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A Cell-Penetrating Peptide from a Novel pVII–pIX Phage-Displayed Random Peptide Library

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Abstract—A novel random peptide library was constructed using a phage-display format on the coat proteins pVII and pIX of filamentous bacteriophage. Panning against B-lymphocyte WI–L2 cells yielded one unique peptide-phage, denoted CHL8, that specifically bound to and penetrated the cells. Studies of each peptide derived from CHL8, denoted pep7 and pep9, established that only pep7 mediated the observed activity and only as a homodimer. Peptide libraries displayed on pVII–pIX should serve as a novel source of bioactive ligands for a variety of applications.

Introduction

A strategy for peptide and protein display on the surface of filamentous bacteriophage was first presented by Smith in the mid-1980s.[1,2] The phage-display method is based on the fact that polypeptides fused to the capsid or 'coat' proteins of bacteriophage are displayed for targeted selection on the phage particles that also encapsulate the cognate genes. Hence, the structural linkage between a polypeptide sequence expressed on the phage, and the DNA encoding that sequence, permits a functional linkage between target recognition and sequence replication that facilitates the rapid screening and identification of polypeptides with novel and desirable properties. Since its inception, phage display has matured as a widespread technology for harnessing the chemical and structural diversity of peptide and protein libraries and has revolutionized the discovery of molecules for many endeavors in academia and industry.[3–14]

In this regard, peptides and proteins that can be internalized by cells have been the subject of intensive study in recent years, since translocation across the cell membrane is critical for drug delivery and gene therapy.[15] Filamentous bacteriophage, which exhibit no natural tropism for mammalian cells,[16] have been genetically engineered to confer mammalian cell-specific tropism.[17–21] Significantly, the advent of phage display has provided a powerful technique to confer this property to bacteriophage and, in the process, select in vitro the peptide or protein that facilitates cell penetration, perhaps by triggering receptor-mediated endocytosis.[22–27] The phage-display method has been also extended to the in vivo selection of peptides that target tumor blood vessels, with the peptides then used to enhance the efficacy of the anticancer drug doxorubicin against a human breast cancer xenograft in mice.[28,29] Developments in these areas of research should have a great impact on the future of targeted therapies.

To date, peptides that bind to cells and trigger internalization have been obtained from peptide libraries that used either pIII phage genome or pVIII phagemid multivalent display formats.[22,23,26,30] We previously showed that the phage coat proteins pVII and pIX could be used to display the antibody variable heavy-chain region (V_H) and variable light-chain region (V_L), respectively, and that this heterodimeric presentation afforded a viable Fv with fully functional binding and catalytic activities.[31] In the present study, we constructed

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a random 15-mer peptide library using display on both pVII and pIX, and demonstrated the direct selection of a unique cell-binding and internalizing peptide utilizing whole cell panning methods.

Results

Library construction and selection

In order to further explore the potential applications of our pVII–pIX phage-display format, we sought to isolate novel peptides that penetrate a specific cell type, perhaps via receptor-mediated endocytosis. To this end, a random peptide library was constructed displaying 15-mer peptides on phage pVII and pIX (Fig. 1). A series of 50 electroporative transformations of *Escherichia coli* XL1-Blue cells with the phagemid afforded a large peptide-phage library containing $\sim 7\times10^9$ members. To examine the integrity of the library, 20 clones were picked at random, and all were found to contain 15-mer peptides on pVII and pIX and with diverse DNA sequences (data not shown). The peptide library was subjected to five rounds of panning against the B-lymphocyte cell line WI–L2 based solely on selecting for the property of internalization. Nonspecific binding peptide-phage were washed away with HBSS buffer, and tightly bound peptide-phage on the cell surface were removed by using a low pH glycine buffer. Finally, the cells were lysed with 100 mM TEA to release any internalized peptide-phage. The internalized peptide-phage were amplified from the cell lysate by infection of freshly prepared *E. coli* XL1-Blue cells. The output peptide-phage number was $\sim 5\times10^5$ cfu in the first round of panning and $\sim 1\times10^9$ cfu in the fifth round of panning that yielded a >1000-fold enrichment. After the fifth round of panning, 15 clones were randomly picked and DNA sequence analysis revealed the same clone in all cases, denoted CHL8, containing two different peptide sequences. These two 15-mer peptide sequences displayed by pVII and pIX were SDLWEMMMV-SLACQY, denoted pep7, and GEAHIPTSEMREKGW, denoted pep9, respectively. Database searches revealed no significant similarities between these two peptides and other known protein sequences.

We also investigated whether specific selection conditions would favor the isolation of peptide-phage that underwent different internalization pathways. Hence, in parallel to the above panning experiment, a second protocol was also used in which chloroquine, a lysosomotropic agent, was present in the cell culture medium during panning. The use of chloroquine would be expected to favor survival and recovery of peptide-phage transported into lysosomes. Panning was done in the presence of 50 μM chloroquine, the cell numbers were reduced from $\sim 5\times10^7$ in the first round to $\sim 1\times10^7$ in the fifth round, and the incubation time with peptide-phage was decreased from 6 h in the first round to 4 h in the fifth round of panning. Of 15 clones that were randomly picked after the fifth round, all contained the pep7 and pep9 sequence pair found in the absence of chloroquine. In addition, the peptide-phage output was $\sim 6\times10^5$ cfu in the first round and $\sim 8\times10^7$ cfu in the fifth round, or only ~ 100-fold enrichment. Consequently, the results showed that chloroquine did not likely alter the initial peptide-phage and WI–L2 cell binding and internalization events, and did not increase the enrichment of peptide-phage that might have specifically translocated into the cell using a lysosomal pathway.

Characterization of CHL8 peptide-phage

The overall endpoint internalization of CHL8 peptide-phage into WI–L2 cells was first assayed by infection of *E. coli* XL1-Blue with WI–L2 cell lysates and then peptide-phage titering. A DNA-binding peptide-phage, denoted DNA19, selected from the same library was tested in

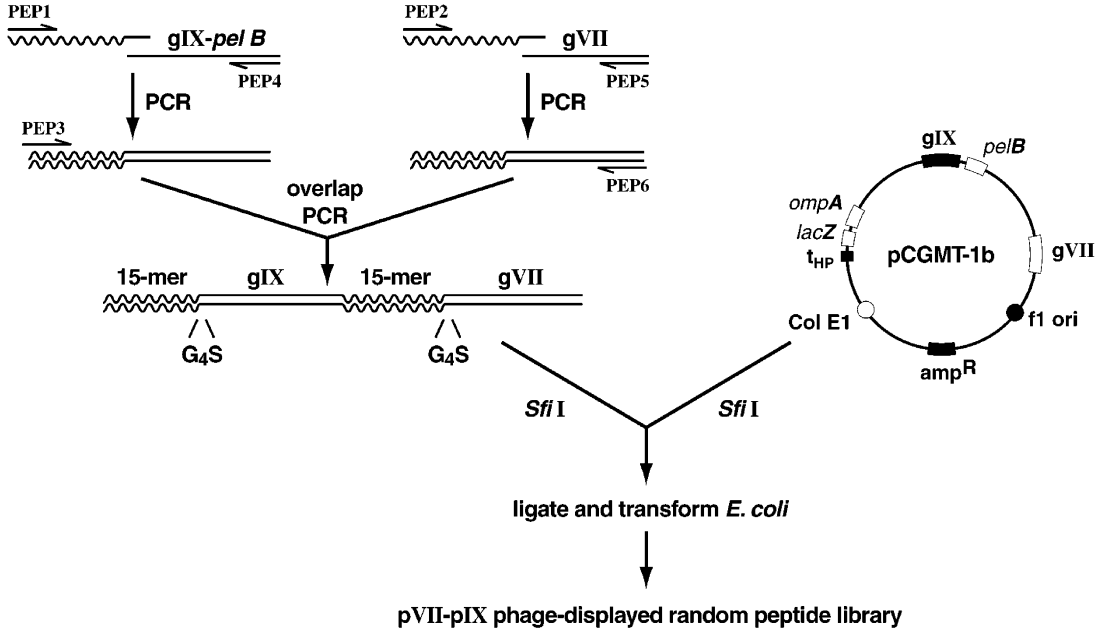

Figure 1. Construction of the phage-displayed pVII–pIX random peptide library.

parallel as a negative control. It was shown that the recovered CHL8 peptide-phage number (>200 cfu/cell) was ~200-fold greater than that of DNA19 peptide-phage (<1 cfu/cell). Since binding is a prerequisite for internalization, the binding event itself for CHL8 peptide-phage and WI–L2 cells was further studied by flow cytometry. As expected, the CHL8 peptide-phage showed an increased fluorescence shift relative to DNA19 peptide-phage (Fig. 2). The results demonstrated that CHL8 peptide-phage had a specific binding and internalization activity against WI–L2 cells.

We further examined the internalization of CHL8 in a more direct fashion, and also obtained evidence that CHL8 peptide-phage were actively endocytosed into WI–L2 cells. The phage particles were stained inside the cells, using a biotinylated mAb that recognized the phage major coat protein pVIII in conjunction with a FITC–streptavidin conjugate, that allowed direct visualization with confocal laser scanning microscopy (Fig. 3). The WI–L2 cells incubated with CHL8 peptide-phage at 37 °C showed a strong intracellular staining, while no staining was observed using DNA19 peptide-phage. Furthermore, no intracellular staining was observed with CHL8 peptide-phage when the incubation with WI–L2 cells was performed at 4 °C (data not shown).

Functional activity of peptide-phage mediated by pep7

We investigated whether both pep7 and pep9 were required for the cell-binding and internalization activity of CHL8 peptide-phage. To this end, the two peptide-phage constructs, CHL8-p7 and CHL8-p9, displaying each of the peptide sequences individually, were tested for internalization activity with WI–L2 cells, in a parallel comparison with other peptide-phage. The titering method described above revealed the following output peptide-phage numbers (cfu): CHL8 (4.6×10^9), CHL8-p7 (9.4×10^8), CHL8-p9 (9.0×10^5), CHL8-p7-VIII (2.6×10^6), DNA19 (1.2×10^6). Surprisingly, the results showed that CHL8-p7 had only a five-fold decrease in activity compared to CHL8, while all other peptide-phage showed a ~1000-fold lower and comparable level of activity (~10^6 cfu). Apparently, only the pVII sequence of the CHL8 peptide-phage was essential for activity and could independently effect peptide-phage cell penetration. Notably, the multivalent display of pep7 on the phage coat protein pVIII in the CHL8-p7-VIII peptide-phage was devoid of activity. This suggested that high-density displays of pep7 were detrimental to activity, but did not rule out the possibility that perhaps lower valent displays, such as two pep7 sequences on pVII (five copies on phage) could retain activity and act as a homodimer.

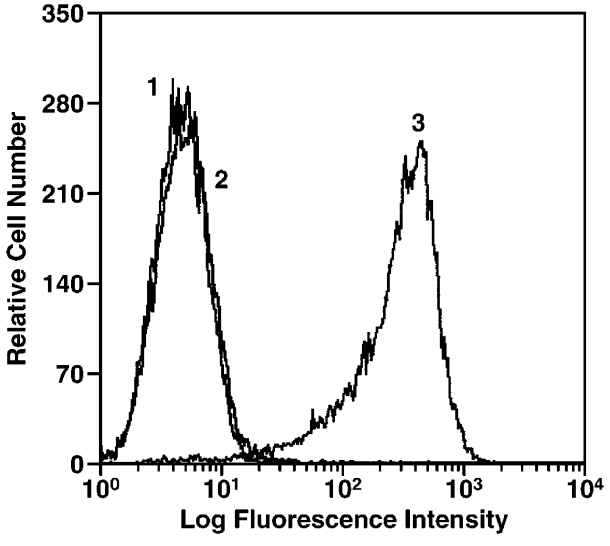

Figure 2. Flow cytometry of peptide-phage binding to B-lymphocyte WI–L2 cells. The WI–L2 cells were first incubated with the respective peptide-phage, followed by incubation with anti-M13 phage mAb, and finally with FITC-conjugated $F(ab')_2$ goat anti-mouse antibody. (1) control, no peptide-phage; (2) control, DNA19 peptide-phage; (3) CHL8 peptide-phage.

Cell binding of a pep7-Jun homodimer

To address the question of whether pep7 was active as a homodimer, a peptide fusion protein was constructed which consisted of pep7-(G_4S)-Jun-FLAG. The Jun dimerization domain was utilized to form the homodimer in which the pep7 chains could interact in a potentially structurally and functionally specific fashion. Expression of the protein occurred as an inclusion body in *E. coli* and refolded as the expected dimer (data not shown). Whole cell enzyme-linked immunosorbent assay (ELISA) showed that the pep7-Jun homodimer bound to WI–L2 cells, and flow cytometry confirmed the binding and a high level of interaction with nearly a

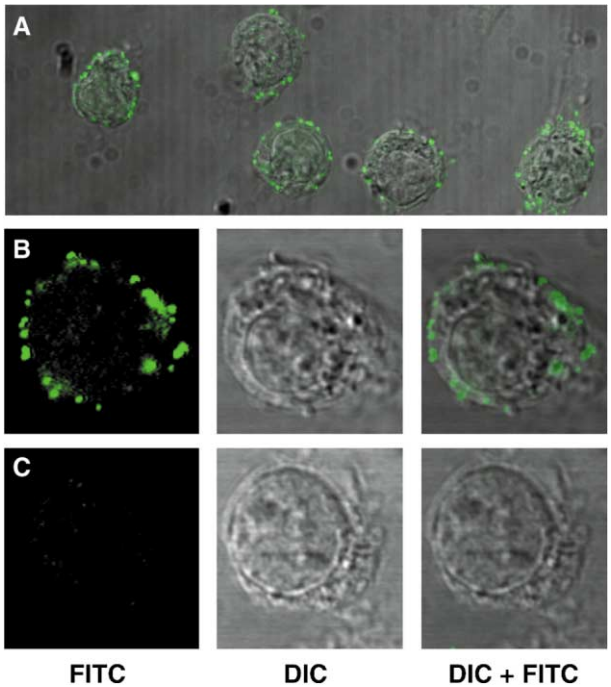

Figure 3. Internalization of peptide-phage in B-lymphocyte WI–L2 cells viewed by confocal laser scanning microscopy. Fixed and permeabilized cells were incubated with biotinylated anti-M13 phage mAb, followed by FITC–streptavidin. (A) overview of intracellular CHL8 peptide-phage; (B) CHL8 magnified views with FITC visualization and differential interference contrast (DIC); (C) control, DNA19 peptide-phage.

100-fold shift in fluorescence intensity compared to the control (Fig. 4). This was similar to the activity observed for CHL8 peptide-phage (Fig. 2).

Cell penetration of a pep7-Jun homodimer

The internalization of the pep7-Jun homodimer into WI–L2 cells was detected by immunofluorescence using deconvolution fluorescence microscopy. The intracellular pep7-Jun fusion protein was stained directly using FITC–streptavidin, since the pep7-Jun was biotinylated, and also with primary/secondary antibody labeling using an anti-FLAG M2 mAb and a goat anti-mouse FITC–F(ab′)$_2$ conjugate. It was observed that the internalized pep7-Jun had an uneven distribution within the cells and was predominantly clustered on one side of the nuclei (Fig. 5). The uneven distribution of peptide-phage within cells was previously observed by other workers.[32]

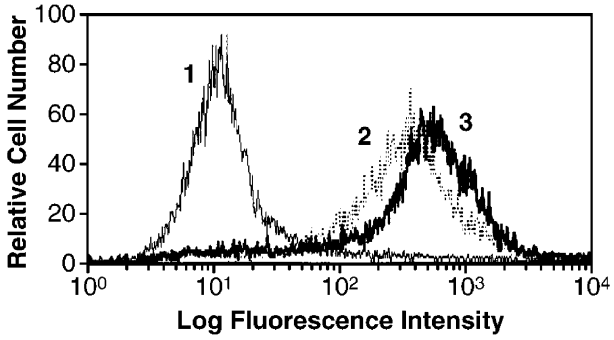

Figure 4. Flow cytometry of pep7-Jun binding to B-lymphocyte WI–L2 cells. The WI–L2 cells were first incubated with biotinylated pep7-Jun, followed by incubation with labeling reagents. (1) control, no pep7-Jun; (2) FITC–streptavidin; (3) anti-Flag M2 mAb, then FITC-conjugated F(ab′)$_2$ goat anti-mouse antibody.

The pep7-Jun/pep9-Fos heterodimer. Similar to the Fab antibody expression system from our laboratory, two leader sequences, *pelB* and *ompA*, were used to transport the untagged pep7-Jun and FLAG-tagged pep9-Fos fusion proteins into the periplasm, respectively. The pep9-Fos could be isolated by affinity gel chromatography free of pep7-Jun homodimer. Native-PAGE identified two protein bands at 8.5 and 17 kDa corresponding to the pep9-Fos monomer and pep7-Jun/pep9-Fos heterodimer, respectively. Whole cell ELISA showed no binding activity to WI–L2 cells (data not shown).

Synthetic pep7 monomer. The synthetic pep7 did not competitively inhibit the binding of either CHL8 peptide-phage or the pep7-Jun homodimer to WI–L2 cells (data not shown).

Discussion

A novel random peptide library displayed by phage coat proteins pVII and pIX was constructed and used for panning a B-lymphocyte WI–L2 cell line based on internalization as an enforced selection parameter, and with no predefined knowledge of a membrane target or endocytic pathway. The aim was to identify peptides that could penetrate the cell membrane, perhaps by triggering receptor-mediated endocytosis as the mechanism of action. After five rounds of panning, a unique pair of sequences, pep7 and pep9, on peptide-phage clone CHL8 was selected from the library. Flow cytometry showed that CHL8 peptide-phage satisfied the criterion of first binding to the surface of WI–L2 cells. Analysis of confocal sections by fluorescence microscopy then firmly established that CHL8 peptide-phage were also able to enter the cell.

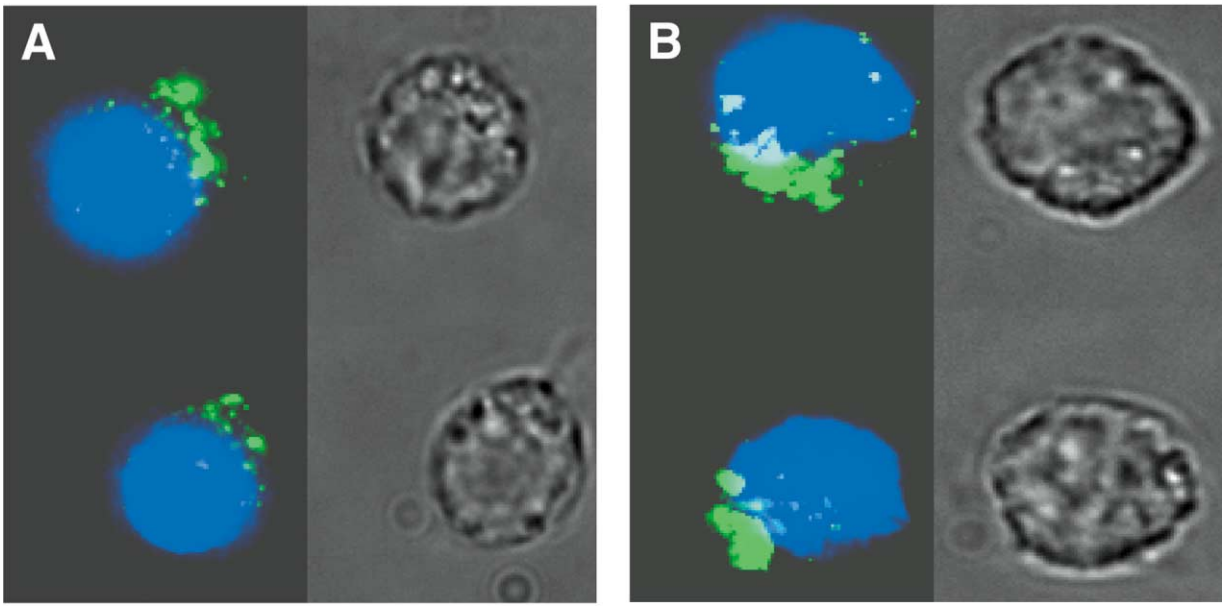

Figure 5. Internalization of pep7-Jun in B-lymphocyte WI–L2 cells viewed by deconvolution fluorescence microscopy. Fixed and permeabilized cells were incubated with biotinylated pep7-Jun, followed by incubation with labeling reagents. The WI–L2 cell nucleus was dyed blue with Hoest before mounting and observation. (A) FITC–streptavidin; (B) anti-Flag M2 mAb, then FITC-conjugated F(ab′)$_2$ goat anti-mouse antibody.

To ascertain the structural requirements for the activity of CHL8 peptide-phage, the cell-binding and internalization of peptide-phage displaying the individual peptides, pep7 and pep9, were investigated. It was found that pep7, as part of its original pVII construct, showed activity comparable to the parent CHL8 peptide-phage based upon phage titering, after incubation with the WI–L2 cells followed by cell lysis. On the other hand, pep9, as part of its original pIX construct, showed no activity. Furthermore, a peptide-phage construct in which pep7 was displayed on coat protein pVIII was also inactive, suggesting that higher-order clustering of pep7 was detrimental.

At this point, we speculated as to minimum requirements for pep7 activity, namely whether pep7 operated only as a monomer or perhaps a homodimer. Although the peptide library was constructed using a phagemid approach, which should yield, on the average, monodisplay of peptides on each of the coat protein fusions, two copies of one or both peptides on a large fraction of the phage population would not be unexpected in the presence of IPTG induction.[31] In this regard, the bacteriophage possesses five copies each of pVII and pIX that are actually closely packed, hydrophobic peptides of 33 and 32 amino acids, respectively,[4,31] and three native copies are likely to be sufficient for virion integrity. Since the pep7 sequence was found to contain a cysteine residue at position 13, it seemed plausible that two pep7 chains in close proximity on pVII could form a disulfide-linked homodimer. To address some of these issues, we prepared phage-free peptide constructs.

A pep7-Jun fusion protein homodimer was prepared by fusing pep7 with the Jun dimerization domain. This domain contains a leucine zipper motif which is characterized by a pattern of leucine residues repeating every seventh amino acid. The leucine motif mediates protein dimerization through the formation of parallel α-helical dimers as in the two-stranded coiled coils of fibrous proteins.[33] In addition, calculations (http://cubic.bioc.columbia.edu) predict that pep7 has a very high helical content of 88%, so that a defined association of pep7 chains was perhaps intimately a part of the homodimeric structure. It was found that the homodimer not only had strong binding activity to WI–L2 cells, but was also internalized. We then constructed a heterodimer, as a phage-free mimetic for a monodisplayed, heterodimeric CHL8 clone, composed of a pep7-Jun monomer and a similarly constructed pep9-Fos fusion protein. Jun and Fos form a heterodimer that is actually more stable than the Jun homodimer, whereas Fos itself exists either as a monomer or a very unstable homodimer.[33,34] The isolated pep7-Jun/pep9-Fos heterodimer, containing some pep9-Fos monomer, was devoid of cell-binding activity as measured by whole cell ELISA. These results, taken together with those above from the peptide-phage, established that the originally displayed and selected pep7 on the phage surface acted as a homodimer, and not as a heterodimeric pep7-pep9 species, and that pep9 did not contribute to the investigated cell-binding and internalization activity.

Whether the pep7 homodimer functioned as a purely noncovalent complex, a complex stabilized by a disulfide bond, or simply two chains joined by the disulfide, was not fully elucidated. However, it seemed that the special condition of 'proximity' and a high effective concentration, as occurred on pVII or through Jun interaction, was necessary for two pep7 chains to have biological function. The fact that the synthetic pep7 15-mer did not bind to WI–L2 cells suggested that pep7 association and/or disulfide formation did not occur at the micromolar concentrations at which pep7-Jun was active, or at even higher concentrations (200 μM), and provided additional evidence, together with the pep7-Jun/pep9-Fos result, that a single pep7 chain could not mediate activity. We previously showed that antibody Fv fragments, which are notorious for being unstable in solution, were very stable on the surface of phage when V_H and V_L were displayed on pVII and pIX, respectively.[31] Future work will be aimed at obtaining a completely synthetic pep7 homodimer, as well as fluorescently labeled conjugates, containing the cysteine-13 disulfide bridge, and evaluating these for activity.

The multivalent display of peptides has been hypothesized as an important factor influencing the translocation efficiency of specific phage constructs into mammalian cells in vitro.[32] Internalization by cells is increased by multivalent ligand display, either on pVIII or pIII.[32,35] For example, the internalization of phage particles with multivalent display of scFvs against epidermal growth factor was more efficient than internalization of monovalent phage produced by an equivalent vector.[35] This increased internalization probably results from enhanced avidity and/or the stimulation of receptor dimerization or clustering and subsequent endocytosis. Indeed, peptides displayed as multiple copies on phage, or by artificial dimerization of the peptide, are often found to be more active than the free synthetic peptide.[36,37] In contrast, high multivalency or mosaic-type clustering afforded by pVIII was not suitable for the activity of pep7 in our investigations.

In the active pep7 homodimer, it is conceivable that the chains are independent and have secondary α-helical character and that each binds to a separate receptor molecule, which triggers a requisite receptor dimerization. Alternatively, pep7 could function as a true homodimer in which a paired α-helical tertiary structure is critical for specific binding to a receptor site, or in other modes of cell-membrane penetration postulated for membrane-active peptides.[38–40] The general agreement is that such peptides utilize either a receptor-based entry or in some way disrupt the cell membrane bilayer structure. Some of these peptides only penetrate the membrane and leave the cell intact, and others often operate via cell lysis in which the membrane is perturbed to the point of cytosol leakage. Although many different sequences have been identified, characteristics often include a large net positive charge, hydrophobicity, and an amphipathic α-helical structure. Many of these peptides show no high degree of secondary structure in solution, yet helical or even β-sheet formation is induced or stabilized upon membrane binding.

Detailed structural studies of pep7 and pep7 homodimers will provide insight into a possible mechanism of action. What is clear based on the enforced selection strategy and the acquired data, is that a pep7 homodimer penetrates WI–L2 cells, but does not have lytic activity.

Studies by other workers showed that phage internalized by mammalian cells enter two distinct subcellular compartments.[32] The clathrin-independent internalization of RGD-phage resulted in the immediate translocation of the phage into a nonacidic intracellular compartment, which was followed by the gradual relocation of the phage into an acidic endosomal compartment associated with phage inactivation. Inclusion of the lysosomotropic agent chloroquine significantly increased the recovery of internalized phage, suggesting that internalized phage were indeed degraded upon uptake.[32] However, in our experiments, chloroquine offered no advantage and, in fact, somewhat decreased the peptide-phage enrichment after five rounds of panning. The reasons for this are uncertain, but might derive from the unique display of peptides which enlist a receptor and translocation pathway that does not invoke delivery to a lysosome, or any endocytic vesicle where pH alteration by chloroquine is of consequence. It is also possible that chloroquine effected the efficiency of uptake of peptide-phage by WI–L2 cells. Some pharmacological agents have resulted in the selective inhibition of various pinocytic pathways.[41] In any event, the internalization of CHL8 peptide-phage did not occur when incubated with WI–L2 cells at 4 °C. This indicated that normal cell metabolism, namely cellular turnover of cytosolic and membrane proteins, aspects bearing greatly on receptor-mediated endocytosis, were important in the mechanism of cellular uptake of CHL8 peptide-phage. Further studies will seek to isolate a putative receptor and determine the endocytic pathway, and this, together with continued exploitation of the pVII–pIX peptide library, will provide a foundation for developing peptide-based reagents and therapeutics that specifically target and penetrate tumor cells or other cells of interest.

Experimental

Cell line

B-lymphocyte WI–L2 cells (American Type Culture Collection CRL-8062) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) in 6.5% CO_2 at 37 °C.

Library construction

Primers shown below and used in library construction were purchased from Operon Inc. The phage-display vector pCGMT-1b[31] was used as a template for the generation of the peptide-pVII and peptide-pIX fusion gene repertoires. Briefly, primers PEP1 and PEP4 were used to amplify the DNA fragments encoding 15 amino acid peptide and pVII fusion gene repertoires. Primers PEP2 and PEP5 were employed to amplify the DNA fragments encoding 15 amino acid peptide and pIX fusion gene repertoires. Finally, equimolar amounts of the above polymerase chain reaction (PCR) products were mixed and assembled by overlap PCR with primers PEP3 and PEP6 to generate the dual-display peptide gene repertoire. The peptide genes were digested with restriction enzyme *Sfi* I, agarose gel-purified, and ligated into pCGMT-1b, which was cut with the same restriction enzyme. The ligated products were electroporated into *E. coli* XL1-Blue cells, plated on modified 2YT medium (15 g tryptone, 10 g yeast extract, 10 g MOPS, pH 7.0, and 15 g agar per liter, containing 1% glucose, 100 μg/mL carbenicillin, and 10 μg/mL tetracycline) in 100 dishes, and then incubated overnight at 30 °C. The clones were scraped off the plates into super broth (SB) medium with 10% glycerol and subsequently stored at −70 °C. PEP1: 5′-CAA CCA GCC ATG GCC NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK TGT TCT GGC GGC GGC GGC TCC-3′ PEP2: 5′-GTG GCC CAG GCG GCC NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK TGT TCT GGT GGT GGT GGT TCT-3′ PEP3: 5′-GCT GGT TTC GCT ACC GTG GCC CAG GCG GCC-3′ PEP4: 5′-GAT CTG GCC CGC GAG GCC TTA TTA TCT TTG ACC CCC AGC GAT TAT-3′ PEP5: 5′-GGC CAT GGC TGG TTG GGC AGC-3′ PEP6: 5′-CTC GTC GAC TGG AAT TCA GAT CTG GCC CGC GAG GCC-3′

Rescue of peptide-phage

To rescue the peptide-phage library, 1 L of SB medium containing 2% glucose, 100 μg/mL of carbenicillin, and 10 μg/mL of tetracycline was inoculated with $\sim 10^{11}$ cells from the glycerol stock library. The culture was shaken at 37 °C until $OD_{600} \sim 0.5$. Then, $\sim 5\times 10^{13}$ plaque forming units of VCSM13 helper phage and 2 mL of 0.5 M isopropyl β-D-thiogalactopyranoside (IPTG) were added. After a 30 min incubation at room temperature without shaking, the culture was diluted into 4 L of SB medium and grown for 2 h at 30 °C. Then, 70 μg/mL of kanamycin was added and the culture was allowed to grow overnight at 30 °C. The peptide-phage were purified by two rounds of standard polyethylene glycol precipitation.[42,43]

Selection of peptide-phage against WI–L2 cells

We used a slightly modified literature procedure.[24,25] Briefly, WI–L2 cells were grown in tissue culture flasks to logarithmic phase. Culture medium was renewed 2 h prior to the addition of the peptide-phage. In the experiments with chloroquine, the cells were pre-incubated with culture medium containing 50 μM chloroquine for 2 h before adding the peptide-phage. To $\sim 5\times 10^7$ cells were added $\sim 10^{13}$ colony forming units (cfu) of peptide-phage and incubated for 6 h at 37 °C with slow shaking (100 rpm) in the first round of panning. Following the incubation, the internalization events were stopped by immersion of the flasks in ice for 10 min. The cells were washed six times with Hanks' Balanced Salt Solution (HBSS) (Invitrogen) and three times for

10 min each with 10 mL of low pH buffer (50 mM glycine, pH 2.5, 0.5 M NaCl) at room temperature. After two additional washes with phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) (PBS), the cells were lysed with 1 mL of 100 mM triethylamine (TEA) and neutralized with 0.5 mL of 1 M Tris–HCl, pH 7.4. The lysate was used to infect 10 mL of *E. coli* XL1-Blue cells and internalized peptide-phage were rescued as described above and used in the next round of selection.

Construction of phagemids displaying only pep7 on pVII, pep9 on pIX, and pep7 on pVIII

Two phagemid vectors, denoted CHL8-p7 and CHL8-p9, that contained the peptide-pVII (pep7-pVII) and peptide-pIX (pep9-pIX) fusion genes, respectively, from clone CHL8, were constructed. The pep7-pVII and pep9-pIX fusion gene fragments were obtained by digesting CHL8 DNA using restriction enzymes *Nco* I/*Nhe* I and *EcoR* I/*Xba* I, respectively. The fragments were ligated into the vector pCGMT[31] that was cut by *Nco* I/*Nhe* I and *EcoR* I/*Nhe* I.

For the construction of a phagemid with pep7 displayed on the major coat protein pVIII, denoted CHL8-p7-VIII, the pep7 sequence was amplified by PCR from CHL8 DNA with primers ARAHF and PCHL8–7R shown below. The pVIII gene was amplified from helper phage VCSM13 DNA with primers P8F and P8R, and the pep7-pVIII fusion gene was then assembled by overlap PCR with primers ARAHF and P8R. The PCR product was digested with *Nco* I/*Nhe* I and ligated into the pCGMT vector digested with the same enzymes. ARAHF: 5′-GCC TAC GGC AGC CGC TGG ATT GTT ATT ACT-3′ PCHL8–7R: 5′-AGA GCC ACC GCC ACC AGA ACA ATA CTG ACA CGC CAA AGA-3′ P8F: 5′-TCT GGT GGC GGT GGC TCT GCT GAG GGT GAC GAT CCC GCA AAA-3′ P8R: 5′-TAC AAT GCT AGC TTA GCT TGC TTT CGA GGT GAA TTT CTT-3′

The correct constructs were further confirmed by DNA sequencing and CHL8-p7, CHL8-p9, and CHL8-p7-VIII peptide-phage were individually prepared as described above.

Expression and purification of a pep7-Jun fusion protein

The fusion protein, denoted pep7-Jun, consisted of pep7 at the N-terminus, followed by a short G_4S linker, the Jun dimerization domain, and a FLAG tag at the C-terminus. First, the pep7-linker gene fragment was amplified by PCR with primers PJun1 and PJun2, and by using CHL8 DNA as the template. The Jun dimerization domain-FLAG gene fragment was amplified by PCR with primers PJun3 and PJun4, and by using the Jun gene (provided by Dr. Peter Vogt, The Scripps Research Institute, La Jolla, CA, USA) as the template. The pep7-linker and Jun-FLAG gene fragments were assembled by overlap PCR with primers PJun1 and PJun4 to generate the final fusion gene. The fusion gene was subcloned into pET15b (Novagen). The proteins were expressed as inclusion bodies in *E. coli* B834 (DE3) (Novagen) and the inclusion bodies were refolded and purified as described.[44] PJun1: 5′-CCA GCC ATG GCC TCG GAT TTG TGG GAGATG-3′ PJun2: 5′-AGA ACC ACC ACC ACC AGG ACA ATA CTG ACA CGC-3′ PJun3: 5′-TCC GGT GGT GGT GGT TCT CTC GAG GAA AAA GTG AAA ACT-3′ PJun4: 5′-TCG TAT ACT AGT TTA TTA TTT GTC ATC GTC ATC TTT GTA GTC AAA CGT TTG CAA CTG TTG TGT-3′

Construction of a pep7-Jun/pep9-Fos heterodimer

The pep-7-Jun fusion protein was constructed without the FLAG tag. The pep7 gene fragment was first amplified by PCR with primers PJun1 and PJun2 using CHL8 DNA as the template. The Jun dimerization domain gene fragment was amplified by PCR with primers PJun3 and PJun5, and by using the Jun gene as the template. Then, the pep7 and Jun gene fragments were assembled by overlap PCR with primers PJun1 and PJun5 to generate the final fusion gene. The PCR product was digested with restriction enzymes *Nco* I/*Spe* I and ligated into pIWPY, a vector constructed from pET15b (Novagen) and the polylinker region from pComb3H[45] used for Fab expression.

A pep9-Fos fusion protein was constructed containing a linker and FLAG tag analogous to the original pep7-Jun fusion. The pep9 gene fragment was amplified from the CHL8 vector by using PFos1 and PFos2 primers. The primers PFos3 and PFos4 were then used to amplify the Fos dimerization domain from the Fos gene (provided by Dr. Peter Vogt, The Scripps Research Institute, La Jolla, CA). Finally, the pep9 gene and Fos gene fragments were assembled by overlap PCR with primers PFos1 and PFos4 to generate the final fusion gene. The PCR product was digested with *Sac* I/*Xba* I and subcloned into vector pIWPY containing the insert of the pep7-Jun (without Flag) fusion gene. PJun5: 5′-CAA AAT ACT AGT TTA AAA CGT TTG CAA CTG TTG TGT-3′ PFos1: 5′-ATC TCA GAG CTC GGG GAG GCG CAT ATT CCG-3′ PFos2: 5′-GGA GCC GCC GCC GCC AGA CCA ACC CTT CTC-3′ PFos3: 5′-TCT GGC GGC GGC GGC TCC CTG CAG GCG GAG ACG GAC-3′ PFos4: 5′-GTT TGG TCT AGA TTA TTT GTC ATC GTC ATC TTT GTA GTC CTC CTC GGG CAT CTT GCA GGC-3′

To produce the heterodimer, the plasmid was introduced into *E. coli* B834 (DE3). The transformed cells were plated onto Luria–Bertani agar containing carbenicillin (100 μg/mL) and incubated overnight at 37 °C. The clones were scraped off the plates and inoculated into 5 L of SB medium containing carbenicillin (100 μg/mL). The culture was grown at 37 °C with shaking to OD_{600} ~0.7–0.9, then IPTG was added to a final concentration of 0.5 mM to induce the T7-polymerase gene placed under the control of the *lac*UV5 promoter. The culture was then incubated overnight at 28 °C. The FLAG-tagged heterodimeric fusion protein was purified on anti-FLAG M2 affinity agarose (Sigma) from the periplasmic extracts and medium according to the manufacturer's instructions.

Peptide-phage internalization

The endpoint internalization activity was assessed by titering the phage number. First, $\sim 5\times10^6$ WI–L2 cells were incubated with $\sim 10^{11}$ cfu of peptide-phage for 4 h at 37 °C with slow shaking (100 rpm). After six washes with PBS, the cells were washed three more times for 10 min each with low pH glycine buffer, followed by two additional PBS washes. Second, the cells were lysed with 0.5 mL of 100 mM TEA and neutralized with 0.25 mL of 1 M Tris–HCl, pH 7.4. Finally, the lysate was used to infect 10 mL of *E. coli* XL1-Blue cells and internalized phage were titered by counting the bacterial colonies.

Flow cytometry

The specific cell binding activities of peptide-phage and pep7-Jun were measured by FACScan (Becton Dickinson). The WI–L2 cells were grown in tissue culture flasks to a late logarithmic phase. Culture medium was renewed 2 h prior to the addition of the peptide-phage. To $\sim 5\times10^5$ WI–L2 cells were added $\sim 10^{10}$ cfu of peptide-phage and incubated for 2 h at 4 °C with slow shaking (100 rpm). After three washes with PBS/1% bovine serum albumin (BSA), 5 μL of goat serum was added and incubated for 10 min to block nonspecific binding sites. The WI–L2 cells were then incubated with anti-M13 phage mAb (Serotec) diluted 1:500 in PBS for 30 min. The cells were washed again and incubated with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-mouse antibody (Pierce) diluted 1:100 in PBS. After three additional washes, cells were resuspended in PBS/1% BSA/1% formaldehyde and analyzed using FACScan (Becton Dickinson).

For the pep7-Jun homodimer, similar procedures were followed. The pep7-Jun was first biotinylated in the Jun region for detection purposes to complement the FLAG tag. Biotinylation used NHS-LC-LC-Biotin (Pierce) according to the instructions. After incubation of the WI–L2 cells with 5 μg of pep7-Jun and washing, FITC–streptavidin (Amersham Pharmacia) was added and incubated for 1 h at 4 °C to afford direct labeling. For detection using the FLAG tag, the WI–L2 cells were incubated with pep7-Jun, washed, and then incubated with anti-FLAG M2 mAb (Sigma) for 1 h at 4 °C. The cells were washed again and incubated with FITC-conjugated $F(ab')_2$ goat anti-mouse antibody (Pierce) for 1 h at 4 °C. The cells were prepared and analyzed as described above.

Immunofluorescence microscopy

The cell penetration of peptide-phage and pep7-Jun were observed using fluorescence microscopy. The WI–L2 cells were grown in tissue culture flasks to a logarithmic phase. Culture medium was renewed 2 h prior to the addition of the peptide-phage. To $\sim 1\times10^7$ WI–L2 cells were added $\sim 10^{12}$ cfu of peptide-phage and incubated in 5% CO_2 for 10 h at 37 °C. The cells were washed six times with PBS, three times with low pH glycine buffer, and twice with PBS. The cells were resuspended in 1 mL of PBS and attached onto a poly-L-lysine coated coverslip for 30 min. After washing with PBS, the attached cells were fixed in 4% formaldehyde for 10 min and permeabilized with 0.05% saponin for 15 min at room temperature. Permeabilized cells were blocked in PBS containing 10% normal goat serum and 3% BSA for 1 h, then incubated with biotinylated anti-M13 phage mAb (Serotec) for 1 h, followed by FITC–streptavidin (Amersham Pharmacia) for 1 h at room temperature. After washing with PBS, the coverslip was mounted onto a slide. The cells were examined using a Bio-Rad MRC 1024 scanning laser confocal microscope.

When studying the biotinylated pep7-Jun homodimer, $\sim 2.5\times10^6$ WI–L2 cells were incubated with a final concentration of 1 μM pep7-Jun in 10 mL of RPMI 1640 medium supplemented with 10% FCS in 6.5% CO_2 for 4 h at 37 °C. The cells were washed six times with PBS, three times with low pH glycine buffer, and twice with PBS. The cells were resuspended in 1 mL of PBS and attached onto a poly-L-lysine coated coverslip for 30 min. After washing with PBS, the attached cells were fixed in 4% formaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. Permeabilized cells were blocked in PBS containing 10% normal goat serum and 3% BSA for 1 h, then incubated with anti-FLAG M2 mAb (Sigma) for 1 h, followed by FITC-conjugated $F(ab')_2$ goat anti-mouse antibody (Pierce) for 1 h at room temprature. The cells were counterstained with Hoest (Molecular Probes) and inverted on a slide on Prolong antifade mounting medium (Molecular Probes). Cells were then viewed with a Delta Vision deconvolution microscope mounted on an Olympus IX-70 inverted microscope using a 60X1.4NA lens together with a Photometric CH350L liquid cooled camera. The data were deconvoluted using a SGI O2 computer and Delta Vision software SoftWoRx version 2.5.

Whole-cell ELISA

To $\sim 5\times10^5$ WI–L2 cells in a 1.5 mL microcentrifuge tube were added peptide-phage or pep7-Jun in 200 μL of PBS/5% skimmed milk for 1 h at 4 °C with slow rotation. Cells were washed five times with PBS and incubated with 200 μL of horseradish peroxidase (HRP)-conjugated anti-M13 phage mAb (Amersham Pharmacia) diluted 1:1000 in PBS/5% skimmed milk for detection of peptide-phage, or cells were incubated with HRP-conjugated anti-FLAG M2 mAb (Sigma) diluted 1:1000 in PBS/5% skimmed milk for detection of pep7-Jun. After incubation for 1 h at 4 °C with slow rotation and five washes with PBS, the cells were resuspended in 150 μL of TMB substrate (Pierce) and developed for 5 min before quenching with 150 μL of 2 M H_2SO_4. The cells were pelleted by centrifugation and the supernatant was transferred to a microtiter 96-well plate. The absorbance was measured at 450 nm on an ELISA plate reader (Molecular Devices).

Synthetic pep7 monomer

The pep7 was synthesized at the Core Facility of The Scripps Research Institute. The pep7 was dissolved in dimethyl sulfoxide and diluted into PBS at different

concentrations for use in competition ELISA. To $\sim 5\times 10^5$ WI–L2 cells were added various concentrations (1–200 μM) and incubated for 30 min at 37 °C. Then $\sim 10^{11}$ cfu of CHL8 peptide-phage or 2 μg of pep7-Jun homodimer were added. The ELISA procedure was as described above.

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