Filamentous Phage Display in the New Millennium

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Received April 1, 2005

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1. Introduction

Since its first description in 1985, publications involving phage display have relied upon the fact that the displayed entity, be it a peptide, protein, or antibody fragment, is physically linked to a viral particle.[1] This coupling allows one to carry out selections rather than screens: a single protein with a desired trait can be captured from a pool containing billions of variants, its gene can be amplified as part of the phage genome, and the protein sequence can be used for whatever downstream purpose might be desired. This review describes some of the myriad ways in which phage display has been applied in the last five years. A number of excellent reviews that describe earlier work are available.[2−4] While we will focus upon the use of the filamentous bacteriophage M13 and its close relative fd, peptides and proteins have been displayed on the surface of other bacteriophage[5−9] and eukaryotic viruses.[10−12] Other display systems are beyond the scope of this review, but techniques such as RNA display,[13,14] ribosome display,[15] yeast display,[16,17] insect cell display,[18,19] pure translation display,[20] DNA-templated organic synthesis,[21] anchored periplasmic expression,[22] and cis display[23] each have unique strengths and should be of interest to those laboratories engaged in selections based on "molecular phenotypes".

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2. The M13 Virion

A great deal is known about the structure of the M13 virion (Figure 1).[24] Wild-type M13 is 65 Å in diameter and 9300 Å in length, giving it the proportions of a "four-foot long pencil" (D. Rodi, personal communication). The bulk of the virion consists of a circular, 6407-base, single-stranded DNA (ssDNA) genome,[25] coated with 2700 copies of the major coat protein, pVIII. Each end of the phage is capped with two different minor coat proteins: five copies each of pVII and pIX comprise one end, while the other end contains five copies each of pIII and pVI.[26] The length of the virion depends on the length of the genome: longer genomes result in longer phage, whereas shorter genomes generate shorter phage. Up to 12 000 bases can be added to the wild-type phage genome without disrupting packaging.[24]

The exact conformation of the DNA inside the phage particle is not known. Interestingly, if pVIII is mutated such that its carboxy-terminal region carries three positive charges rather than four, the virion becomes 30% longer for a given genome size.[27,28] Thus, the virion includes 30% more copies of pVIII, and the additional copies compensate for the lost charge in the mutated pVIII. This variation in length requires that the DNA undergo some conformational alteration. The fact that phage assembly continues unimpaired suggests that the DNA does not play a critical structural role.

In recent years, attention has shifted from studying the complete virion to examining the individual proteins and their domains. This approach has been most successful with pIII. A pIII fragment containing domains N1 and N2 has been crystallized,[29] and N1 has also been crystallized in a complex with a domain from the bacterial co-receptor TolA (Figure 2).[30] In both cases, the amino terminus of N1 extended into solution such that a peptide or protein could be added

10.1021/cr000261r CCC: $53.50
Published on Web 10/21/2005

John Kehoe was introduced to research in the laboratory of Paul Loach at Northwestern University. His undergraduate project applied peptide synthesis and other methods to investigate the structure–function relationships in bacterial light-harvesting complexes. After Northwestern, John went on to receive his Ph.D. at UC–Berkeley with Carolyn Bertozzi. John first worked with phage display during graduate school, in an effort to discover antibodies against sulfated tyrosine residues. John went on to complete a postdoctoral fellowship in the Kay laboratory and has recently accepted a position at Johnson & Johnson.

Brian Kay started his scientific career as a cell and developmental biologist, using *Xenopus laevis* as a model system in which to study oogenesis and muscle differentiation. Approximately 13 years ago, while on the faculty at the University of North Carolina at Chapel Hill, he generated his first phage-displayed combinatorial peptide library with his colleague, Dana Fowlkes. Using the techniques and reagents developed, the author's graduate students and postdoctoral fellows have mapped the protein–protein interactions of protein interaction modules present in numerous signal transduction, endocytic, and cytoskeletal proteins. In 1997, he moved to the Department of Pharmacology at the University of Wisconsin-Madison where he focused on using these peptide ligands for drug discovery. In October of 2001, he moved to the Biosciences Division at the Argonne National Laboratory, where he is a Senior Biochemist and Group Leader, and is currently setting up a high-throughput functional genomics effort using various display technologies. He has authored 110 publications and reviews, co-edited three books, and been issued 15 patents.

without interfering with pIII function. These structures provide an explanation as to why the amino terminus of pIII is so accepting of peptide or protein fusions (vide infra). The structure of detergent-solubilized pVIII was found to consist of two α-helices connected by a flexible hinge region,[31] in agreement with solid-state NMR studies.[32] The minor coat protein pIX seems to adopt a helical conformation in the viral particle.[33,34] However, little is known about the manner in which the capping proteins complete the body of the virion.[35]

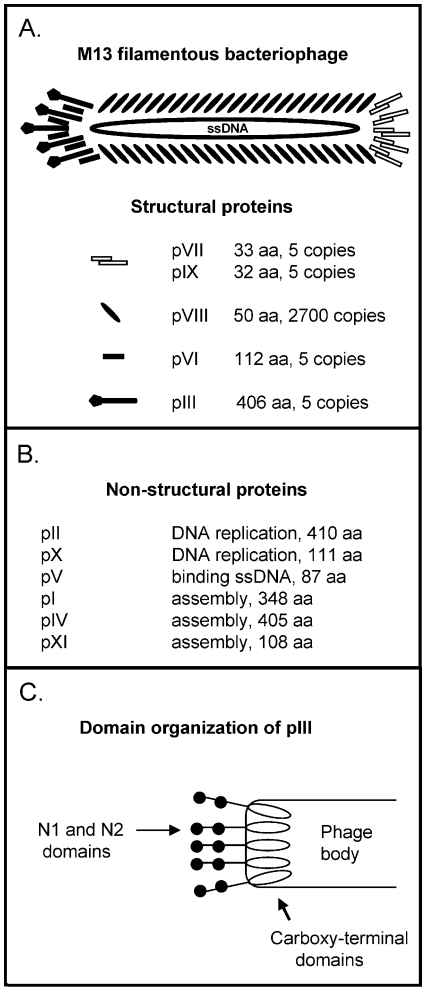

Figure 1. M13 proteins. (A) M13 possesses a very simple structure. The major coat protein, pVIII, encases a single-stranded DNA genome, and the ends of the virion are capped with the minor coat proteins. The carboxy terminus of pVI is exposed on the surface of the phage, and the other coat proteins expose their amino termini. Various peptides and proteins can be fused to these termini and thereby displayed on the surface of the phage (see text for details). (B) M13 produces six proteins that are not part of the virion. (C) The minor coat protein pIII has three domains.

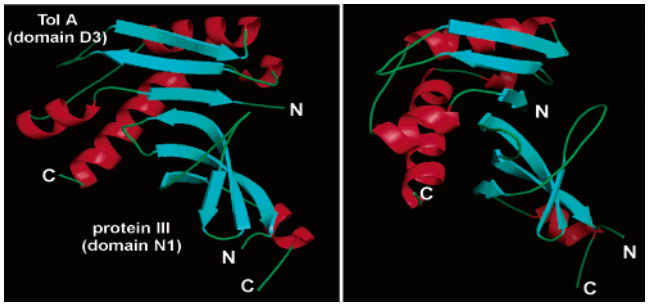

Figure 2. Crystal structure of TolA and the N1 domain from pIII. Peptide and protein fusions to the amino terminus of the N1 domain are removed from the TolA interface. In many cases, these fusions will not interfere with the normal function of pIII.

3. The M13 Lifecycle

The life cycles of M13 and other F pilus-specific filamentous phage are known in some detail (Figure 3).[26] Infection begins when pIII attaches to the F pilus of *Escherichia coli*. The crystal structure of the first

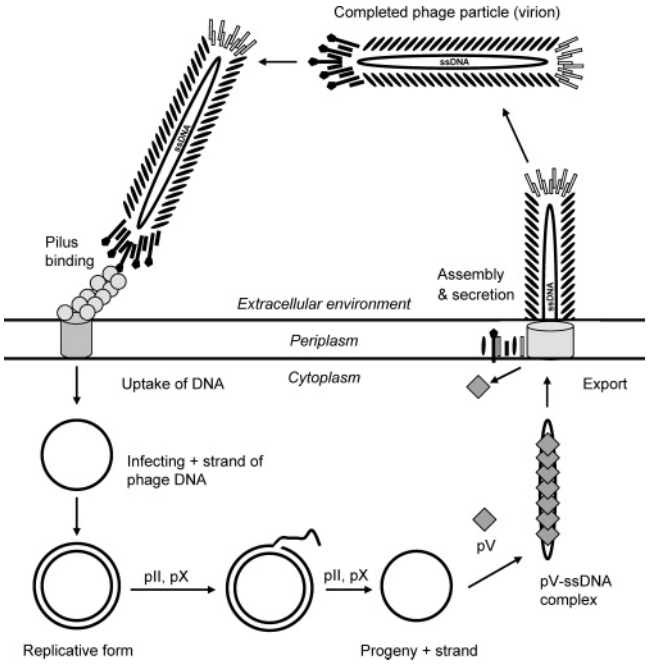

Figure 3. M13 lifecycle. Phage particles bind to *E. coli* through the pilus, which is encoded by genes carried on the F-factor. After binding to the phage, the pilus retracts, bringing the phage particle to the cell surface. There, the N-terminal domain (N1) of pIII interacts with the membrane-bound protein, TolA. The single-stranded DNA (plus strand) of the phage particle enters the bacterial cell through a process that is not well-understood, and it is then converted to double-stranded DNA (replicative form) by *E. coli* proteins. Two virally encoded proteins, pII and pX, are involved in rolling-circle amplification of the double-stranded viral genome, which leads to the production of plus-strand copies of the phage DNA. Such progeny molecules are then coated by the single-strand DNA binding protein, pV. The DNA−protein complex interacts with the export machinery (pI, pIV, pXI, and thioredoxin), which simultaneously assembles and secretes viral particles using a pool of phage coat proteins that have already been inserted into the inner membrane. Finally, the viral particle is released from the cells, without killing them, and pV is recycled.

two pIII domains showed them to have a horseshoe shape, with extensive interdomain interactions.[29] Biochemical data indicate that only the second domain is involved in binding to the pilus[36] and that the pilus-binding site is on the outer edge of the horseshoe.[37] After phage binding, the pilus retracts until the phage reaches the surface of the bacterium and pIII binds to the *E. coli* membrane protein, TolA.[38] It is thought that pilus binding briefly disrupts the interaction between the first and second domains of pIII (N1 and N2), leaving N1 available to bind Tol A (Figure 2).[39,40] Upon contacting the bacterial surface, the phage transfers its genome into the host cell, the infecting phage disassembles, and the coat proteins insert into the bacterial membrane.[41] Once inside the bacterium, the phage's genome is converted to double-stranded DNA (dsDNA) by bacterial enzymes, and the synthesis of M13's 11 proteins begins.

As phage proteins and ssDNA genomes accumulate within the infected bacterium, virion assembly begins. The structural proteins pVIII, pVII, pIX, pVI, and pIII spontaneously insert into the inner bacterial membrane upon synthesis and await the replication of ssDNA genomes.[26] When pV reaches sufficient concentrations, it coats newly synthesized, single-stranded phage genomes and prevents their conversion to dsDNA.[42,43] A small packaging signal, ssDNA that forms an imperfect hairpin, is left free of pV. This hairpin is captured by a complex composed of the integral membrane proteins pIV, pXI, and pI.[44,45] The virion is concurrently assembled and extruded from the bacterium at this complex, commonly described as a membrane pore.[26]

During assembly, pV is stripped from the ssDNA in a process that may involve thioredoxin, and the phage genome is coated with pVIII.[46] An average of five copies each of pVII and pIX are placed at the end of the virion containing the packaging signal; they assist in the initiation of assembly. As the virus is secreted from the infected cell, five copies each of pIII and pVI are incorporated at the opposite end of the virion; pIII assists in the release of phage from the bacterial cell.[47] It is important to note that M13 is a nonlytic bacteriophage, and the host cell is not killed by the infection. Infected host cells continue to grow and to divide indefinitely, albeit at half the rate of uninfected bacteria.[26]

4. Display of Peptides and Proteins

All five capsid proteins in the phage virion have been utilized for display purposes. The most common approach for peptide display is to fuse the foreign sequences to the amino terminus of pIII or pVIII, while proteins are usually displayed from pIII. Peptide and protein fusions to the amino termini of pVII[48] and pIX[49] have been reported, as well as fusions to the carboxy termini of pVI,[50] an artificial pVIII,[51] and pIII.[52] While these structural proteins all insert into the membrane prior to phage assembly, the displayed peptide or protein is completely exposed to the periplasmic environment. This allows disulfide bond formation in both protein and peptide libraries and explains the utility of the periplasmic protein *skp* in allowing increased expression of pIII fusion proteins containing disulfide bonds.[53] Despite these facts, it is possible to display proteins with free cysteines.[54,55] As a general rule, the minor coat proteins will display larger proteins more effectively than pVIII. However, protein display is very idiosyncratic. Some short peptides cannot be displayed, while proteins as large as penicillin G acylase, an 86 kDa heterodimer, will display on both pVIII and pIII.[56] Although a library of polypeptides displayed on the surface of M13 is referred to generically as a phage library, three different systems have actually been invented: phage, hybrid, and phagemid (Figure 4).

4.1. Types of Phage Display Systems

Phage systems are constructed such that the polypeptide fusion is displayed from every copy of its partner. Thus, if a peptide library is displayed from pVIII in a phage system, each virion has a peptide displayed from every copy of pVIII − some 2700 copies per virion. This feat is accomplished by fusing the library DNA with the only copy of pVIII in the

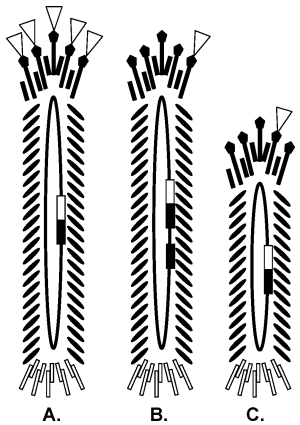

Figure 4. The three types of phage display. (A) Phage systems. The phage genome contains only one copy of pIII (black), and the displayed sequence (white) is fused to that copy. Consequently, every pIII displays the same fusion protein (triangle). (B) Hybrid systems. The phage genome contains two copies of pIII, one with a fusion partner and one without. The promoters for these two genes are generally designed such that the wild-type pIII is expressed at higher levels in the cell. The more common wild-type protein thus appears more frequently in the virion. (C) Phagemid systems. Instead of a phage genome, the virion contains the phagemid, which is generally smaller. Infection with helper phage drives the expression of phage proteins, and the phagemid expresses the pIII fusion library.

phage genome. Peptide libraries can be displayed on pVIII using a phage system, but the size of the peptide is limited, and the "avidity effect" often prevents one from discriminating between low- and high-affinity binders. In fact, this system is ideally suited to the identification of low-affinity binders for difficult targets. Many peptide libraries have been cloned into the phage genome such that they will be displayed at the amino terminus of pIII. This approach modifies every copy of pIII found in the virion, but with only five copies the avidity effect is much smaller. However, even pIII is limited in what it can accept. Significant alterations of every pIII in the virion, such as fusion to a heterologous protein, can prevent viral assembly or block infectivity. Even if the changes are only a minor impediment to viral assembly then selective pressure will increase the number of revertant (i.e., wild-type) phage in the library.

A phagemid is a plasmid that carries an antibiotic resistance marker, bacterial and phage origins of replication, the phage gene from which the library is to be displayed, and a phage packaging signal. The library is cloned into the phagemid such that it will be expressed as a fusion to the phage protein. The phagemid DNA is then used to transform bacteria and can be amplified and isolated in the same fashion as any other plasmid. Production and maintenance of the library in bacteria, which replicate their DNA much more accurately than bacteriophage, is one way in which the diversity of libraries is preserved.

To display peptides or proteins using a phagemid system, bacteria carrying the phagemid DNA are infected with helper phage to produce a viral particle for use in selections. A simple approach is to use packaging-defective phage as the helper. Upon infection of bacteria carrying the phagemid, the helper phage genome initiates synthesis of all the wild-type phage proteins. Since the phagemid DNA carries a phage promoter upstream of the sequence encoding the fusion protein, synthesis of the fusion protein is initiated as well, and the fusion protein is added to the cellular pool of phage proteins. When phage assembly begins, virions will be preferentially assembled using the phagemid DNA, which differs from the helper phage in that it carries a fully functional packaging signal. Small amounts of the fusion protein will be drawn from the cellular pool for phage assembly, and the secreted phage particles will both display a foreign sequence on a coat protein and carry the phagemid encoding that sequence.

Other types of helper phage have been designed to block the growth of phage that lack a fusion protein. Despite the best experimental procedures, the output from every selection will contain a small number of helper phage that do not specifically recognize the target. In selections with few specific binders or specific binders that impede the growth of the phage, it is possible that the helper phage may crowd out the real binders during amplification. This outcome should be preventable if all the phage that lack fusion proteins are unable to amplify themselves. In one approach, the N1 and N2 domains of pIII were deleted from the helper phage genome,[57] leaving the carboxy-terminal (CT) domain, which can still be expressed and incorporated into the viral particle. These helper phage are propagated via electroporation of their genome into a bacterial strain expressing pIII from a plasmid; when a selection is carried out using such helper phage, the only virions that can propagate themselves are those that carry a fusion protein. Alternatively, a proteolysis site can be introduced into the helper phage-encoded pIII and the selection output treated with trypsin.[58]

Hybrid systems do away with the helper phage but still allow for the assembly of phage particles using wild-type protein plus a fusion protein. For example, a second copy of the pVIII gene can be placed in the phage genome under the control of a *tac* promoter with a multiple cloning site.[59] Thus, heterologous DNA added to the 5′-end of the second copy of the gene will appear in the recombinant protein product as an amino-terminal fusion, which will yield a fusion capsid protein that is assembled into virions along with the native pVIII protein. Although this system is applicable to the minor M13 coat proteins, such as pIII,[60] it is most commonly used with pVIII.[61−64]

4.2. Altering M13 for Display

Extensive structural knowledge of M13, combined with the ability to construct libraries and to carry out selections, has induced some investigators to attempt the improvement of M13 as a display system.[65] Much of this work has focused on pVIII. Although peptides of six residues or less are easily displayed on every copy of pVIII in the viral particle, the likelihood that a larger sequence will display decreases with size.[66] This may stem from limitations in pVIII processing.[67] If one switches from phage systems to hybrid or phagemid systems some proteins

(>100 amino acids) will display from the amino terminus of pVIII, but if the protein can be displayed at all, the number of copies per virion often averages less than one.[67] It has been suggested that larger fusions simply will not fit through the integral membrane assembly complex comprised of pIV, pXI, and pI. This inability to display in a multivalent format negates the main advantage of displaying from pVIII. Scientists have addressed this problem with a simple, straightforward approach:[68] they constructed phagemid libraries that fused either human growth hormone or streptavidin to mutagenized pVIII and then carried out selections for protein display. After a number of rounds of affinity selection, the investigators identified numerous mutations in pVIII that increased the display levels of either human growth hormone or streptavidin by up to 100-fold.[69] Similar work has been conducted to improve carboxy-terminal display from pVIII.[70] The discovery that pVIII could accept a wide range of mutations and still be incorporated into the protein coat inspired a daring, yet successful, effort to invent an artificial pVIII that would pack into the bacteriophage coat with an inverse orientation[51] so that the resultant pVIII homologue could display large polypeptides from its carboxy terminus. Subsequent work has identified nine residues in pVIII that are both necessary and sufficient for pVIII incorporation into the phage particle.[71]

5. Production of Libraries

This review contains a great deal of discussion about peptide and protein libraries, yet those who use phage display actually have very little to do with constructing a peptide or protein library. This is the job of the phage-infected bacteria. The investigator must instead be familiar with the production of DNA libraries. While a plethora of techniques exist for the production of DNA libraries,[72] most of the approaches use one of two methodologies: Kunkel mutagenesis or the ligation of polymerase chain reaction (PCR) products into a vector.

5.1. Kunkel Mutagenesis

Kunkel mutagenesis is well suited for peptide libraries or alternative scaffolds (vide infra) with contiguous, randomized positions (Figure 5). In the original protocol, the sequence to be mutated is cloned into the phage genome, and the phage is grown in a bacterial strain that lacks dUTPase and uracil glycosylase (i.e., a *dut*$^-$*ung*$^-$ strain).[73] The resultant phage genomes contain uracil and, as discussed above, are single-stranded. It is thus possible to anneal a chemically synthesized oligonucleotide to the isolated phage genome and enzymatically create a second DNA strand that lacks uridine. This dsDNA product can then be transformed into competent *E. coli* cells with dUTPase and uracil glycosylase, which destroy the uridine-containing strand. Using this type of system, one can create libraries of combinatorial peptides or proteins with randomized regions by annealing a chemically synthesized oligonucleotide with one or more randomized residues, extending it enzymatically to create the second DNA strand, electroporating bacteria with the resultant dsDNA, and collecting the amplified, secreted bacteriophage M13 virions.[74]

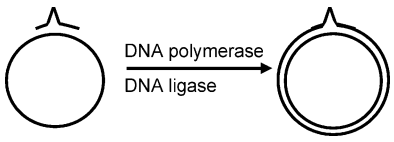

Figure 5. Kunkel mutagenesis for library construction. An oligonucleotide is synthesized such that the randomized segment is flanked by two annealing regions. While the randomized region is not expected to have any particular affinity for the phage genome, the two annealing regions are long enough to compensate. The oligonucleotide is extended with DNA polymerase and then ligated to itself to create a closed circular piece of DNA. For details on the preparation of the DNA template, see text.

Our laboratory has recently published a technological refinement of Kunkel mutagenesis.[75] The requirement for uracil-containing DNA can be burdensome because phage do not grow very well in *dut*$^-$*ung*$^-$ strains. Furthermore, up to 20% of the phage in a library constructed through standard Kunkel mutagenesis may carry the original sequence, rather than a mutation.[74] To avoid both of these problems simultaneously, we constructed a phage vector that carries the amber stop codon, TAG, within the coding region for protein III. This vector can be propagated in a suppressor strain but will not grow in nonsuppressor strains. We can thus isolate TAG-containing phage genomes, anneal and extend an oligonucleotide that replaces the TAG codon and adjacent codons with randomized codons, transform the resultant dsDNA into nonsuppressor bacteria, and obtain phage that are almost exclusively recombinant. We are able to routinely generate libraries of up to 10^{10} transformants in 2 days using this approach. The system is not limited to peptide libraries but can be expanded to any protein that is stably expressed in a phage system.

5.2. PCR-Driven Library Construction

If Kunkel mutagenesis is unsuitable for some reason, the library can be constructed using PCR reactions and ligations. For example, if the sites of randomization are widely distributed, one can use a series of PCR reactions to assemble DNA fragments that carry randomized positions.[76,77] Randomization "cassettes" derived from chemically synthesized oligonucleotides can be ligated into the coding region of a protein.[78] In the case of antibody libraries, there may be little need to introduce diversity through chemically synthesized oligonucleotides. The source immune system, after all, already contains a library of different sequences (i.e., hypervariable regions). The variable domains of the immunoglobulin light and heavy chain mRNAs can be amplified as DNA through reverse transcriptase-PCR and then ligated directly into a phage-display vector. Libraries assembled with PCR products typically reach 10^9 members.[79,80]

5.3. Reduced Codon Sets

A further consideration in the construction of a phage-displayed library is the question of exactly

which amino acids to include in the randomization scheme. Libraries that randomize only a small number of positions can be large enough to encompass every possible sequence (e.g., 5 positions with the 20 common amino acids provides 20^5 or 3.2×10^6 different sequences). The number of possible sequences climbs rapidly as more positions are randomized (e.g., $20^{15} = 3.2 \times 10^{19}$). This situation is complicated by the fact that a NNK (N = G, A, T, or C; K = G or T) randomization scheme actually encompasses 32 different codons with an unequal representation of the 20 amino acids. Since most libraries cannot include every possible sequence, one might ask whether some amino acids are more useful than others. Certainly the human immune system seems to prefer complementarity determining regions (CDRs) that are biased toward a subset of amino acids: tyrosine, serine, and glycine alone account for 39% of the amino acid residues in the third CDR of human heavy chains.[81] This observation inspired the construction of phage-displayed Fab libraries in which the solvent-exposed CDR positions were randomized with a series of codons that only encoded four types of amino acids.[82] Surprisingly, some of these libraries readily provided binders with nanomolar dissociation constants. The amino acid set of tyrosine, alanine, aspartic acid, and serine was especially effective, as well as threonine, arginine, glycine, and alanine.

As the scientific community gains a greater understanding of reduced codon sets, it may become more common to construct libraries using oligonucleotides synthesized from codons, rather than bases.[78] This approach allows precise control over which amino acids are present in a given position. One approach has been to utilize trinucleotide insertion mutagenesis[83] to randomize the hypervariable regions of scFvs,[78] Fabs,[84,85]and ankyrin repeats.[86] Another method is to "blend" oligonucleotides that differ in selected positions by a subset of codons[87] and then clone them into the hypervariable regions of an antibody.[87−89] Both of these approaches have the important benefit of exploring protein sequence space more effectively than would be possible with larger (i.e., NNK) codon combinations.

5.4. Incorporation of Unnatural Amino Acids

While a wide variety of protein structures can be displayed on the surface of M13, the functional groups were, until recently, limited to those of the naturally occurring amino acids. Three approaches have been utilized for the addition of interesting molecules to phage-displayed proteins. Various investigators have used expressed protein ligation to add small fluorescent probes, posttranslational modifications, stable isotopes, and unnatural amino acids to their protein of interest.[90] One group adapted expressed protein ligation to phage display and ligated a chemically synthesized peptide to a library of proteins displayed on either pIII or pVIII.[91] In other cases, the selenocysteine display system may prove helpful.[92] Selenocysteine is normally found in *E. coli*, and its side chain reactivity is orthogonal to other amino acids. Thus, selenocysteines in phage-displayed peptides can be selectively modified. Finally, it is possible to create phage-displayed libraries that display unnatural amino acids in peptides or proteins through the translation of amber codons with suppressor tRNAs charged with unnatural amino acids.[93] The presence of unnatural or modified amino acids within a peptide or protein may expand its binding capabilities for a given target or permit spectroscopic monitoring of binding events. It will be interesting to follow further developments in this area.

6. How To Carry Out Selections

Selections are conceptually simple, although they can be fraught with technical difficulties. A basic selection for binding requires one to immobilize the target, add the phage, wash away those that did not bind to the target, elute the phage that did bind to the target, and amplify the eluted phage for subsequent rounds of selection (Figure 6). After a number of rounds of selection the eluted phage are grown as single plaques for further analysis. There can be a great deal of variation in this approach, in everything from the library that is displayed to the method used to elute phage. There are also numerous means of increasing the affinity of the selected binders.[94−98] Regardless of the differences in the details, selections following the general scheme outlined in Figure 6 have been successful in identifying peptide and

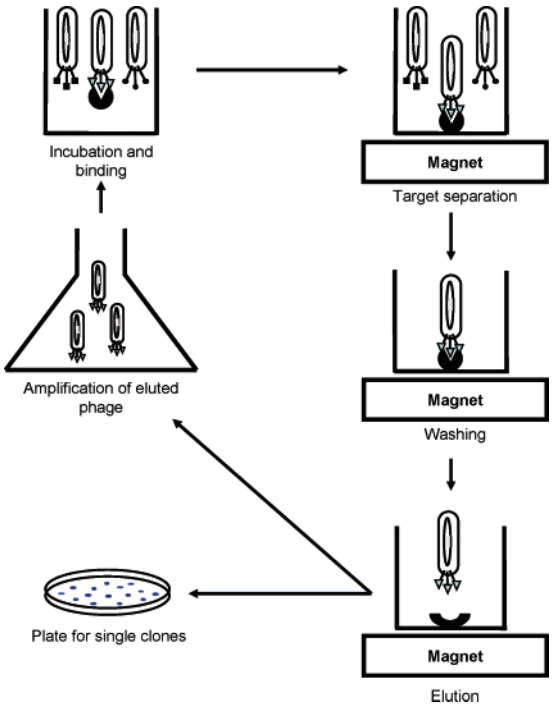

Figure 6. A basic selection for binding. Phage displaying different peptides or proteins are first mixed with the target (incubation and binding, top left). As shown, the target is attached to magnetic beads, although other immobilization techniques have been used (see text). After an hour or so, the beads are captured and washed, and the remaining phage are eluted, generally via brief exposure to extremes of pH. At this point, the phage can either be grown en masse for further rounds of selection, or diluted and plated so as to grow genetically homogeneous, or clonal, populations for further analysis.

protein binders for a wide range of soluble proteins. Affinity reagents that recognize membrane proteins have also been isolated.[99−102] In addition, this approach has successfully isolated binders against small molecules and lipids, such as ampicillin,[103] sialyl Lewis^x,[104] and the glycosphingolipid G_{M3}.[105]

The method by which one immobilizes the target can have a great impact on the success or failure of the selection process. Targets are often immobilized by nonspecific adsorption to a surface, such as nitrocellulose,[106] poly(vinylidene fluoride),[107] or polystyrene.[108] While this is a convenient approach, requiring no modification of the protein and no special preparation of the surface, adsorbing a protein to a surface can change the protein's conformation. This approach has even been used as an experimental system to create and to study molten globules.[109−111] One attractive alternative is the use of in vivo target biotinylation followed by capture on streptavidin-coated plates or resin.[108] The *E. coli* biotin ligase BirA will biotinylate a short peptide tag in vivo or in vitro and can be used to generate homogeneous preparations of biotinylated proteins. These proteins can then be immobilized on a streptavidin-coated surface (e.g., magnetic beads or microtiter plate wells). While some of the streptavidin may denature, this will have no effect on the selection. Biotinylated target can remain largely exposed to solution, tethered to the surface only through a biotinylated amino- or carboxy-terminal fusion tag.

7. Applications of Phage Display

When hundreds of imaginative scientists apply a technique to their favorite problem, the resultant list of applications is far too big to review comprehensively. We have selected 10 topics to discuss in detail. Other common applications that are not addressed here include the discovery of protein−protein interactions,[112−118] the selection of peptide mimotopes for use in vaccines,[119−122] investigation of the immune response,[123−129] and the evaluation of small molecule drug candidates.[130−134]

7.1. Affinity Reagents

The most common type of library is one designed to produce affinity reagents, that is, a peptide or protein that binds to a particular target. While libraries of immunoglobulin fragments are obvious sources of affinity reagents,[135] they are often not ideal. IgGs, the antibody type commonly used in laboratory procedures, are normally secreted from eukaryotic cells. It is very difficult, although possible, to express these proteins in bacteria.[136] In lieu of full-sized antibodies, investigators have turned to single-chain fragments of the variable regions (scFvs)[80] and fragments, antigen-binding (Fabs).[137] Phage-displayed libraries of antibody fragments have been generated with assembled segments of immunoglobulin cDNAs or through randomization of the hypervariable regions with a single scaffold structure.[78,85,138,139]

While scFvs and Fabs are very useful, they are often unstable and difficult to express on their own in bacteria.[140,141] These difficulties have given rise to a search for other proteins that can function in the same manner as antibodies in binding specific targets with high affinity. These proteins are known collectively as "alternative scaffolds", a name that stems from the thought that these proteins can serve as an alternative framework from which an investigator can display random sequences of amino acids. A large number of proteins have been investigated as alternative scaffolds, including affibodies,[142] lipocalins,[76] cytotoxic T lymphocyte-associated antigen 4,[143] knottins,[144] M13 pVIII,[145] the new antigen receptor,[146] autonomous heavy chain variable domains (V_HH),[147] bovine pancreatic trypsin inhibitor,[148] fibronectin type III domains,[149] cytochrome b562,[150] zinc finger domains,[151] Kunitz domains,[152] ecotin,[153] tendamistat,[60] pancreatic secretory trypsin inhibitor,[154] β-lactamase,[155] ankyrin repeats,[86,156] and carbohydrate binding modules.[157]

Selection of scaffold proteins is driven by a number of different factors.[158] Since a portion of the protein surface will be randomized, there must be solvent-accessible loops or a cluster of surface-exposed residues that can be changed without altering the protein fold (Figure 7).[159] These putative binding sites should

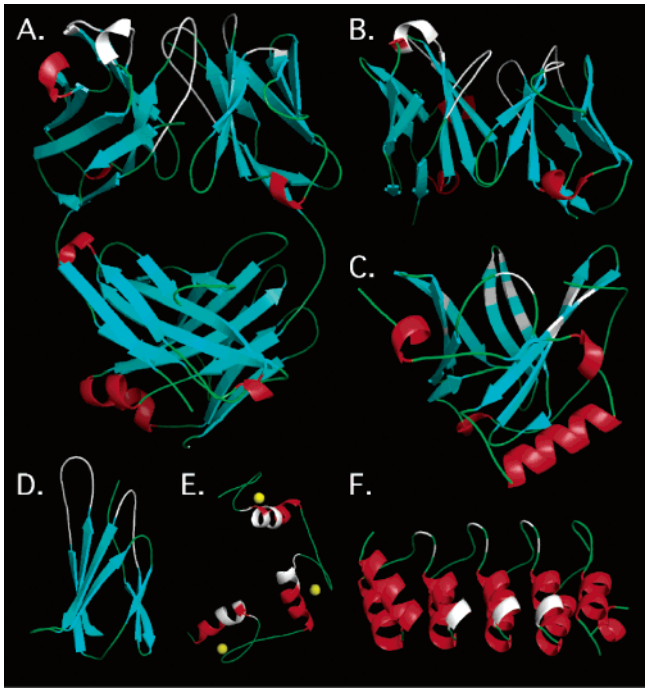

Figure 7. Antibody fragments and alternative scaffolds. (A) Structure of a fragment, antigen binding (Fab), from the herceptin antibody (PDB file 1N8Z). In this structure, and in all other structures in this figure, the regions of variable sequence are colored white. (B) Structure of a single-chain fragment of the variable region (scFv) (PDB file 2AP2). (C) The bilin binding protein (PDB file 1BBP). Residues on the inside of the β-barrel, near the top opening, have been randomized for the production of phage-displayed libraries. (D) The tenth type III cell adhesion module of human fibronectin (PDB file 1FNA). The two loops at the top of the structure have been randomized for the production of phage-displayed libraries. (E) A DNA-binding module comprised of three zinc fingers. (F) Structure of an ankyrin repeat protein (PDB 1SVX). Residues along the upper side of the α-helices and in the β-turns were randomized for the production of a phage-displayed library. See text for details.

be independent of the hydrophobic core, as is the case in antibodies.[158] Of course, it must also be possible to display the protein on phage, and the protein scaffold should express well on its own in bacteria. Various combinations of scaffold size and randomization site have proven successful. Some alternative scaffolds, such as tendamistat, cytotoxic T lymphocyte-associated antigen 4, and the fibronectin type III domain, exhibit immunoglobulin-like folds. These proteins will accept randomization of one or more surface-exposed loops.[60,143,149] The complementarity-determining regions of single-domain antibodies from camelids and the Wobbegong shark can also be randomized and the resultant libraries displayed on the surface of phage.[146,147] Three- and four-helix bundles can accept the randomization of the loops connecting their helices[150] or of a group of amino acids on one face of the protein.[142] The lipocalins can be randomized at one end of their β-barrel structure.[76,160] Reporter proteins themselves can be used as alternative scaffolds. Surface-exposed loops in TEM-1 β-lactamase[155] can be randomized for library production and subsequent selection of protein binders. Alternative scaffolds can also be fused to reporter proteins such as β-galactosidase,[161] alkaline phosphatase,[162] and green fluorescent protein.[163]

While most alternative scaffolds are viewed primarily as protein-binding reagents, it is interesting to note the exceptions. The lipocalins are primarily small-molecule binders. These proteins normally bind hydrophobic small molecules such as chromophores and pheromones.[164] Family members exhibit a β-barrel structure with the binding sites inside the barrel. Libraries of lipocalins with randomized amino acids around the binding site have been displayed on phage and used for the selection of proteins that bind to small molecules with low nanomolar dissociation constants.[76,160,165−167] Similarly, a library of neocarzinostatin variants has recently been displayed on phage and used to discover new binders of small drug-like molecules.[168] The engrailed homeodomain has also been employed as the starting point for generating binders of DNA variants,[169] and extensive work has been done concerning zinc finger libraries (vide infra).

7.2. Inhibitors

Affinity reagents are often observed to inhibit the target's activity, perhaps by blocking an enzyme's active site or by preventing a growth factor from binding to its receptor. Alternatively, an affinity reagent can bind to the target's substrate.[170] Small peptide-based inhibitors and large protein-based inhibitors are both commonly obtained. Although some characteristics of peptides render them less than ideal as therapeutic agents, they are useful in laboratory settings. Peptide inhibitors can serve as biological tools to assist in goals such as target validation,[171] and both peptides and proteins have been used to discriminate between different target conformations.[172−174]

Numerous efforts have been made to maximize the utility of peptide inhibitors isolated from phage-displayed libraries. A general difficulty in using peptides is their low affinity for target proteins. The dissociation constants of both linear and cyclized peptides are rarely below the micromolar range, but the peptides can be converted to multimers or conjugated to carrier proteins to use the avidity effect to increase their affinity for a given target.[171,175] This approach has been used in efforts to develop a clinically useful inhibitor of the anthrax toxin.[176] One limitation to the therapeutic use of peptides is their difficulty in crossing cell membranes. However, some peptide sequences have been observed to drive transport across a cell membrane.[177] Fusion of these transporter sequences to peptide inhibitors should, in theory, allow targeting of any exogenous protein in the cytosol. Such an approach has shown promise in animal models of Huntington's disease[178,179] and against in vitro replication of HIV.[180] If the use of peptides or some peptide variant is not acceptable, one can use the peptide inhibitor as a lead compound for small molecule drug development.[181] Peptide motifs that bind to a given target may be only a few amino acids in length,[182] or they may possess some structure in solution without binding to the target.[183,184] Small, structured peptides are ready starting points for the synthesis of peptidomimetic compounds with improved therapeutic properties.[185−187]

Antibodies are commonly used as inhibitors both in the laboratory and in the clinic.[188] As molecules that have evolved to bind targets, antibodies are better suited to this task than peptides, and the discovery of effective antibody inhibitors is relatively simple. Direct selections from "naïve" phage-displayed libraries can in some cases produce antibodies with nanomolar affinities,[189] while further rounds of mutagenesis and selection may be necessary in other cases.[190] The complete inhibition of especially difficult targets such as botulinum toxin may require the simultaneous use of two or three different antibodies targeting different epitopes on the protein surface.[191] An interesting recent publication details the development of a single immunoglobulin that targets both the epidermal growth factor receptor (EGFR) and the insulin-like growth factor receptor (IGFR).[192] Therapeutics with such bispecific capabilities should be useful in the simultaneous targeting of multiple oncogenic signaling pathways. It is also interesting to note that one can identify antibodies that activate signal transduction.[193]

A great deal of interest has recently focused on "intrabodies", or antibodies that are meant to function inside a cell. The process of selecting a binder to an intracellular target and then expressing that binder inside the cell is, in and of itself, straightforward. The difficulties surrounding the laboratory use of intrabodies stem from the fact that the cytosol is a reducing environment. Both full-sized IgGs and antibody fragments such as scFvs contain disulfide bonds. This is not a problem in the normal use of antibodies, since antibodies and their derivatives are produced in the Golgi compartment of eukaryotic cells or the periplasm of *E. coli*. Both of these compartments are reducing environments that contain enzymes promoting disulfide bond formation. Proper folding of cytosolic scFvs is unlikely without

disulfide bonds, and cytosolic production of a Fab or IgG would also seem unlikely because of the requirement for disulfide bonds to cross-link the heavy and light chains. However, scFvs do not absolutely require disulfide bonds, and some fraction of scFv sequences can fold and bind to their target in the reducing environment of the cytosol. The unusually high stability of these scFvs enables them to function as intrabodies,[194,195] and libraries based on these sequences have been constructed.[196] Intrabodies have shown promise in cellular models of Huntington's disease[197] and HIV infection.[198]

7.3. Mirror Image Phage Display

A clever combination of chemistry and phage display has allowed investigators to discover peptide ligands composed of D-amino acids. The phage virion itself is composed of L-amino acids, and without expressed protein ligation, it is not possible to generate a library of phage that display D-peptides. However, if the target protein is small enough, it can be chemically synthesized from D-amino acids. HIV protease,[199] rubredoxin,[200] and monellin[201] have all been synthesized from D-amino acids and compared to their natural counterparts. As anticipated by some scientists,[199] structural analysis of the D-proteins suggests that they are mirror images of their L-protein counterparts. Importantly, the mirror imagery can be extended to the ligands of these proteins. The D-isomer of HIV protease hydrolyzed the same peptide sequence as the L-isomer and was blocked by previously identified inhibitors of the L-isomer if and only if the substrate and the inhibitors were themselves synthesized with D-amino acids.[199] The fact that the ligands of the D-HIV protease must be mirror images of the natural ligands implies that one can select for L-peptides that bind to a chemically synthesized D-protein target and then synthesize the selected peptide as the D-isomer and use it to bind the natural L-isomer of the target.

This approach has been termed "mirror image" phage display.[202] In the original description of the technique, the D- and L-isomers of the c-Src SH3 domain were used as targets in a selection from a phage-displayed library of 10-residue peptides. Linear peptides that contained the expected polyproline sequences were isolated during the selection against L-SH3, while selection against D-SH3 produced cyclic peptides that did not resemble previously reported SH3 ligands. The D-isomer of one of these cyclic peptides was chemically synthesized and found to bind L-SH3 with a K_D of 63 μM. This approach has also been applied to the HIV protein gp41,[203] cell-surface sugars,[204] and amyloid Aβ(1−42).[205] Mirror image phage display may be of particular value for drug discovery, in that D-peptides are resistant to in vivo proteolysis and can serve as lead compounds for drug development.[206,207]

7.4. Binding to Inorganic Materials and Nanostructures

Selections from various libraries have been used to identify peptides that bind to a wide range of inorganic materials and nanostructures.[208] While it may seem surprising that one can readily isolate peptides that bind to a specific metal or mineral, various organisms are known to create a wide range of complex inorganic materials, and the assembly of these materials is carried out by peptide or protein-based systems.[208−213] Selections for these binders have been carried out in the customary fashion,[214−216] but this is probably not the best approach. Bound phage particles are eluted from protein targets using conditions that are known to disrupt protein−protein interactions, such as protease treatment or changes in pH. Our understanding of the interactions between peptides and metals, minerals, or carbon nanotubes is so limited that we cannot predict what treatment, if any, will serve as a general tool to remove phage from these targets. The limitations of pH-driven elutions have been shown by the identification of certain binding phage that are recalcitrant to acid elution.[217] The best approach may be to either release the bound phage with a protease that recognizes a cleavage site following the library or use PCR to amplify the coding regions of phage stably bound to the target.[217] In fact, the lack of consensus sequences discussed below may be a sign that only phage bearing low-affinity peptides are released from the target.

The peptides isolated in these selections have been subjected to a variety of analyses. In biological systems, selections against a particular target often identify a consensus, or a short sequence that can direct binding to the target regardless of where it is in the selected peptide.[218−221] Sequencing of the peptides selected by inorganic materials and nanostructures has thus far revealed what appear to be hints at a few consensus sequences for a particular target.[214,215,222−224] However, in some publications it is not clear whether the authors have discovered an actual consensus sequence or whether a single bacteriophage has been isolated multiple times. Other selections seem to isolate peptides with unrelated sequences.[216,217] The absence of clear consensus sequences is surprising and may indicate that there is a multitude of mechanisms by which peptides bind to these targets. What these mechanisms might be is not clear. Sano and Shiba have begun to answer this question for a single titanium-binding peptide by investigating the importance of individual amino acids in the peptide−metal interaction.[225] Another unanswered question for many of these peptides is their affinity for their targets. At least in the case of gold-binding peptides, that affinity seems to be moderate.[208]

Free peptides have been used to seed the growth of nanocrystals. Phage-displayed peptides that were selected for their ability to bind to silver are in fact able to induce the formation of silver nanocrystals from a solution of silver nitrate.[214,217] The authors speculate that the peptides interact with preexisting nuclei of silver atoms and produce a reducing environment at the surface of the crystals, thereby enhancing crystal formation. These peptides have subsequently been used to synthesize silver nanocrystals on bis(*N*-α-amido-glycylglycine)-1,7-heptane dicarboxylate nanotubes.[226]

Using the selected peptides in the context of a larger structure is far more common than using free peptides. In some cases, the investigators have evaluated the ability of phage-displayed peptides to guide crystal growth. For example, calcium carbonate crystals have been grown with phage particles that display calcium carbonate-binding peptides.[215] Similar work has been done with semiconductor materials[223] and metal alloys.[222] Phage that display selected peptides have also been used as templates; their long, thin structure has suggested the possibility of using them to guide nanowire and nanofiber construction.[227−229] Phage are also flexible enough to generate rings, so they may serve as templates for the construction of metallic rings.[230]

7.5. Selections for Improved Protein Stability

Although most selections revolve around protein−protein interactions, it is possible to design a selection that identifies polypeptides based solely upon their resistance to proteolysis. While these selections may find some use in the identification of mutations that protect a particular protein from a particular protease, they are more broadly applicable in the tasks of increasing protein stability[231,232] and discovering new structures.[233,234] Folded proteins are more resistant to proteolysis than unfolded proteins, and mutations that improve the energetics of folding should increase resistance to proteolysis.[235] It is, of course, possible to select for improved protein stability using other techniques.[236]

The most common technique for selecting proteolysis-resistant mutants relies upon the fact that the minor coat protein pIII contains multiple domains, all of which are necessary for phage infectivity (Figure 1). The two amino terminal domains, N1 and N2, are responsible for binding to the pilus and TolA, while the carboxy-terminal domain anchors pIII in the viral particle. Surprisingly, large proteins such as β-lactamase can be inserted between N2 and the carboxy-terminal domain without significant loss of infectivity.[237] Phage are known to be unaffected by some proteases under physiological conditions, but the introduction of a foreign sequence into a protein that is essential for infection suggests a simple selection procedure. If a library of protein variants is cloned into pIII between N2 and the carboxy-terminal domain using a phage system and the resultant phage are treated with a protease, then a phage can only infect bacteria and propagate itself if the insert is resistant to proteolysis.[238,239] To prevent selection for deletions of the insert from the capsid, one can add a capture step after the proteolysis.[240]

An alternative approach is to place the library of variant proteins at the amino-terminus of pIII, add an epitope tag to the extreme amino terminus of the displayed sequence, carry out proteolysis, and capture the phage carrying resistant sequences via the epitope tag.[240−242] Phage particles are susceptible to proteolysis under some conditions, such as elevated temperature, but this does not necessarily limit stability testing. If unfolding of the displayed protein is irreversible, the phage can be incubated under a given set of conditions and then brought back to physiological conditions for proteolysis and infection. Phage that have been subjected to a pH range of 4−10, incubation in 50% dimethylformamide, exposure to 4 M guanidinium chloride, or exposure to temperatures as high as 60 °C exhibit full infectivity upon return to physiological conditions.[238,239]

Proteolytic selection has been used in a number of studies concerning protein stability. One interesting result has been that proteins seem to be much less tolerant of mutations in stability-based selections than in selections based on function.[239,242] Furthermore, previous work has shown that mutations that increase stability can sometimes reduce activity.[243] If one wishes to produce a protein that is both more stable and more active it would be prudent to select for both activity and stability simultaneously.[244] It has also been possible to increase protein stability at high temperatures by selecting from a library of variant proteins with randomized surface residues.[245,246] Finally, this technique can be used to ask whether a naturally occurring mutation destabilizes a protein. For example, numerous BRCA1 mutations found in breast cancer patients cause the BRCA1 protein to misfold.[247]

7.6. Rapid Analysis of Mutant Affinities

Phage display can be used for the rapid, facile quantitation of amino acid binding energies at a given protein−protein interface. Alanine scanning has traditionally been used to determine how important a given amino acid is for a protein−protein interaction. While effective, this approach is very labor intensive. It requires making the mutant, verifying it by sequencing, expressing the mutant protein, purifying the mutant protein, and subjecting it to an array of biophysical tests. It has recently been shown that "shotgun alanine scanning" can accomplish the same task much more rapidly.[112,248]

Shotgun alanine scanning relies upon well-established library production protocols and affinity selection approaches. First, a phage-displayed library is made in which the interface amino acids of one binding partner are largely either wild-type or alanine. For most proteins, the total number of different amino acid combinations is usually below the size of the library.[248,249] The library is then subjected to two parallel selections. One selection is designed to isolate properly folded proteins, while the second aims to identify library members that can form a particular protein−protein interaction.

The DNA sequences of phage from both types of selections are then used to quantitate the contribution of individual amino acids to binding.[248] The DNA inserts of hundreds of output phage are sequenced, and the ratio of wild-type amino acid to alanine is recorded at each randomized position. Given that these phage particles are a selection output, the ratio of wild-type to alanine (WT/Ala) is dependent upon the binding ability of the displayed protein. Thus, WT/Ala is assumed to be equivalent to the ratio of equilibrium binding constants. Once this assumption is made, the WT/Ala ratio can be used in the equation $\Delta\Delta G = RT \ln(K_{eq_{WT}}/K_{eq_{Ala}})$, and one can calculate the

change in free energy with respect to both folding ability and binding ability.

Given that a mutation that affects folding may also show a pronounced effect on binding, regardless of whether it is directly involved in the protein−protein interface, one has to control for perturbations to protein structure. The two selection series allow for this comparison. The free energy change for the folding selection is subtracted from the free energy change for the binding selection to yield the free energy change in binding associated with mutating a given amino acid to alanine. When applied separately to human growth hormone and its receptor, shotgun alanine scanning gave results in good agreement with alanine mutagenesis.[248] The technique has since been applied to other protein−protein interactions,[250,251] adapted to carry out homologue scanning,[252,253] and applied to the interaction between streptavidin and biotin.[254] A similar procedure, relying upon ratios of ELISA signals rather than amino acid frequency, has also been published.[255]

7.7. Artificial Transcription Factors

While any scaffold can, in principle, be used as a starting point in a search for a DNA-binding domain, the work already done through millions of years of evolution suggests that natural DNA-binding domains are the preferred starting point. One such domain, the Cys_2His_2 zinc finger, occurs more than 4000 times in the human genome and has emerged as a common experimental system.[256] These domains are roughly 30 amino acids long, and require metal ions to fold into their $\beta\beta\alpha$ architecture; the two antiparallel β-sheets fold onto the α-helix with the metal ion bridging the gap. The opposite side of the α-helix inserts into the major groove of DNA, and most of the DNA contacts involve three consecutive bases in one strand. Because contact with only three nucleotides is insufficient for genome-wide specificity, zinc fingers usually appear in groups, such that a single protein will bind to DNA using two or more zinc fingers.[257]

Structural analysis of protein−DNA complexes has revealed a wealth of information about the mechanism by which zinc fingers bind to DNA.[257] The crystal structure of the Zif268 DNA-binding domain complexed to DNA, the first such structure published, reveals three zinc finger domains connected by short linkers.[258] The zinc fingers all contact the DNA through their α-helices, which follow the major groove. This leads the protein to wrap around the DNA. Although some proteins carry more than three zinc fingers, one cannot assume that all of the domains will bind DNA simultaneously.[259] This may stem from linkers that do not allow the domains sufficient maneuverability.[260] Subsequent studies of other zinc finger proteins have revealed that only some bind to DNA in the same manner as Zif268.[257,261] The information derived from these structures has guided investigators in their efforts to develop zinc fingers that bind to a given DNA sequence.

Identifying zinc fingers that target new DNA sequences is best accomplished through selections from randomized libraries. The crystal structures of the first few zinc finger−DNA complexes suggested that a binding "code" could be developed, because it was thought that changing a few surface-exposed amino acids could alter the specificity of zinc fingers in a predictable manner. While this approach can be successful, the protein−DNA interface is subject to unpredictable context-dependent effects.[262−264] The complexity of these DNA−protein interfaces suggests that selections are necessary to effectively target a zinc finger protein to a particular DNA sequence.

Three major approaches to zinc finger selections have been described: parallel, sequential, and bipartite.[265] In parallel selections, a zinc finger protein is displayed on phage with one zinc finger randomized. Selections are then carried out against a DNA sequence that contains the recognition sites for the nonrandomized zinc fingers, as well as the three bases for which a domain is to be selected.[266−268] The expectation is that the new, selected zinc fingers can function as independent modules, able to be fused in any order to recognize any DNA sequence. Several reports have appeared in which these individually selected zinc fingers have been linked together to produce DNA-binding proteins with nanomolar or better affinities.[267,269] The approach seems to be generally applicable, although some combinations of zinc finger domains do not bind DNA sequences as predicted.[269] In an effort to evade position-dependent effects on zinc finger specificity, some investigators have completed a series of selections for DNA trimers, shuffled the output, and used the shuffled library for selections with the full-length target sequence.[270] Sequential selections are carried out similarly to parallel selections, except that the output of the selections, the new zinc finger, is used as the starting point for subsequent rounds of selections.[271] Thus, if the protein to be constructed carries three zinc finger domains, three different rounds of library construction and selection will be completed, one for each zinc finger. This approach ensures that the resultant protein will have zinc fingers that work well as a unit, but it is obviously much more labor intensive than parallel selections. Bipartite selection is a two-step process in which the target DNA is split in half and zinc fingers are selected separately for each of the two parts.[272] The genes for the zinc fingers selected in this first step are then fused and displayed on phage as a second library, and binders to the complete DNA sequence are selected.

The ability to target proteins to certain sites in the genome opens up an incredible range of possibilities.[273] New restriction enzymes have been designed and used to stimulate homologous recombination.[274−276] Investigators have designed new transcription factors to up- or down-regulate genes in tissue culture cells and have even observed physiological effects in mouse models.[277−284] Viral replication has been inhibited by repressing the transcription of viral proteins.[285−287] Zinc finger proteins have targeted integration of retroviral cDNA.[288] The recent construction of a cell-permeable transcription factor raises the possibility that the therapeutic use of designed zinc finger transcription factors need not await the perfection of gene therapy.[289]

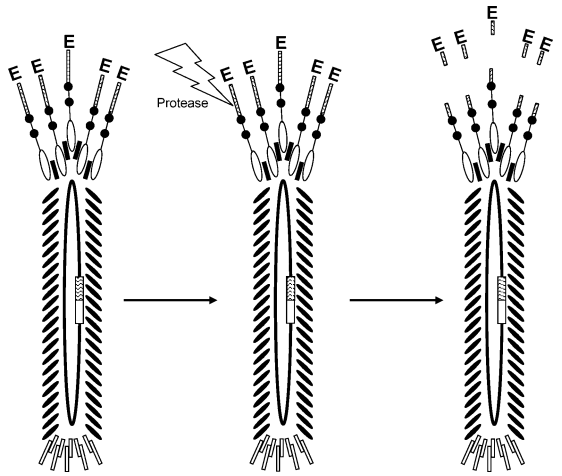

Figure 8. Selections for protease substrates. The library is fused with the amino terminus of pIII and then capped with an epitope tag (E). The phage are captured via the epitope tag, and protease treatment releases phage that display protease substrates.

7.8. Identification of Protease Substrates

Phage-displayed libraries for the identification of protease substrates are constructed such that the randomized amino acids are placed between the coat protein and an epitope tag (Figure 8). In most cases, the phage are immobilized via the epitope tag and then treated with the protease.[290] The expectation is that some of the randomized regions will be susceptible to protease cleavage, and the eluted phage can be grown for further analysis. Alternatively, the phage can be treated with protease, and the resistant phage can be captured and removed.[291,292]

Protease substrates identified with phage-displayed libraries can reveal natural, in vivo substrates of an enzyme. For example, characterization of membrane-type serine protease 1 (MT-SP1) with both chemically synthesized and phage-displayed peptide libraries suggested that protease-activated receptor 2 and the single-chain urokinase-type plasminogen activator are both in vivo substrates of MT-SP1.[293] Biochemical experiments confirmed that this was, in fact, the case. Simple identification of optimal peptide substrates has been carried out for a number of proteases, and in some cases these optimal sequences agree well with known or potential in vivo substrates.[291,294−300]

Peptides that are identified as substrates in these screens can also serve as valuable tools, whether or not they resemble an in vivo substrate. Small peptides that are effectively cleaved by a protease may serve as a starting point for drug design.[301] Substrates that are selectively cleaved by one enzyme can be used to estimate the concentration of that enzyme in a biological sample.[302] Tumor biopsies or other clinical samples may one day be used with substrate libraries to provide snapshots of protease activity.[303,304]

7.9. Enzyme Design

A great deal of work has been focused on the in vivo evolution of enzymatically active antibodies.[305,306] Many of these antibodies were isolated from animals that had been immunized with a transition-state analogue. Similar work has been done to select binders of transition-state analogues from phage-displayed libraries of antibody fragments.[307−309] Antibodies that bind to transition-state analogues can possess catalytic activity, but it is often modest relative to the catalytic capabilities of natural enzymes. In some ways this lack of equivalency is not surprising, because transition-state analogues can be inaccurate mimics of a transition-state structure. A second, underlying problem is that this process does not select for enzymatic activity; it selects for binding. Selections for catalysis would seem to be a better route for developing new catalytic proteins.

It is possible to select proteins from phage-displayed libraries on the basis of their catalytic activity. Many of these selections rely upon substrate homologues that bind to the enzyme covalently during or after catalysis. For example, some aldolases will bind covalently to 1,3-diketones and can thereby be selected from libraries.[310,311] Difluoromethylphenyl phosphate has been used as a capture reagent by way of its susceptibility to nucleophilic attack after phosphate hydrolysis.[139] Previously known suicide inhibitors, such as phosphonylating reagents, have been adapted to selections from serine protease libraries.[312,313] These substrates are similar in nature to those that have been used for the reactive immunization of animals.[314−319] A second approach is to design the selection such that the reaction product becomes attached to the phage. If the product of the desired reaction can be captured, it should be possible to isolate phage displaying the desired enzyme activity. Investigators have thus isolated improved versions of subtiligase,[320] mutants of human O^6-alkylguanine-DNA alkyltransferase that can label themselves (and, by extension, their fusion partners),[321] and mutants of DNA polymerase that can polymerize RNA.[322]

7.10. Cell Targeting

In recent years, there has been a great deal of interest in developing affinity reagents that bind specifically to a single cell type. The most direct approach to this problem is to carry out in vitro selections using cells as targets. Live adherent cells will capture phage from solution in the same manner as an immobilized protein.[323,324] Alternatively, selections can be conducted with suspensions of fixed or live cells.[325,326] While blocking agents such as BSA are occasionally used, the depletion of cross-reactive library members via incubations with multiple cell lines is probably a more effective strategy for the reduction of background.[327,328] As with selections against immobilized proteins, bound phage can be retrieved with extremes of pH.[323,327] If the investigator wishes to isolate phage that have bound to a specific receptor, it may be possible to elute only those phage by adding an excess of the natural ligand for that receptor.[324] Alternatively, one can select for phage that display peptide ligands to cell surface receptors through centrifugation. The biopanning and rapid analysis of selective interactive ligands, termed BRASIL, may prove to be a powerful method for identifying peptide ligands or generating affinity reagents to cell surface receptors.[329]

A more powerful approach to the identification of cell-specific affinity reagents is to carry out the selection in a living animal. Phage can be introduced into the animal's system through a number of means, including injection,[330] ventilation,[331] and gavage.[332] After a brief time period, tissue biopsies are collected, homogenized, and used to infect bacteria for phage amplification and subsequent rounds of selection.[331,333] Regardless of how they were created, cell-specific affinity reagents can be used for the specific delivery of therapeutics or diagnostics.[323,331,334−337]

8. Conclusion

The past two decades have seen an incredible growth in the use of phage display, and a number of related display technologies have recently been developed. The value of the genotype−phenotype linkage, which they all share, is beyond doubt. As we move forward to confront new scientific problems, the utility of these display systems will be limited only by our imagination.

9. Abbreviations

ssDNA single-stranded DNA
dsDNA double-stranded DNA
PCR polymerase chain reaction
CDR complementarity-determining region
EGFR epidermal growth factor receptor
IGFR insulin-like growth factor receptor
IgG immunoglobulin G
scFv single-chain fragment of the variable region
Fab fragment, antigen binding
WT wild-type

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CR000261R