Toward cell-targeting gene therapy vectors: Selection of cell-binding peptides from random peptide-presenting phage libraries

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Ideal gene therapy vectors would be delivered intravenously to transfect only specific cells. Existing vectors only transfect cells *in vivo* in a manner determined by blood flow and the site of introduction. As a general and systematic approach for generating cell-targeting ligands for gene therapy vectors, we have used peptide-presenting phage libraries to select peptides that bind and enter several different cell types. Because of their small size, cell-binding peptides such as these could be incorporated into biological or physical gene therapy vectors. In addition, peptide-presenting phage themselves may also be candidates for gene therapy vectors.

Current gene therapy vectors transfect cells *in vivo* in a manner largely determined by blood flow and the site of introduction[1,2]. Because of this, the ability of these vectors to deliver therapeutic genes to the correct cells *in vivo* is limited. Attempts to generate cell-targeting molecules have focused primarily on monoclonal antibodies. Although extensively studied, this approach has several limitations (reviewed in ref. 3), including the complexity of isolating the appropriate monoclonal antibody and its immunogenicity. Phage libraries have been used previously to select random peptides that bind single proteins[4,5]. More recently, phage that display known integrin-binding peptides have been shown to bind and enter mammalian cells[6]. Peptides have also been selected against platelets by using phage libraries displaying rational derivatives of the thrombin receptor ligand[7].

To produce cell-targeting ligands, we have developed a method of selecting cell-binding and cell-entry peptides from random peptide-presenting phage libraries. Unlike the use of known ligands[6,7], this process requires no prior knowledge of the biology of the target cells. This approach has the advantage that molecular recognition and selection are not influenced by the immunogenicity of candidate targets. As a result, peptide ligands should be more easily isolated and incorporated into biological vectors by cloning or by chemical conjugation to synthetic vectors. For these reasons, selected cell-targeting peptides may be useful for gene therapy and drug-targeting strategies.

Phage libraries

The following proof-of-concept experiments were performed using the peptide-presenting phage libraries ON159.3 (12 random amino acids) and ON543 (20 random amino acids) where the random amino acids are fused to the amino terminus of the pIII protein (Fig. 1*a*). Libraries presenting large peptides were chosen because we reasoned that they might be able to present a variety of secondary structures as well as linear stretches of amino acids.

Fibroblast-binding peptide selection at 4 °C

Phage were initially selected that bound the PEA10 mouse fibroblast cells. Binding was conducted at 4 °C to avoid endocytosis of the phage. Ten library equivalents of the 12-amino acid polymer (12-mer) library (3×10^9 phage from ON159.3) were incubated on the cells, the cells were washed, and the acid-labile phage were eluted from the cell surface and recovered as the acid-eluted fraction. In the first round of selection, approximately 10^{-6} of the input phage were eluted by acid (data not shown). Although peptide-presenting phage are usually recovered from their target molecule by using a low pH wash[4,5], slightly more phage remained associated with the cells following multiple acid washes than were eluted by the acid. This cell-associated fraction was also recovered and amplified because these phage might have higher affinities for the cells or involve hydrophobic interactions. Following six rounds of binding and amplification, individual phage clones were sequenced (Table 1). For the acid-eluted phage, none of the inserted sequences were identical. In contrast, three of four cell-associated phage clones were identical (designated phage 12.1).

Phage 12.1 bound the cells approximately 100 times as efficiently as its parent library did (Fig. 2), indicating that this phage was selected because of higher affinity or avidity for the cells. Similar selection of cell-associated 20-mer phage following binding at 4 °C resulted in the isolation of one phage type after six rounds (phage 20.1, Table 1). As with the selected 12-mer, cell binding by phage 20.1 was approximately 100 times as high as that by the 20-mer library. As negative controls for the involvement of peptides in cell binding, wild-type phage lacking pIII peptides and phage presenting peptides selected for binding the yeast protein Gal4 (20.yeast) were tested. Cell binding by both peptide controls was 1% of the binding mediated by phage presenting the 20.1 peptide (Fig. 2 and data not shown). These data

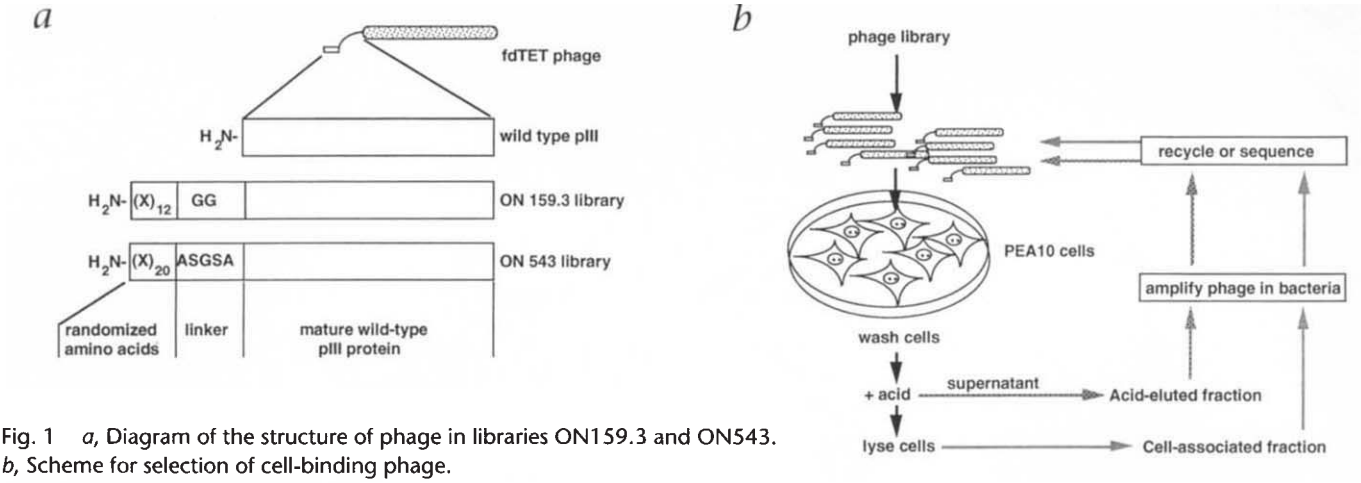

Fig. 1 *a*, Diagram of the structure of phage in libraries ON159.3 and ON543. *b*, Scheme for selection of cell-binding phage.

demonstrate that cell-binding peptides can indeed be generated by peptide-presenting phage libraries.

Fibroblast-binding peptide selection at 37 °C

We next determined whether peptides could be selected following binding at 37 °C under conditions that might allow peptide binding to endocytosing receptors. The phage were incubated on the cells in the presence of chloroquine to block potential degradation of any phage that bound a receptor undergoing endocytosis[8]. After six rounds of selection using the 12-mer library there was no apparent selection of particular peptides (Table 1). By contrast, three rounds of selection were sufficient to identify two peptides when the 20-mer library was subjected to binding and amplification in the same manner (Table 1). Three of four clones sequenced were identical (phage 20.2). Interestingly, the other selected clone was phage 20.1, which was also selected at 4 °C. Following three more rounds of selection, five of five sequenced clones were identical to phage 20.2, suggesting that the 20.2 peptide had a higher affinity for the cell surface or was more readily taken up by the cells. Both 20-mers bound the cells more than 1000-fold as well as their parent library (Fig. 2). This level of binding improvement is similar to that commonly observed following selection of phage against single proteins[4]. At 37 °C, both 20-mers bound the cells ~100 times as well as the selected 12-mer. In other experiments where the 12- and 20-mer libraries were mixed and co-panned, only 20-mer peptides were selected, with no evidence of 12-mer peptides in the population after the second round (data not shown). These observations and the inability of the 12-mer library to provide a peptide at 37 °C, suggests that longer amino-acid libraries may have advantages for binding cells, at least in this system.

Under the same conditions, the selected 20-mers associated with cells about 350 times as well as the irrelevant 20.yeast phage at 37 °C (Fig. 2), demonstrating the specificity of cell binding residues in the selected peptide sequences. Phage 12.1, which was selected at 4 °C, binds three times as well at 4 °C as at 37 °C, suggesting that this 12-mer recognizes a receptor that is not endocytosed and that this binding is unstable at higher temperatures. In contrast, cell-association by phage 20.1 and 20.2 was approximately 100-fold as high at 37 °C as at 4 °C, suggesting that increased cell association could be due to higher affinity or endocytosis of the phage at 37 °C.

Phage binding to other cell lines

In these initial experiments, phage that bound fibroblasts were selected without an attempt to deplete the library of phage binding other cell types. Therefore we were interested in the specificity of the selected peptides. The binding of each selected phage clone was compared across a panel of cell lines including the fibroblasts they were selected against (Fig. 3). Phage 12.1 bound to both fibroblasts and hepatoma cells with little binding to the other cells. Phage 20.1 and 20.2 appeared to bind all of the cells, except

Table 1 Predicted peptides selected for PEA10 cell binding

Phage population*	Clone	Clone peptide sequence†	Incidence‡
4 °C 12-mer, acid, passage 6		GHSSLGISRWVG	1/6
		GNAQRIFSPRSY	1/6
		AAFGSNRVELFM	1/6
		GLLSIIWNSSAR	1/6
		RGAEWYMVNEGD	1/6
		MNLARPNATSDM	1/6
4 °C 12-mer, cell, passage 6	12.1	SALNPWDEYLEL	3/4
		IASVVRTEVAGF	1/4
4 °C 20-mer, cell, passage 6	20.1	TPHSLYEDLKRQMMQLGRHL	5/5
37 °C 12-mer, cell, passage 6		MKVPDPVSNASN	1/5
		WNYTISERATRD	1/5
		NYTSTIMHRGYR	1/5
		SGNGSYFWVFPL	1/5
		GMRQAPDFAV(△)	1/5
37 °C 20-mer, cell, passage 3	20.1	TPHSLYEDLKRQMMQLGRHL	1/4
	20.2	KTLTLEAALRNAWLREVGLK	3/4
37 °C 20-mer, cell, passage 6	20.2	KTLTLEAALRNAWLREVGLK	5/5

*Phage population designates the selection process used to produce the indicated phage sequences. The acid-eluted fraction is referred to as 'acid' and the cell-associated fraction as 'cell'. Designated clones are those phage whose binding was further characterized in the text. †The amino acid sequence of each clone is expressed by single-letter abbreviation; positively charged amino acids are in bold type, whereas negatively charged amino acids are underlined. ‡The incidence refers to the number of times each phage was isolated out of the total number sequenced. △, In-frame deletion.

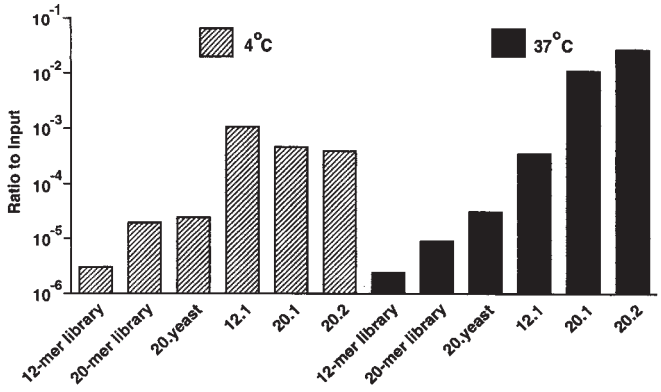

Fig. 2 Binding of library or selected phage to PEA10 cells. Binding was performed at 4 °C (striped bars) or 37 °C (black bars) for each phage indicated. Ratio to input is the number of cell-associated phage divided by the number of total phage applied to the cells.

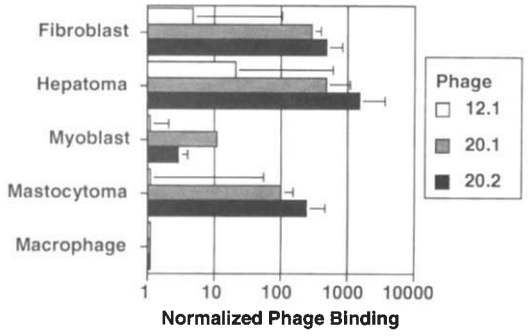

Fig. 3 Relative binding of selected phage clones to a panel of cell lines. Phage binding was performed on the following mouse cell lines: fibroblasts (PEA10), hepatoma (Hepa 1-6), myoblasts (Sol8), mastocytoma (P815), and macrophage (Raw 264.7). Phage were applied in similar numbers from a master solution to all of the cells. Each ratio to input was normalized by dividing it by the ratio to input for the nonspecific phage 20.yeast on each cell type. Error bars, s.d. from 3 experiments.

macrophages, to varying degrees. In all cases, the phage bound the hepatoma cells at higher numbers than the other cells, with 20.2 binding slightly better in all but the myoblast cell line. No phage bound the macrophage cell line. This effect was due to lack of binding rather than destruction of the phage by macrophage phagocytosis, since the binding of phage 20.yeast to these cells was comparable to its binding to any of the other cell types. Considering that the phage were selected against the fibroblasts without an attempt to restrict binding only to fibroblast cells, it is not particularly surprising the selected peptides recognized common cellular receptors.

Phage selection against myoblasts and myotubes

The 12- and 20-mer phage libraries were mixed and selected against C_2C_{12} myoblasts and C_2C_{12} cells differentiated into myotubes to test whether other phage sequences could be selected. Within two rounds of selection, phage 20.1, which was previously selected against fibroblasts, dominated the population of phage selected against the myoblasts (Table 2). This is perhaps not surprising, because this peptide bound myoblasts reasonably well (Fig. 3). However, selection of the mixed library against myotubes produced two new peptides (T.1 and T.2) within five rounds of selection (Table 2). Phage 20.1 was not selected against the myotubes, suggesting that the cellular receptor recognized by 20.1 is downregulated on the differentiated cells. The binding of T.1 and T.2 to myotubes was 10 times that of the starting mixed library. This lower binding relative to the other selected peptides could indicate either poor affinity or a low number of cellular receptors. The data indicate that a variety of peptide sequences can be selected against different cell types. It may also be significant that no 12-mer phage sequences were observed when the 12- and 20-mer libraries were selected together.

Peptide sequence and predicted structural comparison

No obvious linear sequence similarity was apparent between any of the selected peptides, suggesting that the peptides recognized different cell-surface molecules. Although no linear similarity was obvious, peptides 20.1 and 20.2 were both predicted to adopt an amphipathic α-helix where the charged amino acids cover only one side of the helix. Phage 20.1 and 20.2 do not appear to bind the same cellular receptor, as binding of either was uninfluenced by a 100-fold excess of the other phage (data not shown). This also suggests that it is not the amphipathic α-helix *per se* or phage aggregation that causes the phage to bind to mammalian cells. Peptide 12.1 would be predicted to have little secondary structure other than a potential β-turn in the center of the peptide. Nor was there any apparent sequence similarity between fibroblast- and myotube-selected peptides. Peptide T.1 is predicted to be mostly without structure although it may possibly have an α-helical segment in the final eight amino acids. Peptide T.2 is predicted to be without structure.

Mutant peptides derived from the 20.2 peptide

A mutagenic library was constructed based on the 20.2 peptide sequence such that there was a 70:10:10:10 misincorporation of the first two bases of each codon with a G or T in the third position. These mutants were constructed in the pVIII protein as opposed to the selections described above, which were peptides fused to pIII. Rather than having two to five peptides colocalized

Table 2

***a*, Predicted peptide selected for myoblast cell binding**

Phage population*	Clone	Clone peptide sequence†	Incidence‡
37 °C 12- and 20-mer, cell, passage 2	20.1	TP**H**SLYEDL**KR**QMMQLG**RH**L	5/5

***b*, Predicted peptides selected for myotube cell binding**

Phage population*	Clone	Clone peptide sequence†	Incidence‡
37 °C 12- and 20-mer, cell, passage 5	T.1	TGGETSGI**KK**APYASTT**RN**R	3/5
	T.2	S**HH**GVAGVDLGGGADF**K**SIA	2/5

Table item designations are described in Table 1.

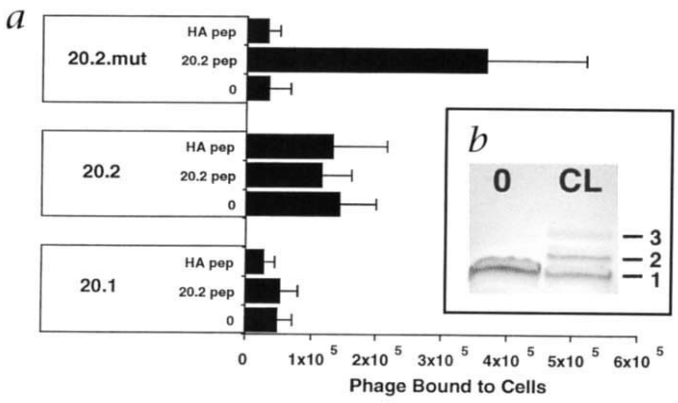

Fig. 4 20.2 peptide effects and dimerization. *a*, Phage were bound to PEA10 fibroblasts as described in Fig. 2 in the absence (0), or presence of the 0.4 mM 20.2 peptide (20.2 pep), or 0.4 mM hemagglutinin peptide (HA pep). Bars indicate the average number of bound phage from 3 experiments. Error bars, s.d. *b*, The 20.2 peptide (1 mg/ml) was incubated in crosslinking buffer in the absence (0) or presence (CL) of oxidizing reagent necessary to initiate Gly-Gly-His crosslinking. The samples were run on a Phast HD gel under denaturing conditions and stained with Coomassie blue. 1, 2 and 3 indicate the number of peptides predicted to be present in each band of the gel.

on one end of the phage, approximately 150 of the ~3000 pVIII molecules per phage present peptide. Therefore there are not only more peptides per phage, but the peptides are relatively physically separated from each other.

The mutagenic library was selected against the fibroblasts as was done with the pIII library, and the cell-associated fractions were cycled. After six rounds of selection, sequencing of random clones revealed partial selection of one peptide designated 20.2.mut (Table 3). Considering that the library should contain more than 1000 copies of the original 20.2 peptide, the fact that we did not observe the starting peptides suggests that 20.2.mut was an improved peptide. However, fibroblast binding by phage presenting 20.2.mut peptide was only ~25% of cell binding by phage presenting 20.2 peptide (Fig. 4). This suggested that the peptides presented separately on pVIII were less effective than those presented on pIII.

Effects of free 20.2 peptide on phage binding

The 20.2 peptide was synthesized and used to test whether it could affect phage binding on the mammalian cells. When incubated on the cells at 1 mg/ml (0.4 mM), the peptide had no inhibitory effect on phage 20.1 or 20.2 binding to fibroblasts (Fig. 4). Likewise, an irrelevant peptide corresponding to the hemagglutinin peptide from influenza had no effect. The lack of peptide competition for pIII-presented 20.2 is perhaps not surprising, because the peptide is out of context of the "scaffold" of the pIII protein, and the cellular target could be undergoing continuous turnover making competitive inhibition difficult. However, another explanation is supported given that addition of the 20.2 peptide to the pVIII 20.2.mut phage increased its binding to the fibroblasts tenfold to a level approximately three times that of the original pIII-presenting 20.2 peptide phage (Fig. 4*a*). By contrast, the hemagglutinin peptide had no effect. The observation that the free 20.2 peptide had no effect on pIII-presenting 20.2 phage, but increased pVIII-presented 20.2.mut binding, suggests that the peptide complements a defect in the binding of the pVIII peptide. The pIII-presented 20.2 peptides exist on the phage in two to five copies in the same locale, whereas the 150 pVIII-presented peptides are "diluted" on the surface of the phage by the other ~3000 wild-type pVIII molecules. Given this, we propose that the 20.2 peptides on pIII bind cells via cooperative interactions, whereas the pVIII peptides are prevented from this by being physically segregated. The observed binding of the 20.2.mut phage with peptide was three times that of the original 20.2 pIII-presented peptide. This would also be consistent with the 20.2.mut peptides being present in greater numbers (~30-fold higher) per phage.

The 20.2 peptide dimerizes in solution

To test whether the 20.2 peptide can form multimeric structures, the free peptide was crosslinked in solution using a Gly-Gly-His oxidative crosslinking reagent[9]. This agent is not itself a crosslinker, but appears to activate hydrophobic residues to form covalent crosslinks between proteins in tight association. Because residues within the protein form the covalent bridge, crosslinking with the Gly-Gly-His reagent has been shown to occur only when proteins are in legitimate association. Random collisions of proteins in high concentrations (that is, 50 mg/ml) produce no crosslinks unless the proteins form stable associations. At concentrations of 1 mg/ml, approximately 25% of the 20.2 peptide crosslinked as a dimer, and a small amount forms trimers as assessed by denaturing gel electrophoresis (Fig. 4*b*). Hemagglutinin peptide and an S10 epitope peptide did not form dimers under the same conditions (data not shown). The formation of dimers and trimers and the enhanced binding by the pVIII-presented peptide strongly suggest that the 20.2 peptide present in two to five copies per phage could form dimers on the phage and that these could be involved in cell binding. A dimeric binding peptide is consistent with the enhancing effects of peptide on pVIII-presenting phage binding, because 20.2.mut is also predicted to form an amphipathic α-helix, which could associate with the 20.2 free peptide. In addition, 20.2 peptide combined with the pVIII-presented peptide gave higher binding than the pIII-presented peptide, which is consistent with the higher number of pVIII peptides than pIII peptides per phage. Coincubation of the crosslinked 20.2 peptide did not block binding by

Table 3 Predicted mutant pVIII peptides derived from peptide 20.2

Phage population*	Clone	Clone peptide sequence†	Incidence‡
Original 20.2 peptide	20.2	KTLTLEAALRNAWLREVGLK	
37 °C pVIII mutant library R6	20.2.mut	M**TL**P**L**V**AA**VQ**N**EA**L**H**EVG**SN	4/7
		M**TL**P**L**DDFR**R**P**A**S**L**EH**VGL**I	1/7
		M**TL**PPHDFR**R**P**A**S**L**EH**VGL**I	1/7
		I**TL**NVVD**A**PWK**AW**MWSGEEN	1/7

Table item designations are as described in Table 1 except that amino acids in bold type are identical to those in the original 20.2 peptide.

20.2 phage or 20.2.mut phage (data not shown), which adds to our argument that dimerization outside of the context of a protein structure is not sufficient for competitive binding.

Phage immunolocalization

Fibroblast-binding phage were immunolocalized following binding to PEA10 cells at 37 °C for 2 hours (Fig. 5). Phage 20.1 and 20.2 both bound strongly to the surface of the cells (not shown and Fig. 5*b*), whereas phage 20.yeast showed no specific binding (Fig. 5*d*). Phage 12.1 was barely detectable on the cell surface, which was consistent with its lower binding ability at 37 °C (not shown). Binding by 20.1 and 20.2 to individual cells was heterogeneous, showing strong, intermediate or no immunolocalization of phage on both attached and unattached cells (Fig. 5*b* and not shown). Incubation of phage-bound cells for 24 hours after removal of unbound phage caused little change in the cell surface binding of phage 20.1, whereas phage 20.2 appeared in "clumps" on or in some of the cells (not shown). Although there appeared to be some endosomal phage protein (arrow), this was not observed in all of the cells.

Discussion

We have shown that phage libraries presenting random peptides can be used to generate cell-binding peptides in the absence of any prior knowledge of cellular receptors. This observation represents a preliminary step toward developing cell-targeting gene therapy vectors at will. Because peptides can be selected in as little as a week using inexpensive reagents, this selection system could supply a high throughput process for generating peptides that recognize a variety of cell types. Cell-binding peptides selected in this manner could then be linked by physical or genetic manipulation to gene therapy vectors that mediate their own endocytosis (for example, adenovirus[10,11]). A slightly different phage selection strategy generated peptides that not only produced much stronger cell-binding peptides, but may also mediate endocytosis. Peptides selected to target endocytosing receptors would be most useful when linked to gene therapy vectors that have no inherent capacity to enter the cell (such as polycation–DNA complexes[8]). Selection of cell-binding peptides by this technique may also provide a means of targeting other bioactive agents, like drugs, to particular cells *in vivo*.

Cell specificity of selected peptides. The selected 12.1 peptide bound only fibroblasts and hepatocytes and did not bind myoblasts, mastocytoma or macrophage cells. By contrast, the 20-mer peptides recognized several different cell types. This is not surprising, because they were not selected to bind only fibroblasts, and it is possible that common cellular targets (for example, Na^+/K^+-ATPase) may have a higher affinity for peptides in the population. Selection of phage against common receptors is supported by the observed selection of the 20.1 peptide following binding to both fibroblasts and myoblasts. These types of promiscuous peptides could be used for targeting cells in general. To select cell-specific peptides, phage libraries could be cleared on unwanted cell types to eliminate promiscuous peptides from the peptide population before panning on the cells of interest. Alternatively, a collection of peptides selected against one cell type could be subsequently screened for the desired cell-binding specificity. The existence of different cell-binding peptides in the libraries was indicated by the selection of two novel peptides when panned against myotubes. In addition, platelet-binding peptides have been selected from random-peptide libraries[12], further indicating the potential for using phage libraries to identify ligands against a variety of cell types.

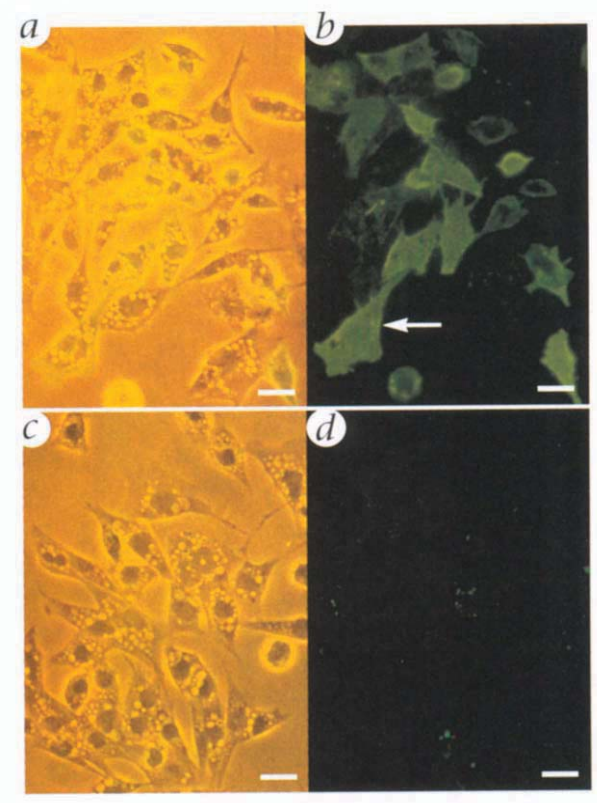

Fig. 5 Immunolocalization of phage on PEA10 cells. Immunolocalization (*b* and *d*) and phase contrast of the same frame (*a* and *c*) of PEA10 cells incubated with the indicated phage. *a* and *b*, cells incubated with phage 20.2, ×200 magnification. The arrow indicates potential endosomal immunolocalization of the phage. *c* and *d*, Cells incubated with 20.yeast, an irrelevant 20-mer phage selected for binding to a yeast protein (×200). Scale bar, 20 μm.

Complementation of phage binding by free peptide. Not surprisingly, the free 20.2 peptide did not compete with binding of the phage-born peptide. What was surprising was that the free peptide increased the binding of the 20.2mut phage to cells up to 50-fold. Our explanation for this effect is that the 20-mer peptides bind most efficiently as dimers or multimers. On pIII this dimerization would be facilitated because the peptides are in close proximity. However, since the peptides are presented on only a fraction of the pVIII proteins, there is less chance for dimerization. Consistent with this scenario is the demonstration that the 20.2 peptide does efficiently form dimers in solution. If dimerization does play a role in binding it may explain why the 20-mer library is more effective than the 12-mer, particularly at 37 °C. It also suggests that libraries with even longer random peptides may be worth exploring.

Random vs. rational cell-binding peptides. An alternate approach to using random peptides to target unknown receptors has recently been used in rationally designed peptide libraries. Phage bearing peptides derived from known sequences that recognize integrin[6] or thrombin receptors[7] are able to bind cells. The integrin-binding peptides are particularly interesting in that they

are able to enter cells in an endosome-independent manner. Peptides generated for binding particular receptors may be useful in those cases where a particular cell-specific receptor is known for the target cell, whereas the random peptide approach described here may be useful when the biology and receptors of target cells are unknown. The random peptide approach may be particularly useful for selecting peptides against cancer cells, because these cells will not be of defined lineage and may present a nearly random set of potential cell-surface receptors. It is conceivable that cell binding peptides selected from random libraries could also be used to isolate or characterize the receptors that they recognize, although this characterization would not be necessary for their utility in cell targeting.

Peptide-presenting phage as gene delivery vehicles. The integrin-binding peptide described above has recently been used out of context of phage to mediate transfection of mammalian cells by forming complexes with plasmid DNA using poly-L-lysine[13]. Likewise, phage carrying reporter plasmids are able to transfect mammalian cells in the presence of DEAE dextran[14] or liposomes (data not shown). In preliminary experiments, we have used phage bearing the 20.2 peptide and a luciferase plasmid to mediate low-level transfection of the PEA10 fibroblast cells (data not shown). We are currently testing the specificity of this transfection as a means of assessing the possible use of peptide-presenting bacteriophage as transfection vehicles. Should these bacterial viruses prove useful, they would be a biological analog of the poly-L-lysine-DNA complexes used for gene therapy. In the phage, modifications to enhance gene delivery (for example, addition of endosome-disrupting agents[15] or integrin-binding peptides[13]) could be introduced by cloning rather than chemical manipulation, because the phage tolerate multiple changes to their proteins[4–6,16,17]. Future work will involve testing phage as gene delivery vectors and developing methods to screen peptides specific for only certain cell types.

Methods

Cell lines. PEA10 mouse fibroblasts were obtained from Joachim Herz (University of Texas, Southwestern, Dallas). Hepa1-6 mouse hepatoma cells, P815 mouse mastocytoma cells, and RAW264.7 monocyte-macrophage cells were obtained from American Type Culture Collection. Myoblasts were differentiated as described in ref. 18.

Phage library construction. pIII libraries were constructed as described in ref. 5 and their basic structure is shown in Fig. 1*a*. Mutagenic pVIII libraries were constructed similarly by insertion of randomly mutated oligonucleotides based on the peptide sequence of phage 20.2 into the pVIII gene in phagemid vector p8V2. Random insertion of mutant bases in positions 1 and 2 of each codon occurred at a ratio of 70:10:10:10 such that 70% of each contained the normal base and 30% contained another base. The third position of each codon was restricted to G or T.

Phage selection. PEA10 cells in 60-mm dishes were incubated in serum-free medium for 2 h before incubation with phage. In the first round of panning, approximately 10 library equivalents (3×10^9 phage from ON159.3 and 4×10^9 phage from ON543) were added to PEA10 cells in a total volume of 2 ml of PBS-BSA (8.2 mM Na_2PO_4, 1.5 mM KH_2PO_4, pH 7.25, 137 mM NaCl, 2.7 mM KCl, 0.1% bovine serum albumin) supplemented with 1 mM $CaCl_2$, 10 mM $MgCl_2$ (PBS-BSA Ca/Mg). The phage were then incubated with the cells for 1 h at 4 °C or 37 °C (with 100 μM chloroquine). The cells were washed 6 times with 5 ml of cold PBS-BSA Ca/Mg for 5 min each. 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 hour on ice. The cell debris was scraped from the plate, spun in a vortex mixer briefly and saved as the cell-associated phage fraction. Phage from each fraction were then amplified as described[5]. For each subsequent round of panning, portions of purified phage were reapplied to PEA10 cells and panning was carried out as described while maintaining fraction specificity (for example, when an acid-eluted fraction of phage was initially recovered, then only the acid fraction was amplified in all subsequent rounds). pVIII-presenting phage were used similarly, except that helper phage was used to propagate the selected phagemids.

Sequencing selected phage. At selected rounds of panning (3 or 6), individual colonies of phage-infected bacteria were isolated from random sites on plates, and each colony was grown overnight in 2 ml YT-TET. The phage were isolated from this solution[5] and lysed in 60 μl of 10 mM Tris, pH 8, 0.1 mM EDTA, 0.5% Triton X-100 by incubation for 15 min at 72 °C. Single-stranded DNA was precipitated by addition of 30 μl of 2.55 M NaOAc and 150 μl of cold 95% ethanol and incubation at –20 °C for 30 min. Phage DNA was recovered by centrifugation for 5 min at 13,000 r.p.m. The DNA was resuspended in 10 μl of TE and 1.5 μl was used for sequencing using an end-labeled primer identical to that described[5] using the fmol sequencing kit (Promega).

Phage binding comparison. Indicated phage clones and libraries were grown up in liquid culture and purified[5]. To test for cell binding, approximately equal numbers of each phage (10^9 to 10^{10}) were diluted into a master solution of PBS-BSA Ca/Mg immediately before each experiment, and aliquots of this solution were added to approximately 10^6 mammalian cells for 1 h at 4 °C or 37 °C as for selection. The cell-associated phage were recovered and their titers were estimated by incubation of a dilution of this sample with 100 μl of concentrated K91 cells for 10 min at 37 °C. The cells were then plated onto 100 mm YT-TET plates and grown overnight. Sample phage titers were then calculated from the number of phage/bacteria colonies and the appropriate dilution factor. For peptide competition, the indicated peptides were added at the same time as phage at the indicated concentrations.

20.2 peptide crosslinking. The 20-amino acid peptide corresponding to the 20.2 peptide was synthesized by Research Genetics Inc. (Huntsville, Alabama). Hemagglutinin (YPYDVPDYA) and S10 (MASMTGGQQMG) peptides previously used for epitope-specific antibody release were used as negative control peptides. Each peptide was diluted to 1 mg/ml in PBS and crosslinked as described[9]. Non-crosslinked control peptides were treated as the crosslinked samples except the MMPP oxidizing agent required for crosslinking was not added.

Phage immunolocalization. PEA10 cells were plated into the wells of an 8-well Lab-Tek permanox chamber slides. The next day, the medium was removed, and the cells were incubated in serum-free medium for 2 h. The medium was removed and approximately 10^9 phage were added to the chamber in 200 μl of PBS-BSA Ca/Mg with 100 μM chloroquine and incubated at 37 °C for 2 h. The cells were then washed 6 times with PBS-BSA and then fixed in 400 μl of 3.7% formaldehyde in PBS for 10 min. The fixed cells were washed 3 times with 5% dried milk in 0.1% saponin in PBS and then incubated for

1 h at room temperature with 200 µl of 1:50 dilution of sheep anti-M13 antibody (5 Prime-3 Prime, Boulder, Colorado) in 5% milk, 4% rabbit normal serum, 0.1% saponin in PBS. The chambers were then washed 6 times during 15 min with 0.1% saponin in PBS and incubated for 1 h at room temperature with 1:500 biotinylated rabbit anti-sheep IgG (Vector Laboratories, Burlingame, California). in 5% milk, 4% rabbit normal serum, 0.1% saponin in PBS. The chambers were washed 6 times with 0.1% saponin in PBS and incubated for 1 h with 1:500 Neutralite avidin-fluorescein conjugate (Molecular Probes, Eugene, Oregon) in 5% milk, 4% rabbit normal serum, 0.1% saponin in PBS. The chambers were washed 6 times with 0.1% saponin in PBS, the solution was removed from the chambers and the chambers, and their gasket were detached from the slide. The slide was overlaid with Vectashield (Vector), and a coverslip was placed on the slide. The cells were visualized using a UV light source on an Olympus BH-2 microscope with fluorescein filters. The cells were photographed on automatic with an Olympus OM-4T camera using Kodak Ektachrome Elite 400-speed color slide film.

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