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The structure of AAVrh32.33, a Novel Gene Delivery Vector

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Abstract

The Adeno-Associated viruses (AAVs) are being developed as gene delivery vectors for therapeutic clinical applications. However, the host antibody immune response directed against their capsid, prevalent in ~40–70% of the general population, depending on serotype, negatively impacts efficacy. AAVrh32.33, a novel vector developed from rhesus macaques isolates, has significantly lower seroprevalence in human populations compared to AAV2 and AAV8, which are both in clinical use. To better understand the capsid determinants of this differential immune response to AAVrh32.33, its structure was determined by X-ray crystallography to 3.5 Å resolution. The capsid viral protein (VP) structure conserves the eight-stranded β-barrel core and αA helix reported for other parvoviruses and the distinct capsid surface topology of the AAVs: a depression at the icosahedral two-fold axis, three protrusions surrounding the three-fold axis, and a depression surround a cylindrical channel at the five-fold axis. A comparison to AAV2, AAV4, and AAV8, to which AAVrh32.33 shares ~61%, ~81%, and ~63% identity, respectively, identified differences in previously defined AAV VP structurally variable regions (VR-1 to VR-IX) which function as receptor attachment, transduction efficiency, and/or antigenic determinants. This structure thus provides a 3D platform for capsid engineering in ongoing efforts to develop AAVrh32.33, as well as other AAV serotypes, for tissue targeted gene-therapy applications with vectors that can evade pre-existing antibody responses against the capsid. These features are required for full clinical realization of the promising AAV gene delivery system.

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Keywords

AAVrh32.33; X-ray crystallography; Parvovirus; Gene therapy; Adeno-associated virus; Virus capsid structure

Introduction

Adeno-associated viruses (AAVs) hold great promise as clinical gene delivery vectors due to their lack of pathogenicity and ability to deliver packaged foreign genes for high levels of protein expression in different human tissues (Gao et al., 2005). Several AAV serotypes, including AAV1, AAV2, and AAV8, have been used in human clinical trials (Maguire et al., 2009; Mingozzi and High, 2011a; Nathwani et al., 2011). However, the host antibody immune response to the AAVs, which is detrimental to therapeutic efficacy, is prevalent in a large portion percent (~40–70%) of the general population (Asokan et al., 2012; Boutin et al., 2010; Calcedo et al., 2009; Mingozzi and High, 2011a; Mingozzi and High, 2011b; Nathwani et al., 2011). These antibodies neutralize AAV vectors in a serotype-specific manner. Thus significant effort is being extended to identify AAV vectors with a low immune prevalence in the human population and to understanding the antigenic structure of the capsid epitopes towards elucidating the epitopes to which these responses are directed (Gurda et al., 2012; Gurda et al., 2013; Huttner et al., 2003; Lochrie et al., 2006; Maheshri et al., 2006; McCraw et al., 2012; Moskalenko et al., 2000; Perabo et al., 2006; Wobus et al., 2000).

A worldwide epidemiology study demonstrated that AAVrh32.33, a hybrid of two rhesus macaque isolates, AAVrh32 and AAVrh33 (Lin et al., 2009), had significantly lower seroprevalence than AAV1, AAV2, AAV7, and AAV8 in the human population. The natural antibody titers for IgG in response to AAVrh32.33 were reported to be 1:20 while the titers for AAV2 and AAV8 were 1:2560 and 1:320, respectively (Calcedo et al., 2009). AAV4, a serotype that is one of the most structurally and serologically different from other human and non-human primate AAVs (Gao et al., 2004; Govindasamy et al., 2006) but shares high (~81%) sequence similarity with AAVrh32.33, was found to have a similar low antibody response (Calcedo et al., 2009). Thus AAVrh32.33 (and AAV4) are attractive for development as a gene delivery vectors with the potential to evade pre-existing host antibody responses against AAV capsids (Calcedo et al., 2009; Li et al., 2012; Mays and Wilson, 2009; Mays et al., 2009). AAVrh32.33 is also being evaluated as a genetic vaccine platform, specifically for human immunodeficiency virus type 1 (HIV-1) and type A influenza virus (Lin et al., 2009). In mouse models, a robust CD8⁺ T-cell response to the HIV gag protein expressed from genes delivered with an AAVrh32.33 vector was observed. Studies in macaques, using vectors expressing gp140 from HIV-1, also reported a robust CD8⁺ T-cell response to gp140 and high titer neutralizing antibodies to HIV-1 (Lin et al., 2009). These studies thus identify AAVrh32.33 as a vector that enables the stimulation of immune responses to expressed transgenes. Interestingly, a comparative study of AAVrh32.33 and AAV8, another non-human primate serotype, showed that the latter induces a minimal CD8⁺ T-cell response (Mays et al., 2009). The AAV capsid is reported to play a major role in this phenotype (Mays et al., 2013).

AAVrh32.33, like other AAVs, belongs to the *Dependovirus* genus of the *Parvoviridae* family. These are non-enveloped viruses which package their 4.7 kb ssDNA genomes into capsids which are ~260 Å in diameter and have T=1 icosahedral symmetry. The capsid is assembled from 60 copies of a combination of three overlapping viral proteins (VPs), VP1, VP2, and VP3, encoded from the *cap* open reading frame of their genome. VP1 is the largest VP at ~81 kDa, has a unique N-terminal region (VP1u) of 137 amino acids, and contains the entire sequence of VP2. VP3, the major capsid protein, is ~60 kDa and contained within VP2 which has an additional 65 amino acids (VP1/2 common region) compared to VP3. The predicted capsid ratio of VP1:VP2:VP3 is 1:1:10 (Buller and Rose, 1978; Johnson et al., 1971; Rose et al., 1971).

The 3D structure of several AAV serotypes have been determined by X-ray crystallography and/or cryo-electron microscopy and image reconstruction (DiMattia et al., 2012; Govindasamy et al., 2006; Govindasamy et al., 2013; Lerch et al., 2010; Nam et al., 2007; Ng et al., 2010; Padron et al., 2005; Xie et al., 2011; Xie et al., 2002). In all these structures, only the VP3 overlapping region has been clearly resolved in electron density maps (Chapman and Agbandje-Mckenna, 2006; Halder, 2012). This VP3 structure contains a conserved eight-stranded anti-parallel β -barrel (designated β B- β I) plus β -strand A (β A) that forms the contiguous capsid shell, alpha helix (α A), and large loops inserted between the β -strands. The loops, which form the majority of the capsid surface, contain small stretches of β -strand structure, and variable regions (VRs) at their apex, designated VR-I to VR-IX, based on the comparison of AAV2 and AAV4 (Govindasamy et al., 2006). The sequence and structure variation in the VRs serve as determinants of differential receptor attachment, transduction efficiency, and antigenicity between the AAVs (DiMattia et al., 2012; Govindasamy et al., 2006; Gurda et al., 2012; Gurda et al., 2013; McCraw et al., 2012; Nam et al., 2007; Ng et al., 2010; Xie et al., 2011). Conserved capsid surface features, formed by the interaction between symmetry related VP3 monomers, are depressions at the icosahedral two-fold symmetry axis and surrounding the five-fold axis, protrusions surrounding the three-fold axes, and a cylindrical channel at the five-fold axis.

Reported here is the structure of AAVrh32.33 determined to 3.5 Å by X-ray crystallography. To better understand the capsid determinants of its differential immune response properties, the structure was compared to those of AAV2, AAV4, and AAV8 to which AAVrh32.33 shares ~61%, ~81%, and ~63% identity, respectively. As with the other AAV structures, only the VP3 common region of AAVrh32.33 is ordered and it conserves the VP topology and surface features described above. Comparison of AAVrh32.33 to the other AAVs showed high similarity to AAV4, with smaller structural variations observed between their VR-I to VR-IX compared to AAV2 and AAV8. This structure thus identifies AAV capsid surface features that can drive ongoing efforts to develop AAVrh32.33, as well as other AAV serotypes, for tissue targeted gene-therapy applications. In addition, it provides information on regions that can be modified to generate vectors capable of evading pre-existing antibody responses against the capsid for improved therapeutic efficacy.

Materials and Methods

Vector production and purification

Recombinant AAVrh32.33 vectors, with a packaged firefly Luciferase gene, rAAVrh32.33_ffluc, was manufactured as described previously (Wang et al., 2005) by PennVector at the University of Pennsylvania (Philadelphia, PA). Briefly, a plasmid containing the Luciferase transgene cDNA driven by the chicken β -actin (CB) promoter and flanked by AAV2 inverted terminal repeats was packaged into the AAVrh32.33 capsid by triple transfection of HEK293 cells using an adenovirus helper plasmid (pAd F6), and a chimeric packaging construct containing the AAV2 *rep* gene and the AAVrh32.33 *cap* gene. Vectors were purified by three rounds of cesium chloride gradient centrifugation. The genome titer (genome copies [GC] per milliliter) was determined by real-time polymerase chain reaction (PCR) as previously described (Wang et al., 2005).

Crystallization, data collation, and data reduction

The hanging-drop vapor-diffusion method (McPherson, 1982) was used to screen for crystallization conditions in VDX 24-well plates with siliconized cover slips [Hampton Research, Laguna Niguel, CA]. Crystallization drops contained 2 μ l sample (at ~2 mg/ml) and 2 μ l precipitant solution and were equilibrated against 1 ml of precipitant solution at RT. The precipitant solution screened polyethylene glycol 8000 (PEG 8000). Crystals belonging to the space group P1 were obtained from a precipitant solution containing 1.3 mM KCl, 0.7 mM KH₂PO₄, 8.2 mM NaCl, 4 mM Na₂HPO₄, 5% glycerol, and 3.5% PEG 8000.

Prior to data collection, crystals were rapidly dipped into cryoprotectant that consisted of the precipitant solution and 30% glycerol (v/v) and flash-cooled in a liquid nitrogen gas stream. The X-ray diffraction data were collected at the F1 beamline at the Cornell High Energy Synchrotron Source (CHESS) Cornell University, Ithaca, NY, USA, on an ADSC Quantum 4 CCD detector. Crystal-to-detector distances of 300–400 nm, an oscillation angle of 0.3°, and exposure times of 2–4 min per image were used. The data was indexed and processed using HKL 2000 and scaled and reduced with SCALEPACK (Otwinowski, 1997).

Structure Determination and Refinement

The orientations of the virus particle in the crystal unit was determined with a self-rotation function using the GLRF program (Tong and Rossmann, 1997) computed with ~10% of the observed data between 10.0 and 4.5 Å resolution as large terms to search $\kappa = 72^\circ$ (five-fold), 120° (three-fold), and 180° (two-fold). There was one virus capsid in the P1 cell, with no coincidental non-crystallographic and crystallographic symmetry axes. To generate initial phases for structure refinement, a cross-rotation function search was conducted with data in the 10.0 – 4.0 Å resolution range in the AMoRe program (Navaza, 2001) using a 60-mer capsid model of AAV4 (PDB accession no. 2G8G). The cross-rotation angles were applied to the model followed by positioning at (0,0,0) for generating non-crystallographic symmetry (NCS) operators for subsequent refinement and averaging procedures using the CNS program (Brunger et al., 1998). Sixty NCS operators were generated from superposition of a reference VP3 monomer onto the 59 other VP3s in the capsid model.

Phases were calculated from the model to 3.5 Å resolution for electron density map averaging in the CNS program (Brunger et al., 1998).

To begin structure refinement, the AAV4 amino acid residues were mutated to those of the AAVrh32.33 sequence for the reference VP3 monomer using the SWISS-MODEL program (Schwede et al., 2003). This VP3 monomer was then refined against the observed data by several cycles of the simulated annealing (first cycle only), energy minimization, individual B-factor (temperature factor) refinement, and anisotropic temperature factor refinement options of the CNS program, constrained by 60-fold NCS. A test data set of 5% of the reflections was partitioned for monitoring of the refinement process. Each refinement step was followed by manual model rebuilding, using the COOT program (Emsley and Cowtan, 2004), into NCS-averaged $2F_o - F_c$ and $F_o - F_c$ electron density maps (F_o represents observed structure factors and F_c represents the calculated structure factors) generated in CNS using a molecular mask.

The thermal parameters (B-factors) of the final VP3 model, containing residues 214 to 733 (VP1 sequence numbering) and an ordered nucleotide (NT) were calculated in the CNS program (Brunger et al., 1998). The geometric properties and root mean square deviations (RMSD) from ideal bond lengths and angles for the VP were calculated using the PROCHECK program (Laskowski et al., 1996). Refinement statistics are given in Table 1. The figures were generated using Chimera (Pettersen et al., 2004) and PyMOL (DeLano, 2008).

Protein structure accession numbers

The refined VP3 coordinates for the rAAVrh32.33 and the data set structure factors have been deposited in the RCSB PDB with accession code 4IOV and RCSB ID code rcsb077022.

Comparison of AAVrh32.33, AAV2, AAV4, and AAV8 VP3 structures

The AAVrh32.33 VP3 structure was compared with those of AAV2 (PDB accession # 1LP3), AAV4 (PDB accession # 2G8G), and AAV8 (PDB accession # 2QA0) using the secondary structure matching (SSM) application in COOT (Krissinel and Henrick, 2004). The program superposes the C α positions of two structures and calculates Å differences in their C α positions. A structure based alignment was generated based, on the outcome of the superposition, in which C α positions that were greater than 1.0 Å apart were offset below the alignment.

Results and Discussion

The AAVrh32.33 structure

The structure of the rAAVrh32.33_{ffluc} was determined to 3.5 Å resolution (Fig. 1) from crystals grown in a triclinic P1 space group. The final R_{factor} and R_{free} values, 0.28, is comparable to those for other parvovirus structures as well as members of other virus families reported at comparable resolutions (see the VIPER database, <http://viperdb.scripps.edu/>). The similarity of R_{factor} and R_{free} for virus structures results from the

high non-crystallographic icosahedral symmetry of the capsid that limits the ability to select random unique reflections for the R_{free} calculation.

The final model consists of amino acid residues 214 to 733 (VP1 numbering), which comprise most of the VP3 of the VP1/VP2/VP3 common region. The NCS-averaged electron density map for this stretch of residues showed that there was no phase bias from the AAV4 phasing model (Fig. 1A and B). Amino acid residues 1 to 213, which includes the first eleven residues of VP3, were not ordered in the density maps. The lack of ordering of the N-terminus region of VP1–VP3 is consistent with all known parvovirus structures determined to date, except for B19 in which the entire VP2 was observed in a structure determined by cryo-electron microscopy (Kaufmann et al., 2004). The lack of ordering is likely a result of the low copy numbers of VP1 and VP2 in the capsid or the possibility that different conformations exist for the N-terminus of VP1, VP2, VP3. The NSC assumed during the structure determination and refinement processes is incompatible with these possibilities. In addition, an intrinsic disorder disposition was predicted for the VP1/2 common region of the AAVs (Venkatakrishnan et al., 2013) which could also hinder ordering of this capsid region.

The AAVrh32.33 VP3 monomer structure contains the core eight-stranded β -barrel motif (labeled β B to β I in Fig. 1C) with loops between the strands and α A (residues 283–294). The loop regions also contain small regions of β -strand and helical structure (Fig. 1C). The loops connecting the β -barrel strands interact between symmetry related monomers to form the surface of the capsid (Fig. 2). The least number of interactions between VP monomers occurs at the two-fold interface at which there is a depression on the capsid surface (Fig. 2A and D). Residues 687 to 700, located after β I, and residues 283 to 288, located on β C, on the two two-fold symmetry related VP monomers, form the two-fold interactions. The three-fold axis of symmetry is surrounded by three protrusions (Fig. 2B and D). Residues from two three-fold symmetry related VP monomers assemble each protrusion. Residues 440 to 454 and 580 to 589 (one monomer, labeled IV and VIII in Fig. 2A and D) between the β G and β H strands, stabilized by small β strands, make up the two finger-like projections. These flank residues 477 to 507 (other monomer, labeled V in Fig. 2A and D) to make up the three-fold protrusion. The peak of each protrusion is $\sim 46\text{\AA}$ from the other two protrusions and is offset by $\sim 20\text{\AA}$ from the three-fold axis of symmetry. The five-fold axis of the capsid contains a cylindrical channel and is surrounded by a depression on the capsid surface (Fig. 2C and D). The channel is formed by residues 313 to 327 between the β D and β E strands (DE loop) which form a β -ribbon (Fig. 1C, 2C and D). Residues 651 to 665 between β H and β I form the HI loop that overhangs residues 242 to 245, 359 to 365, and 671 to 673 of an adjacent VP monomer to form the floor of the depression surrounding the fivefold axis (Fig. 1C, 2C, and D). The five-fold interactions also consists of residues 216 to 223 and 392 and 398 located inside the capsid interior. Low signal threshold density (0.5σ) was observed inside the fivefold channel that could not be reliably modeled. This observation is reminiscent of the report for the AAV8 crystal structure (Nam et al., 2011; Nam et al., 2007). This channel is the proposed site for the externalization of the VP1u for its phospholipase A2 function and for the VP1 and VP1/VP2 nuclear localization signals (Grieger et al., 2006; Sonntag et al., 2006). Thus it is possible that the weak density seen is

for these VP regions that are less ordered due to the reasons already discussed above or that are transiently exposed.

The AAV DNA nucleotide binding site is conserved in the AAVrh32.33 capsid

An NT is ordered in a pocket inside the capsid interior close to the icosahedral threefold axis that is surrounded by residues 409 to 414, 625 to 630, 634 to 637 (Fig. 3). The sigma threshold for the density of the purine base ($\sim 3\sigma$ in the F_o -FS map), which inserts into the capsid, was higher than for the deoxyribose sugar and phosphate group ($\sim 2\sigma$ and ~ 1 , respectively in the F_o -F_c map). The base was modeled as purine due to the size of density and as adenine because there was no obvious amino group density at the C2 position (Fig. 3A). In addition to van der Waal contacts, the N1, N6, and N7 atoms of the NT are involved in polar interactions with the main- and side-chain atoms of residues P628 (O), S629 (OG), and G636 (O). One of the phosphate oxygen O3P interacts with the NE2 atom of H625 from a three-fold symmetry related VP monomer.

In the crystal structures of AAV1 (PDB accession # 3NG9), AAV3b (PDB accession # 3KIC), AAV4 (PDB accession # 2G8G), AAV6 (PDB accession # 3OAH), and AAV8 (PDB accession # 2QA0), density interpretable as a single dAMP nucleotide was also observed inside the capsid near the threefold axis (Govindasamy et al., 2006; Lerch et al., 2010; Nam et al., 2007; Ng et al., 2010) (Fig. 3B). The base of the AAVrh32.33 NT is located in essentially the same position and involved in the same interactions as those reported for AAV4 and AAV8 (Govindasamy et al., 2006; Nam et al., 2007). As discussed previously for the AAVs, this ordering of DNA density was not expected. This is because the capsid only packages one copy of the genome which is incompatible with NCS averaging. The NT density was observed in both the non-averaged and NCS-averaged F_o -F_c maps, thus the observation is not an artifact of icosahedral averaging. However, the non-averaged F_o -F_c map showed density for more than one NT while only the modeled NT could be built confidently into the averaged density map. Thus it is likely that not all 60 of the binding sites in the capsid are occupied or that the NT ordered in all 60 binding sites is not the same.

Significantly, unlike the other AAV structures reported to date, this AAVrh32.33 structure is the first determined for a vector packaging a firefly luciferase gene. Thus the observation of an NT in the same pocket as that in which NTs were observed for AAV structures packing wild type, GFP, or baculovirus or insect cell DNA points to a yet to be determine function for this interaction. This could be a role in capsid/DNA stabilization or genome packaging or capsid assembly. The ordering of a similar NT from different DNA sequences suggests that the DNA sequence is not the determinant of the interaction, but rather the conserved binding pocket is important as previously proposed (Ng et al., 2010). Characterizing this interaction could lead to the improvement of DNA packaging efficiency and warrants further studies.

Structural comparison of AAV capsid structures to AAVrh32.33

The differential transduction efficiencies and immune responses exhibited by AAVs result from differences in their capsid structure. And, as already stated above, there is a high prevalence of capsid-targeted pre-existing neutralizing antibodies in the general human

population. Thus a comparison of the AAVrh32.33, with a low immunoprevalence, to those with high reactivity could highlight VP regions that can be targeted for modification for engineering antibody escape vectors.

A structural alignment of AAVrh32.33, AAV2, AAV4, and AAV8 VP3, based on a superposition in the COOT program (Emsley et al., 2010), showed conservation of the regions forming the secondary structure elements, including the β -barrel and α A helix (Fig. 4A and 5). This also highlighted the differences in the nine AAV variable regions (VRI-IX) described when AAV2 and AAV4 were compared (Govindasamy et al., 2006). Of the AAVs compared, the most similar to AAVrh32.33 was AAV4, with an RMSD value of 0.71 Å between C α atoms for residues 214 to 733 of AAVrh32.33 superposed onto residues 211 to 734 of AAV4. For AAV2 and AAV8, the RMSD for superposition residues 214 to 733 of AAVrh32.33 and 217 to 735 of AAV2 (VP1 numbering) and 214 to 733 of AAVrh32.33 and 220 to 738 of AAV8 was similar at 1.1 Å.

As expected based, on the higher sequence similarity, the structures of AAVrh32.33 and AAV4 were the most similar and also the most distinct from the other AAV structures compared (Fig. 4). Small C α distance differences were observed in all the previously defined AAV capsid VR regions, except VR-I, with the largest stretches localized to residues 544 to 554 within VRVII and residues 577 to 590 within VR-VIII at 2.85 Å and 2.69 Å, respectively (Table 2, Fig. 4A). This results in both viruses structures having similar capsid surfaces with minor local differences at the protrusions that surround the icosahedral three-fold axis of symmetry (Fig. 4B).

All the nine AAV capsid VRs differed between AAVrh32.33 and AAV2 with most of the C α position distances being greater than 3.7 Å. The most prominent differences were in VR-IV, VR-V, VR-VI, and VR-IX, respectively (Fig. 4A and 5). VRI, VR-III, and VR-VII assemble the two/five-fold wall between the depressions at the two-fold and surrounding the five-fold axes (Fig. 4B and 6). VR-II is in the turn of DE loop and located at the top of the exterior opening of the five-fold channel (Fig. 4A, B and 6). VR-VI is located to the base of the three-fold protrusions (Fig. 4B and 6). VR-IV and VR-VIII from one monomer together with VR-V from a symmetry related VP monomer assemble the three-fold protrusions (Fig. 4A, B and 6). VR-X is localized adjacent to the two-fold axis (Fig. 4A, B and 6). The clustering of these VRs on the assembled capsids result in the distinctly different surface topologies observed for these two viruses, including rounder protrusions in AAVrh32.33 (Fig. 4B and 6). These VRs contain receptor attachment, transduction efficiency, and antibody recognition sites in AAV2 (Gurda et al., 2013; Levy et al., 2009; Lochrie et al., 2006; O'Donnell et al., 2009; Pulicherla et al., 2011; Xie et al., 2002).

In the comparison to AAV8, like with AAV2, all nine VRs differed since these structures are very similar (Nam et al., 2007) (Table 2, Fig. 4A). Consistently, distances between C α positions between AAV8 and AAVrh32.33 were similar to those described above for AAV2. Significantly, mutagenesis studies and the structure determined for an AAV8 bound to a neutralizing antibody ADK8, has identified VR-IV, VR-V, and VR-VIII as being important determinants of tissue transduction and antigenicity (Gurda et al., 2012; Gurda et al., 2013; Pulicherla et al., 2011; Raupp et al., 2012). In particular, VR-VIII was reported to

dictate liver tropism for this virus and mutations to other AAV8 VR sequences can abrogate antigenic recognition while retaining tropism for different tissues (Gurda et al., 2012; Gurda et al., 2013; Pulicherla et al., 2011; Raupp et al., 2012).

Summary—The crystal structure of AAVrh32.33 conserved the features previously reported for the AAV capsid. However, it identified capsid surface regions which differ from serotypes actively being developed for gene delivery applications (Fig. 6). Significant for this virus, the CD8⁺ T-cell response mediated by this virus has been mapped to the VP3 region, and based on comparative studies with AAV8, is localized to the VRs, although not VR-I, VR-II, or VR-V (Mays et al., 2013). VR-IV was also observed to be an important muscle transduction determinant for this virus. Furthermore, the observation of differences between closely related AAV members, such as AAVrh32.33 and AAV4, can be used to further pinpoint regions of the capsid that may be dictating functional phenotypes. Thus the low prevalence of AAVrh32.33 in the human population, beyond the fact it was derived from non-human primates, is likely due to its distinct structure compared to other serotypes, including AAV2, the most seroprevalent, and its highly inflammatory nature which may be more selected against, leading to reduced prevalence.

A detailed understanding of vector capsid structure combined with functional information is required for engineering improved efficacy. This structure thus provides a visual platform for ongoing efforts to develop AAVrh32.33 and other AAVs as tissue specific targeted vectors for vaccination or corrective gene delivery through the grafting of desired functions. These efforts can also improve vector efficacy by ablation of antigenic epitopes, which are can be predicted to be capsid surface regions differing between AAVrh32.33 and other AAVs, while maintaining parental tropism.

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Figure 1.

Structure of AAVrh32.33. (A and B) Section of the 2Fo-Fc electron density map (gray mesh) for residues 463 to 467 contoured at a threshold of 1σ for residues that differ between AAVrh32.33 and AAV4. The AAVrh32.33 residues are colored according to atom type: C – orange, N – blue, O – red; the AAV4 residues are in red in stick form. AAVrh32.33 residue labels are underlined. (C) A ribbon diagram of the AAVrh32.33 VP3 monomer. β -strand regions are shown as orange ribbons, helical regions are in red and loop regions are in black. The core eight-stranded β -barrel formed by the β BIDG and β CHEF sheets, conserved α A, the DE loop between β A and β E, and the HI loop (between β H and β I), the first N-terminal residue observed (214) and the C-terminal residue (733) are labeled. The sides of the VP monomer forming the capsid interior and exterior surfaces are also labeled. The approximate icosahedral two-, three-, and five-fold axes are indicated by the filled oval, triangle, and

pentagons, respectively. These images were generated with the program PyMOL (<http://www.pymol.org>)

Figure 2.

AAVrh32.33 VP symmetry related interactions and capsid surface topology. (A) VP dimer (viewed down the two-fold axis) showing the VP interactions. The DE and HI loop, as defined in figure 1, are labeled. The reference (Ref) and two-fold (2F) related monomers are labeled. (B) VP trimer (viewed down the three-fold axis) showing VP interactions. (C) VP pentamer (viewed down the five-fold axis) showing VP interactions and the channel at the five-fold axis surrounded by the HI loop. (D) Capsid image, viewed approximately down the icosahedral two-fold axis. The image shows a radial coloring, orange to blue, from the

particle center. Example two-, three-, and five-fold symmetry axes are indicated. Panels A-C was generated with the PyMol program (<http://www.pymol.org>). Panel D was generated in the Chimera program (Yang et al., 2012).

Figure 3.

AAVrh32.33 NT binding site. (A) Stereo view of the ordered density NT inside the capsid interpreted as a deoxyadenylate (dA) and some of the surrounding VP amino acids (black mesh, contoured at a threshold of 1.0σ). For the NT C atoms is in blue, O in red, phosphate group is in orange. The example surrounding amino acids are shown with C in orange, N in blue, O in red, and labeled. (B) Comparison of the AAVrh32.33 NT binding site (orange) with that in AAV1 (purple), AAV3B (yellow), AAV4 (red), AAV6 (hot pink), and AAV8 (green). The NT binding site is conserved in these viruses. AAVrh32.33 amino acid positions

are labeled. (C) Surface representation of a trimer (as in Fig. 2C) but showing the inside surface of the capsid and the location of the ordered NT in AAVrh32.33. This DNA is located in a pocket that resides under the protrusions surrounding this axis. These images were generated with the program PyMOL (<http://www.pymol.org>).

Figure 4.

Comparison of AAV structures. (A) Superposition of coil representations of the VP3 monomer structure of AAVrh32.33 (orange) with those for AAV2 (blue), AAV4 (red) and AAV8 (green). The surface of AAVrh32.33 is shown in translucent orange. The approximate two-, three-, and fivefold axis are indicated as in Figure 1. The position of the AAV VRs are indicated with labels I to IX. The conserved α A and β BIDG and β CHEF forming an eight-stranded β -barrel, are indicated with arrows and labeled. (B) Radially color-cued image of the AAV2, AAV4, AAVrh32.33, and AAV8 capsid (blue to green to

yellow to red). These images highlight the capsid surface variations due to the VRs which cluster from symmetry related monomers (see Figure 6) to produce local differences. Panel A was generated with the program PyMOL (<http://www.pymol.org>), panel B was generated by the program Chimera (Pettersen et al., 2004).

Figure 5.

A structure-based sequence alignment of AAVrh32.33, AAV4, AAV2, and AAV8 VP3. The core β strands forming the β -barrel are represented as arrows on top of the alignment, and VR-I to VR-IX I are indicated above the sequence. VP regions that differ structurally between AAVrh32.33 and the other viruses are offset below the alignment.

Figure 6.

Variable region locations on the AAVrh32.33 capsid surface. (A) Capsid surface and (B) Roadmap (Xiao and Rossmann, 2007), respectively, of AAVrh32.33. Variable regions are colored: purple (VR-I), blue (VR-II), yellow (VR-III), red (VR-IV), gray (VR-V), magenta (VR-VI), cyan (VR-VII), green (VR-VIII), and brown (VR-IX). A viral asymmetric unit, boarded by a five-fold (filled pentagon), two three-fold (filled triangle) axes with a two-fold

in the middle) is shown in black in panels A and B. Panel A was generated with the program PyMOL (<http://www.pymol.org>).

Table 1

Crystal data-collection and processing statistics

Parameter	Value
Resolution (Å)	40-3.5 (3.63-3.5)
Space Group/crystal system	P1
Unit cell (Å, °)	$a = 246.4$ $b = 247.5$ $c = 250.3$, $\alpha = 70.4$, $\beta = 65.4$, $\gamma = 60.2$
R_{merge}^a	0.16 (0.39)
Completeness (%)	68.5 (66.0)
I/σ	2.98 (1.65)
R_{factor}^b	0.28
R_{free}^c	0.28
No. of observed reflections	727,358
No. of unique reflections	397,503
No. of protein atoms	4117
RMSD for bond lengths (Å)	0.014
RMSD for bond angles (°)	1.82
Avg B factor, main chain (Å ²)	41.1
Avg B factor, side chain (Å ²)	41.4
Avg B factor, NT (Å ²)	55.8
Residues in the most/additional/ generously allowed regions (%)	88.5/11.5/0

^a Calculated as $\sum |I_{hkl} - \langle I_{hkl} \rangle| / \langle I_{hkl} \rangle$, where I_{hkl} is a single value of the measured intensity of the hkl reflection and $\langle I_{hkl} \rangle$ is the mean of all measured values of the intensity of the hkl reflections.

^b Calculated as $\sum ||F_O| - |F_C|| / |F_O|$, where F_O and F_C are the observed and calculated structure factors, respectively.

^c Same as R_{factor} , but calculated with a 5% randomly selected fraction of the reflection data not included in the refinement.

Table 2

Amino acid residue ranges localized in the AAV VRs for AAV2, AAV4, AAV8, and AAVrh32.33

	AAVrh32.33	AAV2	AAV4	AAV8
VR I	T257-S260	Q263-D269	S257-S260	G264-D272
VR II	T316-T321	Q325-T330	T316-T321	Q328-K333
VR III	I371-D381	L380-G388	L371-D382	L383-G391
VR IV	L435-A465	L442-R471	L436-S466	L445-A474
VR V	Q480-Y503	Q486-G504	Q481-Y504	Q489-A507
VR VI	A525-L537	H526-L540	A526-L538	H529-L543
VR VII	G541-L555	K544-V557	G542-L556	K547-V560
VR VIII	I577-T590	V579-T592	L578-V591	V582-I595
VR IX	S700-W710	S702-F712	S701-W711	S705-F715