

Optimization of stealth adeno-associated virus vectors by randomization of immunogenic epitopes

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ABSTRACT

Therapeutic gene transfer by adeno-associated virus of serotype 2 (AAV-2) vectors is hampered in patients with pre-existing immunity. Molecular engineering was recently used to identify key immunogenic amino acid residues of the viral capsid and generate mutants with decreased antibody recognition. Here we explored the importance of finely tuning amino acid identity at immunogenic sites to optimize vector phenotype. A capsid library was generated by codon randomization at five positions where substitutions were shown to yield antibody evading phenotypes. Screening this library to isolate immune-escaping mutants allowed an exhaustive scan of combinations of the 20 natural amino acids at each position and yielded variants that remained infectious when incubated with serum or IVIG concentrations that completely neutralize AAV-2. Clones obtained replacing different residues at the same positions displayed strikingly different phenotypes, demonstrating that a precise choice of amino acid substitutions is fundamental to optimize immune-escaping, packaging ability, infectivity and tropism.

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Introduction

Adeno-associated viruses have become popular vectors for therapeutic gene transfer and clinical trials are being performed employing different AAV serotypes (Mueller and Flotte, 2008). Since no known pathology has been associated to AAV infection, and because the viral capsid elicits only mild immune responses, these vectors offer a robust safety profile (Buning et al., 2008). Furthermore, AAV vectors can be produced at high titers and transduce a number of different tissue types due to their wide tropism (Wu et al., 2006). However, viruses belonging to this genre are widespread in nature and the majority of the human population possess antibodies that would neutralize or reduce vector transduction rates (Chirmule et al., 1999; Fisher et al., 1997; Moskalenko et al., 2000; Xiao et al., 2000). Moreover, even naive patients will generate anti-AAV antibodies upon vector application, hindering a second successful vector application when required. Use of vectors derived from alternative serotypes that do not cross-react with neutralizing antibodies can circumvent this problem in some cases (Boissier et al., 2007; Calcedo et al., 2009; Wu et al., 2006), but differences in viral tropism between

natural capsid variants restrict the viability of this solution. Therefore, different techniques have been recently employed to generate “stealth” viral mutants that escape recognition by human antibodies. In these studies, structural modifications are genetically introduced in the capsid, resulting in disruption of immunogenic epitopes and in reduced antibody binding affinity. Mutations introduced to redirect AAV-2 tropism for example, have been shown to decrease binding and neutralization by pre-existing antibodies (Huttner et al., 2003). This principle has been eventually exploited to generate ad hoc immune-escaping viral mutants by amino acid substitutions at the viral capsid surface by rational design or by directed evolution approaches (Huttner et al., 2003; Lochrie et al., 2006; Maheshri et al., 2006; Perabo et al., 2006a). These works identified several amino acid residues whose substitution decreased binding of human or rabbit antibodies and improved transduction efficiencies after incubation with diluted sera or IVIG. However, even if combinatorial libraries allow screening larger numbers of mutations than rational design approaches, scanning of amino acid substitutions on the capsid surface is not exhaustive when randomization of large capsid protein fragments is required. In the study of Perabo and colleagues for example, a fragment of 414 amino acids was randomized resulting in a pool of clones carrying 5 mutations per clone (Perabo et al., 2006a). Since the number of possible clones carrying n amino acid substitutions over a fragment of L residues is $[L! / (L-n)!] \times 1/n! \times 20^n$, a

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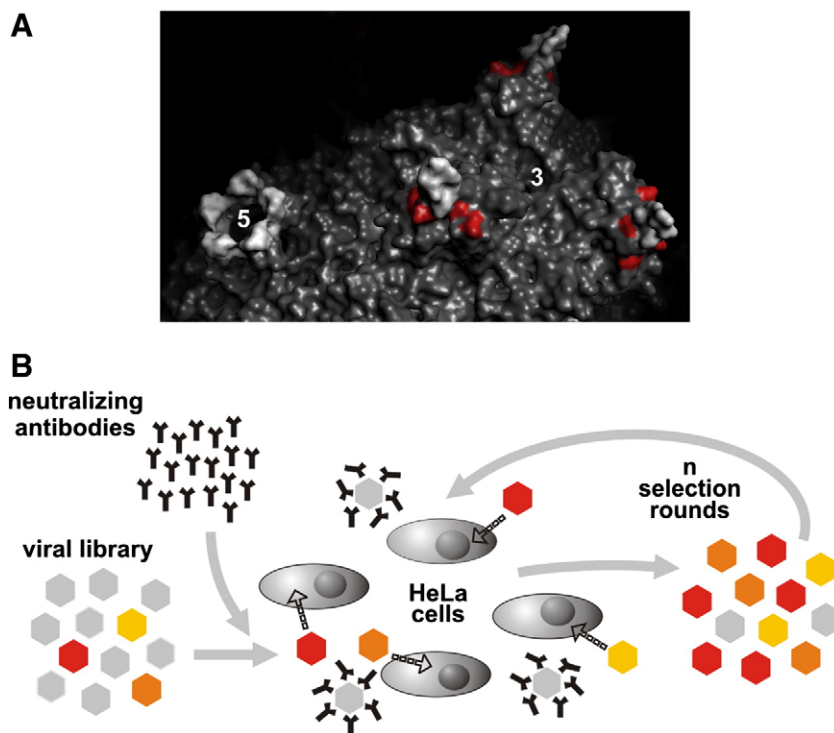


Fig. 1. (A) Location of the five randomized positions (red). Protruding residues at the three- and five-fold symmetry regions (indicated by numbers) are depicted in white to facilitate topology visualization. (B) Library panning procedure.

library containing 3.2×10^{17} different clones would have been required to represent all possible amino acid combinations, while 9.9×10^{10} clones would be required to represent one single amino acid substitution at each site. Since technical limitations currently restrict biodiversity of capsid libraries to a maximum of approximately 10^7 – 10^8 different clones, only a minimal fraction of the possible amino acid combinations at each position is represented. Therefore, despite being a powerful method to identify relevant immunogenic epitopes, error prone PCR randomization and selection protocols are likely to identify amino acid substitutions that are suboptimal in terms of stealth phenotype, transduction efficiency, stability or other important characteristics of the vector.

To prove the importance of optimizing amino acid composition of immune-escaping mutants, here we combined rational design and evolutionary approaches randomizing five previously identified immunogenic residues located at positions 449 and 458 (our own unpublished results), 459 and 551 (Perabo et al., 2006a) and 493 (Maheshri et al., 2006) of the structural proteins ORF (*cap*) of AAV-2 (Fig. 1A). We obtained a library of approximately 6×10^6 different clones which is likely to represent the majority of the possible combinations of 20 amino acids at these five positions (20^5). The library was panned to isolate mutants that efficiently infect target cells in the presence of high concentrations of neutralizing antibodies. Selected clones were characterized with respect to immune-escaping phenotype, packaging ability and tropism.

Results

Selection of immune-escaping mutants

A capsid library containing approximately 6×10^6 different clones was produced by randomization of five codons corresponding to positions 449, 458, 459, 493 and 551 (VP1 numbering) of the structural proteins of AAV-2. The pool was panned performing four selection rounds as depicted in Fig. 1B. Briefly, HEK293 cells were infected with the capsid library in the presence of an AAV-2

neutralizing human serum (S1) and viral progeny collected from cell lysates 48 h after infection. Forty-one clones of the original library, and 43 clones of the pool collected after four selection rounds were sequenced (Fig. 2).

Even if no predominant clone was observed (only a single mutant was recovered twice), compared to the initial library the diversity of different amino acids at each position decreased substantially as result of the selective pressure applied during the screening procedure (Table 1). The relative amount of small hydrophilic amino acids increased, while the fraction of large, hydrophobic amino acids decreased at all five positions. This presumably reflects the greater likelihood of small, hydrophilic amino acids to be tolerated by the capsid architecture, a tendency also suggested by previous experiments (Perabo et al., 2006b). In addition, the increase of small and hydrophilic amino acids at position 493 is in agreement with previous experiments showing that a A493G substitution halved neutralization of the viral capsid by rabbit anti-AAV-2 serum (Maheshri et al., 2006). The abundance of alkaline amino acids decreased at all positions, however was well tolerated at position 459 (~20%), in accordance to previous selection experiments that identified stealth mutants with a R459K substitution (Perabo et al., 2006a). Interestingly, while acidic amino acids completely disappeared from positions 449, 458, 459 and 493 after selection, their frequency doubled at position 551. This is in accordance with previous results showing that substitution of the original asparagine with aspartate at position 551 resulted in an immune-escaping phenotype (Perabo et al., 2006a).

Packaging ability of selected mutants

Four clones of the original library, four clones collected after two selection rounds, and eight clones collected after four rounds were chosen randomly, packaged as GFP-expressing vectors and characterized with regard to their packaging capacity (Table 2). These mutants were compared to AAV-2, AAV-1 (a natural serotype with 120 amino acid differences on the capsid protein that does not cross-react with anti-AAV-2 antibodies (Sun et al., 2003; Xiao et al., 1999)),

B
SRTNTPSGTTTQSLRQFSQAGASDIRDQSBNWLPGPCYRQQRVSKTSADNNNSEYSWTGATKYHLNGRDSDLVNPGPAMASHKODEEKFFPQSGVLIFCKQGSEKTNVHIE
T.....PQ.....S.....D.
R.....PG.....T.....G.
T.....PQ.....T.....
T.....PQ.....T.....A.
T.....PQ.....T.....K.
T.....PQ.....T.....
T.....PQ.....G.....G.
A.....TQ.....T.....T.
Q.....CQ.....T.....G.
Q.....QV.....S.....A.
Q.....VA.....
A.....QS.....
S.....QV.....T.....G.
G.....NG.....
G.....NG.....
G.....NG.....G.
G.....NG.....T.....A.
S.....GG.....S.....G.
G.....RG.....S.....Q.
G.....RG.....T.....
A.....YG.....T.....E.
S.....GS.....D.
S.....GS.....G.
S.....GV.....T.....A.
T.....MC.....G.....E.
. . . G . . T . . G .
G . . G . . G . . D .
G . . GV . . T . .
G . . L . .
. . PL . . G .
S . . CK . . S . . G .
C . . TK . .
C . . HL . .
A . . C . . M . .
. . AL . . P . .
S . . RL . . S . . G .
S G . . E .
S . . RS . . G . .
C . . A . . G . . G .
S . . GS . . F . .
S . . RS . . P . . G .
G . . NG . . T . .

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Table 1

Percentage variation of occurrence of different amino acid types between library ($n = 41$) and selected pool ($n = 43$) at each randomized position.

Amino acid position	449	458	459	493	551
wt	N	S	R	A	N
Biodiversity	−56%	−36%	−47%	−56%	−41%
Small, hydrophilic	+118%	+20%	+55%	+94%	+6%
Big, aromatic, hydrophobic	−71%	−43%	−32%	−15%	−40%
Alkaline	−100%	−58%	−20%	−100%	−71%
Acidic	−100%	−100%	−100%	−100%	+100%

and K4, the best mutant isolated from an error prone PCR library in our previously published experiments (Perabo et al., 2006a).

Capsid titers (ranging from 1.6×10^8 to 8.2×10^9), genomic titers (ranging from 2.2×10^6 to 5.2×10^8) and their ratio (between 3 and 23) were comparable to AAV-2, suggesting that the selection procedure eliminates clones with substitutions that interfere with the packaging process. Accordingly, the eight clones isolated in the fourth selection round yielded on average higher genomic titers than those selected after two rounds, which in turn had higher titers than those chosen among the unselected pool (Table 2). Since capsid titers remained similar before and after selection, the proportion of full particles increased during selections. Noteworthy, the mutant that yielded the lowest titer included two cysteines, a methionine and an aspartate among substituted residues, which being bulky (Cys and Met) or acidic (Asp), accordingly to the observations reported in the previous section are expected to interfere with the fitness of the capsid.

Transduction ability in the presence of neutralizing sera

To test their immune-escaping phenotype, clones A11, B2, B10, C6, H6 (carrying non-wt amino acids at all five positions, A11, B2 and B10 were chosen for comparison since they are similar), A2 (carrying a single wt residue at 458), AAV-1, AAV-2 and the K4 mutant were used to transduce HeLa cells. Infections were performed in the presence of increasing concentrations of the human serum used in the selection procedure (S1), a different AAV-2-neutralizing human serum (S2), or a highly concentrated intravenous immunoglobulin pool (IVIG) derived from more than thousand healthy individuals and representing an immunologic average of the human population. Infections were conducted using identical amounts of genomic particles, total capsids

and iodixanol concentrations to make sure that differences between viral preparations did not influence the outcome of the experiments. Transduction rates were quantified by flow cytometry 48 h post infection (Fig. 3). The amount of serum needed to halve transduction rates (N50) was extrapolated from this data set in order to quantify the immune-escaping ability of the mutants (Table 3). Relative N50 values (rN50) were calculated dividing the N50 value of the mutants by the N50 value of AAV-2 to quantify the beneficial effect of the mutations with regard to immune-escaping. Relative infectivity values were calculated dividing the number of cells transduced by the mutants in the absence of serum, by the number of cells transduced by AAV-2.

Depending on the type of substitutions at the same five sites, relative N50 of analyzed clones ranged from 0.5 to 5.1 while relative infectivity ranged from 0.39 to 1.14, demonstrating the importance of the exact nature of amino acid exchange. Also noteworthy in this regard are the remarkable differences in N50 and infectivity values observed between mutants A11, B2 and B10 despite their similarity. N50 values obtained for IVIG were lower than those obtained for S1 and S2. This is likely to depend on the higher concentration of immunoglobulins in IVIG (50 mg/ml) than in human serum (approximately 5–18 mg/ml). AAV-1 showed the highest relative N50 values on all sera, but when tested on the serum used in the selection procedure (S1), the values of the selected mutants were closer to those obtained for AAV-1, suggesting that the screening procedure identifies mutations that are optimized for the specific serum used for the selection. Accordingly, even if immune-escaping phenotypes were similar on S1, S2 and IVIG for B10, C6, A11, H6 (rN50 values >1) and A2 (rN50 values <1), serum-specific oscillations can be observed and remarkably, B2 yielded rN50 values >1 on S2 and <1 on IVIG (rN50 of B2 was not determined on S1 due to serum exhaustion).

The comparison of N50 and infectivity values with the neutralization profiles of each mutant underlines that the ability to escape antibody neutralization results from a combination of two factors: the ability to elude antibody binding and the efficiency of cell transduction. For example, while the N50 of B10 on S2 is 1.05-fold better than H6, due to its higher infectivity H6 infects a higher amount of cells in the presence of neutralizing antibodies. Conversely, despite being generally less infectious, AAV-1 infects more cells than H6 at high concentrations of S2 thanks to higher N50 values (Fig. 3). In all other settings, combining good N50 values and superior infectivity, H6 yielded the highest transduction rates in the absence or presence of all tested sera.

Table 2

Amino acid substitution, genomic and capsid packaging titers of the selected clones and ratio of empty to full capsids of packaged GFP-vectors.

Vector	AA at position 449, 458, 459, 493, and 551	Genomic titer (/μl)	Mean genomic titer (/μl)	Capsid titer (/μl)	Mean capsid titer (/μl)	Capsids/Genome	Mean capsids/Genome
AAV-2	NSRAN	$(1.90 \pm 0.05) \times 10^8$		1.18×10^9		6.2	
AAV-1	–	$(2.91 \pm 0.17) \times 10^7$		n.d.		n.d.	
K4	NSKAD	$(2.05 \pm 0.06) \times 10^7$		4.61×10^8		23	
B6	AGSLG	$(1.43 \pm 0.06) \times 10^7$	2.08×10^7	n.d.	1.31×10^8	n.d.	16.5
B8	TARSA	$(1.14 \pm 0.10) \times 10^7$		1.60×10^8		14	
C4	CQCGK	$(5.55 \pm 0.09) \times 10^7$		1.03×10^9		19	
C7	ICMDC	$(2.24 \pm 0.05) \times 10^6$		n.d.		n.d.	
A2^a	ASCMG	$(4.63 \pm 0.21) \times 10^7$	7.17×10^7	9.44×10^8	2.59×10^8	20	11.975
A11^a	AYGGD	$(5.10 \pm 0.40) \times 10^7$		8.18×10^9		16	
B2^a	AGGGQ	$(1.32 \pm 0.08) \times 10^8$		1.01×10^9		8	
B10^a	AGTTG	$(5.74 \pm 0.19) \times 10^7$		2.29×10^8		3.9	
C6^b	QQVSA	$(4.50 \pm 0.35) \times 10^8$	3.05×10^8	1.74×10^9	1.45×10^8	4	8.625
E3 ^b	TPQSD	$(1.15 \pm 0.12) \times 10^7$		2.38×10^8		21	
E4 ^b	AYGTE	$(5.17 \pm 0.58) \times 10^8$		2.90×10^9		6	
E10 ^b	THCGE	$(1.91 \pm 0.12) \times 10^7$		3.06×10^8		16	
F8 ^b	GNGTA	$(3.59 \pm 0.16) \times 10^8$		1.86×10^9		5	
H6^b	SGKSG	$(1.56 \pm 0.06) \times 10^8$		1.57×10^9		10	
H8 ^b	TPQTK	$(4.73 \pm 0.18) \times 10^8$		1.38×10^9		3	
H11 ^b	SASPG	$(4.54 \pm 0.19) \times 10^8$		1.59×10^9		4	

^a Recovered after 2nd selection round.

^b Recovered after 4th selection round. Bold letters indicate clones selected for detailed immune-escaping characterization.

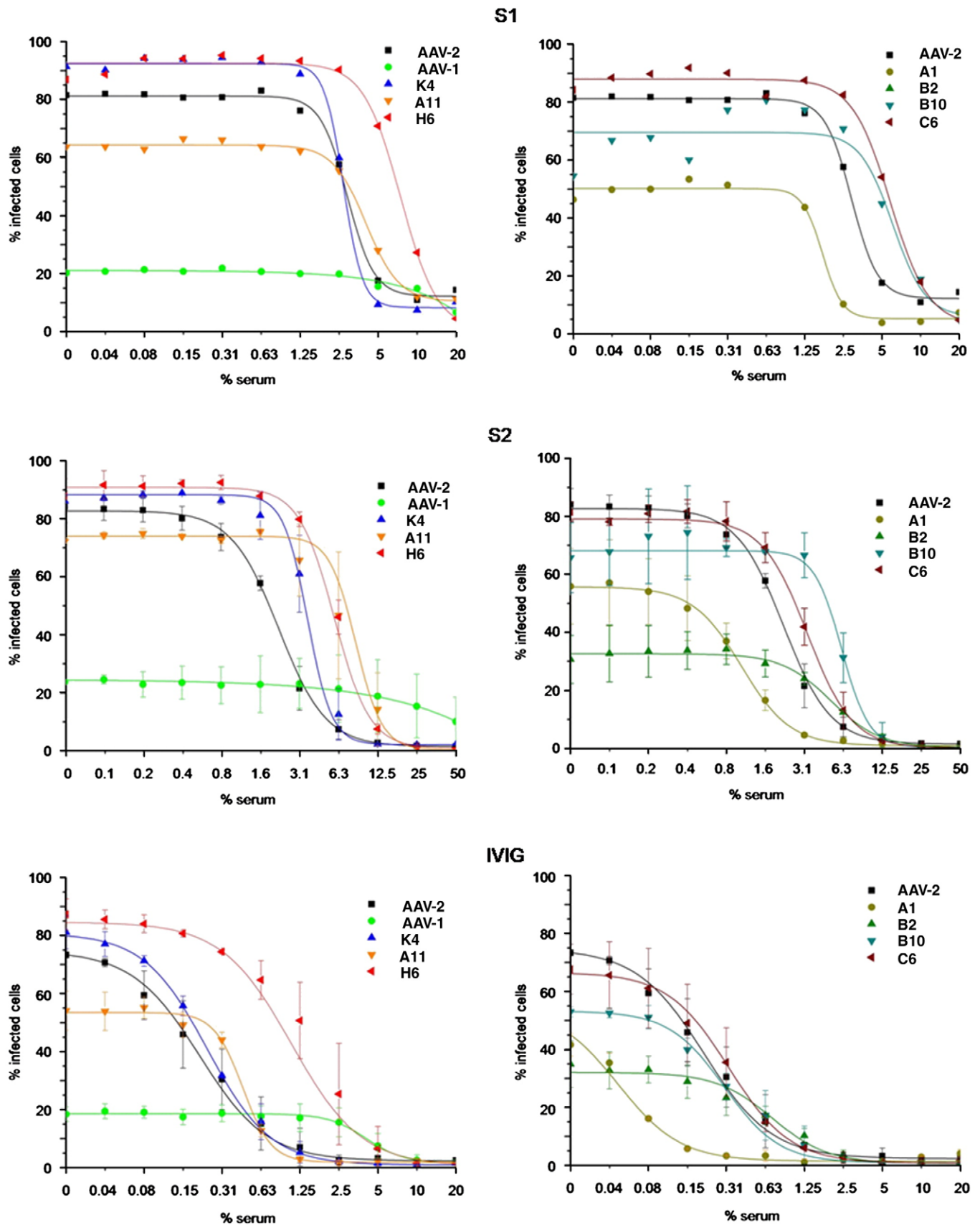


Fig. 3. Neutralization assay. Prior to infection GFP-vectors were incubated with increasing amounts of S1 (upper panels), S2 (center panels) or IVIG (lower panels). In left panels, AAV-2 (black) is shown together with AAV-1 (green), K4 mutant (blue), A11 mutant (orange) and H6 mutant (red). On the right, AAV-2 is shown with A2 (dark yellow), B2 (olive), B10 (cyan) and C6 (dark red). Measurements with S1 were performed as single experiment because the serum stock was exhausted after the selection rounds. The curve of A2 with S1 could not be calculated correctly since two data points were missing.

Table 3

N50 values, rN50 values and r infectivity (compared to AAV-2) of the tested vectors on S1, S2 and IVIG.

	AAV-2	AAV-1	K4	A2	B2	B10	A11	C6	H6
N50 (S1)	2.89	14.51	2.70	1.72	n.d.	6.08	4.02	5.81	7.45
rN50 (S1)	1	5.0	0.9	0.6	n.d.	2.1	1.4	2.0	2.6
r infectivity (S1)	1	0.26	1.14	0.62	n.d.	0.86	0.79	1.08	1.14
N50 (S2)	2.16	37.78	3.61	1.02	5.02	6.06	8.48	3.29	5.79
rN50 (S2)	1	17.5	1.7	0.5	2.3	2.8	3.9	1.5	2.7
r infectivity (S2)	1	0.30	1.07	0.67	0.39	0.82	0.89	0.96	1.10
N50 (IVIG)	0.21	4.05	0.24	0.20	0.05	0.70	0.45	0.33	1.08
rN50 (IVIG)	1	19.3	1.1	0.1	0.2	3.3	2.1	1.6	5.1
r infectivity (IVIG)	1	0.25	1.08	0.73	0.43	0.71	0.72	0.89	1.13

B2 could not be tested on S1 due to serum exhaustion.

Decoy effect of selected capsid mutants

Since they showed the best N50 values, two mutants A11 (best on S2) and H6 (best on S1 and IVIG) were further investigated for their ability to bind human antibodies independently from their infectivity, packaging efficiency and efficiency of transgene expression. To quantify their binding to anti-AAV-2 antibodies, same amounts of GFP-expressing AAV-2 vectors were mixed with different amounts of AAV-2, A11 or H6 empty particles and incubated with increasing amounts of S2. Transduction rates were quantified by flow cytometry (Fig. 4). In this setting, empty virions act as decoys for neutralizing antibodies contained in the serum and diminish the neutralizing power of the serum proportionally to their affinity for the capsids. N50 values obtained were extrapolated from this data set (Table 4). While the addition of AAV-2 empty capsids increased N50 values proportionally in a dose-dependent manner (11.51-fold after addition of the highest empty capsids dose), the increase obtained by addition of empty H6 capsids was weaker (8.53-fold) and the addition of A11 capsids resulted in no significant increase (1.63-fold). These data demonstrate that while wt efficiently binds anti-AAV-2 antibodies, H6 capsids are bound at reduced efficiency and A11 capsids escape recognition and are very inefficient decoys.

Consequences of amino acid substitutions on vector tropism

The transduction ability of mutants A11, H6, AAV-1 and AAV-2 was assessed on a panel of cell lines: human melanoma cells (BLM and MV3), human breast cancer cells (MDA), human embryonic kidney cells (293), human keratinocytes (HaCat), and murine embryonic fibroblasts (NIH3T3) (Fig. 5A). These results were used to calculate the relative infectivity of the mutants, dividing for each cell line the transduction rates of a vector by the transduction rate of AAV-2 (Fig. 5B). Compared to AAV-2, H6 yielded higher transduction rates in all tested cell types (from 1.15- to 1.71-fold higher). Conversely, A11 yielded lower relative transduction rates than AAV-2 (0.09- to 0.23-fold lower). AAV-1 yielded lower transduction rates on most cell lines (between 0.1- and 0.27-fold), but was comparable to AAV-2 on HEK293 cells (0.89-fold). Therefore, while large structural differences distinguishing different natural AAV serotypes result in a radically different tropism, immune-escaping mutants carrying a limited number of appropriate mutations, depending on the identity of the introduced amino acids can retain a tropism that resembles (or even outperforms) the original. This hypothesis was corroborated infecting HeLa cells in the presence or absence of soluble heparin, an analog of HSPG, the primary receptor of AAV-2 and measuring transduction rates 48 h later (Fig. 6). While the five amino acid substitutions did not disrupt the ability of the capsid to interact with the primary receptor of AAV-2, the over 120 amino acid differences of AAV-1 abolished the ability of AAV-1 to recognize and interact with HSPG.

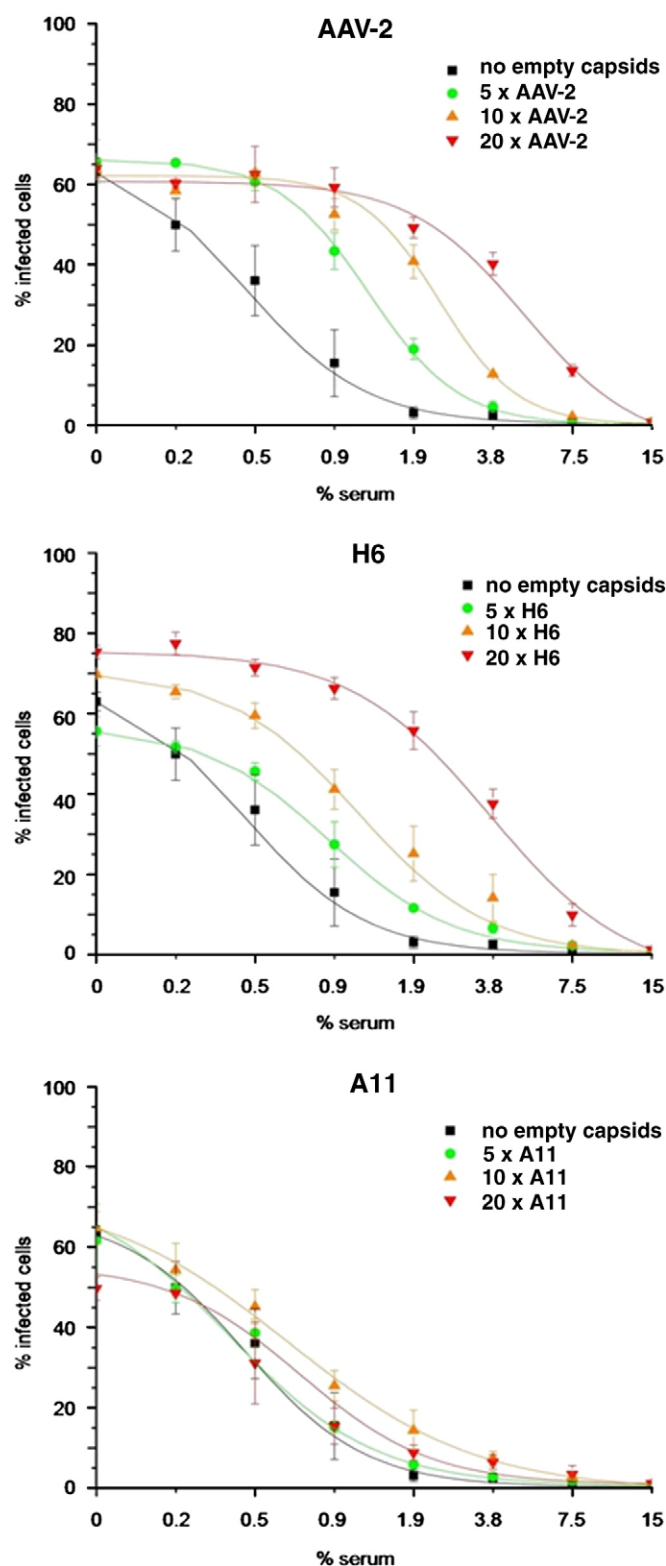


Fig. 4. Decoy assay for AAV-2, A11 and H6 mutant. Same amounts of AAV-2 vectors were mixed with 5-, 10- and 20-fold empty particles of AAV-2 (top), H6 (middle) or A11 (bottom). After incubation with serial dilutions of S2, HeLa cells were infected and transduction rates measured 48 h later.

Discussion

The possibility to modify by genetic engineering the molecular topology of vector particles and disrupt immunogenic domains has opened the possibility to tailor a new generation of virions that escape

Table 4
Analysis of decoy efficiencies of AAV-2, H6 and A11 empty particles.

Type of empty capsids	N50 increase (x-fold)		
	5×	10×	20×
AAV-2	2.95	5.48	11.51
H6	2.08	2.70	8.53
A11	0.88	1.46	1.63

N50 values after addition of 5×, 10× or 20× empty capsids were extrapolated from Fig. 4 and the x-fold increase in comparison to the N50 obtained without addition of empty particles was calculated.

neutralization in seropositive patients. Proof of this principle has been provided by pioneering studies that identified several immunogenic residues on the viral surface and described capsid mutants with diminished recognition by antibodies (Lochrie et al., 2006; Maheshri et al., 2006; Moskalenko et al., 2000; Perabo et al., 2006a; Wobus et al., 2000; Wu et al., 2000). These results suggest the presence of a main immunogenic region located around the characteristic peaks at the 3-fold symmetry region of the AAV-2 capsid and have shown that combination of mutations of several residues in the same clone increases the immune-escaping phenotype. Directed evolution high throughput protocols have been recently introduced to take advantage of the possibility to generate by error prone PCR millions of different clones, and to screen them by relatively simple procedures. These studies contributed to the identification of important immunogenic epitopes that can be mutated to decrease antibody recognition, but the biodiversities of the used libraries allowed representations of a minimal fraction of possible combinations of different mutations at each site. Since different amino acids can confer very diverse biochemical properties to the capsid, we speculated that phenotype of viral particles could be considerably improved by optimizing the specific amino acid

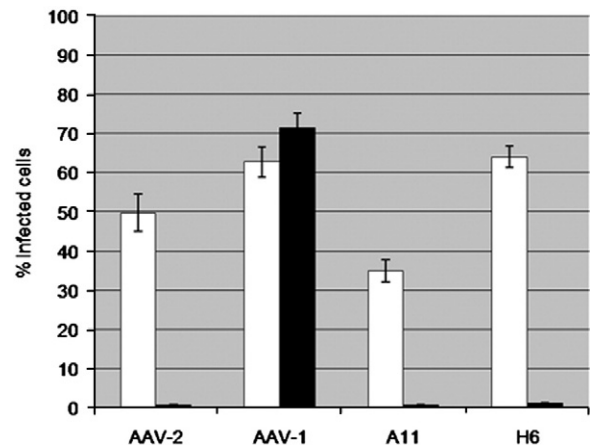


Fig. 6. Transduction efficiencies of AAV-2, A11, AAV-1 and H6 on HeLa cells in the presence (black bars) or absence (white bars) of soluble heparin.

substituted at each site in order to isolate the best combinations. Restricting randomization to five amino acid positions, whose mutation was previously shown to influence binding of neutralizing antibodies, allowed the generation of a library that represents most possible combinations of natural amino acids at these positions.

The first demonstration of the importance of this approach is the statistical predominance of particular amino acids at the randomized positions after selection. Besides providing valuable information of structural requirements of the virus surface for future capsid engineering attempts, the preference for amino acid with particular biochemical characteristics (e.g. acidic residues at position 551, small and hydrophilic residues at all other positions) demonstrates that a precise choice of amino acid substitutions is pivotal for the optimization of engineered capsids. Accordingly, detailed characterization and comparison of several mutants isolated after the selection procedure carrying different substitutions at the same five positions highlighted dramatic differences in immune-escaping ability, infectivity and tropism. Although the packaging ability of the viral mutants is expected to be strongly influenced by different amino acid substitutions, all tested clones packaged at high titers, reflecting the fact that inefficiently assembling clones are rapidly eliminated from the viral pool independently of the selection conditions. Conversely, with regard to infectivity and immune-escaping ability, a wide spectrum of phenotypes was recovered after selection because during the selection process, mutants with low infectivity could be rescued by a strong immune-evading phenotype and vice versa. In addition, screening procedures are “leaky” and occasional surviving of inefficient clones occurs. This could explain the isolation after two selection rounds of mutant A2, which showed poor infectivity and was neutralized more efficiently than AAV-2 by all tested antibodies. Noteworthy, mutants B10 and H6, showing the best N50 values on S1 and IVIG host at positions 459, 493 and 551 different substitutions than those most strongly selected in previous experiments (Maheshri et al., 2006; Perabo et al., 2006a), suggesting the importance of fine tuning amino acid identity.

AAV natural serotypes can be used as alternatives to circumvent pre-existing immunity (Boissier et al., 2007; Calcedo et al., 2009; Wu et al., 2006) because major sequence and structural differences confer to these variants the ability to escape neutralization by antibodies directed against another serotype. In this study for example, AAV-1, a natural occurring variant with different amino acid composition at 120 sites, clearly outperformed all mutants selected in these experiments in terms of antibody elusion. However, major capsid modifications result in dramatic tropism alterations as demonstrated in experiments depicted in Fig. 5. Conversely, modifying a restricted number of residues is less likely to affect tropism of the resulting particle. Infection tests conducted on a panel of cell lines

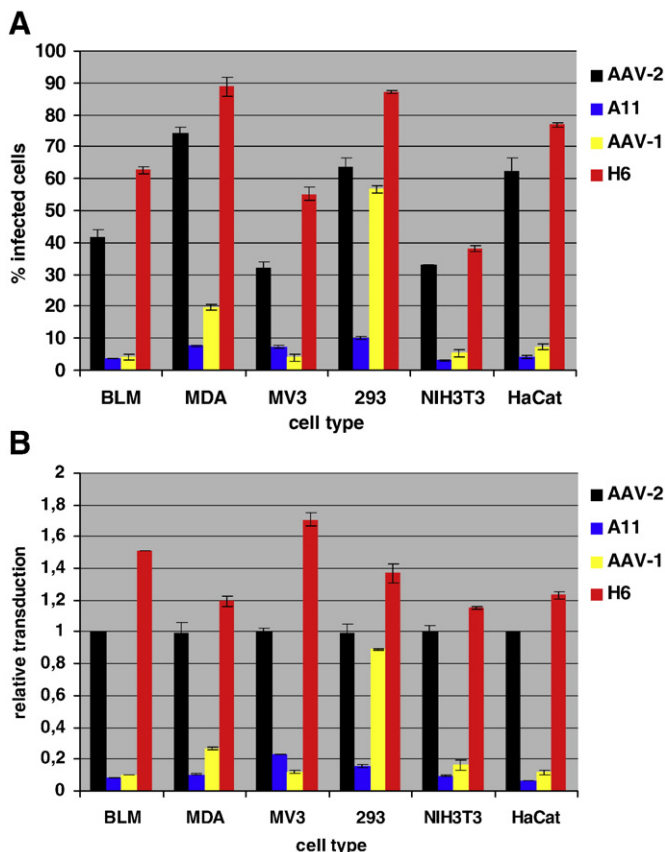


Fig. 5. Absolute (A) and relative (B) infectivities of AAV-2, A11, AAV-1 and H6 on various cell lines.

demonstrated that H6 conserved a tropism that is very similar to AAV-2. Interestingly, even if different mutations of the same five positions carried by mutant A11 and H6 did not affect the ability to bind HSPG, they influenced infectious rates on HeLa cells (Fig. 5) and yielded strongly diminished infectivity for mutant A11 (Fig. 6). The 3-fold symmetry region of AAV-2 capsid has been shown to play an important role in capsid/receptor interactions. For example, two arginines located at positions 585 and 588 are responsible for binding HSPG (Kern et al., 2003; Opie et al., 2003; Xie et al., 2002) and the virus primary receptor (Summerford and Samulski, 1998). Another domain located at position 511–513 has been shown to be responsible for the binding of the capsid to $\alpha 5 \beta 1$ integrins (Asokan et al., 2006). Depending on the nature of the introduced amino acid, substitutions at the five positions could influence these or other still unknown receptor binding domains and dramatically alter viral tropism. Although the exact mechanism responsible for tropism alterations requires further investigation, tropism differences between AAV-2, H6 and A11 demonstrate once more the importance of the specific amino acid substitution.

Despite enhancing antibody evasion, all mutants identified in this study were fully neutralized by serum concentrations above 20% (Fig. 3). The generation of AAV particles with desired tropism that escape neutralization in vivo remains an ambitious goal that will be eventually achieved by combining a higher number of different mutations, resulting in a cumulative effect. Combining separate substitutions is a complex task because they are usually not compatible with one another when merged on the same clone. However, advances in understanding capsid biology and the development of powerful mutagenesis and screening procedures are bringing us closer to this goal. The diversity of phenotypes obtained by randomizing exhaustively only five sites strongly suggests the potential of fine tuning amino acid identity of substituted residues for optimizing vector characteristics for therapeutic application. Recently, another powerful solution has been described to obtain stealth phenotype and tropism modulation by swapping entire antigenic domains between viral serotypes (Grimm et al., 2008; Koerber et al., 2008; Li et al., 2008). It can be anticipated that combining rational design, error prone PCR, domain swapping between serotypes and codon randomization will yield soon a new generation of modified gene therapy vectors that circumvent the current limitations.

Materials and methods

Cell culture

The human epitheloid cervix carcinoma cell line HeLa, the human embryonic kidney cell line 293, the human keratinocyte cell line HaCat, the human breast cancer cell line MDA and the murine embryonic fibroblast cell line NIH3T3 were maintained as monolayer cultures at 37 °C and 5% CO₂ in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. BLM and MV3 melanoma cell lines were maintained in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 1% MEM non-essential amino acid solution.

Plasmids and viruses

The complete randomization library plasmid pdegen5Lib was generated by consecutive PCR steps using following primers to randomize positions 449, 458, 459, 493 and 551 respectively: GCAGAACANNSACTCAAGTGAACACCACGACGAGNNSNNSCTTCAGTTT, CGCCATGCTACTTATCTACG, AAAGTGAAGSNNSNCTGCGTGGTGGTTC-CACITGGAGTSNNTGTTCTGC, GACATCTNNSGATAACAACAACAGTGAA-TACTCG, CGAGTATTCACTGTTGTTGTTATCSNAGATGTC, GGCTCAGAGAAACANNSGTGGACAATG and TCCACSNNTGTTTTCTCT-GAGCCTTGC. The procedure for the amplification of the plasmid library was described previously (Perabo et al., 2003, 2006a). The biodiversity

of the library was estimated by counting bacterial colonies after plasmid ligation and transformation. pXX6, pRC, pscAAV/GFP and the protocol for the production of AAV were described previously (Perabo et al., 2003; Xiao et al., 1998). Briefly, HEK293 cells were seeded at 80% confluence and co-transfected by calcium phosphate (2:3 ratio) of pdegen5Lib and pXX6 for the production of the AAV-2 library. For the production of scGFP containing vectors, cells were transfected with a 1:1:3 ratio of pscAAV/GFP, AAV2pRC and pXX6. At 48 h post-transfection, cells were harvested and resuspended in 150 mM NaCl–50 mM Tris–HCl (pH 8.5), freeze–thawed three times, and treated with Benzonase for 30 min at 37 °C. Cell debris was spun down at 3700 × g for 20 min at 4 °C, and the supernatant loaded onto a discontinuous iodixanol gradient and centrifuged at 60,000 rpm using a Sorvall T-865 rotor. Genomic titers of vector stocks were determined by quantitative PCR (Roche, Germany) after purifying viral DNA with the DNeasy kit (Qiagen, Germany). Selected clones were sequenced (Qiagen, Germany) after purification with DNeasy Kit, amplification by PCR with Primers cgcatgtactatctacg and taccagctccgtagctctcgg and cloning as previously described (Perabo et al., 2006a). The capsid titers were estimated by A20 ELISA assay (Grimm et al., 1999). To produce GFP-expressing vectors of selected mutants, the modified region of each mutant was cloned into the pRC plasmid by BsiWI/SnaBI digestion and ligation.

Selection protocol

For selection, the AAV library was incubated for 2h at 37° with different amounts of human neutralizing serum (33% in the first and second selection round, 50% in the third and fourth). Sera were obtained with informed consent from healthy individuals and tested to assess their ability to neutralize AAV-2 infection (Huttner et al., 2003). 5×10^6 HeLa cells were infected with a concentration of 100 viral genomic particles/cell. At the same time, cells were super-infected with adenovirus type 5 at a concentration of 700 pfu/cell. Cells were harvested 48 h post infection by scraping and lysed by three cycles of freeze–thaw. Adenovirus was heat inactivated at 60 °C for 30 min. The genomic titer of these preparations was measured by qPCR as described above and eventually used for further selection rounds.

Transduction assays

In 48-well plates, 2×10^4 HeLa cells were seeded and infected 24 h later with same genomic particles, total capsids and iodixanol amounts of GFP-expressing AAV vectors after incubation with serial dilutions of human neutralizing sera or IVIG (Intratect®, Biotest) for 2 h at RT. After 48 h, cells were harvested and GFP expression measured by flow cytometry (FACSCalibur, BD Biosciences, USA) analyzing at least 5000 cells. For determination of the decoy activity of AAV-2 and mutant capsids, 2×10^4 HeLa cells were seeded in 48 well plates 24 h before infection. 10^5 , 2×10^5 or 4×10^5 of either AAV-2 or mutant empty particles (A11 and H6) were added to 10^4 AAV-2 genomic particles per cell. Transduction rates were measured 48 h later by flow cytometry were performed as described above. N50 values were calculated by Origin 8 software using the Hill sigmoidal curve fit: $y = \text{START} + (\text{END} - \text{START}) * x^n / (k^n + x^n)$ (START: max. y-data, END: min. y-data, $n = 2$, $k = x$ at $y50$).

Heparin competition assay

Twenty-four hour prior to infection 2×10^4 HeLa cells per well were seeded in 48 well plates. 6×10^3 genomic particles of GFP-expressing viral vectors per cell (6×10^4 genomic particles per cell for AAV-1) were incubated in a total volume of 500 µl DMEM/10% FCS in the presence or absence of 1.7% (85 I.E./ml) soluble heparin (B. Braun Melsungen AG, Germany) at 37 °C for 30 min. This solution was then used to infect cells. Forty-eight hours post infection, the percentage of transduced cells was determined by flow cytometry.

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