Identification of an adeno-associated virus binding epitope for AVB sepharose affinity resin

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Recent successes of adeno-associated virus (AAV)–based gene therapy have created a demand for large-scale AAV vector manufacturing and purification techniques for use in clinical trials and beyond. During the development of purification protocols for rh.10, hu.37, AAV8, rh.64R1, AAV3B, and AAV9 vectors, based on a widely used affinity resin, AVB sepharose (GE), we found that, under the same conditions, different serotypes have different affinities to the resin, with AAV3B binding the best and AAV9 the poorest. Further analysis revealed a surface-exposed residue (amino acid number 665 in AAV8 VP1 numbering) differs between the high-affinity AAV serotypes (serine in AAV3B, rh.10, and hu.37) and the low-affinity ones (asparagine in AAV8, rh.64R1, and AAV9). The residue locations within a surface-exposed, variable epitope flanked by highly conserved residues. The substitution of the epitope in AAV8, rh.64R1, and AAV9 with the corresponding epitope of AAV3B (SPAKFA) resulted in greatly increased affinity to AVB sepharose with no reduction in the vectors’ in vitro potency. The presence of the newly identified AVB-binding epitope will be useful for affinity resin selection for the purification of novel AAV serotypes. It also suggests the possibility of vector engineering to yield a universal affinity chromatography purification method for multiple AAV serotypes.

INTRODUCTION

Adeno-associated virus (AAV) is a single-stranded DNA virus which is so far not linked to human disease. In recent years after decades of persistent effort, the field of AAV vector-based gene therapy has enjoyed several successes. Alipogene tiparvovec (trade name: Glybera), an AAV1-LDL vector treating lipoprotein deficiency, was approved in Europe in 2012. In addition, good outcomes have been reported from clinical trials in the treatments of Leber’s congenital amaurosis and hemophilia B with AAV vectors. With more AAV vector–based therapeutic candidates entering the pipeline, and with the discovery of novel AAVs with versatile tropisms and their increasing applications in clinical trials, the need for large-scale GMP-grade AAV vector-manufacturing processes with the capability of producing and purifying a number of AAV serotypes is acute.

The purification phase of the AAV gene therapy vector-manufacturing process is critical for guaranteeing the safety, potency, and overall yield of the drug product. Various methods for the purification of AAV vectors have been explored, mainly based on buoyant density (ultra)centrifugation using cesium chloride and iodixanol gradients, as well as chromatography-based methods including size exclusion, ion exchange, and affinity-based methods and combinations thereof. Ceramic hydroxyapatite has also been used in the purification of AAV2 (ref. 12) and AAV9. Various ligands have been used for affinity chromatography including heparin, mucin, A20 monoclonal antibody, and AVB Sepharose High Performance. Other less conventional methods involving manipulation of the AAV capsid have also been proposed to purify AAV vectors by displaying certain well-studied binding epitopes on the surface of the AAV capsid and then capturing the vector with ligands to those epitopes. Examples include biotinylated AAV and hexa-histidine-tagged AAV captured with avidin and Ni-NTA columns, respectively.

The ligand of the AVB resin is a single-chain Llama antibody produced from yeast which is conjugated to Sepharose beads. The resin is reported by the manufacturer to capture AAV serotypes 1, 2, 3, and 5 but has also been recently used to purify AAV1 through AAV8, AAVrh.10, and AAV12 from crude cell lysates of the baculovirus-insect cell AAV production platform. In the same study, the authors were unable to demonstrate binding of AAV9 and AAV11 serotypes.

During development of purification protocols using the AVB resin for the purification of hu.37, AAV8, rh.64R1, AAV3B, and AAV9 serotypes, we found that although the AVB resin was able to capture these serotypes, their respective affinities were quite different. In the course of investigating the molecular determinants of these differences using a combined approach of sequence alignment, structural analysis, and AVB-binding studies, we successfully identified an AAV capsid epitope specific for the AVB resin. The presence or absence of this epitope determines the differential binding of the

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Vector genome distribution among the AVB column fractions. AAV vectors were diluted in binding buffer AVB.A (for AAV3B, culture supernatant was buffer-exchanged into the binding buffer) and then loaded onto the AVB column. Fractions from flow through (FT), AVB.A wash (W1), AVB.C wash (W2), and elution (AVB.B) (E) were collected. Vector genome copies were determined by real-time PCR.

In Figure 1, 84% of the loaded rh.10 vector genome was present in the elution fraction. The affinity of the hu.37 vector was similar to rh.10, with 82% in the elution fraction. On the contrary, both AAV8 and rh.64R1 vectors bound AVB resin poorly, with only 20 and 22% in the elution fraction, respectively. The affinity of AAV3B for AVB resin was remarkable, with 98% of vector genomes recovered in the elution fraction.

Sequence alignment and structure analysis showed that the amino acid region 665–670 (AAV8 VP1 numbering) was the most diverse region on the capsid surface between the high AVB-affinity AAV serotypes, AAV3B, rh.10, and hu.37, and the low-affinity serotypes, AAV8 and rh.64R1. Among the residues exposed on the surface of the AAV8 capsid (PDB accession number: 2QA0 (ref. 24)), the following 26 residues are identical between rh.10 and hu.37 serotypes but different from AAV8 (numbering format: AAV8 residue-AAV8 VP1 numbering-rh.10/hu.37 residue): A269S, T453S, N459G, T462Q, G464L, T472N, A474S, N475A, T495L, G496S, A507G, N517D, I542V, N549G, A551G, A555V, D559S, E578Q, I581V, Q594I, I595V, N665S, S667A, N670A, S712N, V722T. Among the 26 residues, only residue 665 (AAV8 VP1 numbering) is identical among AAV1, 2, 3B, AAV5, rh.10, and hu.37. As shown in Figure 1, all the poor-affinity AAV serotypes (AAV8, rh.64R1, and AAV9) have an Asn residue at this position while the high-affinity serotypes have Ser. The 665 residue is located in a small variable patch region 665–670 (AAV8 VP1 numbering) was the most diverse region on the capsid surface between the high AVB-affinity AAV serotypes, AAV3B, rh.10, and hu.37, and the low-affinity serotypes, AAV8 and rh.64R1. Among the residues exposed on the surface of the AAV8 capsid (PDB accession number: 2QA0 (ref. 24)), the following 26 residues are identical between rh.10 and hu.37 serotypes but different from AAV8 (numbering format: AAV8 residue-AAV8 VP1 numbering-rh.10/hu.37 residue): A269S, T453S, N459G, T462Q, G464L, T472N, A474S, N475A, T495L, G496S, A507G, N517D, I542V, N549G, A551G, A555V, D559S, E578Q, I581V, Q594I, I595V, N665S, S667A, N670A, S712N, V722T. Among the 26 residues, only residue 665 (AAV8 VP1 numbering) is identical among AAV1, 2, 3B, AAV5, rh.10, and hu.37. As shown in Figure 1, all the poor-affinity AAV serotypes (AAV8, rh.64R1, and AAV9) have an Asn residue at this position while the high-affinity serotypes have Ser. The 665 residue is located in a small variable patch 665–670, AAV8 VP1 numbering) of the AAV capsid. The entire patch is exposed at the capsid surface, near the pore region (Figure 2b), and this whole epitope was therefore selected for swapping experiments. Because the affinity of the AAV3B serotype for AVB resin is very good, we chose the SPAKFA epitope from AAV3B to swap into the AAV8, rh.64R1, and AAV9 serotypes using site-specific mutagenesis. The resulting mutants were denoted as AAVx-SPAKFA. As a control, a reverse swap mutant was made where the corresponding epitope of AAV9 (NKDKLN) was swapped into the AAV3B capsid; the resulting mutant was named AAV3B-NKDKLN. The vector production yield of the SPAKFA epitope mutants was 81% (AAV8), 82% (rh.64R1), and 137% (AAV9) of their wild-type counterparts. The yield of AAV3B-NKDKLN was 28% of AAV3B.

**Figure 1.** Vector genome distribution among the AVB column fractions. AAV vectors were diluted in binding buffer AVB.A (for AAV3B, culture supernatant was buffer-exchanged into the binding buffer) and then loaded onto the AVB column. Fractions from flow through (FT), AVB.A wash (W1), AVB.C wash (W2), and elution (AVB.B) (E) were collected. Vector genome copies were determined by real-time PCR.

**Figure 2.** AAV serotype sequence alignment. (a) The alignment was performed with Vector NTI using ClustalW algorithm. The 665–670 region is shown with the SPAKFA epitope of AAV3B underlined. (b) The region corresponding to SPAKFA is shown in black on AAV8 capsid.

**Figure 3.** Substitution mutant vector genome distribution among the AVB column fractions. AAV vectors and their SPAKFA mutants were loaded onto an AVB column. Fractions for flow through (FT), DPBS wash (W1), AVB.C wash (W2), and elution (E) were collected for real-time PCR titration and represented as percent genome copies of the total. Each AAV and its mutant were compared head-to-head from production to titration. For AAV8, AAV9, and rh.64R1, mutants were made by substituting the corresponding region to SPAKFA based on sequence alignments shown in Figure 2a. For the AAV3B mutant, the SPAKFA epitope was replaced by NKDKLN.
SPAKFA epitope exchange greatly improved the AVB affinity of AAV8, rh.64R1, and AAV9 serotypes
As shown in Figure 3, after SPAKFA substitution, clear improvement in the affinity of AAV8, rh.64R1, and AAV9 serotypes was shown, with the percentage recovery of loaded vector in the elution fraction rising from 30, 18, and 0.6% of total fractions to 93, 91, and 51%, respectively. In contrast, when the NKDKLN epitope of AAV9 was swapped into AAV3B, the elution fraction yield decreased from 98 to 87%, and the fractions of flow-through (FT), wash 1 (W1) and wash 2 (W2), rose from 1.3 to 8.2%, 0.0 to 0.1%, and 0.3 to 4.6%, respectively. The majority of AAV3B-NKDKLN was still in the elution fraction however, indicating the existence of other epitope(s) which are involved in binding to AVB resin.

SPAKFA epitope exchange did not lower the in vitro infectivity of AAV8, rh.64R1, and AAV9 serotypes
One key question was whether the epitope swapping performed impaired the potency of the recipient vector. To address this question, an in vitro infectivity assay was performed with the epitope substitution mutants in Huh7 cells. A range of vector concentrations were used for infection in order to avoid the possible saturation of transduction pathways at high multiplicity of infection. For AAV3B and AAV3B-NKDKLN, the vector concentration used for infection was 1 log lower than that for the other AAV vectors due to the very high Huh7 cell infectivity of the AAV3B serotype (data not shown) and its sibling, AAV3.25-28 As shown in Figure 4, the infectivity of the AAV8-, rh.64R1-, and AAV9-SPAKFA mutants was 130, 128, and 108% relative to the corresponding wild-type AAV vectors. Conversely, the infectivity of AAV3B-NKDKLN was only 59% of AAV3B.

**DISCUSSION**
A simple, efficient, generic, and easily scalable purification protocol that can be used for all AAV serotypes is highly desirable. Affinity resins such as AVB will likely play an important role in enabling such a process as recently demonstrated in a study by Mietzsch et al.,23 in which 10 serotypes (AAV1-8, rh.10, and AAV12) were purified in a single step from clarified crude lysate using the AVB resin. However, our present study shows that although AAV8, rh.64R1, hu.37, rh.10, and AAV3B can be captured by AVB resin, the affinity of the resin for these different serotypes is very different, with AAV3B having a strong affinity and AAV8 and rh.64R1 binding more poorly. While further optimization of buffers and flow rate can improve binding of AAV8 in our hands (data not shown), conditions and the resulting resin capacity are still not optimal for process scale-up.

The variation in AVB affinity for AAV serotypes rh.10, AAV8, hu.37, and rh.64R1 was intriguing since they all belong to Clade E and display a high degree of sequence similarity. By contrast, another serotype, AAV5, binds well to AVB but is distantly related to Clade E members. These observations led us to speculate that some subtle sequence differences may play a role in the different binding affinities of these serotypes to AVB. Sequence alignment and structure analysis of the VP3 capsid proteins of these serotypes led us to narrow in on residue 665. At this position, AAV8 and rh.64R1 are asparagine, while rh.10, hu.37, and AAV5 are serine. Because the sequence patch around residue 665 is a small variable region, it was decided to swap the whole patch of AAV8, rh.64R1, and AAV9 with the patch (SPAKFA) from AAV3B. The clear improvement in affinity observed following these substitutions indicates that the SPAKFA sequence patch is an epitope of the AVB resin. Importantly, the substitutions did not impair capsid fitness, in terms of lowering yield or in vitro infectivity.

**Figure 4.** Huh7 cell transduction of AAVs and their SPAKFA mutants. The transgene cassette was CB7.CI.fluciferase. Huh7 cells were infected with AAV vectors (filled circles) and their SPAKFA mutants (empty circles) at various concentrations (x-axis). The substitution mutant for AAV3B was AAV3B-NKDKLN. Luciferase expression was read 3 days after infection and denoted as RLU/s. gc, vector genome copies; RLU, relative luminescence unit.
Another interesting observation was made when the corresponding sequence patch from the AAV9 serotype, NKDKLN, was substituted in place of the SPAKFA epitope in the AAV3B capsid. While the affinity of the AAV3B-NKDKLN vector was apparently weakened, as evidenced by the appearance of the vector in the flow-through fraction, the majority still bound to the column. This result, in conjunction with the fact that substitution of the SPAKFA epitope into AAV9 did not produce the affinity observed with AAV3B, suggests that there are other epitopes besides SPAKFA in the AAV3B VP3 amino acid sequence which contribute to AVB binding. One epitope candidate is the region containing residues 328–333 (Figure 5). This region is at the outside surface of the pore wall and is spatially close to the region containing residues 665–670. Residue 333 is especially close in spatial terms to the region containing residues 665–670 and for weak AVB binders such as AAV8, rh64R1, and AAV9, this residue is Lysine, while in stronger binding serotypes such as AAV3B, it is threonine. The hypothesis suggested by these observations is that the regions containing residues 665–670 and 328–333 both contribute to AVB binding, although residues 665–670 make the major contribution. The AVB-binding data generated in this study, in addition to the AAV3B-NKDKLN data described above, support this hypothesis. Serotypes with high SPAKFA homology in the 665–670 region and a threonine residue at position 333 bind best to AVB (AAV3B, AAV1, AAV2, and AAV5). Serotypes with low SPAKFA homology and a lysine residue at position 333 bind poorly (AAV8, rh64R1, and AAV9). Intermediate cases such as serotypes rh10, hu37, and epitope-substituted mutants which contain SPAKFA but have lysine rather than threonine at position 333 (AAV8-SPA and rh64R1-SPA) do bind to AVB resin but less well than serotypes such as AAV3B. Further mutagenic analysis of the 328–333 region and confirmation of its role in AVB binding is complicated because it overlaps the coding sequences for the assembly-activation protein (AAP) in another reading frame. However, with careful mutagenesis design, this problem might be overcome and would further enhance our understanding of the necessary interactions.

The discovery of the SPAKFA epitope might be useful in predicting whether AVB is a suitable resin for purification of some of the less commonly used AAV serotypes. For example, among the clade E members, rh.8, rh.43, and rh.46 serotypes have sequences very similar to AAV8 at residues 665–670 and so their affinity for AVB will probably be low. On the other hand, rh.39, rh.20, rh.25, AAV10, bb.1, bb.2, and pi.2 serotypes are likely to bind well because their sequences in this region are identical (or very similar) to rh.10. Similarly, for many clade D members, the 665–670 amino acid sequence is TPAKFA, and thus, these serotypes are likely to display high affinity to AVB, while the rh.69 serotype is likely to bind poorly since the 665–670 amino acid sequence is NQAKLN.

An intriguing possibility suggested by the studies presented here is that substitution of the SPAKFA epitope into the capsids of poor-affinity AAV serotypes such as AAV9 would permit for the use of AVB as a universal affinity chromatography resin for all AAV serotypes. While such an approach would provide process uniformity and simplicity, it is important to establish that the epitope substitutions do not affect vector yields, infectivity, and tropism. In the studies presented here, yields and infectivity of vectors receiving the SPAKFA sequence were not adversely affected; however, the impact on tropism was not investigated since it was beyond the scope of this work. However, there are reports which show that the tropism of AAV8 vectors relates mainly to hyper-variable region VII (AAV8 549–564) and IX (AAV8 708–720), and/or the subloop 1 (AAV8 435–482) and subloop 4 (AAV8 574–643) of the AAV8 capsid. Neutralizing antibody mapping data also supports the notion that the pore structure of AAV capsids and its nearby regions, which are responsible for binding to AVB resin, are not involved in cell transduction and therefore tropism. Neutralizing epitopes identified so far mainly locate around the threefold protrusion of the AAV capsid. Indeed, for AAV2, switching the tip (RGNR) of the threefold protrusion resulted in drastic changes in the tropism of the vector. Another relevant antibody study was performed with monoclonal mouse antibody 3CS raised against AAV5. This antibody is not neutralizing, and one of its epitopes locates in the 665–670 region. This observation therefore suggests that antibody binding in this region does not affect cell transduction and by extension, tropism.

In summary, the work presented here increases our understanding of the AAV capsid sequences necessary for binding to the commercially available affinity resin AVB sepharose. The use of AAV serotypes other than those commonly vectored for gene therapy applications might be a useful approach to attain specifically desired tropisms and may be valuable in avoiding immune responses in cases where readministration of a vector transgene is required. One factor in the selection of an appropriate AAV serotype for such gene therapy applications may be the ease by which it can be purified. In this regard, the ability to screen for AVB resin binding based upon the primary amino acid sequence as suggested by the data here, would greatly facilitate the selection process. For those serotypes where AVB resin binding is predicted to be poor, the substitution of the SPAKFA epitope may present a viable solution and enable the institution of a universal purification process for multiple serotypes.

**MATERIALS AND METHODS**

**Plasmids**

Constructs pAAV2/8, pAAV2/rh.64R1, pAAV2/9, and pAAV2/3B expressing the AAV8, rh.64R1, AAV9, and AAV3B capsid protein respectively were used. Mutagenesis of these plasmids was carried out with QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, CA), following...
Sequence alignment and structure analysis

Sequence alignments were generated with the ClustalW algorithm by the AlignX component of Vector NTI Advance 11.0 (Invitrogen, CA). The protein sequences were: AAV1 (accession: NP_049542), AAV2 (accession: YP_680426), AAV3 (accession: NP_043941), AAV8 (accession: AA895452), AAV5 (accession: YP_068409), rh.10 (accession: AA088201), hu.37 (accession: AA599285), AAV8 (accession: YP_077180), rh.64R1 (accession: ACB55316), AAV9 (accession: AA599264). Structure analysis was performed with the Chimera program 16,17 and the AAV8 capsid structure (PDB: 2QA0 (ref. 24)).

CONFLICT OF INTEREST

M.L. and M.R.A. are inventors on patents of AAVs licensed to various biopharmaceutical companies, including RegenX. J.M.W. is an advisor to RegenXBio, Dimension Therapeutics, Solid Gene Therapy, and Alexion and is a founder of, holds equity in, and is an inventor on patents licensed to various biopharmaceutical companies. Q.W., A.J.P., and B.P. have nothing to disclose.

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