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DNA-ENCODED PEPTIDE LIBRARIES AND DRUG DISCOVERY

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Summary

Over the past decade, several methods have been developed for the construction of DNA-encoded peptide libraries. The common principle behind all these methods is the establishment of a physical linkage between a displayed peptide and its encoding DNA. Vast libraries can be generated, binding peptides can be isolated with simple selections, and the sequences of selected peptides can be rapidly determined from the sequence of the linked DNA. As a result, DNA-encoded libraries can provide specific ligands for essentially any protein. These ligands can be used to determine the natural binding specificities of protein–protein interactions, and this information can be used to identify natural binding partners or to aid the design of organic mimics. Binding peptides can also be used for target validation and the development of high-throughput screens for small-molecule libraries. Finally, binding peptides themselves could prove useful as drugs.

1. Introduction

Over the past decade, several methods have been developed for the construction of DNA-encoded, combinatorial peptide libraries. These technologies depend on one basic principle: the establishment of a physical linkage between a displayed polypeptide and the DNA sequence that encodes it. Simple molecular biology techniques can be used to first construct combinatorial libraries of vast size ($>10^{12}$) and subsequently to amplify library pools or individual members. These libraries can be used to isolate peptides that bind with high specificity and affinity to virtually any protein of interest. Most importantly, the sequences of selected peptides can be determined by rapid and efficient sequencing of the encoding DNA.

In this chapter, we describe the various DNA-encoded systems for polypeptide display. We also outline the main applications for combinatorial peptide libraries, and we describe relevant examples from the recent literature. It is a testament to the breadth of this field that no single review can hope to be comprehensive. Thus, in each section we direct the reader to more focused reviews in particular areas of interest.

2. Methods for DNA-Encoded Peptide Display

Several methods have been used to link polypeptides to their encoding DNA. In phage display, peptides fused to viral coat proteins are displayed on the surfaces of bacteriophage particles that also encapsulate the encoding DNA.

"Peptides on plasmids" is an alternative method in which peptides are fused to a DNA-binding protein that binds specifically to plasmid DNA. A third approach is provided by microorganism surface display systems in which peptides are displayed directly on the surfaces of yeast or bacterial cells. Finally, "ribosome display" methods use *in vitro* transcription and translation reactions to couple mRNA molecules to their encoded polypeptides.

The displayed libraries can be used in binding selections with immobilized ligands (Fig. 1). Selective pressure is applied to pooled library members to enrich for displayed peptides with desired binding specificities and affinities. The enriched pool can be amplified and passed through additional rounds of selection, or at any point, selectants can be grown as individual clones to produce DNA for sequence analysis. In the following sections, we describe the different display methods and discuss the advantages and disadvantages of each approach.

A. Phage Display

The concept of phage display was first demonstrated by George Smith when he showed that peptides could be functionally displayed on the surface of M13 bacteriophage particles that also encapsulated the encoding DNA (Smith, 1985). Phage display remains the most widely used system for DNA-encoded polypeptide display, and M13 bacteriophage remains the predominant phage display scaffold. However, display systems based on other bacteriophage have also been developed, and these may offer some advantages for specialized applications. While the practical limit for phage-displayed library diversities was long believed to be about 10^{10} (Roberts, 1999; Schaffitzel *et al.,* 1999), recent refinements of established methods (Dower *et al.,* 1988) have extended the limit to about 10^{12} for M13 display (Sidhu *et al.,* 2000). In addition to the peptide display applications described here, phage display has been used in many protein engineering studies (reviewed by Clackson and Wells, 1994; Smith and Petrenko, 1997; Johnsson and Ge, 1999), and antibody display in particular has had an enormous impact on biotechnology (reviewed by Griffiths and Duncan, 1998; Vaughan *et al.,* 1998; Hoogenboom *et al.,* 1998; Dall'Acqua and Carter, 1998).

1. M13 Phage Display

M13 is a filamentous bacteriophage that infects *Escherichia coli* in a nonlytic life cycle. Both the viral assembly process and the structure of the assembled phage particle have been extensively studied (reviewed by Webster, 1996; Marvin, 1998). M13 phage assembly occurs at the host cell membrane where viral DNA is extruded through a pore complex and concomitantly surrounded by membrane-associated coat proteins. The assembled phage particle is approximately 1 μm in length but less than 10 nm in diameter, and it consists of a single-stranded, closed circular DNA core encapsulated in a coat composed of five different coat proteins (Fig. 2). The length of the particle is covered by about 3000 copies of the major coat protein (protein-8, P8). One end of the particle is capped by approximately five copies each of two minor coat proteins (protein-7 and protein-9, P7 and P9), and the other end is similarly capped by two other minor coat proteins (protein-3 and protein-6; P3 and P6). While all five coat proteins contribute to the structural integrity of the phage particle, P3 is also responsible for host cell recognition and infection. Consequently, it is the largest and most complex of the coat proteins, containing 406 residues and 3 distinct domains.
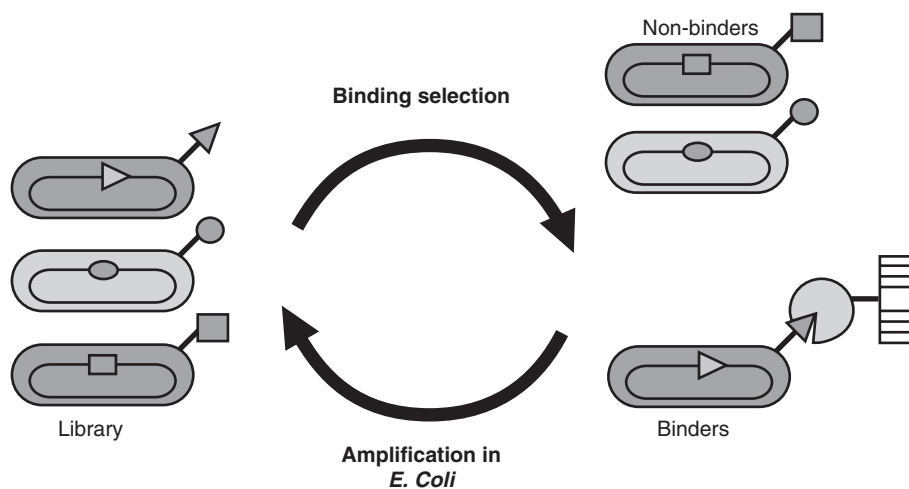

FIGURE 1 Isolating specific ligands from phage-displayed peptide libraries. Peptides are displayed on the phage surface while the encoding DNA is encapsulated in the phage particle. A library of peptide phage is exposed to an immobilized target. Nonbinding phage are removed by washing while bound phage are retained. Bound phage are then eluted and amplified by infection of an *E. coli* host. After several rounds of selection, the sequences of individual binding peptides can be determined by sequencing the cognate DNA. (See Section 2 for additional details.)
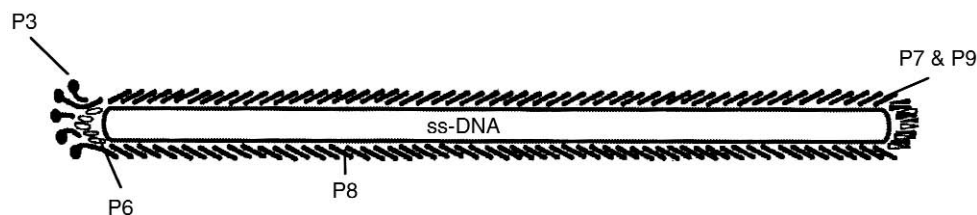
FIGURE 2 Diagram of an M13 phage particle. The single-stranded DNA genome is encapsulated in a coat composed primarily of P8, the major coat protein, which surrounds the length of the particle. One end of the particle is capped by five copies each of P7 and P9, whereas the other end is similarly capped by P3 and P6. Polypeptides can be displayed as fusions to the N terminus of P3, P8, P7, or P9. Alternatively, polypeptides have also been displayed as C-terminal fusions with P6 or P8. (See Section 2.A.1 for additional details.)

M13 phage has long been used as a versatile cloning vector because even large DNA inserts are readily tolerated by the viral genome (Sambrook *et al.,* 1989). An increase in the genome size is accommodated by a corresponding increase in the phage particle length and a concomitant increase in the number of P8 molecules. Phage display extends from the observation that foreign DNA fused in frame to a coat protein gene can result in the display of foreign peptides on the phage surface (Smith, 1985). Each of the five M13 coat proteins has been used for phage display (Fig. 2). Initial examples of phage display involved fusions to the N terminus of P3 (Scott and Smith, 1990; Devlin *et al.,* 1991; Cwirla *et al.,* 1991). Shortly thereafter, the viability of N-terminal P8 fusions was also demonstrated (Greenwood *et al.,* 1991; Felici *et al.,* 1991). More recently, the display of polypeptides fused to the C terminus of P6 (Jespers *et al.,* 1995) or P8 (Fuh *et al.,* 2000) has also been reported. Finally, it has been shown that fusions to the N terminus of P7 or P9 also result in display (Gao *et al.,* 1999).

The different display formats are useful for different applications. Because there are several thousand copies of P8 in each virion, N-terminal peptide fusions usually result in highly polyvalent display (Greenwood *et al.,* 1991). In contrast, fusions to one of the minor coat proteins usually result in lower valency or even monovalent display in which the average display level is actually less than one polypeptide per phage particle (Bass *et al.,* 1990). These high- and low-valency display formats have complementary properties that have been exploited in the selection and affinity maturation of binding peptides (Sidhu *et al.,* 2000). A large and diverse naïve peptide library is first presented in a polyvalent format on P8. Multivalent binding produces an avidity or "chelating" effect that allows for the isolation of low-affinity peptides with affinities in the high micromolar range. These low-affinity leads are then transferred to a low-valency P3 format where they can be matured to higher affinity through the introduction of mutations and additional rounds of affinity selection. C-terminal P6 and P8 display formats have also proven useful in applications not suited to N-terminal display, including the display of cDNA libraries (Jespers *et al.,* 1995)

and studies of protein–protein interactions that involve free C termini (Fuh *et al.,* 2000).

Early M13 phage display vectors used single-gene systems in which peptides were fused to a coat protein in the viral genome (Scott and Smith, 1990; Greenwood *et al.,* 1991). These systems were limited by the fact that fusions could not be displayed if they compromised coat protein function. In particular, the display of large proteins was severely limited (Iannolo *et al.,* 1995). The introduction of two-gene phagemid systems solved the problem (Bass *et al.,* 1990; Lowman *et al.,* 1991). In such systems, peptides are fused to a coat protein encoded by the phagemid vector. Phagemid DNA can be packaged into phage particles by using a helper phage that supplies all the proteins necessary for phage assembly, including wild-type copies of all the coat proteins. The resulting phage particles contain predominantly wild-type coat proteins from the helper phage, but they also contain some copies of the phagemid-encoded coat protein. Thus, peptide display is achieved by incorporation of the phagemid-encoded coat protein, while the deleterious effects of displayed peptides are attenuated by the predominance of wild-type coat protein from the helper phage.

These advances in library construction methods, vector design, and display formats have made M13 phage an extremely robust vehicle for peptide and protein display. In addition, the long history of M13-based vectors in molecular biology has made it very easy for many laboratories to adopt the system. As a result, the majority of published DNA-encoded library studies (and most of the examples in this chapter) have used M13 phage display.

2. Other Phage Display Systems

The success of M13 phage display has prompted the development of alternative display systems based on λ phage (Maruyama *et al.,* 1994; Sternberg and Hoess, 1995) and T4 phage (Efimov *et al.,* 1995; Ren *et al.,* 1996). λ phage and T4 phage assemble in the *E. coli* cytoplasm and are released by cell lysis. Thus, these systems may be particularly suited for the display of intracellular proteins that have evolved to fold in the reducing, cytoplasmic environment. In contrast,

M13 phage assembly is a membrane-associated process, and prior to assembly the coat proteins reside in the membrane with their N termini in the periplasm and their C termini in the cytoplasm (Webster, 1996). As a result, proteins fused to the N terminus of M13 coat proteins must be secreted through the membrane and fold in the oxidizing environment of the periplasm. Such conditions are ideal for naturally secreted proteins, but they may limit the display of intracellular proteins. Thus, the λ phage and T4 phage display systems may prove useful for the display of proteins that cannot be displayed as fusions to the N termini of M13 coat proteins. However, promising results with the display of polypeptides fused to the C termini of M13 coat proteins may extend the utility of M13 phage display to these same applications (Jespers *et al.,* 1995; Fuh *et al.,* 2000).

C. Cell Surface Display

In cell surface display, polypeptides are displayed directly on the surface of bacterial or yeast cells that also harbor the encoding plasmid DNA (reviewed by Georgiou *et al.,* 1997; Stahl and Uhlen, 1997; Cereghino and Cregg, 1999). Display is achieved by fusing gene fragments to genes encoding host membrane proteins. The resulting gene product remains associated with the outer cell surface, and the fusion is accessible for binding selections. Yeast display systems have used *Saccharomyces cerevisiae* and most bacterial display systems have used *E. coli*. However, several gram-positive bacterial strains have also been developed for specialized applications (Stahl and Uhlen, 1997).

Cell surface display libraries can be used in binding selections analogous to those used for phage display (Fig. 1), but a major advantage of cell surface display libraries is that they can also be screened by fluorescence-activated cell sorting (FACS). In contrast, the small size of bacteriophage particles precludes the use of FACS with phage-displayed libraries. FACS sorting is an extremely sensitive technique that allows for very efficient enrichment of binding clones over nonbinding clones. Furthermore, FACS enables direct discrimination of binding affinities so that even subtle differences can be reliably selected (VanAntwerp and Wittrup, 2000). However, FACS is limited by the throughput speed of cell sorters (about 4×10^7 cells per hour), whereas there is no such limiting factor in panning selections.

DNA transformation efficiencies for yeast are significantly lower than for *E. coli;* consequently, the practical limits to yeast library sizes are also comparatively low. Thus, *E. coli* has been the preferred host organism for most biological libraries. However, the yeast *S. cerevisiae* provides certain advantages in specialized applications. In particular, yeast is a eukaryotic organism with protein folding and secretory machinery very similar to that of mammalian cells (Boder and Wittrup, 1997). Thus, yeast surface display can be used to study proteins that are not amenable to *E. coli* expression or

phage display, and promising results have been obtained with yeast-displayed T-cell receptors (Kieke *et al.,* 1999) and G-protein-coupled receptors (Pausch, 1997).

D. Peptides on Plasmids

The peptides-on-plasmids system relies on a plasmid that encodes the DNA-binding protein LacI repressor and also contains LacI binding sites (Cull *et al.,* 1992). In an *E. coli* host, LacI repressor binds tightly to the LacI binding sites on the plasmid and thus establishes a link between the protein and its encoding DNA. Peptide libraries can be displayed as fusions to the LacI repressor C terminus, and the protein–plasmid complexes can be used in affinity selections (Fig. 3). Since the LacI repressor is a homotetramer, peptides-on-plasmids libraries are displayed in a polyvalent format. However, a monovalent version of the system has also been developed by deleting the tetramerization domain and fusing two tandem DNA binding domains to a single peptide (Gates *et al.,* 1996).

In principle, the peptides-on-plasmids system is analogous to M13 phage display, but the methods differ in the requirements for library purification and propagation. M13 phage particles are secreted directly into the media, and they can be easily purified with a precipitation procedure. In contrast,
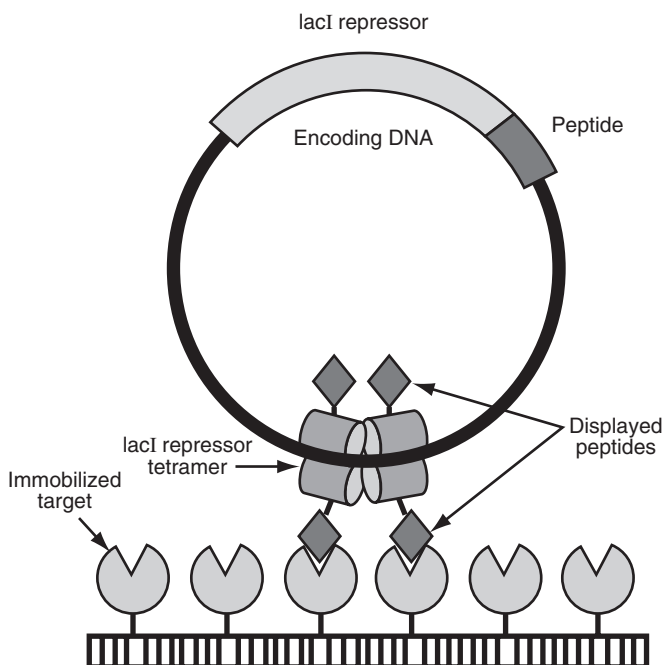

FIGURE 3 Peptides on plasmids. The display plasmid contains LacI binding sites and also a gene encoding the LacI repressor with a peptide displayed as a C-terminal fusion. In an *E. coli* host, the LacI repressor tetramer binds to the LacI binding sites and thus the displayed peptides are linked to their encoding DNA. Host cell lysis releases the peptide–plasmid complexes, which can then be used in binding selections with immobilized target proteins. (See Section 2.C for additional details.)

protein–plasmid complexes are obtained by bacterial lysis and thus remain associated with soluble *E. coli* proteins. Following binding selection, phage particles can be amplified by highly efficient infection of a suitable host strain. The introduction of plasmids into *E. coli* requires less efficient and more complicated transformation procedures.

E. Ribosome Display

In the methods described thus far, library construction requires a transformation step that introduces recombinant DNA into microorganisms. Subsequent transcription and translation within host cells produces the DNA-encoded polypeptide library. In such systems, library size is limited by the transformation efficiency. The most efficiently transformable organism is *E. coli,* and the practical size limit for *E. coli*-derived libraries is about 10^{12} (Sidhu *et al.,* 2000). Ribosome display methods circumvent the limiting transformation step and, as a result, they can be used to access library sizes in excess of 10^{13} (reviewed by Hanes and Pluckthun, 1999; Roberts, 1999).

Ribosome display was first reported by Mattheakis *et al.* (1994) and subsequently optimized by Hanes and Pluckthun (1997). Ribosome display uses a special DNA cassette that contains an open reading frame preceded by sequences that signal efficient transcription and translation. The open reading frame encodes a variable N-terminal polypeptide library followed by a constant C-terminal spacer. The DNA cassette is used as the template for *in vitro* transcription followed by *in vitro* translation of the open reading frame. The open reading frame does not contain a stop codon, and this inhibits dissociation of the translation complex. As a result, the translated polypeptides remain associated with the encoding mRNA, with the ribosome acting as a noncovalent linker (Fig. 4A). At appropriate salt concentrations and low temperatures, these complexes are stable enough to enable functional selections with the displayed proteins (Hanes *et al.,* 1998). Following selection, eluted mRNA can be converted to DNA by reverse transcription, and the DNA can then be amplified with the polymerase chain reaction (PCR) to produce template for additional rounds of selection. In this way, the entire selection process can be conducted *in vitro*.

Two groups have sought to further improve ribosome display by establishing a more stable, covalent linkage between the displayed polypeptide and its cognate mRNA (Nemoto *et al.,* 1997; Roberts and Szostak, 1997). Both groups used puromycin, an antibiotic that enters the ribosome and forms a stable amide bond with the nascent polypeptide. mRNA molecules were generated with puromycin covalently coupled to their 3′ ends with an intervening DNA spacer. *In vitro* translation with such a template produces the mRNA-encoded polypeptide, but the ribosome stalls at the RNA–DNA junction. At this point, puromycin enters the ribosome and forms a covalent bond with the C terminus of the
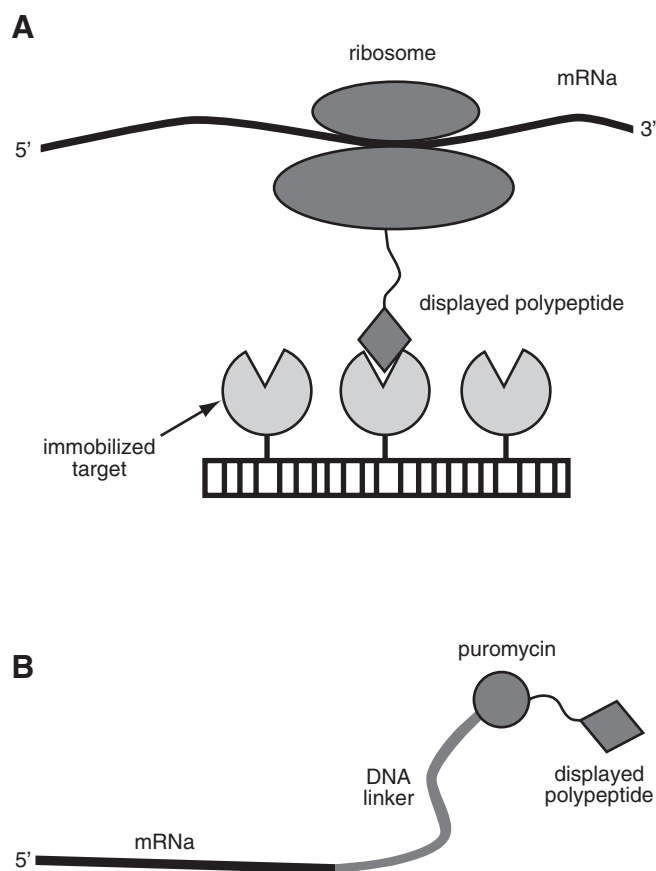
FIGURE 4 **(A)** Ribosome display. A special DNA cassette encoding a polypeptide library is transcribed and translated *in vitro*. The lack of a stop codon inhibits dissociation of the translation complex, resulting in a complex consisting of a displayed polypeptide and its encoding mRNA linked noncovalently by a stalled ribosome. Under appropriate conditions, these complexes are stable enough to be used in binding selections with immobilized targets. **(B)** Covalently linked mRNA–polypeptide fusion. Puromycin is attached to the 3′ end of mRNA with an intervening DNA spacer. During *in vitro* translation, the ribosome stalls at the RNA/DNA junction, and the puromycin moiety forms a covalent bond with the nascent peptide. (See Section 2.D for additional details.)

polypeptide, resulting in a covalently linked protein–mRNA complex (Fig. 4B) that can be used in selection experiments.

3. Applications for DNA-Encoded Peptide Libraries

Therapeutically relevant targets for ligand peptides can be divided into three main groups. The first group involves protein–protein interactions with binding contacts spread over large surfaces. Many extracellular protein–protein interactions are of this type, including the interactions between the extracellular domains of single-transmembrane signaling receptors and their ligands. The second group consists of proteins that bind to small, continuous stretches of amino acids within

other proteins, and this group includes many of the intracellular protein–protein interactions involved in signal transduction. The third group consists of the enzymes that catalyze the numerous chemical reactions essential to biological systems. DNA-encoded peptide libraries have been used to obtain ligands that not only bind specific targets but often also modulate biological activity. In this section, we discuss some of the important applications in each of these areas.

A. Peptide Mimics of Extracellular Protein–Protein Interactions

Many extracellular protein–protein interactions involve contact surfaces that are large and flat. Such interactions typically bury between 600 and 1300 Å^2 of protein surface area, and they include many intermolecular contacts involving 10–30 side chains from each protein (de Vos *et al.,* 1992; Clackson and Wells, 1995). Consequently, small-molecule screening efforts that work well in identifying ligands for concave surfaces have not been successful in targeting these interactions (Dower, 1998; Cochran, 2000). These failures have led to the belief that surfaces evolved to bind large proteins cannot bind small molecules with high affinity because such surfaces are solvent exposed and critical molecular contacts are spread over a large area. However, detailed mutagenic analyses of the interface between human growth hormone and its receptor have revealed that only a small subset of side chains from each protein make significant contributions to binding energy, and furthermore, these side chains are clustered together near the center of the interface (Cunningham and Wells, 1989; Clackson and Wells, 1995). These findings have led to the controversial but compelling suggestion that it may be possible to design small inhibitors that mimic this smaller "functional epitope" (Clackson and Wells, 1995). Recent results with phage-displayed peptide libraries support this concept.

There are numerous examples of phage-derived peptides that bind with high affinity to extracellular protein surfaces (reviewed by Kay *et al.,* 1998; Dower, 1998; Cochran, 2000; Sidhu *et al.,* 2000). It is notable that while most of these ligands were selected from unbiased naïve libraries, they generally bind to sites that coincide with natural ligand binding sites (Kay *et al.,* 1998; DeLano *et al.,* 2000). As a result, many of these selected peptides act as antagonists of natural protein–protein interactions. Even more remarkably, some binding peptides act as agonists that potently mimic the biological activity of the much larger natural ligands. Disulfide-constrained peptide libraries have proven most useful in these studies because a structural constraint promotes a discrete structure in solution. This in turn reduces the entropy of the free peptide and makes high-affinity binding more likely. Furthermore, structured peptides may be amenable to nuclear magnetic resonance (NMR) analyses that can provide invaluable insights into structure–function relationships. Indeed, NMR has been used to determine several free-solution structures, and crystal structures have also been determined for several peptides in complex with their cognate protein ligands (Fig. 5).
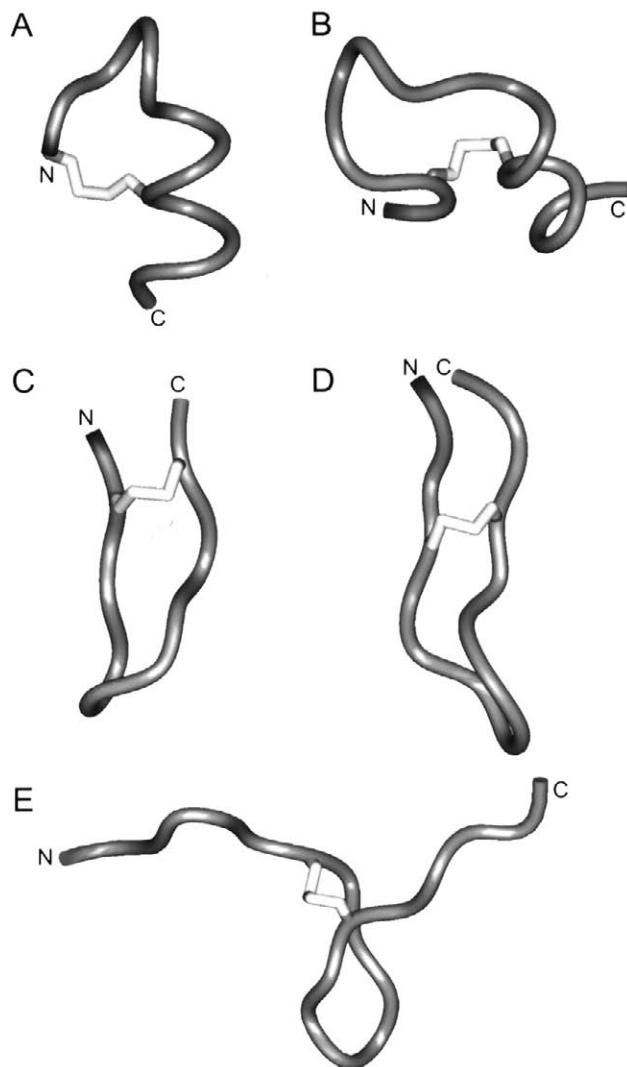

FIGURE 5 Structures of free or bound peptides isolated from phage-displayed peptide libraries. Main chains are shown as dark gray ribbons and disulfide bonds are shown in light gray; other side chains have been omitted for clarity. The N and C termini are labeled. Structures A and B were determined by NMR with the unbound peptides in solution. Structures C, D, and E were determined by x-ray crystallography with peptides bound to their cognate protein ligands. **(A)** The structure of the IGFbp1-binding peptide (bp101) shows a turn-helix conformation (Lowman *et al.,* 1998). **(B)** Within the disulfide-bonded loop, the structure of the FVIIa-binding peptide (E-76) consists of a distorted type I reverse turn and an irregular turn of an helix that extends to the C terminus (Dennis *et al.,* 2000). **(C)** A peptide that binds to the IgG-Fc (Fc-III) has a β-hairpin structure (Delano *et al.,* 2000). **(D)** As shown, the monomer structure of the EMP1 peptide is a β-hairpin, but the peptide forms a non-covalent, symmetrical dimer in complex with two molecules of EPO receptor (Fig. 6) (Livnah *et al.,* 1996). **(E)** The structure of the VEGF-binding peptide (v108) consists of a disulfide-bonded loop and an extended N terminus that makes main chain hydrogen-bonding interactions with VEGF (Weismann *et al.,* 1998). (See Section 3.A for additional details.)

1. Antagonist Peptides

The first report of a small, potent peptide antagonist of a cytokine receptor was published in 1996. Yanofsky *et al.* (1996) targeted a receptor for interleukin-1 (IL-1), an inflammatory cytokine implicated in many immune responses. Phage display was used to first isolate low-affinity leads that were subsequently affinity matured to yield unconstrained, 15-residue peptides that bound the IL-1 receptor with IC_{50} values of about 2 nM. Substitution of a proline residue with a proline analogue (azetidine), along with blocking of the N and C termini, produced a peptide with fourfold improved binding affinity. Several of these peptides were shown to be potent antagonists of IL-1-mediated cellular responses.

Phage-displayed libraries of disulfide-constrained libraries have yielded several antagonist peptides for which three-dimensional structures have been determined. Fairbrother *et al.* (1998) reported three classes of peptides that bound to vascular endothelial growth factor (VEGF), a primary modulator of vascular neogenesis, angiogenesis, and vessel permeability. All three classes inhibited the binding of VEGF to its receptors, and the highest affinity peptide antagonized VEGF-induced proliferation of primary human umbilical vascular endothelial cells. The crystal structure of one of these peptides complexed with VEGF revealed an extended conformation in which the N-terminal residues formed a β strand paired with a β strand in VEGF (Weismann *et al.,* 1998) (Fig. 5E). Because VEGF-induced angiogenesis is associated with pathologic processes, such as tumor growth and metastasis (Ferrara, 1995), VEGF antagonists could have broad therapeutic applications.

Insulin-like growth factor 1 (IGF-1) is a hormone with both metabolic and mitogenic activities that are mediated through binding to cell surface receptors. However, IGF-1 also binds to several distinct IGF-binding proteins, and these binding events block or modulate *in vivo* activity (Jones and Clemmons, 1995). Lowman *et al.,* (1998) isolated disulfide-constrained peptides that bound to one of these IGF-binding proteins (IGFbp-1) and in so doing prevented binding of IGF-1. Such antagonist peptides may act as indirect agonists *in vivo* by binding to IGFbp-1 and thus freeing IGF-1 for interactions with its signaling receptors. NMR analysis of a 14-residue peptide revealed a well-defined free-solution structure that could aid in the design of therapeutically useful compounds (Fig. 5A).

The constant fragment of immunoglobulin G (IgG-Fc) contains a consensus binding site that interacts with at least four structurally distinct natural proteins. Delano *et al.* (2000) showed that this site was also dominant for binding of peptides from naïve phage-displayed libraries. The crystal structure of a 13-residue, disulfide-constrained peptide complexed with IgG-Fc revealed that the peptide adopts a β-hairpin structure that is different from any known Fc-binding motif (Fig. 5C), yet it binds to the same consensus site. A detailed analysis showed that the different ligands use a number of similar binding interactions, and the consensus binding site on IgG-Fc is highly accessible, adaptive, and hydrophobic. The authors speculated that such properties may predispose binding sites for interactions with multiple binding partners and, furthermore, that small peptides can target such a site by mimicking the interactions of much larger, natural ligands with completely different structural scaffolds.

2. Agonist Peptides

Phage display has also been used to select peptides that act as agonists for growth factor receptors. The first example of this remarkable activity was a disulfide-constrained peptide that was selected for binding to the signaling receptor for erythropoietin (EPO), a growth factor that controls the production of red blood cells (Wrighton *et al.,* 1996). In cell-based assays, the peptide acted as an agonist that mimicked EPO activity. The crystal structure of an agonist peptide complexed with the EPO receptor showed that the peptide adopts a simple β-hairpin structure (Fig. 5D) that noncovalently dimerized with itself and two EPO receptors (Fig. 6) (Livnah *et al.,* 1996). Thus, it appears that the peptide activates the EPO receptor by mimicking the natural receptor dimerization event induced by EPO itself. Two different strategies were used to produce covalently linked agonist peptide dimers that were about 100-fold more potent than the monomeric peptide (Wrighton *et al.,* 1997; Johnson et al. 1997).

Agonist peptides have also been selected to mimic the activity of thrombopoietin (TPO), a growth factor that stimulates platelet production (Cwirla *et al.,* 1996). In this case, phage display was first used to select peptide ligands that bound the TPO receptor with low affinity and acted as agonists in a cell-based assay. These low-affinity leads were then affinity matured with peptides-on-plasmids display and ribosome display. Finally, screening of individual clones identified a 14-residue, unconstrained peptide that performed well in a competition binding assay (IC_{50} = 2 nM) but had poor potency in a cell proliferation assay. However, as in the case of the EPO mimetic peptides, covalent dimerization of this peptide produced a more potent agonist. In fact, the potency of the dimer was equal to that of the natural growth factor TPO and 4000-fold greater than that of the monomer.

B. Peptide Ligands for Intracellular Protein Binding Domains

There are several distinct families of intracellular domains that bind to other proteins and thus influence cellular function (reviewed by Pawson, 1995; Pawson and Scott, 1997; Cowburn, 1997). These domains differ in their tertiary fold and also in the nature of the sequences to which they bind. Interestingly, most intracellular protein binding domains recognize small continuous stretches of primary sequence in their binding partners. Small, linear peptides are excellent

FIGURE 6 Complex of the extracellular domain of the EPO receptor and the agonist peptide EMP1 (Livnah *et al.,* 1996). The main chains are shown as ribbons, and the side chains have been omitted for clarity. The complex consists of a peptide dimer (dark gray) and two EPO-binding proteins (light gray). (See Section 3.A.2 for additional details.)

mimics of such interactions; therefore, these domains are ideal targets for combinatorial peptide libraries. Indeed, there has been considerable success in isolating peptide antagonists of intracellular protein–protein interactions, and often, these synthetic ligands have been found to be homologous to the natural ligands. In such systems, combinatorial peptide libraries provide ligands that can be used to inhibit natural binding interactions as well as to elucidate natural binding specificities.

1. Domains That Bind Polyproline-Rich Sequences

Numerous intracellular protein domains direct intracellular signaling by binding to short proline-rich stretches within proteins (reviewed by Pawson and Scott, 1997). The best characterized of these belong to the family of Src homology 3 (SH3) domains. Several studies have reported the use of phage-displayed peptide libraries to identify small, linear

peptide ligands for SH3 domains (Sparks *et al.,* 1994, 1996; Rickles *et al.,* 1994, 1995). In all cases, proline-rich sequences were isolated that often matched sequences found in the natural SH3 ligands (Sparks *et al.,* 1996). It was also possible to obtain peptides that bound particular SH3 domains with greater affinity than peptide sequences derived from natural ligands (Rickles *et al.,* 1995). Furthermore, some selected peptides antagonized interactions between SH3 domains and their natural ligands *in vivo* (Sparks *et al.,* 1994). Phage display has also been used to study the binding specificity of WW domains, a structurally distinct family that also recognizes proline-rich sequences (Linn *et al.,* 1997).

2. Domains That Bind Phosphotyrosine-Containing Peptides

Many intracellular signaling pathways are regulated by reversible phosphorylation and/or dephosphorylation of particular tyrosine residues. These reversible modifications modulate enzymatic activities and also create or eliminate specific protein–protein interactions. Consequently, eukaryotic cells contain numerous protein domains that bind to specific phosphotyrosine-containing sequences and, in so doing, regulate signal transduction pathways (reviewed by Pawson, 1995; Cowburn, 1997). Two distinct structural classes of phosphotyrosine binding domains have been identified: the SH2 domains and the phosphotyrosine binding (PTB) domains.

It has been difficult to investigate phosphotyrosine-mediated binding interactions using DNA-encoded libraries because there is no genetic codon for phosphotyrosine. However, it has been shown that phage-displayed peptide libraries can be phosphorylated *in vitro* and that these modified libraries can be used to isolate phosphotyrosine-containing peptide ligands. While tyrosine kinases normally exhibit specificity for the sequences flanking the substrate tyrosine, two groups have demonstrated that prolonged exposure of phage-displayed peptide libraries to kinases results in virtually complete phosphorylation of tyrosine-containing peptides (Dante *et al.,* 1997; Gram *et al.,* 1997). Furthermore, these experiments showed that phosphorylation of naturally occurring tyrosines in wild-type phage coat proteins was not significant enough to interfere with subsequent selection experiments.

The phosphorylated libraries were used to investigate the binding specificities of a PTB domain (Dante *et al.,* 1997) and an SH2 domain (Gram *et al.,* 1997). Tyrosine-containing consensus sequences were identified in each case, and for the SH2 domain it was further demonstrated that phosphotyrosine-containing synthetic peptides corresponding to the selected sequences actually bound with greater affinity than peptides derived from the natural ligand. These results suggest that *in vitro* phosphorylation, and other posttranslational modifications, could further extend the utility of DNA-encoded peptide libraries.

3. PDZ Domains That Bind C-Terminal Peptide Sequences

PDZ domains are found in a large and diverse set of proteins (reviewed by Cowburn, 1997; Fanning and Anderson, 1999). In general, PDZ domain-containing proteins are responsible for clustering and assembling other proteins at specialized subcellular sites, such as epithelial cell tight junctions and neuronal synaptic densities. Many PDZ domains function by binding to the extreme C termini of their protein ligands, and thus, PDZ domains should be ideal targets for DNA-encoded peptide libraries. However, a complicating factor has been that while most PDZ domains interact with the free C termini of their ligands, the C terminus is blocked by fusion to the display scaffold in most DNA-encoded libraries. Nonetheless, two DNA-encoded approaches have been used to study the interactions between PDZ domains and ligands with free C termini.

Peptides-on-plasmids libraries are displayed as C-terminal fusions to the LacI repressor, and thus the displayed peptides have free C termini. This system has been used to map the binding specificities of PDZ domains from several different proteins (Stricker *et al.,* 1997; van Huizen *et al.,* 1998; Wang *et al.,* 1998). Recently, M13 phage-displayed peptide libraries fused to the C terminus of P8 have also proven effective in the analysis of PDZ domain–ligand interactions (Fuh *et al.,* 2000). In all of these studies, specific peptide ligands were identified, and these sequences were used to search genetic databases for natural proteins that may interact with particular PDZ domains. Furthermore, synthetic peptide analogues based on the selected sequences were used to map in detail the binding interactions between a PDZ domain and its high-affinity ligand (Fuh *et al.,* 2000). It was shown that the last four ligand amino acids provide almost all of the binding energy, and alanine scanning of a tetrapeptide ligand showed that all four side chains contribute favorably to PDZ binding, with the final two side chains being most important. These studies support a model in which the peptide main chain makes β-sheet interactions with a β strand in the PDZ domain, and the terminal carboxylate and the last four side chains all make productive binding contacts (Fig. 7). Thus, PDZ domains are able to bind tetrapeptides with high affinity and specificity by using binding contacts with essentially every functional group on the tetrapeptide.

C. Peptides as Enzyme Inhibitors

Enzymes have proven to be very good targets for phage-displayed peptide libraries. This is likely because enzymes typically have many deep clefts and binding pockets, including the active site and allosteric regulatory sites. In a recent study, Hyde-DeRuyscher *et al.* (1999) attempted to isolate peptide inhibitors for representatives from seven distinct enzyme classes. The results were very encouraging in that
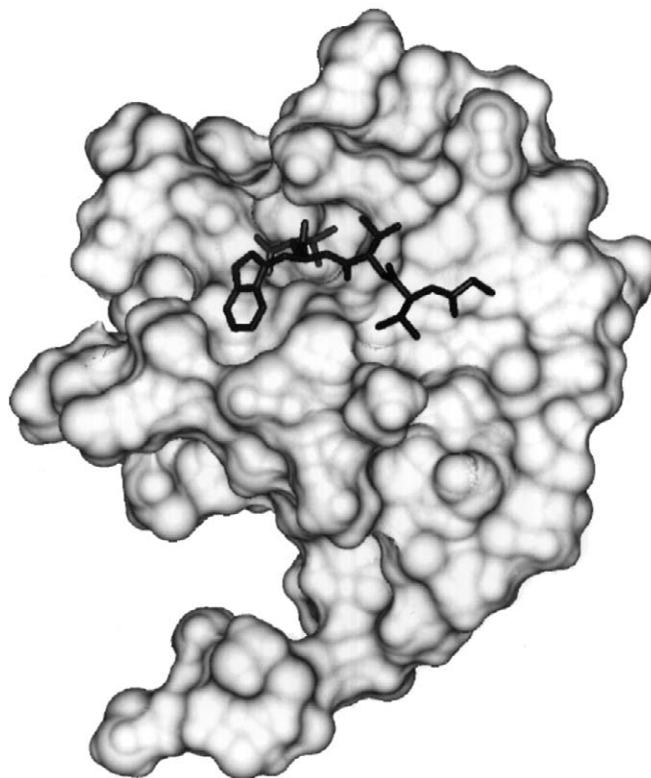
FIGURE 7 Molecular surface of a modeled PDZ domain complexed with a high-affinity pentapeptide ligand (Gly-Val-Thr-Trp-Val) (Fuh *et al.,* 2000). The model was based on the crystal structures of two homologous PDZ domains (Morais-Cabral *et al.,* 1996; Doyle *et al.,* 1996). The peptide ligand forms a β strand that intercalates between a β strand and an α helix of the PDZ domain. The terminal carboxylate and all four peptide side chains make binding contacts with the protein. (See Section 3.B.3 for additional details.)

specifically binding peptides were identified for all seven targets. In each case, many of the peptides bound to the same site, and the majority inhibited enzyme function. These results support the notion that proteins possess "preferred binding sites" (see Section 3.A.1) that are predisposed for ligand binding and that many of these sites are functionally relevant. The authors further demonstrated that inhibitory peptides could be used as detection reagents to discover small-molecules that bind to a protein of interest. In this application, small-molecule binding is detected by the displacement of bound peptide from the protein. Such competitive binding assays can be formatted with various detection systems, and they are readily adaptable to automation and high-throughput screening (Hyde-DeRuyscher *et al.,* 1999).

Dennis *et al.* (2000) used phage display to isolate peptides that bind the serine protease factor VIIa (FVIIa), a key regulator of the blood coagulation cascade. They obtained an 18-residue disulfide-constrained peptide (E-76) that not only bound FVIIa but also inhibited activity in a noncompetitive manner with exquisite specificity and high potency (K_i ~1
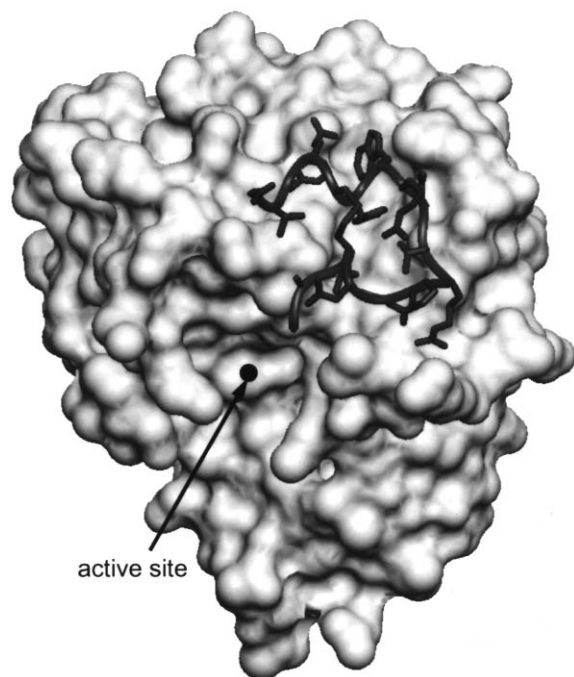
FIGURE 8 Crystal structure of peptide E-76 in complex with FVIIa (Dennis *et al.,* 2000). The molecular surface of FVIIa is shown in light gray. E-76 is in dark gray, with the side chains depicted in stick format. E-76 binds at an exosite distinct from the active site and noncompetitively inhibits enzyme activity. (See Section 3.C for additional details.)

nM). Consistent with a noncompetitive inhibition mode, the crystal structure revealed that E-76 binds to FVIIa at an "exosite" distinct from the active site (Fig. 6), and apparently, it inhibits activity by an allosteric mechanism. The solution structure for the free peptide was also determined by NMR, and interestingly, the main chain fold was found to be almost identical to that in the bound state. Thus, the phage display not only provided a potent FVIIa inhibitor; it also led to the discovery of a hitherto unknown binding site and mode of inhibition. These results are of great therapeutic relevance because, despite the importance of anticoagulant therapy, it has been extremely difficult to selectively inhibit the myriad proteases of the coagulation cascade (Hirsch and Weitz, 1999; Dennis *et al.,* 2000).

4. Conclusions

DNA-encoded peptide libraries can provide small, specific ligands for essentially any protein, and such ligands have several key applications in drug discovery research. First, the natural binding specificities of protein–protein interactions can be inferred from selected sequences, and this information can be used to identify natural binding partners (Section 3.B) or to aid the design of organic mimics (reviewed by Dame-

wood, 1996; Cunningham and Wells, 1997; Cochran, 2000). Second, binding peptides can be used for target validation, that is, to assess the potential therapeutic effects of blocking specific protein–protein interactions. This is especially important in the current genomics era because DNA sequencing efforts are revealing thousands of new proteins with unknown functions (Kay *et al.,* 1998). Third, binding peptides may be used as detection reagents in high-throughput screens for small-molecule inhibitors (Section 3.C). Finally, despite considerable obstacles, peptides themselves could be used as drugs (Latham, 1999).

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