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AAV6 K531 serves a dual function in selective receptor and antibody ADK6 recognition

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ABSTRACT

Adeno-associated viruses (AAVs) are being developed as vectors for the treatment of genetic disorders. However, pre-existing antibodies present a significant limitation to achieving optimal efficacy for the AAV gene delivery system. Efforts aimed at engineering vectors with the ability to evade the immune response include identification of residues on the virus capsid important for these interactions and changing them. Here K531 is identified as the determinant of monoclonal antibody ADK6 recognition by AAV6, and not the closely related AAV1. The AAV6-ADK6 complex structure was determined by cryo-electron microscopy and the footprint confirmed by cell-based assays. The ADK6 footprint overlaps previously identified AAV antigenic regions and neutralizes by blocking essential cell surface glycan attachment sites. This study thus expands the available repertoire of AAV-antibody information that can guide the design of host immune escaping AAV vectors able to maintain capsid functionality.

1. Introduction

The Adeno-associated viruses (AAVs), small single stranded DNA (ssDNA) viruses with a diameter of ~260 Å, belong to the *Dependoparvovirus* genus of the *Parvoviridae* (Agbandje-McKenna and Chapman, 2006; Chapman and Agbandje-McKenna, 2006). The AAV capsid is assembled with a T = 1 icosahedral architecture (Agbandje-McKenna and Chapman, 2006; Chapman and Agbandje-McKenna, 2006) from 60 copies (in total) of three overlapping capsid viral proteins (VPs), VP1, VP2, and VP3, coded from the *cap* open reading frame by use of alternative transcripts and start sites. The VP sequences are stochastically incorporated into the T = 1 capsid in a ratio of 1:1:10 for VP1:VP2:VP3 (Buller and Rose, 1978; Rose et al., 1971; Snijder et al., 2014).

The AAVs exhibit several features which make them attractive as gene delivery vectors: they are non-pathogenic, can package non-genomic ssDNA and self-complementary DNA (scDNA) sequences, can infect dividing and non-dividing cells, and exhibit long-term gene expression (Buning et al., 2004). Currently, AAVs represent ~10% of gene therapy clinical trials worldwide (ClinicalTrials.gov) and are being developed for the treatment of several different monogenic diseases, including cystic fibrosis, hemophilia, and muscular dystrophy (Mendell et al., 2009; Nathwani et al., 2011; Wagner et al., 1999). Significantly, an AAV serotype 1 (AAV1) based vector packaging the lipoprotein lipase gene was the only approved gene therapy treatment marketed under the name Glybera™ (Gaudet et al., 2012) until the recent FDA approval of an AAV2 vector, Luxterna™, packing the RPE65 gend for the treatment of biallelic RPE65 mutation-associated retinal dystrophy. However, although the AAV vector system shows significant promise for gene delivery, one of the limitations is the pre-existing B-cell immunity against different AAV serotypes observed in the general population (Hurlbut et al., 2010; Li et al., 2012; Manno et al., 2006; Zadori et al., 2001). Epidemiological studies report a prevalence of 40–70% seropositivity for AAV in people depending on serotype (Boutin et al., 2010). This antibody response has the ability to neutralize AAV vectors when tested in vitro and in vivo, and eligibility for inclusion in recent clinical trials requires that patients be naïve for anti-AAV antibodies (Nathwani et al., 2011). Thus, significant effort is extended to overcoming the effects of this response. This includes the isolation of novel

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AAV sequences from non-human primates, other mammals, and non-mammalian species, the use of directed evolution in the presence of antibodies, and rational design of capsid variants guided by information on antigenic footprints (reviewed in (Tseng and Agbandje-McKenna, 2014)). In all these efforts, there is a need to preserve the ability of the vectors to infect and transduce the target/desired cells. Thus it is important that functionally mapped regions, including receptor binding sites and transduction determinants, which often overlap with antigenic footprints, are minimally modified in the engineered variants (reviewed in (Tseng et al., 2015)).

There are currently over 150 genomic sequences of AAV isolated from humans, non-human primates, and other mammalian species. Thirteen "serotypes" have been described that represent the range of sequence identity between these viruses (Gao et al., 2004, 2002; Mori et al., 2008; Schmidt et al., 2008). The AAVs are divided onto different groups (group A to F) and clonal isolates (AAV4 and AAV5) based on sequence identities that tends to trend with tissue tropism (Gao et al., 2004). The closely related AAV1 and AAV6, which have ~98% sequence identity, are the representative members of group A. These viruses differ by only 6 out of 736 amino acids in their VPI sequence, with one of the differences (residue 129) located in VP1u (the unique sequence of VP1, not present in VP2 or VP3), and the remaining five located within the VP3 common region (Wu et al., 2006). Previously, structures determined for AAV1 and AAV6, in which only the VP3 region is ordered, showed two of the differing residues (418 and 642) were located in the interior surface of the capsid while the remaining three residues (531, 584, and 598) were located on the exterior capsid surface (Ng et al., 2010). All five VP3 residues are clustered around the icosahedral 3-fold axis of the capsid associated with receptor attachment and/or transduction determinants for several AAVs (Huang et al., 2016). Importantly, comparative analysis of single amino acid variants in AAV1 and AAV6 at the 6 positions, identified K531 as a determinant of heparan sulfate (HS) receptor binding and liver tropism, while other studies identified K531 as a transduction determinant when combined with AAV1's L129 (Limberis et al., 2009; Wu et al., 2006). Residues involved in sialic acid (SIA) receptor binding by AAV1 and AAV6 are identical and have been mapped to the base of the protrusions surrounding the 3-fold axis (Huang et al., 2016).

Several capsid antibodies generated against AAV1 cross-react with AAV6 and capsid-antibody complex structures have shown similar footprints on the AAV1 and AAV6 capsids (Kuck et al., 2007; Gurda et al., 2012). One of these antibodies, ADK1a, which neutralizes both AAV1 and AAV6, has a footprint overlapping with the SIA binding site suggesting a mechanism of neutralization. However, ADK6, generated against the AAV6 capsid, does not recognize AAV1 (Sonntag et al., 2011). In this study, the determinant of the selective reactivity of AAV6 to ADK6 was identified by cryo-electron microscopy (cryo-EM) and image reconstruction (cryo-reconstruction). This was confirmed with site-directed mutagenesis followed by native immunoblots and in vitro neutralization assays. The goal was to determine which of the three AAV1/AAV6 capsid surface amino acids (E531K, F584L, A598V; AAV/AAV6 residue types (Ng et al., 2010)) conferred this selective recognition. Residue 531 was identified as the determinant of this differential recognition despite the ADK6 footprint covering all three AAV1/6 differing residues and containing residues which overlap with the previously mapped footprints for anti-AAV1 capsid antibodies (Tseng et al., 2015). Significantly, as mentioned above, K531 is the determinant of HS binding by AAV6, a phenotype that is absent in AAV1, and dictates liver tropism (Wu et al., 2006). The ADK6 footprint also overlapped the previously mapped common SIA binding site residues, N447, S472, V473, N500, T502, and W503, for AAV1 and AAV6. These observations indicate a block of both HS and SIA binding as the mechanism of ADK6 neutralization of AAV6. This study thus provides residue level information that can aid future efforts to engineer AAV1 and AAV6 vectors with desired tissue tropism and antibody escape properties.

2. Materials and methods

2.1. Production and purification of AAV6 virus-like particles

The production and purification of AAV6 virus-like particles (VLPs) using the *Baculovirus*/SF9 expression system has been previously described (DiMattia et al., 2005; Miller et al., 2006; Ng et al., 2010). Briefly, a baculovirus packaging a gene containing the DNA for expressing the AAV6 VP2 and VP3 was made using the Bac-to-Bac system according to the manufacturer's instructions (Invitrogen) and used to infect SF9 cells. The cell pellet from the infection was resuspended in TNTM buffer (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.2% Triton X-100, 2 mM $MgCl_2$), freeze/thawed 3× with Benzonase (Promega) treatment at 37 °C after the second thaw, and clarified by centrifugation at 10,000 rpm in a JA-20 rotor at 4 °C for 20 min. The supernatant was loaded on a 20% sucrose cushion (w/v of sucrose in TNTM buffer) and the sample centrifuged at 45,000 rpm on a Ti70 rotor at 4 °C for 3 h. The supernatant was discarded and the pellet was resuspended in TNTM and left stirring overnight at 4 °C. The resuspended pellet was loaded onto a 5–40% (w/v) sucrose step gradient and the sample centrifuged at 35,000 rpm in an SW41 rotor for at 4 °C for 3 h. The VLP containing fraction were collected by fractionation, dialyzed into Buffer A (20 mM Tris-HCl, pH 8.5, 15 mM NaCl) and the sample further purified by ion exchange chromatography before use.

A 1 ml anion exchange (Q column, GE Healthcare) was equilibrated with Buffer A and Buffer B (20 mM Tris-HCl, pH 8.5, 500 mM NaCl). The sample was loaded on the column at a flow rate of 0.5 ml/min, the column was washed with 10 column volume (CV) of Buffer A, and the sample was eluted with a 5 CV gradient from 0 to 100% Buffer B (Zolotukhin et al., 2002). Five hundred microliter fractions were collected and screened to identify the fractions containing AAV6 VP. These fractions were pooled, buffer exchanged into PBS, concentrated to 1 mg/ml, and analyzed by SDS PAGE and negative stain electron microscopy (EM) to check for purify and capsid integrity, respectively.

2.2. Purification of ADK6 IgG antibodies

The ADK6 immunoglobulin G (IgG) antibody was produced by the University of Florida Hybridoma Core Lab as previously described (Kuck et al., 2007; Tseng et al., 2016). The ADK6 hybridoma supernatant was diluted 1:5 with PBS and loaded onto a 1 ml HiTrap protein G HP column (GE Healthcare), washed with 10 CV of PBS, eluted with 0.5 ml of Glycine-HCl at pH 2.7, and neutralized with 50 µl of neutralization buffer (1 M Tris-HCl pH 10). The purified IgG was buffer exchanged into 20 mM Sodium Phosphate pH 7.0, 10 mM EDTA, and concentrated for papain cleavage.

2.3. ADK6 fragment antibody (FAb) generation and purification

Cysteine HCl was added to the papain digestion buffer (20 mM sodium phosphate pH 7.0, 10 mM EDTA) immediately prior to use. Concentrated IgGs were incubated with immobilized papain (Pierce) at an enzyme:substrate ratio of 1:160 at 37 °C for 16 h. An equal volume of papain stop buffer (10 mM Tris-HCl pH 7.5) was added to stop the cleavage process and the mixture was centrifuged at 1500 × g for 2 min to separate the sample from the immobilized papain. The FAbs were separated from the undigested IgG and fragment crystallizable (Fc) fragments on a HiTrap Protein A column (GE Healthcare). The FAbs were collected in the wash and flowthrough fractions, buffer exchanged into PBS, and concentrated for use.

2.4. AAV6-ADK6 FAb complex formation

AAV6 VLPs at a concentration of 1 mg/ml and ADK6 FAbs at a concentration of 1 mg/ml were mixed at a molar ratio of 1:1 and 2:1 FAb: VP binding site, and the mixture was incubated at 4 °C for 1 h. The