Identification of PDGFR as a receptor for AAV-5 transduction

Giovanni Di Pasquale, Beverly L Davidson, Colleen S Stein, Inês Martins, Dominic Scudiero, Anne Monks & John A Chiorini

Understanding the process of vector transduction has important implications for the application and optimal use of a vector system for human gene therapy. Recent studies with vectors based on adeno-associated virus type 5 (AAV-5) have shown utility of this vector system in the lung, central nervous system, muscle and eye. To understand the natural tropism of this virus and to identify proteins necessary for AAV-5 transduction, we characterized 43 cell lines as permissive or nonpermissive for AAV-5 transduction and compared the gene expression profiles derived from cDNA microarray analyses of those cell lines. A statistically significant correlation was observed between expression of the platelet-derived growth factor receptor (PDGFR-α-poly peptide) and AAV-5 transduction. Subsequent experiments confirmed the role of PDGFR-α and PDGFR-β as receptors for AAV-5. The tropism of AAV-5 in vivo also correlated with the expression pattern of PDGFR-α.

Gene transfer vectors based on AAV combine a number of attractive features. AAV is naturally defective for replication and is considered nonpathogenic. It can transduce both mitotic and post-mitotic cells, transfer genes to a number of cell types efficiently and mediate long-term gene expression.

The AAVs (genus Dependovirus) were originally classified according to size, structure and dependence upon a helper virus for replication. Because the majority of AAV isolates were first identified as contaminants of laboratory stocks of adenovirus, little is known about their natural tissue tropism. Currently, eight isolates have been cloned and their initial characterization indicates that each serotype has unique binding and cell tropism characteristics. Most preclinical studies and current phase 1 clinical trials use vectors derived from AAV-2. This serotype has a broad tropism in vitro and can transduce both dividing and post-mitotic cells. AAV-2-permissive cells express a number of factors, including heparin sulfate proteoglycan, fibroblast growth factor receptor-1, the β3 subunit of αvβ3 integrin and a novel 150-kDa protein.

Other serotypes are potentially useful vectors for gene transfer applications because of their improved transduction efficiencies in vitro compared with AAV-2, and their resistance to AAV-2-neutralizing antibodies. Vectors based on AAV-5 can more efficiently transduce cells in the central nervous system, eye, muscle and lung compared with AAV-2 (refs. 12–15). The difference in transduction efficiencies suggests a difference in the mechanism of uptake for these serotypes, which is supported by in vitro studies and structural comparison of the viral capsids.

Screening of patient samples for AAV-5-neutralizing antibodies revealed a very low level of seropositive individuals in a normal volunteer sample population. Unlike AAV-2, AAV-5 transduction is not inhibited by soluble heparin and requires α2,3-N-linked sialic acid for efficient binding and transduction. While a number of other viruses require an interaction with a charged carbohydrate such as sialic acid or heparan sulfate for infection, membrane proteins may also be required for efficient binding and cell entry.

To identify proteins involved in AAV-5 transduction, we compared the relative transduction efficiency for AAV-5 with gene expression data from cDNA microarrays developed for a panel of human tumor cell lines. These data are available from the Developmental Therapeutics Program (DTP) at the National Cancer Institute website (http://dtp.nci.nih.gov/). A statistically significant correlation was observed between PDGFR expression and permissiveness for AAV-5 transduction. In this study, we report that modulation of PDGFR expression by transfection of expression plasmids or treatment with inhibitors or competitors alters virus binding and transduction. In addition, coprecipitation experiments indicate a direct interaction between AAV-5 and PDGFR-α. This study confirms the usefulness of comparative microarray technology as an adjunct for functional genomic studies and gene discovery. In addition, these observations should have important implications in defining the use of AAV-5 vectors for gene transfer.

RESULTS

PDGFR expression correlates with AAV-5 transduction

Our previous research showed that cell surface α2,3-N-linked sialic acid is required for efficient transduction with AAV-5 vectors. To identify proteins involved in AAV-5 transduction, we attempted...
to correlate gene expression profiles in AAV-5-permissive and nonpermissive cells using cDNA microarrays. The DTP has carried out extensive biochemical and genetic profiling of 60 human tumor cell lines (NCI60 panel). Forty-three of the adherent cell lines from the panel were transduced with serially diluted recombinant AAV-5 vectors containing the Rous sarcoma virus long terminal repeat promoter and a nuclear-localized β-galactosidase reporter gene (RSV-NLS-LacZ). Sixty hours after transduction, cells were stained for β-galactosidase. The relative transduction efficiencies of the 43 cell lines were expressed as a pattern (Fig. 1), which we then used as a seed file for comparison to the patterns in the DTP database using the COMPARE algorithm. This algorithm determines the similarity of patterns between the given ‘seed’ and others within a database by creating a scalar index of similarity expressed quantitatively as a Pearson correlation coefficient. The output of the COMPARE program is a rank-ordered list (by Pearson correlation coefficient) of the most highly correlated patterns from the database. Available databases include drug response databases, molecular target databases of protein or gene expression, and a searchable database of microarray data. The microarray database contains gene expression values of approximately 10,000 genes or transcripts in the 60 cell lines, based on comparative hybridization of cDNA from the cells to cDNA transcripts on a microarray chip, relative to expression of pooled cDNA from 12 cell lines. A statistically significant correlation was observed between the pattern of AAV-5 transduction and the level of expression of PDGFR-α in the microarray database (Fig. 1). Statistical analysis calculated a Pearson correlation coefficient of 0.5, with a statistical significance of $P < 0.0005$.

PDGFR-α and PDGFR-β belong to a subgroup of receptor tyrosine kinases that includes stem cell factor receptor, c-Kit, colony-stimulating factor-1 (CSF-1) receptor and c-Fms. These receptor tyrosine kinases contain an extracellular ligand-binding portion containing immunoglobulin-like regions, a transmembrane domain and an intracellular kinase region. The extracellular domains of PDGFR-α and PDGFR-β are structurally and functionally similar but differ in ligand binding. Whereas the ligand PDGF-BB can bind and trigger activation of either PDGFR-α or PDGFR-β, the ligand PDGF-AA can only bind and trigger activation of PDGFR-α. Binding of PDGF to its receptor is followed by internalization and degradation of the ligand-receptor complex.

The α- and β-forms of the receptor have different expression profiles in the 43 cell lines tested, and comparison of AAV-5 transduction efficiency with PDGFR-β expression did not show a significant correlation (Fig. 1). However, either PDGFR-β or PDGFR-α was expressed in 100% of the AAV-5-permissive cell lines examined in the NCI60 panel (Fig. 1).

**PDGFR inhibition blocks AAV-5 transduction and binding**

To test the correlation of PDGFR expression with AAV-5 transduction, several permissive cell lines from the NCI60 panel were treated with SB203580, a p38 mitogen-activated protein kinase (MAPK) inhibitor reported to suppress transcription of PDGFR-α.
in rat lung fibroblasts. Western blots were prepared using equivalent numbers of cells from three cell lines in the panel list (no. 14, H9578T; no. 38, SNB19; and no. 43, SF295). Cells treated overnight with SB203580 (6 μM) confirmed the downregulation in expression reported for PDGFR-α. PDGFR-β expression was also inhibited in the H9578T and SF295 cells that expressed PDGFR-β (Fig. 2a). Actin was used as a loading control (Fig. 2a). Treatment of cells with SB203580 also inhibited AAV-5 transduction and binding (Fig. 2b,c). Before treatment, all three cell lines had similar transduction efficiencies with AAV-5 vectors (3 × 10^5 transducing units per ml). After treatment with SB203580, this titer decreased three- to sixfold for all cell types (Fig. 2b). Binding of AAV-5 to the cell surface also decreased threefold for all cell types after treatment with SB203580 (Fig. 2c). AAV-2 has been shown to enter cells by a pathway distinct from that used by AAV-5 (ref. 2), and treatment of the cells with SB203580 did not inhibit AAV-2 transduction or cell binding (Fig. 2b,c). Therefore, whereas treatment with SB203580 inhibited PDGFR-α and PDGFR-β expression and AAV-5 transduction, it did not decrease AAV-2 transduction.

Overexpression of PDGFR increases AAV-5 transduction
Inhibition of p38 MAPK activity by treatment with SB203580 can affect the expression of a number of mRNA transcripts or proteins in the cell. To test more specifically the effects of PDGFR-α or PDGFR-β expression on AAV-5 transduction, HeLa cells were stably transfected with expression plasmids for PDGFRα or PDGFRβ, and polyclonal pools of cells were tested for a change in transduction efficiency. HeLa cells are transduced by AAV-5 vectors, but at a lower efficiency compared with Cos cells or HeLa cells transfected with AAV-2 (ref. 2). Transfection with the PDGFRα and PDGFRβ plasmids increased AAV-5 transduction efficiency six- to tenfold, respectively, compared with the parental cells (Fig. 3a). In contrast, AAV-2 transduction efficiency was unchanged compared with parental cells (Fig. 3a). We have also tested stable transfectants of porcine aortic endothelial cells and A549 cells engineered to express either PDGFR-α or PDGFR-β, and observed similar results (data not shown).

The interleukin-3-dependent 32D myeloid progenitor cell line does not express endogenous PDGFR-α or PDGFR-β. Engineered cell lines expressing either receptor have been extensively studied as model systems for the characterization of the PDGF signal transduction pathway. Transduction of these cells with recombinant AAV-5 expressing RSV-GFP (1,000 DNAse-resistant particles (DRP) per cell) showed a strong dependence on PDGFR expression. Transduction efficiency substantially increased in cells expressing PDGFR-α (32D-αR) or PDGFR-β (32D-βR), compared with parental cells (Fig. 3b-d). In addition, the 32D cells did not express endogenous epidermal growth factor receptor (EGFR) tyrosine kinase. Like PDGFR, EGFR has a large extracellular domain that is glycosylated and contains sialic acid. In contrast to the increase in transduction efficiency we observed with 32D cells transfected with PDGFR-α or PDGFR-β, the transduction efficiency of 32D-EGFR cells was similar to that of parental 32D cells (Fig. 3b,e). This result suggests that cell-surface sialic acid alone is not sufficient for AAV-5 transduction.

Figure 3 AAV-5 transduction of HeLa and 32D cells expressing PDGFR-α, PDGFR-β or EGFR. (a) Polyclonal populations of HeLa cells expressing either PDGFR-α (αR) or PDGFR-β (βR) were selected with G418 and assayed for changes in transduction efficiencies compared with parental cells using either AAV-5 or AAV-2 (n = 3). WT, wild type. (b–e) Parental 32D cells (b) or 32D cells expressing PDGFR-α (c), PDGFR-β (d) or EGFR (e) were transduced with AAV-5 RSV-GFP. Equivalent numbers of cells were mounted on glass slides and photographed with a fluorescent microscope 48 h after transduction. Original magnification, ×100.

Figure 4 Ligand inhibition of AAV-5 binding and transduction. (a) Binding assays were conducted by incubating equal numbers of 32D-αR or 32D-βR cells with an excess of PDGF-AA followed by chilling of the cells and addition of recombinant AAV. Bound virus was measured by Q-PCR using a primer-probe set specific for the viral genome (n = 4). +, ligand-treated; –, mock-treated. (b) Fold change in transduction was determined by incubating cells with ligand, followed by addition of recombinant AAV-5 RSV-GFP (n = 3). (c) For blocking experiments, AAV-2, AAV-4 or AAV-5 was incubated with purified soluble PDGFR-α (3 μM) for 20 min before addition of virus (n = 3).
recombinant His-tagged PDGFR-α produced in 293 cells. After incubation with the virus, the beads were washed and bound virus was quantitated by Q-PCR (Q-PCR) using primers specific for the reporter gene cassette. As a control, the amount of bound virus was also quantitated from beads incubated with membranes from untransfected 293 cells (n = 3).

binding and transduction, and that AAV-5 transduction is specific for PDGFR expression. No transduction with AAV-2 was observed with any of the 32D cells tested (data not shown).

**PDGF-AA or sPDGFR-α block AAV-5 binding and transduction**

Ligand binding to PDGFR-α triggers activation and internalization in 32D-αR cells, reducing the amount of receptor on the cell surface. To test whether the addition of ligand could block AAV-5 binding to the cell surface, 32D-αR cells were preincubated with PDGF-AA (12 nM), followed by chilling and the addition of recombinant AAV-5 expressing RSV-GFP (5,000 DRP/cell). After extensive washing, the amount of followed by chilling and the addition of recombinant AA V-5 expressing RSV-GFP (5,000 DRP/cell). After extensive washing, the amount of

... PDGF-AA ligand is specific for PDGFR-α. The ligand binds and triggers internalization of PDGFR-α, but does not bind or trigger activation of PDGFR-β2. To test the specificity of PDGF-AA inhibition, 32D-βR cells were treated with PDGF-AA and assayed for a change in AAV-5 binding and transduction. As with the 32D-αR cells, ~130 particles/cell bound to the surface of the untreated cells. In contrast to the results with the 32D-αR cells, however, treatment of 32D-βR cells with PDGF-AA did not decrease, but rather slightly increased, AAV-5 binding compared with untreated cells (Fig. 4a). No change was observed in the transduction of 32D-βR cells with AAV-5 after treatment with PDGF-AA (Fig. 4b).

Figure 5 AAV and PDGFR-α coprecipitation. Recombinant AAV-2 or AAV-5, containing an RSV-β-galactosidase expression cassette, was incubated with recombinant His-tagged PDGFR-α produced in 293 cells. After incubation with the virus, the beads were washed and bound virus was quantitated by Q-PCR with primers specific for the reporter gene cassette. As a control, the amount of bound virus was also quantitated from beads incubated with membranes from untransfected 293 cells (n = 3).

The addition of a soluble form of PDGFR-α had a similar effect on AAV-5 transduction. Preincubation of AAV-5 vector with 3 μM of the purified extracellular domain of PDGFR-α, produced in mouse cells, inhibited AAV-5 transduction of Cos cells fourfold compared with cells incubated with media alone (Fig. 4c). The inhibition was specific for AAV-5 and did not block the transduction of AAV-2 or AAV-4, another sialic acid–binding AAV isolate (Fig. 4c).

Although the data presented thus far strongly suggest a direct interaction between PDGFR and AAV-5, PDGFR could be functioning as part of a complex or using the same trafficking pathway as PDGFR, and not directly interacting with AAV-5. To assay for direct interaction between AAV-5 and PDGFR, a C-terminal 6xHis-tagged version of full-length PDGFR-α was transfected into 293 cells, and the recombinant protein was isolated from the membrane fraction using Ni-NTA His-Bind beads. The PDGFR-α-coated beads were then incubated with purified recombinant AAV-2 or AAV-5 viral particles both containing RSV-NLS-LacZ expression cassette. After incubation with the virus, the beads were washed and the bound virus was quantitated by Q-PCR with primers specific for the reporter gene cassette. As a control, the amount of bound virus was also quantitated from beads incubated with membranes from untransfected cells. Compared with the control lysate or AAV-2, there was a six- to tenfold increase in the amount of bound AAV-5 when incubated with the PDGFR-α-transfected membrane lysates (Fig. 5). The binding of AAV-4 particles to the PDGFR-α-coated
beads was at background levels (data not shown). Thus, AAV-5 does seem to directly interact with PDGFR-α.

**PDGFR-α expression and AAV-5 transduction in brain**

Our earlier research showed that AAV-5 can mediate gene transfer to extensive areas of the brain after direct intraparenchymal injection. We therefore assessed whether transduced cells expressed PDGFR-α. Hippocampi of mice were injected with 1 μl of recombinant AAV-5 with the RSV-NLS-LacZ (1 × 10^9 DRPs). Fifteen weeks later, we analyzed hippocampal sections for β-galactosidase and PDGFR-α expression using immunofluorescence. AAV-5 efficiently transduced neurons throughout the CA1 to CA3 regions and the dentate gyrus of the hippocampus (Fig. 6a–e). These transduced cells also stained robustly with PDGFR-α-specific antibodies. Quantitation of cells in the dual-stained sections showed that >75% of the AAV-5-transduced cells also expressed PDGFR-α. Together, these in vivo and in vitro data suggest that PDGFR-α has an important role in allowing AAV-5 entry into cells.

**DISCUSSION**

The development of cDNA microarrays has permitted the investigation of global gene expression patterns within a cell and allowed for comparison between cell populations, as demonstrated by the National Cancer Institute's anticancer drug screening of a panel of 60 human tumor cell lines. To determine whether the permissiveness of cell lines to AAV-5 transduction could be associated with expression of a particular target in the cell, we used the well-documented correlative analysis COMPARE (http://dtp.nci.nih.gov/docs/compare/compare.html). This tool allows for available patterns of relative drug response or gene expression to be correlated across 60 human tumor cell lines. Previously, this approach has uncovered a correlation between multidrug resistance-1 expression and rhodamine efflux, and between a mutant allele of the RAS oncogene and sensitivity to cytosine arabinoside and certain topoisomerase II inhibitors.

Using that approach, PDGFR-α was identified as being potentially associated with AAV-5 transduction and was confirmed in follow-up experiments as a crucial factor for mediating AAV-5 transduction. We believe this is the first time that COMPARE has been used to identify a new function for a gene, and demonstrates its usefulness as an adjunct to functional genomic studies.

AAV-5 can effectively transduce central nervous system, eye and lung cells, which correlates well with the tissue distribution of PDGFR. PDGFR-β is highly expressed in uterus and ovary. Previous work suggested that herpes simplex virus could function as a helper virus for AAV-5, which is also tropic for these tissues, and further supported the circumstantial association previously reported for AAV-5 serum conversion and herpes simplex virus infection. Based on the PDGFR expression profile, AAV-5 may also be a useful vector for gene therapy applications in pancreas, liver, spleen, adrenal glands and some types of Burkett lymphoma. Previously, we reported that AAV-5 efficiently transduced Purkinje cells in the cerebellum, and in the present study we show high-level transduction of hippocampal neurons. These findings correlate well with the expression pattern of PDGFR-α by neurons in adult mouse brain and further support the use of this vector in therapies directed at neurodegenerative diseases. PDGFRs are sialoglycoproteins containing both N-linked and O-linked oligosaccharide chains with sialic acid. Our previous work showed that cell-surface α-2,3-N-linked sialic acid is important for AAV-5 binding and transduction and can be blocked by treatment with neuraminidase. In contrast, PDGF-AA binding to PDGFR-α does not require sialic acid or the carbohydrate portion of the receptor, and it is insensitive to neuraminidase. Therefore, AAV-5 may bind these receptors in a manner distinct from the ligand. Alternatively, the requirement for sialic acid may indicate that an interaction of AAV-5 with an additional protein is required for efficient binding and transduction.

Although the inefficient binding and poor transduction by AAV-5 in cells that do not express PDGFR suggests that PDGFR could mediate AAV-5 attachment and entry, it remains to be determined whether this interaction is sufficient for viral entry or whether other events are necessary for entry.

Transduction is a complex process. Although our data does show a direct interaction between PDGFR and AAV-5 particles, other factors may be necessary for efficient transduction. Additional cellular interactions required for AAV transduction include escape from the endosome, nuclear entry, uncoating and second-strand synthesis. Inhibition at any of these steps could result in a nonpermissive phenotype or poor transduction efficiency despite expression of PDGFR, which we propose has a role in the early stages of transduction. As noted earlier, other proteins may also function as receptors for AAV-5. PDGFR-β is one example for which we have confirmed a role in AAV-5 transduction. For both AAV-2 and adenovirus, an interaction with integrins is important for viral entry. After activation with ligand, PDGFR is internalized and could therefore be responsible for binding and internalization of AAV-5. PDGFR is known to interact with other proteins, so PDGFR expression alone may not be sufficient for AAV-5 binding and internalization.

The intracellular trafficking of PDGFR resembles that of AAV-5. PDGFR is internalized in clathrin-coated vesicles that concentrate near the nucleus and are associated with the Golgi apparatus. Similarly, studies with HeLa cells indicate that AAV-5 also enters the cell through clathrin-coated vesicles and passes through the cytoplasm to form cap-like structures in the trans-Golgi network adjacent to the nucleus. AAV-5 has shown promise as a vector for gene transfer, but our understanding of its natural tropism and the molecular events that determine efficient gene transfer are limited. The characterization of PDGFR as a crucial factor mediating transduction is an important step in understanding how AAV-5 associates with cells and will help direct research on the application and optimal use of AAV-5 for gene therapy.

**METHODS**

**Cell culture, plasmids and recombinant AAV production.** Cos, 293T and HeLa cells were maintained as previously described (see Supplementary Methods online). The 32D cells have been previously described and were kindly provided by N. Loker (Millenium Pharmaceuticals) and M. Alimandi (University of Rome La Sapienza). HeLa cells were transfected with PDGFR-α or PDGFR-β expression plasmids using FuGENE 6 (Roche) according to the manufacturer's protocol. Forty-eight hours after transfection, cells were split and plated at low density and selected with G418 at a final concentration of 400 μg/ml. After 10 d of selection, untransfected control cells had died and the polyclonal pool of cells was assayed for its relative transduction efficiency compared with the parent cells. An expression plasmid for PDGFR-β was provided by C. Engler (National Cancer Institute) and has been previously described. The PDGFR-α expression plasmid was purchased from Stratagene. Recombinant AAV-2 and AAV-5 were produced using a three-plasmid procedure previously described.

**Correlation of transduction with microarray gene expression pattern.** NCI60 cell lines were maintained as previously described and transduced with recombinant virus containing an RSV-NLS-LacZ expression cassette in serial dilution, starting at 2 × 10^9 DRP/cell, for 2 h. The cells were washed to remove unbound virus and stained for β-galactosidase activity 60 h after transduction.
AAV-5 and PDGFR-α coprecipitation assay. We transfected 293 cells with a 6His-tagged PDGFR-α expression plasmid (Stratagene) using FuGENE 6 (Roche) according to the manufacturer’s protocol. Forty-eight hours after infection, cells were collected and lysed in buffer (50 mM NaH₂PO₄ pH 8.0; 150 mM NaCl; 1% Triton X-100; 0.5% deoxycholic acid; protease inhibitor cocktail (Sigma)); 20 μl of QBI DNease (Promega); and 20 mg/ml RNase) and incubated for 30 min at 4 °C with agitation. After incubation, the lysate was centrifuged for 30 min at 13,000 g, and the supernatant was recovered and incubated for 4 h at 4 °C with Ni-NTA His-Bind beads (Novagen). After incubation, the beads were washed twice with lysis buffer and once with Ni-NTA wash buffer (50 mM NaH₂PO₄ pH 8.0; 150 mM NaCl; and 30 mMimidazole). Control beads were prepared in the same manner but were incubated with lysate from untransfected cells. For coprecipitation experiments, AAV-2 or AAV-5 (4×10⁵ particles) were incubated with PDGFR-α-bound beads in 250 μl of binding buffer (50 mM NaH₂PO₄ pH 8.0; 150 mM NaCl; and 1 mg/ml BSA) for 4 h at 4 °C with gentle agitation. The beads were washed five times with wash buffer (50 mM NaH₂PO₄ pH 8.0 and 150 mM NaCl) and virus-bound beads were quantitated by Q-PCR as described above.

Injection of mice and staining of tissue sections. Adult C57BL/6 mice (Jackson Laboratory) were surgically prepared and injected as previously described. The injection protocol was approved by the animal care and use committee of the University of Iowa (see Supplementary Methods online).

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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