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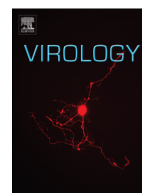

AAV8 capsid variable regions at the two-fold symmetry axis contribute to high liver transduction by mediating nuclear entry and capsid uncoating

Rebeca M. Tenney, Christie L. Bell, James M. Wilson *

Gene Therapy Program, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

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Adeno-associated virus serotype 8 (AAV8) is a promising vector for liver-directed gene therapy. Although efficient uncoating of viral capsids has been implicated in AAV8's robust liver transduction, much about the biology of AAV8 hepatotropism remains unclear. Our study investigated the structural basis of AAV8 liver transduction efficiency by constructing chimeric vector capsids containing sequences derived from AAV8 and AAV2 – a highly homologous yet poorly hepatotropic serotype. Engineered vectors containing capsid variable regions (VR) VII & IX from AAV8 in an AAV2 backbone mediated near AAV8-like transduction in mouse liver, with higher numbers of chimeric genomes detected in whole liver cells and isolated nuclei. Interestingly, chimeric capsids within liver nuclei also uncoated similarly to AAV8 by 6 weeks after administration, in contrast with AAV2, of which a significantly smaller proportion were uncoated. This study links specific AAV capsid regions to the transduction ability of a clinically relevant AAV serotype.

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Introduction

Vectors based on the non-pathogenic parvovirus adeno-associated virus (AAV) are currently among the most promising gene delivery systems for use in human gene therapy, owing to an ability to mediate safe and stable transgene expression in many non-dividing mammalian cell types (Kotin, 1994). AAV vectors consist of a single-stranded DNA genome packaged in a non-enveloped capsid of icosahedral symmetry (Xie et al., 2002). The capsid protein includes a group of conserved strands and several highly diversified interstrand loop regions termed “variable regions” that define the various AAV serotypes (Govindasamy et al., 2006).

One of the first AAVs to be discovered and the most prominently investigated serotype has been AAV2, both in studies aimed at elucidating the cell biology of AAV and in studies investigating AAV's potential as a therapeutic vector (Bartlett et al., 1998). However, in the past decade, a large number of other AAV serotypes with varying properties and tropisms have been identified (Wu et al., 2006; Gao et al., 2002; Lochrie et al., 2005).

A notable example is AAV serotype 8, which has emerged as a clinically relevant alternative to AAV2 owing to its remarkable ability to mediate robust transgene expression in tissues not efficiently transducible by AAV2, such as liver (Wang et al., 2005; Hurlbut et al., 2010), retina (Vandenberghe et al., 2008, 2011) and skeletal muscle (Louboutin et al., 2005). AAV8 consistently achieves 10–100-fold improvement in gene transfer efficiency over AAV2 in hepatocytes (Gao et al., 2002), with minimal lag between vector administration and onset of expression (Nakai et al., 2002).

The biological basis for high liver transduction with AAV8 is not fully understood, although an ability to efficiently uncoat vector particles is currently suggested as a potential feature accounting for this supremacy (Thomas et al., 2004). More recently, the kinetics of AAV uncoating have also been proposed as a rate-limiting step in the transduction mediated by self-complementary AAV6 in cardiac cells (Sipo et al., 2007), and uncoating efficiency has been linked to stability of transient dsDNA AAV2 and AAV8 genomes (Wang et al., 2007). It remains unclear how structural differences between AAV serotypes might be affecting their characteristic uncoating rates. This is especially perplexing for the case of AAV2 and AAV8, given that the AAV8 capsid is 82% homologous in primary sequence and displays high conservation in tertiary structure to AAV2 (Nam et al., 2007), which is a poor liver transducer.

Chimeric vectors constructed by swapping of variable capsid protein domains between AAV serotypes have been employed to identify structural determinants of characteristic serotype tropisms, and this approach has been successful in mapping AAV1's

* Correspondence to: Director, Gene Therapy Program, University of Pennsylvania, 125 S. 31st Street, Suite 2000 TRL, Philadelphia, PA 19104-3403.
Tel.: +1 215 898 0226.
E-mail address: wilsonjm@mail.med.upenn.edu (J.M. Wilson).

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muscle tropism, for example (Hauck and Xiao, 2003). Domain swap experiments have indicated that many residues located within the longest interstrand loop – the GH loop, which contains five of the nine variable regions – to be the key determinants of serotype- and context-specific transduction ability (Van Vliet et al., 2008; DiMattia et al., 2012). Similar strategies aimed at uncovering capsid regions responsible for the hepatotropism of AAV8 have likewise implicated variable portions located at the three-fold symmetry axis. A recent report also identified regions on the AAV9 capsid that may aid in delaying blood clearance (Kotchey et al., 2011). To date, no domain-swapping studies have evaluated parameters other than transduction, such as intracellular genome copy number or uncoating state (Shen et al., 2007; Raupp et al., 2012; Gurda et al., 2012).

In the current study, we identify two variable regions facing the two-fold symmetry axis as possible capsid structural determinants of AAV8's remarkable liver transduction and, for the first time, correlate specific AAV8 capsid portions with cell entry, nuclear entry and vector uncoating ability. We show that an AAV2-based chimeric vector containing these variable regions from AAV8 achieves significantly improved cellular and nuclear entry over AAV2, and is able to fully uncoat by 6 weeks after injection, unlike its parent vector. This chimeric vector retained heparin-binding ability akin to AAV2, supporting the notion that transduction differences are effected at post-attachment steps. Additionally, the chimera displayed an altered antigenic profile *in vitro* compared to its parental serotypes, indicating that its capsid surface could harbor changes extending beyond the exact mutated regions.

Results

Rational design, generation and in vitro testing of chimeric AAV2 vectors containing putative capsid transduction domains from AAV8

Previous work had suggested that the capsid portion mediating high liver transduction by AAV8 was located in variable portions surrounding the three-fold axis of symmetry, in large VP3 areas encompassing VR IV, V, and VII as well as a few other disparate amino acids (Shen et al., 2007; Raupp et al., 2012). In an effort to further pinpoint the key capsid residues effecting AAV8 transduction, we began by generating chimeric vectors containing narrower swaps that replaced only the most surface-exposed loops of VR IV, V and VIII on an AAV2 backbone. Mice were injected intravenously with chimeric vectors expressing firefly luciferase from the CB7 promoter. None of the chimeras amenable to scaled production and purification for *in vivo* testing yielded liver transduction greater than that achieved by AAV2 (Fig. S1a), which corroborated most of the published results mentioned above for those particular residues. Expression was generally low with AAV2, AAV8 and all the chimeras in spleen and lung (Fig. S1c and d). AAV8-mediated expression in heart was one log higher than AAV2, with four out of the six chimeras tested showing expression similar to that observed with AAV8 (Fig. S1b). The divergence in performance of the chimeras between heart and liver is interesting and underscores the documented involvement of three-fold protrusions in vector–cell interactions; however, the poor performance of all chimeras in liver led us to search for a domain of interest residing outside the three-fold axis.

We next focused on the two-fold symmetry axis, generating seven more AAV2-based chimeras containing variable regions VR I, VII and IX – which face the two-fold axis – from AAV8, either alone or in every possible combination. Fig. 1a indicates the locations of these regions in a structural amino acid alignment of AAV2 and AAV8. *In vitro* experiments assessing chimeric vector assembly as well as transduction ability in HEK293 cells showed that all these AAV2 chimeric vectors containing small regions from AAV8 were able to assemble into functional particles and mediated high, AAV2-like transduction ability *in vitro* (Fig. S2).

An AAV2 chimera containing VR VII & IX from AAV8 transduces mouse liver nearly as well as AAV8

After testing the performance of these seven chimeras *in vitro*, chimeric vectors were produced in larger scale and purified through iodixinal or cesium chloride gradients for *in vivo* testing. Two of the vectors – the chimera containing all three regions swapped and the one containing regions I & VII – did not withstand the production process, and were therefore not tested in mice. For the remaining chimeras, *in vivo* transduction was assessed for the liver, heart, spleen and lung of 6–8 week-old male C57BL/6 mice following intravenous injection of 10^{11} GC of vectors encoding firefly luciferase under the CB7 promoter. All chimeras containing AAV8 regions VII and/or IX gave rise to vectors with ~100-fold increased expression over AAV2 in liver as well as in heart lysates, where the expression mediated by AAV8 is typically high. In lung and spleen, where expression is typically low both with AAV8 and AAV2, modest ($<$10-fold) increases in expression were observed with some chimeras, although results for these organs were more highly variable across animals and therefore not indicative of a marked change in tropism (Fig. 2).

The best-performing chimera in liver was the VR VII & IX chimera, and we proceeded to further characterize this vector in an effort to obtain insight into biological mechanisms accounting for AAV8 liver transduction ability. Analysis of this chimera's expression over a time course revealed that this vector also exhibited fast expression kinetics, achieving its maximal luciferase activity by day 4, similarly to AAV8 (Fig. 3a). Fig. 1b shows the location of regions VII and IX in a structural representation of the assembled capsid. This chimera is entirely identical to AAV2 except for 17 amino acids in VP3. Additionally, further evaluation of chimera expression following intramuscular injection (Fig. S3) and subretinal injection (Fig. S4) showed that the chimera did not mediate high, AAV8-like transgene expression levels in muscle or eye, indicating that effects of the AAV8 variable regions VII & IX on the chimera to be chiefly liver- and heart-specific in terms of *in vivo* transduction.

Chimeric vector genomes exhibit increased cell entry, nuclear entry and persistence over AAV2 in mouse liver

In an attempt to determine at which step in the vector transduction cycle the chimeric vector might be distinct from AAV2 in the liver, we first quantified vector genome copy number in DNA extracted from liver cell homogenates using a Taqman qPCR assay recognizing the vector's polyadenylation sequence (Fig. 3b). Typically, we detect substantially higher amounts of AAV8 genomes in liver compared to AAV2 when dosed with equivalent amounts of vector (Gao et al., 2002) [Wang and Wilson, unpublished results], an observation that was once again replicated in this experiment. Genome copy assessment in nuclei isolated from freshly dissected livers revealed that AAV8 also greatly surpassed AAV2 in nuclear entry (Fig. 3c). As for mice injected with chimera, both the total liver and the nuclear genome copy numbers were substantially greater than those reached following AAV2 injection, although still lower than what was observed with AAV8 (Fig. 3b and c).

Nearly all chimeric capsids that localize to liver nuclei eventually undergo uncoating

The results described above show that this chimeric vector appears to have a heightened ability to enter the liver compared

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