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Construction of a Hexapeptide Library using Phage Display for Bio-panning

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Random hexapeptide library on the surface of filamentous bacteriophage was constructed using the SurfZAP vector. The size of the library was approximately 10^5. The peptide insert was flanked by two cysteines to constrain the peptide structure with a disulfide bond. This library was screened for the topoisomerase II binding peptide. Dramatic enrichment of the fusion phage over the VCS M13 helper phage was demonstrated by bio-panning affinity selection.

Key words: hexapeptide library, filamentous bacteriophage, SurfZAP vector, topoisomerase II, bio-panning

Phage display (16) is a technique in which a foreign protein or peptide is presented at the surface of a bacteriophage by fusion to the N-terminus of gIII or gVIII coat proteins. This system was originally used to create large libraries of antibodies (21) for the purpose of selecting those that strongly bind to a specific antigen. Recently, it has also been used to present peptides, domains of proteins, and intact proteins at the surface of a phage (1). Libraries of phages displaying random peptide sequences are increasingly being used to identify peptide ligands specific for a wide spectrum of target molecules, for example, streptavidin (4), the urokinase receptor (6), the antagonist to the thrombin receptor (3), and even specific sequences of DNA (19). Phage-displayed peptide libraries consisting of a large number of clones expressing different short peptide sequences can also be used to design vaccines, identify genes, and map epitopes (2, 14, 15).

Topoisomerases are ubiquitous enzymes which act to resolve topological problems that arise during various processes of DNA metabolism, including transcription, recombination, replication, and chromosome partitioning during cell division (20). These enzymes are now known to be the primary cellular targets for some of the widely prescribed antibiotics and anticancer drugs used in the treatment of human disease (5).

We constructed a random hexapeptide library displayed on the gene III protein of filamentous bacteriophage by the use of a SurfZAP vector derived from a bacteriophage lambda vector (8, 9, 10, 13). The library was made by inserting degenerated oligonucleotides which encode 6 variable amino acids into the N-terminal region of the phage gene protein. The peptide insert was flanked by two cysteines to constrain the peptide structure with a disulfide bond. This library, which contained over 10^5 different phages, was screened for peptides which had high affinity to topoisomerase II.

Materials and Methods

Construction of an oligonucleotide library

Three oligonucleotides were used to construct the hexapeptide library. The oligonucleotide sequences were as follows: PEP-C1 (5'-GGCCGCAGGTCTCTC CTCTTAGCAGCACAACCAGCAATGGCCNNTGC $(NNK)_6$TGCGGAGGCGGTGGAA-3'), in which N is A, C, G or T (equimolar), and K is G or T (equimolar); S-PEP-1 (5'-CTAGTTCCACCGCCTCC-3'); and S-PEP-2 (5'-AAGAGGAGGAGACCTGC3'). The three oligonucleotides were annealed in 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, and 50 mM NaCl, with heating to 65°C for 5 min and slow cooling to room temperature (11). The SurfZAP vector was added to the annealed oligonucleotides. These fragments were ligated by adding of T4 ligase and were incubated for 2 days at 4°C. The intended final products were packaged *in vitro*. The titer of the library was determined by infection of *E. coli* XL1-Blue cells (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F' proAB lacIqZΔM15 Tn10(Tet^r)*]) and plating on LB

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plates containing tetracycline (12.5 μg/ml).

Mass library *in vivo* excision

The primary lambda library was amplified and mixed with *E. coli* strain XL1-Blue cells at a multiplicity of infection (MOI) of 0.1. ExAssist helper phages were added to the mixture at a 1:10 helper phage-to-cell ratio. The culture was diluted to 100 ml of LB supplemented with 50 μg/ml ampicillin and incubated for 6 h with gentle shaking. The culture was then incubated at 70°C for 20 min to lyse the lambda particles and the XL1-Blue cells. The cells were centrifuged and the supernatant containing the phage particles was transferred to a fresh tube. The titer of the excised particles was determined by infection of *E. coli* strain SOLR cells (*e14*$^-$ (*mcrA*) Δ (*mcrCB-hsdSMR-mrr*) *171 sbcC recB recJ umuC Tn5*(*kan*^{r}) *uvcC lac gyrA96 relA1 thi-1 endA1* λ^R [*F' proAB lacq ΔM15*] *Su*$^-$) and plating on LB plates containing ampicillin (50 μg/ml). Rescued colonies were selected for plasmid preparation and DNA analysis (17). The titered supernatant containing excised phagemid particles was mixed with *E. coli* SOLR cells (OD_{600}=1.0) at a cell-to-phagemid ratio of 1:1. The culture was then diluted to 100 ml using LB supplemented with 50 μg/ml ampicillin and incubated for 6 h with shaking. The cells were centrifuged and resuspended in 10 ml of 10 mM $MgSO_4$.

Preparation of a phage-displayed peptide library

The amplified excised phagemid library in SOLR cells was resuspended in 10 ml of LB and infected with VCS M13 helper phages at a MOI of 10. After 15 min at 37°C the culture was diluted to 100 ml using LB supplemented with 50 μg/ml ampicillin and incubated for another 5 h. After centrifugation the titer of the library was determined by infection of *E. coli* XL1-Blue cells and plating on LB plates containing amplicillin (50 μg/ml).

Library screening (bio-panning)

Microplates with 96 wells were coated overnight at 4°C with 60 μl of 200 μg/ml polyclonal rabbit antibody to p170 topoisomerase (TopoGEN, INC) in 0.1 M carbonate buffer (pH 9.5). The wells were then blocked by incubation with PBS (135 mM NaCl, 2.5 mM KCl, 10 mM $NaHPO_4$, pH 7.2) supplemented with 1% bovine serum albumin (BSA). After washing three times with TBST buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.5 % (v/v) Tween 20), human p170 topoisomerase II in TBST buffer was added to each well and the microplates were incubated for 12 h at 4°C with gentle agitation. As a control, additional microplates were coated with a blocking buffer. Following three washes with distilled water, 50 μl of the phagemid library in PBS containing 0.1% BSA was added to each well and the microplates were incubated for 12 h at 4°C with gentle agitation. Following 10 washes with TBST buffer bound phages were eluted by incubation with 50 μl of 0.1 M glycine (pH 2.2) with gentle shaking for 15 min. The eluates from each well were pooled and neutralized by addition of 3 μl of 2 M Tris-base. For biopanning against both topoisomerase and blocking buffer, the VCS M13 helper phage was used as a control. Eluted phages were used to infect XL1-Blue cells and amplified for the next cycle of panning. An aliquot of an appropriately diluted solution of each elution was used to infect *E. coli* XL1-Blue cells in the stationary phase. After incubation for 15 min at 37°C the infected cells were plated on LB plates containing amplicillin(50 μg/ml). For the second panning, PBS containing 5% skim milk was used as a blocking solution in place of BSA in order to exclude BSA-binding phages. Yields were derived using the following formula:

No. of phage particles in eluate / No. of phage particles applied × 10^5

Results

Construction of a phage-displayed peptide library

The SurfZAP vector (Fig. 1) was used to generate surface expression libraries. The high ligation and packaging efficiencies of lambda result in the construction of very large primary libraries. Because the lambda bacteriophage grows lytically, expressed proteins are also readily detectable with a variety of filter screening methods. The SurfZAP vector was designed to allow *in vivo* excision of plasmid DNA from the lambda phage vector.

Proteins in the SurfZAP vector are expressed as a

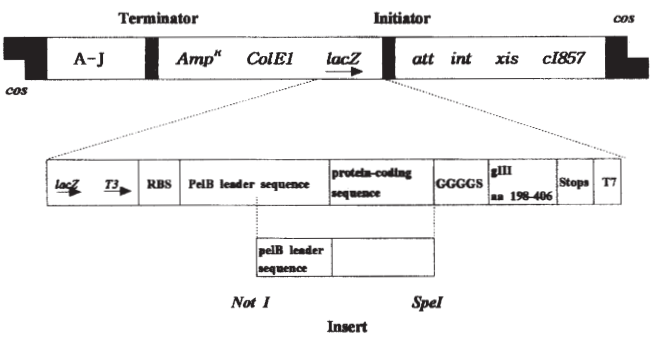

Fig. 1. Schematic representation of the SurfZAP vector used for phage display.

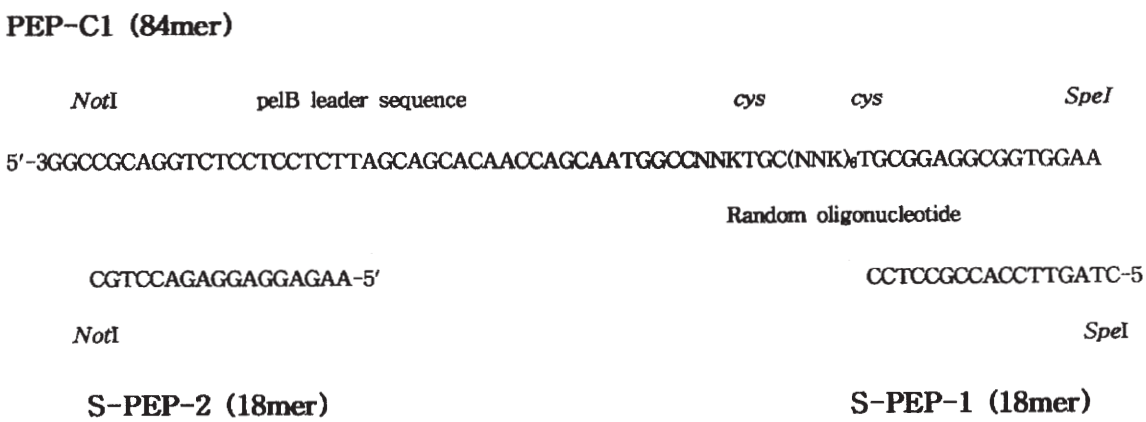

Fig. 2. Oligonucleotides used for the construction of a hexapeptide library.

fusion with amino acids 198-406 of gIII, which contain the membrane anchoring domain. The structure of oligonucleotides to be cloned into the SurfZAP vector is shown in Fig. 2. The 5' and 3' ends each had a fixed sequence. The central portion contained a variable region. Half-site oligonucleotides were hybridized to the 5' and 3' ends of oligonucleotide PEP-C1 to form appropriate *Not*I and *Spe*I cohesive ends.

The hybridized structure was then ligated to the *Not*I and *Spe*I sites of the SurfZAP vector. The ligation products yielded 1.01×10^5 recombinant plaques on the XL1-Blue strain. The primary lambda libraries were amplified and then converted to phagemid libraries (Fig. 3) by *in vivo* mass excision with the ExAssist helper phage (18). The ExAssist helper phage is not propagated in the SOLR strain (*supO*) because its genome contains an amber mutation. The excised phagemid particles were amplified in SOLR cells and recombinant phagemid particles for biopanning were produced by single-stranded rescue with VCS M13 helper phages that contained no amber mutations. When phagemid-harboring SOLR cells were infected with helper phages the phagmid genomes were replicated and packaged into M13 particles. As phagemids were extruded from the cell, fusion proteins were incorporated into the surface coat along with three to five copies of wild type gp III encoded by helper phages. The final products were a mixture of wild type helper phages and recombinant phagemid particles, which contained the fusion proteins and carried DNA encoding the cloned insert. The majority of the phage particles carrying the phagemid genome generally displayed a single copy of the fusion protein. A recombinant genome which encodes an active binding protein can be readily isolated from large libraries by biopanning and amplified in *E. coli* for further characterization.

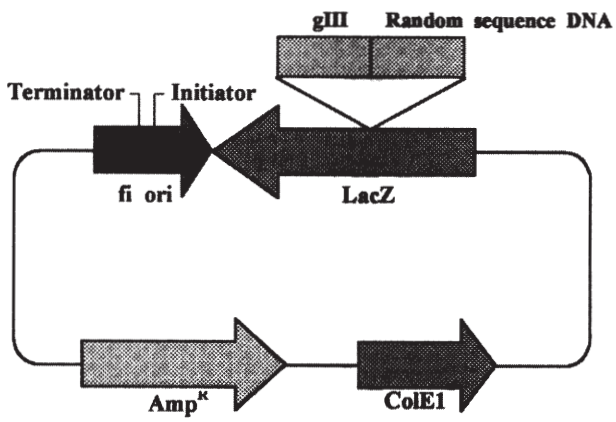

Fig. 3. Schematic representation of the pSurfscript SK (–) Phagemid.

Library screening (bio-panning)

Bio-panning against immobilized topoisomerase II was performed to test whether topoisomerase II binding phages could be selectively enriched from the phage-displayed peptide library. Aliquots of the fusion phage were allowed to bind to the immobilized protein. They were then washed and eluted at a low pH. Eluted phages were amplified by reinfection into *E. coli* and harvested phages from this amplification were used for the next cycle of binding and enrichment. The results are shown in Fig. 4.

Discussion

Screening of potential ligands against a target molecule of interest was greatly simplified using phage libraries which displayed random peptides. Epitope identification using a random peptide library has the advantage of speed and simplicity, as neither cloning of the gene nor prior knowledge of

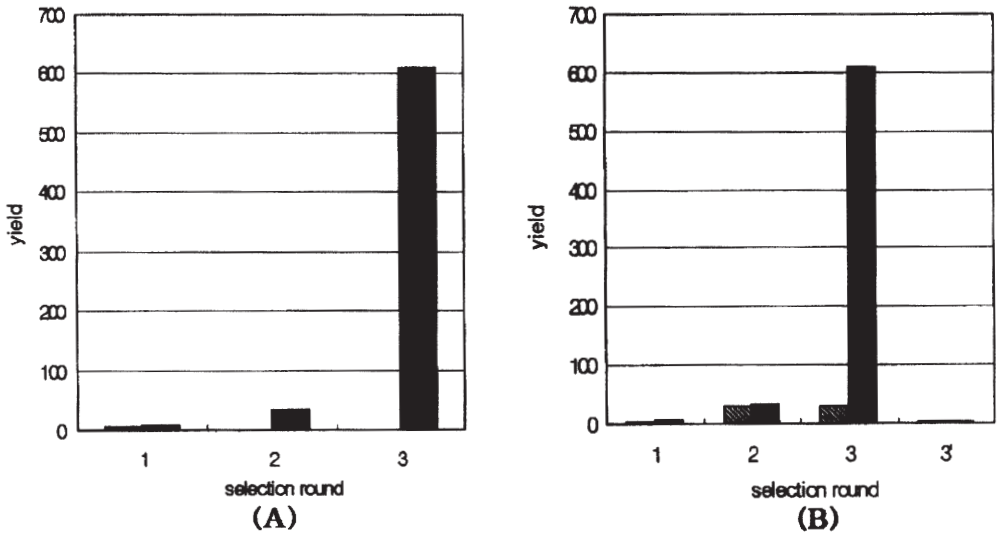

Fig. 4. Affinity selection of the phage-displayed peptide library. The phage library was screened through three cycles of enrichment and amplification (1° to 3°). At each cycle, phage were titered with *E. coli* XL1-Blue cells. (A) The recombinant phagemid particles (black) or VCS M13 (hatched, negative control) helper phage were pipetted into topoisomerase II-coated plates. (B) The recombinant phagemid particles were pipetted into topoisomerase II (black)-or BSA (hatched, negative control)-coated plates. In the third panning for the control experiment (3'), the recombinant phagemid particles eluted from topoisomerase II-coated plates were pipetted into BSA-coated (black) plates, and the recombinant phagemid particles eluted from BSA-coated plates were pipetted into topoisomerase II coated plates (hatched).

the protein sequence is necessary. However, the selected peptides often have affinities which are too low for practical purposes. To overcome this problem, Lowman and Wells prepared a monovalent display system in which only one copy of a peptide is displayed per phage. Using this system a high affinity variant of the human growth hormone was produced (12). It has been suggested that the structure of the displayed peptide should be constrained by a disulfide bond to obtain peptide having high binding affinity (7).

A fusion phage library was constructed in the SurfZAP vector derived from a bacteriophage lambda vector. The relative ease of use makes lambda phage packaging an alternative to electroporation techniques that have been previously used to create phage-displayed peptide libraries. The library was constructed by inserting degenerate oligonucleotides into the N-terminal region of the phage gene III protein. The peptide inserts were flanked by two cysteines to constrain the peptide structure with a disulfide bond. Surface expression of the phage gene III fusion protein allowed for selective binding and subsequent enrichment of infectable virions bearing unique and random amino acid sequences.

We found that topoisomerase II-binding phages were specifically enriched from a pool of background phages after bio-panning against immobilized topoisomerase II. A 610-fold enrichment was observed after the third panning for the phage-displayed peptide library, while the VCS M13 helper phage did not display any enrichment. As shown in Fig. 4 (B), no enrichment was seen for a phage-displayed peptide library selected from random peptides (blocking solution). Controls confirmed that BSA-binding phages were excluded (Fig. 4).

Results presented herein show that a library of fusion-phages provides a general tool for identifying and studying protein-protein interactions. The direct use of phage peptides in a functional assay greatly reduces the amount of time and money spent on peptide synthesis for functional assays.

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