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High Copy Display of Large Proteins on Phage for Functional Selections

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We have isolated mutations in the major coat protein P8 of M13 phage that greatly increase the surface display of monomeric or oligomeric proteins. The monomeric protein, human growth hormone (hGH), was fused to the N terminus of P8; libraries of P8 variants were constructed and variants that increased hGH display were selected by binding to the extracellular domain of the hGH receptor. The hGH-P8 fusion protein was found to be extremely tolerant of mutations, and a number of P8 variants were found that increased display to levels that improved detection of the hGH-P8 fusion by almost 100-fold. The increased display likely results from better accommodation of the hGH-P8 fusion protein in the phage coat. Using this high copy display format, it was possible for the first time to detect variants of hGH with very weak affinities for the hGHbp ($K_d > 1$ μM). The display of a tetrameric protein, streptavidin (≈50 kDa), was also increased, suggesting the approach may be general to many proteins. The initial product of a natural or invented selection from a naive library is often a weakly functioning protein. These improvements in high copy display should facilitate the broader goal for selection of proteins with novel functions.

Keywords: major coat protein; mutagenesis; M13; phage display

Introduction

Through a two-step process, the immune system generates high-affinity antibodies for essentially any protein (Alberts *et al.*, 1983). The initial immune response is dominated by decavalent antibodies of the IgM class, a polyvalent format that facilitates the selection of low-affinity ligands through avidity or ''chelate'' effects. Low-affinity IgM selectants are switched to the bivalent IgG format and affinity is improved through somatic mutation and clonal selection. This two-step approach, based on switching from high to low valency, is very economical; to identify such high-affinity ligands in one step would require massively large numbers of genes equal to the product (instead of the sum) of the initial IgM and secondary IgG libraries.

Phage display is a powerful approach to engineering peptides or proteins for binding to target proteins of interest (Smith, 1985; Scott & Smith, 1990; Cwirla *et al.*, 1990; Lowman *et al.*, 1991; Wrighton *et al.*, 1996; Fairbrother *et al.*, 1998). Ligands are expressed as fusions to phage coat proteins and their respective genes are packaged within the phage. The ''fusion-phage'' having the best binding properties are selected by binding to an immobilized receptor protein *in vitro*.

Phage display for selection of naive binding peptides has been made to mimic the two-step avidity switch process used by the immune system (Wrighton *et al.*, 1996; Fairbrother *et al.*, 1998). Initial low-affinity peptides ($K_d > 1$ μM) can be selected from naive libraries displayed in a polyvalent format on the major coat protein, protein-8 (P8), which is present in about 2700 copies per virion (Marvin, 1998). Low-affinity selectants are matured to high affinity (K_d in the range of 1-100 nM) by display in a low-valency format; peptides are fused to the minor coat protein, protein-3,

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Abbreviations used: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbant assay; hGH, human growth hormone; hGHbp, human growth hormone binding protein; P3, protein-3, the gene-3 minor coat protein of M13 phage; P8, protein-8, the major coat protein of M13 phage; PBS, phosphate-buffered saline; PEG, polyethylene glycol.

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(P3), which is present in only three to five copies per virion.

Proteins have been displayed in a low copy format by fusing to P8 or P3 in a phagemid vector and providing wild-type P8 and P3 *in trans* from a helper phage (Bass *et al*., 1990). However, polyvalent display on P8 has been difficult because the display levels vary with fusion length and sequence (Malik *et al*., 1996). Moreover, when display is directed from a single P8 gene-fusion (without a wild-type P8 gene present), phage are unstable when the fusion peptides are generally greater than ten residues (Iannolo *et al*., 1995). Investigators have been able to express larger peptide-P8 fusions using phagemids, and providing an abundant supply of wild-type P8 protein *in trans* from a helper phage. However, even in a phagemid system, the fusion protein (>100 residues) is usually displayed in no more than a single copy per phage particle (Kretzschmar & Geiser, 1995). Thus, while monovalent phage display (on P3 or P8) has been used to affinity mature many different proteins from moderate to high affinity (Clackson & Wells, 1994), polyvalent display on P8 for selection of naive properties has not been practical for large proteins (>100 residues). It has been possible to select antibodies from naive libraries displayed on P3; these initial selectants often have affinities in the nanomolar range, probably because weaker-affinity molecules cannot be rescued in the low display format (Vaughan *et al*., 1996).

Here, we show that the display of two different proteins (22 kDa and 50 kDa) can be greatly increased by simply mutating the P8 gene to which the proteins are fused. This dramatic improvement in display is likely due to better accommodation of the fusion protein in the phage coat. Access to a high copy display format should allow weaker protein functions to be selected initially from naive libraries with much the same success as it has been used for small peptides. This should expand the potential for selecting new or existing protein functions.

Results

Human growth hormone (hGH) display on P8 or P3

Initially, we attempted to increase the display of hGH on P8 by constructing a phagemid vector (pS1607) in which transcription of the hGH-P8 fusion was controlled by the IPTG-inducible P_{tac} promoter (Amman & Brosius, 1985). To assay the level of hGH display, we titered the phage in microtiter wells containing the hGHbp and measured the amount of hGH-phage bound using an ELISA (see Materials and Methods for details). In the absence of IPTG, pS1607 phage particles displayed hGH at a level comparable to that obtained with an analogous construct (pS1602) in which hGH was fused to P3 (Figure 1). Induction with 10 μM IPTG caused a twofold reduction in the yield of pS1607 phage particles and a greater than 100-fold reduction in the yield of pS1602 phage particles; hGH display on pS1607 particles increased slightly, resulting in a fivefold improvement in hGH detection (Figure 1). Further induction with 100 μM IPTG reduced the yield of both pS1607 and pS1602 phage particles by more than 100-fold. Thus, increasing hGH display through IPTG induction proved impractical for P3 display and only moderately improved P8 display.

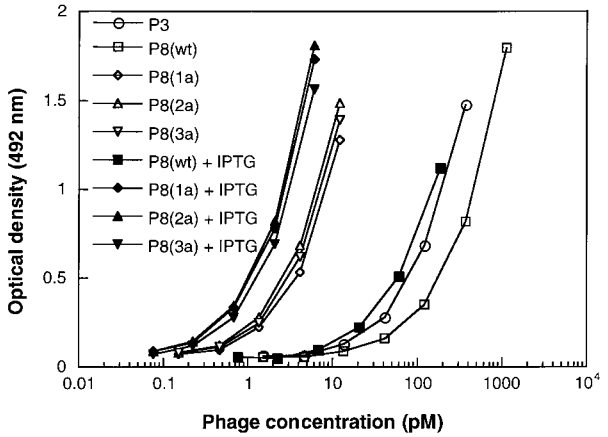

Figure 1. Phage ELISAs for hGH display. Serial dilutions of hGH-P8 phage solutions were incubated in wells containing the immobilized hGHbp as the capture target. Non-specifically bound phage were removed by washing, and bound phage were detected spectrophotometrically (492 nm) using a reaction catalyzed by horseradish peroxidase/anti-M13 antibody conjugate. Concentrations of input phage solutions are plotted along the *x*-axis and the absorbance at 492 nm, which is proportional to the amount of bound phage, is plotted along the *y*-axis. hGH was fused to: the C-terminal domain of P3 (circles), wild-type P8 (squares) or to P8 selectants: P8(1a) (diamonds), P8(2a) (triangles), or P8(3a) (inverted triangles) (see Figure 3). Phage were produced from cultures that were either uninduced (open) or induced with 10 μM IPTG (filled). See Materials and Methods.

P8 variants provide increased hGH display

We reasoned that packing of the P8-fusion in the phage coat may improve display. To test this, five ten residue libraries were constructed that together spanned the 50 residue P8 sequence (Figure 2). Most positions within each ten residue block were either fully or partially randomized. Lysine residues were not mutated, because the positively charged side-chains may interact favorably with the negatively charged viral DNA, based on the structure of the phage coat (Symmons *et al*., 1995). Very large libraries were produced containing at least 3×10^9 independent transformants using a high transformation efficiency strain of *Escherichia coli* (Sidhu *et al*., 2000).

Each of the five libraries was cycled through five separate rounds of binding selection on plates

coated with the hGHbp, and selected clones were sequenced (Figure 3). In general, only a small number of wild-type residues were highly conserved in zones 1-3. Only one wild-type residue (Ala10) was completely conserved, and six residues (Ala7, Leu14, Ala18, Ile22, Met28, and Val30) were generally retained as wild-type. Eight positions showed consensus to a non-wild-type sequence (E2K, D4E, P6F, K8R, FllY, G23R, A27T, and V29Y). The zone 4 and zone 5 selectants yielded contaminants from other libraries, and no consensus mutation was found in these regions. These signs are typical that improvements are unlikely to be found. It would

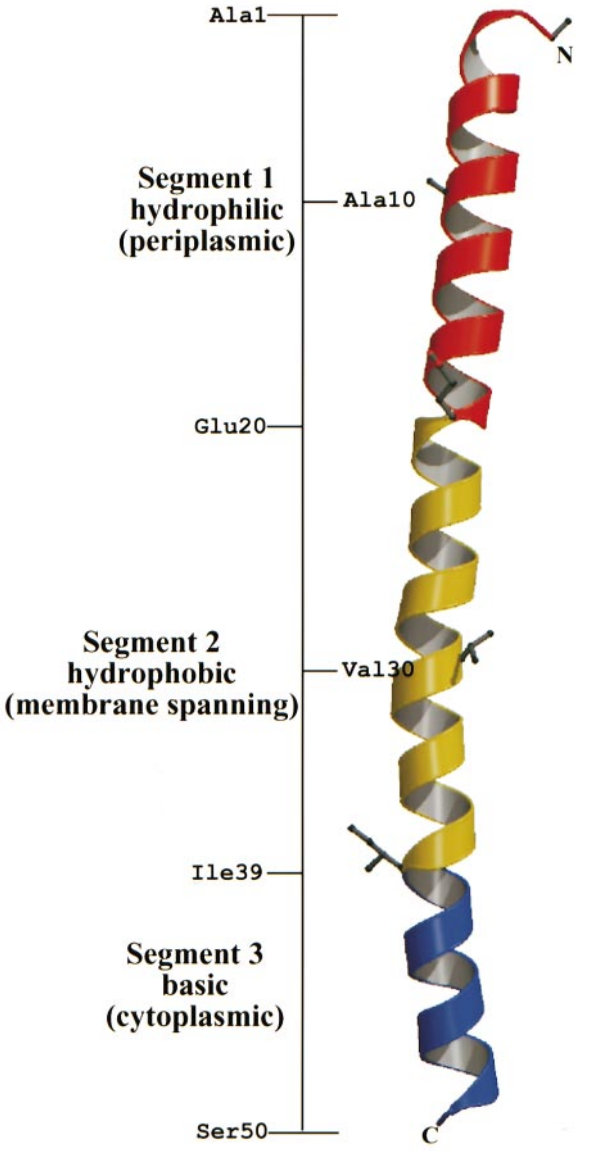

Figure 2. The gene-8 major coat protein. The P8 mainchain is shown as a helical ribbon. P8 can be divided into three distinct segments. In the phage particle, segment 1 (red) forms the outer surface, segment 2 (yellow) forms the hydrophobic core and segment 3 (blue) interacts with the DNA core. Side-chains are shown for residues at about ten residue intervals and labeled to the left of the protein. The depicted P8 is from Ff bacteriophage, but it differs from the M13 P8 in only one position (Asn12 in M13 P8 is an Asp residue in Ff P8). Atomic coordinates were obtained from the Brookhaven Protein Data Bank (entry 1IFJ) and represented using programs MOLSCRIPT-II (Kraulis, 1991) and RASTER-3D (Merritt & Murphy, 1994).

A

	1	2	3	4	5	6	7	8	9	10
wt	A	E	G	D	D	P	A	K	A	A
1a	D	K	S	E	K	F	-	R	D	-
1b	I	K	D	E	Q	F	-	R	-	-
1c	I	Y	I	K	E	T	S	-	N	-
1d	N	Y	V	-	Q	V	S	-	N	-
1e	-	K	A	E	E	F	-	E	-	-
1f	-	D	I	-	-	F	-	R	S	-
Consensus		K		E		F	A	R		A

B

	11	12	13	14	15	16	17	18	19	20
wt	F	N	S	L	Q	A	S	A	T	E
2a	Y	E	A	-	E	D	I	-	-	N
2b	Y	E	A	-	E	D	I	-	-	N
2c	Y	E	A	-	E	D	I	-	-	N
2d	Y	D	V	-	-	I	A	-	I	N
2e*	L	K	D	-	K	-	T	V	I	Q
2f*	Y	E	T	I	K	D	D	I	V	K
2g*	L	Q	N	I	H	S	-	I	S	K
2h*	Y	K	T	V	-	G	A	I	A	K
2i*	Y	K	T	I	K	S	I	-	N	K
2j*	Y	Q	-	-	-	I	I	-	A	Q
2k*	-	Q	-	-	K	D	T	-	D	-
2m*	-	E	N	-	-	-	T	I	-	K
Consensus	Y			L				A		

C

	21	22	23	24	25	26	27	28	29	30
wt	Y	I	G	Y	A	W	A	M	V	V
3a	L	F	F	L	L	G	T	V	H	L
3b	-	Y	L	N	I	L	-	V	Y	-
3c	F	-	R	V	T	-	T	-	Y	-
3d	V	-	R	-	V	M	S	-	Y	-
Consensus		I	R				T	M V	Y	V

Figure 3. P8 variants selected for increased display of fusion proteins. (a), (b), and (c) Selected sequences from libraries for zones 1, 2, and 3, respectively. Residues identical with the wild-type are represented by dashes. Consensus sequences are those that occur in at least half of the selectants. *Selected for SAV display; all others were selected for hGH display.

appear that the first half of the P8 protein is extremely tolerant of substitutions, whereas the back half is not so tolerant. We therefore focused on mutants from zones 1, 2, and 3.

All the selected variants from zones 1-3 that were tested increased hGH display; data for the best selectant in each zone are shown in Figure 1. These data indicate that, on average, these selectants had display levels that improved hGH detection about 100-fold relative to the wild-type hGH-P8 fusion. For example, the hGH-P8 selectants produced detectable ELISA signals with phage concentrations in the picomolar range, whereas the wild-type hGH-P8 fusion produced a detectable ELISA signal with phage concentrations in the 100 picomolar range. Similar results were obtained when hGH-phage were captured with an anti-hGH monoclonal antibody instead of the hGHbp (data not shown), suggesting the increase was not due to the mutated P8 somehow improving the affinity for the hGHbp.

IPTG induction of the hGH-P8 variants produced results similar to those obtained with wild-type hGH-P8: 10 μM IPTG induction caused a two-fold reduction in phage yield and a further fivefold improvement in hGH detection (Figure 1), while 100 μM IPTG induction reduced phage yields more than 100-fold. These data suggest that these fusions are toxic to *E. coli* and their over-expression exacerbates the problem.

Display and detection of hGH mutants with attenuated binding affinities

To evaluate the limits for isolating mutants with weakened affinities, we generated hGH variants whose receptor binding site 1 affinity was reduced 100 or 500-fold for the hGHbp (Pearce *et al*., 1999). The hGH variants were displayed as fusions either to the wild-type P8 or to the best P8 selectant from zone 1. As expected, reductions in binding affinity produced corresponding reductions in the phage ELISA signal strength (Figure 4). When fused to wild-type P8, the lowest-affinity interaction ($K_d = 820$ nM; ≈500-fold weaker than wild-type) was barely detectable at phage concentrations in the nanomolar range. However, the same hGH mutant provided a robust signal when displayed on the P8 selectant. In fact, display of the lowest-affinity hGH mutant on the P8 selectant provided a stronger ELISA signal than that of wild-type hGH displayed on wild-type P8. Given the size of the signal, it is reasonable to expect that hGH variants with K_d values of the order of 10 μM would be readily detectable in the high display format.

P8 variants provide increased display of streptavidin

To demonstrate the generality of this approach, we tested the P8 display of the biotin-binding protein SAV. When SAV is fused to wild-type P8 it is barely detectable in a phage ELISA with biotinylated-BSA (Figure 5). IPTG induction did not increase SAV display (data not shown). The SAV gene was fused to gene-8 containing random mutations in zone 1, 2 or 3. The libraries were pooled together and sorted for five rounds of binding selection with a polyclonal anti-SAV antibody as the capture target. This target was chosen over biotinylated-BSA because it was easier to release the bound phage from the antibody. Individual clones were sequenced revealing that all of the selectants were from the zone 2 library (Figure 3).

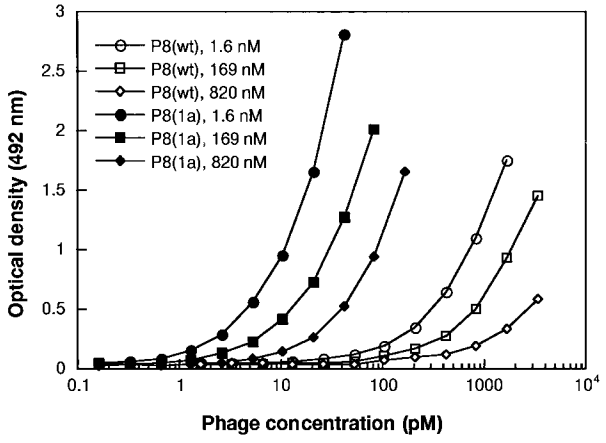

Figure 4. Phage ELISAs for display of hGH mutants. Display was measured by titering phage for wild-type hGH (circles, $K_d = 1.6$ nM), hGH(Y164A/R178A) (squares, K_d 169 nM), and hGH(K172A/R178A) (diamonds, $K_d = 820$ nM) fused to either wild-type P8 (open) or the selectant P8(1a) (filled). Phage were produced from uninduced cultures. See Figure 1 for further details. Affinities for hGH site 1 binding to hGHbp are from Pearce *et al*. (1999).

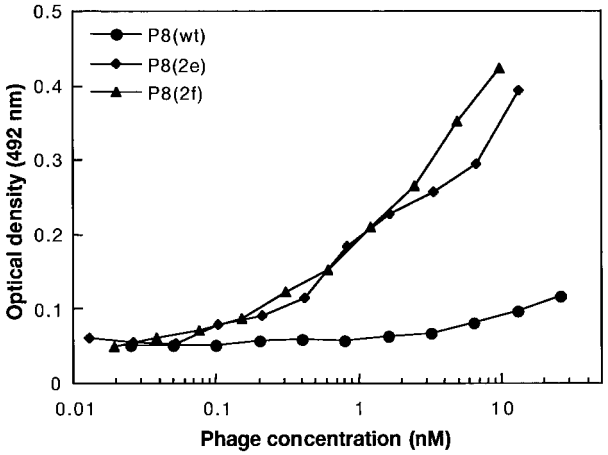

Figure 5. Phage ELISA for SAV display. Immobilized, biotinylated BSA was used as the capture target. Display was measured by titering phage for SAV fused to wild-type P8 (circles) or to P8 selectants: P8(2e) (diamonds) or P8(2f) (triangles) (see Figure 3). Phage were produced from uninduced cultures. See Figure 1.

This is the zone that showed the greatest consensus in the hGH selection. Although the sequence consensus was minimal, all the SAV selectants showed an increased display of SAV. To quantify the level of SAV display, we titered the phage onto microtiter wells containing biotinylated-BSA and measured the amount of SAV-phage bound using an ELISA, as described in Materials and Methods. The P8 selectants increased the signal strength by at least 50-fold (Figure 5).

Discussion

In practice, it has been possible to display small peptides but not large proteins in a polyvalent format on P8 (Scott & Smith, 1990; Cwirla *et al.*, 1990; Wrighton *et al.*, 1996; Fairbrother *et al.*, 1998; Iannolo *et al.*, 1995). The fact that phage will not tolerate large peptide fusions on P8 has necessitated the use of phagemid constructs where the phagemid provides the P8 fusion protein and a helper phage produces the wild-type P8 *in trans*. However, even in phagemid systems, the display levels decrease sharply with increasing fusion size (Malik *et al.*, 1996). In our experience, most large proteins display well below one copy per phage particle.

We show here that increased transcription from the fusion gene increased display somewhat, but the gains for hGH were modest (Figure 1) and there was virtually no effect on SAV display (data not shown). Further increases in transcription greatly reduced the phage yields from both P8 and P3 display systems. These data suggest that transcription is not the limiting factor for high copy display, and that over-expression of these fusions is toxic to *E. coli* or the phage assembly process.

Phage assembly is a dynamic process; P8 transitions from its independent membrane form to its final role as a polymeric coat protein (Webster, 1996). In the phage particle, each helical P8 associates with other P8 molecules to form a highly stable sheath surrounding the viral DNA (Marvin, 1998). We speculated that the fusion of proteins to the N terminus of P8 may hinder the incorporation of the linked P8 during assembly. Thus, low level display of large proteins may be due to the bulky N-terminal fusions interfering with phage assembly and allowing the wild-type P8 to out-compete the P8-fusion.

To test if compensatory mutations could be isolated, we constructed five libraries of P8 variants with N-terminal hGH fusions (Figure 2) and selected for those that showed increased display (Figure 1). The selection yielded P8 variants that were extremely divergent from the wild-type (Figure 3). An analogous selection for increased display of SAV produced similar results: the selectants showed little consensus amongst themselves or with wild-type (Figure 3(b)), yet all improved SAV display (Figure 5).

The structure of P8 can be divided into three distinct regions (Figure 2; Marvin, 1998). Prior to phage assembly, the first 20 residues comprise a polar, periplasmic domain; following assembly, they form the outer surface of the phage particle. The next 19 residues form a hydrophobic, membrane-spanning helix; in the mature virion, these helices from adjacent P8 molecules pack together and provide structural support through hydrophobic interactions. The C-terminal 11 residue stretch resides in the cytoplasm; it contains four positively charged lysine residues that are believed to interact with the negatively charged DNA in the assembled phage particle.

Our results suggest the first 30 residues are very tolerant to mutations; the protein-P8 selectants exhibited minimal homology with wild-type P8 or with each other. Variants from the library spanning residues 21-30 (zone 3) were particularly interesting. Although this hydrophobic segment is believed to reside in the membrane, hydrophilic substitutions occur in all selectants. A previous study (Deber *et al.*, 1993) also produced mutants with polar substitutions in this region, and these investigators suggested that residues 21-27 are solvent-accessible.

Libraries spanning the final 20 residues did not yield consensus selectants. Instead, contaminants from other libraries were obtained, suggesting this region is very intolerant to substitution. These results are consistent with a previous study in which the N-terminal half of P8 was found to be more mutable than the C-terminal half (Williams *et al.*, 1995).

Previous selections for P8 variants have demonstrated that wild-type P8 can accommodate mutations (Williams *et al.*, 1995), and several triple mutants have been described (Deber *et al.*, 1993). The selectants described here are surprising, in that they averaged seven mutations within ten varied positions; two variants contained mutations at all ten varied positions (Figure 3). What makes these results more striking is that the libraries were mildly mutagenized and thus were biased towards the wild-type: most varied positions encoded only eight possibilities and the wild-type was always included. Thus, a selective pressure for increased incorporation into the phage coat (i.e. increased fusion display) caused extreme divergence from the wild-type sequence. This strongly suggests that the wild-type P8 sequence is not optimal for display of fusion proteins, and that display can be improved by mutating P8, especially in zone 2.

Earlier selections for mutations in P8 required production of viable phage from a single P8 gene (Deber *et al.*, 1993; Williams *et al.*, 1995). For a mutation to be selected in such a phage system, the mutant P8 must be capable of incorporating into the phage coat and the resulting homo-polymeric coat must be stable. Since a phage particle contains several thousand copies of P8, the structural effects of a single mutation are greatly ampli-

fied in a homo-polymeric coat, and the range of viable mutations is consequently narrow.

The phagemid display system here contains two P8 genes: a wild-type gene from the helper phage and a P8-fusion gene from the phagemid vector. Thus, selection is driven for the ability of the P8-fusion protein to be tolerated among the wild-type P8 in the assembled coat. (Even polyvalent display with ten displayed ligands per phage requires less than 1 % of the fusion protein incorporated into the phage coat.) With the wild-type P8 maintaining the integrity of the phage coat, the P8-fusion gene can mutate more freely without compromising phage viability. Thus in a phagemid system, P8 variants can be maintained regardless of their ability to function as independent coat proteins.

The hGH-P8 selectants could be detected at phage concentrations 100-fold below the detection limit for hGH fused to wild-type P8 (Figure 1). Furthermore, hGH mutants with reduced affinity for hGHbp were readily detected when fused to a P8 variant (Figure 4). Display of the biotin-binding protein SAV was particularly challenging, since the protein exists as a tetramer (Weber *et al*., 1989) and is poorly secreted by *E. coli*. Functional display on phage requires the incorporation of SAV-P8 into the virion coat, and the assembly of tetrameric SAV. An amber stop codon was inserted between the segments encoding SAV and P8, and phage were produced in an amber supressor *E. coli* strain. In this system, most translation terminates at the amber stop to produce free SAV monomers, while a small amount of readthrough produces SAV-P8 fusions. It is likely that the majority of displayed tetrameric SAV consists of three free SAV monomers complexed with one SAV-P8 fusion incorporated into the phage coat. When fused to wild-type P8, the display of functional SAV was barely detectable above background. Selected P8 variants provided a 50-fold increase in signal strength, and display was increased from an impractical level to levels that should allow for functional selection (Figure 5).

In conclusion, we have demonstrated that mutations in P8 can be used to increase the display of large proteins on the surface of M13 phage. Since only the fused P8 is mutated, the displayed protein is not altered. High level display allows for the isolation of low-affinity interactions that could not be detected using either wild-type P8 or P3. This technology should expand our ability to select for naive protein functions that are often very weak in the initial pool of variants.

Materials and Methods

Materials

Reagents for dideoxynucleotide sequencing were from United States Biochemicals. Enzymes and plasmid pMal-p2 were from New England Biolabs. Maxisorp immunoplates were from NUNC (Roskilde, Denmark). *E. coli* XL1-Blue and M13-VCS were from Stratagene. *E. coli* SS320 is deposited with the American Type Culture Collection (Manassas, VA). Bovine serum albumin (BSA), Tween 20, and *o*-phenylenediamine dihydrochloride were from Sigma. Horseradish peroxidase/anti-M13 antibody conjugate was from Pharmacia Biotech. *Streptomyces avidinii* was from ATCC (accession number 27419). Goat anti-streptavidin polyclonal antibody was from Zymed Laboratories (South San Francisco, CA).

Oligonucleotides

DNA degeneracies are represented in the IUB code (K = G/T, N = A/C/G/T, R = A/G, S = G/C, W = A/T, Y = C/T). The following oligonucleotides were used:

hGH-1: *GGGTATCTAGAGGTTGAG*
hGH-2: TGGAGCTCCCGGATCCTCCACCGCTCTGGAAGCCACAGCTGCCCTC
IPTG-1: AAAAGAATTCCCGACACCATCGAATGGTGC
IPTG-2: ACCAGATGCATAAGCCGAGGCGGAAAACATCATCG
SAV-1: GGCTATCGGAATGCATCGGGCATCACCGGCACCTG
SAV-2: *GAGTCATAGTCGTCAGGCGCCTCCTCCGGATCCTCCACCCAC*CTTGGTGAAGGTGTCGTGG
g8stop1: GGATCCGGGAGCTCCAGCTGATGAGGTGACGATCCCGCAAAA
g8stop2: *GATCCCGCAAAAGCGGCCTGATGATCCCTGCAAGCCTCAGCG*
g8stop3: *CAAGCCTCAGCGACCGAATGATGAGGTTATGCGTGGGCGATG*
g8stop4: *GCGTGGGCGATGGTTGTTTGATGAGTCGGCGCAACTATCGGT*
g8stop5: *GCAACTATCGGTATCAAGTGATGAAAGAAATTCACCTCGAAA*
g8V1: *GGATCCGGGAGCTCCAGCRNTNASRNTNASNASNYCRNTR*NARNTRNTTTTAACTCCCTGCAAGCC
g8V2: *GATCCCGCAAAAGCGGCCNWTNASRNTNYTNASRNTRNTRNT*RNTNASTATATCGGTTATGCGTGG
g8V3: *CAAGCCTCAGCGACCGAANWCNWCNKTNWCNYYTNKGNYTNKGN*WTNWTGTCATTGTCGGCGCAACTATC
g8V4: *GCGTGGGCGATGGTTGTTNWTNWCNWTNKTNYTNYTNNTNNT*NNTAAGCTGTTTAAGAAATTCACC
g8V5: *GCAACTATCGGTATCAAGNNGNNSAAGAAANNSNNGNNGAAANN*GNNGTGATAAACCGATACAATTAAAGGC
Y164A/R178A: AACTACGGGCTGCTCGCTTGCTTCAGGAAGGACATGGACAAGGTCGAGACATTCCTGGCTATCGTGCAGTG-CCGC
K172A/R178A: TTCAGGAAGGACATGGACGCTGTCGAGACATTCCTGGCTATCGTCCAGTGCCGCTCT

Phagemids for phage display of hGH on P8 or P3

A DNA fragment containing the gene for hGH was amplified using the PCR (Saiki *et al*., 1988), with a derivative of plasmid pB0475 (Cunningham *et al*., 1989) as template and oligonucleotides *hGH-1* and *hGH-2* as primers. The DNA fragment was digested with *Nsi*I and cloned into a P8 display phagemid (Lowman *et al*., 1998) that had been digested with *Kas*I and treated with phage T4 DNA polymerase to produce blunt ends, and subsequently digested with *Nsi*I. The resulting phagemid was designated pS135a. In a second PCR, we used primers *IPTG-1* and *IPTG-2* to amplify a 1.6 kbp fragment of pMal-p2 containing the *lacI*^{q} gene and a gene fragment encoding the signal peptide from maltose binding protein under the control of the P_{tac} promoter. The DNA fragment was digested with *Eco*RI and *Nsi*I, and ligated with the large fragment resulting from a similar digestion of pS135a. The resulting phagemid (designated pS1607) encodes the following fusion product under the control of the IPTG-inducible P_{tac} promoter (Amman & Brosius, 1985): the maltose binding protein signal peptide, followed by hGH, a Gly/Ser-rich linker peptide (QSGGGSGSSS), and mature P8 of *E. coli* bacteriophage M13. In addition, pS1607 also contains the *lacI*^{q} gene (Muller-Hill *et al*., 1968) for effective transcription repression in the absence of IPTG. An analogous phagemid (designated pS1602) was constructed to encode hGH fused to the C-terminal domain of P3 (Lowman *et al*., 1991). The phagemids pS1607 and pS1602 permit display of hGH on P8 or P3, respectively.

A phagemid for phage display of streptavidin on P8

A derivative of pS1607 was constructed and designated pS657a. pS657a differs from pS1607 in two respects. First, the gene encoding hGH has been replaced by sequence encoding a pentapeptide (GGRPV). Second, the introduction of an *Xba*I site into the linker preceding P8 changes the codon encoding glutamine to an amber (TAG) stop codon. Digestion with *Nsi*I and *Xba*I excises the pentapeptide-encoding sequence and allows for the directional cloning of appropriately digested DNA fragments into a position analogous to that of the hGH gene in pS1607.

A PCR was performed with *Streptomyces avidinii* genomic DNA as template and oligonucleotides SAV-1 and SAV-2 as primers. Primer design was based on a published sequence for the SAV gene (Argarana *et al*., 1986). The amplified DNA fragment contained codons 16 to 133 of the SAV gene open reading frame, flanked by an *Nsi*I site at the 5′ end and an *Xba*I site at the 3′ end. The fragment was digested with *Nsi*I and *Xba*I, and cloned into similarly digested phagemid pS657a. The resulting phagemid (pW277e) encodes a fusion product similar to that encoded by pS1607, except that hGH has been replaced by SAV. Also, an amber stop codon has been positioned between the segments encoding SAV and P8. In an amber supressor *E. coli* strain such as XL1-Blue, the production of both free and P8-associated SAV monomers facilitates the assembly of functional, tetrameric SAV on the phage surface.

Construction of mutant P8 libraries

For library constructions, P8 was divided into five zones containing approximately ten contiguous residues each (Figure 2). Complete coverage of a library with ten fully randomized codons would require 20^{10} library members, which far exceeds the practical limits of phage display. Therefore, we used restricted codon sets over the ten residue segments to generate libraries with theoretical diversities of 1.1×10^9. Although we cannot be sure every possible variant is present in each library, the actual diversities exceeded the theoretical diversity in all cases (see below). The zone 1, zone 2, and zone 3 libraries each encompassed ten residues, which were represented by degenerate codons encoding eight amino acids each. The degenerate codons were chosen on the basis of the wild-type sequence (DNA degeneracies are represented in the IUB code: K = G/T, N = A/C/G/T, R = A/G, S = G/C, W = A/T, Y = C/T). The Ala, Gly, Ser or Thr codon was replaced by an RNT codon, except in zone 3, where Ala codon was replaced by an NYT codon and Gly codon was replaced by an NKT codon. The Asp, Asn, Glu or Gln codon was replaced by an NAS codon. The Phe or Val codon was replaced by an NWT codon. The Ile or Tyr codon was replaced by an NWC codon. The Met or Trp codon was replaced by an NKG codon. The Leu codon was replaced by an NYT codon. The Pro codon was replaced by an NYC codon. The zone 4 library mutated only nine residues. The first six positions were mutated as described above, while the final three were substituted by an NNT codon, which encodes 16 amino acids. The zone 5 library spans 11 contiguous residues, but only the seven non-lysine residues were mutated. In this case, the Phe codon was replaced by an NNS codon that encodes all 20 natural amino acids, while the remaining five positions were replaced by an NNG codon that encodes 16 amino acids.

Libraries were constructed using a modified version (Sidhu *et al*., 2000) of a previously described method (Lowman, 1998). Briefly, for each zone, an oligonucleotide (*g8stopn*, where *n* is the zone number) was used with either pS1607 (for hGH display) or pW277e (for SAV display) as a template to introduce two consecutive TGA stop codons within the zone, using Kunkel mutagenesis (Kunkel *et al*., 1987). The resulting phagemid was used as a template in a second round of Kunkel mutagenesis with a degenerate oligonucleotide (*g8Vn*, where *n* is the zone number) designed to introduce mutations at the desired sites, as described above.

Libraries were constructed for each zone (Figure 2) in P8 within the hGH-P8 fusion product of pS1607. The diversities of these libraries were as follows: zone 1, 2.5×10^{10}; zone 2, 2.5×10^{10}; zone 3, 2.5×10^{10}; zone 4, 1.3×10^{10}; and zone 5, 5.0×10^9. Libraries were constructed for zone 1, zone 2, and zone 3 of the P8 moiety within the SAV-P8 fusion product of pW277e. The diversities of these libraries were as follows: zone 1, 3.0×10^9; zone 2, 6.8×10^9; and zone 3, 8.6×10^9.

Selection of P8 variants that increase fusion protein display

Phage from the hGH-P8 libraries described above were cycled through rounds of binding selection with the human growth hormone binding protein (hGHbp) (Fuh *et al*., 1990) coated on 96-well Maxisorp immunoplates as the capture target. All libraries were sorted separately. Phage were propagated in *E. coli* SS320 cells with M13-VCS helper phage. After five rounds of binding selection, individual phage were isolated and analyzed in a phage ELISA by capturing the hGH-phage with hGHbp immobilized on a plate, and detecting bound phage (see below). Phage exhibiting strong signals in the phage ELISA were subjected to sequence analysis.

In a similar fashion, the SAV-P8 libraries were pooled and binding selection was performed with an anti-SAV polyclonal antibody as the capture target. Phage were propagated in the *SupE E. coli* strain XL1-Blue in which the amber stop codon is suppressed as glutamine (Bullock *et al*., 1987).

Site-directed mutagenesis

Mutagenesis was performed using Kunkel mutagenesis (Kunkel *et al*., 1987). The mutagenic oligonucleotide *Y164A/R178A* or *K172A/R178A* was used to introduce the mutations Y164A/R178A or K172A/R178A into the hGH gene.

Phage ELISA for determining relative levels of fusion protein display

Phage ELISA protocols were adapted from a previous work (Pearce *et al*., 1997). Cultures of *E. coli* XL1-Blue harboring phagemids were grown for eight hours at 37 °C in 1 ml of 2YT, 50 μg/ml carbenicillin, 10 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 IPTG at the appropriate concentration, and grown overnight at 37 °C. Phage were harvested from the culture supernatant by precipitation twice with PEG/NaCl (Lowman *et al*., 1998) and resuspended in 1.0 ml of BSA blocking buffer (phosphate-buffered saline, 0.2 % (w/v) BSA, 0.1 % (v/v) Tween 20). Phage concentrations were determined spectrophotometrically ($\varepsilon_{268} = 1.2 \times 10^8\ M^{-1}cm^{-1}$).

Maxisorp immunoplates (96-well) were coated with capture target protein for two hours at room temperature (100 μl at 5 μg/ml in 50 mM carbonate buffer (pH 9.6)). The plates were then blocked for one hour with 0.2 % BSA in phosphate-buffered saline (PBS) and washed eight times with PBS, 0.05 % Tween 20. Phage particles were serially diluted into BSA blocking buffer and 100 μl was transferred to coated wells. After one hour, plates were washed eight times with PBS, 0.05 % Tween 20, incubated with 100 μl of 1:3000 horseradish peroxidase/anti-M13 antibody conjugate in BSA blocking buffer for 30 minutes, and then washed eight times with PBS, 0.05 % Tween 20 and twice with PBS. Plates were developed using an *o*-phenylenediamine dihydrochloride/H_2O_2 solution (100 μl), stopped with 2.5 M H_2SO_4 (50 μl), and absorbance measured at 492 nm.

References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J. D. (1983). The Immune System. In *The Cell*, pp. 951-1012, Garland Publishing Inc., New York.

Amman, E. & Brosius, J. (1985). ''ATG vectors'' for regulated high-level expression of cloned genes in *Escherichia coli*. *Gene,* **40**, 183-190.

Argarana, C. E., Kuntz, I. D., Birken, S., Axel, R. & Cantor, C. R. (1986). Molecular cloning and nucleotide sequence of the streptavidin gene. *Nucl. Acids Res,* **14**, 1871-1882.

Bass, S., Greene, R. & Wells, J. A. (1990). Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins: Struct. Funct. Genet.* **8**, 309-314.

Bullock, W. O., Fernandez, J. M. & Short, J. M. (1987). XL1-Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. *Biotechniques,* **5**, 376-379.

Clackson, T. & Wells, J. A. (1994). *In vitro* selection from protein and peptide libraries. *Trends Biotechnol.* **12**, 173-184.

Cunningham, B. C., Jhurani, P., Ng, P. & Wells, J. A. (1989). Receptor and antibody epitopes in human growth hormone identified by homolog-scanning mutagenesis. *Science,* **243**, 1330-1336.

Cwirla, S. E., Peters, E. A., Barrett, R. W. & Dower, W. J. (1990). Peptides on phage: a vast library of peptides for identifying ligands. *Proc. Natl Acad. Sci. USA,* **87**, 6378-6382.

Deber, C. M., Khan, A. R., Li, Z., Joensson, C., Glibowicka, M. & Wang, J. (1993). Val to Ala mutations selectively alter helix-helix packing in the transmembrane segment of phage M13 coat protein. *Proc. Natl Acad. Sci. USA,* **90**, 11648-11652.

Fairbrother, W. J., Christinger, H. W., Cochran, A. G., Fuh, G., Keenan, C. J., Quan, C., Shriver, S. K., Tom, J. Y. K., Wells, J. A. & Cunningham, B. C. (1998). Novel peptides selected to bind vascular endothelial growth factor target the receptor-binding site. *Biochemistry,* **37**, 17754-17764.

Fuh, G., Mulkerrin, M. G., Bass, S., McFarland, N., Brochier, M., Bourell, J. H., Light, D. R. & Wells, J. A. (1990). The human growth hormone receptor. Secretion from *Escherichia coli* and disulfide bonding pattern of the extracellular binding domain. *J. Biol. Chem.* **265**, 3111-3115.

Iannolo, G., Minenkova, O., Petruzzelli, R. & Cesarini, G. (1995). Modifying filamentous phage capsid: limits in the size of the major capsid protein. *J. Mol. Biol.* **248**, 835-844.

Kraulis, P. J. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallog.* **24**, 946-950.

Kretzschmar, T. & Geiser, M. (1995). Evaluation of antibodies fused to minor coat protein III and major coat protein VIII in bacteriophage M13. *Gene,* **155**, 61-65.

Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**, 367-382.

Lowman, H. B. (1998). Phage display of peptide libraries on protein scaffolds. In *Methods in Molecular Biology* (Cabilly, S., ed.), vol. 87, pp. 249-264, Humana Press, Inc., Totawa, NJ.

Lowman, H. B., Bass, S. H., Simpson, N. & Wells, J. A. (1991). Selecting high-affinity binding proteins by monovalent phage display. *Biochemistry,* **30**, 10832-10838.

Lowman, H. B., Chen, Y. M., Skelton, N. J., Mortensen, D. L., Tomlinson, E. E., Sakick, M. D., Robinson, I. C. A. F. & Clark, R. G. (1998). Molecular mimics of insulin-like growth factor 1 (IGF-1) for inhibiting IGF-1:IGF-binding protein interactions. *Biochemistry,* **37**, 8870-8878.

Malik, P., Terry, T. D., Gowda, L. R., Langara, A., Petukhov, S. A., Symmons, M. F., Welsh, L. C., Marvin, D. A. & Perham, R. N. (1996). Role of capsid structure and membrane protein processing in determining the size and copy number of peptides displayed on the major coat protein of filamentous bacteriophage. *J. Mol. Biol.* **260**, 9-21.

Marvin, D. A. (1998). Filamentous phage structure, infection and assembly. *Curr. Opin. Struct. Biol.* **8**, 150-158.

Merritt, E. A. & Murphy, M. E. P. (1994). Raster3D version 2.0. A program for photorealistic molecular graphics. *Acta Crystallog. sect. D,* **50**, 869-873.

Muller-Hill, B., Crapo, L. & Gilbert, W. (1968). Mutants that make more lac repressor. *Proc. Natl Acad. Sci. USA,* **59**, 1259.

Pearce, K. H., Jr, Potts, B. J., Presta, L. G., Bald, L. N., Fendly, B. M. & Wells, J. A. (1997). Mutational analysis of thrombopoietin for identification of receptor and neutralizing antibody sites. *J. Biol. Chem.* **272**, 20595-20602.

Pearce, K. H., Jr, Cunningham, B. C., Fuh, G., Teeri, T. & Wells, J. A. (1999). Growth hormone binding affinity for its receptor surpasses the requirements for cellular activity. *Biochemistry,* **38**, 81-89.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science,* **239**, 487-491.

Scott, J. K. & Smith, G. P. (1990). Searching for peptide ligands with an epitope library. *Science,* **249**, 386-390.

Sidhu, S. S., Lowman, H. B. & Wells, J. A. (2000). Phage display for selection of novel binding peptides. *Methods Enzymol.* In the press.

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science,* **228**, 1315-1317.

Symmons, M. F., Welsh, L. C., Nave, C., Marvin, D. A. & Perham, R. N. (1995). Matching electrostatic charge between DNA and coat protein in filamentous bacteriophage. Fiber diffraction of charge-deletion mutants. *J. Mol. Biol.* **245**, 86-91.

Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., McCafferty, J., Hodits, R. A., Wilton, J. & Johnson, K. S. (1996). Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nature Biotechnol.* **14**, 309-314.

Weber, P. C., Ohlendorf, D. H., Wendoloski, J. J. & Salemme, F. R. (1989). Structural origins of high-affinity biotin binding to streptavidin. *Science,* **243**, 85-88.

Webster, R. E. (1996). Biology of the filamentous bacteriophage. In *Phage Display of Peptides and Proteins* (Kay, B. K., Winter, J. & McCafferty, J., eds), pp. 1-20, Academic Press, San Diego.

Williams, K. A., Glibowicka, M., Li, Z., Li, H., Khan, A. R., Chen, Y. M. Y., Wang, J., Marvin, D. A. & Deber, C. M. (1995). Packing of coat protein amphipathic and transmembrane helices in filamentous bacteriophage M13: role of small residues in protein oligomerization. *J. Mol. Biol.* **252**, 6-14.

Wrighton, N. C., Farrell, F. X., Chang, R., Kashyap, A. K., Barbone, F. P., Mulcahy, L. S., Johnson, D. L., Barrett, R. W., Jolliffe, L. K. & Dower, W. J. (1996). Small peptides as potent mimetics of the protein hormone erythropoietin. *Science,* **273**, 458-463.

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