Chapter 2

Antibody phage display technology and its applications

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Immunotechnology **4**(1), 1-20 (1998)

Abstract

In recent years, the use of *display* vectors and *in vitro* selection technologies has transformed the way in which we generate ligands, such as antibodies and peptides, for a given target. Using this technology, we are now able to design repertoires of ligands from scratch and use the power of phage selection to select those ligands having the desired (biological) properties. With phage display, tailor-made antibodies may be synthesized and selected to retain the desired affinity of binding and specificity for *in vitro* and *in vivo* diagnosis, or for immunotherapy of human disease. This review addresses recent progress in the construction of, and selection from phage antibody libraries, together with novel approaches for screening phage antibodies. As the quality of large naïve and synthetic antibody repertoires improves and libraries becomes more generally available, new and exciting applications are pioneered such as the identification of novel antigens using differential selection and the generation of receptor a(nta)gonists. Combining the design and generation of millions to billions of different ligands, together with phage display for the isolation of binding ligands and with functional assays for identifying (and possibly selecting) bio-active ligands, will open even more challenging applications of this inspiring technology, and provide a powerful tool for drug and target discovery well into the next decade.

1. Introduction

The generation of new drugs has long involved the search amongst hundreds of thousands of components using well defined *in vitro* screening tests, the output of which was chosen to mimic as closely as possible the desired *in vivo* activity of the new drug. Now new library methodologies offer many alternative and at least as powerful routes, by combining the creation of billions of components with a fast screening or selection procedure to identify the most interesting lead candidates. One of the most widely used library methodologies is based on the use of filamentous phage [1], a bacteriophage that lives on *Escherichia coli*. Phage-display has proven to be a very powerful technique to display libraries containing millions or even billions of different peptides or proteins. One of the most successful applications of phage display has been the isolation of monoclonal antibodies using large phage antibody libraries [2]. Indeed, in the last few years, very efficient techniques have been developed to design and build large libraries of antibody fragments, and ingenious selection procedures have been established to derive antibodies with the desired characteristics. Here, we review the progress made in this rapidly developing field, and discuss a broad range of applications, including the use of large

phage antibody libraries to discover novel targets on cells and methods for selection of biologically active ligands. Finally, we address the potential of combining phage display with complementary technologies, to increase the scope and range of applications of this technology.

2. Antibody Phage display

2.1. The phage display principle

The power of the phage display system is illustrated in Fig. 1. DNA encoding millions of variants of a certain ligands (e.g. peptides, proteins or fragments thereof) is batch-cloned into the phage genome as a fusion to the gene encoding one of the phage coat proteins (pIII, pVI or pVIII). Upon expression of the ligands, the coat protein fusion will be incorporated into new phage particles that are assembled in the bacterium. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the ligand being presented on the phage surface, while its genetic material resides within the phage particle. This linkage between ligand genotype and phenotype allows the enrichment of antigen-specific phage, e.g. using selection on immobilised antigen. Phage that display a relevant ligand will be retained on a surface coated with antigen, while non-adherent phages will be washed away. Bound phages can be recovered from the surface, reinfected into bacteria and re-grown for further enrichment, and eventually for analysis of binding. The success of ligand phage display hinges on the combination of this display and enrichment method, with the creation of large combinatorial repertoires on phage.

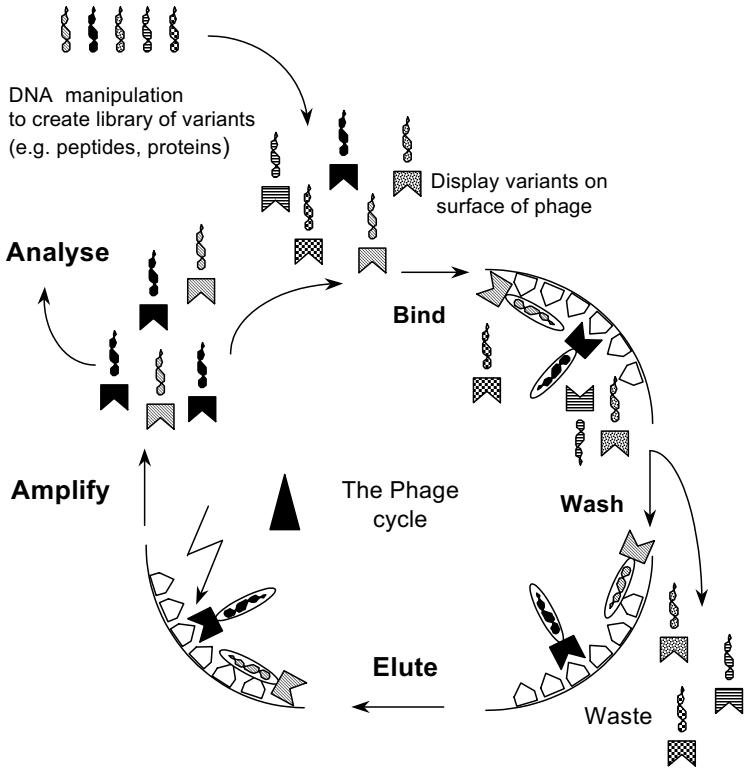

Figure 1

The phage display cycle

DNA encoding for millions of variants of certain ligands (e.g. peptides, proteins or fragments thereof) is batch-cloned into the phage genome as part of one of the phage coat proteins (pIII, pVI or pVIII). Large libraries containing millions of different ligands can be obtained by force cloning in *E.coli*. From these repertoires phage carrying specific binding ligands can be isolated by a series of recursive cycles of selection on antigen, each of which involves binding, washing, elution and amplification.

2.2 Filamentous phage biology and display

By far the most popular phage that has been used for display is the filamentous bacteriophage. The non-lytic filamentous phage fd or M13 infect strains of *E. coli* containing the F conjugative plasmid. Phage particles attach to the tip of the F pilus that is encoded by genes on this plasmid, and the phage genome, a circular single-stranded DNA molecule, is translocated into the cytoplasm. The genome is replicated involving both phage and host derived proteins, and packaged by the infected cell into a rod-shaped particle, which is released into the media. All virion proteins will undergo transport to the cell periplasm prior to assembly and extrusion. Several filamentous phage coat proteins have been used for display of ligands (for review, see an excellent issue of Methods in Enzymology [3] and a book on phage biology and display applications [4]). By far the most extensively used is the pIII phage protein, which is involved in bacterial infection and is present in three to five copies per phage particle.

2.3 Basic display methodology

Antibodies were the first proteins to be displayed successfully on the surface of phage [5]. This was achieved by fusing the coding sequence of the antibody variable (V) regions encoding a single-chain Fv (scFv) fragment to the amino terminus of the phage minor coat protein pIII. The antibody was displayed using a phage vector, based on phage fd-tet [6] and its gene III as fusion partner. In this vector, the genes encoding an antibody scFv fragment were cloned in frame with gene III and downstream of the gene III signal sequence, which normally directs the adsorption protein to the periplasm. Here, the VH and VL domains will fold correctly, both stabilised by a intramolecular disulphide-bridge, and pair to form a functional scFv [7, 8]. Initially, phage vectors that carry all the genetic information required for the phage life cycle were used [5, 9]. Now, phagemids, have become a more popular type of vector for display. Phagemids are small plasmid vectors that carry gene III with appropriate cloning sites and a phage packaging signal [10-12]. In phagemids, the scFv may be fused at the N-terminus of the mature gene III protein [5, 11] or at the N-terminus of a truncated pIII lacking the first two N-terminal domains [10, 13]. Phagemids have high transformation efficiencies and are therefore ideally suited for generating very large repertoires, as well as providing a format for direct secretion of the unfused antibody fragment, without subcloning [11].

Many phagemids utilize the *lac*Z promoter to drive expression of the antibody-pIII fusion [11, 13-15]. For display of the antibody-pIII product, either the catabolic repressor (glucose) of the *lac*Z promoter is removed or depleted, leading to expression of sufficient fusion product to derive 'monovalent' phage particles. Whenever expression-mediated toxicity is an issue (which is the case for some, mostly hybridoma-derived, antibody fragments [16]), it may be required to

regulate the expression more tightly. The use of a *lac*Z promoter with an additional transcriptional terminator [17] or of the phage shock promoter (psp) [18] may allow display of relatively toxic products and reduce expression-mediated library bias.

The phagemid DNA encoding the antibody-pIII fusion will be preferentially packaged into phage particles using a helper phage such as M13KO7 or VCS-M13, which supplies all structural proteins. Since helper phage also encode wild-type pIII, typically over 90% of rescued phage display no antibody at all and the vast majority of the rescued phage particles that do display the fusion product will only contain a single copy. Ideally, more efficient, even multivalent display would therefore be preferable when selecting very large antibody libraries to guarantee selection with a limited number of phage particles per clone. Monovalent display, on the other hand, may be essential when selecting antibodies of higher affinity. Therefore the use of inducible promoters [19] or the use of a helper phage with gene III deleted [20, 21], which may be produced efficiently in cells containing gIII under control of psp [18], may in the future allow the modulation of the valency of displayed antibodies.

2.4 Formats for antibody display

Effective display formats for antibodies are scFv [5, 9, 22], Fab fragments [10, 11, 13, 23], Fv's with an engineered intermolecular disulphide bond to stabilize the V_H-V_L pair ('dsFv's: [24]), and diabody fragments [25, 26]. The smaller size of the scFv format makes these libraries genetically more stable than Fab libraries. However, many scFv's can form higher molecular weight species including dimers and trimers, which can complicate selection and characterisation (see [25]). Fab fragments, on the other hand, lack this tendency to dimerise, which facilitates for example assays to screen the kinetics of binding (see 5.2). To display Fab fragments on phage, either the light or heavy (Fd) chain is fused *via* its C-terminus to pIII and the partner chain is expressed unfused and secreted into the periplasmic space, where it can associate to form an intact Fab fragment, as illustrated in Fig. 2. A similar method is used to express bispecific diabodies [26]. Such bispecific dimers of scFv's were displayed on phage by expression from a bicistronic cassette containing two VH-VL fusion products, one of which is also fused to gIII. The advantage of the diabody format is that either bivalent antibodies may be isolated, a feature that could be used for 'functional' screening (see 5.4) or that large panels of bispecific molecules may be generated, avoiding extensive recloning after selection [26].

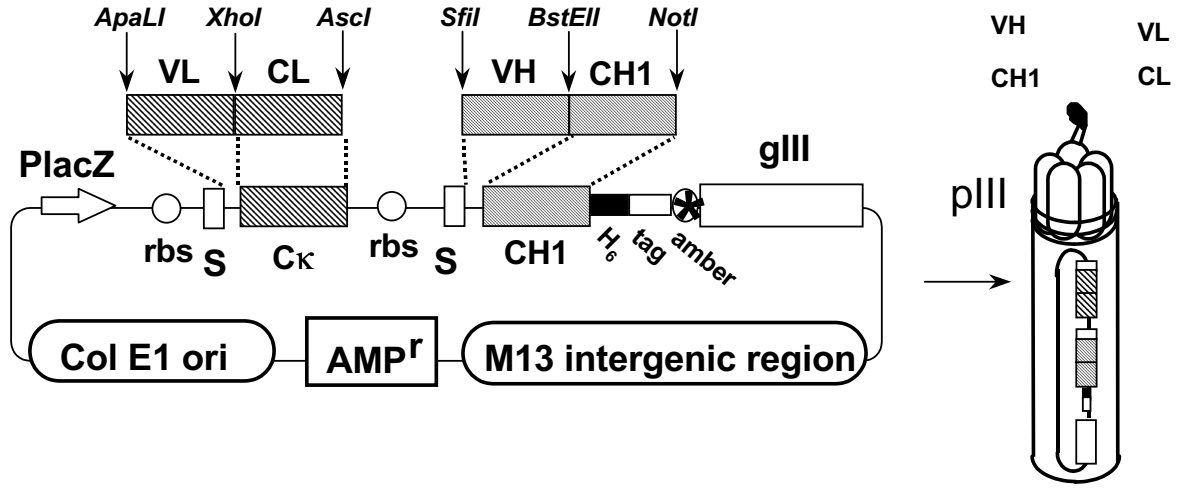

Figure 2

Display of Fab fragments on filamentous phage

Fab fragments may be displayed on phage using phagemids (pCES1 is shown asan example) that express the heavy chain (Fd) fragment containing the variable domain and the first constant domain fused to a coat proteingene, gene III, of filamentous phage fd, in combination with separate expression of the partner (light) chain. Bacteria harbouring this phagemid vector are superinfected with helper phage to drive production of phage particles carrying the Fab fragment, as a fusion product with the phage coat protein pIII, on the surface, and the DNA encoding the immunoglobulins inside the particle. Abbreviatirons : rbs, ribosome binding site; AMPr, ampicilling resistance, H_6 and tag, Histidine stretch and peptide tag respectively, for purification and detection purposes; ambercodon (TAG) that allows expression of soluble antibody fragment in non-suppressor strains; gIII, gene III for phage fd; S, signal sequence directing the expressed protein to the bacterial periplasm.

3. Antibody libraries

One of the most successful applications of phage display has been the isolation of monoclonal antibodies from large phage antibody libraries (Fig. 3). We will discuss the three types of such phage libraries, immune, naïve and synthetic antibody libraries.

3.1 Antibody libraries from immunized animals or immune donors

Repertoires may be created from the IgG genes of spleen B-cells of mice immunised with antigen [9] or from immune donors. An immune phage antibody repertoire will be enriched in antigen-specific antibodies, some of which will also have been affinity matured by the immune system [9, 27]. This method gives access to more and sometimes antibodies with higher affinity than obtained from hybridomas, as was reported for an anti-CEA antibody [28]. Other advantages of this procedure are that, compared to the hybridoma technology, many more antibodies may be accessed from the material of a single immunised donor, and selected antibodies can be rapidly derived or further manipulated.

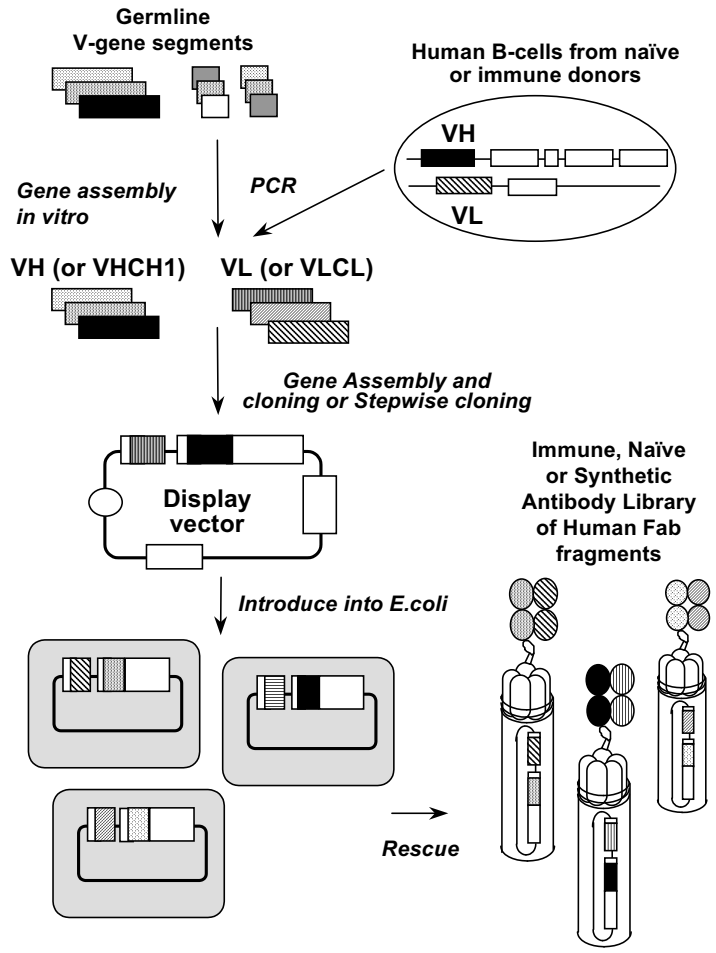

Figure 3

Construction of a human antibody library diplayed on phage.

cDNA fragments encoding for the heavy and the light variable regions of antibodies (V_H, V_L) are amplified from human B-cells by PCR and assembled. The assembled genes are inserted in a phagemid vector in frame with the gene encoding the coat protein pIII. The vector is introduced into *E. coli* . After rescue with helper phage, the random combinatorial library of antibodies is displayed on phage, and selection performed.

The construction of immune libraries from a variety of species has been reported, including mouse [9, 28, 29], human [30, 31], chicken [32, 33], rabbit [34], and camel [35]. Active immunization is, however, not always possible due to ethical constraints, neither always effective due to tolerance mechanisms towards or toxicity of the antigen involved. Tolerance mechanisms may be put to use in some cases, for example to deplete antibodies to certain antigens *in vivo* before immunization and selection of the new phage library on antigen *in vitro* [31]. Provided suitable sources of antibody producing B-cells or plasma cells are accessible, immune phage libraries are useful in analyzing natural humoral responses, for example in patients with autoimmune disease [36-38], viral infection (for review see [39]) or neoplastic diseases [31, 40, 41] (R.C.R. *et al*., in preparation), or to study *in vitro* immunisation procedures [42]. In addition, when studying specific (e.g. mucosal) humoral responses, mRNA coding for specific Ig isotypes (e.g. IgA) may be selectively used for library synthesis [43].

3.2 Single pot repertoires

From immune libraries, antibodies can be obtained only against the set of antigens to which an immune response was induced, which necessitates repeated immunization and library

construction. Ideally, universal, antigen-unbiased libraries would be available, from which very high affinity antibodies to any chosen antigen may directly be selected, independent of the immune history. At present, several of such 'single-pot' libraries have been described (reviewed in [2, 44]), which are particularly useful for the selection of human antibodies since these are very difficult to establish with more traditional techniques. We discriminate 'naïve' and 'synthetic' antibody libraries, depending on the source of immunoglobulin genes. For most applications, the availability of large pre-made collections of non-immune repertoires has thus superseded the use of immune repertoires.

3.2.1 Antibody libraries from non-immunised donors

The primary (unselected) antibody repertoire contains a large array of IgM antibodies that recognize a variety of antigens. This array can be cloned as a 'naïve' repertoire of rearranged genes, by harvesting the V-genes from the IgM mRNA of B-cells of unimmunized human donors, isolated from peripheral blood lymphocytes [22], spleen (de Haard, H.R.H., *et al*, unpublished), bone marrow or tonsil B-cells [45], or from similar animal sources [46]. In theory, the use of possibly heavily mutated and antigen-biased IgG V-genes should be avoided. However, even when using random priming to include mRNA of all Ig isotypes, a repertoire with excellent performance has been synthesized [45]. Libraries could also be made from the possibly more "naïve" pool of IgD mRNA.

The V-genes are amplified from the cDNA using family-based oligonucleotides [47], and heavy and light chains are randomly combined and cloned to encode a combinatotial library of scFv or Fab antibody fragments. This procedure generates access to antibodies that have not yet encountered antigen, although the frequency of those genuine 'germline antibodies' will depend heavily on the source of B-cells [48]. A single 'naïve' library, if sufficiently large and diverse, can indeed be used to generate antibodies for a large panel of antigens, including self, non-immunogenic and relatively toxic antigens [20, 22, 45].

The affinity of antibodies selected from a naïve library is proportional to the size of the library, ranging from 10^{6-7} M^{-1} for a small library, with 3×10^{7} clones [20, 22], to 10^{8-10} M^{1} for a very large repertoire with 10^{10} clones made by brute force cloning [45], a finding which is in line with theoretical considerations [49]. Other large naïve human scFv libraries (6.7×10^{9} clones) (J.D. Marks, unpublished) and a very large Fab library (4.1×10^{10} clones) (de Haard, H.R.H., *et al*., unpublished), made via an efficient 2-step restriction fragment cloning procedure, also seem to perform very well.

3.2.2 Synthetic antibody libraries

In the second type of 'single pot' repertoires, the antibodies are built artificially, by *in vitro* assembly of V-gene segments and D/J segments. V-genes may be assembled by introducing a predetermined level of randomization of CDR regions (and possibly also of bordering framework-regions) into germline V-gene segments [50], or rearranged V-genes [51]. The regions and degree of diversity may be chosen to correspond to areas of highest natural diversity of the antibody repertoire. Most natural structural and sequence diversity is found in the loop most central to the antigen combining site, the CDR3 of the heavy chain, while the five other CDRs have limited variation [52]. This has therefore been the target for introduction of diversity in the first synthetic libraries.

In the first synthetic antibody library constructed according to these principles [50], a set of 49 human VH-segments was assembled via PCR with a short CDR3 region (encoding either 5 or 8 amino acids) and a J-region, and cloned for display as a scFv with a human lambda light chain. From this repertoire many antibodies to haptens and one against a protein antigen, were isolated [50]. Subsequently, the CDR3-regions were enlarged (ranging from 4 to 12 residues) to supply more length diversity in this loop [53]. Other original designs have used only one (cloned) rearranged V-gene pair with a single-size randomized CDR3 region in the heavy chain [51], or have used complete randomization of all 3 CDR-loops in one antibody V-domain [54, 55]. Some of these libraries have yielded antibodies against many different antigens, including haptens [50, 51], proteins [53], and cell-surface markers [56], but their affinities are typically in the micromolar range.

Antibodies with nanomolar affinity were eventually isolated from a synthetic antibody library which combined a novel synthesis method to construct combinatorial libraries, *in vivo* recombination, with an effort to maximally mimic natural antibody diversity (i.e. to optimally use ‘sequence space’). In the largest synthetic library made to date [57], the 49 human heavy chain segments that were used earlier [50] were combined with a collection of 47 human kappa and lambda light chain segments with partially randomised CDR3 regions. The heavy and light chain V-gene repertoires were combined on a phage vector in bacteria using the *lox*-Cre site-specific recombination system to create a large 6.5×10^{10} clone repertoire of Fab fragments displayed on phage. The library yielded antibodies with in some cases nanomolar affinity against numerous different antigens [36, 57]. This phage library proved to be difficult to re-propagate without significant loss of diversity. However, a more stable, large 1.2×10^{9} clone scFv phagemid library made by standard cloning methods and using the same synthetic V-genes was recently shown to be equally effective (Heather Griffin, personal communication).

It would seem desirable to synthesize even larger collections of antibodies. However, there are physical limitations to the enrichment that may be achieved in the selection procedure, which limits the size of accessible genetic diversity. With enrichment factors for a single selection round never reported to be higher than 10^5 per round and a typical phage titre of 10^6 clones eluted in the first, critical, round of selection, the total genetic diversity accessed by the selection procedure would be at the most 10^{11} clones. If selection conditions are so stringent that very few phage particles are recovered in the first round (typical for, for example, panning on cells followed by sorting via flow cytometry [56]), chances are that different subsets of antibodies will be selected every time the selection is repeated. It therefore appears that it becomes more crucial to optimise the quality of the displayed antibodies (with regard to display and expression level), and the selection procedure itself. This is where synthetic antibody libraries will have a major advantage over naïve libraries using naturally rearranged V-genes. For example: the choice of V-gene segments for the construction of synthetic antibody repertoires may be guided by factors that will increase the overall performance of the library, such as good expression and folding and low toxicity in *E.coli*; this will increase the functional library size. Large differences in V-gene usage both *in vivo* and in phage repertoires [57] also suggest that some scaffolds may be better suited to form antigen-binders than others. Such a promising second generation synthetic antibody library is being built by MorphSys, using V-gene segments based on 'master' frameworks representing each of the Kabat subclasses, incorporating in principle only well expressed scaffolds. To overcome the problem of introducing stop-codons within the area of diversity, which would also decrease the functional library size, they assembled V-genes with oligonuceotides made from trinucleotides instead of from single bases [58] (MorhoSys, unpublished).

It is likely that further thoughtful design will continue to improve the performance of these libraries. For example, pre-selection of amplified and displayed synthetic V-domains on Ig-domain binding proteins (protein A for V_H, protein L for V_κ_etc. [59]) would remove clones with stop codons and frameshifts, as well as select for functional expression (Tomlinson, unpublished). Finally, an exciting idea is to combine the complementary diversity of the primary (germline) and secundary (somatic hypermutation) antibody libraries in one single phage antibody library. This may be feasible, since only a few residues are known to be hotspots for the hypermutation machinery. These developments may well eventually establish a 'super' library, which may contain antibodies of a superior affinity than what nature-made B-cells have on offer [44, 60].

3.3 Secondary phage libraries for affinity maturation

Although the antibodies selected from many of the immune and even the large single pot repertoires may be extremely useful for the researcher for ELISA, Western blot, immunofluorescence etc., their affinity is often not sufficiently high for therapeutic applications as immunotherapy, viral neutralization, or for use in sensitive diagnosis. Sufficient gain in apparent affinity may be achieved by simply constructing multivalent molecules, as reviewed in [61, 62]; however there will be situations in which *in vitro* affinity maturation of the selected antibodies is required.

The process essentially involves three steps, (1) introduction of diversity in the V-genes of the anibody (or antibodies) chosen to mature, creating a 'secondary' library, (2) selection of the higher affinity from the low affinity variants, and (3) screening to allow discrimination of antibody variants with differences in affinity or kinetics of binding. Diversity in the antibody genes may be introduced using a variety of methods, either more or less random using mutator strains [63, 64], error-prone PCR [65], chain shuffling [9, 66], and DNA shuffling [67], or directed to defined residues or regions of the V-genes using codon based mutagenesis, oligonucleotide-directed mutagenesis and PCR techniques [58, 68-71]. The non-directed approaches have been used to mature some antibodies with relatively low starting affinity [63, 66, 70, 72]. Once antibodies with nanomolar affinities are used as starting leads, it appears that CDR-directed approaches are more successful. For example, residues that modulate affinity may be randomised, ideally 4-6 residues at a time to allow efficient sampling of the sequence space. Such residues that contact the antigen or influence other residues contacting the antigen may be defined experimentally by chain shuffling [16], by alanine-scanning of the CDR-regions [73], by parsimonious mutagenesis [74, 75], or by modeling [73]. Targeting CDRs in parallel has been carried out [71], but additive effects of mutants are frequently unpredictable. The most successful approaches report improvements of affinity to below 100 pM, by saturation mutagenesis and affinity selection of CDR3 of heavy and light chain which form the centre of the antibody combining site [70, 71]. A detailed study of the sequence diversity of human antibodies created in the primary and secondary immune responses also suggests other key residues for targeting in affinity maturation studies [60, 76]. Several chapters in [77] give detailed protocols for creating secondary phage libraries; the selection and screening for clones with affinity differences is discussed below.

3.4. Beyond phage display libraries

Before we discuss selection and screening procedures in detail, we report on a very recent development that may well allow even larger repertoires of biomolecules to be made, as well as

facilitate antibody affinity maturation. A display method has been described in which proteins are entirely *in vitro* translated by, displayed- and selected on ribosomes [78, 79]. The polysome complex containing the encoding mRNA and translated amino acid sequence are utilized for selection with a ligand. The mRNA from selected polysomes is converted into cDNA and used for the next transcription, translation and selection round. Antibody fragments, and in particular scFv's, may form functional molecules in several cell-free translation systems ([80] and ref. in [79]). This approach has therefore recently been used for the display [79, 81] and evolution of a scFv antibody *in vitro* [79]. This system has the major advantage that the diversity of any repertoire of proteins will not be limited by the host cell/phage life cycle. The size of repertoire that can be sampled is potentially unlimited, and its generation would be very fast and greatly simplified. It remains to be seen whether these *in vitro* methods will compete with the rather robust *in vivo* display technologies, but the fact that they are more amenable to automation will definitely favour a swift development.

4. Phage antibody selection procedures and applications

4.1. Diversity in selection methods

Phage antibody selections involve the sequential enrichment of specific binding phage from a large excess of non-binding clones. This is achieved by multiple rounds of phage binding to the target, washing to remove non-specific phage and elution to retrieve specific binding phage (a schematic outline is depicted in Fig. 1). Any method that separates clones that bind from those that do not, can be used as a selection method, and as such, many different selection methods have been used. In Fig. 4, top panel, the most popular procedures are listed. These include biopanning on immobilised antigen coated onto plastic plates, columns or BIAcore sensorchips [9, 22, 57, 82], selection using biotinylated antigen [65], panning on fixed prokayotic cells [83] and on mammalian cells [31, 56], subtractive selection using sorting procedures [56], enrichment on tissue sections or pieces of tissue [84], and, in principle selections using living animals, as was reported for peptide phage libraires [85]. The selection methods described in Fig. 4, panel A-F, have been extensively described and were reviewed elsewhere [2, 44, 77].

Phage antibodies bound to antigen can be eluted in different ways: with (one step or gradients of) acidic solutions such as HCl or glycine buffers [86, 87], with basic solutions like triethylamine [22], with DTT when biotin was linked to antigen by a disulphide bridge [20], by enzymatic cleavage of a protease site engineered between the antibody and gene III [88], or by competition with excess antigen [9] or antibodies to the antigen [89].

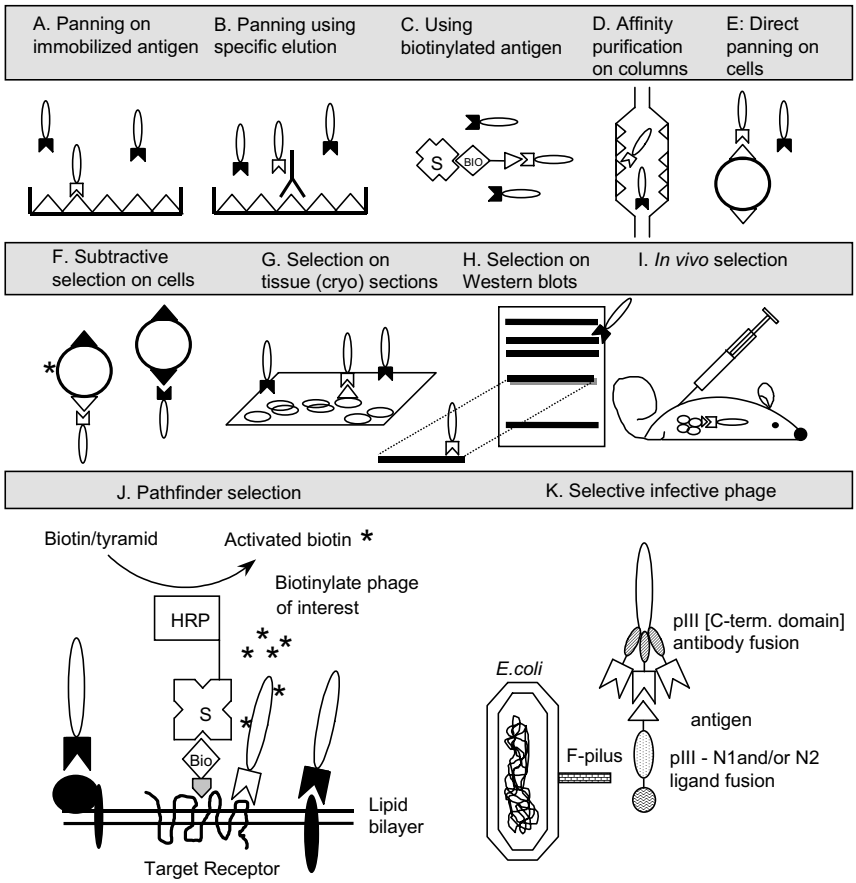

Figure 4

Selection strategies for obtaining specific phage ligands.

Affinity selection of phage (antibody) libraries by (a) panning on antigen adsorbed onto a solid support. After washing, specific phage is eluted with acid or basic solutions; (b) alternatively elution with antibody or an excess of the antigen is possible. (c) To avoid conformational changes during coating, selection of specific antibodies to biotinylated antigen in solution is more favoured. Bound and unbound phage antibodies are separated using streptavidin-coated magnetic beads. (d) Antigen can be immobilized onto a column for affinity selection. (e) Selection on cells can be done directly by panning on cell monolayers or cells in suspension. (f) Subtraction via FACS: the cells of interest are fluorescently labeled and separated from theothers by cell sorting. (g) Tissue and organ specific phage antibodies might be obtained by selection on tissue slides. (h) Non-purifiable or unknown antigens may be separated on SDS-PAGE and blotted onto membranes for selection. (i)*In vivo* selection. (j) The procedure of 'Pathfinder' selection, and (k) infection-mediated selection. Specific phage carry a white ligand, irrelevant phage a black one.

Phage background binding to the matrix or antigen itself inevitably necessitates the repetition of the selection procedure. Background problems may diminish when repeated selections of phage libraries are carried out without amplification step, by reusing the eluted and neutralized polyclonal phage ligands directly for selection, as reported for protease inhibitors [90]. Some recent evidence indicates that anti-hapten antibodies may also by isolated by 3 rounds of panning without re-amplification, using a very large naïve Fab library (de Haard, H.R.H., *et al.,* unpublished). If *in vivo* re-amplification steps could be by-passed, it might be possible to fully automate the isolation of antibody fragments from very large libraries. During affinity maturation studies direct selection without re-amplification may speed up the procedure and reduce selection of aberrant clones with a growth but no affinity advantage [16, 65].

4.2 Infection-based selection methods

Alternative selection methods have been described that aim at the co-selection of antibodies and their cognate antigens. In the 'selectively infective phage' (SIP) method, an antibody library is made using non-infective phage particles, by deleting part of the gene III protein. Infection will be restored by binding a fusion protein consisting of the cognate antigen and the two first amino terminal domains of gene III which are responsible for pilus binding (Fig. 5) [91-93], reviewed in [94]. The fusion product may be supplied *in vitro*; infection of the antibody-displaying phage will be mediated in a small concentration window. In the alternative, *in vivo,* system, both antibody and antigen are encoded on the same phage genome. Both affinity and folding properties have been shown to influence the selection of antibodies with the SIP methodology [95]. Finally, in a recently reported procedure, bacteria expressing epitopes within the context of the F pilus were proven to be infected by phage only when they displayed an antibody recognising the epitope [96]. It still remains to be seen whether these selection systems will be more efficient than standard procedures in isolating antibodies from very large naïve or synthetic antibody libraries. A better understanding of the infection process itself [97] may eventually lead to more generic application of these procedures in isolating interacting protein-ligand pairs.

4.3 Selection for affinity

In a similar fashion to what happens *in vivo* during B-cell selection, phage antibodies with higher affinity may be enriched during successive rounds of selection by decreasing the concentration of antigen used for selection. The selection may be chosen to favour affinity or kinetic parameters such as off-rate; this hinges on the use of limited and decreasing amounts of antigen and on performing the selections in solution rather than by avidity-prone panning on coated antigen [65]. When selecting from a secondary phage library (see section 3.3), the antigen concentration is typically reduced below the K_d of the parent clone to allow preferential selection of higher affinity mutants [65]. In one of the most thorough studies on antibody affinity maturation carried out to date [73], it was necessary to empirically determine the antigen concentration to be used for selection, as well as the elution condition for phage retrieval, using BIAcore [98]. The SIP procedure may also be used to enrich antibodies for certain kinetic parameters [95, 99].

4.4 Selection on complex antigens

Most successes with selections have been reported using purified antigen. Selections on impure antigens are significantly more difficult, due to the problem of limited amount of target antigen present in the mixture, and of enrichment of phage antibodies specific for non-relevant

antigens. Examples of complex, "difficult" antigens are those that cannot easily be purified from contaminants with similar properties, or cell surface receptors that are only functionally retained in lipid bilayers. Depletion and/or subtraction methods, competitive elution with an antibody or the antigen itself [89], or selection by alternating between different sources of antigen [100] may be used. Theoretical, as well as experimental studies may help to understand the extent to which different parameters govern the outcome of subtractive selection processes [101-103]. The enrichment of phage antibodies specific for the target antigen is also influenced by the background binding of non-specific phage particles, which necessitates reiteration of the selection procedure. Background problems may diminish when repeated selections of phage libraries are carried out without amplification step, by reusing the eluted and neutralized polyclonal phage ligands directly for selection, as reported for protease inhibitors [90]. During affinity maturation studies, direct selection without re-amplification may speed up the procedure and reduce selection of abberant clones with growth advantages [65]. Some recent evidence indicates that anti-hapten antibodies may also by isolated by 3 rounds of panning without re-amplification, using a very large naïve Fab library (de Haard, H.R.H., *et al.,* unpublished). If *in vivo* re-amplification steps could be by-passed, it might be possible to fully automate the isolation of antibody fragments from very large libraries. Background binding of phage has also been a major obstacle to carry out selection on antigens blotted onto nitrocellulose or other membranes. Such a procedure could be applied to batch-select phage antibodies to very large collections of (denatured) antigens, and possibly to isolate antibodies to small quantities of partially purified proteins. Using combinations of detergents, we have recently succeeded in obtaining a 10-50-fold enrichment on Western blots using a low affinity phage antibody (10^7 M^{-1}) to the U1A protein (Fig. 4H; R.M.H. *et al.,* in preparation).

4.5. Selection on cells

A special case form the antigens present on cell surfaces. Direct panning on cell surfaces carrying the antigen may be carried out on adherent cells grown in monolayers, or on intact cells in suspension (Fig. 4E) [31, 104, 105]. This may fortuitously select for antigen-specific phage antibodies, in particular when using immune libraries in which the frequency of irrelevant phage antibodies will be lower [31, 104]. Depletion and/or subtraction methods, cell sorting (Fig. 4F) using flow cytometry [56] or magnetic bead systems [106], competitive elution with an antigen-specific ligand or antibody, or selection by alternating between different cell types all carrying the antigen, are possible, at least in theory. However, it should be noted that in many cases, antigens will be present at very low densities on the cell surface, and antigen concentrations during selections will reach values much lower than the K_d of any antibody in the library. Even when antigen concentration is sufficient for antibody binding and retrieval, antigen inaccessibility

through steric hindrance caused by the presence of other proteins or glycosylation may prevent the selection of antibodies specific for the target antigen. This may be illustrated by an example of a naïve library selection [45] that we carried out on cell transfectants, which expressed relatively high levels (200.000 copies per cell) of one of two very different membrane antigens. Strikingly different results were obtained, depending on the structure and nature of the transmembrane protein. Selections on CHO cells one of the G-protein coupled seven-helix transmembrane receptors for somatostatin were unsuccessful, despite extensive pre-absorption of phage with receptor-negative cells. On the other hand, direct panning (without depletion) on cells carrying the transmembrane glycoproteins CD36 at similar surface density, generated a large collection of antigen-specific antibodies to a selection-dominant epitope on the antigen (Lutgerink, H.R.H., *et al.*, in preparation). It is therefore difficult to assess the value of subtractive methods, i.e. cell sorting by flow cytometry [56] or magnetic activated cell separation [106], without a direct comparison with cell panning. Nevertheless, for most applications, it is likely that more refined subtraction methods will be required to home in on antigen-specific phage antibodies.

The feasibility of selecting antibodies to "difficult" complex antigens and in particular to cell surface molecules would expand the utility of phage antibody libraries tremendously. However, for such antigens the selection conditions including pre-treatment of the samples, incubation conditions, washing procedure and phage retrieval, need to be established empirically. We have developed a model that can compare and determine the relative efficacies of the various enrichment procedures on complex sources of antigen. Phage carrying a scFv specific for the tumor-associated antigen epithelial glycoprotein-2 (EGP-2) were mixed with an excess of irrelevant phage and the enrichment factor and recovery of specific phage were determined after a single round of selection. As antigen source we used a tumour cell line, a tissue cryosection of primary colon carcinoma and (subcutaneous) *in vivo* grown solid tumours in mice (Fig. 4E, G and I). Our results, summarised in Table 1, show that there are major differences with regards to selection efficacy; however, antibody-displaying phage were enriched in all but one selection method.

The efficacy of the procedure depends on the antigen amount and concentration (Table 1; estimated to decline from top to bottom) and on antigen accessibility. The most efficient procedure, recovery of phage using panning on cells in suspension (2-5% recovery of the input phage), reaches an enrichment factor similar to what has been reported for purified antigens [102]. For the *in vivo* selection, antibody-displaying phage are enriched over non-binding phage only when phage are directly injected into the tumour interstitium, but not when injected

intravenously. Efficient *in vivo* selection may thus be suited only for antigens that are in direct contact with the blood stream i.e. endothelial cell antigens [107]. Such model selections help in defining the optimal experimental parameters for selections on complex antigens.

Table 1
Enrichment factors and phage recovery after a single round of selection on different complex antigens [1]

Antigen source	Mode of selection	Enrichment	Recovery of specific phage (% of input)
Colon cancer cell line	Panning on cells in suspension	10,000	2-5
Tissue cryosection	Panning on glass slide-mounted cryosections	80	0.02
In vivo grown tumour	Injection of phage into tumour interstitium	10	0.005
In vivo grown tumour	Injection of phage into the tail vein	none	0.001

[1] Phage mixtures of an excess of control phage over specific (anti EGP-2) phage were selected on a number of different antigen sources. Since antigen-specific and control phage confer a different antibiotic resistance to the bacterial host upon infection (ampicillin (AMP) and tetracyclin (TET) respectively), enrichment and recovery of binding phage can easily be determined by titration and parallel selection on both antibiotics. Recovery of specific phage was calculated as the amount (percentage) of AMP-resistant colonies (cfu), recovered after one round of selection. Enrichment factors were calculated as the product of ratios of AMP and TET-resistant colonies before and after selection, according to the formula (in = input titer; out = output titer): enrichment factor = $(({}^{in}cfu_{tet} / {}^{in}cfu_{amp}) * ({}^{out}cfu_{amp} / {}^{out}cfu_{tet}))$.

4.6 Finding new antigens with large phage libraries

Selection from phage antibody libraries provides a new tool for the isolation of novel self antigens, such as disease- (e.g. tumour-) associated antigens. Both the *de novo* combined V-domain pairs in naïve and synthetic antibody libraries (but particularly the latter) are not shaped by the constraints of the immune system, and avoid library bias caused by *in vivo* tolerance mechanisms. Therefore, antibodies to unique self-epitopes can be isolated, provided powerful cell depletion or subtraction methods are available. To date, this application has been used for probing lymphocyte [56] and tumor [31] cell surfaces, yielding antibodies to known antigens and a number of promising but as yet uncloned new cell-type specific antigens. We have recently

generated panels of anti-epithelial cell antibodies by panning a large naïve antibody library on tumour cell lines, but also with libraries derived from the B-cells of a tumour draining lymph node of a patient with colorectal cancer (R.C.R. *et al*., in preparation). These studies will help to study the natural humoral immune response of cancer patients to the autologous tumour and will possibly identify alternative targets for active or passive immunotherapy.

A recently described method, called 'Pathfinder' selection, might be suitable to overcome several difficulties associated with phage antibody selections, i.e. the use of complex antigens like cell surfaces for selection and the preferential selection of antibodies to dominant epitopes on a given antigen [108]; the procedure is schematically depicted in Fig. 4K. The method uses a peroxidase-conjugated ligand ("lead") to deposit biotin-tyramine free radicals in a local area around the binding site of the lead. If phage are bound within this radius (of approximately 25nm), they will be biotinylated and therefore retrievable on streptavidin-coated beads. The target antigen in this procedure can be anything from a purified protein to a receptor on a cell surface, or an antigen fixed on tissue sections. The procedure was examplified by selecting antibodies to TGF-β1, CEA and a cell surface receptor, CC-CKR5. The method may overcome problems of 'selection-dominant' epitopes, as well as provide a means to select phage antibodies to rare cell surface receptors of orphan ligands.

5. Phage antibody screening procedures and applications

5.1 Basic screening assays

The outcome of any selection procedure is a mixture of binding ligands with differing properties. It may be necessary to screen large numbers of antibodies to identify those variants with the most optimal characteristics. The best screening assays are fast, robust, amenable to automation (96-well format), and use unpurified phage antibodies, or the soluble antibody fragments from the bacterial supernatant. The screening assay should also be linked as closely as possible to the ultimate (functional) requirements of the ligand. Binding of poly- or monoclonal phage antibodies to the antigen has been tested with diverse assays, ranging from a simple ELISA with coated antigen [22], to bioassays that screen for direct neutralization upon binding [109], and whole cell ELISA or flow cytometry. Typically, for a first screen ELISA-based assays are used, in combination with restriction-fingerprinting of the antibody-DNA to identify different clones [22]. Further, specificity of antibodies may be tested using immunoprecipitation [36] or immunocyto- or histochemistry [84, 110].

To speed up screening procedures, phagemid vectors that incorporate a dual purpose have been developed. These allow both monovalent display of antibody fragments and the production of soluble antibody fragments for screening without the necessity to subclone the antibody V-genes. In such systems, an amber codon is positioned between the antibody and pIII genes; in *E. coli* suppressor strains, expression of the fusion product for incorporation into the phage coat is possible; in non-suppressor strains, this amber serves as a stop codon yielding soluble antibody fragments [11] (due to incomplete suppression, this will also happen to some extend in suppressor strains). A variety of tags have been described that are appended to the antibody fragment for detection, including the myc-derived tag recognised by the antibody 9E10 [22], and the Flag sequence [111, 112]. This set-up will allow the use of unpurified phage antibodies or antibody fragments, present in crude supernatant or periplasmic extracts, for screening assays. Finally, it is possible to fuse, for example in between the antibody and gIII, a Histidine-encoding tag, for purification of soluble antibodies using Immobilised Metal Affinity Chromatography [113, 114] and detection with antibodies.

5.2 Screening for affinity or kinetics of binding

After selection of the higher affinity from the low affinity variants, the screening assay will need to differentiate between antibody variants with differences in affinity or kinetics of binding. ELISA-based methods have been described as well as screening using BIAcore [71, 73]; for extensive discussion see [115] and various chapters in [77]). Affinity and dissociation rate screening on the BIAcore is particularly straight forward when using antibody Fab fragments. These lack the problem of the multimerisation behaviour inherent to many scFv formats [61], which complicates measurement of the kinetics of antigen binding of unpurified scFv fragments. We have developed on- and off-rate screening assays for panels of unpurified Fab fragments using periplasmic extracts (A. Reurs, H.R.H., in preparation; an example of an off-rate screen is shown in Fig. 5A). In combination with BIAcore-based methods to determine the amount of Fab in these crude preparations (necessary for accurate determination of the association rate constants [116]), the affinity of large panels of antibodies may be determined. A routine, reliable determination of affinity constants is very important in deciding which molecules to use in further analysis and affinity maturation steps. It should be kept in mind that it will be necessary to test biological potency, cross-reactivity and expression level to further assess the potential of the affinity matured candidates, as these parameters may change with changing affinity [117, 118].

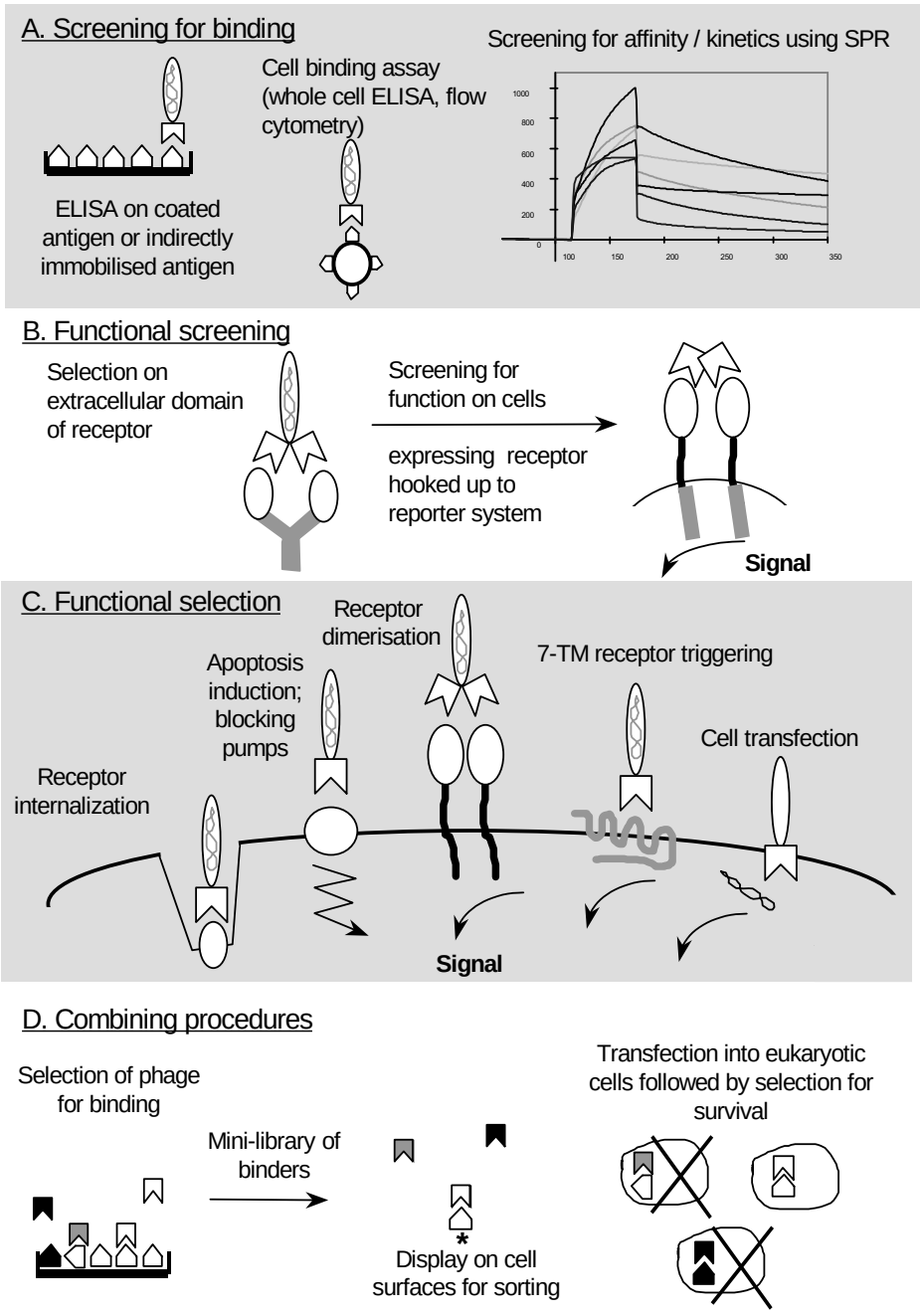

Figure 5

Multiple strategies for selecting and screening phage ligands.

(a) Primary screens of selected phage ligands frequently involve tests to measure binding, including ELISA, cell binding methods and BIAcore screening. (b) Alternatively, selected phage ligands may be tested for bioactivity, for example by selecting on a receptor-immunoadhesin, and screening for receptor triggering on cells. (c) Direct selection of phage ligands for a particular function may be envisaged using a variety of methods; depicted here are cell-related methods only. (d) Example of a combined procedure, involving selection of antigen-binding phage ligands, and a secundary selection in an alternative system, i.e. using a prokayotic or eukaryotic cell system, either for affinity sorting, or for selection based on cell survival (see text for details).

5.3 Recloning selected phage antibodies for expression in other hosts

One drawback for analysis after the *first* screen is that antibody expression levels in *E.coli* are dependent on the primary sequence of the individual antibody, and can be extremely variable (from 10 μg to 100 mg/L). Unless expression is at sufficiently high levels, consideration should be given to re-cloning the antibody into another expression system (for review see [61]). To re-format selected antibodies, however, fast recloning methods are needed in those cases where

large numbers of clones need to be screened. Recently a eukaryotic expression vector was described that may be used for one-step re-cloning of V-genes derived from any phage repertoire, and cloned for expression as Fab fragments or whole antibody, or for targeting to different intracellular compartments [119, 120]. This permits facile and rapid, one-step cloning of antibody genes for either transient or stable expression in mammalian cells. By carefully choosing restriction sites that are rare in human V genes, the immunoglobulin genes of selected populations may be batch-cloned into these expression vectors. All the important elements in the vectors -promoter, leader sequence, constant domains and selectable markers- are flanked by unique restriction sites, allowing simple substitution of elements, and further engineering. By design of the correct promoter cassette, ribosome binding site, 'consensus' signal sequences and by using 'intron space' appropriately, it should be possible to create vectors that mediate both phage display of antibody fragments in prokaryotic hosts, as well as expression of antibody fragments or whole antibodies from eukaryotic hosts (A. Bradbury and H.R.H., unpublished). Such shuttle vectors would be suitable for linking selection of panels of binding antibodies, with a screening assay based on a particular format of the antibody, to be expressed in eukaryotic cells. In addition, it would allow the combination of different methods of display or combinatorial library screening. For example, a pre-selection for binding from a very large bacteriophage display library, followed by a fine-tuned affinity selection by means of flow cytometry of the medium-sized library using yeast or bacterial surface display (Fig. 5D) can be envisaged. In a milestone study which is further discussed in section 5.5, Gargano and Cattaneo [121] demonstrate the power of such combined methods for retrieving biologically active anti-viral agents.

5.4 Bioactivity assays with phage antibodies and peptides

Fast read-out is particularly required for screening methods where the influence of affinity or kinetic behaviour is unclear or less important than the functional result of binding, e.g. virus or cytokine neutralization and receptor blocking or triggering. In such assays many different factors besides epitope recognition or antibody affinity will determine antibody. Methods have been reported to quickly test phage derived Fab or scFv fragments for their blocking or enhancing effect on the activity of a growth factor [109], or for their direct receptor triggering effects based on receptor dimerisation [122]. In the latter case, high affinity anti-MuSK antibodies were selected from a large naïve antibody library by selection on an MuSK-Fc immuno-adhesin, and scFv-agonists were identified by screening on cells expressing a chimeric MuSK-Mp1 receptor (Fig. 5B). An elegant study with Epidermal Growth Factor displayed on phage demonstrates that phage particles themselves may also induce receptor triggering [123]. The applicability of this screening method for phage antibodies has to be proven still, and will depend on the mechanism

of receptor triggering and receptor accessibility.

In the example of MuSK, receptor dimerization is required for signal transduction, thus the ligand needs to be dimeric, either by multivalent display on phage or via natural dimerisation, like has been noted for scFv fragments [122]. The use of repertoires of bivalent ligands, such as 'diabody' libraries [26], would be preferable for these applications. Phage-mediated receptor triggering is, however, also feasible for receptors that do not require dimerisation for activation, in particular for phage that recognise G-protein coupled receptors with multiple membrane spanning regions, which are normally triggered directly via ligand interaction. Such receptors have a wide range of activities and have therefore been used as target for ligand screening using chemical peptide and other libraries. We have recently obtained evidence that peptide ligand-displaying phage themselves may act as receptor agonists (Rousch, H.R.H. *et al.*, submitted), a feature which will dramatically simplify screening for phage-based ligands in search of a(nta)gonistic lead compounds. Phage carrying somatostatin, a 14-mer cyclic peptide, were shown to be enriched via panning on cells expressing one of its receptors, and also scored positive in flow cytometry, whole cell ELISA and ELISA using anti-ligand sera. Upon cell binding, this phage lowered intracellular cAMP concentration and reduced adenylyl cyclase activity, providing evidence for specific triggering of this G-protein coupled 7-TM receptor. To date only one other study has addressed phage-mediated triggering of 7-TM receptors [124]: in this study the melanocortin receptor was triggered using one of the receptor's natural ligands displayed on phage. It should thus be feasible to isolate receptor-specific ligands from phage libraries, using panning on cells that overexpress the target receptor, and screen the selected phage directly for activity. After selection for binding, individual phage clones may be screened for receptor triggering effects, to differentiate agonists from antagonists from irrelevant binders.

6. New avenues for phage libraries

6.1 Selection for function

With large libraries at hand, we may go beyond the *in vitro* binding interaction itself, and select for a particular function. For example, provided reporter systems with sufficient sensitivity are used, it may eventually be possible to sort cells which have been triggered by a phage particle displaying an a(nta)gonistic ligand. Such sorting procedures could allow the direct selection of phage particles with agonist or antagonist activity for a given receptor directly from the phage library. With new reporter genes and sensitive fluorescent read-out methods under development (for review, see Ref. [125]), we envisage that such 'functional selection' schemes will be useful tools for drug discovery. Such methods may be used to identify peptide ligands for orphan

receptors (such as the many related opioid receptors), for which a function but not a natural ligand is known. Examples of already demonstrated functional *selection* methods are found in retrieving catalytic antibodies [126]; as yet underexplored routes are selection for cell internalization [127], cell survival or killing (induction of apoptosis) upon ligand binding, cell transfection, specific inhibition of certain cell surface molecules such as drug pumps, (inhibition of) viral entry, and, finally, receptor cross-linking or triggering. The list of these novel applications, some of which are depicted in Fig. 5C, will keep growing as access to the technology widens.

6.2 Combining phage display with other procedures

Functional selection may also be carried out, after preselection of antigen-specific phage antibodies, using selection schemes in other cellular systems. In Fig. X two of such possible procedures are described. The first hypothetical application is to display the selected library on the surface of particles which are large enough to allow affinity sorting (bacteria, yeast or mammalian cells [128], reviewed in [129]). This could possibly provide a rapid method for antibody affinity maturation. The other application involves a selection step for bioactivity, which allows to select for a subset of phage library-derived ligands from a mini-library of ligands that interact with the target antigen. An elegant example of this, using intracellular selection, was described by Gargano and Cattaneo [121]. They developed a model system showing that intracellularly (cytosolic) expressed antibody fragments that block the activity of reverse transcriptase (RT), can inhibit integration of a retrovirus containing the Herpes Simplex Thymidine Kinase gene, resulting in the selective survival of transfected cells through protection against the cytotoxic action of Gancyclovir. Using pools of antibody fragments, cloned in expression vectors that mediate cytosolic expression of the antibodies, it was possible to select the antibody with neutralizing activity for reverse transcriptase from a polyclonal population of antibodies that just bound (but not blocked) RT. This suggests that it may be possible to combine the power of the large phage libraries, to select pools of antigen-specific phage antibodies together with their genetic material, with a subsequent selection of the population using a mammalian host cell [121], or other cell systems [130]. This combination of technologies may indeed optimally utilize the advantages of each system and also by-pass some of the disadvantages of individual methods.

7. Beyond antibodies

The ideas on functional selections and screening procedures may be expanded to the use of alternative proteins or protein domains for constructing binding molecules (reviewed in [131]).

Scaffolds different from antibodies have been reported to form suitable binding ligands for many types of antigens. There are ample examples of 'host scaffolds' that contain sufficient permissive regions to accomodate a reasonable numbers of substitutions, which may be used to generate a library of localised variability. Alternative scaffolds reported to date include β-sheet proteins [132, 133], α-helical bundle proteins [134-138], combinations of these two [139, 140], a separate group of highly constrained protease inhibitors [141-143], and, most recently, Green Fluorescent Protein (GFP) [144]. Since secreted as well as cytoplasmic and nuclear proteins have been displayed on phage (for review see [3]), display on phage is often the first strategy to define permissive sites for randomization and to generate ligand-binding variants [145]. Alternatively, the use of lambda [146], bacterial [129] or eukaryotic cell display methods has been reported. In regards of 'functional selection' methods, choosing other types of molecule besides antibodies is validated by the fact that antibody expression and folding may be impaired in the subcellular location where the desired functional activity is required. It would be advantageous to engineer the antibody for intracellular expression, for example by building stable disulphide free antibodies [147], or to use libraries of scaffolds that are naturally produced in the targeted cell organelle, provided that effective structural diversity may be obtained.

8. Perspectives

This review highlights the advantages and possible applications of phage display for the development of antibodies. With this technology, antibody engineering for the first time may be used to design antibodies from scratch, with an option to choose its building blocks, its affinity (up to the picomolar range), its format (size and valency) and its effector function (natural (IgG) or novel (enzymes etc.) [148]. Tailor-made reagents may thus be generated, for *in vitro* or *in vivo* diagnosis and for therapy. We expect that the number and quality of naïve and synthetic phage-antibody libraries will increase over the next few years. The extent of the use of phage antibody libraries in academic research will benefit from a virtual unrestricted availability of such very large and stable phage libraries. The libraries may be used for the search for new drug targets, cell receptors and their ligands, which will interface with the human genome project and functional genomics. Combining the design and generation of millions to billions of different ligands, with a function-based selection procedure rather than mere selection for binding, will open even more challenging applications of this inspiring technology, and provide a powerful tool for drug and target discovery well into the next decade.

Acknowledgements

This work has been supported by grants from the Profileringsfonds of the University Hospital Maastricht (grant PF37), the European Community, Biotechnology Programme 5.1 (PL950252), and receives financial support from the Netherlands Technology Foundation (STW) in a project coordinated by the Life Sciences Foundation (SLW) (project 805.17.753).

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