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REVIEW

Receptor targeting of adeno-associated virus vectors

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Adeno-associated virus (AAV) is a promising vector for human somatic gene therapy. However, its broad host range is a disadvantage for in vivo gene therapy, because it does not allow the selective tissue- or organ-restricted transduction required to enhance the safety and efficiency of the gene transfer. Therefore, increasing efforts are being made to target AAV-2-based vectors to specific receptors. The studies summarized in this review show that it is possible to target AAV-2 to a specific cell. So far, the most promising approach is the genetic modification of the viral capsid. However, the currently available AAV-2 targeting vectors need to be improved with regard to the elimination of the wild-type AAV-2 tropism and the improvement of infectious titers. The creation of highly efficient AAV-2 targeting vectors will also require a better understanding of the transmembrane and intracellular processing of this virus.

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Introduction

The development of safe and efficient gene transfer vehicles is critical for the success of gene therapy. One of the most promising viral vectors is based on adeno-associated virus type 2 (AAV-2), a member of the parvovirus family. AAV-2 was discovered as a coinfecting agent during an adenovirus outbreak, without any apparent pathogenicity contributed by AAV-2.[1–3] Until now, no human disease caused by AAV-2 has been detected. Moreover, AAV-2 seems to be protective against bovine papillomavirus and adenovirus-mediated cellular transformation.[4–6] AAV-2 does not induce cytotoxic effects and does not elicit a cellular immune response as commonly seen with other viral vectors.[7] Finally, AAV-2 has the unique potential to integrate site specifically into the q-arm of human chromosome 19.[8,9]

AAV-2 has a broad tissue tropism infecting diverse organs such as brain, liver, muscle, lung, retina and heart muscle. This makes AAV-2 attractive for *in vitro* gene transfer into various tissues.[7] AAV-2 vectors are now successfully used for *in vivo* gene transfer.[10,11] However, the studies reported so far clearly demonstrate that clinically relevant gene expression can be reached only in the liver, unless vectors are administered directly into the target tissue or organ. These results emphasize the need for a targeting of AAV vectors in order to overcome the apparent limitations of a broad tissue tropism. In addition, the targeting of AAV vectors would also enhance the safety and efficiency of AAV-mediated gene transfer *in vivo*. Therefore, increasing efforts are being made to retarget AAV-2-based vectors to specific receptors and to generate selective, tissue- or organ-restricted vectors. The studies summarized in this review show that it might become possible to target AAV-2 to a specific cell type or organ. However, the targeting vectors still need to be optimized by a further reduction of the wild-type AAV-2 tropism, or with an increase in infectious titer. With the rapidly increasing knowledge about the functional domains on the AAV-2 capsid involved in receptor binding and subsequent steps of transmembrane and intracellular processing of the virion, we feel justified to predict that the creation of highly efficient AAV-2 targeting vectors will become possible in the near future.

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Organization of the AAV-2 genome

AAV-2 is a single-stranded, replication deficient non-enveloped DNA virus[12] composed of an icosahedral protein capsid and a viral genome of 4680 nucleotides. The AAV-2 genome encodes the two large open reading frames (ORF) *rep* and *cap*. It is flanked at both ends by the 145 bp inverted terminal repeat sequences (ITR). The ITRs are required for encapsidation of the viral genome and seem to have enhancer and/or weak promoter activity. They are, besides the viral Rep proteins, necessary for the site-specific integration of wild-type AAV-2 and for the rescue of proviruses. The 5′ open reading frame *rep* encodes four overlapping, multifunctional proteins (Rep78, Rep68, Rep52 and Rep40) controlled by two different promoters.[13] The large Rep proteins (Rep78 and its splice variant Rep68) are controlled by the p5 promoter and are necessary for viral DNA replication, transcriptional control and site-specific integration. Rep52 and its splice variant Rep40 are known as small Rep proteins. They are transcribed

from the p19 promoter and play an essential role in the accumulation of single-stranded progeny genomes used for packaging. The 3′ ORF *cap* accommodates the three capsid proteins VP1 (90 kDa), VP2 (72 kDa) and VP3 (60 kDa), which form the 60 subunits of the AAV-2 viral capsid at a 1:1:20 ratio.[14] They are controlled by the p40 promoter, share the same stop codon, but differ because of alternative splicing and different initiation codons resulting in progressively shorter proteins from VP1 to VP3. All three capsid proteins are necessary for the generation of infectious particles, although capsids are formed in the absence of VP1.[15–17] The capsid assembly itself occurs in the nucleus.[18,19] The N-terminus of VP2 contains a nuclear localization sequence by which it transports VP3 into the nucleus.[20,21] The encapsidation of the AAV-2 genome probably takes place in the nucleoplasm and Rep-tagged DNA seems to initiate packaging by interaction with capsid proteins.[22]

If *rep* and *cap* are provided *in trans* on a helper plasmid, 96% of the wild-type AAV genome can be removed and replaced by a transgene, because the ITRs are the only *cis* elements necessary for the generation of recombinant AAV (rAAV).[7] The protocols to generate high titer and highly purified viral preparations have undergone continuous improvements.[23,24] Until now, rAAV is commonly produced by transfection of a vector plasmid (containing the ITR flanked transgene) and a helper plasmid (encoding *rep* and *cap*) into HeLa or 293 cells, followed by superinfection with adenovirus type 5. Alternatively, a triple transfection of vector-, helper- and an adenovirus helper plasmid can be used.[25–28] After harvesting, AAV is purified using iodixanol or CsCl gradient ultracentrifugation and/or chromatography.[24,29–31] After purification, *infectious* particle titers of AAV-2 of $>10^9$/ml are easily reached, which is sufficient for most *in vitro* and *in vivo* experiments, at least in smaller rodents. However, when it comes to larger animals or human beings in clinical applications, it is strongly desirable to enhance the target specificity of AAV vectors by receptor retargeting in order to reduce the amount of vector particles to be administered.

Three-dimensional structure of AAV-2

Recently, Xie *et al*[32] were able to determine the atomic structure of AAV-2 to a 3-Å resolution by X-ray crystallography. Like Kronenberg *et al*,[33] who investigated empty capsids by electron cryo-microscopy and icosahedral image reconstruction, Xie *et al*[32] observed substantial differences in the surface topology between AAV-2 and other parvoviruses. The inner surface of the AAV-2 capsid is composed of a jelly-roll β-barrel motif, comprising two antiparallel β-sheets. This motif is common in virus capsids and has also been described for other parvoviruses like canine parvovirus (CPV),[34] feline panleukopenia virus,[35] minute virus of mice[36] or the human parvovirus B19.[37] However, the interstrand loops located between the strands of the core β-barrel have quite different structures in the different parvoviruses and are the regions responsible for interactions with antibodies and cellular receptors. The most prominent features of the AAV-2 surface topology are the 'three-fold proximal' peaks. These peaks cluster around each icosahedral three-fold rotation axis. Unique for AAV-2 is that neighboring subunits interact intimately at this three-fold axis. Additional, but more modest interactions, were also observed for residues in the HI, BC and EF loop of neighboring subunits. The three-fold proximal peaks are mainly formed by a so-called GH loop. This loop is missing in densoviruses (insect parvoviruses) and is structurally different in CPV. This loop is, as expected, mainly involved in binding to the primary receptor of AAV-2 (see below) and contains the epitope recognized by the neutralizing antibody C37-B. Moreover, the most promising position for the insertion of receptor-specific peptides (amino acid position 587) is also located in this loop.

Before the characterization of the three-dimensional structure of the AAV-2, capsid potential insertion sites as well as the determination of antibody and receptor binding regions were identified by epitope mapping, mutagenesis studies of capsid proteins, as well as sequence alignments of AAV-2 and related parvovirus including other serotypes. Although it was possible by these approaches to determine functionally relevant regions of the AAV-2 capsid, the now solved three-dimensional structure will accelerate this process by using a more rational, structure-based approach.

Infectious pathway of wild-type AAV-2

A successful viral infection is a multistep process starting with the attachment of the virus to the cell surface, followed by viral uptake, intracellular trafficking and, in most of the cases, nuclear transport and deposition or replication of the viral genome in the cell nucleus. In the current model of infection of permissive cells by AAV-2 (Figure 1a), AAV-2 first binds to heparan sulfate proteoglycans (HSPG), which act as primary or attachment receptors.[38] Since all adherent cells express glycosaminoglycans on their surface, this offers a simple explanation for the broad tropism of AAV-2. No distinct heparin binding motif was identified so far in the AAV-2 capsid. However, Wu *et al*[39] mapped two regions involved in HSPG binding by alanine substitution and insertion of the haemagglutinin (HA) epitope YPVDVPDYA. These regions encompass amino acids 509–522 and 561–591 and cluster around the three-fold proximal peaks.[32] The alanine substitution mutant 585-RGNR-588 and the HA insertion mutant 591, for example, are located on the side of the three-fold proximal peak facing the valley that separates this peak from its neighbor, whereas the alanine insertion mutant 509 is on the floor of the valley. The other two mutants generated by Wu *et al*[39] were mapped at the base of (alanine substitution at 561–565) and underneath (HA insertion at 522) the peak facing the two-fold axis. Xie *et al*[32] assume that mutations in the regions underneath the peak (insertion at 522[39] and 519[40]) are not directly affecting the HSPG binding of AAV-2.

Two types of AAV-2 coreceptors have been identified, $\alpha_v\beta_5$ integrin and fibroblast growth factor receptor 1 (FGFR1).[23,41,42] It is postulated that FGFR1 enhances the attachment process.[41,42] Antibodies against $\alpha_v\beta_5$ integrin do not interfere with cell binding but inhibit endocytosis. Therefore, $\alpha_v\beta_5$ integrins seem to be required for endocytosis of AAV-2,[43] which is mediated mainly by clathrin-coated pits.[41,44] This endocytotic process and the

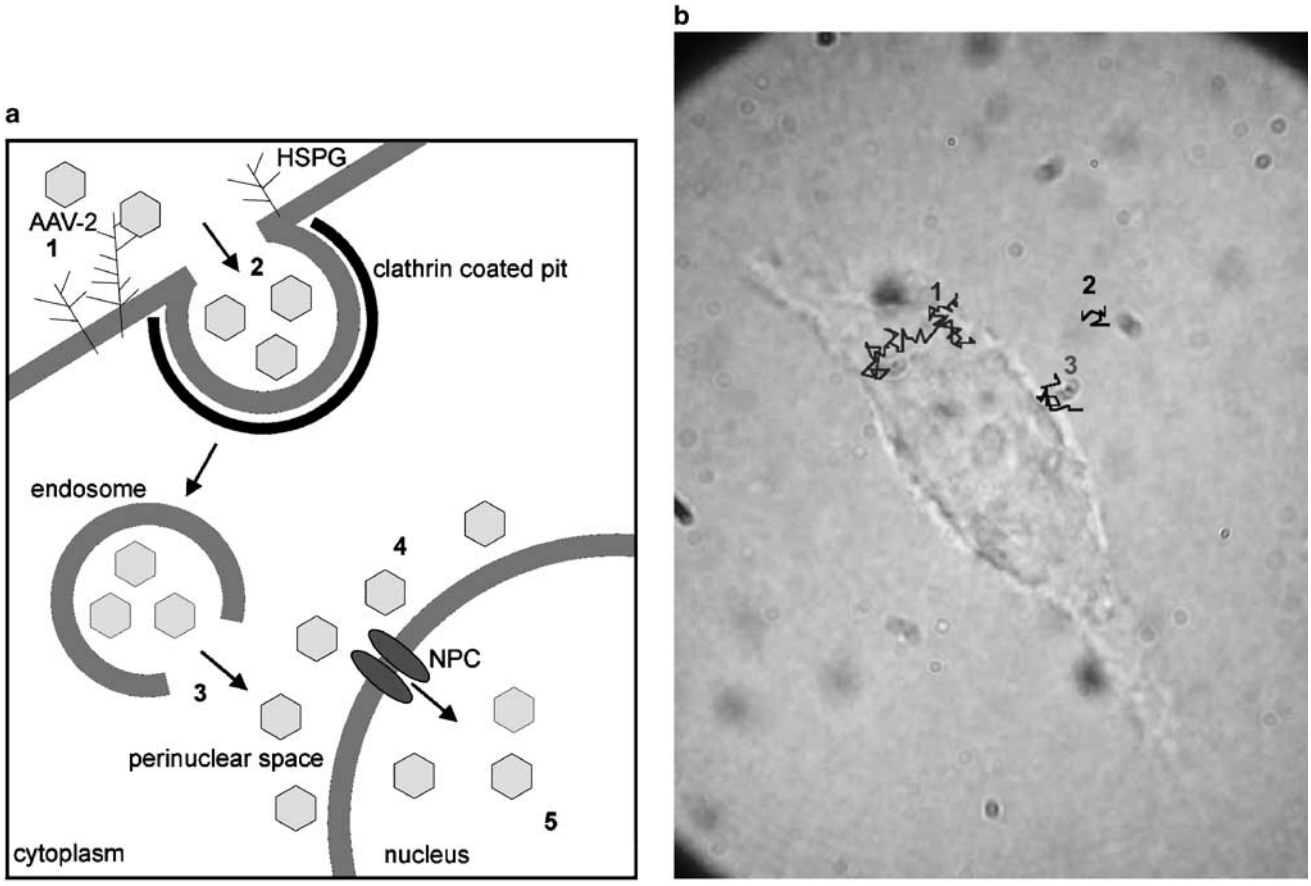

Figure 1 *Infectious pathway of wild-type AAV-2. (a) Schematic representation of AAV entry and endocytic trafficking in HeLa cells as seen by fluorescent and confocal laser microscopy.[41] (Following binding to heparan sulfate proteoglycans (HSPG) on the cell surface (1), AAV is rapidly internalized via clathrin-coated pits (2) through a process involving $\alpha_v\beta_5$ integrin, dynamin and Rac1.[23, 41, 44] Following endocytosis, the activation of phosphatidylinositol-3 kinase seems to support the initiation of the intracellular movement.[43] After acidification, AAV is released from the endosome into the cytoplasm (3), where it is found in a perinuclear localization (4). Then AAV slowly enters the nucleus (5) probably via nuclear pore complexes (NPC). (b) Trajectories of single AAV-Cy5 particles analyzed by single virus tracing.[52] The traces show single diffusion of different virus particles at different times. In this figure, three examples of the various stages of the AAV-2 infection are visible (diffusion in the cytoplasm after cell entry (1), diffusion in solution (2) and touching at the cell membrane (3)). Normal diffusion could be measured for AAV-2 outside the cell (2). A deceleration of AAV-2 near the cell could be observed. When approaching the cell, a repetitive touching of the cell membrane by AAV-2 occurred, which was interrupted by a short diffusion path in the vicinity of the cell surface (3). Inside the cell AAV movements using direct motion, anomalous and normal diffusion were observed (1). This method also allowed to distinguish between free viruses and AAV inside endosomes by calculating the diffusion constants of the particles traced by SVT.*

subsequent steps are still poorly understood. However, it is possible to assume that as for adenovirus α_v integrin clustering facilitates the localization of virus particles to coated pits.[45] This could then activate the endocytosis in which dynamin, a 100 kDa cytosolic GTPase, is involved. Although the precise function of dynamin in vesicle formation remains controversial,[46,47] it is known that it is essential for scission of newly formed vesicles from the plasma membrane.[46] For AAV-2 it was shown that the introduction of a dynamin mutant results in the decrease of AAV-mediated transduction,[1,44] although it was not possible to abolish the AAV-2 infection. However, Sanlioglu *et al*[43] assumed that the binding to the integrin could have an additional effect, which is the activation of Rac1. They propose that Rac1 activation results in the stimulation of phosphoinositol-3 kinase (PI3K), which facilitates the rearrangements of microfilaments and microtubuli. These rearrangements are necessary to support the initiation of the intracellular movements of AAV-2 to the nucleus after endocytosis. The release of AAV-2 from the endosome at the early-to-late endosomal transition requires a low endosomal pH.[41,48] As with other viruses,[49] the low pH is likely to induce conformational changes of key viral proteins necessary for a successful endosomal release or nuclear entry. Interestingly, the unique region of VP1 contains a potential phospholipase A2 (PLA2) domain,[50] which might be involved in this process. PLA2 inactivating point mutations do not influence the capsid assembly, packaging, cell binding or entry of AAV-2, but delay the onset and reduce the amount of early gene expression. Thus, the PLA2 activity in the N-terminus of VP1 may be required for the exit of AAV-2 from the endosome and the transfer of the viral genome to the nucleus. A PLA2 domain with similar function has been found in porcine parvovirus.[51] The destiny of AAV-2 after this endosomal release is mostly unclear. Some studies have observed a perinuclear accumulation of AAV-2 particles,[41] before the virus slowly enters the nucleus, probably via nuclear pore complexes. However, the perinuclear accumulation of AAV-2 observed by conventional fluorescent and confocal laser microscopy may suffer from some

methodological shortcomings such as interference effects and the cellular virus overload required for these conventional imaging studies (see the next paragraph). These results need to be confirmed by independent methods.

The development of a novel technique, called *single virus tracing* (SVT), which allows the visualization of an individual virus in a living cell with high spatial and temporal resolution, may permit a more detailed analysis of specific steps of the cellular infection[52] (Figure 1b). Using this technique, Seisenberger *et al*[52] observed AAV-2 movements towards the cell surface, which were followed by repetitive touching and short diffusion paths in the vicinity of the cell surface. The touching events were clearly visible as short periods of immobility at the cell surface, with a mean touching time of t_t=62 ms. Inside the cell, three different kinds of AAV-2 movements were observed in the cytoplasm and the nuclear area, namely directed motion, anomalous diffusion and normal diffusion. In agreement with the current model of AAV-2 infection, most virions followed a normal diffusion in endo- or lysosomal particles. However, in marked contrast to the above findings with conventional microscopic techniques, neither a nuclear accumulation nor a slow penetration of the nuclear membrane was observed by SVT. Interestingly, the total time measured for membrane penetration, trafficking through the cytoplasm and entry into the nuclear area was much shorter than determined by other methods.[52] By this new method it was, for example, possible to detect at least one Cy5-labelled AAV-2 in the nucleus of 50% of the cells 15 min after adding virus to the cells.

AAV serotypes other than AAV-2

Most AAV vectors are based on the AAV-2 serotype, as it was the first serotype from which an infectious clone was available.[53] A total of 50–96% of the population is seropositive for AAV-2. Five additional primate AAV serotypes (AAV-1, AAV-3, AAV-4, AAV-5 and AAV-6) have been characterized at the nucleotide level.[54–57] With the exception of AAV-6, which has a >99% amino acid homology with AAV-1, all serotypes show a significantly different amino-acid sequence of the capsid proteins, which is most prominent in VP3[14] and most obvious for AAV-4 and AAV-5. It remains to be determined how these differences influence the binding of neutralizing antibodies, the viral tropism and the intracellular processing.

An investigation of the humoral immunity against AAV performed with a cohort of 85 human volunteers revealed that none of the sera contained neutralizing antibodies against AAV-5, although neutralizing antibodies against AAV-1 and AAV-2 were detected in 19 and 25% of the sera, respectively.[58] Furthermore, neutralizing antibodies against AAV-4 or AAV-5 do not crossreact.[14] Serum from mice immunized with AAV-2 vectors did not neutralize AAV-6 infection in tissue culture, neutralized AAV-3 only partially, but inhibited AAV-2 almost completely.[59] Similar results were obtained with AAV-3 used for immunization. Serum from AAV-6-immunized animals did not crossreact with AAV-2 or AAV-3 and neutralized the infection by AAV-6 only weakly.

In AAV-2, VP3 is responsible for receptor binding and therefore mainly determines the viral tropism. Differences in this region should result in a different receptor usage and viral tropism. Therefore, it was not unexpected that AAV-4 and AAV-5, whose VP3 shows the lowest similarity to AAV-2, use α2-3 *O*-linked (AAV-4) and *N*-linked (AAV-5) sialic acid for cell binding[60] instead of HSPG. AAV-6, whose VP3 has a homology of about 60% to AAV-4 and AAV-5,[61] also binds to sialic acid,[62] whereas AAV-3 binds to HSPG.

The various AAV serotypes show a different tissue or cell tropism.[58,59,63] AAV-1 is more efficient than AAV-2 for the transduction of skeletal muscle.[64] AAV-3 is superior for the transduction of megacaryocytes.[65] Compared to AAV-2, AAV-5 and AAV-6 infect apical airway cells more efficiently.[66,67] AAV-2, AAV-4 and AAV-5 transduce cells of the central nervous system, but differences in the distribution and the target cell types[68] exist. It can be anticipated that further work on AAV serotypes will result in the identification of all domains involved in receptor binding and uptake. This knowledge will be very useful for the creation of AAV retargeting vectors.

Receptor Targeting of AAV-2

In principle, at least two different strategies are possible to achieve a receptor targeting of AAV[69]:

1. *Indirect targeting*: In contrast to wild type (Figure 2a), the interaction between the viral vector and the target cell is mediated by an associated molecule (e.g., a glycoside molecule or a bispecific antibody), which is bound to the viral surface and interacts with a specific cell surface molecule[70] (Figure 2b).
2. *Direct targeting*: The cell-specific targeting of the vector is mediated by a ligand that is directly inserted into the viral capsid[71] (Figure 2c).

For indirect targeting (Figure 2b), it is not necessary to know the three-dimensional structure of the viral surface if high-affinity viral surface binding molecules such as monoclonal antibodies are available. For this strategy, the stability of the interaction of the virus with the intermediate molecule and the efficiency by which the complex is generated are rate limiting. In addition, the intermediate molecules must bind to cell-specific receptors, which allow the uptake and correct intracellular processing of the virus.

A combination of two important parameters is required for the successful generation of a targeting vector by direct modifications of the capsid (Figure 2c). The first parameter is a good choice of the insertion site to ensure that packaging of the mutant remains efficient and the inserted ligand is exposed on the virus surface. Until now two alternative strategies have been used for AAV-2 to identify candidate positions for insertion of heterologous ligand: (a) sequence alignment between AAV-2 and other parvoviruses for which the X-ray crystal structure is known,[25,72] and (b) a systematic, insertional mutagenesis of the whole AAV-2 capsid.[39,40,73] The second important parameter is the choice of the targeting peptide. It is difficult to predict the secondary structure of the ligand inserted into the AAV capsid. Therefore, the ligand should be structure independent and not too large to avoid the destabilization of the entire capsid. Moreover, the ligand should be cell-type specific. Finally, the ligand–receptor complex should be inter-

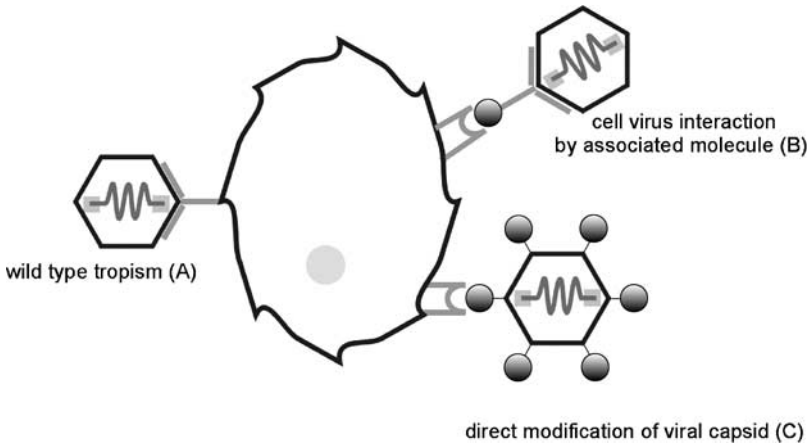

Figure 2 *Possibilities of targeting viral vectors. Viral vectors with wild-type tropism (a) show a direct binding of structural capsid components to the cell surface receptor. In targeting vectors, the virus–cell interaction is mediated by a molecule associated with the capsid (indirect targeting, (b) or by a ligand directly inserted into the capsid (direct targeting, c).*

nalized in a way that allows an efficient transport of the virus and the release of the viral DNA in the cell nucleus.

Both approaches and a combination thereof have been used to retarget AAV-2[25,39,72–77] and will be described in the following paragraphs.

Targeting by bispecific antibodies

The feasibility to target AAV-2 using a bispecific antibody that mediates the interaction between virus and target cell (Figure 2b) was first shown by Bartlett *et al*.[75] The antibody used was generated by a chemical crosslink of the Fab arms of monoclonal antibodies against the $\alpha_{IIb}\beta_3$ integrin (AP-2 antibody) and the intact AAV-2 capsid (A20 antibody[18]).[75] The major ligand for $\alpha_{IIb}\beta_3$ is fibrinogen, which becomes internalized via endocytosis. Therefore, AAV-2 targeted to this integrin was expected to become internalized via receptor-mediated endocytosis, similar to wild-type virus. This targeting vector transduced MO7e and DAMI cells, which are not permissive for wild-type AAV-2 infection (70-fold above background). In contrast, a 90% reduction in AAV-2 transduction was seen on cells negative for the targeting receptor. It remains to be determined whether this reduction was because of steric hindrance or some other mechanism. Another issue that remains to be resolved is the stability of the virus-bispecific antibody complexes *in vivo*.

Targeting by insertion of single-chain antibodies or receptor-specific ligands at the N-terminus of VP proteins

The first attempt to alter the tropism of AAV-2 was described by Yang *et al*.[74] They inserted a single-chain antibody against human CD34, a cell surface molecule expressed on haematopoietic progenitor cells, at the 5′ ends of VP1, VP2 and VP3. Using a transcription and translation assay, they could express all three different single-chain fragment variable region (scFv)-AAV-2 capsid fusion proteins. However, they failed to produce detectable rAAV-2 particles when using either all three scFv-VP fusion proteins or one scFv-VP fusion with two other unmodified capsid proteins. Therefore, they had to use all three wild-type AAV-2 capsid proteins for the packaging process in addition to one of the three single scFv-VP fusion proteins. Using this procedure, intact viral particles could be generated that were able to infect HeLa cells and showed an increased transduction of CD34-positive KG-1 cells. Although this approach provided the first demonstration that targeting of AAV-2 by direct modification of the capsid is possible, the virus titers (1.9×10^2 transducing units/ml on KG-1) were extremely low. Moreover, very heterogeneous viral preparations consisting of an unknown mixture of chimeric, targeting and wild-type AAV-2 particles were produced.

Wu *et al*[39] inserted the HA epitope YPVDVPDYA into the N-terminal regions of VP1, VP2 and VP3 and the C-terminus of the *cap* ORF. They observed that the insertion of this and other epitopes at the N termini of VP1 (VPN1) and VP3 (VPN3) and at the C-terminus of the *cap* ORF (VPC) resulted in either no detectable particles (VPN3 and VPC), or in a 2–3 log decrease of infectious and physical particle titers. In agreement with Yang *et al*,[74] only the insertion at the N-terminus of VP2 (amino acid position 138) was tolerated.[39] Moreover, exchanging the HA epitope by the serpin receptor ligand KFNKPFVFLI78 resulted in a 15-fold higher infection of the lung epithelial cell line IB3 than by wild-type AAV-2. The fact that the N-terminal insertion of different peptides is tolerated in VP2 and allows targeting, albeit at low efficiency, probably reflects the exposure of the N-terminus of VP2 at the viral surface analogous to CPV.[79,80] This assumption was further confirmed by the results of Shi *et al*,[73] who inserted a six amino-acid peptide (TPFYLK) from bovine papillomavirus (BPV) at position 139 and were able to detect this epitope on the capsid surface by monoclonal antibodies against BPV. In addition, the insertion of a 10 amino-acid peptide (HCSTCYYHKS) derived from the human luteinizing hormone (LH) increased the infection efficiency of an LH-receptor-positive human ovarian adenocarcinoma cell line, OVCAR-3.

Targeting of rAAV-2 vectors by insertion of ligand coding sequences into the capsid genes

The first successful demonstration that a genetic capsid modification (direct targeting) can be used to retarget AAV-2 was described by Girod *et al*.[25] A sequence alignment of AAV-2 and CPV identified six sites (amino acid positions 261, 381, 447, 534, 573, 587) that were expected to be exposed on the surface of the virus capsid and to accept the insertion of a ligand without disrupting functions essential for the viral life cycle (Figure 3a).

At these positions, the sequence for the 14 amino-acid peptide L14 (QAGTFALRGDNPQG) was inserted into the capsid gene. The L14 peptide contains the RGD motif of the laminin fragment P1,[81] is the target for several cellular integrin receptors, and can also serve as a viral receptor.[81,82] In addition, no specific secondary structure is required for the recognition of the receptor.[81] All six mutants could be packaged with an efficiency similar to wild-type AAV-2 and showed an intact capsid structure in electron microscopy images.[19,83] Using an ELISA with an anti-L14 polyclonal antibody, it was demonstrated that the L14 epitope was exposed at the viral surface when inserted at amino-acid positions 261, 381, 447, 573 and 587. In a cell binding assay, insertion mutants I-447 and I-587 were able to bind B16F10 (mouse melanoma) and RN22 (rat swannoma) cell lines, which did not bind to and were not infected by wild-type AAV-2. An efficient transduction of B16F10 cells was observed using the AAV insertion mutant I-587 expressing Rep or β-galactosidase.

The same site, 587, was also successfully used for the insertion of an endothelial-specific peptide isolated by phage display, and allowed the generation of an AAV-2 mutant able to infect endothelial cells such as human umbilical vein endothelial cells (HUVEC) and human saphenous vein endothelial cells (HSVEC).[76] In contrast to wild-type AAV-2 infection, the infection of the endothelial-specific cells by the mutant was not blocked by heparin, showing that the infection did not depend on HSPG. Moreover, heparin binding studies showed that the mutant was not retained in a heparin column, in contrast to wild-type AAV-2. The specificity of the binding was shown by infection studies using different non-endothelial-specific cell lines such as HepG2. Furthermore, the mutant seemed to follow an intracellular route different from wild-type AAV-2 since compounds such as bafilomycin A2 (an inhibitor of endosomal acidification) did not inhibit transduction. Taken together, all studies underline the potential value of the 587 site of the AAV-2 capsid, which is positioned at the tip of the GH loop[32] for the generation of cell-specific AAV-2 vectors by the direct targeting approach (Figure 2C).[25,76,77]

This strategy was successfully repeated by Grifman *et al*.[72] They also used a sequence alignment approach to identify potential targeting sites of the AAV-2 capsid by expanding their comparisons to parvoviruses other than CPV and to the other AAV serotypes (AAV-1, AAV-3, AAV-4 and AAV-5). They identified identical regions to Girod *et al*,[25] and finally used sites 448 and 587 for their studies. Grifman *et al*[72] inserted the Myc epitope and a CD13 (NGR receptor expressed on angiogenic vasculature and in many tumor cell lines) specific peptide with the sequence NGRAHA, identified by phage display. The insertion of NGRAHA at 587 allowed a cell-specific targeting to different cell lines (KS1767 (Kaposi sarcoma) and RD (rhabdomyosarcoma)). Interestingly, deletion of the six amino acids (GNRQAA) at position 586–591 resulted in the loss of heparin binding, whereas the insertion of the targeting peptide (NGRAHA) restored the heparin binding ability. Taking into account that HSPG has a negative charge, the R at position 588 might have an essential role for HSPG binding.

For a systematic characterization of functional domains of the AAV capsid proteins, Wu *et al*[39] constructed 93 mutants at 59 different positions on the AAV-2 capsid by site directed mutagenesis. They identified several putative regions, which were involved in HSPG binding and/or exposed on the capsid surface, with the potential to tolerate the insertion of a ligand. These positions were 34 (in VP1), the N-terminus of VP2 (138), as well as 266, 328, 447, 522, 553, 591, 664 (in VP3). Although all VP3 insertion mutants were precipitated by an antibody against the inserted HA epitope, only 266, 447, 591 and 664 were still infectious. For insertion mutant 522, this could be explained by the loss of the HSPG binding

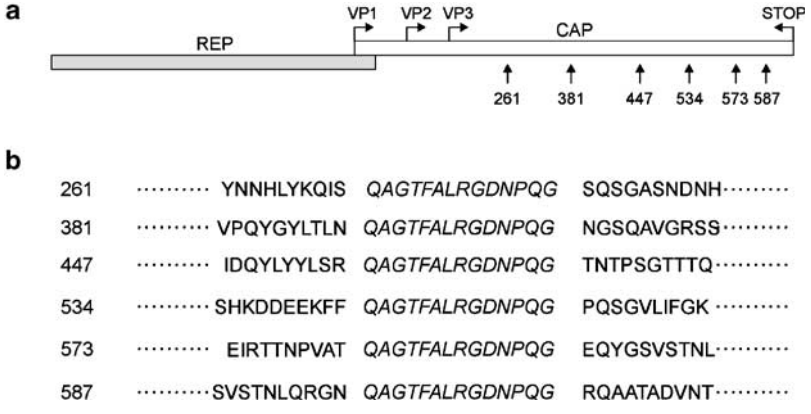

Figure 3 *L14 peptide insertion sites in the AAV2 capsid. (**a**) Schematic diagram of the two open reading frames rep and cap. Cap encodes the three capsid proteins VP1, VP2 and VP3. Sites of the L14 insertion described by Girod et al[25] are marked by arrows (numbers are the amino acid positions N-terminal of the insertion). (b) Sequence of L14 and flanking amino acids at the different insertion sites tested. The insertion mutant I-587 displayed the L14 peptide on the surface and was able to retarget the mutant to the mouse melanoma cell line B16F1025 (numbering starts at the start codon of VP1).*

ability.[39,40] For the other mutants, a simple explanation is lacking. Wu *et al*[39] tested only position 34 in the VP1 sequence (FVFLI substitution) and the N-terminus of VP2 (KFNKPFVFLI insertion) for targeting of AAV-2 to IB3 cells via serpin receptor. It was shown that targeting and infection was possible. In this approach, the N-terminal VP-2 insertion mutant was 15-fold and the VP1 mutant approximately 62-fold more infectious than the wild type. In both cases, the insertions were placed outside the potential HSPG binding regions. Therefore, it was not surprising that the transduction of the target cells by these mutants was blocked by heparin, suggesting that the serpin-tagged mutants continued to use HSPG as primary receptor and used the serpin receptor as alternative (co-)receptor.

Using insertional mutagenesis, Shi *et al*[73] also tried to identify positions in the AAV-2 capsid that might tolerate the insertion of heterologous peptide ligands. In addition to the VP2 N-terminal insertion mutants mentioned above, mutants with insertions into the capsid sequences were generated. These mutants contained either an insertion of two amino acids (TG or AG) or of longer epitopes derived from the BPV (TPFYLK), from the human LH (HCSTCYYHKS) or a cyclic RGD-containing peptide specific for α_v integrins (4C-RGD, CDCRGDCFC). None of these mutants were tested for retargeting, but three important observations were made:

(1) Five different capsid regions were identified that allowed the surface display of the BPV peptide ligand. These were 139 (N-terminus of VP2), 161 (in the VP2 region), 459, 584 and 587 (in the VP3 region). The positions 139 and 587 confirmed earlier results.[25, 39] The other sites remain to be tested for functional targeting, because the surface display of a ligand alone is a prerequisite but not sufficient for a ligand-dependent infection by the virus mutant.[25]

(2) The scaffold sequences flanking the heterologous ligand are important for epitope display, HSGP binding ability and titers. Using, for example, the amino acids ALS to flank the BPV ligand inserted at 584 resulted in mutants that showed surface display of the epitope, HSPG binding and the production of infectious particles. In contrast, LLA and GLS used as scaffold sequences did not allow the production of infectious particles and reduced the surface display of the BPV ligand. In contrast, the GLS scaffold sequence was better tolerated than LLA or ALS at position 587.

(3) Not every ligand, even if comparable in length, is tolerated at a specific insertion site. Shi *et al*[73] inserted either the BPV or the LH ligand both flanked by the GLS scaffold sequences at site 459 and observed that the BPV ligand (a six amino-acid insertion) generated fully infectious particles, whereas the insertion of the 10 amino-acid LH peptide created noninfectious virus particles. Our laboratory made a similar observation when trying to insert multimers of the L14 sequence at position 587: larger insertions at position 587 resulted in a decrease of the packaging efficiency although the insertion of a 34 amino acid containing the Z34C protein A domain of *Staphylococcus aureus* (see below) was well tolerated. These results show that the maximal length of the peptide tolerated at this position depends on the sequence itself. The precise determinants of this phenomenon are unknown.

Generation of universal targeting vectors by combining two principles of vector targeting

Inspired by an earlier attempt for Sindbis virus,[84] we tried to use a general targeting vector using a truncated 34 amino-acid peptide, Z34C, from protein A of *Staphylococcus aureus*.[77] Protein A recognizes and binds the Fc part of immunoglobulins (Ig), but not the variable Ig domain, which therefore remains free to bind the antigen. Z34C is derived from the protein A subunit B, which encompasses 56 amino acids and binds the Fc portion with a dissociation constant of about 10–50 nM.[85] A 38 residue truncation of this domain, selected by phage display, was further truncated and stabilized by insertion of disulphide bonds and showed thereafter a dissociation constant of 20 nM. The insertion of Z34C at position 587 in the AAV-2 capsid (587Z34C) resulted in a 10-fold decrease of packaging efficiency in comparison to wild-type AAV-2. In contrast, the combination of the insertion with a nine amino-acid deletion (587Δ9Z34C) resulted in a packaging efficiency similar to wild-type AAV-2. Electron microscopy and A20-ELISA revealed a wild-type capsid morphology for both mutants, although empty capsids were observed three-fold more frequently. Interestingly, the wild-type tropism of the Z34C insertion mutants decreased by four orders of magnitude, in agreement with the results of Nicklin *et al*[76]. The insertion of Z34C at position 587 allowed a functional expression of the IgG binding domain, as shown by binding studies using various antibodies. Interestingly, the capsid mutant 587Z34C bound antibodies more efficiently than 587Δ9Z34C, maybe because the binding domain was less accessible with the nine amino-acid deletion. In agreement, Grifman *et al*[72] showed that a substitution at 587 was less efficient than an insertion. Coupling 587Z34C virus with antibodies against CD29 (β1-integrin), CD117 (c-kit-receptor) or CXCR4 resulted in a specific, antibody-mediated transduction of haematopoietic cell lines. No transduction could be detected without antibody, whereas the targeted infection was blocked with soluble protein A or with IgG molecules. In addition, no inhibition of transduction by the targeting vector was observed with heparin, demonstrating that the interaction of the 587Z34C mutants with the natural AAV-2 receptor HSPG was not essential for infection or transduction. Taken together, this targeting approach shows that a universal AAV targeting vector can be generated and loaded with different targeting molecules to transduce the desired cells via specific receptors. However, this approach leaves room for improvement, since the titers obtained with these vectors were relatively low.

Future prospects: understand the infectious biology of AAV-2

To efficiently retarget AAV vectors, a better understanding of the infectious biology of AAV will be required. This includes the virus–cell surface interactions, mechanisms of uptake, endosomal processing

and release, nuclear transport and mechanisms leading to gene expression. The structure determination of the AAV-2 capsid[32] will dramatically enhance our knowledge of the location and function of different capsid domains.

The identification of HSPG as the primary attachment receptor for AAV-2 was an important achievement. However, no distinct binding motif within the capsid has been identified so far, despite some useful information presented in the work of Rabinowitz *et al*[40] and Wu *et al*.[39] Such knowledge will be required to specifically modify the natural viral tropism of AAV-2.

A better understanding of the intracellular processing of AAV targeting vectors will be essential, because AAV targeting vectors may be transferred into a cellular compartment from which they will never be released, or in which they will be processed in ways preventing nuclear processing or gene expression. Therefore, the success of creating AAV targeting vectors will ultimately depend on our ability to unveil the detailed mechanisms of AAV transport and processing. Some pieces of the puzzle are already known,[41,43,52] but the picture is not complete. In this regard, single virus tracing[52] will be an important tool to understand which receptors and cellular compartments need to be used to efficiently retarget AAV. On the other hand, the technique of AAV vector targeting will help to uncover some important, basic functions of AAV capsid proteins, as well as mechanisms of the infectious biology of AAV.

Another important issue is the identification of the optimal ligand or targeting receptor. For the genetic modification strategy chosen by our group, the length and sequence of the ligand are critical, as the insertion of a peptide may result in profound alterations of the three-dimensional capsid structure. One possibility to overcome this problem is the combination of the insertion with one or more deletions. Another possibility is the insertion of a sequence that is able to form its own secondary structure, for example, a loop closed by a cysteine bridge.

These difficulties are overcome when using an antibody or another bridging molecule to mediate the interaction between the viral surface and the target cell. However, this approach will encounter other problems such as the stability of the virus–ligand complex, limitations to scale up the vector production, and steric hindrance of the virus uptake by large virus–ligand complexes.

To identify new ligands, phage display is a valid approach. However, the ligand sequences are selected in an architectural context that is different from that of the final vector. This means that once inserted in the context of AAV, they could destabilize the capsid structure (resulting in low packaging efficiency) or lose their biological properties (resulting in low infectious titers). To overcome these difficulties the screening for new 'retargeting' peptides to be inserted might be done more efficiently in the context of the AAV capsid itself (vector display), where a pool of randomized peptide sequences is inserted into the capsid sequence and the viral pool is then screened directly on the target cells.[86] The exciting results obtained by this approach[86] together with the rapidly advancing knowledge of the structure and biology of AAV raise the expectation of dramatic improvements of AAV vector technology in the near future.

The ultimate goal of all these attempts will be the generation of a recombinant AAV vector, which allows gene delivery exclusively to the desired cells or tissue, thereby widening the therapeutic window of this vector for clinical application.

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