Antibody Phage Display Technology

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ABSTRACT

In the past decade, the drive to develop completely human antibodies for human therapy has led to the development of phage display technology. This technology is able to deliver the ultimate in antibody engineering, that is, high-affinity fully human antibodies to any antigen of choice. Here, this application of phage display technology is reviewed, and the many other antibody engineering avenues this technology offers are highlighted as well.

Keywords: antibody, phage display, technology

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RESUMEN

Tecnología de presentación de anticuerpos en la superficie de fagos. Durante la pasada década los impulsos por desarrollar anticuerpos humanos con fines terapéuticos contribuyeron al desarrollo de la tecnología de presentación de proteínas en la superficie de fagos. Esta tecnología ofrece las mejores ventajas para la ingeniería de anticuerpos: anticuerpos totalmente humanos de alta afinidad para cualquier antígeno seleccionado. Este trabajo aborda la aplicación de la tecnología de presentación de anticuerpos sobre la superficie de los fagos, y destaca las perspectivas que ofrece para la terapia con anticuerpos, la genómica y la proteómica funcionales.

Palabras claves: anticuerpo, presentación de anticuerpos en la superficie de fagos, tecnología

*I*ntroduction

The idea of using antibodies for therapy, as a "magic bullet", is now 100 years old. The crucial technology making this idea feasible was made 25 years ago by Köhler and Milstein who described a technique on how to make monoclonal antibodies [1]. However, for almost 20 years, despite extensive efforts, only a handful of antibody-based clinical products have been approved. The major obstacle to this goal has been the immune reaction of patients against these murine molecules. Moreover, it became obvious that a large molecule like an immunoglobulin is not ideal for certain clinical applications, such as solid tumor targeting, due to a weak penetration of the tumor. The progress of molecular biology and antibody engineering has now made it possible to make these mouse molecules look more human. In the mid-eighties, the first chimaeric molecules, human antibodies with murine variable domains, allowed to greatly reduce these human anti-mouse antibody (HAMA) reactions. A few years later, a further improvement was made with humanized antibodies having only small parts (the complementarity determining regions or CDRs) still murine. As a consequence, since 1997, several chimaeric and humanized antibodies have been approved as therapeutic products. As of the early nineties, the appearance of phage display is having a major impact in this field [2]. The simplicity and the power of phage display now allows to very rapidly select fully human antibody fragments against any antigen without the need for immunization. Consequently, it is expected that more human antibodies will be undergoing clinical trials, and for the first time, it seems that antibodies may finally hold their promise as magic bullets in many different medical applications. This article will describe the current state-of-the-art of this technique and discuss the possible future developments.

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*A*ntibody Phage Display

Principle

The general principle of display methodologies is to link a ligand (peptide, protein) to the gene coding for this ligand. In the phage display technique, this is obtained by fusing the ligand gene to the gene coding for a coat protein of a filamentous phage. The recombinant phage genome is then introduced into *Escherichia coli* where the hybrid protein will be expressed together with all the other phage proteins. The fusion protein will then be incorporated into the phage coat containing the phage genome (containing the ligand gene). The secreted phage particle displaying the ligand can be selected on an immobilized target while all the non-binding phages are washed away. After an elution step, the recovered phage is used to infect *E. coli* to allow the amplification of this phage for a new round of selection and eventually for the binding analysis. Large collections of antibody variants can thus be screened for the presence of an antigen-binding clone in the library (Figure 1).

The filamentous phage

The most popular phage used for display is phage fd or M13. It infects *E. coli* containing the F conjugative plasmid by attaching itself to the tip of the pilus and translocating into the cytoplasm. The genome is replicated by both phage and host proteins, and packaged into a rod-shaped particle that is released into the medium without lysing the infected cell. All coat proteins are secreted into the periplasm prior to assembly and extrusion. Several coat proteins have been used for display including p8 and p6 but p3 is by far the most extensively used one. P3, present in three to five copies at the tip of the phage particle, is involved in bacterial infection.

Phage or phagemids

The first proteins to be displayed were antibody fragments named scFv (single chain Fv fragment), consisting of the VH and the VL domains linked together by a peptide linker. Originally, the gene coding for this construct was cloned between the signal sequence and the gene of the p3 coat protein, into the whole phage genome. After translation, the fusion is sent into the periplasm, where the antibody domains can be correctly folded and where disulfide bonds, essential for the antibody fragment activity, can be formed. At present, phagemids have become more popular [3]. They are small plasmid vectors carrying the p3 gene and a phage-packaging signal. Upon infection with a helper phage allowing the expression of all the other phage proteins, the phagemid is replicated and incorporated into the virion like a normal phage genome. The helper phage genome carries a mutated origin and is not replicated efficiently which ensures that most particles will contain the phagemid. Phagemids have a much higher transformation efficiency, which is very important for combinatorial approaches, and allows the direct production of the unfused antibody fragments [3].

In most described phagemid-display systems, the antibody-p3 fusion expression is driven by the lacZ promoter. For the production of phage particles, the catabolic repressor (glucose) is removed from the medium, leading to a sufficient amount of fusion. Since the helper phage genome encodes wild type p3, typically 90% of the rescued phage does not display any antibody-p3 fusion. The vast majority of the phages displaying an antibody will be monovalent and only a very small fraction will be multivalent. This monovalence allows for the selection for high affinity antibodies by minimizing avidity effects [4].

Format of the antibody fragment

Antibody fragments have been displayed on phages using different formats. The most common formats are the scFv [5] and the Fab fragment [3]. However, other possibilities have been used including the dsFv where the VH and VL domains are linked via a disulfide bond [6], and the diabody (dimer of scFv, bivalent or bispecific) [7]. The scFv's are usually easier to produce in *E. coli*, but their tendency to form multimers may complicate their selection from libraries and characterization. Fab fragments do not have this drawback and are our preferred format for both antibody display and screening. For example, the Fab format allows fast kinetic screens of large numbers of unpurified fragments selected from phage libraries [2, 4], and is generally a more stable protein when purified from bacterial cultures. Fab's are displayed on the phage by linking either the fd fragments or the light chain to the p3 while the other chain is secreted freely into the periplasm and can interact to form an intact Fab fragment.

The same strategy is used to display diabodies [8]. These molecules are formed by the dimerization of a scFv fragment made with a short linker avoiding the interaction of the two domains of a single molecule. The VH of one molecule, linked to p3, is thus forced to interact with the VL of a second one secreted into the periplasm, and vice versa. This format can be used to create libraries of bivalent molecules that might be very useful in selection for bioactivity [3, 4], but also to create libraries of diabody fragments, thereby avoiding extensive recloning used to create these molecules from scFv or Fab [7].

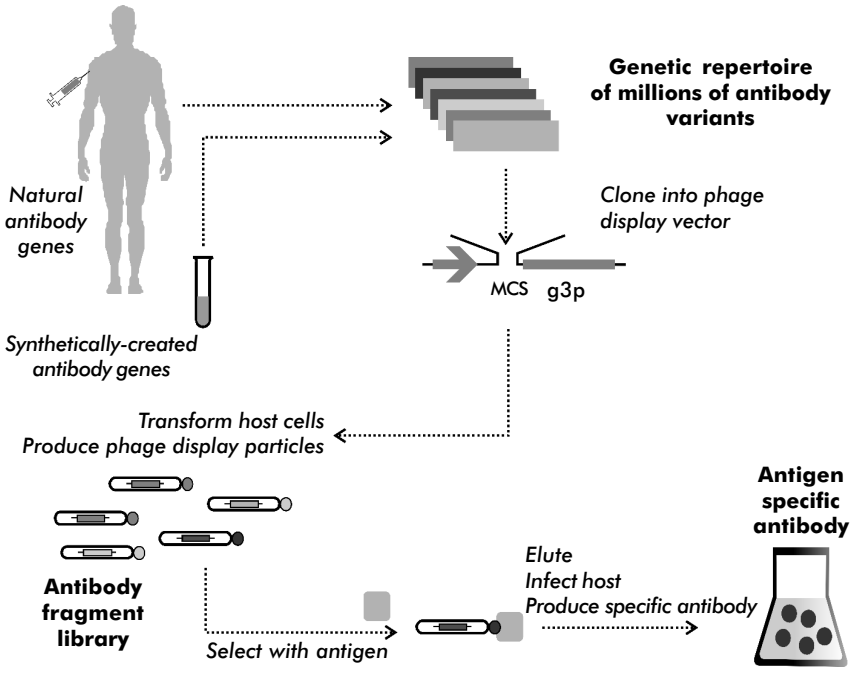

Figure 1. Antibody library technology. Synthetic or natural V genes are amplified by PCR and cloned into a phage display vector. The repertoire is transformed into *E. coli* and phage particles expressing the antibody fragments on their surface that are produced by infection with a helper phage. The phage-antibody library is selected against the antigen of choice and the selected phages are used to re-infect *E. coli* to produce new phage particles for a subsequent round and to eventually produce clones for further characterization.

Different Kinds of Antibody Libraries

Libraries from immunized animals or donors

Immune phage antibody repertoires are constructed by using B cells coming from immunized individuals [2], having several advantages. First of all, the B cells will be enriched in antigen specific cells and thus a small library will have a high chance to lead to binders. Furthermore, these B cells have undergone affinity maturation, which implies that high affinity clones will be easily selected. In some cases, the resulting clones have even higher affinities than clones obtained by the traditional hybridoma technology.

This type of library is mainly used to study the humoral response of patients presenting a viral infection, suffering from neoplastic diseases or autoimmune disorders, or to build large collections of antibodies to particular antigens or cell types [9]. RNA isolated from the B cells of patients is used to build antibody phage libraries that are subsequently selected for the antigen of interest. These libraries may also provide human virus-neutralizing antibodies, or occasionally anti-tumor antibodies with therapeutic interest.

Naïve and synthetic libraries

The drawback of immune libraries is that one has to immunize and build a new library for each antigen, which is very time-consuming. Moreover, antibodies against self, conserved, or toxic antigens cannot

be easily obtained in this way. A universal library from which high affinity human antibodies can be selected against any antigen is much more desirable. These kinds of "single pot" libraries have been built and are now extensively used [10]. These libraries are made from B cells isolated from peripheral blood lymphocytes, spleen, bone marrow cells or the tonsils of non-immunized donors [11]. Heavy and light chain genes are amplified, randomly combined and cloned to produce a combinatorial library (Figure 1). Antibodies against virtually any antigen have been selected from these libraries. The only limitation in this case is the size of the repertoire, which to a large extent determines the range of the affinities for the selected antibodies. Several "naïve" repertoires of more than 1010 clones have been built by brute force cloning [11, 12]. Alternatively, using *in vivo* cre recombination in a single bacterium, Sblattero *et al.* could obtain a repertoire of 3×10^{11} clones [13].

The second type of "single pot" repertoire is made by *in vitro* PCR-based artificial assembly of V gene segments and D/J segments, introducing a pre-determined level of randomization, generally in CDR, and especially in CDR3 regions [14, 15]. These repertoires have led to antibodies against many different antigens but with affinities in the micromolecular range. Similarly, the size of the library governs the range of the antibody affinities retrieved. Therefore, nanomolar affinity antibodies were readily retrieved from a very large "synthetic" antibody library made by using *in vivo* recombination to create a 6.5×10^{10} clone repertoire [16]. However the genetic instability of this repertoire has limited its widespread use.

Nevertheless, it must be kept in mind that the first round of selection yields typical 10^6 clones. Since the maximum enrichment factor published is around 10^5, the limit in size allowing a full sampling of the repertoire would be 10^11. Therefore, instead of building very large repertoires that may display only a minor fraction of well-behaved antibody fragments, it may be more efficient to create a repertoire with a large majority of functional antibodies. Such an improvement on synthetic antibody library design was proposed by Pluckthun [17], in which diversity was introduced in all of the six CDR regions using "designed" V-gene segments, and by Tomlinson, suggesting to preselect individual V-gene libraries for functional expression (display), before their complete assembly into the scFv fragment. Tomlinson pre-selected a synthetic library based on a unique scaffold on protein A and L, thereby selecting for the well folded VH and VL domain (Tomlinson and Winter, manuscript in preparation). This increased the functional size of the repertoire and as a side effect, also pre-selected efficiently produced antibody fragments. Moreover, the selected fragments can be purified, and detected via protein L and A interactions and the tri-dimensional structure modeling of the selected clones is facilitated since all the clones share a same scaffold. Thus, this kind of approach presents several advantages and may represent the future of antibody repertoires [10]. In both cases the affinity of the selected antibodies from such "designer" synthetic libraries should be increased due to the higher frequency of functional antibodies in these libraries; this must still be verified.

Second generation library for affinity maturation

Antibodies selected by large repertoires often have an affinity in the 10-100 nM range [12]. Although it may already have different usages, its application in therapy, such as immunotherapy, would often require a higher affinity antibody. Several techniques have been developed for this. The principle is to use the selected clone as a scaffold to create a secondary library by introducing diversity in the V genes. This new library is affinity-selected against the antigen and the selected variants are screened using a method based on kinetic measurements. Two types of approaches have been described to introduce diversity. The first one is a random based approach such as error prone PCR, mutator strains, chain shuffling, DNA shuffling [18-21]. Although usually successful, these approaches rarely lead to very high affinities. More rational approaches have been developed to obtain better affinities. In this case, the diversity is introduced into CDRs or mutational hot spots [22], after having selected interesting residues by alanine scanning, parsimonious mutagenesis or modeling. Impressive affinities in the picomolar range have been obtained by these techniques [23].

*S*election

Classical selection procedures

The selection principle is to retain binders on a solid surface coupled with the antigen while the non-binding phages are washed away. Many selection procedures have been described (Figure 2A). The most extensively used method called panning is to immobilize the antigen on a plastic surface by adsorption [2]. Although it is very simple, this technique has severe drawbacks, the main one being a frequent denaturation of proteins upon adsorption [24]. To circumvent this problem, the antigen can be chemically biotinylated and incubated with the phage repertoire. The binder-antigen complexes are then retrieved using streptavidin coated magnetic beads (Figure 2A) [25]. Another major advantage of this approach resides in the possibility of accurately controlling the concentration of the antigen, which is crucial in affinity maturation processes. In this case, antigen concentrations below the desired kD are typically chosen in order to favor the selection of the best binders. Other techniques based on purified antigens have been described, including selection on columns or on BIAcore chips [26, 27].

The binders are recovered by an elution step usually consisting of an incubation with an acidic (HCl, glycine) or basic buffer (TEA) [26]. However several methods have been described including the use of chaotropic agents, DTT (when biotin is linked to the antigen by a disulfide bond) [28], enzymatic cleavage of a site placed between the antibody fragment and p3 [29], competition with an excess of antigen [2] or simply by putting directly bound phages in contact with *E. coli.*

Selection on cells and complex antigens

In some cases, the antigen cannot be easily purified, or consists of a membrane antigen or a complex of

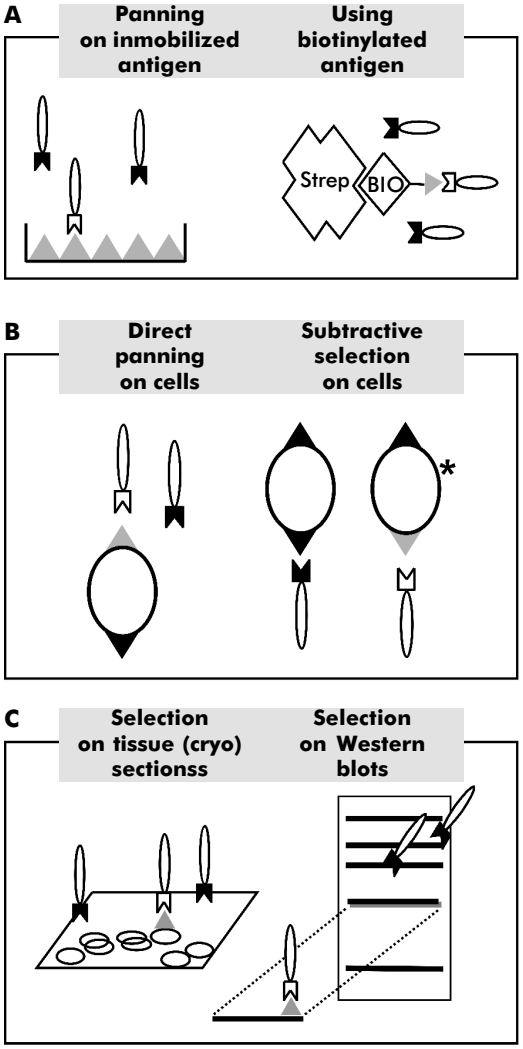

Figure 2. Phage library selection procedures. Selection of binders can be done in several ways. (A) The most extensively used methods use panning against coated purified antigens or purified biotinylated antigens. In the latter, the complex phage antibody-antigen is retrieved by incubation with streptavidin coated magnetic beads. (B) When the antigen cannot be produced as a purified protein, selection can be done on living cells. If the antigen is not abundant, it is often necessary to deplete the phage antibody library for pan cell binders against cells that do not express the antigen of interest. The library is then incubated with the same cell line presenting the antigen. (C) More original methods have been proposed, such as selection on tissue section or Western blot membrane. In the latter, a cell lysate is separated by SDS-PAGE, transferred to a membrane. After the incubation of the library, the band containing the protein of interest (with bound phages) is cut out and used to reinfect *E. coli*.

polypeptides. Direct panning on cells in suspension or in monolayers have proven to be feasible (Figure 2B) [30, 31]. Depletion and/or subtraction on cells, competitive elution using an antigen specific ligand that does not express the antigen are usually used to eliminate pan reactive binders. Although several groups succeeded in selecting antigen-specific antibodies, it has to be kept in mind that this technique is sensitive to the density of the antigen on the cell surface; chances to select antibodies against poorly expressed antigens being rather small. A second crucial parameter is the accessibility of the antigen on the cell surface. Proteins presenting a large extracellular domain may present a diversity of epitopes available for binding. Conversely, proteins such as seven transmembrane receptors, displaying only small extracellular loops, are very difficult targets for phage antibodies [32] but not for phage peptides [33].

A method called "pathfinder" has been developed to increase the chance of finding an antigen-specific antibody on the cell surface [34]. In a first step, cells are incubated with a known peroxidase-labeled ligand of the target. The phage repertoire is then incubated and cells are washed. Biotin tyramid free radicals are then used to biotinylate all proteins in the vicinity of the peroxidase, including target-specific phage-coat proteins. These phages are finally retrieved using streptavidin beads.

Other original techniques have been proposed, including selection on tissue sections and selection on Western blot membranes (Figure 2C). However these techniques still need to be optimized and are not yet frequently used.

Infection based selection

An alternative way of selecting binders called SIP for selectively infective phage uses the antigen-antibody interaction to restore infectivity. Therefore only antigen-binders have the possibility of being amplified in an *E. coli* culture. The p3 is deleted from one or two domains and then fused to the antibody fragment. The antigen, linked to the missing part of p3 is then supplied in the culture or encoded in the phage genome [35].

This technique has the advantage of being very simple (basically an overnight growth of *E. coli*) but has demonstrated to be very sensitive to the affinity of the interaction. Although powerful in principle, this technology must still be improved to lead to better results than the traditional approaches.

Screening

Basic screening

Selection leads to a mixture of specific binders and phages binding in a non-specific, or non direct way against the desired target (streptavidin binders for example, are efficiently selected by direct binding on the magnetic beads). Thus screening procedures allowing the identification of specific binders are crucial.

Several features have been added to antibody fragments to facilitate this step. First of all, an amber stop codon is often introduced between the antibody gene and the g3p to allow the production of a phage-antibody or unfused antibody fragment for the same construct (Figure 3) [3]. Several tags are also usually fused at the C-terminal part of the fragments, allowing an easy detection (C-myc tag, FLAG tag) and purification (hexa-histidine tag for metal affinity chromatography) [26, 36].

The typical assay used to confirm the specificity of selected clones is an ELISA using the immobilized antigen and the unpurified phage-antibody or the fragment excreted into the culture supernatant [26]. Whole

cell ELISA and FACS experiments are used to identify binders after selection on cells [4]. More specific assays such as immunoprecipitation [37] and immunohistochemistry [38] have been used to further confirm the fine antibody specificity (Figure 3).

Kinetic based selection

In many instances, the screening procedure following affinity maturation has to be based not only on specificity but also on the affinity of the selected variants. Some ELISA methods have been proposed to determine the affinity of phage-antibodies, but the use of biosensors allowing the determination of the on and off rate is rapidly developing [39]. For many applications, including immunotherapy, a good off-rate is more important than the overall affinity. For this purpose, the Fab format is more suitable since it cannot lead to the dimerization effects observed with scFv fragments that complicate off-rate measurements. Unpurified Fabs produced in the culture supernatant can be used to rapidly evaluate the outcome of a selection, thereby guiding the future affinity maturation steps [12].

Screening for biological activity

One important application of antibody-phage display could be the selection of a molecule that is capable of triggering or blocking specific receptors on the cell surface [40]. In this approach, phage-antibodies first have to be selected for their simple binding to the receptor, mainly through cell selection, since many membrane receptors cannot be produced in a soluble form. The next step is to screen the binders to discriminate between simple binding, the antagonist or agonist effect. Several groups have demonstrated the feasibility of this approach. It must be noticed that the format of the displayed antibody is an issue here, as some receptors are triggered via dimerization [40, 41]. In this case, multivalency is essential and can be provided either by the use of phage vectors leading to multivalent display (as opposed to phagemid vectors) or using the bivalent diabody format. This dimerization-dependent agonist effect of scFv fragments has been demonstrated by the selection of antibodies capable of triggering the MuSK-Mp1 receptor. The screen used by these authors was based on the ability of the binders to induce proliferation in a factor dependent cell line through a chimeric receptor, MuSK-Mpl [41].

Another study used a luciferase reporter assay to screen epidermal growth factor receptor (EGFR) binding molecules for their ability to stimulate or inhibit signal transduction [42]. In this case, the phage particle displaying the EGF molecules were able to trigger the receptor, demonstrating that phage particles themselves can also be used in this kind of activity-based screens.

*P*hage display: perspectives

We have just seen that it is possible to screen preselected binders for biological activity. It would be much more powerful to directly select for these capabilities, instead of screening. As fluorescence-based cell sorting techniques are becoming more and more sensitive, it becomes possible to use a reporter system to select for phage-antibodies that are able to trigger a receptor instead of only binding to it. Indeed, the agonist phage would trigger the receptor, and these fluorescent cells would be sorted by FACS [43].

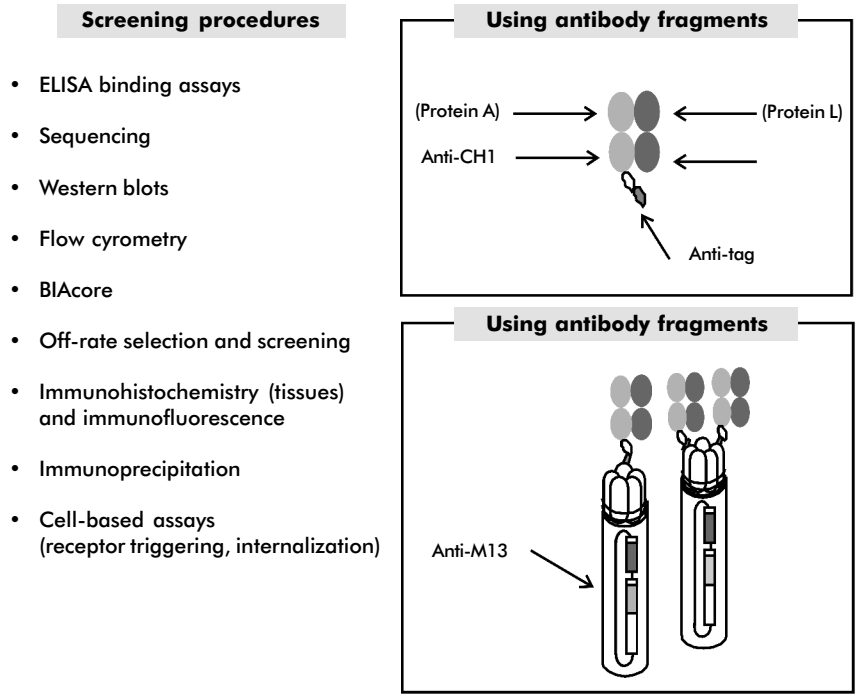

Figure 3. Phage antibody screening procedures. Several methods have been described to screen for binders after selection rounds. This screen can be performed using either the phage particles or the antibody fragments isolated from the cell periplasm or more conveniently from culture supernatants. Phage particles have the advantage of displaying several antibody fragments per particle, which allow an avidity effect dramatically increasing the apparent affinity. Moreover, the use of anti-M13 antibodies for detection leads to very powerful signal amplifications. On the other hand, antibody fragments do not suffer from the high background that may be observed with phage particles and are thus more versatile. They are the format of choice for high affinity variant selection, especially in the case of Fab fragments, a format that does not lead to dimerization.

Recently, this idea has been efficiently used to select phage for internalization into mammalian cells [44]. The group of Larocca have enriched a phage displaying EGF that is capable of delivering the gene coding for GFP in COS cells. Using four rounds of cell sorting and PCR to recover the peptide sequences, they could enrich the EGF sequence 1 million-fold over a background of peptide expressing phages [45].

The group led by Marks has shown that this concept can be applied to phage-antibodies. Using wild-type C6.5 scFv displaying monovalently on a phagemid, they demonstrated that anti-ErbB2 phage antibodies can undergo receptor-mediated endocytosis, proving that it is possible to select for endocytosable antibodies in mammalian cells [46]. This has interesting applications for receptor-mediated gene transfer to mammalian cells.

Phage antibody selection may also be used together with another selection system. For example, selections using bacteria display or yeast display are potentially very interesting since these organisms can be sorted by FACS using a fluorescent antigen, among other applications, for affinity maturation [47]. However it has been shown to be very difficult to obtain and manipulate large naïve or synthetic repertoires using these techniques. Thus a pre-selection on the immobilized antigen can be done using phage display and the small repertoire of binders

obtained this way can be recloned for bacterial or yeast display.

The same strategy can be used to select efficient intrabodies. The intracellular expression of an antibody fragment to block a defined target has a big potential for gene-therapy or as a phenotypic knock-out to elucidate the function of a protein. However, the intracellular behavior of fragments selected *in vitro* is very difficult to predict. The group led by Cattaneo has shown that it was possible to select a neutralizing antibody from a pool of antibodies binding a reverse transcriptase using intracellular expression [48]. Thus phage display could be used to select a pool of binders against a purified intracellular target *in vitro* and to reclone this pool of binders for further selection *in vivo* using intracellular expression. This process should rapidly lead to efficient intrabodies.

Finally one of the most promising applications of phage display could be in the field of genomics and proteomics. The genome-sequencing projects have discovered thousands of open reading frames coding for unknown products [49]. Phage display is rapidly evolving toward automation that will allow selection against multiple antigens in short periods. Thus a way of obtaining information on a given unknown protein is to select antibodies against peptides deduced from the sequence and having a high probability of being exposed to the solvent (Figure 4). These antibodies might be used in immunohistochemistry to obtain information on the expression and localization of the protein of interest, to purify it by affinity chromatography or immunoprecipitation, and it may help to elucidate protein function. Finally, antibodies directed against disease-related antigens might have a therapeutic interest.

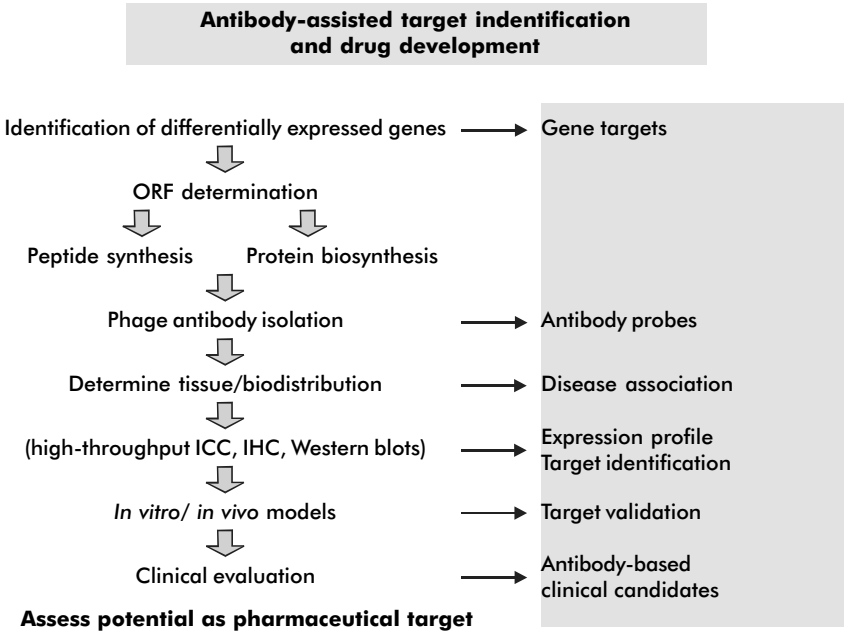

Figure 4. Phage display and drug development. One of the promising applications of phage display is fast drug development. Human genome sequencing and techniques such as differential display or DNA microarrays can identify genes involved in diseases. Phage display can be used to select antibodies against peptides or recombinant proteins corresponding to these genes. The antibodies are then used as a probe to determine the expression pattern of the gene product and to confirm an association with a disease. Eventually these antibodies may be used as a targeting or blocking reagent in animal models and clinical trials. Thus phage display might have the potential to rapidly identify interesting targets and deliver the corresponding drugs in a highly thorough manner.

Conclusions

Antibody phage display has rapidly evolved into a robust and widely used technique allowing the selection of human antibody fragments in a few days or weeks against any antigen, including toxic or conserved antigens. Furthermore, antibody engineering gives the possibility to choose the size, valency, avidity, affinity (up to picomolar range) and effector functions of the molecule. The availability of large antibody repertoires and new selection techniques should permit the rapid isolation of molecules for drug targeting, receptor blocking or triggering. Consequently, the use of phage-displayed antibody libraries will play an increasingly important role in therapeutic antibody development, as well as in the fields of functional genomics and proteomics, both for target discovery and target validation.

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