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A point mutation in the CH3 domain of human IgG3 inhibits antibody secretion without affecting antigen specificity

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Abstract

Immunoglobulins (Ig) require correct folding and assembly of both heavy (H) and light (L) chains to form a functional H_2L_2 dimer that is secreted from plasma cells. This process is dependent upon the endoplasmic reticulum (ER) chaperone BiP, which targets improperly, folded or assembled Ig molecules for degradation. While investigating the mechanism of low IgG3 secretion, we identified a missense mutation L368P in the CH3 region of the human γ3 H-chain that was associated with impaired secretion of intact and functional Ig. The non-secreted H-chains displayed slower electrophoretic migration than secreted H-chains, consistent with them being glycosylated in the ER but not fully processed in the golgi apparatus and secretory pathway. Reversion of the mutated codon to wild type restored secretion of the IgG3, which displayed the same fine specificity for antigen as non-secreted IgG3. However, the non-secreted IgG3 was not opsonic in an in vitro phagocytosis assay. The results indicate that correct IgG3 CH3 domain folding is essential for secretion and effective function but does not affect specificity for antigen.
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1. Introduction

The technology and methodology for the production of monoclonal antibodies has progressed at a remarkably fast pace since the original description of the hybridoma technique (Kohler and Milstein, 1975). It is now possible to utilize recombinant DNA technology to produce monoclonal antibodies with desired specificities from mammalian cells as well as bacteria and yeast, and in transgenic animals and plants (Anon., 1999). All of these methods still rely on the ability of the mammalian immune system to create antibody specificity but use quite different techniques to identify and produce specific antibodies.

The difficulties, both technical and inherent, in adapting the hybridoma strategy to the production of human monoclonal antibodies, particularly those targeting human proteins, has led to the adoption of protein engineering techniques for reducing the immunogenicity of murine monoclonal antibodies to the human immune system. This necessity of the use of recombinant DNA techniques to modify monoclonal antibodies has led to the production of antibodies from transfected mammalian cells, becoming the most common source of therapeutic antibodies. Other strategies for generating human monoclonal antibodies, such as SLAM

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or phage display, also require systems for the production of the recombinant immunoglobulins (Ig). For example, once the B-lymphocyte producing the antibody of desired specificity is identified, cloning of the relevant heavy (H) and light (L) chain Ig genes enables the unlimited production of monoclonal antibodies of the same specificity (Babcook et al., 1986). With this method, the rearranged Ig variable region genes (VH and VL) of choice that were amplified from selected antibody forming cells can be used in immunoglobulin expression vectors that encode functional Ig of any class. Likewise phage display strategies generate VH and VL genes that must be expressed as Ig for many applications (Barbas et al., 2001). Plasmid expression vectors have been described that contain constant (C) region genes for many of the human Ig isotypes (Coloma et al., 1992; Norderhaug et al., 1997; McLean et al., 2000) and the methods for the introduction of such vectors into mammalian cells and for harvesting of antibodies from culture supernatants are well established. However, despite the power of this technology, the production of antibodies in this way can be problematic. The recombinant antibodies are generally secreted at lower rates than traditional hybridomas, thus reducing the overall yield. These difficulties may be overcome by the use of alternative expression systems such as bacteria and insect cells but these bring additional complications, such as alterations in post-translational processing of proteins. Furthermore, loss of secretion or alterations in biological activity due to potential incompatibility of the V and C regions can also occur, highlighting the delicate nature of this technique (Yoo et al., 2002).

Previously we synthesized a matched set of Ig expression vectors designed to produce fully human, fully murine or chimeric recombinant antibodies from mammalian cells (McLean et al., 2000). All of these vectors, except the vector used for the production of human or chimeric IgG3, produced functional, secreted Ig molecules. Recombinant chimeric mouse–human IgG3 (chIgG3) was detected at only very low levels in the culture supernatant, although chIgG3 that retained antigen specificity could be isolated from cell-lysates (McLean et al., 2002). Analysis of the IgG3 C region of this vector revealed two nucleotide differences from what was expected from the published germ-line sequence. Here we describe that mutation of these two nucleotides to the published sequence resulted in secretion of chIgG3 molecules from this expression vector, suggesting that our original sequence had been affected by PCR-induced errors during construction. Our results provide some insight into the effects on secretion of recombinant antibodies through protein folding.

2. Materials and methods

2.1. Immunoglobulin expression vectors and site-directed mutagenesis

Expression vectors (pLC-huC$_\kappa$, pHC-huC$_\gamma$1, pHC-huC$_\gamma$3) used for the production of chIgG1 and chIgG3 were exactly the same as described previously (McLean et al., 2000). The Ig V domains for the murine monoclonal antibody 18B7 (Casadevall et al., 1998) were used to construct mouse–human chIgG1 and chIgG3; these sequences have been described previously (McLean et al., 2002). To alter Pro 136 and Pro 368 residues in the human C$_\gamma$3 sequence (GenBank accession number AJ294732), site-directed mutagenesis was performed on the pHC-huC$_\gamma$3 plasmid. The method was based on the Quik-Change protocol using high fidelity Pfu polymerase (Stratagene, Carlsbad CA). Briefly, PCR was performed using pHC-huC$_\gamma$3 as a template with the following conditions 95 °C for 30 s, 55 °C for 30 s, and 68 °C for 14 min for 16 cycles. Primers for converting Pro 136 to Ser were; ccaggagcacc**t**ctgggggcacag and ctgtgcccccag**a**ggtgctcctgg, and for converting Pro 368 to Leu were; gcctgacctgcc**t**ggtcaaaggcttc and gaagcctttgacc**a**ggcaggtcaggc (altered nucleotides are shown in bold). Successful mutation of codons encoding Pro 136 to Ser and Pro 368 to Leu in the plasmids was confirmed by bi-directional sequencing.

2.2. Production and purification of chimeric IgG

HEK 293 cells were transiently transfected with plasmids encoding L-chain and various H-chains to produce chIgG1 and chIgG3. Briefly, 3 μg of each plasmid was mixed with lipofectamine reagent (Invitrogen, Carlsbad, CA) and added to a monolayer of 4×10^6 HEK 293 cells for 6 h at 37 °C. Complete media (DMEM including 10% FCS) was then added, harvested and re-added every 24 h for up to 5 days. To harvest non-secreted chimeric IgG3, cells were collected by centrifugation at 24 and 48 h post-transfection and lysates were made using PBS containing 1% IGEPAL (Sigma, St. Louis, MO). Recombinant chIgG1 and chIgG3 were purified from either cell supernatants or cell-lysates using a protein-G affinity column (Amersham Biosciences, Uppsala, Sweden). Following elution with 100 mM glycine pH 2.5, the purified chIgG was dialyzed over 2 days versus three changes of PBS. Preparations were quantitated for IgG level by BCA protein assay (Pierce Biotechnology, Rockford, IL).

2.3. ELISA

This was performed as previously described for both GXM binding and chIgG production (McLean et al., 2002). Intact chIgG was assayed by capture with anti-L-chain antibodies and detected by using anti-H-chain antibodies. Anti-human γ chain, anti-human κ chain, both unlabelled and conjugated to alkaline phosphatase were from Southern Biotechnology Associates (Birmingham, AL).

2.4. Immunoblotting

Lysates of transfected 293 cells were prepared using RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS) containing protease

inhibitors (Complete Mini, EDTA-free) (Roche Diagnostics GmbH, Mannheim, Germany) and the protein content quantified by BCA protein assay (Pierce Biotechnology, Rockford, IL). For secretors 30 μg, and for non-secretors 75 μg of whole cell lysate was separated by SDS-PAGE (12%), transferred to nitrocellulose membranes, and analyzed by blotting with anti-human γ chain conjugated to peroxidase (Southern Biotechnology Associates, Birmingham, AL).

2.5. Immunofluorescence staining

Transfected 293 cells were fixed in 4% paraformaldehyde, blocked, and permeabilized in PBS containing 4% FCS and 0.2% Triton-X. Anti-human golgin-97 (Molecular Probes, Eugene, OR) was used for Golgi staining. Alexa 488-conjugated (anti-mouse IgG) (Molecular Probes, Eugene, OR) or Texas red-conjugated (anti-human IgG) (Southern Biotechnology Associates, Birmingham, AL) secondary antibodies were used according to the recommendations of the manufacturer. Images were acquired and analyzed as described (Noseda et al., 2004).

2.6. Cryptococcal assays

Both the immunofluorescence (IF) staining and phagocytosis assays were performed as described previously (McLean et al., 2002). Enriched chIgG preparations from supernatants or cell-lysates were used for IF staining whilst protein-G purified chIgG3 was used for the phagocytosis assay.

2.7. Homology modelling of human IgG3 Fc region

The amino acid sequence of human IgG3 was aligned with sequences of proteins deposited in the Protein Data Bank (PDB) using BLAST (http://www.ncbi.nlm.gov/BLAST/). PDB entry 1HZH (human anti-HIV B12 antibody; Saphire et al., 2001) was identified as a protein with 94% amino acid sequence identity to IgG3 in the Fc region. WT IgG3 and L368P IgG3 amino acid sequences were used as target sequences. The coordinates of the 1HZH_B chain and 1HZH_K chain were used as templates to generate a dimeric model with the help of the web-based Swiss-Model software (Peitsch, 1995; Guex and Peitsch, 1997; Schwede et al., 2003). The results are displayed using PyMol graphic software (DeLano Scientific LLC, San Carlos CA, USA http://www.pymol.org).

3. Results

3.1. Human IgG3 constant region cDNA clones

The single clone of human IgG3 C region cDNA (IGHG3) we had previously amplified by PCR contained two nucleotide differences from the closest IGHG3 sequence in nucleotide databases. Our IGHG3 cDNA had been amplified from cells expressing a mouse–human chIgG3 antibody (Sandlie et al., 1989) and has been described previously as part of the Ig expression vector pHC-huC$_\gamma$3 (McLean et al., 2000). Thus, nucleotides 59 and 893 of the C region cDNA were both found to be c instead of t, resulting in predicted amino acid substitutions of Ser to Pro in CH1 and Leu to Pro in CH3, at positions 136 and 368 (Eu numbering; Edelman et al., 1969), respectively. This sequence (GenBank accession number AJ294732) was closest to that of IGHG3*01, containing exons for CH1, CH2 and CH3 as well as four hinge exons (GenBank accession number X03604) (Huck et al., 1986), one of the 19 alleles displayed in the IMGT database (http://imgt.cines.fr) (Lefranc et al., 1999). Recombinant Ig molecules expressed using our human C region were not secreted from the cells. Defective secretion was also observed when the chIgG3 H-chains were co-expressed with either murine κ chains (McLean et al., 2000) or chimeric mouse–human κ chains (McLean et al., 2002), although functional Ig was isolated from cell-lysates in the latter case. The associated VH region was not the cause of the secretion defect, since a similar failure of secretion was observed when the C$_\gamma$3 region was expressed together with two different murine VH domains, R45 and 18B7. Furthermore, the secretion defect was not cell type specific since we observed a lack of secreted chIgG3 from both a murine plasmacytoma cell line (NS0) and human embryonic kidney cells (HEK 293).

All human IgG isotypes (IgG1, IgG2, IgG3, and IgG4) contain Ser at position 136 and Leu at position 368, suggesting the importance of these residues in the protein structure. We suspected that the substitution of Pro at these positions in our IgG3 molecules were responsible for the absence of Ig secretion. To explore this hypothesis, we altered these nucleotide differences in our IGHG3 cDNA sequence of pHC-huC$_\gamma$3 and evaluated their effect on the secretion of chIgG3 molecules. Site-directed mutagenesis was performed to revert the nucleotide sequence to the sequence of allele IGHG3*01. We constructed three new IGHG3 cDNA sequences and thus, pHC-huC$_\gamma$3 plasmids, with either or both of the Pro residues changed to the Ser or Leu present in IGHG3*01 (Fig. 1). All pHC-huC$_\gamma$3 constructs contained the VH domain of the murine monoclonal antibody 18B7 (GenBank accession number AJ309276) specific for *Cryptococcus neoformans* capsule glucuronoxylomannan (GXM) (Casadevall et al., 1998).

3.2. Secretion analysis of chIgG3 molecules

The various pHC-huC$_\gamma$3-18B7VH constructs and the control pHC-huC$_\gamma$1-18B7VH construct were co-transfected with pLC-huC$_\kappa$-18B7V$_\kappa$ (18B7V$_\kappa$ GenBank accession number AJ309277, human C$_\kappa$ GenBank accession number AJ294735) into HEK 293 cells to monitor transient expression of chIgG3. Culture supernatants and cell-lysates were harvested at both 24 and 48 h following transfection. These Ig preparations were tested by ELISA for production of chIgG and for binding to GXM. The IgG3 secretion results are

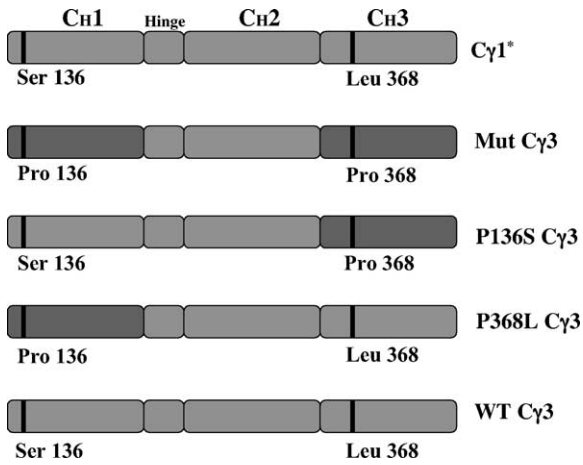

Fig. 1. Schematic diagram of constant regions for the H-chain chIgG clones. Mutated (P136, P368) and wild type (S136, L368) residues are indicated at the corresponding positions of each C_γ region. Darker shading represents a domain with a mutated residue. * The human $C_\gamma1$ differs from human $C_\gamma3$ in that the hinge region is shorter but is not displayed as such in this figure.

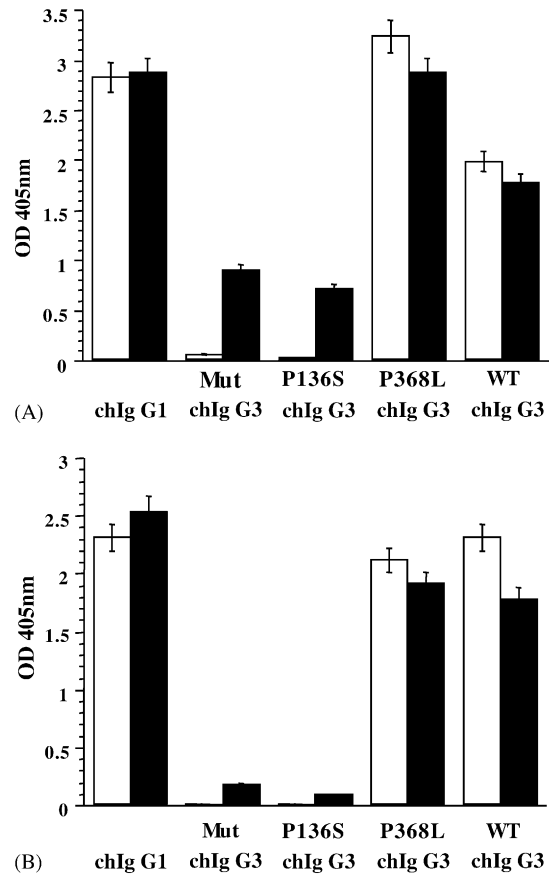

Fig. 2. chIgG3 with the L368P mutation are not secreted from cells. Supernatants (open columns) and cell-lysates (closed columns) of 293 cells, co-transfected with the indicated plasmid constructs encoding H-chains and L-chains for production of chIgG, were analyzed after (A) 24 h and (B) 48 h for chIgG production by capture ELISA.

shown in Fig. 2. Control chIgG1 was efficiently secreted into the supernatant at both 24 and 48 h following transfection whereas the original chIgG3 (Mut IgG3) was not secreted although it was detectable in cell-lysates at 24 h post-transfection (Fig. 2A and B), consistent with previous results. This confirms that the Mut IgG3 H-chains were produced by the transfected cells and were paired with the chimeric L-chains but that the Ig molecules were not secreted from cells despite the presence of identical heavy chain Ig leader sequences, VH regions and L-chains. chIgG3 expressed with just the CH1 Pro residue reverted to wild type Ser (P136S IgG3), behaved like Mut IgG3 and were not secreted, whereas chIgG3 expressed with the CH3 Pro residue reverted to wild type Leu (P368L IgG3) or both Pro residues which reverted to the canonical (WT IgG3) were both efficiently secreted from cells (Fig. 2A and B). Calculations of the ratio of secreted IgG to internal IgG from these cells confirm an approximate 1:1 ratio for secretors which is reduced by 10- to 20-fold for non-secretors (Table 1). These results support the contention that the presence of the Pro residue at position 368 in CH3 of human IgG3 negatively affected secretion of these proteins while the Pro residue at position 136 of CH1 had no such adverse effect on protein secretion.

To ascertain whether the chimeric $C_\gamma3$ H-chains produced by transfected cells had the expected molecular mass, we

Table 1
Analysis of anti-*C. neoformans* chIgG secretion and function

chIgG clone	Amino acid		Secreted/internal IgG index[a]	GXM ELISA[b]	*C.neoformans* IF pattern	Phagocytosis index[c]
	@ 136	@ 368				
IgG1	Ser	Leu	0.98	++	Annular[d]	+++[d]
Mut IgG3	Pro	Pro	0.07	++	Punctate	+/–
P136S IgG3	Ser	Pro	0.04	+++	nd[e]	+/–
P368L IgG3	Pro	Leu	1.13	++++	Punctate	++
WT IgG3	Ser	Leu	1.12	++++	Punctate	++

[a] Data are summarized from Fig. 2. IgG was harvested 24 h post-transfection and diluted 1:4 for ELISA analysis. Results are expressed as the ratio of OD 405 nm obtained for secreted IgG/internal IgG.

[b] Data are summarized from Fig. 4. IgG was protein-G purified and normalized for protein content before ELISA analysis for GXM binding. The OD 405 nm values achieved with 1 μg/ml chIgG were assigned as follows: 0–0.2 +, 0.2–0.4 ++, 0.4–0.6 +++, 0.6–0.8 ++++.

[c] The ability of chIgG to opsonize *C. neoformans* was measured as previously described (McLean et al., 2002). Phagocytosis index is assigned as follows: no opsonization –, weak opsonization +, medium opsonization ++, strong opsonization +++.

[d] Data previously published (McLean et al., 2002).

[e] Not done.

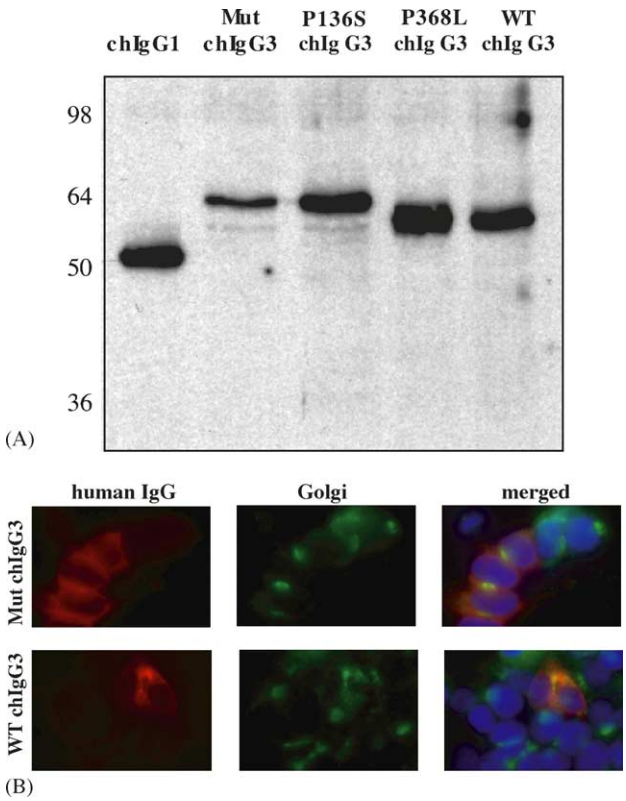

Fig. 3. Secreted and non-secreted chIgG3 H-chains have different molecular masses and localize to different subcellular compartments. (A) Whole cell-lysates were made 24 h after co-transfection with the indicated plasmid constructs encoding H-chains and L-chains for production of chIgG. H-chains were assessed by immunoblot with anti-human γ chain. M: molecular mass. (B) Immunofluorescence staining of HEK 293 cells transfected to express either Mut chIgG3 (upper panels) or WT chIgG3 (lower panels) was performed using anti-human γ chain or anti-human golgin-97. Merged images with Dapi staining of nuclei are shown at the far right.

analyzed cell-lysates by western blotting. Interestingly, we found that the mobility of the non-secreted H-chains was slower than that of secreted H-chains despite almost identical molecular weight predicted for the protein product from the cDNA (Fig. 3A). Thus, the electrophoretic molecular weight for the non-secreted H-chains (Mut and P136S), secreted H-chains (P368L and WT) and control $C_\gamma1$ H-chain was ∼60, ∼57, and ∼52 kDa, respectively. The most straightforward interpretation of these results is that the non-secreted $C_\gamma3$ chains were glycosylated differently than the secreted $C_\gamma3$ chains. To confirm our suspicion that the increased molecular weight of non-secreted H-chains was the result of unprocessed N-linked carbohydrate chains due to blockade early in the secretory pathway, we performed IF staining of transfected 293 cells to compare the localization of secreted and non-secreted H-chains (Fig. 3B). We found that both non-secreted and secreted H-chains were expressed throughout the cytoplasm whilst secreted H-chains tended to co-localize with the golgi apparatus marker, unlike the non-secreted H-chains (Fig. 3B, lower right panel). This result is consistent with the non-secreted H-chains being incorrectly folded and unable to traffic to the golgi, hindering their passage through the secretory pathway.

3.3. Reactivity of chIgG3 with GXM

We next evaluated the functionality of the chIgG by testing their binding to the cognate antigen GXM. To facilitate the analysis of GXM binding by all the chIgG clones, the Ig's were purified from supernatants and cell-lysates by protein-G chromatography. Preparations of purified IgG were normalized for protein content and tested for binding to GXM by ELISA. Here, significant binding of both the secreted and non-secreted chIgG3, as well as the control chIgG1 was observed (Fig. 4). Taken together, these results confirm that chIgG3 with the described amino acid differences in either C_H1 or C_H3, that have opposing effects on protein secretion, are still bound to GXM in ELISA experiments with characteristics similar to those of canonical chIgG3 and chIgG1.

3.4. Immunofluorescence patterns of chIgG3 with C. neoformans

Since all of the purified chIgG3 preparations were bound to GXM by ELISA, we decided to re-evaluate the fine specificity of binding to *C. neoformans* cells by immunofluorescence (IF). We have previously shown that the fine specificity of antibodies, expressing mAb 18B7 V regions, for the polysaccharide capsule of *C. neoformans* differed depending on the antibody isotype (McLean et al., 2002). In this system, differences in IF pattern produced by antibody binding to the polysaccharide capsule reflect differences in antibody fine specificity and two major patterns known as annular or punctate have been described. Thus, the murine mAb 18B7 (IgG1-κ) and chIgG1, chIgG2, chIgG4, each bound in an annular pattern to the *C. neoformans* capsule, whereas chIgG3, with identical 18B7 V_H and V_κ region sequences, produced a punctate pattern. This result was interpreted as indicative of C region induced changes in fine specificity of antigen

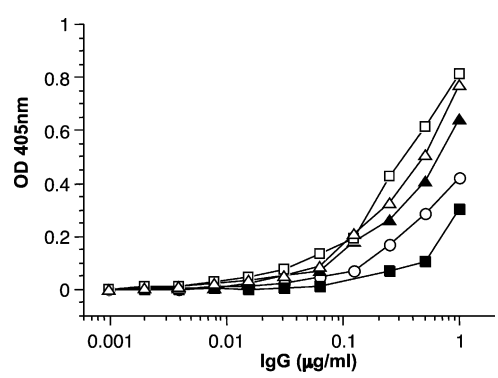

Fig. 4. Secreted and non-secreted chIgG3 bind to GXM. Direct ELISA to *C. neofomans* GXM with protein-G purified chIgG. The various chIgG are designated as follows. Secreted chIgG (open symbols), chIgG1 (circle), P368L chIgG3 (square), WT chIgG3 (triangle). Non-secreted chIgG (closed symbols), Mut chIgG3 (square), P136S chIgG3 (triangle).

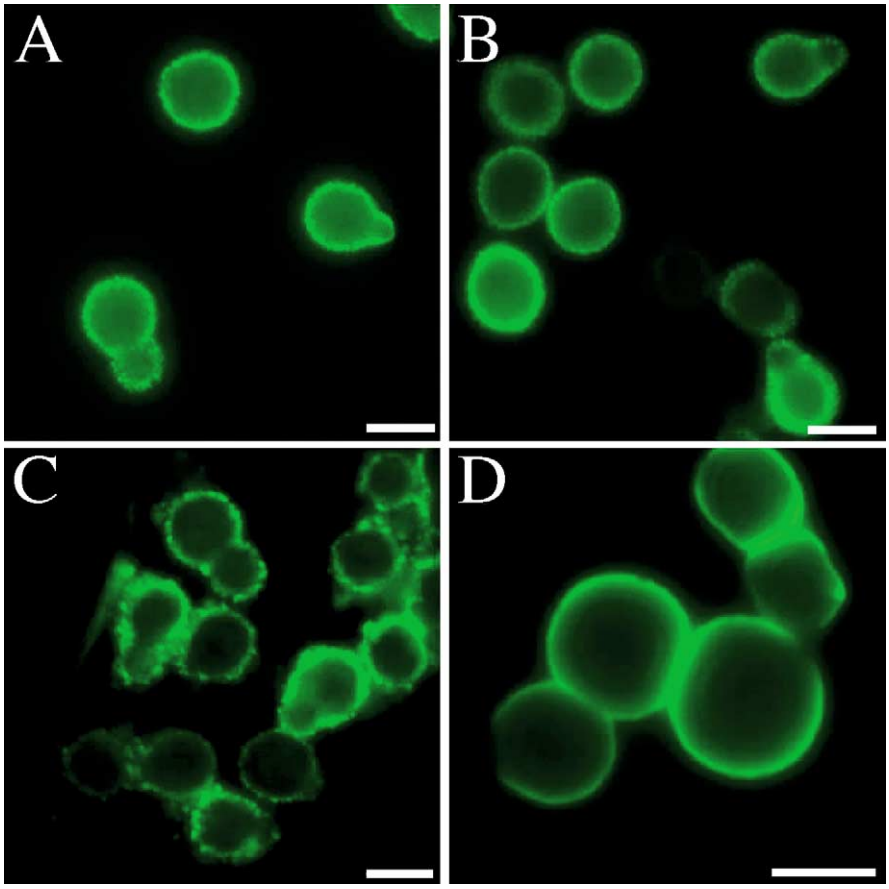

Fig. 5. Secreted and non-secreted chIgG3 immunofluorescence staining of *C. neoformans* cells is punctate. *C. neoformans* cell suspensions were incubated with secreted ((A) P368L chIgG3; (B) WT chIgG3) and non-secreted ((C), Mut chIgG3) chIgG3 antibodies and detected as previously described (McLean et al., 2002). Control staining with chIgG1 was done to display annular IF pattern for comparison (D). The scale bar represents 5 μm.

binding. However, the finding that the IgG3 C region contained two point mutations, that impeded secretion and normal glycosylation, raised the possibility that abnormal IgG3 structure may have been responsible for punctate staining. However, we observed here that antibodies with either our original IgG3 C region or the canonical one produced punctate IF patterns, demonstrating that the Pro residues were not responsible for the altered specificity relative to other isotypes (Fig. 5). Thus, secreted chIgG3 where Pro 368 was reverted to Leu 368 (P368L IgG3 and WT IgG3), both produced punctate IF (Fig. 5A and B) and the non-secreted chIgG3 clone (Mut IgG3), when purified from cell-lysates, also produced punctate IF (Fig. 5C) confirming the previously reported results with non-secreted chIgG3 (McLean et al., 2002). Importantly, the chIgG1 produced the expected annular IF (Fig. 5D). These results are consistent with our previously reported novel finding that antibody fine specificity to a polysaccharide antigen depends on the associated C region (McLean et al., 2002).

3.5. Opsonic efficacy of chIgG3

We assessed the ability of the various chIgG3 proteins to opsonize *C. neoformans*. Opsonic ability correlated with secretion efficiency. Thus, the secreted chIgG3 clones (P368L IgG3 and WT IgG3) and control chIgG1 showed significant opsonic efficacy, whereas the non-secreted chIgG3 (Mut IgG3 and P136S IgG3) were not opsonic (Table 1). These results are consistent with the hypothesis that the correct folding and/or glycosylation of the chIgG3 CH3 domain are required not only for secretion from cells but also for opsonic efficiency.

3.6. Human IgG3 Fc region model

We performed homology modeling of the canonical and the original chIgG3 Fc domain to pinpoint the contribution of Pro 368 to the non-secretion phenotype (Fig. 6). The relative positions of the N-linked glycosylation site of CH2 (Asn 297) and the Tyr 407 and Pro 368 residues in the CH3 dimer interface are indicated (Fig. 6A). Closer inspection of the CH3 dimer interface (Fig. 6B and C) reveals that the sidechains of Leu 368 form a close interaction with the sidechains of Tyr 407. One possible reason for the lack of secretion of chIgG3 containing Pro 368 is that this residue contributes to a lack of dimeric domain stability. Our model supports this possibility by predicting that the substitution of Pro 368 resulted in a

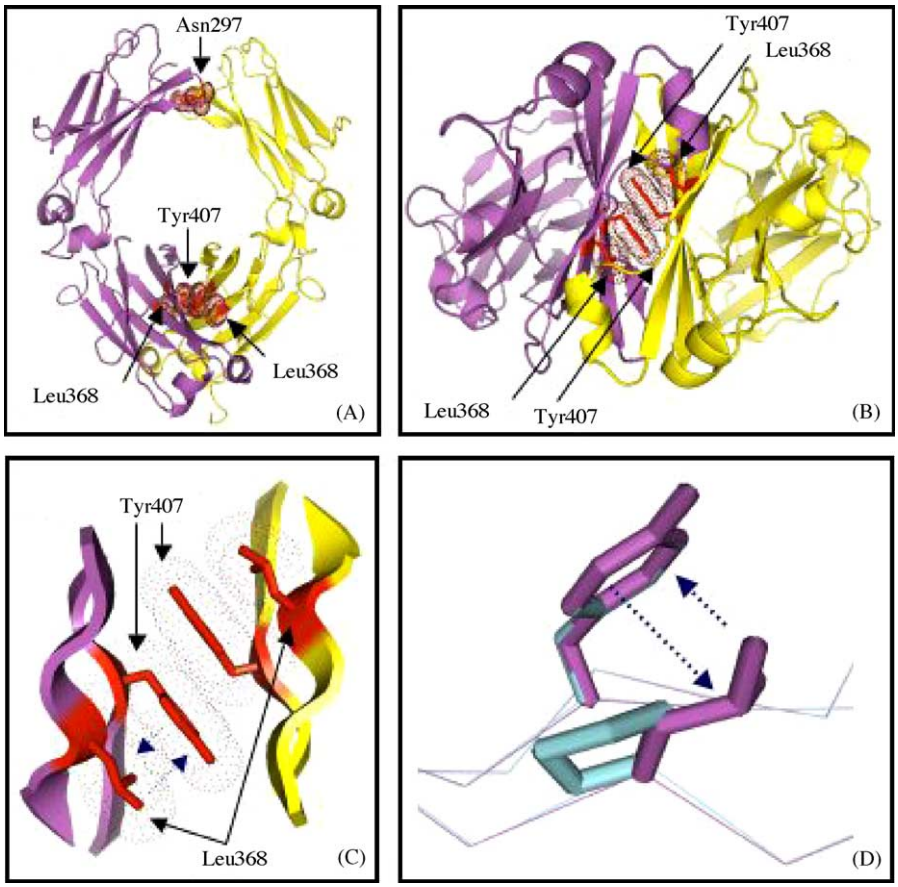

Fig. 6. Structural analysis of IgG3 Fc region and L368P mutant by homology modeling. (A) View perpendicular to the 2-fold rotation axis between the H-chain dimer interface of WT IgG3 Fc region. The locations of residues Asn297 (glycosylation site), Leu368 (mutant residue), and Tyr407 (structurally important) from each monomer are displayed in red with the van der Waals surfaces of their side-chains represented by dots. (B) View along the 2-fold rotation axis between the H-chain Fc dimer looking down the interface. Residues Leu368 and Tyr407 from each monomer are displayed in red with the van der Waals surfaces of their sidechains represented by dots. (C) Blow up of Tyr407 stacking and Leu368 clamp. (D) A close-up of WT model backbone (magenta) and the L368P model backbone (cyan). The sidechains of residues Leu368 and Pro368 as well as Tyr407 are displayed and indicate the loss of "clamping" on Tyr407 caused by the L368P mutation.

lack of clamping to the stacking interaction of Tyr 407 at the interface between the two CH3 monomers (Fig. 6D). This clamping would normally be provided by the sidechain of Leu 368. Lower stability of this domain, due to the presence of Pro 368, may block entry of the proteins into the secretory pathway from the ER, where they remain destined for degradation.

4. Discussion

Prior studies had shown that our pHC-huC$_\gamma$3 expression vector produced intact chIgG3 that remained within the cell and was not secreted (McLean et al., 2002). To investigate this problem we analyzed the C$_\gamma$3 plasmid sequence and noted two nucleotide differences from the closest human C$_\gamma$3 sequence in the database that resulted in replacement amino acid substitutions. We considered the possibility that our mutations represented polymorphisms or that perhaps the database sequence was in error. Establishing the correct sequence was important because one or both of these amino acid differences could be responsible for the impaired secretion observed with this vector. Furthermore, the observation complicated interpretation of our finding that chIgG3 anti-cryptococcal antibodies had different fine specificity than other isotypes with identical V regions (McLean et al., 2002). Thus it was conceivable that the alteration in pattern of IF was a consequence of the C region mutation and did not reflect a general property of chIgG3. Therefore, we analyzed the effect of these two amino acid differences on secretion and on IF pattern. Restoration of the nucleotides in our original construct to those in the canonical sequence resulted in a marked increase in the amount of secreted chIgG3, and corrected the secretion defect associated with the original pHC-huC$_\gamma$3 expression vector.

Our observations showed that the CH1 domain mutation Pro 136 was not associated with defective secretion. In contrast, the Pro 368 mutation in CH3 greatly reduced chIgG3 secretion. This result raised the possibility that the structure of the CH3 domain was affected by Pro 368 but the CH1 domain

structure was not affected by Pro 136. Homology modelling of the original and canonical IgG3 Fc domain helped us to determine, in a structural context, the contribution of Pro 368 to the non-secretion phenotype. The model predicts that the presence of Pro 368 destabilizes the CH3 dimer interface via loss of interaction with important stacking Tyr 407 residues from each H-chain. This destabilized domain structure would be identified by ER chaperones, such as BiP, leading to the block of chIgG3 export and subsequent degradation within the ER.

Glycosylation has been implicated in the proper assembly and secretion of IgG molecules (Taylor and Wall, 1988) and human IgG3 contains a biantennary complex N-linked carbohydrate at residue Asn 297 within CH2 (Rademacher et al., 1986). We noted for the non-secreted chIgG3 H-chains differences in electrophoretic migration compared to that of the secreted chIgG3, suggesting that the non-secreted H-chains due to CH3 instability were retained in the ER, preserving the initial high mannose and unprocessed carbohydrate side chains. In fact, our experiments showed that the non-secreted H-chains could not localize to the golgi apparatus, unlike secreted H-chains. This is supported by the recent demonstration that a recombinant IgG with VH-associated N-linked carbohydrate side chains that remain high in mannose, is not secreted and is retained in the ER (Gala and Morrison, 2004).

Our results raised the possibility that the presence of not fully processed carbohydrate in the non-secreted chIgG3 isolated from within cells could alter the ability of these antibodies to carry out effective function and/or alter their fine specificity for antigen. Interestingly, all chIgG3 bound efficiently to the purified cryptococcal capsular polysaccharide GXM, including the non-secreted chIgG3. Previously we had shown that the anti-cryptococcal chIgG3 isolated from cell-lysates, since it was not secreted, produced a punctate IF pattern (McLean et al., 2002). Prior work has established two major patterns of antibody binding to the *C. neoformans* polysaccharide capsule, annular and punctate, which are named based on their microscopic fluorescence appearance. Antibody 18B7 produces annular IF, so to determine whether this change in fine specificity was associated with the amino acid differences within the C region, we repeated the IF using the repaired chIgG3. When these chIgG3 were compared for binding to *C. neoformans* cells by IF, we noted that the staining pattern was punctate rather than the typical annular pattern previously described for mAb 18B7 (Casadevall et al., 1998). Our finding that repaired chIgG3 expressing the wild type sequence produced the same IF upon binding *C. neoformans* indicate that the changes in fine specificity for the non-secreted chIgG3, were not a result of alterations in primary C region sequence. These results are consistent with, and supportive of, the notion that V regions may differ in fine specificity when associated with different C regions (Cooper et al., 1993; Pritsch et al., 1996; McLean et al., 2002). Importantly, the CH1 mutation Pro 136, that did not inhibit secretion, also did not affect fine specificity for *C. neoformans*. We conclude that the presence of Pro at positions 136 and 368 in chIgG3 does not affect fine specificity for antigen whereas the Pro at position 368 affects chIgG secretion.

Non-secreted chIgG3 did not display effector function in an in vitro phagocytosis assay while secreted chIgG3 could opsonize *C. neoformans*, suggesting that, despite normal antigen specificity the instability and aberrant glycosylation of the Fc region may interfere with Fc receptor interaction and hence Ig effector function. In this regard, it is noteworthy that IgG C region N-glycan structures are critically important for the interaction of immunoglobulin with the Fc receptor (Mimura et al., 2001; Radaev et al., 2001). Furthermore, the Fc receptor-binding site of IgG is in the lower hinge and hinge-proximal region of CH2 (Jefferis and Lund, 2002), which is distant from the Pro 368 residue in CH3 but nearby the N-linked glycosylation site at Asn 297. An alternative explanation for the lack of opsonic ability of these chIgG3 preparations could simply be related to insufficient Ig present, since we could only purify relatively small amounts of the non-secreted chIgG3 protein and consequently could not perform a detailed dose–response evaluation. Further investigation with higher concentrations of the non-secreted chIgG3 will be required to demonstrate whether their apparent inability to opsonize *C. neoformans* is due to the loss of interaction with the Fc receptor or not. Previously, antibodies displaying annular IF pattern on *C. neoformans* had stronger opsonic activity than antibodies that produced punctate IF (Nussbaum et al., 1997; Cleare and Casadevall, 1998). The results of this study suggest that the association between punctate IF and non-opsonization is not ubiquitous in that some antibodies that produce punctate IF can opsonize equally as well as antibodies that produce annular IF.

An association has been made between annular IF and IgM-mediated phagocytosis in the absence of complement (Taborda and Casadevall, 2002). That phenomenon presumably involves a structural change in the capsule that promotes phagocytosis through the complement receptor by direct polysacchride-CD18 interactions (Taborda and Casadevall, 2002). In contrast, IgM mAb's that produce punctate IF are not opsonic by this mechanism, presumably because the necessary capsular structural changes that allow the interaction with the complement receptor does not occur. In this study we noted that the secreted chIgG3 was opsonic despite producing a punctate IF pattern on *C. neoformans* capsules. We interpret this phenomenon as indicative of the fact that chimeric IgG3, unlike IgM, can interact with Fc receptor and promote phagocytosis without engaging complement receptors. Hence, the association between punctate binding and lack of opsonic efficacy does not apply to antibodies that can engage receptors directly.

The findings presented herein describe the restoration of secretion of recombinant chIgG3 molecules from the expression vector pHC-huC$_\gamma$3. This was important for two reasons: (1) a reliable system for producing functional, secreted recombinant IgG3 was required and (2) to confirm that our previous observation that the alteration of fine specificity of 18B7-chIgG3 was not due to amino acid differences within

the $C_{\gamma}3$ sequence. We have achieved these aims and presented the results in this report. The implications of this work are enhanced by the fact that mAb 18B7 is currently in clinical evaluation and that mouse–human chimeric antibodies are increasingly being used in human therapy.

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