PHAGE DISPLAY: A MOLECULAR FASHION SHOW

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The display of peptides and proteins on the surfaces of bacteriophages is a powerful technique for the identification of specific ligands. Today, phage display technology is a well-established method that has revolutionized our capability of selecting specific molecules. This technology, which was introduced in 1985 with the display of peptides on the surface of the filamentous bacteriophage M13 (100), has now expanded unimaginably, touching every field of science. A plethora of display vehicles and well-developed systems are available, and peptides of all kinds have been successfully displayed and used in diverse applications. The power of display technology emanates from the fact that there is a direct physical linkage between the peptide or protein displayed on the surface of the vehicle and the DNA encoding that peptide. As a result, while one selects for a binding partner based on the peptide (phenotype) displayed on the phage particle, the identity of the peptide (genotype) is encapsulated within the same particle. Also, since the selected particle can replicate, the displayed molecule can be decoded simply by sequencing the encapsulated DNA.

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APPLICATIONS OF PHAGE DISPLAY TECHNOLOGY

Phage display technology has enjoyed enormous success in recent years and has been used in countless studies. The following text highlights some of the major applications of this technology.

(i) The earliest example of phage display was given by George Smith for the display of peptides and proteins on the bacteriophage M13 surface (95, 100, 101). Today, almost all kinds of protein and peptide sequences have been displayed successfully on phages. Libraries of random peptide sequences are commercially available and can be used to obtain peptide binders to virtually any ligand.

(ii) Phage display technology has enjoyed phenomenal success in the fields of antibody discovery and antibody engineering (102). Besides providing a convenient

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technology for fast, high-throughput identification of antibodies and elucidation of their epitopes, phage display has made the production of completely human antibodies directed against every possible antigen a reality. Coupled with antibody engineering and intrabody production, phage display of antibodies provides a route to the development of future molecular medicines (39, 40, 56, 80, 109).

(iii) Phage display methods, in combination with other protein engineering technologies such as DNA shuffling and random or directed mutagenesis, have expanded the natural repertoire of protein structure and function, enabling the synthesis of custom-made or "designer" peptides and proteins (26, 32, 46).

(iv) Phage-displayed peptide libraries have been used for rapid determinations of the specificities (15) of proteases that are important in various diseases and to understand the importance of each residue in the identified substrate (76, 84).

(v) Phage display has been used to create novel DNA recognition domains, such as designed transcription factors for selectively regulating gene expression, for potential therapeutic applications (24, 79, 106, 113). This approach allows either upregulation or downregulation of transcription, unlike antisense or RNA interference approaches, which only inhibit gene expression.

(vi) Phage display libraries have been used to identify small peptide ligands and antibodies which can be used to detect biological threat agents, including toxins, bacteria, spores, and viruses (79, 106).

(vii) Gene therapy holds promise for the treatment of diseases for which effective pharmacological therapies are insufficient or unavailable. The currently used vectors for gene transfer show low efficiencies due to their lack of tissue selectivity. The greatest challenge for all delivery systems is to find ligands that have higher affinities for target cells than for normal cells. Phage display technology has been used to identify peptide ligands that are selective for specific cell types and to engineer novel gene transfer vectors incorporating these selected peptides, thereby improving the performance of gene delivery vehicles (1, 2). Phage display has applications in receptor-based research that include the identification of cell-, tissue-, and disease-specific receptors and their ligands and of peptides and antibodies which modulate receptor activity for use in drug design (26, 81).

(viii) Display libraries have been used to identify autoantibody diversity in autoimmune diseases, to map autoantigenic epitopes (111), to identify peptide mimics of non-protein antigens such as anti-DNA antibodies in systemic lupus erythematosus (14) as well as capsular polysaccharide antibodies in meningitis and other diseases (68, 70, 81), and to identify peptide antagonists of various cellular interactions (10, 36).

(ix) Recently, phage-displayed combinatorial peptide and cDNA libraries have been used to study protein-protein interactions (17), to identify drug targets, and to validate target-drug interactions (65, 85).

(x) Phage display technology is being used to study the appearance of novel antigens and patient immune responses in a variety of medical conditions related to aging, viral and bacterial infections, cancer, hematology (57, 73, 110), and allergy (12, 23).

(xi) Phage display is also being used to develop tailor-made synthetic ligands to develop affinity chromatography methods for the production of therapeutic plasma products (5).

(xii) In vivo phage display is being widely used to isolate organ- and tumor-homing peptides for use in the targeted delivery of chemotherapeutics, peptides, growth factors, and cytokines (4, 51). Cell-specific

peptides will be useful not only as drug delivery vehicles but also as diagnostic agents, affinity reagents for cell purification, gene delivery agents, probes to study the cell surface, and reagents for radio imaging and radiotherapy.

VARIOUS SURFACE DISPLAY PLATFORMS

With the increasing realization of the potential of phage display technology, there appears to be a need to develop display platforms that endow the user with the flexibility of displaying a diverse repertoire of peptides and proteins that vary in size and composition. The most popular platform for surface display has been the filamentous bacteriophage M13, wherein fusions are made to the major coat protein gpVIII or to the minor coat protein gpIII, and in a few examples, to other coat proteins such as gpVI (97). However, certain deficiencies and limitations of the M13 system (discussed below) have led to the development of surface displays by the use of other biological systems. Alternative phage display systems utilizing bacteriophages such as λ, T4, and T7, which assemble in the host cytosol and are released via cell lysis, have now been developed (8).

Recently, microbial cell surface displays for peptide library screening, bioadsorption, and live vaccine development have been reported (63). Outer membrane proteins of gram-negative and gram-positive bacteria have been used as vehicles to display foreign peptides on the bacterial cell surface (61). Peptide display on bacterial flagella (112), the staphylococcal surface (103), and the streptococcal surface by fusion to an M6 protein (78) has been used to develop whole-cell diagnostic devices and live vaccines and to study ligand-receptor interactions.

The requirement of posttranslational modifications for a variety of applications has led to the development of eukaryotic display systems. A large number of proteins have been displayed on the surfaces of *Saccharomyces cerevisiae* cells for studies of protein-protein interactions. Based on the concept of bacteriophage display, eukaryotic viruses, which allow large gene insertions, are easy to propagate, and are nonpathogenic to humans, are being engineered for surface display. These include baculovirus, adenovirus, retroviruses, and tobacco mosaic virus (31, 53, 107). Besides these, an in vitro selection system based on ribosome display has become available and is being used to select various high-affinity reagents (35a). Nevertheless, bacteriophages continue to be the most widely used display platforms, and various available display systems using bacteriophages are discussed below.

M13 DISPLAY SYSTEM

The filamentous bacteriophage M13 is the most popular and widely used display vehicle. The Ff class of filamentous bacteriophages (f1, fd, and M13) contain a circular single-stranded DNA genome encapsulated in a capsid cylinder made up of several proteins. These phages use the F pilus of *Escherichia coli* as a receptor for infection and are thus specific to *E. coli* strains containing the F plasmid. The phage genome carries 11 genes (named genes *I* to *XI*) which encode 11 functional proteins (gpI to gpXI). gpII, gpV, and gpX are involved in replication of the phage genome, while gpI, gpXI, and gpIV are involved in the assembly of the phage. gpVIII is the major capsid protein, while gpIII, gpVI, gpVII, and gpIX are the minor capsid proteins of the phage. Phage assembly occurs in the periplasmic space, where gpVIII assembles around the phage DNA genome, with gpVII and gpIX present at the emerging tip of the capsid (Fig. 1). The gpVIII protein continues to polymerize around the DNA until the end of the DNA is reached, when assembly is terminated by the addition of gpVI and gpIII (88–90).

All five capsid proteins of M13 have been shown to be suitable for functional display, including in most cases fusions to both the N and C termini of the capsid proteins (28, 45). Of the five coat proteins, three have been shown to tolerate the insertion of foreign peptides well. The major coat protein, gpVIII, is capable of displaying hundreds of peptide copies on the phage surface, gpIII can display up to five copies, and

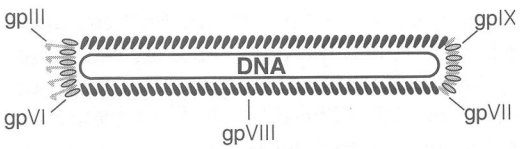

FIGURE 1 Schematic representation of the filamentous bacteriophage M13. The single-stranded circular DNA core is coated with five viral coat proteins. The schematic locations of the different proteins are shown. The gpVIII protein is present at about 2,700 copies, while gpIII, gpVI, gpVII, and gpIX are present at about 5 copies each. All of the coat proteins can be used as platforms for protein display. With the exception of gpIII, the capsid proteins are small, with 33 to 112 amino acids.

gpVI, as opposed to the first two, can display peptides as fusions only with its C terminus. However, gpVIII and gpIII are the most widely used display partners. For display as a fusion to gpIII and gpVIII, the DNA sequence encoding the peptide to be displayed is inserted between the sequence for the signal peptide and the amino-terminal coding region of the mature capsid protein. The insertion leads to the expression of a fusion protein which is directed to the periplasm and incorporated into the assembling phage particle, leading to the production of phage particles which display a peptide on their surface that is accessible for interaction in the surrounding milieu.

Several user-friendly M13-based vectors for displaying peptides and proteins fused to the coat proteins gpVIII and gpIII have been developed (35). These vector systems theoretically allow three to five copies of a foreign protein or peptide per phage particle to be displayed by the minor coat protein (gpIII) and up to 2,700 copies by the major coat protein (gpVIII). However, because of the potential for degradation of the fusion peptide, the real numbers displayed are likely to be somewhat lower. Also, the major coat protein does not permit the fusion of a peptide larger than about six to eight amino acids (33), probably as a result of interference of the displayed peptide with the process of phage assembly (42). Hybrid phages are therefore often used for display on either gpIII or gpVIII (25, 33). In this system, the vector backbone is derived from a phagemid carrying a cassette consisting of a regulated promoter followed by a signal sequence and cloning sites for inserting foreign DNA at the 5′ end of the gene that codes for gpIII or gpVIII. Phage particles are produced by infecting phagemid-transformed *E. coli* cells with a native phage. During assembly, the phagemid-encoded fusion proteins are incorporated into phage particles along with native coat proteins produced by the helper phage. Statistically, fewer than one fusion protein is generally displayed per individual particle (11). A large number of manuals and research papers describe established protocols for use of the M13 display system for a variety of applications, and a few of them are listed at the end of this chapter (3, 9, 20, 50, 77).

The success of any display technology depends on the efficiency with which polypeptides can be displayed on the phage surface. With a need for high-density display being increasingly felt, there has been a continuous effort to improve display densities of both gpIII and gpVIII display vectors. Appreciable improvements in gpIII-based phagemid systems have been reported as a result of using helper phage variants which provide no or very low-level expression of wild-type gpIII under selective conditions during display (55, 86). However, at maximum, only five copies of the fusion protein can be displayed per phage particle.

A different approach towards improving the display density of proteins fused to gpVIII in a phagemid-based system was the isolation of mutants of coat proteins that allowed improved display (98). Libraries of gpVIII mutants were created by either partially or fully randomizing 10-residue blocks of gpVIII. It was found that the N-terminal half of the protein was extremely tolerant to substitutions, whereas the C-terminal half was less accommodating. Mutant coat protein-bearing virions showed several orders of magnitude more binding in enzyme-linked immunosorbent assays than the corresponding phage particles displaying the same protein fused to wild-type gpVIII. The number of fusion proteins per phage particle was, however, not determined. These studies have demonstrated that

the M13 phage coat is extremely malleable and can be engineered for better display, expanding the utility of phage display as a powerful tool in biotechnology.

In one example, a selection system using a gpIII-based M13 library of *Staphylococcus aureus* DNA resulted in 20 to 40% positive clones after two rounds of selection. When the number of fusion proteins per phage particle was enhanced by use of a gpVIII-based display, the frequency of correct clones increased to 75 to 100% (44). Rapid progress is now being made in engineering coat proteins of phages to improve them as display platforms.

The M13 system, though extremely popular, suffers from several drawbacks. The requirement of periplasmic secretion of the display peptide imposes a constraint on the sequence repertoire that can be efficiently displayed. Also, a display density of several hundred copies of a variety of peptides and protein domains has not been achieved with M13 systems. The use of M13 vectors for the display of cDNA-encoded libraries is also not well established because of the lack of efficient C-terminal display in M13. Alternative systems based on large-genome phages, mainly T7, T4, and λ, have been used recently.

T7 PHAGE DISPLAY VECTORS

T7 is a double-stranded DNA phage that assembles inside the *E. coli* cell and is released by cell lysis (22). Studier and coworkers have described display vectors based on bacteriophage T7. This system has the capacity to display peptides of up to 50 amino acids at a high copy number (415 per phage) and peptides or proteins of up to 1,200 amino acids at a low copy number (0.1 to 1 per phage). The T7Select phage display system (available from Novagen) uses the T7 capsid protein to display peptides or proteins on the surface of the phage (87). The capsid protein of T7 is normally made in two forms, 10A (344 amino acids) and 10B (397 amino acids), and is present as 415 copies per capsid. 10B is produced by a translational frameshift at amino acid 341 of 10A and comprises up to 10% of the capsid protein. There are two basic types of T7Select phage display vectors, namely, the T7Select 415-1 vector, for high-copy-number display of peptides, and the T7Select-1 and -2 vectors, for low-copy-number display of peptides or larger proteins. In all vectors, the natural translational frameshift site within the capsid gene has been removed, and a series of multiple cloning sites following residue 348 of the 10B protein have been inserted.

The T7Select 415 vector has been used for the display of functional peptides of up to 39 amino acids. Expression of the T7Select 415 capsid gene is controlled by a strong phage promoter (ϕ10), and the translational initiation site (S10) is the same as that in the wild-type phage. The capsid-peptide fusion protein is produced in large amounts during infection. The capsid shell of the phage is composed entirely of the capsid-peptide fusion proteins, thereby displaying 415 copies of peptides on the surface of the phage. The low-copy-number display vector T7Select-1 does not carry the promoter of the capsid gene, and the translational initiation site has been altered. The capsid RNA is still produced from phage promoters located further upstream of the gene, but the production of capsid protein is greatly reduced. T7Select-1 phage are grown on a complementing host (BLT 5403 or BLT 5615) that provides a large amount of the 10A capsid protein from a plasmid clone.

As reported above, the T7Select 415 system has been used to display (10 to 39 residues) peptides at high copy numbers (415 per phage). The T7Select-1 vectors have been used to display peptides and proteins at low copy numbers. The copy number per phage, as measured by Western blot analysis, has been 0.5 for herpes simplex virus Tag, 0.3 for the T7 single-stranded DNA binding protein, 0.2 for β-galactosidase, and 0.1 for T7 RNA polymerase (87).

In the T7 system, the elution of bound phage after biopanning cannot be done by use of a low pH, as in the case of filamentous phage-based display vectors. Therefore, elution with 1% sodium dodecyl sulfate is recommended. However, the eluted phage must be suitably diluted before amplification. An alternative approach involves the addition of mid-log-phase host cells

to the bound phage. Fusion proteins displayed on the capsid do not interfere with tail assembly or the infectivity of bound phage. Alternatively, T7 display vectors can be modified to contain a protease site between 10A and the fusion protein so that specific proteases can be used for the elution of phage bound to the solid surface following biopanning. The T7 phage is extremely robust, and a variety of agents can be used for selection procedures. The phage remains infective even after treatment with 1% sodium dodecyl sulfate, 5 M NaCl, 4 M urea, 2 M guanidine-HCl, 10 mM EDTA, or alkalinity up to pH 10. Another property that makes T7 an attractive display vector is its rapid replication time. Plaques form within 3 h at 37°C and cultures lyse within 2 h after infection. This enables multiple rounds of selection to be performed in a short time. Also, T7 is easy to grow, its DNA can be easily purified for cloning, and high-efficiency in vitro packaging systems are available.

T7 phage display-based cDNA libraries have been used for the identification and characterization of a novel angiotension-binding protein (47), vaccine candidates of *Brugia malayi* (30), and bacterial ribonuclease inhibitors (54), for the study of protein interactions (29, 41), and for the cloning of RNA binding proteins (13). The commercial availability of the T7 phage display system, optimized protocols, and ready-made libraries for selections has led to an increasing use of this system for a variety of studies.

T4 PHAGE DISPLAY VECTORS

The phage T4 capsid is composed of three essential capsid proteins, namely, the major capsid protein gp23 (960 copies per phage particle) and the two minor capsid proteins gp24 (vertex protein; 55 copies per particle) and gp120 (portal vertex protein; 12 copies per particle). In addition, the outer surface of the capsid is coated with two nonessential outer capsid proteins, HOC (molecular mass, 40 kDa) and SOC (molecular mass, 9 kDa). The following features of these two proteins make them suitable for the display of peptides and proteins. (i) HOC and SOC are not essential for T4 capsid morphogenesis, but if available, they bind with high affinities to sites on the outer surface of the capsid after the completion of capsid assembly but prior to DNA packaging. (ii) HOC and SOC are present at high copy numbers, with 160 copies of HOC and 960 copies of SOC per capsid particle. (iii) The elimination of one or both proteins by mutation does not affect phage productivity, viability, or infectivity. Apparently, these proteins provide additional stability to T4 phage under adverse conditions such as extreme pH or osmotic shock.

Because of these properties, both HOC and SOC have been employed for the display of a variety of peptides and proteins. For the display of peptides and proteins fused to SOC, first a gene fusion is created in a plasmid and then incorporated into the T4 genome via homologous recombination between the phage and the plasmid on each side of the plasmid *soc* gene. The T4 SOC display system has been used to display a 43-amino-acid domain (V3) of the gp120 protein of human immunodeficiency virus type 1 (HIV-1), with a large number of copies per capsid. The V3-displaying phage were highly antigenic in mice and produced antibodies reactive to native gp120 (83). The system was also used for the display of the poliovirus VP1 capsid protein (312 residues), although with a smaller copy number. T4 phage displaying SOC-VP1 were isolated from a 1:106 mixture by two cycles of a simple biopanning procedure, indicating that proteins of at least 35 kDa may be accommodated. A 271-residue heavy and light chain fused anti-egg white lysozyme immunoglobulin G antibody has been displayed in its active form attached to the carboxy terminus of the SOC capsid protein. Similarly, the other nonessential protein of the T4 capsid, HOC, was used to display the HIV-1 CD4 receptor of 183 amino acids, which was detectable by monoclonal antibodies against human CD4 domains 1 and 2 (82). The number of protein molecules displayed per phage particle was small (10 to 40 per phage), but the displayed molecules possessed a native conformation. Jiang and coworkers used T4 SOC and HOC to display a 36-amino-acid PorA peptide from *Neisseria meningitidis* as an N-

terminal fusion (45a). The displayed peptide was detectable by specific monoclonal antibodies in an enzyme-linked immunosorbent assay. They also showed that more than one subtype-specific PorA peptide can be displayed on the capsid surface and that the peptide can also be displayed on a DNA-free empty capsid. It was also demonstrated that PorA-HOC and PorA-SOC recombinant phages were highly immunogenic in mice and elicited strong anti-peptide antibody titers, even with a weak adjuvant such as Alhydrogel or with no adjuvant at all, suggesting that the T4 HOC-SOC system may be used for the construction of the next generation of multicomponent vaccines. With a bipartite phage T4 display system, random peptide libraries were created by fusing DNAs encoding five randomized amino acids at the 3′ end of *soc* and the 5′ end of *hoc* (66).

The description above shows that T4 phage may be used as a high-density display system. However, the cloning strategies required are not very simple, and no published report describes the construction of any library displaying large domains of proteins to select desired phage by biopanning.

LAMBDA DISPLAY SYSTEM

One of the lambda proteins used for display is the capsid stabilizing protein gpD. The product of the D gene of λ, gpD is a small protein (11.4 kDa) that is found as 405 to 420 copies per capsid (7). During phage morphogenesis, the packaging of λ DNA in the preformed prohead is followed by expansion of the prohead, which exposes sites for the binding of gpD to the prohead (43). gpD exists as a trimer on the phage head (114) and binds to the underlying molecules of gpE that form the prohead (Fig. 2). gpD acts to stabilize the phage head and is dispensable for heads containing <82% of the λ genome. This conditional requirement of gpD suggests that it can be used as a display vehicle and that the fused peptides and proteins would not interfere with phage assembly. A second protein that has been used for λ display is the tail protein gpV. It has been reported that the carboxy-terminal portion of gpV, which forms protrusions on the outer surface of the tail, is dispensable (48, 49). This makes it an ideal platform for the display of peptides and proteins fused at its truncated C terminus.

Several studies in recent years have shown displays of peptides fused to gpD and gpV. In one of the earliest studies, Sternberg and Hoess (104) demonstrated the display of proteins fused to the N terminus of gpD and the selective capturing of displaying phage with a reagent recognizing the fusion partner. Subsequently, Mikawa et al. (69) showed that fusions can be made to both the N and C termini of gpD and that proteins as large as β-lactamase and tetrameric β-galactosidase can be displayed in a functionally active form. Based on the enzyme activity, it was calculated that about 34 copies of β-galactosidase were displayed per capsid. In a recent study, the number of molecules displayed per capsid was found to be size dependent, and small peptides of about 10 residues were present at as many as 405 copies per phage (34).

Fusions to the carboxy terminus of gpV were first made with β-galactosidase and *Bauhinia purpurea* plant agglutinin. The displaying phages were affinity selected by the use of specific antibodies, and the displayed β-galactosidase was shown to be present in a tetrameric, functionally active form (67). Several peptides and larger protein fragments have been displayed as fusions to gpV (18, 19, 21).

Lambda display has been used for epitope mapping of monoclonal antibodies against a large number of human and microbial proteins (34, 58, 59, 72, 105). It has also been used to determine the DNA binding specificities of displayed DNA binding proteins (115). One of the most promising potential uses of lambda display is the construction of cDNA-encoded library displays. In one study, a hepatitis C virus cDNA library was displayed on lambda and used for affinity selection with monoclonal antibodies and patient sera (92). Libraries from the HeLa and HepG2 cell lines have also been constructed (75) and used for affinity selection with sera from patients with a chronic autoimmune disorder. In that study, new autoantigens

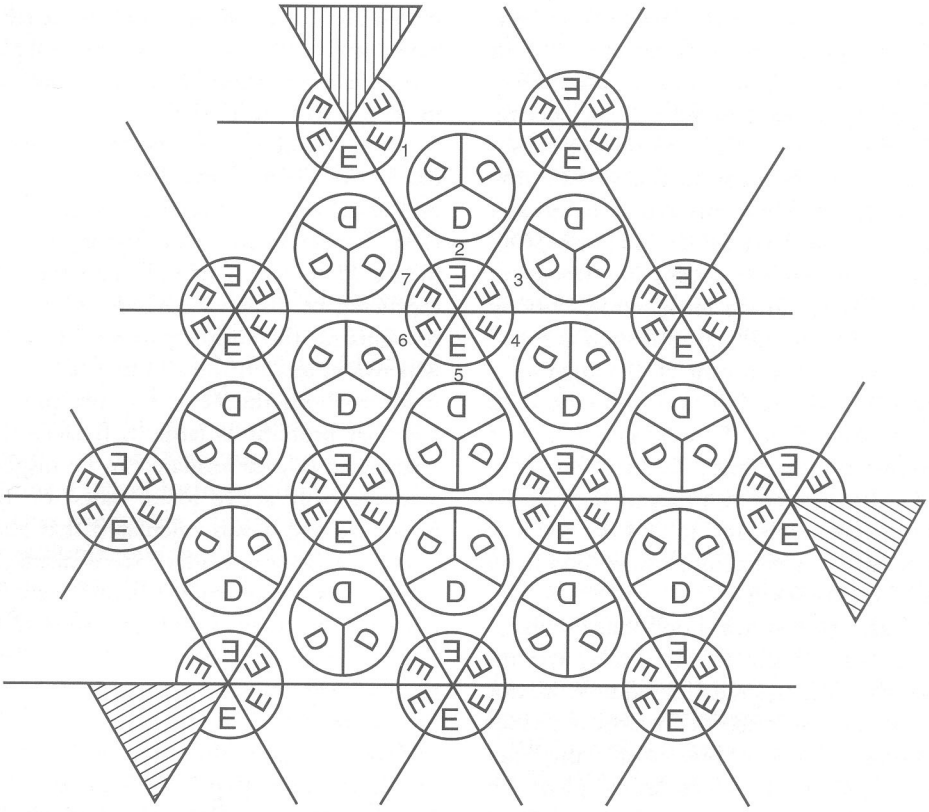

FIGURE 2 Arrangement of the bacteriophage lambda head proteins D and E. Both the D and E proteins are present at over 400 copies. The D protein serves as an efficient platform for display. Arrangements of the trimeric D protein surrounding the hexameric E protein are shown. The figure shows one face of the icosahedral head, with the vertices of the E pentamers. The three corners of the face are shown as triangles.

responsible for a specific syndrome were identified (115). A lambda-displayed yeast genomic library has been used to study protein interactions and to select clones that bind to a heat shock factor (64).

In comparison to filamentous phage display, lambda display has been used for a limited number of applications. This is attributed to several reasons: (i) lambda phage biology is more complex than that of M13 phage; (ii) the lambda genome is very large (48 kb), making the isolation of viral DNA, the insertion of user-defined restriction sites, the cloning of foreign fragments, and the packaging of the ligated product in vitro to form lambda particles difficult and the achieved library sizes smaller than those obtained with phagemid-based M13 vectors (96); and (iii) the intracellular assembly of phage may not allow disulfide bond formation in the molecule to be displayed.

However, lambda is an attractive display vehicle, as it has the ability to display multimeric proteins, has no requirement for the secretion of displayed fusion proteins, and allows the means to vary the valency of the displayed fusion proteins. It is being increasingly used for cDNA-encoded display (37). Large insertions can be tolerated in lambda without affecting phage morphogenesis and the display of encoded peptides.

Recently, a bacteriophage lambda system for the display of peptides and proteins fused at the

C terminus of the head protein gpD of phage lambda has been described wherein, by a highly efficient process of phage infection and in vivo recombination, the sequence encoding the peptide to be displayed on the lambda surface is integrated into the lambda genome and the peptide encoded by the cloned sequence is displayed as a gpD fusion protein on the surfaces of progeny lambda phage particles (34). In this strategy, DNA encoding the foreign peptide or protein is first inserted at the 3′ end of a DNA segment encoding gpD under the control of the *lac* promoter in a plasmid vector (donor plasmid), which also carries $loxP_{wt}$ and $loxP_{511}$ mutant recombination sequences. The site-specific recombinase (Cre)-expressing cells are transformed with this plasmid and subsequently infected with a recipient lambda phage that carries a stuffer DNA segment flanked by $loxP_{wt}$ and $loxP_{511}$ sites. Recombination occurs in vivo at the *lox* sites, and Amp^r cointegrates are formed. The cointegrates produce recombinant phage that display the foreign protein fused at the C terminus of gpD. Because the cloning is done in a plasmid, high transformation efficiencies (by electroporation) can be achieved. Also, the recombinations occur in vivo, eliminating the need to isolate lambda DNA, clone DNA sequences into it, and package the recombinant lambda phage in vitro. The frequency of recombinants (with inserts) at the plasmid level as well as at the phage level (cointegrates) is >90%, as opposed to the 3 to 15% reported for direct cloning in lambda display vectors (75).

This system uses two noncompatible *lox* recombination sequences, $loxP_{wt}$ and $loxP_{511}$ (38). Recombination can occur only in *trans*, resulting in the integration of the plasmid sequence into the lambda DNA. This strategy circumvents the problem of excision of the integrated plasmid from lambda DNA due to the presence of two compatible $loxP_{wt}$ sites in the cointegrate. The recombinants (cointegrates) formed can be easily selected for antibiotic resistance conferred by the integrated plasmid. The cointegrates contain the cloned foreign DNA sequence as part of the genome, and the corresponding phage display the encoded peptide or protein as a gpD fusion protein on their surfaces. With this system, >75% of cointegrates are double-crossover cointegrates and are inert to any recombination in *cis*. The single-crossover cointegrates have two $loxP_{wt}$ and two $loxP_{511}$ sites and can undergo recombination in a Cre^- host (91), which can result in either the formation of a double crossover or the loss of the integrated plasmid.

This lambda system was able to display proteins of different sizes (72, 156, and 231 amino acids), and the number of copies of each protein per phage particle on lambda was 2 to 3 orders of magnitude higher than that for display on M13 phage as a fusion to gpVIII or gpIII. The high-density display on lambda phage resulted in the selective enrichment of epitope-bearing clones from gene fragment libraries. The single-chain Fv fragment of an antibody was also displayed in a functional form on lambda, demonstrating that correct disulfide bond formation takes place during the cytosolic assembly of lambda particles in *E. coli*.

IMPORTANCE OF DISPLAY DENSITY

The screening of phage-displayed peptide and protein libraries is an effective method for finding much-needed "needles" in a vast molecular "haystack." An important property of display platforms in this regard is the copy number of the peptide or protein displayed on the surface of a single particle; this property is referred to as display density. Display densities as low as one copy per particle are important for protein engineering when one needs to isolate improved binders to bait, e.g., antibody molecules with high affinities for a ligand. Displays of 3 to 10 copies of the molecule are sufficient to allow the isolation of specific binders to bait when the strength of the interaction is in the nanomolar to micromolar range. However, for the study of weaker interactions (micromolar to millimolar range), the display density needs to be on the order of few hundred molecules per particle. In fact, a high display density is an essential requirement for a large number of upcoming applications, such as the use of display vehicles for

targeted gene delivery, in vivo diagnostics, the isolation of tissue-specific peptides, tissue imaging, etc.

Molecular interactions in biological systems are dependent on the concentrations and affinities of the interacting partners. Phage display gives us a way to modulate these parameters. It allows for variations in the concentrations of the reactants by varying the valency of the displayed partner and also allows for the evolution of higher affinity partners. For all of these parameters, it is important to first identify interacting molecules in the cell; there are numerous examples describing the use of phage-displayed peptide and/or cDNA libraries for this purpose, a few of which were listed earlier in this chapter. Analyses of the results obtained for several of these studies indicate the plausible benefits of the use of high-density display and the potential advantages afforded by increased display densities. Some of the future applications of surface display for which high densities of display will be beneficial are briefly mentioned below.

Phage display is used to study the specificities of immune responses of patients with various diseases, cancers, autoimmune disorders, and age-related conditions (14, 16, 93, 99, 111). This involves the use of random peptide libraries or gene/genome fragment libraries and cDNA libraries to identify peptides against which immune responses are elicited by patients. In the majority of these studies, the titers of antibodies in patient sera that are specific to the particular disease condition are relatively low, which may lead to difficulty in the identification of specific epitopes. High-density display on phages will be useful for selection procedures and the enrichment of specific binders from a large population of nonbinders (34). The identification of serum antibody-binding ligands will facilitate the design of diagnostic assays and therapeutic vaccines.

Intact phages displaying specific peptides or antibodies are used as biodetectors for a variety of threat agents, such as viruses, bacteria, spores, and toxins. For this use, phages displaying a dense array of the peptide or antibody will be more sensitive detectors because the detection limit will increase with the density of the detecting peptide (79).

Targeted gene delivery exploits specific receptor-ligand interactions and is therefore concentration, time, and affinity dependent. While it is not possible to modify these properties for the cellular receptor of the gene delivery vehicle, the vehicle can be evolved to incorporate all of these parameters for fruitful delivery. Phage display technology is used not only to identify high-affinity targeting peptides but also to evolve efficient delivery vehicles. Phages are being recognized as better gene delivery vehicles than existing systems because they are easy to handle, economical to produce to high titers, have no intrinsic tropism for various cell types (a drawback of several existing vehicles), and are relatively simple to genetically manipulate and modify per user requirements.

However, for effective gene delivery vehicles, it is also important to have sufficient copies of the membrane transporter peptide displayed on the outer surface of the gene transfer complex. Lambda phage encapsulates a large duplex DNA in a capsid shell with an approximately 55-nm diameter and can display various peptides on this capsid without any interference to capsid assembly and particle stability. Therefore, recombinant lambda phage particles which display peptides that facilitate transport across the cell membrane and encapsulate the therapeutic nucleic acid will be effective gene delivery vehicles (74).

It has been shown that multivalent display on phages increases the transduction efficiency from as low as 1 to 2% to 45% in certain cell lines (62). These studies showed that phage vectors can be improved by genetic modifications for enhanced cell targeting, increased stability, reduced immunogenicity, reduced nonspecific tropism, and other properties which are desirable for efficient gene delivery. Such delivery vehicles will also be useful for drug and vaccine delivery to specific cells (60). Phage displaying targeting peptides will also be useful as in vivo imaging agents.

Live vaccine development using heterologous carriers involves the use of viruses or other microbes displaying a candidate peptide on their surfaces as immunogens. In this case also, "landscape" vehicles, i.e., those which have a very dense display of the candidate peptide so as to cover the entire vehicle surface, will be better immunogens. Similarly, applications involving the use of surface display vehicles as biocatalysts or for bioadsorption will benefit from high-density display.

Phage cDNA libraries are useful for defining natural binding interactions. In this case, weak interactions can be more efficiently studied if the concentration of the displayed molecule is high. Recent studies have shown a novel utility of phage display. Bacteriophages expressing cell-specific peptides are internalized by mammalian cells, and the foreign genes harbored in the phage genome are delivered into the cells. This system would again benefit from a high display density of targeting peptide and will be a useful method for the intracellular expression of foreign peptides (108). Phage vectors can be engineered for receptor-mediated gene transfer to mammalian cells (71).

In a unique application of phage-displayed proteins, Frenkel and Solomon (27) showed that filamentous phage were able to penetrate the central nervous system and were able to deliver phage-displayed anti-β-amyloid antibodies via intranasal administration into the brains of mice. In another recent study, filamentous phage displaying cocaine-binding proteins on their surfaces reached the brains of mice after intranasal application and could sequester cocaine in the brain (6). This study suggested the application of this protein-based treatment for drug abuse syndrome. It is conceivable that for such brain-targeted delivery, a high density would be beneficial and phage lambda could be more useful. However, it remains to be seen if lambda phage will reach the brain after intranasal administration.

Experimental approaches using phage display are evolving rapidly. For example, the M13 phage display system was recently used for the isolation of peptides that home to white fat vasculature (52). After four rounds of in vivo selection, proapoptotic peptides specific for adipose sequences were isolated. These peptides were found to be associated with prohibitin and were found to be effective for fat tissue resorption. Furthermore, recent developments in genomic high-throughput target protein purification and immobilization in the native conformation (94) provide for rapid and direct selection of phages from phage-displayed combinatorial libraries. Such experimental systems combine high-level expression with in vivo protein biotinylation followed by binding to streptavidin-coated magnetic beads.

CONCLUSIONS

Phage display technology has come of age and will be a critical tool in the ongoing era of proteomics. Various display systems described in this chapter are undergoing refinement to increase the scope of this technology. Improvements in DNA cloning methods should enable larger libraries to be made in high-density display systems such as lambda, if desired, so that deficiencies of the low-density M13 system can be overcome. This will improve the cDNA libraries for a variety of interaction studies. High-density display systems could be explored for their utility as gene transfer agents and peptide delivery vehicles. Clearly, phage display technology can benefit a large number of upcoming areas of research in the near future.

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