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Longer Inactivating Sequence in Peptide Lock Improves Performance of Synthetic Protease-Activatable Adeno-Associated Virus

Maria Y. Chen^{†,‡}, Tawana M. Robinson[§], Junghae Suh^{*,†,||}

[†] Department of Bioengineering, Rice University, 6100 Main Street, Houston, Texas 77005, United States

[§] Department of Chemistry, Rice University, 6100 Main Street, Houston, Texas 77005, United States

^{||} Systems, Synthetic and Physical Biology Program, Rice University, 6100 Main Street, Houston, Texas 77005, United States

[‡] Medical Scientist Training Program, Baylor College of Medicine, 1 Baylor Plaza, Houston, Texas 77030, United States

Abstract

Adeno-associated viruses (AAVs) are promising gene therapy vectors but may exhibit off-target delivery due to broad tissue tropism. We recently developed a synthetic protease-activatable AAV vector, named provector, that transduces cells preferentially in environments rich in matrix metalloproteinases (MMPs) which are elevated in a variety of diseases, including various cancers and heart diseases. The provector displays peptide locks made up of MMP recognition sites flanking an inactivating sequence (IS) composed of four aspartic acid residues (D4). When present, the IS prevents AAV from binding cell receptors and no transduction occurs (OFF state). High levels of MMPs cleave the recognition sequences and release the IS from the capsid surface, restoring cell receptor binding (ON state). The AAV9 provector prototype is not optimal as it displays baseline OFF transduction at 5–10% of that of the wild-type capsid, which can lead to off-target delivery. We hypothesized that changes to the IS may decrease OFF state transduction. We created a provector panel with IS of lengths 0 (D0) to 10 (D10) aspartic acid residues and characterized this panel in vitro. Notably, we find that the D10 provector has an OFF transduction of less than 1% of wild-type capsid and an ON/OFF transduction ratio of 27, the best outcome achieved for any provector thus far. In summary, our results enable us to define new design rules

*Corresponding Author: jsuh@rice.edu.

Author Contributions

M.Y.C. and T.M.R. conceived of the study. M.Y.C. planned and conducted experiments and collected and analyzed data. J.S. supervised the project. M.Y.C. wrote the manuscript with input from all authors.

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Supporting Information

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Table S1 lists primers used to create the provector plasmid constructs; Figure S1 shows transduction efficiency of provector viruses in ON and OFF states; Figure S2 demonstrates the linear relationship between transduction index and multiplicity of infection; and Figure S3 illustrates the amino acid “scars” on AAV provector capsid post-proteolysis (PDF)

for the provector platform, specifically that (1) the IS is necessary for provector locking and (2) increasing the number of aspartic acid residues in this sequence improves locking.

Graphical Abstract

Keywords

adeno-associated virus; AAV; gene delivery; stimulus-responsive; enzyme-responsive; synthetic virology

Numerous clinical trials have shown that adeno-associated virus (AAV) is a promising gene therapy vector that has low immunogenicity and is nonpathogenic.¹ Additionally, AAV can be engineered as a biocomputing nanoplatfrom capable of delivering genetic material in response to specific stimuli.^{2,3} Controllable and targeted gene delivery vectors are necessary to decrease negative off-target side effects and limit toxicity to healthy tissue. Such vectors also allow for systemically delivered therapeutics to act preferentially at specific locations in the body. To achieve more controlled and targeted gene delivery, we previously developed a protease-activatable AAV platform, called provector.^{4,5} The provector has switchable behavior and transduces cells only in the presence of matrix metalloproteinases (MMPs), which are overexpressed in many diseased states, such as various cancers,^{6–8} stroke,⁹ congestive heart failure,^{10,11} and atherosclerosis.¹²

The first generation provector was based on AAV serotype 2 (AAV2) and created by inserting a “peptide lock” into the capsid after location G586 near the heparan sulfate proteoglycan (HSPG) binding domain.⁴ The peptide lock is composed of MMP recognition sequences flanking an inactivating sequence (IS) (Figure 1). The IS serves to interfere with virus–cell receptor interactions, thus greatly decreasing transduction when it is present on the capsid surface. Upon exposure to MMPs, the peptide lock is cleaved and IS removed from the capsid surface. The provector concept was then translated to AAV9 via incorporation of a peptide lock after G453 near the galactose binding domain.⁵ Cell surface glycans terminating in galactosyl residues is a major receptor for AAV9, and inhibition of virus–galactose interactions significantly reduces its ability to infect cells.¹³ The switchable behavior of the provector in response to MMP is robust, with the ON state being able to achieve up to 11.3-fold increased transduction compared to the OFF state for AAV2 provector¹⁴ and 5-fold increase for AAV9 provector.⁵ Further improvement of the ON/OFF

ratio would allow for a more effective gene delivery vector with greater specificity to target tissues. In particular, the provectors currently transduce up to 10% of that of the wild-type capsid in the OFF state,^{4,5} which may lead to substantial off-target gene expression in vivo. Therefore, decreasing the OFF transduction level would further minimize nontarget delivery and increase the provector specificity.

All provector platforms thus far have incorporated an IS that is four amino acids in length, with the tetra-aspartic acid motif (D4) being the most well-characterized. This motif was hypothesized to interfere with the electrostatic forces driving the interaction between AAV2 and HSPG.^{15,16} Further studies showed that steric hindrance and electrostatics both contribute to the disruption of virus–receptor interactions in the AAV2 provector.¹⁷ Additionally, the majority of IS variants besides the D4 motif tested thus far result in significant decreases in AAV2 provector production or capsid stability.¹⁷ For AAV9-based provectors, it is currently unclear how the IS affects binding between AAV9 and galactose. In order to further optimize the provector platform and to facilitate its translation to other AAV serotypes, the design rules for the peptide lock must be elucidated.

In this study, we set out to define design rules regarding the IS for protease-activatable AAV9 vectors. We created a panel of AAV9 provectors with IS variants ranging from 0 (D0) to 10 (D10) aspartic acids in length. We characterized the physical properties (e.g., virus formation and stability) and the functional properties (e.g., protease cleavability and transduction activatability) of this panel of viral variants. In doing so, we were able to create an improved provector variant with an OFF transduction level of less than 1% of wild-type capsid and an ON/OFF transduction ratio of 27, the best achieved thus far.

RESULTS

Effect of Inactivating Sequence Length on Virus Formation and Genome Protection Ability

ProvIS (provector inactivating sequence) AAV mutants with inactivating sequences (IS) containing aspartic acid residues ranging from size 0 (D0, no IS) to size 10 (D10) were created to test the effect of IS length on provector formation and function (Table 1). The S1 mutant contains a scrambled MMP cleavage sequence that is not recognized by MMPs and thus acts as a negative control vector. Alteration of the IS changes not only the length but also the electrostatic charge of the inserted peptide lock sequence. The peptide lock sequences in the ProvIS mutants add from +2 (D0) to −8 (D10) charges to each viral protein (VP) subunit in the AAV capsid, which may affect virus formation and stability.

To quantify the formation of the ProvIS mutants, cell lysates from ten 15 cm tissue culture plates were pooled, extracted using iodixanol gradient, and titered with qPCR. There are no statistically significant differences in vector production titers between wild-type (wt) AAV9 capsid vector and most of the ProvIS mutants (Figure 2a). The only exception is the mutant with no IS (i.e., D0), which has a significantly decreased virus titer. Next, an equal number of viral genomes of each ProvIS mutant was visualized on a Western blot with B1 antibody, which binds to a C-terminal epitope common to all three VPs (Figure 2b). Insertion of the peptide lock adds 22–32 amino acids to all VP subunits in the viral capsid, with WT9 subunits having the smallest subunits and fastest migration on the protein gel, while mutant

subunits from D0 to D10 undergo increasingly slower migration on the gel corresponding to increasingly larger molecular weight. S1 adds the same number of amino acids as D4 and both shift a similar distance. VP1/VP2/VP3 capsid protein ratios appear roughly the same as those of wt AAV9 capsid. No truncation products are seen, which suggests that the mutant viruses are stable at the level of each individual VP subunit.

To test if the mutants are able to protect their encapsidated genomes from nuclease digestion, a benzonase assay was performed (Figure 2c). If the capsid is unstable, incubation with benzonase will digest ill-protected viral genomes. Genome protection was calculated as the ratio of viral titers remaining after benzonase treatment compared to no benzonase treatment. A plasmid-only control was included to ensure that the treatment conditions were adequate to digest all unprotected DNA. S1 shows a slight reduction in genome protection, whereas D0–D10 mutants have protection levels similar to those of the wt AAV9 capsid. Taken together, these data suggest that completely abolishing the IS has a negative effect on virus formation but does not significantly affect genome protection, and extension of the IS length does not negatively affect virus formation or genome protection ability.

Effect of Inactivating Sequence Length on Proteolytic Susceptibility

The MMP recognition sequences in the peptide lock flank the IS (Figure 1), and we do not expect proteolytic cleavage to be affected by elongation of the central aspartic acid motif. To test this property, purified ProvIS mutants were incubated with MMP2, MMP7, and MMP9, and the cleavage results were visualized on a silver stain alongside the wt capsid (Figure 3). The expected VP fragments due to proteolysis are illustrated in Figure 3a. The control sham-treated samples were incubated with enzyme storage buffer containing no active enzyme, and thus we see all VP subunits intact (Figure 3b). Examination of the cleavage products shows that ProvIS mutants D0–D10 are effectively cleaved by MMP2, MMP7, and MMP9. No cleavage is visible for the wt AAV9 capsid. Trace amounts of S1 subunits are cleaved by MMPs, with a substantial majority remaining uncleaved. Taken together, the results show that changing the IS length does not greatly affect the proteolytic susceptibility of the viruses.

Protease-Activated Transduction Ability of ProvIS Mutants

The ability of ProvIS mutants to transduce cells was tested in vitro in the CHO-Lec2 cell line (Figure 4). Viruses carrying scGFP were treated with sham buffer or proteases and added to cells, and flow cytometry was used to quantify transduction efficiency. The transduction index (TI) is calculated as the percent of GFP positive cells multiplied by the geometric mean fluorescence intensity ($TI = \%GFP \times gMFI$) (Supplemental Figure S1). TI is the preferred measure of transduction efficiency because it scales linearly with transduction MOI (Supplemental Figure S2). This linear relationship allowed for retitering of proteolyzed virus samples to account for pipetting errors by using the titering results to normalize TI values (Figure 4a).

Both wt AAV9 capsid and S1 control behave as expected and do not display protease-activatable behavior. D0 and D2 also do not display significant activatable behavior. The ratio of ON transduction versus OFF transduction (i.e., ON/OFF) increases as more aspartic

acids are included in the IS from D4 to D10, ranging from 8.1-fold for D4 to 27-fold for D10. The D10 variant shows the greatest knock-down in transduction when locked, with a TI that is 0.9% of wt (Figure 4b). Although there is a significant decrease in TI from wt to locked D0, and from locked D0 to locked D2, the difference in transduction between locked D2–D10 and S1 is not statistically significant. Upon unlocking via proteolytic cleavage, the ProvIS variants do not regain a wt level of transduction efficiency (Figure 4c). Unlocked D0 transduces best, at 37% of wt, though there are no statistically significant differences between the TIs of unlocked D0–D10 variants. Additionally, there are no statistically significant differences between MMP2-, MMP7-, and MMP9-treated groups within each virus for the ProvIS mutants and S1. Taken together, these data show that the IS length does not impact virus unlocking but is necessary for locking the virus, and that increasing the number of aspartic acids in the IS increases the level of transduction knock-down in the OFF state.

Cell Receptor Usage by ProvIS Mutants

AAV9 binds to cell surface galactose in order to internalize into cells.²⁰ As the peptide lock is located near the galactose binding site of the AAV9 capsid, we hypothesized that virus locking occurs primarily via interruption of galactose binding. Lectin competitively inhibits galactose binding by AAV, and thus the lectin competition assay was used to probe the galactose binding sensitivity of ProvIS mutants. As previously shown⁵ and reaffirmed here, the wt AAV9 capsid's transduction decreases with increasing lectin concentration (Figure 5a). There is no statistically significant difference in lectin sensitivity between sham- and MMP-treated wt capsid virus, indicating that MMP treatment does not alter native viral galactose binding. In the ON state, the D0 and D4 ProvIS mutants respond as expected to lectin inhibition, with transduction efficiencies decreasing with increasing lectin concentration, leading to complete abolition of transduction at the highest lectin concentration (Figure 5b,c). Sham-treated D4 remains locked regardless of lectin concentration. These data suggest that the transduction efficiency restored by MMP cleavage in the D4 mutant is primarily due to restoration of galactose binding ability.

Surprisingly, the sham-treated and MMP-treated D0 vectors transduce substantially differently in the lectin competition assay (Figure 5c) compared to the previous transduction assay (Figure 4). Under normal transduction conditions in which the viruses were incubated with cells for 24 h, sham- and MMP-treated D0 vectors transduce at above 30% of wt AAV9 (Figure 4). Under the lectin competition pulse-transduction protocol, which involves a 1 h virus–cell incubation on ice followed by a PBS (phosphate-buffered saline) wash, sham-treated D0 transduction remains at above 30% of wt, whereas MMP-treated D0 transduction drops to less than 10% of wt (Figure 5c,d). It is expected that the overall transduction efficiency would be reduced with a shorter virus–cell incubation time (24 h for regular transduction compared to 1 h for lectin competition). However, it is unexpected that this decrease in transduction is not proportional for the sham- versus MMP-treated D0 mutant. This difference suggests that the cellular transduction mechanism of D0 may differ between sham- and MMP-treated conditions.

Transduction Kinetics of ProvIS Mutants

To further characterize the difference between ON and OFF ProvIS mutants, we performed a timed transduction study in which CHO-Lec2 cells were incubated with virus for various times, rinsed with PBS to remove unbound virus, and transduction quantified via flow cytometry at 48 h post-transduction (Figure 6). The TI for each incubation duration was also normalized to the maximum TI obtained for each virus at 24 h to find TI_{50} , the time at which the virus reaches 50% of the full transduction level (Figure 6d,e). Sham-treated D4 displays low transduction efficiencies at all incubation times tested, and the difference in TI at 1 versus 24 h of incubation is not statistically significant. Sham-treated D0 displays transduction kinetics similar to those of wt capsid but experiences an overall reduction in transduction efficiency. MMP-treated D0 and D4 have similar transduction profiles where the TI_{50} occurs at a much later time (~16 h) compared to sham-treated D0 and the wt AAV9 capsid (TI ~ 8 h). In other words, the unlocked ProvIS vectors need to be in contact with cells for twice as long to achieve half of their maximum transduction levels compared to the wt capsid as well as to the locked D0 vector. This result helps to explain the previous discrepancies seen in TI values between transduction characterization (Figure 4) and lectin competition assay (Figure 5) and suggests that the cleavage scars left behind on unlocked ProvIS mutants cause a slowdown in transduction kinetics.

DISCUSSION

The ProvIS mutants offer insights on the effect of IS length on the formation and switchability of protease-activatable AAV vectors. As lengthening the IS by increasing the number of aspartic acids results in incorporation of a greater number of charged residues into the capsid, we expected increased disturbance to the native virus capsid structure and thus decreased virus production and genome protection ability. Surprisingly, D0—the variant with no aspartic acids—forms at significantly lower titer, whereas the remaining mutants are similar to the wt AAV9 capsid (Figure 2a). This result demonstrates that a larger insert with more negative charge is not necessarily detrimental to virus formation. It is possible that elongation of the provector lock allows for more flexibility in the inserted peptide and thus puts less strain on the capsid attachment points and improves virus formation. None of the aspartic-acid-based ProvIS variants yields spontaneous VP truncation products (Figure 2b) as has been previously seen when four lysines were used instead of aspartic acids.¹⁷

Changing the IS length does not affect cleavage by MMP at the conditions tested in this study (Figure 3). This is expected because the MMP cleavage sequence is distinct from the IS and separated by glycine linkers. However, the conditions for the enzymatic reactions used in this study were carried out to completion based on previous provector testing.⁴ Thus, it is possible that the kinetics of MMP cleavage is affected by lengthening the distance between the two MMP cleavage motifs and/or by the increase in IS negative charge, and further enzymatic studies are needed to investigate these potential changes more closely.

The IS appears to be essential for provector locking in the OFF state, with OFF state transduction decreasing with increasing IS size (Figure 4). In the panel tested here, no aspartic acids (D0) in the IS leads to least effective locking, with the locked and unlocked D0 achieving the same level of transduction (Figure 4). Two aspartic acid residues (D2) are

also not enough to provide sufficient locking. Thus, the provector cannot be sufficiently locked without a dedicated IS that, if using consecutive aspartic acid motifs, must be four amino acids or longer.

A limitation of the panel tested in this study is that changes in electric charge are coupled to changes in IS length. Therefore, it is difficult to conclude which property contributes more significantly to provector locking. Lectin competition assay with D0 and D4 suggests the restoration of transduction upon cleavage involves galactose binding (Figure 5). Although hydrophobic interactions have been shown to be important for galactose binding,^{21,22} galactose may also form hydrogen bonds with polar and charged amino acids.²¹ Aromatic residues that can contribute to hydrophobic interactions as well as polar/charged amino acids that can form hydrogen bonds can be found near the galactose binding site on AAV9.¹³ Thus, it is conceivable that disruption of either force can significantly affect galactose binding. Further studies on the IS would allow us to draw more concrete conclusions regarding which forces are dominant in inhibiting galactose binding in locked AAV9 provectors.

Another aspect of provector function is restoration of transduction in the ON state. Interestingly, all cleaved ProvIS mutants achieve the same level of transduction in the ON state at around 30–40% the level of wt capsid (Figure 4c). This decrease in transduction has been previously reported^{4,5,14} and is most likely due to the few amino acids of the lock that remain on the capsid after cleavage (“cleavage scars”) (Supplemental Figure S3). D0 is the only variant that has significant transduction in the OFF state, and timed transduction studies show that locked D0 has the same transduction kinetics profile as the wt capsid (Figure 6d). However, the transduction profiles of unlocked D0 and D4 have different transduction kinetics compared to wt or locked D0 capsid (Figure 6), with the unlocked viruses approaching their maximal transduction at a later time point. Therefore, locked and unlocked D0 reach similar transduction levels but have different kinetic profiles (Figure 6d), suggesting they operate through different mechanisms of transduction. Kinetic analysis of other members of the ProvIS panel besides D0 and D4 was not explored but may provide further insight on the effects of different IS motifs on transduction kinetics. It is possible that the cleavage scars confer the virus with the ability to transduce via an alternative unidentified pathway. It is also possible that the presence of the uncleaved peptide lock on sham-treated D0 provides an alternative transduction pathway. The existence of an alternate transduction pathway may also explain why provector transduction does not recover to the wt level after unlocking. Transduction of unlocked D0 and D4 mutants is still susceptible to lectin inhibition, however, suggesting that galactose binding is still a major component of their transduction pathway. The impact of the cleavage scar on avidity of AAV9 binding to galactose was not quantitatively explored in this study, and further examination of the cleavage scars is necessary to explain the altered provector transduction profile.

Finally, the newly optimized D10 can be used as a basis for further improvement of the provector platform. Previous studies have shown that incorporation of wt subunits into a provector with a D4 IS can improve ON transduction levels.¹⁴ However, this incorporation also results in increased off-target transduction in the OFF state.¹⁴ The incorporation of wt subunits into D10 rather than D4 may result in an increase in ON transduction while

maintaining an acceptable level of OFF transduction, further enhancing the ON/OFF switchability of the provector.

CONCLUSIONS

The IS of the provector peptide lock has been hypothesized to be necessary to block virus binding to cellular receptors, but its importance and impact had not been systematically investigated before. This study shows that the IS is necessary to block native receptor binding but does not affect transduction levels in the ON state. An aspartic acid motif of at least four residues in length is necessary for sufficient locking of the AAV capsid, with increasing length corresponding to better locking. The longest aspartic-acid-based motif tested thus far, D10, is optimal and yields a 27-fold ON/OFF transduction ratio, with OFF transduction at about 1% of that of the wt capsid. The remaining cleavage scars on unlocked provectors appear to negatively affect virus transduction and may cause the virus to transduce cells through an alternate pathway. In sum, this study outlines a basic design rule establishing the importance and impact of the IS in the provector peptide lock. More extensive studies would allow for further improvement of the protease-activatable AAV platform.

MATERIALS AND METHODS

Plasmid Cloning

ProvIS plasmids were constructed based on the pAAV9-453-L001 plasmid,⁵ which has AAV2 *rep* and AAV9 *cap* with a peptide lock inserted in the *cap* gene after G453. Forward primers attaching downstream of the inactivating sequence and reverse primers attaching upstream of the inactivating sequence (Supplemental Table S1) containing various numbers of aspartic acid codons were used in polymerase chain reaction (PCR) with Phusion HF polymerase (New England BioLabs) to generate ProvIS mutants containing 0 (D0) to 10 (D10) aspartic acids in the inactivating sequence (IS). The 5' ends of primers were phosphorylated using T4 polynucleotide kinase (PNK; New England BioLabs) under manufacturer recommended conditions. T4 PNK was inactivated by incubating at 65 °C for 20 min. The optimized two-step PCR conditions for generating D0–D8 were as follows: 1× Phusion HF buffer, 0.2 mM dNTPs, 0.5 μM forward primer, 0.5 μM reverse primer, 0.2 ng/μL template DNA (pAAV9-453-L001), 3% DMSO, and 0.4 units of Phusion polymerase, with thermocycler conditions (98 °C for 30 s for initial denaturation, then 25 cycles of denaturation at 98 °C for 10 s and annealing/extension at 72 °C for 2 min 30 s, and final extension at 72 °C for 10 min). D10 was generated using the same PCR conditions except pAAV9-453-L001-D8 was used as the template. PCR products were run on 0.7% agarose gel with GelStar stain (Lonza) and extracted using Zymoclean gel DNA recovery kit (Zymo Research). Blunt-end ligation was performed using T4 DNA ligase (New England BioLabs) overnight at 16 °C, and the ligated product was transformed into NEB10-β-competent *Escherichia coli*. All plasmids were sequence-verified by an external vendor (GeneWiz).

Virus Production

Virus was produced using triple-plasmid transfection in 1.6:2:2 molar ratio of (1) pXX6-80, helper plasmid encoding adenoviral genes, (2) self-complementary GFP (scGFP) transgene cassette containing ITRs, and (3) plasmid containing *rep* and *cap* including the appropriate ProvIS mutation. HEK293T cells were seeded on poly-L-lysine-coated 15 cm plates and grown using DMEM medium with 10% fetal bovine serum (FBS; Gemini Bio-Products) and 1% penicillin–streptomycin (P/S, Thermo Fischer Scientific). At 80–90% confluency, 40 μ g of total DNA was added with 7.5 μ M polyethylenimine (Polysciences). Medium was changed at 24 h post-transfection, and cells were harvested at 48 h posttransfection. Protease inhibitor cocktail (Sigma-Aldrich) was added to collected cells; cells were lysed with three freeze–thaw cycles, and virus was extracted using iodixanol gradient and ultracentrifugation as previously described.¹⁸ Virus was diluted in 1 $\times$ gradient buffer (GB, 10 mM Tris, 10 mM MgCl₂, 150 mM NaCl) with 0.001% Pluronic F-68 (Sigma-Aldrich) and buffer-exchanged and concentrated using Amicon Ultra-15 100 kDa centrifugation units (Millipore).

Virus Titer Quantification

The genomic titer of viruses was measured using quantitative polymerase chain reaction (qPCR) as described previously.¹⁹ Briefly, a denatured and diluted virus sample was mixed with SYBR green (Applied Biosystems) and primers against the CMV promoter (forward: 5'-TCACGGGGATTTCCTCAAGTCTC-3' and reverse: 5'-AATGGGGCGGAGTTGTTACGA-3'), and the reaction quantified using the Bio-Rad C1000 thermal cycler.

Cellular Transduction Assay

To measure the cellular transduction efficiency of OFF versus ON ProvIS viruses in vitro, CHO-Lec2 cells were seeded on poly-L-lysine-coated 48-well plates at 5×10^4 cells/well. At 70–80% confluency, viruses were added at multiplicity of infection (MOI) 5000 in serum-free MEM- α medium with 1% P/S. After 24 h of incubation at 37 °C, the medium was replaced with MEM- α containing 10% FBS and 1% P/S. The cells were harvested at 48 h for flow cytometry with FACSCanto II flow cytometer (BD Biosciences).

Nuclease Protection Assay

To estimate the degree of viral genome protection against nuclease digestion, virus was first incubated in 1 $\times$ GB for 20 h at 37 °C, and then capsid protection of its genome was measured using the benzonase protection assay. Virus in endo buffer (15 mM MgCl₂, 5 mg/mL BSA, 500 mM Tris pH 8.0) was treated with benzonase (250 units/ μ L, Sigma) or sham buffer (50% glycerol, 20 mM Tris pH 8.0, 20 mM NaCl, 2 mM MgCl₂) for 30 min at 37 °C. The reaction was terminated with 0.5 μ M EDTA, and remaining viral genomes were quantified via qPCR as described above.

Western Blot and Silver Stain

Western blotting was used to visualize viral capsid proteins. A total of 3×10^9 viral genomes of iodixanol-extracted virus was heated at 75 °C for 15 min with LDS and NuPAGE sample reducing agent (Thermo Fisher Scientific). The sample was separated via electrophoresis on

a NuPAGE 4–12% bis-Tris gel (Life Technologies) and transferred onto a nitrocellulose membrane. The common C-terminal of capsid proteins was probed using B1 primary antibody (ARP American Research Products) and goat anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology). Viral capsid proteins and proteolyzed VP fragments were visualized using silver staining. A total of 1.5×10^9 viral genomes was denatured for 75 °C for 15 min with LDS, separated via electrophoresis on NuPAGE 4–12% bis-Tris gel, and stained using the SilverQuest silver staining kit (Life Technologies).

Proteolysis

Viruses were treated with matrix metalloproteinases (MMPs) to probe for cleavage susceptibility. Concentrated viruses were diluted 1:1 in MMP buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 5 mM CaCl₂) and treated with 0.317 μ M of recombinant human MMP2, MMP7, or MMP9 (catalytic domains, Enzo Life Sciences) or sham buffer (50 mM Tris-HCl pH 7.5, 1 mM CaCl₂, 300 mM NaCl, 5 μ M ZnCl₂, 0.1% Brig-35, 15% glycerol) for 20 h at 37 °C. The reaction was terminated with MMP stop buffer (1 $\times$ GB with 0.001% Pluronic F-68 and 20 mM EDTA).

Lectin Competition Assay

The galactose binding ability of ProVIS viruses was tested using a lectin competition assay. CHO-Lec2 cells were seeded on poly-L-lysine-coated 48-well plates at 5×10^4 cells/well. At 70–80% confluency, serum-free MEM- α medium with 1% P/S and 0–50 μ g/mL of unconjugated *Erythrina cristagalli* lectin (Vector Laboratories) dissolved in PBS was added and incubated with the cells on ice for 30 min. Virus was added at MOI 5000, and the cells were incubated for another 1 h on ice. The cells were then rinsed with sterile PBS and incubated at 37 °C for 24 h with serum-free medium. At 24 h, serum-free medium was replaced with serum-containing medium. At 48 h post-transduction, cells were harvested for flow cytometry (FACSCanto II) to measure GFP expression.

Statistical Analysis

Data presented in Figure 4 and Figure S1 were obtained in three independent experiments, with duplicates in each experiment. All other data were obtained in two independent experiments with duplicates in each experiment. The mean and standard error of the mean are plotted. Statistical analysis was done using Graph Pad Prism 7. As indicated in the figure legends, statistically significant differences were calculated using either one-way analysis of variance (ANOVA) or two-way ANOVA with Bonferrini post hoc test with a significance level of 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Provector overview. (a) Composition of peptide locks inserted into the AAV9 capsid after amino acid G453. The provector inactivating sequence (ProvIS) is in red and is flanked by glycine linkers and the MMP cleavage sequence, VPMSMRGG. The NgoMIV/KasI restriction sites (RE) make up the beginning and end of the peptide lock. (b) Overview of provector function. In the locked state, the peptide lock sequence blocks cell receptor binding and prevents the virus from transducing cells. When MMPs are prevalent, the lock sequences are cleaved and the virus is once again able to interact with and transduce cells.

Figure 2.

Structural characterization of ProvIS viruses. (a) Titer of 10 plate preparations of ProvIS viruses separated via iodixanol gradient ultracentrifugation and measured by qPCR. Standard error of mean (SEM) of two qPCR experiments are shown. (b) Visualization of ProvIS VP subunits via Western blotting. A total of 3×10^9 viral genomes was loaded on a 4–12% bis-Tris gel and probed with B1 antibody. (c) Genome protection assay of ProvIS viruses with plasmid control (Plas). Viruses were incubated with benzonase for 30 min. Genome protection is quantified as the ratio of intact viral genomes postincubation to sham-treated control. Two independent experiments were conducted, and error bars are SEM. Statistically significant difference from wt AAV9 capsid levels was calculated with one-way ANOVA and indicated with * for $P < 0.05$.

Figure 3.

Proteolytic cleavage of ProvIS mutants. (a) Schematic of VPs illustrating the location of the peptide lock and expected cleavage fragments upon proteolysis. The peptide lock is inserted after amino acid G453 in the *cap* sequence. Thus, cleavage by MMP would result in distinct N-terminal cleavage fragments for each VP (VP1', VP2', VP3') and a common C-terminal cleavage product for all three VPs (C-term). Figure not drawn to scale. (b) Silver stain of sham (S)-treated and MMP2-, MMP7-, and MMP9-treated ProvIS viruses. Viruses were incubated with the indicated treatment for 20 h. Viral genomes (1.5×10^9) were denatured and run on a 4–12% bis-Tris gel.

Figure 4.

Transduction efficiency of ProvIS viruses in OFF and ON states. (a) Transduction indices (TI, calculated as $\% \text{ GFP}^+ \times \text{gMFI}$) normalized by the viral genomes added to cells, as quantified by titrating sham-treated virus. Ratio of increase in TI for MMP9 unlocked virus compared to sham-treated locked virus is marked and indicated with * for $P < 0.05$ by two-way ANOVA. Normalized TI of ProvIS viruses in (b) sham-treated OFF state and (c) MMP9-treated ON state. CHO-Lec2 cells were incubated with MOI (multiplicity of infection) 5000, and transduction efficiency was quantified with flow cytometry at 48 h post-transduction. Three independent experiments were conducted, and error bars are SEM; * indicates significance compared to wt AAV9 capsid, and + indicates significance compared to D0 by two-way ANOVA, $P < 0.05$.

Figure 5.

Lectin competition transduction assay of D0 and D4 ProvIS mutants. Lectin inhibits transduction in (a) AAV9, (b) D4, and (c) D0. CHO-Lec2 cells were incubated with varying amounts of lectin to block galactose sites on the cell surfaces. Virus was incubated with sham buffer or MMP9 and then added to lectin-treated cells (MOI 5000) in a pulse transduction manner. Specifically, cells were incubated with the virus for 1 h on ice, after which cells were rinsed and then returned to 37 °C. Flow cytometry was performed at 48 h to measure transgene expression. The * indicates significant difference from the corresponding 0 μg/mL lectin condition as determined by two-way ANOVA, $P < 0.05$. Unlabeled bars are not significantly different from 0 lectin. (d) Sham- and MMP-treated viruses were subjected to pulse transduction with no lectin present. Normalized transduction index (TI) was calculated relative to sham- or MMP9-treated wild-type AAV9. Two independent experiments were conducted, and error bars are SEM.

Figure 6.

Kinetics of sham- versus MMP9-treated provector transduction. (a) Schematic of experimental timeline. CHO-Lec2 cells were seeded, and MOI 5000 of virus was added for varying amounts of time followed by PBS wash and media change. Flow cytometry was performed at 48 h post-transduction to quantify (b,c) TI and (d,e) TI at each time point normalized by TI of each virus at 24 h. Data for the D4 sham is not shown in (d) because there is no statistically significant difference in TI after 1 h versus after 24 h incubation as measured by the student *t* test. Two independent experiments were conducted. Error bars are SEM and are only shown above each symbol. The * in (d,e) indicates significant difference compared to WT9 sham at each time point as determined by two-way ANOVA, $P < 0.05$.

Table 1.

Peptide Lock Sequences of ProvIS Mutants Generated in the Study^a

name	lock sequence
wt	N/A
D0	AG – <u>VPMS↓MRGG</u> – G – G – <u>VPMS↓MRGG</u> – GA
D2	AG – <u>VPMS↓MRGG</u> – G – DD – G – <u>VPMS↓MRGG</u> – GA
D4	AG – <u>VPMS↓MRGG</u> – G – DDDD – G – <u>VPMS↓MRGG</u> – GA
D6	AG – <u>VPMS↓MRGG</u> – G – DDDDDD – G – <u>VPMS↓MRGG</u> – GA
D8	AG – <u>VPMS↓MRGG</u> – G – DDDDDDDD – G – <u>VPMS↓MRGG</u> – GA
D10	AG – <u>VPMS↓MRGG</u> – G – DDDDDDDDDD – G – <u>VPMS↓MRGG</u> – GA
S1	AG – <u>SMVGMRPG</u> – G – DDDD – G – <u>SMVGMRPG</u> – GA

^a All locks are flanked by NgoMIV/KasI restriction enzyme cut sites and are located after capsid location G453. MMP cleavage sequence is underlined, with arrow indicating cleavage location. Inactivating sequence is shown in bold. S1 contains the SMVGMRPG scrambled cleavage sequence, which is not cleavable by MMPs.