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EDITORIAL

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Bacteriophage epitope libraries

The generation of specific binding proteins and peptides in vitro

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Abstract New concepts and methodologies that can be used to generate proteins, such as specific variable regions of immunoglobulins and other binding peptides in an in vitro selection system are reviewed. These technologies can also be used to alter the kinetics, affinity and avidity of various binding interactions. The nature of epitopes recognized by specific antibodies or receptors can be delineated using selected epitopes displayed on bacteriophages. The basic principle of the technology is predicated upon the belief that if one has a large enough variety of keys, one can open any given lock. The range of utility of these systems to generate new reagents will impact upon the development of new diagnostic and therapeutic reagents. This technology should allow for a much wider range of probes which may have increased binding capacity and allow the development of more sensitive assays with higher signal to noise ratios. These reagents can be produced more efficiently without the use of animals and will be used in diagnostic and experimental pathology. This brief review presents a concise description of the concepts and uses of this new technology. Selected references and reviews are given as sources for further details.

Key words Bacteriophages · Filamentous phage Epitope libraries · Recombinant antibodies · Panning

Protein-protein interactions are necessary for numerous biochemical processes and the formation of structural networks. An approach to studying the mechanisms involved in these processes is to generate proteins or peptides that specifically interact with the target proteins. These generated peptides or proteins can be used to study the kinetics of reactions or to locate the target protein in tissues or body fluids. Some of these studies have been performed using polyclonal antibodies, monoclonal antibodies (mAb) or peptides prepared as cleavage fragments or synthesized by solid phase methods. However, recently molecular biological techniques have been designed whereby a large number of variant proteins and protein fragments can be displayed on the surface of filamentous bacteriophages such as M13 or fd (Smith 1985; see Table 1). Other modified phagemid vectors have also been constructed for a higher efficiency of transformation. However, certain constructs of phagemids are defective and must be rescued by superinfection with a helper phage. The DNA coding for the inserted peptide or protein is spliced into the phage DNA in a position which allows the expression of this peptide or protein adjacent to the amino terminus of the phage surface proteins (Fig. 1). Each phage particle contains the genetic information for one unique inserted protein or peptide. Phage particles, which exhibit specific binding to a target, are selected using a variety of methods such as affinity chromatography or panning. Clones of the selected phages are purified and characterized.

The methods used for the construction and selection of useful phage clones and their applications are as follows.

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Generation of phage libraries

A. Generation of variable recombinant single chain antibody fragments (scFv):

1. Isolation of mRNA from lymphocytes.
2. cDNA preparation using reverse transcriptase.
3. Polymerase chain reaction (PCR) amplification of the DNA of the variable regions of the heavy and light chains with subsequent separation of the product DNAs on agarose gels.
4. Random linkage of the 3′ end of the heavy with the 5′ end of the light immunoglobulin (Ig) chain PCR

Table 1 Application of phage display in binding studies

A. Synthetic peptide libraries inserted into phage and used for ligand binding to:	
Monoclonal antibodies (mAb) specific for endorphins	Cwila et al. 1990
Streptavidin	Devlin et al. 1990
HLA-DRI	Hammer et al. 1992
Anti-Interleukin-1B	Felici et al. 1991
Anti-B gulactosidase	Parmley and Smith 1988
mAb A2, mAb_{33} and mAb B1312 (anti-myohemerythrin)	Scott and Smith 1990 Gong et al. 1993
B. Selection of recombinant antibodies using the phage display methodology:	
Anti-hepatitis B surface antigen	Zebedee et al. 1992
Anti-tetanus toxoid	Barbas et al. 1991 Duchosal et al. 1992
Anti-HIV (human immunodeficiency virus)	Burton et al. 1991 Tsunetsugu-Yokoto et al. 1991
Antimalarial (anti-*Plasmodium falciparum* circumsporozoite protein)	Greenwood et al. 1991
Antilysozyme	McCafferty et al. 1990
Anti-interleukin-2 receptor (p55)	Chang et al. 1991
Anti-4D5 [HER 2(new)receptor]	Garrard et al. 1991
Haptens:	
Anti-phox (2-phenyl-5-oxazolone)-bovine serum albumin (BSA)	Hoogenboom and Winter 1992 Clackson et al. 1991 Marks et al. 1991
Anti-NIP (4-hydroxy-3-iodo-5-nitrophenyl) acetyl-BSA	Hoogenboom and Winter 1992 Hawkins and Winter 1992 Hawkins et al. 1992
Anti-NPN (*p*-nitrophenyl phosphonamidate)-BSA	Kang et al. 1991
Antiprogesterone-3-(O-carboxymethyl) oxime-BSA	Gram et al. 1992
Anti-fluorescein-17-BSA	Barbas et al. 1992
C. Non-antibody proteins displayed in the phage binding system:	
Human growth hormone	Bass et al. 1990 Lowmann et al. 1991 Wells et al. 1993
Escherichia coli alkaline phosphatase	McCafferty et al. 1991
Bovine pancreatic trypsin inhibitor	Roberts et al. 1992a, b
External domain of platelet derived growth factor receptor	Jackson et al. 1993
CD4	

products with an intervening flexible linker. (This generates a large number of recombinatorial sequences whose product proteins are single chains which can fold into antigen binding sites). These have been called scFv.

5. Further amplification of the products using primers anchored to specific restriction site sequences.
6. Insertion, into phage or phagemid DNA, in a site directing expression into the pili (pIII) or coat protein (cpVIII) and transfection of the ligated DNA into *Escherichia coli* (*E. coli*).

B. Random peptide phage libraries

Random sequences of oligonuceotides, which code for random peptides with 6–15 amino acids, can be synthesized with the appropriate tail sequence necessary for ligation into a site adjacent to the genes for the coat proteins pIII or cpVIII of the phage. The resultant DNA library can code for a large number of combinations of amino acids (peptides). Most libraries will not be complete in terms of the theoretical complexity, but this may not be necessary for obtaining usable products.

Selection of phages expressing inserted proteins or peptides with affinity to specific targets

This is commonly performed by a produce called panning. A target protein, which can be an antibody, a receptor, an enzyme, or a structural protein or peptide, can be affinity tagged or immobilized and used to select for specific phages through binding interactions with the unique proteins expressed by the phage. The target protein is usually labeled with biotin. The phage binding to the biotin-labeled target is selected by panning with streptavidin-coated plates and washing away unwanted constructs (Parmley and Smith 1988; Diamandis and Christopoulos 1991). When the selected phage is segregated from the non-binding phage particles it is released from the affinity matrix and propagated in *E. coli*. The conditions for washing and elution can be constructed so as to select for binding proteins with different properties. The selection procedure is repeated for several cycles.

The phage library may initially contain millions of variant proteins or peptides in its repertoire. The insertion map and the phenotypic expression of inserted

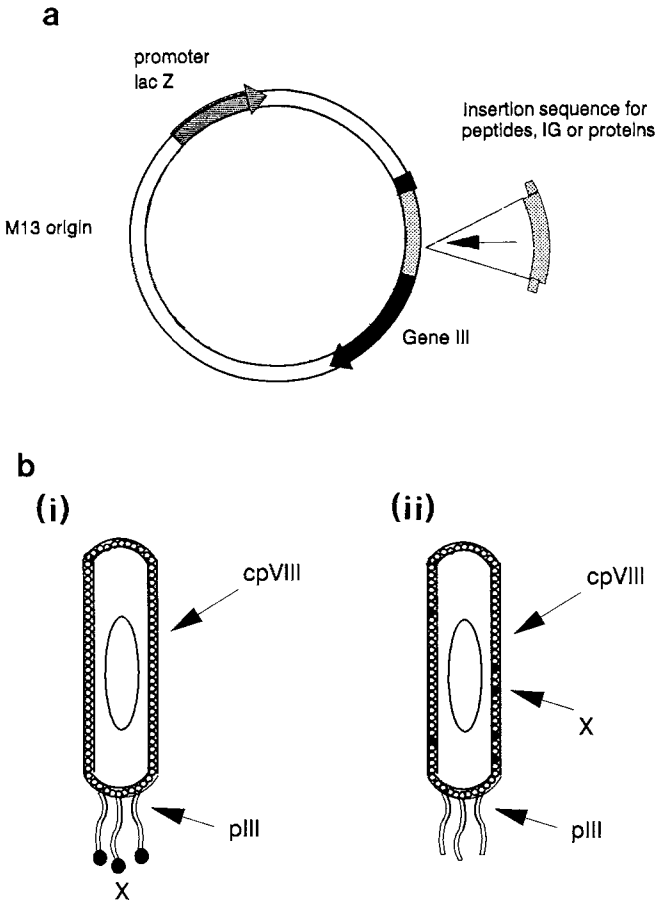

Fig. 1 **a** Map of bacteriophage M13 with insert in gene III. **b** Bacteriophage M13 with expression of a peptide or protein. *X* may be: random peptides, variable regions of immunoglobulins, hormones or enzymes which may be inserted adjacent to (i) pIII, of which there are 3 copies per phage or (ii) cp VIII, of which there are 2,500 copies per phage

genes on the filamentous phage M13 are shown in Fig. 1a, b. Typically the inserted test peptide or protein has been on the exposed adsorption protein pIII (Marks et al. 1992). Once the phage clone of interest is identified, the inserted region corresponding to the variable portions of the Ig or other inserted proteins can be sequenced and further mutagenized to alter the nature of their binding to the target. After mutagenesis, the Ig variable fragment or other proteins can be reselected by panning techniques (Gram et al. 1992; Wells et al. 1993). Using this approach, the binding constants of ligands to receptors can be markedly increased and the amino acid substitutions effecting the change can be delineated (Bass et al. 1990; Lowman et al. 1991).

Uses and advantages of phage display libraries

The rationale in the in vitro selection of Ig scFv mimics the antigen driven expansion and maturation of antibody producing cells that occurs in vivo in that it relies upon binding, subsequent selection and amplification (multiplication). A major difference is that the selective forces can be controlled in vitro. The repertoire of in vitro generated Ig fragments is not limited by host selection pressures which prevent the formation of autoantibodies. Several other advantages of in vitro selection of Ig and binding peptides are listed in Table 2. A major asset of these systems is that the library generated, whether it be a peptide library or a recombinatorial Ig library from pre-immunized or immunized B cells can be used over and over. The problems of animal experimentation are thus avoided.

Analogous experimental protocols to those described above can also be used to display and modify specific portions of enzymes, receptors or structural molecules in order to determine specific functionality of these fragments. The list in Table 1 gives some examples of proteins and peptides which have been put into the phage display. An example of the use of phage display is the placement of the human growth hormone (HGH) into pIII and panning with immobilized HGH receptor (Bass et al. 1990; Lowman et al. 1991; Wells et al. 1993). This system, along with site mutagenesis after panning cycles, has led to the production of HGH with a 400 times greater binding constant for the HGH receptor, leading to a better understanding of the hormone-receptor interactions. The selected HGH may have implications in designing antagonists to treat acromegaly or gigantism.

The epitope on a specific protein recognized by a specific monoclonal antibody can be defined using the phage display system (Fig. 2). The coding region for the antigen is cleaved with endonucleases or PCR amplified

Table 2 Advantages of in vitro selection of immunoglobulins and binding peptides

1. Lack of limitation (selective pressures) by the host immunoregulatory systems
2. Ability to set the conditions of binding (such as the number of exposed peptides or protein groups on each phage particle or the concentration of the target protein in the selection step) may be used to select for interactions with varied affinity or avidity
3. Rapidity of development (days instead of weeks) and decreased production costs
4. Ability to mutagenize and alter selected phage clones and to generate antibody fragments which have alterations in their binding properties and possibly even catalytic activity
5. Ability to aid in the identification of specific epitopes recognized by specific monoclonal antibodies
6. Useful in defining characteristics of peptides in hormone-ligand or other protein-protein interactions and possible role as pharmacological agents
7. Ability to add peptides that facilitate separation, purification, identification and quantification and to make fusion products with toxins which may be useful in killing selected target cells

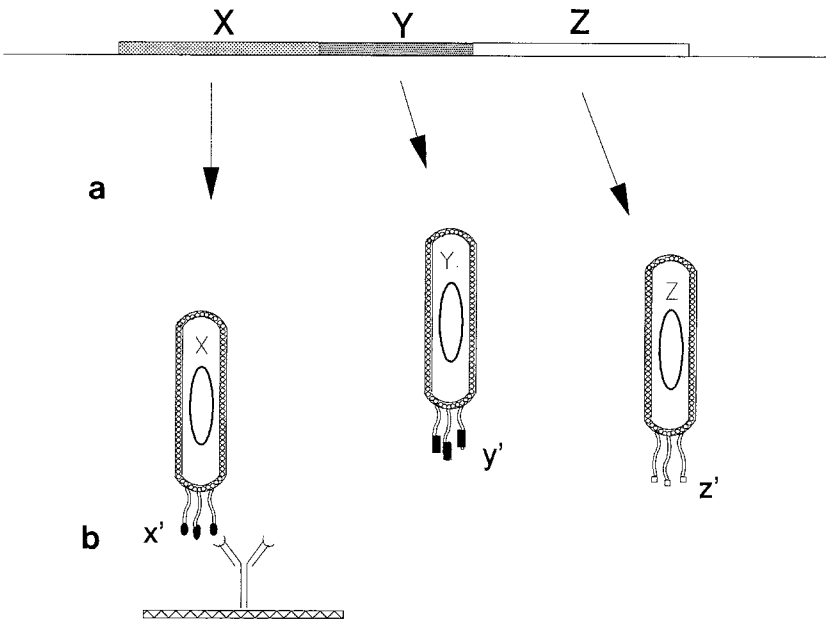

Fig. 2a, b Schematic approach for the localization of the epitope *x'* recognized by an monoclonal antibody against an antigen using the phage display methodology. Endonuclease digestion of the antigen-coding DNA or polymerase chain reaction with specific primers to obtain smaller fragments of DNA (*X, Y, Z*) which are then inserted in frame with the phage gene III (or gene VIII) for surface display (*a*). Identification of the phage bearing the epitope *x'* by panning with the monoclonal antibody and sequencing the DNA insert in phage *X* to reveal the peptide sequence of epitope *x'* (*b*)

to generate small fragments of DNA for insertion into the phage vector. The reading frame of the inserted DNA has to be in phase with the phage DNA in order for the epitope to be properly expressed. Identification of the recognition epitope is performed by panning for phage expressing fragments of the antigen protein with the selected mAb and sequencing the inserted DNA. This technology has been used on several mAb.

An exciting potential use for peptide epitope display libraries is the identification of ligands for proteins where the natural ligand is not as yet known. In studies where a mAb or a receptor with a known ligand was used as the panning agent, the nucleic acid sequence coding for the epitopes displayed in the selected phages had motifs homologous to the nucleic acid sequence coding for the natural ligands. The differences were often in single base substitutions in the coding sequence for the selected epitopes. The in vitro selected peptides may represent a mimicking of natural selective processes and evolution. The selected epitopes may be those nature has either already tried or those that may evolve depending on which have the most favorable selective advantages. The only selective force used to identify the chosen epitopes in most of these experiments is that of binding between the epitope displayed and the chosen target protein. Variants of this sequence can be selected which have either increased or decreased binding constants to the protein under study (Roberts et al. 1992a, b). Studies using the peptides generated from phage epitope libraries have shown that the peptides selected seem to interact preferentially with the regions of the target proteins thought to be exposed by models generated from X-ray crystallographic data (Gong et al. 1993). These peptides may be useful in confirming protein structure and in delineating the functional domains of proteins.

A new area of research is involved in redesigning antibodies to allow for the selection of antibody fragments that have catalytic activity (Iverson et al. 1989). A knowledge of the structure of certain Ig variable regions is allowing a redesigning of these Ig fragments using site directed mutagenesis to enhance specific catalytic properties or increase the binding constants (Gram et al. 1992). A similar approach may be used to alter the specificity of enzymes (Carter and Wells 1987; Wells et al. 1993). In addition to industrial applications, these catalytic antibody fragments may have uses in in vivo reactions for correction of enzyme deficiencies.

Other genetic manipulations can be applied in conjunction with the phage displays system. These include the placement of specific modifier sequences adjacent to the coding region for the protein displayed. These modifier sequences can be: (1) amber mutations which allow selective termination of the insert peptide when the vector phage is propagated in non-suppressor *E. coli* strains; (2) specific endonuclease cleavage sites; (3) specific sequences for oligonucleotide hybridization and subsequent PCR amplification; or (4) sequences specifying peptides which allow rapid affinity isolation and purification of the selected displayed protein. These tags or fusion proteins may also be useful as affinity ligands for localization and/or quantification of the isolated protein in immunohistochemical reactions of enzyme-linked immuno-sorbent assays.

There are several other reasons for the development of in vitro systems for generating antibodies. These include the generation of bifunctional reagents containing either heterologous binding sites or binding sites with detector enzyme activity. The genes for certain Ig fragments, which have specific binding to cancer cells, have been spliced with genes for toxins. The antibody fragments which have been produced have been used in

attempts to deliver the toxins to specific tumor cells (Waldmann 1991; Pastan 1993). Some hybrid antibodies are being used in developing new in vitro diagnostic assays or in immunohistochemical studies.

There are other techniques for detecting and selecting peptides which bind to proteins. One method uses random synthetic peptides linked to beads. In this method a fluorescent target protein can be used to select for beads with the highest binding and the peptide on the bead can be micro sequenced. In this type of selection, the repertoire (approximately 10^5 combinations for a hexapeptide) is usually smaller than that in phage libraries (Lam et al. 1991), usually due to the physical restraints of the bead size. Because this system does not have the DNA component (or genetic information) for replication in a biological system, there is no opportunity for subsequent mutagenesis and reselection. An advantage of this non-biological system, however, is that substituted amino acids such as *D*-amino acids can be used in the selection library. These may have advantages in being resistant to proteases and may be more suitable as pharmacological agents. In addition to peptides on beads, specifically located peptides on a matrix are being used to find interactions with proteins (Fodor et al. 1991). Peptides bound to oligonucleotide tags, which are primers for PCR reactions, are being used to locate and identify which peptides bind to target proteins. Vast numbers of combinations of nucleic acids in oligomers of RNA (Irvine et al. 1991) and DNA (Brenner and Lerner 1992) are also being used to find specific binding interactions with target proteins (Amato 1992).

The phage display technology is in its infancy. It is possible within the next several years that the methodologies will evolve so that many of the protein or peptide reagents that are produced for diagnostics or therapeutics will be selected or produced in bacteriophage systems. Recently, commercial packages have become available which include all the reagents for inserting the DNA sequence for Ig scFv or other peptides into a phagemid (Pharmacia Biotech, Almeda, Calif., USA). The user does not have to be extensively trained as a phage geneticist to generate usable products. These kits may be analogous to early software for computers which allowed computer usage by those of us who were not professional programmers. Improvements in the phage display system will include the development of predictive modeling data bases for protein-protein interactions which will allow smaller repertories to be used in selection. This, along with automation, will facilitate rapid selection characterization and scaled up production. The range of applications will only be limited by the imagination of the investigator and the commercial utility of the products. For further details the reader is referred to Winter and Milstein (1991), Dower (1992), French (1992), Huse (1992), Jackson et al. (1992), Joyce (1992) and Marks (1992).

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