Efficient phage display of polypeptides fused to the carboxy-terminus of the M13 gene-3 minor coat protein

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Abstract **We report that, contrary to common belief, polypeptides fused to the carboxy-terminus of the M13 gene-3 minor coat protein are functionally displayed on the phage surface. In a phagemid display system, carboxy-terminal fusion through optimized linker sequences resulted in display levels comparable to those achieved with conventional amino-terminal fusions. These findings are of considerable importance to phage display technology because they enable investigations not suited to amino-terminal display, including the study of protein–protein interactions requiring free carboxy-termini, functional cDNA cloning efforts, and the display of intracellular proteins.**

Key words: M13 bacteriophage; Carboxy-terminus; Phage display; Minor coat protein

1. Introduction

Phage display is a powerful technology for engineering polypeptides that bind virtually any target, and also for investigating the binding specificities of natural protein–ligand interactions [1–3]. The method is based on the observation that polypeptides fused to M13 bacteriophage coat proteins are displayed on phage particles that also encapsulate the cognate genes [4]. In this way, a physical linkage is established between phenotype and genotype. 'Fusion-phage' displaying ligands with desired binding specificities can be selected by binding to immobilized targets, and the polypeptide sequence can be deduced from the sequence of the encapsulated DNA.

A significant limitation of M13 phage display has been the paucity of robust carboxy-terminal display formats. While the M13 coat contains five different proteins, the vast majority of phage display has relied on fusions to the amino-terminus of either the gene-3 minor coat protein (protein-3, P3) or the gene-8 major coat protein (protein-8, P8) [1,2]. A single report has detailed the display of proteins fused to the carboxy-terminus of the gene-6 minor coat protein, but display levels were about 100-fold lower than those observed with amino-terminal P3 display [5]. Another report has indicated that the two remaining coat proteins (the gene-7 and gene-9 minor coat proteins) both support amino-terminal fusions but not carboxy-terminal fusions [6]. Crameri et al. [7] have also developed a heterodimeric display system in which one member of a heterodimer pair is displayed as a fusion to the amino-terminus of P3, and the second member is co-expressed with a carboxy-terminal fusion. The heterodimer assembles on the phage surface and thus enables display of polypeptides with free carboxy-termini. To our knowledge, the levels of display achieved with this method have not been compared with other phage display methods.

While it is widely believed that neither P3 nor P8 can support carboxy-terminal fusions [7–9], this belief is based on circumstantial evidence. In the case of P8, the X-ray fiber structure of the M13 phage coat reveals that the amino-terminus is exposed on the particle surface, while the carboxy-terminus is buried in the core [10]. However, the phage coat contains several thousand P8 molecules organized in a repeating array, and we have recently demonstrated that fusions to a small fraction of the P8 molecules can be tolerated in a coat predominantly composed of wild-type P8 [11]. This is readily accomplished in a phagemid display system where wild-type P8 from a helper phage forms the vast majority of the phage coat. In such a format, peptides fused to the carboxy-terminus of a phagemid-encoded P8 moiety are displayed, and they are accessible for binding to ligands.

The case against P3 carboxy-terminal display is based on the presumption that the carboxy-terminus is buried in the phage particle, and that as a result, carboxy-terminal fusions would hinder incorporation into the phage coat [7]. P3 contains three distinct domains [12]. The two amino-terminal domains mediate host recognition and infection [13,14]. The carboxy-terminal domain (residues 253–406) interacts with other phage coat components, and is thus responsible for the incorporation of P3 into the phage particle [15]. The structure of the P3 carboxy-terminal domain is not known, and thus, the role of the P3 carboxy-terminus within the phage coat has not been rigorously defined. However, it has recently been shown that a heptapeptide fused to the carboxy-terminus of P3 did not affect phage viability, but larger fusions were not tolerated [15].

We thought it worthwhile to investigate whether fusions to the carboxy-terminus of P3 can be functionally displayed in a phagemid system. We report that polypeptides fused to the carboxy-terminus of P3 are displayed on the surface of the phage particle in a manner that permits functional selection. With selected linker sequences of eight or 10 residues, carboxy-terminal fusions were displayed at levels comparable to those achieved with conventional amino-terminal fusions.

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Abbreviations: P3, protein-3, the gene-3 minor coat protein of M13 phage; P8, protein-8, the gene-8 major coat protein of M13 phage; truncated P3, P3 residues 257–406

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2. Materials and methods

2.1. Materials

Reagents for dideoxynucleotide sequencing were from United States Biochemical Corp. Enzymes were from New England Biolabs. Maxisorp immunoplates were from Nunc (Roskilde, Denmark). *Escherichia coli* XL1-Blue and M13-VCS were from Stratagene. *E. coli* SS320 has been described previously [3]. Bovine serum albumin (BSA) and Tween 20 were from Sigma. Horseradish peroxidase/anti-M13 antibody conjugate was from Amersham Pharmacia Biotech. Anti-tetra-His antibody was from Qiagen. 3,3′,5,5′-Tetramethyl-benzidine/H_2O_2 substrate was from Kirkegaard and Perry Laboratories Inc.

2.2. Library construction

Standard molecular biology techniques were used to delete the hGH gene from pS1602, a previously described phagemid designed for the phage display of hGH [16]. The resulting phagemid (pS1428d) contained an open reading frame encoding the maltose binding protein signal peptide fused to the carboxy-terminal domain of P3 (residues 257–406). Co-infection of *E. coli* with pS1428d and a helper phage produces phage particles that contain single-stranded pS1428d DNA and P3 moieties derived from both pS1428d and the helper phage.

With phagemid pS1428d as the template, a previously described method [3] was used to fuse polypeptide libraries to the carboxy-terminus of P3. The libraries consisted of linkers containing six, eight, or 10 randomized amino acids followed by a hexa-His tag. The randomized positions were represented by degenerate NNS codons (where N = A/C/G/T and S = C/G) which encode all 20 natural amino acids. The six, eight, and 10 residue linker libraries contained 3.5×10^{10}, 1.3×10^{10}, and 2.8×10^{10} individual clones, respectively.

2.3. Selection for polypeptide display

Phage from the libraries described above were pooled together and cycled through rounds of binding selection [3] with an anti-tetra-His antibody coated on Maxisorp immunoplates as the capture target. Phage were propagated in *E. coli* SS320 cells with M13-VCS helper phage. After two or three rounds of binding selection, individual phage clones were analyzed in a phage ELISA by capturing the phage with the anti-tetra-His antibody and detecting bound phage (see below). Phage exhibiting strong signals in the phage ELISA were subjected to DNA sequence analysis.

2.4. Phage ELISAs for detecting polypeptide display

Phage ELISA protocols were adapted from a previous work [3]. Cultures of *E. coli* SS320 harboring phagemids were grown for 8 h at 37°C in 1 ml of 2YT, 50 μg/ml carbenicillin, 5 μg/ml tetracycline. The cultures were transferred to 30 ml of the same medium supplemented with M13-VCS helper phage (10^{10} phage/ml), and grown overnight at 37°C. Phage were harvested from the culture supernatant by precipitation with PEG/NaCl and resuspended in 1 ml of phosphate-buffered saline (PBS), 0.2% BSA, 0.1% Tween 20 (BSA blocking buffer). Phage concentrations were determined spectrophotometrically ($\varepsilon_{268} = 1.2\times10^8$ M^{-1} cm^{-1}).

Maxisorp immunoplates (96-well) were coated with capture target for 2 h at room temperature (100 μl at 5 μg/ml in 50 mM carbonate buffer, pH 9.6). The plates were then blocked for 1 h with 0.2% BSA in PBS and washed eight times with PBS, 0.05% Tween 20. Phage particles were serially diluted into BSA blocking buffer and 100 μl were transferred to coated wells. After 1 h, plates were washed eight times with PBS, 0.05% Tween 20, incubated with 100 μl of 1:3000 horseradish peroxidase/anti-M13 conjugate in BSA blocking buffer for 30 min, and then washed eight times with PBS, 0.05% Tween 20 and two times with PBS. Plates were developed using a 3,3′,5,5′-tetramethyl-benzidine/H_2O_2 substrate (100 μl), quenched with 1.0 M H_3PO_4 (100 μl), and read spectrophotometrically at 450 nm.

3. Results and discussion

3.1. Peptide display

For our experiments, we used a standard phagemid display system in which the phagemid encodes a P3 moiety containing only the carboxy-terminal domain (truncated P3) [17]. Phagemid DNA can be packaged into phage particles by super-infection with a helper phage which supplies all the proteins necessary for viral assembly. The resulting phage contain copies of both full-length P3 from the helper phage and also truncated P3 from the phagemid [17,18]. The presence of full-length P3 confers infectivity, and it also attenuates any deleterious effects due to the presence of heterologous proteins fused to the phagemid-encoded P3 moiety.

A

1		Y	S	S	A	E	T	D	R	
2		L	L	D	M	S	E	G	R	
3		K	S	W	V	P	P	Q	E	
4		L	P	V	P	G	G	E	N	
5		L	P	I	T	Q	R	D	H	
6		P	N	V	E	S	R	G	N	
7		T	D	R	G	C	H	H	N	
8		S	P	S	T	S	H	R	R	
9		K	G	P	L	A	S	T	E	
10		P	T	T	S	A	W	G	G	

B

11		E	I	F	R	R	G	Q	E	G	G
12		K	M	G	S	S	Q	I	E	S	D
13		G	K	P	W	V	R	C	T	F	R
14		E	S	T	P	A	S	E	V	D	H

C

15		**R**	I	**R**	I	**L**	Q	**K**	G	**K**	E
16		**R**	K	**K**	A	**W**	T	**K**	E	**M**	E
17		**R**	A	**K**	I	**V**	Q	**I**	C	**K**	E
18	G	**R**	A	**R**	I	**V**	Y	**R**	N	**K**	
19	V	**R**	A	**R**	Q	**L**	G	**E**	D	**K**	
20	Q	**Y**	G	**A**	G	**V**	L	**R**	N	**K**	

Fig. 1. Linker sequences selected for carboxy-terminal P3 display. The sequences were selected from a library in which a hexa-His tag was fused to the carboxy-terminus of truncated P3 using random linkers containing six, eight, or 10 amino acids. A: Eight-residue linkers. B: Non-homologous 10-residue linkers. C: Ten-residue linkers exhibiting homology (bold text).

We first determined whether small peptides fused to the P3 carboxy-terminus were displayed on the phage surface, and if so, what fusion sequences and lengths would result in optimal display. We constructed three libraries consisting of a hexa-His tag epitope fused to the carboxy-terminus of truncated P3 through intervening linkers containing six, eight, or 10 random amino acids. The libraries were combined in a pool containing 6.5×10^{10} individual clones. Phage particles displaying the hexa-His tag were isolated from the library by binding to an anti-tetra-His antibody immobilized on plastic plates. After washing to remove unbound phage, bound phage were eluted, amplified by passage through an *E. coli* host, and cycled through another round of binding selection. After two rounds of selection, binding assays revealed that two-thirds of the clones displayed the hexa-His tag. By the third round, essentially all of the clones ($>95\%$) were positive for display. Twenty clones exhibiting strong display were subjected to DNA sequence analysis.

DNA sequence analysis revealed many diverse linker sequences connecting the hexa-His tag to the P3 carboxy-terminus. In fact, all the sequenced clones were unique (Fig. 1). The selected linkers contained either eight or 10 amino acids; linkers containing six residues were not observed. This suggests that there is a minimum length required to display the carboxy-terminal fusion in a manner accessible for binding. A similar length requirement has also been observed for the display of carboxy-terminal P8 fusions [11]. While no consensus sequence was evident in the eight-residue linkers (Fig. 1A), a

subset of the 10-residue linkers exhibited significant homology (Fig. 1C). In this subset, every second residue is homologous, resulting in a common motif with a central hydrophobic residue flanked by two positively charged residues on either side. We speculate that this motif may facilitate display through interactions with the phage coat, but it is not absolutely required, because non-homologous linkers also provide comparable levels of peptide display.

We compared the levels of polypeptide display achieved with amino- or carboxy-terminal fusions to either P3 or P8 (Fig. 2A). Surprisingly, hexa-His tag display levels with the selected P3 carboxy-terminal fusions were similar to, or even greater than, display levels achieved with amino-terminal fusions to either P3 or P8. In contrast, display with the carboxy-terminal P8 fusion was reduced about 10-fold relative to the other formats. In a phagemid system, many different sequences are well tolerated as carboxy-terminal P3 fusions (Fig. 1), and eight-residue linkers are sufficient to render fused polypeptides fully accessible for binding to immobilized ligands. Taken together, our results not only demonstrate the viability of carboxy-terminal P3 display, they also question the supposition that the P3 carboxy-terminus is inaccessibly buried in the phage coat. Certainly, the exact role and position of the P3 carboxy-terminus within the phage particle merits further investigation.

3.2. Protein display

We next investigated the feasibility of displaying proteins fused to the P3 carboxy-terminus. For this purpose, we used Flt-1 domain-2 (residues 129–229), the second extracellular domain of the Fms-like receptor tyrosine kinase which binds to vascular endothelial growth factor (VEGF) [19]. We displayed the 12-kDa Flt-1 domain-2 as a fusion to either the carboxy- or amino-terminus of truncated P3. The two formats displayed protein at comparable levels, as evidenced by the capture of phage particles with immobilized VEGF (Fig. 2B) which only binds to correctly folded Flt-1 domain-2 [19].

3.3. Uses for carboxy-terminal phage display

Carboxy-terminal phage display has significant advantages over amino-terminal display in several important applications. Protein–protein interactions that require a free carboxy-terminus are not suited to amino-terminal display, because the carboxy-terminus is blocked by fusion to the phage coat protein. Carboxy-terminal display is ideal for such studies, as we have previously demonstrated in studies of PDZ domain–ligand interactions with carboxy-terminal P8 display [11]. In another important application, phage-displayed cDNA libraries can be used to rapidly identify natural ligands for biologically important molecules [8], but again, such studies are better suited to carboxy-terminal display rather than amino-terminal display. This is because the natural stop codons that terminate open reading frames prevent amino-terminal display but not carboxy-terminal display [7]. Carboxy-terminal display may be particularly useful for the study of intracellular proteins. Prior to phage assembly, P3 and P8 span the *E. coli* inner membrane with their amino-termini in the periplasm and their carboxy-termini in the cytoplasm [20]. As a result, amino-terminal fusions must pass through the host secretion machinery and fold in the periplasm, while carboxy-terminal fusions bypass the secretion pathway and fold in the cytoplasm. Thus, carboxy-terminal display may enable the display of intracellular proteins that are not evolved for secretion and/or folding in an oxidizing environment. Finally, it may be possible to simultaneously display amino- and carboxy-terminal fusions on the same P3 moiety. Such bi-functional formats

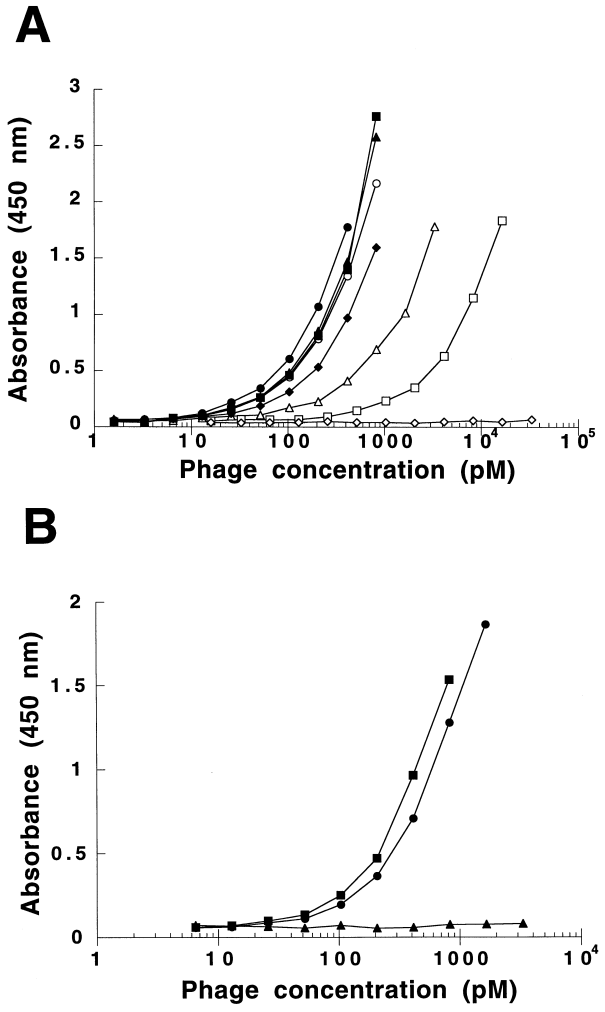

Fig. 2. Phage ELISAs for polypeptide display. Serial dilutions of phage solutions were incubated in wells containing immobilized capture target. Non-specifically bound phage were removed by washing, and bound phage were detected spectrophotometrically (450 nm) using a reaction catalyzed by horseradish peroxidase/anti-M13 antibody conjugate. Concentrations of input phage solutions are plotted along the *x*-axis and the absorbance at 450 nm, which is proportional to the amount of bound phage, is plotted along the *y*-axis. See Fig. 1 for selected linker sequences and Section 2 for additional details. A: Hexa-His tag display. An anti-tetra-His antibody was used as the capture target. The hexa-His tag was fused to: the carboxy-terminus of truncated P3 using selected linker 1, 2, 3, or 15 (filled circles, squares, triangles, or diamonds, respectively), the amino-terminus of truncated P3 (open circles), the carboxy-terminus of P8 (open squares), or the amino-terminus of P8 (open triangles). The amino-terminal P3 fusion used a glycine/serine-rich linker, while the P8 fusions used a previously described linker sequence (AWEENIDSAP) selected for carboxy-terminal P8 display [11]. A non-displaying phage is shown as a negative control (open diamonds). B: Flt-1 domain-2 display. Immobilized VEGF was used as the capture target. Standard molecular biology techniques were used to construct phagemids for the display of Flt-1 domain-2 fused to the truncated P3 carboxy-terminus (circles) or amino-terminus (squares). The carboxy-terminal fusion used selected linker 1, while the amino-terminal fusion used a glycine/serine-rich linker. A non-displaying phage is shown as a negative control (triangles).

could be useful in studies of heterodimeric protein–protein interactions [6] and enzyme–substrate interactions [21].

4. Conclusions

In a phagemid system, polypeptides fused to the P3 carboxy-terminus can be displayed on the phage surface at levels comparable to amino-terminal fusions. Thus, this format appears to be a robust platform for carboxy-terminal M13 phage display. The results are of considerable importance to phage display technology because they enable investigations not suited to amino-terminal display. These include the study of protein–protein interactions requiring free carboxy-termini, functional cDNA cloning efforts, and perhaps the display of intracellular proteins.

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