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Increased In Vitro and In Vivo Gene Transfer by Adenovirus Vectors Containing Chimeric Fiber Proteins

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Alteration of the natural tropism of adenovirus (Ad) will permit gene transfer into specific cell types and thereby greatly broaden the scope of target diseases that can be treated by using Ad. We have constructed two Ad vectors which contain modifications to the Ad fiber coat protein that redirect virus binding to either α_v integrin [AdZ.F(RGD)] or heparan sulfate [AdZ.F(pK7)] cellular receptors. These vectors were constructed by a novel method involving E4 rescue of an E4-deficient Ad with a transfer vector containing both the E4 region and the modified fiber gene. AdZ.F(RGD) increased gene delivery to endothelial and smooth muscle cells expressing α_v integrins. Likewise, AdZ.F(pK7) increased transduction 5- to 500-fold in multiple cell types lacking high levels of Ad fiber receptor, including macrophage, endothelial, smooth muscle, fibroblast, and T cells. In addition, AdZ.F(pK7) significantly increased gene transfer in vivo to vascular smooth muscle cells of the porcine iliac artery following balloon angioplasty. These vectors may therefore be useful in gene therapy for vascular restenosis or for targeting endothelial cells in tumors. Although binding to the fiber receptor still occurs with these vectors, they demonstrate the feasibility of tissue-specific receptor targeting in cells which express low levels of Ad fiber receptor.

Adenovirus (Ad) has been widely used as a vector to deliver genes to a number of tissues, including lung, vascular, neuronal, and muscle tissue, in vivo (7, 11, 31, 41). Ad grows to high titer, its genome is well characterized, and it delivers and expresses genes in nonproliferating cells. However, previous studies have found a low level of receptor-mediated binding of Ad to many cells and tissues, including primary bronchial epithelium (8), smooth muscle (49), endothelium (49), and macrophage and T cells (24). This reduced binding has been found to result in lowered transduction efficiencies (49). Therefore, one potential improvement to this system would be to target the virus to receptors on specific cell types. This improvement would increase the delivery efficiency to the target tissue and reduce nonspecific transduction of nontarget tissue, thus allowing lower vector doses to be administered with fewer unwanted side effects (7, 35).

Host cell infection by Ad involves two of its coat proteins which interact with distinct cellular receptors (47). The fiber protein, alone, mediates viral attachment to recently identified cellular receptors. Not one but two unrelated cellular receptors for the fiber have recently been reported (3, 23, 43). One receptor, termed the coxsackievirus-Ad receptor because it functions as a receptor for both Ad and coxsackievirus, contains two immunoglobulin domains and has not been previously identified in any protein databases (3, 43). The $\alpha 2$ domain of major histocompatibility complex class I has also been reported to mediate Ad attachment via the fiber (23). Following fiber-mediated attachment to cells, penton base binds via an RGD motif to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors, which then mediate virus internalization via receptor-mediated endocytosis (2, 33, 37, 45, 47). The Ad type 5 (Ad5) penton base does not function in virus attachment to host epithelial cells, which is likely due to the steric hindrance imposed by the 32-nm-long fiber protein (40, 47).

Due to the limited expression of coxsackievirus-Ad receptor in many tissues, Ad tropism can be expanded by targeting its binding to a broadly expressed receptor (48). For example, heparan sulfate-containing receptors are broadly expressed on many cell types and are known to bind to stretches of the positively charged amino acids lysine and/or arginine (13, 26). Another approach to modify tropism is to target Ad binding to a more narrowly expressed or tissue-specific receptor, in order to limit vector binding and transduction to a particular cell type. For example, the α_v integrin receptors are not expressed in many cells unless they are stimulated by cytokines or growth factors, which are produced as a result of wounding, infection, or inflammation (17). For example, granulocyte-macrophage and macrophage colony-stimulating factors (GM-CSF and G-CSF) and basic fibroblast growth factor/vascular endothelial growth factor have been shown to upregulate α_v integrin expression in monocytes (9) and endothelial cells (12), respectively. In addition, α_v integrins can also be aberrantly expressed in invasive glioblastomas and metastatic melanomas (1, 16).

Other successful strategies to target Ad have used antibodies to redirect the virus to new receptors (10, 49). We previously have found that a bispecific antibody which binds to a modified Ad and directs its binding directly to α_v integrins enhances vector binding and transduction of endothelial and smooth muscle cells (49). However, drawbacks to this method include the additional production and characterization of the bispecific antibodies, the potential clearance of virus by the Fc receptor, and the potential activation of the complement system. The most direct way to target Ad is to incorporate receptor-binding motifs into the penton base or fiber coat proteins (36, 46). While Ad5 vectors containing the fiber proteins from other serotypes have been developed (14, 29), high-titer Ad vectors that are targeted to known receptors through the addition of defined receptor-binding motifs to the fiber protein have not been reported.

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In this study, we report a novel system that has been used to produce two Ads containing modifications to their fiber protein. These viruses can be grown to high titer and can target specific receptors through the incorporation of high-affinity peptide motifs into the fiber protein. One virus contains a high-affinity, α_v integrin-binding motif (28) which preferentially increases the transduction of endothelial and smooth muscle cells. A second vector contains a polylysine, heparin-binding motif (13, 48) which increases the transduction of multiple cell types, including macrophage, endothelium, smooth muscle, glioblastoma, and T cells. These vectors demonstrate the feasibility of designing and producing Ads which can target tissue-restricted receptors to increase the efficiency and/or specificity of gene transfer.

MATERIALS AND METHODS

Cells. Human alveolar carcinoma cells (A549), human embryonic kidney cells (293), bovine endothelial cells (CPAE), primary human intestinal smooth muscle cells (HISM), rat smooth muscle cells (A-10), mouse melanoma (B16-F1), human glioblastomas (U-118 and A172), human monocyte-like cells (THP-1 and U-937), and primary human foreskin fibroblasts (Hs68) were obtained from the American Type Culture Collection (Rockville, Md.). The α_v-293 cells were produced by transfecting 293 cells with the α_v and β_3 integrin subunit genes. A549, CPAE, A-10, 293, and α_v-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum (Gibco BRL, Grand Island, N.Y.). The primary cells, HISM and Hs68, were maintained in MCDB medium (Gibco BRL) supplemented with 10% fetal bovine serum and bovine pituitary extract (Gibco BRL). Peripheral blood T lymphocytes were isolated from buffy coats or leukopacks from normal NIH Blood Bank donors by Ficoll-Hypaque density gradient sedimentation, plastic adherence, and nylon wool adsorption (15). Resting cells were obtained from a discontinuous 30 and 40% Percoll gradient (15, 39) as the high-buoyant-density fraction. Fluorescence-activated cell sorting using monoclonal antibody (MAb) OKT3, specific for the CD3 receptor, showed that greater than 90% of the cells isolated in this manner were $CD3^+$. Peripheral blood monocytes were isolated from the fraction of peripheral blood cells adhering to plastic. The monocytes were cultured for 10 to 14 days to allow differentiation into macrophages prior to transduction experiments. All cells and cell lines were maintained in RPMI 1640 supplemented with 10% calf serum (Gibco BRL).

Viruses. The E1- and E3-deleted Ad, AdZ, contains the β-galactosidase gene under a cytomegalovirus (CMV) promoter (GenVec, Inc., Rockville, Md.). AdZ was grown in human embryonic kidney (293) cells which contain the complementary E1 region for virus growth (19). AdZ.F(pK7) and AdZ.F(RGD) were derived from the E4-minus vector, AdZ.11A, to incorporate the additional amino acids present on the C termini of their fiber proteins (see below). AdZ.11A was constructed by exchanging the E1 expression cassette of AdCFTR.11A to a CMV expression cassette expressing β-galactosidase. AdCFTR.11A and AdZ.11A contain a complete deletion of E4 and inclusion of the E4 spacer element as previously described (5). These vectors express a high level of β-glucuronidase from the E4 promoter in 293 or 293-ORF6 cells.

All viruses were purified from infected 293 cells at 2 days postinfection by three freeze-thaw cycles followed by three successive bandings on CsCl gradients (30). Purified virus was dialyzed into 10 mM Tris–150 mM NaCl (pH 7.8) containing 10 mM $MgCl_2$ and 3% sucrose and frozen at −80°C until required for use. Radiolabeled Ad was made by infecting cells at a multiplicity of infection (MOI) of 5 and adding 50 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, Ill.) per ml to the medium of infected cells at 20 h postinfection. The infected cells were then harvested at 60 h postinfection, and the virus was purified as described above by two successive bandings on CsCl gradients. The activity of the labeled viruses was approximately 10^4 virus particles/cpm. Infectious particle titer (in fluorescent focus units [FFU]) was determined by using a fluorescent focus assay on 293 cells (42).

The virus yield from infected 293 cells was determined by infecting 10^6 293 cells with 0.2 ml of virus for 1 h in 6-cm-diameter plates at an MOI of 10 on day 0. The cells were harvested on 1, 2, and 3 days postinfection. The cells were spun down and resuspended in 1 ml of phosphate-buffered saline (PBS) for AdZ and AdZ.F(RGD). AdZ.F(pK7) was lysed in 1 ml of PBS containing 2 M NaCl to facilitate release of virus particles from the cells.

Construction of transfer plasmids. The transfer plasmid, pNS 83-100, was constructed by cloning from pGBS 59-100 the Ad5 *Nde*I-to-*Sal*I fragment, which spans the region of the Ad5 genome from map units 83 to 100, into plasmid pNEB193 (New England Biolabs, Beverly, Mass.). The *Nde*I-*Mun*I fragment was replaced with a synthetic oligonucleotide comprising a *Bam*HI site, which was flanked by a 5′ *Nde*I site and a 3′ *Mun*I site to facilitate cloning. The double-stranded synthetic oligonucleotide fragment was created from the overlapping synthetic single-stranded sense and antisense oligonucleotides, i.e., primers TAT GGA GGA TCC **AAT AAA** GAA TCG TTT GTG TTA TGT TTC AAC GTG TTT ATT TTT C and AAT TGA AAA ATA AAC ACG TTG AAA CAT AAC ACA AAC GAT TCT **TTA TT**G GAT CCT CCA, respectively. The ends of the overlapping oligomers were made to have overhangs compatible for direct cloning into the *Nde*I and *Mun*I sites. The resultant vector, pNS (ΔF), lacks all of the coding sequences for the fiber gene but contains the entire Ad E4 coding sequence. The plasmid retains the AATAAA polyadenylation signal (boldface) included in the synthetic *Nde*I/*Mun*I oligonucleotide and also incorporates a new *Bam*HI restriction site (underlined).

The transfer plasmid, pNS (F5*), which contains a mutated fiber gene with a *Bam*HI site between the last fiber protein codon and the fiber protein stop codon, was constructed from pNS (ΔF). The mutated fiber gene was incorporated into the fiber-minus (ΔF) pNS plasmid, using synthetic sense and antisense oligonucleotide primers to amplify the fiber gene by PCR while incorporating a *Bam*HI site following the last codon of the fiber gene to create the mutant fiber gene. This *Bam*HI site also serves to code for the amino acids glycine and serine. The primers used to amplify from the *Nde*I site to the C-terminal coding regions of the fiber gene from Ad5 genome DNA were antisense primer T CCC CCC GGG TCT AGA **TTA** GGA TCC TTC TTG GGC AAT GTA TGA (stop site in boldface; *Bam*HI site underlined) and the sense primer CGT GTA TCC ATA TGA CAC AGA (*Nde*I site underlined). The PCR product was then cut with *Nde*I and *Bam*HI and cloned into the *Nde*I/*Bam*HI sites of pNS (ΔF).

The mutant transfer plasmids containing sequences encoding an amino acid glycine-serine (GS) repeat linker, a targeting sequence, and a stop codon were made by cloning synthetic oligonucleotides into the *Bam*HI site of pNS (F5*). The overlapping synthetic oligonucleotides used to make the transfer plasmid pNS (F5) pK7 were GA TCA GGA TCA GGT TCA GGG AGT GGC TCT AAA AAG AAG AAA AAG AAG AAG TAA G (sense) and GA TCC TTA CTT CTT CTT TTT CTT CTT TTT AGA GCC ACT CCC TGA ACC TGA TCC T (antisense). The oligonucleotides used to make the transfer plasmid pNS (F5) RGD were GA TCA GGA TCA GGT TCA GGG AGT GGC TCT GCC TGC GAC TGT CGC GGC GAT TGT TTT TGC GGT TAA G (sense) and GA TCC TTA ACC GCA AAA ACA ATC GCC GCG ACA GTC GCA GGC AGA GCC ACT CCC TGA ACC TGA TCC T (antisense). The sense and antisense oligonucleotides were mixed in equimolar ratios and cloned into the *Bam*HI site of pNS (F5*) to create pNS (F5) pK7 and pNS (F5) RGD. Sequencing in both directions across the region of the inserts verified that the clones contained the appropriate sequence.

A third version of pNS (F5*) was also created to allow multiple targeting sequences to be inserted following a preexisting poly(GS) spacer. The sense and antisense oligonucleotides used to make the vector pNS pGS were GA TCC GGT TCA GGA TCT GGC AGT GGC TCG ACT AGT TAA A and GA TCT TTA ACT AGT CGA GCC ACT GCC AGA TCC TGA ACC G, respectively. This sequence encoded amino acids GSGSGSGSGSTS and contained an *Spe*I site in the TS codons, which facilitated the direct cloning of targeting sequences following the poly(GS) spacer region. Other oligonucleotide sense and antisense pairs were synthesized to clone into the *Spe*I site of pNS pGS. The sense and antisense pairs encoding the poly(arginine-glycine-aspartate [RGD]) sequence $(GRGDTF)_3$ were CT AGT GGA AGA GGA GAT ACT TTT GGC CGC GGC GAC ACG TTC GGA AGG GGG GAT ACA TTT T and CT AGA AAA TGT ATC CCC CCT TCC GAA CGT GTC GCC GCG GCC AAA AGT ATC TCC TCT TCC A, respectively. The sense and antisense pairs encoding the poly (YIGSR) sequence $(GYIGSR)_3$ designed to target the high-affinity laminin receptor (18) were CT AGT GGA TAC ATC GGC AGT CGC GGT TAC ATT GGG TCC CGA GGA TAT ATA GGC TCA AGA T and CT AGA TCT TGA GCC TAT ATA TCC TCG GGA CCC AAT GTA ACC GCG ACT GCC GAT GTA TCC A, respectively. The sense and antisense pairs encoding the E-selectin-binding sequence DITWDQLWDLMK (32) were CT AGA GAC AAT ACC TGG GAC CAG CTT TGG GAC CTT ATG AAG A and CT AGT CTT CAT AAG GTC CCA AAG CTG GTC CCA GGT AAT GTC T, respectively. The sense and antisense pairs encoding the laminin receptor-binding sequence $(SIKVAV)_2$ (27) were CT AGT GCC GCC AGC ATT AAG GTG GCT GTC TCG ATC AAA GTT GCG GTA TAA GAC GT and C TTA TAC CGC AAC TTT GAT CGA GAC AGC CAC CTT AAT GCT GGC GGC A, respectively. The sense and antisense pairs encoding the FLAG peptide sequence DYKDDD DK were CT AGA GAC TAC AAG GAC GAC GAT GAT AAG A and CT AGT CTT ATC ATC GTC GTC CTT GTA GTC T, respectively. Sequencing in both directions across the region of the inserts verified that the clones contained the appropriate sequences.

Construction of chimeric Ad vectors. The plasmid DNAs from pNS (F5) pK7, pNS (F5) RGD, and the other pNS transfer plasmids were linearized with *Sal*I, purified, and transfected by using calcium phosphate into 293 cells (20). The transfected 293 cells had been preincubated with the E1-, E3-, and E4-deleted construct AdZ.11A (GenVec) at an MOI of 1 FFU per cell, 1 h prior to transfection with the pNS plasmids (5). Recombination of the $E4^+$ pNS plasmid with the E4-deleted vector resulted in the rescue of an $E1^-$ $E3^-$ $E4^+$ vector capable of replication in 293 cells. The resultant vectors, AdZ.F(pK7) and AdZ.F(RGD), and the other modified vectors were isolated in two successive rounds of plaque purification on 293 cells. Each vector was verified to contain the correct insert by sequencing PCR products derived from virus DNA template by using primers that span the region of the insert DNA. Restriction analysis of Ad DNA from each of the viruses showed that the viruses were pure and contained the *Bam*HI or *Spe*I restriction site unique to the correctly constructed virus (22).

TABLE 1. Construction and isolation of Ad vectors containing fiber C-terminal peptides

Peptide addition	Target	No. of white plaques picked	No. of plaques positive	Binding of target
$(GS)_5$ACDCRGDCFCG	α_v Integrins	5	5	Yes
$(GS)_5$KKKKKKK	Heparan sulfate	5	5	Yes
$(GS)_5$TRDYKDDDDKTS	Anti-FLAG MAb	5	5	Yes[a]
$(GS)_5$TS$(GRGDTF)_3$SS	α_v Integrins	5	5	No[b]
$(GS)_5$TS$(GYIGSR)_3$SS	Laminin receptor	5	5	No[c]
$(GS)_5$TRSDITWDQLWDLMKTS	E-selectin	0	0	ND[d]
$(GS)_5$TSAA$(SIKVAV)_2$	Laminin receptor	0	0	ND

[a] 293 cells infected by this virus were positive by immunofluorescence with the anti-FLAG MAb M2.
[b] Compared to unmodified virus, this virus did not increase the transduction of α_v-expressing 293 cells preincubated with fiber. The other RGD-modified virus comprising the sequence ACDCRGDCFCG did increase transduction in these cells preincubated with fiber.
[c] Compared to unmodified virus, this virus did not increase the transduction of B16-F1, macrophage, HISM, or A549 cells preincubated with fiber.
[d] ND, not determined.

Expression of recombinant fiber proteins by using the baculovirus expression system. Recombinant fiber and penton base were produced in baculovirus as previously described (47). High-titer recombinant fiber and penton base baculovirus stocks were used to produce the recombinant protein in TN 5 cells. Baculovirus-infected cells were pelleted at 3 days postinfection and resuspended in PBS containing the protease inhibitors leupeptin (5 μg/ml), aprotinin (5 μg/ml), and phenylmethylsulfonyl fluoride (1 mM). The cell suspension was subjected to three freeze-thaw cycles and then cleared by centrifugation at $15,000 \times g$ for 15 min. The recombinant proteins were then purified from the cellular proteins as previously described (47).

Immunoblot analysis of virus particles. Purified virus particles (2×10^{10}) in a volume of 10 μl were diluted 1:1 in running buffer and loaded onto a 0.1% sodium dodecyl sulfate (SDS)–9% acrylamide gel. The gel was run at 150 mV, and the protein was then transferred to nitrocellulose. The nitrocellulose was blocked with 5% dry milk and then probed with a combination of rabbit polyclonal antiserum against denatured Ad5 virions (1:1,000) and against fiber protein (1:5,000). Proteins were then detected by using anti-rabbit-peroxidase (1:5,000) and the ECL Western blotting analysis system (Amersham). A control blot probed with only polyclonal serum to the fiber protein verified that the shifted bands in the previous immunoblot were fiber protein.

Binding assays using recombinant Ad vectors. Confluent monolayers of 293, CPAE, and A-10 cells in collagen-coated 24-well plates were preincubated with 300 μl of DMEM–20 mM HEPES containing recombinant fiber protein (5 μg/ml), heparin (3 mg/ml), penton base (50 μg/ml), a combination of fiber plus heparin, a combination of fiber plus penton base, or no competitor for 1 h at 4°C. ^{3}H-labeled AdZ, AdZ.F(RGD), or AdZ.F(pK7) (5,000 to 25,000 cpm) in a volume of 10 μl was added to the wells, which were then rocked for 90 min at room temperature. The cells were washed three times with PBS containing 1 mM $MgCl_2$, solubilized in 200 μl of a 1% SDS solution, and counted in a scintillation counter. The bound counts were then expressed as a percentage of the radioactive counts added per well.

Gene expression assays. Approximately 0.5×10^6 to 1.0×10^6 A549, HISM, CPAE, Hs68, or macrophage cells were seeded onto 6-cm-diameter plates 1 to 2 days prior to experiments. Increasing doses of 10^7, 10^8, 10^9, or 10^{10} AdZ, AdZ.F(RGD), or AdZ.F(pK7) virus particles were incubated with the cells in a volume of 0.2 ml for 1 h. For the human high-density T cells, the experiments were performed by incubating the cells in suspension in a volume of 0.3 ml for 1 h. The cells were then washed three times with DMEM and cultured in DMEM containing 10% calf serum at 37°C. After 2 days of culture, the cells were lysed in 1 ml of 1× reporter lysis buffer containing 10 mM EDTA (Promega, Madison, Wis.). Galactosidase activity in the cell lysates was then assayed by using a Galactolight fluorometric assay kit (Tropix, Bedford, Mass.).

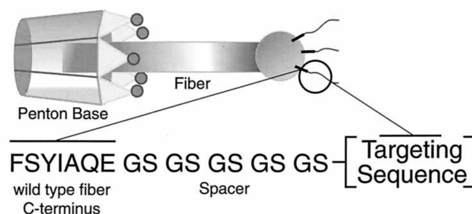
In vivo gene expression. Male domestic pigs (12 to 15 kg; Walter Whippo, Enon Valley, Pa.) were placed under general anesthesia, and bilateral common iliac arteries were exposed through a midline laparotomy incision. After obtaining distal and proximal vascular control, arterial injury was performed with a 4-French Fogarty catheter, inserted through a distal side branch of the common iliac artery, and inflated to 2 atm for 5 min. Ad solution (2×10^{10} particles/ml, approximately 500 μl/iliac artery) was instilled for 30 min. After infection, the Ad solution was evacuated, the side branch was ligated, and blood flow was reestablished. AdZ.F(pK7) was instilled in the right common iliac artery, while AdZ was instilled in the left. No anticoagulation or antiplatelet agents were administered.

Three days after viral transduction, animals were euthanized with potassium chloride and sodium pentothal overdose, and the iliac arteries were collected. The arteries were opened on their long axes, fixed in 2% paraformaldehyde in PBS for 1 h, and then stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) overnight. Vessels were then imaged by light microscopy (Nikon light microscope) for the amount of blue-staining cells on the luminal surface. In addition, some vessels were cross-sectioned (10-μm thickness) after X-Gal staining and counterstained with eosin prior to imaging.

RESULTS

Construction of Ad particles containing chimeric fiber proteins. An Ad transfer plasmid was constructed to allow the convenient addition of ligands onto the C terminus of the Ad fiber protein. The transfer plasmid contained the Ad5 DNA sequence from map units 83 to 100 of the Ad5 genome, including most of the fiber gene as well as the complete E4 region. A unique *Bam*HI restriction site was inserted between the last amino acid codon and the fiber protein termination codon to allow the cloning of sequence encoding receptor-binding ligands directly onto the end of the fiber.

Multiple peptide motifs were chosen to add onto the C terminus (Table 1). Of these motifs, at least two were known to interact with high affinity to heparin sulfate proteoglycans or to α_v integrins (Fig. 1). One motif contained an RGD integrin-binding sequence, ACDCRGDCFCG, which has been shown to bind to α_v integrins with approximately 100-fold-higher affinity than the motif GRGDSP from fibronectin (28). The second motif incorporated a string of seven lysines (KKKK KKK) which contains multiple overlapping consensus motifs that allow high-affinity binding to heparin and heparan sulfate



Vector Name	Target Receptor	Target Sequence
AdZ.F(RGD)	α_V Integrins	ACDC**RGD**CFCG
AdZ.F(pK7)	Heparan sulfate-containing receptors	KKKKKKK

FIG. 1. Diagram depicting the linker and two ligand sequences used to target Ad binding to adhesion molecules. The vector AdZ.F(RGD) contains a targeting sequence with high affinity for α_v integrins. The vector AdZ.F(pK7) contains a stretch of seven lysines.

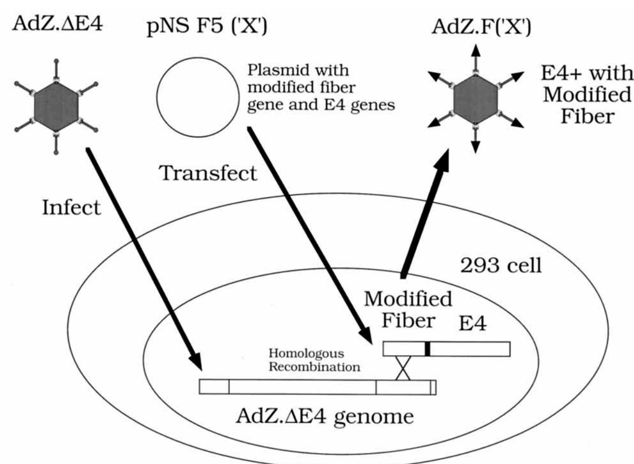


FIG. 2. Schematic of the E4 rescue vector construction method used to create Ad vectors containing modified fiber proteins. A base transfer plasmid comprising the Ad5 sequence from the *Nde*I site (map unit 83) to the *Sal*I site (map unit 100) and a chimeric fiber gene encoding the fiber, a linker sequence, and a targeting sequence was transfected into 293 cells by using calcium phosphate. The cells were infected at a low MOI with the E4-deleted construct AdZ.11A (AdZ.ΔE4). Homologous recombination produced a vector containing the E4 region plus the modified fiber gene. Recombinant $E1^-$ $E4^+$ vectors were isolated by plaquing on 293 cells.

(13). Oligonucleotides encoding either the RGD motif or the polylysine motif plus a short spacer encoding five GS repeats were synthesized and cloned onto the end of the fiber gene. The spacer sequence was encoded before the targeting sequence to optimize accessibility of the ligand to a target receptor. The other receptor or ligand-binding sequences chosen to incorporate into the C terminus of the fiber protein are shown in Table 1. These sequences have been reported to mediate binding to the anti-FLAG MAb (49), E-selectin (32), and laminin receptors (18, 27), as well as a sequence that was comprised of three tandem repeats of a lower-affinity RGD motif.

Recombinant vectors containing the fiber chimeras were efficiently produced by using an E4 rescue technique (Fig. 2). An E4-deleted vector, AdZ.11A, containing the *lacZ* gene driven by the CMV promoter was used to infect 293 cells that had been transfected with the transfer plasmids containing the F(RGD), F(pK7), or other chimeric fiber genes. A single, homologous recombination event between the E4-deleted Ad and the E4-positive plasmid produced a recombinant virus that was replication competent in the 293 cells. The resultant recombinant vector was isolated with high efficiency following plaque purification on 293 cells. Because only E4-expressing vectors replicate in 293 cells, this method resulted in highly efficient recovery of the recombinant vector (Table 1). In addition, the AdZ.11A vector contains the β-glucuronidase gene under control of the E4 promoter. Consequently, only the recombinant plaques remain white after the infected-transfected cell lysates are plaqued on 293 cells in the presence of the substrate X-Glu, which turns glucuronidase-expressing cells blue. Six of the eight infections-transfections resulted in white-plaque isolates at the 10^{-2} dilution of the infection-transfection cell lysate. Of those that were further analyzed, all were shown by PCR to contain the C-terminal addition to the fiber gene. In addition, there was no detectable β-glucuronidase activity in 293 cells infected with isolates that had been twice plaque purified. Two of the constructs could not be isolated despite repeated attempts. The inability to isolate

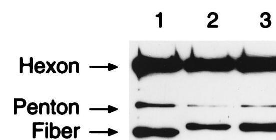


FIG. 3. Immunoblot analysis showing the mobility shift of chimeric fiber proteins compared to wild-type fiber. Equal particle numbers of all vectors (2×10^{10} particles/lane) were diluted in Laemmli running buffer, boiled, and run on a 0.1% SDS–9% acrylamide minigel. The proteins were transferred to nitrocellulose and detected by using a rabbit polyclonal antibody to denatured Ad particles. Lane 1, AdZ; lane 2, AdZ.F(pK7); lane 3, AdZ.F(RGD).

these constructs suggests that the peptides were incompatible with the correct folding of the fiber protein. These constructs encoded C-terminal additions that were five or six amino acids shorter than the two longest constructs that were successfully made. These observations suggest that the targeting peptide composition, rather than absolute peptide length, is most critical for making short peptide additions to fiber C terminus.

Characterization of the chimeric fiber proteins incorporated into Ad vectors. Plaque isolates of the two recombinant vectors AdZ.F(RGD) and AdZ.F(pK7) were amplified and characterized to verify that they encoded the desired receptor-binding ligands on the C terminus of the fiber gene. Sequencing of viral DNA confirmed that AdZ.F(RGD) and AdZ.F(pK7) encoded the correct insert sequence. To confirm the increased size of the modified fiber proteins on the virus, equal numbers of CsCl-purified AdZ, AdZ.F(RGD), and AdZ.F(pK7) particles were loaded on an SDS gel and evaluated by immunoblot analysis (Fig. 3). The proteins were probed with a combination of antibodies directed against whole Ad5 and against fiber. The combination of antibodies detected the major coat proteins, hexon, penton base, and fiber. The mobilities of the hexon and the penton proteins were the same in AdZ.F(RGD) and AdZ.F(pK7) as in AdZ. However, the decreased mobility of the fiber proteins of AdZ.F(RGD) and AdZ.F(pK7) relative to AdZ were consistent with AdZ.F(RGD) and AdZ.F(pK7) containing the appropriate additions. Immunoblot analysis using an antifiber polyclonal antibody confirmed that the shifted bands were fiber (data not shown).

Characterization of vector growth kinetics. Virus growth kinetics of the AdZ.F(RGD), AdZ.F(pK7), and AdZ vectors were assessed to determine whether the ligand additions to the fiber protein had any adverse effects on active virus particle assembly (Fig. 4). 293 cells were infected at an MOI of 5 active

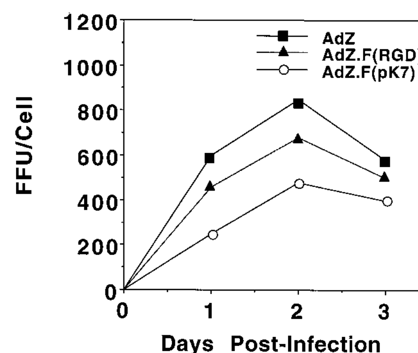


FIG. 4. Virus growth kinetics of AdZ, AdZ.F(pK7), and AdZ.F(RGD) in 293 cells. On day 0, 1 million 293 cells were infected with AdZ, AdZ.F(pK7), or AdZ.F(RGD) at an MOI of 5 FFU/cell. At 1, 2, or 3 days postinfection, the cells were harvested and freeze-thawed three times. The number of active particles (FFU) produced per cell was determined.

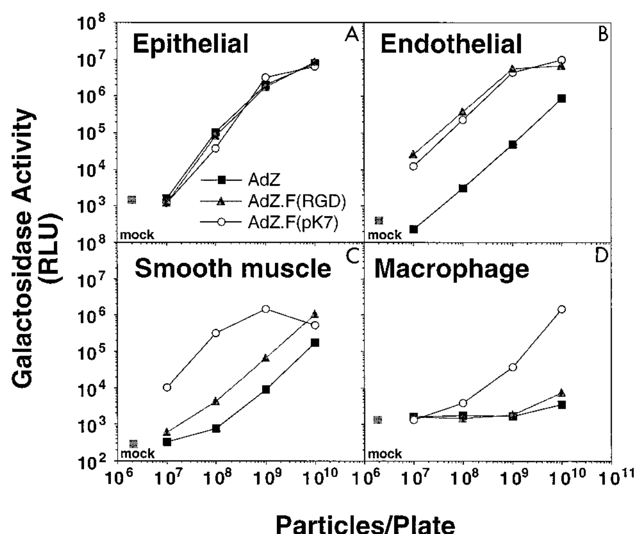


FIG. 5. Comparison of transduction of different cell lines by AdZ, AdZ.F (pK7), and AdZ.F(RGD). The indicated cells (0.5×10^6 to 1.0×10^6 per 6-cm-diameter plate) were seeded 1 day prior to incubation with increasing concentrations of virus in a total volume of 0.2 ml for 1 h at 37°C. The cells were washed three times with DMEM and cultured for 2 to 3 days in DMEM–10% fetal bovine serum. The cells were lysed in 1.0 ml of reporter lysis buffer, and β-galactosidase activity was determined by using a chemiluminescence assay. Enzyme activity is reported in relative light units (RLU). Reported RLU represent the average of duplicate measurements. (A) A549 alveolar epithelial cells; (B) CPAE endothelial cells; (C) human primary smooth muscle cells; (D) human peripheral blood macrophages.

virus particles/cell with either AdZ, AdZ.F(RGD), or AdZ.F (pK7), and the number of infectious particles (FFU) produced per cell was determined at 1, 2, and 3 days postinfection. The peak titers of AdZ.F(RGD) and AdZ.F(pK7) were 80 and 56%, respectively, of that of AdZ. These results indicate that the growth kinetics of the two modified vectors were not dramatically affected by the addition of ligands onto the end of the fiber protein.

Gene delivery by AdZ, AdZ.F(pK7), or AdZ.F(RGD) to multiple cell types. The relative transduction efficiencies of the modified vectors were compared on different cell types (Fig. 5). Increasing total particle doses of AdZ, AdZ.F(pK7), or AdZ.F(RGD) were incubated with cells, and the resulting β-galactosidase gene expression was determined 2 days later. A549 cells were used as a control to verify that each vector equivalently transduced cells expressing high levels of the fiber receptor. All three vectors equally transduced A549 cells, indicating that the activity of the vector particles was not negatively affected by the addition of ligands onto the end of the fiber protein (Fig. 5). At the highest dose of vector, corresponding to approximately 10,000 virus particles/cell, the transduction did not linearly increase with vector concentration. This corresponding decrease in transduction efficiency was associated with an apparent cytotoxic effect caused by all three of the vectors not observed at the lower doses. This cytotoxic effect was noted despite each vector containing less than 1 replication-competent Ad in 10^8 active particles (not shown).

When the three vectors were tested on the other cell lines, the results were dramatically different. AdZ.F(pK7) and AdZ.F(RGD) transduced the endothelial cell line CPAE approximately 100-fold more efficiently than AdZ. These results indicate that CPAE cells lack high levels of fiber receptor but do express sufficient levels of the receptors recognized by the polylysine and RGD-containing ligands present in AdZ.F(pK7) and AdZ.F(RGD), respectively.

The transduction levels achieved by the three vectors were all different on primary smooth muscle cells. The AdZ.F(pK7) was 650- and 170-fold more efficient than AdZ in transducing the smooth muscle cells at approximately 100 and 1,000 particles per cell, respectively. The transduction by AdZ.F(RGD) was between that by AdZ and AdZ.F(pK7) and averaged sevenfold higher than that by AdZ at 100, 1,000, and 10,000 particles per cell. These results suggest that the level of receptor expression plays a role in the level of transduction that is observed.

AdZ.F(pK7), but not AdZ.F(RGD), transduced human primary macrophages over 100-fold more efficiently than AdZ at the two highest doses of vector. The lack of increase by the AdZ.F(RGD) vector indicates that macrophages lack high levels of α_v integrins. This result is not unexpected, as macrophages isolated from peripheral blood do not normally express high levels of α_v integrins, except in response to GM-CSF and G-CSF (9). This assumption seemed to be correct, as culturing human macrophages in a combination of GM-CSF and G-CSF increased their transduction by AdZ.F(RGD) 4.3-fold over that by AdZ. In addition, activation of the macrophage-like cell lines THP-1 and U-937 by phorbol myristate acetate, which has been shown to upregulate α_v integrin expression in these cells, likewise increased transduction by AdZ.F(RGD) relative to AdZ 1.6- and 2.0-fold, respectively (data not shown).

The transduction of resting peripheral blood T lymphocytes, fibroblasts (Hs68), and glioblastoma cells (U-118 and A172) with 100 particles per cell was also evaluated (data not shown). Relative to the AdZ vector, AdZ.F(RGD) increased transduction of the Hs68 cells 6.6-fold but did not increase the transduction of the glioblastoma cells. The AdZ.F(pK7) vector increased the transduction of T, Hs68, A172, and U-118 cells 40-, 109-, 5.5, and 4.2-fold, respectively.

Gene delivery by the other targeted vectors to multiple cell types. The four other vectors that could be made were also evaluated for binding to their target molecules (Table 1). Immunofluorescence studies showed that AdZ.F(FLAG)-infected, but not AdZ-infected, 293 cells were recognized by the anti-FLAG MAb M2, similar to results of previous studies with an Ad vector containing the FLAG peptide in the penton base (49). Furthermore, the FLAG peptide expressed on the fiber C terminus was also able to mediate direct binding of AdZ.F (FLAG) to α_v integrins in assays using a bispecific antibody with specificities for the FLAG peptide and for α_v integrins, similar to previous results with the penton-modified vector (data not shown) (49).

Two other vectors, AdZ.F$(GRGDTF)_3$ and AdZ.F $(GYIGSR)_3$, each containing C-terminal additions of 32 amino acids, could be efficiently grown to high titer. However, neither vector appeared to recognize the targeted receptor with high enough affinity to mediate increased virus binding and transduction (Table 1). AdZ.F$(GRGDTF)_3$, which contains three lower-affinity RGD motifs in tandem at the end of each fiber monomer, did not increase delivery to α_v integrin-expressing cells relative to the unmodified vector, AdZ. This result was in contrast to that for the vector AdZ.F(RGD), which contains a single high-affinity RGD motif and which increased the transduction of α_v integrin-expressing endothelial cells (Fig. 5). Similarly, the vector expressing three tandem YIGSR motifs, which have been shown to interact with the high-affinity laminin receptor, did not increase the transduction of B16-F1 melanoma cells or other cell lines known to express high levels of the high-affinity laminin receptor. These results suggest that a critical receptor-ligand affinity is required to achieve transduction via the targeted receptor.

TABLE 2. Comparison of AdZ and AdZ.F(pK7) binding to three cell lines[a]

Protein	Bound counts[b]					
	α_v-293		CPAE		A-10	
	AdZ	AdZ.F(pK7)	AdZ	AdZ.F(pK7)	AdZ	AdZ.F(pK7)
Control	7.6	18.2	0.19	2.32	0.72	9.90
Fiber	1.7	13.3	0.22	2.07	0.23	8.30
Heparin	9.0	5.1	0.06	0.13	0.53	0.41
Fiber/heparin	0.3	0.9	0.05	0.13	0.10	0.20

[a] Confluent cell monolayers in collagen-coated 24-well plates were preincubated with the indicated competitors for 1 h at 4°C. ^{3}H-labeled AdZ or AdZ.F(pK7) was then added to cells for 90 min at room temperature.

[b] Expressed as a percentage of the radioactive counts added per well. Error for all values was less than 10%.

AdZ.F(pK7) binding to cells expressing high or low levels of fiber receptor. Virus binding assays were performed to determine whether the increases in transduction observed on certain cell lines using AdZ.F(pK7) and AdZ.F(RGD) were due to increased binding mediated by heparan sulfate proteoglycans and α_v integrins, respectively (Tables 2 and 3). The binding properties of radiolabeled AdZ.F(RGD), AdZ.F(pK7), and AdZ were evaluated on cells expressing high levels of fiber receptor and α_v integrins (α_v-293 cells), cells expressing very low levels of fiber receptor (CPAE endothelial cells), and cells expressing low but detectable levels of Ad fiber receptor (A-10 cells). For AdZ.F(pK7), the specificity of binding to the fiber receptor or to heparan sulfate-containing receptors was assessed through competition with soluble recombinant fiber protein, soluble heparin, or a combination of fiber and heparin (Table 2). The inhibition of AdZ.F(pK7) binding by these competitors was measured relative to their inhibition of AdZ binding. Overall, the binding to 293 cells by AdZ.F(pK7) compared to AdZ was comparable in the absence of inhibitors, although AdZ.F(pK7) binding was approximately double that of AdZ. Fiber, but not heparin, inhibited the binding of AdZ. However, fiber had only a marginal effect on AdZ.F(pK7) binding, whereas heparin alone reduced binding by over two-thirds. Only the combination of heparin plus fiber was able to completely abrogate the binding of AdZ.F(pK7) to 293 cells. These results strongly suggest that AdZ.F(pK7) binds to cells via two interactions: the fiber receptor interaction blocked by soluble fiber and a second class of receptors whose interaction is blocked by soluble heparin.

CPAE cells bound 10-fold-higher levels of AdZ.F(pK7) than AdZ. Soluble fiber had no significant effect on the binding of AdZ, confirming that CPAE cells lack detectable levels of functional fiber receptor. These results demonstrate that the polylysine modification in AdZ.F(pK7) significantly increases its binding to cells that express low or undetectable levels of functional fiber receptor. Furthermore, the increased binding is blocked by heparin, indicating that the polylysine addition to the fiber mediates Ad binding to a heparin- or heparan-containing cellular receptor. These results demonstrate that the increased transduction of the fiber receptor-deficient cells by AdZ.F(pK7) is facilitated through the increased binding of the virus via its polylysine-modified fiber protein.

AdZ.F(RGD) binding properties to cells expressing high or low levels of fiber receptor. AdZ.F(RGD) specificity of binding to the fiber receptor or to α_v integrins was assessed through competition with soluble recombinant fiber protein, soluble penton base, or a combination of fiber and penton base (Table 3). Penton base was used as a competitor since it has been shown to block α_v integrin interaction via its RGD motif. Like AdZ.F(pK7), the inhibition of AdZ.F(RGD) binding by these competitors was determined relative to their inhibition of AdZ binding.

While fiber blocked binding of AdZ to α_v-293 cells, it had no significant effect on the binding of AdZ.F(RGD) to these cells. This result indicated that AdZ.F(RGD) bound to cells via an additional interaction. Penton base had no significant effect on AdZ.F(RGD) binding; however, the combination of fiber plus penton base significantly blocked AdZ.F(RGD) binding. Fiber had no effect on AdZ.F(RGD) binding to CPAE cells, whereas penton base significantly blocked binding close to the levels observed for AdZ. These results demonstrate that AdZ.F(RGD), unlike AdZ, can bind directly to α_v integrins on cells expressing or lacking fiber receptor expression. In addition, compared to AdZ, the additional interaction of AdZ.F(RGD) with α_v integrins significantly increases virus binding to cells which express little or no detectable fiber receptor. These results demonstrate that the increased transduction of the endothelial and smooth muscle cells by AdZ.F(RGD) is due to increased binding of the virus to α_v integrins which are expressed on these cells.

Increased in vivo transduction by AdZ.F(pK7). The greatly increased transduction efficiency of smooth muscle cells in tissue culture using the AdZ.F(pK7) vector suggested that it should increase LacZ gene delivery to vascular smooth muscle cells in vivo. To investigate this, the left and right pig iliac arteries were subjected to balloon catheter injury and then transduced for 30 min with either AdZ.F(pK7) or AdZ (Fig. 6). After transduction, blood flow was reestablished in the arteries; after 3 days, the arteries were removed and stained for β-galactosidase expression. Arteries transduced with AdZ.F(pK7) (Fig. 6C and D) showed increased expression compared to the corresponding arteries transduced with AdZ (Fig. 6A and B). These results demonstrate that the AdZ.F(pK7) vector transduces smooth muscle cells, in vivo, with higher efficiency than the unmodified vector, AdZ.

DISCUSSION

Depressed fiber receptor expression has been shown to reduce gene transfer by decreasing viral adsorption. To overcome this problem, we have shown that high-titer stocks of modified Ad particles which increase adsorption and gene transfer efficiency to cells both in vitro and in vivo can be made. While Ad has been shown to transduce a number of tissues and cell types, this study and previous studies have identified multiple cell types which express low levels of fiber receptor, in-

TABLE 3. Comparison of AdZ and AdZ.F(RGD) binding to three cell lines[a]

Protein	Bound counts[b]					
	α_v-293		CPAE		A-10	
	AdZ	AdZ.F(RGD)	AdZ	AdZ.F(RGD)	AdZ	AdZ.F(RGD)
Control	7.6	12.7	0.19	0.84	0.72	1.68
Fiber	1.7	12.3	0.22	1.06	0.23	1.40
Penton base	9.0	9.7	0.20	0.37	0.80	0.62
Fiber/penton base	1.0	3.7	0.21	0.46	0.20	0.41

[a] Confluent cell monolayers in collagen-coated 24-well plates were preincubated with the indicated competitors for 1 h at 4°C. ^{3}H-labeled AdZ or AdZ.F(RGD) was then added to cells for 90 min at room temperature.

[b] Expressed as a percentage of the radioactive counts added per well. Error for all values was less than 10%.

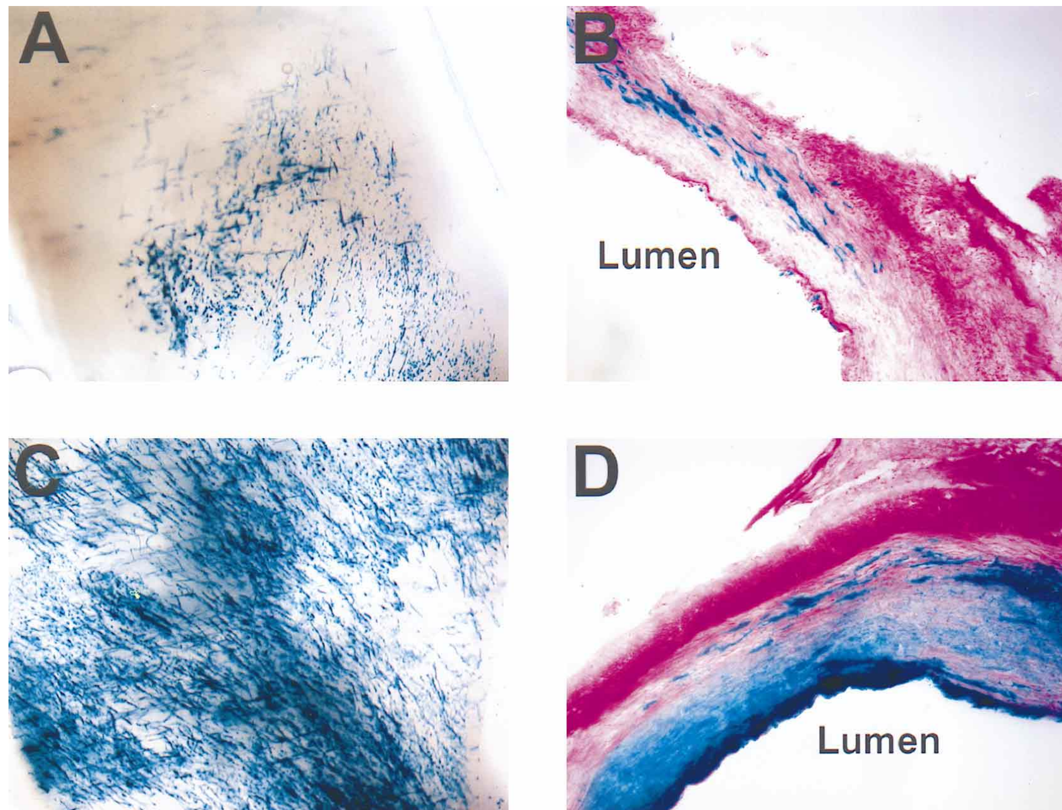


FIG. 6. Comparison of in vivo gene transfer efficiencies in assays using AdZ and AdZ.F(pK7). Pig iliac arteries were subjected to balloon catheter injury and then transduced for 30 min with 2×10^{10} particles of either AdZ or AdZ.F(pK7) per ml. After transduction, viral solutions were evacuated and blood flow was reestablished through the iliac arteries. Animals were euthanized on postoperative day 3, and the iliac arteries were fixed and stained with X-Gal. (A and B) AdZ-treated vessels; (C and D) AdZ.F(pK7)-treated vessels. Panels A and C are en face images (magnification, ×4) of the lumenal surface of the arteries, while panels B and D are cross-sections (magnification, ×10) of the stained vessels. Blue-staining cells represent positively transduced cells.

cluding endothelium, smooth muscle, melanoma, glioblastoma, primary bronchial epithelium, macrophages, and T cells (8, 24, 25, 40, 49). Therefore, modified Ad vectors with increased adsorption and transduction efficiency could have a profound impact upon the diseases treatable by gene therapy.

Vascular smooth muscle cells are a primary target for gene therapy to prevent aberrant smooth muscle cell proliferation (6, 21, 44). Smooth muscle cell proliferation has been implicated in the pathogenesis of atherosclerosis and in clinically significant restenosis in 30 to 40% of patients following balloon angioplasty of the coronary arteries (34). High levels of Ad particles have typically been required to achieve significant transduction of the smooth muscle. Our results demonstrate that smooth muscle transduction by Ad vectors can be dramatically increased by using the modified polylysine vector AdZ.F (pK7). Decreased doses of therapeutic Ad in smooth muscle gene therapies are likely to decrease side effects often associated with Ad gene therapy.

Ad coat proteins can be modified to redirect the virus to ubiquitously expressed receptors which then mediate the efficient binding and transduction by Ad to a broad spectrum of tissues. Polylysine has been shown to interact with heparan sulfate (13) as well as other polyanions such as DNA (8). Because heparan sulfate and other polyanionic molecules such as chondroitin sulfate and mucins are broadly expressed on cells, the pK7 vector is universal in the cell types that it can transduce. Thus, in terms of its cell tropism the pK7 vector is actually nontargeted because the receptor targeted by the virus is broadly expressed. The significant increases in binding and transduction of cells lacking high fiber receptor expression levels shown here demonstrate that the addition of peptide receptor-binding motifs into the coat proteins of Ad can expand the range of tissues amenable to efficient adenovirus-mediated gene therapy.

A vector similar to AdZ.F(pK7) has previously been constructed by incorporating a frameshift mutation into the fiber stop codon, which resulted in polylysine addition to the fiber C terminus (48). The absence of an in-frame stop codon permits translation to proceed into the poly(A) tail of the message which encodes polylysine. However, the frameshift mutation also significantly depresses fiber protein synthesis which is associated with a 100-fold decrease in virus titer (unpublished results). Here we have shown that the addition of a defined peptide linker and polylysine sequence onto the C terminus results in both efficient transduction via the polylysine and relatively unaffected vector growth properties.

Coat protein modifications can also be made to direct the virus to tissue-specific receptors. Such modifications then allow the virus to efficiently bind and transduce only select tissues. The α_v integrins are promising receptors for targeted gene transfer. While many cells in the body are capable of expressing α_v integrins, they are often highly expressed in response to only certain cytokines and growth factors which are produced during infection, wounding, or inflammation. This characteristic of α_v integrins makes them an ideal receptor for targeting injured vasculature following angioplasty or for targeting pro-

liferating endothelial cells in tumors (4, 12). Bispecific antibodies have been previously shown to target adenovirus to α_v integrin-expressing cells (49). However, drawbacks to this approach include the additional production and characterization of the bispecific antibodies, the potential clearance of virus by the Fc receptor, and the potential activation of the complement system. By expressing the high-affinity RGD ligand as a fiber fusion protein, it is possible to produce comparable titers of the targeted vector in standard packaging cell lines without the drawbacks associated with bispecific antibodies.

The affinity of the C-terminal peptide motif for its receptors appears to be a critical factor in directing significant virus binding to cells via the targeted receptor. AdZ.F(RGD), which incorporates multiple, linear RGD motifs, did not mediate detectable binding to cells via α_v integrins. We have similarly found that incorporating multiple YIGSR motifs into the fiber C terminus does not increase binding to cells expressing the high-affinity laminin receptor. The ACDCRGDCFCG motif, that was incorporated into the AdZ.F(RGD) vector, was previously identified by using phage display technology (28). This motif has an approximately 100-fold-higher affinity than other RGD motifs and is believed to form a tight loop through a pair of disulfide bonds between the cysteines. Therefore, although several receptor-binding peptide motifs have been identified, the low affinities of many of these motifs suggest that few will mediate significant increases in adenovirus binding to cells expressing the targeted receptor. Furthermore, vector binding to cells does not necessarily indicate that the vector will efficiently internalize into the cells.

The E4 rescue technique that we used to produce fiber-modified viruses was much more efficient and reliable than other vector construction methods, and contaminating virus background was virtually absent. This system also allowed blue/white selection of the appropriate plaques by using the X-Glu substrate. This selection is possible because the AdZ.11A virus expresses β-glucuronidase from the E4-deleted region (blue plaques), while the E4-wild type recombinant does not contain the β-glucuronidase gene (white plaques). Using some peptide motifs in this system, we found that we could not isolate viable recombinant vectors. The most likely explanation for this is that certain peptide motifs might prevent proper folding of the fiber protein or interfere with trimerization.

While the AdZ.F(RGD) vector is not truly targeted to α_v integrins because it can still bind to the fiber receptor, the increased transduction by Ad vectors with expanded receptor-binding repertoires demonstrates the feasibility of targeting vectors to specific cell types via tissue-specific receptors. In fact, the potential of tissue-specific targeting using high-affinity peptides has recently been demonstrated. Phage displaying the same high-affinity RGD motif as in AdZ.F(RGD) were found to home to tumor endothelial cells when injected intravenously into tumor-bearing mice (38). Therefore, the efficient targeting of a single cell type or tissue will necessitate Ad vectors which lack fiber receptor-binding activity. Such vectors may likewise require special receptor-expressing cell lines in order to propagate them. In any case, it will be important to determine the levels of Ad receptors in tissues targeted for gene therapy. If fiber receptor expression is low in a given tissue, targeting Ad to receptors that are expressed by the tissue is likely to increase the efficiency and specificity of gene transfer.

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