Selection of Muscle-Binding Peptides from Context-Specific Peptide-Presenting Phage Libraries for Adenoviral Vector Targeting

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Adenoviruses are arguably one of the most robust vectors for gene delivery. The attraction for the use of these viruses as gene delivery vectors stems from their ability to transduce a wide range of dividing and nondividing cell types. Adenovirus type 2 (Ad2) and Ad5 bind and enter cells through a multistep process usually involving the combined interactions of the fiber and penton base proteins with cellular receptors. The trimeric fiber protein forms a shaft structure at the vertices of the virus icosahedron and is thought to mediate initial cell binding (10). Cells lacking the fiber receptor (the coxsackievirus-adenovirus receptor [CAR]) are relatively resistant to infection (4). Alterations in the vasculature of the liver.

Production of cell-targeting vectors in part involves the addition of new targeting ligands to the vector to mediate binding to the cells of interest. For viral vectors, the ideal approach is to genetically engineer new ligands into the capsid proteins of the virus to generate a single agent to mediate therapy. Although this is ideal, this insertion of an exogenous ligand from one structural context into the differing structural context of a capsid protein can ablate the function of the ligand or disrupt viral assembly and function. To address this context problem for adenoviral vectors, we have engineered a “context-specific” peptide-presenting phage library. We have displayed a 12-amino-acid (12-mer) random peptide library between the H and I sheets of the fiber protein of adenovirus type 5 on the pIII protein of fd bacteriophage. This library was used for peptide selection against C2C12 mouse skeletal muscle cells. Five rounds of selection combined with four rounds of clearing on nontarget cells selected one primary peptide designated 12.51, which bound target C2C12 cells approximately 100-fold better than the positive control RGD peptide. Translation of 12.51 back into the fiber protein produced a ligand-modified adenoviral vector that mediated 14-fold-better transduction of target C2C12 cells. These data suggest context-specific peptide-presenting libraries may allow selection of compatible peptide ligands for functional translation into viral vectors for retargeting.

Adenoviral vectors also transduce a variety of nondividing cells including myoblasts and myofibers. First-generation adenoviral vectors carrying a 6.3-kbp mini-dystrophin gene mediated expression in as many as 50% of myofibers after direct injection into the muscle bed of mdx mice (19). This promising result was observed in very young (5- to 7-day-old) mice and used fairly large amounts of Ad (>10^9 PFU). Subsequent work showed this high-efficiency delivery occurred only in very young mice (1). This decreased transfection with age appears to be due in part to the loss of the CAR on the muscle cells (7, 24).

One approach to avoid these problems is to develop cell-targeting vectors by replacing the promiscuous or ineffective cell-binding native ligands present on vectors with cell-specific ligands to generate vectors with enhanced cell specificity and activity. Proof of principle for this approach for muscle has been demonstrated by using Ad vectors modified with ligands such as polylysine (5).

Although polylysine does increase transduction, this ligand is nonspecific and increases transduction on many cell types. To identify other ligands for vector targeting, we and others have applied peptide-presenting phage libraries to select cell binding peptides (reviewed in reference 3). Although many cell-binding peptides can be identified from phage libraries, two unpredictable problems can arise when inserting ligands into capsid proteins: (i) insertion of the ligand can destroy capsid and vector function (31), and (ii) the ligand may fail when translated into the heterologous structure of the virus (16). These “context” problems are fundamental, since an ideal candidate peptide ligand may be identified but cannot be...
applied because the ligand destroys the vector or the vector destroys the ligand. This translation problem stems in part from the fact that peptides isolated from phage libraries are selected in the protein structural context of the phage pIII protein and are then translated into different protein structures of a viral capsid protein.

Given this "context" problem, we have engineered viral "context-specific" phage libraries by introducing the H and I sheets of the adenovirus knob domain on to the pIII protein of filamentous bacteriophage. A 12-amino-acid (12-mer) random peptide library was constructed by insertion between the H and I sheets in the normal position of the HI loop. Selection of this HI loop context-specific peptide library against C2C12 myoblasts with preclearing against nontarget cells generated a peptide designated 12.51 with binding substantially better than that of the positive control integrin-binding ligand RGD.

When this peptide was translated back into the knob domain of an Ad5 vector, this vector was functional, and 12.51 mediated improved muscle cell transduction compared to wild-type Ad5. These data suggest context-specific phage libraries may be used to identify compatible peptide ligands for viral vector targeting.

**MATERIALS AND METHODS**

**Cells.** C2C12 mouse myoblasts were purchased from American Type Tissue Culture (Manassas, VA). C2C12 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic/antimycotic. C2C12 myotubes were grown in DMEM supplemented with 2% FBS and 25 μg/ml Penicillin and 25 μg/ml Streptomycin (CHO). HeLa, DU-145, MDA-MB-231, and Hepa 1-6 cells were purchased from American Type Tissue Culture and maintained in RPMI or DMEM supplemented with 10% FBS and antibiotic/antimycotic.

**Construction of phagemids with test peptides.** pUC18/H-BAP-pIII and pUC18/H-RGD-pIII were constructed as phagemids expressing the 14-amino-acid biotin acceptor peptide and RGD integrin-binding motif, respectively, within the H and I sheets of the adenoviral knob on the N terminus of the pHII phage protein. HI-BAP displaying a biotin acceptor peptide (BAP) and HI-RGD displaying this integrin-binding motif were PCR amplified from pA2B5-H-HBAP and pA2B5-H-HRGD, respectively. The resulting PCR products were gel purified and ligated at a 1:5 mass ratio with 20 μg of each pUC18knobpIII phagemid vector digested with BamHI and XbaI. The ligation was purified by phenol-chloroform extraction and transformed into XLI-Blue electrocompetent cells. Transformation efficiency was determined by titration, and library diversity was confirmed by sequencing. Phage was prepared as described above.

**Phage library selection.** Peptide selection was performed as described earlier (2, 27). Direct affinity selection of phage without clearing was performed essentially as described previously (2) with the following variations. Briefly, each cell line was grown to confluence in 60-mm dishes. The cells were then washed once with serum-free RPMI and incubated in serum-free medium for 2 h prior to incubation with phage. In the first round of panning, approximately 10 library equivalents (10^8 phage) were added to target cells in a total volume of 2 ml of HBSS-BSA. The phage were then incubated with the cells for 1 h at 37°C with 100 μM chloroquine with protease inhibitor cocktail (Complete Protease Inhibitors; Roche). The cells were washed six times with 5 ml of room temperature HBSS-BSA. The cells were then incubated for 10 min on ice with 2 ml of 0.1 M HCl (pH 2.2) (by glycine). This acid-eluted fraction of phage was saved and neutralized with 400 μl of 1 M Tris (pH 8). The cells were lysed in 1 ml of 30 mM Tris (pH 8)–1 mM EDTA for 1 h on ice. The cell debris was scraped from the plate, vortexed briefly, and saved as the cell-associated phage fraction. Phage from each fraction were then amplified as described previously (2). For each subsequent round of panning, portions of purified phage were reapplied to the target cells, and panning was carried out as described above while maintaining fraction specificity (e.g., when an acid-eluted fraction of phage were recovered, then only the acid fraction was amplified in all subsequent rounds).

**Phage library selection with clearing.** To attempt to remove promiscuous cell-binding peptides from the peptide selection, the phage libraries were bound to nontarget competitor cells. Phage libraries were first selected for one round against the target cells as described above to enrich the peptide population with cell-binding peptides (~10,000-fold enrichment). At each round of selection after the initial round, the phage from the previous round were added back to the nontarget competitor cells grown in monolayers in 60-mm dishes for 1 h at 37°C. For the present study, phage were cleared in mass on a mixture of HeLa, 293, DU-145, Hepa 1-6, CHO, MDA-MB-231, and RAW264.7 cells, where 10^9 of each cells was mixed and used for clearing. Once the phage population had incubated with the nontarget clearing cells, the supernatant containing the specific binders was harvested and transferred to the target C2C12 myoblasts. These cleared phage were incubated for 1 h at 37°C on the target cells and cell-binding phage were recovered and amplified as described above. Clearing and affinity panning were repeated for all rounds after round one.

**Sequence selected phage.** After five rounds of panning, individual colonies of phage-infected bacteria were isolated from random sites on plates, and each colony was grown overnight in 3 ml of YT-AMP (YT medium with 50 μg of aminoglycoside). Phage DNA was isolated from the phage stock pellets on M13 DNA purification columns (QIAGEN, California). The DNA was PCR amplified with M13 forward and reverse primers and prepared for sequencing.

**Phage binding comparison.** Indicated phage clones and libraries were grown up in liquid culture and purified. To test for cell binding, equal numbers of each phage (10^10) were diluted into a master solution of HBSS-BSA immediately prior to each experiment, and aliquots of this solution were added to approximately 10^5 target cells in 24-well plates for up to 1 h at 37°C as described earlier (2). The cell-associated phage were recovered, and their titers were estimated as described above.

**Peptide repertaging of Ad vectors.** One of the selected peptides, designated 12.51, was genetically engineered into the HI loop of pL29FiberStop expression vector by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (QIAGEN). Briefly, PCR product was amplified by using phosphorilated oligonucleotides encoding the 12.51 peptide. After DpnI digestion, the PCR products were gel purified, self-ligated, and transformed in XLI-Blue electrocompetent cells. Successful clones were grown up, the vector was digested with BglII and KpnI, and the resulting insert containing the 12.51 peptide was genetically engineered into the HI loop of pL29FiberStop vector. After confirmation by PCR screening, DNA was purified using a QiaFilter Midi kit (QIAGEN). Adenoviral production was done according to the manufacturer's recommendations (Strategene). Briefly, pAd-HI-12.51 and wild-type Ad were digested with PacI, and 5 μg of each was transfected into 10-cm tissue culture
plates seeded with 293A cells grown to 70% confluence by using Lipofectamine (Invitrogen). About 14 days posttransfection, plaque formation was observed, and plaques were picked, subjected to small-scale amplification. Large-scale amplification was done by using a cell factory (Corning, New York). After a cytopathic effect was observed, cell lysate was collected and subjected to three freeze-thaw cycles. After centrifugation, viral supernatant was purified by two rounds of cesium chloride (CsCl) density gradient ultracentrifugation at 20,000 rpm for 3 h. Collected viruses were desalted by using DG10 desalting columns (Bio-Rad, California). The genomic titer of the viral particles was quantitated by real-time PCR (Rotorgene; Corbett, Sydney, Australia) using Stratagene Mas-

RESULTS

Display of peptides within the fiber HI loop on phage. A BAP and RGD integrin-binding motif were incorporated into the HI loop of the Ad5 fiber as negative and positive control cell-binding peptides, respectively (17; data not shown). The H and I sheets along with intervening peptides were then subcloned for display on the N terminus of the mature pIII protein on fd bacteriophage (phage designated HI-BAP and HI-RGD). To determine the functionality of the displayed peptides, HI-BAP was biotinylated with the biotin protein ligase, BirA. Western blotting of HI-BAP and HI-RGD de-

TABLE 1. Sequence diversity of “context-specific” HI loop 12-mer library

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FIG. 1. Phage binding of HI-RGD on an integrin-expressing cell line. HI-RGD phage was bound to integrin-expressing human embry-onic kidney 293A cell line compared to control VCSm13 helper phage and HI-BAP phage. A total of 10⁶ input phage of each was bound to 293A cells for 1 h, and titers of collected cell fractions were determined on YT-AMP plates in serial tenfold dilutions.
ies where no significant data has been obtained by alignment of peptides to the nucleic acid and protein databases (2, 27).

Cell binding of 12.51 selected peptide on phage. Since the 12.51 sequence was most frequently displayed from the selected clones, the 12.51 phage was tested for its ability to bind C2C12 cells compared to negative control phage, such as HI-BAP and HI-RGD. Phage were bound to C2C12 myoblasts and were collected, and titers were determined as described previously (2, 27). 12.51 phage-selected peptide bound C2C12 myoblasts approximately 100- and 1,000-fold better than HI-RGD and HI-BAP phage, respectively (Fig. 2).

Retargeting of adenoviral gene therapy vectors with muscle-selected peptides. Given that 12.51 appeared to bind C2C12 cells, this peptide was genetically engineered in between the H and I sheets of the Ad5 fiber, and this modified fiber gene was recombined into a dsRed2 red fluorescent protein-expressing Ad5 genome by using Red recombinase system (Fig. 3) (6, 14). This recombinant Ad5 was then transfected into 293 cells and formed plaques within 14 days (data not shown). Given that the virus formed plaques at the same rate as unmodified Ad5, this suggested that the 12.51 peptide was tolerated within the viral context and did not disrupt viral assembly and function. Large-scale amplification and purification yielded normal amounts of viral particles. Particle/PFU ratios were 23 for unmodified Ad and were 64 for Ad-HI-12.51, indicating a slight reduction in transduction of nonmuscle cells by the peptide-modified vector. To assess transduction, unmodified Ad and Ad-HI-12.51 were added to C2C12 myoblasts, and transduction was assessed 48 h later. In this case, Ad-HI-12.51 mediated ~14-fold increase in transduction to C2C12 myoblasts compared to unmodified Ad (Fig. 4A, B, and E). Ad-HI-12.51 mediated twofold-lower transduction on differentiated C2C12 myotubes than against myoblasts (Fig. 4C, D, and F). Nonetheless, the 12.51-modified vector still mediated fourfold higher transduction than unmodified Ad5. These data therefore suggest that 12.51 may be useful for increasing transduction of both myoblasts and differentiated muscle cells. These data suggest that translation of this “context”-selected peptide back into its native context in the adenoviral fiber can enhance transduction to target cells.

DISCUSSION

Adenoviruses have proved to be an attractive method for gene delivery due to their ability to transduce a wide variety of dividing and nondividing cells. However, their ability to successfully target a given cell type is limited by its evolution to infect a multitude of cell types. As one approach to develop more specific vectors, we and others have used peptide-presenting phage libraries to identify cell-binding peptides to be engineered into gene therapy vectors (2, 13, 16, 21, 22, 27, 29). However, two problems can arise from
incorporation of selected peptide ligands into viral vectors for retargeting: the selected peptide ligand can ablate viral assembly or function, and the virus can debilitate the binding and/or internalization ability of the peptide ligand. In order to circumvent this ligand compatibility problem, we generated here a “context-specific” filamentous phage library in which random peptides were displayed between the H and I sheets from the Ad5 fiber protein, a location previously used on the virus for ligand display (20). By this approach, we demonstrate the selection and functional translation of a skeletal muscle cell-binding peptide back into Ad5 vectors to increase transduction of these cells.

Previous work reported the display of the intact knob domain from the fiber on filamentous phage (18). In our hands, similar display of intact knob phage was toxic to bacteria and with only 1 out of 100 displaying the knob (3). We speculate that this toxicity was due to incompatibility of knob during its secretion from the bacteria on the pIII protein. A more recent study has demonstrated intact knob peptide libraries on lyogenic lambda phage rather than the secreted filamentous

FIG. 4. Transduction of mouse C2C12 myoblasts and myotubes. Wild-type Ad5 or Ad-HI-12.51 expressing red fluorescent protein was applied to the indicated C2C12 myoblasts or myotubes. Cells were transduced with 1,000 particles/cell, and transduction was analyzed 48 h later by fluorescence microscopy (A to D) and flow cytometry (E and F). Flow cytometry results are expressed as the percent positive cells transduced by respective virus after 60 min of incubation. The standard deviation of each is indicated. (A) Ad5 on myoblasts; (B) Ad-HI-12.51 on myoblasts; (C) Ad5 on myotubes; (D) Ad-HI-12.51 on myotubes; (E) transduction of myoblasts measured by flow cytometry; (E) transduction of myotubes measured by flow cytometry.
phage (9). In this case, a 2 × 10⁵ member semirandom 14-mer peptide library was displayed in the HI loop of knob. Selection of this library against NIH 3T3 cells selected peptides that increased transduction on the 3T3 cells when translated into the virus by 100-fold (9). The advantage of this lambda library approach is that legitimate knob trimers can be displayed on the phage. The advantage of the filamentous phage display used in our study is that substantially larger peptide libraries can be generated to theoretically allow more peptide ligands to be screened to perhaps find higher affinity ligands (3). Like all phage-displayed libraries, one limitation of the “context-specific” library is the possible selection of phage particles that are truncated, deleted, or frameshifted of the peptide ligands (8, 26). Work is currently under way to optimize library design to overcome these ligand display problems.

Our data suggest that “context-specific” peptide libraries can be used to identify ligands that are compatible when translated back into the viral capsid context. Ad-HI-12.51 exhibited 14-fold increase in transduction compared to wild-type Ad in vitro. In our studies, we observed poor transduction of mouse muscle cells by unmodified Ad5, which is likely due to the low level of CAR expression on these cells (15). Consequently, the efficacy of Ad transduction is limited. Upon incorporation of selected 12.51 peptide into the Ad HI loop, we observed significant increase in transduction.

In summary, we demonstrate proof of principle for the use of relatively large repertoire “context-specific” peptide-presenting phage libraries as a potential approach to generate and identify compatible ligands when incorporated back into the viral capsid for retargeting. We are currently testing this library for its utility in selecting novel peptides against human skeletal muscle cells and other target cells for gene therapy.

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