

Mutations on the External Surfaces of Adeno-Associated Virus Type 2 Capsids That Affect Transduction and Neutralization

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Mutations were made at 64 positions on the external surface of the adeno-associated virus type 2 (AAV-2) capsid in regions expected to bind antibodies. The 127 mutations included 57 single alanine substitutions, 41 single nonalanine substitutions, 27 multiple mutations, and 2 insertions. Mutants were assayed for capsid synthesis, heparin binding, in vitro transduction, and binding and neutralization by murine monoclonal and human polyclonal antibodies. All mutants made capsid proteins within a level about 20-fold of that made by the wild type. All but seven mutants bound heparin as well as the wild type. Forty-two mutants transduced human cells at least as well as the wild type, and 10 mutants increased transducing activity up to ninefold more than the wild type. Eighteen adjacent alanine substitutions diminished transduction from 10- to 100,000-fold but had no effect on heparin binding and define an area (dead zone) required for transduction that is distinct from the previously characterized heparin receptor binding site. Mutations that reduced binding and neutralization by a murine monoclonal antibody (A20) were localized, while mutations that reduced neutralization by individual human sera or by pooled human, intravenous immunoglobulin G (IVIG) were dispersed over a larger area. Mutations that reduced binding by A20 also reduced neutralization. However, a mutation that reduced the binding of IVIG by 90% did not reduce neutralization, and mutations that reduced neutralization by IVIG did not reduce its binding. Combinations of mutations did not significantly increase transduction or resistance to neutralization by IVIG. These mutations define areas on the surface of the AAV-2 capsid that are important determinants of transduction and antibody neutralization.

Adeno-associated virus type 2 (AAV-2) is a parvovirus belonging to the *Dependovirus* genus (16). AAV-2 has a linear, single-stranded DNA genome containing 4,679 nucleotides that encodes two genes called *rep* and *cap*. The *rep* gene encodes four proteins that play roles in viral DNA replication, DNA packaging, and the regulation of transcription and splicing. The *cap* gene encodes three proteins (VP1, VP2, and VP3) that package viral DNA and determine viral tropism. The external surface of the capsid is also the target for neutralizing antibodies. To date, about 180 different AAV capsids have been identified. The three capsid proteins are produced from the same translational reading frame by means of alternative splicing and translation initiation sites.

The C termini, each of which is typically about 530 amino acids long, are identical and represent VP3. VP3 is the most abundant capsid protein, representing about 80% of the protein in an assembled capsid. VP2 is typically about 65 amino acids longer than VP3 at the amino terminus and is dispensable for most AAV-2 functions (51). VP1 is typically about 135 amino acids longer than VP2 at the amino terminus. This N-terminal domain is located inside the capsid (26) and encodes the phospholipase A2, which is required for transduction in vitro (14).

AAV-2 has been used as a human gene therapy vector for a variety of reasons (4, 8, 11, 46). Vectors that lack all viral genes and can be produced in the absence of helper viruses or wild-

type AAV contamination have been generated (29). AAV-2 can infect a wide range of cell types, including nondividing cells. It infects a variety of mammals so that the same vector used in animal models can be used in human clinical trials. AAV-2 is not pathogenic in any human or animal. Although recombinant AAV genomes can integrate into genomic DNA at a very low frequency, most transgene expression appears to come from episomal forms of AAV, adding to its favorable safety profile. Expression of transgenes delivered by AAV vectors to cells that divide infrequently can be stable in animals for years. Finally, the AAV-2 vector can be produced at high levels, be readily purified by scalable methods, and remain exceptionally stable over a wide range of physical and chemical conditions, making it well suited for the production, storage, and transportation requirements of a pharmaceutical. To date, 25 human gene therapy protocols that use AAV-2 have been submitted to the Office of Biotechnology Activities for review (<http://www4.od.nih.gov/oba/rac/PROTOCOL.pdf>).

Although AAV-2 has many desirable properties as a gene transfer vector, one property of AAV-2 that needs improvement is its sensitivity to antibody-mediated neutralization (49). Treatment of a naive animal or exposure of a naive human to AAV often generates neutralizing anti-AAV antibodies that prevent subsequent treatments by AAV vectors with the same capsid (17, 30, 35, 56). While nonprimate animals are naive with respect to AAV-2 exposure, most humans are not (1a, 10, 12, 17, 21, 23, 50, 56). Neutralizing anti-AAV-2 antibodies have been found in at least 20 to 40% of humans (6, 23, 30). We have found that out of a group of 50 hemophiliacs, approximately 40% had AAV-2-neutralizing capacities exceeding 1×10^{13} viral par-

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ticles/ml of sera (R. Surosky, personal communication). This corresponds to about 5×10^{16} viral particles per circulating blood volume, an amount of AAV that is substantially greater than doses used in current human trials. Humans also have neutralizing antibodies against other primate-derived AAV serotypes, such as AAV-1, -3, -4, -5, -6, and -8. Furthermore, after administration of AAV-2 to humans, the titer of neutralizing antibodies to AAV-2, as well as other serotypes, increases (R. Surosky, personal communication).

Various approaches have been taken to generate AAVs that are more resistant to neutralization. One approach suggested mixing peptide competitors of neutralizing antibodies with the gene therapy vector (30). Another approach utilized the insertion of peptides in or near neutralizing epitopes (23). Simultaneous recombination and mutagenesis of the AAV-2 capsid gene followed by selection in the presence of human antisera has generated multiple mutants with increased resistance to neutralization (28). Isolation of novel AAV capsid genes from humans (42), nonhuman primates (12), bovines (43), goats (1), and mice (M. Lochrie, submitted for publication) has also identified AAVs that are more resistant than AAV-2 to neutralization. Chimeric capsids consisting of mixtures of capsomeres from different AAV serotypes (18, 38) or consisting of a single chimeric capsomere (19) are also more resistant to neutralization. A problem with some of these studies is that mouse or rabbit serum was used to assess neutralization, and the relevance of those results to neutralization by human sera is questionable. In addition, novel AAVs are not well characterized and have not been used in any human clinical trials.

There are also several other methods that could be used to generate AAVs with reduced neutralization susceptibility. However, these methods have not been used. For example, a marker rescue method (3) may allow the selection of biologically active, neutralization-resistant AAVs. Chemical modifications such as the attachment of a strand of polyethylene glycol have been considered for some viruses, such as adenovirus (7), but there are no published neutralization studies of AAV to which polyethylene glycol is attached. Traditionally, neutralization-resistant viruses have been selected during replication in the presence of a neutralizing antibody. However, using such an approach for AAV is complicated by the fact that AAV replication is helper virus dependent, and therefore, most human sera will contain antibodies that will neutralize the helper virus. This could potentially be overcome by isolating AAV-2 variants that are helper independent.

Although many of the methods mentioned above can result in decreased neutralization, they may be impractical from the viewpoint of gene therapy vector development since some approaches can result in vectors that are difficult to manufacture or have significantly altered properties compared to those of AAV-2. Due to these difficulties, we sought to identify mutants of AAV-2 that are resistant to neutralization by human antibodies but that also have minimal changes in sequence or biological properties. Previous studies with other viruses, such as poliovirus and rhinovirus, have shown that viral receptors and neutralizing antibodies bind to a distinct set of amino acids and that it is possible to identify mutants at particular positions on viral capsids that reduce the binding of neutralizing antibodies but not binding by receptors or other functions needed

for viral infection. Mutations, even in single amino acids, can result in significant resistance to antibody neutralization.

Many mutations have been made in the AAV-2 capsid (2, 5, 9, 13–15, 20, 22, 25, 27, 31–34, 39–41, 44, 45, 52, 54, 55, 58, 59), but few single mutations on the external surface have been made. This is because most mutations made in the AAV-2 capsid were made prior to the determination of the high-resolution structure of the AAV-2 capsid. With the determination of a high-resolution structure for the AAV-2 capsid (57), it becomes feasible to attempt a rational design of mutations that might circumvent neutralization.

In this study, we had two goals. First, we wanted to extend current knowledge about antibody binding and neutralizing epitopes on the AAV-2 capsid, since only a limited number of studies have attempted to identify neutralizing epitopes on AAV-2 capsids (23, 28, 30, 53). Second, we wanted to characterize areas on the external surface of the AAV-2 capsid that influence its relevant biological properties in more detail.

MATERIALS AND METHODS

Materials. HepG2 cells, HeLa cells, 293 cells, Eagle's minimal essential medium, and adenovirus type 5 were obtained from the American Type Culture Collection. All cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (HyClone). The B1 and A20 monoclonal antibodies were obtained from Maine Biotechnology Services (Portland, ME). Etoposide was obtained from Calbiochem and dissolved in dimethyl sulfoxide to a concentration of 20 mM. Individual sera from human hemophiliacs were obtained from Mark Kay (Stanford University). Pooled, purified, human, intravenous immunoglobulin G (IgG) (Panglobulin), also called IVIG, was purchased from the American Red Cross and dissolved in water to a concentration of 100 mg/ml. All oligonucleotides used for mutagenesis, quantitative PCR (Q-PCR), and DNA sequencing were obtained from Operon except for the Q-PCR probe for *lacZ*, which was obtained from Applied Biosystems.

Structural analysis of AAV-2 capsid. Coordinates for the monomeric AAV-2 capsid protein were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) (<http://www.rcsb.org>; identification number, 1LP3). The structure was analyzed using Swiss PdbViewer version 3.7b1 (<http://www.expasy.org/spdbv/>), Vector NTI 3D-Mol version 8.0 (Invitrogen, Inc.), RasMol (<http://www.umass.edu/microbio/rasmol/>), or Chime (MDL Information Systems, Inc.; <http://www.umass.edu/microbio/chime/getchime.htm>). Various portions of the AAV-2 capsid were generated by using the oligomer generator program on the Virus Particle Explorer website (<http://viprdb.scripps.edu/>), by using the coordinate transformation functions of Swiss PdbViewer in conjunction with matrix coordinates in the PDB 1LP3 file, or by downloading files from the protein quaternary structure database at the European Bioinformatics Institute (<http://pqqs.ebi.ac.uk/pqqs-bin/macmol.pl?filename=1lp3>). Possible antibody binding sites on AAV-2 were analyzed by manually docking a murine IgG2a monoclonal antibody (PDB identification number, 1IGT) to various portions of the AAV-2 capsid using Swiss PdbViewer. Distances, amino acid clashes, and contact areas between IgG2a and the AAV-2 capsid were assessed using the appropriate tools within Swiss PdbViewer. To generate a model of the fibroblast growth factor receptor 1 (FGFR1)/basic FGF (bFGF)/heparin complex binding to AAV-2, PDB file 1FQ9 was used. This structure contains the β -isoform of FGFR1, which lacks the D1 Ig domain.

Mutagenesis. Mutagenesis of the AAV-2 capsid gene in pHLP19 was done using the QuikChange II kit (Stratagene) to make single amino acid changes and the Multi Change kit (Stratagene) to simultaneously make multiple amino acid changes. The entire gene of each capsid mutant was sequenced on both strands by Elim Biopharmaceuticals, Inc., using an Applied Biosystems model 3730xl DNA sequencer.

rAAV-2 *lacZ* preparation. Recombinant AAV-2 virions containing the β -galactosidase gene (rAAV-2 *lacZ*) were prepared in 293 cells using a calcium phosphate-mediated, triple-plasmid transfection procedure (29). The three plasmids used in the transfection were pladen5 (which supplies adenovirus helper genes E2a, E4, VA I, and VA II), pVmLacZ (which provides the β -galactosidase gene under the control of a cytomegalovirus promoter and flanked by AAV-2 inverted terminal repeats), and pHLP19 (which supplies AAV-2 *rep* and *cap* genes) or pHLP19 with mutated capsid genes. pladen5 and pVmLacZ are

described in U.S. patent 6,004,797 (6a). pHLP19 is described in U.S. patent 6,841,357 (50a). Transfection was for 6 h, the DNA mixture was removed from the cells, fresh cell culture medium without fetal bovine serum was provided, and the cells were incubated for an additional 66 h. After the cells were transfected, they were lysed by three cycles of freezing on solid carbon dioxide and thawing in a 37°C water bath to generate a crude lysate. DNA-containing virions were purified from non-DNA-containing virions using two successive CsCl gradients.

Capsid synthesis assay. To determine the total cellular level of capsids made by the mutants, Western blotting of crude lysates was performed. After gel electrophoresis and electrophoretic blotting onto nylon membranes (Hybond-P; GE Healthcare), the blots were probed with an anti-AAV capsid antibody (monoclonal clone B1) at a dilution of 1:20 and then with a sheep anti-mouse antibody coupled to horseradish peroxidase (GE Healthcare) at a dilution of 1:12,000. Capsid proteins were detected using the ECL Plus detection system (GE Healthcare). The membranes were exposed to X-ray film (BioMax MS; Kodak) for 1 to 5 min. The signals were quantified, by comparison to standards consisting of twofold dilutions of CsCl gradient-purified AAV-2, using an AlphaImager 3300 (Alpha Innotech). None of the mutations affected the sequence of the B1 epitope (53) except for S498A/R729K.

Viral titer assay. Q-PCR was used to titer rAAV-2 *lacZ* virions. Virions were diluted 100-fold and digested with DNase I to remove any plasmid (used in transfection) that might result in a false-positive signal. The sequences of the primers, located in the *lacZ* gene, used for amplification are 5'-TGCCACTCG CTTTAATGAT-3' and 5'-TCGCCGCACATCTGAACCT-3'. The sequence of the probe is (5'-6-carboxyfluorescein-AGCCTCCAGTACAGCGCGGCTGA-6-carboxytetramethylrhodamine-3'). Amplification was done using an Applied Biosystems model 7000 sequence detection system using the standard amplification program. A standard curve was constructed using fourfold dilutions of linearized pVmLacZ with copy numbers ranging from 61 to 1,000,000. The copy numbers of packaged rAAV-*lacZ* genomes in each sample were calculated from the cycle threshold values obtained from amplification plots using the Prism 7000 sequence detection system version 1.0 software. Titers are expressed in units of viral genomes (VG) per milliliter.

Heparin binding assay. Heparin binding by viruses in crude lysates was performed by mixing 20 μ l of crude cell lysate with 20 μ l of a 50% slurry of heparin beads. The heparin beads (ceramic Hyper D M hydrogel-heparin; Pall) were 80 μ m in diameter and had 1,000-Å pores. The beads were washed thoroughly in phosphate-buffered saline (PBS) prior to use. The beads and virions were incubated at 37°C for 60 min. The beads were pelleted. The supernatant containing unbound virions was saved. The beads were washed two times with 500 μ l PBS. The supernatants were combined, and capsid proteins were precipitated with 10% trichloroacetic acid. Precipitated capsid proteins were denatured, fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then detected by Western blotting as described above.

In vitro transduction assay. Human HeLa or HepG2 cells were plated in 24-well dishes at 1×10^5 cells per well in 1 ml of media. After 24 h, 10-fold dilutions of control wild-type or mutant viruses were added to the cells along with either adenovirus 5, 20 μ M etoposide (for HepG2 cells), or 80 μ M etoposide (for HeLa cells). The titer of adenovirus used was determined previously and shown to maximally stimulate rAAV-2 *lacZ* transduction. After 24 h at 37°C, the cells were fixed and stained for β -galactosidase activity for 24 h at 37°C. The numbers of blue cells in four random microscopic fields were counted and averaged for each well. The probability of a difference between the transduction efficiency of the wild type and that of a mutant virion was assessed by using a two-tailed Student *t* test by assuming unpaired, independent samples. A *P* value of <0.05 was considered to be significant.

Antibody, serum, and IVIG neutralization assays. HepG2 cells were plated in 24-well dishes at 1×10^5 cells per ml per well for 24 h at 37°C. Twofold dilutions of the A20 antibody, human sera, or IVIG were made in Eagle's minimal essential medium and 0.1% bovine serum albumin (BSA). Wild-type and mutant viruses were diluted to 1×10^9 VG/ml in Eagle's minimal essential medium and 0.1% BSA. Equal volumes (15 μ l) of virus and antibody were mixed and incubated at 37°C for 1 h. Half of each reaction mixture was added to the cells along with 20 μ M etoposide. After 24 h at 37°C, the cells were fixed and stained for β -galactosidase activity for 24 h at 37°C. The numbers of blue cells in four random microscopic fields were counted. The percent neutralization of transduction versus antibody dilution was graphed. A four-parameter logistic equation was fit to the data (Sigma plot) to determine the antibody titer, defined as the dilution of antibody at which 50% of the virus was neutralized. For IVIG, a concentration of 10 mg/ml, which is within the normal range of IgG concentration in human blood, was considered to be undiluted.

ELISAs. An AAV-2 capsid enzyme-linked immunosorbent assay (ELISA) kit (American Research Products) that uses a monoclonal antibody (A20) to cap-

ture and detect AAV-2 was used to measure AAV-2 capsid binding to A20. The kit was used exactly according to the manufacturer's instructions.

To measure the binding of AAV capsids to IVIG, microtiter plates (96-well enzyme immunoassay/radioimmunoassay flat-bottom, high-binding polystyrene; Costar) were coated with 100 μ l (1×10^{10} VG) AAV per well in 0.1 M sodium bicarbonate buffer, pH 9.2, for 16 h at 20°C. Plates were blocked with 200 μ l PBS, 1% BSA, and 0.05% Tween 20 per well for 1 h at 20°C. IVIG (10 mg in 100 μ l) was added and incubated for 1 h at 37°C. Unbound IVIG was washed off using three 200- μ l aliquots of PBS and 0.1% Tween 20. Then, 100 μ l of donkey, anti-human IgG, coupled to horseradish peroxidase (GE Healthcare) and diluted 1:5,000, was added and incubated for 1 h at 37°C. Unbound secondary antibody was washed off using three 200- μ l aliquots of PBS and 0.1% Tween 20. Horseradish peroxidase substrates (ImmunoPure TMB; Pierce) were added and incubated for 15 min at 20°C. The reaction was stopped with 100 μ l 2 M sulfuric acid, and optical density was measured in a Spectramax 340PC plate reader (Molecular Devices) at a 450-nm wavelength. For each ELISA, the amount of virus required to result in a half-maximal optical density reading was determined and used to compare the results from different samples.

RESULTS

Location and selection of mutations. Areas on the external surfaces of parvoviruses have been given names based on their shape (57). Such names facilitate discussions of capsid structure and will be used here (Fig. 1B). For example, the "cylinder" is located at the fivefold axis of symmetry. "Spikes" are protrusions located around the threefold axis of symmetry. On AAV-2, there are three distinct spikes around each threefold axis of symmetry. "Canyons" are areas that surround the cylinder and the spikes. The "dimple" is a part of the canyon that is at the twofold axis of symmetry. There is another feature on the AAV-2 surface that has not been given a name. For the purpose of this study, we have named this feature the "plateau" since it is relatively flat. On AAV-2, plateaus are located between the spikes, surrounding the threefold axis of symmetry.

Several criteria were used to select which amino acids to mutate. One goal of this study was to identify single amino acid mutations that would reduce antibody binding and neutralization. By docking a murine IgG2a to the AAV-2 capsid, we determined that the cylinder, spike, and plateau are likely to be the most accessible to antibody binding. The canyon surrounding the cylinder is somewhat accessible, and the dimple is inaccessible. Therefore, mutations were made at positions on the spike, cylinder, and plateau. Although part of the canyon may be capable of contacting an antibody, it appeared that only a small number of amino acids in the canyon could bind to an antibody, and therefore, no amino acids in the canyon were changed. Amino acids that had a solvent-accessible surface area of less than 20 Å² were not mutated because they would be expected to make minimal contributions to antibody binding, given that the surface area between antibodies and protein antigens is typically 600 to 900 Å². About 145 amino acids in each AAV-2 capsomere have some external exposure. Applying the above criteria resulted in the selection of 64 positions that would be most likely to bind antibodies. These positions encompass about 55% of the external surface area of AAV-2. The positions that were mutated and their locations on the AAV-2 capsid surface are indicated in Fig. 1B and listed in Table 1. A total of 127 mutants that include these positions were constructed.

Mutagenesis strategy. Previous attempts to make AAV-2 capsid mutants that retain normal function have not been highly successful. In the past, only about 15% of the mutants that were

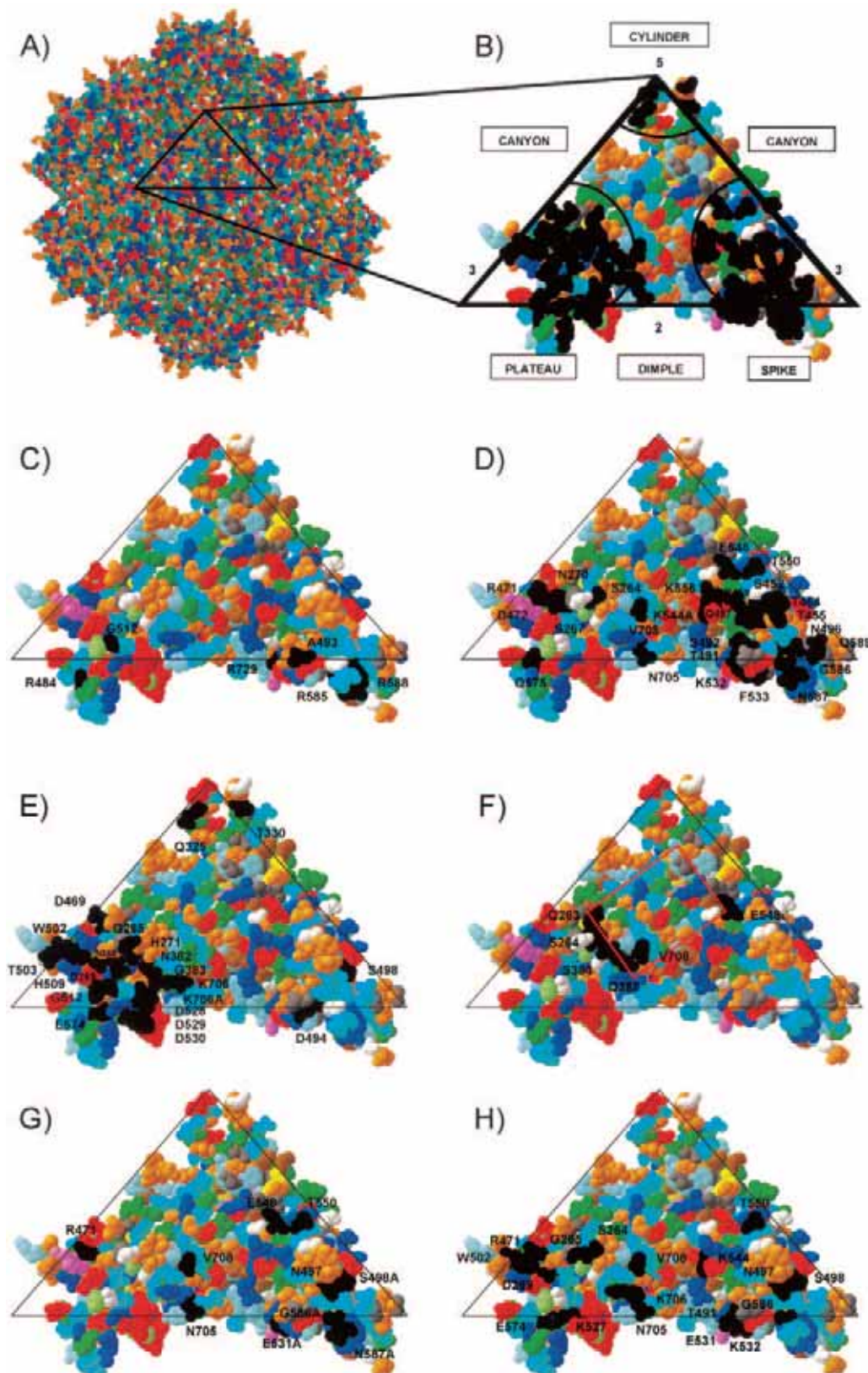


FIG. 1. Structure of AAV-2 capsid and location of mutations. (A) Space-filling diagram of AAV-2 capsid. Amino acids are colored as follows: red, residues D and E; pink, W; brown, P; orange, S and T; yellow, C and M; dark blue, R and K; medium blue, N and Q; light blue, F and Y; light green, H; dark green, I, L, and V; gray, A; and white, G. The black triangle defines the boundary of one asymmetric structural unit. There are 60 asymmetric structural units per capsid. Approximately 145 amino acids out of 735 amino acids in VP1 are on the surface. (B) Positions

made retained normal transducing activity. Most of these mutations were deletions, insertions, and multiple amino acid changes. This suggested that multiple amino acid changes in the AAV-2 capsid often result in inactive virions. Therefore, we decided to make primarily single mutations. Furthermore, since alanine substitutions are often tolerated at many positions in proteins, most of the mutations that were made were changes to alanine.

Amino acids were also replaced with lysines at several positions, a strategy that we term "lysine scanning." The idea behind lysine scanning was to replace a small amino acid with a larger one in order to interfere with antibody binding. This is in contrast to replacing a larger amino acid with a smaller one, such as alanine, an operation that might reduce but not eliminate antibody binding. Lysine was chosen because its side chain is large and it is almost always found on protein surfaces.

Substitution of alanine and glycine was given special consideration. One alanine (A493) and six glycines (G265, G328, G383, G466, G512, and G586) met our selection criteria. In all cases, except at positions A493 and G586, we chose an amino acid substitution that was found at the same position in AAV-1, -3, -4, -5, or -6. Both A493 and G586 are close to the heparin binding site. In AAV-2, the heparin binding site is arginine rich. A493 was changed to arginine because it was thought that an A493R mutant may have improved heparin binding and transduction efficiency and that the larger amino acid might interfere more effectively with neutralization. G586 was changed to alanine because, although it is serine or asparagine in other AAVs, it is between R585 and R588 in AAV-2. R585 and R588 are required for heparin binding and in vitro transduction. However, they are not found in AAVs that are not closely related to AAV-2. Therefore, G586 was mutated to alanine, the amino acid closest in size to glycine.

In addition to single mutations, combinations of up to nine single mutations were made in order to determine whether biologically active, multiple mutations could be made and whether they would result in more resistance to neutralization than single mutations. Also, six double mutants (Q263A/S264L, S267A/N268A, H497H/S498A, S498A/N495K, S498A/S631P, and S498A/R729K mutants) and two insertion mutants (Δ 265ins1 and R471A/N497K/E531A/ins2 mutants) were unintentionally isolated in addition to the desired mutants. These were presumably a result of polymerase or primer hybridization errors during the mutagenesis procedure. Although they were not intentionally constructed, they were assayed like other mutants in case they had interesting properties.

Biological properties of AAV-2 capsid mutants. The capsid synthesis, heparin binding, and in vitro transduction properties of the mutants are summarized in Table 1. The total level of capsid proteins made by the mutants was between 20-fold lower and 4-fold higher than that of the wild type.

All of the mutants except for seven (A493R, G512P, R585A, R588A, S498A/S631P, S498A/R729K, and R484C/G586A/N587A mutants) bound heparin beads as efficiently as native AAV-2 capsids (Table 1; Fig. 2A). Previous studies (25, 34) have identified five basic amino acids (R484, R487, K532, R585, R588) as being important for heparin binding by AAV-2. All of the mutations identified here that reduce heparin binding are located in or near that region (Fig. 1C). Substitution of K527, a basic amino acid that is near the heparin binding site, has not been reported. We found that the K527A mutant binds heparin normally (Fig. 2A).

Fourteen of the mutants transduced HepG2 cells in vitro from 1.3- to 8.6-fold better than the wild type, 50 transduced at 10 to 100% of the wild-type transduction level, 32 transduced at 1 to 10% of the wild-type level, and 31 transduced at less than 1% of the wild-type level (Table 1). The in vitro transduction data shown in Table 1 were generated by transducing the human liver-derived HepG2 cell line in the presence of etoposide. However, there was less than a 10-fold difference between these results and the results obtained by transducing HeLa cells in the presence of either adenovirus type 5 or etoposide or by transducing HepG2 cells in the presence of adenovirus (data not shown).

Ten single mutants (S267A, S267T, R459A, R471A, D494E, N496A, N497H, K532A, R585K, and R588K mutants) and four multiple mutants (S498A/R729K, R471A/G586A, R471A/N587A, and R471A/G586A/N705A mutants) had significantly more transducing activity than the wild type. The S267T mutant exhibited the largest increase in transduction (ninefold). Another mutant with a mutation at the same position (S267A mutant) had a fourfold-increased transduction efficiency.

The mutant that was most defective for transduction was the D529V mutant. Its transduction efficiency was below the level of detection ($<0.0005\%$). A function for D529V has not been described, but it is highly conserved in primate-derived AAV capsid genes.

Lysine substitutions in place of smaller amino acids were made at eight positions (N497K, E530K, E531K, E548K, G586K, N587K, N705K, V708K). Lysine scanning was not as successful as alanine scanning. Of these eight mutants, only

mutated. The locations of positions that were mutated are colored black. The locations of major surface features (canyon, spike, cylinder, plateau, dimple) are indicated, and the approximate boundaries of these features within an asymmetric unit are indicated by circular black lines. The numbers 2, 3, and 5 represent the two-, three-, and fivefold axes of symmetry, respectively. (C) Heparin binding site. The locations of mutations on the capsid surface that reduced heparin binding are colored black and labeled. Note that although G512 and R484 are located on the plateau in this diagram, they would be adjacent to other heparin binding amino acids on the spike within the context of a fully assembled capsid. (D) Normal mutants. The locations of 25 single alanine substitutions on the capsid surface that do not significantly reduce capsid synthesis, heparin binding, or in vitro transduction are colored black and labeled. (E) Dead zone. The locations of single alanine substitutions in the dead zone that reduced in vitro transduction by more than 10-fold but did not reduce heparin binding are colored black and labeled. The position of the H509A mutation, characterized by Opie et al. (34), is included. Note that although D494 and S498 are located on the spike in this diagram, they would be adjacent to other amino acids in the dead zone on the plateau within the context of a fully assembled capsid. (F) A20. The locations of mutations on the capsid surface that significantly reduce binding or neutralization by mouse monoclonal antibody A20 are colored black and labeled. The red rectangle represents the approximate size of an antibody binding site on a protein antigen (25 Å by 35 Å). (G) Human sera. The locations of mutations that significantly reduce binding or neutralization by three human sera are colored black and labeled. (H) IVIG. The locations of mutations that significantly reduce binding or neutralization by IVIG are colored black and labeled.

TABLE 1. Properties of AAV-2 capsid mutants

Mutation ^a	Location ^b	Capsid synthesis ^c	Heparin binding ^d	Transduction ^e	Mutation ^a	Location ^b	Capsid synthesis ^c	Heparin binding ^d	Transduction ^e
None (wild type)		++	++	100 ± 11	D529I	P	++	++	1 ± 0.03*
Q263A	P	++	++	39 ± 4*	D529N	P	+++	++	0.2 ± 0.01*
S264A	P	++	++	72 ± 5	D529V	P	++	++	<0.0005*
G265A	P	++	++	0.4 ± 0.1*	E530A	P	++	++	0.9 ± 0.02*
G265D	P	++	++	1 ± 0.01*	E530D	P	++	++	121 ± 15
S267A	P	++	++	378 ± 23*	E530K	P	+++	++	0.02 ± 0.0*
S267T	P	++	++	864 ± 42*	E530Q	P	++	++	1 ± 0.04*
N268A	P	++	++	0.003 ± 0.0003*	E531A	P	++	++	41 ± 4*
N268Q	P	++	++	0.02 ± 0.002*	E531K	P	++	++	2 ± 0.1*
D269A	P	++	++	1 ± 0.1*	K532A	S	++	++	157 ± 18*
D269E	P	++	++	1 ± 0.01*	F533A	S	+++	++	83 ± 9
D269N	P	+++	++	9 ± 1*	K544A	S	+++	++	79 ± 12
N270A	P	+++	++	94 ± 7	E548A	S	++	++	105 ± 15
H271A	P	++	++	10 ± 1*	E548K	S	++	++	1 ± 0.2*
H271F	P	++	++	0.02 ± 0.001*	T550A	S	+++	++	93 ± 15
H271Q	P	+++	++	2 ± 0.1*	T550K	S	++	++	34 ± 1*
H271T	P	++	++	0.6 ± 0.05*	E555A	S	+++	++	20 ± 1*
Q325A	C	++	++	8 ± 0.2*	K556A	S	++	++	206 ± 37
D327A	C	++	++	39 ± 3*	E574A	P	+++	++	2 ± 0.9*
G328S	C	++	++	7 ± 1*	Q575A	P	++	++	106 ± 10
T330A	C	++	++	0.9 ± 0.03	R585A	S	+++	—	0.2 ± 0.02*
N382A	P	++	++	3 ± 0.3*	R585K	S	++	++	134 ± 5*
G383A	P	+++	++	0.1 ± 0.0*	G586A	S	++	++	70 ± 11
S384A	P	+	++	25 ± 3*	G586K	S	++	++	5 ± 0.3*
Q385A	P	++	++	14 ± 0.6*	N587A	S	++	++	134 ± 12
S452A	S	++	++	94 ± 8	N587K	S	+++	++	1 ± 0.2*
T454A	S	++	++	99 ± 6	R588A	S	+++	—	1 ± 0.1*
T455A	S	++	++	91 ± 8	R588K	S	++	++	139 ± 16*
Q457A	S	++	++	91 ± 4	Q589A	S	++	++	118 ± 5
R459A	S	++	++	271 ± 23*	N705A	P	++	++	98 ± 19
G466R	S	++	++	1 ± 0.2*	N705K	P	++	++	11 ± 1*
S468A	P	+++	++	61 ± 5*	K706A	P	+++	++	3 ± 0.5*
D469A	P	++	++	5 ± 0.2*	V708A	P	++	++	111 ± 11
R471A	P	+++	++	318 ± 79*	V708K	P	+++	++	74 ± 7*
D472A	P	+++	++	75 ± 6	Δ265ins1	P	++	++	0.002 ± 0.0001*
R487K	S	+++	++	24 ± 2*	Q263A/S264L	P	++	++	0.004 ± 0.0009*
T491A	S	+++	++	99 ± 13	S267A/N268A	P	++	++	0.003 ± 0.0*
S492A	S	++	++	107 ± 15	N497H/S498A	S	+++	++	32 ± 2*
S492T	S	+++	++	103 ± 16	S498A/N495K	S	++	++	0.004 ± 0.0004*
A493R	S	++	+/-	2 ± 0.03*	S498A/S631P	S	++	+	24 ± 2*
D494A	S	++	++	9 ± 2*	S498A/R729K	S	++	+	250 ± 39*
D494E	S	++	++	302 ± 23*	R471A/N497K	P, S	++	++	1 ± 0.2*
D494N	S	++	++	5 ± 0.3*	R471A/G586A	P, S	++	++	132 ± 4*
D494Q	S	++	++	3 ± 0.1*	R471A/N587A	P, S	++	++	373 ± 47*
N496A	S	++	++	191 ± 38*	R471A/N705A	P	++	++	90 ± 10
N497A	S	++	++	16 ± 1*	N497K/N587A	S	++	++	0.1 ± 0.01*
N497H	S	++	++	177 ± 20*	E548A/T550A	S	++	++	95 ± 6
N497K	S	++	++	0.1 ± 0.0*	G586A/N587A	S	+++	++	97 ± 2
S498A	S	++	++	0.4 ± 0.1*	G586A/N705A	P, S	++	++	117 ± 10
E499A	S	+++	++	40 ± 3*	G586K/N705K	P, S	++	++	0.02 ± 0.002*
W502A	P	+++	++	5 ± 1*	N705A/V708A	P	++	++	65 ± 5*
W502F	P	+++	++	5 ± 0.6*	R471A/N497K/E531A	P, S	++	++	0.8 ± 0.2*
T503A	P	+++	++	0.004 ± 0.0003*	R471A/N497K/G586K	P, S	+++	++	0.01 ± 0.001*
T503S	P	++	++	26 ± 2*	R471A/G586A/N705A	P, S	++	++	424 ± 12*
H509A	P	NT	++ ^f	0.008 ^f	R471A/G586K/N705K	P, S	++	++	3 ± 0.3*
H509F	P	++	++	7 ± 1*	R484C/G586A/N587A	P, S	++	+	0.02 ± 0.002*
H509K	P	++	++	93 ± 10	R471A/N497K/N587A	P, S	++	++	0.3 ± 0.01*
H509N	P	++	++	106 ± 11	R471A/N497K/E531A/N587A	P, S	+++	++	1 ± 0.02*
H509Q	P	++	++	71 ± 6	R471A/N497K/E531A/ins2	P, S	++	++	0.001 ± 0.0001*
G512A	P	++	++	3 ± 0.6*	R471A/N497K/G586K/N705K	P, S	++	++	0.001 ± 0.0001*
G512P	P	+++	+	0.005 ± 0.0001*	E548A/T550A/G586A/N587A	S	+++	++	114 ± 5
D514A	P	+++	++	57 ± 5*	E548A/T550A/G586A/N587A/	P, S	+++	++	20 ± 1*
K527A	P	+++	++	53 ± 7*	N705A/V708A				
D528A	P	++	++	2 ± 0.1*	R471A/N497K/E531A/E548A/	P, S	++	++	0.02 ± 0.001*
D529A	P	++	++	0.002 ± 0.0004*	T550A/G586A/N587A/				
D529E	P	+++	++	20 ± 2*	N705A/V708A				

^a In mutations, the first letter is the amino acid in the wild-type AAV-2 capsid, the number is the VP1 amino acid position that was mutated, and the last letter is the mutant amino acid. For Δ265ins1, amino acid 265 was deleted and the sequence DASNDNLSSQSD inserted in its place. ins2 indicates an insertion of the sequence HKDDEAKFFPQ after position 536. Slashes separate the single mutations that are in multiple mutants.

^b Location of mutation. C; cylinder; P, plateau; S, spike.

^c Levels of capsid synthesis were determined by Western blotting of crude lysates. +++, 100 to 400% of the wild-type level; ++, 10 to 100% of the wild-type level; +, 1 to 9% of the wild-type level; NT, not tested.

^d Heparin binding levels. ++, 50 to 100% of the wild-type level; +, 10 to 49% of the wild-type level; +/-, 1 to 9% of the wild-type level; —, <1% of the wild-type level.

^e Transduction of human HepG2 cells in the presence of etoposide. Data are expressed as percentages of wild-type transduction (means ± standard errors of the means). Data are averages from two independent transfections to produce the AAV-2 vector and two transductions to measure titer. *, statistically different from wild-type results ($P < 0.05$). The lower limit of detection was 0.0005% of wild-type transduction.

^f Data from Opie et al. (34).

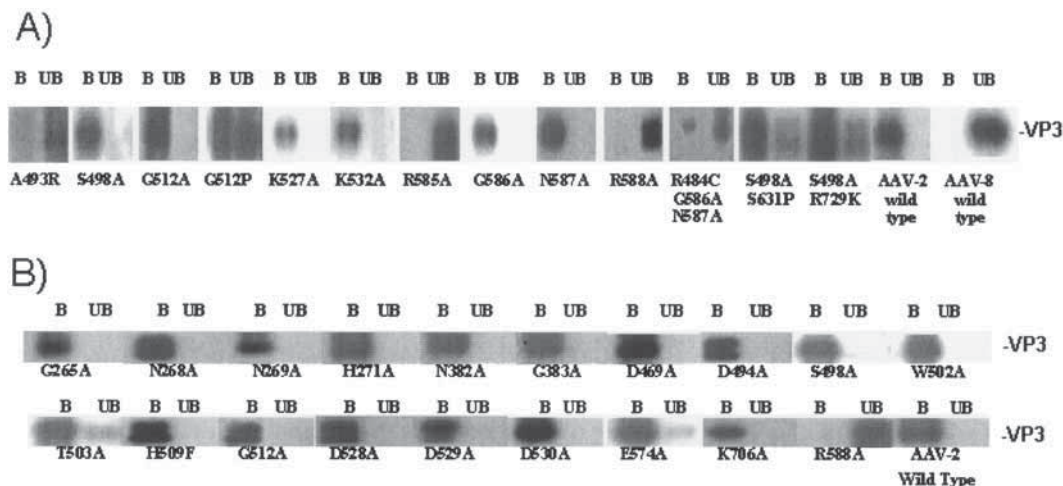


FIG. 2. Heparin binding. (A) Heparin binding by capsids with mutations in or near the heparin binding site. Wild-type AAV-2 and wild-type AAV-8 were used as positive and negative controls, respectively. (B) Heparin binding by mutants with mutations in the dead zone. These mutants have <10% of the wild type's transduction in vitro. The R588A mutant and wild-type AAV-2 were used as negative and positive controls, respectively. B, bound to heparin; UB, unbound. Only VP3 is shown.

one (V708K mutant) had more than 50% of the wild type's transduction efficiency.

Out of 127 mutants, 44 were identified as having levels of capsid synthesis, heparin binding, and in vitro transduction that were indistinguishable from or better than those of wild-type AAV-2 capsids. Of these 44 mutants, 25 had single alanine substitutions. The locations of these 25 mutations are shown in Fig. 1D. Single alanine substitutions could be made at many locations on the spike, a few locations on the plateau, and no locations on the cylinder without affecting the biological properties that were assayed.

Of 28 multiple mutants, 9 made capsids, bound heparin, and transduced at levels equal to or greater than those of the wild type. The largest number of mutations that could be combined without a loss of normal properties was four (in the E548A/T550A/G586A/N587A mutant). Multiple mutants that were defective in transduction usually transduced at levels similar to that of the single mutant within a multiple mutant which had the lowest level of transduction. For example, all nine multiple mutants that contained the N497K mutation, which by itself reduced transduction to 0.1% of that of the wild type, also had low levels of transduction (0.001 to 1%).

Identification of an area distinct from the heparin binding site that is required for AAV-2 transduction. Of 19 single-alanine-substitution mutants that bound heparin as well as the wild type but had less than 10% of the transduction activity of the wild type (G265A, N268A, D269A, H271A, Q325A, T330A, N382A, G383A, D469A, D494A, S498A, W502A, T503A, G512A, D528A, D529A, D530A, E574A, and K706A mutants), only 2 (Q325A and T330A mutants) were not located on the plateau in an area that we named the "dead zone" (Fig. 1E). The dead zone is adjacent to the heparin binding site, and although mutants in the dead zone are defective in transduction, all of them bind heparin beads as well as wild-type AAV-2 capsids (Table 1; Fig. 2B). Therefore, the dead zone defines an area distinct from the heparin binding site that is required for in vitro transduction of human cells. The H509A

mutation, previously characterized by Opie et al. (34), is also transduction defective and binds heparin normally. Therefore, the dead zone, defined as an area where single alanine substitutions decrease the in vitro transduction of human cells by at least 10-fold but do not affect heparin binding, consists of 18 amino acids. The dead zone is roughly circular in shape and is about 3.5 nm in diameter. It covers about 15% of the AAV-2 surface. Of the charged residues in the dead zone, 7 are acidic and 1 is basic.

To explore which amino acids can be tolerated in the dead zone, 24 substitutions other than alanine were made at 11 positions (265, 268, 269, 271, 494, 502, 503, 509, 512, 529, 530) in the dead zone (Table 1). In some cases, charge was conserved, while in other cases, size was conserved. At these 11 positions, we could find substitutions that restored transduction to normal levels at only 3 positions (494, 509, 530). For three acidic amino acids (D494, D529, E530), substitution with another acidic amino acid was most effective at increasing transduction compared to the transduction of the alanine mutant. At the two histidines (H271, H509), substitution of amino acids with an amine or amide group (Q, K, N) restored function, while substitution with an amino acid of similar size (F) did not. In most cases, the most conservative substitutions in the dead zone resulted in AAV-2 virions with the best transduction efficiency.

Binding and neutralization of AAV-2 capsid mutants by monoclonal antibody A20. The A20 monoclonal antibody neutralizes AAV-2 and AAV-3 and is used in ELISAs to quantify AAV-2 and AAV-3 capsids. To characterize the epitope recognized by A20 and determine whether mutations in the AAV-2 capsid could reduce neutralization by an antibody that binds at a single epitope, all mutants were screened for binding to A20, and all mutants with more than 10% of the wild type's transduction efficiency were screened for neutralization by A20. Mutations at six positions (Q263, S264, S384, Q385, E548, V708) that reduced binding or neutralization by A20 were identified (Table 2). There was a good correlation between the

TABLE 2. Binding and neutralization of AAV-2 capsid mutants by mouse monoclonal antibody A20

Mutant	Fold decrease in A20 binding ^a	Fold increase in resistance to A20 neutralization ^b
Q263A mutant	1	3*
S264A mutant	64*	60*
S384A mutant	2*	2*
Q385A mutant	2*	6*
E548A mutant	NT	3*
V708A mutant	1	2*
V708K mutant	10*	220*

^a Measured by ELISA as described in Materials and Methods. NT, not tested; *, significantly different from wild-type results ($P < 0.05$).

^b Measured in vitro on HepG2 cells as described in Materials and Methods. The neutralization titer of A20 for rAAV-2 *lacZ* virions with a wild-type capsid was $1:507 \pm 126$. *, statistically different from wild-type results ($P < 0.05$).

effect of a mutation on A20 binding and its effect on A20 neutralization. Two mutations in particular (S264A, V708K) had a large effect on both binding and neutralization. It is notable that while the V708A mutation had no effect on A20 binding and reduced neutralization by 2-fold, the V708K mutation reduced A20 binding by 10-fold and reduced A20 neutralization by 220-fold.

Five of the mutants that are more resistant to A20 neutralization (Q263A, S264A, S384A, Q385A, and V708K mutants) have mutations located next to each other on one edge of the plateau (Fig. 1F). The amino acids at these five positions are identical in AAV-3, which is also neutralized by A20, but are different in other AAV capsids (AAV-1, -4, -5, -6, and -8) that are not bound or neutralized by A20. The other mutation (E548A) is located on the spike. However, E548 is close enough to the other five mutations to be part of a single epitope (Fig. 1F). E548 in AAV-2 is analogous to T548 in AAV-3, and even though these amino acids are not identical, an amine group on A20 could hydrogen bond to both, for example.

Neutralization of AAV-2 capsid mutants by human sera. All single alanine mutants and one double mutant (N497H/S498A mutant) with more than 10% of the transduction activity of wild-type AAV-2 capsids were screened for neutralization by three human antisera that neutralize AAV-2. Since we were interested in treating factor IX-deficient hemophiliacs with AAV-2 factor IX gene therapy, sera from factor IX-deficient hemophiliacs were used for these experiments. The results are shown in Table 3.

Ten mutants that were significantly (2- to 42-fold) more resistant to neutralization than the wild type AAV-2 capsid were identified. A different set of mutants was resistant to each serum. However, we identified two mutants (the R471A and N587A mutants) that were more resistant to neutralization by all three antisera, and the R471A mutant was the most resistant to each. Eight other mutants (N497A, N497H/S498A, E531A, E548A, T550A, G586A, N705A, and V708A mutants) were more resistant to neutralization by one or two of the sera. Notably, two of the mutants (the E548A and V708A mutants) were also more resistant to neutralization by the A20 monoclonal antibody.

The locations of the mutations that confer neutralization resistance to these three human sera are shown in Fig. 1G.

They are spread over a large area, one that is too large to be contained in a single epitope. However, several of these mutations are near or adjacent to each other.

Binding and neutralization of AAV-2 capsid mutants by IVIG. To identify AAV-2 capsids that are not bound or neutralized by IgG from a larger portion of the human population, we used IVIG as a reagent. IVIG is purified human IgG prepared from thousands of blood donors and is thought to represent about 99% of all immune responses within the donor population (North America in this case). Twenty-five mutants were tested for binding to IVIG, and 41 mutants were tested for resistance to neutralization by IVIG. The results are shown in Table 4.

An IVIG-based ELISA was developed and used to assess the binding of the mutants to IVIG. One mutant (V708K) that bound to IVIG 10-fold less than wild-type AAV-2 capsids was identified. The binding of V708K to A20 was also reduced 10-fold (Table 2). When an A20 (capture)/IVIG (detect) sandwich ELISA format was used, the binding of the V708K mutant was reduced 100-fold compared to that of the wild type, confirming that the binding of the V708K mutant to A20 and IVIG was reduced 10-fold for each.

A total of 21 mutants (S264A, G265A, D269N, R471A, T491A, N497H/S498A, W502A, K527A, E531K, K532A, K544A, T550K, E574A, G586A, N705A, K706A, V708A, R471A/G586A, R471A/N705A, N705A/V708A, and R471A/G586A/N705A mutants) that were more resistant to neutralization by human IVIG than the wild-type AAV-2 capsid were identified. A subset of the mutants (R471A, N497H/S498A, G586A, N705A, and V708A mutants) that were resistant to neutralization by IVIG were also resistant to neutralization by

TABLE 3. Neutralization of AAV-2 capsid mutants by human sera

Human serum	Wild type or mutant ^a	Fold increase in resistance to neutralization ^b
HA2	Wild type	1
	R471A mutant	42*
	E531A mutant	11*
	T550A mutant	9*
	N587A mutant	5*
	N497H/S498A mutant	2*
HA151	Wild type	1
	R471A mutant	16*
	E531A mutant	6*
	E548A mutant	6*
	G586A mutant	4*
	N587A mutant	3*
HA165	V708A mutant	3*
	Wild type	1
	R471A mutant	15*
	N497A mutant	4*
	N587A mutant	3*
	N705A mutant	2*

^a Mutations are as described in Table 1, footnote a.

^b Titers were determined as described in Materials and Methods. Data are presented as ratios of the titer of the serum against virions with mutant capsids to the titer of the serum against virions with wild-type capsids. The neutralization titer of these sera for rAAV-2 *lacZ* virions with a wild-type capsid was about 1:100. Only those mutants whose results were statistically different from wild-type results (*, $P < 0.05$) are shown.

TABLE 4. Binding and neutralization of AAV-2 capsid mutants by IVIG

Mutation ^a	Fold decrease in binding	Fold increase in resistance to neutralization ^b
None (wild type)	1	1
S264A	NT	2*
G265A	NT	4*
S267A	1	1
S267T	1	1
D269N	1	4*
N270A	NT	1
H271Q	1	1
G383A	1	NT
R471A	1	2*
T491A	NT	2*
D494E	1	2
D494N	NT	2
N497H	1	NT
N497H/S498A	NT	2*
W502A	NT	10*
H509K	1	1
H509Q	1	NT
G512P	NT	2
D514A	NT	2
K527A	NT	2*
E530D	1	NT
E531A	NT	1
E531K	NT	2*
K532A	NT	5*
F533A	NT	2
K544A	NT	3*
T550K	1	3*
E555A	NT	1
E574A	NT	2*
Q575A	NT	1
R585K	NT	1
G586A	1	3*
N587A	NT	1
Q589A	NT	1
N705A	1	2*
N705K	1	NT
K706A	NT	4*
V708A	1	4*
V708K	10*	1
R471A/G586A	1	4*
R471A/N705A	1	2*
E548A/T550A	1	NT
G586A/N705A	1	2
N705A/V708A	1	2*
R471A/G586A/N705A	1	3*
E548A/T550A/G586A/N587A	1	1
E548A/T550A/G586A/N587A/ N705A/V708A	NT	1

^a Mutations are as described in Table 1, footnote *a*.^b Titers were determined as described in Materials and Methods. Data are presented as ratios of the titer of the serum against virions with mutant capsids to the titer of the serum against virions with wild-type capsids. The neutralization titer of IVIG (10 mg/ml) for rAAV-2 *lacZ* with a wild-type capsid was 1:312 ± 126. *, statistically different from wild-type results (*P* < 0.05); NT, not tested.

the individual human sera (Table 3). The locations of mutations conferring resistance to neutralization by IVIG are shown in Fig. 1H. They are spread over a large area that cannot be contained in a single epitope.

The level of resistance to IVIG neutralization (2- to 10-fold) is not as great as that observed when individual sera were tested. For example, while the R471A mutant was 15- to 42-

fold more resistant to three hemophilic sera, it was only 2-fold more resistant to neutralization by IVIG. Notably, the V708K mutant was not resistant to neutralization by IVIG even though its binding to IVIG was reduced by 90%.

Certain combinations of mutations that conferred resistance to neutralization by the three individual human sera were constructed and tested for neutralization by IVIG. None of these multiple mutants had a significantly increased level of resistance to neutralization by IVIG.

DISCUSSION

Mutations on the external surface of AAV-2 that affect in vitro transduction efficiency and binding and neutralization by murine and human antibodies are described.

Transduction. Of 127 mutants made, 44 were indistinguishable or better than the wild type with regard to in vitro transduction. In previous studies, about 250 capsid mutants have been made, and only about 15% were able to transduce at least as well as the wild type. This is probably because in previous studies, insertions, deletions, and multiple mutations were made. Also, some of those mutations were made in areas of the capsid, such as in the conserved jelly roll motif, that would be expected to be disruptive. The results of this study show that even on the external surface of AAV-2, mutations in some areas affect transduction more than others. For example, the spike appears to be very tolerant to mutation; the cylinder is less tolerant; and the dead zone and heparin binding site are very intolerant of mutation.

A second area on the surface of AAV-2 that is required for in vitro transduction in addition to the heparin binding site was identified. Single alanine substitutions at 18 adjacent positions, in an area that we call the dead zone, had <10% of the wild type's transduction activity. Although dead zone mutants transduced less efficiently in vitro, all of them bound heparin as efficiently as the wild type. This phenotype can be contrasted with that of mutants with mutations in the heparin binding site which are defective in both transduction and heparin binding. The dead zone is adjacent to the heparin binding site and is very acidic. It is sensitive to changes in amino acid sequence, since conservative substitutions at several positions in the dead zone are still defective for transduction. The dead zone is large enough to be a protein binding site. Besides heparan sulfate (48), the $\alpha_5\beta_1$ integrin receptor (47), the fibroblast growth factor receptor FGFR1 (37), and the hepatocyte growth factor receptor (24) have also been proposed to be receptors required for AAV-2 transduction. The areas on AAV-2 to which these receptors may bind have not been identified. It is possible that one or more may bind to the dead zone. The dead zone is located next to the heparin binding site. Therefore, if a protein binds to the dead zone, then it may also bind heparin. Since heparin is required for basic FGF binding to FGFR1, FGFR1 or bFGF may bind to the dead zone. Notably, the shape of the extracellular portion of FGFR1 (Fig. 3B) is complementary to the shape of the dimple on AAV-2 (Fig. 3A). When a dimeric heparin/bFGF/FGFR1 complex is docked into the twofold symmetric dimple, the heparin in that complex lies very close to the heparin binding site of AAV-2 (Fig. 3C). In such a model, FGFR1 is predicted to contact part of the dead zone. Although bFGF would be predicted to be about 10 angstroms

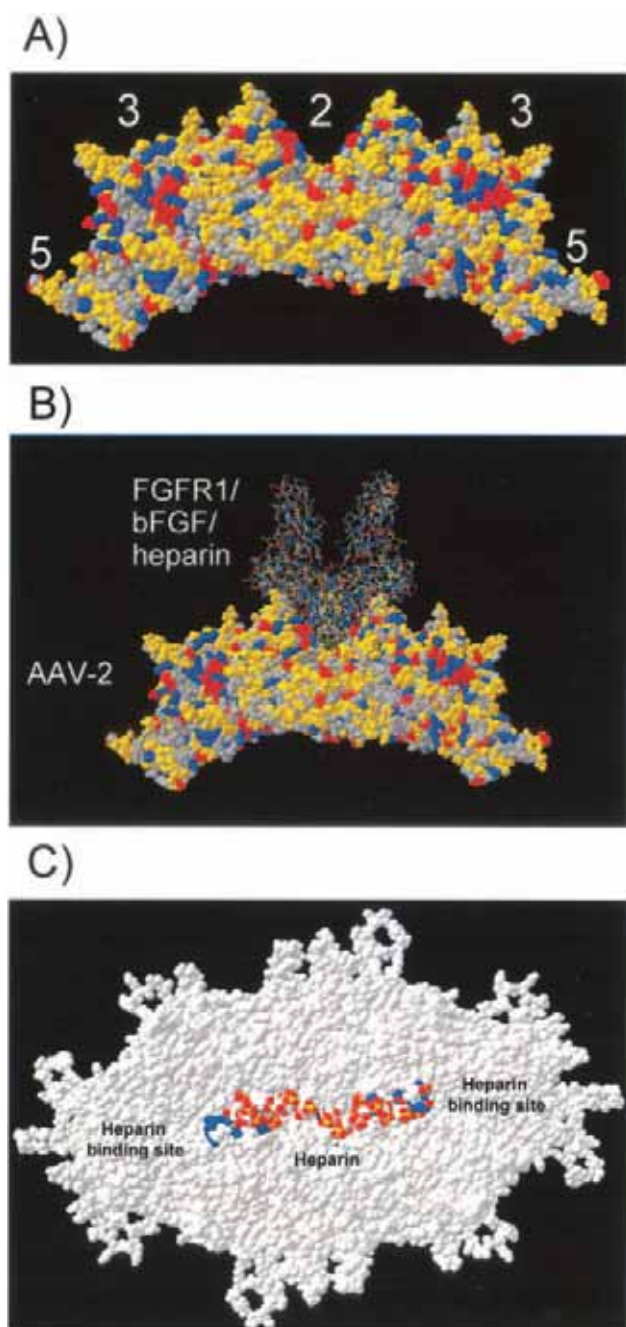


FIG. 3. Model of AAV-2 capsid binding to a fibroblast growth factor receptor 1/basic fibroblast growth factor/heparin complex. (A) Two trimers of the AAV-2 capsid monomer. Axes of symmetry (2, 3, and 5) are indicated. The AAV-2 capsid is shown in space-filling format and colored as follows: red, acidic amino acid; blue, basic; yellow, polar; and gray, hydrophobic. The dimple is centered over the twofold axis of symmetry and is located at the top of the figure. (B) Docking of a fibroblast growth factor receptor 1/basic fibroblast growth factor/heparin complex into the dimple. The receptor complex is shown in the wire frame format. (C) Location of heparin in the model. Two trimers of the AAV-2 capsid monomer are shown in white and rotated 90° toward the reader relative to their positions in Fig. 3A. The heparin is shown in Corey, Pauling, and Koltun colors. The five basic amino acids that comprise the AAV-2 heparin binding site are colored blue.

above the dead zone in such a model, structural changes that could occur after AAV-2 binding may allow bFGF to contact the dead zone. Notably, the dead zone has about the same diameter as bFGF. The structures of α_v integrin (complexed with β_3 integrin) and the HGF receptor are also known. However, since they are asymmetric, it is more difficult to propose a model for how they may bind to AAV-2.

Only a subset of the AAV capsids in clade B (related to AAV-2) have dead zones that are identical to the AAV-2 dead zone. Therefore, if a protein binds to the dead zone, then it may be uniquely required for transduction by the AAVs that are most closely related to AAV-2. In the future, it will be interesting to determine whether any of the mutants in the dead zone are unable to bind to any of the protein receptors proposed to mediate AAV-2 entry. It may also be of interest to determine whether mutations in the analogous regions of other AAV subtypes affect transduction.

Fourteen mutations resulted in increased in vitro transduction efficiency. All of these mutations except one (R459A) are adjacent to the dead zone or the heparin binding site. Strikingly, a conservative S267T mutation resulted in the largest transduction increase. The cellular mechanism of this increased transduction has not been explored. However, these mutants transduced at the same level whether HepG2 or HeLa cells were used to assay transduction or whether etoposide or adenovirus 5 was used to enhance transduction. Therefore, these mutations, for example, may affect common intracellular transduction pathways or enable more efficient uncoating. Given that in vitro transduction efficiency by AAV often does not correlate with in vivo transduction efficiency, it would be interesting to determine whether these mutants also exhibit increased in vivo transduction. In preliminary in vivo experiments, the S267T mutant transduces murine retina cells with the same efficiency as wild-type AAV-2 (P. Campochiaro, personal communication).

Multiple mutants had transduction efficiencies similar to that of the most defective single mutant used to construct the multiple mutant. This may be because the locations of the single mutations that were assembled to make multiple mutations are widespread and mutations at any single position may have an effect independent of other mutations. Inclusion of a mutation that increased transduction in the context of other mutations that decreased transduction did not compensate for the defective transduction. Combinations of those mutations that increased transduction the most were not tested, but it would be interesting to determine whether such combinations would be synergistic.

Insertion sites. The results reported here suggest new areas where peptide insertions might be made without affecting function. An active area for improving AAV vectors involves the insertion of peptide ligands to retarget AAV tropism (5, 13, 15, 27, 31–33, 36, 40, 44, 45, 52, 54, 58). Typically, the insertion of targeting ligands has been made near or within the heparin binding site so that heparin tropism is eliminated and simultaneously replaced with a new tropism. However, insertions there and at other sites have usually resulted in substantial losses in infectivity. There are few positions, other than the N terminus of VP2 (51), where peptides have been inserted without reducing infectivity. Furthermore, sequence-specific effects have been observed. Therefore, identification of other inser-

tion sites that could accept a variety of sequences with no effect on other capsid properties would be desirable. In some cases, insertions have been made between amino acids that are buried under the surface or near amino acids critical for function. Therefore, an ideal site for insertion might be one where amino acids adjacent to the insertion site are surface exposed, where mutation of amino acids adjacent to the insertion site has no effect on critical biological properties, and where amino acids adjacent to the insertion site do not interact with any other amino acids. Applying these criteria, one site between positions T454 and T455 on the top of the spike emerges as a position where insertions might be made without any disruption of function. Since T454 is also the highest point on the surface of AAV-2, insertions there may also be more accessible than insertions at other places. Another candidate insertion site would be between D327 and G328, which are located on top of the cylinder. If elimination of heparin binding is desired, then it would be preferable to accomplish that by the mutation of one amino acid in the heparin binding site. Insertions or deletions in the heparin binding site may eliminate the heparin binding tropism but may also interfere with the function of the dead zone.

Heparin binding. Seven mutants were defective for heparin binding, and all of these are in or near a previously described heparin binding site (25, 34). The A493R and G512P mutants have not been previously described as being defective in heparin binding but are located 1 amino acid away from the previously described heparin binding site. Since basic amino acids often bind heparin, it is interesting that the A493R mutant is defective in heparin binding. An arginine at position 493 may sterically interfere with heparin binding.

Two substitutions were made at position 512. The G512P mutant was defective in heparin binding, but the G512A mutant was not. It is possible that the secondary amide of the G512 mutant, which is a tertiary amide in the G512P mutant, is required for heparin binding. Alternatively, substitution of G512 with proline may result in a disruption of structure that indirectly reduces heparin binding.

Three heparin-binding-defective mutants (R484C/G586A/N587A, S498A/R729K, and S498A/S631P mutants) are multiple mutants. G586A, N587A, and S498A alone had no effect on heparin binding (Fig. 2A). If one assumes that the individual mutations in these multiple mutants act independently, then R347C, S494P, and R592K may result in the heparin binding defect observed in these multiple mutants. R347 has previously been described as being required for heparin binding (25, 34). R592 is on the surface of AAV-2 and is 2 amino acids away from K532 in the previously described heparin binding site. S494 is located under the heparin binding site but on the inside surface of the capsid.

The K532A mutant was reported to bind heparin by Opie et al. (34) and to not bind heparin by Kern et al. (25), while we found that it bound heparin. Each group used different methods to analyze the mutant's heparin binding ability. Since none of the heparin bead binding methods used are quantitative, it is possible that differences in virus input, heparin density, or washing conditions could result in different heparin binding results with the same mutant in different labs.

Binding and neutralization by murine antibodies. The A20 antibody is widely used to detect or neutralize AAV-2. There-

fore, an understanding of the epitope recognized by A20 would be useful. We identified six single mutations at positions 263, 264, 384, 385, 548, and 708 that reduced binding or neutralization by the A20 antibody. All of these positions except 548 are immediately adjacent to each other, which makes a compelling argument that they are part of the A20 epitope. In addition, these amino acids are conserved in AAV-3, which is also recognized by A20. To our knowledge, no other single mutations have been reported to affect recognition of AAV-2 by A20.

The location of the A20 epitope has been suggested from the results of several studies (13, 30, 44, 53, 55) that used a variety of methods. However, the results of those studies do not agree. Peptide competition methods were used in two studies (30, 53). Wobus et al. (53) identified four peptides corresponding to amino acids 270 to 279, 368 to 377, 532 to 541, and 565 to 574 that competed with AAV-2 binding to A20 in an ELISA. These peptides are all located on the plateau on opposite sides of the dead zone. Two of these peptides (encompassing amino acids 270 to 279 and 368 to 377) are near mutations that we identified as affecting A20 binding. Moskalenko et al. (30) identified a pool of peptides, consisting of amino acids 305 to 324, 321 to 340, 329 to 348, 337 to 356, 401 to 420, and 441 to 460, that prevented neutralization of AAV-2 by A20. However, individual peptides were not tested. These peptides are located on the cylinder and the spike. None of these peptides, except for those consisting of amino acids 441 to 460, which are near position 548, are near the mutations that we identified or the peptides that Wobus et al. identified as affecting A20 binding. Peptides encompassing amino acids 401 to 420 and 441 to 460 are completely buried in the crystal structure of AAV-2. As such, it is hard to imagine how they could be epitopes unless the capsid can exist in a conformation different from the crystal structure, which is possible.

Insertions, ranging from 9 to 14 amino acids in length, after positions 34 (55), 261 (13), 266 (55), 319 (44), 381 (13), 457 (44), and 496 (44) reduced A20 binding to AAV-2. Several of these sites (261, 266, 381, 457) are very close to the positions that we identified as reducing A20 binding or neutralization. (Note that position 457 is close to position 548.) Notably, a 2-amino-acid insertion at position 457 did not inhibit A20 binding while an 11-amino-acid insertion did (44), suggesting that the A20 epitope is near but may not include position 457.

Each of the methods used to map the A20 epitope has its strengths and weaknesses. Peptide competition can be a result of nonspecific binding. Large peptide insertions are a crude way to map epitopes. In addition, mutagenesis can alter capsid structure. We have not determined whether the mutations that we made induced conformational changes that affected the A20 epitope from a distance. However, the mutations that reduced A20 binding did not affect in vitro transduction or heparin binding, which argues that they did not cause global changes in capsid structure. The best method for determining the exact and complete composition of the A20 epitope may be to determine the crystal structure of an AAV-2/A20 antibody complex.

Hauck and Xiao (19) constructed chimeric capsid proteins consisting of sections from AAV-1 placed into AAV-2. They found that when amino acids 213 to 423 from AAV-1 were placed into AAV-2, the chimeric capsid was not neutralized as

efficiently by a mouse anti-AAV-2 polyclonal serum. There are nine amino acids in this region that are different between AAV-1 and AAV-2. Four of these are on the surface of AAV-2 and correspond to Q263, an insertion of threonine after S264, Q325, and T329. Consistent with those results, we found that Q263A and S264A reduced neutralization by A20. Q325 and T329 are adjacent to each other on the cylinder. Therefore, mice may recognize at least two antigenic areas on AAV-2. One may correspond to the A20 epitope, and another one may be located in the cylinder.

Wobus et al. (53) have shown that A20 does not block the binding of AAV-2 to heparin. The results reported here support these data since mutations that affected heparin binding are located far from mutations that affected A20 neutralization. Although A20 does not block heparin binding, it does prevent AAV-2 from entering cells. It is possible that A20 does not interfere with binding to a "docking receptor," such as heparin, but instead interferes with the binding of AAV-2 to an "entry receptor." As mentioned above, several proteins have been proposed to be receptors that mediate AAV-2 entry. If one of these binds to the AAV-2 dimple, then binding of A20 on the plateau could block binding of an entry receptor and explain the observation that A20 neutralizes a step in transduction subsequent to heparin binding.

Neutralization by human sera. Several mutants that were more resistant to neutralization by individual human sera were identified. A different set of mutants was resistant to each serum, suggesting that different humans can produce neutralizing antibodies to different sets of epitopes on AAV-2. Two mutants (the R471A and N587A mutants) that were more resistant to neutralization by all sera tested were identified. These positions may be part of one or more dominant epitopes recognized by most humans. Two mutants (the E548A and N708A mutants) were also resistant to neutralization by A20, which suggests that humans may have an immune response to at least one neutralizing epitope on AAV-2 that is similar to the immune response in mice.

The clustered locations of mutations that reduced neutralization by human sera suggest that humans may recognize at least three neutralizing epitopes on the AAV-2 capsid. One epitope (including adjacent positions N497, S498, G586, and N587 and nearby E531) may be next to the heparin binding site. Supporting this notion, Huttner et al. (23) found that insertion mutations near the heparin binding site after position 587 reduced neutralization by 15 human sera an average of 15-fold. Another epitope (including adjacent positions E548 and T550) may be on the side of the spike closest to the cylinder. A third epitope (including positions N705 and V708) may be on the plateau. R471 is not adjacent to any of the other positions. However, it is close to E548 and T550 and may be part of a neutralizing epitope that includes them. Alternatively, it is also possible that R471, N705, and V708 are part of a neutralizing epitope that spans the dead zone.

Binding and neutralization by human IVIG. Twenty-one mutants that are more resistant to the neutralization of the AAV-2 capsid by IVIG were identified. The mutations that constitute those mutants are located at various positions across the surface of AAV-2 but are in areas close to those that resulted in resistance to individual sera. The size of the area they cover is about three times the size of an average epitope,

implying that there may be at least three neutralization epitopes recognized by IVIG.

There was not a correlation between mutations that affected binding to IVIG and those that affected neutralization by IVIG. For example, as assessed in an ELISA, V708K reduced the binding of IVIG by 10-fold but had no effect on neutralization by IVIG. This might be explained by the possibility that an ELISA detects the binding of only higher-affinity antibodies, which may not be neutralizing. Although the V708K mutant is not neutralization resistant in an *in vitro* neutralization assay, it might be neutralization resistant in an *in vivo* assay because neutralization mechanisms *in vivo* can be more complex. We have developed a murine model of human IVIG neutralization that may allow the effect of V708K on IVIG neutralization to be assessed *in vivo* (42a).

W502A reduced IVIG neutralization by 10-fold but had no effect on IVIG binding. Huttner et al. (23) also identified an insertion mutation that reduced neutralization by human sera but had no detectable effect on binding. Mutations that affect neutralization may not have a detectable effect on binding because neutralizing antibodies may be a small fraction of the total amount of binding antibodies.

Some mutants were more resistant to neutralization by IVIG than to neutralization by individual sera (e.g., the W502A mutant). Some mutants were more resistant to neutralization by individual sera than to neutralization by IVIG (e.g., the E548A mutant). Such results could be explained by differences in the frequencies or affinities of anti-AAV antibodies between individual sera and IVIG. This emphasizes the importance of using both types of reagents to study viral neutralization. Furthermore, our survey of three hemophilic sera cannot be considered representative or unbiased.

Huttner et al. (23) used six insertion mutations to map sites that may represent binding sites for human antibodies. They screened a panel of 29 human sera for binding. Two mutations reduced binding by the largest amount and in the most sera. Those insertions are located after positions 534 and 573, both of which are next to the heparin binding site. However, 10 of the 29 sera that they tested bound all of the mutants, suggesting that at least one binding epitope was not affected by those insertions. Based on our results, that epitope may be located near R471, E548, or T550.

Summary. To summarize, 127 mutants that include 98 mutants with single mutations were made at 64 positions on the external surface of the AAV-2 capsid. Prior to this study, only nine single mutations had been made on the external surface of AAV-2. Mutations that reduce binding and neutralization by polyclonal human and monoclonal murine antibodies were identified. Mutations that reduce, have no effect on, or increase transduction were identified. A region called the dead zone, which contains at least 18 amino acids, that is required for transduction in addition to the heparin binding site was identified. Taken together, the results reported here shed new light on transduction by and neutralization of AAV-2.

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