Protein Engineering vol.15 no.11 pp.943–950, 2002

α-Helically constrained phage display library

V.A.Petrenko[1,2], G.P.Smith[3], M.M.Mazooji[3] and T.Quinn[4]

[1]Department of Pathobiology, College of Veterinary Medicine, Auburn University, AL 36849 and [3]Division of Biological Sciences and [4]Department of Biochemistry, University of Missouri, Columbia, MO 65211, USA

[2]To whom correspondence should be addressed.
E-mail: petreva@vetmed.auburn.edu

The library described here is a collection of phages with six degenerate codons in gene VIII, specifying amino acids 12, 13, 15–17 and 19 of the major coat protein. The randomized positions are surface exposed in the wild-type protein and thus might be expected to tolerate a great diversity of side chains without compromising phage viability. In agreement with this supposition, the new library showed great diversity of amino acids at the randomized positions and diversity did not diminish noticeably during repeated subculture. Despite their diversity, however, the randomized positions should be strongly constrained conformationally because they lie in an extended α-helical portion of the protein, stabilized by numerous inter- and intra-subunit contacts—a presupposition corroborated by circular dichroism spectroscopy of many library members. To reflect this conformational homogeneity and the fact that random amino acids subtend a major fraction of the surface 'landscape' of the particle, we call the new construct an alpha landscape library. It can be used as a source of α-helical ligands and substitute antibodies.

Keywords: fibrinogen binding/landscape library/nanotechnology/phage display/substitute antibody

Introduction

The major coat protein pVIII of filamentous phages (including strains fd, f1 and M13) is coded by gene VIII and constitutes 98% of the protein mass and 87% of total mass of the virion (Berkowitz and Day, 1976). About 2700 copies of this 50-residue protein assemble in a helical lattice to form the tubular capsid, whose lumen encloses the viral DNA. Each pVIII subunit is largely α-helical and rod-shaped; its axis lies at a shallow angle to the long axis of the virion, its N-terminus exposed on the outer surface and its C-terminus in the lumen (Marvin, 1998). Neighboring pVIII subunits make numerous inter-subunit contacts that impart remarkable physical stability to the structure. The length of the capsid is directly proportional to the length of viral DNA to be enclosed and inversely proportional to the number of positively charged residues at the luminal (C-terminal) end of the pVIII polypeptide (Hunter *et al.*, 1987). Thus there appear to be no geometrically specific contacts between the DNA and the luminal wall of the capsid, but rather a requirement for a certain overall ratio of positive charges on pVIII to negative charges on the DNA.

The major coat protein in viable filamentous phages tolerates different point mutations (Williams *et al.*, 1995), deletion of N-terminal amino acids (V.A.Petrenko, unpublished work) and insertion of short peptides into the N-terminus (Ilyichev *et al.*, 1989; Felici *et al.*, 1991; Greenwood *et al.*, 1991; Kishchenko *et al.*, 1991; Iannolo *et al.*, 1995, 1997; Petrenko *et al.*, 1996; Terry *et al.*, 1997). Foreign N-terminal peptides fused to every copy of pVIII can subtend as much as 25–30% of the virion surface, dramatically changing the particle's surface architecture and properties. Depending on the particular foreign peptide sequence, such phages can bind organic ligands, proteins, antibodies and cell receptors (Petrenko *et al.*, 1996; Iannolo *et al.*, 1997; Petrenko and Smith, 2000; Romanov *et al.*, 2001), interact with proteases (Terry *et al.*, 1997), induce specific immune responses in animals (Minenkova *et al.*, 1993; di Marzo Veronese *et al.*, 1994; Perham *et al.*, 1995; De Berardinis *et al.*, 2000), resist stress factors such as chloroform or high temperature (Petrenko *et al.*, 1996) or migrate differently in an electrophoretic gel (V.A.Petrenko, unpublished work). Recombinant phages with foreign peptides fused to all pVIII subunits are called 'landscape' phages and collections of such phages are called 'landscape libraries' (Petrenko *et al.*, 1996; Petrenko and Smith, 2000).

Here we describe a new type of landscape library, the 'alpha' landscape library, in which the randomized amino acids lie within the α-helical portion of pVIII rather than at the N-terminus and are thus conformationally homogeneous (Bianchi *et al.*, 1995; Nord *et al.*, 1995). Our results show that phages tolerate multiple substitutions at these positions as long as they do not disturb general α-helical architecture of major coat protein. The new library can serve as a source of α-helical ligands and substitute antibodies and can be used in conjunction with another landscape library to construct a 'mosaic' library whose member particles simultaneously display different foreign peptides on their surface (V.A.Petrenko, unpublished work).

Materials and methods

Solutions, preparations and reagents

Solutions, preparations and reagents referred to in this paper are described in Table I.

General procedures

Escherichia coli and phage strains, general methods of phage display, titering infective particles as tetracycline transducing units (TU), spectrophotometric quantitation of phage particles, transfection, preparation of replicative form (RF) and viral single-stranded circular DNA, propagation and processing of phages, DNA sequencing and other standard microbiological methods have been described (Smith and Scott, 1993; Yu and Smith, 1996; Barbas *et al.*, 2001) and are also detailed at the website (http://www.biosci.missouri.edu/SmithGP/index.html). DNA was extracted with phenol–chloroform in Phage Lock Gel tubes (5 Prime → 3 Prime, Inc.) and precipitated with ethanol as outlined by Sambrook *et al.* (Sambrook *et al.*, 1989).

Table I. Solutions, preparations and reagents

Solution or preparation	Description
AP-SA	Alkaline-phosphatase-conjugated streptavidin; Jackson ImmunoResearch Laboratories 016-050-084; dissolved at a concentration of 500 μg/ml in 5 mM Tris–HCl (pH 8), 125 mM NaCl, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 50% (v/v) glycerol; stored at 4°C
AP-SA diluent	1 mg/ml BSA, 0.1% Tween 20, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ in TBS
BLOTTO	5 g Carnation non-fat milk in 100 ml TBS containing 0.02% NaN_3
BSA	Bovine serum albumin, Fraction V; Sigma Chemical A2153; 50 mg/ml stock is filter-sterilized and stored at 4°C
dNTPs	Mixture of equal concentrations of dATP, dGTP, dCTP and dTTP
Elution buffer	0.1 M HCl, 1 mg/ml BSA, pH adjusted to 2.2 with glycine; made by mixing water, 50 mg/ml BSA and 0.4 M HCl, pH adjusted to 2.2 with glycine; filter-sterilized and stored at room temperature
40× GBB	1.68 M Tris, 0.8 M sodium acetate, 72 mM Na_2EDTA, pH adjusted to 8.3 with glacial acetic acid
10× Klenow buffer	0.5 M Tris–HCl, pH 7.8, 0.1 M $MgCl_2$
λ DNA markers	*Bst*EII digest of phage λ DNA: 8453, 7242, 6369, 5687, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702, 224 and 117 base pairs (bp)
5× Ligase buffer	150 mM Tris–HCl, pH 7.5, 150 mM NaCl, 37 mM $MgCl_2$, 10 mM DTE, 1 mM EDTA, 5 mM spermidine, 100 μg/ml acetylated BSA, 1.25 mM ATP
NAP buffer	80 mM NaCl, 50 mM $NH_4H_2PO_4$, pH adjusted to 7.0 with NH_4OH; autoclave and store in refrigerator or room temperature
NPP substrate	Just before use, add 10 μl 1 M $MgCl_2$ and 100 μl of 50 mg/ml *p*-nitrophenyl phosphate (stored at –20°C) to 10 ml 1 M diethanolamine, pH adjusted to 9.8 with HCl
NZY medium	Dissolve 10 g NZ amine A, 5 g yeast extract and 5 g NaCl in 1 l water; adjust pH to 7.5 with NaOH; autoclave; store at room temperature
NZY plates	Autoclave 11 g Bacto-agar in 500 ml water in a 2 l polypropylene flask; without cooling, add 500 ml 2× NZY medium at room temperature; add supplements such as Tc as required; swirl to mix; pour about 25 ml per 100 mm Petri dish.
PBS	0.15 M NaCl, 5 mM NaH_2PO_4, pH 7.0 adjusted with NaOH
TBS	50 mM Tris–HCl (pH 7.5), 0.15 M NaCl; autoclave
TBS–Tween	0.5% (v/v) Tween 20 in TBS; autoclave
Tc (20 mg/ml)	1:1 (v/v) mixture of filter-sterilized 40 mg/ml tetracycline (Tc) and autoclaved glycerol (cool before mixing); store at –20°C
Tc medium	NZY medium supplemented with 20 μg/ml tetracycline
Tc plates	NZY plates supplemented with 40 μg/ml tetracycline
TcKan plates	NZY plates supplemented with 40 μg/ml tetracycline and 100 μg/ml kanamycin
TE	10 mM Tris–HCl (pH 8), 1 mM Na_2EDTA
TTDBA	1 mg/ml dialyzed BSA, 200 μg/ml NaN_3 in TBS–Tween

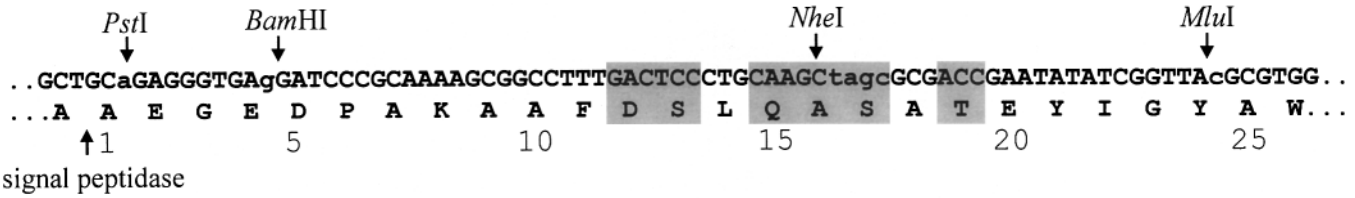

Fig. 1. Structure of vector f8–5. Nucleotide and amino acid sequences correspond to the beginning of the mature form of pVIII. Only the viral strands of DNA (anti-complementary to mRNA) are shown. Mutated nucleotides are shown as small letters. The site of cleavage with signal peptidase between C-terminus of the leader peptide and N-terminus of the pVIII is shown with an arrow.

Vector f8–5

The 9183 bp vector f8–5 (GenBank Accessions bankit439334) was constructed by standard methods and is illustrated in Figure 1. It has *Pst*I, *Bam*HI, *Nhe*I and *Mlu*I cloning sites in gene VIII and confers tetracycline resistance on the host cell.

Propagation and purification of individual phage clones

We used E.coli K91BlueKan cells (Yu and Smith, 1996) for propagation of phages. For sequencing, phage was propagated in 2 ml of culture and separated by PEG precipitation (Haas and Smith, 1993). For ELISA, phage was propagated in 20 ml of culture and purified by double PEG precipitation. For CD analysis, phage was grown in 200 ml of culture and purified by double PEG precipitation followed by ultracentrifugation in a gradient of CsCl as described (Smith and Scott, 1993)

Construction of the f8α library

Double-stranded f8–5 RF DNA (600 μg) was cleaved with *Bam*HI (1340 units) and *Mlu*I (670 units) in 10 ml of NEB buffer 3 (both enzymes and buffer from New England Biolabs), diluted with 400 μl of 250 mM EDTA, treated with phenol–chloroform, precipitated with ethanol and dissolved in 12 ml of TE buffer. A short stuffer fragment that lies between *Bam*HI and *Mlu*I sites was removed by ultrafiltration of 2 ml portions of DNA solution through six Centricon 100 kDa units (Amicon) followed by washing of each unit with 6×2 ml of TE buffer. DNA in a total volume of 5 ml was treated with phenol and chloroform and precipitated with ethanol; the yield was 365 μg. Meanwhile, the degenerate insert was prepared by annealing two partially complementary synthetic oligonucleotides, 1 and 2 (Table II), filling in the gaps at each end with DNA polymerase and digesting the product with *Bam*HI and *Mlu*I as follows. A mixture of 768 pmol of 32-mer 1 and 624 pmol of 73-mer 2 in 80 μl of 3.75× Klenow buffer, divided into three equal portions, was incubated in PCR microtubes for 5 min at 70°C, 10 min at 37°C and 10 min at 20°C. The tubes containing 1 μl of 5 mM dNTPs, 50 units of Klenow fragment and water to 100 μl were incubated at 30°C for 45 min. Reactions were stopped with 4 μl of 250 mM EDTA; mixtures were pooled, adjusted to 800 μl with TE; DNA was treated in two portions with phenol and chloroform and precipitated with ethanol. The resulting 85-mer degenerate duplex was digested in 660 μl of NEBuffer 3 with 1400 units of *Bam*HI and 700 units *Mlu*I, reactions being controlled by

Table II. Oligonucleotides

Oligonucleotide	Sequence	Use
1	5′-CAGAGGGTGAGGATCCCGCAAAAGCTGCCTTT-3′	Synthesis of degenerate insert for f8α library
2	5′-ACCATCGCCCACGCGTAACCGATATATTCmnnCGC-$(mnn)_3$CAG$(mnn)_2$AAAGGCAGCTTTTGCGGGAT-3′	As above
3	5′-GGAGCCTTTAATTGTATCGG-3′	Sequencing

Table III. Occurrence of amino acids in random peptides of f8α library

Amino acids	Occurrence at position (%), primary library/amplified library[a]						Expected (%)[b]
	12	13	15	16	17	19	
Charged (RKHYCDE)	38/47	26/26	44/23	33/30	23/26	15/39	29
Acidic (DE)	38/45	15/22	12/11	29/20	11/3	5/21	6
Basic (KR)	0/1	3/2	18/9	3/4	12/21	6/4	13
Polar (NCQSTY)	38/35	47/43	52/61	24/39	44/52	46/48	29
Hydrophobic (AILFWV)	16/11	21/27	3/11	38/25	23/15	32/11	32
A	6/6	5/6	0/6	**18/11**	9/6	9/4	6
C	0/0	0/0	0/0	0/0	0/0	0/0	3
D	**17/12**	8/6	0/0	12/7	2/0	0/4	3
E	20/32	8/16	12/11	17/13	9/3	5/18	3
F	0/0	3/1	2/0	0/1	0/0	2/0	3
G	8/5	5/3	0/2	$\frac{3}{4}$	0/0	2/1	6
H	0/2	3/2	12/4	2/4	0/1	3/14	3
I	2/1	2/2	2/1	5/2	5/0	5/3	3
K	0/1	2/1	8/4	2/0	6/6	3/2	3
L	5/2	8/10	0/1	12/7	6/4	3/1	10
M	2/2	6/2	$\frac{3}{4}$	2/5	9/8	8/2	3
N	9/11	3/8	9/11	9/7	3/7	11/9	3
P	0/0	0/0	0/0	0/0	0/0	0/0	6
Q	9/8	8/9	**18/26**	3/12	21/18	8/13	3
R	0/0	2/1	11/4	2/4	6/15	3/2	10
S	9/6	**9/17**	12/9	8/6	12/22	8/14	10
T	9/10	23/9	11/14	5/11	8/4	**18/12**	6
V	3/2	5/8	0/3	3/4	$\frac{3}{4}$	14/3	6
W	0/0	0/0	0/0	0/0	0/0	0/0	3
Y	0/0	5/0	2/0	0/3	0/1	2/0	3

[a]Occurrences of wild-type amino acids are shown in bold.
[b]Calculated as portion of nucleotide triplets encoding this amino acid.

electrophoresis in a 12% polyacrylamide gel along with pGEM DNA markers (Promega). The insert was excised from a preparative 12% polyacrylamide gel (Yu and Smith, 1996), electroeluted in an ISCO device on to Whatman DE81 paper, eluted from the paper with 3×25 µl of 1 M NaCl and precipitated with ethanol. The *Bam*HI/*Mlu*I-cut RF DNA (13.8 µg) was ligated with a 2.7-fold molar excess of gel-purified degenerate insert in 1.5 ml Ligase buffer with 380 units of T4 ligase (Boehringer) at 16°C for 16 h. Reaction was stopped with 60 µl of 250 mM EDTA; DNA product was isolated by phenol–chloroform extraction and ethanol precipitation. It was treated with *Nhe*I to destroy traces of uncut vector, isolated by phenol–chloroform extraction and ethanol precipitation and dissolved in 40 µl of water. Four 100 µl portions of frozen electrocompetent cells of *E.coli* MC1061 (Yu and Smith, 1996) (competence 9.8×10^8 TU per µg of f8–5 RF DNA) were electroporated with 10 µl of ligation mixture as described (Yu and Smith, 1996). Transformed cells were inoculated into four 1 l cultures of NZY medium containing 20 µg/ml tetracycline. After spreading 200 µl portions of appropriate dilutions on Tc plates to determine the number of independent transformed clones (4.4×10^8), the bulk cultures were shaken vigorously overnight at 37°C. Meanwhile, TcKan plates were seeded with K91BlueKan cells (Yu and Smith, 1996) and 100 transformed MC1061 colonies were grided on to the seeded plate. In order for a colony to grow at a grid-point, the corresponding MC1061 colony (resistant to tetracycline by virtue of the phage it carries, but sensitive to kanamycin) must have released infectious phage particles that could transduce the kanamycin-resistant K91BlueKan cells to tetracycline resistance. This occurred at 40% of the grid-points, indicating that 40% of the original transformed clones release infectious phages; 55% of these clones were shown by sequence analysis to harbor random peptides.

To get rid of the background wild-type clones, the primary library was propagated in bacterial cells, as described (Smith and Scott, 1993) and wild-type RF DNA was inactivated with *Nhe*I as follows. RF DNA was purified by alkaline lysis and CsCl equilibrium density centrifugation in ethidium bromide as described (Smith and Scott, 1993); a portion of RF DNA (100 µg) was treated in 120 µl of buffer B with *Nhe*I (400 units, Promega), which should cleave insertless vector molecules but not the desired insert-bearing molecules (previous paragraph); according to gel electrophoresis in 0.8% agarose–4× GBB, about half the RF was cut to the linear double-stranded form. Sixteen 100 µl portions of electro-

competent cells MC1061 (competence 9.8×10^8 transformants per μg f8–5 RF DNA) were transfected with the *Nhe*I-cut RF (4×12 μg and 12×3 μg) and propagated with vigorous shaking overnight at 37°C in eight 1 l cultures of NZY medium containing 20 μg/ml tetracycline. The yield was determined as before by counting colonies from small pre-growth samples and amounted to 2.7×10^9 transfectant clones (1.8×10^9 clones from 48 μg and 0.9×10^9 clones from 36 μg of *Nhe*I-cut DNA). Virions in the culture supernatant, constituting the f8α library, were purified by double PEG precipitation (Smith and Scott, 1993). Sequence analysis of 120 individual library clones showed that two clones (1.7%) were insertless vector molecules. The distribution of the amino acids in the foreign peptides displayed by the remaining 118 clones is presented in Table III.

Repeated propagation of libraries without affinity selection

A 50 ml culture of NZY was inoculated with 1 ml of an overnight culture of K91BlueKan cells, shaken at 37°C until the optical density at 600 nm reached 1.0, infected with ~2×10^{10} TU of f8–8mer or f8α library phages, shaken gently for 15 min at 37°C, transferred to a 3 l flask containing 500 ml of NZY supplemented with 0.2 μg/ml tetracycline and shaken vigorously for 35 min at 37°C. Additional tetracycline was added to a final concentration of 20 μg/ml, a portion was withdrawn and dilutions of that portion were spread on Tc plates; the total yield of transductants was 2.1×10^{10} and 5×10^{10} TU for f8α and f8–8mer libraries, respectively. Meanwhile, the 500 ml cultures were shaken vigorously overnight at 37°C. Two 1 ml portions were withdrawn and cleared of cells by two 5 min centrifugations in a microfuge. The resulting first-round supernatants contained ~5×10^{10} TU/ml for f8α and f8–8mer libraries. They served as inputs for a second round of propagation carried out in the same way, yielding second-round supernatants containing ~5×10^{10} TU/ml. These served in turn as inputs for a third round of propagation, yielding third-round supernatants. Individual clones in the third-round supernatants were propagated and their viral DNAs sequenced in the relevant regions to determine sequences of their displayed guest peptides.

Affinity selection of fibrinogen-binding phages

Six 35 mm Petri dishes were coated with 400 μl of 10 μg/ml bovine fibrinogen (Sigma) in TBS for 16 h at 4°C. These were used for three rounds of affinity selection from the f8α library (2×10^{10} virions) and the f8–8mer library (2×10^{11} virions) as described (Petrenko and Smith, 2000). Phages from 40 individual clones were propagated in 20 ml and partially purified by double PEG precipitation (first precipitation from 20 ml of supernatant and second from 1 ml of TBS solution to obtain a titer of ~2×10^{12} in 200 μl of 1/20 TE) for sequence analysis and binding studies.

Biotinylated fibrinogen

Bovine fibrinogen (38 mg dissolved in 9 ml of water; Sigma Chemical) was dialyzed against PBS, mixed with 1 ml of 1 M $NaHCO_3$ and 644 μl of freshly prepared 1.8 mM sulfo-succinimidyl 6-(biotinamido)hexanoate (Biotin-LC-NHS; Pierce Chemical) and reacted at 20°C for 2 h. To quench the residual reagent, 2.5 ml of 1 M Tris–HCl, pH 8.9 was added and the mixture incubated for an additional 1 h at 20°C. After dialysis against TBS, the solution was concentrated to 3.2 ml using a Centriprep-30 Concentrator (Amicon), diluted with an equal volume of glycerol and stored at –20°C; the concentration was 3 mg/ml protein, assuming that an A_{280} of 1 corresponds to 0.61 mg/ml.

ELISA of fibrinogen binders

Phages, 5×10^{11} virions/ml in 40 μl TBS, were absorbed in wells of 96-well polystyrene ELISA dishes for 16 h at 4°C. The wells were washed, blocked with BLOTTO solution, washed, filled with various amounts of biotinylated bovine fibrinogen in 40 μl of TBS, incubated for 30 min at 20°C, washed and developed with alkaline phosphatase-conjugated streptavidin and *p*-nitrophenyl phosphate as described (Yu and Smith, 1996). In competition ELISA, biotinylated fibrinogen at a fixed concentration of 76 nM was preincubated for 45 min with various amounts of inhibitor phage before being added to the wells.

Results and discussion

In this paper we describe a new library of phages displaying a randomized α-helical segment of the major coat protein. We called this library 'alpha library' to emphasize conformational homogeneity of exposed peptides. We studied the evolution of the library during its propagation and tested the alpha library as a source of substitute antibodies and potential partners in forming the mosaic phages, carrying two or more different guest peptides on their surface.

Construction of the alpha library

The major coat protein of filamentous phages consists of four functional domains A–D:

```
          A         B         C         D
          1        10        20        30        40        50
M13       AEGDDPAKAAFNSLQASATETIGTAWAMVVVIVGATIGIKLFKKFTSKAS
f1        ----------D-----------------------------------------
fd        ----------D-----------------------------------------
f8α       ----------XX-XXX-X-----------------------------------
```

N-Terminal mobile surface domain A (Ala1 to Asp5) appears to be in a non-helical, possibly disordered conformation (Kishchenko *et al.*, 1994). Domain B is an amphipathic, slowly curving α-helix, extending from Pro6 to about Tyr24 (Glucksman *et al.*, 1992). Domain C, a highly hydrophobic helix extending from Ala25 to Ala35, is entirely buried in the interior of the protein coat. The remainder of the protein, D, from Thr36 to Ser50, constitutes an amphipathic helix forming the inside wall of the protein coat. Four basic residues near the C-terminus interact with the viral DNA.

Foreign peptides fused to N-terminal domain A can adopt various conformations depending on their sequence (Ilyichev *et al.*, 1989; Felici *et al.*, 1991; Greenwood *et al.*, 1991; Kishchenko *et al.*, 1994; Iannolo *et al.*, 1995; Petrenko *et al.*, 1996; Petrenko and Smith, 2000). In this work we found that unlike promiscuous N-terminal peptides, foreign peptides loaded into domain B submit to α-helical architecture of wild-type protein.

According to an α-helical model of segment B, amino acids K8, D12, S13, Q15, A16, S17, T19, E20, Y21, G23 and Y24 belong to a polar area and are exposed on the surface of the phage, while another amino acids belong to a non-polar region and interact with phage body (Makowski, 1993; Marvin *et al.*, 1994; Williams *et al.*, 1995). We designed a 10^8 clone library f8α (see the structure above) of phages randomized in the polar area of the B segment (amino acids 12, 13, 15–17 and 19).

Composition of f8α library and its evolution

It was interesting to compare the evolution of the phage library *in vitro* and the natural evolution of filamentous phages. Phages f1, fd and M13 presumably have arisen through independent mutations of a common ancestor (Hill and Petersen, 1982). Their evolution resulted in accumulation of dozens of mutations in DNA, which, however, did not change substantially the primary structures of the capsid proteins. In mature forms of the major coat protein there is only one difference between the wild-type phages: D12 in phage f1 and fd is replaced for N12 in phage M13, as shown above, which probably is not essential for phage reproduction. In phage libraries, however, some peptide inserts can confer a selective growth advantage or disadvantage for the host phage, as shown by Iannolo *et al.* (Iannolo *et al.*, 1997). In our 'evolution experiments' we used populations of phages from f8α library, mutated in positions 12, 13, 15–17 and 19 and, for comparison, f8–8 library of clones having random multiple mutations in the N-terminus (deletion of EGD sequence with insertion of random 8-mers) (Petrenko *et al.*, 1996). 10^8 phage clones from the f8α library were propagated three times in liquid media with an excess of bacterial cells. The proportion of the wild-type phages in the phage population did not increase substantially during propagation: 2/120 (1.7%) before and 7/160 (4.3%) after three rounds of propagation. Data on the diversity of amino acids in 64 randomly chosen clones from the initial library and 114 clones from the propagated library are summarized in Table III. There are some clear biases in the distribution of some amino acids in the libraries. For example, cysteine, proline and tryptophan are prohibited in all positions, while the occurrence of negatively charged and polar amino acids is considerably higher than expected. However, the composition of the library is not dramatically changed through the propagation of the clones, indicating that clones from the alpha library have about equal advantages in the growing process. It is interesting to note that natural phage M13 successfully uses the less advantaged asparagine instead of the commonly dominant aspartic acid present in the strains fd and f1.

Evolution of the f8α library contrasted dramatically with that of the landscape library f8–8, composed of the clones having insertions of foreign peptides in N-terminal part of the major coat protein (Petrenko *et al.*, 1996; Petrenko and Smith, 2000). To characterize the primary library f8–8 we determined the structure of the foreign peptides in 140 clones among which 13 clones (11%) were identified as wild-type phages (vector). Occurrences of different amino acids in foreign peptides in 73 non-wild-type phages are shown in Table IV. The distribution of amino acids in the inserts does not differ dramatically from random, with some exceptions: cysteine is absent and proline and threonine are favored in all positions.

After three rounds of amplification of the f8–8 library, 77 clones were sequenced and 65 were found to be vector (84%). Random non-wild-type phages have the following structures of N-terminal part of major coat protein:

aEL**S**N**TTTS**dpak....
aA**ST**V**STS**Edpak...
aEN**S**NPL**TT**dpak...
aAQE**SS**MVPdpak...
aDR**S**LIDG**T**dpak...
aG**T**EDAALPdpak...
aD**S**GAPRYEdpak...
aGADGDdpak...
aVPdpak...
aegedpak... – vector

where non-wild-type amino acids are shown as capitals and dominant serine and threonine are bold.

After four rounds of amplification, 40 clones were sequences and 35 (88%) were found to be vector. The structure of the other five clones are as follows:

aAxN**TS**A**TT**dpak...
aD**T**A**TS**R**ST**dpak...
aD**ST**AAGL**T**dpak
aEPGQDdpak...
aEdpak...

These results clearly indicate powerful censoring during growth of phages from the f8–8 library in favor of the wild-type phages and probably weak or no censoring of the f8α library.

α-Helical conformation of random peptides in f8α library

The absence of proline and cysteine and the frequent appearance of glutamic acid and glutamine in the random peptides of the f8α library agree with their α-helical conformation (Chou and Fasman, 1974). More detailed analysis using Protean 4.0 Expert Sequence Analysis Software from DNASTAR showed that, according to the Chou–Fasman and Garnier–Robson algorithms, in all 18 randomly chosen clones the exposed part of the major coat protein between 9 and 21 amino acids preferably appears in an α-helical conformation. In contrast, not one of 10 randomly chosen fusion peptides from the f8–8 library, used as a control, was present in an α-helical conformation. An intense positive band at 194 nm along with two negative bands at about 223 and 208 nm in the CD spectra of randomly chosen clones from the f8α library (Figure 2) also demonstrate that they are exposed in an α-helical conformation (Johnson, 1990; Williams and Deber, 1996). As a control we used phages fused to the peptide identical with a loop segment in concanavalin A (Hardman *et al.*, 1982; Petrenko *et al.*, 1996). The lower magnitude of positive and negative bands in its CD spectrum, as expected, corresponds to lower α-helicity of the fusion protein in phage capsid in comparison with clones from the alpha library (Toumadje and Johnson, 1993).

Alpha library as a source of substitute antibodies and helically constrained ligands

It can be expected that peptides of the alpha library, which are constrained in the α-helical conformation, are not promiscuous as peptides from another phage libraries, which are flexible enough to bind various ligands and receptors in their native conformations (Smith and Petrenko, 1997). However, the rigid conformation of the displayed peptides can provide the entropic advantage of binding with the matching molecule, thus increasing the affinity of binding. It can give also precise information on the structure of the complex, which can be used for the design of organic peptidomimetics (Bianchi *et al.*, 1995).

We compared the repertoires of the binding phages from two libraries, alpha library f8α and landscape library f8–8 (Petrenko *et al.*, 1996; Petrenko and Smith, 2000), using bovine fibrinogen as a model acceptor of the phage clones. Fibrinogen is a 45 nm long rod-like 340 kDa glycoprotein composed of six polypeptide chains joined by disulfide bonds (Doolittle *et al.*, 1998). To select fibrinogen-binding phages, fibrinogen was absorbed on plastic dishes and treated with landscape libraries. After three rounds of selection (Petrenko and Smith, 2000), fibrinogen-binding clones were propagated and sequenced (Table V). The fibrino-

Table IV. Occurrence of amino acids in random peptides of f8–1/8 library

Amino acid	Amino acid occurrence (found/predicted) in random peptides X_8 (Xa–Xh)								
	At position								
	Xa[a]	Xb	Xc	Xd	Xe	Xf	Xg	Xh[a]	Average
Ala	1.2	1.7	0.8	0.4	1.3	1.1	1.5	0.6	1.1
Arg	0/0	0.3	0.6	0.7	0.9	0.4	0.6	0.4	0.6
Asn	0/0	3.8	0.8	0.8	0.8	0.8	0	0/0	1.2
Asp	1.9	1.3	0.4	0.8	1.3	1.3	0.4	0/0	1.1
Cys	0/0	0	0	0	0	0	0	0/0	0
Gln	0/0	0.4	1.3	0.8	0.8	2.5	3.0	1.8	1.5
Glu	0.8	0.8	0.4	0.8	1.3	0.8	0.4	1.6	0.9
Gly	0.9	0.4	0.8	0.8	0.4	0.6	0.4	0.6	0.6
His	0/0	0	1.3	2.1	2.1	0.8	0.8	0/0	1.2
Ile	0/0	0.8	0.8	0.8	1.3	0.4	1.7	0/0	1.0
Leu	0/0	0.6	0.8	1.1	0.7	1.0	0.8	0.9	0.9
Lys	0/0	0.4	0.4	0.4	0.4	0.4	1.3	0.4	0.5
Met	0/0	0.4	1.3	0.8	1.3	0.4	0.4	0.4	0.7
Phe	0/0	1.3	1.3	1.3	0.4	0.4	0	0/0	0.8
Pro	0/0	2.1	1.7	1.7	2.3	2.5	2.1	2.7	2.2
Ser	0/0	1.1	1.7	2.0	0.7	0.8	1.0	1.4	1.2
Thr	0/0	1.9	2.5	1.3	2.5	1.9	2.5	1.8	2.1
Trp	0/0	0.8	0.4	0.4	0.8	0.4	0.4	0.4	0.5
Tyr	0/0	0.8	0	0.4	0	0	0.4	0/0	0.3
Val	0.6	0.8	0.6	0.6	0.2	1.3	0.4	0.4	0.6

[a]0/0 means that no amino acids were found and none were expected in these positions.

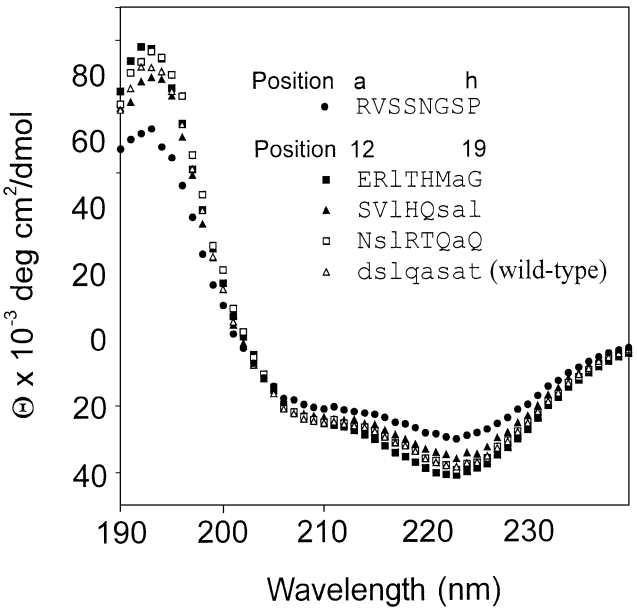

Fig. 2. CD spectra of control phage fd (second bond from the bottom at 223 nm), phage with a concanavalin A epitope RVSSNGSP inserted into the N-terminus of major coat protein (upper band at 223 nm) and phages from the alpha library with random peptides in positions 12–19: ERLTHMAG, NSLRTQAQ and SVLHQSAL, where α-helical areas according to Chou and Fasman are underlined (first, third and fourth bands from the bottom at 223 nm).

gen-binding phage displayed five families of guest peptides with dominant motifs AYLADRAD, FDLQLLAE, EAGPRXXP, (D/E)G(Y,F)LRP(E/D)Z and DSSVRFTG, where the positions marked X are occupied with an unusually high proportion (50%) of S and T and positions marked Z have P, S or T. The majority of clones selected from the alpha library displayed peptides AYLADRAD or FDLQLLAE, while clones selected from the landscape library f8–8 had diverse structures of guest peptides.

Table V. Fibrinogen-binding phage

Fibrinogen-binding clones selected from		
Alpha library f8–5/6	Landscape library f8–1/8	
AYLADRAD[3 4]	**EAGPRS**A**P**	**EGYLRPD**T
AYLLL**RAD**	**EAGPRS**S**P**[2]	**DGFLRPE**
FDLQLLAE[4]	**EAGPRS**N**P**	**EGYLRPE**S
	A**AGPR**PTS	**EGYMRPD**P
	EAGPRSTQ	**DGFLRPD**P
	V**AGPR**EV**P**	**DGFLRPD**S
	V**AGPR**MTE	**EGF**T**RP**SP
	V**AGPR**EVS	
	EAGPRAA**P**[3]	**DSSVRFTG**
	EAGPRST**P**[3]	
	EAGPRAS**P**	
	AGPREPNL	
	EAGPRSQ**P**	
		AG**GPRT**A**P**

Superscript numbers indicate the number of selected clones.

Complexes of selected phages with fibrinogen were characterized by ELISA and inhibition ELISA (Goldberg and Djavadi-Ohaniance, 1993; Yu and Smith, 1996). Isotherms of absorption of fibrinogen with some of the selected phages (Figure 3) demonstrate dose-dependent specific binding of fibrinogen to the selected phages. Inhibition curves (Figure 4) indicate that there are probably two binding sites in fibrinogen molecule. One site binds phage peptide FDLQLLAE from the f8α library, which competes for binding with phage peptides EAGPRSAPP and AYLADRAD from the f8–8 and f8α libraries. Fibrinogen-binding peptides with consensus motif GPR were identified by Bonnycastle *et al.* (Bonnycastle *et al.*, 1997) by screening the panel of peptide libraries. They mimic the N-terminal peptide of the α-chain of fibrin, so-called

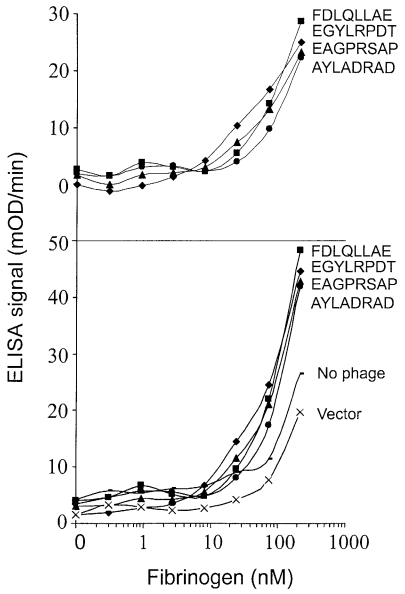

Fig. 3. Binding of fibrinogen to immobilize phages as measured by direct ELISA (see Materials and methods). Phages displaying the indicated peptides were immobilized in the wells of microtiter dishes and reacted with graded concentrations of biotinylated fibrinogen. Antigen remaining bound after washing was quantified with alkaline phosphatase conjugated with streptavidin as described in Materials and methods. The bottom graph shows signals observed with the loaded phages, the control wild-type phages and wells covered only with BLOTTO solution without any phages. The latter two controls indicate essential non-specific binding of fibrinogen to the blocked wells. This effect, which was also observed with different blocking reagents (Pierce, not shown), reflects the natural stickiness of the fibrinogen preparation. The top graph shows the net effect of binding.

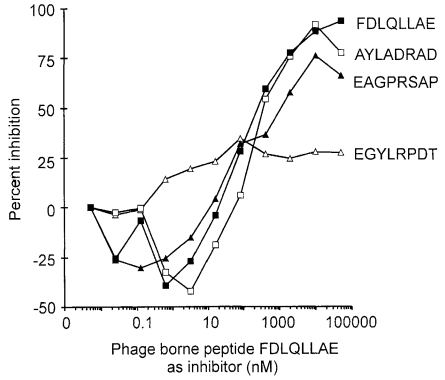

Fig. 4. Binding of fibrinogen to soluble phage inhibitors as measured by inhibition ELISA (see Materials and methods). Phages displaying indicated peptides were immobilized in the wells of ELISA dishes. Meanwhile, biotinylated fibrinogen was preincubated with graded concentrations of the competitor phage displaying peptide FDLQLLAE. The fibrinogen–competitor mixture was reacted with the phage-coated wells and fibrinogen remaining bound to the wells after washing was quantified with alkaline phosphatase–streptavidin conjugate as described in Materials and methods. Binding of fibrinogen to the soluble competitor decreases binding to immobilized phages if they interact with the same site on the fibrinogen molecule and does not affect significantly the ELISA signal if they interact with different non-overlapping sites.

A-knobs, which are known to bind 'holes' in the γ-chain of fibrinogen in the vicinity of Tyr363 (Doolittle *et al.*, 1998). Their homologues AGPRGAP and AGPRPSP are present in collagen α1-chain and β4 integrin subunit, hinting at a possible interaction of fibrinogen with these proteins in the hemostasis process. Phage peptide EGYLRPDT from the phage family (D/E)G(Y,F)LRP(E/D)Z does not compete for binding with fibrinogen with peptide FDLQLLAE and binds another site on the fibrinogen. It might mimic B-knocks, the N-terminal part of thrombin-processed β-chains, having the structure GHRP and interacting with the β-chain pocket of fibrinogen during its polymerization (Everse *et al.*, 1999).

Comparison of f8α and f8–8 libraries

Earlier we demonstrated that landscape phages, harboring foreign peptides fused to all copies of major coat protein, can demonstrate emergent properties intrinsic to the whole phage architecture, such as the ability to bind small organic compounds and protein antigen, resistance to stress conditions, mobility in an electric field, etc. (Petrenko *et al.*, 1996; Petrenko and Smith, 2000). The architecture of landscape phages depends on the structure of the guest peptide and the mode of its fusion to the major coat protein. For example, Terry *et al.* (Terry *et al.*, 1997) showed that peptide bonds located approximately three residues or more from the N-terminus of the major coat protein are resistant to proteases, presumably because of their proximity to the viral surface and conformational constraints. On the other hand, amino acids 1–12 of the major coat protein serve as antigenic epitopes and interact with antibodies (Kneissel *et al.*, 1999). In phages from the f8–8 library the foreign octamers are introduced into the N-terminus of the major coat protein between amino acids A-1 and D-5. The C-terminal part of the insert is probably accessible for binding but can be conformationally constrained. Furthermore, in some clones whole peptides can be constrained, being squeezed between neighboring subunits, as was shown by Kishchenko *et al.* (Kishchenko *et al.*, 1994). In contrast to the f8–8 library, whose clones are promiscuous enough to bind different receptors and ligands (Petrenko *et al.*, 1996; Petrenko and Smith, 2000), the f8α library contains a repertoire of clones with a strongly constrained conformation of guest peptides, which allows them to bind only receptors and ligands having a flexible conformation, matching the shape of α-helically constrained peptides, such as fibrinogen, used in this work as a model antigen.

The two libraries differ dramatically in their evolution. Most of the clones in the f8–8 library with foreign inserts in the N-terminal part of the major coat protein replicate in bacterial cells considerably slower than wild-type phages and are lost during several rounds of amplification. Different mechanisms of the biases intrinsic to the libraries, such as protein synthesis, inner membrane insertion, signal peptide cleavage, assembly, etc., can be considered (Rodi and Makowski, 1999). In contrast, the diversity of the f8α library is not changed much after three rounds of amplification, leading to the conclusion that clones in this library, having multiple mutations in the central displayed part of the major coat protein, apparently have similar replication rates.

Conclusion

The alpha library is a new type of phage display library, in which biological selection helps to generate a great variety of conformationally biased α-helical ligands. Strong biological censoring during phage growth probably prevents the appearance of amino acids which disturb the α-helical conformation of the phage major coat protein. Phage-borne α-helical peptides have a very rigid structure and cannot be used with sure success for the selection of ligands for any receptors and antibodies. However, if selected they provide precise information about the structure of the ligand, including its conformation, and give a clue for the design of lead compounds for

this receptor. Furthermore, in combination with other landscape libraries, the alpha library is a partner for the generation of mosaic phage libraries with a very high diversity of antigen-binding sites, reminding antibodies and limited only by the volume of infected bacteria, 10^{12} clones being a realistic number. The alpha library is a source of new fiber materials for nanotechnology with emergent physical and chemical properties. There is a need for such materials, for example, in the construction of diagnostics, chemical and biological detectors, vaccines, hemostatics, hemosorbents, affinity sorbents, gene- and drug-delivery vehicles, matrices for artificial tissues or transplants, etc. (Smith and Petrenko, 1997; Koivunen *et al.*, 1999).

Acknowlegements

We thank Natalia I.Petrenko for excellent technical assistance. This work was supported by US Army Research Office grants DAAG55-98-1-0258 and DAAD19-01-10454 to V.A.P., NIH grant DHHS 5 R21 RR12647-02 to V.A.P. and the State of Alabama through Auburn University AU Detection and Food Safety Peak of Excellence (to V.A.P.).

References

Barbas,C.F.,III, Barton,D.R., Scott,J.K. and Silverman G.J. (eds) (2001) *Phage Display. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Berkowitz,S.A. and Day,L.A. (1976) *J. Mol. Biol.*, **102**, 531–547.

Bianchi,E. *et al.* (1995) *J. Mol. Biol.*, **247**, 154–160.

Bonnycastle,L.L.C., Brown,K.L., Tang,J. and Scott,J.K. (1997) *Biol. Chem.*, **378**, 509–515.

Chou,P.Y. and Fasman,G.D. (1974) *Biochemistry*, **13**, 211–222.

De Berardinis,P., Sartorius,R., Fanutti,C., Perham,R.N., Del Pozzo,G. and Guardiola,J. (2000) *Nature Biotechnol.*, **18**, 873–876.

di Marzo Veronese,F., Willis,A.E., Boyer-Thompson,C., Appella,E. and Perham,R.N. (1994) *J. Mol. Biol.*, **243**, 167–172.

Doolittle,R.F., Spraggon,G. and Everse,S.J. (1998) *Curr. Opin. Struct. Biol.*, **8**, 792–798.

Everse,S.J., Spraggon,G., Veerapandian,L. and Doolittle,R.F. (1999) *Biochemistry (Mosc.)*, **38**, 2941–2946.

Felici,F., Castagnoli,L., Musacchio,A., Jappelli,R. and Cesareni,G. (1991) *J. Mol. Biol.*, **222**, 301–310.

Glucksman,M.J., Bhattacharjee,S. and Makowski,L. (1992) *J. Mol. Biol.*, **226**, 455–470.

Goldberg,M.E. and Djavadi-Ohaniance,L. (1993) *Curr. Opin. Immunol.*, **5**, 278–281.

Greenwood,J., Willis,A.E. and Perham,R.N. (1991) *J. Mol. Biol.*, **220**, 821–827.

Haas,S.J. and Smith,G.P. (1993) *Biotechniques*, **15**, 422–431.

Hardman,K.D., Agarwal,R.C. and Freiser,M.J. (1982) *J. Mol. Biol.*, **157**, 69–86.

Hill,D.F. and Petersen,G.B. (1982) *J. Virol.*, **44**, 32–46.

Hunter,G.J., Rowitch,D.H. and Perham,R.N. (1987) *Nature*, **327**, 252–254.

Iannolo,G., Minenkova,O., Petruzzelli,R. and Cesareni,G. (1995) *J. Mol. Biol.*, **248**, 835–844.

Iannolo,G., Minenkova,O., Gonfloni,S., Castagnoli,L. and Cesareni,G. (1997) *Biol. Chem.*, **378**, 517–521.

Ilyichev,A.A., Minenkova,O.O., Tatkov,S.I., Karpyshev,N.N., Eroshkin,A.M., Petrenko,V.A. and Sandakhchiev,L.S. (1989) *Dokl. Biochem. (Proc. Acad. Sci. USSR) Engl.Transl.*, **307**, 196–198.

Johnson,W.C.,Jr. (1990) *Proteins*, **7**, 205–214.

Kishchenko,G.P., Minenkova,O.O., Ilyichev,A.A., Gruzdev,A.D. and Petrenko,V.A. (1991) *Mol. Biol. Engl. Transl.*, **25**, 1171–1176.

Kishchenko,G., Batliwala,H. and Makowski,L. (1994) *J. Mol. Biol.*, **241**, 208–213.

Kneissel,S., Queitsch,I., Petersen,G., Behrsing,O., Micheel,B. and Dubel,S. (1999) *J. Mol. Biol.*, **288**, 21–28.

Koivunen,E., Arap,W., Rajotte,D., Lahdenranta,J. and Pasqualini,R. (1999) *J. Nucl. Med.*, **40**, 883–888.

Makowski,L. (1993) *Gene*, **128**, 5–11.

Marvin,D.A. (1998) *Curr. Opin. Struct. Biol.*, **8**, 150–8.

Marvin,D.A., Hale,R.D., Nave,C. and Citterich,M.H. (1994) *J. Mol. Biol.*, **235**, 260–286.

Minenkova,O.O., Ilyichev,A.A., Kishchenko,G.P. and Petrenko,V.A. (1993) *Gene*, **128**, 85–88.

Nord,K., Nilsson,J., Nilsson,B., Uhlen,M. and Nygren,P.-A. (1995) *Protein Eng.*, **8**, 601–608.

Perham,R.N., Terry,T.D., Willis,A.E., Greenwood,J., di Marzo Veronese,F. and Appella,E. (1995) *FEMS Microbiol. Rev.*, **17**, 25–31.

Petrenko,V.A. and Smith,G.P. (2000) *Protein Eng.*, **13**, 589–592.

Petrenko,V.A., Smith,G.P., Gong,X. and Quinn,T. (1996) *Protein Eng.*, **9**, 797–801.

Rodi,D.J. and Makowski,L. (1999) *Curr. Opin. Biotechnol.*, **10**, 87–93.

Romanov,V.I., Durand,D.B. and Petrenko,V.A. (2001) *Prostate*, **47**, 239–251.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Smith,G.P. and Petrenko,V.A. (1997) *Chem. Rev.*, **97**, 391–410.

Smith,G.P. and Scott,J.K. (1993) *Methods Enzymol.*, **217**, 228–257.

Terry,T.D., Malik,P. and Perham,R.N. (1997) *Biol. Chem.*, **378**, 495–502.

Toumadje,A. and Johnson,W.C.,Jr. (1993) *Anal. Biochem.*, **211**, 258–260.

Williams,K.A. and Deber,C.M. (1996) *Biochemistry*, **35**, 10472–10483.

Williams,K.A., Glibowicka,M., Li,Z., Li,H., Khan,A.R., Chen,Y.M., Wang,J., Marvin,D.A. and Deber,C.M. (1995) *J. Mol. Biol.* **252**, 6–14.

Yu,J.N. and Smith,G.P. (1996) *Methods Enzymol.*, **267**, 3–27.

Received April 12, 2002; revised July 17, 2002; accepted July 26, 2002