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CD9 promotes adeno-associated virus type 2 infection of mammary carcinoma cells with low cell surface expression of heparan sulphate proteoglycans

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Abstract. Recombinant adeno-associated virus (AAV) is a promising non-pathogenic vector in the emerging field of gene therapy. For AAV serotype 2 (AAV-2) infection, experimental evidence points to an involvement of heparan sulphate proteoglycans (HSPG), but also to the existence of additional receptors. We investigated a potential role of the tetraspanin CD9 in AAV-2 infection of breast cancer cells mainly because it binds to the heparin-binding EGF-like growth factor, suggesting that it may also interact with a heparin-binding virus. Among breast cancer cell lines, expression of HSPG or potential AAV-2 (co)-receptors was not found to correlate with transduction efficiency. In complete accordance with the role of CD9, blocking with anti-CD9 antibodies resulted in drastically decreased AAV-2 transduction efficiencies in cell lines with low expression of HSPG. Furthermore, specific inhibition of CD9 expression with siRNA resulted in fewer transgene-positive cells, whereas overexpression of CD9 in the breast cancer cell line T47D as well as in BT8Ca and BT12Ca rat glioma cells (with low background expression of HSPG and CD9) increased the number of AAV-transduced cells. The minimal epitope recognized by antibody 72F6, which most efficiently blocked AAV-mediated transgene expression, was deduced from the specific binding to peptides immobilized on colour-encoded microspheres consisting of the amino acid sequence PKKDV located in the large extracellular loop of CD9. Our results clearly point to an involvement of CD9 in the attachment, uptake or processing of AAV-2 by target cells expressing low amounts of HSPG, which may help to define cell populations accessible in AAV-based therapeutic applications.

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Introduction

Recombinant adeno-associated virus (AAV) is one of the most promising vectors in the emerging field of gene therapy of monogenic diseases and complex pathogenic processes such as the destructive expansion of malignant tumours. Optimized purification processes (1) and the introduction of a plasmid coding for all required adenoviral helper proteins (2,3) allowed the generation of pure high titer stocks of this non-pathogenic, replication defective and, therefore, helper virus-dependent single-stranded DNA virus as a basis for gene transfer experiments and potential clinical application. Despite the rapid increase in reports confirming the feasibility of AAV-based gene therapy in various model systems, few mechanistic details of the cell attachment of AAV, its internalization and intracellular processing have been revealed.

Concerning AAV-2, the most extensively studied AAV serotype, an involvement of heparan sulphate proteoglycans (HSPG) in cell attachment was concluded from the inhibition of AAV binding and AAV-mediated transduction by soluble heparan sulphate (HS; heparin) and confirmed by experiments with cell lines defective in proteoglycan synthesis that showed reduced AAV attachment and transduction (4). Direct binding of AAV serotypes 2 and 3, but not AAV-1 and AAV-5, to a heparan sulphate-coated surface was demonstrated by atomic force microscopy (5). The heparin-binding motif on AAV-2 capsids was identified by its different protein subunits by mutagenesis (6), but AAV variants in which certain arginines determined to be crucial for heparin binding were replaced, still showed substantial infectivity in various tissues. In accordance with these observations, Qiu *et al* (7) reported that both AAV binding and recombinant AAV-2 transduction did not correlate with the amount of HS on the cell surface, as determined by flow cytometric analyses of 23 cell lines. They also determined a low affinity (K_d, ~2 nM) of AAV to heparin and suggested the presence of an additional high-affinity receptor mediating AAV-2 infection. In this concept, all AAV serotypes require

high-affinity receptors for cell attachment and efficient infection whereas low-affinity interaction with cell surface HS supports the viral entry of serotypes 2 and 3. In accordance with a limited role of HSPG, the removal of cell surface HSPG by enzymatic cleavage did not abolish AAV-2 transduction in airway epithelial cells (8).

Three cell surface proteins, integrin $\alpha_V ß_5$, fibroblast growth factor receptor 1 (FGFR1) and hepatocyte growth factor receptor were reported to be AAV-2 co-receptors (9-11). For AAV-5 this role was assigned to the PDGF receptor (12). Although direct binding of AAV-2 to the denatured integrin subunit $ß_5$ was demonstrated in a virus overlay assay (9), neither the interaction of AAV-2 with purified or recombinant integrin $\alpha_V ß_5$ nor the inhibition of AAV-2 transduction by addition of purified integrin, RGD peptides or an anti-integrin antibody was observed by Qiu and Brown (13). On the contrary, endocytosis, but not the attachment of AAV-2 to HeLa cells, was blocked with an anti $\alpha_V ß_5$ antibody, suggesting a major role of this integrin (14). The FGF receptor was considered a candidate AAV-2 co-receptor because of its intimate interaction with HS proteoglycan which is required for the efficient binding of the ligand. Co-transfection experiments in which both HSPG and FGFR1 are expressed in M07e and Raji cells clearly showed that these molecules can cooperatively enhance AAV-2 binding and transduction (10). For which cell types AAV-2 infection depends on the presence of the FGFR1 co-receptor remains to be determined. To summarize the previous work on AAV-2 receptors, cell surface HS clearly plays an important role in various cell types whereas the relative contributions of reported AAV-2 co-receptors still need to be defined for the cell types that are targets for gene therapeutic intervention.

CD9 is the most extensively investigated member of the tetraspanin protein family of adaptor molecules in cell membranes that are involved in numerous fundamental cellular processes and frequently found to be altered during malignant conversion and tumor progression (15). We investigated a potential involvement of CD9 in AAV-2 infection of mammary carcinoma-derived cell lines because: a) tetraspanins have been identified as cell surface receptors for other viruses; the most important example is CD81, the receptor for human hepatitis C virus (16); b) CD9 is part of functional membrane protein complexes with integrins which were suggested to play a role in this process; c) CD9, like HSPG, is ubiquitously expressed; and d) CD9 binds to the HS-binding domain of the heparin-binding EGF-like growth factor (HB-EGF) (17) suggesting that it may also bind to other heparin-binding molecules. In accordance with a role as an AAV-2 co-receptor, we report an inhibition of AAV-2-mediated transduction by blocking with anti-CD9 antibodies and a reduced transduction efficiency after down-regulation of CD9 with inhibitory siRNAs.

Materials and methods

Cell lines. Mammary carcinoma-derived cell lines and epithelial HBL-100 cells, established from the milk of an apparently healthy woman, were initially obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Monolayer cultures were grown at 37˚C in an atmosphere containing 5% CO_2 in RPMI medium (PAA Laboratories, Linz, Austria) supplemented with 10% fetal calf serum (FCS; PAA Laboratories), 2 mM L-glutamine, 3.3 mM arginine, 1.5 mM asparagine, 100 U/ml penicillin, and 100 μg/ml streptomycin (cell lines MDA MB 157, MDA MB 231 and MDA MB 361). This medium was additionally supplemented with 10 μg/ml insulin (cell lines HS 578t, MDA MB 436, and T47D), or with 10 μg/ml insulin and 0.1 mM non-essential amino acids (MCF-7 cells), respectively. Rat neuroectodermal BT8Ca and BT12Ca cells (18), from the collection of cell lines and tumor tissues at the Institute of Cell Biology, University of Essen, Germany, were cultivated in DMEM/10% FCS (PAA Laboratories). Testing of all cultivated cell lines for a potential mycoplasma infection was performed regularly and prior to AAV transduction experiments with a Mycoplasma detection kit (Roche Diagnostics, Mannheim, Germany).

Antibodies and reagents. We used commercially available monoclonal antibodies (mAbs) directed against HSPG (unlabeled and FITC-conjugated; Seikagaku, Tokyo, Japan); CD9 (unlabeled 72F6 from Novocastra, Newcastle, UK and PE-labeled M-L13 from BD Pharmingen, Heidelberg, Germany) and integrin $\alpha_V ß_5$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the previously described mAbs NCA-1 and NCA-4 (19) recognizing both rat and human CD9. In all experiments, the specificity of these antibodies was confirmed by parallel processing with murine isotype-matched control antibodies: IgG_1 and IgG_{2a} from Southern Biotechnology, Birmingham, AL, USA; IgM, FITC-labeled IgM, and PE-labeled IgG from BD-Pharmingen. For epitope mapping with the Luminex-100 system, we used R-phycoerythrin-labeled $F(ab')_2$-fragments of a polyclonal goat anti-mouse IgG (H+L) antibody (Dianova, Hamburg, Germany).

Flow cytometric analyses. For immunofluorescence staining of CD9, integrin $\alpha_V ß_5$ and HSPG, cells were harvested, washed twice with PBS/10% FCS and suspended in 80 μl of this washing buffer (WB) containing 0.8 μg of the fluorochrome-conjugated mAb specific for the cell surface antigen to be determined. After 20 min incubation, cells were washed with 1 ml WB and analyzed after re-suspension in 0.5 ml PBS by means of a FACScalibur fluorescence activated cell sorter (BD Pharmingen) with Cell Quest Pro 2.0 software for data acquisition and analyses. To quantify the expression of cell surface determinants, the antigen-specific mean fluorescence intensity (MFI) was calculated from the MFI of cells stained with a fluorochrome-conjugated specific mAb by subtraction of the MFI of cells incubated with a similarly conjugated isotype-matched control antibody. Cells expressing EGFP after AAV/EGFP transduction were detected by comparison of fluorescence signals with auto-fluorescence of the parental carcinoma cells. Cells showing higher fluorescence intensities than 99% of the non-transduced cells were defined as EGFP-positive.

AAV-2 transduction. Recombinant AAV-2 containing the coding sequence of EGFP (AAV/EGFP), produced and characterized as previously described (20,21), were kindly provided by H. Buening and M. Hallek (University of Cologne,

ell of a 24-well plate and incubated for 24 h in 400 µl of the appropriate medium supplemented with 10% FCS. An amount of infectious AAV resulting in an MOI (multiplicity of infection) of 30 was diluted to a final volume of 100 µl with medium and added to the cells. After incubation for 48 h, cells were pre-evaluated by fluorescence microscopy (Zeiss Axiovert 25), harvested and prepared for flow cytometric assessment of transgene expression. Parallel samples were processed with medium containing additional heparin (Ratiopharm, Ulm, Germany) at a final concentration of 100 U/ml to block HSPG-dependent infection by AAV-2. Potential inhibition of AAV transduction was tested by incubation of the cells for 1.5 h with 100 µl medium containing 1 µg of antibodies specific for CD9, HSPG, or integrin $\alpha_V\beta_5$, before AAV/EGFP (in 400 µl medium) was added.

CD9 silencing with siRNAs. Four CD9-targeting synthetic double-stranded siRNAs (Qiagen, Hilden, Germany) were initially tested and the most efficient one (corresponding DNA target sequence AAGAGCATCTTCGAGCAAGAA) was chosen to analyse the effect of CD9 down-regulation on AAV-mediated transduction. Experimental conditions for efficient siRNA transfection of MCF-7 and T47D cells were optimized with a fluorescein-labeled control siRNA (Qiagen). Cells were seeded at a density of $8x10^4$ cells/well in a 24-well tissue-culture plate and incubated for 24 h. The medium was then replaced for 24 h by 100 µl of fresh medium supplemented with 6 µl RNAiFect (Qiagen) and 67 pmol double-stranded siRNA. After incubation under standard conditions for additional 48 h, AAV/EGFP was added at MOI 30. EGFP fluorescence indicating AAV-mediated transduction as well as red fluorescence of the PE-labelled anti-CD9 antibody bound to the cell surface was measured two (MCF-7 cells) or three days (T47D cells) later by flow cytometry.

Generation of transfectant cell lines. The complete coding region of CD9 cDNA was amplified by PCR from IMAGE clone 5758827 (Invitrogen, Karlsruhe, Germany) with primers containing additional terminal regions for recombinase-mediated insertion into the Creator donor vector pDNRDual (In-Fusion cloning kit, Clontech, Heidelberg, Germany). Recombination of this donor vector with pLPCMVneo in the presence of Cre recombinase (Clontech) according to the supplier's protocol yielded a plasmid for the CMV promoter-driven expression of CD9. This plasmid was transfected as recommended by the manufacturer into recipient cells with Lipofectamine 2000 (Invitrogen). After two days, cells were treated with medium containing 500 µg/ml G-418 (Invitrogen) for two weeks. Remaining clones were harvested and re-cultivated in medium supplemented with 100 µg/ml G-418. Expression of CD9 was determined by flow cytometry and immunofluorescence staining.

Synthesis and immobilization of CD9 peptides. Peptides with purities >70% were synthesized on a multiple peptide synthesizer (Syro II, MultiSynTech) on resins from PepChem (Tuebingen, Germany) using the Fmoc strategy. After every coupling step, the remaining amino groups were capped with acetic anhydride. Cleavage from the resin and deprotection of peptides was performed by treatment with 82.5% trifluoroacetic acid, 5.0% water, 5.0% phenol, 2.5% ethandithiol, and 5.0% thioanisole. The identities of all peptides were confirmed by mass spectrometry on a Micormass ESI-Q-TOF (Waters, Milford, MA, USA). Overlapping 13mers with an offset of 2 amino acids were synthesized to cover the two extracellular domains of human CD9 (amino acids 21-61 and 99-197, Swiss-Prot P21926). Cysteines in 19 of the 59 peptides were replaced by 2-amino butyric acid. All peptides were synthesized as C-terminal amides and, for linking the peptides to a carrier protein, a hydrophilic spacer (8-amino-2,6-dioxa-octanoic acid) and a cysteine were added to the N-terminal ends.

A covalent immobilisation of the peptides to Luminex microspheres was performed in a two-step procedure using standard EDC/NHS chemistry. Carboxylated polystyrene beads (5.6 µm; Luminex, Austin, TX, US.) were coated with BSA to which HS-peptides were attached in a second step. Briefly, 3,750,000 beads were coated with 37.5 µg of bovine serum albumin (BSA; Roth, Karlsruhe, Germany) in a volume of 375 µl according to the manufacturer's protocol. For each coupling reaction, 250,000 BSA-beads were used in one well of a 96-well filterplate (1.2 µm Durapore PVDF; Millipore, Schwalbach, Germany). Beads were initially washed with 100 µl PBS in a vacuum manifold (Millipore). Activation with sulfo-SMPB (Interchim, Montflucon, France; 1.66 mM in 5.0% DMSO/PBS) was performed in a volume of 50 µl in a Thermomixer (Eppendorf, Hamburg, Germany) for 60 min at 23˚C and 650 rpm. Peptides were dissolved in 40% acetonitrile in PBS (~600 µM). By adding 2 volumes of 300 µM tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Fluka, Buchs, Switzerland) in PBS, peptides were then completely reduced in 20 min at 23˚C and 250 rpm. Maleimide-activated beads were washed three times with 100 µl PBS and incubated with the reduced peptide for 60 min at 23˚C and 650 rpm. After washing (three times with 100 µl PBS) the peptide-loaded microspheres were stored in PBS with 1.0% BSA and 0.05% sodium azide.

Epitope mapping. To map epitopes of CD9-specific antibodies, we used a multiplexed suspension array containing 7 or 26 different populations of peptide-coupled beads in assay buffer (PBS with 1.0% BSA). The number of beads of each type was adjusted to 100/µl. Mix 1 contained peptides a1-a15 (small loop) and b1-b11 (large loop), mix 2 contained b12-b37, and mix 3 peptides b38-b44. As a control, beads loaded with a control peptide (c-myc, EQKLISEEDL) were added to each mix.

The mixtures of beads were transferred to 96-well filterplates (1,000 beads of each type per well), mixed with 30 µl of the antibody to be mapped, and incubated for 120 min at 23˚C and 650 rpm. Different antibody dilutions (1:40-1:4000) or concentrations (10.0-0.1 µg/ml) were tested. Beads were washed three times with 100 µl PBS before 0.15 µg PE-labeled detection antibody in 30 µl was added. After incubation for 60 min at 23˚C and 650 rpm, beads were washed again three times and the measurement of bead type-specific fluorescence was performed with the Luminex 100 system.

To confirm that the identified epitopes were recognized, 10 nmol soluble peptides containing the consensus sequence

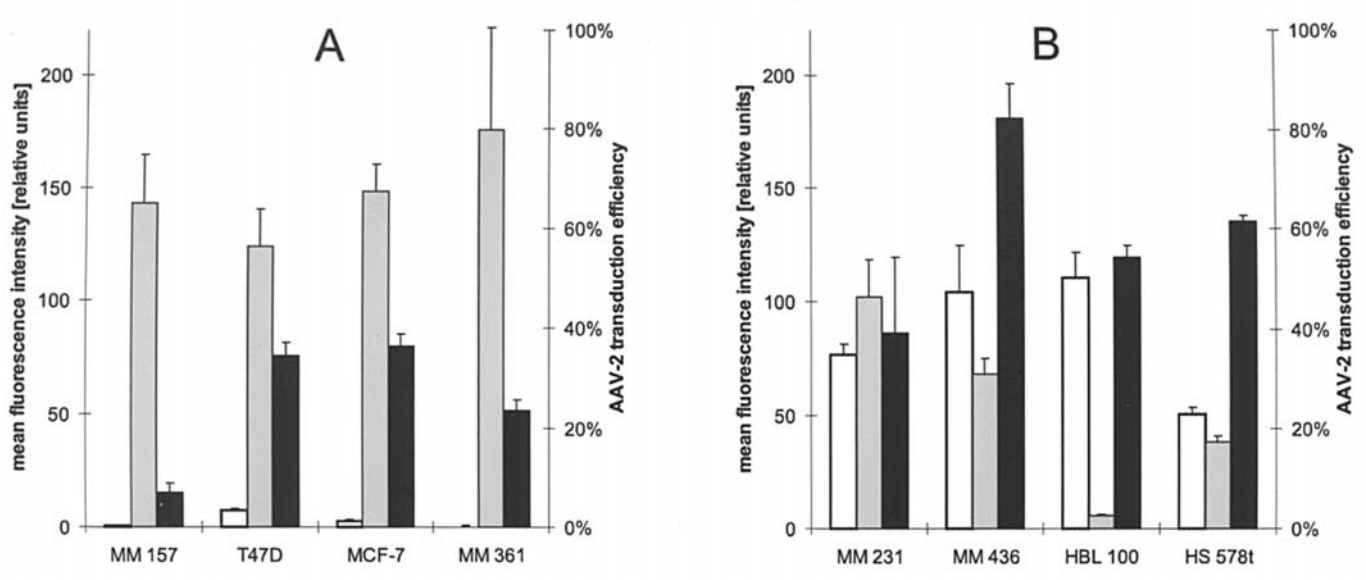

Figure 1. Expression of potential AAV-2 receptors by breast cancer cells. Expression of HSPG (empty bars, left scales of mean fluorescence intensities) and CD9 (grey bars) by mammary carcinoma-derived cell lines and the fractions of EGFP-positive cells after AAV-2-mediated gene transfer in these cells (black bars, right scales) were determined by flow cytometry. The analyzed cell lines were divided into two groups with low (A) or high (B) expression of HSPG.

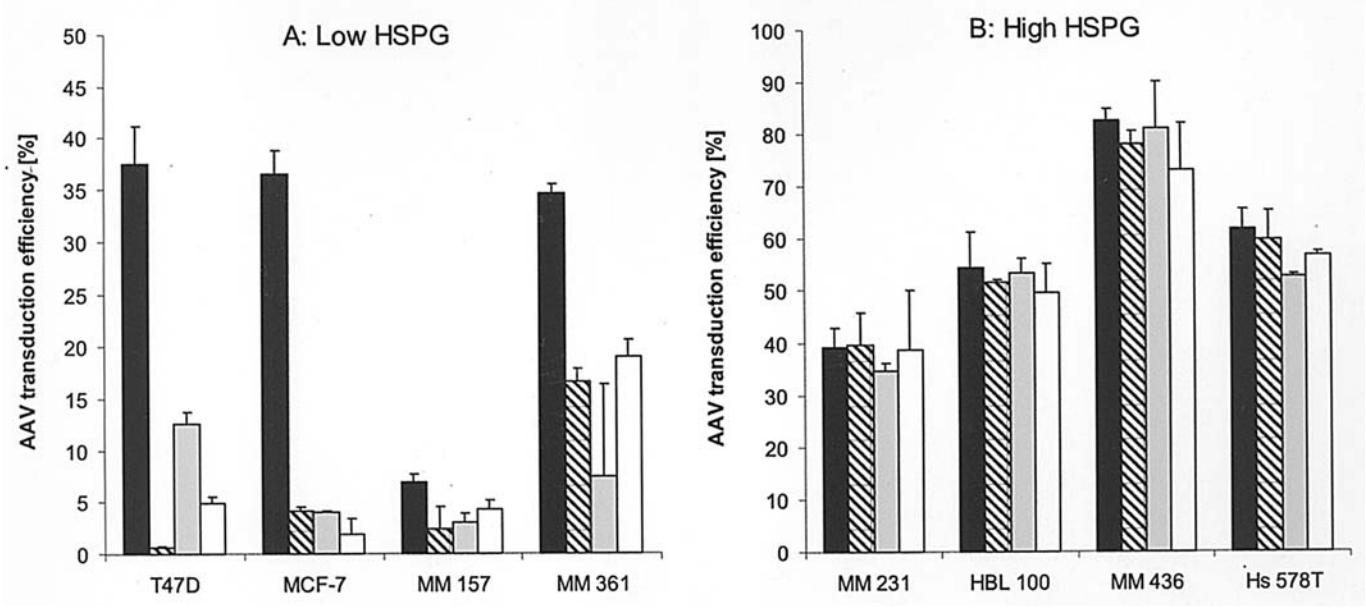

Figure 2. Inhibition of AAV-2-mediated transgene expression with anti-CD9 antibodies. Pre-incubation with anti-CD9 antibodies 72F6 (white bars), NCA-1 (hatched bars) or NCA-4 (grey bars) resulted in a dramatically reduced fraction of transgene-positive cells in the four cell lines showing weak expression of HSPG (A) but not in cell lines strongly expressing HSPG (B). Transduction efficiency was not affected by incubation with an isotype-matched control antibody (black bars).

or randomly selected control peptides were added to suspensions of $2.5x10^5$ cells prior to flow cytometric assessment of cell surface CD9 with 2 μg of the corresponding antibody.

Results

Cell lines derived from malignant breast tumours and HBL-100 cells were initially characterized in terms of susceptibility to AAV-2 transduction and expression of HSPG, other cell surface molecules described to act as AAV-2 co-receptors, and CD9. We observed a high degree of variability of cell surface expression of HSPG and AAV-2-mediated transduction of reporter EGFP. Expression of HSPG or potential AAV co-receptors was not found to correlate with transduction efficiency. Notably, cell lines MCF-7, T47D and MM 361 were efficiently transduced despite low levels of HSPG (Fig. 1A). However, AAV/EGFP transduction of all cell lines which strongly expressed HSPG resulted in a considerable fraction of transgene-expressing cells (Fig. 1B). All analyzed cell lines invariably expressed considerable amounts of the potential AAV co-receptor integrin $\alpha_V ß_5$, whereas FGF receptor I could not be detected on their surfaces by flow cytometry (not shown). This suggests the

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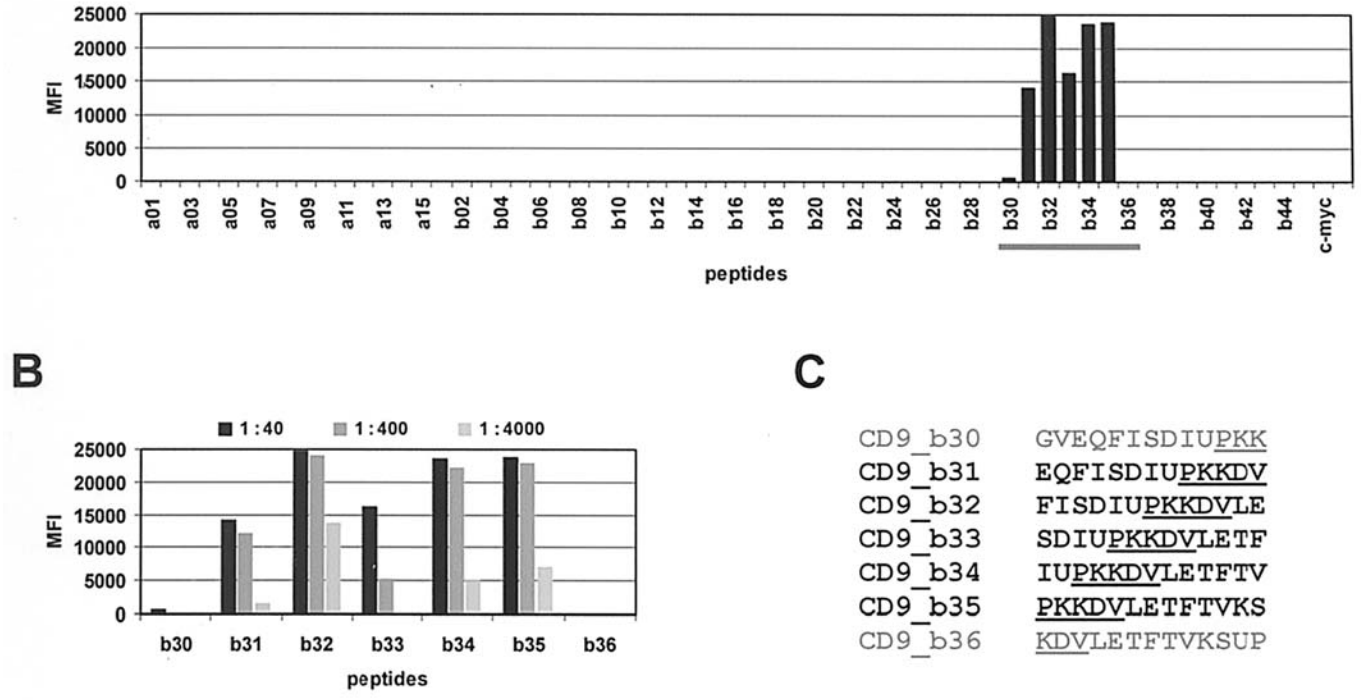

Figure 3. Mapping of the epitope recognized by anti-CD9 antibody 72F6 with a multiplexed suspension array. (A) Interaction of the 72F6 antibody with immobilized CD9 peptides was measured with the Luminex-100 system. For every peptide, mean fluorescence intensity (MFI) signals are shown as black bars. (B) Signals indicating interaction with peptides b30 to b36 at three different antibody dilutions. (C) Amino acid sequences (containing Us for cysteines replaced by 2-amino butyric acid) of peptides b30 to b36. The putative minimal epitope (PKKDV, amino acids 167-171) within the second extracellular loop of CD9 is underlined.

existence of an alternative receptor involved in cell surface attachment of AAV-2 to cells displaying low amounts of HSPG.

Inhibition of AAV-2 transduction with anti-CD9 antibodies. CD9 is a member of the tetraspanin family of adaptor proteins of which several have previously been identified as cell surface determinants involved in the attachment of other viruses. Therefore, CD9 was considered a candidate receptor potentially utilized by AAV-2 for infection of MCF-7, T47d and MM361 cells, and the effects of anti-CD9 antibodies on AAV-2 transduction were determined as described in Materials and methods.

Despite expression of only small amounts of HSPG in their plasma membranes, AAV transduction of MCF-7, T47D and MM361 cells at MOI 30 resulted in fractions of transgene-positive cells between 24% and 37%. In these cells, AAV-mediated expression of EGFP was considerably reduced by pre-incubation with three different anti-CD9 monoclonal antibodies (NCA-1, NCA-4, and 72F6) whereas transduction efficiency was not affected by isotype-matched control antibodies (Fig. 2). Inhibition of AAV-2-mediated transgene expression was also observed in MM 157 cells, a breast cancer cell line weakly expressing HSPG which showed a low susceptibility to AAV-2 transduction. In contrast, AAV-2 transduction of all HSPG strongly expressing cell lines was very efficient and not influenced by anti-CD9 antibodies (Fig. 2B).

Mapping of the epitope recognized by blocking antibody 72F6. To identify the epitope recognized by the anti-CD9 antibody 72F6 which most efficiently blocked AAV-mediated transgene expression, we synthesized a set of overlapping peptides covering both extracellular loops of CD9. These peptides were immobilized on colour-encoded microspheres and combined to multiplexed suspension arrays used to detect antibody-specific binding to peptides. At different antibody concentrations, strong fluorescence signals (up to 24,000 relative units) were observed for a series of five overlapping peptides (b31 to b35, amino acids 159-179) in the large extracellular loop (Fig. 3A). Signal intensities for all other peptides were low (<45 units) in the range of the background fluorescence. Signals for peptides b31 to b35 were not observed for other tested antibodies, and the blank control signals (peptide-coupled beads plus detection system only) for all beads were at background level (data not shown), indicating that the observed antibody-peptide interaction was specific. Furthermore, the intensities of the specific signals but not the sort of interacting peptides were dependent on the concentration of the antibody in the assay (Fig. 3B). The putative minimal epitope recognized by antibody 72F6 was deduced to consist of the amino acid sequence PKKDV (Fig. 3C). Accordingly, the addition of peptides b32 (FISDIUPKKDVLE) and b34 (IUPKKDVLETFTV) interfered with binding of mAb 72F6 to MCF-7 and T47D cells, as indicated by a reduction of CD9-specific fluorescence to 3.3% and 20.5%, respectively. Control peptides chosen

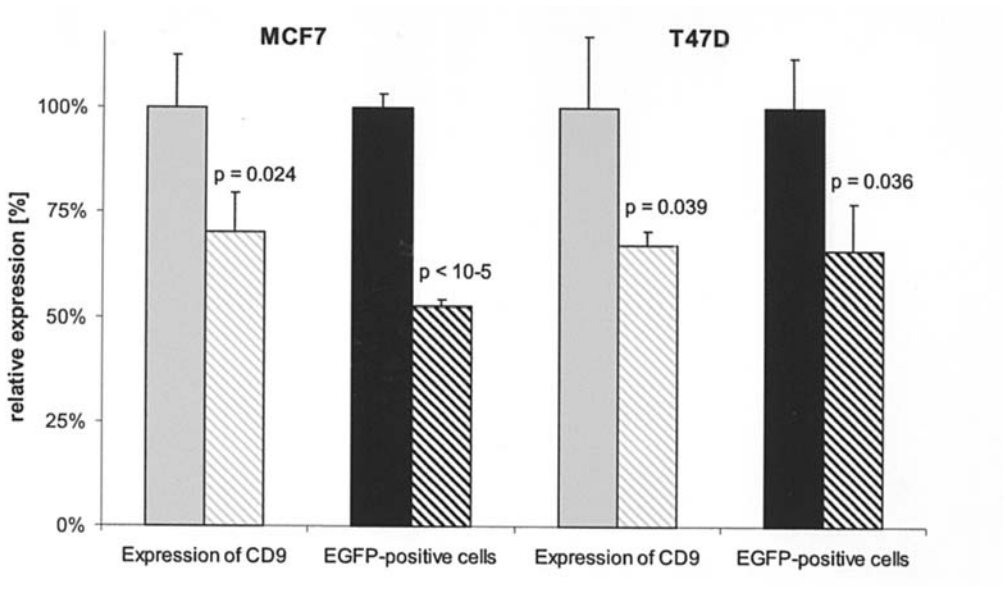

Figure 4. Down-regulation of cell surface expression of CD9 in MCF-7 and T47D cells resulted in reduced AAV-2 transduction efficiency. Expression of CD9 (filled grey bars) and AAV-2 transduction efficiency (filled black bars) were not affected by control siRNA. After treatment with CD9-specific siRNA, the observed reduction of both cell surface expression of CD9 (hatched grey bars, measured by flow cytometry) and of the fraction of transgene-expressing cells (hatched black bars) was significant, as calculated with the unpaired, Two-tailed t-test.

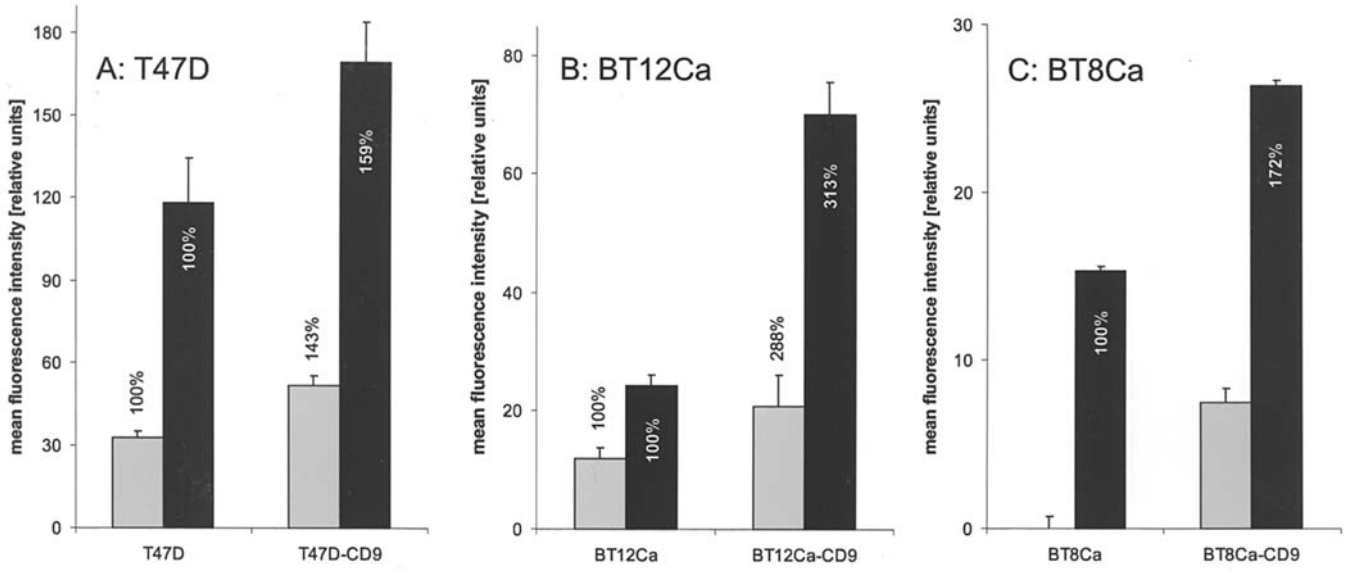

Figure 5. Overexpression of CD9 in T47D breast cancer cells and rat glioma cells (BT8Ca, BT12Ca) resulted in an increased AAV-2 transduction efficiency. (A) Despite considerable basal expression, an increased cell surface expression of CD9 corresponded with a higher susceptibility to AAV-2 transduction as shown for one representative clone. (B) Overexpression of CD9 in BT12Ca cells increased AAV-2-mediated transgene expression. (C) Overexpression of CD9 in BT8Ca cells lacking this tetraspanin increased AAV-2-mediated transgene expression. Specific mean fluorescence intensities indicating expression of CD9 (grey bars) and of transgenic EGFP (black bars) were measured by flow cytometry. Also shown for each pair of values is the calculated relative increase.

from the first (a15, NNNNSSFYTGVYI) and second (b40, VKSUPDAIKEVFD) extracellular loop of CD9 did not significantly affect anti-CD9 immunoreactivity.

CD9 silencing decreased AAV transduction rates. Breast cancer cell lines MCF-7 and T47D were treated with synthetic double-stranded siRNA to investigate the effect of CD9 down-regulation on AAV susceptibility. Compared to control cells processed in parallel, the amounts of cell surface CD9, determined by flow cytometry after transfection with specific siRNA, were considerably reduced to 70% and 67%, respectively. AAV-mediated transduction was reduced likewise to 53% and 66% (Fig. 4) supporting a role of CD9 in this process.

Overexpression of CD9 increased AAV transduction rates. The breast cancer cell line T47D was chosen for the generation of CD9 overexpressing transfectant cell lines despite a high basal expression and AAV transduction rate because effects of anti-CD9 antibodies and CD9-specific siRNA on these cells

observed. Increased cell surface expression of CD9 transfectant T47D cells correlated well with the higher number of EGFP-positive cells after AAV/EGFP transduction (Fig. 5A). In addition, we generated a set of CD9-overexpressing cell lines originating from BT8Ca and BT12Ca rat glioma cells which were reported to lack expression of CD9. Immunofluorescence staining confirmed that CD9 was not present on the cell surface of BT8Ca cells and only weakly expressed by BT12Ca cells. As observed for the T47D line, CD9-transfectant BT8Ca and BT12Ca cells that expressed substantial amounts of CD9 on their surfaces also increased their susceptibility to AAV-transduction (Fig. 5B and C).

Discussion

Adeno-associated viruses are promising vehicles for therapeutic gene transfer in a wide variety of cell types. For several of the identified AAV serotypes, the mechanisms of initial attachment to the cell surface and subsequent internalisation of the viral particles and expression of a transgene have been suggested (22). Convincing evidence has been published indicating that HSPG is generally involved in AAV-2 binding to cells (23,6). In addition, co-receptors appear to be needed and, especially for AAV-2 infection of cells expressing low amounts of HSPG, alternative routes have to be revealed. Analyses of the expression of previously proposed AAV-2 (co)-receptors integrin $\alpha_V ß_5$ and FGFR-1 (9,10) and their interpretation in view of the observed host range of AAV-2 suggest that additional cell surface molecules mediating attachment and entry of AAV-2 might exist. We found such a discrepancy in a set of mammary carcinoma-derived cell lines (Fig. 1) and investigated a potential role of the candidate receptor/co-receptor CD9. In complete accordance with the proposed role of CD9 in AAV-2 transduction, blocking with anti-CD9 antibodies resulted in drastically decreased transduction efficiency in two of the tested cell lines which was also achieved by specific inhibition of CD9 expression by treatment with siRNA. Furthermore, overexpression of CD9 in the breast cancer cell line T47D and in rat neuroectodermal cell lines BT8Ca and BT12Ca (with a low background expression of endogenous CD9) increased the number of AAV-transduced cells.

Inhibition of AAV-2-mediated transduction by CD9-specific antibodies was not observed in cell lines with strong cell surface expression of HSPG. In these cell lines, binding of AAV particles to HSPG apparently results in their CD9-independent attachment and subsequent internalization. When a threshold value of HSPG expression is exceeded, sufficient internalization of attached AAV might be provided even by un-triggered endocytotic processes of membrane homeostasis. For AAV-mediated transduction of mammary carcinoma cells with low expression of HSPG, specific receptors associated with efficient mechanisms of membrane transition may play an important role. Very strong suppression of AAV-mediated transduction in two cell lines by treatment with anti-CD9 antibodies suggests that this tetraspanin is critically involved in the HSPG-independent attachment of AAV to the cell surface, its internalization or intracellular processing. In accordance with this assumption, CD9 gene silencing resulted in a decrease, and overexpression of CD9 in an increase of the AAV transduction rate. In view of previously described functions of members of the tetraspanin protein family, an involvement of CD9 in all steps of AAV transduction seems possible. So far the most extensive investigation of a virus-tetraspanin interaction was initiated by Pileri *et al* (16) who reported direct binding of the hepatitis C virus (HCV) envelope protein E2 to the major extracellular loop of CD81. Subsequent analyses with a HIV pseudotyping system (24) and other HCV pseudotype particles displaying the E1/E2 glycoproteins confirmed the critical role of CD81 in HCV infection of heptatocytes but also supported the assumption that CD81 alone is not sufficient to allow viral entry into hepatocytes. In a similar approach, inhibition of viral entry by an anti-CD81 antibody was demonstrated to take place after initial attachment of HCV to the cell surface requiring a yet unidentified primary receptor. Recently, a role of the tetraspanin CD63 in CCR5-mediated human immunodeficiency virus 1 infection of macrophages has been suggested, but in this case, productive infection was blocked by treatment with an anti-CD63 antibody at a later stage between virus-induced cell fusion and reverse transcripton (25). Antibodies binding to CD9 were previously reported to inhibit productive infection with feline immunodeficiency virus (26,27) and canine distemper virus (28,29). Initial mechanistic analyses indicated that the attachment and uptake of these viruses are not affected by anti-CD9 antibodies, and an interference with FIV assembly or release was suggested. This would be in accordance with an emerging body of evidence that tetraspanins are localized not only in the plasma membrane, but also in different types of vesicles that contribute to intracellular trafficking (30-32). Although we found a clear correlation of AAV transduction and the amount of cell surface CD9 determined by flow cytometry, when CD9 expression was modulated, similarly regulated intracellular CD9 may have contributed significantly to the observed effects. However, CD9 can modulate ligand-receptor interactions, e.g. as a protein directly bound to the diphtheria toxin receptor HB-EGF (33), and other cell surface processes in complexes with other proteins. The exact role of CD9 in AAV-mediated transduction remains to be resolved. Our initial observations do not allow us to conclude whether CD9 is a primary AAV-2 binding site on the cell surface or an AAV-2 co-receptor, similar to CD81 in HCV infection, or is involved in intracellular trafficking, as suggested for interactions of CD9 with FIV and CDV.

We clearly identified the binding site of the anti-CD9 antibody that efficiently blocked AAV transduction in the large extracellular loop of CD9. In initial experiments (not shown), direct binding of AAV-2 to the immobilized peptides used to map the epitope could not be demonstrated, indicating that there is a native binding site not represented by single short peptides or interference of the antibody with an associated primary AAV receptor. Assuming that there is a yet unidentified primary high-affinity AAV-2 receptor, an involvement of a tetraspanin adaptor protein in formation of functional protein complexes at the cell surface is an attractive hypothesis. Among the identified binding partners of CD9, integrins have properties and expression profiles in accordance with an AAV receptor function. Targeting integrins with

modified AAV-2 particles containing RGD motifs resulted in increased HSPG-independent AAV transduction (34) and it will be important to learn if antibodies binding to CD9 can interfere with the binding and uptake of such modified viruses.

AAV is an ideal vector for the therapeutic transfer of genes into a variety of cell types that is currently under evaluation in preclinical and early clinical trials. Identification of CD9 as a cell surface molecule critically involved in AAV-2 transduction will most likely help to define fields for potential therapeutic applications and provide a rationale for the design of novel vectors containing binding motifs for CD9 binding partners expressed by target cells. The frequently observed down-regulation of CD9 during progression of malignant diseases (35,36) might be a limiting factor for approaches with AAV-2 in cancer therapy, supporting decisions in favour of CD9-independent AAV serotypes, variants, or other viral vectors.

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