson's Disease (PD) patients.

Fig. 4 is a Western blot of affinity-purified conditioned media from a cell line which overexpresses APP. The affinity-purified material from the conditioned media of human mixed-brain cell cultures which had been further purified by reversed-phase chromatography.

Fig. 5 is an autoradiogram demonstrating the presence of soluble βAP in the culture fluid of human kidney 293 cells. The βAP was immunoprecipitated from the culture fluid with a βAP-specific antibody to residues 1-40 of βAP.

Fig. 6 is an autoradiogram demonstrating that βAP in the media of human kidney 293 cells is fully soluble and that it remains in the post-10^5xg supernatant and is not found in the post-10^5xg pellet after ultracentrifugation.

Fig. 7 shows the quantitation of βAP (left panel) and the secreted β-amyloid precursor protein (APPs) (right panel) in conditioned media in transiently transfected 293 cells using two distinct sandwich ELISAs. Each column represents the mean of four transfection experiments with normal or variant APP constructs with the exception of the mock column, which is based on three transfection experiments.

Fig. 8 is an autoradiogram demonstrating the levels of soluble βAP in the culture fluid of human kidney 293 cells transfected with normal or variant APP_{695} constructs.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

[0072] The present invention results from the discovery that detectable amounts of soluble β-amyloid peptide (βAP) and βAP fragments are generated continuously at low concentrations by a wide variety of mammalian cells. In particular, it has been found that such βAP peptides are generated *in vitro* by cultured mammalian cells and may be measured in the conditioned culture medium of numerous mammalian cell lines. It has been further found that βAP peptides are present in the body fluids of various mammalian hosts, and that elevated levels of βAP peptides are associated with βAP-related conditions, such as Alzheimer's Disease and Down's Syndrome.

[0073] Based on this discovery, the present invention provides both methods for drug screening to identify potential βAP generation inhibitors and methods for diagnosing and monitoring βAP-related conditions. Both methods rely on the measurement of very low βAP concentrations in a fluid sample, typically in the range from 0.1 ng/ml to 10 ng/ml, with the present invention further providing highly sensitive and specific methods for performing such measurements. In particular, detection methods of the present invention provide for measurement of βAP at threshold concentrations of 0.1 ng/ml and below, and are sufficiently specific to distinguish βAP from other fragments of the β-amyloid precursor protein (APP) which contain precursor amino acids in addition to the 39-43 amino acids that comprise the βAP region.

[0074] The mechanism of βAP and βAP fragment generation is not presently understood. It is possible that intact or full length βAP is produced intracellularly and thereafter released or secreted into the extracellular fluid, i.e., body fluids *in vivo* and conditioned cell culture medium *in vitro.* Alternatively, it is possible that a precursor protein or fragment, which may be the entire APP or a portion thereof containing the βAP region, is secreted or released from the mammalian cells and processed outside of the cellular source. Regardless of the particular mechanism, the present invention relies on the detection and measurement of the concentrations or amounts of βAP and βAP fragments in extracellular fluids, including conditioned culture medium and body fluids, as discussed in more detail below.

[0075] The term "β-amyloid peptide (βAP) as used herein refers to an approximately 4.2 kD protein which, in the brains of AD, Down's Syndrome, HCHWA-D and some normal aged subjects, forms the subunit of the amyloid filaments comprising the senile (amyloid) plaques and the amyloid deposits in small cerebral and meningeal blood vessels (amyloid angiopathy). βAP can occur in a filamentous polymeric form (in this form, it exhibits the Congo-red and thioflavin-S dye-binding characteristics of amyloid described in connection therewith). βAP can also occur in a non-filamentous

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form ("preamyloid" or "amorphous" or "diffuse" deposits) in tissue, in which form no detectable birefringent staining by Congo red occurs. A portion of this protein in the insoluble form obtained from meningeal blood vessels is described in U.S. Patent No. 4,666,829. βAP when used in connection with this invention, specifically refers to an approximately 39-43 amino acid peptide that is substantially homologous to the form of the protein produced by the method described in the patent of Glenner et al., but which, according to the instant invention, can be found in and purified from the extracellular fluid (medium) of cultured cells grown *in vitro* or from body fluids of humans and other mammals, including both normal individuals and individuals suffering from βAP-related conditions. Thus, βAP also refers to related βAP sequences that result from mutations in the βAP region of the normal gene. In whatever form, βAP is an approximately 39-43 amino acid fragment of a large membrane-spanning glycoprotein, referred to as the β-amyloid precursor protein (APP), encoded by a gene on the long arm of human chromosome 21. βAP is further characterized by its relative mobility in SDS-polyacrylamide gel electrophoresis or in high performance liquid chromatography (HPLC). Its 43-amino acid sequence is:

1
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr

11
Glu Val His His Gln Lys Leu Val Phe Phe

21
Ala Glu Asp Val Gly Ser Asn Lys Gly Ala

31
Ile Ile Gly Leu Met Val Gly Gly Val Val

41
Ile Ala Thr

or a sequence that is substantially homologous thereto.

[0076] The term "βAP peptides" as used herein refers to intact or full length βAP as well as to fragments and degradation products of βAP which are generated at low concentrations by mammalian cells. Particular βAP fragments have a molecular weight of approximately 3 kD and are presently believed to consist of amino acid residues 11-40 and 17-40 of βAP.

[0077] The term "βAP junction region" as used herein refers to a region of βAP which is centered at the site between amino acid residues 16 and 17 (Lys^{16} and Leu^{17}) which is a target for normal proteolytic processing of APP. Such normal processing results in a variety of APP fragments which are potentially immunologically cross-reactive with the intact βAP molecule and fragments of βAP which are to be identified in the methods of the present invention. The junction region will span amino acid residues 10 to 35, preferably spanning amino acid residues 15 to 30, with antibodies raised against a synthetic peptide consisting of amino acid residues 13-28 having been found to display the requisite specificity.

[0078] The term "β-amyloid precursor protein" (APP) as used herein is defined as a polypeptide that is encoded by a gene of the same name localized in humans on the long arm of chromosome 21 and that includes βAP within its carboxyl third. APP is a glycosylated, single-membrane-spanning protein expressed in a wide variety of cells in many mammalian tissues. Examples of specific isotypes of APP which are currently known to exist in humans are the 695-amino acid polypeptide described by Kang et al. (1987) Nature 325:733-736 which is designated as the "normal" APP; the 751-amino acid polypeptide described by Ponte et al. (1988) Nature 331:525-527 (1988) and Tanzi et al. (1988) Nature 331:528-530; and the 770-amino acid polypeptide described by Kitaguchi et al. (1988) Nature 331:530-532. Examples of specific variants of APP include point mutations which can differ in both position and phenotype (for review of known variant mutations see Hardy (1992) Nature Genet. 1:233-234).

[0079] The term "APP fragments" as used herein refers to fragments of APP other than those which consist solely of βAP or βAP fragments. That is, APP fragments will include amino acid sequences of APP in addition to those which form intact βAP or a fragment of βAP.

[0080] The term "βAP-related condition" as used herein is defined as including Alzheimer's Disease (which includes familial Alzheimer's Disease), Down's Syndrome, HCHWA-D, and advanced aging of the brain.

[0081] The terms "conditioned culture medium" and "culture medium" as used herein refer to the aqueous extracellular fluid which surrounds cells grown in tissue culture (*in vitro*) and which contains, among other constituents, proteins

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and peptides secreted by the cells.

[0082] The term "body fluid" as used herein refers to those fluids of a mammalian host which will be expected to contain measurable amounts of βAP and βAP fragments, specifically including blood, cerebrospinal fluid (CSF), urine, and peritoneal fluid. The term "blood" refers to whole blood, as well as blood plasma and serum.

[0083] According to the present invention, βAP and βAP fragments may be detected and/or measured in a variety of biological and physiological samples, including *in vitro* samples, such as conditioned medium from cultured cells, including transfected cell lines and endogenous cell lines, and *in vivo* patient samples, typically body fluids. Detection and measurement of βAP peptides may be accomplished by any technique capable of distinguishing βAP and βAP fragments from other APP fragments which might be found in the sample. Conveniently, immunological detection techniques may be employed using binding substances specific for βAP, such as antibodies, antibody fragments, recombinant antibodies, and the like, which bind with specificity and sensitivity to βAP. In particular, it has been found that antibodies which are monospecific for the junction region of βAP are capable of distinguishing βAP from other APP fragments. The junction region of βAP is centered at amino acid residues 16 and 17, typically spanning amino acid residues 13-28, and such junction-specific antibodies may be prepared using synthetic peptides having that sequence as an immunogen. Particularly suitable detection techniques include ELISA, Western blotting, radioimmunoassay, and the like.

[0084] A preferred immunoassay technique is a two-site or "sandwich" assay employing a junction-specific antibody as the capture antibody (bound to a solid phase) and a second labeled antibody which binds to an epitope other than that bound to by the capture antibody. The second labeled antibody preferably recognizes the amino terminus of βAP and may be conveniently raised against a synthetic peptide consisting essentially of amino acid residues 1-16 of βAP. Particular methods for preparing such antibodies and utilizing such antibodies in an exemplary ELISA are set forth in the Experimental section hereinafter.

[0085] Other non-immunologic techniques for detecting βAP and βAP fragments which do not require the use of βAP specific antibodies may also be employed. For example, two-dimensional gel electrophoresis may be employed to separate closely related soluble proteins present in a fluid sample. Antibodies which are cross-reactive with many fragments of APP, including βAP, may then be used to probe the gels, with the presence of βAP being identified based on its precise position on the gel. In the case of cultured cells, the cellular proteins may be metabolically labeled and separated by SDS-polyacrylamide gel electrophoresis, optionally employing immunoprecipitation as an initial separation step. A specific example of the latter approach is described in the Experimental section hereinafter.

[0086] Antibodies specific for the βAP may be prepared against a suitable antigen or hapten comprising the desired target epitope, such as the junction region consisting of amino acid residues 13-28 and the amino terminus consisting of amino acid residues 1-16. Conveniently, synthetic peptides may be prepared by conventional solid phase techniques, coupled to a suitable immunogen, and used to prepare antisera or monoclonal antibodies by conventional techniques. Suitable peptide haptens will usually comprise at least five contiguous residues within βAP and may include more than six residues.

[0087] Synthetic polypeptide haptens may be produced by the well-known Merrifield solid-phase synthesis technique in which amino acids are sequentially added to a growing chain (Merrifield (1963) J. Am. Chem. Soc. 85:2149-2156). The amino acid sequences may be based on the sequence of βAP set forth above.

[0088] Once a sufficient quantity of polypeptide hapten has been obtained, it may be conjugated to a suitable immunogenic carrier, such as serum albumin, keyhole limpet hemocyanin, or other suitable protein carriers, as generally described in Hudson and Hay, *Practical Immunology,* Blackwell Scientific Publications, Oxford, Chapter 1.3, 1980, the disclosure of which is incorporated herein by reference. An exemplary immunogenic carrier utilized in the examples provided below is α-CD3∈ antibody (Boehringer-Mannheim, Clone No. 145-2C11).

[0089] Once a sufficient quantity of the immunogen has been obtained, antibodies specific for the desired epitope may be produced by *in vitro* or *in vivo* techniques. *In vitro* techniques involve exposure of lymphocytes to the immunogens, while *in vivo* techniques require the injection of the immunogens into a suitable vertebrate host. Suitable vertebrate hosts are non-human, including mice, rats, rabbits, sheep, goats, and the like. Immunogens are injected into the animal according to a predetermined schedule, and the animals are periodically bled, with successive bleeds having improved titer and specificity. The injections may be made intramuscularly, intraperitoneally, subcutaneously, or the like, and an adjuvant, such as incomplete Freund's adjuvant, may be employed.

[0090] If desired, monoclonal antibodies can be obtained by preparing immortalized cell lines capable of producing antibodies having desired specificity. Such immortalized cell lines may be produced in a variety of ways. Conveniently, a small vertebrate, such as a mouse is hyperimmunized with the desired immunogen by the method just described. The vertebrate is then killed, usually several days after the final immunization, the spleen cells removed, and the spleen cells immortalized. The manner of immortalization is not critical. Presently, the most common technique is fusion with a myeloma cell fusion partner, as first described by Kohler and Milstein (1975) Nature 256:495-497. Other techniques including EBV transformation, transformation with bare DNA, e.g., oncogenes, retroviruses, etc., or any other method which provides for stable maintenance of the cell line and production of monoclonal antibodies. Specific techniques

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for preparing monoclonal antibodies are described in *Antibodies: A Laboratory Manual,* Harlow and Lane, eds., Cold Spring Harbor Laboratory, 1988, the full disclosure of which is incorporated herein by reference.

[0091] In addition to monoclonal antibodies and polyclonal antibodies (antisera), the detection techniques of the present invention will also be able to use antibody fragments, such as F(ab), Fv, V_L, V_H, and other fragments. In the use of polyclonal antibodies, however, it may be necessary to adsorb the anti-sera against the target epitopes in order to produce a monospecific antibody population. It will also be possible to employ recombinantly produced antibodies (immunoglobulins) and variations thereof as now well described in the patent and scientific literature. See, for example, EPO 8430268.0; EPO 85102665.8; EPO 85305604.2; PCT/GB 85/00392; EPO 85115311.4; PCT/US86/002269; and Japanese application 85239543, the disclosures of which are incorporated herein by reference. It would also be possible to prepare other recombinant proteins which would mimic the binding specificity of antibodies prepared as just described.

[0092] *In vivo* detection of βAP in patient samples can be used for diagnosing and monitoring of Alzheimer's Disease and other βAP-related conditions, such as Down's Syndrome and HCHWA-D. Suitable patient samples include body fluids, such as blood, CSF, urine, and peritoneal fluid. The presence of the βAP-related condition will generally be associated with elevated levels of βAP in the fluid when compared to those values in normal individuals, i.e., individuals not suffering from Alzheimer's Disease or any other βAP-related condition. Diagnostic concentrations of βAP in blood are in the range from 0.1 ng/ml to 10 ng/ml or higher, more generally 0.1 ng/ml to 3 ng/ml. Diagnostic concentrations of βAP in CSF are in the range from 0.1 ng/ml to 25 ng/ml or higher, more generally 0.1 ng/nl to 5 ng/ml.

[0093] In addition to initial diagnosis of the βAP-related condition, the measured concentrations of βAP may be monitored in order to follow the progress of the disease, and potentially follow the effectiveness of treatment (when such treatments become available). It would be expected that levels of βAP would decrease with an effective treatment regimen.

[0094] *In vitro* monitoring of βAP levels in conditioned culture medium from a suitable cell culture may be used for drug screening. By growing cells under conditions which result in the accumulation of βAP in the conditioned culture medium, and exposing the cultured cells to test compounds, the effect of these test compounds on βAP production may be observed. It would be expected that test compounds which are able to diminish the amount of βAP accumulation would be candidates for testing as inhibitors of βAP generation. Suitable cell lines include human and animal cell lines, such as the 293 human kidney cell line, human neuroglioma cell lines, human HeLa cells, primary human endothelial cells (e.g. HUVEC cells), primary human fibroblasts or lymphoblasts, primary human mixed brain cells (including neurons, astrocytes, and neuroglia), Chinese hamster ovary (CHO) cells, and the like.

[0095] Preferred for use in drug screening methods according to the present invention are cell lines capable of expressing APP variants which overproduce βAP. By "overproduce," it is meant that the amount of βAP produced from the variant APP will be greater than the amount produced from any or all of the normal APP isoforms, e.g., the 695, 751, and 770 amino acid isoforms which have been previously described. Particularly preferred are APP variants having one or several amino acid substitutions directly amino-terminal of the βAP cleavage site. For example, as shown in the Experimental section herein, K293 cells which express an APP DNA bearing a double mutation (Lys^{595}->Asn^{595} and Met^{596}->Leu^{596}) found in a Swedish FAD family produce approximately six-to-eightfold more βAP than cells expressing normal APP. The mutation at residue 596 appears to be principally responsible for the increase.

[0096] Similarly, *in vivo* monitoring of βAP in animal models, such as the mouse animal model disclosed in WO 91/19810, the disclosure of which is incorporated herein by reference, and animal models expressing other APP isotypes and/or variants, may also be used to screen compounds for therapeutic effectiveness (usually for testing of compounds which have previously been identified by an *in vitro* screen, such as the *in vitro* screen described above). The test compound(s) are administered to the animal and the level of βAP or βAP fragment in a body fluid observed. Test compounds which reduce the level of the βAP in certain body fluids are considered to be candidates for further evaluation.

[0097] The test compounds can be any molecule, compound, or other substance which can be added to the cell culture without substantially interfering with cell viability. Suitable test compounds may be small molecules, biological polymers, such as polypeptides, polysaccharides, polynucleotides, and the like. The test compounds will typically be administered to the culture medium at a concentration in the range from about 1 nM to 1 mM, usually from about 10 μM to 1 mM. Test compounds which are able to inhibit generation, accumulation, or secretion of βAP are considered as candidates for further determinations of the ability to decrease βAP production in cells and/or animals.

[0098] The present invention further comprises methods for inhibiting β-amyloid production in cells, where the method includes administering to the cells compounds selected by the method described above. The compounds may be added to cell culture in order to inhibit βAP production by the cultured cells. The compounds may also be administered to a patient in order to inhibit the deposition of amyloid plaque associated with Alzheimer's and other βAP-related diseases.

[0099] The present invention further comprises pharmaceutical compositions incorporating a compound selected by the above-described method and including a pharmaceutically acceptable carrier. Such pharmaceutical compositions

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should contain a therapeutic or prophylactic amount of at least one compound identified by the method of the present invention. The pharmaceutically acceptable carrier can be any compatible, non-toxic substance suitable to deliver the compounds to an intended host. Sterile water, alcohol, fats, waxes, and inert solids may be used as the carrier. Pharmaceutically acceptable adjuvants, buffering agents, dispersing agents, and the like may also be incorporated into the pharmaceutical compositions. Preparation of pharmaceutical conditions incorporating active agents is well described in the medical and scientific literature. See, for example, Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania, 16th Ed., 1982, the disclosure of which is incorporated herein by reference.

[0100] The pharmaceutical compositions just described are suitable for systemic administration to the host, including both parenteral, topical, and oral administration. The pharmaceutical compositions may be administered parenterally, i.e. subcutaneously, intramuscularly, or intravenously. Thus, the present invention provides compositions for administration to a host, where the compositions comprise a pharmaceutically acceptable solution of the identified compound in an acceptable carrier, as described above.

[0101] Frequently, it will be desirable or necessary to introduce the pharmaceutical compositions directly or indirectly to the brain. Direct techniques usually involve placement of a drug delivery catheter into the host's ventricular system to bypass the blood-brain barrier. Indirect techniques, which are generally preferred, involve formulating the compositions to provide for drug latentiation by the conversion of hydrophilic drugs into lipid-soluble drugs. Latentiation is generally achieved through blocking of the hydroxyl, carboxyl, and primary amine groups present on the drug to render the drug more lipid-soluble and amenable to transportation across the blood-brain barrier. Alternatively, the delivery of hydrophilic drugs can be enhanced by intraarterial infusion of hypertonic solutions which can transiently open the blood-brain barrier.

[0102] The concentration of the compound in the pharmaceutical carrier may vary widely, i.e. from less than about 0.1 % by weight of the pharmaceutical composition to about 20% by weight, or greater. Typical pharmaceutical composition for intramuscular injection would be made up to contain, for example, one to four ml of sterile buffered water and one μg to one mg of the compound identified by the method of the present invention. The typical composition for intravenous infusion could be made up to contain 100 to 500 ml of sterile Ringer's solution and about 1 to 100 mg of the compound.

[0103] The pharmaceutical compositions of the present invention can be administered for prophylactic and/or therapeutic treatment of diseases related to the deposition of βAP, such as Alzheimer's disease, Down's syndrome, and advanced aging of the brain. In therapeutic applications, the pharmaceutical compositions are administered to a host already suffering from the disease. The pharmaceutical compositions will be administered in an amount sufficient to inhibit further deposition of βAP plaque. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Such effective dose will depend on the extent of the disease, the size of the host, and the like, but will generally range from about 0.01 μg to 10 mg of the compound per kilogram of body weight of the host, with dosages of 0.1 μg to 1 mg/kg being more commonly employed.

[0104] For prophylactic applications, the pharmaceutical compositions of the present invention are administered to a host susceptible to the βAP-related disease, but not already suffering from such disease. Such hosts may be identified by genetic screening and clinical analysis, as described in the medical literature (e.g. Goate (1991) Nature 349: 704-706). The pharmaceutical compositions will be able to inhibit or prevent deposition of the βAP plaque at a symptomatically early stage, preferably preventing even the initial stages of the β-amyloid disease. The amount of the compound required for such prophylactic treatment, referred to as a prophylactically-effective dosage, is generally the same as described above for therapeutic treatment.

[0105] The following examples are offered by way of illustration, not by way of limitation.

EXPERIMENTAL

Materials and Methods

1. Antibody Preparation.

a. Monoclonal Antibodies to the βAP Junction Region.

[0106] Monoclonal antibodies to the junction region of βAP were prepared using a synthetic peptide spanning amino acid residues 13-28 (βAP_{13-28}). βAP_{13-28} was conjugated to an immunogen (α-CD3∈ antibody; Clone No. 145-2C11, Boehringer-Mannheim) using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) according to the manufacturer's (Pierce) instructions.

[0107] A/J mice were immunized initially intraperitoneally (IP) with the βAP conjugate mixed with complete Freund's adjuvant. Fourteen days later, the mice were boosted IP with the βAP conjugate mixed with phosphate buffered saline (PBS) at 14 day intervals. After six total boosts, the mice were finally boosted intravenously with βAP conjugate mixed

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with PBS and fused 3 days later. Fusion of spleen cells with P3.653 myeloma cells was performed according as described in Oi and Herzenberg, *Selective Methods in Cellular Immunology,* Mishell and Shigii, Eds., W.H. Freeman and Company, San Francisco, Chapter 17 (1980). Serum titers and initial screens were performed by the RIA method described below. Several clones were expanded to a 24 well plate and subjected to further analysis as described below. Clones of interest were produced in mouse ascites.

[0108] The RIA method used to screen serum bleeds and fusion hybridoma supernatants was based upon a method developed by Wang et al. (1977) J. Immunol. Methods 18:157-164. Briefly, the supernatant (or serum) was incubated overnight at room temperature on a rotator with ^{125}I-labeled βAP_{1-28} and Sepharose® 4B beads to which sheep anti-mouse IgG had been coupled via cyanogen bromide. The beads from each well were harvested onto glass fiber filter discs with a cell harvester and washed several times with PBS. The filter discs were then transferred to gamma tubes and the bound radioactivity was counted in a gamma counter.

[0109] All hybridomas were tested for binding to βAP_{1-28} using the method described above in the initial screen, and then retested 3 days later. βAP_{1-28} positive clones were further characterized for reactivity to ^{125}I-labeled βAP_{1-16} using the RIA method described above. No clones were found to bind βAP_{1-16}. In a peptide capture ELISA, all clones were found to react with βAP_{13-28} while no clones reacted to βAP_{17-28}. Therefore, it was determined that all clones had an epitope within the junction region spanning amino acids 16 and 17.

[0110] Based on results of the above assays, several clones were expanded into 24 well plates. These clones were further characterized by saturation analysis. Supernatants at the 50% titer point (as determined by the RIA method described above) were added to wells containing Sepharose®-sheep anti-mouse IgG beads, a constant amount of ^{125}I-labeled βAP_{1-28}, and varying amounts of unlabeled βAP_{13-28} or βAP_{17-28}. The concentration of cold peptide for 50% inhibition was determined for each antibody. For the βAP_{17-28}, no inhibition was seen at 100 ng/well for any clones. The 50% inhibition point for βAP_{13-28} ranged from 10-80 ng/well. The clones were also characterized based on reactivity in Western blots. Based on titer point, sensitivity (as determined by the 50% inhibition point), and reactivity on Western blot, several clones were produced in ascites.

[0111] Antibodies from hybridomas designated 067, 266, 297, and 361 were selected for use as a capture antibody in the assays described below.

b. Monoclonal Antibodies to the N-terminal Region of βAP.

[0112] Monoclonal antibodies to the N-terminal region of βAP were prepared using a synthetic peptide spanning amino acid residues 1-28 (βAP_{1-28}). βAP_{1-28} was chemically coupled using disuccimidyl suberate (DSS) to rabbit serum albumin (RSA) using a 20:1 molar ratio of peptide to protein in 50 mM sodium phosphate, pH 7.0, 150 mM NaCl, overnight at 21°C using 1 mM DSS (Hyman et al. (1992) J. Neuropath. Exp. Neuro. 51:76).

[0113] Antibodies 10D5 and 6C6 were obtained from a fusion where mice had received 5 injections of βAP_{1-28} coupled to RSA via DSS at 100 μg/ml. The initial injection was in complete Freund's adjuvant (CFA) followed by second and subsequent injections in incomplete Fruend's adjuvant (IFA) every 10-14 days. Three days before the fusion, mouse 4 which had a titer of 1/70,000 as measured by ELISA against βAP_{1-28}, received 100μg of βAP_{1-28} RSA in PBS intraperitoneally as a final boost. Screening was done by ELISA and on paraffin-fixed AD brain sections. The coating concentration of βAP_{1-28} was 1 μg/well. 10D5 and 6C6 were positive by ELISA and AD brain tissue section.

[0114] Antibodies from hybridomas designated 10D5 and 6C6 were selected for use as a reporter antibody in the assays described below.

c. Polyclonal Antibodies.

[0115] Polyclonal antibodies were raised against synthetic peptides βAP_{1-38}, βAP_{1-40}, and βAP_{1-42}, and were designated anti-βAP_{1-38} (antiserum Y), anti-βAP_{1-40} (antiserum 1280) and anti-βAP_{1-42} (antiserum HM). Rabbits were immunized with 0.5-3.0 mg of one of these peptides (unconjugated) in complete Freund's adjuvant intradermally. The rabbits received booster injections of 0.1-0.5 mg peptide 3 weeks after primary immunization and at approximately 2-4 week intervals thereafter until high titers of anti-peptide reactivity could be detected in samples of the rabbit serum. These antisera were then used in immunological assays at dilutions ranging from 1:300 to 1:1,500.

2. ELISA Assay.

a. Binding of Capture Antibody to Microtiter Wells.

[0116] Monoclonal antibody 266 was diluted to a concentration of 5 μg/ml, and monoclonal antibody 067 to 10 μg/ml in a buffer containing $NaH_2PO_4 \cdot 7H_2O$, 26.2 g/L; NaN_3, 1 g/L; pH 8.3. One hundred μl/well of this solution was then dispensed in a 96 well polystyrene transparent COSTAR plate and incubated overnight at room temperature. Following

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coating, the remaining solution was aspirated and the non-specific antibody binding sites were blocked with 0.25% human serum albumin (HSA) dissolved in a buffer containing $NaH_2PO_4 \cdot H_2O$, 1 g/L; $Na_2HPO_4 \cdot 7H_2O$, 10.8 g/L; NaN_3, 0.5 g/L and sucrose, 25 g/L; pH 7.4. These coated/blocked plates were used immediately or dried in a desiccator and stored in a dry container at 4°C for a maximum of 5 days.

b. Assay Protocol.

[0117] Calibrators containing known amounts of βAP and samples from various bodily or extra bodily fluids were then added to the plate at 100 µl/well. The samples were added undiluted or diluted in a buffer containing $NaH_2PO_4 \cdot H_2O$, 0.2 g/L; $Na_2HPO_4 \cdot 7H_2O$, 2.16 g/L; NaN_3, 0.5 g/L; BSA (globulin free) 6 g/L; Triton X-405, 0.5 mL/L; NaCl, 8.5 g/L; pH 7.4. Samples and calibrators were incubated in the wells for 1 hour at room temperature, subsequently aspirated, and the wells washed with 300 µl/well of a solution containing NaCl, 80 g/L; KCl, 3.8 g/L; Tris base, 5.85 g/L; Tris HCl, 31.75 g/L; and 0.05% Tween® 20; pH 7.5 (TBS).

[0118] NHS-biotin (15 mg) was dissolved in 0.25 ml dimethylsulfoxide, and 10 µl of this solution was added to 1 mg of 10D5 or 6C6 antibody suspended in 1 ml of sodium carbonate solution, 50 mM, pH 8.5. The mixture was incubated in the dark for 1½ hours at room temperature and then dialyzed against phosphate buffered saline, pH 7.4 for 48 hours at 4°C, to produce biotinylated reporter antibody. One hundred µl/well of the biotinylated reporter antibody (10D5 or 6C6) diluted to 3 µg/ml was then added to each well and incubated for another hour at room temperature. The antibody diluent consisted of Trizma base, 1.21 g/L; NaCl, 29.22 g/L; NaN_3, 1.5 g/L, Triton X 405, 0.5 ml/L; PEG (MW 3350), 40 g/L; Mg $Cl_2 \cdot 6H_2O$, 0.095 g/L; $ZnCl_2$, 0.014 g/L; fetal bovine serum 100 ml/L; and BSA 2.5 g/L, pH 7.4.

[0119] After 1 hour incubation at room temperature with the reporter antibody (10D5 or 6C6) the supernatant was aspirated and the wells were washed three times with 300 µl/well of TBS. Streptavidin alkaline phosphatase (100 µl/well, diluted 1:2000 in the conjugate diluent buffer) was added and incubated for another hour at room temperature. The supernatant was then aspirated and washed 3 times with 300 µl/well TBS. Fluorescent substrate (4-methyl-umbellipheryl phosphate in 2-amino-2-methyl propranolol buffer; pH 9.5; (100 µl/well) was added) and fluorescence read and expressed as relative fluorescent units (FSU) after 15 minutes using a Cytofluor 2300 from Millipore, with 360/40 excitation filter and 460/40 emission filter.

3. Cultured Cells.

[0120] Human cells (and cells from other mammals) were cultured under standard cell culture conditions in plastic dishes or multi-well microtiter plates. In particular, human embryonal kidney carcinoma 293 cells (hereinafter designated K293 cells) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics. K293 cells that had previously been transfected with a recombinant DNA construct containing the full coding region of the β amyloid precursor protein (APP) were utilized in addition to untransfected K293 cells (Selkoe et al. (1988) Proc. Acad. Sci. USA 85:7341-7345; and Oltersdorf et al. (1990) J. Biol. Chem. 265:4492-4497). The transfected cells express high levels of the APP protein, compared to the usual background levels of endogenous APP characteristic of K293 cells.

[0121] Several other cell types were also cultured, including human umbilical vein endothelial cells (HUVEC); a human megakaryocytoid leukemic cell line designated DAMI; Chinese hamster ovary (CHO) cells, primary human fibroblasts, and primary mixed brain cell cultures (including neurons, astrocytes, and microglia) established from human or rodent brain.

[0122] These various cell lines were grown at 37°C in a tissue culture incubator containing an atmosphere of 95% oxygen and 5% carbon dioxide. The cells were routinely subcultured by providing fresh culture medium at regular intervals. The extracellular fluid surrounding the cells (conditioned medium) was harvested from cells grown either under standard resting conditions or following various biochemical treatments of the cells. All cultured cells and their derived media samples were handled under aseptic conditions.

[0123] cultures of human-mixed brain cells for use in immunoaffinity chromatography studies were prepared as follows. Fetal neural tissue specimens were obtained from 12-14 week old fetal cadavers. Samples of cerebral cortex were rinsed twice with Hank's Balanced Saline solution (HBSS). Cortical tissue (2-3 grams) was placed in 10 mls of cold HBSS to which 1 mg of DNase (Sigma Chemical Co., St. Louis, MO D3427) was added. The triturated suspension was filtered through Nitex nylon screens of 210 µm then 130 µm, as described by Pulliam et al. (1984) J. Virol. Met. 9:301.

[0124] Cells were harvested by centrifugation and resuspended in neuronal medium (MEM fortified with 10% fetal bovine serum, 1% glucose, 1 mM Na pyruvate, 1 mM glutamine, 20 mM KCl). Polyethyleneimine coated 100 mm dishes were seeded with 1.5×10^7 cells in 8 mls of neuronal medium. The medium was harvested and fresh medium added twice weekly. The conditioned medium from the cells (HFBC-CM) was frozen until use.

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4. Innunoprecipitation/Autoradiography Assay for βAP.

a. Metabolic Labeling and Immunoprecipitation.

[0125] K293 cells grown under standard culture conditions underwent metabolic labeling of newly synthesized proteins by addition of ^{35}S-radiolabeled methionine to the culture medium. During this step, the medium contained no unlabeled ("cold") methionine but was otherwise identical to the standard medium used to culture K293 cells. Amounts of radioactive methionine varying from 50-300 μCi/ml of media were used in the labeling experiments. Cells were incubated for approximately 10-20 hours. Thereafter, the medium containing any radiolabeled proteins released from the cell was collected.

[0126] A polyclonal antibody produced to a synthetic βAP peptide comprising the amino acids *Asp*-1 through *Val*-40 (βAP_{1-40}) was added to the collected media and incubated for periods varying from 2-10 hours. This allowed antigen-antibody complexes to form between the anti-βAP antibody and any βAP peptide present in the culture media. Thereafter, a protein A-Sepharose® reagent capable of binding to immunoglobulins (antibodies) was added, and this mixture was further incubated for varying periods of 2-10 hours. This incubation enabled the protein A-Sepharose® beads to bind to the anti-βAP antibodies which in turn were bound to βAP peptide. The conditioned media was then centrifuged at 12,000 xg for 10 minutes to pellet the antigen-antibody-protein A-Sepharose® bead complexes.

b. SDS-Polyacrylamide Gel Electrophoresis (PAGE) of the Immunoprecipitate.

[0127] The imnunoprecipitates of the media from metabolically labeled cells was electrophoresed on 10-20% Tris-tricine gels, which have the advantage of resolving low molecular weight proteins (such as βAP) well. The gels were then dried and exposed to X-ray film to produce an autoradiogram or fluorogram. Exposure times varied but were usually in the range of 2-7 days. Following development of the X-ray film, any radiolabeled proteins that were precipitated from the cell media by the anti-βAP antibody were visualized as dark bands at the appropriate molecular weight (i.e., 4 kD).

5. Preparation of 266 Resin.

[0128] Antibody 266 (15 mls at 0.85 mg/ml) was dialyzed versus 10 mM Na acetate, 15 mm NaCl, pH 5.5 and then coupled to Affi-Gel® Hz Hydrazide (Bio-Rad, Richmond, CA) according to the manufacturer's protocol, using approximately 5 mls of resin. One ml of the resin was placed in a 1 x 10 cm column for the purification of βAP from 4 liters of conditioned medium.

6. Western Blotting.

[0129] Samples were subjected to SDS-PAGE on 10% - 20% Tricine gels (Novex) and transferred to PVDF membranes (Pro-blot, Applied Biosystems) at 40 volts, overnight, in the buffer system described by Towbin, et al. (1979) Proc. Natl. Acad. Sci. USA 76:4350-4354. Visualization of immunoreactive proteins employed the TROPIX chemiluminescence system according to the manufacturer's directions for the AMPPD substrate. The primary antibody used was 10D5 at a concentration of 5 μg/ml.

7. Construction and Analysis of the 'Swedish' FAD Mutation.

[0130] The Swedish mutation involves two adjacent base pair conversions: nucleotide 1785 G to T and nucleotide 1786 A to C which leads to two amino acid exchanges: Lys->Asn^{595} and Met->Leu^{596} (all numbering based on APP_{695}).

[0131] To analyze the biochemical effect of this mutation on the metabolism of APP *in vitro* it was introduced by *in vitro* mutagenesis in an expression vector for eukaryotic expression of APP molecules (described in Selkoe et al., 1988 *supra).* In this case, both forms of the vector carrying the 695 and the 751 amino acid forms of APP were used. Mutagenesis was performed by use of two oligonucleotide primers derived from the APP sequence and polymerase chain reaction (PCR). Primer 1 (sense) is a 30-mer and has the sequence GAG GAG ATC TCT GAA GTG AAT CTG GAT GCA. This primer contains a BglII restriction endonuclease site (AGA TCT) corresponding to the BglII site in position 1770 of APP and contains the two described nucleotide exchanges at positions 24 and 25 of the primer. Primer 2 (antisense) is a 29-mer with the sequence AAT CTA TTC ATG CAC TAG TTT GAT ACA GC. The primer contains a SpeI restriction endonuclease site (ACT AGT) corresponding to position 2360 of APP. Using a normal APP cDNA for a template, the two primers allow the creation of a DNA fragment of approximately 600 basepairs in length by standard PCR (reagents and protocols from Perkin Elmer). The obtained fragment as well as the expression vector containing the normal APP cDNA were cleaved with restriction endonucleases BglII and SpeI.

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[0132] BglII and SpeI were chosen as they are both single cut sites in this vector and therefore allow the simple removal of the non-mutated restriction fragment corresponding to the fragment created by PCR. Accordingly the approximately 600 basepair vector fragment was replaced by the PCR generated fragment of equal length carrying the mutation by standard techniques. DNA of recombinant bacterial clones was obtained by standard methods which was screened for the absence of an Mbo II restriction endonuclease site. Then the DNA sequence was confirmed by sequencing of the complete region that had undergone the PCR reaction.

[0133] In addition to the construction of the double mutation, the effects of each of the two mutations were separately examined. Using appropriate primers or olignucleotides specific for either the 595 Lys->Asn substitution or the 596 Met->Leu substitution, DNA constructs were prepared and used to transfect K293 cells as described.

[0134] Analysis of the effects of the mutation was carried out by transient expression of the obtained mutated clone in 293 cells. The DOTAP reagent transfection method was used according to the manufacturer's specifications (Boehringer Mannheim, Indianapolis, IN). Conditioned medium was harvested 48 hours after transfection. Transfection efficiency was assessed by a sandwich ELISA for APPs in the conditioned medium with affinity purified polyclonal antibodies B5 (biotinylated) to a bacterial fusion protein of $APP_{444-592}$ and the capture antibody B3, to a bacterial fusion protein of APP_{20-304}. βAP levels were measured by the ELISA described above in Example 2. As described below, the measurement of βAP in the conditioned media of transiently transfected K293 cells expressing the Swedish variant form of APP shows a 6-7 fold increase in production of βAP.

Results

[0135] The βAP ELISA assay was used to detect known amounts of synthetic βAP peptides $βAP_{1-38}$ and $βAP_{1-40}$. The assay employing 266 capture antibody and 10D5 reporter antibody was able to detect the peptides at 0.1 ng/ml. See Fig. 1. Moreover, the 266/10D5 assay was found not to significantly cross-react with full length APP, secreted APP 695 or 751, recombinant APP fragments constructs 15 or 6, βAP fragments 15-20, 11-18, 13-18, or 13-20, or fibrinogen (Table I).

Table I

βAP Cross-Reactivity in 266/10D5 ELISA		
Molecule	ng/ml	%Cross-Reactivity
Full Length APP	1-100	0
Secreted APP 695	1-100	0
Secreted APP 751	1-100	0
Construct 15*	1-100	0
Construct 6**	1-100	<0.1
βAP Fragment 15-20	1-1000	0
βAP Fragment 11-18	1-1000	0
βAP Fragment 13-18	1-1000	0
βAP Fragment 13-20	1-1000	0.2
Fibrinogen Type I	1-1000	0
Fibrinogen Type II	1-1000	0
Fibrinogen Type III	1-1000	0

*Described in Sinha et al. (1991) J. Biol. Chem. 266:2104-21013.

**Residues 590-695, as numbered in 695 isoform.

[0136] The ELISA assay was used to screen blood and CSF samples from humans, dogs, guinea pigs, and rats. Detectable amounts of βAP were found, with concentrations in the ranges set forth in Table II.

Table II

βAP Level in Plasma and CSF of Various Species		
Species	CSF βAP (ng/ml)	Plasma βAP (ng/ml)
Human	0.1-20.0	0.1-30.0
Dog	2.0 - 10.0	2.0
Guinea Pig	2.5 - 8.0	4.0 - 5.0

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Table II (continued)

βAP Level in Plasma and CSF of Various Species		
Species	CSF βAP (ng/ml)	Plasma βAP (ng/ml)
Rat	1.5	ND
Rabbit	1.5 - 9.0	0.5 - 3.0

[0137] A comparative study was carried out in groups of normal individuals and AD patients, both in plasma and CSF. Samples were screened using the ELISA assay, with the results for plasma set forth in Fig. 2A and the results for CSF set forth in Fig. 2B. βAP levels in CSF for normal individuals (C), AD patients, cerebrovascular accident (CVA), and Parkinson's Disease (PD) are set forth in Fig. 3. A CSF pool of approximately 1000 individuals had a mean value of 2.5 ng/ml in CSF, circled in Fig. 3, row C. The rest of the control individuals had a variety of non-AD neuronal degenerative diseases. The AD mean values are well above the control pool value.

[0138] A number of transfected and non-transfected cultured cells were tested for release of βAP using the ELISA assay. All cells tested were found to release βAP into the culture medium, with the APP transfected cells releasing higher concentrations than released from non-transfected cultured cells, as set forth in Table III.

Table III

Release of βAP by Cells in Culture		
Cell Type	Transfection	βAP(ng/ml)
K293	---	0.1 - 0.4
K293	695	1.6 - 2.5
K293	751	1.2 - 2.5
Mixed brain cells	---	4.0
CHO	751	2.0 - 9.0

[0139] Using the immunoprecipitation/autoradiography assay described above in Example 4, it was shown that polyclonal antibody 1280 immunoprecipitated a 4 kD protein from K293 cell media that comigrated precisely with a standard sample of radioiodinated synthetic βAP_{1-40} peptide. In addition, a 3 kD protein was simultaneously precipitated. This appears to be a fragment of βAP lacking the first 10 or 16 amino acids. When the 1280 antibody was preabsorbed with synthetic βAP_{1-40} peptide to block its activity, no 3 kD or 4 kD bands were precipitated from the K293 cell media. When the conditioned medium of K293 cells overexpressing APP was centrifuged at 100,000 xg for 2 hours (to pellet any insoluble proteinaceous material), immunoprecipitation/autoradiography with the 1280 antibody showed that substantially all of the 3 kD and 4 kD proteins remained in the supernatant, (see Fig. 6). This experiment demonstrates the βAP found in culture media is a soluble molecule, in contrast to previous reports about βAP in postmortem human brain tissue (see for example, Glenner and Wong (1984), *supra).*

[0140] The precipitation of the 4 kD βAP comigrating peptide from the media of K293 cells transfected with βAPP cDNA was confirmed by utilizing additional βAP antibodies. Antibody Y to synthetic βAP_{1-38} peptide precipitated the 4 kD protein in identical fashion to antibody 1280. Also, antibody HM to synthetic βAP_{1-42} precipitated the 4 kD protein. As a control, each of these antibodies was preabsorbed with its synthetic peptide antigen, thereby neutralizing its activity. Thereafter, the antibodies no longer immunoprecipitated the 4 kD βAP peptide from conditioned media. As an additional control, the preimmune sera (i.e., a sample of normal serum taken from each rabbit used to raise the polyclonal antibodies prior to the actual immunization) did not immunoprecipitate the 4 kD peptide from the media.

[0141] To ascertain the immunochemical specificity of the 4 kD βAP comigrating peptide precipitated from media, other antibodies to regions of APP flanking the βAP region were used in the above immunoprecipitation/autoradiography assay. For example, an antibody to the 20-amino acid region immediately amino-terminal to the beginning of the βAP region of APP failed to precipitate the 4 kD peptide from media. Likewise, an antibody to the last 20 amino acids at the carboxyl terminus of APP (60 amino acids beyond the βAP region) also failed to precipitate the 4 kD peptide. In contrast, an antibody to the first 15 residues of βAP successfully precipitated the 4 kD band. Likewise, antibodies to the middle portion of βAP also precipitated the 4 kD peptide, but not the 3 kD peptide discussed above. These various antibody precipitations demonstrate that the 4 kD peptide present in the media of cultured cells (e.g., K293 cells) shows the specific immunochemical reactivities characteristic of βAP. The 3 kD peptide in the media shows the specific immunochemical reactivity of βAP lacking the first 10 or 16 residues. Evidence that the antibodies were active in each reaction was provided by the co-precipitation from the same media of the normal secreted fragment of APP (designated "soluble APP" or "APP_s") whenever the precipitation reactions were carried out with antibodies to that region of APP.

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This large soluble APP fragment is known to be present normally in the media of cultured cells expressing APP. Its coprecipitation by anti-βAP antibodies thus represents a positive control reaction demonstrating the intact activity of the antibodies used in this assay.

[0142] As an additional control reaction to demonstrate that the immunoprecipitated 4 kD protein comigrating with synthetic βAP indeed represented authentic βAP, the media of cells transfected with an APP cDNA (and thus overexpressing APP) were compared to the media of untransfected cells. Immunoprecipitation/autoradiography showed an increased amount of precipitable 4 kD protein in the media of the transfected versus non-transfected cells, as expected from their increased production of the APP precursor molecule. See Fig. 5. Similarly, the media of the transfected cells showed more of the APP_s soluble fragment of APP than the media of the non-transfected cells; this positive control reaction was observed simultaneously in the immunoprecipitates that contained the 4 kD protein.

[0143] The sane result was obtained when a different cell type, chinese hamster ovary (CHO) cells, was used. Comparison of CHO cells either untransfected or transfected with APP cDNA showed increased levels of the 4 kD 1280-precipitable peptide in the media of the latter cells. These results provided further evidence that the 4 kD protein in the cell media was bona fide βAP.

[0144] Authentic, native βAP was extracted from autopsied human cerebral cortex of patients who died with AD. This sample of Aβ from AD brain tissue comigrated with the 4 kD 1280-precipitable peptide from cultured cell media when analyzed by SDS-polyacrylamide gel electrophoresis. This comigration provided further support for the identity of the 4 kD peptide as βAP.

[0145] Four liters of human fetal brain culture-conditioned media (HFBC-CM) were thawed and filtered through a 0.45 μm filtering flask. Leupeptin (1 μg/ml) and PMSF (35 μg/ml) taken from a 35 mg/ml stock in isopropanol were added to the HFBC-CM immediately prior to affinity chromatography. The material was run through the 266-affinity column at a flow rate of approximately 2 ml/min at 4°C. The column was then washed with 500 mls of PBS. Elution of material specifically bound to the resin was achieved with 0.2 M glycine pH 2.0. A total of 9 mls was used.

[0146] The eluted material was subjected to two steps of reversed phase liquid chromatography using a Vydac C4 [0.21 x 15 cm] reversed phase column and a solvent system containing 0.1% TFA in buffer "A" and 0.1% TFA/80% acetonitrile in buffer "B". The affinity-purified material was loaded onto the reversed phase column at 200 μl/min and then washed with 80% buffer (A) and 20% buffer "B" at 200 μl/min for 60 min and 50 μl/min for 42 min to equilibrate the column and stabilize the baseline. A gradient from 20% to 70% "B" was executed over 50 min at 50 μl/min, and the eluant was monitored at OD_{220}. Fractions of 100 μl were collected and assayed by both Western blot and ELISA. Based on these results, fractions 11 and 12 were pooled and rechromatographed under nearly identical conditions except that a 150 min gradient from 10 to 40% "B" was employed followed by a 20 min gradient from 40 to 100% "B".

[0147] Fraction 77 from the second reversed phase chromatography step was found to be reactive against the 10D5 antibody by both Western blot (Fig. 4) and ELISA.

[0148] An aliquot of the material in fraction 77 was microsequenced and shown to have the N-terminal sequence of βAP, beginning with Asp. Microsequencing was performed on an Applied Biosystems Model 477 protein sequencer using a microscale reaction cartridge and Applied Biosystems's MICFST program cycles.

[0149] A second aliquot was subjected to electrospray ionization mass spectrometry, performed at M-SCAN, Inc., revealing a mass peak at 4329.81 (+/- 1.27 SD) which would correspond to the expected mass of βAP 1-40 (theoretical MW of 4330.4). N-terminal sequencing of several of the A_{220} peaks was positive for βAP. The ELISA revealed the βAP sequence to be present in fractions 65, 70, 75 in addition to the major peak in fraction 77. Fractions 65 and 70 contained an additional sequence of a previously undescribed βAP fragment beginning at βAP residue 11 (Glu). In fraction 65, sufficient material was present to sequence through βAP residue 33.

[0150] Western Blot (Fig. 4) of RPLC fractions of 266-affinity purified material from HFBC-CM. 3 μl of the indicated fractions were diluted with 15 μl of SDS-PAGE sample buffer and neutralized with 1 μl of 1 M NaOH before boiling and processing as described above. The material loaded in the respective lanes was as follows:

Lane	RPLC fraction
1	65
2	70
3	71
4	74
5	75
6	77
7	78
8	79

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(continued)

Lane	RPLC fraction
9	83
10	84
11	85

[0151] Lane 12 was buffer only; lane 13 contained 20 ng of βAP 1-38; lane 14 contains 100 ng of βAP 1-38; lane 15 contains low molecular weight Rainbow® standards (Amersham). Note the approximate 4 kD band in lane 6 which co-migrates with the βAP standard in lane 14.

[0152] Parallel experiments to those described for HFBC-CM were performed using 4 liters of human CSF to structurally characterize the βAP immunoreactivity from this source. Sequencing data confirmed the presence of N-terminal sequences beginning with βAP residue 1 (Asp) and βAP residue 11 (Glu).

[0153] The increase in βAP levels in the media of the Swedish transfectants was quantitated using the βAP-specific ELISA with monoclonal antibodies 266 and 6C6. The sandwich ELISA for APP_s used polyclonal antibodies B5 and B3. For each ELISA, increasing amounts of purified synthetic $βAP_{1-40}$ or purified APP_s from conditioned media of K293 cells transfected with cDNA for the APP 695 isoform were used to construct a standard curve. Quantitation of βAP (left panel) and APP_S (right panel) in conditioned media in the transiently transfected K293 cells is shown in Fig. 7. Each column represents the mean of four transfections with the exception of the mock column, which is based on three cultures. Error bars indicate the standard deviation. For columns without error bars, the standard deviation was less than 0.01 units.

[0154] As shown in Fig. 7, cells expressing the Swedish variant APP 695 construct produced 6-7 fold more βAP in their media than identically transfected cells expressing normal APP (695 isoform). Moreover, a similar 7-8 fold increase was observed in cultures expressing the Swedish mutation in the APP 751 isoform (Fig. 7). Similar increases in βAP levels were documented using a second method of quantitation: phosphor inager analysis of the 4 kD βAP band in gels of 1280 immunoprecipitates. This method further demonstrated that the 3 kD fragment was decreased several fold in the media of the Swedish transfectants. CHO and K293 cells stably transfected with cDNA the Swedish mutation in the APP 751 isoform also showed marked increases in the levels of βAP in their cultured media.

[0155] To study the mechanism responsible for the increased βAP production, the effects of each single mutation (Lys->Asn^{595} and Met-Leu^{596}) in the APP 695 isoform were separately examined. Fig. 8 shows conditioned media of radiolabeled K293 cells transiently transfected with no DNA (lane 1); normal APP (lane 2), Swedish mutant APP 695 KM-NL (lane 3), variant APP 695 K-N (lane 4), variant APP 695 M-L (lane 5), APPΔC (APP cytoplasmic domain deletion, lane 6) and APPΔC KM-NL (Swedish mutations and cytoplasmic domain deletions, lane 7) and immunoprecipitated with 1280. The βAP and 3 kD bands are indicated by arrows. ^{125}I-labeled synthetic βAP (1-40) was run as a size marker on the same gel (lane 8). Cells expressing the Met->Leu substitution had increased levels of βAP in their medium, whereas cells expressing the Lys->Asn substitution had levels similar to normal transfectants (Fig. 8, lanes 2-5). This finding suggests that the 596 mutation results in more proteolytic cleavage of APP at the Leu-Asp peptide bond than at the normal Met-Asp bond. It is possible that the Lys->Asn switch at 595 may further enhance the cleavage when coupled with the Met->Leu substitution at 596.

[0156] Cells containing the Swedish mutation together with the deletion of the cytoplasmic domain of APP deletion, thus removing the Asn-Pro-X-Tyr lysosomal targeting consensus sequence, still produced substantially more βAP in their media than normal transfectants but showed increased levels of the 3 kD peptide (Fig. 8, lanes 6 and 7). This result indicates that the effect of the Swedish mutations does not require an intact cytoplasmic domain and that generation of APP is unlikely to require processing of APP in late endosomes/lysosomes.

[0157] These findings provide experimental evidence that point mutations in the APP gene found in FAD kindreds can result directly in increased generation of βAP. Analysis of the Swedish mutations demonstrates the utility of measuring βAP production *in vitro* from APP bearing a particular mutation (e.g., mutations at residue 717 immediately following the βAP region and those within the βAP region) as not only a route to elucidating the mechanism of accelerated β-amyloidosis in familial forms of AD, but also to the utility of APP variants in the methods of the present invention.

[0158] Screening assays for test compounds capable of inhibiting βAP production in cell lines possessing the Swedish mutation were performed as follows. Two cell lines (human kidney cell line K293 and Chinese hamster ovary cell line (CHO) were stably transfected with gene for APP751 containing the double mutation Lys-651-Met-652 to Asn651-Leu-652 (APP-751 numbering) commonly called the Swedish mutation using the method described in Citron et al. (1992) Nature 360:672-674. The transfected cell lines were designated as 293 751 SWE and CHO 751 SWE, and were plated in Corning 96 well plates at $2.5X10^4$ cells per well respectively in Dulbecco's minimal essential media plus 10% fetal bovine serum. Following overnight incubation at 37°C in an incubator equilibrated with 10% carbon dioxide (CO_2), the media were removed and replaced with 200 µl per well of media containing a test protease inhibitor.

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After a two hour pretreatment period, the media were again removed and replaced with fresh media containing the same protease inhibitor, and the cells were incubated for an additional two hours.

[0159] Protease inhibitor stocks were prepared in DMSO such that at the final concentration used in the treatment, the concentration of DMSO did not exceed 0.5% After treatment, plates were centrifuged in a Beckman GPR at 1200 rpm for five minutes at room temperature to pellet cellular debris from the conditioned media. From each well, 100 μl of conditioned media were transferred into an ELISA plate precoated with antibody 266 against βAP-13-28 as described above and stored at 4°C overnight. An ELISA assay employing labelled antibody 6C6 (against βAP-1-16) was run the next day to measure the amount of βAP produced.

[0160] Cytotoxic effects of the compunds were measured by a modification of the method of Hansen et al. (1989) J. Immun. Meth. 119:203-210. To the cells remaining in the tissue culture plate was added 25 μl of a 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) stock solution (5 mg/ml) to a final concentration of 1 mg/ml. Cells were incubated at 37°C for one hour, and cellular activity was stopped by the addition of an equal volume of MTT lysis buffer (20% w/v sodium dodecylsulfate in 50% DMF, pH 4.7). Complete extraction was achieved by overnight shaking at room temperature. The difference in the OD_{562nm} and the OD_{650nm} was measured in a Molecular Devices UV_{max} microplate reader as an indicator of the cellular viability.

[0161] The results of the βAP ELISA were fit to a standard curve and expressed as ng/ml βAP peptide. In order to normalize for cytotoxicity, these βAP results were divided by the MTT results and expressed as a percentage of the results from a drug-free control.

[0162] The test compounds were assayed for βAP production inhibition activity in cells using this assay. The results presented in Tables IV and V, below, are the mean and standard deviation of at least six replicate assays.

TABLE IV

βAP Production Inhibition Activity in Cells	
Example	βAP Production Inhibition
41	-6.2 ± 6.2%
42	13.1 ± 7.5%
43	16.2 ± 8.5%
45	3.3 ± 1.1%
48	1.6 ± 19.8%

TABLE V

Test Compound	βAP Production* Inhibition
1	37.5 ± 2.2%
2	53.4 ± 1.4%
3	53.5 ± 1.5%
4	57.4 ± 3.1%
5	58.1 ± 2.8%
6	65.4 ± 3.9%
7	69.1 ± 6.8%
8	77.5 ± 6.0%
9	89.3 ± 7.7%
10	89.8 ± 4.9%
11	110.3 ± 3.3%
12	111.7 ± 9.1%

* Negative test results are not shown in this Table.

[0163] Although the foregoing invention has been described in detail for purposes of clarity of understanding, it will be obvious that certain modifications may be practiced within the scope of the appended claims.

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Claims

1. A method for detecting a soluble β-amyloid peptide (βAP) in a fluid sample in the presence of β-amyloid precursor protein (APP) or fragments thereof, said method comprising:

   capturing soluble βAP from the sample using a first binding substance under conditions in which the first specific binding substance binds to an epitope at a junction region on the βAP and which is substantially free from cross-reactivity with APP and fragments thereof other than βAP, wherein the junction region is centered at the site between βAP amino acid residues 16 and 17 and is a target for normal proteolytic cleavage; and detecting capture of the soluble βAP using a labelled second binding substance which binds to an epitope on a second region of the soluble βAP other than the epitope on the βAP which is bound by the first binding substance.

2. A method as claimed in claim 1, wherein the βAP is intact βAP.

3. A method as claimed in claim 1 or claim 2, wherein the soluble βAP is captured on a solid phase, and the capture is detected by exposing the solid phase to the labelled second binding substance and thereafter detecting the presence of the label on the solid phase.

4. A method for detecting a soluble β-amyloid peptide (βAP) in a fluid sample, said method comprising:

   exposing the sample to a first binding substance specific for an epitope at a junction region on soluble βAP disposed between amino acid residues 13 to 28; and

   detecting binding between the first binding substance and the soluble βAP under conditions where the first binding substance binds to the βAP without substantial cross-reactivity with β-amyloid precursor protein (APP) and fragments thereof other than βAP.

5. A method as claimed in claim 4, wherein the first binding substance is an antibody, optionally a monoclonal antibody, raised against a peptide consisting of amino acid residues 13 to 28 of βAP.

6. A method as claimed in claim 4 or claim 5, wherein binding of the first binding substance and the soluble βAP is detected by separating bound complexes of the binding substance and βAP exposing the separated bound complexes to a labelled second binding substance specific for the N-terminal region of βAP, and detecting the presence of label on the bound complexes.

7. A method as claimed in claim 6, wherein the second binding substance is an antibody, optionally a monoclonal antibody, raised against a peptide consisting of amino acid residues 1-16 of βAP.

8. A method for detecting a soluble β-amyloid peptide (βAP) in a fluid sample which may contain βAP and βAP fragments of β-amyloid precursor protein (APP) other than βAP, said method comprising:

   exposing the fluid sample to a first binding substance under conditions in which the first binding substance will bind to an epitope on soluble βAP and βAP fragments but will not bind to epitopes on APP fragments which may be present in the sample; and

   detecting binding between the first binding substance and the soluble βAP and βAP fragments.

9. A method as claimed in claim 8, wherein the first binding substance binds specifically to intact βAP or to an approximately 3 kD fragment of βAP.

10. A method as claimed in claim 8 or claim 9, wherein the first binding substance is immobilized on a solid phase, and binding is detected by exposing the solid phase to a labeled second binding substance and thereafter detecting the presence of the label on the solid phase.

11. A method as claimed in any preceding claim, wherein the fluid sample is selected from the group consisting of culture medium, blood, CSF, urine, and peritoneal fluid.

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12. A method for identifying β-amyloid peptide (βAP)production inhibitors said method comprising culturing mammalian cells in a culture medium under conditions which result in production of a soluble βAP peptide which can be detected in the culture medium; exposing the cultured cells to a test compound; and determining whether the test compound affects the amount of soluble βAP peptide present in the culture medium.

13. A method as claimed in claim 12, wherein the determination of βAP peptide is specific for soluble βAP present in the culture medium.

14. A method as claimed in claim 12 or claim 13, wherein the effect of the test compound is determined by comparing the accumulation of βAP in the culture medium over a time period in the absence of the test compound with the accumulation of βAP in the culture medium in the presence of the test compound over the same time period.

15. A method for assaying a test compound for the ability to inhibit β-amyloid peptide (βAP) production by cells, said method comprising culturing a first population of mammalian cells in a culture medium under conditions which result in generation of a soluble βAP peptide which can be detected in the culture medium; culturing a second population of the same mammalian cells in a second culture medium under identical conditions to the first population, except that the test compound is present in the second culture medium; measuring the amounts of soluble βAP present in the culture media of the first population and the second population of cells; and comparing the measured amounts of βAP to determine whether the test compound has had an effect on soluble βAP peptide generation by the cultured cells.

16. A method as claimed in claim 15, wherein the measurement is specific for soluble βAP present in the culture media of the first and second population of cells.

17. A method as claimed in any of claims 12 to 16, wherein the cultured mammalian cells are from a cell line comprising DNA encoding an amyloid precursor protein (APP) isotype or variant which provides overproduction of βAP in the cultured mammalian cells.

18. A method as claimed in any one of claims 12 to 17, wherein the cultured cells are from a human cell line.

19. A method as claimed in any preceding claim, wherein the test compound is present at a concentration in the range 1 nM to 1mM.

20. A method for identifying β-amyloid (βAP) production inhibitors, said method comprising administering a test compound to a mammalian transgenic host comprising DNA encoding an amyloid precursor protein (APP) isotype or variant which provides overproduction of βAP in cultured mammalian cells comprising said DNA; and determining whether the test compound affects the amount of a soluble βAP peptide present in a body fluid.

21. A method as claimed in claim 20, wherein the effect of the test compound is determined by measuring a baseline amount of soluble βAP peptide in a body fluid prior to administering the test compound and remeasuring the amount of soluble βAP in the body fluid after said administering.

22. A method as claimed in claim 20 or claim 21, wherein the mammalian transgenic host is selected from the group consisting of monkeys, dogs, rabbits, guinea pigs, rats and mice.

23. A method as claimed in any one of claims 20 to 22, wherein the mammalian transgenic host is a transgenic host having enhanced susceptibility to βAP plaque deposition compared to a mammal of the same species not comprising said DNA.

24. A method as claimed in any one of claims 20 to 23, wherein the body fluid is selected from the group consisting of blood, CSF, urine and peritoneal fluid.

25. A method as claimed in any one of claims 20 to 24, wherein the test compound is administered orally, topically, intramuscularly, intravenously, subcutaneously intraventricularly, or intraperitoneally.

26. A method as claimed in any preceding claim, wherein the soluble βAP peptide is intact βAP or an approximately 3 kD fragment of βAP.

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27. A method as claimed in claim 26, wherein the βAP fragment consists of amino acid residues 11-40 or 17-40 of βAP.

28. A method as claimed in any preceding claim, wherein said DNA encodes an APP having one or several amino acid substitutions directly amino-terminal of the βAP cleavage site.

29. A method as claimed in any preceding claim, wherein said DNA encodes an APP comprising leucine as the residue immediately amino N-terminal to βAP cleavage site.

30. A method as claimed in any preceding claim, wherein said DNA encodes an APP comprising the Swedish mutation (a double mutation $Lys^{595} \rightarrow Asn^{595}$ and $Met^{596} \rightarrow Leu^{596}$).

31. A β-amyloid peptide (βAP) production inhibitor that affects the amount of soluble βAP peptide present in the culture medium of a cell line of mammalian cells that are cultured under conditions which result in soluble βAP which can be detected in the culture medium.

32. An inhibitor as claimed in claim 31, wherein the cultured mammalian cells are from a cell line optionally human comprising DNA encoding an amyloid precursor protein (APP) isotype or variant which provides overproduction of βAP in the cultured mammalian cells.

33. An inhibitor as claimed in claim 31 or claim 32, wherein the cultured cells are from a human cell line.

34. An inhibitor as claimed in any of claims 31 to 33, wherein the inhibitor affects the amount of soluble βAP in the culture medium of the mammalian cell line when present at a concentration in the range 1 nM to 1mM.

35. A β-amyloid (βAP) production inhibitor that when administered to a non-human mammalian host affects the amount of a soluble βAP peptide present in a body fluid of that host.

36. An inhibitor as claimed in claim 35, wherein the mammalian host is transgenic and has DNA encoding an amyloid precursor protein (APP) isotype or variant which provides overproduction of βAP in cultured mammalian cells comprising said DNA.

37. An inhibitor as claimed in claim 35 or claim 36, wherein the non-human mammalian host is selected from the group consisting of monkeys, dogs, rabbits, guinea pigs, rats and mice.

38. An inhibitor as claimed in claim 36 or claim 37, wherein the non-human mammalian transgenic host has enhanced susceptibility to βAP plaque deposition compared to a mammal of the same species not comprising said DNA.

39. An inhibitor as claimed in any one of claims 35 to 38, wherein the body fluid is selected from the group consisting of blood, CSF, urine and peritoneal fluid, optionally wherein the inhibitor is administered orally, topically, intramuscularly, intravenously, subcutaneously intraventricularly, or intraperitoneally.

40. An inhibitor as claimed in any preceding claim, wherein the soluble βAP peptide is intact βAP or an approximately 3 kD fragment of βAP.

41. An inhibitor as claimed in claim 40, wherein the βAP fragment consists of amino acid residues 11-40 or 17-40 of βAP.

42. An inhibitor as claimed in any preceding claim, wherein said DNA encodes an APP having one or several amino acid substitutions directly amino-terminal of the βAP cleavage site.

43. An inhibitor as claimed in any preceding claim, wherein said DNA encodes an APP comprising leucine as the residue immediately amino N-terminal to βAP cleavage site.

44. A β-amyloid peptide (βAP) production inhibitor as claimed in any preceding claim for use as a pharmaceutical.

45. The use of a β-amyloid peptide (βAP) production inhibitor of any of claims 31 to 44 for the manufacture of a medicament for the treatment of Alzheimer's Disease or other βAP-related conditions.

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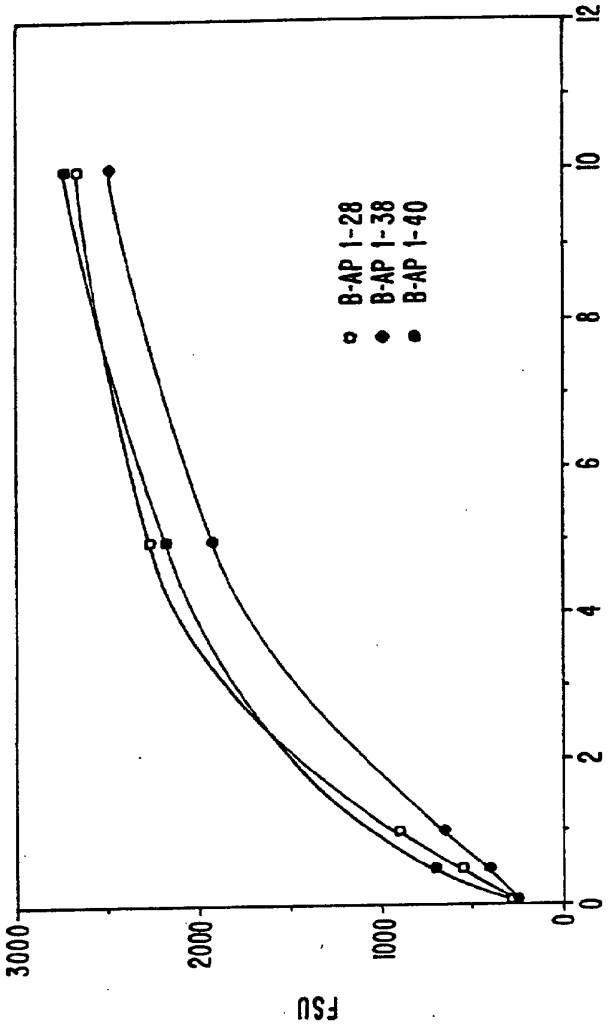


FIG. 1.

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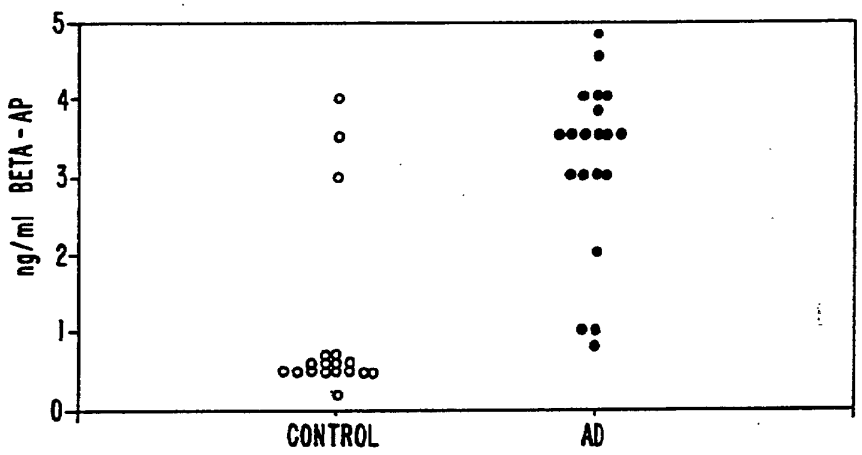


FIG. 2A.

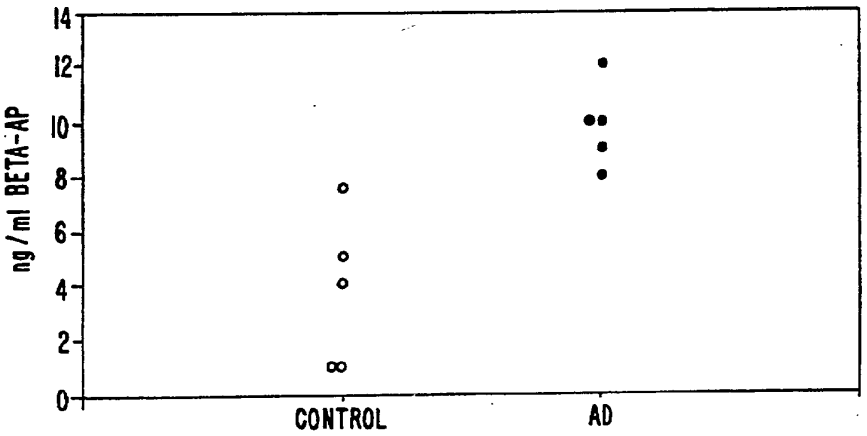


FIG. 2B.

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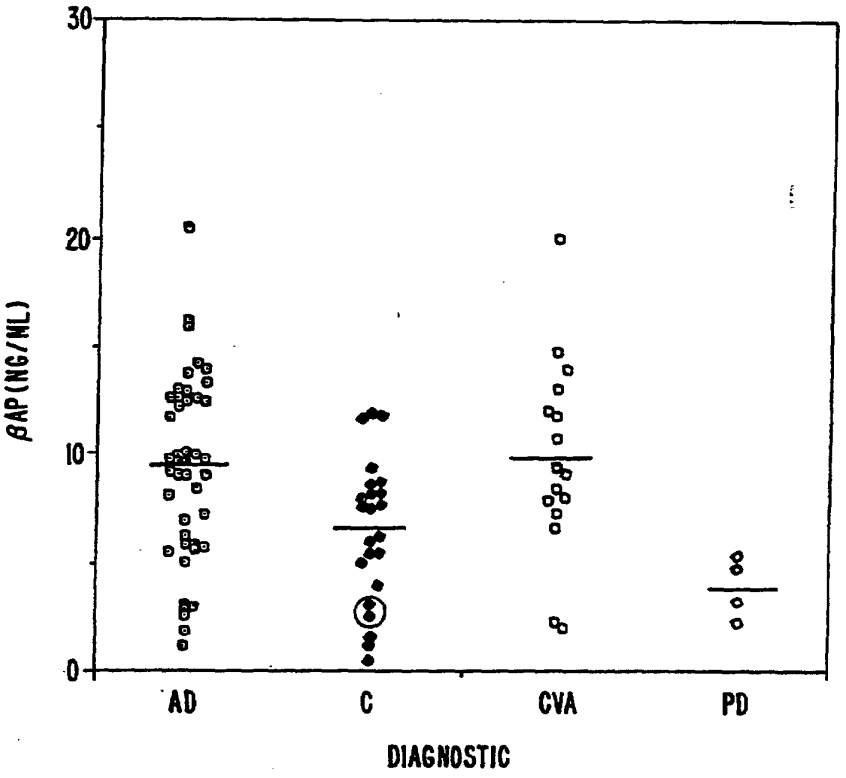


FIG. 3.

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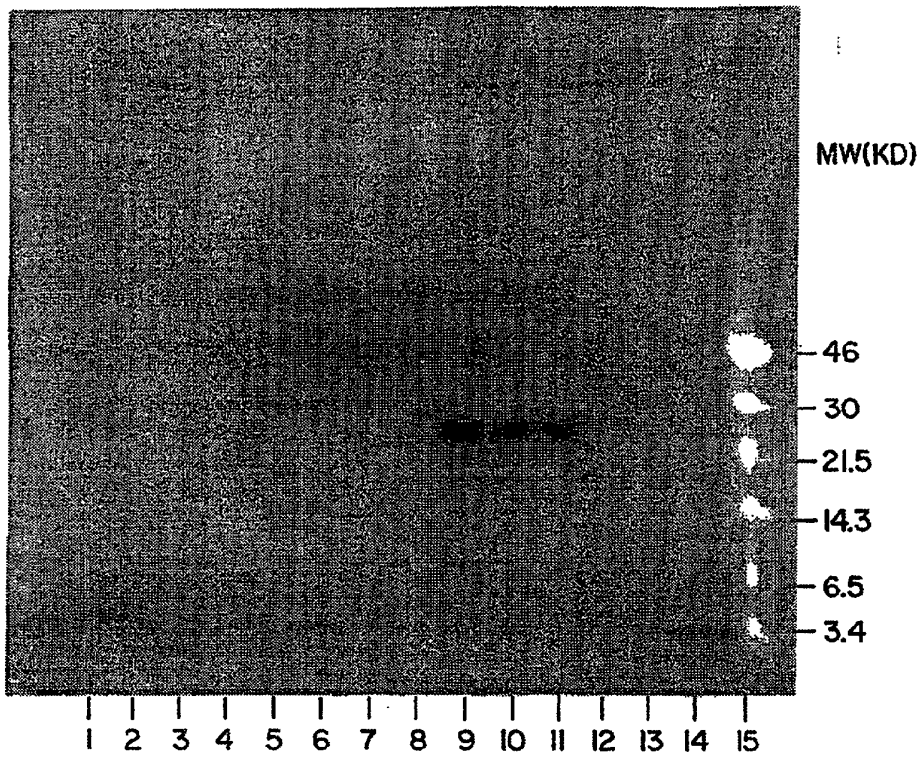


FIG. 4.

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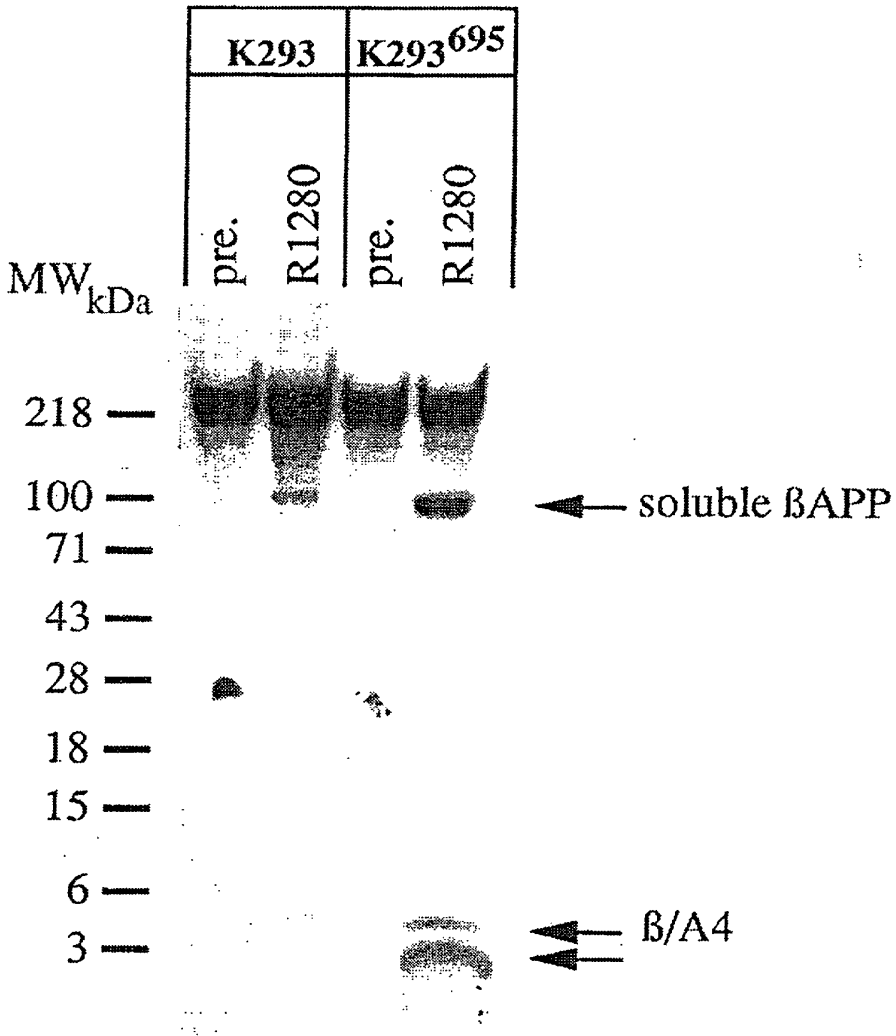


FIG. 5.

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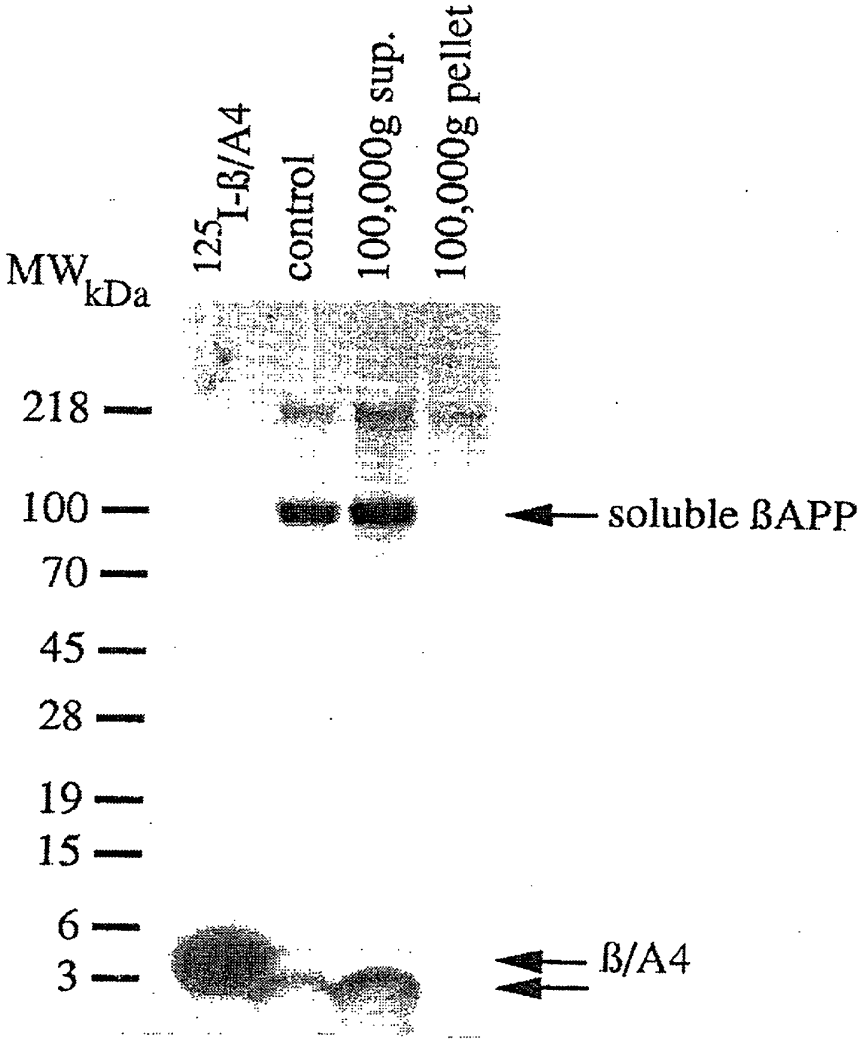


FIG. 6.

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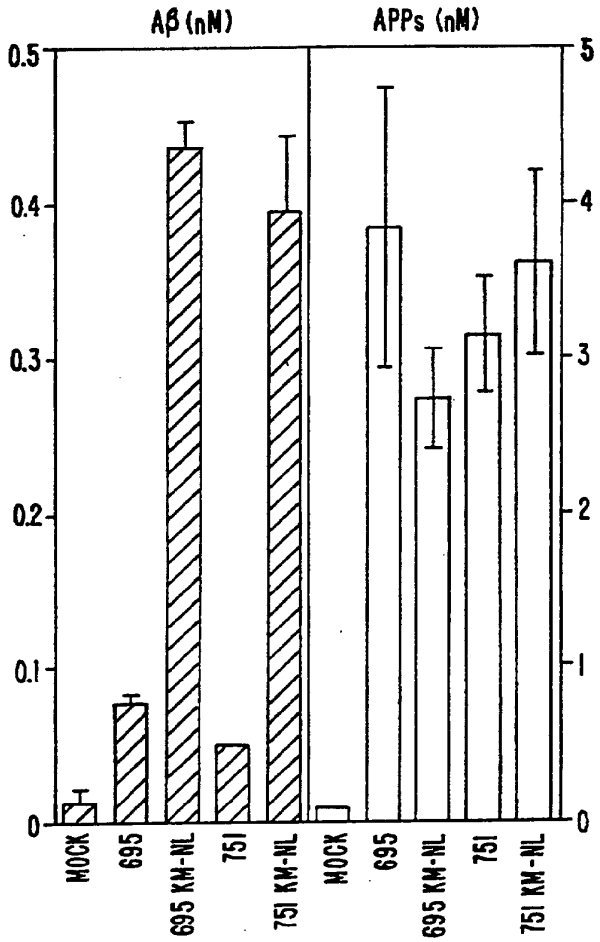


FIG. 7.

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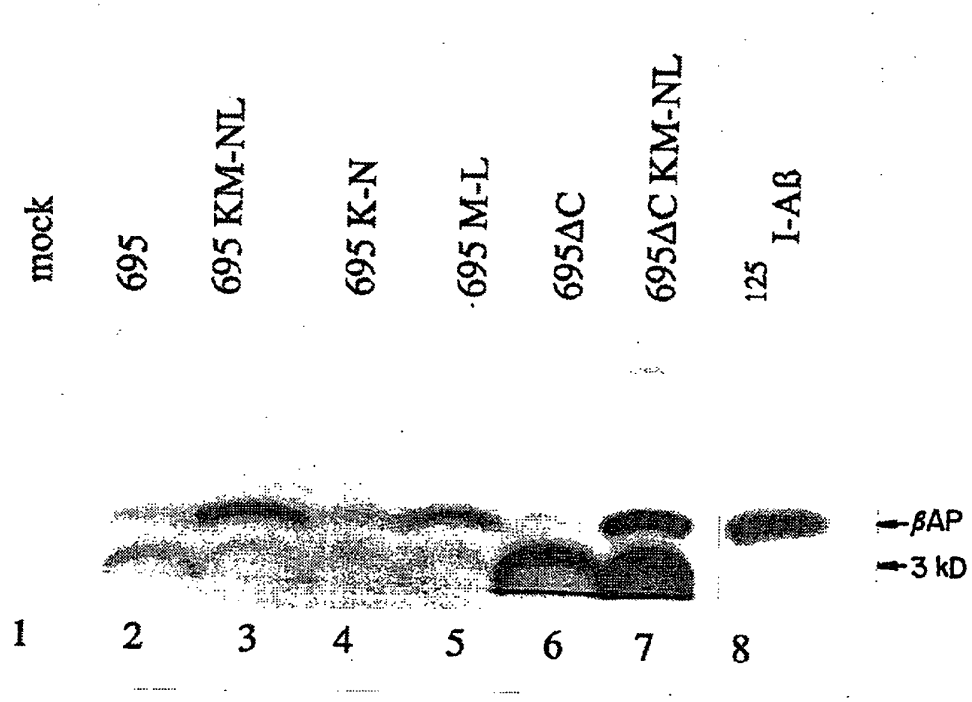


FIG. 8.

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Remarks:
Claims 11-17, 19-38, 41-42 are deemed to be abandoned due to non-payment of the claims fees (Rule 45(3) EPC).

(54) Anti-amyloid antibodies and their use in diagnosis and therapy of amyloid diseases

(57) The invention concerns antibodies to amyloid fibrillar and non-fibrillar polypeptides. The invention further concerns the use of such antibodies in diagnosis, treatment and/or prevention of amyloid diseases. The present invention also provides preparations and methods for preventing and treating, in a mammal, a disease characterized by amyloid formation and/or aggregation, preferably by promoting a non-fibrillar aggregation.

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Description

[0001] The invention concerns antibodies to amyloid fibrillar and non-fibrillar polypeptides. The invention further concerns the use of such antibodies in diagnosis, treatment and/or prevention of amyloid diseases. The present invention also provides preparations and methods for preventing and treating, in a mammal, a disease characterized by amyloid formation and/or aggregation, preferably by promoting a non-fibrillar aggregation.

[0002] Amyloid diseases or amyloidosis refers to a pathological condition in a mammal characterized by the presence of amyloid fibers. Amyloid is a generic term referring to a group of diverse but specific protein deposits. All amyloid deposits have common morphologic properties, stain with specific dyes (e.g. Congo red), and have a characteristic red-green birefringent appearance in polarized light after staining. Different amyloids are also characterized by the type of protein present in the deposit. For example, neurodegenerative diseases such as scrapie, bovine spongiform encephalitis, Creutzfeldt-Jakob disease and the like are characterized by the appearance and accumulation of a protease-resistant form of prion protein in the nervous system. Similarly, Alzheimer's disease, another neurodegenerative disorder, is characterized by neuritic plaques and neurofibrillary tangles. In this case, the plaque and blood vessel amyloid is formed by the deposition of fibrillar beta-amyloid protein.

[0003] Amyloid material deposition (also referred to as amyloid plaque formation) is also a central feature of a variety of unrelated pathological conditions including Alzheimer's disease, prion-related encephalopathies, type II diabetes mellitus, familial amyloidosis and light-chain amyloidosis.

[0004] Amyloid material is composed of a dense network of rigid, nonbranching proteinaceous fibrils of indefinite length that are about 80 to 100 A in diameter. Amyloid fibrils contain a core structure of polypeptide chains arranged in antiparallel or parallel beta-pleated sheets lying with their long axes perpendicular to the long axis of the fibril.

[0005] Approximately twenty amyloid fibril proteins have been identified in-vivo and correlated with specific diseases. These proteins share little or no amino acid sequence homology, however the core structure of the amyloid fibrils is essentially the same. This common core structure of amyloid fibrils and the presence of common substances in amyloid deposits suggest that data characterizing a particular form of amyloid material may also be relevant to other forms of amyloid material and thus can be implemented in template design for the development of drugs against amyloid-associated diseases such as type II diabetes mellitus, Alzheimer's dementia or diseases and prion-related encephalopathies.

[0006] Furthermore, amyloid deposits do not appear to be inert in vivo, but rather are in a dynamic state of turnover and can even regress if the formation of fibrils is halted.

[0007] Thus, therapies designed to inhibiting amyloidosis may be useful for treating amyloid associated diseases.

[0008] Inhibition or modification of the formation of amyloid fibrils--Amyloid, including islet amyloid, contains potential stabilizing or protective substances, such as serum amyloid P component, apolipoprotein E, and perlecan. Blocking their binding to developing amyloid fibrils could inhibit amyloidogenesis, as could treatment with antibodies specific for certain parts of an amyloidogenic protein.

[0009] Accordingly, there exists the need for new therapies and reagents for the treatment of amyloid disease, in particular, therapies and reagents capable of effecting a therapeutic benefit at physiologic doses.

[0010] The present invention features new immunological reagents, in particular, therapeutic peptides/binding peptides or antibody reagents for the prevention and treatment of amyloidogenic disease. The invention is based, at least in part, on the identification and characterization of antibodies that specifically bind to amyloid fibrillar/non-fibrillar polypeptides and are effective at reducing plaque burden and/or reducing the neuritic dystrophy associated with amyloidogenic disorders. Structural and functional analysis of these antibodies leads to the design of various humanized antibodies for prophylactic and/or therapeutic use. In particular, the invention features humanization of the variable regions of these antibodies and, accordingly provides for humanized immunoglobulin or antibody chains, intact humanized immunoglobulins or antibodies, and functional immunoglobulin or antibody fragments, in particular, antigen binding fragments, of the featured antibodies. The invention also provides methods for treating or preventing diseases associated with amyloid/beta-amyloid deposit in an individual, such as Alzheimer's disease, Down's syndrome, multi-infarct dementia, mild cognitive impairment, cerebral amyloid angiopathy, depression, Creutzfeldt-Jakob disease, and dementia with Lewy body in a subject by administering to the subject an effective amount of a pharmaceutical composition comprising an antibody, a polypeptide, or a polynucleotide encoding the antibody or the polypeptide described herein.

[0011] Also featured are antibodies (e.g., humanized antibodies) having altered effector functions, and therapeutic uses thereof.

[0012] Surprisingly, the peptide of the invention, characterized in that it is selected from the group comprising:

a) a peptide consisting of the amino acid sequence according to SEQ ID No. 1, preferably SEQ ID No. 2, 3 and/or 4.

b) a peptide consisting of an amino acid sequence having sufficient homology to be functionally analogous/equivalent to an amino acid sequence in accordance with a);

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c) a peptide according to an amino acid sequence a) or b) which is modified by deletions, additions, substitutions, translocations, inversions and/or insertions and functionally analogous/equivalent to an amino acid sequence in accordance with a) or b);

d) a peptide according to amino acid sequence a), b) or c) which is modified by branch or extension with the same or another peptide according to amino acid sequence a), b) or c) to form a homooligomeric or heterooligomeric peptide,

interacts in a specific manner with amyloid fibrillar/non-fibrillar polypeptides.

[0013] In the meaning of the invention the term "to be functionally analogous" or "equivalent" to the above-mentioned sequences means that the homologues exhibit a behavior in amyloid diseases such that reliable and effective use thereof is possible in diagnosis and/or therapy of such diseases or pathogenic conditions associated with said diseases. Functionally analogous sequences in the meaning of the invention are all those sequences which can be identified as equally effective by a person skilled in the art, using routine tests.

[0014] Even if the substance of the invention consists of the amino acid sequences SEQ ID No. 1, 2, 3 and/or 4, wherein SEQ ID Nos. 2, 3 and 4 are preferably binding domains, it is known to those skilled in the art that the substance of the invention may be modified by addition, deletion or substitution without essentially altering the polypeptide. The modified amino acid sequence is not essentially altered as long as it fulfils the same function as the sequences SEQ ID No. 1, 2, 3 and/or 4 in essentially the same way, leading to essentially the same outcome. In the meaning of the invention, the modified amino acid sequence thus essentially corresponds particularly to the amino acid sequences SEQ ID No. 1, 2, 3 and/or 4, if applying it does not essentially influence the solution of the problem of the invention and if this is obvious for a person skilled in the art, who realises that the inventors do not intend to limit their teaching to the wording of the claims - that is the sequences according to SEQ ID No. 1, 2, 3 and/or 4 or its modifications.

[0015] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (M. J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J. E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R. I. Freshney, ed., 1987); Introduction to Cell and Tissue Culture (J. P. Mather and P. E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J. B. Griffiths, and D. G. Newell, eds., 1993-1998) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Handbook of Experimental Immunology (D. M. Weir and C. C. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J. M. Miller and M. P. Calos, eds., 1987); Current Protocols in Molecular Biology (F. M. Ausubel et al., eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Current Protocols in Immunology (J. E. Coligan et al., eds., 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C. A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: a practical approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal antibodies: a practical approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); Using antibodies: a laboratory manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995).

[0016] In certain embodiments, the present invention concerns novel compositions comprising at least one protein or peptide. As used herein, a protein or peptide generally refers, but is not limited to, a protein of greater than about 200 amino acid; a polypeptide of greater than about 100 amino acids; and/or a peptide of from about 3 to about 100 amino acids. For convenience, the terms "polypeptide", "oligopeptide", "peptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. It is understood that, because the polypeptides of this invention are based upon an antibody, the polypeptides can occur as single chains or associated chains.

[0017] In certain embodiments the size of the protein or peptide may comprise, but is not limited to, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, about 110, about 120, about 130, or greater amino acid residues.

[0018] As used herein, an "amino acid residue" refers to any naturally occurring amino acid, any amino acid derivative or any amino acid mimic known in the art. In certain embodiments, the residues of the protein or peptide are sequential,

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without any non-amino acid interrupting the sequence of amino acid residues. In other embodiments, the sequence may comprise one or more non-amino acid moieties. In particular embodiments, the sequence of residues of the protein or peptide may be interrupted by one or more non-amino acid moieties.

[0019] Accordingly, the term "protein or peptide" encompasses amino acid sequences comprising at least one of the 20 common amino acids found in naturally occurring proteins, or at least one modified or unusual amino acid, 2-Aminoadipic acid, N-Ethylasparagine, 3-Aminoadipic acid, Hydroxylysine, beta-alanine, beta-Amino-propionic acid, allo-Hydroxylysine, 2-Aminobutyric acid, 3-Hydroxyproline, 4-Aminobutyric acid, piperidinic, 4-Hydroxyproline acid, 6-Aminocaproic acid, Isodesmosine, 2-Aminoheptanoic acid, allo-Isoleucine, 2-Aminoisobutyric acid, N-Methylglycine, sarcosine, 3-Aminoisobutyric acid, N-Methylisoleucine, 2-Aminopimelic acid, 6-N-Methyllysine, 2,4-Diaminobutyric acid, N-Methylvaline, Desmosine, Norvaline, 2,2'-Diaminopimelic acid, Norleucine, 2,3-Diaminopropionic acid, Ornithine and/or N-Ethylglycine.

[0020] Proteins or peptides may be made by any technique known to those of skill in the art, including the expression of proteins, polypeptides or peptides through standard molecular biological techniques, or the chemical synthesis of proteins or peptides. Alternatively, various commercial preparations of proteins, polypeptides and peptides are known to those of skill in the art and proteins or peptides of specific sequence may be obtained from a variety of commercial vendors known in the art (e.g., Midland Certified Reagents, Midland, Tex.). Peptides containing derivatized amino acid residues may be prepared by incorporating such residues into the peptide chain during synthesis. Alternatively, modified amino acid residues may be prepared by chemical derivatization after peptide synthesis, for example using side-chain specific chemical modifying agents well known in the art.

[0021] Another embodiment for the preparation of polypeptides according to the invention is the use of peptide mimetics. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure. See, for example, Johnson et al., "Peptide Turn Mimetics" in BIOTECHNOLOGY AND PHARMACY, Pezzuto et al., Eds., Chapman and Hall, New York (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule. These principles may be used to engineer second generation molecules having many of the natural properties of the peptides of the invention (= binding peptides) disclosed herein, but with altered and even improved characteristics.

[0022] In certain embodiments, the present invention may concern fusion proteins. These molecules may have all or a substantial portion of a binding peptide of the invention, linked at the N- or C-terminus, to all or a portion of a second polypeptide or protein. For example, fusions may employ leader sequences from other species to permit the recombinant expression of a protein in a heterologous host. Inclusion of a cleavage site at or near the fusion junction may be used to facilitate removal of the extraneous polypeptide after purification. In other cases, a leader sequence may be included to facilitate intracellular targeting of the binding peptide of the invention. Other useful fusions may include linking of functional domains, such as active sites from enzymes or transmembrane regions. These examples are not meant to be limiting and it is contemplated that within the scope of the present invention virtually any protein or peptide could be incorporated into a fusion protein comprising the binding peptide of the invention. Methods of generating fusion proteins are well known to those of skill in the art. Such proteins can be produced, for example, by chemical attachment using bifunctional cross-linking reagents, by de novo synthesis of the complete fusion protein, or by attachment of a DNA sequence encoding the anti-amyloid binding peptide to a DNA sequence encoding the second peptide or protein, followed by expression of the intact fusion protein.

[0023] Certain embodiments may concern isolation and/or purification of the anti-amyloid binding peptide of the invention. Protein purification techniques are well known to those of skill in the art. The protein or peptide of interest may be purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, gel exclusion chromatography, polyacrylamide gel electrophoresis, affinity chromatography, immunoaffinity chromatography and isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography (FPLC) or even HPLC. For short peptides, reverse-phase HPLC may be of use.

[0024] Various techniques suitable for use in protein purification are well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like, or by heat denaturation, followed by: centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of these and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

[0025] There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed

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utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

[0026] Affinity chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule to which it can specifically bind to. This is a receptor-ligand type of interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (e.g., altered pH, ionic strength, temperature, etc.). The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand.

[0027] In a preferred embodiment, the invention relates to a peptide wherein the amino acid sequence specified under b) has at least 40% homology to any of the amino acid sequences specified under a).

[0028] In another preferred embodiment, the invention relates to a peptide wherein the amino acid sequence specified under b) has at least 60%, preferably 70%, more preferably 80%, especially preferably 90% homology to any of the amino acid sequences specified under a).

[0029] In another preferred embodiment, the invention relates to a peptide which consists of the amino acid sequence SEQ ID No. 1.

[0030] In a preferred embodiment, the invention relates to a peptide which comprises or preferably consists of the amino acid sequence SEQ ID No. 2.

[0031] In another preferred embodiment, the invention relates to a peptide which comprises or preferably consists of the amino acid sequence SEQ ID No. 3.

[0032] In another preferred embodiment, the invention relates to a peptide which comprises or preferably consists of the amino acid sequence SEQ ID No. 4. In a more preferred embodiment, the sequences SEQ ID No. 2, 3 and/or 4 are binder to the amyloid.

[0033] In another preferred embodiment, the invention relates to a peptide comprising an amino acid sequence wherein the linker and/or a spacer are selected from the group comprising: α-aminocarboxylic acids as well as homo- and heterooligomers thereof, α,ω-aminocarboxylic acids and branched homo- or heterooligomers thereof, other aliphatic and/or aromatic amino acids as well as linear and branched homo- or heterooligomers; amino-oligoalkoxyalkylamines; maleinimidocarboxylic acid derivatives; oligomers of alkylamines; 4-alkylphenyl derivatives; 4-oligoalkoxyphenyl or 4-oligoalkoxyphenoxy derivatives; 4-oligoalkylmercaptophenyl or 4-oligoalkylmercaptophenoxy derivatives; 4-oligoalkylaminophenyl or 4-oligoalkylaminophenoxy derivatives; (oligoalkylbenzyl)phenyl or 4-(oligoalkylbenzyl)phenoxy derivatives, as well as 4-(oligo-alkoxybenzyl)phenyl or 4-(oligoalkoxy-benzyl)phenoxy derivatives; trityl derivatives; benzyloxyaryl or benzyloxyalkyl derivatives; xanthen-3-yloxyalkyl derivatives; (4-alkylphenyl)- or m-(4-alkylphenoxy)alkanoic acid derivatives; oligoalkyl-phenoxyalkyl or oligoalkoxyphenoxyalkyl derivatives; carbamate derivatives; amines; trialkylsilyl or dialkylalkoxysilyl derivatives; alkyl or aryl derivatives and/or combinations thereof.

[0034] In another preferred embodiment, the invention relates to a peptide comprising an amino acid sequence X1-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-X15-X16-X17-X18-X19-X20-X21-X22-X23-X24-X25-X26-X27-X28-X29-X30-X31-X32-X33-X34-X35-X36-X37-X38-X39-X40-X41-X42-X43-X44-X45-X46-X47-X48-X49-X50-X51-X52-X53-X54-X55-X56-X57-X58-X59-X60-X61-X62-X63-X64-X65-X66-X67-X68-X69-X70-X71-X72-X73-X74-X75-X76-X77-X78-X79-X80-X81-X82-X83-X84-X85-X86-X87-X88-X89-X90-X-1-X92-X93-X94-X95-X96-X97-X98-X99-X100-X101-X102-X103-X104-X105-X106-X107-X108-X109-X110-X111-X112-X113-X114-X115-X116-X117-X118-X119-X120-X121-X122-X123-X124-X125-X126-X127-X128-X129-X130-X131 wherein

X1 = amino group, amide, acetyl group, biotin group, marker, spacer, linker, GKK, SGKK or deletion,
X2 = E*
X3 = V#
X4 = Q#
X5 = L#
X6 = V#
X7 = E#
X8 = S#
X9 = G#
X10 = G#
X11 = G#

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(continued)

X12 = L#
X13 = V#
X14 = Q#
X15 = P*
X16 = G*
X17 = G#
X18 = S#
X19 = L#
X20 = R#
X21 = L#
X22 = S#
X23 = C#
X24 = T#
X25 = A#
X26 = S#
X27 = G#
X28 = Y*
X29 = T*
X30 = F*
X31 = S*
X32 = H#, R#, K#
X33 = R#, H#, K#
X34 = Y#, W#, F#, I#, L#, V#, T#, S#
X35 = H#, R#, K#
X36 = R#, H#, K#
X37 = W#
X38 = F#
X39 = R#
X40 = Q#
X41 = A#
X42 = P*
X43 = G*
X44 = K*
X45 = E#
X46 = R#
X47 = E#
X48 = I#
X49 = V#
X50 = A#
X51 = V#
X52 = I#
X53 = S#, T#, W#, V#, I#, L#
X54 = Q, N
X55 = S, T
X56 = G, A
X57 = M#, C#, V#, I#, L#, Y#, W#, F#
X58 = R#, K#, H#
X59 = T#
X60 = Y#
X61 = Y#
X62 = A#

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(continued)

X63 =	D*
X64 =	S*
X65 =	V*
X66 =	K*
X67 =	G*
X68 =	R#
X69 =	F#
X70 =	T#
X71 =	I#
X72 =	S#
X73 =	R#
X74 =	D#
X75 =	N*
X76 =	A*
X77 =	K*
X78=	N*
X79 =	T#
X80 =	V#
X81 =	Y#
X82 =	L#
X83 =	Q#
X84 =	M#
X85 =	N#
X86 =	S*
X87 =	L*
X88 =	K*, R*, S*, T*, A*, C*, D*, E*, F*, G*, H*, I*, L*, M*, N*, P*, Q*, V*, W*, Y*
X89 =	P*
X90 =	E*
X91 =	D*
X92 =	T#, I#, Y#, F#, V#, C#, M#, W#, Q#, E#, S#, K#, R#, H#, L#
X93 =	A#
X94 =	M#
X95 =	Y#
X96=	Y#
X97 =	C#
X98 =	A#
X99 =	A#
X100 =	G#, A#
X101 =	T#, S#, F#, W#, I#, L#, V#
X102 =	R, K, H
X103 =	K, H, R
X104 =	N, Q
X105 =	V, I, L, M
X106 =	W, F, Y
X107 =	T, S
X108 =	R, K, H
X109 =	Q, N
X110 =	H, K, R
X111 =	P*
X112 =	F*
X113 =	D#

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(continued)

X114 = Y#
X115 = W#
X116 = G#
X117 = Q#
X118 = G#
X119 = T#
X120 = Q#
X121 = V#
X122 = T#
X123 = V#
X124 = S#
X125 = S*
X126 = A*
X127 = S*
X128 = G*
X129 = A*
X130 = E*
X131 = amino group, amide, acetyl group, biotin group, marker, spacer, linker, GKK, SGKK or deletion, wherein

in a preferred embodiment, the CDR-regions are underlined. Amino acids, which are marked with a star (*), are variable. In some preferred embodiments, the amino acids marked with a star are modified to any one of amino acids A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, Y. In another preferred embodiment, the amino acids marked with a star, can be deleted. In one aspect, the invention relates to an antibody which comprises a peptide with the general amino acid sequence X1 to X131, wherein the amino acid according to (i) about X1 to X31, (ii) about X37 to X52, (iii) about X59 to X99 and (iv) about X111 to X131 are substantially identical or homologue to the above-mentioned sequences (i) to (iv) and unrestrictedly variable in the part of about X32 to X36, about X53 to X58 and about X100 to X110. Amino acids, which are marked by a hash sign (#) are beta-strands; in a preferred embodiment, the introduction of singular beta-sheet breakers may not have any influence. At least 15%, 20%, 25%, 30%, 35%, 40%, or 50% of the amino acids which are not underlined can be exchanged by any type of natural and/or artificial amino acid without influencing the biological activity according to the invention, that is the interaction with amyloid fibrillar/non fibrillar polypeptides.

[0035] In another preferred embodiment, the invention relates to a peptide which is used as a medical active substance.

[0036] In another preferred embodiment, the invention relates to a peptide which is bound by amyloid fibrillar and/or non-fibrillar polypeptides of patients suffering from amyloid disease.

[0037] In a preferred embodiment, the peptide binds amyloid with much higher affinity than Congo red. The peptide is a conformation-sensitive amyloid binder, which interacts with pathogenic aggregates, soluble oligomeres or protofibrils.

[0038] In another preferred embodiment, the invention relates to a peptide which is immobilized and/or fixed to magnetic, paramagnetic and/or non magnetic nanoparticles.

[0039] In certain embodiments of the invention, one or more labels may be attached to the peptide of the invention. A number of different labels may be used, such as fluoro-phores, chromophores, radioisotopes, enzymatic tags, antibodies, bioluminescent, electroluminescent, phosphorescent, affinity labels, nanoparticles, metal nanoparticles, gold nanoparticles, silver nanoparticles, magnetic particles, spin labels or any other type of label known in the art.

[0040] Non-limiting examples of affinity labels include an antibody, an antibody fragment, a receptor protein, a hormone, biotin, DNP, and any polypeptide/protein molecule that binds to an affinity label.

[0041] Non-limiting examples of enzymatic tags include urease, alkaline phosphatase or peroxidase. Colorimetric indicator substrates can be employed with such enzymes to provide a detection means visible to the human eye or spectrophotometrically.

[0042] Non-limiting examples of photodetectable labels include Alexa 350, Alexa 430, AMCA, aminoacridine, BODIPY 630/650, BODIPY 650/665, BODIPY-FL, BODIPY-R6G, BODIPY-TMR, BODIPY-TRX, 5-carboxy-4',5'-dichloro-2- ',7'-dimethoxy fluorescein, 5-carboxy-2',4',5',7'-tetrachlorofluorescein, 5-carboxyfluorescein, 5-carboxyrhodamine, 6-carboxyrhodamine, 6-carboxytetramethyl amino, Cascade Blue, Cy2, Cy3, Cy5,6-FAM, dansyl chloride, Fluorescein, HEX, 6-JOE, NBD (7-nitrobenz-2-oxa-1,3-diazole), Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, phthalic acid, terephthalic acid, isophthalic acid, cresyl fast violet, cresyl blue violet, brilliant cresyl blue, para-aminobenzoic acid, erythrosine, phthalocyanines, azome-thines, cyanines, xanthines, succinylfluoresceins, rare earth metal cryp-

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tates, europium trisbipyridine diamine, a europium cryptate or chelate, diamine, dicyanins, La Jolla blue dye, allopycocyanin, allococyanin B, phycocyanin C, phycocyanin R, thiamine, phycoerythrocyanin, phyco-erythrin R, REG, Rhodamine Green, rhodamine isothiocyanate, Rhodamine Red, ROX, TAMRA, TET, TRIT (tetramethyl rhodamine isothiol), Tetramethylrhodamine, and Texas Red. These and other luminescent labels may be obtained from commercial sources such as Molecular Probes (Eugene, O-reg.).

[0043] In other embodiments of the invention, labels of use may comprise metal nanoparticles. Methods of preparing nanoparticles are known. Nanoparticles may also be obtained from commercial sources. Modified nanoparticles are available commercially, such as Nanogold.RTM. nanoparticles from Nanoprobes, Inc. (Yaphank, N.Y.).

[0044] In some embodiments of the invention, proteins may be labeled using side-chain specific and/or selective reagents. Such reagents and methods are known in the art. Non-limiting exemplary reagents that may be used include acetic anhydride (lysine, cysteine, serine and tyrosine); trinitrobenzenesulfonate (lysine); carbodiimides (glutamate, aspartate); phenylglyoxal (arginine); 2,3-butanedione (arginine); pyridoxal phosphate (lysine); p-chloromercuribenzoate (cysteine); 5,5'-dithiobis(2-nitro-benz- oic acid) (cysteine); diethylpyrocarbonate (lysine, histidine); N-bromosuccinimide (tryptophan) and tetrani-tromethane (cysteine, tyrosine). Various methods for attaching labels to nucleic acids and/or oligonucleotides are known in the art and may be used. For example, watersoluble carbodiimides may be used to cross-link the phosphate groups of nucleic acids or oligonucleotides to various labels. Amino or sulfhydryl modified oligonucleotides or nucleic acids may be attached to labels using known bifunctional crosslinking reagents.

[0045] In alternative embodiments of the invention, various cross-linking reagents known in the art, such as homobifunctional, hetero-bifunctional and/or photoactivatable cross-linking reagents may be used. Non-limiting examples of such reagents include bisimidates; 1,5-difluoro-2,4-(dinitrob- enzene); N-hydroxysuccinimide ester of suberic acid; disuccinimidyl tartarate; dimethyl-3,3'-dithio-bispropionimidate; N-succinimidyl-3-(2-pyridyldithio)propionate; 4-(bromoaminoethyl)-2-nitro-phenylazide; and 4-azidoglyoxal. Such reagents may be modified to attach various types of labels, such as fluorescent labels. The skilled artisan will realize that such cross-linking reagents are not limited to use with proteins, but may also be used with other types of molecules.

[0046] In another preferred embodiment, the invention relates to a peptide which is bound to a solid phase.

[0047] The invention also relates to an isolated nucleic acid molecule selected from the group comprising

a) a nucleic acid molecule comprising a nucleotide sequence which encodes at least one peptide of the invention, particularly according to SEQ ID No. 1 and/or SEQ ID No. 2, 3 and/or 4.;

b) a nucleic acid molecule which is complementary to a nucleotide sequence in accordance with a);

c) a nucleic acid molecule which undergoes hybridization with a nucleotide sequence according to a) or b) under moderately or preferred stringent conditions;

d) a nucleic acid molecule comprising a nucleotide sequence having sufficient homology to be functionally analogous/equivalent to a nucleotide sequence according to a), b) or c);

e) a nucleic acid molecule which, as a consequence of the genetic code, is degenerated into a nucleotide sequence according to a) through d); and

f) a nucleic acid molecule according to a nucleotide sequence of a) through e) which is modified by deletions, additions, substitutions, translocations, inversions and/or insertions and functionally analogous/equivalent to a nucleotide sequence according to a) through e).

[0048] The present invention also relates to a nucleic acid preparation comprising a nucleic acid sequence capable of encoding the peptide/peptide variant of the invention a fragment thereof. The nucleic acids of the invention can be DNA or RNA. A DNA molecule is "capable of expressing" a polypeptide, such as the antibody of the invention or the N-terminal fragment thereof or the C-terminal fragment thereof, if it contains nucleotide sequences which contain transcriptional and translational regulatory information, and such sequences are operably linked to nucleotide sequences which encode the peptide/polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The regulatory regions needed for gene expression, in general, include a promoter region as well as the DNA sequences which, when transcribed into RNA, will signal the initiation of protein synthesis. Such regions will normally include those 5'-non-coding sequences involved in initiation of transcription and translation. A promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. The nucleic acid segment encoding the peptide or peptide variant of the invention or the N-terminal fragment thereof or the C-terminal fragment thereof should thus be linked to regulatory elements, such as a promoter and enhancer, that allow expression of the nucleic acid

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segment in the intended host. For expression in blood cells, for example, as is desirable for induction of an immune response in a mammal, promoter and enhancer elements from light or heavy chain immunoglobulin genes or the CMV major intermediate early promoter and enhancer are suitable to direct expression.

[0049] The linked regulatory elements and coding sequences are often cloned into a vector. A vector is a nucleic acid, preferably a DNA molecule, capable of autonomous replication in a cell, to which a nucleic acid segment (e. g. a gene or polynucleotide, preferably DNA) can be operatively linked so as to bring about replication of the attached segment. Vectors capable of directing the expression of nucleic acid (preferably DNA) segments (genes) encoding for one or more proteins are referred to as "expression vectors". An expression vector thus is any plasmid or virus into which a foreign nucleotide sequence (preferably DNA) may be inserted or expressed. A number of viral vector systems are available, including (but not limited to) retroviral systems (Lawrie and Tumin, 1993), adenoviral vectors (Bett et al., 1993), adeno-associated virus vectors (Zhou et al., 1994), viral vectors from the pox family including vaccinia virus and the avian pox viruses, viral vectors from the alpha virus genus such as those derived from Sindbis and Semliki Forest Viruses (Dubensky et al., 1996), and papillomaviruses (Ohe et al., 1995; WO 94/12629; Xiao and Brandsma, 1996).

[0050] As used herein, "stringent conditions" or "high stringency conditions" are those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50 degree C.; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42 degree C.; or (3) employ 50% formamide, 5 times SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 times Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42 degree C., with washes at 42 degree C. in 0.2 times SSC (sodium chloride/sodium citrate) and 50% formamide at 55 degree C., followed by a high-stringency wash consisting of 0.1 times SSC containing EDTA at 55 degree C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

[0051] It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the present invention. Further, alleles of the genes comprising the polynucleotide sequences provided herein are within the scope of the present invention. Alleles are endogenous genes that are altered as a result of one or more mutations, such as deletions, additions and/or substitutions of nucleotides. The resulting mRNA and protein may, but need not, have an altered structure or function. Alleles may be identified using standard techniques (such as hybridization, amplification and/or database sequence comparison).

[0052] The polynucleotides of this invention can be obtained using chemical synthesis, recombinant methods, or PCR. Methods of chemical polynucleotide synthesis are well known in the art and need not be described in detail herein. One of skill in the art can use the sequences provided herein and a commercial DNA synthesizer to produce a desired DNA sequence.

[0053] For preparing polynucleotides using recombinant methods, a polynucleotide comprising a desired sequence can be inserted into a suitable vector, and the vector in turn can be introduced into a suitable host cell for replication and amplification, as further discussed herein. Polynucleotides may be inserted into host cells by any means known in the art. Cells are transformed by introducing an exogenous polynucleotide by direct uptake, endocytosis, transfection, F-mating or electroporation. Once introduced, the exogenous polynucleotide can be maintained within the cell as a non-integrated vector (such as a plasmid) or integrated into the host cell genome. The polynucleotide so amplified can be isolated from the host cell by methods well known within the art. See, e.g., Sambrook et al. (1989).

[0054] In a preferred embodiment of the invention, the nucleotide sequence specified under d) has at least 40% homology to any of the nucleotide sequences specified under a) through c).

[0055] In another preferred embodiment of the invention, the nucleotide sequence specified under d) has at least 60%, preferably 70%, more preferably 80%, especially preferably 90% homology to any of the nucleotide sequences specified under a) through c).

[0056] "Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, cabamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pen-

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dant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, ply-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups.

[0057] Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'--O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, .alpha.-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), "(O)NR.sub.2 ("amidate"), P(O)R', P(O)OR', CO or CH.sub.2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (--O--) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

[0058] In another preferred embodiment of the invention, the nucleic acid molecule of the invention is a genomic DNA, a cDNA and/or an RNA.

[0059] The invention also relates to a vector comprising the nucleic acid molecule of the invention. As used herein, "vector" means a construct, which is capable of delivering, and preferably expressing, one or more gene(s) or sequence (s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells.

[0060] In another aspect, the invention relates to a host cell comprising the vector of the invention. A "host cell" includes an individual cell or cell culture that can be or has been a recipient for vector(s) for incorporation of polynucleotide inserts. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in genomic DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. A host cell includes cells transfected in vivo with a polynucleotide(s) of this invention. The invention also relates to an animal comprising said host cell.

[0061] In yet another aspect, the invention relates to a recognition molecule or binding protein (e. g. an antibody) comprising the peptide of the invention.

[0062] In a preferred embodiment, the antibody is a camel antibody or a part thereof, for example a camelid VHH-antibody domain. The antibody of the invention advantageously binds amyloid with high affinity but shows little interactions with disaggregated, monomeric precursor polypeptides. In a preferred embodiment, the antibody possesses the remarkable property for it recognizes amyloid irrespective of the underlying polypeptide sequence and whether amyloid samples come from in vitro or in vivo sources. This is possible because the basic polypeptide conformation of amyloid is always the same. Although this conformation is a beta-sheet structure, it is distinct from beta-sheets in native proteins. The antibody of the invention does advantageously not recognize the beta-sheets in native proteins. The antibody of the invention advantageously discriminates between amyloid and native protein conformations. The antibody of the invention is so far the only protein known to do so. Diagnosis of extracerebral amyloidosis differs from diagnosis of cerebral amyloidosis, because the former are clinically far more heterogeneous and diverse in origin. By definition, amyloid can only be diagnosed by histological investigation of a tissue sample using an amyloid-specific stain to reveal the presence of amyloid. After *diagnosis* has been obtained *amyloid classification* reveals the type of amyloid (i.e. which protein forms the deposit). Currently, amyloid diagnosis requires Congo red polarising microscopy. Several severe problems are associated with this method: its low sensitivity makes early diagnosis very difficult and creates significant sampling errors with small amyloid deposits, results are non-quantitative, different laboratories use significantly different (and often incorrect) Congo red staining protocols, correct amyloid diagnosis requires a polarisation microscope that is not generally available in pathology departments. This has lead to numerous cases of misdiagnosis. Different types of amyloidosis differ by the protein forming the fibrils. Altogether there are more than 25 amyloidosis-associated proteins. Amyloid classification is performed normally with sequence-specific antibodies, but only specialised labs hold antibodies for more than a handful of amyloidoses. Moreover, some amyloidoses are notoriously difficult to classify, such as AL.

[0063] Historically, the most investigated binding proteins are antibodies. Antibodies normally display binding sites composed of two separate polypeptide chains, assembled as tetrameric protein in the immunoglobulin IgG molecule. More recently, it has become possible to produce single polypeptide chain binding proteins, in which binding is mediated by a single protein domain. Such binding proteins can be based on the same or highly related protein scaffolds or sequence, yet display highly divergent binding specificities. Preferably, the peptides of the invention are defined as a

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single polypeptide chain binding proteins. Frequently, they are made by first providing a certain level of diversity in a chosen monomeric protein scaffold or fold, which itself can have a natural origin or synthetic basis, and then using molecular selection or screening methods to identify amongst the protein variants those that show a desirable binding specificity. Alternatively, they are harvested from nature, such as the "heavy chain only" camelid, shark and/or llama antibodies.

[0064] The term specific recognition, specifically recognizing, specifically binding with, specifically reacting with or specifically forming an immunological reaction with refers to a binding reaction by the antibody to the amyloid fibrillar and/or non-fibrillar polypeptides or fragments thereof respectively in the sample tested, in the presence of a heterogeneous population of other proteins and/or other biologics. Thus, under the designated immunoassay conditions, the specified antibody preferentially binds to the amyloid fibrillar and/or non-fibrillar polypeptides or fragments thereof.

[0065] As used herein, an "antibody" generally refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD, and IgE, respectively. The basic immunoglobulin (antibody) structural unit is known to comprise a tetramer or dimer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids, primarily responsible for antigen recognition. The terms "variable light chain" and "variable heavy chain" refer to these variable regions of the light and heavy chains respectively. Optionally, the antibody or the immunological portion of the antibody, can be chemically conjugated to, or expressed as, a fusion protein with other proteins.

[0066] Antibodies of the invention include, but are not limited to polyclonal, monoclonal, bispecific, human, humanized or chimeric antibodies, single variable fragments (ssFv), single chain fragments (scFv), Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic antibodies and epitope-binding fragments of any of the above, provided that they retain the original binding properties. Also mini-antibodies and multivalent antibodies such as diabodies, triabodies, tetravalent antibodies and peptabodies can be used in a method of the invention. The immunoglobulin molecules of the invention can be of any class (i.e. IgG, IgE, IgM, IgD and IgA) or subclass of immunoglobulin molecules.

[0067] Various adjuvants may be used to enhance the immunological response of the antibody of the invention, depending on the host species, including, but not limited to, complete or incomplete Freund's adjuvant, a mineral gel such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyol, a polyanion, a peptide, an oil emulsion, keyhole limpet hemocyanin, dinitrophenol, or an adjuvant such as BCG (bacille Calmette-Guerin) or corynebacterium parvum. For the preparation of monoclonal antibodies, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. Hyperimmunization of an appropriate donor, generally a mouse, with the antigen is undertaken. Isolation of splenic antibody producing cells is then carried out. These cells are fused to a cell characterized by immortality, such as a myeloma cell, to provide a fused cell hybrid (Hybridoma) which can be maintained in culture and which secretes the required monoclonal antibody. The cells are then cultured in bulk and the monoclonal antibodies harvested from the culture media for use. Specific techniques include but are not limited to the hybridoma technique developed by Kohler and Milstein (1975), the human B-cell hybridoma technique (Kozbor et al., 1983) or the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985). Screening for the desired antibody can be done by techniques known in the art, such as ELISA. Thus, in a particular embodiment, the present invention provides an antibody that binds with greater affinity (particularly at least 2-fold, more particularly at least 5-fold, still more particularly at least 10-fold greater affinity) to amyloid fibrillar and/or non-fibrillar polypeptides or fragments thereof than to another protein. In another preferred embodiment, the present invention provides an antibody that binds with greater affinity (particularly at least 2-fold, more particularly at least 5-fold, still more particularly at least 10-fold greater affinity) to an N-terminal truncated and/or post-translationally modified amyloid fibrillar and/or non fibrillar polypeptide or fragments thereof.

[0068] While various antibody fragments are defined in terms of enzymatic digestion of an intact antibody with papain, pepsin or other proteases, one of skill will appreciate that such antibody fragments as well as full size antibodies may be synthesized de novo either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibodies and antibody fragments either produced by the modification of whole antibodies or synthesized de novo using recombinant DNA methodologies.

[0069] Heteroconjugate antibodies, comprising two covalently joined antibodies, are also within the scope of the invention. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents and techniques are well known in the art, and are described in U.S. Pat. No. 4,676,980.

[0070] Chimeric or hybrid antibodies also may be prepared in vitro using known methods of synthetic protein chemistry, including those involving cross-linking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-

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4-mercaptobutyrimidate.

[0071] Humanized antibody comprising one or more CDRs of antibodies of the invention or one or more CDRs derived from said antibodies can be made using any methods known in the art. For example, four general steps may be used to humanize a monoclonal antibody. These are: (1) determining the nucleotide and predicted amino acid sequence of the starting antibody light and heavy variable domains (2) designing the humanized antibody, i.e., deciding which antibody framework region to use during the humanizing process (3) the actual humanizing methodologies/techniques and (4) the transfection and expression of the humanized antibody. See, for example, U.S. Pat. Nos. 4,816,567; 5,807,715; 5,866,692; 6,331,415; 5,530,101; 5,693,761; 5,693,762; 5,585,089; 6,180,370; 5,225,539; 6,548,640.

[0072] The term humanized antibody means that at least a portion of the framework regions of an immunoglobulin is derived from human immunoglobulin sequences. The humanized versions of the mouse monoclonal antibodies can, for example, be made by means of recombinant DNA technology, departing from the mouse and/or human genomic DNA sequences coding for H and L chains or from cDNA clones coding for H and L chains. Humanized forms of mouse antibodies can be generated by linking the CDR regions of non-human antibodies to human constant regions by recombinant DNA techniques (Queen et al., 1989; WO 90/07861). Alternatively the monoclonal antibodies used in the method of the invention may be human monoclonal antibodies. Human antibodies can be obtained, for example, using phage-display methods (WO 91/17271; WO 92/01047). In these methods, libraries of phage are produced in which members display different antibodies on their outer surfaces. Antibodies are usually displayed as Fv or Fab fragments. Phage displaying antibodies with a desired specificity are selected by affinity enrichment to amyloid fibrillar polypeptides. Human antibodies against amyloid fibrillar and/or non-fibrillar polypeptides or fragments thereof can also be produced from non-human transgenic mammals having transgenes encoding at least a segment of the human immunoglobulin locus and an inactivated endogenous immunoglobulin locus (W093/12227; WO 91/10741).

[0073] As used herein, humanized antibodies refer also to forms of non-human (e.g. murine, camel, llama, shark) antibodies that are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences, but are included to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. Antibodies may have Fc regions modified as described in WO 99/58572. Other forms of humanized antibodies have one or more CDRs (one, two, three, four, five, six) which are altered with respect to the original antibody, which are also termed one or more CDRs "derived from" one or more CDRs (e. g. SEQ ID No. 2, 3 and/or 4) from the original antibody, preferably a camel antibody with the sequence of SEQ ID No. 1 and CDRs according to SEQ ID No. 2, 3 and/or 4.

[0074] As used herein, human antibody means an antibody having an amino acid sequence corresponding to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies known in the art or disclosed herein. This definition of a human antibody includes antibodies comprising at least one human heavy chain polypeptide or at least one human light chain polypeptide. One such example is an antibody comprising murine light chain and human heavy chain polypeptides. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan et al., 1996, Nature Biotechnology, 14:309-314; Sheets et al., 1998, PNAS, (USA) 95:6157-6162; Hoogenboom and Winter, 1991, J. Mol. Biol., 227:381; Marks et al., 1991, J. Mol. Biol., 222:581). Human antibodies can also be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. This approach is described in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016. Alternatively, the human antibody may be prepared by immortalizing human B lymphocytes that produce an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or may have been immunized in vitro). See, e.g., Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boemer et al., 1991, J. Immunol., 147 (1):86-95; and U.S. Pat. No. 5,750,373.

[0075] Human antibodies can be selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a particular mouse antibody. Such antibodies are particularly likely to share the useful functional properties of the mouse antibodies. Human polyclonal antibodies can also be provided in the form of serum from humans immunized with an immunogenic agent. Optionally, such polyclonal antibodies can be concentrated by affinity purification using

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amyloid fibrillar and/or non-fibrillar polypeptides or fragments thereof as an affinity reagent. Monoclonal antibodies can be obtained from serum according to the technique described in WO 99/60846.

[0076] A variable region of an antibody refers to the variable region of the antibody light chain or the variable region of the antibody heavy chain, either alone or in combination. The variable regions of the heavy and light chain each consist of four framework regions (FR) connected by three complementarity determining regions (CDRs) also known as hypervariable regions. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies. There are at least two techniques for determining CDRs: (1) an approach based on cross-species sequence variability (i.e., Kabat et al. Sequences of Proteins of Immunological Interest, (5th ed., 1991, National Institutes of Health, Bethesda Md.)); and (2) an approach based on crystallographic studies of antigen-antibody complexes (Allazikani et al (1997) J. Molec. Biol. 273:927-948)). As used herein, a CDR may refer to CDRs defined by either approach or by a combination of both approaches.

[0077] A "constant region" of an antibody refers to the constant region of the antibody light chain or the constant region of the antibody heavy chain, either alone or in combination.

[0078] The term "Fc region" is used to define a C-terminal region of an immunoglobulin heavy chain. The "Fc region" may be a native sequence Fc region or a variant Fc region. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The numbering of the residues in the Fc region is that of the EU index as in Kabat. Kabat et al., Sequences of Proteins of Imunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md., 1991. The Fc region of an immunoglobulin generally comprises two constant domains, CH2 and CH3.

[0079] As used herein, "Fc receptor" and "FcR" describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the Fc.gamma.RI, Fc.gamma.RII, and Fc.gamma.RIII subclasses, including allelic variants and alternatively spliced forms of these receptors. Fc.gamma.RII receptors include Fc.gamma.RIIA (an "activating receptor") and Fc.gamma.RIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. FcRs are reviewed in Ravetch and Kinet, 1991, Ann. Rev. Immunol., 9: 457-92; Capel et al., 1994, Immunomethods, 4:25-34; and de Haas et al., 1995, J. Lab. Clin. Med., 126:330-41. "FcR" also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., 1976, J. Immunol., 117:587; and Kim et al., 1994, J. Immunol., 24:249). Complement dependent cytotoxicity" and "CDC" refer to the lysing of a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (Clq) to a molecule (e.g. an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., J. Immunol. Methods, 202:163 (1996), may be performed. A "functional Fc region" possesses at least one effector function of a native sequence Fc region. Exemplary "effector functions" include Clq binding; complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down-regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g. an antibody variable domain) and can be assessed using various assays known in the art for evaluating such antibody effector functions. A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification, yet retains at least one effector function of the native sequence Fc region. Preferably, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will preferably possess at least about 80% sequence identity with a native sequence Fc region and/or with an Fc region of a parent polypeptide, and most preferably at least about 90% sequence identity therewith, more preferably at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% sequence identity therewith.

[0080] As indicated above, the present invention also provides preparations and methods for preventing and treating, in a mammal, a disease characterized by amyloid formation and/or aggregation, preferably by promoting a non-fibrillar aggregation or in another preferred embodiment by induction of an immune response in a mammal or human patient. Accordingly, the present invention also provides a vaccine composition or a therapeutic composition comprising a peptide of the invention as referred to above, comprising an additional antibody or a T-cell specific for an N-terminal truncated and/or post-translationally modified A beta peptide, or comprising a nucleic acid encoding an N-terminal truncated post-translationally modified A beta peptide and/or amyloid fibrillar/non-fibrillar polypeptides.

[0081] As used herein, the term "preventing a disease" means inhibiting or reversing the onset of the disease, inhibiting or reversing the initial signs of the disease (i.e. formation and/or aggregation of amyloid peptides), inhibiting the appearance of clinical symptoms of the disease. As used herein, the term "treating a disease" includes substantially inhibiting

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the disease, substantially slowing or reversing the progression of the disease, substantially ameliorating clinical symptoms of the disease or substantially preventing the appearance of clinical symptoms of the disease.

[0082] The mammal examined in the present invention may be a non-human mammal, such as (but not limited to) a cow, a pig, a sheep, a goat, a horse, a monkey, a rabbit, a hare, a dog, a cat, a mouse, a rat, an elk, a deer, or a tiger. In a preferred embodiment, the mammal is a primate. In another preferred embodiment the mammal is a human, more preferably the mammal is a human adult.

[0083] The vaccine or therapeutic compositions of the present invention induce an immune response against the amyloid fibrillar and/or non-fibrillar polypeptides or fragments thereof. The induction of an immune response is "active" when an immunogen is administered to induce antibodies or T-cells reactive against the immunogen. The induction of an immune response is "passive" when an antibody is administered that itself binds to the amyloid fibrillar and/or non-fibrillar polypeptides or fragments thereof in the mammal.

[0084] In a preferred embodiment of the invention, the recognition-molecule is an antibody-fragment of an antibody or an antibody.

[0085] In another preferred embodiment of the invention, said antibody or the fragment thereof is a chimeric antibody or a fragment thereof.

[0086] In another preferred embodiment of the invention, said chimeric antibody or the fragment thereof is a (partially) humanized antibody or a fragment thereof.

[0087] In another preferred embodiment of the invention, said fragment of the antibody is a variable fragment of a single-chain of an antibody.

[0088] In another preferred embodiment of the invention, said single-chain is a heavy chain variable domain.

[0089] Preferably, the antibody of the invention comprises the sequences SEQ ID No. 2, 3 or 4, e. g. as CDR-region.

[0090] The CDR portions of the antibody according to SEQ ID No. 1 are preferably SEQ ID No. 2, 3 and/or 4 or other parts of the sequence. Determination of CDR regions is well within the skill of the art. It is understood that in some embodiments, CDRs can be a combination of the Kabat and Chothia CDR (also termed "combined CDRs" or "extended CDRs"). In some embodiments, the CDRs are the Kabat CDRs. In other embodiments, the CDRs are the Chothia CDRs. In other words, in embodiments with more than one CDR, the CDRs may be any of Kabat, Chothia, combination CDRs, or combinations thereof.

[0091] In some embodiments, the invention provides a polypeptide (which may or may not be an antibody) which comprises at least one CDR, at least two, at least three, or more CDRs that are substantially identical to at least one CDR, at least two, at least three, or more CDRs of the antibody or peptide of the invention. Other embodiments include antibodies which have at least two, three, four, five, or six CDR(s) that are substantially identical to at least two, three, four, five or six CDRs of the antibodies of the invention or derived from the antibodies of the invention. In some embodiments, the at least one, two, three, four, five, or six CDR(s) are at least about 85%, 86%, 87%, 88%, 89%, 90%, 95%, 96%, 97%, 98%, or 99% identical to at least one, two or three CDRs of the antibody of the invention. It is understood that, for purposes of this invention, binding specificity and/or overall activity is generally retained, although the extent of activity may vary compared to said antibody (may be greater or lesser).

[0092] The invention also provides a polypeptide (which may or may not be an antibody) which comprises an amino acid sequence of SEQ ID No. 1, 2, 3 and/or 4 or its variants that has any of the following: at least 3 contiguous amino acids, at least 4 contiguous amino acids, at least about 5 contiguous amino acids, at least about 10 contiguous amino acids, at least about 15 contiguous amino acids, at least about 20 contiguous amino acids, at least about 25 contiguous amino acids of a sequence of SEQ ID No. 1 or its variants, wherein at least 3 of the amino acids are from a sequence of SEQ ID No. 2, 3 and/or 4 or its variants. In a preferred embodiment, the sequences of SEQ ID No. 2, 3 or 4 represent the variable region of the antibody, more preferably the CDR-region. In one embodiment, the variable region is from a light chain, in another embodiment, the variable region is from a heavy chain of the antibody.

[0093] The binding affinities of the antibodies and polypeptides of the invention may vary, and need not be (but can be) a particular value or range, as the exemplary embodiments described below. The binding affinity (K_D) of the antibodies and polypeptides of the invention to amyloid fibrillar and/or non-fibrillar polypeptides can be about 0.10 to about 0.80 nM, about 0.15 to about 0.75 nM and about 0.18 to about 0.72 nM. In some embodiments, the binding affinity is about 2 pM, about 5 pM, about 10 pM, about 15 pM, about 20 pM, about 40 pM, or greater than about 40 pM. In one embodiment, the binding affinity is between about 2 pM and 22 pM. In other embodiments, the binding affinity is less than about 10 nM, about 5 nM, about 4 nM, about 3 nM, about 2 nM, about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 200 pM, about 150 pM, about 100 pM, about 90 pM, about 80 pM, about 70 pM, about 60 pM, about 50 pM, about 40 pM, about 30 pM, about 10 pM. In some embodiment, the binding affinity is about 10 nM. In other embodiments, the binding affinity is less than about 10 nM, less than about 50 nM, less than about 100 nM, less than about 150 nM, less than about 200 nM, less than about 250 pM, less than about 500 nM, or less than about 1000 nM. In other embodiments, the binding affinity is less than about 5 nM. In other embodiments, the binding affinity is less than about 1 nM. In other embodiments, the binding affinity is about 0.1 nM or about 0.07 nM. In other embodiments, the binding affinity is less than about 0.1 nM or less than about 0.07 nM. In other

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embodiments, the binding affinity is from any of about 10 nM, about 5 nM, about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 200 pM, about 150 pM, about 100 pM, about 90 pM, about 80 pM, about 70 pM, about 60 pM, about 50 pM, about 40 pM, about 30 pM, about 10 pM to any of about 2 pM, about 5 pM, about 10 pM, about 15 pM, about 20 pM, or about 40 pM. In some embodiments, the binding affinity is any of about 10 nM, about 5 nM, about 1 nM, about 900 pM, about 800 pM, bout 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 200 pM, about 150 pM, about 100 pM, about 90 pM, about 80 pM, about 70 pM, about 60 pM, about 50 pM, about 40 pM, about 30 pM, about 10 pM. In still other embodiments, the binding affinity is about 2 pM, about 5 pM, about 10 pM, about 15 pM, about 20 pM, about 40 pM, or greater than about 40 pM. In a more preferred embodiment, the binding affinity is about 7 nM.

[0094] The invention encompasses modifications to the antibody of the invention, including functionally equivalent antibodies which do not significantly affect their properties and variants which have enhanced or decreased activity and/or affinity. For example, amino acid sequence of the antibody may be mutated to obtain an antibody with the desired binding affinity to amyloid polypeptides. Modification of polypeptides of the invention is routine practice in the art and need not be described in detail herein. Examples of modified polypeptides include polypeptides with conservative substitutions of amino acid residues, one or more deletions or additions of amino acids which do not significantly deleteriously change the functional activity, or use of chemical analogs.

[0095] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody fused to an epitope tag. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody of an enzyme or a polypeptide which increases the serum half-life of the antibody.

[0096] Substitution variants have at least one amino acid residue in the antibody molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

TABLE 1 Amino Acid Substitutions

Original Residue	Conservative Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Asg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Asp, Lys; Arg
Asp (D)	Glu	Glu; Asn
Cys (C)	Ser	Ser; Ala
Gln (Q)	Asn	Asn, Glu
Glu (E)	Asp	Asp; Gln
Gly (G)	Ala	Ala
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; Norleucine
Leu (L)	Ile	Norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Tyr	Leu; Val; Ile; Ala; Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr; Phe
Tyr (Y)	Phe	Trp; Phe; Thr; Ser

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(continued)

Original Residue	Conservative Substitutions	Exemplary Substitutions
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; Norleucine

[0097] Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) Non-polar: Norleucine, Met, Ala, Val, Leu, Ile;

(2) Polar without charge: Cys, Ser, Thr, Asn, Gln;

(3) Acidic (negatively charged): Asp, Glu;

(4) Basic (positively charged): Lys, Arg;

(5) Residues that influence chain orientation: Gly, Pro; and

(6) Aromatic: Trp, Tyr, Phe, His.

[0098] Non-conservative substitutions are made by exchanging a member of one of these classes for another class.

[0099] Any cysteine residue not involved in maintaining the proper conformation of the antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant cross-linking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability, particularly where the antibody is an antibody fragment such as an Fv fragment.

[0100] Amino acid modifications can range from changing or modifying one or more amino acids to complete redesign of a region, such as the variable region. Changes in the variable region can alter binding affinity and/or specificity. In some embodiments, no more than one to five conservative amino acid substitutions are made within a CDR domain. In other embodiments, no more than one to three conservative amino acid substitutions are made within a CDR domain.

[0101] Modifications also include glycosylated and nonglycosylated polypeptides, as well as polypeptides with other post-translational modifications, such as, for example, glycosylation with different sugars, acetylation, and phosphorylation. Antibodies are glycosylated at conserved positions in their constant regions (Jefferis and Lund, 1997, Chem. Immunol. 65:111-128; Wright and Morrison, 1997, TibTECH 15:26-32). The oligosaccharide side chains of the immunoglobulins affect the protein's function (Boyd et al., 1996, Mol. Immunol. 32:1311-1318; Wittwe and Howard, 1990, Biochem. 29:4175-4180) and the intramolecular interaction between portions of the glycoprotein, which can affect the conformation and presented three-dimensional surface of the glycoprotein (Hefferis and Lund, supra; Wyss and Wagner, 1996, Current Opin. Biotech. 7:409-416). Oligosaccharides may also serve to target a given glycoprotein to certain molecules based upon specific recognition structures. Glycosylation of antibodies has also been reported to affect antibody-dependent cellular cytotoxicity (ADCC). In particular, CHO cells with tetracycline-regulated expression of beta (1,4)-N-acetylglucosaminyltransferase III (GnTIII), a glycosyltransferase catalyzing formation of bisecting GlcNAc, was reported to have improved ADCC activity (Umana et al., 1999, Mature Biotech. 17:176-180).

[0102] Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine, asparagine-X-threonine, and asparagine-X-cysteine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

[0103] The following methods may be used for adjusting the affinity of an antibody and for characterizing a CDR. One way of characterizing a CDR of an antibody and/or altering (such as improving) the binding affinity of a polypeptide, such as an antibody, termed "library scanning mutagenesis". Generally, library scanning mutagenesis works as follows. One or more amino acid positions in the CDR are replaced with two or more (such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) amino acids using art recognized methods. This generates small libraries of clones (in some embodiments, one for every amino acid position that is analyzed), each with a complexity of two or more members

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(if two or more amino acids are substituted at every position). Generally, the library also includes a clone comprising the native (unsubstituted) amino acid. A small number of clones, e.g., about 20-80 clones (depending on the complexity of the library), from each library are screened for binding affinity to the target polypeptide (or other binding target), and candidates with increased, the same, decreased or no binding are identified. Methods for determining binding affinity are well-known in the art. Binding affinity may be determined using BIAcore surface plasmon resonance analysis, which detects differences in binding affinity of about 2-fold or greater. BIAcore is particularly useful when the starting antibody already binds with a relatively high affinity, for example a K.sub.D of about 10 nM or lower. Screening using BIAcore surface plasmon resonance is described in the Examples, herein.

[0104] Binding affinity may be determined using Kinexa Biocensor, scintillation proximity assays, ELISA, ORIGEN immunoassay (IGEN), fluorescence quenching, fluorescence transfer, and/or yeast display. Binding affinity may also be screened using a suitable bioassay.

[0105] In some embodiments, every amino acid position in a CDR is replaced (in some embodiments, one at a time) with all 20 natural amino acids using art recognized mutagenesis methods (some of which are described herein). This generates small libraries of clones (in some embodiments, one for every amino acid position that is analyzed), each with a complexity of 20 members (if all 20 amino acids are substituted at every position).

[0106] In some embodiments, the library to be screened comprises substitutions in two or more positions, which may be in the same CDR or in two or more CDRs. Thus, the library may comprise substitutions in two or more positions in one CDR. The library may comprise substitution in two or more positions in two or more CDRs. The library may comprise substitution in 3, 4, 5, or more positions, said positions found in two, three, four, five or six CDRs. The substitution may be prepared using low redundancy codons. See, e.g., Table 2 of Balint et al., (1993) Gene 137(1):109-18).

[0107] The CDR may be CDRH3 and/or CDRL3. The CDR may be one or more of CDRL1, CDRL2, CDRL3, CDRH1, CDRH2, and/or CDRH3. The CDR may be a Kabat CDR, a Chothia CDR, or an extended CDR.

[0108] Candidates with improved binding may be sequenced, thereby identifying a CDR substitution mutant which results in improved affinity (also termed an "improved" substitution). Candidates that bind may also be sequenced, thereby identifying a CDR substitution which retains binding.

[0109] Multiple rounds of screening may be conducted. For example, candidates (each comprising an amino acid substitution at one or more position of one or more CDR) with improved binding are also useful for the design of a second library containing at least the original and substituted amino acid at each improved CDR position (i.e., amino acid position in the CDR at which a substitution mutant showed improved binding). Preparation, and screening or selection of this library is well known in the state of the art.

[0110] Library scanning mutagenesis also provides a means for characterizing a CDR, in so far as the frequency of clones with improved binding, the same binding, decreased binding or no binding also provide information relating to the importance of each amino acid position for the stability of the antibody-antigen complex. For example, if a position of the CDR retains binding when changed to all 20 amino acids, that position is identified as a position that is unlikely to be required for antigen binding. Conversely, if a position of CDR retains binding in only a small percentage of substitutions, that position is identified as a position that is important to CDR function. Thus, the library scanning mutagenesis methods generate information regarding positions in the CDRs that can be changed to many different amino acid (including all 20 amino acids), and positions in the CDRs which cannot be changed or which can only be changed to a few amino acids.

[0111] Candidates with improved affinity may be combined in a second library, which includes the improved amino acid, the original amino acid at that position, and may further include additional substitutions at that position, depending on the complexity of the library that is desired, or permitted using the desired screening or selection method. In addition, if desired, adjacent amino acid position can be randomized to at least two or more amino acids. Randomization of adjacent amino acids may permit additional conformational flexibility in the mutant CDR, which may in turn, permit or facilitate the introduction of a larger number of improving mutations. The library may also comprise substitution at positions that did not show improved affinity in the first round of screening.

[0112] The second library is screened or selected for library members with improved and/or altered binding affinity using any method known in the art, including screening using BIAcore surface plasmon resonance analysis, and selection using any method known in the art for selection, including phage display, yeast display, and ribosome display.

[0113] The invention also relates to solid phases for affinity chromatography or solid-phase extraction consisting of organic, inorganic, synthetic polymers or of mixed polymers, preferably cross-linked agarose, cellulose, silica gel, polyamide and polyvinyl alcohols, which are optionally chemically activated, with peptides or a recognition-molecule of the invention immobilized on the surface of the solid phase.

[0114] In a preferred embodiment of the invention, the peptides of the solid phases are bound to the solid support phase covalently or by adsorption.

[0115] In another preferred embodiment of the invention, the peptides of the solid phases are covalently bound to the solid phase on any of positions X1 to X135.

[0116] In another preferred embodiment of the invention, the peptides of the solid phases are distanced from the

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support surface by linkers/spacers.

[0117] The invention also relates to a device for removing amyloid fibrillar and/or non-fibrillar polypeptides from samples on solid phases, wherein the device contains a solid phase of the invention, and means for the entry of samples are provided.

[0118] In yet another aspect, the invention relates to a pharmaceutical composition comprising a nucleic acid molecule according to the invention, a vector of the invention, a host cell of the invention, a peptide according to the invention, a recognition-molecule according to the invention and/or a solid phase according to the invention; optionally together with a pharmaceutically tolerable carrier.

[0119] In a preferred embodiment of the invention, the pharmaceutical agent of the invention comprises a carrier selected from the group comprising fillers, disintegrants, binders, humectants, extenders, dissolution retarders, absorption enhancers, wetting agents, adsorbents and/or lubricants.

[0120] In another preferred embodiment of the invention, said agent is a capsule, a tablet, a coated tablet, a suppository, an ointment, a cream, an injection solution and/or an infusion solution.

[0121] In another preferred embodiment of the invention, said agent is an oral, vaginal, rectal, nasal, topical, subcutaneous, intravenous, intramuscular, intraperitoneal agent, suppository, pad and/or foam.

[0122] The invention also relates to a kit comprising a nucleic acid molecule according to the invention, a vector according to the invention, a host cell according to the invention, a peptide according the invention, a recognition-molecule according to the invention, a solid phase according to the invention and/or a pharmaceutical composition according to the invention, optionally together with instructions for combining the contents of the kit and/or providing a formulation. The kits may comprise, in suitable container means, one or more binding peptides and/or antibodies, a control protein or peptide (e.g., A beta 42 or amyloid polypeptides), a control peptide that is not recognized by the antibody of the invention and various buffers, reagents, enzymes and other standard ingredients well known in the art.

[0123] The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the probes and/or primers may be placed, and preferably, suitably aliquoted. Where an additional component is provided, the kit will also generally contain additional containers into which this component may be placed. The kits of the present invention will also typically include a means for containing the peptides or proteins and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

[0124] In a preferred embodiment of the invention, the kit is used for the therapy of amyloid diseases.

[0125] In another preferred embodiment of the invention, the kit is used for in vitro or in vivo diagnosis of amyloid diseases.

[0126] In another aspect, the kit of the invention is used as an amyloid-histology kit, an amyloid-ELISA kit, an amyloid affinity kit and/or as an amyloid classification kit. The binder, that means the peptide or the recognition-molecule, e. g. antibody of the invention, interacts with a structural epitope, common and specific to all amyloid fibrils. The peptides and especially the antibody of the invention can advantageously be used as conformation-sensitive amyloid binders. The conformation-dependent binders of the invention (e. g. the antibody) can advantageously be used in the non-invasive in vivo amyloid diagnosis or for amyloid therapy. Since extracerebral amyloidoses primarily affect parenchymal organs such as heart, liver and kidney, these deposits are also easier to reach diagnostically and therapeutically than those from cerebral amyloidoses (no blood-brain barrier, extracellular deposition). The antibody of the invention has a significantly higher amyloid-affinity than molecules of the state of the art. The kit of the invention allows a prescreen of patients before taking biopsies and for readily evaluating therapeutic strategies. These assays of the invention are based on a protein that binds a conformational epitope specific for and common to all amyloid fibrils. Its phosphatase moiety allows direct detection in histology and ELISA and signal amplifications, wherein a secondary antibody is not needed.

[0127] In prophylactic applications, pharmaceutical or vaccine compositions are administered to a mammal susceptible to, or at risk of developing a disease associated with amyloid formation and/or aggregation in an amount sufficient to eliminate or reduce the risk or delay the ontset of said disease. In therapeutic applications, compositions are administered to a mammal suspected of, or already suffering from such a disease in an amount sufficient to cure, or at least partially arrest, the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as a therapeutically- or pharmaceutically-effective dose. In both prophylactic and therapeutic regimes, agents are usually administered in several dosages until a sufficient immune response has been achieved. Typically, the immune response is monitored and repeated dosages are given if the immune response starts to fade.

[0128] For passive immunization with an antibody, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg of the host body weight. Doses for nucleic acids encoding the peptide/recognition-molecule of the invention range from about 10 ng to 1 g, 100 ng to 100 mg, 1 μg to 10 mg, or 30 to 300 μg DNA per patient. Doses for infectious viral vectors vary from 10 to 10.sup.9 or more virions per dose. Effective doses of the therapeutic compositions (e. g. vaccine) of the present invention, vary depending upon many different factors, including means of administration, target site, physiological state of the mammal, whether the patient is a human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Treatment dosages need to be titrated to optimize

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safety and efficacy. The amount of peptides/recognition molecules of the invention depends on whether adjuvant is also administered, with higher dosages being required in the absence of adjuvant. The amount of peptides/recognition molecules of the invention for administration sometimes vary from 1-500 μg per mammal and more usually from 5-500 μg per injection for human administration. Occasionally, a higher dose of 1-2 mg per injection is used. Typically about 10, 20, 50 or 100 μg is used for each human injection. The timing of injections can vary significantly from once a day, to once a year, to once a decade. On any given day that a dosage of the peptides/recognition molecules of the invention is given, the dosage is greater than 1 μg/patient and usually greater than 10 μg/patient if adjuvant is also administered. In the absence of adjuvant, the dosage is greater than 10 μg/patient and usually greater than 100 μg/patient.

[0129] Agents for inducing an immune response (e. g. antibody for passive immunization) can be administered by parenteral, topical, intravenous, oral, subcutaneous, intraperitoneal, intranasal, or intramuscular means for prophylactic and/or therapeutic treatment. The most typical route of administration is subcutaneous, although others can be equally effective. The next most common is intramuscular injection. This type of injection is most typically performed in the arm or leg muscles. Intravenous injections as well as intraperitoneal injections, intraarterial, intracranial, or intradermal injections are also effective in generating an immune response. In some methods, agents are injected directly into a particular tissue where deposits are accumulated.

[0130] Therapeutic compositions of the invention can optionally comprise other agents that are at least partly effective in treatment of diseases associated with amyloid formation and/or aggregation. In the case of Alzheimer's and Down's syndrome, in which amyloid aggregation occurs in the brain, the vaccine or therapeutic composition of the invention may also comprise other agents that increase passage of the active components of the composition of the invention across the blood-brain barrier. A peptide, protegrin PG-1, belonging to the family of beta-stranded antimicrobil peptides, for example, has been successfully used to deliver therapeutic compounds into eucaryotic cells (Drin and Temsamani, 2002). Other strategies to enhance drug delivery across the blood brain barrier, especially vector-mediated strategies, have been reviewed by Temsamani et al. (2001).

[0131] The preferred form of the vaccine and/or therapeutic composition depends on the intended mode of administration and application. The compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like.

[0132] The vaccine or pharmaceutical compositions can also include large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids and copolymers (such as latex functionalized sepharose, agarose, cellulose, and the like), polymeric amino acids, amino acid copolymers, and lipid aggregates (such as oil droplets or liposomes). Additionally, these carriers can function as immunostimulating agents (i.e., adjuvants).

[0133] For parenteral administration, the compositions of the invention can be administered as injectable dosages of a solution or suspension of the substance in a physiologically acceptable diluent with a pharmaceutical carrier that can be a sterile liquid such as water, oils, saline, glycerol, or ethanol. Also encapsulation into biodegradable microparticles can be used as a parenteral delivery system (Brayden et al., 2001).

[0134] Additionally, auxiliary substances, such as wetting or emulsifying agents, surfactants, pH buffering substances and the like can be present in the vaccine and/or therapeutic compositions. Other components of pharmaceutical compositions are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, and mineral oil. In general, glycols such as propylene glycol or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions.

[0135] Typically, compositions are prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The preparation also can be emulsified or encapsulated in liposomes or micro particles such as polylactide, polygly-colide, or copolymer for enhanced adjuvant effect, as discussed above (Langer, 1990; Langer et al, 1997). The compositions of this invention can be administered in the form of a depot injection or implant preparation which can be formulated in such a manner as to permit a sustained or pulsatile release of the active ingredient.

[0136] Additional formulations suitable for other modes of administration include oral, intranasal, and pulmonary formulations, suppositories, and transdermal applications. For suppositories, binders and carriers include, for example, polyalkylene glycols or triglycerides. Such suppositories can be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%. Oral formulations include excipients, such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, and magnesium carbonate. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%.

[0137] Alternatively, transdermal delivery can be achieved using a skin path or using transferosomes (Paul et al., 1995; Cevc et al., 1998).

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[0138] Further techniques for formulation and administration of drugs can also be found in "Remington's Pharmaceutical Sciences".

[0139] The vaccine and therapeutic compositions of the present invention can thus be used for the prevention and/or treatment of any disease associated with amyloid formation and/or aggregation. In a preferred embodiment the vaccine and therapeutic compositions of the invention are used for the prevention and/or treatment of Alzheimer's disease, Down's syndrome or other amyloid diseases.

[0140] In non-demented humans, prophylaxis can begin at any age (e.g., 10, 20, 30 years). Usually, however, it is not necessary to begin prophylaxis until a patient reaches the age of 40, 50, 60, or 70 years. Prophylaxis typically encompasses multiple dosages over a period of time. If the response falls, a further dosage is indicated.

[0141] Accordingly, the present invention also encompasses a method for the prevention and/or treatment, in an mammal, of a disease associated with beta-amyloid formation and/or aggregation, said method comprising the administration, to said mammal, of a vaccine composition or a therapeutic composition as described above.

[0142] In another aspect, the invention also relates to an apparatus for chromatography, comprising the recognition-molecule according to the invention.

[0143] In a preferred embodiment, the recognition-molecules of the apparatus are bound to a solid phase according to the invention.

The invention also relates to the use of a nucleic acid molecule according to the invention, a vector according to the invention, a host cell according to the invention, a peptide according to the invention, a recognition-molecule according to the invention, a solid phase according to claims the invention, a pharmaceutical composition according to the invention, a kit according to the invention, an apparatus according to the invention in the prophylaxis, diagnosis, therapy, follow-up and/or aftercare of amyloid diseases. In a preferred embodiment of the invention, said amyloid diseases are selected from the group comprising

- Systemic amyloidosis
    - Primary amyloidosis
        - Mutations in lysozyme, transthyretin, apolipoprotein B, fibrinogen
        - AL amyloidosis (immunoglobulin light chains, as seen with multiple myeloma)
    - Secondary amyloidosis
        - AA amyloidosis (serum amyloid A protein, an acute-phase protein due to chronic inflammation)
        - Gelsolin amyloidosis (plasma gelsolin fragments).
    - Familial or Hereditary amyloidosis
        - Most commonly caused by mutations in the transthyretin protein, but in rare occurrences can also be caused by apolipoprotein AI, gelsolin, fibrinogen, and lysozyme mutations.
        - Primarily caused by genetics, believed to be autosomal dominant, high probability of passage to offspring
        - Appalachian type amyloidosis
        - Shar Pei fever for amyloidosis in Shar Peis
- Organ-specific amyloidosis
    - Familial or Hereditary amyloidosis
    - Diabetes mellitus type 2 (amylin, also known as IAPP)
    - Neurology
        - Alzheimer's disease (Aβ 39-42)
        - Parkinson's disease (alpha-synuclein)
        - Huntington's disease (huntingtin)
        - Transmissible spongiform encephalopathies (prion protein, PrP)
            - Creutzfeldt-Jakob disease (PrP in cerebrum)
            - Kuru (diffuse PrP deposits in brain)
            - Fatal Familial Insomnia (PrP in thalamus)
            - Bovine spongiform encephalopathy (PrP in cerebrum of cows)

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■ Congophilic angiopathy (Amyloid beta)

- Cardiac amyloidosis

■ congestive heart failure; some instances (PrP or transthyretin in heart)

- Inclusion body myositis

- Iatrogenic conditions

- insulin amyloidosis (injection-administered insulin)

[0144] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypo-dermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

[0145] In yet another aspect, the invention relates to a method for the treatment of the above-referenced amyloid diseases by binding and/or removal of the amyloid fibrillar polypeptides or the non-fibrillar polypeptides by means of the recognition-molecules according to the invention.

EXAMPLES

[0146] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

[0147] N-biotinylated, disaggregated Aβ(1-40) peptide or Aβ(1-40) fibrils were captured on different flow cells of a SA sensor chip (Biacore). 22C4 and B10AP were injected in 10 mM HEPES buffer (pH 7.4), 0.15 M NaCl, 3 mM ethylenediamine-tetraacetic acid, 5 μM surfactant P20 at a flow rate of 10 μl/min. Regeneration was done with 10 mM glycine/HCl, 1 M NaCl (pH 1.5) for 1 min. K_Ds were calculated from the concentration dependent steady-state binding of B10 or B10AP using the 1:1 steady state affinity model. Molar stoichiometry of the antibody/Aβ(1-40) fibril complexes was estimated from the measured maximum binding capacity of the fibril coated sensor surface, the response level of immobilized Aβ fibrils and the molecular mass of the antibody (15725 Da) and Aβ(1-40) (4330 Da) .

[0148] Polypeptide was blotted onto 0,45 μm nitrocellulose membrane (GE Healthcare or Schleicher & Schuell) and blocked with 2 % bovine serum albumin in Tris-buffered saline (pH 7.4, TBS) at room temperature for 1 h. Equal loading was confirmed with Ponceau red or Coomassie staining of control membranes. Membranes were washed 2 x in H_2O and 1 x in TBST (TBS with 0.1 % Tween 20) for 5 min, before they were incubated for 1 h at room temperature with 0.5 μg/ml of the antibodyAP in TBS. Competition studies were performed by preincubation of the antibodyAP for 1 h with Aβ(1-40) fibrils at 1:0.5, 1:5, 1:10 w/w ratios of Aβ to AA or AL fibrils. After the antibodyAP incubation, membranes were washed 2 x with H_2O and 1 x TBST for 5 min. Alternatively, washing and blocking may be performed in TBS containing 300 mM NaCl. The antibodyAP binding was visualised with NBT/BCIP (Pierce). Densitometric quanti-fications used TotalLab 100 software.

[0149] The figures show the following:

Fig. 1: Surface plasmon resonance sensorgrams of 100 nM 22C4 or antibodyAP to biosensors covered with fibrils or disaggregated Aβ(1-40). In the lower panel the blue curve is scaled to 1/10th of its original value.

Fig. 2: AntibodyAP staining of disaggregated and fibrillar Aβ(1-40) blotted onto nitrocellulose, quantitated by densit-

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ometry with standard error from 3 experiments.

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SEQUENCE LISTING

<110> Leibniz-Insitut für Naturstoff-Forschung und Infektionsbiologie Hans-Knöll-Institut
Leibniz-Insitut für Altersforschung Fritz-Lippmann-Institut e.V.

<120> Anti-amyloid antibodies and their use in the therapy of amyloid diseases

<130> XII 397-07

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20 25 30

Tyr His Arg Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Ile Val
35 40 45

Ala Val Ile Ser Gln Ser Gly Met Arg Thr Tyr Tyr Ala Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Met Tyr Tyr Cys
85 90 95

Ala Ala Gly Thr Arg Lys Asn Val Trp Thr Arg Gln His Pro Phe Asp
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Tyr Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser Ala Ser Gly Ala
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1 5 10

Claims

1. A peptide,
characterized in that
it is selected from the group comprising:

a) a peptide consisting of the amino acid sequence according to SEQ ID No. 1, particularly according to SEQ ID No. 2, 3 and/or 4;
b) a peptide consisting of an amino acid sequence having sufficient homology to be functionally analogous/equivalent to an amino acid sequence in accordance with a);
c) a peptide according to an amino acid sequence a) or b) which is modified by deletions, additions, substitutions, translocations, inversions and/or insertions and functionally analogous/equivalent to an amino acid sequence in accordance with a) or b);
d) a peptide according to amino acid sequence a), b) or c) which is modified by branch or extension with the same or another peptide according to amino acid sequence a), b) or c) to form a homooligomeric or heterooligomeric peptide.

2. The peptide according to claim 1,
characterized in that

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the amino acid sequence specified under b) has at least 40% homology to any of the amino acid sequences specified under a).

3. The peptide according to claim 1 or 2,
characterized in that
the amino acid sequence specified under b) has at least 60%, preferably 70%, more preferably 80%, especially preferably 90% homology to any of the amino acid sequences specified under a).

4. The peptide according to any of claims 1 to 3,
characterized in that
it consists of the amino acid sequence SEQ ID No. 1, 2, 3 and/or 4.

5. The peptide according to any of claims 1 to 4 comprising an amino acid sequence X1-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-X15-X16-X17-X18-X19-X20-X21-X22-X23-X24-X25-X26-X27-X28-X29-X30-X31-X32-X33-X34-X35-X36-X37-X38-X39-X40-X41-X42-X43-X44-X45-X46-X47-X48-X49-X50-X51-X52-X53-X54-X55-X56-X57-X58-X59-X60-X61-X62-X63-X64-X65-X66-X67-X68-X69-X70-X71-X72-X73-X74-X75-X76-X77-X78-X79-X80-X81-X82-X83-X84-X85-X86-X87-X88-X89-X90-X-1-X92-X93-X94-X95-X96-X97-X98-X99-X100-X101-X102-X103-X104-X105-X106-X107-X108-X109-X110-X111-X112-X113-X114-X115-X116-X117-X118-X119-X120-X121-X122-X123-X124-X125-X126-X127-X128-X129-X130-X131 wherein

X1 = amino group, amide, acetyl group, biotin group, marker, spacer, linker, GKK, SGKK or deletion,
X2 = E*
X3 = V#
X4 = Q#
X5 = L#
X6 = V#
X7 = E#
X8 = S#
X9 = G#
X10 = G#
X11 = G#
X12 = L#
X13 = V#
X14 = Q#
X15 = P*
X16 = G*
X17 = G#
X18 = S#
X19 = L#
X20 = R#
X21 = L#
X22 = S#
X23 = C#
X24 = T#
X25 = A#
X26 = S#
X27 = G#
X28 = Y*
X29 = T*
X30 = F*
X31 = S*
X32 = H#, R#, K#
X33 = R#, H#, K#
X34 = Y#, W#, F#, I#, L#, V#, T#, S#

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(continued)

X35 = H#, R#, K#
X36 = R#, H#, K#
X37 = W#
X38 = F#
X39 = R#
X40 = Q#
X41 = A#
X42 = P*
X43 = G*
X44 = K*
X45 = E#
X46 = R#
X47 = E#
X48 = I#
X49 = V#
X50 = A#
X51 = V#
X52 = I#
X53 = S#, T#, W#, V#, I#, L#
X54 = Q, N
X55 = S, T
X56 = G, A
X57 = M#, C#, V#, I#, L#, Y#, W#, F#
X58 = R#, K#, H#
X59 = T#
X60 = Y#
X61 = Y#
X62 = A#
X63 = D*
X64 = S*
X65 = V*
X66 = K*
X67 = G*
X68 = R#
X69 = F#
X70 = T#
X71 = I#
X72 = S#
X73 = R#
X74 = D#
X75 = N*
X76 = A*
X77 = K*
X78= N*
X79 = T#
X80 = V#
X81 = Y#
X82 = L#
X83 = Q#
X84 = M#
X85 = N#

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(continued)

X86 = S*
X87 = L*
X88 = K*, R*, S*, T*, A*, C*, D*, E*, F*, G*, H*, I*, L*, M*, N*, P*, Q*, V*, W*, Y*
X89 = P*
X90 = E*
X91 = D*
X92 = T#, I#, Y#, F#, V#, C#, M#, W#, Q#, E#, S#, K#, R#, H#, L#
X93 = A#
X94 = M#
X95 = Y#
X96= Y#
X97 = C#
X98 = A#
X99 = A#
X100 = G#, A#
X101 = T#, S#, F#, W#, I#, L#, V#
X102 = R, K, H
X103 = K, H, R
X104 = N, Q
X105 = V, I, L, M
X106 = W, F, Y
X107 = T, S
X108 = R, K, H
X109 = Q, N
X110 = H, K, R
X111 = P*
X112 = F*
X113 = D#
X114 = Y#
X115 = W#
X116 = G#
X117 = Q#
X118 = G#
X119 = T#
X120 = Q#
X121 = V#
X122 = T#
X123 = V#
X124 = S#
X125 = S*
X126 = A*
X127 = S*
X128 = G*
X129 = A*
X130 = E*
X131 = amino group, amide, acetyl group, biotin group, marker, spacer, linker, GKK, SGKK or deletion,

wherein particularly binding or CDR-regions are underlined;
wherein particularly amino acids, which are marked with a star (*) can be modified to any one of amino acids selected from the group comprising A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, Y or can be deleted;
wherein amino acids, which are marked by a hash sign (#) are in particular beta-strands.

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6. The peptide according to any of claims 1 to 5,
characterized in that
the linker and/or a spacer are selected from the group comprising:

α-aminocarboxylic acids as well as homo- and heterooligomers thereof, α,ω-aminocarboxylic acids and branched homo- or heterooligomers thereof, other aliphatic and/or aromatic amino acids as well as linear and branched homo- or heterooligomers; amino-oligoalkoxyalkylamines; maleinimidocarboxylic acid derivatives; oligomers of alkylamines; 4-alkylphenyl derivatives; 4-oligoalkoxyphenyl or 4-oligoalkoxyphenoxy derivatives; 4-oligoalkylmercaptophenyl or 4-oligoalkylmercaptophenoxy derivatives; 4-oligo-alkylaminophenyl or 4-oligoalkylaminophenoxy derivatives; (oligoalkylbenzyl)phenyl or 4-(oligoalkylbenzyl)phenoxy derivatives, as well as 4-(oligo-alkoxybenzyl)phenyl or 4-(oligoalkoxy-benzyl)phenoxy derivatives; trityl derivatives; benzyloxyaryl or benzyloxyalkyl derivatives; xanthen-3-yloxyalkyl derivatives; (4-alkylphenyl)- or ω-(4-alkylphenoxy)alkanoic acid derivatives; oligoalkylphenoxyalkyl or oligoalkoxyphenoxyalkyl derivatives; carbamate derivatives; amines; trialkylsilyl or dialkylalkoxysilyl derivatives; alkyl or aryl derivatives and/or combinations thereof.

7. The peptide according to any of claims 1 to 6 for use as a medical active substance.

8. The peptide according to any of claims 1 to 7,
characterized in that
the peptide is bound by amyloid fibrillar and/or non-fibrillar polypeptides of patients suffering from amyloid disease.

9. The peptide according to any of claims 1 to 8,
characterized in that
the peptide is immobilized and/or fixed to magnetic, paramagnetic and/or non magnetic nanoparticles.

10. The peptide according to one of the preceding claims,
characterized in that
the peptide is bound to a solid phase.

11. An isolated nucleic acid molecule selected from the group comprising:

a) a nucleic acid molecule comprising a nucleotide sequence which encodes at least one peptide selected from the group consisting of peptides according to claims 1 to 5, particularly according to SEQ ID No. 1, preferably according to SEQ ID No. 2, 3 and/or 4;
b) a nucleic acid molecule which is complementary to a nucleotide sequence in accordance with a);
c) a nucleic acid molecule which undergoes hybridization with a nucleotide sequence according to a) or b) under stringent conditions;
d) a nucleic acid molecule comprising a nucleotide sequence having sufficient homology to be functionally analogous/equivalent to a nucleotide sequence according to a), b) or c);
e) a nucleic acid molecule which, as a consequence of the genetic code, is degenerated into a nucleotide sequence according to a) through d); and
f) a nucleic acid molecule according to a nucleotide sequence of a) through e) which is modified by deletions, additions, substitutions, translocations, inversions and/or insertions and functionally analogous/equivalent to a nucleotide sequence according to a) through e).

12. The nucleic acid molecule according to claim 11,
characterized in that
the nucleotide sequence specified under d) has at least 40% homology to any of the nucleotide sequences specified under a) through c).

13. The nucleic acid molecule according to claim 11,
characterized in that
the nucleotide sequence specified under d) has at least 60%, preferably 70%, more preferably 80%, especially preferably 90% homology to any of the nucleotide sequences specified under a) through c).

14. The nucleic acid molecule according to any of claims 11 to 13,
characterized in that
it is a genomic DNA, a cDNA and/or an RNA.

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15. A vector comprising a nucleic acid molecule according to any of claims 11 to 14.

16. A host cell comprising the vector according to claim 15.

17. A peptide, said peptide being encoded by a nucleic acid molecule according to any of claims 11 to 14.

18. A Recognition-molecule comprising the peptide according to claims 1 to 5.

19. The Recognition-molecule according to claim 18,
characterized in that
the recognition-molecule is an antibody-fragment of an antibody or an antibody.

20. The Recognition-molecule according to claims 18 or 19,
characterized in that
the antibody or the fragment thereof is a chimeric antibody or a fragment thereof.

21. The Recognition-molecule according to any of claims 18 to 20,
characterized in that
the chimeric antibody or the fragment thereof is a partially humanized antibody or a fragment thereof.

22. The Recognition-molecule according to any of claims 18 to 21,
characterized in that
the fragment of the antibody is a variable fragment of a single-chain of an antibody.

23. The Recognition-molecule according to any of claims 18 to 22,
characterized in that
the single-chain is a heavy chain variable domain.

24. Solid phases for affinity chromatography or solid-phase extraction consisting of organic, inorganic, synthetic polymers or of mixed polymers, preferably cross-linked agarose, cellulose, silica gel, polyamide and polyvinyl alcohols, which are optionally chemically activated, with peptides or a recognition-molecule according to at least one of claims 1 to 10 or 17 to 23 immobilized on the surface of the solid phase.

25. The solid phases according to claim 24, wherein the peptides are bound to the solid support phase covalently or by adsorption.

26. The solid phases according to claim 24 or 25,
wherein the peptides are covalently bound to the solid phase on any of positions X1 to X135.

27. The solid phases according to any of claims 24 to 26, wherein the peptides are distanced from the support surface by linkers/spacers.

28. A device for removing amyloid fibrillar and/or non-fibrillar polypeptides from samples on solid phases, wherein the device contains a solid phase according to any of claims 24 to 25, and means for the entry of samples are provided.

29. A pharmaceutical composition comprising a nucleic acid molecule according to any of claims 11 to 14, a vector according to claim 15, a host cell according to claim 16, a peptide according to any of claims 1 to 10, a recognition-molecule according to claims 18 to 23 and/or a solid phase according to claims 24 to 27, optionally together with a pharmaceutically tolerable carrier.

30. The pharmaceutical agent according to the preceding claim,
characterized in that
the carrier is selected from the group comprising fillers, disintegrants, binders, humectants, extenders, dissolution retarders, absorption enhancers, wetting agents, adsorbents and/or lubricants.

31. The pharmaceutical agent according to any of the preceding claims,
characterized in that
said agent is a capsule, a tablet, a coated tablet, a suppository, an ointment, a cream, an injection solution and/or

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an infusion solution.

32. The pharmaceutical agent according to any of the preceding claims,
characterized in that
said agent is an oral, vaginal, rectal, nasal, topical, subcutaneous, intravenous, intramuscular, intraperitoneal agent, suppository, pad and/or foam.

33. A kit comprising a nucleic acid molecule according to any of claims 11 to 14, a vector according to claim 15, a host cell according to claim 16, a peptide according to any of claims 1 to 10, a recognition-molecule according to claims 18 to 23, a solid phase according to claims 24 to 27 and/or a pharmaceutical composition according to claim 29, optionally together with instructions for combining the contents of the kit and/or providing a formulation.

34. Kit according to claim 33,
characterized in that,
the antibody comprises phosphatase moiety which allows in a preferred embodiment a direct detection of amyloidoses.

35. Use of the kit according to claims 33 or 34 for diagnosis of amyloid diseases.

36. Use of the kit according to claims 33 or 34 for conformation-sensitive detection of amyloid.

37. An apparatus for chromatography, comprising the recognition-molecule according to any of claims 18 to 23.

38. The apparatus according to claim 37,
characterized in that
the recognition-molecules are bound to a solid phase according to claims 24 to 27.

39. Use of a nucleic acid molecule according to any of claims 11 to 14, a vector according to claim 15, a host cell according to claim 16, a peptide according to any of claims 1 to 10, a recognition-molecule according to claims 18 to 23, a solid phase according to claims 24 to 27, a pharmaceutical composition according to claim 29 to 32, a kit according to claim 33 or 34, an apparatus according to claim 37 or 38 in the prophylaxis, diagnosis, therapy, follow-up and/or aftercare of amyloid diseases.

40. The use according to claim 39, wherein the peptide, the nucleic acid molecule according to any of claims 11 to 14, the vector according to claim 15, the host cell according to claim 16, the recognition-molecule according to claims 18 to 23, the solid phase according to claims 24 to 27, the pharmaceutical composition according to claim 29 to 32, the kit according to claim 33 or 34, the apparatus according to claim 37 or 38 modify the aggregation of amyloid proteins, in particular by promoting a non-fibrillar aggregation.

41. The use according to claim 39 or 40,
characterized in that
the amyloid diseases are selected from the group comprising

- Systemic amyloidosis
  - Primary amyloidosis
    - Mutations in lysozyme, transthyretin, apolipoprotein B, fibrinogen
    - AL amyloidosis
  - Secondary amyloidosis
    - AA amyloidosis
    - Gelsolin amyloidosis
  - Familial or Hereditary amyloidosis
    - Caused by mutations in the transthyretin protein or caused by apolipoprotein A1, gelsolin, fibrinogen,

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and lysozyme mutations
■ Appalachian type amyloidosis
■ Shar Pei fever for amyloidosis in Shar Peis

- Organ-specific amyloidosis

• Familial or Hereditary amyloidosis
• Diabetes mellitus type 2
• Neurology

■ Alzheimer's disease (Aβ 39-42)
■ Parkinson's disease (alpha-synuclein)
■ Huntington's disease (huntingtin)
■ Transmissible spongiform encephalopathies (prion protein, PrP)

- Creutzfeldt-Jakob disease (PrP in cerebrum)
- Kuru (diffuse PrP deposits in brain)
- Fatal Familial Insomnia (PrP in thalamus)
- Bovine spongiform encephalopathy (PrP in cerebrum of cows)

■ Congophilic angiopathy (Amyloid beta)

• Cardiac amyloidosis

■ congestive heart failure

• Inclusion body myositis

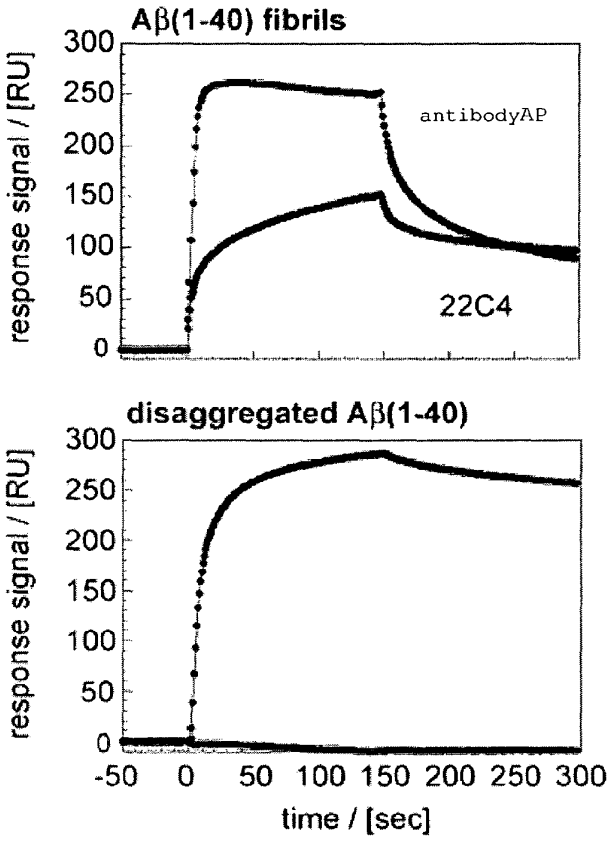
- Iatrogenic conditions

• insulin amyloidosis.

42. A method for the treatment of said amyloid diseases by binding and/or removal of the amyloid fibrillar polypeptides or the non-fibrillar polypeptides by means of the recognition-molecules according to any of claims 18 to 23.

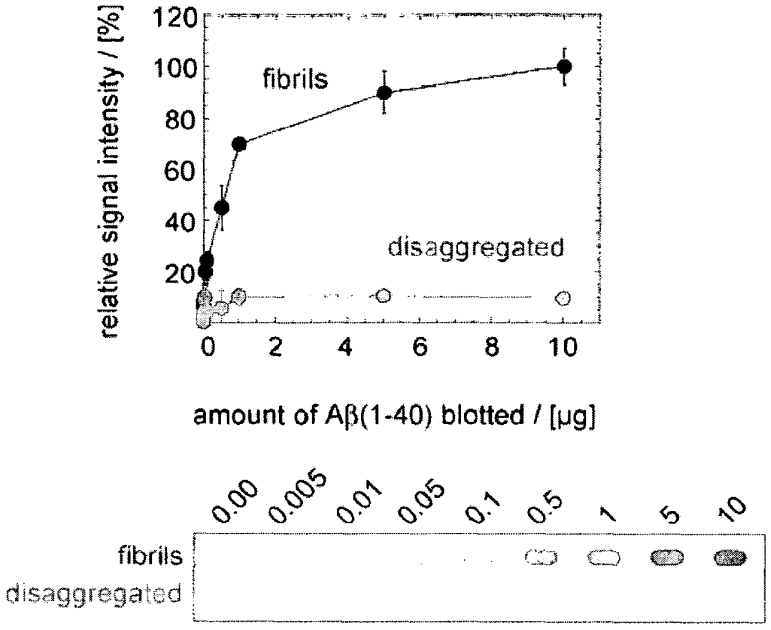
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Fig. 1



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Fig. 2



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European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 07 09 0073

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X Y	WO 2006/040153 A (ABLYNX N V [BE]; LAUWEREYS MARC [BE]; VAN LEUVEN FRED [BE]; VAN DER AU) 20 April 2006 (2006-04-20) * sequences 158, 156, 152, 148, 140, 130, 129, 123, 122, 114 * -----	1-3,18, 39,40 1-10,18, 39,40	INV. C07K16/18 A61K39/395 G01N33/563 C12N15/13
Y	WO 2004/044204 A (PASTEUR INSTITUT [FR]; CENTRE NAT RECH SCIENT [FR]; ROUGEON FRANCOIS [) 27 May 2004 (2004-05-27) * the whole document * -----	1-10,18, 39,40	
Y	WO 2007/022416 A (UNIV RAMOT [IL]; GERAGHTY ERIN [US]; SOLOMON BEKA [IL]; COHEN-KUPIEC R) 22 February 2007 (2007-02-22) * the whole document * -----	1-10,18, 39,40	
	-/--		

TECHNICAL FIELDS SEARCHED (IPC)

C07K

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
Munich	25 September 2007	Weikl, Martina

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

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European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 07 09 0073

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (IPC)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
Y	SOLOMON B: "Immunotherapeutic strategies for prevention and treatment of Alzheimer's disease" DNA AND CELL BIOLOGY, NEW YORK, NY, US, vol. 20, no. 11, November 2001 (2001-11), pages 697-703, XP002236936 ISSN: 1044-5498 * the whole document *	1-10,18, 39,40	
Y	ARBABI GHAHROUDI M ET AL: "Selection and identification of single domain antibody fragments from camel heavy-chain antibodies" FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 414, no. 3, 15 September 1997 (1997-09-15), pages 521-526, XP004261105 ISSN: 0014-5793 * the whole document *	1-10,18, 39,40	TECHNICAL FIELDS SEARCHED (IPC)

EPO FORM 1503 03.82 (P04C10)

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European Patent Office

INCOMPLETE SEARCH
SHEET C

Application Number
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Although claims 39 and 40 are directed to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition. Although claims 39 and 40 are directed to a diagnostic method practised on the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 07 09 0073

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

25-09-2007

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(54) Title: METHODS AND COMPOSITIONS FOR TREATMENT AND DIAGNOSIS OF ALZHEIMER DISEASE AND OTHER DISORDERS

(57) Abstract

The present invention is directed to methods for treating Alzheimer disease and other disorders associated with the presence of neurofibrillary tangles (NFTs) by increasing the activity of a phosphatase towards abnormal hyperphosphorylated tau ("AD P-tau") present in the NFTs of paired helical filaments in the neurons of patients having Alzheimer disease or other NFT-associated disorder. Pharmaceutical compositions and diagnostic methods are also provided. The inventions provide methods of treatment by admnistering to a subject a therapeutically effective amount of a composition comprising a molecule which increases protein phosphatase activity toward AD P-tau, a phosphatase which dephosphorylates AD P-tau, or a nucleic acid encoding such a phosphatase.

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METHODS AND COMPOSITIONS FOR TREATMENT AND DIAGNOSIS OF ALZHEIMER DISEASE AND OTHER DISORDERS

This invention was made in part with government support under grants NS18105, AG05892, AG08076, and AG04220 awarded by the National Institutes of Health. The government has certain rights in the invention.

1. INTRODUCTION

The present invention is directed to methods for treatment and diagnosis of Alzheimer disease (AD) and other disorders, and therapeutic and diagnostic compositions. In particular, the invention relates to methods of treatment by administration of molecules which increase the activity of protein phosphatases towards abnormal hyperphosphorylated tau, the major protein subunit of paired helical filaments in neurofibrillary tangles.

2. BACKGROUND OF THE INVENTION

2.1. ALZHEIMER DISEASE

Alzheimer disease, which is the single major cause of dementia in adults in industrialized societies, is a degenerative brain disorder characterized clinically by a progressive loss of memory, confusion, dementia and ultimately death. Histopathologically, Alzheimer disease is characterized by the presence in the neocortex, especially the hippocampus of two brain lesions, the neurofibrillary tangles (NFTs) of paired helical filaments (PHF) in the neurons and the neuritic (senile) plaques of ß-amyloid in the extracellular space. In addition to the neurofibrillary tangles in the neuronal perikarya, the PHF also accumulate in the dystrophic neurites surrounding the extracellular deposits of ß-amyloid in the neuritic plaques, and in the dystrophic neurites of the neuropil as neuropil threads (Braak et al., 1986, Neurosci. Lett. 65:351-355). Many of the neurons with neurofibrillary changes may be only partially functional, and in some areas of the brain such as neocortex, many of them may eventually die, leaving behind tangled masses of abnormal fibrils,

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the "ghost tangles". The ß-amyloid also accumulates in the wall and the lumen of the brain vessels. Deposits of ß-peptide, polymers of which form amyloid, are also seen as diffuse plaques throughout the affected areas of the brain.

Neither the neurofibrillary tangles nor the plaques are unique to Alzheimer disease. Neurofibrillary tangles of PHF are also found in great abundance in Guam-Parkinsonism dementia complex, dementia pugilistica, postencephalitic parkinsonism, and adults with Down syndrome and in small number in a few cases of subacute sclerosing panencephalitis, Hallervorden-Spatz disease, and neurovisceral lipid storage disease (for review, see Wisniewski et al. 1979, Ann. Neurol. 5:288-294; Iqbal and Wisniewski, 1983, in *Alzheimer's Disease*, B. Reisberg, ed., The Standard Reference, The Free Press, NY, pp. 48-56). The neuritic (senile) plaques are also seen in Down syndrome and aged humans and in some species of animals. Unlike the tangles, which are present in only very small numbers in non-demented elderly and absent in animals, the plaques are seen frequently in both aged human and animal brains. The numbers of plaques in non-demented aged humans are sometimes similar to those seen in Alzheimer disease cases (Katzman et al., 1988, Ann. Neurol. 23:138-144). Recent studies have shown that most of the plaques found in non-demented elderly, unlike in Alzheimer disease, are free of PHF in the dystrophic neurites (Dickson et al., 1988, Am. J. Pathol. 132:86-101; Barcikowska et al., 1989, Acta. Neuropathol. (Berl.) 78:225-231).

At present, the etiology and the pathogenesis of Alzheimer disease are not established. Alzheimer disease probably has polyetiology, which includes genetic, environmental, and metabolic factors. The major form of Alzheimer disease is sporadic and has a late onset, whereas a small percentage of cases are familial and have an early onset. Some of the familial cases of Alzheimer disease are strongly associated to one or more mutations at different sites on the ß-amyloid precursor protein, the gene of which lies on

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chromosome 21. Whether these mutations are the cause of Alzheimer disease in the affected patients, however, has not been as yet proven experimentally.

2.2. ABNORMAL PHOSPHORYLATION OF TAU AND DISRUPTION OF MICROTUBULES

In Alzheimer disease brain there are two general populations of PHF, the PHF I and the PHF II (Iqbal et al., 1984, Acta. Neuropathol. (Berl.) 62:167-177). PHF I are readily soluble in sodium dodecyl sulfate, whereas PHF II are solubilized by repeated heat extractions in sodium dodecyl sulfate and ß-mercaptoethanol or by ultrasonication followed by extraction in the detergent (Iqbal et al., 1984, Acta. Neuropathol. (Berl.) 62:167-177). PHF I and PHF II probably represent early and late maturation stages, respectively, of the neurofibrillary tangles. The major protein subunit of PHF is the microtubule associated protein tau (Grundke-Iqbal et al., 1986, J. Biol. Chem. 261:6084-6089; Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci. USA 83:4913-4917; Grundke-Iqbal et al., 1988, Mol. Brain Res. 4:43-52; Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650; Lee et al., 1991, Science 251:675-678). Some of the tau in PHF II and not in PHF I is ubiquitinated (Grundke-Iqbal et al., 1988, Mol. Brain Res. 4:43-52; Morishima-Kawashima et al., 1993, Neuron 10:1151-1160; Köpke et al., 1993, J. Biol. Chem. 268:24374-24384).

Tau is a family of several closely related neuronal polypeptides which are generated from a single gene by alternative splicing (Goedert and Jakes, 1990, EMBO J. 9:4225-4230). In adult human brain there are six isoforms of tau which differ from one another in containing three or four tubulin binding repeat domains and the presence or absence of two amino terminal inserts of 29 amino acids each (Goedert and Jakes, 1990, EMBO J. 9:4225-4230). Tau in PHF is abnormally phosphorylated (Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci. USA 83:4913-4917; Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650). The abnormal phosphorylation of

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tau apparently precedes its polymerization into PHF/neurofibrillary tangles because (a) there is a pool of non-PHF and non-ubiquitinated soluble abnormally phosphorylated tau that can be isolated from Alzheimer disease brain and (b) some of the non-tangle bearing neurons in Alzheimer disease brain and normal aged but not young adult cases are stained immunocytochemically for the abnormal tau (Köpke et al., 1993, J. Biol. Chem. 268:24374-24384; Bancher et al., 1989, Brain Res. 477:90-99; Bancher et al., 1991, Brain Res. 539:11-18).

The abnormally phosphorylated tau from Alzheimer disease brain contains 6-12 moles phosphate per mole of the protein, which is two- to six-fold the level in normal tau; normal tau contains 2-3 moles phosphate per mole of the protein (Iqbal and Grundke-Iqbal, 1991, in *Alzheimer's Disease: Basic Mechanisms, Diagnosis and Therapeutic Strategies*, Iqbal et al., eds., John Wiley & Sons Ltd., pp. 173-180; Köpke et al., 1993, J. Biol. Chem. 268:24374-24384; Ksiezak-Reding et al., 1992, Brain Res. 597:209-219). To date, nine abnormal phosphorylation sites on PHF tau have been recognized (Table 1).

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TABLE 1

Phosphorylation sites of abnormally phosphorylated AD tau

P-amino acid[a]	Phos. Site[b]	Antibody Used[c]	Reference
Ser 46	KE**S**P	102c	Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650
Thr 123	HV**T**Q	TP30	Brion et al., 1991, Biochem. J. 273:127-133
Ser 199	TS**S**P	Tau-1, AT8	Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci. USA 83:4913-4917; Biernat et al., 1992, EMBO J. 11:1593-1597
Ser 202	PG**S**P	Tau-1, AT8	Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci USA 83:4913-4917; Biernat et al., 1992, EMBO J. 11:1593-1597;
Thr 231	VR**T**P	--	Hasegawa et al., 1992, J. Biol. Chem. 267:17047-17054
Ser 235	PK**S**P	SMI33	Lichtenberg-Kraag et al., 1992, Proc. Natl. Acad. Sci USA 89:5384-5388; Hasegawa et al., 1992, J. Biol. Chem. 267:17047-17054
Ser 262	IG**S**T	--	Hasegawa et al., 1992, J. Biol. Chem. 267:17047-17054
Ser 396	YK**S**P	PHF-1, T3P	Greenberg et al., 1992, J. Biol. Chem. 267:564-569; Lee et al., 1991, Science 251:675-678
Ser 404	DT**S**P	ptau 2	Kanemaru et al., 1992, J. Neurochem. 58:1667-1675

[a] The phosphoamino acid is shown in bold print and underlined.

[b] The phosphorylation site is numbered according to the largest isoform of human tau, tau_{441}.

[c] The antibody used to map the phosphorylation site.

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To date, only phosphorylation of serines and threonines has been shown in normal tau and Alzheimer disease abnormally phosphorylated tau. Phosphoseryl/phosphothreonyl protein phosphatases are classified into four types, termed PP-1, PP-2A, PP-2B and PP-2C (for review, see Cohen, 1989, Annu. Rev. Biochem. 58:453-508). All four protein phosphatases are present in brain tissue (Gong et al., 1993, J. Neurochem. 61:921-927; Ingebritsen et al., 1983, Eur. J. Biochem. 132:297-307; Cohen, 1983, Eur. J. Biochem. 132:297-307). However, it is not known whether the abnormal hyperphosphorylation of tau in AD is a result of an increase of protein kinase activities or an impairment of protein phosphatase activities, or both, or the identities of any such involved kinases or phosphatases. Hence it is essential to identify the protein kinase(s) and phosphatase(s) involved in the regulation of tau phosphorylation. Recently, several protein kinases have been reported to phosphorylate tau *in vitro* at some of the sites which are abnormally phosphorylated in PHF-tau (*e.g.* Drewes et al., 1992, EMBO J. 11:2131-2138). However, the identity of the protein phosphatase(s) that can dephosphorylate these abnormal phosphorylation sites are presently not known. Although *in vitro* several of these phosphorylation sites are accessible to alkaline phosphatase, the overall accessibility to the phosphatase in PHF is less than in normal microtubule tau (Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650; Iqbal and Grundke-Iqbal, 1990, J. Neuropathol. Exp. Neurol. 49:270 (Abstract)). The aberrant phosphorylation in Alzheimer disease brains might be selective to a few neuronal proteins and not be a part of a generalized hyperphosphorylation. Levels of both total free phosphate and phosphoprotein phosphate are normal in Alzheimer disease brain (Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650; Iqbal and Grundke-Iqbal, 1990, in *Molecular Biology and Genetics of Alzheimer Disease*, Miyatake et al., eds., Elsevier, Amsterdam pp. 47-56).

One of the vital functions of the neuron is the transport of materials between the cell body and the nerve endings, and microtubules are

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required for this axonal transport. Tau stimulates microtubule assembly by polymerizing with tubulin (Weingarten et al., 1975, Proc. Natl. Acad. Sci. USA 72:1858-1862) and maintains the microtubule structure (Drubin and Kirschner, 1986, J. Cell Biol. 103:2739-2746). Phosphorylation of tau depresses tau's ability to promote microtubule assembly (Lindwall and Cole, 1984, J. Biol. Chem. 259:5301-5305). In Alzheimer disease brain, the levels of normal tau in the cytosol are decreased by around 40%, whereas the total tissue levels are increased several-fold and this increase is in the form of the abnormally phosphorylated protein (Khatoon et al., 1992, J. Neurochem. 59, 750-753). Binding of guanosine triphosphate (GTP) to the β-subunit of tubulin, which initiates microtubule assembly, is stimulated by tau. Lack of functional tau in Alzheimer disease brain might lead to decreased GTP binding and, consequently, decreased assembly of microtubules (Khatoon et al., 1990, Neurobiol. Aging 11:279 (Abstract)). Microtubules are rarely seen in neurons with neurofibrillary tangles and microtubules are not assembled from brain cytosol of Alzheimer disease cases (Iqbal et al., 1986, Lancet 2:421-426; Iqbal et al., 1987, Lancet 1:102). Both PHF-tau and soluble abnormally phosphorylated tau require dephosphorylation to stimulate *in vitro* assembly of tubulin into microtubules (Iqbal et al., 1991, J. Neuropathol. Exp. Neurol. 50:316 (Abstract)).

2.3. PROTEIN PHOSPHORYLATION/ DEPHOSPHORYLATION SYSTEMS INVOLVED IN HYPERPHOSPHORYLATION OF TAU

The mechanism by which tau in Alzheimer disease brain is abnormally hyperphosphorylated is not as yet established. The state of phosphorylation of substrate proteins depends on the relative activities of protein kinases and phosphoprotein phosphatases.

Seven of the nine phosphorylation sites identified in the abnormally phosphorylated sites to date, are cannonical sites for the proline-directed protein kinases (PDPK). These seven PDPK sites are Ser 46, Ser

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199, Ser 202, Ser 231, Ser 235, Ser 396, and Ser 404; the two non-PDPK sites are Thr 123 and Ser 262 (the amino acid numbering is according to the amino acid sequence of the largest isoform of human tau, tau_{441}). These findings indicate that most likely more than one protein kinase might be involved in the abnormal phosphorylation of tau in the diseased brain. Phosphorylation of tau at some of the abnormal sites by mitogen-activated protein (MAP) kinases (Roder and Ingram, 1991, J. Neurosci. 11:3325-3343; Drewes et al., 1992, EMBO J. 11:2131-2138), glycogen synthase kinase-3 (Lesdesma et al., 1992, FEBS Lett. 308:218-224; Ishiiguro et al., 1992, J. Biol. Chem. 267:10897-10901) and cyclin-dependent cell cycle regulatory kinase, $p34^{cdc2}$ (Vulliet et al., 1992, J. Biol. Chem. 267:22570-22574; Ishiiguro et al., 1992, J. Biol. Chem. 267:10897-10901) have been observed *in vitro*. However, the time kinetics of the abnormal phosphorylation obtained with these kinases are very slow, requiring up to 24 hours. These findings suggest that either an interaction of the substrate with other protein(s) or a combination of kinases, *i.e.* site-site interactions might be required for the abnormal phosphorylation of tau.

Studies (Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci. USA 83:4913-4917; Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650; Iqbal and Grundke-Iqbal, 1990, J. Neuropathol. Exp. Neurol. 49:270 (Abstract)) have shown the dephosphorylation of the abnormally phosphorylated sites of tau after treatment with alkaline phosphatase *in vitro*. The activity of MAP (mitogen activated protein) kinase, which might be involved in the phosphorylation of some of the abnormal sites (Roder and Ingram, 1991, J. Neurosci. 11:3325-3343; Drewes et al., 1992, EMBO J. 11:2131-2138), is inhibited by both PP-2A and phosphotyrosine protein phosphatase (Pelech and Sanghera, 1992, Science 257:1355-1356). Goedert et al. (1992, FEBS Lett. 312:95-99) reported that $PP\text{-}2A_1$ but not PP-2B could dephosphorylate a MAP-kinase-phosphorylated tau. PP-2A and PP-2B have been shown to dephosphorylate tau phosphorylated by Ca^{2+}/calmodulin-

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dependent protein kinase and cAMP-dependent protein kinase (PKA) (Yamamoto et al, 1988, J. Neurochem. 50:1614-1623; Goto et al., 1985, J. Neurochem. 45:276-283).

Citation of a reference hereinabove shall not be construed as an admission that such reference is prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention is directed to methods for treating Alzheimer disease and other disorders associated with the presence of neurofibrillary tangles (NFTs) by increasing the activity of a phosphatase towards abnormal hyperphosphorylated tau ("AD P-tau"). Pharmaceutical compositions and diagnostic methods are also provided.

As used herein, AD P-tau shall mean that hyperphosphorylated form of tau as present in the NFT of PHF in the neurons of patients having AD or other NFT-associated disorders (as described in detail in the Examples Sections *infra*).

The inventions provide methods of treatment by administering to a subject a therapeutically effective amount of a composition comprising (i) a molecule which increases protein phosphatase (PP) activity toward AD P-tau, (ii) a phosphatase which dephosphorylates AD P-tau, or (iii) a nucleic acid encoding such a phosphatase.

3.1. ABBREVIATIONS

As used herein, the following abbreviations shall have the meanings indicated:

AD = Alzheimer disease

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AD P-tau = abnormally phosphorylated tau as found in AD and other disorders associated with NFTs

MAP kinase = mitogen-activated protein kinase

NFT = neurofibrillary tangle

PHF = paired helical filaments

PP = protein phosphatase

SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

4. DESCRIPTION OF THE FIGURES

Figure 1: A model scheme showing the mechanism of neurofibrillary degeneration in Alzheimer disease. Tau is phosphorylated by several protein kinases including MAP kinase. Because of a decrease in the activities of protein phosphatase 2A (PP-2A) and phosphotyrosine protein phosphatases (PTP) in affected neurons, some of the protein kinases, including the MAP kinase, may remain active for extended periods of time, thereby producing hyperphosphorylated tau. The latter (a) does not bind to tubulin to form microtubules, (b) competes with tubulin in binding to normal tau and inhibits the microtubule assembly, and (c) becomes stabilized and polymerizes into PHF. The affected neurons degenerate both as a result of the breakdown of the microtubule system, and because of the accumulation of PHF as Alzheimer neurofibrillary tangles (ANT) filling the entire cell cytoplasm, leaving behind ghost tangles in the extracellular space.

Figure 2. Dephosphorylation of AD P-tau by PP-1, PP-2A and PP-2B. Immunoblots of AD P-tau were carried out after incubation either without (lane 1) or with 1.2 units/ml PP-1 (lane 2), PP-$2A_1$ (lane 3) or PP-2B (lane 4) at 30°C for 60 min as described in Section 6.1; lane 5 shows untreated normal human tau for comparison. Reaction mixtures for PP-2A also contained 1.0 mM $MnCl_2$, whereas for PP-2B, 1.0 μM calmodulin, 1.0

mM $CaCl_2$ and 1.0 mM $NiCl_2$ were included. Six phosphorylation-dependent antibodies were used for immunoblotting as shown above each panel. Tau-1, 102c and SMI33 recognize dephosphorylated forms whereas SMI31, SMI34 and PHF-1 recognize phosphorylated forms of tau at specific sites. Molecular weight (kDa) markers are indicated at left of panels.

Figure 3. Time course of dephosphorylation of AD P-tau by PP-2B. Immunoblots of AD P-tau were carried out after incubation either without (lane 1) or with 1.2 units/ml PP-2B as described in Fig. 2 at 30°C for different time intervals (lane 2 - 10); lane 11 is untreated normal human tau. As in Fig. 2, six phosphorylation-dependent antibodies were used to show the site-specific dephosphorylation. Rabbit antiserum, 92e, which recognizes phosphorylation-independent epitopes on tau, was used to show mobility shift. Molecular weight (kDa) markers are indicated at the left.

Figure 4. Effect of calmodulin and divalent cations on dephosphorylation of AD P-tau by PP-2B. AD P-tau was subjected to immunoblotting with Tau-1 antibody after incubation either without (lane 1) or with 1.2 units/ml PP-2B at 30°C for different time intervals (lane 2 - 7); lane 8 contains untreated normal human tau. Dephosphorylation of AD P-tau was carried out in the presence of 1.0 mM $MnCl_2$ (A); 1.0 mM $NiCl_2$ (B); 1.0 mM $CaCl_2$, 1.0 μM calmodulin and 1.0 mM $MnCl_2$ (C); 1.0 mM $CaCl_2$, 1.0 μM calmodulin and 10 mM $MgCl_2$ (D); or 1.0 mM $CaCl_2$, 1.0 μM calmodulin and 1.0 mM $NiCl_2$ (E). No apparent dephosphorylation of AD P-tau was observed in the presence of only 1.0 mM $CaCl_2$ and 1.0 μM calmodulin (not shown).

Figure 5. Dephosphorylation of AD P-tau by PP-2B at various concentrations of Mn^{2+}. AD P-tau was subjected to immunoblotting with Tau-1 antibody after incubation either without (lane 1) or with 1.2 units/ml PP-2B (lanes 2-7), at 30°C for 60 min as described in Section 6.1. Reaction mixtures also contained 1.0 μM calmodulin, 1.0 mM $CaCl_2$ and various concentrations (μM) of $MnCl_2$ as indicated under each lane. Not shown in

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this Figure is that similar data were obtained when $MnCl_2$ was substituted with $NiCl_2$.

Figure 6. Dephosphorylation of AD P-tau by variable amounts of PP-$2A_1$, PP-$2A_2$ and PP-2B. Dephosphorylation of AD P-tau by variable concentration of PP-$2A_1$ (○), PP-$2A_2$ (●) and PP-2B (△) was carried out at 30°C for 30 min, as described in Section 7.1. After reaction, samples were subjected to immunoblotting with Tau-1, and immunoblots were scanned in a densitometer. Dephosphorylation is represented as a percentage of the maximum.

Figure 7. Immunoblots of AD P-tau after dephosphorylation by variable amounts of PP-$2A_1$, PP-$2A_2$ and PP-2B. AD P-tau was subjected to immunoblotting after incubation either without enzyme (lane 1 of each panel) or with 0.5 U/ml(lane 2 of A, D and G), 5.0 U/ml (lane 3 of A, D and G) or 10.0 U/ml (lane 4 of A, D and G) of PP-$2A_1$; 0.5 U/ml (lane 2 of B, E and H), 5.0 U/ml (lane 3 of B, E and H) or 10.0 U/ml (lane 4 of B, E and H) of PP-$2A_2$; or 0.15 U/ml (lane 2 of C, F and I), 1.5 U/ml (lane 3 of C, F and I) or 3.0 U/ml (lane 4 of C, F and I) of PP-2B. Dephosphorylation reactions were carried out at 30°C for 30 min, as described in Section 7.1. Antibodies 102c (A, B and C), Tau-1 (D, E and F) and PHF-1 (G, H and I) were used to monitor the dephosphorylation; they recognize Ser-46, Ser-199/Ser-202 and Ser-396, respectively. Molecular weight (kDa) markers are indicated at left of panels.

Figure 8. Dephosphorylation of AD P-tau at specific sites by PP-$2A_1$ and PP-$2A_2$. Immunoblots of AD P-tau were carried out after incubation either without (lane 1) or with 5.0 U/ml PP-$2A_1$ (lane 2) or PP-$2A_2$ (lane 3) at 30°C for 60 min, as described in Section 7.1; lane 4 shows untreated normal human tau for comparison. Seven phosphorylation-dependent antibodies and one phosphorylation-independent antibody (92e) were used for immunoblotting, as shown above each panel. Tau-1, 102c and SMI33 recognize dephosphorylated epitopes, whereas AT8, SMI31, SMI34

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and PHF-1 recognize phosphorylated epitopes of tau at specific sites, as described in the text. Molecular weight (kDa) markers are indicated at left of panels.

Figure 9. Treatment of AD P-tau with PP-$2A_2$ in the presence of okadaic acid and protease inhibitors. Immunoblot of AD P-tau was carried out with mAb Tau-1 (lanes 1-4) or SMI31 (lane 5) after incubation either without (lanes 1 and 5) or with 5.0 U/ml PP-$2A_2$ (lanes 2 - 4) at 30°C for 60 min as described in Section 7.1. Reaction mixtures also included 1.0 μM okadaic acid for lane 3 and protease inhibitor cocktail (2.0 μg/ml each of aprotinin, leupeptin and pepstanin, and 2.0 mM benzamidine) for lane 4, respectively. Molecular weight (kDa) markers are indicated at left of the blot. The slowest moving tau band in lanes 2 and 4 is also seen in overloaded normal tau preparations (see Köpke et al., 1993, J. Biol. Chem. 268:24374-24384).

Figure 10. Time course of dephosphorylation of AD P-tau by PP-$2A_1$ and PP-$2A_2$. Immunoblots of AD P-tau were carried out after incubation either without (lane 1) or with 5.0 U/ml PP-$2A_1$ (A, C, E and G) or PP-$2A_2$ (B, D, F and H), as described in Section 7.1 for different time intervals (lanes 2 - 10); lane 11 is untreated normal human tau. Antibodies 102c (A and B), Tau-1 (C and D), SMI-31 (E and F) and PHF-1 (G and H) were used to monitor the dephosphorylation at Ser-46, Ser-199/Ser-202, Ser-396/Ser-404 and Ser-396, respectively. Molecular weight (kDa) markers are indicated at the left.

Figure 11. Effect of Mn^{2+}, Mg^{2+} and polylysine on dephosphorylation of AD P-tau by PP-$2A_1$ and PP-$2A_2$. AD P-tau was subjected to immunoblotting with Tau-1 antibody after incubation either without (lane 1) or with 5.0 U/ml PP-$2A_1$ (A, C, E and G) and 5.0 U/ml PP-$2A_2$ (B, D, F and H) at 30°C for different time intervals (lanes 2 - 7); lane 8 contains untreated normal human tau. Dephosphorylation of AD P-tau was

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carried out in the presence of 1.0 mM EDTA (A and B), 2.0 mM $MnCl_2$ (C and D), 2.0 mM $MgCl_2$ (E and F) and 10 μM polylysine (G and H).

Figure 12. PP-1 (upper panel) and PP-2C (lower panel) activities in the presence or absence of various divalent metal ions. Dephosphorylation reactions were carried out using [^{32}P]phosphorylase kinase as substrate as described in Section 8.1, and in the presence of 1.0 mM EDTA (▵), 1.0 mM $MnCl_2$ (▲) or 10 mM $MgCl_2$ (■). The open circles (○) indicate assays in the absence of the protein phosphatases.

Figure 13. Dephosphorylation of AD P-tau by PP-1, PP-2B and PP-2C. Immunoblots of AD P-tau were carried out after incubation either without (lane 1) or with 2.0 units/ml PP-1 (lane 2), PP-2B (lane 3) or PP-2C (lane 4) at 30°C for 60 min as described in Section 8.1; lane 5 shows untreated normal human tau for comparison. Reaction mixtures for PP-1 and PP-2C also contained 1.0 mM $MnCl_2$ and 10 mM $MgCl_2$, respectively. For PP-2B, 1.0 μM calmodulin, 1.0 mM $CaCl_2$ and 1.0 mM $MnCl_2$ were included. Five phosphorylation-dependent antibodies were used for immunoblotting as shown above each panel to monitor dephosphorylation of the specific sites of AD P-tau. 102c (A), Tau-1 (B) and SMI33 (C) recognize dephosphorylated forms, whereas SMI31 (D) and PHF-1 (E) recognize phosphorylated forms of tau at specific sites as described in Section 8.1. Molecular weight (kDa) markers are indicated at left margin of the figure.

Figure 14. Time course of dephosphorylation of AD P-tau by PP-1. Immunoblots of AD P-tau were carried out after incubation either without (lane 1) or with 1.0 unit/ml PP-1 as described in Fig. 13 at 30°C for different time intervals (lane 2 - 10). Phosphorylation-dependent antibodies Tau-1 (A), SMI31 (B) and PHF-1 (C) were used to monitor the dephosphorylation. Molecular weight (kDa) markers are indicated at the left of each panel.

Figure 15. Effect of Mn^{2+} and Mg^{2+} on dephosphorylation of AD P-tau by PP-1. AD P-tau was incubated with 1.0 unit/ml PP-1 in the

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presence of either 1.0 mM EDTA (○), 1.0 mM Mn^{2+} (●) or 10 mM Mg^{2+} (▲) at 30°C for different time intervals as described in Materials and Methods. After incubation, AD P-tau was subjected to immunoblotting with monoclonal antibody PHF-1 which stains only phosphorylated forms of tau, followed by densitometric scanning. Dephosphorylation is expressed by percentage of remaining PHF-1 staining.

Figure 16. Dephosphorylation of PKA-phosphorylated tau by PP-1, PP-2B and PP-2C. PKA-phosphorylated tau (0.1 mg/ml) was incubated either without (o) or with 0.4 unit/ml of PP-1 (●), PP-2B (□) or PP-2C (▲) at 30°C for different time intervals as described in Section 8.1. The reaction mixtures also included 1.0 mM $MnCl_2$ for PP-1, 1.0 mM $CaCl_2$, 1.0 μM calmodulin and 1.0 mM $MnCl_2$ for PP-2B, and 10 mM $MgCl_2$ for PP-2C.

Figure 17. Western Blots of the Cytosolic Acid-soluble Tau and AD P-tau without (A) and with (B) Alkaline Phosphatase Treatment. The amounts of tau loaded onto the gels were 2 μg protein per lane. Blots were immunodeveloped with monoclonal antibody (mAb) Tau-1. Positions of the molecular weight markers in kilodaltons are indicated on the left of panel A. Increased staining on dephosphorylation shows abnormally phosphorylated tau. In neither AD nor control cytosolic acid-soluble preparations was any increase in immunostaining seen in the alkaline phosphatase-treated blot (compare panel A with B), suggesting an absence of AD P-tau in these preparations. As expected, the AD P-tau sample was intensely labeled with Tau-1 antibody after alkaline phosphatase treatment of the blot.

Figure 18. Electron Micrographs Showing the Products of Microtubule Assembly Negatively Stained with Phosphotungstic Acid. Microtubule assembly was carried out from rat brain tubulin by the addition of: a, control acid-soluble tau; b, AD acid-soluble tau; c, AD P-tau; d, AD P-tau after dephosphorylation. Aliquots of each sample were taken at steady state of polymerization. Only an occasional microtubule was seen with tubulin alone (figure not shown) and with AD P-tau (c), and a large number of

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microtubules was observed in all of the other situations above (a,b,d). No ultrastructural differences could be seen amongst microtubules assembled with tubulin and normal control tau, AD cytosolic tau or dephosphorylated AD P-tau.

Figure 19. Effect of Alkaline Phosphatase Treatment on AD Acid-soluble and on AD P-tau on Microtubule Assembly-promoting Activity. The microtubule assembly-promoting activity of AD P-tau (B) but not of AD acid-soluble tau (A) was increased after the alkaline phosphatase treatment (before, 2; after, 1).

Figure 20. Effect of AD P-tau on Microtubule Assembly. Polymerization of tubulin was determined as described in Materials and Methods, except that a mixture of normal tau and AD P-tau was used. The assembly reaction was carried out using 0.1 mg/ml of normal tau either mixed with 0.1 mg/ml (4) or 0.2 mg/ml (5) of AD P-tau. For comparison, normal tau was used in different amounts, 0.1 mg/ml (3), 0.2 mg/ml (2), and 0.3 mg/ml (1). AD P-tau inhibited the microtubule assembly-promoting activity of normal tau (compare curves 2 and 3 with 4; and 1 and 3 with 5).

Figure 21. Interaction of AD P-tau with Normal Tau and Tubulin. AD P-tau was dotted on nitrocellulose strips and overlaid with tubulin (■) or normal tau (●). The nitrocellulose strips were developed with either anti-tubulin antibody DM1A (■) or with Tau-1 antibody (●). The inset shows the binding of tubulin to normal tau. The amount of tubulin or tau bound is expressed as the relative amount of radioactivity from the radioimmunoassay. Normal tau bound to AD P–tau (●) and tubulin bound to normal tau (inset), but had only background binding to AD P-tau (■).

Figure 22. Relationship of the Ratio of Sedimentable Non-hyperphosphorylated Tau/ Supernatant Tau (s.nP-tau/sup.tau) to the Levels of AD P-tau. The levels of tau were determined in the 200,000 x g supernatant (sup.tau) and 27,000 - 200,000 x g pellet (s.nP-tau and AD P-tau) from brain homogenates of four AD (●) and four control (□) cases by radioimmuno-slot-

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blot assay with or without alkaline phosphatase treatment. AD P-tau was calculated from the increase in immunoreactivity after the dephosphorylation (see Section 9.1). The AD P-tau values are expressed as cpm of radioactivity bound per μg of the protein; sn.P-tau/sup.tau ratios were obtained from the means of triplicate assays of these pools of tau determined at two different concentrations. The levels of the non-hyperphosphorylated tau correlate directly with the levels of AD P-tau in the 27,000 x g to 200,000 x g fraction (Sperman $R = 0.824$, $p < 0.012$), and levels of sup. tau in the 200,000 x g supernatant correlate inversely with AD P-tau (Sperman $R = -0.748$, $p < 0.032$). The ratio of sn.P-tau/sup.tau shows a highly significant (Sperman $R = 0.913$, $p < 0.002$) direct correlation with the AD P-tau levels.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to methods for treating Alzheimer disease and other disorders associated with the presence of neurofibrillary tangles (NFTs) by increasing the activity of a phosphatase towards abnormal hyperphosphorylated tau ("AD P-tau"). Pharmaceutical compositions and diagnostic methods are also provided.

As used herein, AD P-tau shall mean that hyperphosphorylated form of tau as present in the NFT of PHF in the neurons of patients having AD or other NFT-associated disorders (as described in detail in the Examples Sections *infra*).

As described in the Examples sections *infra*, it has been discovered that AD P-tau isolated from Alzheimer disease brain is dephosphorylated by the phosphoseryl/phosphothreonyl protein phosphatases PP-2B (calcineurin), PP-2A, and PP-1 (but not PP-2C) and that these enzyme reactivities are markedly increased in the presence of either of the divalent cations Mn^{2+} and Ni^{2+}. The order of the level of the phosphatase activity towards AD P-tau is PP-2B > PP-2A > PP-1. Furthermore, PP-2B dephosphorylates the tau abnormally phosphorylated sites Ser 46, Ser 199/Ser

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202, Ser 235, Ser 396, and Ser 404, whereas PP-2A dephosphorylates all of these sites except Ser 235, and PP-1 dephosphorylates only Ser 199/Ser 202 and Ser 396.

The invention provides various therapeutic methods, which, while not intending to be bound mechanistically, are believed to exert their therapeutic effect by decreasing the level of phosphorylation of AD P-tau, and thus allowing normal microtubule function in the affected neurons of patients. It is believed that (1) that the protein phosphorylation-dephosphorylation system is defective in Alzheimer disease brain, leading to abnormally phosphorylated tau and some other neuronal proteins and (2) that the abnormal phosphorylation of tau contributes to a microtubule assembly defect and consequent impairment of axoplasmic flow and neuronal degeneration (Figure 1).

The subject which is treated according to the methods of the invention has or is suspected of having a disease or disorder associated with the presence of NFTs of PHF in the neurons. Such a disease or disorder is selected from the group including but not limited to Alzheimer disease, Guam-Parkinsonism dementia complex, dementia pugilistica, postencephalitic parkinsonism, Down's syndrome, subacute sclerosing panencephalitis, Hallervorden-Spatz disease, and neurovisceral lipid storage disease (for a review concerning these disorders, see Wisniewski et al., 1979, Ann. Neurol. 5:288-294; Iqbal and Wisniewski, 1983, Neurofibrillary tangles, in *Alzheimer's Disease*, Reisberg, B., ed., The Standard Reference, The Free Press, NY, pp. 48-56).

The inventions provide methods of treatment by administering to a subject a therapeutically effective amount of a composition comprising a molecule which increases PP activity toward AD P-tau, a phosphatase which dephosphorylates AD P-tau, or a nucleic acid encoding such a phosphatase. In a preferred aspect, the foregoing therapeutics are substantially purified. In a specific embodiment, administration is repeated over time. The subject is

an animal, including but not limited to animals such as cows, pigs, chickens, etc., and is preferably a mammal, and most preferably human. Preferably, the methods of the invention also inhibit the activities of (by dephosphorylating) proline-directed protein kinases such as the MAP kinase, which may participate in the abnormal phosphorylation of AD P-tau.

5.1. ADMINISTRATION OF MOLECULES WHICH INCREASE PROTEIN PHOSPHATASE ACTIVITY

In one embodiment, the invention provides methods of treating disorders associated with the presence of NFTs by administering a therapeutically effective amount of a composition comprising a molecule which increases the activity of a protein phosphatase (PP) towards AD P-tau. The PP has the ability to dephosphorylate one or more of the phosphorylation sites of AD P-tau shown in Table 1 hereinabove; the more sites which the PP can dephosphorylate, the more it is preferred. In a preferred aspect, the PP can dephosphorylate at least six of the phosphorylated sites shown in Table 1. In a specific aspect, the PP is selected from the group consisting of PP-1. PP-2A, PP-2B (calcineurin), and related PPs. By "related PPs" is meant PPs which have substantially the same catalytic subunit as one of the foregoing PPs. The terms PP-1, PP-2A, and PP-2B are meant to include the different isotypes for each PP, *e.g.*, PP-$2A_1$ and PP-$2A_2$ for PP-2A. In a specific embodiment, the PP type whose activity is increased according to the invention is generally detectable in neurons of the brain. PP-1, PP-2A and PP-2B are detectable in neurons of the brain, whereas alkaline phosphatase is not. In the methods of the invention, a molecule which increases the activity of PP-2B is most preferred, followed by PP-2A, and then PP-1 in decreasing order of preference. In a preferred aspect, the molecule increases the activity of at least two of the aforesaid PPs, and most preferably all three types. Molecules which can be used therapeutically according to the invention include but are not limited to metals such as Mn^{2+} and Ca^{2+}, and polylysine,

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with Mn^{2+} most preferred. The effect of various metals and of polylysine on AD P-tau dephosphorylation by PP-1, PP-2A, and PP-2B is shown in Table 2.

TABLE 2

Effect of Metal and Polylysine on AD P-tau Dephosphorylation by PPs

Effector	PP-1	PP-2A	PP-2B
Mn^{2+}	↑↑	↑↑	↑↑
Mg^{2+}	↓	→	↑
Ni^{2+}			↑↑
Ca^{2+}			↑
Al^{3+}			↓
Polylysine		↑	

↑: Increase in enzyme activity
↓: Decrease in enzyme activity
→: No effect

Other molecules which potentially can be administered for therapeutic effect according to the invention include but are not limited to those listed in Table 3, which are known to increase activity of the indicated PP toward a substrate other than AD P-tau; these potential therapeutics should thus be tested in an appropriate *in vitro* assay for their effect upon PP activity towards AD P-tau (*e.g.*, in an assay as described in the Examples Sections *infra*) prior to therapeutic use.

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TABLE 3

Protein Phosphatase Activators

Activator	PP	Reference
Ceramide	PP-2A (heterotrimer only)	Dobrowsky et al., 1993, J. Biol. Chem. 268(21):15523-15530
HSP-70 kDa	PP-1 and/or PP-2A	Mivechi et al., 1993, Biochem. Biophys. Res. Commun. 192(2):954-963
Insulin	PP-1	Begum et al., 1993, J. Biol. Chem. 268(11):7917-7922; Chan et al., 1988, Proc. Natl. Acad. Sci. USA 85(17):6257-6261
Chromostatin	PP-2A	Galindo et al., 1992, Proc. Natl. Acad. Sci. USA 89(16):7398-7402
cdc2	PP-1	Villa-Moruzzi et al., 1992, FEBS Lett. 304(2-3):211-215
Basic proteins	PP-2A	Ballou and Fischer, 1987, *The Enzyme* 17:311-361
Polyamines	PP-2A	Ballou and Fischer, 1987, *The Enzyme* 17:311-361
Insulin	PP-2A	Speth and Lee, 1984, J. Biol. Chem. 259:4027-4030
Calpain	PP-2B	Wang et al., 1989, Biochem. Cell Biol. 67(10):703-711
Growth factor	PP-1	Chan et al., 1988, Proc. Natl. Acad. Sci. USA 85(17):6257-6261
Spermine	PP-2A	Damuni et al., 1987, J. Biol. Chem. 262(11):5133-5138

Trypsin	PP-2B	Wolff and Sued, 1985, J. Biol. Chem. 260(7):4195-4202

In a specific embodiment, the molecule increases the activity towards AD P-tau of at least one of the foregoing PPs and does not inhibit any such activity of the foregoing PPs. In another specific embodiment, the molecule is not Mg^{2+}. The metal can be in ionic form, salt form, or conjugate. The manganese is preferably in the form of a water-soluble salt such as but not limited to manganese chloride, manganese sulfate, manganese acetate, manganese gluconate, manganese lactate, and manganese citrate. The manganese may also be in the form of other compounds such as manganese hypophosphite, manganese silicate, manganese sulfide, manganese iodide, manganese phosphate, manganese borate, manganese bromide, manganese oleate, manganese nitrate, manganese carbonate, manganese carbonyl, manganese difluoride, manganese trifluoride, manganese oxalate, manganese oxide, manganese dioxide, manganese selenide, manganese sesquioxide, etc. In a specific embodiment, the manganese is not in the form of manganese pyruvate or a manganese chelate of an alkylamino-ester of phosphoric acid (*e.g.*, manganese aminoethyl phosphate). Preferably, such molecules are administered orally, although any form of administration known in the art can be used (see Section 5.5 *infra*).

Molecules which increase the activity of a PP toward AD P-tau can be identified as having such activity by any appropriate *in vitro* assay, preferably an assay described in the Examples Sections *infra*.

Molecules demonstrated to have the desired activity *in vitro* can then be tested further *in vitro* if desired, and then *in vivo* to demonstrate therapeutic efficacy. For example, such molecules can be tested in suitable cell culture systems for their effect on AD P-tau in cultured cells, and in animal systems prior to testing in humans, including but not limited to rats,

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mice, chicken, cows, monkeys, rabbits, etc. Suitable model systems, where available in the art, can be used.

5.2. ADMINISTRATION OF PROTEIN PHOSPHATASES AND NUCLEIC ACIDS ENCODING THE SAME

In another embodiment, the invention provides methods of treating disorders associated with the presence of NFTs by administering a therapeutically effective amount of a phosphatase which dephosphorylates (at least some phosphorylated residues of) AD P-tau. Such a phosphatase preferably is active toward AD P-tau to a greater extent than normal tau. In a specific aspect, such a phosphatase is selected from the group consisting of PP-1, PP-2A, PP-2B, related PPs, and functionally active derivatives and analogs thereof. By "related PPs" is meant PPs which have substantially the same catalytic subunit as one of the foregoing PPs. The terms PP-1, PP-2A, and PP-2B are meant to include the different isotypes for each PP, *e.g.*, $PP\text{-}2A_1$ and $PP\text{-}2A_2$ for PP-2A. In a specific embodiment, the phosphatase which is administered is a PP whose activity can be detected in neurons of the brain. PP-2B is most preferred for use, followed by PP-2A and then PP-1 in decreasing order of preference. In specific embodiments, one, two, or three of the aforesaid phosphatases can be administered in combination.

Phosphatases can be purified from biological sources by methods known in the art (see *e.g.*, Examples Sections *infra*), or purchased where commercially available (see Examples Sections *infra*), or expressed by recombinant methods known in the art from host cells containing a cloned gene encoding a PP. Functionally active derivatives and analogs can be obtained by chemical or enzymatic modification of the phosphatase (*e.g.*, acetylation, carboxylation, amidation, phosphorylation, cleavage, etc.) or recombinant manipulation of the gene encoding a PP, all by methods commonly known in the art.

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PPs which have the desired activity toward AD P-tau can be identified by an *in vitro* assay such as described in the Examples Sections *infra*.

In another embodiment, nucleic acids encoding one or more of the aforesaid PPs can be administered *in vivo* such that the encoded PP is expressed for therapeutic effect. The cloning and/or nucleotide sequences of PPs are available in the art, *e.g.*, as described in the following publications. For PP-1: Sasaki et al., 1990, Jpn. J. Cancer Res. 81:1272-1280. For PP-1α: Berndt et al., 1987, FEBS Lett. 223:340-346. For PP-2A: Kitagawa et al., 1988, Biochim. Biophys. Acta 951:123-129; Kitagawa et al., 1988, Biochem. Biophys. Res. Commun. 157:821-827; Sasaki et al., 1990, Biochem. Biophys. Res. Commun. 170:169-175. For PP-2B: Muramatsu and Kincaid, 1993, Biochim. Biophys. Acta 1178:117-120; Guerini and Klee, 1989, Proc. Natl. Acad. Sci. USA 86:9183-9187; Kincaid et al., 1990, J. Biol. Chem. 265:11312-11319; Kincaid et al., 1988, Proc. Natl. Acad. Sci. USA 85:8983-8987.

5.3. METHODS OF DIAGNOSIS

The present invention also provides methods of diagnosing the presence, staging the progression, and monitoring treatment of diseases and disorders associated with the presence of NFTs by detecting or measuring the levels of AD P-tau in a sample from a subject having or suspected of having such a disease or disorder. Preferably, the sample is cerebrospinal fluid (CSF), which can be obtained by a spinal tap as commonly performed in the art. The detection or measurement of AD P-tau levels is preferably carried out by contacting any AD P-tau in the sample with an antibody (or antibodies) which specifically bind to phosphorylated epitopes of AD P-tau (and do not substantially bind to nonphosphorylated epitopes of tau) such that immunospecific binding can occur, and detecting or measuring the amount of immunospecific binding that occurs. An increased level of AD P-tau which is

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thus observed relative to subjects not having the disease or disorder indicates the presence of the disorder in the subject. Increased levels over time indicate disease progression. It is believed that decreased levels after treatment will indicate treatment efficacy.

The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. In specific embodiments, the immunoassay is carried out by sandwich immunoassay, immunoprecipitation, or dot blot methods, as are well known in the art. Antibodies which can be used, which recognize phosphorylated epitopes of AD P-tau, are known in the art and include but are not limited to those listed in Table 1, Section 2.2 hereinabove, or described in the Examples Sections *infra*. Antibodies can also be generated by standard methods commonly known in the art, by use of AD P-tau as immunogen.

In one embodiment, the sandwich assay is carried out by binding (as capture antibody) an antibody which recognizes AD P-tau (which need not be specific to a phosphorylated epitope) to a solid substrate (*e.g.* a plastic dish), incubating with sample (*e.g.* CSF); and incubating with (as detection antibody) an antibody which specifically recognizes a phosphorylated epitope of AD P-tau. Substances which do not immunospecifically bind are removed by one or more washing steps, commonly known in the art.

In another specific embodiment, the dot blot approach, the biological sample (*e.g.*, CSF or proteins obtained therefrom) is applied to a membrane filter, washed, and then contacted with a composition containing the antibody to a phosphorylated epitope of AD P-tau.

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In one embodiment, the antibody which binds to the phosphorylated epitope of AD P-tau is labeled (*e.g.*, by an enzyme, radionuclide, fluorescent tag), and the presence of the label is detected or measured. In another embodiment, such antibody is unlabeled, and a labeled specific binding partner to the antibody is added, allowed to bind to the antibody, preferably a washing step is performed, and then the label of the binding partner is detected or measured.

The antibody(ies) can be polyclonal or monoclonal.

5.4. PHARMACEUTICAL COMPOSITIONS

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a therapeutic of the invention (a molecule which increases the activity of a PP toward AD P-tau, a phosphatase which dephosphorylates AD P-tau, or a nucleic acid encoding such a phosphatase), and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. These

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compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, cellulose, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a specific embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric

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hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, the physical condition of the subject, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous protein administration are generally about 20-500 micrograms of active molecule per kilogram body weight. Suitable dosage ranges for intranasal administration of protein are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from the dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

In a preferred aspect in which manganese is the therapeutic, the dosage of a composition comprising manganese (in salt or conjugate form) which is administered is so as to achieve a level of Mn^{2+} in the brain greater than 10 μM (basal levels of Mn^{2+} in the brain are about 6-10 μM), and preferably so as to achieve a level of Mn^{2+} in the brain in the range of 20-100 μM, and most preferably 40-100 μM. The dosages for achieving 20-100 μM Mn^{2+} concentration in the brain are believed to be in the range of 2.5-12.5 mg manganese compound/kg body weight/day when oral administration is used. To achieve 40-100 μM Mn^{2+} concentration in the brain, the dosages are believed to be in the range of 5-25 μg manganese compound/kg body weight/day when intravenous administration is used.

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The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

5.5. THERAPEUTIC ADMINISTRATION

Various delivery systems are known and can be used to administer a therapeutic of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, expression by recombinant cells, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a therapeutic nucleic acid as part of a retroviral or other vector, etc. The therapeutics of the invention, particularly those with the ability to cross the blood-brain barrier (*e.g.*, manganese, nickel), can be administered systemically, and more preferably parenterally, *i.e.*, via an intraperitoneal, intravenous, perioral, subcutaneous, intramuscular, intraarterial, etc. route, in order to treat disease. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. In a specific

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aspect, the compounds are directly administered to the cerebrospinal fluid by intraventricular injection. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent (for example, in the administration of manganese ion, or protein therapeutic, etc.).

In a specific embodiment, it may be desirable to administer the therapeutics of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

In another embodiment, the therapeutic compound can be delivered in a vesicle, in particular a liposome (*see* Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; *see* generally *ibid.*)

In yet another embodiment, the therapeutic compound can be delivered in a controlled release system. In one embodiment, a pump may be used (*see* Langer, *supra*; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (*see* Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); *see also* Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the

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brain, thus requiring only a fraction of the systemic dose (*see*, *e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

In a specific embodiment where the therapeutic is a nucleic acid encoding a protein phosphatase, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see *e.g.*, Joliot et al., 1991, Proc. Natl. Acad. Sci USA 88:1864-1868), etc. Alternatively, a nucleic acid therapeutic can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

6. EXAMPLE: ALZHEIMER DISEASE ABNORMALLY PHOSPHORYLATED TAU IS DEPHOSPHORYLATED BY PROTEIN PHOSPHATASE-2B (CALCINEURIN)

As described herein, we have examined the site-specific dephosphorylation of abnormal hyperphosphorylated tau (AD P-tau) from Alzheimer disease brains by different protein phosphatases. Tau dephosphorylation was monitored by its interaction with several phosphorylation-dependent antibodies. AD P-tau was dephosphorylated by brain protein phosphatase-2B at the abnormally phosphorylated sites Ser-46, Ser-199, Ser-202, Ser-235, Ser-396 and Ser-404, and its relative mobility on SDS-PAGE shifted to that of normal tau. Protein phosphatases-1 and -2A could dephosphorylate only some of the above six phosphorylation sites.

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6.1. MATERIALS AND METHODS

AD abnormally phosphorylated tau and normal human tau were isolated from autopsied brains as described by Köpke et al. (1993, J. Biol. Chem. 268:24374-24384). Phosphorylase kinase was purified from rabbit skeletal muscle by the method of Cohen (1973, J. Biochem. 34:1-14). Rabbit skeletal muscle PP-1 was purchased from Upstate Biotechnology Inc., Lake Placid, NY. Rat brain PP-2A_1 and PP-2A_2 were kindly provided by Dr. S. Jaspers of University of Massachusetts. PP-2B (holoenzyme) was purified from bovine brain according to the method of Sharma et al. (1983, Meth. Enzymol. 102:210-219). Phosphorylase and calmodulin were purchased from Sigma, St. Louis, MO. Polyclonal antibodies 102c and 92e were raised as previously reported (Grundke-Iqbal et al., 1988, Mol. Brain Res. 4:43-52; Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650). Monoclonal antibodies Tau-1 and PHF-1 were kindly provided by Drs. L. I. Binder (Binder et al., 1985, J. Cell Biol. 101:1371-1378) and S. Greenberg (Greenberg et al., 1992, J. Biol. Chem. 267:564-569), respectively; SMI33, SMI31 and SMI34 were purchased from Sternberger Monoclonals Inc., Baltimore, MD. Alkaline phosphatase-conjugated goat anti-mouse and anti-rabbit IgG were purchased from Bio-Rad, Hercules, CA.

Phosphorylase (2.0 mg/ml) was phosphorylated in 40 mM Tris-HCl, pH 8.5, 20 mM ß-mercaptoethanol, 0.2 mM $CaCl_2$, 15 mM $MgCl_2$, 10 μg/ml phosphorylase kinase and 0.5 mM [γ-^{32}P]ATP. After incubation at 30°C for 10 min, [^{32}P]phosphorylase (0.9 mol ^{32}P incorporated/95,000 g) was separated from free ATP on Sephadex G-50 column. [^{32}P]phosphorylase kinase (1.9 mol ^{32}P incorporated/335,000 g) was prepared as reported previously (Gong et al., 1993, J. Neurochem. 61:921-927). The activities of PP-1, PP-2A and PP-2B were measured by counting the radioactivity released from [^{32}P]substrate as previously described (Gong et al., 1993, J. Neurochem. 61:921-927). The reaction mixtures contained 50 mM Tris, pH 7.0, 20 mM ß-mecaptoethanol, 2.0 mM $MnCl_2$ and 2.0 μM [^{32}P]phosphorylase for PP-1

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and PP-2A; and 50 mM Tris, pH 7.0, 20 mM ß-mecaptoethanol, 1.0 mM $CaCl_2$, 1.0 μM calmodulin and 1.0 μM [^{32}P]phosphorylase kinase for PP-2B. One unit of protein phosphatase activity is defined as that amount which catalyzes the release of 1.0 nmol phosphate per min from [^{32}P]substrate at 30°C.

Unless otherwise stated, dephosphorylation of AD P-tau was carried out at 30°C in 50 mM Tris, pH 7.0, 10 mM ß-mecaptoethanol, 0.1 mg/ml BSA, 50 μg/ml AD P-tau and PP-1, PP-2A or PP-2B. The reaction was started by addition of enzyme and stopped by addition of 5 volumes of cold acetone. The precipitated protein samples were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and heated at 95°C for 4 min, followed by 10% SDS-PAGE. Immunoblotting was carried out as described previously (Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci. USA 83:4913-4917). The primary antibodies for immunoblotting and their epitopes have all been previously characterized. Antibodies 102c (Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650), Tau-1 (Biernat et al., 1992, EMBO J. 11:1593-1597 Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci. USA 83:4913-4917) and SMI33 (Lichtenberg-Kraag et al., 1992, Proc. Natl. Acad. Sci. USA 89:5384-5388) recognize dephosphorylated form of tau at sites Ser-46, Ser-199/Ser-202 and Ser-235 respectively. While antibodies SMI31, SMI34 (Lichtenberg-Kraag et al., 1992, Proc. Natl. Acad. Sci. USA 89:5384-5388) and PHF-1 (Lang et al., 1992, Biophys. Res. Commun. 187:783-790) recognize tau phosphorylated at Ser-396/Ser-404, Ser-235/Ser-396 and Ser-396, respectively. Antiserum 92e is a phosphorylation-independent antibody which recognizes both AD P-tau and normal tau. These antibodies were used at dilution of 1:100 for 102c, SMI31 and SMI34, 1:500 for SMI33 and PHF-1, 1:5,000 for 92e, and 1:500,000 for Tau-1.

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6.2. RESULTS

Dephosphorylation of AD abnormally phosphorylated tau (AD P-tau) by different protein phosphatases

PP-2B (1.2 units/ml) was found to change epitopes of all six antibodies (Fig. 2), suggesting that it can dephosphorylate AD P-tau at Ser-46, Ser-199, Ser-202, Ser-235, Ser-396 and Ser-404. Equivalent activities (1.2 units/ml) of PP-1 and PP-2A did not change the epitopes appreciably. After prolonged incubation with PP-1, PP-$2A_1$ or PP-$2A_2$ (1.2 units/ml) or after the addition of 1.0 mM Mn^{2+}, the epitopes of some of the six antibodies were changed (data not shown). These results indicate that PP-1 and PP-2A can dephosphorylate only some of above six abnormal phosphorylated sites studied. Thus PP-2B seems to be a preferential AD P-tau phosphatase. Hence the dephosphorylation of AD P-tau only by PP-2B was further examined in this study.

Time course of dephosphorylation of AD P-tau by PP-2B

The rates of dephosphorylation of various sites were rapid but nonidentical (Fig.3). After only 1 min incubation, the staining of tau by 102c, Tau-1 and SMI33 was already apparent, and staining by SMI31, SMI34 and PHF-1 began to disappear. Epitopes of SMI33, Tau-1 and 102c were almost completely unblocked in 3 min, 15 min and 20 min, respectively. Blocking of the epitopes of antibodies SMI31, PHF-1 and SMI34 was practically complete in 6 min, 6 min and 15 min, respectively. These results indicate that in hyperphosphorylated AD P-tau the preferential substrate sites for PP-2B are pSer-235 > pSer-396/pSer-404 > pSer-199/pSer-202 > pSer-46.

Phosphorylated and dephosphorylated tau are known to have different relative mobilities on SDS-PAGE (Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci. USA 83:4913-4917). After only 1 min incubation with PP-2B, a mobility shift of different tau species is already apparent. Maximal

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shift of all isoforms, except the slowest one, is achieved in 15 min (Fig. 3, a, b and g).

Effects of calmodulin and divalent cations on dephosphorylation of AD P-tau by PP-2B

We have investigated the effects of different divalent cations on the dephosphorylation of AD P-tau by PP-2B (Fig. 4). The highest activity of PP-2B towards AD P-tau was observed in the presence of Ca^{2+}, Ni^{2+} and calmodulin (Fig. 4, E). When Mn^{2+} and Mg^{2+} were used instead of Ni^{2+}, the activity was lower (Fig. 4, compare C and D with E). In the presence of either Mn^{2+} or Ni^{2+} alone, the magnitude of dephosphorylation was lower compared to the inclusion of Ca^{2+} and calmodulin in reaction mixtures (Fig. 4, compare A with C and B with E). In the presence of Ca^{2+} and calmodulin alone, however, no apparent dephosphorylation of tau by PP-2B was detected (data not shown). We further examined the required concentration of divalent metal ions for the dephosphorylation of AD P-tau by PP-2B and found that in the presence of 1.0 mM Ca^{2+} and 1.0 μM calmodulin, 10 μM of either Mn^{2+} or Ni^{2+} activated the dephosphorylation. Maximal dephosphorylation was achieved in the presence of 100 μM of either Mn^{2+} or Ni^{2+} (Fig. 5).

6.3. DISCUSSION

In the present study, we found that PP-2B can dephosphorylate all the six abnormal phosphorylation sites studied as well as change relative electrophoretic mobility of AD P-tau into a normal state. This *in vitro* dephosphorylation required the presence of 10 - 100 μM of either Mn^{2+} or Ni^{2+}. The physiological level of Mn^{2+} was reported as 5 - 11 μM in brain (Friberg et al., 1986, Handbook on the toxicology of methods, Vol. 2, Nordberg and Vouk, eds., Elsevier Science Publishers, New York, pp. 264-366), and therefore the dephosphorylation of AD P-tau by PP-2B may have physiological significance. This study indicates that PP-2B might be

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involved in dephosphorylation of AD P-tau *in vivo*. The following observations from other laboratories strengthen this suggestion. First, PP-2B has the highest expression in brain, and the amount of PP-2B in brain tissue is about 1% of total proteins, higher than that of other protein phosphatases (Cohen, 1989, Annu. Rev. Biochem. 58:453-508; Kuno et al., 1992, J. Neurochem. 58:1643-1651). Second, immunohistochemical and immunochemical studies have shown that PP-2B is located on microtubules and is plentiful in the cerebral cortex and hippocampus (Kuno et al., 1992, J. Neurochem. 58:1643-1651). Third, it was recently reported that PHF-like tau was generated after incubation of fresh brain slices with 5 - 20 μM okadaic acid (which inhibits PP-2B as well as PP-2A and PP-1), and that PP-2B can reverse the effect of okadaic acid (Harris et al., 1993, Ann. Neurol. 33:77-87).

Goedert et al. (1992, FEBS Lett. 312:95-99) have reported that MAP kinase-phosphorylated tau could be dephosphorylated by PP-$2A_1$, one of three subtypes of PP-2A, but not by PP-2B. A cause of this discrepancy between these two studies might be the use of different substrates. AD P-tau (physiological substrate) (which we employed) and MAP kinase-phosphorylated tau which requires 16 - 24 hours for *in vitro* phosphorylation (Drewer et al., 1992, EMBO J. 11:2131-2138; Goedert et al., 1992, FEBS Lett. 312:95-99) might behave as different substrates for PP-2B. This is because only some and not all abnormal sites are phosphorylated by MAP kinase (Biernat et al., 1993, Neuron 11:153-163), and non-MAP kinase sites might alter the kinetics of the dephosphorylation.

In a previous study measuring protein phosphatase activities using [^{32}P]phosphorylase kinase as substrate (Gong et al., 1993, J. Neurochem. 61:921-927), we found that PP-1 and PP-2A activities were significantly decreased in AD brains as compared with controls. We did not, however, find such a decrease in activity of PP-2B. One possible reason for this may be that PP-2B activity was not measured using tau as substrate.

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7. EXAMPLE: DEPHOSPHORYLATION OF ALZHEIMER DISEASE ABNORMALLY PHOSPHORYLATED TAU BY PROTEIN PHOSPHATASE-2A

As described herein, the site-specific dephosphorylation of abnormally phosphorylated Alzheimer tau (AD P-tau) by protein phosphatase-2A was examined by its interaction with several phosphorylation-dependent antibodies to various abnormal phosphorylation sites. Protein phosphatase-2A was able to dephosphorylate AD P-tau at Ser-46, Ser-199, Ser-202, Ser-396, and Ser-404, but not at Ser-235 (the amino acids are numbered according to the largest isoform of human tau, tau_{441}). Two major types of protein phosphatase-2A -- $PP\text{-}2A_1$ and $PP\text{-}2A_2$ -- dephosphorylated AD P-tau at approximately the same rate. After AD P-tau was dephosphorylated by protein phosphatase-2A, its relative mobility on SDS-PAGE increased. The dephosphorylation of AD P-tau by $PP\text{-}2A_1$ and $PP\text{-}2A_2$ was markedly stimulated by Mn^{2+}. These results indicate that tau dephosphorylation is catalyzed by both types of protein phosphatase-2A, $PP\text{-}2A_1$ and $PP\text{-}2A_2$.

Several protein kinases have been reported to phosphorylate tau *in vitro* at some of the same sites at which PHF-tau is abnormally phosphorylated (Drewes et al., 1992, EMBO J. 11:2131-2138; Ishiguro et al., 1992, J. Biol. Chem. 267:10897-10901; Ledesma et al., 1992, FEBS Lett. 308:218-224; Mandelkow et al., 1992, FEBS Lett. 314:315-321; Vulliet et al., 1992, J. Biol. Chem. 267:22570-22574). However, the *in vitro* conditions required to obtain the abnormally hyperphosphorylated tau identical to that in Alzheimer disease are still unknown. Hence, we used hyperphosphorylated tau isolated from Alzheimer brain as a substrate to study the potential protein phosphatases that may be involved in the dephosphorylation of tau. Monitoring by immunoblotting with several phosphorylation-dependent antibodies 102c, Tau-1, SMI33, SMI31, SMI34 and PHF-1, we found that protein phosphatase-2B (PP-2B) can rapidly dephosphorylate Alzheimer tau at sites Ser-46, Ser-199, Ser-202, Ser-235,

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Ser-396 and Ser-404 *in vitro* (see Section 6 above). In this Section, we report that protein phosphatase-2A (PP-2A), in addition to PP-2B, can dephosphorylate AD P-tau at phosphorylation sites Ser-46, Ser-199, Ser-202, Ser-396 and Ser-404 but not at Ser-235.

7.1. EXPERIMENTAL PROCEDURES

Materials

Phosphorylase kinase was purified from rabbit skeletal muscle by the method of Cohen (1973, Eur. J. Biochem. 34:1-14). PP-$2A_1$ and PP-$2A_2$ were purified from rat brain basically as described by Cohen et al. (1988, Methods in Enzymol. 159:390-408) and kindly provided by Dr. S. Jaspers of the University of Massachusetts. PP-2B was purified from bovine brain according to the method of Sharma et al. (Sharma et al., 1983, Methods Enzymol. 102:210-219). Phosphorylase, calmodulin, and poly-L-lysine (molecular weight 4,000 - 15,000) were purchased from Sigma, St. Louis, MO. Production of rabbit polyclonal antibodies 102c and 92e was reported previously (Grundke-Iqbal et al., 1988, Mol. Brain Res. 4:43-52; Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650). Mouse monoclonal antibodies Tau-1 (Binder et al., 1985, J. Biol. Chem. 101:1371-1378), AT8 (Mercken et al., 1992, Neuropathol. 84:265-272), and PHF-1 (Greenberg et al., 1992, J. Biol. Chem. 267:564-569), were kindly provided by Drs. L. I. Binder, University of Alabama, Birmingham, AL; A. Van de Voorde, Innogenetics, Industriepark Zwijinaarde, Belgium; and S. Greenberg, Burke Medical Research Institute, White Plains, NY, respectively. SMI33, SMI31, SMI34, goat anti-mouse IgG and peroxidase-anti-peroxidase complex were purchased from Sternberger Monoclonals Inc., Baltimore, MD. Alkaline phosphatase-conjugated goat anti-mouse and anti-rabbit IgG were purchased from Bio-Rad, Hercules, CA.

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Isolation of tau

Abnormally phosphorylated Alzheimer tau (AD P-tau) and normal human tau were isolated by the method of Köpke et al. (1993, J. Biol. Chem. 268:24374-24384) from autopsied brains of a 70-year-old male with Alzheimer disease and a 51-year-old male normal case, respectively. Briefly, AD P-tau was isolated from a non-neurofibrillary tangle pool, the 27,000 *g* to 200,000 *g* fraction of the Alzheimer brain homogenate by extraction in 8 M urea, followed by dialysis against Tris buffer. This AD P-tau is readily soluble in buffer and abnormally phosphorylated as PHF-tau (Köpke et al., 1993, J. Biol. Chem. 268:24374-24384). Normal human tau was purified from 35-45% ammonium sulfate precipitates of the 200,000 *g* brain supernatant, followed by acid treatment (pH 2.7) and chromatography on a phosphocellulose column (Cellulose Phosphate P11, Whatman) (Köpke et al., 1993, J. Biol. Chem. 268:24374-24384).

Protein concentrations were determined by a modified Lowry assay (Bensadoun and Weinstein, 1976, Anal. Biochem. 70:241-250).

Preparation of [^{32}P]substrates and determination of protein phosphatase activities

PP-2A and PP-2B activities were measured as described above by using [^{32}P]phosphorylase (0.9 mol ^{32}P incorporated/95,000 g) and [^{32}P]phosphorylase kinase (1.9 mol ^{32}P incorporated/335,000 g) as substrates, respectively (see Section 6). Phosphorylase and phosphorylase kinase phosphorylation were catalyzed by phosphorylase kinase and catalytic subunit of cAMP-dependent protein kinase, respectively. One unit of protein phosphatase activity is defined as that amount which catalyzes the release of 1.0 nmol phosphate per min from [^{32}P]substrate at 30°C.

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Dephosphorylation of abnormally phosphorylated Alzheimer tau by PP-2A and PP-2B

Unless otherwise stated, dephosphorylation of AD P-tau by PP-$2A_1$ or PP-$2A_2$ was carried out at 30°C in 50 mM Tris, pH 7.0, 20 mM ß-mecaptoethanol, 0.1 mg/ml BSA, 1.0 mM $MnCl_2$, 50 μg/ml AD P-tau and 5.0 U/ml enzyme. In some experiments, Mn^{2+} in the reaction mixture was replaced by other effectors (see Figure legends). In the reaction mixture for PP-2B, $MnCl_2$ was substituted with 1.0 mM $NiCl_2$, 1.0 mM $CaCl_2$ and 1.0 μM calmodulin. The reaction was started by the addition of enzymes. After appropriate incubation times (see Figure legends), reactions were stopped by the addition of 5 volumes of cold acetone to precipitate proteins. Dephosphorylation was monitored by immunoblotting with the phosphorylation-dependent antibodies described below.

SDS-polyacrylamide gel electrophoresis and immunoblotting

The precipitated protein samples were dissolved in SDS-PAGE sample buffer and heated at 95°C for 4 min, followed by 10% SDS-PAGE (1.0 μg tau protein/lane) as described by Laemmli (1970, Nature 227:680-685). Immunoblotting was carried out as described previously (Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci. USA 83:4913-4917). The primary antibodies for immunoblotting and their epitopes have all been previously characterized. Antibodies 102c, Tau-1 and SMI33 recognize the dephosphorylated form of tau at Ser-46 (Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650), Ser-199/Ser-202 (Biernat et al., 1992, EMBO J. 11:1593-1597; Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci. USA 83:4913-4917; Szendrei et al., 1993, J. Neurosci. Res. 34:243-249), and Ser-235 (Lichtenberg-Kraag et al., 1992, Proc. Natl. Acad. Sci. USA 89:5384-5388) respectively; whereas antibodies AT8, SMI31 and PHF-1 recognize tau phosphorylated at Ser-199/Ser-202 (Biernat et al., 1992, EMBO J. 11:1593-1597), Ser-396/Ser-404 (Lichtenberg-Kraag et al., 1992, Proc.

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Natl. Acad. Sci. USA 89:5384-5388) and Ser-396 (Greenberg et al., 1992, J. Biol. Chem. 267:564-569; Lang et al., 1992, Biochem. Biophys. Res. Commun. 187:783-790), respectively. These antibodies were used at dilutions of 1:100 for 102c, SMI31 and SMI34, 1:500 for SMI33 and PHF-1, 1:5,000 for 92e, and 1:500,000 for Tau-1. Antibody AT8 was used at a concentration of 2.0 μg/ml. The blots were developed by alkaline phosphatase staining (for 102c, Tau-1, SMI33, PHF-1 and 92e) or peroxidase staining (AT8, SMI31 and SMI34). The intensity of immunostaining of some blots with Tau-1 was scanned by using a Shimadzu dual-wavelength flying-spot scanner.

7.2. RESULTS

Comparison of dephosphorylation of AD P-tau by PP-2A$_1$, PP-2A$_2$ and PP-2B

In a previous study, we found rapid dephosphorylation of AD P-tau by purified PP-2B but not by an equivalent amount of PP-2A (see Section 6). When incubated either for longer times at 30°C or in the presence of Mn^{2+}, PP-2A was also able to dephosphorylate AD P-tau at some of the abnormal phosphorylation sites. Therefore, in optimal conditions for both PP-2A and PP-2B, we have further compared the dephosphorylation of AD P-tau by different amounts of PP-2A (PP-2A$_1$ and PP-2A$_2$) and PP-2B (Fig. 6). Dephosphorylation of AD P-tau (Tau-1 epitope) was observed with a lower amount of PP-2B than PP-2A. Maximal dephosphorylation of AD P-tau was observed with about 3.0 U/ml PP-2B and about 5.0 U/ml of either PP-2A$_1$ or PP-2A$_2$. No significant differences in the dephosphorylation of AD P-tau between PP-2A$_1$ and PP-2A$_2$ were observed.

Dephosphorylation of AD P-tau by different amounts of PP-2A$_1$, PP-2A$_2$ and PP-2B was also determined by immunoblotting with antibodies 102c and PHF-1, which monitor the dephosphorylation at phosphorylation sites Ser-46 and Ser-396 of the protein, respectively. The results indicated that, as with the Tau-1 sites, the dephosphorylation of the

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102c and PHF-1 sites of AD P-tau required higher amounts of PP-2A_1 or PP-2A_2 than of PP-2B (Fig. 7).

Site-specific dephosphorylation of AD P-tau by PP-2A_1 and PP-2A_2

Site-specific dephosphorylation of AD P-tau by either 5.0 U/ml PP-2A_1 or 5.0 U/ml PP-2A_2 was studied. Eight antibodies, seven of which are phosphorylation-dependent, were used for this experiment. Antibodies 102c, Tau-1 and SMI33 recognize the dephosphorylated form of tau at the sites Ser-46, Ser-199/Ser-202 and Ser-235, respectively. In contrast, antibodies AT8, SMI31 and PHF-1 recognize the phosphorylated form of tau at the sites Ser-199/Ser-202, Ser-396/Ser-404 and Ser-396, respectively. As shown in Fig. 8, dephosphorylation of AD P-tau either by PP-2A_1 or PP-2A_2 altered the accessibilities of the antibodies 102c, Tau-1, AT8, SMI31, SMI34 and PHF-1, but not of antibody SMI33. This finding suggests that PP-2A_1 and PP-2A_2 can dephosphorylate AD P-tau at Ser-46, Ser-199, Ser-202, Ser-396 and Ser-404, but not at Ser-235. The two types of PP-2A, PP-2A_1 and PP-2A_2, showed almost no difference in dephosphorylation of AD P-tau (compare lanes 2 with 3 of panels A-G of Fig. 8).

Normal human tau has a higher relative mobility on SDS-PAGE than abnormally phosphorylated tau isolated from Alzheimer brain and tau phosphorylated by several protein kinases (Baudier and Cole, 1987, J. Biol. Chem. 262:17577-17583; Drewes et al., 1992, EMBO J. 11:2131-2138; Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci. USA 83:4913-4917; Iqbal et al., 1986, Lancet 2:421-426; Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650; Mandelkow et al., 1992, FEBS Lett. 314:315-321; Vulliet et al., 1992, J. Biol. Chem. 267:22570-22574). The mobility shift of tau on SDS-PAGE has been used previously as a criterion of hyperphosphorylation of tau. The seven phosphorylation-dependent antibodies above can recognize either dephosphorylated (Tau-1 and SMI33) or untreated (AT8, SMI31, SMI34 and PHF-1) AD P-tau, except that 102c also weakly

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stains untreated AD P-tau. We therefore used a phosphorylation-independent tau antibody, 92e, which recognizes both normal and AD P-tau, to monitor the mobility shift of AD P-tau after dephosphorylation by PP-2A (Fig. 8, H). This mobility shift was apparent after dephosphorylation both by PP-$2A_1$ and PP-$2A_2$ (Fig. 8, H). Similar results were also seen on immunoblots with antibody 102c (Fig. 8, A).

We have investigated the effects of a PP-2A inhibitor, okadaic acid, and protease inhibitors on the changes of relative electrophoretic mobility and of epitopes of above antibodies after treatment of AD P-tau with PP-2A. As shown in Fig. 9, incubation of AD P-tau with PP-2A in the presence of 1.0 μM okadaic acid did not unmask Tau-1 epitope (lane 3), whereas both in the presence (lane 4) and the absence (lane 2) of protease inhibitor cocktail the Tau-1 epitope was unmasked. However, protease inhibitor cocktail could block the appearance of the lowest faint band (compare lane 2 with lane 4), indicating that this band was contributed by proteolysis. Similar results were also observed by immunoblots with other antibodies (Figure not shown). This result confirmed that the epitope changes of AD P-tau resulted from dephosphorylation by PP-2A and the mobility shift of AD P-tau is mainly contributed by dephosphorylation.

Time course of dephosphorylation of AD P-tau by PP-$2A_1$ and PP-$2A_2$

To observe the relative dephosphorylation rates of each specific site of AD P-tau by PP-$2A_1$ and PP-$2A_2$, we studied the time course of site-specific dephosphorylation of AD P-tau by these two types of PP-2A. The dephosphorylation was tested by antibodies 102c, Tau-1, SMI31 and PHF-1 (Fig. 10). Both PP-$2A_1$ and PP-$2A_2$ rapidly changed the epitopes of the four antibodies above. After 18-30 min, the change of staining density on blots was almost at its maximum. No significant difference of the dephosphorylation rates was observed when either PP-$2A_1$ or PP-$2A_2$ was used. These results indicate that the PP-2A isozymes have almost the same

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activity towards phosphorylated sites Ser-46, Ser-199, Ser-202, Ser-396 and Ser-404 of AD P-tau. A mobility shift accompanying dephosphorylation was also clearly seen on immunoblots with antibody 102c (Fig. 10, A and B).

Effects of Mn^{2+}, Mg^{2+} and polylysine on dephosphorylation of AD P-tau by PP-2A$_1$ and PP-2A$_2$

It is known that PP-2A activity is affected by divalent cations and basic proteins (Ballou and Fischer, 1987, The Enzymes 17:311-361; Cohen, 1989, Annu. Rev. Biochem. 58:453-508). Mn^{2+} and polylysine can activate PP-2A. These effects on PP-2A activity are variable, depending on the substrate used. In contrast, Mg^{2+} might slightly stimulate, inhibit or have no effect on PP-2A activity, also depending on the subtypes of the enzyme as well as the substrates used. We therefore investigated the effects of Mn^{2+}, Mg^{2+} and polylysine on the dephosphorylation of AD P-tau by PP-2A$_1$ and PP-2A$_2$ (Fig. 11). In the absence of metals and polylysine (1.0 mM EDTA present), PP-2A$_2$ had very little activity towards AD P-tau (Fig. 11, B), whereas PP-2A$_1$ had some activity (Fig. 11, A). Mn^{2+} markedly stimulated the activities of both PP-2A$_1$ and PP-2A$_2$ (Fig. 11, compare C with A, and D with B). Polylysine also strongly activated PP-2A$_1$ (Fig. 11, compare G with A), but only slightly activated PP-2A$_2$ (Fig. 11, compare H with B and G). Mg^{2+} had no detectable effect on the activities of either PP-2A$_1$ or PP-2A$_2$ (Fig. 11, compare E with A, and F with B). In comparison, when [^{32}P]phosphorylase was used as a substrate, Mn^{2+} had almost no effect on PP-2A$_1$ activity, whereas polylysine slightly stimulated the activity; under similar conditions, Mn^{2+} stimulated PP-2A$_2$ much more strongly than polylysine did (data not shown). These results suggest that the modulation of AD P-tau phosphatase activity of PP-2A by Mn^{2+} and polylysine is different from that of other substrates.

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7.3. DISCUSSION

In the present study, we examined the dephosphorylation of AD P-tau by PP-2A$_1$ and PP-2A$_2$ in comparison with that by PP-2B. We found that both PP-2A and PP-2B could dephosphorylate AD P-tau, as determined by immunoblotting with phosphorylation-dependent anti-tau antibodies. Approximately twice as much PP-2A as PP-2B was needed for comparable dephosphorylation of AD P-tau.

PP-2A and PP-2B dephosphorylate different sites on AD P-tau. PP-2B can dephosphorylate AD P-tau at Ser-46, Ser-199, Ser-202, Ser-235, Ser-396 and Ser-404 (see Section 6 hereinabove), whereas PP-2A failed to dephosphorylate Ser-235 of AD P-tau. Furthermore, the rate of dephosphorylation of different sites by PP-2A was almost the same, but that by PP-2B was nonidentical (see Section 6). Interestingly, Ser-235 was more rapidly dephosphorylated by PP-2B than other sites (see Section 6), whereas this site could not be dephosphorylated by PP-2A. These results suggest that probably both PP-2A and PP-2B are involved in the dephosphorylation of AD P-tau.

We have found that PP-2A can dephosphorylate AD P-tau at Ser-46, Ser-199, Ser-202, Ser-396 and Ser-404. Using [^{32}P]phosphorylase kinase as substrate, we previously observed that the PP-2A activity of Alzheimer disease brains was lower than that of age-matched controls (Gong et al., 1993, J. Neurochem. 61:921-927). Taken together, these results suggest that a deficiency of PP-2A might contribute to the abnormal hyperphosphorylation of tau in Alzheimer disease.

PP-2A may also be associated with tau phosphorylation indirectly. Recent studies have shown that PP-2A can inactivate mitogen-activated protein (MAP) kinase (Pelech and Sanghera, 1992, Science 257:1355-1356) and cdc2 (Clarke et al., 1993, Mol. Cell. Biol. 4:397-411). These two kinases have been reported to phosphorylate tau at some of the abnormally phosphorylated sites known to be present in AD P-tau (Drewes et

al., 1992, EMBO J. 11:2131-2138; Vulliet et al., 1992, J. Biol. Chem. 267:22570-22574). Therefore, decreased PP-2A activity may result in MAP kinase and cdc2 kinase remaining in activated states for extended periods. Under these conditions, it is possible that tau may become hyperphosphorylated by these kinases.

PP-2A is present *in vivo* in two major forms, termed PP-$2A_1$ and PP-$2A_2$ (Cohen, 1989, Annu. Rev. Biochem. 58:453-508). PP-$2A_1$ generally has lower activity than PP-$2A_2$ towards a variety of substrates (Cohen, 1989, Annu. Rev. Biochem. 58:453-508), but this depends on the substrates used. Using phosphorylase as substrate to standardize PP-$2A_1$ and PP-$2A_2$ activities, we have found that they both had almost the same activities towards AD P-tau. The specificities for different sites on AD P-tau were also identical. Goedert et al. (1992, FEBS Lett. 312:95-99) have recently reported that the tau phosphatase : phosphorylase phosphatase activity ratio of PP-$2A_1$ was 7- to 8-fold higher than that of PP-$2A_2$. In their study, the tau phosphatase activity was determined by using [^{32}P]tau phosphorylated *in vitro* by MAP kinase. Comparison of their results with ours indicates that AD P-tau and MAP kinase-phosphorylated tau are different substrates for PP-2A. In fact, not all abnormal sites of AD P-tau are phosphorylated by MAP kinase (Drewes et al., 1992, EMBO J. 11:2131-21383), and non-MAP kinase sites may alter the conformation of AD P-tau in such a way that it behaves as a different substrate.

In conclusion, PP-2A can dephosphorylate AD P-tau *in vitro* at some of the abnormal sites. PP-$2A_1$ and PP-$2A_2$ have almost the same activity towards AD P-tau. The dephosphorylation of AD P-tau by PP-2A is markedly stimulated by Mn^{2+}. Regulation of tau dephosphorylation may be carried out by a combination of PP-2A and PP-2B. The deficiency of either PP-2A or PP-2B, or both, might result in abnormal hyperphosphorylation of tau in Alzheimer disease brain.

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8. EXAMPLE: DEPHOSPHORYLATION OF MICROTUBULE-ASSOCIATED PROTEIN TAU BY PROTEIN PHOSPHATASE-1 AND -2C AND ITS IMPLICATION IN ALZHEIMER DISEASE

As described herein, dephosphorylation of tau by protein phosphatase-1 and -2C was examined, using both AD P-tau and [^{32}P]tau labelled by cAMP-dependent protein kinase as substrates. Dephosphorylation of AD P-tau was monitored by its interaction with the following phosphorylation-dependent antibodies: 102c, Tau-1, SMI33, SMI31 and PHF-1. The abnormally phosphorylated sites Ser-199, Ser-202, Ser-396 and Ser-404 but not Ser-46 and Ser-235 of AD P-tau (the amino acids are numbered according to the largest isoform of human tau, tau_{441}) were found to be dephosphorylated by protein phosphatase-1 and this dephosphorylation was activated by Mn^{2+}. In contrast, protein phosphatase-2C did not dephosphorylate any of these sites. Both protein phosphatase-1 and -2C had high activities towards [^{32}P]tau phosphorylated by cAMP-dependent protein kinase. These results suggest that both protein phosphatase-1 and -2C might be associated with the normal phosphorylation state of tau, but only the former and not the latter phosphatase is involved in its abnormal phosphorylation in Alzheimer disease.

8.1. MATERIALS AND METHODS

Materials

Phosphorylase kinase was purified from rabbit skeletal muscle by the method of Cohen (1973, Eur. J. Biochem. 34:1-14). cAMP-dependent protein kinase (PKA) was purchased from Sigma, St. Louis, MO, USA. Rabbit skeletal muscle PP-1 was purchased from Upstate Biotechnology Inc., Lake Placid, NY. PP-2B (holoenzyme) was purified from bovine brain according to the method of Sharma et al. (1983, Meth. Enzymol.

102:210-219). PP-2C was purified from bovine kidney as previously described (Amick et al., 1992, Biochem. J. 287:1019-1022).

Polyclonal antibodies 102c were raised as previously reported (Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650). Monoclonal antibodies Tau-1 and PHF-1 were kindly provided by Drs. L. I. Binder (Binder et al., 1985, J. Cell Biol. 101, 1371-1378) and S. Greenberg (Greenberg et al., 1992, J. Biol. Chem. 267:564-569), respectively; SMI33, SMI31, goat anti-mouse IgG and peroxidase-anti-peroxidase complex were purchased from Sternberger Monoclonals Inc., Baltimore, MD. Alkaline phosphatase-conjugated goat anti-mouse and anti-rabbit IgG were purchased from Bio-Rad, Hercules, CA.

Isolation of tau

AD P-tau and normal human tau were isolated from autopsied brains of a 70-year-old male with Alzheimer disease and a 51-year-old male normal case, respectively (Köpke et al., 1993, J. Biol. Chem. 268:24374-24384). Briefly, AD P-tau was isolated from a non-neurofibrillary tangle pool, the 27,000 *g* to 200,000 *g* fraction of the Alzheimer brain homogenate was extracted in 8 M urea, followed by dialysis against Tris buffer. This AD P-tau is readily soluble in buffer and abnormally phosphorylated as PHF-tau (Köpke et al., 1993, J. Biol. Chem. 268:24374-24384). Normal human tau was purified according to Köpke et al. (1993, J. Biol. Chem. 268:24374-24384) from the 35 - 45% ammonium sulfate precipitates of 200,000 *g* brain supernatant, followed by acid treatment (pH 2.7) and chromatography on a phosphocellulose column (Cellulose Phosphate P11, Whatman).

Protein concentrations were determined by a modified Lowry assay (Bensadoun and Weinstein, 1976, Anal. Biochem. 70, 241-250).

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Preparation of [^{32}P]phosphorylase kinase and determination of protein phosphatase activities

[^{32}P]phosphorylase kinase (1.9 mol ^{32}P incorporated/335,000 g) phosphorylated by catalytic subunit of cAMP-dependent protein kinase was prepared as reported previously (Gong et al., 1993, J. Neurochem. 61:921-927). The activities of PP-1, PP-2B and PP-2C were measured by counting the radioactivity released from [^{32}P]substrate as previously described (Gong et al., 1993, J. Neurochem. 61:921-927). The reaction mixtures contained 50 mM Tris, pH 7.0, 20 mM ß-mecaptoethanol, 1.0 mM $MnCl_2$ and 1.0 μM [^{32}P]phosphorylase kinase for PP-1. For PP-2B and PP-2C activities, $MnCl_2$ was substituted by 1.0 mM $CaCl_2$ and 1.0 μM calmodulin, and 10 mM $MgCl_2$, respectively. One unit of protein phosphatase activity is defined as that amount which catalyzes the release of 1.0 nmol phosphate per min from [^{32}P]phosphorylase kinase at 30°C.

Treatment of AD P-tau with protein phosphatases

Unless otherwise stated, dephosphorylation of AD P-tau was carried out at 30°C in 50 mM Tris, pH 7.0, 10 mM ß-mecaptoethanol, 0.1 mg/ml BSA, 50 μg/ml AD P-tau and PP-1, PP-2B or PP-2C. In some experiments, several effectors were added in the reaction mixture (see Results section). The reaction was started by addition of the enzyme. After appropriate incubation times (see Figure legends), reactions were stopped by addition of 5 volumes of cold acetone to precipitate proteins. The precipitated protein samples were dissolved in SDS-PAGE sample buffer (60 mM Tris-HCl, pH 6.8, 3% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.05% bromophenol blue) and heated at 95°C for 4 min, followed by 10 % SDS-PAGE. Immunoblotting was carried out as described previously (Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci. USA 83:4913-4917). The primary antibodies for immunoblotting and their epitopes have all been previously characterized. They are phosphorylation-dependent as well as site-

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specific. Briefly, antibodies 102c (Iqbal et al., 1989, Proc. Natl. Acad. Sci. USA 86:5646-5650), Tau-1 (Grundke-Iqbal et al., 1986, Proc. Natl. Acad. Sci. USA 83:4913-4917; Biernat et al., 1992, EMBO J. 11, 1593-1597; Szendrei et al., 1993, J. Neurosci. Res. 34:243-249) and SMI33 (Lichtenberg-Kraag et al., 1992, Proc. Natl. Acad. Sci. USA 89:5384-5388) recognize dephosphorylated form of tau at sites Ser-46, Ser-199/Ser-202 and Ser-235, respectively. Antibodies SMI31 (Lichtenberg-Kraag et al., 1992, Proc. Natl. Acad. Sci. USA 89:5384-5388) and PHF-1 (Lang et al., 1992, Biochem. Biophys. Res. Commun. 187:783-790) recognize tau phosphorylated at Ser-396/Ser-404 and Ser-396, respectively. These antibodies were used at dilutions of 0.4 μg/ml for 102c, 1:100 for SMI31, 1:500 for SMI33 and PHF-1, and 1:500,000 for Tau-1.

Preparation of [^{32}P]tau and dephosphorylation of [^{32}P]tau by protein phosphatases

Tau purified from normal human brain was phosphorylated with [^{32}P]ATP by PKA as described by Scott et al. (1993, J. Biol. Chem. 268:1166-1173). About 2 mol ^{32}P/mol tau was incorporated by PKA. Dephosphorylation of [^{32}P]tau by PP-1, PP-2A and PP-2B was carried out employing the same conditions as when AD P-tau was used as a substrate. The phosphatase activities were measured by counting the radioactivity released from [^{32}P]tau as previously described (Gong et al., 1993, J. Neurochem. 61:921-927).

8.2. RESULTS

Definition of PP-1 and PP-2C activities and their modulation by Mn^{2+} and Mg^{2+}

To observe and compare the potential dephosphorylation of AD P-tau by various protein phosphatases, the same amount of enzyme activity of

each phosphatase should be used. Hence we employed [^{32}P]phosphorylase kinase as a substrate to standardize the activities of PP-1 and PP-2C. PP-1 and PP-2C activities are modulated by cations and each enzyme preparation responds differently to divalent cations (Ballou and Fisher, 1987, The Enzymes 17:311-361). We therefore determined PP-1 and PP-2C activities in the absence and presence of either Mn^{2+} or Mg^{2+} using [^{32}P]phosphorylase kinase as a substrate. As shown in Fig. 12, PP-1 was activated by 1.0 mM Mn^{2+} but inhibited by 10 mM Mg^{2+}. PP-2C was Mg^{2+}- or Mn^{2+}-dependent, and no activity was detected in the absence of Mg^{2+} or Mn^{2+}. Highest activities were obtained using 1.0 mM Mn^{2+} for PP-1 and 10 mM Mg^{2+} for PP-2C. Hence these conditions were used to study the *in vitro* dephosphorylation of AD P-tau and PKA-phosphorylated tau.

Treatment of AD P-tau with PP-1 and PP-2C

We have previously shown that PP-2A dephosphorylated abnormal phosphorylation sites Ser-46, Ser-199, Ser-202, Ser-396 and Ser-404 of AD P-tau (see *supra*), and that in addition to above sites, PP-2B also dephosphorylated another abnormal phosphorylation site, Ser-235 (see *supra*). In the present study, using immunoblots with five site-specific phosphorylation-dependent antibodies which recognize six abnormal phosphorylation sites of AD P-tau, we have further examined whether PP-1 and PP-2C can also dephosphorylate these abnormal phosphorylation sites at optimum *in vitro* conditions. PP-2B was employed as a positive control. We found that PP-1 unmasked the epitope of antibody Tau-1 and blocked the epitopes of antibodies SMI31 and PHF-1, but failed to unblock the epitopes of antibodies 102c and SMI33 (Fig. 13). PP-2C did not change any of these epitopes. These results indicate that PP-1 dephosphorylates abnormal phosphorylation sites Ser-199, Ser-202, Ser-396 and Ser-404 but not Ser-46 and Ser-235 of AD P-tau. Whereas PP-2C had no effect on dephosphorylation of above sites.

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The rate of dephosphorylation of Ser-199/Ser-202, Ser-396/Ser-404 and Ser-396 of AD P-tau by PP-1 was determined using immunoblots with Tau-1, SMI31 and PHF-1, respectively. The time course showed a rapid change of epitopes of AD P-tau towards these three antibodies (Fig. 14). Within 20 - 30 min incubation of AD P-tau with PP-1, staining of Tau-1 became maximal and those of both PHF-1 and SMI31 disappeared completely. These results suggested that the four phosphorylation sites of AD P-tau can be easily hydrolyzed by PP-1.

We have also investigated the dephosphorylation of AD P-tau at various conditions (Fig. 15). In the absence of metal (1.0 mM EDTA present), PP-1 could also dephosphorylate AD P-tau at Ser-396 but the activity was low. Dephosphorylation of AD P-tau by PP-1 was strongly activated by 1.0 mM Mn^{2+} but inhibited by 10 mM Mg^{2+}. We further investigated the required concentration of Mn^{2+} for this activation. The activation was observed at 10 μM Mn^{2+} and it reached maximum at about 100 μM Mn^{2+} (data not shown).

Dephosphorylation of [^{32}P]tau by PP-1 and PP-2C

Tau protein is known to be phosphorylated *in vitro* at Ser-214, Ser-324, Ser-356, Ser-409 and Ser-416 by PKA (Scott et al., 1993, J. Biol. Chem. 268:1166-1173). So far none of these sites have been reported to be abnormally phosphorylated in Alzheimer disease brain, but they may be involved in normal phosphorylation of tau. We therefore asked whether these non-abnormal phosphorylation sites of tau can be dephosphorylated by either PP-1 and PP-2C. Interestingly, even though PP-1, PP-2B and PP-2C had obviously different effects on dephosphorylation of abnormal phosphorylation sites of AD P-tau, they had similar high activities towards [^{32}P]tau phosphorylated by PKA (Fig. 16).

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8.3. DISCUSSION

We have found that PP-2A and PP-2B rapidly dephosphorylated AD P-tau *in vitro* (Sections 6, 7 *supra*). The present study shows that PP-1 also dephosphorylates AD P-tau at some of the sites whereas PP-2C has no activity towards any of the sites studied. Although three of four types of protein phosphatases can dephosphorylate AD P-tau, they have different site specificities. Six of nine known abnormal phosphorylation sites of AD P-tau have been examined in these studies. They are Ser-46, Ser-199, Ser-202, Ser-235, Ser-396 and Ser-404. All of them can be dephosphorylated by PP-2B; PP-2A can dephosphorylate all except S-235; and PP-1 dephosphorylates Ser-199, Ser-202, Ser-396 and Ser-404 but neither Ser-46 nor Ser-235. Hence at least four abnormal phosphorylation sites, Ser-199, Ser-202, Ser-396 and Ser-404, can be dephosphorylated by the three enzymes, PP-1, PP-2A and PP-2B. These results indicate that the regulation of phosphorylation level of tau is very complex and more than one protein phosphatase might be involved in hyperphosphorylation of tau in AD.

When [^{32}P]phosphorylase kinase was used as a substrate to determine protein phosphatase activities, PP-1 activity was found about 20-fold higher than PP-2C activity in human brain extracts (Gong et al., 1993, J. Neurochem. 61:921-927). In this study, also using [^{32}P]phosphorylase kinase as a substrate to define the activities of both PP-1 and PP-2C, 1.0 unit/ml of PP-1 almost completely dephosphorylated Ser-199, Ser-202, Ser-396 and Ser-404 of AD P-tau in 20 min, but 2.0 units/ml of PP-2C did not dephosphorylate AD P-tau at any sites studied at the optimal *in vitro* conditions. Taken together, these results suggest that AD P-tau is not a substrate for PP-2C.

Tau isolated from adult brain normally contains 2 - 3 moles of phosphate per mole of the protein (Selden and Pollard, 1983, J. Biol. Chem. 258:7064-7071; Ksiezak-Reding et al., 1992, Brain Res. 597:209-219; Köpke et al., 1993, J. Biol. Chem. 268:24374-24384). However, neither the

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phosphorylation sites nor the responsive kinase(s) have yet been fully elucidated. Normal tau might be partially phosphorylated at Ser-202 and Ser-404 (Arioka et al., 1993, J. Neurochem. 60:461-468; Poulter et al., 1993, J. Biol. Chem. 268:9636-9644), but to date other sites have not been excluded to be phosphorylated. PKA was known to phosphorylate tau at Ser-214, Ser-234, Ser-356, Ser-409 and Ser-416 (Scott et al., 1993, J. Biol. Chem. 268:1166-1173). PP-2C as well as PP-1 and PP-2B can release about 80% radioactivity from PKA-phosphorylated [^{32}P]tau in 60 min, suggesting that these phosphatases dephosphorylate most of these phosphorylation sites of tau. Therefore, even if it is not involved in abnormal phosphorylation of AD P-tau, PP-2C may be associated with the regulation of phosphorylation level of normal tau.

The present study also shows that PKA-phosphorylated tau and AD P-tau serve as different substrates for protein phosphatases. AD P-tau can be dephosphorylated by PP-1, PP-2A and PP-2B but not by PP-2C, whereas PKA-phosphorylated tau is almost an equally good substrate for PP-1, PP-2B, and PP-2C. The completely different behavior of AD P-tau and PKA-phosphorylated tau as a substrate for PP-2C may be due to different phosphorylation sites and/or due to different protein conformations of the *in vitro*-phosphorylated vs. the pathological AD P-tau. The pathological conformation of AD P-tau might make tau easily polymerize into PHF in Alzheimer brain. The results of this study also suggest that caution ought to be used when interpreting data obtained employing [^{32}P]tau as a model substrate to study potential protein phosphatase(s) involved in the pathogenesis of AD. This is important, especially because so far no protein kinase including mitogen-activated protein kinase has been reported to phosphorylate tau at all the known abnormal phosphorylation sites. These different phosphorylation sites between ^{32}P-labelled tau and AD P-tau could result in different conformations so that ^{32}P-labelled tau and AD P-tau serve as different substrates for protein phosphatases.

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9. EXAMPLE: ROLE OF ABNORMALLY PHOSPHORYLATED TAU IN THE BREAKDOWN OF MICROTUBULES IN ALZHEIMER DISEASE

As described herein, to understand the role of the abnormal phosphorylation of tau in microtubule disruption in AD brain, we studied the ability of the normal cytosolic, and the AD P-tau to bind to tubulin and to promote microtubule assembly and investigated the effect of alkaline phosphatase treatment of tau on microtubule assembly. Tau isolated from a 2.5% perchloric extract of AD brain had almost the same activity as that obtained from control brain, and this activity did not change significantly on dephosphorylation. Abnormally phosphorylated tau (AD P-tau) isolated from brain homogenate of AD cases had little activity, and on dephosphorylation with alkaline phosphatase, its activity increased to approximately the same level as the acid-soluble tau. Addition of AD P-tau to a mixture of normal tau and tubulin inhibited microtubule assembly. AD P-tau bound to normal tau but not to tubulin. These studies suggest that the abnormal phosphorylation of tau might be responsible for the breakdown of microtubule in affected neurons in AD not only because the altered protein has little microtubule-promoting activity, but also because it interacts with normal tau, making the latter unavailable for promoting the assembly of tubulin into microtubules.

9.1. MATERIALS AND METHODS

Tissue Source and Preparation of Brain Cytosol. Six brains with histopathologically confirmed AD diagnosis and, as a control, six Huntington disease brains obtained between 3 to 5 h postmortem and stored frozen at −75°C were used. Cytosol was obtained by centrifugation (100,000 x g for 1 h) of frontal cortex homogenate (1 g/0.5 ml) in microtubule assembly buffer (see below) containing protease inhibitors (Köpke et al., 1993, J. Biol. Chem. 268:24374-24384).

Antibodies. Monoclonal antibody (mAb) Tau-1, ascites (Binder et al., 1985, J. Cell Biol. 101:1371-1378), and antiserum 92e to bovine tau (Grundke-Iqbal

et al., 1988, J. Mol. Brain Res. 4:43-52) were used at a dilution of 1:25,000 and 1:5,000, respectively; mAb DM1-A (Sigma, St. Louis, MO) against tubulin was used at a 1:1,000 dilution.

Isolation of AD P-tau and Acid-soluble Tau. AD P-tau was isolated by the method of Köpke et al. (1993, J. Biol. Chem. 268:24374-24384). For acid-soluble tau AD and control brains were homogenized with an Omnimixer at 4°C in 2% perchloric acid (10 ml/ g of tissue) containing protease inhibitors, as described previously (Köpke et al., 1993, J. Biol. Chem. 268:24374-24384). The homogenates were spun at 100,000 x g for 30 min. The supernatant was brought to 2.5% perchloric acid and centrifuged again for another 30 min. The supernatant was concentrated approximately 10 times by Amicon filtration and dialyzed against 20 mM sodium acetate buffer, pH 5.6. After dialysis, the extract was spun for 10 min at 100,000 x g, and the supernatant was subjected to carboxyl methyl chromatography using Millipore Mem Sep CM 1010 disk (Millipore, Bedford, MA). The protein sample (25-40 mg/ 50 ml) was loaded at a flow rate of 0.5 ml/min, and tau was eluted with 0.25 M NaCl in 20 mM sodium acetate buffer, pH 5.6. The eluate was analyzed by absorbance at 254 nm and immunoslot blot using antiserum 92e to tau. The tau peak was pooled and dialyzed against 5 mM MES buffer, pH 6.7, containing 0.05 mM EGTA. Aliquots of approximately 500 μl, containing 120 μg of protein, were dried in a Speed Vac concentrator (Savant, Farmingdale, NY). For each assay, the lyophilized tau preparation was reconstituted in 1/10 vol. water immediately before use.

Protein Determination, Immunoblots, Radioimmuno-slot-blot, and Dephosphorylation. Protein concentrations were estimated by the method of Bensadoun and Weinstein (Bensadoun and Weinstein, 1976, Anal. Biochem. 70:241-250). Sample preparation and immunoblots were carried out as described previously (Grundke-Iqbal et al., 1984, Acta Neuropathol. (Berl.) 62:259-267). The levels of normal and AD P-tau were determined by the radioimmuno-slot-blot method of Khatoon et al. (1992, J. Neurochem.

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59:750-753). To detect AD P-tau, the blots were pretreated with alkaline phosphatase, 86 μg/ml in 0.1 M Tris, pH 8.0, and 1 mM phenylmethylsulfonyl fluoride for 15 h prior to immunostaining with mAb Tau-1.

Isolation of Tubulin. Rat brain tubulin was isolated through two temperature-dependent cycles of microtubule polymerization-depolymerization (Shelanski et al., 1973, Proc. Natl. Acad. Sci. USA 70:765-768) followed by phosphocellulose ion-exchange column chromatography (Sloboda and Rosenbaum, 1979, Biochemistry 18:48-55).

Microtubule Assembly-disassembly Assay. Tau (0.1 - 0.4 mg/ml) was mixed at 4°C with purified rat brain tubulin (2 mg/ml) and 1 mM GTP, all in polymerization buffer (100 mM MES, pH 6.7, 1 mM EGTA and 1 mM $MgCl_2$). Tau was added last to initiate the reaction. After rapid mixing, the samples were pipetted into quartz microcuvettes and equilibrated at 37°C in a thermostatically-controlled Cary 1 recording spectrophotometer. Solution turbidity was continuously monitored at 350 nm. Steady state values were determined by measuring the total absorbance change after a turbidity plateau was reached. For the disassembly assay, after the steady state was reached, the reaction mixture was cooled to 6°C, and the turbidity was monitored. The state of assembly of microtubules was confirmed by negative stain electron microscopy (Wisniewski et al., 1984, J. Neuropathol. Exper. Neurol. 43:643-656).

In vitro dephosphorylation of tau was carried out by using the following conditions: acid-soluble tau from AD and control brains, and AD P-tau from AD brains were reconstituted with water to a final protein concentration of 0.1-0.2 mg/ml and then dialyzed against 0.1 M Tris, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride. The dialyzed samples were treated with alkaline phosphatase (500 Units/ml) and a mixture of protease inhibitors (10 μM leupeptin, 0.31 μM aprotinin, and 1.46 μM pepstatin) at 37°C for 15 h. After this incubation, samples were dialyzed against 5 mM

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MES, pH 6.7, containing 0.05 mM EGTA, boiled for 5 min to inactivate the alkaline phosphatase, and then centrifuged at 15,000 x g for 10 min. The phosphatase-control samples were treated identically, except that alkaline phosphatase was omitted and the samples were kept at 4°C. Unless otherwise stated, all steps were carried out at 4°C.

Dot Overlay Assay. AD P-tau interaction was carried out as described by Kremer et al. (1988, Anal. Biochem. 175:91-95). Different amounts (0.5, 1, 2 and 3 μg) of AD P-tau were dotted on nitrocellulose paper and overlaid with either normal human tau (8 μg/ml) or tubulin (10 μg/ml). All the incubations, blocking, and washing were done as previously described (Kremer et al., 1988, Anal. Biochem. 175:91-95). Tau was detected by using Tau-1 antibody, whereas DM1-A antibody was used for tubulin.

9.2. RESULTS

Microtubule Assembly-promoting Activity of 2.5% Perchloric Acid-Soluble Tau From AD and Control Brains Is Similar. Acid-soluble tau was isolated from 6 AD and 6 control brains. No AD P-tau was detected, and the pattern of tau isotypes was very similar in all the preparations (Fig. 17), although the yield of tau from AD brains was approximately 30% lower than that from the control brains (AD brains: 0.020 $\pm$ 0.004 mg/ g of tissue; control brains: 0.029 $\pm$ 0.002 mg/ g of tissue). The total protein composition of the tau preparations was also very similar (data not shown). The microtubule assembly-promoting activity of AD acid-soluble tau was not significantly different from that of control tau, as determined by the amount of microtubules formed at steady state and the rates of assembly and disassembly (Table 4). Furthermore, no ultrastructural differences, either in length or appearance, were detected between the microtubules obtained with the two tau preparations (Fig. 18a and b).

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TABLE 4*

Microtubule Assembly-Promoting and Stabilizing Activities of Cytosolic Acid-Soluble Tau from AD and Control Brain

	Alzheimer	Control
Assembly (%)	97 ± 14 (6)[a]	100 ± 16 (6)
Rate of assembly (min^{-1})	0.10 ± 0.02 (5)	0.10 ± 0.02 (6)
Rate of disassembly (min^{-1})	0.19 ± 0.06 (5)	0.15 ± 0.03 (6)

* The assembly assay was performed using fresh rat tubulin and AD or control acid-soluble tau. Values of OD (0.100 to 0.400) at steady state of polymerization were normalized by using those obtained with the control preparations as 100%. The rate of assembly was calculated from the initial slope. For the rate of disassembly, the temperature was set at 6°C, and the turbidimetric changes were recorded at 350 nm. The rate was calculated from the slope of disassembly. Both rates were determined by using the same amount of tau.

[a]Mean ± SD of cases studied in four different experiments carried out with three different concentrations of tau (0.1, 0.2, and 0.4 mg/ml). None of the differences between AD and control groups were statistically significant.

Dephosphorylation Increases the Microtubule Assembly-promoting Activity of AD P-tau but Not That of AD Acid-Soluble Tau. *In vitro* phosphorylation of tau diminishes its ability to promote the assembly of tubulin into microtubules (Lindwall and Cole, 1984, J. Biol. Chem. 259:5301-5305). Experiments were performed to determine whether dephosphorylation of AD acid-soluble tau and AD P-tau affects this property.

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The amount of the AD P-tau in the acid-soluble preparations was undetectable, as judged by the increase of Tau-1 immunoreactivity after dephosphorylation on Western blots (Fig. 17) and by immuno-slot-blot assay (data not shown). In contrast, the AD P-tau was labeled intensely with Tau-1 on immunoblots treated with alkaline phosphatase and was hardly detectable before dephosphorylation (Fig. 17). The dephosphorylation treatment had no effect on the microtubule assembly-promoting activity of AD acid-soluble tau, whereas it increased markedly the activity of AD P-tau, bringing it to approximately the same level as that obtained with the acid-soluble tau (Fig. 19). Before alkaline phosphatase treatment, only an occasional microtubule could be seen by electron microscopy (Fig. 18c). After the alkaline phosphatase treatment, many microtubules with no ultrastructural differences from those formed with AD acid-soluble tau were observed (Fig. 18d).

AD Cytosolic Fraction Is Able to Promote Microtubule Assembly. The effect of dephosphorylation of tau on microtubule assembly was also studied in brain cytosol. The concentration of normal tau in AD brain cytosols was approximately 65% of the corresponding value in the control cases (Table 5).

TABLE 5

Tau Levels and the Effect of Dephosphorylation on the Microtubule Promoting Activity of AD and Control (Ct) Brain Cytosols

	[a]N tau (cpm)	[b]P-tau (%)	[c]Assemb. (cpm)	[d]Inc. Assemb. (%)
AD(6)	1910 ± 458[f]	77 ± 53[g]	3800 ± 1300[h]	70 ± 29[i]
Ct(6)	2923 ± 649[f]	1.8 ± 7.5[g]	6330 ± 1134[h]	2.2 ± 9.7[i]

[a]The concentration of normal tau (N tau) and [b]abnormally phosphorylated tau (P-tau) were determined by radioimmunoassay (Khatoon et al., 1992, J. Neurochem. 59:750-753). The values of N tau are expressed as

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the amount of radioactivity bound per μg of cytosol protein; P-tau values are expressed as % of N tau.

[c]Fresh rat brain tubulin was incubated for 20 min at 37°C with cytosolic extracts from control and AD brains in the polymerization buffer. The samples were then centrifuged at 100,000 x g for 30 min at 37°C, and the pellets were dissolved in 50 mM MES buffer, kept in ice for 30 min, and centrifuged at 100,000 x g at 4°C, and the amounts of tubulin present in these supernatants were assayed by radioimmuno-slot-blot using DM1-A tubulin antibody. The amount of assembly is expressed as cpm.

[d]The increase in the amount of tubulin assembly with the alkaline phosphatase-treated cytosols was calculated by taking the value obtained without dephosphorylation as 100%.

[f]$p<0.01$; [g]$p<0.004$; [h]$p<0.006$; [i]$p<0.004$; using the nonparametric Mann-Whitney U test.

Because it is well known that tubulin in frozen tissue loses its ability to polymerize, we added fresh rat brain tubulin to a cytosolic fraction of either AD or control brain and assayed polymerization of tubulin; the concentration of tau in both cytosols was adjusted to the same level by dilution with the buffer. A high background resulting from the use of cytosol did not allow a reliable measure of turbidimetric changes, and therefore, the polymerization of tubulin was measured by immuno-assaying the amount of the cold-disassembled protein following the assembly at 37°C for 20 minutes. The cytosolic fraction of AD brain was effective in promoting microtubule assembly, although this activity was approximately 60% less than that of the control cytosolic fraction, as judged by the amount of tubulin in the cold-disassembled fraction obtained after the incubation (Table 5).

Dephosphorylation with alkaline phosphatase treatment dramatically increased the microtubule assembly-promoting activity of AD

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cytosols, but this increase was negligible in control cases (Table 5). The microtubules obtained with both preparations were of similar length and appearance (figure not shown).

AD P-tau Inhibits Microtubule Assembly and Binds to Normal Tau. Because even after adjusting the normal tau levels to control values the AD brain cytosol was significantly less (*i.e.*, 40%) active than the corresponding control fraction in promoting assembly of microtubules (Table 3), we investigated further whether and how AD P-tau inhibited the activity of normal tau. Different concentrations of AD P-tau were added to normal tau before it was mixed with tubulin, and the assembly was determined as described above. AD P-tau inhibited microtubule assembly, and this inhibition was almost total when the concentration of AD P-tau was two times that of normal tau (Fig. 20).

To study if the inhibition observed with AD P-tau was caused by its interaction with tubulin or normal tau, protein-binding studies were carried out by an overlay dot assay. AD P-tau was dotted on a nitrocellulose paper and overlaid either with tubulin or normal tau, followed by an incubation with anti-tubulin antibody or Tau-1 antibody. In the case of the strip overlaid with tubulin, there was no detectable binding, whereas there was a considerable binding of normal human tau to AD P-tau (Fig. 21). In these assays, tubulin was bound to normal tau when it was dotted (Fig. 21, inset), and no binding was observed when bovine serum albumin was used as a negative control (figure not shown). These studies suggest that AD P-tau inhibits the microtubule assembly probably through its interaction with normal tau, and not with tubulin.

Levels of Sedimentable Tau Correlate with Levels of AD P-tau. Previously, we have shown that some of the tau that is non-phosphorylated at Tau-1 epitope sediments at 200,000 x g and the levels of this sedimentable tau

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are markedly higher in AD than in control brains (Köpke et al., 1993, J. Biol. Chem. 268:24374-24384). In light of our findings in the present study on the binding of AD P-tau to normal tau, we investigated whether the 27,000 x g to 200,000 x g fraction where the soluble AD P-tau sediments also contains proportionally higher levels of the non-hyperphosphorylated tau. We determined the levels of the non-hyperphosphorylated tau and AD P-tau in the 27,000 x g - 200,000 x g fraction and tau in 200,000 x g supernatants from four AD cases. We also carried out the above studies on four control cases. The levels of the non-hyperphosphorylated tau showed a direct correlation with the levels of AD P-tau in the 27,000 to 200,000 x g fraction, whereas the levels of tau in the 200,000 x g supernatant had an inverse correlation. Therefore, the ratio of the sedimentable non-hyperphosphorylated tau to tau in the supernatant directly and strongly correlated with the amount of AD P-tau in the 27,000 to 200,000 x g fraction (Fig. 22). The control brains did not contain any detectable levels of the abnormally phosphorylated tau and had only background levels of tau in the 27,000 x g to 200,000 x g fraction. These findings are consistent with the studies in the previous section showing that AD P-tau binds to normal tau.

9.3. DISCUSSION

In the present study, we have investigated a cause and a mechanism of this breakdown of the microtubule system in neurons with NFT of PHF in AD brains. We have found (i) that the abnormally phosphorylated tau is functionally inactive in binding to tubulin and stimulating the assembly of microtubules, (ii) that the microtubule assembly-promoting activity of the abnormal tau is restored by dephosphorylation, (iii) that levels of normal/functional tau in brain cytosol of AD cases are approximately 35% lower than those in non-AD control cases, (iv) that the abnormally phosphorylated tau inhibits tau-promoted assembly of tubulin into

microtubules, and (v) that the abnormal tau binds to normal tau and not to tubulin, suggesting that it inhibits the assembly by interacting with normal tau.

In AD brains, the levels of tau are several-fold higher than in age-matched control brains, and this increase is in the form of the abnormally phosphorylated protein (Khatoon et al., 1992, J. Neurochem. 59:750-753). However, in the present study, when tau was isolated from AD brains with 2.5% $HClO_4$ extraction, only non-abnormally phosphorylated tau was obtained. AD P-tau is probably denatured by 2.5% $HClO_4$ treatment and is not extracted. This finding is in agreement with our previous observations (Köpke et al., 1993, J. Biol. Chem. 268:24374-24384). Although the yield of the acid-soluble tau from AD brains was approximately 70% of that of the control cases, the microtubule-promoting activity of this tau was not significantly different from that of control tau, both in the total amount of microtubules formed and the rates of assembly and disassembly.

On the other hand, AD P-tau isolated from AD cases showed minimal, if any, microtubule-promoting activity. When this tau was dephosphorylated, the activity increased to approximately the same level as occurs with the acid-soluble tau. These results suggest that the abnormal phosphorylation of tau diminishes its microtubule-promoting activity, which can be recovered after dephosphorylation. Dephosphorylation of AD brain cytosol with alkaline phosphatase led to an increase in the microtubule-promoting activity, suggesting that AD P-tau in the extract also could be reactivated by dephosphorylation. However, in a brain cytosolic extract tau is not the only protein that can promote microtubule assembly; microtubule associated protein 2(MAP 2) might also be present, and its activity is also modulated by its degree of phosphorylation. Thus, the increase in microtubule assembly obtained with the dephosphorylated cytosol cannot rule out the involvement of proteins in addition to tau. Recovery of tau activity by dephosphorylation was also obtained with PHF-tau by Iqbal et al. (1991, J. Neuropathol. Exp. Neurol. 50:316 (Abstract)) and Bramblett et al. (1993,

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Neuron 10:1089-1099); the latter study, however, employed the binding of tau to taxol-stabilized microtubules, and not the microtubule assembly promoting activity.

Because AD P-tau has minimal microtubule-promoting activity, this protein did not contribute to the assembly-promoting activity found when AD cytosol extracts were used, although AD P-tau is present there in considerable amounts. Furthermore, we suspected that the altered protein could be inhibiting the assembly because the levels of microtubules formed with AD extracts were lower than those formed with control extracts. The putative inhibitory effect of AD P-tau was confirmed in a system of purified tubulin and normal tau in which AD P-tau inhibited the tau-promoted assembly of tubulin. This inhibitory effect of AD P-tau might be the reason for the low level of polymerization found with AD cytosolic extract.

We studied the mechanism by which AD P-tau might be inhibiting the microtubule assembly, testing the interactions between AD P-tau, normal tau, and tubulin. We found that AD P-tau was able to bind normal tau and not tubulin. These results indicate that the inhibition of microtubule assembly might be caused by an interaction of AD P-tau with normal tau in the purified system. It is also possible that the inhibition seen in the assembly with the AD cytosolic extracts is the result of an interaction of AD P-tau with normal tau. This possibility is supported by the findings of Iqbal et al. (1986, The Lancet 421:426), who were able to see polymerization of tubulin in AD extracts when they replaced tau with DEAE-dextran, showing that in AD brains tubulin is not compromised and is able to polymerize.

In conclusion, the present study suggests that the abnormal phosphorylation of tau probably causes microtubule disruption by decreasing the levels of functional tau in two ways: (i) directly, by diminishing its microtubule-promoting activity and (ii) indirectly, by binding to normal tau and making it unavailable for promoting microtubule assembly.

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Dephosphorylation restores this tau functional deficit. It appears that avoiding the hyperphosphorylation of tau can result in the prevention of microtubule disruption in neurons with neurofibrillary degeneration in AD.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

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WHAT IS CLAIMED IS:

1. A method of treating a subject having a disease or disorder associated with the presence of neurofibrillary tangles comprising administering to the subject a therapeutically effective amount of a composition comprising a molecule, which molecule increases the activity of at least one protein phosphatase towards abnormal hyperphosphorylated tau, which protein phosphatase is selected from the group consisting of PP-2A, PP-2B, PP-1, and related protein phosphatases.

2. The method according to claim 1 in which the disease or disorder is Alzheimer disease.

3. The method according to claim 1 in which the molecule is selected from the group consisting of manganese, calcium, and polylysine.

4. The method according to claim 2 in which the molecule is selected from the group consisting of manganese, calcium, and polylysine.

5. The method according to claim 3 in which the molecule is manganese, in salt, conjugate, or ionic form, with the proviso that the molecule is not manganese pyruvate or a manganese chelate of an alkylamino-ester of phosphoric acid.

6. The method according to claim 4 in which the molecule is manganese, in salt, conjugate, or ionic form, with the proviso that the molecule is not manganese pyruvate or a manganese chelate of an alkylamino-ester of phosphoric acid.

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7. The method according to claim 4 in which the molecule is selected from the group consisting of manganese chloride, manganese sulfate, manganese acetate, manganese gluconate, manganese lactate and manganese citrate.

8. The method according to claim 7 in which said administering is oral.

9. The method according to claim 1 in which the protein phosphatase is PP-2B.

10. The method according to claim 1 in which the protein phosphatase is PP-2A.

11. The method according to claim 1 in which the protein phosphatase is PP-1.

12. The method according to claim 1 in which the protein phosphatase is PP-2B, PP-2A, and PP-1.

13. The method according to claim 1 or 2 in which the subject is human.

14. The method according to claim 6 or 7 in which the subject is human.

15. A method of treating a subject having a disease or disorder associated with the presence of neurofibrillary tangles comprising administering to the subject a therapeutically effective amount of a composition comprising at least one protein phosphatase which

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dephosphorylates abnormal hyperphosphorylated tau, which protein phosphatase is selected from the group consisting of PP-2A, PP-2B, PP-1, and related protein phosphatases.

16. The method according to claim 15 in which the disease or disorder is Alzheimer disease.

17. The method according to claim 15 in which the phosphatase is PP-2B.

18. The method according to claim 16 in which the phosphatase is PP-2B.

19. The method according to claim 15 in which the phosphatase is PP-2A.

20. The method according to claim 16 in which the phosphatase is PP-2A.

21. The method according to claim 15 in which the phosphatase is PP-1.

22. The method according to claim 16 in which the phosphatase is PP-1.

23. The method according to claim 16 in which the composition comprises PP-2B, PP-2A, and PP-1.

24. The method according to claim 15 or 16 in which the subject is human.

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25. A method of treating a subject having a disease or disorder associated with the presence of neurofibrillary tangles comprising administering to the subject a therapeutically effective amount of a composition comprising a nucleic acid encoding a protein phosphatase which dephosphorylates abnormal hyperphosphorylated tau, which protein phosphatase is selected from the group consisting of PP-2A, PP-2B, PP-1, and related protein phosphatases.

26. The method according to claim 25 in which the disease or disorder is Alzheimer disease.

27. The method according to claim 25 in which the phosphatase is PP-2B.

28. The method according to claim 26 in which the phosphatase is PP-2B.

29. The method according to claim 25 in which the phosphatase is PP-2A.

30. The method according to claim 26 in which the phosphatase is PP-2A.

31. The method according to claim 25 in which the phosphatase is PP-1.

32. The method according to claim 26 in which the phosphatase is PP-1.

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33. The method according to claim 25 or 26 in which the subject is human.

34. A pharmaceutical composition comprising a therapeutically effective amount of a phosphatase which dephosphorylates abnormal hyperphosphorylated tau, and a pharmaceutically acceptable carrier; in which the protein phosphatase is selected from the group consisting of PP-2A, PP-2B, PP-1, and related protein phosphatases.

35. The composition of claim 34 in which the phosphatase is PP-2B.

36. The composition of claim 34 in which the phosphatase is PP-2A.

37. The composition of claim 34 in which the phosphatase is PP-1.

38. The composition of claim 34 in which the composition comprises PP-2B, PP-2A, and PP-1.

39. A pharmaceutical composition comprising a therapeutically effective amount of a nucleic acid encoding a phosphatase which dephosphorylates abnormal hyperphosphorylated tau, and a pharmaceutically acceptable carrier; in which the protein phosphatase is selected from the group consisting of PP-2A, PP-2B, PP-1, and related protein phosphatases.

40. The composition of claim 39 in which the phosphatase is PP-2B.

41. The composition of claim 39 in which the phosphatase is PP-2A.

42. The composition of claim 39 in which the phosphatase is PP-1.

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43. A pharmaceutical composition comprising a therapeutically effective amount of a manganese ion, manganese salt, or manganese conjugate, in a slow-release formulation.

44. A method of diagnosing the presence in a subject of a disease or disorder associated with the presence of neurofibrillary tangles comprising:

(a) contacting a sample derived from the subject with an antibody to a phosphorylated epitope of abnormal hyperphosphorylated tau under conditions such that immunospecific binding can occur; and

(b) detecting or measuring the amount of any immunospecific binding which occurs of a component in the sample to the antibody,

in which increased levels of immunospecific binding relative to the level of immunospecific binding which occurs in a subject not having the disease or disorder, indicates the presence of the disease or disorder in the subject.

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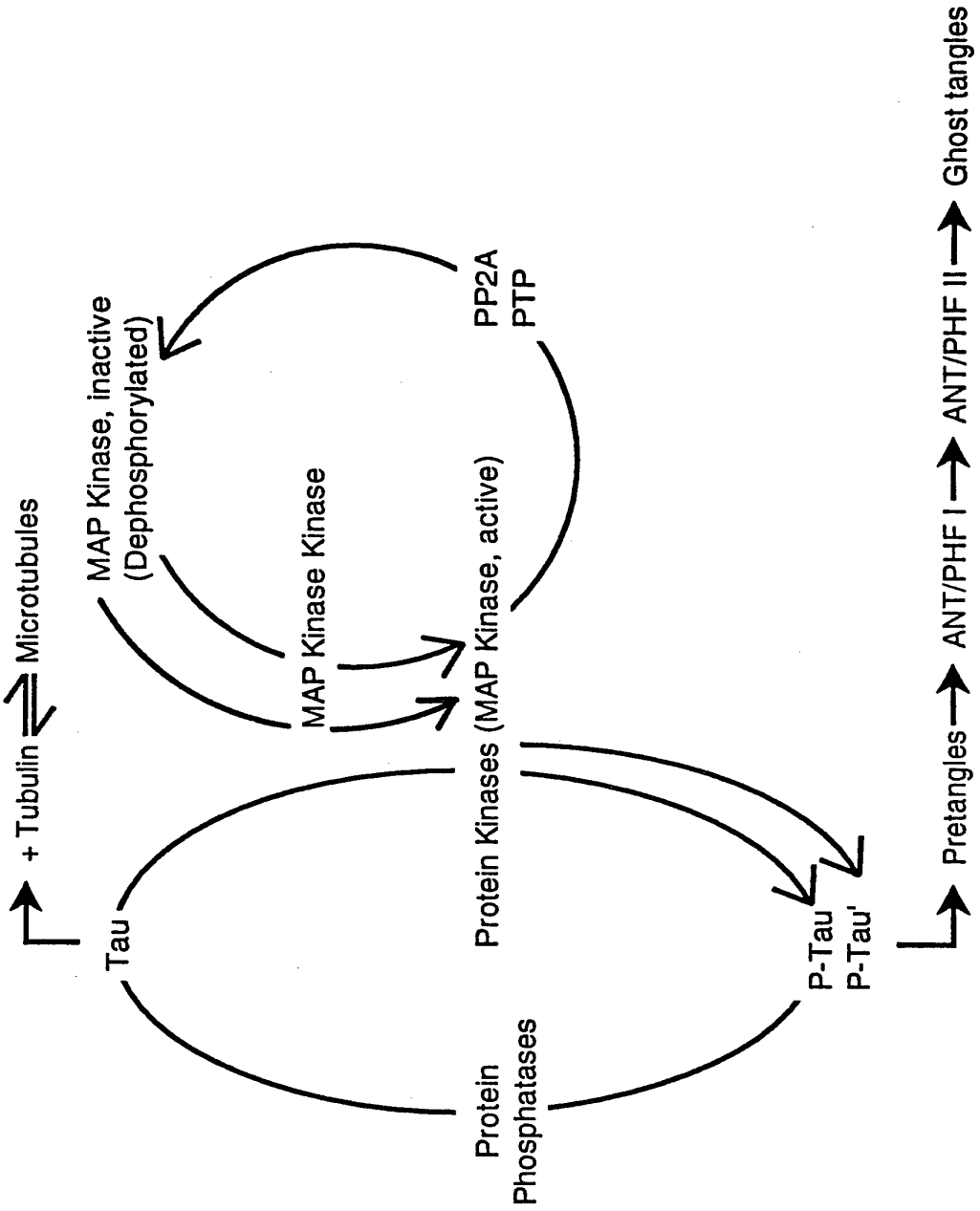


FIG. 1

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FIG.2A

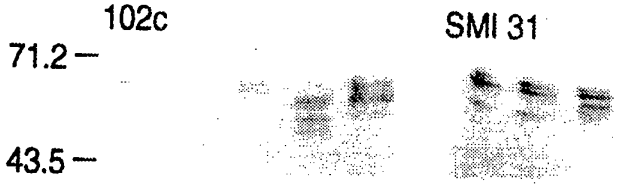


FIG.2B

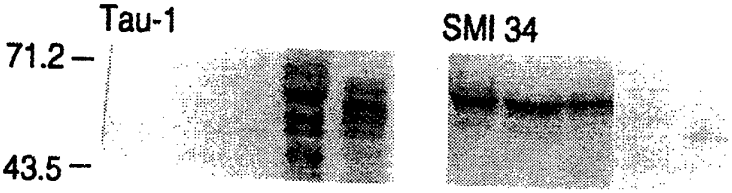
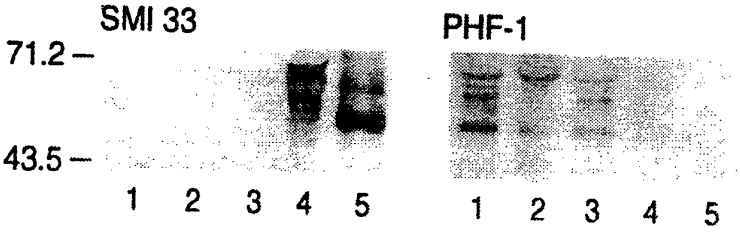


FIG.2C



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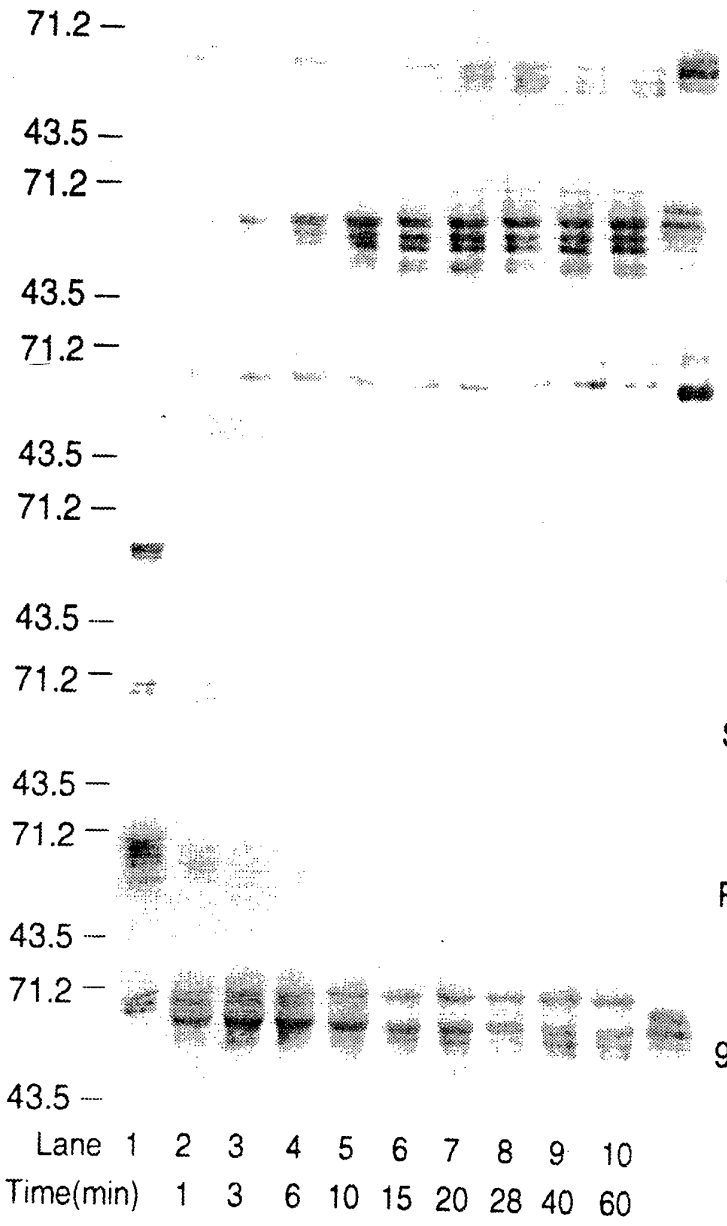


FIG.3A

FIG.3B

FIG.3C

FIG.3D

FIG.3E

FIG.3F

FIG.3G

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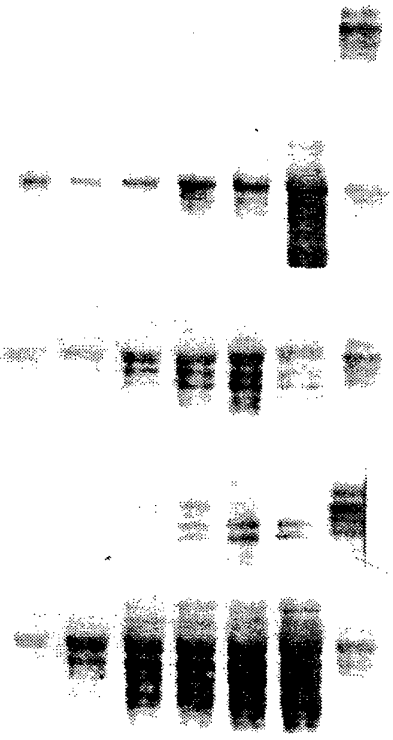
FIG.4A

FIG.4B

FIG.4C

FIG.4D

FIG.4E



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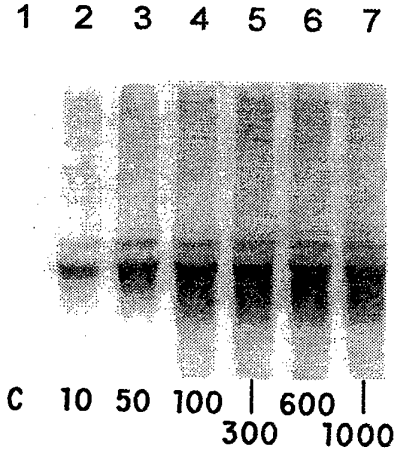


FIG.5

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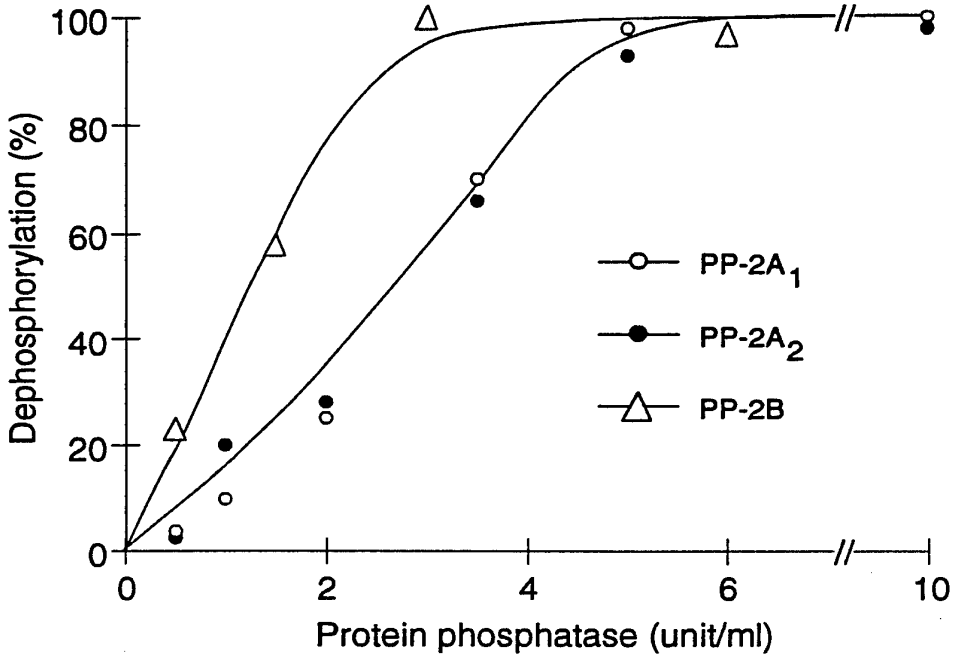


FIG. 6

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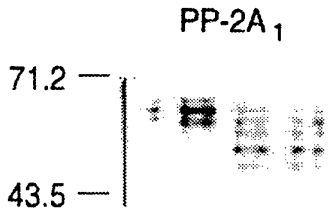


FIG.7A



FIG.7B



FIG.7C



FIG.7D

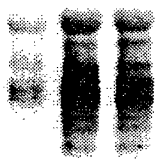


FIG.7E



FIG.7F

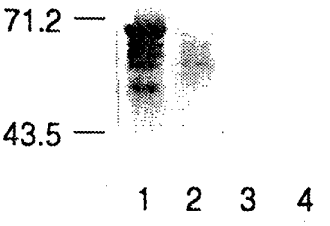


FIG.7G

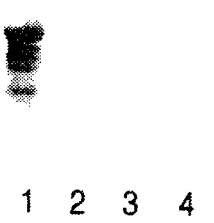


FIG.7H

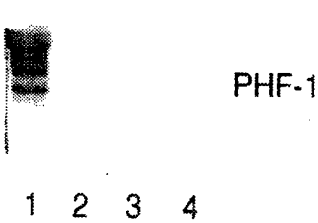


FIG.7I

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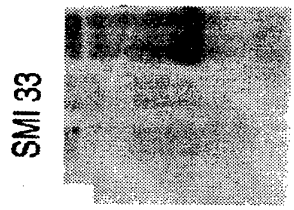


FIG.8D



FIG.8C

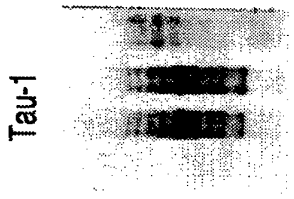


FIG.8B

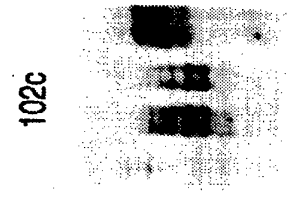


FIG.8A

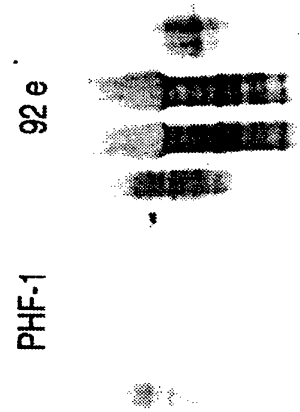


FIG.8G

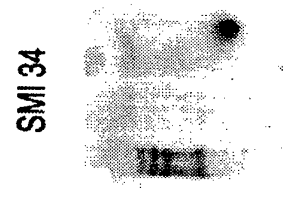


FIG.8F

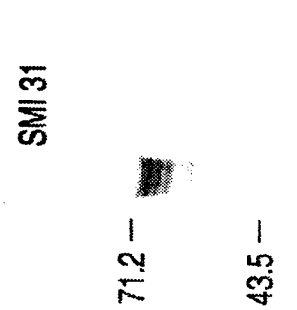


FIG.8E

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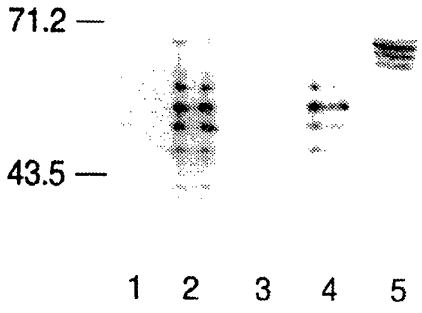


FIG.9

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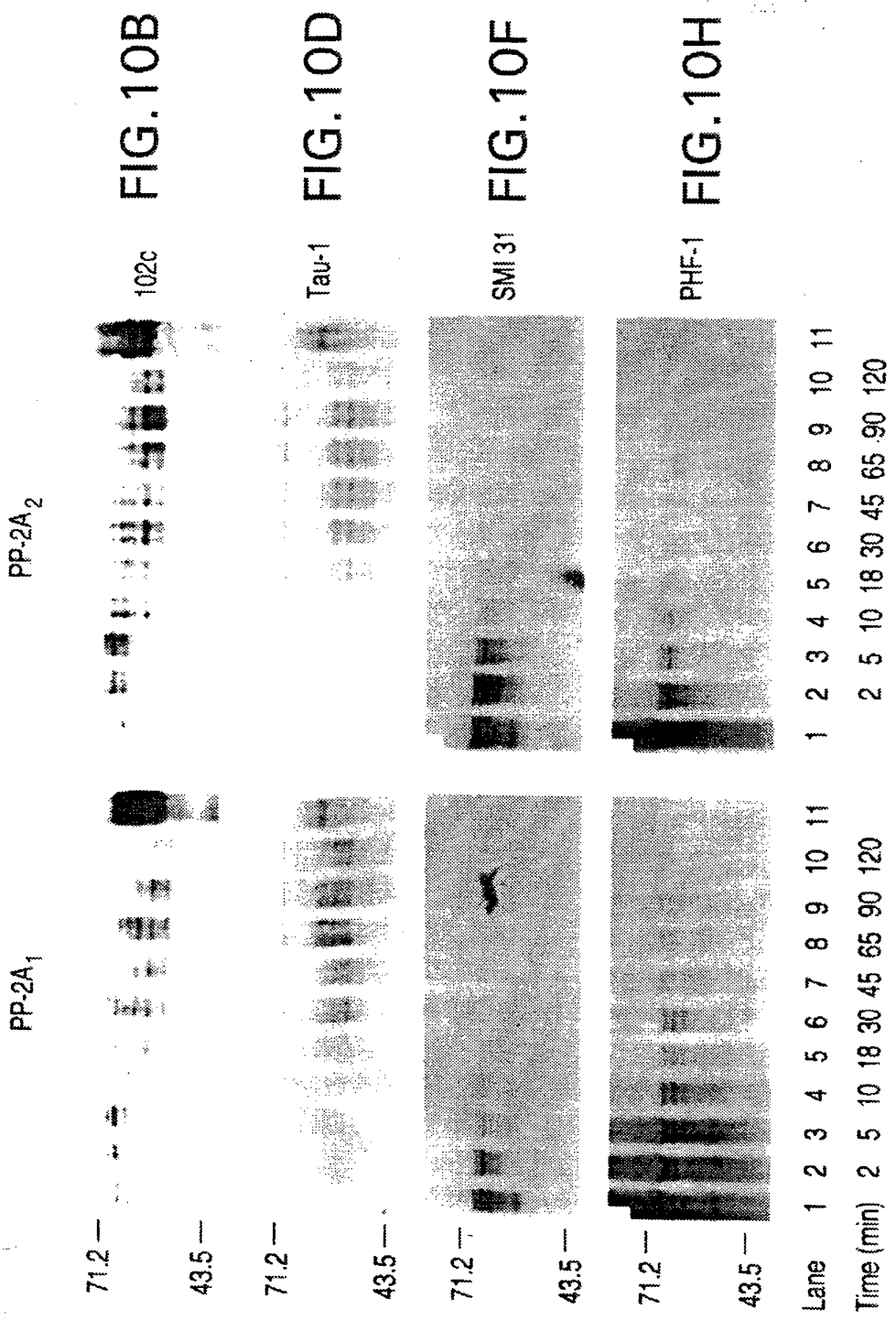


FIG.10A FIG.10B FIG.10C FIG.10D FIG.10E FIG.10F FIG.10G FIG.10H

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PP-2A$_1$ PP-2A$_2$



FIG.11A FIG.11B

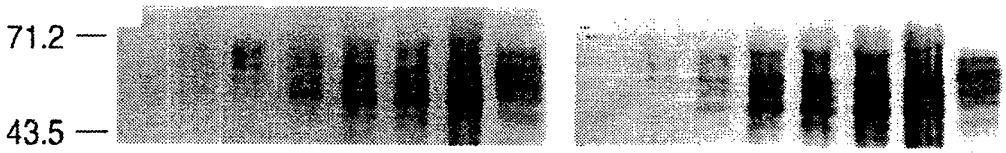


FIG.11C FIG.11D



FIG.11E FIG.11F



FIG.11G FIG.11H

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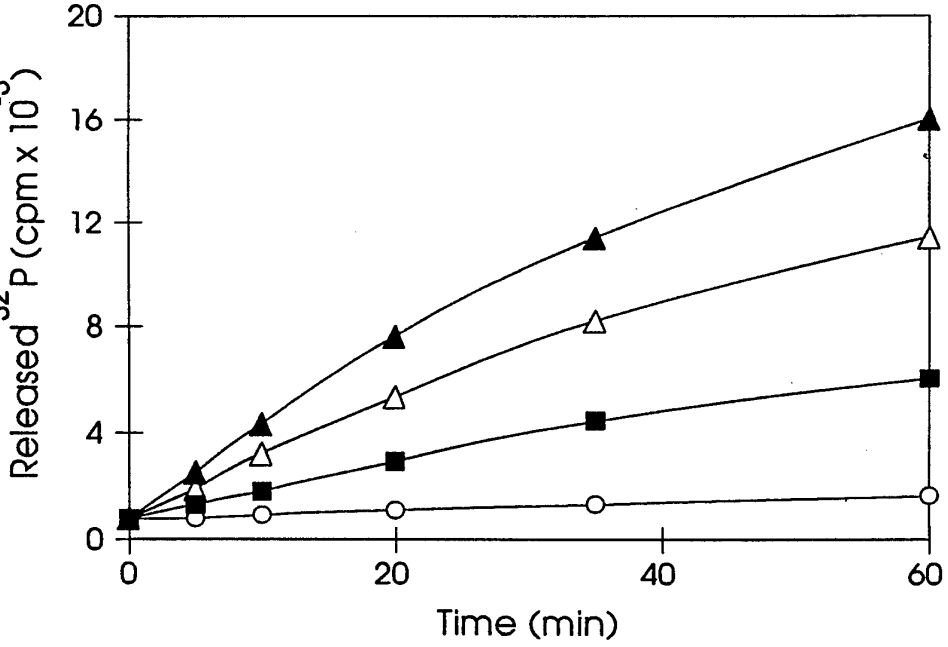


FIG. 12A

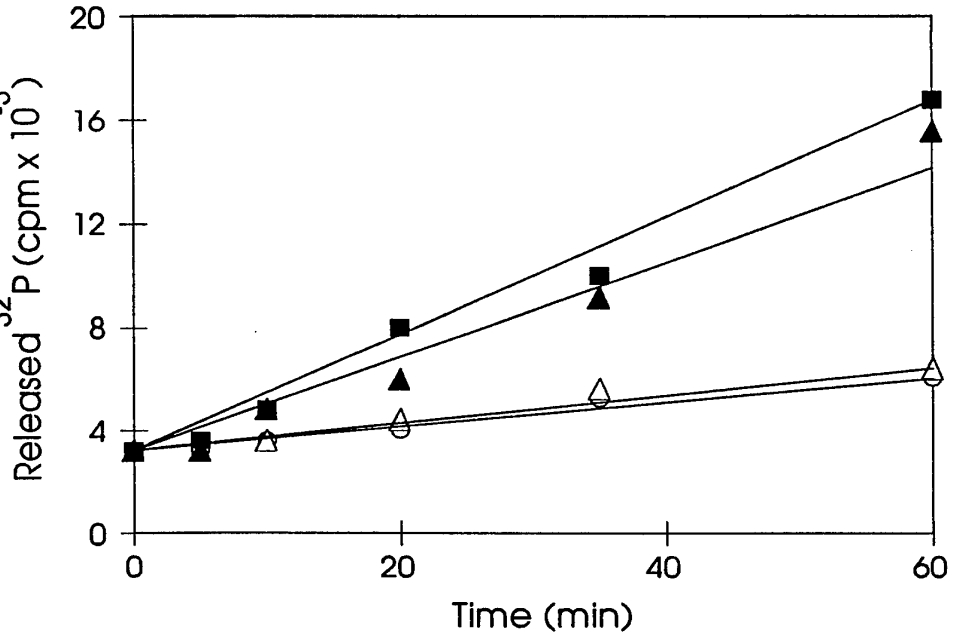


FIG. 12B

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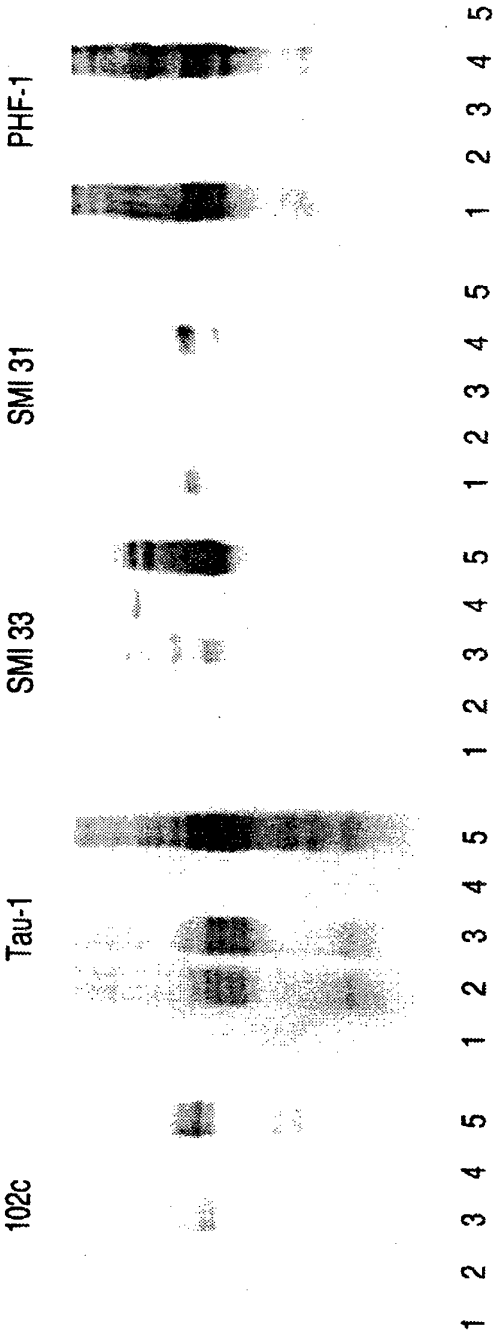


FIG.13A FIG.13B FIG.13C FIG.13D FIG.13E

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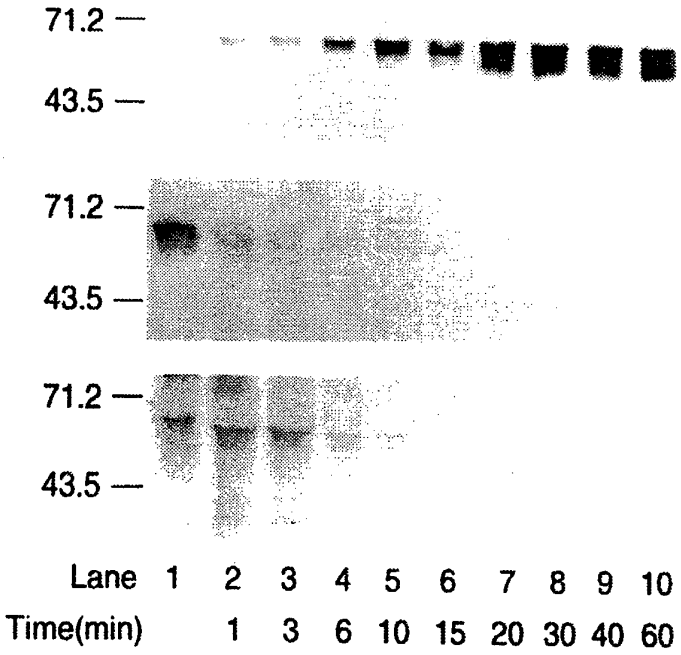


FIG.14A

FIG.14B

FIG.14C

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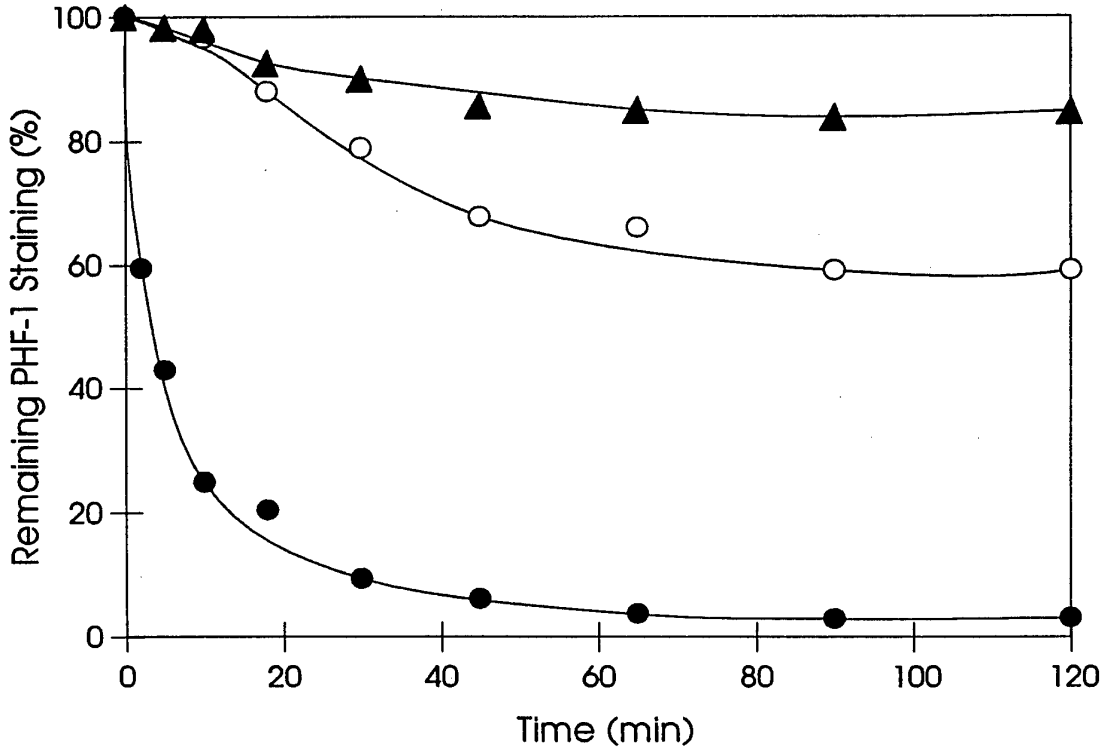


FIG. 15

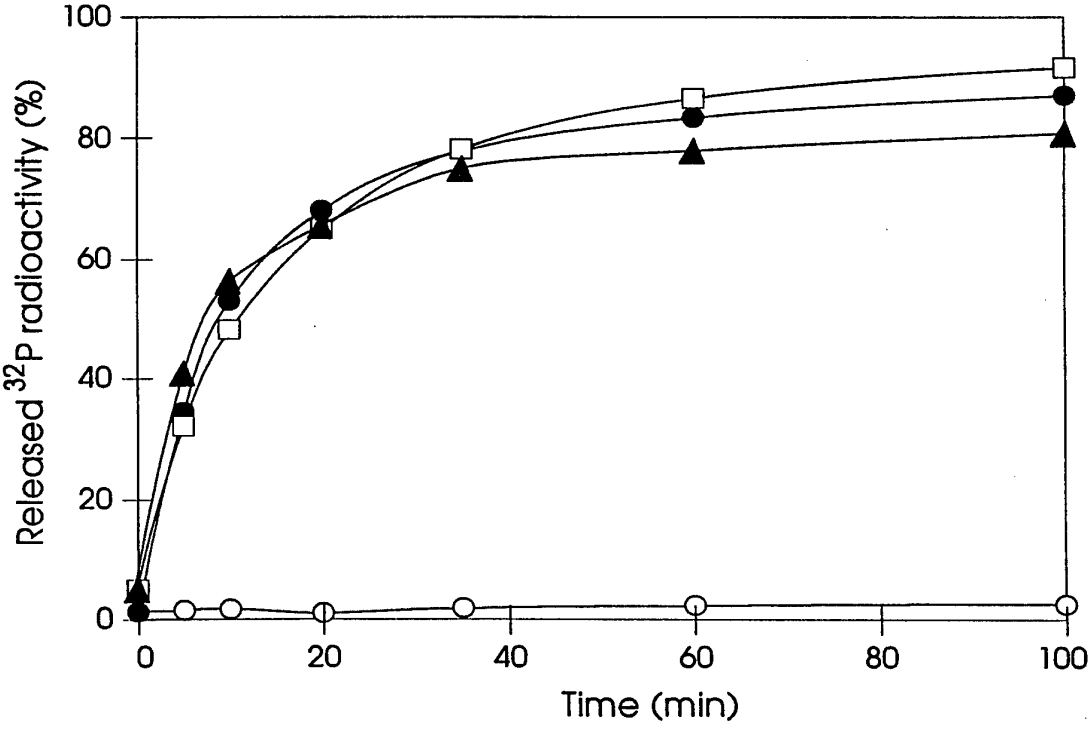


FIG. 16

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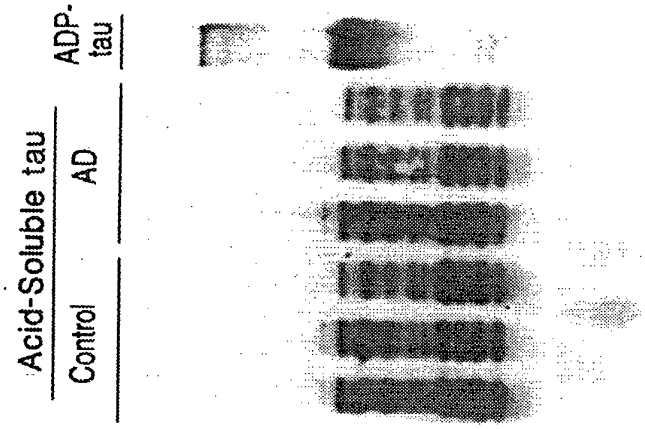


FIG. 17B

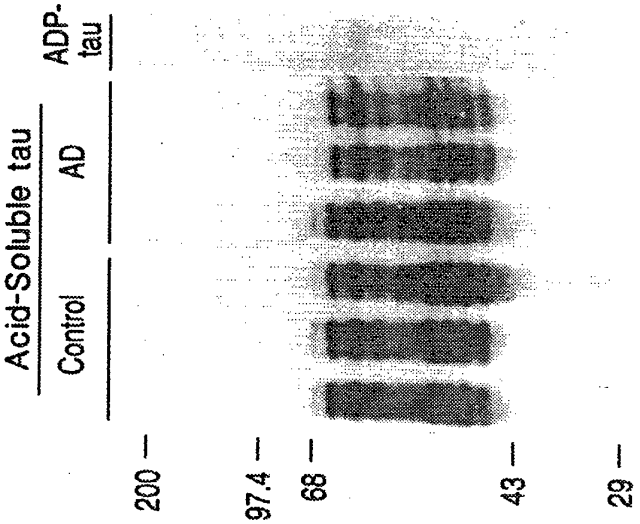


FIG. 17A

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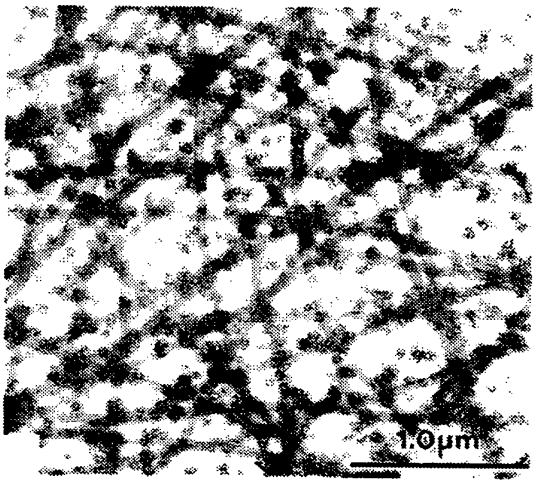


FIG.18A

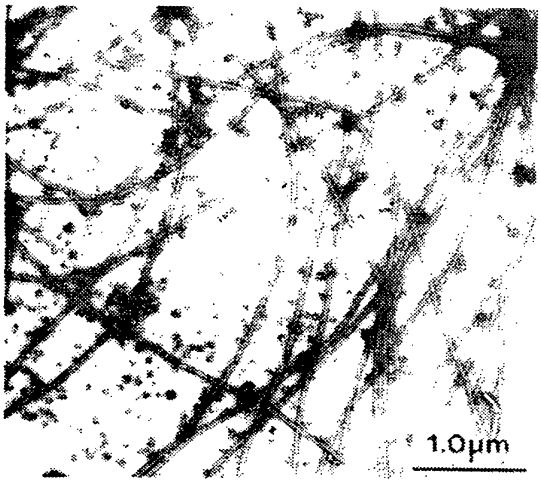


FIG.18B

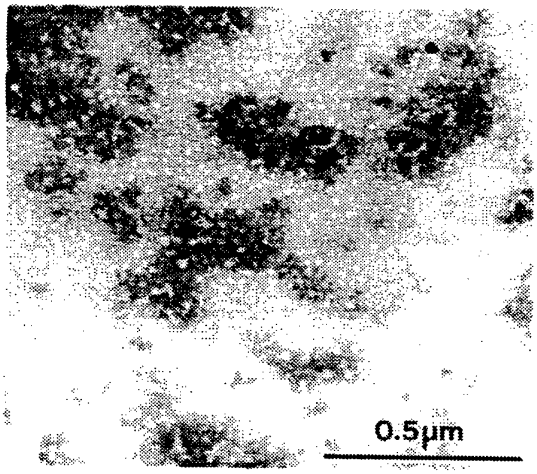


FIG.18C

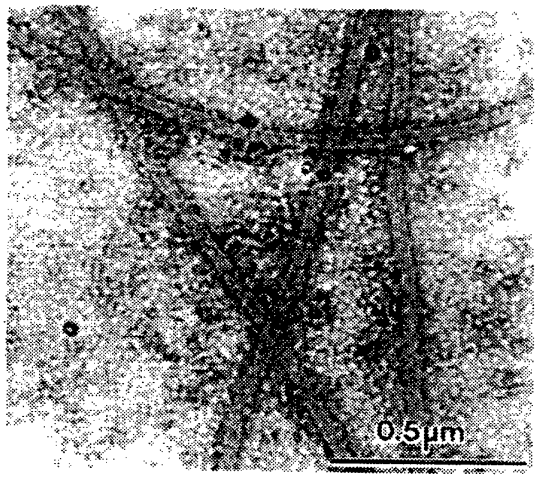


FIG.18D

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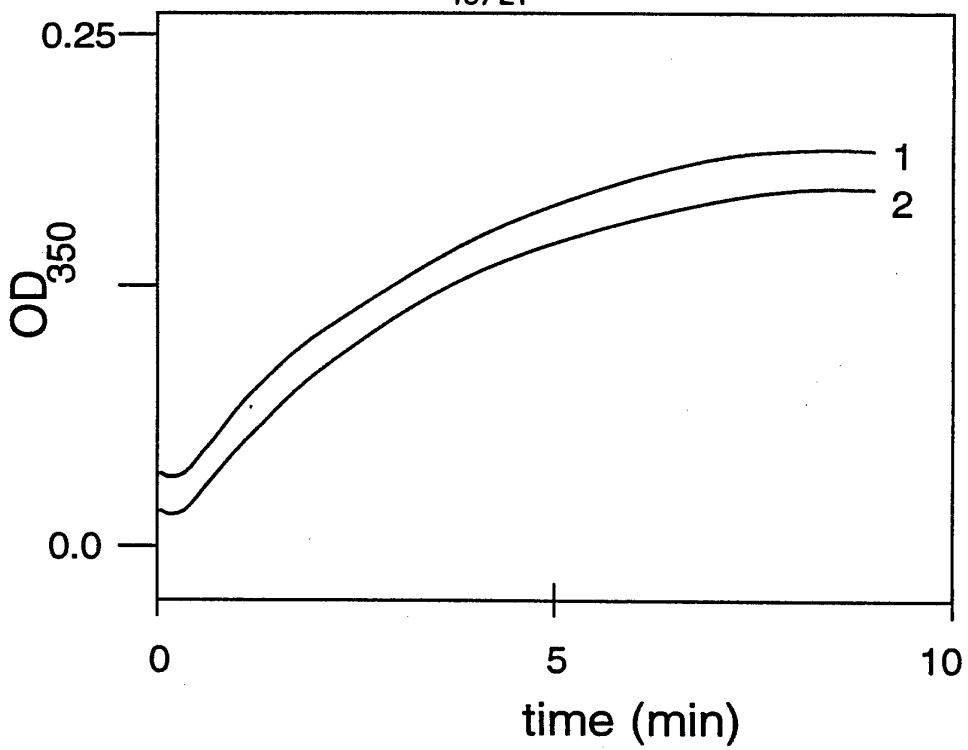


FIG. 19A

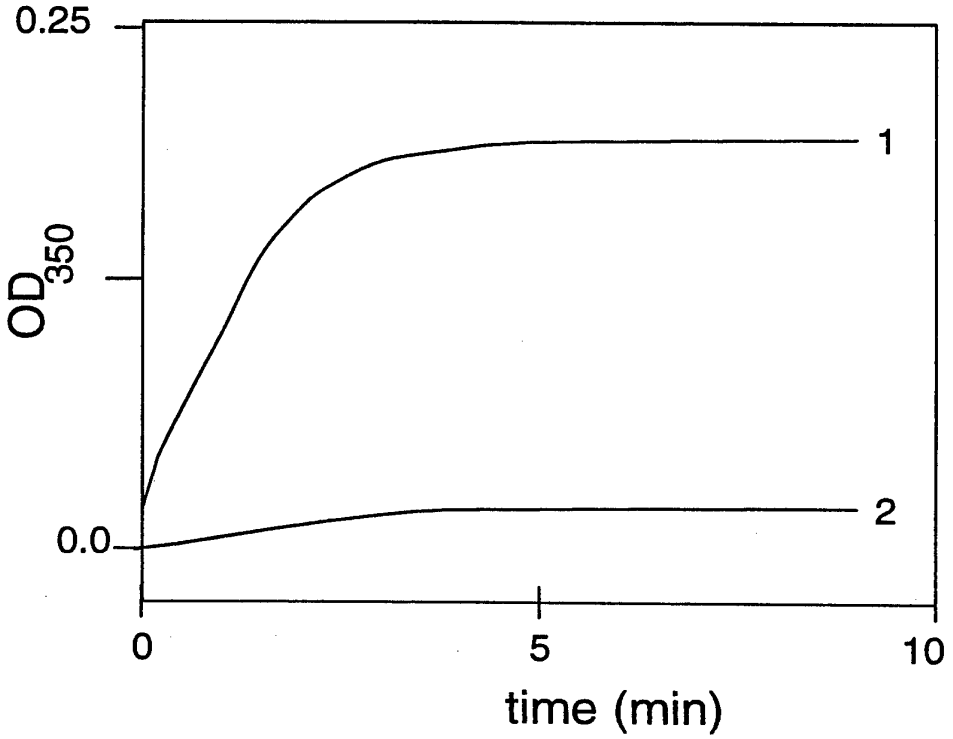


FIG. 19B

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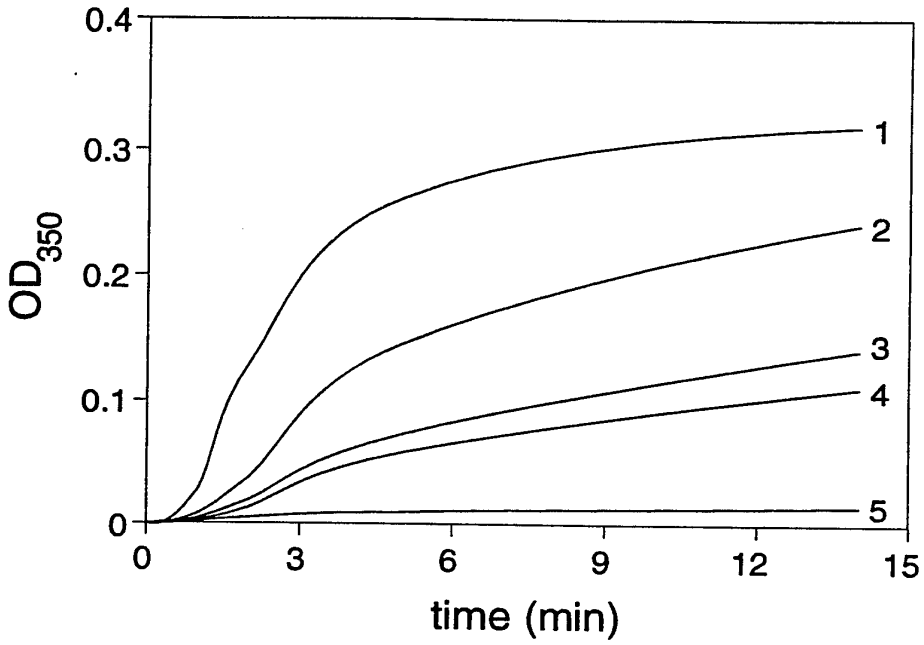


FIG. 20

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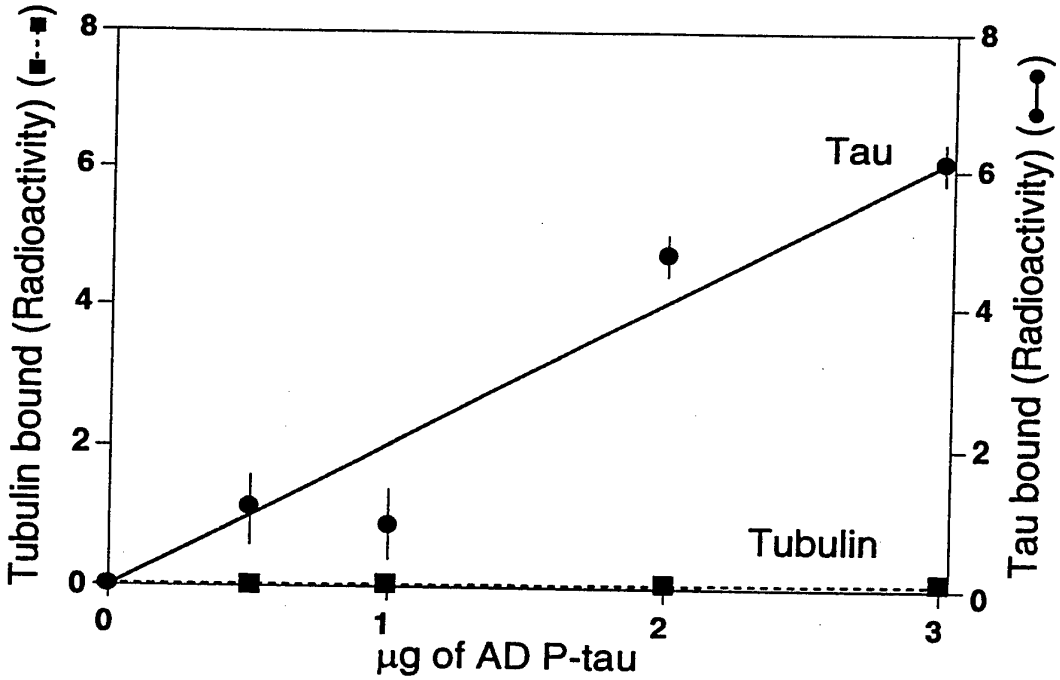


FIG. 21A

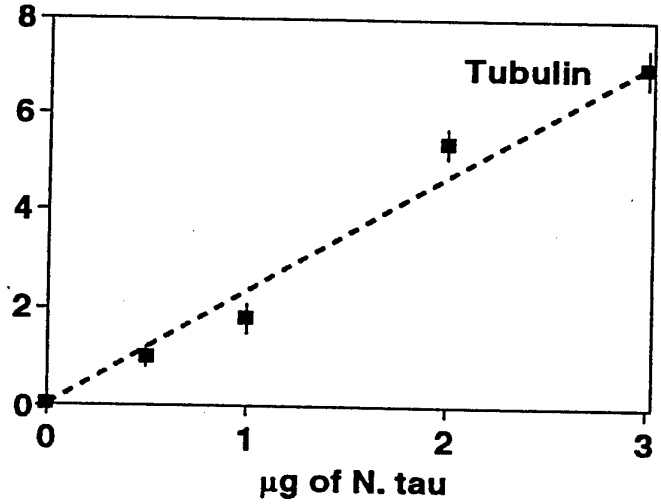


FIG. 21B

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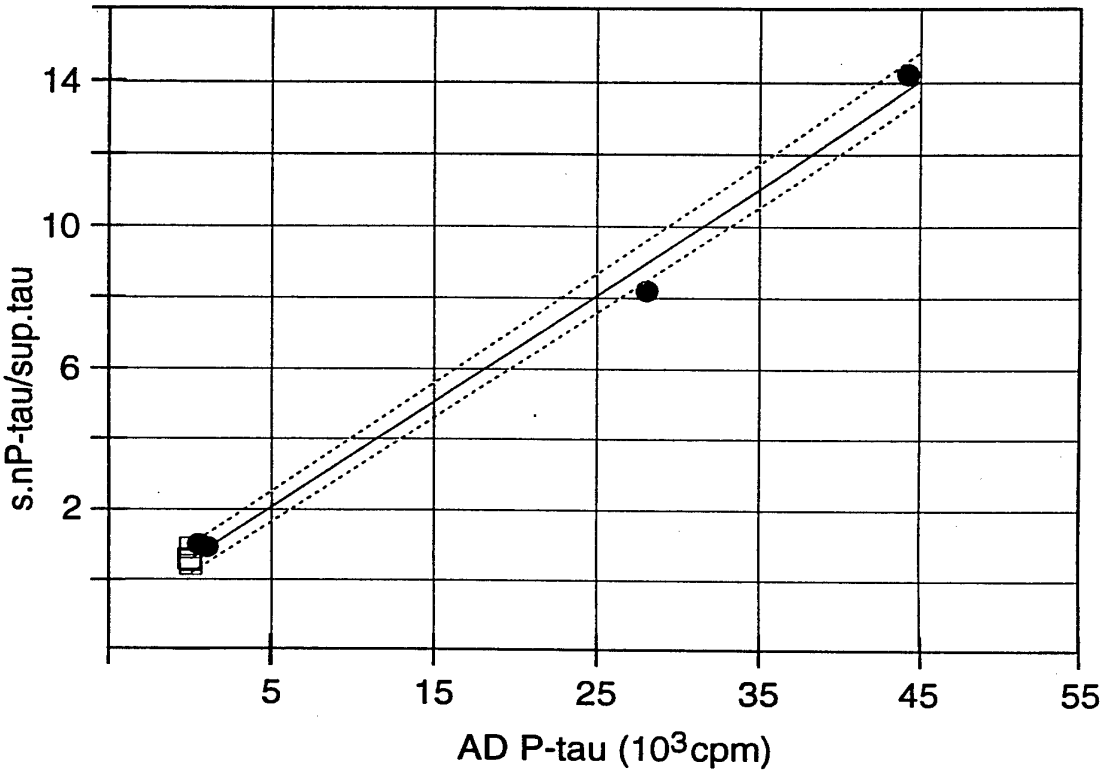


FIG. 22

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00480

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 33/32
US CL :424/639

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/639

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN ONLINE
search terms: (alzheimer? or (neurofibrillary tangle#)) and manganese

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US, A, 5,068,228 (KOHLER) 26 November 1991, see the entire document.	1-4, 9-13, 43 --- 5-8, 14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 MAY 1995	Date of mailing of the international search report 22MAY1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEAN C. WITZ Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00480

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-14 and 43 - manganese species

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00480

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-14 and 43, drawn to a method of treating a subject having a disease or disorder associated with the presence of neurofibrillary tangles comprising administering to the subject a therapeutically effective amount of a composition which increases the activity of at least one protein phosphatase towards abnormal hyperphosphorylated tau and a composition comprising a manganese salt, ion or conjugate.

Group II, claims 15-24 and 34-38, drawn to a method and composition for treating a subject having a disease or disorder associated with the presence of neurofibrillary tangles comprising administering to the subject a therapeutically effective amount of at least one protein phosphatase which dephosphorylates abnormal hyperphosphorylated tau.

Group III, claims 25-33 and 39-42, drawn to a method and composition for treating a subject having a disease or disorder associated with the presence of neurofibrillary tangles comprising administering to the subject a therapeutically effective amount of a nucleic acid encoding a protein phosphatase which dephosphorylates abnormal hyperphosphorylated tau.

Group IV, claim 44, drawn to a method of diagnosing the presence in a subject of a disease or disorder associated with the presence of neurofibrillary tangles.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The inventions are disclosed as different combinations which are not connected in design, operation or effect. The inventions have different modes of operation and each require the administration of different compositions. Accordingly, the inventions are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

Claims 1-14, the three species are:
Manganese
Calcium
Polylysine

Claims 15-24 and 34-38, the three species are:
Phosphatase PP-2B
Phosphatase PP-2A
Phosphatase pp-1

Claims 25-33 and 39 42, the three species are:
Nucleic acid encoding for Phosphatase PP-2B
Nucleic acid encoding for Phosphatase PP-2A
Nucleic acid encoding for Phosphatase PP-1

The claims that are deemed to correspond to the species listed above are set forth above.

The following claims are generic: 1 and 25

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reason: Each species has its own separate chemical and biological properties and modes of operation. Each species does not share a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

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PCT WORLD INTELLECTUAL Internal

WO 9604309A1

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 16/18, C12N 5/20, C07K 14/47, C12N 15/06, C12P 21/08, G01N 33/577, 33/68, C12N 9/12

A1

(11) International Publication Number: **WO 96/04309**

(43) International Publication Date: 15 February 1996 (15.02.96)

(21) International Application Number: PCT/EP95/03032

(22) International Filing Date: 31 July 1995 (31.07.95)

(30) Priority Data:
94870131.3 29 July 1994 (29.07.94) EP
(34) Countries for which the regional or international application was filed: AT et al.

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(57) Abstract

The present invention relates to a monoclonal antibody which forms an immunological complex with a phosphorylated epitope of a particular subclass or form of phosphorylated tau protein without forming an immunological complex with (i) fetal tau or (ii) biopsy or autopsy derived brain material from patients having died or suffering from diseases in which NFT is not a pathological hallmark. The invention also relates to a process for diagnosing brain diseases involving monoclonal antibodies of the invention. The invention also relates to a region of the tau molecule which is specifically recognized by the monoclonal antibodies of the invention. The invention also relates to kinases or phosphorylases which specifically react with the epitope recognized by these monoclonal antibodies as well as to a method for screening compounds which interfere with the activity of these kinases and phosphorylases.

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MONOCLONAL ANTIBODIES SPECIFIC FOR AN EPITOPE OF A PARTICULAR SUBCLASS OR FORM OF PHOSPHORYLATED TAU, HYBRIDOMAS SECRETING THEM, ANTIGEN RECOGNITION OF THESE ANTIBODIES AND THEIR APPLICATIONS

Field of the invention

The present invention relates to the field of diagnosis of Alzheimer's disease.

The invention relates to new monoclonal antibodies specific for a particular *type of* phosphorylated epitopes present in a particular form or subclass of phosphorylated tau, to the hybridomas secreting these monoclonal antibodies, and to the antigen recognition pattern of these monoclonal antibodies and their applications. The invention also relates to a process for diagnosing brain diseases involving the monoclonal antibodies of the invention, more particularly in brain and body fluid such as cerebrospinal fluid (CSF). The invention also relates to a region of the tau molecule modifiable in vivo by the process of phosphorylation, which is found to be associated with the formation of "neurofibrillary tangles" (NFT) and "paired helical filaments" (PHF) as they occur in several types of dementia and which is specifically recognized by the monoclonal antibodies of the invention.

Background of the invention

Alzheimer's disease (AD) is the most common form of adult-onset dementia. At present, no reliable biochemical test is available for antemortem diagnosis of AD. The disease is usually diagnosed clinically on the basis of exclusion of other forms of dementia. The diagnosis can only be confirmed irrevocally by neuropathologic examination and the demonstration of large amounts of neuritic (senile) plaques and neurofibrillary tangles (NFT) in particular brain regions (McKhann et al, 1984), the latter appearing to correlate better with the severity and the duration of AD.

Neurofibrillary tangles consist of paired helical filaments (PHF). The microtubule-associated protein tau is a major protein component of PHF and NFT (Brion et al., 1985b; Delacourte and Defossez, 1986; Grundke-Iqbal et al., 1986; Kosik et al., 1986; Wood et al., 1986; Kondo et al., 1988).

Tau protein exists in different isoforms, of which 4 to 6 are found in adult brain but only 1 isoform is detected in fetal brain. The diversity of the isoforms is generated from

a single gene on human chromosome 17 by alternative mRNA splicing (Himmler, 1989; Goedert et al., 1989; Andreadis et al., 1992). The most striking feature of tau protein, as deduced from molecular cloning, is a stretch of 31 or 32 amino acids, occurring in the carboxy-terminal part of the molecule, which can be repeated either 3 or 4 times. Additional diversity is generated through 29 or 58 amino acid-long insertions in the NH_2-terminal part of tau molecules (Goedert et al., 1989). For simplicity, all numbering in this patent application refers to the human tau variant htau40 containing all exons (441 amino acids long) according to Goedert et al (1989).

In vivo tau promotes microtubule assembly and stability in the axonal compartment of neurons by interactions involving its microtubule binding domain which is localized in the repeat region of tau (255-381) (Lewis et al, 1990).

In normal circumstances adult brain contains 2 à 3 mol phosphate per mole of tau (Selden and Pollard, 1983; Ksiezak-Reding et al, 1992). Phosphorylation of different sites in normal tau as studied in rat and humans is dependent on the developmental state (Lee et al, 1991; Bramblett et al, 1993; Goedert et al, 1993a). Tau variants of 60, 64 and 68 kDa arising as a consequence of phosphorylation have been detected in brain areas showing neurofibrillary tangles (Delacourte et al, 1990; Goedert et al., 1992; Flament et al., 1990b, Greenberg & Davies, 1990). In tau isolated from PHF (PHF-tau), phosphorylation can occur at several positions (Iqbal et al., 1989; Lee et al., 1991; Hasegawa et al., 1992).

Sofar, the detection of PHF-tau in brain extracts, either via antibodies (Mab Alz50: Ghanbari et al., 1990; Mab Ab423: Harrington et al., 1991; Mab AT120 : Vandermeeren et al., 1993; Mab AT180; Mab AT270 : International application No. PCT/EP 94/04146 filed on 14/12/1994 and Mab AT8 : International application published under WO 93/08302), or via the change in molecular weight (Flament et al., 1990, Delacourte et al., 1993), or else by functional assay (Bramblett et al., 1992) has been used to discriminate dementia with altered cytoskeletal properties from normal aged subjects or from patients with other types of dementia. A combination of monoclonal antibodies each recognizing non-phosphorylated epitopes of tau has been used to detect the presence of tau and PHF-tau in CSF (Van de Voorde et al., 1995). However, this assay lacks the necessary discriminative power to distinguish AD patients from patients suffering from other neurodegenerative disorders.

Aims of the invention

The aim of the present invention is to provide monoclonal antibodies which allow the sensitive detection of a particular form or a particular subclass of phosphorylated tau present in freshly isolated brains of patients having died from Alzheimer's disease. Said particular form or subclass of phosphorylated tau is indicative for AD and is distinct from other phosphorylated tau variants which can be demonstrated by the use of monoclonal antibodies such as Tau1, AT8, T3P, PHF1, AT180, AT270 (Kosik et al., 1988; Trojanowski et al., 1989, Lee et al., 1991; Goedert et al., 1994; Mercken et al., 1992,; Binder et al., 1985; Greenberg & Davies, 1990; International application No. PCT/EP 94/04146 filed on 14/12/94; International application published under WO 93/08302).

The invention also aims at providing the hybridomas which secrete the above-said monoclonal antibodies.

The invention furthermore aims at providing the epitopes of the phosphorylated tau protein present in brain homogenates or in body fluids such as cerebrospinal fluid, which are specifically recognized by said monoclonal antibodies.

The invention also aims at providing a process for the detection or diagnosis *in vitro* of brain diseases detected by means of these monoclonal antibodies.

Finally, the present invention aims at providing kinases or phosphorylases responsible for the phosphorylation or the dephosphorylation of the epitopes recognized by the monoclonal antibodies of the present invention.

The present invention also aims at a process for the detection *in vitro* of substances which are capable to interfere with the kinases or the phosphorylases which are implicated in the phosphorylation or the dephosphorylation of the epitopes recognized by the monoclonal antibodies of the present invention.

Summary of the invention

The present invention relates to the new and surprising finding that adult human tau isolated from fresh surgical biopsy samples of Alzheimer patients is phosphorylated at a particular site different from those previously designated as "abnormally phosphorylated" or "PHF-tau", since this site could not be detected in autopsy-derived adult human tau from normal individuals or from non-AD patients.

Previously, PHF-tau or AD-tau was thought to be phosphorylated at unique sites

("abnormal phosphorylation"), different from the "normal" phosphorylation as found in adult tau of non-AD patients. The present invention discloses the finding that several of these previously designated "abnormally phosphorylated" sites also occur in fresh biopsy-derived tau from non-AD patients. The monoclonal antibodies of the invention (similar to AT100), however, are capable of detecting a phosphorylation site that is unique to PHF-tau from the AD brain and that does not detect any of the fresh biopsy- or autopsy-derived brain tau preparations from normal individuals or non-AD patients.

All previously identified monoclonal antibodies that react with PHF-tau appear to be not truly PHF-tau specific when tested upon fresh biopsy-derived and fetal samples from normal individuals or non-AD patients. The monoclonal antibodies of the present invention are thus said to detect only a subset of phosphorylated tau proteins which are truly indicative of AD in fresh biopsy samples.

Detailed description of the invention

The present invention relates more particularly to a monoclonal antibody which forms an immunological complex with a phosphorylated epitope present in a particular form or subclass of phosphorylated tau and which does not form such an immunological complex with (i) fetal tau or (ii) tau isolated from biopsy- or autopsy-derived brain material from the brains of patients having died or suffering from diseases in which the occurrence of NFT is not a pathological hallmark (also referred to as "normal biopsy- or autopsy-derived tau" derived from "normal individuals", "non-AD patients" and "non-demented control individuals" in the remaining part of the present invention).

Presently known tau monoclonal antibodies recognize phosphorylation-dependent or phosphorylation-independent epitopes present on normal tau derived from freshly isolated brain biopsies or from autopsies derived from the brains of patients having died of diseases in which the occurence of NFT is not a pathological hallmark, confirming that these epitopes are not specific for the particular form or the particular subclass of phosphorylated tau as derived from the brains of patients having died from Alzheimer's disease.

Surprisingly, the present inventors have isolated and characterized a monoclonal antibody, AT100, which is recognizes specifically a subclass of phosphorylated tau and does not cross-react with biopsy- or autopsy-derived tau preparations of patients having died of diseases in which the occurrence of NFT is not a pathological hallmark. The present

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invention thus relates to any monoclonal antibody which has the reactivity characteristics in common with the monoclonal antibody AT100 as teached in the Examples section.

The monoclonal antibodies of the invention (AT100 or any other similar monoclonal antibody according to the present invention) may be selected from a range of monoclonal antibodies obtained by direct immunization with tau, extracted from human brain tissue derived from Alzheimer patients as illustrated in the examples section. More particularly, the monoclonal antibodies of the invention are characterized by the fact that they specifically bind to a phosphorylated epitope which is present in a particular subclass or form of phosphorylated tau and which is absent or non-detectable by the present methods (see examples section) in (i) fetal tau or in (ii) tau isolated from autopsy- or biopsy-derived brain material taken from individuals not suffering or having died from diseases in which NFT are a pathological hallmark (also referred to as non-AD patients, see above).

Further analysis of the epitopes recognized by these monoclonal antibodies showed that they are particularly directed at a phosphorylated epitope confined to a region of the tau molecule spanning positions 146 to 251 of the tau molecule as indicated in SEQ ID NO 1 (see below), more particularly to a region of the tau molecule spanning positions 198 to 251.

The monoclonal antibodies of the invention are further characterized by the fact that they recognize epitopes which are different from the epitope of the monoclonal antibody AT8 as defined in Goedert et al. (1993) and described in WO 93/08302 and *the epitope* of the monoclonal antibodies AT180 and AT270 as defined by Goedert et al. (1994) (described in International application PCT/EP 94/04146 filed on 14/12/94) and different from the epitopes recognized by the monoclonal antibodies BT2/Tau1 (Kosik et al., 1988), HT7 (Mercken et al., 1992a) or AT120 (International application published under WO 94/13795).

The monoclonal antibodies of the present invention are further characterized in that they specifically recognize a particular form or subclass of phosphorylated tau either on brain sections, on immunoblots of tau extracted from the brains of AD patients, in ELISA of tau extracted from the brains of AD patients or in body fluids such as CSF, either alone or in combination with other phosphorylated tau specific antibodies.

Said monoclonal antibody is being characterized by the the fact that it is capable of specifically detecting this epitope of phosphorylated tau which may be present in brain samples or body fluids such as cerebrospinal fluid (CSF).

The monoclonal antibodies of the invention are further characterized in that they

specifically bind to and are capable to detect a particular phosphorylated epitope of the region of phosphorylated tau present in the brains of patients having died from AD (representing a particular subclass or form of phosphorylated tau) as specified in SEQ ID NO 1:

```
          146                 150
   NH2- Asp Gly Lys Thr Lys Ile Ala Thr Pro Arg

                   160                                         170
Gly Ala Ala Pro Pro Gly Gln Lys Gly Gln Ala Asn Ala Thr Arg

                                       180
Ile Pro Ala Lys Thr Pro Pro Ala Pro Lys Thr Pro Pro Ser Ser

                   190                                         200
Gly Glu Pro Pro Lys Ser Gly Asp Arg Ser Gly Tyr Ser Ser Pro

                                       210
Gly Ser Pro Gly Thr Pro Gly Ser Arg Ser Arg Thr Pro Ser Leu

                   220                                         230
Pro Thr Pro Pro Thr Arg Glu Pro Lys Lys Val Ala Val Val Arg

                                       240
Thr Pro Pro Lys Ser Pro Ser Ser Ala Lys Ser Arg Leu Gln Thr

                   250
Ala Pro Val Pro Met Pro COOH
```

(SEQ ID NO 1)

The present invention relates more particularly to a monoclonal antibody as defined above, characterized by the fact it specifically forms an immunological complex:

- either with a phosphorylated epitope located within the sequence as defined in SEQ ID NO 1,
- or with any other phosphorylated peptide capable of specifically forming an immunological complex with a monoclonal antibody, which itself is capable of forming a complex with a phosphorylated epitope located in the human tau protein region as shown in SEQ ID NO 1. The latter monoclonal antibody is characterized by the fact that it does not detect (i) fetal tau or (ii) biopsy- or autopsy-derived brain material from individuals not suffering or having died from diseases in which NFT is a pathological hallmark as seen in post-mortem samples collected at short (< 24 h) post-mortem intervals or as seen in biopsy samples taken without post-surgical delay from patients suffering or having died

from diseases in which NFT is not a pathological hallmark.

A preferred monoclonal antibody of the invention, AT100, is produced by the hybridoma deposited at ECACC (European Collection of Animal Cell Cultures, Vaccine Research and Production Laboratory, Public Health and Laboratory Service (PHLS), Centre for Applied Microbiology and Research, Porton Down, GB-Salisbury, Wiltshire SP4 OJG), on 21 April 1994, under No 94042117.

The monoclonal antibodies of the invention are obtainable by a process involving obtention and isolation of hybridomas which secrete these monoclonal antibodies.

It is demonstrated in the examples section of the present invention, that the preferred monoclonal antibody of the invention allows the detection of a subclass of phosphorylated tau associated with AD whilst not detecting (ii) biopsy- or autopsy-derived tau from normal individuals or (ii) fetal tau.

A process for obtaining the hybridomas of the invention involves the steps of:

(a) starting from spleen cells of an animal, e.g. mouse or rat, previously immunized *in vivo* or *in vitro* with an antigen being chosen from the group of:

- phosphorylated tau present in brain extracts derived from the brains of patients having died of AD, or,
- a phosphorylated human tau peptide, or,
- immunoaffinity purified phosphorylated tauy extracted and purified from a human brain sample of a patient having died from Alzheimer's disease,

recognized by the monoclonal antibodies of the invention;

(b) fusing said immunized cells with myeloma cells under hybridoma-forming conditions; and

(c) selecting those hybridomas which secrete the monoclonal antibodies which are capable of specifically recognizing a phqsphorylated epitope of tau present in a particular subclass or form of phosphorylated tau without recognizing (i) biopsy- or autopsy-derived tau from normal individuals or (ii) fetal tau from the brains of patients with diseases in which the occurence of NFT is not a pathological hallmark by means of Western blot analysis or ELISA.

The antigen of the invention is advantageously contained in brain and brain extracts, in the cerebrospinal fluid or the serum of a patient having Alzheimer's disease, Down syndrome, Pick's disease, subacute sclerosing panencephalitis (SSPE) or other neurological diseases in which NFT are a pathological hallmark; this antigen provokes an immunological reaction with the monoclonal antibody of the invention.

More particularly, the present invention relates also to monoclonal antibodies as defined above, obtainable by a process comprising at least the following steps:

(a) starting from the spleen cells of a mouse previously immunized with an antigen chosen from the following group:

- phosphorylated tau extracted and purified from a human brain sample of a patient having died from Alzheimer's disease (as disclosed in the examples section), or,
- a phosphorylated human tau peptide, or,
- immunoaffinity-purified phosphorylated tau extracted and purified from a human brain sample of a patient having died from Alzheimer's disease

capable of reacting with the monoclonal antibodies of the invention,

(b) fusing said immunized cells with myeloma cells under hybridoma-forming conditions,

(c) selecting those hybridomas which secrete monoclonal antibodies which specifically recognize (a particular subclass or form of) phosphorylated tau without recognizing an epitope of (i) fetal tau or (ii) biopsy- or autopsy-derived tau material from the brains of normal individuals (as defined above) or patients having died or suffering from diseases in which NFT is not a pathological hallmark as determined by means of Western blot analysis or ELISA,

(d) culturing the selected hybridomas as indicated above in an appropriate culture medium; and,

(e) recovering the monoclonal antibodies secreted by said selected hybridoma; or alternatively,

(f) implanting the selected hybridoma into the peritoneum of a mouse and, when ascites has been produced in the animal;

(g) recovering the monoclonal antibodies then formed from said ascites.

A process for producing the monoclonal antibodies of the invention involves at least the steps of:

(a) culturing the selected hybridomas as indicated above in an appropriate culture medium; and,

(b) recovering the monoclonal antibodies secreted by said selected hybridoma; or alternatively,

(c) implanting the selected hybridoma into the peritoneum of a mouse and, when ascites has been produced in the animal;

(d) recovering the monoclonal antibodies then formed from said ascites.

The monoclonal antibodies of the invention can be prepared by conventional *in vitro*

techniques such as the culturing of immobilized cells using e.g. hollow fibers or microcapsules or such as the culturing of cells in homogeneous suspension using e.g. airlift reactors or stirred bioreactors.

The invention also relates to a peptide capable of forming an immunological complex with the monoclonal antibodies of the invention, with said peptide being in the phosphorylated form, and

- with the sequence of said peptide comprising, or consisting of phosphorylated parts or derivatives of the sequence as shown in SEQ ID NO 1, or,
- with the sequence of said peptide comprising, or consisting of the sequence of any phosphorylated peptide being capable of specifically forming an immunological complex with the monoclonal antibodies according to the invention.

Said phosphorylated peptides are preferably from 6 to 100 amino acids long. The peptides according to this embodiment of the invention can be prepared by classical chemical synthesis. The synthesis may be carried out in homogenous solution or in solid phase according to any of the techniques well known in the art.

Phosphorylated peptides are prepared according to any technique known in the art, (f.i. de Bont et al., 1990a; de Bont et al., 1990b; Perich, 1991; Otvos et al., 1989)

According to yet another embodiment, the present invention relates to a phosphorylated peptide as defined above, which is capable of generating a monoclonal antibody according to the present invention upon immunization.

The peptides used for immunization are preferentially in the form in which they are joined to a biotin molecule or any other carrier molecule in order to achieve a good immunogenic response. Such carrier molecules are well known in the art and are coupled to the peptide via linker groups, which are also comprised in the art.

The invention also relates to a process for *in vitro* the detection or diagnosis in vitro of a brain/neurological disease involving a particular form or subclass of phosphorylated tau, such as Alzheimer's disease, which comprises at least the following steps:

- contacting a monoclonal antibody of the invention with a preparation of NFT, PHF or a detergent-extracted brain homogenate isolated from a patient having had Alzheimer's disease or any other disease involving phosphorylated tau protein under conditions suitable for producing an antigen-antibody complex;
- detecting the immunological binding of said antibody to said brain homogenate, and possibly separating said complex and possibly recovering the antigen sought in a purified form.

Recovering the antigen sought may be done by first washing the immobilized antibody-antigen complex then formed;

- treating this complex with a solution (e.g. 3 M potassium thiocyanate, 2.5 M magnesium chloride, 0.2 M citrate-citric acid, pH 3.5 or 0.1 M acetic acid) capable of producing the dissociation of the antigen-antibody complex; and;

- recovering the antigen in a purified form.

The invention relates also to a process for the detection or diagnosis *in vitro* of brain/neurological disease involving a particular form or subclass of phosphorylated tau protein, such as in Alzheimer's disease, which includes:

- bringing a sample of CSF, more preferably unconcentrated CSF, or a sample of serum, or proteins or polypeptides as a result of an extraction procedure starting from brain tissues, cerebrospinal fluid or serum known to those skilled in the art (Ibqal et al., 1984; Greenberg & Davies, 1990) from a patient suspected of suffering from brain disease involving NFT, more particularly Alzheimer's disease, into contact under *in vitro* conditions with a monoclonal antibody of the invention, with said conditions being suitable for producing an antigen-antibody complex; and,

- detecting the immunological binding of said antibody to said sample of brain extract, cerebrospinal fluid or serum.

Advantageously, the monoclonal antibodies of the invention are in an immobilized state on a suitable support such as a resin. Alternatively, the present process may be put into practice by using any other immunoassay format known to the person skilled in the art.

The process for the detection of the antigen can then be carried out by bringing together said antigen-antibody complex formed by the antigen and the antibodies of the invention with:

a) a second antibody

* which can be a monoclonal antibody recognizing an epitope of tau protein or phosphorylated tau protein, or of any tau peptide carrying an epitope, with said epitope being different from the one of the invention, or

* which can be a polyclonal antibody recognizing tau protein or phosphorylated tau or a polyclonal antibody recognizing a tau peptide, with said polyclonal antibody being capable of forming an immunological complex with epitopes which are different from the epitope of the invention, with said polyclonal antibody being preferably purified by immunoaffinity chromatography using

immobilized tau protein or phosphorylated tau protein;

(b) a marker either for specific tagging or coupling with said second antibody, with said marker being any possible marker known to the person skilled in the art;

(c) appropriate buffer solutions for carrying out the immunological reaction between the monoclonal antibody of the invention and a test sample on the one hand, and the bound second antibody and the marker on the other hand, and,

(d) possibly also a peptide carrying a phosphorylated epitope of tau comprised in the region spanning SEQ ID NO 1 for standard purposes, or for competition purposes with respect to the antigen which is sought.

Advantageously, the second antibody itself carries a marker or a group for direct or indirect coupling with a marker.

The monoclonal antibodies of the invention enable the diagnosis of Alzheimer's disease (AD) and of any disease involving the formation of NFT on the basis of CSF, brain extract, serum samples or brain sections (i.e. to detect particular subclasses of phosphorylated tau).

The results obtained with the monoclonal antibodies of the invention indicate that the epitope recognized by the monoclonal antibodies of the invention is specifically found in phosphorylated tau derived from the brains of patients having died or suffering from Alzheimer's disease, but may occur also in other neurological diseases where the presence of NFT constitutes a pathological hallmark and are not found in preparations of tau derived from biopsy or autopsy brain material of normal individuals (as defined above) or patients having a disease in which NFT is not a pathological hallmark.

According to another embodiment, the present invention relates to a kit for the diagnosis *in vitro* of one of the following diseases: Alzheimer's disease, Down's syndrome, Pick's disease and other neurological disorders in which phosphorylated tau protein, NFT or paired helical filaments are implicated, characterized in that the kit comprises:

- at least a microplate for deposition thereon of at least a monoclonal antibody of the invention;
- a second antibody
  * which can be a monoclonal antibody recognizing an epitope of tau protein or phosphorylated tau protein, or of any phosphorylated tau peptide carrying an epitope, with said epitope being different from the one of the invention, or
  * which can be a polyclonal antibody recognizing tau protein or phosphorylated tau or a polyclonal antibody recognizing a peptide carrying an epitope of tau, with said

polyclonal antibody being capable of forming an immunological complex with epitopes which are different from the epitope of the invention, with said polyclonal antibody being preferably purified by immunoaffinity chromatography using immobilized tau protein;

- a marker either for specific tagging or coupling with said second antibody;
- appropriate buffer solutions for carrying out the immunological reaction between the monoclonal antibody of the invention and a test sample on the one hand, and the bound second antibody and the marker on the other hand,
- possibly also a peptide carrying a phosphorylated epitope of tau comprised in the region spanning SEQ ID NO 1 for standard purposes, or for competition purposes with respect to the antigen which is sought.

The present invention also relates to a kinase which upon acting on non-phosphorylated tau is capable to specifically introduce a phosphorylation in the region as specified in SEQ ID NO 1, thereby giving rise to the epitope recognized by the monoclonal antibody of the invention.

The invention also relates to a phosphorylase which is capable to react specifically with the epitope recognized by a monoclonal antibody according to the present invention comprised in SEQ ID NO 1, thereby destroying the epitope in such a way that an immunological reaction can no longer take place between said peptide and the monoclonal antibody of the invention.

The present invention further relates to a method for screening for compounds which interfere with the activity of the above-mentioned kinase comprising the steps of:

- bringing into contact non-phosphorylated tau or a non-phosphorylated peptide of which the sequence comprises or consists of the sequence specified in SEQ ID NO 1,
- with a kinase as defined above capable of phosphorylating tau protein or a polypeptide which contains at least the sequence as specified in SEQ ID NO 1, or derivatives thereof, thereby producing the epitope recognized by the monoclonal antibody of the invention,
- and any compound which is capable to interfere with the process of phosphorylation of said epitope by said kinase,
- and detecting the amount of phosphorylation using the monoclonal antibody of the present invention as defined above.

The present invention further relates to a method for screening for compounds

which interfere with the activity of the above-mentioned phosphorylase comprising the steps of :

- bringing into contact phosphorylated tau or a phosphorylated peptide of which the sequence comprises or consists of the phosphorylated sequence specified in SEQ ID NO 1 or phosphorylated derivatives thereof, and which is recognized by the monoclonal antibodies of the invention,
- with a phosphorylase capable to remove phosphate from said phosphorylated tau or said phosphorylated peptide,
- and detecting the amount of dephosphorylation by means of e.g. and ELISA assay comprising the monoclonal antibody of the invention as defined above.

Definitions

The expression "phosphorylated tau" as used in the present invention refers to PHF-tau (also known as "abnormally phosphorylated tau") and biopsy-derived normal tau. The latter has been shown by the present inventors to be also phosphorylated, in contradiction to what was previously thought to be the case.

The expression "specifically bind to and are capable to detect a phosphorylated epitope present in a particular form or subclass of phosphorylated tau" corresponds to the fact that the monoclonal antibodies of the invention detect a particular form or subclass of phosphorylated tau which is predominantly present in PHF derived from brains from AD patients and which is absent, or not detectable by the present methods, in the brains of human foetuses, of non-demented control patients as seen in postmortem samples collected at short (< 24 h) post-mortem intervals or in biopsy samples taken without post-surgical delay (< 15 min.). Non-demented control patients or normal individuals are further defined as patients not suffering from diseases in which NFT are a pathological hallmark and which are defined as normal according to the NINCDS-ADRA (McKahn et al., 1984) or the DSM (Diagnostic and Statistical Manual of Mental Disorders - III, American Physchiatric Association, Washington DC, 1987).

The expression "an epitope which is absent or non-detectable by the present methods" refers to the methods as described in the examples section, such as ELISA, competition ELISA, RIA, and other well known methods to the person skilled in the art.

The expression "form an immunologically complex with" is to be interpretated such that the monoclonal antibody of the invention binds to the above-said antigen under conditions

as mentioned in one of the following techniques:

- Light immunomicroscopy

Post-mortem tissue was obtained from histologically confirmed Alzheimer patients. Immediately after excision, the brain biopsy was immersed in ice-cold phosphate buffered-saline and processed for: (1) the preparation of enriched tau fractions without post-surgical delay; (2) for immunocytochemistry following fixation in isotonic 70% ethanol. Following fixation, the tissue samples were infiltrated and embedded in paraffin (Trojanowski et al., 1989), and 6 μm thick sections were cut from paraffin blocks of the biopsy and autopsy brain samples. Immunohistochemistry using the peroxidase anti-peroxidase method, and the microscopic assessment of the tissue to identify evidence of AD or other types pathological abnormalities were performed as described previously (Bramblett et al., 1992; Lee et al., 1993; Trojanowski et al., 1989).

Other brain tissue samples, of e.g. Alzheimer patients obtained at surgery or autopsy, were fixed by immersion in 4% formalin or Bouin's fixative and embedded in paraffin for sectioning. The monoclonal antibodies of the invention are applied in conjunction with a technique to visualize the formed immune complexes such as the avidin-biotinylated peroxidase complex technique (Hsu et al., 1981) using 3,3'-diaminobenzidine tetrahydrochloride for development of color. Sections were counterstained with Harris haematoxylin stain.

- Immunoblotting procedures

For immunoblotting, fractions enriched in phosphorylated tau are prepared as described (Greenberg and Davies, 1990). Typically, biopsy or postmortem tissue, consisting mostly of gray matter from the frontal and temporal cortex, was homogenized with 10 volumes of cold buffer H (10 mM Tris/1 mM EGTA/0.8 M NaCl/10% sucrose, pH 7.4) in a Teflon/glass Potter S (Braun, Germany) homogenizer. After centrifugation of the homogenate in a 60 Ti MSE rotor at 27,000 x g for 20 min at 4°C, the pellet was removed and the supernatant was adjusted to 1% (wt/vol) N-laurosylsarcosine and 1% (vol/vol) 2-mercaptoethanol and incubated while rotating on a mixer for 2.5 hours at 37°C. The supernatant mixture was centrifuged at 108,000 x g for 35 min at 20°C. The PHF-tau containing pellet was gently washed with PBS and finally suspended in 1 ml of the same buffer.

Normal tau was isolated from brain samples derived from non-demented persons or from patients suffering from diseases in which the presence of NFT is not a pathological hallmark. These samples were homogenized in ice cold reassembly (RA) buffer (0.1 M

MAS, 0.5 mM $MgSO_4$, 1 mM EGTA, 2 mM dithiothreitol [pH 6.8]) containing 0.75 M NaCl, and a cocktail of protease and phosphatase inhibitors (2 mM phenylmethysulfonyl fluoride, 20 mM NaF, 0.5 mM sodium orthovanadate, and TPCK, TLCK, leupeptin, pepstatin, soy bean trypsin inhibitor and apotinin, each at 1 µg/ml. After centrifugation at 50,000 x g for 30 min at 4°C, the supernatants were boiled for 10 min, and recentrifuged at 50,000 x g for 30 min.

SDS-polyacrylamide electrophoresis is performed under reducing conditions on 12% gels (Laemmli, 1970). After electrophoresis, the proteins are either fixed and stained with Coomassie brilliant blue, or transferred (Towbin et al., 1979) to nitrocellulose sheets (Hybond-C, Amersham) or Immobilon filters (Millipore).

After transfer, the filters are presoaked in PBS containing 0.05% (v/v) Tween 20 (Tween-PBS) and then incubated for 1 h in Tween-PBS containing 5% (w/v) skimmed dried milk and 10% (v/v) newborn calf serum (blocking buffer). Next, the filters are treated overnight at 4°C with the monoclonal antibody of the invention appropriately diluted in blocking buffer.

The filters are then washed three times in Tween-PBS and treated for 1.5 h at room temperature with horseradish peroxidase-labeled rabbit anti-mouse IgG (Dakopatts, Denmark) diluted 1/3000 in blocking buffer. After three washes in Tween-PBS, streptavidine-biotinylated horseradish peroxidase complex (Amersham), diluted 1/250 in blocking buffer, is applied for 1.5 h at room temperature. Thereafter, the filters are washed three times in Tween-PBS and once in PBS. The filters are then incubated in PBS containing 0.05% (w/v) diaminobenzidine and 0.03% (v/v) hydrogen peroxide until background staining develops.

It should be clear that the formation of an immunological complex between the monoclonal antibodies and the antigen is not limited to the precise conditions described above, but that all techniques that respect the immunochemical properties of the antibody and antigen binding will produce similar formation of an immunological complex.

The terms "recognizing", "detecting", "forming an immunological complex with" or "reacting" as used in the present invention particularly relate to specifically recognizing, detecting or reacting.

The expression "phosphorylated human tau peptide" refers to a peptide comprising in its amino acid sequence a phosphorylated sequence of human tau protein extracted from the brains of a patient having died from AD and with said sequence being characterized by the fact that it can specifically form an immunological complex with the antibodies of

the invention.

The term derivatives as used in the present invention designates any known protein or peptide derivative known in the art which mimics the immunological reactivity of the protein or peptide it is derived from.

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Examples

Example 1.

Preparation of the monoclonal antibodies AT100 using tau isolated from an AD patient as antigen

1. Preparation of the antigen for immunization

Tau from an AD patient (or PHF-tau) was partially purified by a modification of the method of Greenberg and Davies (1990). Postmortem tissue, consisting mostly of gray matter from the frontal and temporal cortex, was obtained from histologically confirmed Alzheimer patients. This Alzheimer gray matter brain sample (5-10 g) was homogenized with 10 volumes of cold buffer H (10 mM Tris/1 mM EGTA/0.8 M NaCl/10% sucrose, pH 7.4) in a Teflon/glass Potter S (Braun, Germany) homogenizer. After centrifugation of the homogenate in a 60 Ti MSE rotor at 27,000 x g for 20 min at 4°C, the pellet was removed and the supernatant was adjusted to 1% (wt/vol) N-laurosylsarcosine and 1% (vol/vol) 2-mercaptoethanol and incubated while rotating on a mixer (Swelab, Sweden) for 2.5 hours at 37°C. The supernatant mixture was centrifuged at 108,000 x g for 35 min at 20°C. The PHF-tau containing pellet was gently washed with PBS and finally suspended in 1 ml of the same buffer.

The antigen preparation was evaluated by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blotting using polyclonal rabbit anti-human normal tau antiserum (Mercken et al., 1992a).

2. Immunization protocol and fusion procedure

Balb/c mice were primed subcutaneously with 100 μg of a tau preparation derived from the brain of a patient having died from AD in complete Freund's adjuvant and boosted intraperitoneally 3 times thereafter at 3-week intervals with 100 μg of the same antigen in incomplete Freund's adjuvant. On days 3 and 2 before the fusion, mice were boosted with 100 μg PHF-tau in saline.

Mouse spleen cells were fused with SP2/0 myeloma cells, using a modified procedure of Köhler and Milstein (1975), with PEG 4000.

The cells of the fusion experiment were suspended at a density of 4.5×10^4 spleen

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cells/well on 96-well plates preseeded with mouse peritoneal macrophage cells as a feeder layer. These wells were screened after 12 days of continuous growth for anti-PHF-tau antibody production by means of a sandwich ELISA as detailed below.

Hybridoma growth was performed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum, sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), and nonessential amino acids. All products were purchased from Gibco, (Paisley, U.K.). Cells were incubated in a humidified CO_2-air incubator.

3. Sandwich ELISA for screening for antibodies which react with phosphorylated tau of a patient having died from AD

The screening ELISA used for the detection of monoclonal antibodies directed to phosphorylated epitopes of tau was a sandwich ELISA system with affinity-purified polyclonal rabbit anti-human tau antibodies (Mercken et al., 1992a) in the coating phase. To this end, purified human normal tau, prepared as described in Mercken et al. (1992a) was used for the preparation of an immuno-affinity column using covalent immobilization on cyanogen bromide-activated Sepharose (Pharmacia, LKB Sweden). The affinity-bound anti-tau fraction was eluted from this column with a 0.1 M citric acid buffered solution at pH 2.5. After neutralization, the anti-tau-containing fractions were pooled and coated overnight (1 µg/ml) at 4°C on high-binding microtiter plates (Nunc, Gibco, Paisley, UK) in coating buffer (10 mM Tris, 10 mM NaCl, 10 mM NaN_3, pH 8.5). After overcoating for 30 min with 125 µl 10%-saturated casein in PBS to reduce non-specific binding, the plates were incubated with 100 µl of an appropriately diluted phosphorylated tau isolated from a patient having died from AD preparation and incubated for 60 min at 37°C. The plates were washed 3 times with PBS-0.05% Tween 20 (v/v); 100 µl hybridoma supernatant was added and incubation was continued for 1 h at 37°C. After washing, the bound monoclonal antibodies were detected with peroxidase-conjugated rabbit anti-mouse serum (Dakopatts, Glostrup, Denmark). All reagents were diluted in PBS with 10% casein. After final washing, 100 µl 0.42 mM 3,5,3',5'-tetramethylbenzidine, 0.003% H_2O_2 v/v in 100 mM citric acid, 100 mM disodium hydrogen phosphate, pH 4.3, was added as peroxidase substrate. The reaction was stopped with 50 µl of a 2 M H_2SO_4 solution. Absorbance was read in a Titertek Multiscan (Flow Laboratories, Eflab, Oy, Finland) at 450 nm.

From such a fusion experiment, using the screening procedure as described in

section 3 above, 28 positive cultures (i.e. anti-PHF-tau antibodies-secreting cultures) were retrieved out of a total of 1440 cultures. These positive cultures were arbitrarily designated AT1 to AT28 (some of these hybridoma cultures, i.e. AT1 to AT14 are described by Mercken et al., 1992b). As in this initial screening round positive cultures were mostly found to be composed of mixed clones as seen by visual inspection of the wells (usually between 1 and 4 clones per well), all hybridoma cultures were further subcloned by limiting dilution, a technique well known to those skilled in the art, finally resulting in pure hybridoma clones secreting antibodies with a homogeneous idiotype. Some of these pure hybridoma clones were further tested with respect to their reactivity patterns on normal and phosphorylated tau in ELISA as described in Example II and by Western blotting and to the location of their epitopes. From these results AT100 was selected.

The Western blotting procedure was carried out as follows:

Purified normal human tau and phosphorylated tau derived from the brain of patients having died from Alzheimers disease were applied to 10% SDS-polyacrylamide gels and run under denaturing conditions according to Laemmli (1970).

After SDS-PAGE, the transfer to nitrocellulose (Hybond-C, Amersham, Brussels, Belgium) was carried out in 10 mM NaHCO3, 3 mM Na2CO3, pH 9.9 for 120 min at 55 V with cooling. After blotting, the nitrocellulose was equilibrated to phosphate buffered saline (PBS), and protein binding sites were blocked with blot buffer (PBS supplemented with 5% w/v skimmed dried milk and 10% v/v newborn calf serum). Blotted proteins were incubated overnight at 4°C with the antibody of the respective hybridoma. After three washings with PBS-0.05% Tween 20 (v/v), horseradish peroxidase-labeled rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) were used at a dilution of 1/3000 and were incubated for 90 min at room temperature. All antisera were diluted in blot buffer. The blots were then washed three times in PBS/Tween and developed with substrate solution (PBS, 0.05% w/v 3,3'-diaminobenzidine, 0.03% v/v H2O2) after which the reaction was stopped in H20.

As a result of these analyses, 8 hybridomas out of 28 (including AT3, AT8 (Mercken et al.), AT100, AT180 and AT270 (European Patent Application N° 94303133.7) were found to recognise phosphorylated tau isolated from a patient having died from AD (PHF-tau).

Finally the monoclonal antibodies secreted by the hybridomas AT8, AT100, AT180 and AT270 were screened with extracts of brain samples obtained from fetal brain, adult brain and brain biopsies of non-demented patients or post-mortem tissue of patients having

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died from AD and their reactivity patterns were compared with those obtained with other well-characterized monoclonal antibodies such as T41/46, T1, AT8, T3P, PHF1, AT180 and AT270 (see above). Western blotting analysis indicated that AT100 secreted monoclonal antibodies were clearly distinct from well-known other monoclonal antibodies in that the monoclonal antibodies of the invention reacted only with phosphorylated tau as extracted from AD brain and not with tau and phosphorylated tau which can be found in the brain of human foetuses, of adults or of biopsies derived from patients not suffering from a disease in which the presence of NFT is a pathological hallmark.

4. Determination of the antibody class and subclass

The antibody class and subclass was determined by Inno-LIA (Innogenetics, Ghent, Belgium). The antibodies secreted by AT100 appeared to be of the IgG1, kappa subtypes.

Example 2.

Characterization of antibodies which react with phosphorylated tau extracted from a patient having died from AD and their epitopes

1. Discrimination of phosphorylated tau from AD patients from normal tau in ELISA

The preparation of affinity purified normal tau is described in Mercken et al. (1992b) and for phosphorylated-tau isolated from the brain of a patient having died from AD is essentially as described in Greenberg and Davies (1990); Mercken et al. (1992a). Purity of normal tau and phosphorylated-tau standards was determined by SDS-PAGE. The samples were also analyzed on 420 A/H amino acid analyzer (Applied Biosystems B.V., Maarssen, The Netherlands) according to the manufacturer's instructions. Both normal and phosphorylated tau showed the expected amino acid compositions. The exact protein concentration of both affinity purified normal and phosphorylated tau was determined using an internal standard peptide.

The monoclonal antibodies derived from the hybridomas AT100 and purified from serum-free conditioned medium by Protein G column chromatography, were coated overnight at 4°C on high-binding microtiter plates (Nunc, Gibco, Paisley, UK) in coating buffer at 3 μg/ml (10 mM Tris, 10 mM NaCl, 10 mM NaN_3, pH 8.5). After overcoating for

30 min with 150 µl 10%-saturated casein in PBS to reduce non-specific binding, the plates were incubated with 100 µl of an appropriately diluted tau preparation or of a phosphorylated tau preparation derived from the brain of a patient having died from D as standards and incubated for 60 min at 37 °C. The plates were washed 5 times with PBS-0.05% Tween 20 (v/v) and 100 µl of two biotinylated antibodies (AT120 and HT7, Vandermeeren et al., 1993; Mercken, Ph. D. thesis) at a final concentration of 0.2 µg/ml was added and incubated for 1 hr at room temperature. After washing, horse-radish peroxidase conjugated streptavidine (Jackson, Innogenetics, Belgium) at a dilution of 1/10000 was added for 30 min at room temperature. Following a final washing with PBS/Tween 20, 100 µl of 0.42 mM 3,5,3',5'-tetramethylbenzidine, 0.003 % (vol/vol) H_2O_2 in 100 mM citric acid, 100 mM Na_2HPO_4, pH 4.3 were added as peroxidase substrate for 30 min at room temperature. The reaction was stopped with 50 µl of a 2 M H_2SO_4 solution. Absorbance was read in a Titertek Multiscan (Flow Laboratories, Eflab Oy, Finland) at 450 nm.

Under conditions in which 500 ng antigen was present, AT100 did not react with tau derived from patients having died of diseases in which NFT are not a pathological hallmark.

2. Mapping of the AT100 epitope to the proline-rich region

100 µg purified AD specific tau proteins (Greenberg and Davies, 1990 or Bramblett et al., 1993) was digested with Endoprotease AspN (Boehringer) according to the manufacturer's instructions. The digested proteins were loaded on an SDS-tricine acrylamide gel (16.5%, Schägger et al., 1987). Gels were blotted on PVDF membranes, blocked and AT100 reactive bands were revealed. Two bands were immunoreactive and N-terminally sequenced. Results of the sequence analysis is shown in Table 1. Based on these results, the epitope of AT100 can be condluded to lie in the region spanning positions 146 to 251, more particularly in the region spanning positions 198 to 251, of the tau protein.

Example 3.

Reactivity of monoclonal antibodies each recognising a different phosphorylated tau epitope on extracts of human tau derived from AD brain, fetal brain or biopsies obtained from patient suffering of diseases for which the presence of NFT is not a pathological hallmark.

Brain extracts were prepared from human biopsy samples, obtained from lateral temporal lobe, from autopsy-derived fetal brain, from autopsy-derived human adult tau (11 hr postmortem time) or from autopsy-derived human brain from patients having died of AD as described above. Samples of these extracts were loaded onto 10 % SDS-PAGE gels and after electrophoresis were blotted onto nitrocellulose sheets (Wester, blotting). The sheets were probed with each of the following antibodies

Antibody code	Recognition site/epitope
AT270	phosphothreonine (S181)
AT8	phosphoserine (S202, T205)
AT180	phosphothreonine (S231)
T3P	phosphoserine (S396, S404)

and counterstained with ^{125}I-labeled goat anti-mouse IgG. Under these circumstances, a clear signal is obtained with extracts from autopsy-derived human brain from patients having died from AD, a statistically non-significant signal is present for biopsy-derived tau, while fetal-derived and adult-derived tau are also negative or non-significant. In contrast, other monoclonal antibodies are positive for fetal-derived and/or biopsy-derived adult tau, as well as for phosphorylated tau derived from Alzheimer disease brains.

Table 1

Band	MW	AT100	Sequence	Positions
1	2 kD	-	NNNNGTKIG G L	259-282
2	2 kD	-		
3	3 kD	-		
4	4 kD	-	DNIRRIP KNVHAA GAVH ARKG P	146-197
5	14 kD	+	DGTRVIAGVSXXKGG SPE AST K L	146-251
6	15 kD	-		
7	16 kD	-		
8	17 kD	-		
9	18 kD	+	DGPEQXD ERA L	54-251

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CLAIMS

1. A monoclonal antibody which forms an immunological complex with a phosphorylated epitope of an antigen present in a particular subclass or form of phosphorylated tau protein without forming an immunological complex with (i) fetal tau or (ii) biopsy or autopsy derived brain material from individuals suffering or having died from diseases in which NFT is not a pathological hallmark.

2. A mononclonal antibody according to claim 1, which is further characterized in that it forms an immunological complex with a phosphorylated epitope of a particular subclass or form of phosphorylated tau protein, with said epitope residing in the region spanning positions 146-251 with the following amino acid sequence:

```
      146             150
NH2- Asp Gly Lys Thr Lys Ile Ala Thr Pro Arg

                  160                                         170
Gly Ala Ala Pro Pro Gly Gln Lys Gly Gln Ala Asn Ala Thr Arg

                                          180
Ile Pro Ala Lys Thr Pro Pro Ala Pro Lys Thr Pro Pro Ser Ser

                  190                                         200
Gly Glu Pro Pro Lys Ser Gly Asp Arg Ser Gly Tyr Ser Ser Pro

                                          210
Gly Ser Pro Gly Thr Pro Gly Ser Arg Ser Arg Thr Pro Ser Leu

                  220                                         230
Pro Thr Pro Pro Thr Arg Glu Pro Lys Lys Val Ala Val Val Arg

                                          240
Thr Pro Pro Lys Ser Pro Ser Ser Ala Lys Ser Arg Leu Gln Thr

                  250
Ala Pro Val Pro Met Pro COOH
```

(SEQ ID NO 1)

3. A monoclonal antibody according to claim 1, characterized by the fact it specifically forms an immunological complex:

- either with a phosphorylated epitope located within the sequence defined in claim 1 (SEQ ID NO 1),

- or with any other phosphorylated peptide capable of forming an immunological complex with a monoclonal antibody, which itself is capable of forming a complex with a phosphorylated epitope located within the tau protein region as defined in claim 1 (SEQ ID NO 1).

4. A monoclonal antibody termed AT100 according to any of claims 1 to 3 secreted by the hybridoma deposited at ECACC on April 21, 1994 under No. 94042117.

5. A hybridoma, which secretes a monoclonal antibody according to any of claims 1 to 4, more particularly the hybridoma deposited at ECACC on April 21, 1994 under No. 94042117.

6. A phosphorylated peptide capable of forming an immunological complex with a monoclonal antibody according to any of claims 1 to 4, with said peptide being in the phosphorylated form, and - with the sequence of said peptide comprising, or consisting of, phosphorylated parts or derivatives of the sequence as shown in SEQ ID NO 1, or,
- with the sequence of said peptide comprising, or consisting of, peptides capable of forming an immunological complex with a monoclonal antibody according to any of claims 1 to 4.

7. A phosphorylated peptide according to claim 6, which is capable of generating a monoclonal antibody according to any of claims 1 to 4 upon immunization.

8. A process for obtaining and isolating a hybridoma according to claim 5, secreting a monoclonal antibody according to any of claims 1 to 4, characterized in that it involves at least the steps of:
(a) starting from the spleen cells of an animal, e.g. mouse or rat, previously immunized *in vivo*, or from spleen cells of such cells previously immunized *in vitro*, with an antigen being chosen from the group of:
- phosphorylated tau present in brain extracts derived from the brains of patients having died from AD, or,
- a phosphorylated human tau peptide according to claim 6 or 7, or,
- immunoaffinity purified phosphorylated tau isolated from the brains of a patient having died from AD,

recognized by a monoclonal antibody as defined in any of claims 1 to 4;

(b) fusing said immunized cells with myeloma cells under hybridoma-forming conditions; and,

(c) selecting those hybridomas which secrete monoclonal antibodies which specifically recognize a phosphorylated epitope of a particular subclass or form of phosphorylated tau as determined by western blot or by ELISA without recognizing an epitope of biopsy or autopsy derived brain tau material from patients having died or suffering from diseases in which NFT is not a pathological hallmark.

9. Monoclonal antibodies according to any of claims 1 to 4 obtainable by a process comprising at least the following steps:

(a) starting from the spleen cells of a mouse previously immunized *in vivo*, or from spleen cells of such cells previously imunized *in vitro,* with an antigen chosen from the following group:

- phosphorylated tau extracted and purified from a human brain sample of a patient having died from AD, or,
- a phosphorylated human tau peptide according to any of claims 6 or 7, or,
- immunoaffinity-purified phosphorylated tau from the brain of a patient having died from AD,

recognized by a monoclonal antibody according to any of claims 1 to 4;

(b) fusing said immunized spleen cells with myeloma cells under hybridoma-forming conditions,

(c) selecting those hybridomas which secrete monoclonal antibodies which specifically recognize a particular form or subclass of phosphorylated tau without recognizing an epitope of biopsy or autopsy derived brain tau material derived from patients having died or suffering from diseases in which NFT is not a pathological hallmark as determined by western blot analysis or ELISA,

(d) culturing the selected hybridomas according to claim 5 or the hybridomas such as obtained through the process of claim 8, in an appropriate medium culture; and

(e) recovering the monoclonal antibodies excreted by said selected hybridomas; or alternatively:

(f) implanting the selected hybridomas of claim 5, or the hybridomas obtained through the process of claim 8, into the peritoneum of a mouse and, when ascites has been produced by the animal,

(g) recovering the monoclonal antibodies then formed from said ascites.

10. A process for producing monoclonal antibodies according to any of claims 1 to 4 which involves at least the steps of:
(a) culturing the selected hybridomas according to claim 5 or the hybridomas such as obtained through the process of claim 8, in an appropriate medium culture; and
(b) recovering the monoclonal antibodies excreted by said selected hybridomas; or alternatively:
(c) implanting the selected hybridomas of claim 5, or the hybridomas obtained through the process of claim 8, into the peritoneum of a mouse and, when ascites has been produced by the animal,
(d) recovering the monoclonal antibodies then formed from said ascites.

11. Process for *in vitro* detection or diagnosis of brain/neurological disease involving a aprticular subclass or form of phosphorylated tau protein, such as Alzheimer's disease, which comprises at least the following steps:
(a) contacting a monoclonal antibody according to any of claims 1 to 4, with a preparation of NFT, PHF or a detergent-extracted brain homogenate isolated from a patient having had Alzheimer's disease or any other disease involving phosphorylated tau under conditions suitable for producing an antigen-antibody complex; and
(b) detecting the immunological binding of said antibody to said brain homogenate, and possibly separating the antigen from said complex and possibly recovering the antigen sought in a purified form.

12. Process for the detection or diagnosis *in vitro* of brain disease involving a particular subclass or form of phosphorylated tau protein, such as Alzheimer's disease, which includes:
- bringing a sample of CSF, preferably unconcentrated CSF, or of serum from a patient suspected of suffering from a neurological disorder involving NFT, more particularly Alzheimer's disease, or proteins or polypeptides extracted therefrom, into contact under *in vitro* conditions with a monoclonal antibody according to any of claims 1 to 4, with said conditions suitable for producing an antigen-antibody complex; and,
- detecting the immunological binding of said antibody to said sample of cerebrospinal fluid, or of serum, or extract thereof.

13. Process for the detection of AD according to claim 12, further chracterized in that said detection step comprising at least the steps of bringing together said antigen-antibody complex formed by the antigen and the antibodies of the invention with:

(a) a second antibody

* which can be a monoclonal antibody recognizing an epitope of tau protein or phosphorylated tau protein, or of any tau peptide carrying an epitope, with said epitope being different from the one of the invention, or

* which can be a polyclonal antibody recognizing tau protein or phosphorylated tau or a polyclonal antibody recognizing a tau peptide, with said polyclonal antibody being capable of forming an immunological complex with epitopes which are different from the epitope of the invention, with said polyclonal antibody being preferably purified by immunoaffinity chromatography using immobilized tau protein or phosphorylated tau protein;

(b) a marker either for specific tagging or coupling with said second antibody, with said marker being any possible marker known to the person skilled in the art;

(c) appropriate buffer solutions for carrying out the immunological reaction between the monoclonal antibody of the invention and a test sample on the one hand, and the bound second antibody and the marker on the other hand, and,

(d) possibly also a peptide carrying a phosphorylated epitope of tau comprised in the region spanning SEQ ID NO 1 for standard purposes, or for competition purposes with respect to the antigen which is sought.

14. Kit for the diagnosis *in vitro* of one of the following diseases: Alzheimer's disease, Down's syndrome, Pick's disease and other neurological disorders in which phosphorylated tau protein or paired helical filaments are implicated, characterized in that the kit comprises:

(a) at least a microplate for deposition thereon of at least a monoclonal antibody according to any of claims 1 to 4;

(b) a second antibody

* which can be a monoclonal antibody recognizing an epitope of tau protein or phosphorylated tau protein, or of any phosphorylated tau peptide carrying an epitope of phosphorylated tau, with said epitope being different from the one of the invention, or

* which can be a polyclonal antibody recognizing tau protein or phosphorylated tau

or a polyclonal antibody recognizing a peptide carrying an epitope of phosphorylated tau, with said polyclonal antibody being capable of forming an immunological complex with epitopes which are different from the epitope of the invention, with said polyclonal antibody being preferably purified by immunoaffinity chromatography using immobilized tau protein;

(c) a marker either for specific tagging or coupling with said second antibody;

(d) appropriate buffer solutions for carrying out the immunological reaction between the monoclonal antibody of the invention and a test sample on the one hand, and the bound second antibody and the marker on the other hand,

(e) possibly a peptide carrying an epitope of phosphorylated tau comprised in the region as defined in claim 1 (SEQ ID NO 1) for standard purposes, or for competition purposes with respect to the antigen which is sought.

15. Kinase which upon acting on non-phosphorylated tau is capable of specifically introducing a phosphorylation in the region as specified in SEQ ID NO 1, thereby giving rise to the epitope recognized by a monoclonal antibody according to any of claims 1 to 4.

16. Phosphorylase which is capable of specifically reacting with an epitope comprised in SEQ ID NO 1, with said epitope being recognized by a monoclonal antibody according to any of claims 1 to 4.

17. Method for screening for compounds which interefere the activity of a kinase acccording to claim 15, comprising at least the following steps:

- bringing into contact non-phosphorylated tau or a non-phosphorylated peptide of which the sequence comprises or consists of at least parts of the sequence specified in SEQ ID NO 1,
- with a kinase capable of phosphorylating this tau protein sequence, thereby producing the epitope recognized by a monoclonal antibody according to any of claims 1 to 4,
- and any compound which is capable to interfere with the process of phosphorylation of said epitope by said kinase,
- and detecting the amount of phosphorylation by means of an assay detecting the reactivity with a monoclonal antibody according to any of claims 1 to 4.

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18. Method for screening for compounds which interefere with the activity of the phosphorylase of claim 16 comprising at least the steps of:

- bringing into contact phosphorylated tau or a phosphorylated peptide of which the sequence comprises or consists of at least parts of the sequence specified in SEQ ID NO 1,
- with a phosphorylase capable to remove phosphate from said phosphorylated tau or said phosphorylated peptide,
- and detecting the amount of dephosphorylation by means of a method detecting the reactivity with a monoclonal antibody according to any of claims 1 to 4.

INTERNATIONAL SEARCH REPORT

Internatio Application No
PCT/EP 95/03032

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/18 C12N5/20 C07K14/47 C12N15/06 C12P21/08
G01N33/577 G01N33/68 C12N9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N C12P G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ACTA NEUROPATHOLOGICA, vol. 84, no. 3, 1992 BERLIN, GERMANY, pages 265-272, M. MERCKEN ET AL. 'Monoclonal antibodies with selective specificity for Alzheimer Tau are directed against phosphatase-sensitive epitopes.' cited in the application see the whole document ---	1-14
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO,A,93 11231 (MAX PLANCK GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.) 10 June 1993 see table 1 see examples 7,10,12 see claims	1-18
X	EP,A,0 544 942 (MAX PLANCK GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.) 9 June 1993 see table 1 see examples see claims	1-18
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF NEUROCHEMISTRY, vol. 61, no. 5, November 1993 NEW YORK NY, USA, pages 1828-1834, M. VANDERMEEREN ET AL. 'Detection of tau proteins in normal and Alzheimer's disease cerebrospinal fluid with a sensitive sandwich enzyme-linked immunosorbent assay.' cited in the application see page 1829, left column, line 35 - line 44 see abstract ---	1-14
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 89, no. 12, June 1992 WASHINGTON DC, USA, pages 5384-5388, B. LICHTENBERG-KRAAG ET AL. 'Phosphorylation-dependent epitopes of neurofilament antibodies on tau protein and relationship with Alzheimer tau.' see the whole document ---	1-14
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A	WO,A,94 13795 (N.V. INNOGENETICS S.A.) 23 June 1994 see examples see claims ---	1-14
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat[illegible] Application No
PCT/EP 95/03032

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WO 01/90757 A1

(54) Title: ALZHEIMER'S DISEASE ASSAY

(57) Abstract: The present invention relates to a method for the detection of a marker protein (acetylcholinesterase or amyloid beta precursor protein) in a sample of saliva, that may be used as a diagnostic test for Alzheimer's disease or as marker of response to therapeutic intervention of a patient.

ALZHEIMER'S DISEASE ASSAY

The present invention is concerned with a method for the detection of a marker protein that may be used as a diagnostic test for Alzheimer's disease or as a marker of response to current therapeutic intervention.

Alzheimer's disease (AD) is a chronic neurodegenerative disorder. While there is widespread nerve cell death, the neurons affected primarily are those producing the neurotransmitter acetylcholine (ACh) (Whitehouse, et al., 1981, Struble, et al., 1982, Mesulam and Geula, 1988). This plays a major role in the processes underlying the generation of memory and learning and the early stages of the disease are characterised primarily by a loss of short-term memory (Reisberg, 1984).

The current therapy available for AD is to inhibit the enzyme that breaks down ACh called acetylcholinesterase (AChE) (Rogers, et al., 1996, Jann, 2000). These agents are only useful in the early stages of the disease while the cholinergic neurons are still partially active. In the later stages, however, there is a complete loss of these neurons and the drugs no longer have a therapeutic potential (Rosler, et al., 1999).

There is currently no suitable test available that may provide an indication as to whether a patient will respond to treatment. Since the severity of the clinical symptoms does not always correlate with the biochemical events associated with the disease, it is difficult to predict the level of degeneration and the level of responsiveness of a given patient to a specific therapeutic approach.

Previous studies have suggested a potential change in the activity of acetylcholinesterase (AChE) in serum and cerebrospinal fluid in people with AD. However, there has been no consensus among these findings. The level of serum AChE has been reported in different studies to be decreased (Yamamoto, et al., 1990) or unchanged (McQueen, 1995). A similar inconsistency has been demonstrated in cerebrospinal fluid (CSF) where there are reports of a

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decrease (Soininen, et al., 1981, Appleyard, et al., 1983, Nakano, et al., 1986) although these results were not observed in other studies (Lal, et al., 1984, Huff, et al., 1986). Furthermore, there is no evidence for a correlation of CSF AChE with disease progression (Elble, et al., 1987). More recently, there have been reports of an alteration in the chemical properties (glycosylation) of AChE in the cerebrospinal fluid of people with AD (patent application no. WO99/15695, Saev-Valero, et al., 1997, Saez-Valero, et al., 1999). However, the acquisition of these samples is a particularly invasive procedure and is unlikely to be suitable for routine clinical use. There have been no reports to date of altered enzyme glycosylation being detected in serum samples of people with AD.

There remains a need for the development of a robust diagnostic test for AD that is reliable but yet minimally-invasive, to permit the assay to be carried out on a routine basis. There is a further requirement for a test to predict the reaction of individual people to the current therapeutic strategies available. Thirdly, the development of such an assay system may be useful to chart the rate of disease progression and to monitor the effectiveness of future therapeutic interventions.

It is an object of the present invention to obviate and/or mitigate at least one of the aforementioned disadvantages.

The present invention is based in part on the observations by the present inventor that acetylcholinesterase and amyloid β precursor protein peptide can be detected in a sample of saliva.

Thus in a first aspect the present invention provides an assay for detecting the presence of a protein in a sample of saliva, wherein altered levels or forms of the protein have been postulated or are known to be involved in and/or associated with Alzheimer's disease, comprising the steps of:

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- obtaining a sample of saliva from a subject;
- contacting the sample of saliva with a reagent or reagents capable of reacting with any of the protein if present in the sample of saliva; and
- detecting any reaction products obtained.

Hitherto, there has been no suggestion in the art that proteins, the altered levels or forms of which have been postulated or are know to be involved in and/or associated with Alzheimer's disease or the progression of Alzheimer's disease could be detected in saliva. Previous wisdom has taught that serum or cerebrospinal fluid samples were the only suitable samples for use in detecting such proteins. However, it will be appreciated that the taking of such samples are generally more invasive or complex than obtaining a sample of saliva. Moreover, due to the medical condition of the patient such prior techniques can be impractical. The present invention therefore provides a more simple and practical assay for conducting on test subjects. Additionally, in view of the simplicity of the assay it may be possible to conduct a comprehensive screening program in order to facilitate the early detection of Alzheimer's disease and consequent early treatment. Additionally, the present assay may be used to ascertain if medical intervention is proving successful, or if medical intervention may be predicted to be of use. For example, previous teachings have shown that acetylcholinesterase may be increased, decreased or unchanged in Alzheimer's disease patients. In patients with increased levels of acetylcholinesterase, it may be appropriate to treat with acetylcholinesterase inhibitors. However, once levels of acetylcholinesterase have significantly decreased treatment with acetylcholinesterase inhibitors may not be appropriate.

Moreover, treatment with acetylcholinesterase inhibitors may be monitored by use of the assay. That is, the effectiveness to decrease acetylcholinesterase activity with acetylcholinesterase inhibitors may be monitored.

However, if a low level of acetylcholinesterase activity is detected using the present assay, it may be desirable to carry out a further immunological test employing for example an antibody to acetylcholinesterase in order to ensure that the low levels as determined by the enzymic assay are due to the effectiveness of the acetylcholinesterase inhibitors, rather than a decrease in the level of acetylcholinesterase, when treatment with acetylcholinesterase inhibitors may no longer be necessary/appropriate. This has hitherto not been practical or possible with the currently available tests.

The protein to be detected may be any protein the altered level or form of which has been postulated or is known to be involved in and/or associated with Alzheimer's disease. For example the protein may be the enzyme acetylcholinesterase or amyloid β precursor protein, as well as variant forms (eg. variants displaying altered glycosylation) or degredative products of any of the proteins examined.

The sample of saliva may be obtained by any suitable means. For example a swab, dipstick or the like may be used, as is well known in the art. Alternatively the subject may be asked to spit into a receptacle. It has been found that relatively small samples of saliva, such as less than 1ml, preferably less that 0.6ml may be obtained and are suitable for testing.

For suitable testing of, for example, acetylcholinesterase activity in saliva, it has been determined that a total protein concentration of between about 15μg to 80μg, preferably 25μg to 75μg, such as about 50μg is appropriate for testing.

The sample of saliva is contacted, typically mixed, with a reagent or reagents capable of reacting with the protein. For example, in the case of an enzyme, such as acetylcholinesterase, the reagent or reagents may comprise a substrate for the enzyme which upon enzymic reaction yields a product which may be detected colourimetrically,

fluorimetrically or the like. Specifically the presence of acetylcholinesterase may be detected by its action on acetylthiocholine, yielding a colour change which may be detected spectrophotometrically.

Alternatively the protein may be detected by way of immunoassay. Thus, the reagent or reagents may comprise an antibody specific for the protein to be detected. The reaction of the protein with the antibody generating a protein/antibody complex which may be detected by suitable means, such as radiometrically, colourmetrically or fluorometrically. For example a test such as an enzyme-linked immunosorbent assay (ELISA) may be developed as is well known to the skilled addressee.

In one embodiment the present invention provides both an enzymic assay and an immunological assay, both of which may be used to test for acetylcholinesterase in order, for example, to see if treatment with acetylcholinesterase inhibitors is effective or inappropriate/unnecessary.

In a further aspect the present invention provides a kit for use in conducting an assay according to the present invention, the kit comprising:

sampling means for obtaining a sample of saliva from a test subject; and

a reagent or reagents capable of reacting with any protein to be detected in the sample of saliva.

The present invention will now be further described by way of example only and with reference to the Figures which show:

Figure 1 shows the detection of acetylcholinesterase in a sample of saliva and the effect of altered incubation times on salivary AChE activity;

Figure 2(a) shows the activity of acetylcholinesterase to be linear in relation to changes in protein levels;

Figure 2(b) shows the detection of acetylcholinesterase by Western blot analysis in a sample of saliva;

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Figure 3 shows the detection of amyloid β precursor protein by Western blot analysis in a sample of saliva;

Figure 4 shows acetylcholinesterase activity with varying protein concentrations;

Figure 5 shows the results of the measurement of acetylcholinesterase activity in individuals saliva samples (n = 4 ± SEM);

Figure 6 shows the effects of smoking cigarettes on acetylcholinesterase activity in saliva (n = 3 ± SEM);

Figure 7 shows acetylcholinesterase activity measured in saliva of people of varying ages (n = 3 ± SEM);

Figure 8 shows a comparison of acetylcholinesterase activity in saliva taken from males and females (n = 3 ± SEM, p = 0.01);

Figure 9 shows a comparison of acetylcholinesterase activity taken from patients thought to have Alzheimer's disease (AD) patients compared against female control samples; and

Figure 10a and b show the effects of varying neostigmine concentration on acetylcolinesterase and Butyrylcholinesterase activity, respectively, in saliva (n = 15).

Example 1: Detection of acetylcholinesterase in saliva

Acetylthiocholine (Sigma). All other chemicals were of AnalaR grade.

Salivary AChE enzyme activity.

Saliva samples were obtained from healthy volunteers of varying age and sex. The samples were collected in a polyethylene sample tube and centrifuged at 15,000 rpm for 3 minutes to pellet any particulate matter. The samples were stored at 4°C for immediate use or at -20°C for long-term storage. Determination of enzyme activity was carried out by a colorimetric assay system using acetylthiocholine as a substrate (Ellman, et al., 1961). The saliva sample (500μl) was added to the reaction buffer (0.3mM dithionotrobenzoic acid (DTNB) in 0.1M phosphate buffer, pH

8.0) in a total volume of 1.0ml. The reaction mixture was equilibrated at 37°C for 10 minutes prior to the commencement of the reaction. The reaction was started by the addition of 10μl of 0.06M acetylthiochcholine and the absorbance at 412nm was determined at 5 minute intervals for up to 30 minutes. The protein content of the sample was assayed according to the method of Bradford et al (1976) and specific enzyme activity normalised to protein content. The expression of specific proteins was determined by Western blot analysis.

The catalytic activity of enzyme was determined to be linear with regards to both the duration of the reaction (Figure 1) as well as to changes in protein levels (Figure 2). A 15-minute time-point was chosen for all subsequent reactions. The enzyme activity was specific as denaturation of the protein sample resulted in a loss of activity. This demonstrates that the breakdown of the acetylthiocholine substrate was enzyme-catalysed rather than a spontaneous even associated with the incubation process. Expression of the enzyme protein in saliva was confirmed by western blot analysis using a commercially-available antibody that is specific for the AChE enzyme (Transduction Laboratories, USA), see Figure 2b.

Multiple samples were obtained from three individual subjects over a period of 3 consecutive days. The samples were obtained at the same time of day before lunch in order to minimise any variation associated with food consumption. The subjects were asked to drink 200ml of mineral water 5 minutes before providing the sample. This assisted in obtaining samples of similar volumes and protein content. There was a only a small inter- and intra-sample variation (see Tables 1 and 2). The freezing of the samples at - 20°C for storage followed by thawing did not alter the enzyme activities in the samples (see Table 3).

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Table 1

Reproducibility of salivary AChE assay. Samples were taken from subject on three consecutive days.

	Subject 1	Subject 2	Subject 3
Day 1	0.030	0.108	0.065
Day 2	0.032	0.140	0.052
Day 3	0.035	0.088	0.039
Mean	0.032	0.112	0.052
SEM	0.001	0.015	0.007

The difference between the individual samples was found to be significant (ANOVA, F=17.957, p=0.029).

Table 2

Repeated assay of single salivary sample:

	Subject 1	Subject 2
Assay 1	0.08	0.014
Assay 2	0.07	0.010
Mean	0.075	0.012
Range	0.005	0.001

Table 3

The effect of freeze-thawing on AChE activity in a single sample:

Prior to freezing:	0.128
After freezing	0.153

Example 2: Detection of amyloid β peptide in saliva

A similar test to that described in Example 1 was conducted to detect amyloid β peptide.

The amyloid β precursor protein (AβPP) is abnormally broken down in AD to generate the Aβ peptide that is the core element of the amyloid plaques, characteristic of the disease. Detectable amounts of the precursor were identified in saliva (Figure 3) using the commercially available 22C11 monoclonal antibody (Roche Diagnostics) thus allowing for the possibility of detecting altered forms of the protein, or the Aβ peptide, in saliva.

Example 3: Modified procedure for acetylcholinesterase detection in saliva

The assay procedure is as follows:

The tubes contain:
Saliva (50μg protein).
10μl 0.1M phosphate buffer.
10μl dithiobisnitrobenzoic acid (DTNB) in phosphate buffer.
Water - to make volume up to 100μl.

Then 10μl of 0.06M acetylthiocholine in phosphate buffer is added to start the reaction (making the final volume 110μl).
Rest of procedure as detailed in Example 1.

Example 4: Determination of appropriate protein concentration for testing

Assays were carried out on saliva samples containing varying concentrations of protein. Figure 4 shows that concentrations less than about 10μg may be problematic and concentrations greater than 50μg are not necessary.

Example 5: Testing of various parameters and influence on acetylcholinesterase activity

Figure 5 shows the variation in measurement of acetylcholinesterase activity in individuals. Although it can be seen that activity can vary quite significantly, mean absorbance is around about 0.04 and the lowest absorbance was about 0.01. This is still significantly higher than the majority of patients thought to have Alzheimer's disease as shown in Figure 9.

Figure 6 shows that smoking cigarettes does not appreciably have an effect on mean acetylcholinesterase activity, although the variation in levels of activity is more acute.

Figure 7 shows that age does not appear to have a correlative effect on levels of acetylcholinesterase activity, although Figure 8 suggests that females generally have lower levels of acetylcholinesterase than males.

Figure 9 as mentioned above compares the acetylcholinesterase activity in saliva taken from patients thought to have Alzheimer's disease compared against female controls. Alzheimer's disease patients were tested. 5 patients show very little or no acetylcholinesterase activity (ie. absorbance equal to negative control). It is unlikely therefore that current treatment with acetylcholinesterase inhibitors would be of any benefit. One patient has a significant level of acetylcholinesterase activity and may not actually have Alzheimer's disease, but some other form of dementia. Alternatively, this patient may have Alzheimer's disease and would benefit from acetylcholinesterase inhibitor treatment.

Figures 10a and 10b show that the hydrolysis of acetylthiocholine is catalysed solely by acetylcholinesterase as all of the enzyme activity is blocked by the specific inhibitor neostigmine. Butyrylcholinesterase (BuChE) is an enzyme with some substrate overlap with acetylcholinesterase. Neostigmine inhibits AChE but not BuChE so a test was made to check

that the enzymatic activity being measured was in fact due to AchE and not BuChE. The assay employed a substrate, acetylthiocholine, that is acted on primarily by BuChE, but also to a lesser extent by AchE. Breakdown of the substrate was inhibited by neostigmine suggesting that any breakdown of acetylthiocholine was, in fact, due to non-specific AchE activity and therefore, by inference, there is minimal BuChE activity in saliva.

In view of the foregoing the results show the usefulness of assaying acetylcholinesterase activity in saliva samples. The assay may be particularly useful in

a) the definitive diagnosis of advanced Alzheimer's disease;

b) identification of patients that would benefit from acetylcholinesterase inhibitor therapy; and/or

c) identification of patients who should stop therapy, probably with an additional immunoassay to confirm or otherwise that a low level of acetylcholinesterase is due to inhibition and not lack of acetylcholinesterase.

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CLAIMS

1. An assay for detecting the presence of a protein in a sample of saliva, wherein altered levels or forms of the protein have been postulated or are known to be involved in and/or associated with Alzheimer's disease, comprising the steps of:

- obtaining a sample of saliva from a subject;
- contacting the sample of saliva with a reagent or reagents capable of reacting with any of the protein if present in the sample of saliva; and
- detecting any reaction product or products obtained.

2. The assay according to claim 1 wherein the protein to be detected is acetylcholinesterase, amyloid β precursor protein, variant forms thereof, or degredative products thereof.

3. The assay according to either of claims 1 or 2 wherein the sample of saliva is less than 1ml.

4. The assay according to any proceeding claim wherein the protein is an enzyme and the reagent or reagents comprise a substrate for the enzyme which upon enzymatic reaction yields a detectable product.

5. The assay according to claim 4 wherein the product is detected by colourimetric or fluorimetric means.

6. The assay according to claim 5 wherein the enzyme is acetylcholinesterase which is detected by its action on acetylcholine, yielding a colour change which is detected spectrophotometrically.

7. The assay according to any one of claims 1 to 3 wherein the protein is detected by way of immunoassay.

8. The assay according to claim 7 wherein the reagent or reagents comprise an antibody specific for the protein to be detected.

9. The assay according to claim 8 wherein the reaction of the protein with the antibody generates a protein/antibody complex which is detectable by radiometric, colourmetric or fluorometric means.

10. The assay according to claim 9 in the form of an enzyme - linked immunosorbent assay (ELISA).

11. A kit for use in conducting an assay according to any preceding claim, the kit comprising:

sampling means for obtaining a sample of saliva from a test subject; and

a reagent or reagents capable of reacting with any protein to be detected in the sample of saliva.

12. A kit for use in conducting an enzymic assay according to any one of claims 4 to 6 and an immunoassay according to any one of claims 7 to 10, the kit comprising:

sampling means for obtaining a sample of saliva from a test subject;

a substrate for the enzyme which upon enzymic reaction yields a detectable product; and

an antibody specific for the protein to be detected.

13. Use of the assay according to any one of claims 1 to 10 as a diagnostic test for Alzheimer's disease in a test subject.

14. Use of the assay according to any one of claims 1 to 10 as a marker of response to therapeutic intervention of a subject.

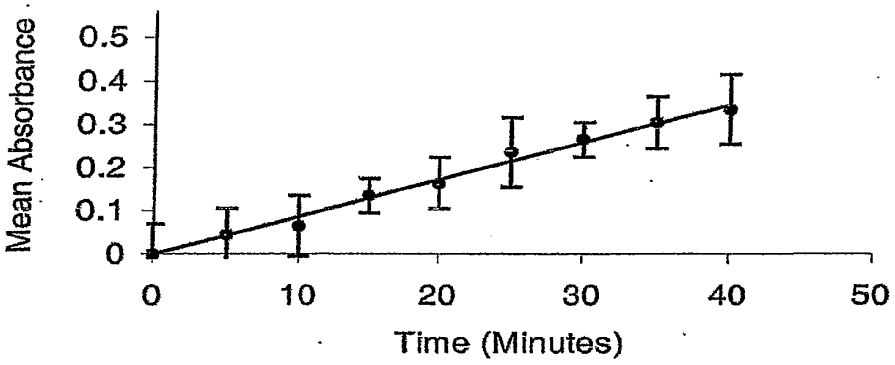
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15. Use of an enzymic assay according to any one of claims 4 to 6 and an immunoassay according to any one of claims 7 to 10 to determine whether or not therapeutic intervention as being provided to a patient is efficacious and/or appropriate.

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Figure 1

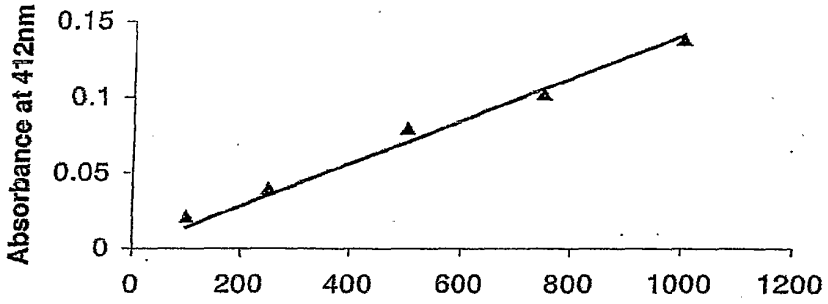
The effect of altered incubation times on salivary AChE activity



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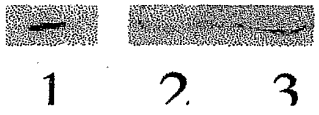
Figure 2

A



B The expression of AChE in saliva as determined by Western blot analysis using a commercially available antibody.

Lane 1: Rat brain (control)
Lane 2: 50µg salivary protein
Lane 3: 100µg salivary protein



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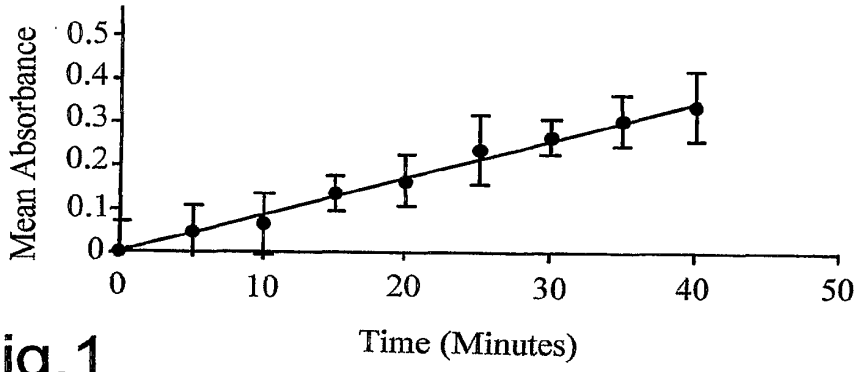


Fig.1

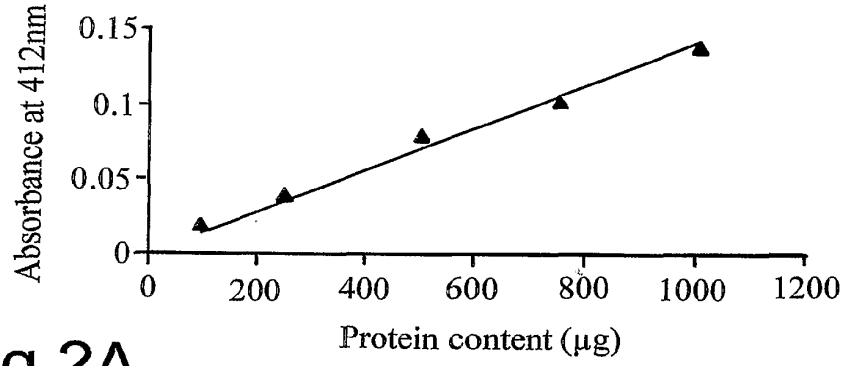
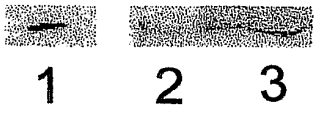


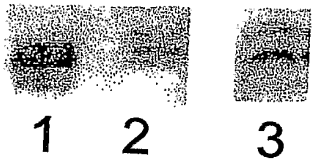
Fig.2A

Fig.2B



Lane 1: Rat brain (control)
Lane 2: 50µ salivary protein
Lane 3: 100µ salivary protein

Fig.3



Lane 1: Rat brain (control)
Lane 2: SHSY-5Y human neuroblastoma cells (control)
Lane 3: Saliva sample

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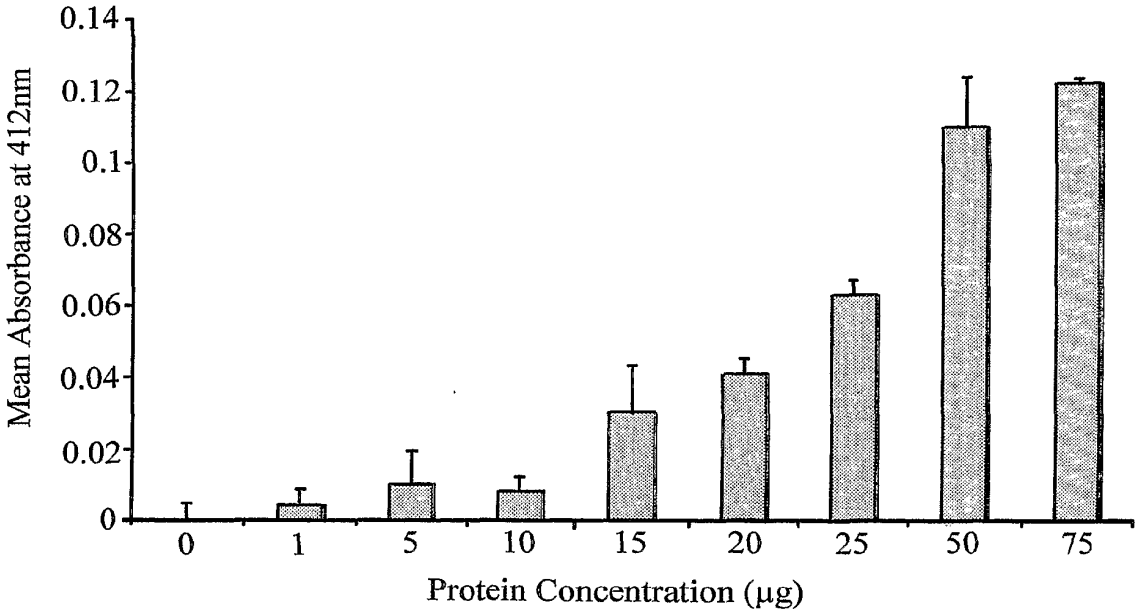


Fig. 4

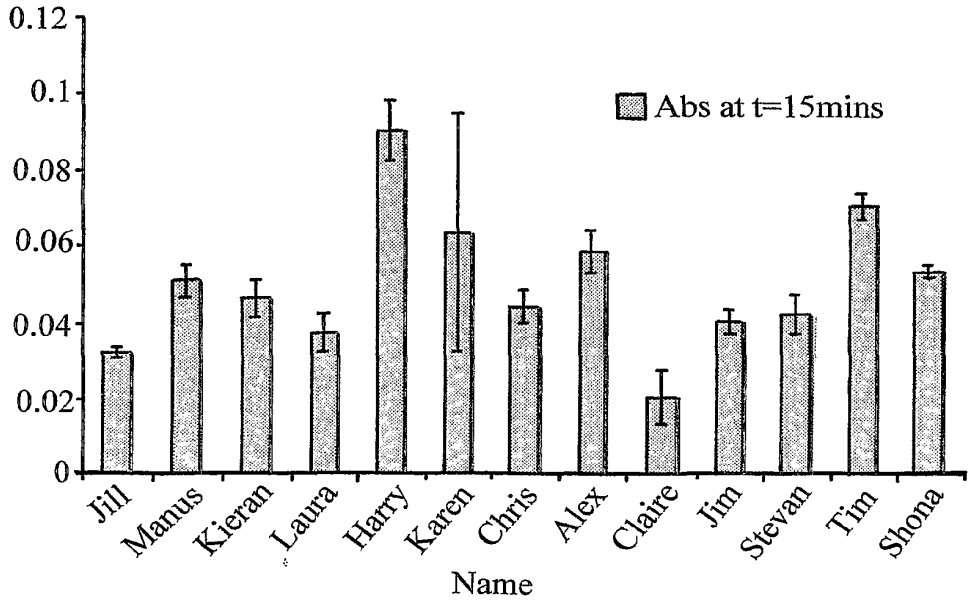


Fig. 5

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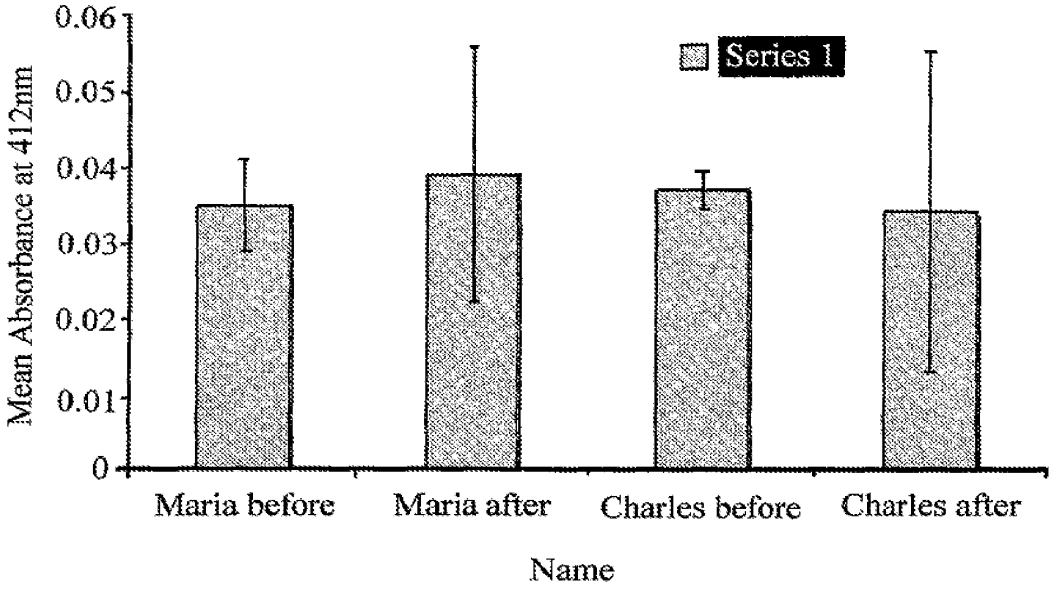


Fig. 6

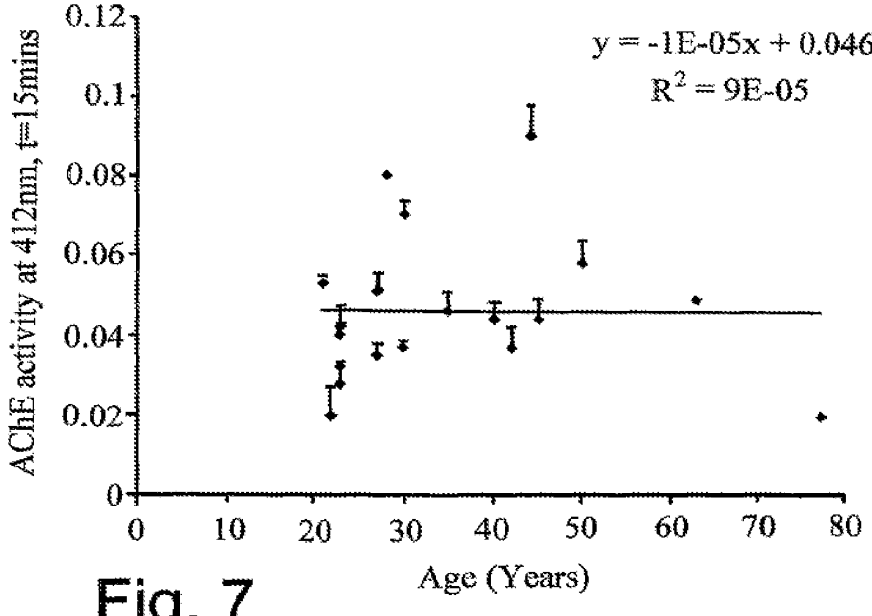


Fig. 7

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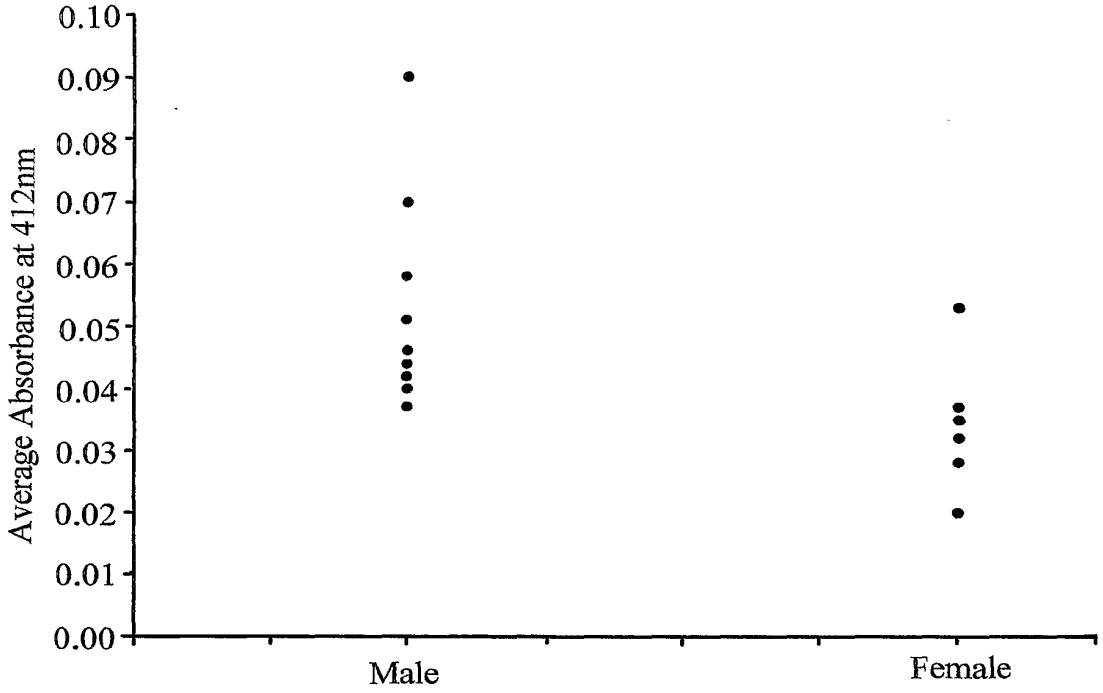


Fig. 8

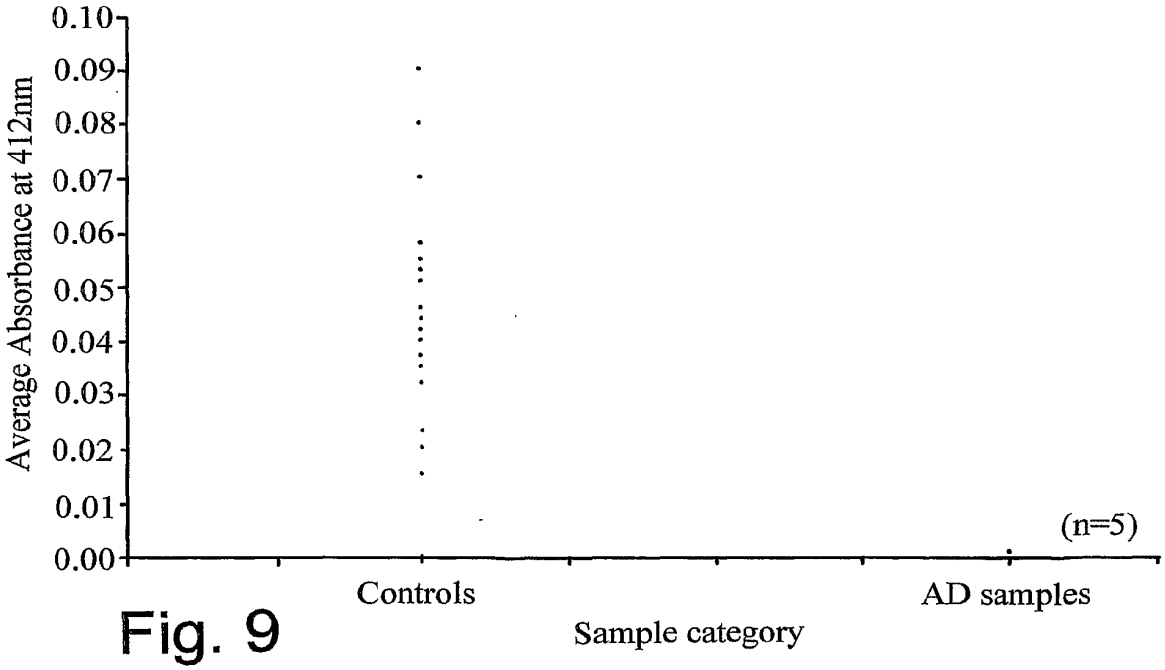


Fig. 9

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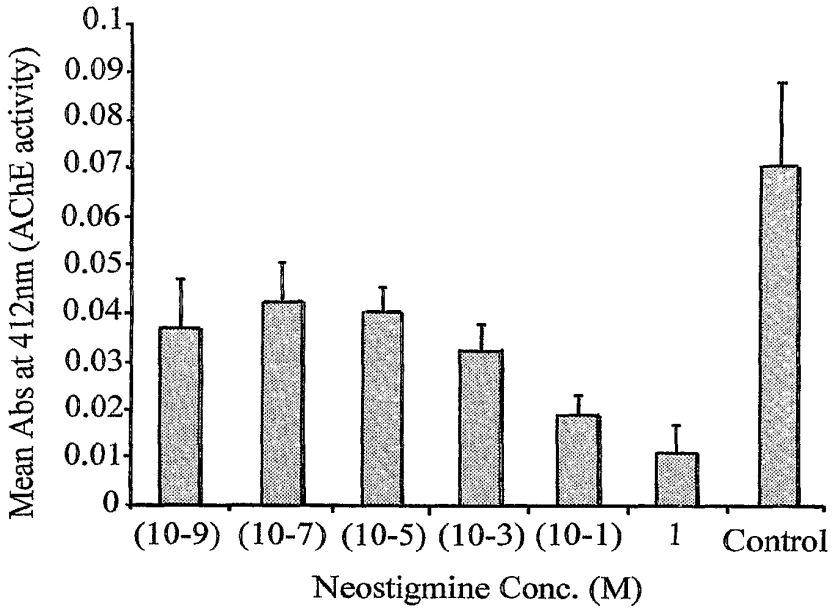


Fig. 10a

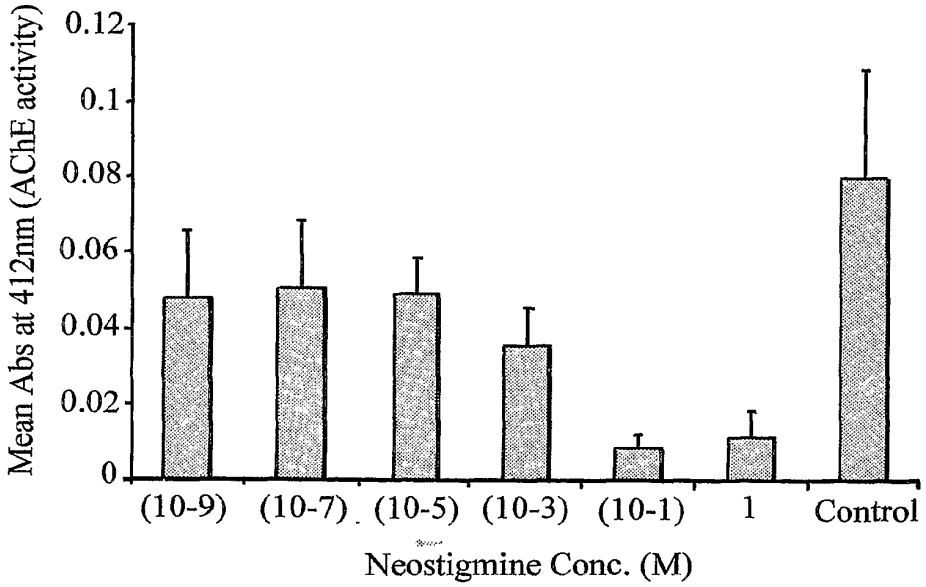


Fig. 10b

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INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/GB 01/02229

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 C12Q1/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 13273 A (ESA INC) 6 August 1992 (1992-08-06) claims 1,3,5,7,10	1-15
X	WO 98 07850 A (PREDDIE ENRIQUE R ;BERGMANN JOHANNA E (DE)) 26 February 1998 (1998-02-26) claim 22	1-15
X	WO 00 25138 A (NITSCH ROGER ;GROWDON JOHN (US)) 4 May 2000 (2000-05-04) the whole document	1-15
A	US 5 976 817 A (DAVIES-HEEREMA THERESA A ET AL) 2 November 1999 (1999-11-02) claim 1	1-15

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
29 October 2001	07/11/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Pellegrini, P

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INTERNATIONAL SEARCH REPORT

Internat Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 459 454 A (SQUIBB & SONS INC) 4 December 1991 (1991-12-04) claim 1	1-15

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INTERNATIONAL SEARCH REPORT

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Internat Application No
PCT/GB 01/02229

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			DE	69120731 D1	14-08-1996
			EP	0459454 A1	04-12-1991

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(54) Title: COMPOSITIONS AND METHODS FOR DIAGNOSING ALZHEIMER'S DISEASE

(57) Abstract: The present invention provides methods and compositions for the diagnosis of Alzheimer's disease. In particular, the present invention provides modified beta-amyloid peptides, antibodies that specifically bind to the modified beta-amyloid peptides, and methods for using these compositions in the diagnosis of Alzheimer's disease, as well as methods to monitor treatment and/or disease progression of Alzheimer's disease in patients. The present invention also provides compositions and methods useful in research involving amyloid precursor protein (APP) metabolism and Alzheimer's disease.

COMPOSITIONS AND METHODS FOR DIAGNOSING ALZHEIMER'S DISEASE

This invention was made during the course of work supported in part by the United States Government, under the National Institutes of Health, Grant Award Number DK 46971. As such, the United States Government may have certain rights to this invention.

FIELD OF THE INVENTION

The present invention provides compositions and methods for diagnosing Alzheimer's disease. In particular, the present invention provides modified beta-amyloid peptides, antibodies that specifically bind to the modified beta-amyloid peptides, and methods of use of these compositions in the diagnosis of Alzheimer's disease, as well as methods to monitor treatment and/or disease progression of Alzheimer's disease in patients.. The present invention also provides compositions and methods useful in research involving amyloid precursor protein (APP) metabolism and Alzheimer's disease.

BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) is the fourth most common cause of death in the United States, next to heart disease, cancer and stroke. It presently afflicts more than four million people, and this number is expected to double during the next forty years with the aging of the population. Alzheimer's disease is also the most common cause of chronic dementia, with approximately two million people in the United States suffering from dementia. At present, it is estimated that ten percent of the population older than 65 years of age have mild to severe dementia. This high prevalence, combined with the rate of growth of the elderly segment of the population, make dementia and particularly Alzheimer's disease, important current public health problems.

To date, Alzheimer's disease is the third most expensive disease in the United States, and costs approximately $100 billion each year. Costs associated with AD include direct medical costs such as nursing home care, direct nonmedical costs such as in-home day care, as well as indirect costs such as lost patient and care-giver productivity. Medical treatment may have economic benefits by slowing the rate of

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cognitive decline, delaying institutionalization, reducing care-giver hours, and improving quality of life.

Alzheimer's disease is a complex multi-genic neurodegenerative disorder characterized by progressive impairments in memory, behavior, language, and visuo-spatial skills, ending ultimately in death. Hallmark pathologies of Alzheimer's disease include granulovascular neuronal degeneration, extracellular neuritic plaques with β-amyloid deposits, intracellular neurofibrillary tangles and neurofibrillary degeneration, synaptic loss, and extensive neuronal cell death. It is now known that these histopathologic lesions of Alzheimer's disease correlate with the dementia observed in many elderly people.

Research on the causes of and treatments for Alzheimer's disease has led investigators down numerous avenues. Although many models have been proposed, no single model of AD satisfactorily accounts for all neuropathologic findings; nor do these models of AD satisfactorily account for the requirement of aging for disease onset. Cellular changes, leading to neuronal loss and the underlying etiology of the disease, remain unknown. Proposed causes include environmental factors (Perl, *Environmental Health Perspective* 63:149 [1985]), metal toxicity (Perl *et al.*, *Science* 208:297 [1980]), defects in beta-amyloid protein metabolism (Shijo *et al.*, *Science* 258:126 [1992]; and Kosik, *Science* 256:780 [1992]), and abnormal calcium homeostasis and/or calcium activated kinases (Mattson *et al.*, *J. Neuroscience* 12:376 [1992]). The mechanisms of disease progression are equally unclear. Considerable human genetic evidence has implicated alterations in production or processing of the human amyloid precursor protein (APP) in the etiology of the disease. However, intensive research has proven that AD is a multifactorial disease with many different, perhaps overlapping, etiologies.

Early detection and identification of Alzheimer's disease facilitate prompt, appropriate treatment and care. However, there is currently no laboratory diagnostic test for Alzheimer's disease. Although studies have suggested that calcium imaging measurement in fibroblasts were of potential clinical use in diagnosing Alzheimer's disease (Peterson *et al.*, *Neurobiology of Aging* 9:261 [1988]; and Peterson *et al.*, *Proc. Natl. Acad. Sci. USA* 83:7999 [1986]), other studies using similar cell lines and techniques have shown no difference in calcium levels in Alzheimer's and normal control fibroblasts (Borden *et al.*, *Neurobiology of Aging* 13:33 [1991]). Thus, there remains a need for diagnostic methods for Alzheimer's disease. In particular, reliable and cost-

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effective methods and compositions are needed to allow reliable diagnosis of Alzheimer's disease.

SUMMARY OF THE INVENTION

The present invention provides compositions and methods for diagnosing Alzheimer's disease. In particular, the present invention provides modified beta-amyloid peptides, antibodies that specifically bind to the modified beta-amyloid peptides, and methods of use of these compositions for the diagnosis of Alzheimer's disease. The present invention also provides compositions and methods useful in research involving amyloid precursor protein (APP) metabolism and Alzheimer's disease.

The present invention provides isolated and modified β-amyloid (βA) peptides comprising at least one malondialdehyde adduct. In one embodiment, the modified βA peptide comprises the amino acid sequence of SEQ ID NO:1. In another embodiment, the modified βA peptide comprises at least one malondialdehyde lysine adduct

The present invention also provides purified antibodies directed against a modified β-amyloid peptide comprising at least one malondialdehyde adduct. In some embodiments, the antibody is a monoclonal antibody, while in other embodiments, the antibody is a polyclonal antibody. In other related embodiments, the invention provides compositions comprising the antibody directed against the modified βA-peptide comprising at least one malondialdehyde adduct.

The present invention further provides methods for detecting at least one modified β-amyloid peptide comprising at least one malondialdehyde adduct, comprising the steps of: providing a sample suspected of containing at least one modified β-amyloid peptide comprising at least one malondialdehyde adduct, and an antibody directed against at least on modified β-amyloid peptide comprising at least on malondialdehyde adduct; contacting the sample with the antibody under conditions such that the antibody specifically binds to the modified β-amyloid peptide comprising at least one malondialdehyde adduct, to form an antigen-antibody complex; and detecting the presence of the antigen-antibody complex. In some embodiments, the sample is selected from the group consisting of blood, plasma, serum, serous fluid, and cerebrospinal fluid. In some preferred embodiments, the sample is from a subject. In particularly preferred embodiments, the subject is a human. In further embodiments, the subject is selected from the group consisting of subjects displaying pathology resulting from Alzheimer's

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disease, subjects suspected of displaying pathology resulting from Alzheimer's disease, and subjects at risk of displaying pathology resulting from Alzheimer's disease. In some particularly preferred embodiments, the methods further comprise the step of diagnosing Alzheimer's disease. In additional particularly preferred embodiments, the Alzheimer's disease diagnosed using the methods of the present invention is selected from the group consisting of late onset Alzheimer's disease, early onset Alzheimer's disease, familial Alzheimer's disease and sporadic Alzheimer's disease. In some preferred embodiments, the methods further comprise the step of monitoring the efficacy of treatment of Alzheimer's disease.

In some preferred embodiments, the methods comprises an enzyme-linked immunosorbent assay. In particularly preferred embodiments, the enzyme-linked immunosorbent assay is selected from the group consisting of direct enzyme-linked immunosorbent assays, indirect enzyme-linked immunosorbent assays, direct sandwich enzyme-linked immunosorbent assays, indirect sandwich enzyme-linked immunosorbent assays, and competitive enzyme-linked immunosorbent assays. In alternative preferred embodiments, the antibody used in the methods of the present invention further comprises a conjugated enzyme, wherein the conjugated enzyme is selected from the group of enzymes consisting of horseradish peroxidases, alkaline phosphatases, ureases, glucoamylases, and β-galactosidases. In some particularly preferred embodiments, the enzyme-linked immunosorbent assay further comprises an alkaline phosphatase amplification system. In alternative preferred embodiments, the methods further comprise at least one capture antibody, while in still further embodiments, the methods further comprise at least one detection antibody wherein the detection antibody is directed against the antibody directed against the modified β-amyloid peptide comprising at least one malondialdehyde adduct. In still further embodiments, the detection antibody further comprises at least one conjugated enzyme selected from the group consisting of horseradish peroxidase, alkaline phosphatase, urease, glucoamylase and β-galactosidase. In still further preferred embodiments, the methods further comprise the step of quantitating the at least one modified β-amyloid peptide comprising at least one malondialdehyde adduct in the sample.

The present invention also provides kits for the detection of at least one modified β-amyloid peptide comprising at least one malondialdehyde adduct, comprising an immobilized support and at least one antibody directed against at least one modified

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modified β-amyloid peptide comprising at least one malondialdehyde adduct. In some preferred embodiments, the kit comprises an enzyme-linked immunosorbent assay kit. In still further embodiments, the kit further comprises components selected from the group consisting of needles, sample collection tubes, 96-well microtiter plates, instructions, at least one modified β-amyloid peptide comprising at least one malondialdehyde adduct, an antibody-enzyme conjugate directed against a modified β-amyloid peptide comprising at least one malondialdehyde adduct, at least one capture antibody, 96-well microtiter plates precoated with the at least one capture antibody, at least one coating buffer, at least one blocking buffer, distilled water, at least one enzyme-linked immunosorbent assay enzyme reaction substrate solution, and at least amplifier system. In some preferred embodiments, the amplifier system is an alkaline phosphatase enzyme-linked immunosorbent assay amplifier system.

The present invention also provides methods for detecting at least one antibody directed against a modified β-amyloid peptide comprising at least one malondialdehyde adduct, comprising: a) providing a sample suspected of containing at least one antibody directed against a modified β-amyloid peptide comprising at least one malondialdehyde adduct; at least one modified β-amyloid peptide having at least one malondiadehyde adduct, and a detection antibody; b) contacting the sample with the modified β-amyloid peptide, under conditions such that the antibody directed against a modified β-amyloid peptide comprising at least one malondialdehyde adduct specifically binds to the modified β-amyloid peptide to form an antigen-antibody complex; c) contacting the antigen-antibody complex with the detection antibody, under conditions such that the detection antibody specifically binds to the complex; and d) detecting the specific binding of the detection antibody to the antigen-antibody complex. In some preferred embodiments, the sample is selected from the group of samples consisting of blood, serous fluid, plasma, serum, cerebrospinal fluid, hybridoma conditioned culture medium, ascites fluid, and polyclonal antiserum. In some particularly preferred embodiments, the sample is from a subject, while in other preferred embodiments, the subject is human. In alternative preferred embodiments, the subject is selected from the group consisting of subjects displaying pathology resulting from Alzheimer's disease, subjects suspected of displaying pathology resulting from Alzheimer's disease, and subjects at risk of displaying pathology resulting from Alzheimer's disease. In still further preferred embodiments, the mehtods further comprise diagnosing Alzheimer's disease in the

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subject. In some preferred embodiments, the Alzheimer's disease is selected from the group consisting of late onset Alzheimer's disease, early onset Alzheimer's disease, familial Alzheimer's disease, and sporadic Alzheimer's disease. In preferred embodiments, the method comprises an enzyme-linked immunosorbent assay. In some preferred embodiments, the enzyme-linked immunosorbent assay is selected from the group consisting of direct enzyme-linked immunosorbent assays, indirect enzyme-linked immunosorbent assays, direct sandwich enzyme-linked immunosorbent assays, indirect sandwich enzyme-linked immunosorbent assays, and competitive enzyme-linked immunosorbent assays. In still further embodiments, the detection antibody further comprises a conjugated enzyme, wherein the conjugated enzyme is selected from the group of enzymes consisting of horseradish peroxidases, alkaline phosphatases, ureases, glucoamylases, and β-galactosidases. In additional embodiments, the enzyme-linked immunosorbent assay further comprises an alkaline phosphatase amplification system.

The present invention also provides kits for the detection of at least one antibody directed against at least one modified β-amyloid peptide comprising at least one malondialdehyde adduct, comprising an immobilized support, at least one purified and modified β-amyloid peptide comprising at least one malondialdehyde adduct, and at least one detection antibody. In some preferred embodiments, the kit is an enzyme-linked immunosorbent assay kit. In some preferred embodiments, the kit comprises components selected from the group consisting of needles, sample collection tubes, 96-well microtiter plates, instructions, at least one purified antibody directed against at least one β-amyloid peptide comprising at least one malondialdehyde adduct, at least one 96-well microtiter plate precoated with at least one β-amyloide peptide comprising at least one malondialdehyde adduct, at least one coating buffer, at least one blocking buffer, distilled water, at least one enzyme reaction substrate solution, and at least one amplifier system. In some particularly preferred embodiments, the amplifier system is an alkaline phosphatase enzyme-linked immunosorbent assay amplifier system.

DESCRIPTION OF THE DRAWING

Figure 1 provides the results of a Western immunoblot assay using a primary antibody having binding specificity for a modified β-amyloid peptide comprising at least one malondialdehyde adduct and brain tissue lysate samples.

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DEFINITIONS

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

As used herein, the terms "peptide," "polypeptide" and "protein" all refer to a primary sequence of amino acids that are joined by covalent "peptide linkages." In general, a peptide consists of a few amino acids, typically from 2-50 amino acids, and is shorter than a protein. The term "polypeptide" encompasses peptides and proteins. In some embodiments, the peptide, polypeptide or protein is synthetic, while in other embodiments, the peptide, polypeptide or protein are recombinant or naturally occurring. A synthetic peptide is a peptide which is produced by artificial means *in vitro* (*i.e.*, was not produced *in vivo*).

The terms "sample" and "specimen" are used in their broadest sense and encompass samples or specimens obtained from any source. As used herein, the term "sample" is used to refer to biological samples obtained from animals (including humans), and encompasses fluids, solids, tissues, and gases. In preferred embodiments of this invention, biological samples include cerebrospinal fluid (CSF), serous fluid, urine, saliva, blood, and blood products such as plasma, serum and the like. However, these examples are not to be construed as limiting the types of samples which find use with the present invention.

As used herein, the terms "beta-amyloid protein," "amyloid beta protein," "βA," "βA peptide" and "βA protein" all refer to a protein produced by neurons and glial cells in the brain. βA is found in the brain plaques of patients with Alzheimer's disease, head trauma and Down's syndrome, and is also found normally in the spinal fluid and blood (*See e.g.*, Haass and Selkoe, *Cell* 75:1039 [1993]; Teller *et al.*, *Nature Med.*, 2:93 [1996]).

As used herein, the term "modified βA peptide" refers to a beta-amyloid peptide. In preferred embodiments, the modified βA peptide has been oxidatively modified. In a particularly preferred embodiment, the modified βA peptide is oxidatively modified by oxidative stress.

As used herein, the terms "modified β-amyloid peptide comprising at least one malondialdehyde adduct" or "βA-MDA" refer to a βA peptide that has been modified by the covalent addition of at least one malondialdehyde group. In some embodiments, the MDA group is added using an oxidative stress-induced modification reaction (*i.e.*, an

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oxidation reaction). In one preferred embodiment, the modified β-amyloid peptide comprising at least one malondialdehyde adduct comprises a malondialdehyde-lysine adduct.

As used herein, the term "oxidative stress" refers to the cytotoxic effects of oxygen radicals (*i.e.*, superoxide anion [$\bullet O_2^-$], hydroxy radical [•OH], and hydrogen peroxide [H_2O_2]), generated as byproducts of metabolic processes that utilize molecular oxygen (*See e.g.*, Coyle *et al.*, *Science* 262:689-695 [1993]).

As used herein, the terms "host," "subject" and "patient" refer to any animal, including but not limited to, human and non-human animals (*e.g.*, rodents, arthropods, insects [*e.g.*, Diptera], fish [*e.g.*, zebrafish], non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, etc.), which is studied, analyzed, tested, diagnosed or treated. As used herein, the terms "host," "subject" and "patient" are used interchangeably.

As used herein, the terms "Alzheimer's disease" and "AD" refer to a neurodegenerative disorder and encompasses familial Alzheimer's disease and sporadic Alzheimer's disease. The term "familial Alzheimer's disease" refers to Alzheimer's disease associated with genetic factors (*i.e.*, demonstrates inheritance) while "sporadic Alzheimer's disease" refers to Alzheimer's disease that is not associated with prior family history of the disease. Symptoms indicative of Alzheimer's disease in human subjects typically include, but are not limited to, mild to severe dementia, progressive impairment of memory (ranging from mild forgetfulness to disorientation and severe memory loss), poor visuo-spatial skills, personality changes, poor impulse control, poor judgement, distrust of others, increased stubbornness, restlessness, poor planning ability, poor decision making, and social withdrawal. In severe cases, patients lose the ability to use language and communicate, and require assistance in personal hygiene, eating and dressing, and are eventually bedridden. Hallmark pathologies within brain tissue include extracellular neuritic β-amyloid plaques, neurofibrillary tangles, neurofibrillary degeneration, granulovascular neuronal degeneration, synaptic loss, and extensive neuronal cell death.

As used herein, the term "early-onset Alzheimer's disease" refers to the classification used in Alzheimer's disease cases diagnosed as occurring before the age of 65. As used herein, the term "late-onset Alzheimer's disease" refers to the classification used in Alzheimer's disease cases diagnosed as occurring after the age of 65.

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As used herein, the terms "subject having Alzheimer's disease" or "subject displaying symptoms or pathology indicative of Alzheimer's disease" "subjects suspected of displaying symptoms or pathology indicative of Alzheimer's disease" refer to a subject that is identified as having or likely to have Alzheimer's disease based on known Alzheimer's symptoms and pathology.

As used herein, the term "subject at risk of displaying pathology indicative of Alzheimer's disease" refers to a subject identified as being at risk for developing Alzheimer's disease (*e.g.*, because of a familial inheritance pattern of Alzheimer's disease in the subject's family).

As used herein, the term "lesion" refers to a wound or injury, or to a pathologic change in a tissue. For example, the amyloid plaque lesions observed in the brains of patients having Alzheimer's disease are considered the hallmark pathology characteristic of the disease.

As used herein, the term "antibody" (or "antibodies") refers to any immunoglobulin that binds specifically to an antigenic determinant, and specifically, binds to proteins identical or structurally related to the antigenic determinant which stimulated their production. Thus, antibodies are useful in assays to detect the antigen which stimulated their production. Monoclonal antibodies are derived from a single clone of B lymphocytes (*i.e.*, B cells), and are generally homogeneous in structure and antigen specificity. Polyclonal antibodies originate from many different clones of antibody-producing cells, and thus are heterogenous in their structure and epitope specificity, but all recognize the same antigen. In some embodiments, monoclonal and polyclonal antibodies are used as crude preparations, while in preferred embodiments, these antibodies are purified. For example, in some embodiments, polyclonal antibodies contained in crude antiserum are used. Also, it is intended that the term "antibody" encompass any immunoglobulin (*e.g.*, IgG, IgM, IgA, IgE, IgD, etc.) obtained from any source (*e.g.*, humans, rodents, non-human primates, lagomorphs, caprines, bovines, equines, ovines, etc.).

As used herein, the terms "auto-antibody" or "auto-antibodies" refer to any immunoglobulin that binds specifically to an antigen that is native to the host organism that produced the antibody (*i.e.*, the antigen is directed against "self" antigens). The presence of auto-antibodies is referred to herein as "autoimmunity."

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As used herein, the term "antigen" is used in reference to any substance that is capable of being recognized by an antibody. It is intended that this term encompass any antigen and "immunogen" (*i.e.,* a substance which induces the formation of antibodies). Thus, in an immunogenic reaction, antibodies are produced in response to the presence of an antigen or portion of an antigen. The terms "antigen" and "immunogen" are used to refer to an individual macromolecule or to a homogeneous or heterogeneous population of antigenic macromolecules. It is intended that the terms antigen and immunogen encompass protein molecules or portions of protein molecules, which contains one or more epitopes. In many cases, antigens are also immunogens, thus the term "antigen" is often used interchangeably with the term "immunogen." In some preferred embodiments, immunogenic substances are used as antigens in assays to detect the presence of appropriate antibodies in the serum of an immunized animal.

As used herein, the terms "antigen fragment" and "portion of an antigen" and the like are used in reference to a portion of an antigen. Antigen fragments or portions typcially range in size, from a small percentage of the entire antigen to a large percentage, but not 100%, of the antigen. However, in situations where "at least a portion" of an antigen is specified, it is contemplated that the entire antigen is also present (*i.e.,* it is not intended that the sample tested contain only a portion of an antigen). In some embodiments, antigen fragments and/or portions therof, comprise an "epitope" recognized by an antibody, while in other embodiments these fragments and/or portions do not comprise an epitope recognized by an antibody. In addition, in some embodiments, antigen fragments and/or portions are not immunogenic, while in preferred embodiments, the antigen fragments and/or portions are immunogenic.

The terms "antigenic determinant" and "epitope" as used herein refer to that portion of an antigen that makes contact with a particular antibody variable region. When a protein or fragment (or portion) of a protein is used to immunize a host animal, numerous regions of the protein are likely to induce the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein (these regions and/or structures are referred to as "antigenic determinants"). In some settings, antigenic determinants compete with the intact antigen (*i.e.,* the "immunogen" used to elicit the immune response) for binding to an antibody.

The terms "specific binding" and "specifically binding" when used in reference to the interaction between an antibody and an antigen describe an interaction that is

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dependent upon the presence of a particular structure (*i.e.*, the antigenic determinant or epitope) on the antigen. In other words, the antibody recognizes and binds to a protein structure unique to the antigen, rather than binding to all proteins in general (*i.e.*, non-specific binding).

As used herein the term "immunogenically-effective amount" refers to that amount of an immunogen required to invoke the production of protective levels of antibodies in a host upon vaccination.

As used herein, the term "adjuvant" is defined as a substance which enhances the immunogenicity of a coadministered antigen. If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant -- or that the same adjuvant, once used, be used for all subsequent immunizations. The present invention contemplates many adjuvants, including but not limited to, keyhole limpet hemocyanin (KLH), agar beads, aluminum hydroxide or phosphate (alum), Freund's adjuvant (incomplete or complete), Quil A adjuvant and Gerbu adjuvant (Accurate Chemical and Scientific Corporation), and bacterins (*i.e.*, killed preparations of bacterial cells, especially mycoplasma).

As used herein, the terms "purified" and "to purify" and "purification" refers to the removal or reduction of at least one contaminant from a sample. For example, antibodies are purified by removal of contaminating non-immunoglobulin proteins. Antibodies are also purified by the removal of immunoglobulin that does not bind to the target molecule. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind to the target molecule results in an increase in the percent of target-reactive immunoglobulins in the sample (*i.e.*, "enrichment" of an antibody).

As used herein, the term "immunoassay" refers to any assay that uses at least one specific antibody for the detection or quantitation of an antigen. Immunoassays include, but are not limited to, Western blots, ELISAs, radio-immunoassays, and immunofluorescence assays. Furthermore, many different ELISA formats are known to those in the art, any of which will find use in the present invention. However, it is not intended that the present invention be limited to these assays. In additional embodiments, other antigen-antibody reactions are used in the present invention, including but not limited to "flocculation" (*i.e.*, a colloidal suspension produced upon the formation of antigen-antibody complexes), "agglutination" (*i.e.*, clumping of cells or

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other substances upon exposure to antibody), "particle agglutination" (*i.e.*, clumping of particles coated with antigen in the presence of antibody or the clumping of particles coated with antibody in the presence of antigen), "complement fixation" (*i.e.*, the use of complement in an antibody-antigen reaction method), and other methods commonly used in serology, immunology, immunocytochemistry, immunohistochemistry, and related fields.

The terms "Western blot," "Western immunoblot" "immunoblot" and "Western" refer to the immunological analysis of protein(s), polypeptides or peptides that have been immobilized onto a membrane support. The proteins are first resolved by polyacrylamide gel electrophoresis (*i.e.*, SDS-PAGE) to separate the proteins, followed by transfer of the protein from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized proteins are then exposed to an antibody having reactivity towards an antigen of interest. The binding of the antibody (*i.e.*, the primary antibody) is detected by use of a secondary antibody which specifically binds the primary antibody. The secondary antibody is typically conjugated to an enzyme which permits visualization of the antigen-antibody complex by the production of a colored reaction product or catalyzes a luminescent enzymatic reaction (*e.g.*, the ECL reagent, Amersham).

As used herein, the term "ELISA" refers to enzyme-linked immunosorbent assay (or EIA). Numerous ELISA methods and applications are known in the art, and are described in many references (*See, e.g.*, Crowther, "Enzyme-Linked Immunosorbent Assay (ELISA)," in *Molecular Biomethods Handbook*, Rapley *et al.* [eds.], pp. 595-617, Humana Press, Inc., Totowa, NJ [1998]; Harlow and Lane (eds.), *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press [1988]; Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, Ch. 11, John Wiley & Sons, Inc., New York [1994]). In addition, there are numerous commercially available ELISA test systems.

One of the ELISA methods used in the present invention is a "direct ELISA," where an antigen (*e.g.*, βA-MDA peptide) in a sample is detected. In one embodiment of the direct ELISA, a sample containing antigen is exposed to a solid (*i.e.*, stationary or immobilized) support (*e.g.*, a microtiter plate well). The antigen within the sample becomes immobilized to the stationary phase, and is detected directly using an enzyme-conjugated antibody specific for the antigen.

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In an alternative embodiment, an antibody specific for an antigen is detected in a sample. In this embodiment, a sample containing an antibody (*e.g.*, an anti-βA-MDA antibody) is immobilized to a solid support (*e.g.*, a microtiter plate well). The antigen-specific antibody is subsequently detected using purified antigen and an enzyme-conjugated antibody specific for the antigen.

In an alternative embodiment, an "indirect ELISA" is used. In one embodiment, an antigen (or antibody) is immobilized to a solid support (*e.g.*, a microtiter plate well) as in the direct ELISA, but is detected indirectly by first adding an antigen-specific antibody (or antigen), then followed by the addition of a detection antibody specific for the antibody that specifically binds the antigen, also known as "species-specific" antibodies (*e.g.*, a goat anti-rabbit antibody), which are available from various manufacturers known to those in the art (*e.g.*, Santa Cruz Biotechnology; Zymed; and Pharmingen/Transduction Laboratories).

In other embodiments, a "sandwich ELISA" is used, where the antigen is immobilized on a solid support (*e.g.*, a microtiter plate) via an antibody (*i.e.*, a capture antibody) that is immobilized on the solid support and is able to bind the antigen of interest. Following the affixing of a suitable capture antibody to the immobilized phase, a sample is then added to the microtiter plate well, followed by washing. If the antigen of interest is present in the sample, it is bound to the capture antibody present on the support. In some embodiments, a sandwich ELISA is a "direct sandwich" ELISA, where the captured antigen is detected directly by using an enzyme-conjugated antibody directed against the antigen. Alternatively, in other embodiments, a sandwich ELISA is an "indirect sandwich" ELISA, where the captured antigen is detected indirectly by using an antibody directed against the antigen, which is then detected by another enzyme-conjugated antibody which binds the antigen-specific antibody, thus forming an antibody-antigen-antibody-antibody complex. Suitable reporter reagents are then added to detect the third antibody. Alternatively, in some embodiments, any number of additional antibodies are added as necessary, in order to detect the antigen-antibody complex. In some preferred embodiments, these additional antibodies are labelled or tagged, so as to permit their visualization and/or quantitation.

As used herein, the term "capture antibody" refers to an antibody that is used in a sandwich ELISA to bind (*i.e.*, capture) an antigen in a sample prior to detection of the antigen. For example, in some embodiments, the polyclonal anti-βA-MDA antibody of

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the present invention serves as a capture antibody when immobilized in a microtiter plate well. This capture antibody binds βA-MDA antigen present in a sample added to the well. In one embodiment of the present invention, biotinylated capture antibodies are used in the present invention in conjunction with avidin-coated solid support. Another antibody (*i.e.*, the detection antibody) is then used to bind and detect the antigen-antibody complex, in effect forming a "sandwich" comprised of antibody-antigen-antibody (*i.e.*, a sandwich ELISA).

As used herein, a "detection antibody" is an antibody which carries a means for visualization or quantitation, which is typically a conjugated enzyme moiety that typically yields a colored or fluorescent reaction product following the addition of a suitable substrate. Conjugated enzymes commonly used with detection antibodies in the ELISA include horseradish peroxidase, urease, alkaline phosphatase, glucoamylase and β-galactosidase. In some embodiments, the detection antibody is directed against the antigen of interest, while in other embodiments, the detection antibody is not directed against the antigen of interest. In some embodiments, the detection antibody is an anti-species antibody. Alternatively, the detection antibody is prepared with a label such as biotin, a fluorescent marker, or a radioisotope, and is detected and/or quantitated using this label.

As used herein, the terms "reporter reagent," "reporter molecule," "detection substrate" and "detection reagent" are used in reference to reagents which permit the detection and/or quantitation of an antibody bound to an antigen. For example, in some embodiments, the reporter reagent is a colorimetric substrate for an enzyme that has been conjugated to an antibody. Addition of a suitable substrate to the antibody-enzyme conjugate results in the production of a colorimetric or fluorimetric signal (*e.g.*, following the binding of the conjugated antibody to the antigen of interest). Other reporter reagents include, but are not limited to, radioactive compounds. This definition also encompasses the use of biotin and avidin-based compounds (*e.g.*, including but not limited to neutravidin and streptavidin) as part of the detection system.

As used herein, the term "signal" is used generally in reference to any detectable process that indicates that a reaction has occurred, for example, binding of antibody to antigen. It is contemplated that signals in the form of radioactivity, fluorimetric or colorimetric products/reagents will all find use with the present invention. In various

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embodiments of the present invention, the signal is assessed qualitatively, while in alternative embodiments, the signal is assessed quantitatively.

As used herein, the term "amplifier" is used in reference to a system which enhances the signal in a detection method, such as an ELISA (*e.g.*, an alkaline phosphatase amplifier system used in an ELISA).

As used herein, the term "solid support" is used in reference to any solid or stationary material to which reagents such as antibodies, antigens, and other test components are attached. For example, in the ELISA method, the wells of microtiter plates provide solid supports. Other examples of solid supports include microscope slides, coverslips, beads, particles, cell culture flasks, as well as many other suitable items.

As used herein, the term "kit" is used in reference to a combination of reagents and other materials which facilitate sample analysis. In some embodiments, the immunoassay kit of the present invention includes a suitable capture antibody, reporter antibody, antigen, detection reagents and amplifier system. Furthermore, in other embodiments, the kit includes, but is not limited to, components such as apparatus for sample collection, sample tubes, holders, trays, racks, dishes, plates, instructions to the kit user, solutions or other chemical reagents, and samples to be used for standardization, normalization, and/or control samples.

As used herein, the term "*in vitro*" refers to an artificial environment and to processes or reactions that occur within an artificial environment. *In vitro* environments consist of, but are not limited to, controlled laboratory conditions. The term "*in vivo*" refers to the natural environment (*e.g.*, an animal or a cell) and to processes or reactions that occur within that natural environment.

DESCRIPTION OF THE INVENTION

The present invention provides compositions and methods for diagnosing Alzheimer's disease. In particular, the present invention provides methods for diagnosing Alzheimer's disease using antibodies that specifically bind to modified beta amyloid peptides indicative of Alzheimer's disease.

Deposition of cerebral amyloid is a primary neuropathologic marker of Alzheimer's disease (AD). This amyloid is composed of a 40-42 amino acid peptide known as the beta amyloid peptide (βA) (Glenner *et al.*, *Biochem. Biophys. Res.*

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Commun., 120:885-890 [1984]). Amyloid deposits in AD are found mainly as components of senile plaques, and in the walls of cerebral and meningeal blood vessels (Robakis *et al.*, *Neurobiol. Aging* 15:S127-129 [1994]). βA is also produced during normal cellular metabolism (Haass *et al.*, *Nature* 359:322-324 [1992]; Shoji *et al.*, *Science* 258:126-129 [1992]).

Molecular cloning showed that βA comprises a small region of a larger amyloid precursor protein (*i.e.*, "APP") (Robakis *et al.*, *Proc. Natl. Acad. Sci. USA* 84:4190-4194 [1987]; and Weidemann *et al.*, *Cell* 57:115-126 [1989]). The APP protein is a type I integral membrane glycoprotein having a large extracytoplasmic portion, a smaller intracytoplasmic region, and a single transmembrane domain. APP undergoes extensive post-translational modifications prior to the secretion of its N-terminal portion (*See e.g.*, Pappolla *et al.*, in "Perspectives in behavioral medicine, Alzheimer's disease and AIDS," Stein *et al.* [eds.], Academic Press, San Diego, California, pp. 3-20 [1995]; and Robakis *et al.*, *Neurobiol. Aging* 15:S127-129 [1994]). Physiologic processing of APP involves cleavage within the βA sequence by an uncloned enzymatic activity, termed alpha-secretase (Anderson *et al.*, *Neurosci. Lett.*, 128:126-129 [1991]). Smaller quantities of APP molecules are cleaved at two other sites that potentially produce amyloidogenic secreted or membrane bound APP (Robakis *et al.*, 1994, *supra*).

Although it is not clear whether beta-amyloid accumulation causes Alzheimer's disease or is an effect of Alzheimer's disease, considerable evidence has strengthened the view that amyloid accumulation is the causative agent of Alzheimer's disease. However, it is not necessary to understand the cause or effect of beta-amyloid accumulation in Alzheimer's disease in order to practice the present invention, nor is it intended that the present invention be limited to any particular mechanism or mechanisms of disease genesis or toxicity. Indeed, an understanding of any of the mechanisms of pathogenesis are not necessary in order to use the present invention.

First, several point mutations within the APP gene which segregate within a subgroup of patients afflicted with a familial form of the disorder have been identified, thereby suggesting a pathogenetic relationship between the APP gene and AD (Chartier-Harlin *et al.*, *Nature* 353:844-846 [1991]; and Kennedy *et al.*, *Brain* 116:3009-324 [1993]). Second, amyloid deposition temporally precedes the development of neurofibrillary changes (Pappolla *et al.*, *Mol. Chem. Neuropathol.*, 28:21-34 [1996]).

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This observation is also consistent with a link between amyloid and neuronal degeneration. Finally, it has been shown that βA has neurotoxic properties (Yankner *et al.*, *Science* 250:279-282 [1990]; and Behl *et al.*, *Biochem. Biophys. Res. Commun.*, 186:944-950 [1992]). Although the mechanism of toxicity is not totally understood, βA neurotoxicity provides a possible connection between amyloid accumulation and neurodegeneration. In addition to free-radicals, increased sensitivity to excitotoxicity and/or disruption of Ca^{2+}, homeostasis seems to be involved (*See e.g.*, Copani *et al.*, *Molecular Pharmacology* 47:890-897 [1995]; Mattson *et al.*, *Trends Neurosci.*, 16:409-414 [1993]; Le *et al.*, *Brain Res.*, 686:49-60 [1995]; and Mark *et al.*, *J. Neurosci.*, 15:6239-6249 [1995]). The magnitude of the damage contributed by each of these factors and the extent of their interaction are not clear (Copani *et al.*, 1995, *supra*). However, it is not necessary to understand the cause or effect of beta-amyloid accumulation in Alzheimer's disease in order to practice the present invention, nor is it intended that the present invention be limited to any particular mechanism or mechanisms of disease genesis or toxicity. Indeed, an understanding of any of the mechanisms of pathogenesis are not necessary in order to use the present invention.

Because of the close association between aging and AD and the similarities in the neuropathology observed in both conditions, oxidative stress has been proposed to play a role in the pathogenesis of Alzheimer's disease. It is believed that oxidative stress introduces oxidative modifications in βA before and/or after its secretion into the extracellular matrix. Furthermore, it is contemplated that circulating βA in individuals having Alzheimer's disease, brain trauma or Down's syndrome, but not in individuals without neurological injury, have specific post-translational chemical modifications. However, an understanding of the mechanism(s) involved in Alzheimer's disease pathogenesis is not necessary in order to practice the present invention, nor is it intended that the present invention be limited to any particular mechanism(s) of disease pathogenesis.

In one embodiment, the present invention provides modified βA synthetic peptides. A commercially available βA synthetic polypeptide 1-42 (SEQ ID NO: 1, cep Sigma, Catalog No. A9810) was chemically modified *in vitro* to produce a βA peptide

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containing malondialdehyde adduct(s) (*See*, Example 1). The unmodified peptide has the sequence:

Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Ly
s-
Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-
Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala (SEQ ID NO:1)

This βA peptide with a malondialdehyde adduct modification is referred to herein as "βA-MDA." It has been shown previously that protein-MDA adducts are formed by the modification of lysine residues. However, it is not intended that the present invention be limited to malondialdehyde adducts formed on lysine residues of the βA peptide. As oxidative stress is implicated in the aging process, it is contemplated that oxidative stress modifications (*e.g.*, the presence of malondialdehyde adduct[s]) are associated with βA peptides in patients having Alzheimer's disease. However, an understanding of the mechanism of oxidative stress modifications or modified βA-peptides is not necessary to make or use the present invention. Also it is not intended that the present invention be limited to this one type of modification.

In another embodiment (*e.g.*, as described in Example 2), synthetic βA-MDA peptide (*e.g.*, produced as described in Example 1) was used to raise anti-βA-MDA polyclonal antiserum in rabbits. Briefly, the βA-MDA peptide was conjugated to keyhole limpet hemocyanin (KLH), mixed with Freund's incomplete adjuvant, and used to immunize New Zealand white rabbits at 8-10 subcutaneous dorsal sites using standard methods. The animals received boosts at 4, 6 and 8 weeks after the initial immunization. Bleeds were obtained at weeks 8, 12 and 16 following the initial immunization. The presence of anti-βA-MDA antibody in the rabbits' sera was tested using a Western immunoblot technique (*e.g.*, as described in Example 4). The polyclonal antibody was purified using a combination of ammonium sulfate precipitation and immunoaffinity purification, as known in the art.

It is not intended that the production of antibody of the present invention be limited to any particular method. Indeed, it is contemplated that the antibody be prepared by any suitable method. In addition, polyclonal as well as monoclonal antibodies find use in the present invention. Indeed, the present invention also

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provides methods for the production of monoclonal antibody directed against βA-MDA peptide (*e.g.*, as described in Example 3). Numerous methods for the production and purification of monoclonal and polyclonal antibodies are well known in the art (*See e.g.*, Sambrook *et al.* (eds.), Molecular Cloning, Cold Spring Harbor Laboratory Press [1989]; Harlow and Lane (eds.), *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press [1988]; Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, p. 11.4.2-11.15.4, John Wiley & Sons, Inc., New York [1994]; Köhler and Milstein, Nature 256:495-497 [1975]; Kozbor *et al.*, *Immunol. Today* 4:72 [1983]; and Cole *et al.*, *in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 [1985]).

In addition, in other embodiments, any suitable modified βA peptide or fragment thereof, is used as an immunogen. In some embodiments, the immunogen is native, while in other embodiments, the immunogen is synthetic (*i.e.*, recombinant or produced by *in vitro* chemical synthesis). Similarly, it is not intended that the present invention be limited to any particular βA-derived immunogen, immunization method, immunization schedule, animal species, test protocol for determining antibody production or antibody purification method.

In some embodiments, the antibody preparation of the present invention is purified from crude antiserum, hybridoma or cell culture supernatant, ascites fluid, or other starting material using any conventional method. Such purification methods include, but are not limited to, protein A affinity, protein G affinity, ammonium sulfate precipitation, ion exchange chromatography, gel filtration, and immunoaffinity chromatography (*See, e.g.*, Harlow and Lane (eds.), *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press [1988]; and Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, Ch. 11, John Wiley & Sons, Inc., New York [1994]).

The antibodies of the present invention find numerous uses, including Western blotting, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), immunofluorescence assays (IFAs), immunoprecipitation, immunohistochemistry and clinical diagnostic applications using methods known in the art (*See e.g.*, Harlow and Lane (eds.), *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press [1988]; Ausubel *et al.*

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(eds.), *Current Protocols in Molecular Biology*, Vol. 1-4, John Wiley & Sons, Inc., New York [1994]; and Laurino *et al.*, *Ann. Clin. Lab Sci.*, 29(3):158-166 [1999]).

The specificity of a polyclonal antibody produced during the development of the present invention was tested in a Western immunoblot (*e.g.*, as described in Example 4, below). In this protocol, purified polyclonal anti-βA-MDA antibody (*e.g.*, produced as described in Example 2) was used as the primary antibody, and a goat-anti-rabbit IgG-horse radish peroxidase (HRPO) conjugated antibody (Santa Cruz) was used as the secondary antibody. Briefly, βA-MDA and unmodified βA synthetic peptide samples were prepared for analysis in Laemmli buffer. Peptide samples were resolved using denaturing polyacrylamide gel electrophoresis (*i.e.*, SDS-PAGE) and electroblotted onto nitrocellulose. Blots were incubated overnight in blocking buffer with anti-βA-MDA antibody at a 1:1,000 dilution. Membranes were washed, then incubated with HRPO-conjugated goat anti-rabbit IgG (Santa Cruz) diluted to 1:5,000 in blocking buffer. Following this incubation, blots were washed 3 times, then incubated in chemiluminescent detection substrate and the signal detected on X-ray film. This experiment demonstrated that the anti-βA-MDA antibody detected βA-MDA synthetic peptide, but not unmodified peptide. The preimmune serum showed no reactivity towards the same samples.

Following the establishment of the specificity of the anti-βA-MDA antibody of the present invention, the antibody was used to detect endogenous βA-MDA forms in human brain cortex tissue from an Alzheimer's patient and a matched control individual without Alzheimer's disease (as described in Examples 5 and 6, below).

Briefly, lysates were produced from these two brain tissues. These brain cortex tissue lysates were then subjected to immunoprecipitation, using 2 μg of an anti-βA antibody (Transduction Laboratories, Affinity Research Products Catalog No. AK6905) and Protein-A-agarose beads. The resulting immunoprecipitates were fractionated using SDS-PAGE and analyzed in a Western immunoblot (*e.g.*, as described in Example 4), where the anti-βA-MDA antibody of the present invention was used as the primary antibody, and an HRPO-conjugated goat-anti-rabbit antibody was the secondary antibody.

As shown in Figure 1, this experiment demonstrates that at least one protein present in the brain tissue βA-immunoprecipitate of an Alzheimer's disease patient

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(lane indicated as "**AD**") was recognized by the anti-βA-MDA antibody. However, no such bands were detected in the lane containing immunoprecipitate from the control (*i.e.*, disease-free individual; lane indicated as "**CONTROL**"). A single, predominant reacting protein band was also observed when using crude brain tissue lysates that had not been immunoprecipitated using the anti-βA antibody.

The specificity of the primary and secondary antibodies were further confirmed by immunoprecipitation and Western blot analysis using an anti-β-galactosidase antibody and brain tissue from an Alzheimer's disease patient. This immunoprecipitate was blotted and probed in parallel with the **AD** and **CONTROL** samples used in the blot shown in Figure 1 using the same anti-βA-MDA primary antibody and the HRPO-conjugated secondary antibody (lane indicated as "***β*-GAL**"). As shown in Figure 1, the anti-βA-MDA primary antibody showed no reactivity towards the anti-β-galactosidase immunoprecipitate from the brain tissue of the Alzheimer's patient. Furthermore, in a separate experiment, it was found that the antibody that detects unmodified βA polypeptide when used as the primary antibody, detected identical bands in the immunoprecipitated brain tissues from an Alzheimer's disease patient and a disease-free control individual.

Thus, polyclonal anti-βA-MDA antibody (*e.g.*, produced as described in Example 2), recognizes a modified endogenous βA-MDA peptide that is found only in the brain of an individual having Alzheimer's disease, but not in the brain of an unaffected individual. Thus, the present invention provides compositions and methods for post-mortem diagnosis of Alzheimer's disease.

It is known that βA is present in human blood and cerebrospinal fluid (CSF) of living subjects. It is contemplated that βA-MDA is also present in the blood, serous fluid and/or CSF of living subjects. It is contemplated that the presence of βA-MDA, or its presence above a threshold level, in these fluids is diagnostic of Alzheimer's disease. Thus, the present invention provides methods and compositions for the diagnosis and prognosis of Alzheimer's disease. Indeed, the present invention provides compositions and methods to analyze disease severity, and the efficacy of Alzheimer's disease therapies. It is contemplated that subjects identified as having higher levels of βA-MDA (*e.g.*, in blood, serous fluid or CSF) have more advanced Alzheimer's disease than subjects showing lower

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