Impact of capsid modifications by selected peptide ligands on recombinant adeno-associated virus serotype 2-mediated gene transduction

Matthias Naumer, Ruth Popa-Wagner and Jürgen A. Kleinschmidt

Vectors based on adeno-associated virus serotype 2 (AAV2) belong to today’s most promising and most frequently used viral vectors in human gene therapy. Like in many other vector systems, the broad but non-specific tropism limits their use for certain cell types or tissues. One approach to screen for transduction-improved vectors is the selection of random peptide libraries displayed directly on the AAV2 capsid. Although the AAV2 library system has been widely applied for the successful selection of improved gene therapy vectors, it remains unknown which steps of the transduction process are most affected and therefore critical for the selection of targeting peptides. Attachment to the cell surface is the first essential step of AAV-mediated gene transduction; however, our experiments challenge the conventional belief that enhanced gene transfer is equivalent to more efficient cell binding of recombinant AAV2 vectors. A comparison of the various steps of gene transfer by vectors carrying a wild-type AAV2 capsid or displaying two exemplary peptide ligands selected from AAV2 random libraries on different human tumour cell lines demonstrated strong alterations in cell binding, cellular uptake, as well as intracellular processing of these vectors. Combined, our results suggest that entry and post-entry events are decisive for the selection of the peptides NDVRSAN and GPOQKNS rather than their cell binding efficiency.

INTRODUCTION

Adeno-associated viruses (AAVs) are members of the family Parvoviridae and belong to the smallest known viruses. The combination of many favourable attributes accelerated the widespread use of AAV-based vectors for human gene therapy (Daya & Berns, 2008; Heilbronn & Weger, 2010). AAV serotype 2 (AAV2) is to date the best studied and characterized serotype, and recombinant forms of this virus (rAAV2) have emerged as one of the leading gene transfer systems in human clinical trials (Kay, 2011; Mingozi & High, 2011; Mueller & Flotte, 2008). Advantages of AAV vectors are their non-pathogenicity, replication deficiency and their transgene delivery to dividing as well as non-dividing, post-mitotic cells. However, the broad but non-specific tissue tropism and the weak transduction of a number of therapeutically interesting target cells remain major hurdles for the application of these gene therapy vectors.

For successful gene transduction, AAV vectors have to overcome several biological barriers which limit transgene delivery. The first step in cell transduction is the attachment of the vector to one or more cell surface receptors or coreceptors, followed by the uptake of the capsid–receptor complex via endocytosis. To date, several potential pathways have been described for the cellular trafficking of rAAV2 vectors, which all agree on the uptake of viral particles into the endosomal compartment after internalization (Ding et al., 2005; Nonnenmacher & Weber, 2012). During endosomal trafficking, the viral capsid undergoes conformational changes leading to the exposure of hidden VP1/VP2 N-terminal domains that are crucial for efficient transduction (Kronenberg et al., 2005; Sonntag et al., 2006). Before it travels to the nucleus, AAV exits from the endosomal compartment and is prone to ubiquitin-dependent proteasomal degradation (Duan et al., 2000; Yan et al., 2002; Zhong et al., 2008). Translocation of rAAV2 across the nuclear membrane seems to be a time-consuming, inefficient process that remains poorly understood but converging evidence suggests that intact AAV particles enter the nucleus (Bartlett et al., 2000; Johnson & Samulski, 2009; Johnson et al., 2010; Sonntag et al., 2006), where transgene expression occurs following the release of the ssDNA genome by capsid uncoating and conversion into dsDNA (Ferrari et al., 1996; Fisher et al., 1996).

Different strategies have been developed to achieve higher transduction efficiency and specificity of AAV-based vectors, mainly through engineering of the viral capsid (Bartel et al., 2012; Michelfelder & Trepel, 2009; Mitchell et al., 2010; Schaffer et al., 2008). A successful screening system for targeted AAV2 vectors was developed by displaying peptides directly on the viral capsid, which allows...
the selection of improved gene therapy vectors even without prior knowledge of the potential binding receptor and corresponding ligands (Müller et al., 2003; Perabo et al., 2003). Combinatorial AAV2 libraries, displaying random peptide ligands at amino acid position 588 of the capsid protein, have been successfully applied for the selection of a number of capsid mutants efficiently transducing various cell types (Michelfelder et al., 2007; 2009; Naumer et al., 2012; Waterkamp et al., 2006; Ying et al., 2010). The strong restriction to particular peptide motifs observed in these studies supports the assumption that target cell binding is only one of several factors affecting the selection process and suggests that peptide ligands inserted into the AAV2 capsid influence different post-binding processes relevant for gene transduction.

Here, we sought to elucidate the impact of two exemplary peptide ligands, selected from AAV2 random libraries on different human tumour cell lines, on the various steps of gene transfer from cell attachment to nuclear transport of the vector. Surprisingly, the selected targeting ligands NDVRSAN and GPQGKNS were found to severely decrease target cell binding compared with the wild-type rAAV2. Instead, differences in vector endocytosis and intracellular processes were found to be responsible for the observed changes in gene transfer efficiency.

RESULTS AND DISCUSSION

AAV2 library selection on human melanoma and glioblastoma cells

Low-passage human melanoma and glioblastoma cell lines were used for in vitro selection of an AAV2 library displaying randomized 7-mer peptide ligands at amino acid position 588 of the viral capsid. For this purpose, \( 1 \times 10^6 \) tumour cells were infected at an m.o.i. of 10 000 genome-containing AAV2 library particles (vg) per cell in the first screening round and superinfected with adenovirus type 5 to enable AAV replication. Progeny viruses were recovered after each selection round and applied onto freshly seeded cells at serial lower m.o.i. in the following screening rounds. Sequence analysis of AAV2 library clones recovered after four rounds of selection revealed a strong enrichment of specific peptide motifs during the selection process on all tumour cells (Table 1). Screening on the melanoma cell lines P Mel A2 and P Mel L resulted in the predominant isolation of one specific clone, NDVRSAN, with 33.3 and 89.8 % recovery after four rounds, respectively. In contrast, selection of the identical AAV2 library on the human glioblastoma cell lines NCH 37 and NCH 199 yielded a set of completely different peptide motifs with slightly lower frequencies of recovery, from 27.3 to 48.0 % regarding the predominantly isolated virus particles displaying the peptides GPQGKNS and EDRTERR, respectively. While the divergent selection outcome on melanoma and glioblastoma cells suggests distinct cellular targets of the respective peptide motifs, the repeated recovery of identical library clones from cells originating from the same tumour type may point towards targeting of identical receptors in these cell lines. Furthermore, the restriction to a relatively small number of selected peptide motifs (compared with the diversity of the AAV2 library of about \( 1.1 \times 10^8 \) individual clones; data not shown) suggests that only a few ligands feature all crucial requirements for successful target cell transduction.

The effect of the most frequently isolated peptide ligands on target cell transduction was analysed using rAAV2 reporter vectors displaying the respective peptide motifs.

### Table 1. Peptides selected from an AAV2 library on human melanoma and glioblastoma cells

Peptide ligands obtained from the selection of an AAV2 random display library on the melanoma cell lines P Mel A2 and P Mel L, as well as the glioblastoma cell lines NCH 37 and NCH 199. Peptide sequences are displayed in one-letter code together with their frequency of recovery (%) after four selection rounds. The numbers in parentheses indicate the absolute count of repeatedly recovered library clones from a total number of approximately 30 sequenced clones.

<table>
<thead>
<tr>
<th></th>
<th>P Mel A2</th>
<th>P Mel L</th>
<th>NCH 37</th>
<th>NCH 199</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>Frequency (%)</td>
<td>Frequency (%)</td>
<td>Frequency (%)</td>
<td>Frequency (%)</td>
</tr>
<tr>
<td>NDVRSAN</td>
<td>33.3 (9 x)</td>
<td>89.8 (26 x)</td>
<td>27.3 (6 x)</td>
<td>48.0 (12 x)</td>
</tr>
<tr>
<td>APVTPRA</td>
<td>14.8 (4 x)</td>
<td>3.4 (1 x)</td>
<td>13.6 (3 x)</td>
<td>24.0 (6 x)</td>
</tr>
<tr>
<td>KDPVRAP</td>
<td>7.4 (2 x)</td>
<td>3.4 (1 x)</td>
<td>9.1 (2 x)</td>
<td>4.0 (1 x)</td>
</tr>
<tr>
<td>NASRIID</td>
<td>7.4 (2 x)</td>
<td>3.4 (1 x)</td>
<td>9.1 (2 x)</td>
<td>4.0 (1 x)</td>
</tr>
<tr>
<td>NRVILDS</td>
<td>7.4 (2 x)</td>
<td>3.4 (1 x)</td>
<td>9.1 (2 x)</td>
<td>4.0 (1 x)</td>
</tr>
<tr>
<td>DLGSGRS</td>
<td>3.7 (1 x)</td>
<td>1.9 (1 x)</td>
<td>9.1 (2 x)</td>
<td>4.0 (1 x)</td>
</tr>
<tr>
<td>DLGSLRS</td>
<td>3.7 (1 x)</td>
<td>1.9 (1 x)</td>
<td>9.1 (2 x)</td>
<td>4.0 (1 x)</td>
</tr>
<tr>
<td>DLRLSLTA</td>
<td>3.7 (1 x)</td>
<td>1.9 (1 x)</td>
<td>9.1 (2 x)</td>
<td>4.0 (1 x)</td>
</tr>
<tr>
<td>LSPVRPG</td>
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<td>1.9 (1 x)</td>
<td>9.1 (2 x)</td>
<td>4.0 (1 x)</td>
</tr>
<tr>
<td>PSSPRMP</td>
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<td>1.9 (1 x)</td>
<td>9.1 (2 x)</td>
<td>4.0 (1 x)</td>
</tr>
<tr>
<td>VSPVRPS</td>
<td>3.7 (1 x)</td>
<td>1.9 (1 x)</td>
<td>9.1 (2 x)</td>
<td>4.0 (1 x)</td>
</tr>
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</table>
and harbouring a luciferase reporter gene cassette under the control of the cytomegalovirus promoter. Transduction analysis of the melanoma cell lines P Mel A2 and P Mel L revealed superior gene transfer efficiencies for vectors displaying the peptides NDVRSAN or NASRIID in comparison with vectors displaying a randomly chosen, non-selected peptide motif (TEWDQPF) or carrying wild-type capsids (Fig. 1a). Another melanoma cell line, P Mel A, which was not included in the AAV2 library selection, showed a similar transduction pattern and was used for further analysis of the transduction process in the following experiments due to its high susceptibility for rAAV2-mediated gene transfer. Vectors displaying the peptides APVTRPA or KDPVRAP, the second and third most frequently recovered motifs from selection on P Mel A2 cells, however, failed to increase gene transfer in comparison with the wild-type vector in all three melanoma cell lines. Similarly, the most frequently selected clones from screening on glioblastoma cells, GPQGKNS and EDRTER, mediated less efficient gene transfer than vectors carrying the wild-type AAV2 capsid in both glioblastoma cell lines (Fig. 1b), demonstrating the difficulty to select transduction-improved library clones on cells that are per se highly susceptible to the parental serotype.

**Insertion of peptide ligands severely decreases the cell binding of rAAV2 vectors**

The first step in cell transduction is the attachment of the viral vector to the cell surface. Modifications of the viral capsid at positions that considerably contribute to the tropism and infectivity of AAV2, especially at amino acid positions 585 and 588, are known to coincide with altered cell binding of the virus (Kern et al., 2003; Opie et al., 2003). To study the effect of the two exemplary targeting peptides NDVRSAN and GPQGKNS on cell binding, we incubated wild-type and peptide-displaying rAAV2 vectors

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**Fig. 1.** Characterization of gene transduction by vectors displaying tumour-cell-selected peptide ligands. Gene transduction of low-passage melanoma cells (a) and human glioblastoma cell lines (b) with rAAV2 luciferase reporter vectors displaying peptides selected on the respective tumour cells. Amino acid sequences of the selected 7-mer peptide inserts are indicated in one-letter code. Non-selected vectors carrying AAV2 wild-type capsids (wt) or displaying the random peptide insert TEWDQPF were used for comparison. Cells were transduced with 10,000 genome-containing vector particles per cell and luciferase expression was determined by light emission measured as relative light units (RLU) at 96 h post-infection. A luciferase standard of known concentration was used to convert RLU into the amount of active luciferase per well, expressed as fg luciferase per well. Data are means ± SD of 12 assays from three independent experiments. *P<0.05 and **P<0.01, Increased transduction efficiency compared with wild-type rAAV2 (wt).
with the respective target cells, P Mel A or NCH 37, for 1 h at 4 °C to enable attachment but prevent entry of the vectors. Cells and supernatant were subsequently collected to quantify the proportion of vectors attached to the cells in comparison with the amount of unbound vector particles using quantitative real-time PCR (qrt-PCR).

Surprisingly, all tested peptide ligands conveyed massive reductions in cell binding to the respective vector constructs compared with vectors carrying a wild-type capsid (Fig. 2). Independent of the tumour cell type, approximately 70% of the recovered vectors carrying a wild-type capsid were able to bind to the cells. In contrast, less than 5% of the vectors displaying the peptide ligand NDVRSAN bound to P Mel A cells, providing evidence that the improvement in transduction achieved with this mutant cannot be explained by increased cell binding. Vectors presenting the glioblastoma-cell-selected peptide GPQGKNS were capable of binding NCH 37 cells at moderate levels of approximately 25%, while modifications of the AAV2 capsid by the randomly chosen ligand TEWDQPF resulted in less than 7% bound vector particles on both tumour cell lines.

Peptide insertions at position 588 are known to interfere with the attachment of AAV2 to one of its natural receptors, heparin sulfate proteoglycan (HSPG). Depending on the size and nature of the ligand, e.g. the net charge of the amino acid side chains, peptide insertions can either disrupt or conserve the ability of the viral capsid to bind heparin (Perabo et al., 2006). To rule out that the observed reductions in cell binding are caused by the inability of the peptide-displaying vectors to bind to HSPG, we compared the HSPG-binding capability of wild-type and capsid-modified vectors via chromatography on heparin agarose (Fig. S1, available in JGV online). Insertion of the randomly chosen ligand TEWDQPF resulted in an almost completely impaired binding to heparin, presumably due to the net negative charge of the inserted peptide which probably prevents binding to the also negatively charged HSPG. In contrast, vectors displaying the selected peptides NDVRSAN or GPQGKNS (neutral or positive net charge, respectively) retained binding to the heparin matrix, which was however, slightly reduced in comparison with the wild-type rAAV2. Therefore it seems unlikely that the strong reduction observed in cell binding of rAAV2 vectors is the result of a hampered HSPG binding of these vectors.

**Presented peptide ligands modulate the cellular uptake of rAAV2 vectors**

Based on the discrepancy between cell binding and transduction efficiency described above, additional steps of the transduction process were analysed in more detail. First, we sought to investigate the cellular uptake of wild-type and peptide-displaying rAAV2 vectors in a time-course experiment by incubating the vectors with the respective target cells for various time spans ranging from 1 to 96 h at 37 °C, before the vector solution was discarded and unbound vectors were washed off. At 96 h post-infection, cells were harvested by intensive trypsin treatment to ensure a complete removal of all non-internalized particles, followed by the quantification of the number of vector genomes present inside the cells (Fig. 3). The unmodified rAAV2 vectors carrying the wild-type capsid showed similar uptake patterns in both melanoma and glioblastoma cells, which were characterized by a moderate increase in the number of vector genomes during the first 12–24 h and relatively stable amounts in the range of approximately 25–30% internalized vectors in the time period thereafter. In contrast, vectors displaying the peptide NDVRSAN showed an initially slower uptake, which then however, led to a continuous and steady accumulation of vector genomes inside P Mel A cells, resulting in approximately threefold higher amounts compared with wild-type rAAV2 after 96 h. Vectors carrying the peptide GPQGKNS accumulated less efficiently than...
Peptide insertions influence the efficiency of intracellular processing of rAAV2 vectors

The overall efficiency of intracellular processing of rAAV2 vectors following cellular uptake was analysed by comparing the ratios of reporter gene activity with the total number of intracellular vector genomes at different time points after transduction (Fig. 4). For this purpose, P Mel A and NCH 37 cells were incubated for 24–72 h in the presence of wild-type rAAV2 or vectors displaying NDVRSAN or GPQGKNS. After incubation, cells were washed and harvested by trypsin treatment before each sample was analysed for the total number of internalized vector genomes, as well as for the corresponding luciferase reporter gene activity (RLU). While only minor differences between the vectors were observed after 24 h of incubation, significant changes in the RLU/vector genome ratios between the wild-type and capsid-modified vectors were observed at 72 h post-transduction, indicating a pronounced effect of peptide insertions on the post-entry processing of rAAV2 vectors. Despite the superior transduction of P Mel A cells by vectors displaying NDVRSAN, the RLU/genome ratio of this vector was approximately twofold lower than that of wild-type rAAV2 after 72 h, suggesting less efficient post-entry processing. On the other hand, rAAV2–GPQGKNS showed an approximately twofold higher RLU/vector genome ratio and therefore more efficient post-entry processing compared with the wild-type vector at 48 and 72 h post-transduction.

Peptide insertions influence the subcellular distribution of rAAV2 vectors

To characterize the influence of selected peptide ligands on the intracellular processing in more detail, we analysed the distribution of rAAV2 vector particles in P Mel A and NCH 37 cells via indirect immunofluorescence and cell compartment fractionation (Fig. 5). For confocal microscopy analysis of vector internalization, 400 000 vg per cell was applied to melanoma or glioblastoma cells for 2 h at 37 °C before the vector solution was exchanged for fresh culture medium. At 6, 12 and 24 h post-infection, cells were fixed, permeabilized and viral capsids were detected by A20 antibody staining (green). At each time point after infection, wild-type capsids could be detected in larger numbers in both cell lines, confirming the advantageous initial uptake of wild-type rAAV2 compared with capsid-modified vectors (Fig. 5a). Only very few vector particles of rAAV2–NDVRSAN were detected, showing that the superior gene transfer efficiency of this vector is based on the continuous endocytosis and accumulation inside the cell over a long time period. No vector particles were visible inside the nuclei of both cell lines.

In order to quantify the cellular distribution of internalized rAAV2 vectors, we fractionated P Mel A and NCH 37 cells after transduction with the respective vectors. Briefly, cells were harvested at 12 h post-transduction by intensive trypsin treatment to remove any non-internalized vectors from the cell surface before the proportion of vector genomes present in the cytosolic fraction, the membrane fraction and the nuclear fraction was quantified by qrt-PCR (Fig. 5b). Proteins extracted from each fraction were analysed by Western blot to validate the separation of cytosolic, membrane and nuclear proteins (Fig. 5c). In P Mel A cells, the majority of vector genomes from wild-type rAAV2 and the NDVRSAN-displaying vector were found in the nuclear fraction, followed by the membrane fraction and the cytosol. In contrast, wild-type and rAAV2–GPQGKNS vectors showed a more even distribution in NCH 37 cells, with relatively high amounts of vector genomes present in the cytosolic and membrane fractions, indicating a distinct, cell-type-dependent processing and routing of rAAV2 vectors in melanoma and glioblastoma cells which was also visible in the immunofluorescence analysis (Fig. 5a). Significant differences between wild-type rAAV2 and the capsid-modified (NDVRSAN) vector were observed in melanoma cells with regard to the nuclear translocation...
(81 versus 59 %, respectively) and the proportion of vector genomes present in the membrane fractions (17 versus 34 %, respectively), indicating a more efficient release of wild-type rAAV2 from the membrane fraction and superior delivery to the host cell nucleus. This may explain the distinct post-entry processing efficiencies between these vectors, as indicated by their different transduction-to-genome ratios (Fig. 4). A similar distribution of vector genomes was observed in P Mel A cells after 48 h (data not shown), indicating an effect of the inserted peptide on the vector distribution, independent of the time point of analysis.

The reduced retention of rAAV2–GPQGKNS in the membrane fraction and the increased cytoplasmic accumulation compared with wild-type rAAV2 indicate an improved processing of the capsid-modified vector until its release into the cytosol. The relatively low proportion of genomes from both vectors in the nuclear fraction of NCH 37 cells suggests a slower nuclear uptake or a decreased intranuclear stability of rAAV2 genomes in NCH 37 cells compared with P Mel A. The striking difference between the accumulation of vector genomes in the nuclear fraction and the lack of detection of vector capsids in the nucleus in immunofluorescence analysis (Fig. 5a) may be explained by an increased destabilization of capsids in the nucleus; however, alternative explanations are also possible (Lux et al., 2005). Inhibition of proteasomal degradation of internalized vector capsids by treatment of the cells with the inhibitor MG132 led to the detachment of the cells, which prevented analysis by immunofluorescence.

**Effects of inserted peptide ligands on transgene release from the viral capsid in vitro and in vivo**

The influence of peptide modifications on capsid integrity and genome release was examined by *in vitro* treatment of rAAV2 vectors at temperatures between 37 and 74 °C. After 5 min incubation at the respective temperature, vector samples were analysed by AAV2 capsid ELISA to determine the proportion of still-intact capsids, detected via A20 antibody binding. Temperature-treated vectors were furthermore subjected to S7 nuclease treatment, leading to the complete degradation of vector DNA that was no longer protected inside an intact capsid. Quantifications of both the number of intact capsids and the amount of vector DNA protected from nuclease treatment, were used to draw conclusions on capsid integrity and the release of the vector genome (Fig. 6a). At physiological temperatures of 37 °C, the integrity of both wild-type and capsid-modified vectors was not affected, as shown by capsid and genome numbers in the 100 % range compared with non-treated vector samples that were kept on ice. Treatment at temperatures above 60 °C resulted in a considerable decrease in the numbers of vector capsids and genomes. Wild-type rAAV2 and vectors modified by the peptide NDVRSAN revealed very similar temperature sensitivities with 70 and 83 % intact capsids after incubation at 62 °C, respectively. In contrast, only 34 % of the vectors displaying the ligand GPQGKNS were detected after treatment at 62 °C and approximately 10 % after incubation at 66 °C, in comparison with 43 and 48 % for wild-type and rAAV2–NDVRSAN, respectively. Hardly any intact capsids could be detected after incubation at temperatures of 70 °C or higher for all tested vector constructs. As mentioned above, the amounts of recovered vector genomes also decreased steadily at higher temperatures. However, no significant differences between the analysed vectors could be observed regarding genome release, indicating that the treatment of rAAV2–GPQGKNS at 62 °C led to a decreased detection of this vector by the A20 antibody, but was not sufficient to induce an increased genome release.

**Fig. 4.** Effects of peptide insertions on post-entry steps of gene transduction. P Mel A and NCH 37 cells were incubated in the presence of 10 000 genome-containing rAAV2 particles carrying a wild-type capsid or displaying the tumour-cell-selected peptide ligands NDVRSAN (P Mel A) or GPQGKNS (NCH 37) for 24, 48 or 72 h, before cells were harvested by trypsin treatment. Each sample was analysed in regard to the total number of internalized vector genomes (normalized to the amount of co-purified cellular DNA by detection of the β-actin gene) using qrt-PCR and the corresponding expression of the encoded luciferase reporter gene measured in relative light units (RLU). Bars represent the ratio of RLU to the number of isolated vector genomes for each time point. Data are means ± SD from three independent experiments. *P*<0.05 compared with wild-type rAAV2. Dark bars, NDVRSAN (P Mel A) or GPQGKNS (NCH 37); light bars, AAV2.
Fig. 5. Subcellular distribution and nuclear translocation of rAAV2 vectors. (a) Confocal microscopy analysis of vector uptake in P Mel A and NCH 37 cells at 6, 12 and 24 h post-transduction. Cells were transduced at m.o.i. of 400,000 genome-containing particles per cell for 2 h at 37 °C before the vector solution was exchanged for medium containing no vectors. rAAV2 capsids were detected by staining with the capsid-specific antibody A20 (green) and nuclei were counterstained with DAPI (blue). (b) Cell compartment fractionation. P Mel A and NCH 37 cells were incubated with 1000 genome-containing vector particles per cell for 1 h at 4 °C to allow vector binding before the cells were shifted to 37 °C. After 2 h the vector solution was removed and replaced by fresh culture medium. At 12 h post-transduction, cells were fractionated into cytoplasm, membrane and nuclei. Vector genomes were isolated from each of the fractions and quantified by qrt-PCR. Vector genome numbers from each fraction were normalized to the amount of cellular DNA (β-actin gene copy numbers) co-purified from the nuclear fraction of the respective fractionation to compensate for variations in cell numbers. Bars indicate the proportion of vector genomes in each fraction related to the total number of isolated genomes at 12 h post-transduction. Data are means ± SD of three independent experiments. **P<0.01; *P<0.05; NS, not significant. Dark bars, NDVRSAN (P Mel A) or GPOGKNS (NCH 37); light bars, AAV2. (c) Western blot analysis of the distribution of cytosolic, membrane and nuclear proteins in subcellular fractions (CF, cytosolic fraction; MF, membrane fraction; NF, nuclear fraction) from representative fractionations of each cell type. Cytosolic, membrane and nuclear proteins were detected with antibodies directed against IκB, Rab5 and LaminB, respectively.
To analyse rAAV2 genome stability in vivo, a pulse–chase experiment was carried out in which P Mel A and NCH 37 cells were incubated in the presence of wild-type rAAV2 or vectors displaying the selected peptides NDVRSAN or GPQGKNS for 24 h (pulse), before unbound vectors were washed off and vector genomes present inside the cells were isolated either directly or after an additional incubation of the cells of up to 48 h in the absence of any vectors (chase). As shown in Fig. 6(b), a comparison of the relative decrease in vector genome numbers revealed no significant differences between wild-type rAAV2 and the vector displaying NDVRSAN. In contrast, insertion of the peptide GPQGKNS resulted in a slightly stronger decrease in the number of viral genomes in NCH 37 cells after 48 h at 37 °C before the vector solution was discarded and cells were washed. After this, vector genomes were isolated from the cells either directly (24 h value) or the cells were incubated for additional time periods of up to 48 h before vectors were recovered (48 and 72 h values). The relative decrease in the number of isolated vector genomes over time was determined for each vector construct in relation to the respective 24 h value which was set to 100 %. Values are means ± sd from three independent experiments. *P<0.05 compared with wild-type rAAV2; ns, not significant. Black lines, NDVRSAN (P Mel A) or GPQGKNS (NCH 37); grey lines, AAV2.

**METHODS**

**Cells and cell culture.** 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % FCS, 2 mM l-glutamine and 100 µg penicillin/streptomycin ml⁻¹. P Mel
A, P Mel A2 and P Mel L cells (Nettelbeck et al., 2004) were cultured in RPMI 1640 supplemented with 10 % FCS, 2 mM l-glutamine, 20 μg gentamicin ml⁻¹ and 250 ng amphotericin ml⁻¹. NCH 37 and NCH 199 cells (Karcher et al., 2006) were maintained in DMEM supplemented with 10 % FCS, 2 mM l-glutamine and 20 μg gentamicin ml⁻¹. All cells were constantly kept in a humidified atmosphere at 37 °C and 5 % CO₂.

AAV2 random peptide library production and selection. A random AAV2 library displaying 7-mer peptide ligands at amino acid position 588 of the viral capsid was produced as previously described (Waterkamp et al., 2006). For selection, 1 × 10⁶ cells were infected with AAV2 library particles at an m.o.i. of 10 000 vg per cell. After 4–6 h incubation at 37 °C, cells were washed and superinfected with adenovirus serotype 5 to enable AAV replication. AAV2 library clones capable of successful infection and replication were harvested from target cells and applied to freshly seeded cells at a 50 % lower m.o.i. in each subsequent selection round.

Production of AAV2 reporter vectors. rAAV2 vectors displaying a selected peptide motif on the capsid surface and harbouring a luciferase reporter gene cassette were produced by transfection of 293T cells via calcium phosphate precipitation as previously described (Naumer et al., 2012) and purified by iodixanol density-gradient centrifugation (Hauswirth et al., 2000). Titres of assembled AAV2 capsid particles (capsids ml⁻¹) were determined by using an A20 antibody-based ELISA, as previously described (Grimm et al., 1999). Genomic titres of AAV2 library and vector productions were determined as the number of viral-genome-containing particles (vg ml⁻¹) by qrt-PCR (Veldwijk et al., 2002).

Luciferase reporter assays. Gene transduction by rAAV2 vectors was analysed in a 96-well format via luciferase reporter gene expression as previously described (Naumer et al., 2012).

Cell binding analysis. To analyse the cell binding of rAAV2 vectors, cells were pre-cooled for 20 min at 4 °C prior to the addition of 10 000 vg per cell in serum-free medium. After 1 h at 4 °C, viral DNA was extracted from cells and supernatant, and was quantified via qrt-PCR.

Vector uptake analysis. Cellular uptake of rAAV2 vectors was assessed by incubating the vectors with the respective cells for different time periods at 37 °C, followed by quantifying the internalized vector particles. Briefly, cells were seeded and infected at an m.o.i. of 10 000 vg per cell in serum-reduced medium. At different time points, the vector solution was removed and cells were washed with PBS and subsequently incubated in complete medium in the absence of vectors. All cells were harvested at 96 h post-infection through a 5 min trypsin treatment, followed by quantification of internalized vector.

Vector stability assay. Capsid stability was examined by in vitro treatment of vectors at temperatures between 37 and 74 °C for 5 min followed by the quantification of intact capsids by ELISA. Vectors were furthermore subjected to S7 nuclease treatment (Roche) prior to the quantification of vector genomes by qrt-PCR. The in vivo stability of rAAV2 vectors was determined by the intracellular amount of viral DNA after various time spans after infection. For this purpose, cells were incubated with 10 000 vg per cell for 24 h before viral DNA was extracted from the cells either directly or after an additional incubation in the absence of vector particles over a period of 24–48 h. Cells were harvested by a 5 min trypsin treatment and washed with PBS, before viral DNA was extracted and quantified by qrt-PCR.

Confocal microscopy. Cells were seeded onto glass coverslips and infected with 400 000 vg per cell for 2 h in serum-free medium. The vector solution was subsequently removed and cells were maintained in complete medium for a total of 6, 12 or 24 h. Afterwards, cells were fixed with 2 % paraformaldehyde, permeabilized and viral capsids were detected by staining with mAb A20 (Wistuba et al., 1995) and Alexa 488-conjugated chicken anti-mouse antibody (Molecular Probes). Nuclei were counterstained with DAPI (Sigma-Aldrich).

Cell compartment fractionation. The subcellular distribution of internalized vectors was analysed using the Qproteome cell compartment kit (Qiagen). Cells were cooled down to 4 °C prior to the addition of 1000 vg per cell in serum-free medium. After 1 h, cells were shifted to 37 °C for 2 h to enable vector internalization before the vector solution was exchanged for medium containing no vectors. At 12 h post-infection, cells were harvested by a 5 min trypsin treatment, washed with PBS and cellular fractions were collected using the respective extraction buffers. Vector genomes were purified from each fraction using DNeasy blood & tissue kit (Qiagen) and quantified by qrt-PCR. Purity of cellular fractions was assayed by Western blot analysis of acetonet-precipitated proteins from each fraction by using the following antibodies from Santa Cruz Biotechnology and appropriate secondary antibodies: anti-IκB rabbit polyclonal antibody (C-21) (cytosolic fraction), anti-Rab5 mouse mAb (D-11) (membrane fraction) and anti-Lamin B goat polyclonal antibody (C-20) (nuclear fraction).

Normalization of qrt-PCR. Samples of purified viral DNA were analysed regarding the amount of co-purified genomic DNA via detection of the β-actin gene. The number of rAAV2 vector genomes determined by qrt-PCR was normalized to the mean amount of co-purified cellular DNA in order to compensate for fluctuations in rAAV2 genome copy numbers due to variations in cell number or loss of DNA during purification. In the case of cell-binding analysis, vector genome copies from supernatant and cells (unbound/bound) were adjusted to the mean amount of cellular DNA co-purified from the respective cell pellet. In the case of the subcellular distribution of rAAV2 vectors, viral genome copy numbers of the membrane, cytoplasmic and nuclear fraction were adjusted to the mean amount of cellular DNA co-purified from the nuclear fraction.

Statistical analysis. All data are expressed as means ± SD. To test for statistical significance, an unpaired Student’s t-test was applied. P<0.05 was regarded as significant.

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