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Peptide affinity reagents for AAV capsid recognition and purification

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Abstract

We report the discovery of AAV capsid-binding peptides identified through phage panning. The heptapeptide motif GYVSRHP selectively recognized AAV serotype 8 capsids and blocked transduction *in vitro*. Recombinant AAV8 vectors were purified directly from crude cell lysate and supernatant through sequential application of peptide affinity and anion exchange chromatography. Peptide affinity reagents may serve as useful alternatives to monoclonal antibodies in AAV capsid recognition, and offer readily scalable solutions for purification of clinical grade AAV vectors.

Keywords

AAV vectors; phage display; affinity column chromatography

INTRODUCTION

Recombinant AAV vectors derived from natural isolates, as well as reengineered strains are rapidly being advanced to the clinic for therapeutic gene transfer applications.[1,2] Current methods for production of preclinical and/or clinical AAV vectors include transient transfection of plasmid DNA in mammalian cell or transduction with baculoviral seed stocks in insect cell systems.[3–7] Following production, iodixanol or cesium chloride gradient ultracentrifugation constitute a critical downstream processing step for clearing cellular debris and protein contaminants. Subsequent purification of iodixanol fractions through anion exchange chromatography has dramatically improved manufacturing efficiency and quality of AAV vector stocks.[8–11] A few affinity-based purification techniques have also been developed such as heparin column chromatography,[8] streptavidin-based purification of biotinylated AAV vectors,[12] immobilized metal-affinity chromatography of hexahistidine tag-modified AAV capsids[13] and protein ligands (AVB Sepharose; GE Healthcare, Piscataway, NJ, USA).[14] Despite unique advantages offered by each of the aforementioned

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CONFLICT OF INTEREST

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strategies, the ever-expanding AAV vector toolkit and need for process scalability present a daunting challenge.

Phage display libraries have been extensively used in identifying highly selective ligands against synthetic and cellular targets. Affinity reagents isolated through phage panning have been used in a battery of applications such as affinity column chromatography, biomarker discovery, therapeutic target validation, epitope mapping, drug design and targeted drug/gene delivery.[15,16] In the current report, we have adapted phage display technology towards development of affinity reagents for AAV capsid recognition. As proof-of-principle, we used a commercially available phage display peptide library to obtain heptapeptide ligands that can recognize AAV serotype 8 capsids. Peptide motifs were then evaluated for their potential to selectively recognize AAV8 capsids, block transduction *in vitro* and *in vivo*, as well as serve as reagents for affinity column chromatography of recombinant AAV8 vectors.

RESULTS AND DISCUSSION

We obtained the consensus motif GYVS(R/H/K)(R/H)(P/S) recognizing AAV8 capsids after three rounds of phage panning using the Ph. D.-7 kit (New England Biolabs, Ipswich, MA, USA) as per manufacturer instructions (Figure 1b). The consensus motif FHENWPS recognizing bovine serum albumin (BSA) was obtained as control (Figure 1c). For solid phase AAV capsid-binding studies, peptide ligands were extended at the C-terminus to include a triglycine linker followed by a cysteine residue (Pep8; H_2N-GYVSRHPGGGC-$CONH_2$ and PepBSA; H_2N-FHENWPSGGGC-$CONH_2$) and conjugated to SulfoLink (Pierce, Rockford, IL, USA) coupling resin. Silver staining of load, wash and eluate fractions demonstrates specific binding of AAV8 capsids to Pep8 (Figure 2a), but not PepBSA (Figure 2b). Further, Pep8 seems to selectively recognize AAV8 capsids, but not other AAV serotypes 1, 2, 5, 6 or 9 (Figure 2c). Competitive elution of AAV8 vectors bound to a Pep8 affinity column using different concentrations of Pep8 suggests that the relative affinity (Kd′) might be in the sub-millimolar range (Supplementary Figure 5). Taken together, the aforementioned results demonstrate the feasibility of generating highly selective peptide affinity reagents for different AAV isolates and reengineered strains.

We evaluated a Pep8-based affinity column chromatography approach for laboratory scale purification of recombinant AAV8 vectors. Briefly, we loaded clarified cell lysate obtained from transfected HEK293 cells directly onto a Pep8 agarose column without previous iodixanol or cesium chloride ultracentrifugation. Silver staining and qPCR analysis (Figures 3a and b, respectively) of different fractions corroborates the recognition of AAV8 capsids by Pep8 and the potential to purify AAV8 vectors directly from cell lysate without ultracentrifugation. Although vector-genome titers ranging from $1–2\times10^{12}$ ml^{-1} were obtained from this initial purification step, protein contaminants were observed in peak eluate fractions (fractions E6 and E7). Similar purity levels are observed following a single iodixanol or cesium chloride density ultracentrifugation step. Nevertheless, it is important to note the improved adaptability of the Pep8 affinity column approach to standard, large-scale protein purification formats, as well as the significant improvement in processing time. A recent study[17] demonstrated that several AAV serotypes including AAV8 are secreted into the media during production. To test whether AAV8 vectors can be purified directly from supernatant, we subjected the media obtained after harvesting transfected HEK293 cells (supernatant) to Pep8 affinity column purification. Silver staining and qPCR analysis of different fractions (Figures 3c and d) demonstrate the ability of the Pep8 column to purify AAV8 vectors from the supernatant. The purity and vector yield obtained from supernatant is higher in comparison with that obtained from cell lysate. (Supplementary Table S1).

Peak eluates obtained from Pep8 affinity column purification of cell lysate were also subjected to anion exchange chromatography to further purify AAV8 capsids. As shown in Supplementary Figure S1, pure AAV fractions with vector-genome titers averaging ~7×10^{11} ml^{-1} were recovered (~40% of Pep8 column yield). Quality of AAV8 particles was further assessed by electron microscopy analysis of peak fractions (Supplementary Figure S2) and by testing infectivity *in vitro* and *in vivo* (Supplementary Figures S3 and S4). The aforementioned results support the feasibility of sequential application of peptide-based affinity column and anion exchange chromatography techniques towards purification of highly pure AAV vector stocks. Although suitable for generating preclinical grade AAV vectors, the aforementioned approach will require significant optimization such as scale-up of peptide agarose column capacity, during production of clinical grade vectors. Further, expansion of the current approach to purify other AAV serotypes and reengineered strains will require phage panning studies for identification of corresponding peptide ligands. Nevertheless, the technology used in the current approach is well-established, commercially available and therefore demonstrates potential for translation to a clinically relevant setting.

Lastly, peptides that block AAV transduction could potentially serve as important reagents in expanding our understanding of AAV biology. As seen in Figure 4b, co-incubation of AAV8 capsids with Pep8 before infection of U87 glioma cells, resulted in a modest (~50%), yet significant decrease in transduction. To potentiate this effect, we synthesized a tetrameric Pep8 construct based on a branched polyethylene glycol reagent (Figure 4a). Briefly, the rationale behind synthesizing a tetrameric Pep8 construct is to obtain enhanced avidity of binding due to multivalent interactions. The latter construct afforded a 60-fold decrease in AAV8 transduction (Figure 4b). Interestingly, pre-incubation of AAV8 vectors with the Pep8 tetramer, before intravenous administration in mice, significantly abrogated transduction *in vivo* (Supplementary Figure S4). Co-administration of peptide-based reagents at different time intervals might yield further insight into mechanisms underlying biodistribution and tissue tropism(s) of AAV vectors.

In summary, we have harnessed the versatility of phage display technology to develop peptide reagents for affinity purification of AAV vectors. In general, the latter approach can also be applied towards identification of peptide ligands specific for other viral vectors. In addition to column chromatography, peptide affinity reagents might be useful alternatives to monoclonal antibodies in understanding AAV infectious pathways and detection of AAV capsids in biological media.

MATERIALS AND METHODS

Cells, plasmids and viruses

HEK 293 cells obtained from the UNC Vector Core were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin/amphotericin B mixture. U87 glioma cells obtained from the UNC Tissue Culture Facility were cultured under similar conditions in DMEM supplemented as mentioned above along with 1% non-essential amino acids and 1% sodium pyruvate.

The AAV8 helper plasmid, pXR8 containing AAV2 *Rep* and AAV8 *Cap* genes and pXX6-80, containing adenoviral helper genes were obtained from the UNC Vector Core. The vector cassette, pTR-CBA-Luc, containing the chicken beta-actin (CBA) promoter-driven luciferase transgene was generated by ligating the luciferase transgene insert flanked by *BamH*I-*Not*I sites into the pTR-CBA backbone. Recombinant AAV8 vectors were produced by the triple-transfection method followed by cesium chloride gradient ultracentrifugation and dialysis as described elsewhere.[18] A subsequent sucrose gradient (5–35%) ultracentrifugation step was carried out to generate high-purity AAV8 stocks suitable

for phage panning studies. Viral titers were determined by qPCR using a Roche Lightcycler (Roche Applied Science, Indianapolis, IN, USA) with primers specific for the Luc transgene region (forward 5′-AAAAGCACTCTGATTGA CAAATAC-3′; reverse 5′-CCTTCGCTTCAAAAAATGGAAC-3′).

Phage panning

Phage panning studies were carried out using the Ph.D.-7 peptide library kit (New England Biolabs) with a diversity of ~1.2×10^9 linear heptapeptides as per manufacturer instructions. Briefly, the library is generated by fusion of randomized peptides to the *N*-terminus of the minor coat protein (pIII) of filamentous bacteriophage M13. First, AAV8 capsids (1×10^{11} vg) or 1% BSA solution in 0.1M sodium bicarbonate buffer (pH 8.6) were incubated in 96-well plates (Corning Inc., Corning, NY, USA) overnight at 4 °C with gentle agitation in a humidified container. Following blocking (with 5 mg ml^{-1} BSA in bicarbonate buffer for 1hr at 4 °C), wells were washed six times with TBS-T (Tris-buffered saline, pH 7.5+0.1% v/v Tween-20). The phage library was then diluted 10 times with TBS-T and ~100 μl added to each coated well (~1×10^{11} pfu per well). Unbound phage was removed after 1-h incubation at room temperature by washing 10 times with TBS-T. Lastly, bound phage was eluted using 0.2 M Glycine-HCl buffer (pH 2.2), then amplified and tittered as per manufacturer instructions. After three rounds of panning, individual clones were processed for DNA isolation and sequenced at the UNC Genome Sequencing Facility.

Synthesis of peptide resin and solid phase-binding studies

For solid phase AAV capsid-binding studies, peptide ligands were extended at the C-terminus to include a triglycine linker followed by a cysteine residue (Pep8; H_2N-GYVSRHPGGGC-$CONH_2$ and PepBSA; H_2N-FHENWPSGGGC-$CONH_2$, Genscript, Piscataway, NJ, USA) and conjugated to SulfoLink coupling resin as per manufacturer instructions. The peptide-agarose resin was then packed into gravity columns (Pierce).

For solid phase-binding studies, AAV8 capsids obtained as described earlier through a sequential cesium chloride-sucrose ultracentrifugation step were loaded onto Pep8 or PepBSA columns. After collecting flow through, columns were washed five times with phosphate-buffered saline and bound virus eluted with 0.2 M Glycine-HCl (pH 2.2). The eluate was immediately neutralized with 1M Tris-HCl (pH 9.0) and all fractions subjected to SDS-PAGE and silver stain analysis (Invitrogen, Carlsbad, CA, USA). To determine serotype selectivity, AAV serotypes 1, 2, 5, 6, 8 and 9 were loaded onto different Pep8 columns and binding studies carried out as described above. Western blot analysis was carried out using the B1 antibody, which recognizes the *C*-terminal end domain (B1 epitope - IGTRYLTR) of VP1, VP2 and VP3 capsid protein subunits of most AAV serotypes.[19,20]

Peptide affinity column purification of AAV8 vectors

HEK 293 cells in 5×15 cm tissue culture dishes were transfected as described earlier. At 72 h post-transfection, cultures were harvested, cell pellets resuspended in 10 ml of TBS-T (Tris-buffered saline, pH 7.5+0.5% v/v Tween-20) and then lysed by sonication. DNase I (Sigma, St Louis, MO, USA) was then added to the cell lysate (100 μl of 10 mg ml^{-1}) and incubated for 30 min at 37°C. The crude lysate is clarified by centrifugation at 10000 *g* for 15 min at 4 °C. Clarified cell lysate was then loaded onto a Pep8-agarose gravity column with 5 ml resin-bed, prepared as described earlier and equilibrated with TBS-T. After collecting flow through, column was washed five times with two column volumes of TBS-T and bound virus eluted with 15 ml of 0.2 M Glycine-HCl, pH 2.2. The eluate was immediately neutralized with 1M Tris-HCl, pH 9.0 (150 μl ml^{-1} of eluate). For purification of AAV8 vectors from supernatant, the media obtained after harvesting transfected HEK293 cells is treated with DNase and subjected to centrifugation as described above. The clarified

supernatant is then directly loaded onto the Pep8 affinity column and wash/elution conditions were identical to those described earlier.

The peak fractions from column-purified cell lysate (E6, E7) were pooled and further purified by a 5-ml HiTrap Q column (GE Healthcare) using a Pharmacia AKTA FPLC system. Briefly, the Q-column was equilibrated with Buffer A (20 mM Tris, 15 mM NaCl, pH 8.5), following which samples were loaded and subjected to linear gradient elution from Buffer A to Buffer B (20 mM Tris, 1 M NaCl, pH 8.5). Peak fractions were monitored using UV absorbance (280 nm). All fractions obtained from cell lysate and supernatant purification steps were subjected to sodium dodecyl sulfate-PAGE, silver stain analysis and viral titers determined by qPCR.

Peptide inhibition assays

To determine whether Pep8 can block AAV8 transduction, we synthesized Pep8 as well as a tetrameric Pep8 construct, (Pep8)×4 (Figure 4a). Briefly, a four-arm polyethylene glycol maleimide construct (20 kDa, Laysan Bio, Arab, AL, USA) was coupled to Pep8 and PepBSA (Genscript) using thiol–maleimide conjugation chemistry. Briefly, 3 mole equivalents of 4-arm polyethylene glycol–maleimide and 18 mole equivalents of Pep8/PepBSA were dissolved in 100 μl of 0.05 M Tris-HCl (pH 7.2) containing 0.01 M TCEP (Pierce) as reducing agent. Following incubation for 2 h at room temperature, the reaction mixture was quenched with cysteine (0.05 M) and dialyzed against phosphate-buffered saline (pH 7.4). For inhibition studies, AAV8 capsids were pre-incubated with Pep8 or (Pep8)×4 for 10 min at room temperature followed by incubation on U87 glioma cells. Luciferase expression levels were determined at 24 h post-infection using a Victor2 luminometer (Perkin Elmer, Waltham, MA, USA). Untreated AAV8 capsids and those pre-incubated with PepBSA or (PepBSA)×4 constructs served as control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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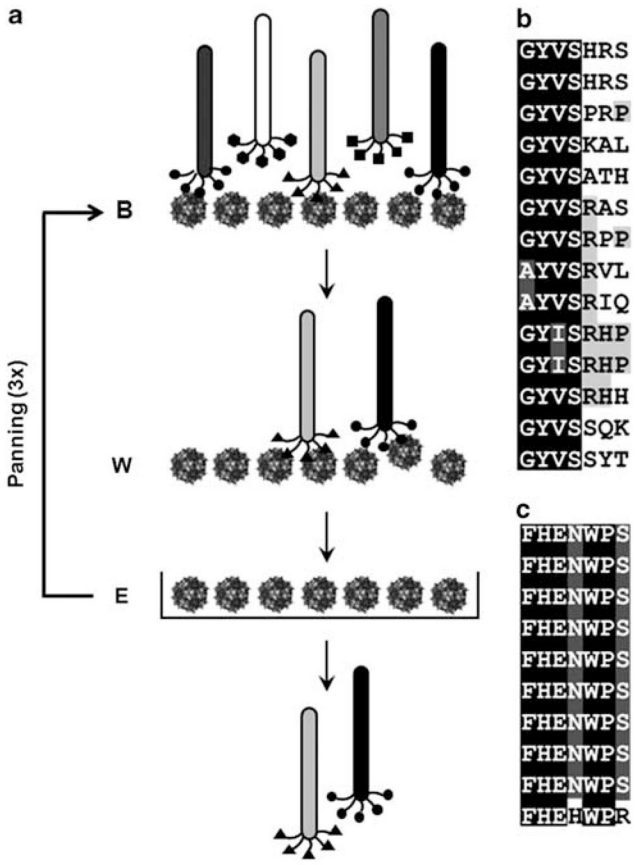

Figure 1.

(**a**) Phage panning procedure. The Ph.D.-7 phage display library was co-incubated with AAV capsids to allow binding (B), followed by washing (W) of unbound phage and elution (E) of bound phage. Bound phage were then amplified and subjected to three rounds of panning to enrich AAV capsid-recognizing phage particles. Phage DNA isolation and sequencing revealed heptapeptide consensus motifs recognizing AAV8 capsids (**b**) and bovine serum albumin (BSA) (**c**) as control.

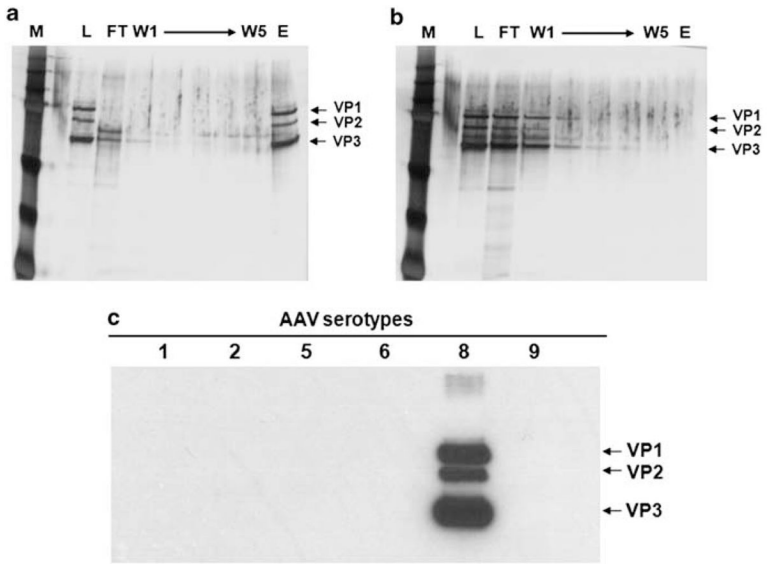

Figure 2.

Solid phase-binding profile of AAV capsids to peptide-agarose beads. Specific binding of AAV8 capsids to Pep8-agarose (**a**), but not PepBSA-agarose (**b**) is shown. Silver stained SDS-PAGE gels containing marker (M), load (L), flow through (FT), wash (W) and eluate (E) fractions are shown. Fractions containing AAV8 capsids show characteristic VP1, VP2 and VP3 protein bands corresponding to 87, 73 and 62-kDa MW species, respectively. (**c**) Western blot showing selective recognition of AAV8 capsids, but not other serotypes by Pep8. Each lane represents an independent binding assay of Pep8 with different AAV serotypes. After loading different AAV serotypes on Pep8-agarose column, the columns were washed and eluate fractions were collected. The eluate fractions were then resolved by SDS-PAGE and subjected to western blot analysis using the B1 antibody.

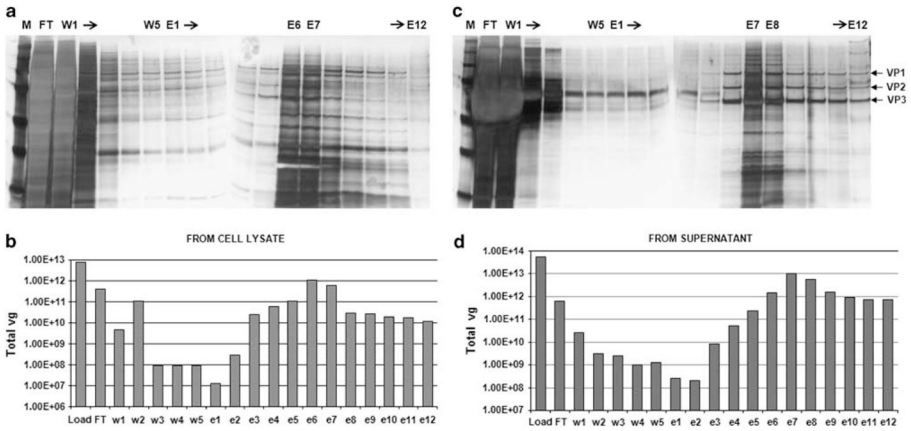

Figure 3.

Affinity column purification of recombinant AAV8 vectors from crude cell lysate (**a**, **b**) and from supernatant (**c**, **d**). Clarified HEK 293 cell lysates were directly loaded onto a Pep8-agarose gravity column with a 5-ml resin bed. SDS-PAGE and silver stain analysis of flow through (FT), wash (W) and eluate (E) fractions obtained from cell lysate (**a**) and supernatant (**c**). Quantitation of vector genome titers obtained in each fraction from cell lysate (**b**) and supernatant (**d**) determined by qPCR. Peak eluate fractions from Pep8 affinity chromatography of cell lysate (E6, E7) and supernatant (E7, E8) are highlighted. Protein bands corresponding to VP1, VP2 and VP3 capsid protein subunits of AAV are highlighted.

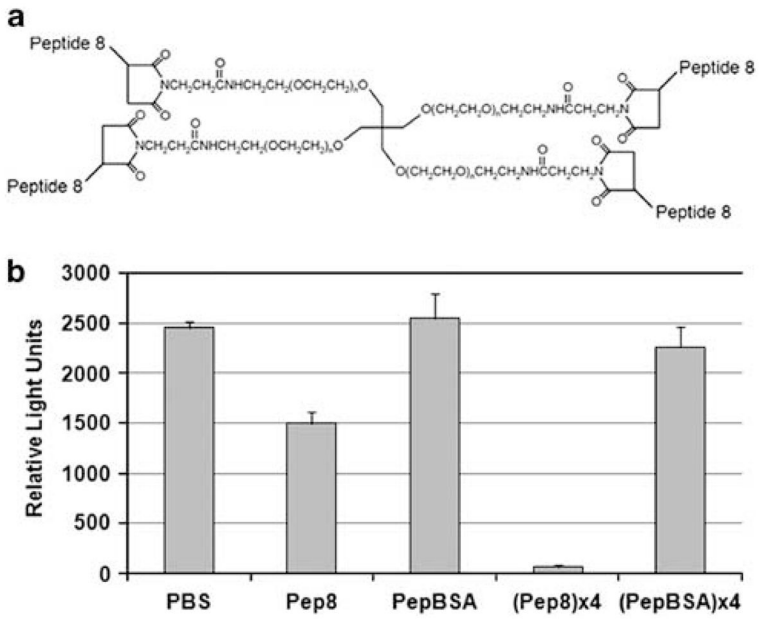

Figure 4.

Inhibition of AAV8 transduction by Pep8 or PepBSA constructs. U87 glioma cells were incubated with recombinant AAV8 vectors packaging a CBA-Luc cassette (MOI=1000), pre-mixed with different peptide-polyethylene glycol conjugates (**a**), peptides alone or phosphate-buffered saline. Analysis of luciferase transgene expression levels at 24-h post-transduction reveals that the tetrameric (Pep8)×4 exhibits highest inhibitory effect on AAV8 transduction (**b**). Error bars represent s.d. (*n*=3).