

Rapid isolation of high-affinity protein binding peptides using bacterial display

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A robust bacterial display methodology was developed that allows the rapid isolation of peptides that bind to arbitrarily selected targets with high affinity. To demonstrate the utility of this approach, a large library (5×10^{10} clones) was constructed composed of random 15-mer peptide insertions constrained within a flexible, surface exposed loop of the *Escherichia coli* outer membrane protein A (OmpA). The library was screened for binding to five unrelated proteins, including targets previously used in phage display selections: human serum albumin, anti-T7 epitope mAb, human C-reactive protein, HIV-1 GP120 and streptavidin. Two to four rounds of enrichment (2–4 days) were sufficient to enrich peptide ligands having high affinity for each of the target proteins. Strong amino acid consensus sequences were apparent for each of the targets tested, with up to seven consensus residues. Isolated peptide ligands remained functional when expressed as insertional fusions within a monomeric fluorescent protein. This bacterial display methodology provides an efficient process for identifying peptide affinity reagents and should be useful in a variety of molecular recognition applications.

Keywords: bacterial display/flow cytometry/library/peptide

Introduction

Polypeptide display technologies have substantially impacted basic and applied research applications ranging from drug discovery to materials synthesis (Clackson and Wells, 1994; Shusta *et al.*, 1999). The strength of these methods derives from the ability to generate libraries containing billions of diverse molecules using the biosynthetic machinery of the cell and, subsequently, to identify rare desired polypeptides using selection or high-throughput screening methods. Display libraries have been applied extensively to isolate and engineer peptides and antibodies for molecular recognition applications. In particular, display of peptides on the surface of filamentous bacteriophage or phage display (Scott and Smith, 1990) has proven a versatile and effective methodology for the isolation of peptide ligands binding to a diverse range of targets (Arap *et al.*, 1998; Norris *et al.*, 1999; Whaley *et al.*, 2000). Recently, interest in peptide libraries has been revitalized by new prospects for peptides as recognition elements in diagnostics and proteomics (Kodadek, 2001), as self-assembly reagents (Whaley *et al.*, 2000; Lee *et al.*, 2002) and as low molecular weight therapeutics capable of high specificity (Nixon, 2002).

Increasing demand for rapid and scalable methods to generate affinity reagents with improved function has prompted continued development of phage display and alternative display systems (Shusta *et al.*, 1999). Alternative systems include mRNA and ribosome display (Wilson *et al.*, 2001), eukaryotic virus display (Bupp and Roth, 2002; Muller *et al.*, 2003) and bacterial and yeast cell surface display (Boder and Wittrup, 1997; Georgiou *et al.*, 1997). Cell surface display methods are attractive since they permit the application of fluorescence-activated cell sorting (FACS) for library analysis and screening (Daugherty *et al.*, 2000a; Georgiou, 2000). FACS provides a quantitative tool for library screening, allowing direct evaluation of library population fitness in terms of affinity and specificity and real-time monitoring and optimization of the screening process (Daugherty *et al.*, 2000b; Olsen *et al.*, 2003). Owing primarily to these features, cell surface display in conjunction with FACS has permitted the directed evolution of a monovalent antibody possessing an equilibrium dissociation constant in the femtomolar range (Boder *et al.*, 2000).

Protein display on the surface of bacterial cells (Brown, 1992; Francisco *et al.*, 1993; Georgiou *et al.*, 1997) holds the potential to simplify and accelerate the process of ligand isolation since experimental procedures with bacteria are efficient and screening can be performed using FACS. Although several different bacterial display systems have been reported (Francisco *et al.*, 1993; Lu *et al.*, 1995; Klemm and Schembri, 2000), their usefulness has been restricted by technical limitations including accessibility on the cell surface, inability to display highly diverse sequences and adverse effects on cell growth and viability (Christmann *et al.*, 1999). Several groups have demonstrated that peptide libraries can be constructed in *Escherichia coli* (Lee *et al.*, 2003) as insertions in extracellular proteins such as pili or flagella subunits (Lu *et al.*, 1995) or as insertions into outer membrane proteins (Camaj *et al.*, 2001). Although these systems have provided encouraging results (Camaj *et al.*, 2001; Tripp *et al.*, 2001), thus far the routine isolation of high-affinity peptide ligands for arbitrary protein targets has not been demonstrated.

To address this need, a robust bacterial display system was developed to allow the isolation of high-affinity binding peptides specific to arbitrary targets. A regulated bacterial display vector was developed, providing high-level expression of peptides accessible to large proteins, cells and surfaces, in a manner that does not compromise cell growth rate and viability. With optimization, this redesigned vector permitted the construction of a library comprised of 5×10^{10} members, the largest reported cell surface display library. Using sequential magnetic selection (MACS) and FACS, peptides with high binding affinity for a variety of protein targets could be isolated in just 2–4 days. The intrinsic speed and simplicity of this methodology should make bacterial display peptide libraries useful for a wide range of existing and emerging molecular recognition applications.

Materials and methods

Bacterial strains, reagents and plasmids

All work was performed in *E.coli* strain MC1061 [F⁻ araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL (Str^R) hsdR2 (r_K⁻ m_K⁺) mcrA mcrB1] (Casadaban and Cohen, 1980), with the exception of YFP expression, which was carried out in FA113 (Bessette *et al.*, 1999). Primers were obtained from Integrated DNA Technology, Operon and Invitrogen. Restriction enzymes were supplied by New England BioLabs. Streptavidin, R-phycoerythrin conjugate was purchased from Molecular Probes. Biotinylated anti-T7-tag monoclonal antibody was obtained from Novagen. Streptavidin-coated magnetic microbeads were obtained from Qiagen, Dynal Biotech or Miltenyi Biotec. Anti-biotin mAb-coated magnetic beads and anti-biotin mAb R-phycoerythrin were supplied by Miltenyi Biotec. Biotinylation and fluorescent labeling with AlexaFluor488 were carried out using the Fluoreporter Mini-biotin-XX Protein Labeling Kit and Alexa Fluor 488 Monoclonal Antibody Labeling Kit, respectively, from Molecular Probes. Human C-reactive protein (Cat. No. C4063) and serum albumin (Cat. No. A3782) were supplied by Sigma. Biotinylated HIV-1 GP120 was obtained from ImmunoDiagnostics (Woburn, MA).

Vector and library construction

To maximize library construction efficiency, asymmetric *SfiI* restriction sites were introduced into an *OmpA* expression vector immediately preceding loop 1 and following loop 4. DNA fragments containing the random epitope insertions were synthesized by polymerase chain reaction (PCR), digested with *SfiI*, ligated into the display vector and transformed into the *E.coli* strain MC1061, which can be made highly transformation competent (Sidhu, 2000) and is *ara*⁻, allowing the use of the *araBAD* promoter for controlled *OmpA* expression.

Plasmid pB33OmpA contains the wild-type *ompA* gene, including the native RBS, inserted downstream of the *araBAD* promoter in plasmid pBAD33 (Guzman *et al.*, 1995). It was constructed by ligation of digested (*KpnI/HindIII*) pBAD33 with a similarly digested *ompA* gene PCR product obtained using MC1061-derived genomic DNA and primers 1 and 2 (see Supplementary data available at PEDS Online). The plasmid pB33OmpA14 contains *SfiI* restriction sites in the *ompA* gene at positions corresponding to the beginning of the first extracellular loop of *OmpA* and at the end of the fourth loop, resulting in mutations F23L, N25G, N26Q and H151G, T152Q, T155Q. Plasmid pB33OmpA14 was made via overlap PCR, using primers 1–4 with pB33OmpA as template. The overlap product was digested (*KpnI/HindIII*) and ligated to similarly digested pBAD33. Plasmids pB33OT1 and pB33OT4 containing the T7-tag epitope inserted into loops 1 and 4, respectively, of *OmpA* were constructed using PCR, with pB33OmpA as template and primer 5 or 6, respectively, with primers 1 and 2. The overlap products were digested with *SfiI* and ligated with *SfiI*-digested pB33OmpA14. Plasmid pB33OS1, containing the streptavidin-binding peptide sequence SAECHPQGPPCIEGR (Giebel *et al.*, 1995), inserted into *OmpA* loop 1, was constructed by overlap PCR using primers 1, 2, 7 and 8 with pB33OmpA14 as template. Products were digested with *SfiI* and ligated into digested pB33OmpA14.

For random 15-mer library construction, primers 9 and 10 were used in a PCR with pB33OmpA as the template. The resulting product was lengthened in a second PCR to permit

efficient digestion, using primers 11 and 12. The product was then digested (*SfiI*) and inserted into the digested (*HincII/SfiI*) pB33OmpA14 vector. Approximately 15 μg of ligated DNA were transformed to the strain MC1061 by electroporation in 10 aliquots. Plating of serial dilutions of the pooled transformation mixture indicated 5×10¹⁰ independent transformants.

The YFP-SA-1 fusion expression plasmid pB33YFP-SA was constructed by overlap extension PCR with an *Aquorea* GFP-based yellow fluorescent protein gene as template (A.Nguyen and P.S.Daugherty, unpublished data) with primers 13–16, resulting in insertion of the 15 amino acid SA-1 peptide in the permissive site between amino acids Y145 and N146 of YFP (Baird *et al.*, 1999).

Magnetic selection

Typically, for the first round of magnetic selection, a frozen aliquot of 2.5×10¹¹ cells was used to inoculate 500 ml of LB medium containing 25 μg/ml chloramphenicol and grown at 37°C with shaking (250 r.p.m.) until the OD₆₀₀ was 1–1.5, at which time L-arabinose was added to a final concentration of 0.02% (w/v). After an additional 2 h of growth, a volume corresponding to 2.5×10¹¹ cells was concentrated by centrifugation (2000 g, 4°C, 15 min) and resuspended in 15 ml of cold PBS. For negative selection, 150 μl of streptavidin-coated magnetic beads (QIAGEN) were added, and the cell–bead mixture was incubated on ice for 30 min, at which time a magnet was applied to the tube and the unbound cells in the supernatant were removed to a new tube. For positive selection, biotinylated antigen (typically 1–100 nM) was added to the supernatant fraction and incubated on ice for 30–60 min. Cells were centrifuged as above and resuspended in 7.5 ml of cold PBS with 150 μl of streptavidin-coated magnetic particles (QIAGEN or Miltenyi). After 30–60 min of incubating the cells on ice with periodic agitation, a magnet was applied to the tube and the supernatant was removed and discarded. The pellet was washed twice in 7.5 ml of cold PBS, repelleted to the magnet each time and finally resuspended in LB medium and grown up overnight at 37°C with shaking in 20 ml of LB with chloramphenicol and 0.2% glucose. For the subsequent rounds of selection or sorting, a volume of cells corresponding to at least 10-fold oversampling of the number of cells retained in the previous round was subcultured to fresh LB with chloramphenicol but without glucose, grown to mid-log phase and induced as above. The volumes used for magnetic selection were reduced, while maintaining the same concentrations. In some cases, subsequent rounds of magnetic selection were carried out with anti-biotin mAb-coated magnetic particles (Miltenyi).

Library analysis and screening by FACS

For flow cytometric analysis and sorting, induced cells were typically labeled with biotinylated or fluorescently labeled antigen in PBS on ice for 45–60 min, followed by centrifugation and removal of the supernatant. When using biotinylated antigens, a secondary labeling was carried out with 6 nM streptavidin–phycoerythrin or 1 nM anti-biotin mAb–phycoerythrin for 30 min on ice, followed by centrifugation and removal of the supernatant. Cells were then resuspended in cold PBS at ~10⁶ cells/ml and immediately analyzed on a Partec PAS III cytometer equipped with a 100 mW argon (488 nm) laser. For analysis, 10⁴–10⁶ cells were interrogated and for sorting at least 10-fold oversampling of the expected clonal diversity was used. Following sorting, retained cells

were either amplified for further rounds of analysis and/or sorting by growing overnight in medium containing glucose or plated directly on agar for isolation of single clones. Typically, 5–15 selected clones were confirmed for antigen binding and the identity of each peptide insert was determined by automated sequencing of the *ompA* gene contained on the isolated plasmid.

Clonal affinity characterization

To obtain equilibrium binding curves, cells were labeled over a range of concentrations (e.g. 0.1–200 nM) of fluorescently conjugated target proteins (streptavidin–phycoerythrin or CRP–AlexaFluor488) and analyzed by flow cytometry, as above. The corresponding mean fluorescence versus concentration data were fitted to a monovalent binding isotherm to obtain the apparent K_D . Dissociation rates of streptavidin-binding clones were measured in the presence of 1–2 μ M biotin. Cells were labeled with 50 nM streptavidin–phycoerythrin for 30 min at room temperature. The cells were then pelleted, resuspended in PBS with biotin and immediately analyzed by flow cytometry. Fluorescence data were collected continuously for \sim 5 min. The dissociation rate constants were then determined as described previously (Daugherty *et al.*, 1998).

For analysis of peptide affinity in a soluble scaffold, streptavidin-binding peptide SA-1 fused within a loop of YFP was prepared by cytoplasmic expression in *E. coli* strain FA113, induced overnight at room temperature. The soluble protein was isolated using B-PER II bacterial protein extraction reagent (Pierce Biotechnology) following the manufacturer's protocol. Approximately 10^7 streptavidin-coated magnetic beads (Dynal) were added to 40 μ l of cell lysate and equilibrated at room temperature for 20 min. The beads were washed once in 2 ml of PBS; biotin was added to a final concentration of 1 μ M and immediately analyzed by flow cytometry as above. Lysate from a strain expressing YFP with a T7-tag insertion at the same location was used as a negative control. BiaCore studies were carried out by the University of Utah Core Facility, using IMAC-purified YFP-SA-1 with a hexahistidine tag. Streptavidin (Sigma, Cat. No. S4762) was immobilized at different densities at 200, 600 or 2200 RU on a CM5 sensor chip using standard amine coupling. A 2-fold dilution series of nine concentrations of YFP-SA-1 starting from 400 nM were injected in triplicate to obtain the affinity.

Results

Design and construction of a large bacterial display peptide library

To allow the construction of a highly diverse bacterial display peptide library, two mobile loops of OmpA were compared for their ability to display a 15 amino acid epitope (Figure 1). *E. coli* OmpA was chosen as a display scaffold since (i) it is monomeric and can be produced at high levels in the outer membrane under certain conditions, (ii) the structures determined using x-ray crystallography and NMR indicate the presence of flexible extracellular loops (Pautsch and Schulz, 2000; Arora *et al.*, 2001) and (iii) it has been shown to accept loop insertions (Freudl, 1989; Mejare *et al.*, 1998; Etz *et al.*, 2001). Since loops 1 and 4 are thought to be relatively flexible, it was reasoned that they would be less likely to adversely impact structural stability. Consequently, a 15-mer insertional fusion containing the 11 amino acid epitope of T7 gene 10 (T7-tag)

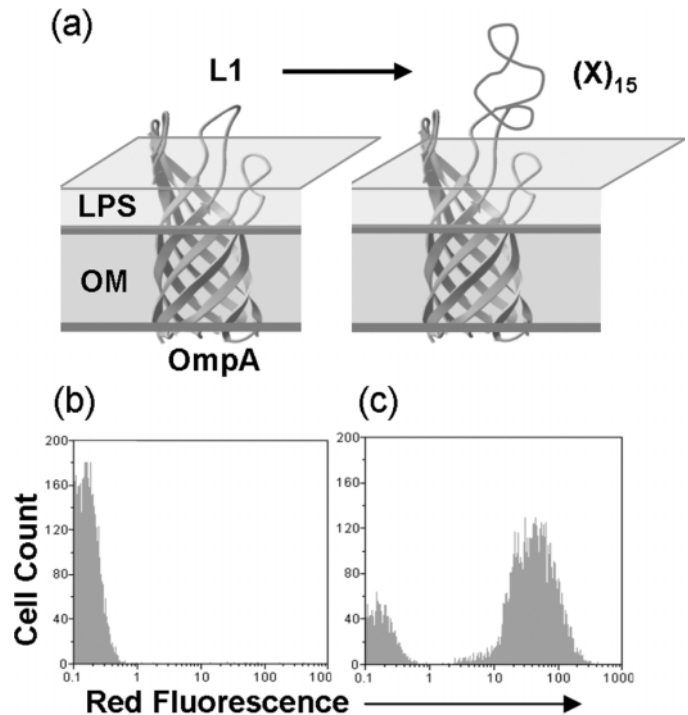


Fig. 1. (a) Schematic representation of display of peptides on the surface of *E. coli* using insertions into the first extracellular loop (L1) of outer membrane (OM) protein A (OmpA). LPS = lipopolysaccharide. (b) Histogram of flow cytometric analysis of clonal population of cells containing plasmid pB33OmpA overexpressing OmpA without any insertions. Cells were induced for 2 h and labeled with 10 nM biotinylated-anti-T7-tag mAb and phycoerythrin-conjugated streptavidin. (c) Cells containing the plasmid pB33OT1 displaying the T7-tag peptide in OmpA loop 1, induced and labeled as in (b).

(MASMTGGQQMG) was made in each loop at positions maximally distant from the cell surface within a sequence region poorly conserved among OmpA homologs. Labeling of whole cells with a biotinylated anti-T7-tag monoclonal antibody (mAb) followed by streptavidin–phycoerythrin (SAPE) demonstrated that both loops were capable of displaying the T7 epitope. Insertions into loop 4 after residue 150 resulted in relatively low-level display, since fluorescence signals were only about 2-fold greater than background cellular autofluorescence. On the other hand, loop 1 epitope insertions after residue 26 (Figure 1) resulted in efficient T7-tag display, with cells exhibiting 300-fold increased fluorescence above background control cells as measured by flow cytometry. Although these experiments were carried out in strain MC1061, which is *ompA*⁺, the over-expression of the engineered OmpA was easily detectable and did not improve in an otherwise isogenic *ompA*⁻ host (data not shown).

In order to allow efficient selection of rare desired cells from a large background of non-target cells, it was necessary to optimize expression and growth conditions. We first determined the effect of plasmid copy number upon cell viability and growth rate after induction of display. The use of a low-copy plasmid utilizing the p15A origin of replication allowed expression without a significant reduction of cell viability. In contrast, an analogous display vector having a pMB1 origin provided high-level expression but resulted in rapid arrest of cell growth shortly after induction (data not shown). Importantly, we utilized a tightly regulatable promoter to prevent loss

of mildly toxic sequences during growth, maintain full library diversity and improve single-round enrichment efficiency (Daugherty *et al.*, 1999). The use of the arabinose inducible promoter from the *araBAD* operon permitted tight repression in the absence of arabinose during library propagation and reproducible induction of surface display of peptide insertions under saturating inducer conditions. High-level display with minimal cell death or growth inhibition (data not shown) was obtained 1–4 h after induction. In subsequent experiments, an induction period of 2 h was typically used before selection or screening to minimize potential toxicity.

For library construction, inserts were chosen to have a length of 15 codons while allowing all possible amino acids (using NNS degenerate codons) at each position. In addition to increasing the physical distance from the cell surface, longer length insert libraries (e.g. 15-mer) offer the advantage of providing more copies of short sequences while allowing for longer binding motifs to emerge. The resulting library of 5×10^{10} independent transformants provides a sparse sampling of the sequence space available to a 15-mer, but would be expected to contain all possible 7-mer sequences (>99% confidence).

Magnetic selection and FACS screening for high-affinity protein binding peptides

To test the library fitness for binding arbitrary protein targets, five unrelated target proteins were chosen: a monoclonal IgG antibody binding to a known epitope (anti-T7-tag mAb), human serum albumin (HSA), human C-reactive protein (CRP), streptavidin and HIV-1 GP120. For each of five protein targets tested, magnetic selection allowed the enrichment of clones displaying protein-binding peptides from non-binding clones. Abundant streptavidin-binding peptides were first depleted from the library using one round of magnetic selection with streptavidin functionalized magnetic particles. The remaining cells were incubated with biotinylated target proteins and subsequently with biotin-binding magnetic particles to capture cells with bound target protein. Each cycle of magnetic selection was followed by overnight growth to amplify the selected population. Flow cytometry was used to monitor the progress of magnetic selection using as fluorescent probes either streptavidin–phycoerythrin or fluorescently conjugated anti-biotin antibodies (Figure 2). One or two rounds of magnetic selection were sufficient to enrich a population containing a significant fraction of binders for each of the five targets tested. In the case of selection for anti-T7-tag mAb binding, one cycle was sufficient to enrich binding peptides to nearly 50% of the population from an initial frequency of about 1 in 50 000—a single round enrichment of 25 000-fold. The initial frequency of T7-tag mAb binding clones indicated that $\sim 10^6$ unique peptide sequences were capable of binding when using a target concentration of 10 nM.

The frequency of target protein binding peptides within the library population was found to vary significantly among different targets, suggesting that the library was more ‘fit’ for binding some antigens. The highest frequency of target binding cells was observed with the anti-T7-tag mAb. Similarly, a high initial frequency of positive cells was observed when using streptavidin and CRP as targets. On the other hand, a reduced frequency ($< 1:10^6$) of GP120-binding clones was observed in the unselected library, possibly reflecting the heavily glycosylated surface of this target. The frequency of target binding clones in the library was consistent with the probability

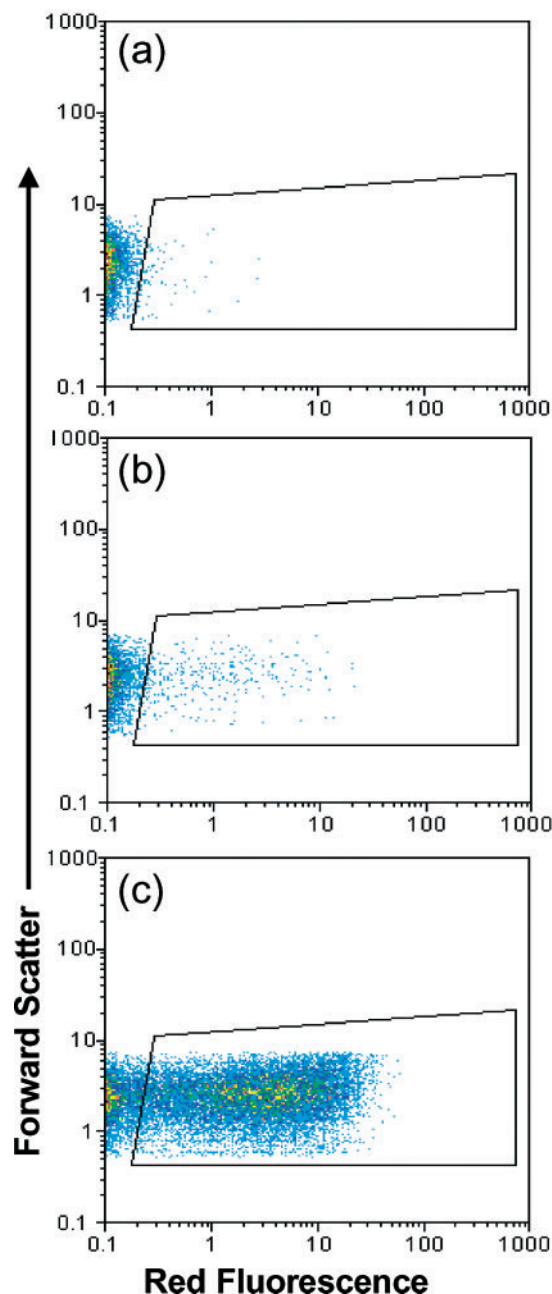


Fig. 2. Enrichment of C-reactive protein binding peptides as measured using flow cytometry. Induced cells were labeled with 10 nM biotin-CRP and 6 nM SAPE. (a) Unselected library population, (b) following two rounds of magnetic selection and (c) following two rounds of magnetic selection and one round of FACS.

of occurrence of certain critical motifs involved in molecular recognition. In the anti-T7-tag mAb selection, for example, the initial frequency of binding clones ($2:10^5$) is consistent with the expected frequency of the identified ‘core’ motif MxP (x/–)QQ of $\sim 2:10^5$. Similarly, for the CRP selection, the consensus motif, NxRGF, is expected to occur at a frequency of $\sim 5:10^5$. Hence cytometric analysis of the library populations prior to screening provided useful statistical information regarding the frequency of target protein-binding peptides.

Cell sorting instrumentation was applied as a quantitative library screening tool to isolate the highest affinity clones from

the magnetically enriched populations (Figure 2), estimated to represent 10^5 – 10^7 unique sequences. Two fundamentally different approaches were applied for quantitative screening, as described previously (Boder and Wittrup, 1998; Daugherty *et al.*, 2000a), on the basis of either equilibrium binding affinity (equilibrium screen) or dissociation rate constants (kinetic screen). In most cases, appropriate antigen concentrations for equilibrium screening were determined by flow cytometric analysis of $\sim 10^6$ clones after labeling with a range of different target protein concentrations. For equilibrium screening, cell populations were labeled with limiting concentrations of the target proteins and all cells exhibiting fluorescence intensities above background autofluorescence were collected (Figure 2). Thus, the ligand concentration and not the lower intensity limit of the sort window was used as the criterion for acceptance. In the case of the streptavidin selection, kinetic screening was performed using free biotin as a competitor. In the absence of biotin, streptavidin-binding peptides exhibited substantially slower dissociation rates, probably owing to rebinding effects. The apparent binding affinities of isolated clones were generally predictable from the antigen concentrations used for screening. Typically, the apparent dissociation constants were roughly 10-fold higher than the ligand concentration used for screening (Table I).

Characterization of selected peptides

Consensus sequences were readily apparent for each of the target proteins after two to three rounds of magnetic selection and one or two rounds of FACS (Table I, Figure 3). One anti-T7-tag mAb binder (Figure 3) possessed seven identities and one similarity with the wild-type T7-tag sequence. Considering codon usage, such a clone would be expected to occur at a frequency of <1 in 10^{10} . Consensus sequences for HSA and for HIV-1 GP120 binding included several hydrophobic residues; a similar abundance of aromatic residues has been observed previously using random libraries on alternative scaffolds (Binz *et al.*, 2004). A high frequency of clones also possessed one or two cysteine residues. In some cases, FACS resulted in enrichment and isolation of putatively cyclic peptides incorporating the consensus sequence. For example, the highest affinity CRP binding clone (CRP-1, Table I) from stringent FACS screening possessed the consensus NxRGF flanked by cysteines—CNDRGFNC. Residues outside of the cyclic constrained consensus also contributed to improved function since two streptavidin-binding clones with identical disulfide loops (CQNV) possessed dissociation rate constants differing by 4-fold (Figure 4b). The overall length of the deduced consensus sequences spanned as many as 10 or 11 residues for anti-T7-tag mAb (SMGPQQM(x/–)AW) or CRP (IxNxRGFxxxV), suggesting that libraries with shorter inserts would not have yielded peptides with comparable affinities or provided equivalent epitope mapping information.

The apparent binding affinities of a subset of the selected peptides were determined using flow cytometric analysis. This method has been shown to enable reliable estimation of both K_D and k_{diss} values (Daugherty *et al.*, 1998). Also, importantly, the relative affinity ranking of selected clones obtained using flow cytometry has been shown to be equivalent to that determined using surface plasmon resonance (Daugherty *et al.*, 1998; Feldhaus *et al.*, 2003). Apparent equilibrium dissociation constants (Figure 4a) were typically in the low nanomolar range ($K_D = 1$ – 10 nM) (Table I), as determined using

Table I. Peptide sequences of isolated clones binding to CRP, streptavidin, HSA and GP120

Clone (K_D)	Sequence	Target concentration (nM) ^a
C-reactive protein		
CRP-1 (1 nM)	EWAC NDRGF NCQLQR	0.1
CRP-2 (3 nM)	FPT YNQRGF ITLASP	0.1
CRP-3	HMRW NTRGFL YPAMS	1 ^b
CRP-4	RY IMNHRGF YIFVPR	1 ^b
CRP-5	VRTW NDRGF QQSVDR	1 ^b
CRP-6 (8 nM)	MT FNSRGFL SLMSSG	10
CRP-7	LMNWRGFM VPRESPK	10
CRP-8	WTK LKNSRGF ELQLD	10
CRP-9	PYL NARGF SVTREQI	10
Consensus	I N RGF	
CRP-10 (5 nM)	YPPRFQ YYR FYYRGP	0.1
CRP-11	TDFLS YYR VYRTPDQ	1 ^b
CRP-12	TFMPS YYR SWGPPPT	1 ^b
CRP-13	TTCK Y LSRWKDL	10
Consensus	SYRYS	
Streptavidin		
SA-1 (10 nM)	RLE ICQ NVCYYLGLT	6 ^c
SA-2 (8 nM)	ICS YVMY TCFLRVY	6 ^d
SA-3 (4 nM)	TVL ICM NICWTGETQ	6 ^d
SA-4	VTSL CM NVCYSLTTY	6 ^d
SA-5	YW VC MNVCMYYTARQ	6 ^d
SA-6	LPVWC V MHVCLTSSR	6 ^d
SA-7	NEW Y CQNVCEMPHS	6
SA-8	IMME CF YVCTIANTQ	6
SA-9	TWV QC TMVYGMSTT	6
SA-10	SIT IC WYTCMVQKTA	6
SA-11	ADT IC WYVCTISVHA	6
Consensus	ICMNV C	
Serum albumin		
HSA-1	NP FC SWYRWR W CTK	100
HSA-2	RHL YC -WT-WR-W CH FKD	100
Consensus	C W WR W	
HSA-3	SYI ST WLN FL FCG QS	100
HSA-4	NN Y SA WL R CL LR AYS	100
Consensus	S WL L S	
HIV-1 GP120		
GP120-1	GDT WV WYCW W TRSI	15
GP120-2	WV CTW NY WTRV W TWCL	15
Consensus	WV WYTR	
GP120-3	P WC W M WT K GRWY Y VVA	0.6
GP120-4	Q I Q WC W V NH R WSP V	0.6
GP120-5	WVAG Y W WC W S VMY RS	15
GP120-6	T WT W CR W R NY I W Q L ST	15
GP120-7	Q EW R Q L TR W CV Q IK	15
GP120-8	Q T AT V S Y W CY W W W K V	15
Consensus	WCW K	

Sequences were aligned using the Clustal W algorithm and consensus residues are shown below each group. For selected clones, the apparent whole cell K_D as measured by flow cytometry is indicated.

^aThe concentration used for the final selection.

^bDissociation in the presence of 100 nM unbiotinylated CRP for 20 min.

^cDissociation in the presence of 1 μ M biotin for 2.5 h.

^dDissociation in the presence of 1 μ M biotin for 6 min.

fluorescently conjugated CRP and SA. Similarly, the best GP120 binding clones exhibited high fluorescence after incubation with 10 nM GP120, indicating that the K_D is <10 nM (data not shown). Apparent dissociation rate constants (k_{diss}) were determined for streptavidin using 1–2 μ M biotin as a competitor to prevent rebinding. Rate constants were found to range from 0.01 s⁻¹ after two cycles of MACS and one cycle of FACS (clones SA-7 to SA-11) to 0.001 s⁻¹ after an additional round of screening (clones SA-1 to SA-6). Although we cannot rule out potential avidity effects for surface

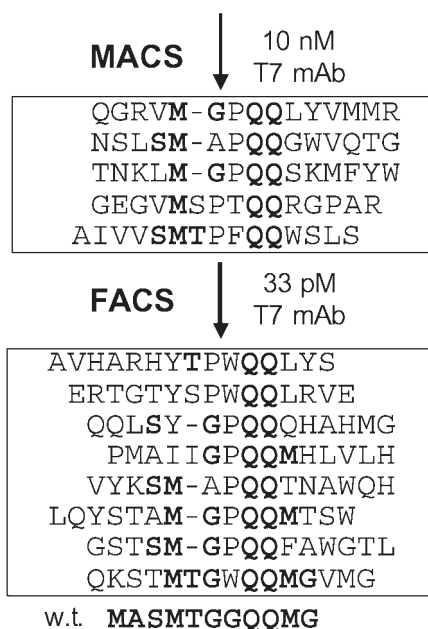


Fig. 3. Representative sequences of surface displayed high-affinity anti-T7-tag mAb binding peptides isolated using magnetic selection and FACS with differing target ligand concentrations. Two rounds of MACS were performed at 10 nM antibody concentration, followed by FACS using 33 pM antibody. Bold residues indicate positions of identity with the wild-type T7-tag epitope, shown at the bottom, against which the antibody was raised.

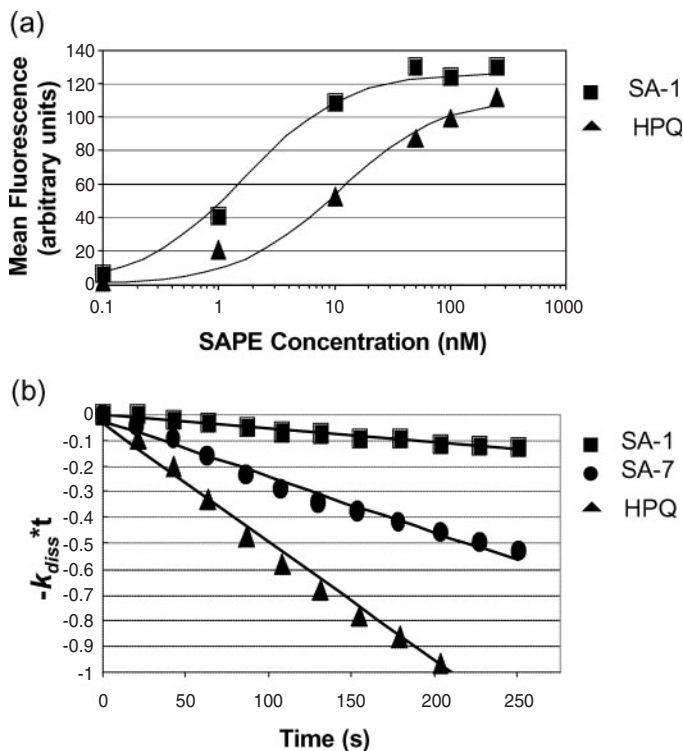


Fig. 4. Measurement of the binding affinities of cell surface-displayed streptavidin-binding peptides using flow cytometry and biotin as a competitor as described in the text. (a) Determination of apparent equilibrium dissociation constants (K_D) and (b) dissociation rate constants (k_{diss}) of cell surface-displayed peptides. Peptide sequences of clones SA-1 and SA-7 are listed in Table I. Clone HPQ contains the sequence SAECHPQPPCIEGR, previously identified by phage display (Giebel *et al.*, 1995), inserted into OmpA loop 1 for comparison.

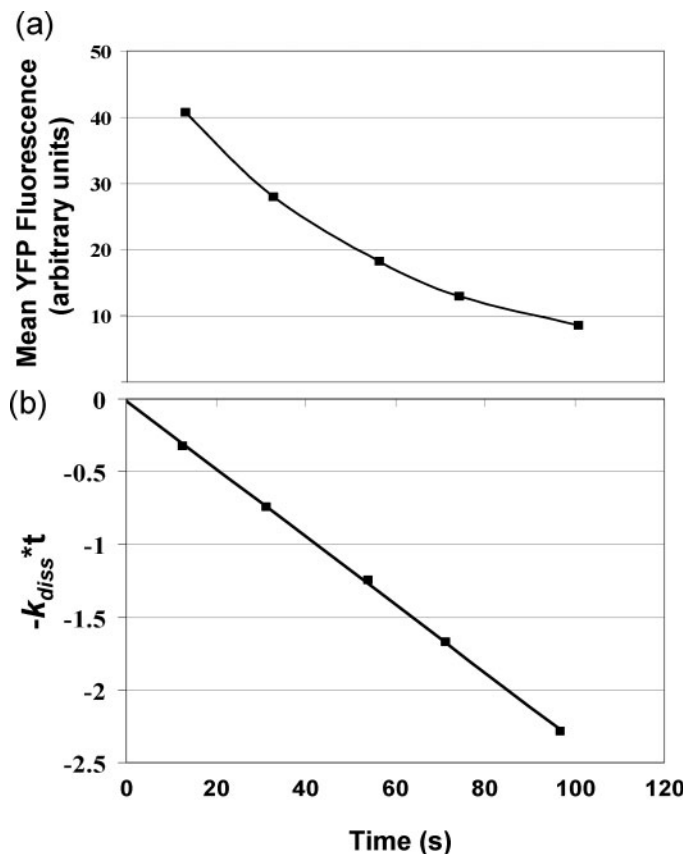


Fig. 5. Measurement of the dissociation rate of streptavidin binding peptide SA-1 constrained in a loop of YFP. Flow cytometry was used to measure the fluorescence of YFP bound to streptavidin-coated 1 μ m beads after the addition of biotin as a competitor.

displayed peptides binding to multimeric target proteins, the dissociation kinetics show excellent agreement with a single exponential decay (Figure 4b), suggesting a 1:1 binding stoichiometry. Furthermore, the apparent equilibrium dissociation constant of the best clone ($K_D = 4$ nM) is in qualitative agreement with the observed k_{diss} of 0.001 s^{-1} , assuming a k_{assoc} value of $\sim 5 \times 10^5$ $M^{-1} s^{-1}$ (Giebel *et al.*, 1995).

To assess the functional contribution of the OmpA scaffold to high-affinity binding, the 15-residue streptavidin binding peptide (SA-1) was genetically inserted into the yellow fluorescent protein immediately following residue Y145 (Baird *et al.*, 1999). The fluorescent protein-peptide fusion was expressed for affinity studies in soluble form in an engineered *E. coli* strain possessing an oxidizing cytoplasm (Besette *et al.*, 1999). This fusion protein retained strong yellow fluorescence, comparable to that of wild-type YFP, and exhibited strong binding to streptavidin-coated polymeric microbeads. Determined using flow cytometry, the dissociation rate constant of the streptavidin binding fluorescent protein was 0.02 s^{-1} (Figure 5). The YFP-SA-1 insertional fusion exhibited an equilibrium dissociation constant of $\sim 5 \times 10^{-7}$ M, as determined using surface plasmon resonance with 200 RU of immobilized streptavidin. Collectively, these data suggest that peptide sequences identified using bacterial display can retain submicromolar affinity, even in the context of scaffolds unrelated to that used for screening.

Peptides that include a simple consensus motif of the amino acids HPQ have been identified in multiple phage display and mRNA display selections against streptavidin

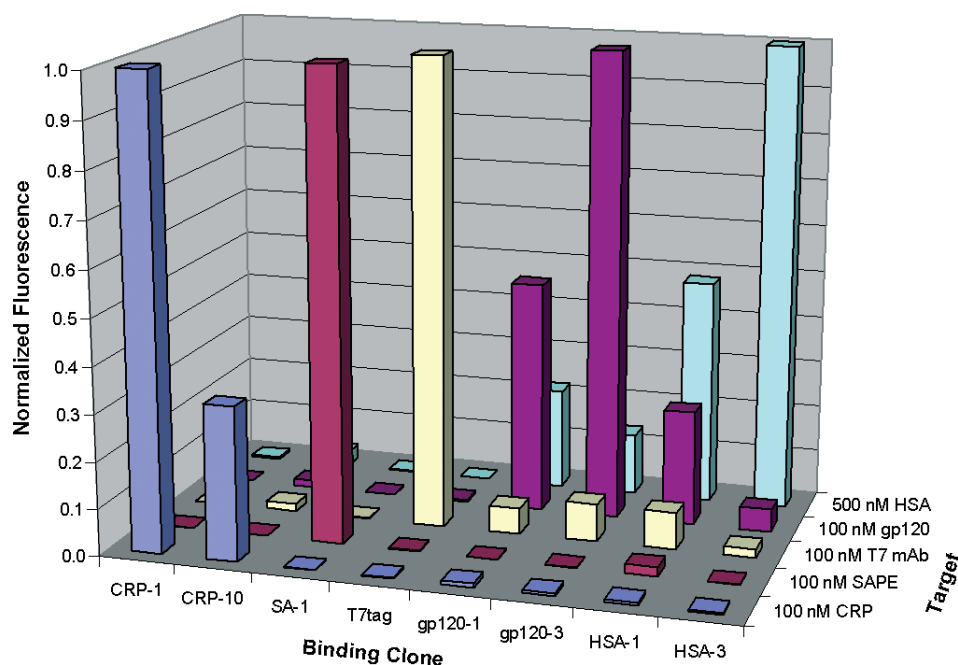


Fig. 6. Specificity of isolated clones for their respective target proteins. Binding clones (see Table I for sequences) were labeled with each of the five target ligands and the resulting fluorescence signals were measured by flow cytometry. The mean fluorescence over background autofluorescence was normalized to the brightest binding clone for each target ligand. For detection, CRP was conjugated with Alexa488. HSA, T7-tag mAb and GP120 were biotinylated and secondary labeling was performed with 10 nM SAPE. For clone SA-1, secondary labeling was carried out with 1 nM anti-biotin mAb PE.

(Giebel *et al.*, 1995); we determined whether these lower affinity sequences previously identified using phage display would provide detectable affinity in the bacterial display format. To allow comparison of the phage and bacterial display peptides, a bacterial display clone was constructed with the insertion SAEHPQGPCIEGR (Giebel *et al.*, 1995), and K_D and k_{diss} were measured in whole cell assays (Figure 4a and b). The phage display-derived peptide containing the disulfide constrained HPQ motif was efficiently displayed on bacteria and possessed a dissociation rate (k_{diss}) 20-fold faster than that of the best peptide isolated using bacterial display (clone SA-1, Figure 4b). In qualitative agreement with this result, the apparent K_D of the cyclic HPQ clone was 5-fold higher than that of the streptavidin binding clone SA-1, confirming the improved affinities of peptides isolated using bacterial display relative to those isolated using phage display.

To investigate the specificity of binding interactions, selected clones from each target selection consensus group, as well as the T7-tag control peptide, were screened for cross-reactive binding to the other four targets (Figure 6). Each target was applied at a higher concentration than that used in the actual library screening process. Clones binding to CRP and streptavidin displayed remarkable specificity in this limited assay. On the other hand, moderate cross-reactivity was observed among HSA and GP120 binders, and also with murine IgG. Importantly, selection and screening steps were not designed to favor specificity. With appropriate blocking conditions or subtractive selections, specificity screening could easily be incorporated into the methodology.

Discussion

Given the substantial potential of bacterial display to simplify ligand isolation while allowing quantitative library analysis

and screening, we sought to develop a simple and robust bacterial display peptide library methodology overcoming technical limitations associated with previously reported bacterial display libraries (Lee *et al.*, 2003). The significance of the methodology described here is illustrated by the fact that it provides the first demonstrated approach permitting routine isolation of high-affinity peptide ligands for arbitrary targets. Protein-binding peptides displayed as loop insertions into the *E.coli* outer membrane protein OmpA were isolated rapidly from a large library using magnetic selection and FACS and typically possessed high affinity. The quantitative capabilities of FACS in library screening allowed fine affinity discrimination and, consequently, identification of unusually strong consensus motifs. When applicable, bacterial display peptide libraries therefore offer advantages in terms of speed and simplicity in peptide ligand isolation.

The emergence of strong consensus sequences while maintaining diversity in the selected populations likely can be attributed to several features of bacterial display. First, since few steps are required for each enrichment cycle, and bacterial display library populations are self-amplifying, there are limited opportunities for clonal biases to arise from steps other than the affinity selection (Zahn *et al.*, 1999; Smith and Fernandez, 2004). Second, the size of the library (5×10^{10}) employed in this work is ~ 10 -fold larger than typical phage display peptide libraries (Lowman, 1997), with some exceptions (Fisch *et al.*, 1996; Deshayes *et al.*, 2002). To our knowledge, the library constructed here is significantly larger than any other reported cell surface display library. Furthermore, the relatively long 15-mer peptide insertions may increase the frequency at which high-affinity binders occur relative to more typical 6–12-mer libraries. Longer insert libraries provide opportunities for longer consensus motifs and secondary structures to emerge (Nakamura *et al.*, 2002).

Table II. Isolated sequences possessing putative disulfide-constrained loops

Streptavidin	TVL I CM N ICWTGETQ 3
	LPV V CV M H V CLTSSR 4
C-Reactive Protein	TTCK Y YL S CRWRKDL 5
	EWAC N DR G F N CQLQR 6
Human Serum Albumin	RHLY C WT R W C HFKD 5
	N P F C SW R WR N W C TK 8
Anti-T7-tag MAb	T C LR G P Q Q T R W C I SR 9
HIV-1 GP120	W V CT W NY W TR V T W CL 10

Finally, as has been demonstrated, cell surface display in conjunction with FACS provides fine discrimination of clonal affinity (Daugherty *et al.*, 1998; Van Antwerp and Wittrup, 2000) and quantitative separations that take advantage of this sensitivity. In the present work, the fine affinity discrimination provided by FACS was important for the isolation of the best sequences binding to streptavidin, CRP and anti-T7-tag mAb.

Remarkably, bacterial display routinely permitted the identification of beneficial cysteine placements to form putative disulfide constrained loops conferring high binding affinity without explicit library design. This feature alleviates the need to construct and screen ≥ 20 different libraries (Deshayes *et al.*, 2002; Nakamura *et al.*, 2002) and removes critical assumptions that have limited the affinities of isolated ligands in earlier studies (Giebel *et al.*, 1995). For example, bacterial display selections for binding to streptavidin yielded a strong preference for CxxVC ligands in all rounds of selection. However, only a single report has described the generation and screening of a CxxxC-type library using phage display technology (Nakamura *et al.*, 2002). Putative disulfide loops were present in peptides binding to all five of the targets tested despite a significantly reduced probability of occurrence (Table II). While a strong consensus sequence of NxRGF was present in clones from the selection for CRP binding, FACS screening of the enriched pool resulted in the isolation of a peptide (CRP-1) having the identical consensus, but flanked by two cysteines (EWA-CNDRGFNC-QLQR). Although a handful of previous studies have reported the identification of peptides with non-designed disulfide bridges (Sahu *et al.*, 1996; Lu *et al.*, 2003), linear libraries most often result in non-cyclic peptides. The fact that cyclic peptides were found among the highest affinity clones for all of the targets tested in this report further underscores the importance of ligand rigidity in high-affinity binding. The present work illustrates that a single library of sufficient size and quality allows the routine isolation of high-affinity cyclic peptides. Even so, intentional library design with disulfide loops (Nakamura *et al.*, 2002),

would be expected to improve further the quality of selected clones since a larger fraction of the 'cyclic peptide sequence space' can be explored for a given library size.

The bacterial display peptide library methodology reported here offers some important advantages over available methods and, therefore, should help to bolster renewed interest in peptides for a variety of applications. Bacterial display provides an approach to generate renewable whole cell binding reagents in non-specialized laboratories since this method is technically accessible and libraries are reusable. This approach has already proven useful for selecting cell-specific binding peptides and for performing diagnostic assays using flow cytometry and fluorescence microscopy (unpublished data). Furthermore, cell surface display libraries can be used for parallel or multiplex ligand isolation (Feldhaus *et al.*, 2003), and clones can be processed with efficient single-cell deposition units present on many cell sorters. Consequently, significant potential exists for proteomic applications, including proteome-wide ligand screens for protein-detecting array development (Kodadek, 2001). Finally, the ability to isolate high-affinity peptides and subsequently to affinity-mature them using sensitive screening instrumentation may provide new prospects for the development of improved peptide therapeutics.

References

- Arap,W., Pasqualini,R. and Ruoslahti,E. (1998) *Science*, **279**, 377–380.
- Arora,A. *et al.* (2001) *Nat. Struct. Biol.*, **8**, 334–338.
- Baird,G.S., Zacharias,D.A. and Tsien,R.Y. (1999) *Proc. Natl Acad. Sci. USA*, **96**, 11241–11246.
- Bessette,P.H. *et al.* (1999) *Proc. Natl Acad. Sci. USA*, **96**, 13703–13708.
- Binz,H.K. *et al.* (2004) *Nat. Biotechnol.*, **22**, 575–582.
- Boder,E.T. and Wittrup,K.D. (1997) *Nat. Biotechnol.*, **15**, 553–557.
- Boder,E.T. and Wittrup,K.D. (1998) *Biotechnol. Prog.*, **14**, 55–62.
- Boder,E.T., Midelfort,K.S. and Wittrup,K.D. (2000) *Proc. Natl Acad. Sci. USA*, **97**, 10701–10705.
- Brown,S. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 8651–8655.
- Bupp,K. and Roth,M.J. (2002) *Mol. Ther.*, **5**, 329–335.
- Camaj,P. *et al.* (2001) *Biol. Chem.*, **382**, 1669–1677.
- Casadaban,M.J. and Cohen,S.N. (1980) *J. Mol. Biol.*, **138**, 179–207.
- Christmann,A. *et al.* (1999) *Protein Eng.*, **12**, 797–806.
- Clackson,T. and Wells,J.A. (1994) *Trends Biotechnol.*, **12**, 173–184.
- Daugherty,P.S. *et al.* (1998) *Protein Eng.*, **11**, 825–832.
- Daugherty,P.S. *et al.* (1999) *Protein Eng.*, **12**, 613–621.
- Daugherty,P.S., Iverson,B.L. and Georgiou,G. (2000a) *J. Immunol. Methods*, **243**, 211–227.
- Daugherty,P.S. *et al.* (2000b) *Proc. Natl Acad. Sci. USA*, **97**, 2029–2034.
- Deshayes,K. *et al.* (2002) *Chem. Biol.*, **9**, 495–505.
- Etz,H. *et al.* (2001) *J. Bacteriol.*, **183**, 6924–6935.
- Feldhaus,M.J. *et al.* (2003) *Nat. Biotechnol.*, **21**, 163–170.
- Fisch,I. *et al.* (1996) *Proc. Natl Acad. Sci. USA*, **93**, 7761–7766.
- Francisco,J.A. *et al.* (1993) *Proc. Natl Acad. Sci. USA*, **90**, 10444–10448.
- Freudl,R. (1989) *Gene*, **82**, 229–236.
- Georgiou,G. (2000) *Adv. Protein Chem.*, **55**, 293–315.
- Georgiou,G. *et al.* (1997) *Nat. Biotechnol.*, **15**, 29–34.
- Giebel,L.B. *et al.* (1995) *Biochemistry*, **34**, 15430–15435.
- Guzman,L. *et al.* (1995) *J. Bacteriol.*, **177**, 4121–4130.
- Klemm,P. and Schembri,M.A. (2000) *Microbiology*, **146**, 3025–3032.
- Kodadek,T. (2001) *Chem. Biol.*, **8**, 105–115.
- Lee,S.W. *et al.* (2002) *Science*, **296**, 892–895.
- Lee,S.Y., Choi,J.H. and Xu,Z. (2003) *Trends Biotechnol.*, **21**, 45–52.
- Lowman,H.B. (1997) *Annu. Rev. Biophys. Biomol. Struct.*, **26**, 401–424.
- Lu,D. *et al.* (2003) *J. Biol. Chem.*, **12**, 12.
- Lu,Z. *et al.* (1995) *Biotechnology (NY)*, **13**, 366–372.
- Mejare,M., Ljung,S. and Bulow,L. (1998) *Protein Eng.*, **11**, 489–494.
- Muller,O.J. *et al.* (2003) *Nat. Biotechnol.*, **3**, 3.
- Nakamura,G.R. *et al.* (2002) *Proc. Natl Acad. Sci. USA*, **99**, 1303–1308.
- Nixon,A.E. (2002) *Curr. Pharm. Biotechnol.*, **3**, 1–12.
- Norris,J.D. *et al.* (1999) *Science*, **285**, 744–746.
- Olsen,M.J. *et al.* (2003) *Methods Mol. Biol.*, **230**, 329–342.
- Pautsch,A. and Schulz,G.E. (2000) *J. Mol. Biol.*, **298**, 273–282.

- Sahu,A., Kay,B.K. and Lambris,J.D. (1996) *J. Immunol.*, **157**, 884–891.
Scott,J.K. and Smith,G.P. (1990) *Science*, **249**, 386–390.
Shusta,E.V., Van Antwerp,J. and Wittrup,K.D. (1999) *Curr. Opin. Biotechnol.*, **10**, 117–122.
Sidhu,S.S. (2000) *Curr. Opin. Biotechnol.*, **11**, 610–616.
Smith,G.P. and Fernandez,A.M. (2004) *Biotechniques*, **36**, 610–618.
Tripp,B.C. *et al.* (2001) *Protein Eng.*, **14**, 367–377.
Van Antwerp,J.J. and Wittrup,K.D. (2000) *Biotechnol. Prog.*, **16**, 31–37.
Whaley,S.R. *et al.* (2000) *Nature*, **405**, 665–668.
Wilson,D.S., Keefe,A.D. and Szostak,J.W. (2001) *Proc. Natl Acad. Sci. USA*, **98**, 3750–3755.
Zahn,G., Skerra,A. and Hohne,W. (1999) *Protein Eng.*, **12**, 1031–1034.

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