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Phage-displayed peptide libraries

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Abstract

Over the past year, significant advances have been achieved through the use of phage-displayed peptide libraries. A wide variety of bioactive molecules, including antibodies, receptors and enzymes, have selected high-affinity and/or highly-specific peptide ligands from a number of different types of peptide library. The demonstrated therapeutic potential of some of these peptides, as well as new insights into protein structure and function that peptide ligands have provided, highlight the progress made within this rapidly-expanding field.

Introduction

The field of phage display was first begun by George P Smith in 1985 [1]. Yet, it was his idea for phage-displayed peptide libraries, published in 1988 [2], that caused a burst of activity in the field, culminating in the publication of three papers describing the first phage-display libraries in 1990 [3–5]. Since then, phage display has developed into a wide-ranging field, full of novel applications and responsible for significant advances in many areas of protein recognition. In preparing this review, we counted over 170 papers published in the field over the past year. (A complete bibliography of this literature search, including an extended, annotated bibliography that covers antibody [Ab], and site-directed mutant libraries, is available on the World Wide Web at URL: http://www.biol.sfu.ca/faculty/scott/phage97-98). Although we cannot do justice to most of this work, we present the results from a handful of selected papers covering the results from screening peptide libraries. Also presented ate advances in library construction and screening methods. To provide the reader an idea of the impact that phage-display technology is making on areas such as drug discovery and cancer therapy, we also include a few ‘follow-up’ reports on exciting, bioactive molecules that were previously selected from phage-display libraries.

Antibody-binding peptides and the structural basis of peptide recognition

A number of groups have screened peptide libraries with monoclonal (M) Abs were produced against protein and non-protein immunogens, and have isolated ‘peptide mimics’ that cross react with the MAbs. In several cases, these ligand peptides were also immunogenic mimics of the antigen ‘target’ against which the screening Ab was made; that

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is, if whole phage were used as immunogens, or if the synthetic counterpart to the phage-displayed peptide was used in a protein-conjugate to immunize, the resulting immune sera cross-reacted with the antigen target. Thus, the polyclonal Ab response induced by an immunogenic-mimic peptide will bind to the same epitope on the target antigen as that recognized by the MAb used to select the peptide. As described below, this type of experiment has now been performed successfully for a number of antigens. The concept of using peptides as 'epitope-mimic' haptens that produce highly-directed Ab responses has held great promise since its inception [6]; yet, the use of 'designed' peptides as immunogenic mimics has not had good success. It is beginning to emerge that small features within the peptide that are involved in Ab binding, but not necessarily in epitope mimicry, may often be responsible for the recent successes. Thus, given a vast set of peptide sequences to choose from, many Abs appear to select effective, immunogenic mimics of their corresponding target epitopes.

Demangel *et al.* [7] previously isolated clones beating linear-epitope-mimic peptides by screening a disulfide-constrained, 6-mer (CX_6C; single letter amino acid code where X may be any amino acid) peptide library with a malaria-specific MAb. On immunization, several clones elicited malaria-binding Abs, including two clones whose peptide sequence bore no homology with the presumed malarial epitope. More recently, this group isolated two MAbs from the malaria-binding, anti-phage response and compared their heavy chain variable region and light chain variable region sequences with those of the parent MAb that was initially used to isolate the phage clone [8•]. They found that there were significant differences in hypervariable-region sequences, although there were similarities in features contributing to the gross structure of the Ab combining site, such as variable-gene and canonical structure use. Thus, the phage-borne peptide and the malarial epitope may elicit Abs sharing gross structural features that allow cross-reactivity, but that differ in their finer specificities. Similarly, in collaboration with M Yu and P Talbot (Institute Armond Frappier, Laval, Quebec), we isolated a number of linear-epitope-mimic clones by screening a panel of 12 peptide libraries [9] with a MAb that neutralizes a murine coronavirus. In this case, all of the clones shared a strong consensus sequence matching a sequence on the viral coat, and all bound the MAb with similar strength; yet, when each of the clones was used to immunize mice, only one clone produced cross-reactive Abs that protected mice from intracerebral challenge with the virus (M Yu, JK Scott and P Talbot, unpublished data). These results indicate that relatively small sequence differences in the peptides (in this case, differences in the regions flanking the consensus sequence) are responsible for driving the fine specificity of the Ab response. Structural studies are imperative for understanding the complexities involved in this process. The molecular basis of immunogenic mimicry can best be revealed by comparing the target epitope and peptide mimic bound to both the screening MAb and to cross-reactive MAbs produced by peptide immunization (see [10] for an excellent example of such a comparison). As, in many experiments of this type, whole phage bearing cross-reactive peptides are used as immunogens, it would also be valuable to know the structure of a given peptide in the milieu of the phage, rather than as a synthetic analog. In some cases, the structure of the phage-borne peptide can be determined, as shown by the NMR study of Jelinek *et al.* [11••].

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There is also evidence that immunogenic-mimic peptides can be isolated by polyclonal, serum Abs. In previous work, Mennuni *et al.* [12] isolated several diabetes-specific peptides by screening two peptide libraries with scrum IgGs from a number of pre-diabetic patients; in turn, several peptides from among these were bound by the serum Abs from a number of patients having full-blown diabetes. Mennuni *et al.* [13••] showed that, on immunization of rabbits, a phage clone bearing one of these peptides elicited Abs that recognized pancreatic-islet β cells. Thus, this immunogenic-mimic peptide may reveal new antigens (or epitopes) involved in the evolution of type-1 diabetes, especially if it appears to produce pathogenic Abs (i.e. Abs that direct inflammatory responses against pancreatic-islet β cells) as a hapten on its own.

There is some controversy as to whether immunogenic-mimic peptides always act as structural mimics of the epitope on the target antigen, by binding MAb through the same mechanism as the target epitope (i.e. by making identical contacts with the Ab combining site). Alternatively, it may be that the mechanism of binding a MAb differs between the target epitope and its corresponding immunogenic-mimic peptide (i.e. with each antigen making different contacts with the Ab combining site). This controversy has been most clearly defined with anti-carbohydrate and anti-DNA antibodies and their peptide ligands, as these types of targets are chemically very different from peptides, and thus would be most likely to bind Ab by a different mechanism from that of a peptide. Phalipon *et al.* [14••] screened two peptide libraries (X_9 and CX_9C) with two MAbs that had been produced against the lipopolysaccharide (LPS) of *Shigella flexneri*. There were 19 related sequences identified by the MAbs, with only one clone cross-reacting with both MAbs. The 19 clones were tested as immunogens, and two clones (the one that cross-reacted with both MAbs and one that did not) elicited significant lipopolysaccharide-binding activity. This indicates that structural mimicry may be involved in immunogenic mimicry (as one clone was recognised by both MAbs and, thus, is consistent with being a structural mimic of LPS); but that structural mimicry is not necessarily required for immunogenic mimicry to occur (as the other peptide was specific for only one of the two MAbs and thus must bind by a different mechanism from LPS and, yet, elicited LPS-binding Ab responses). Similarly, Sibille *et al.* [15••] isolated phage bearing consensus-sequence peptides with two different, polyreactive, anti-DNA MAbs, by screening an $X_4CX_6CX_4$ library. Although the phage isolated by each MAb cross-reacted only with its selecting MAb, several phage clones, on immunization, produced DNA-binding Abs. These studies show that peptides can act as Ab-specific ligands (as they can discriminate between two DNA-binding MAbs), and also act as effective immunogenic mimics of DNA (in eliciting DNA-binding Abs). Taken together, these two studies [14••,15••] support the notion that some immunogenic-mimic peptides do not function as structural mimics of their LPS and DNA targets in eliciting cross-reactive Ab production.

Fine-specificity discrimination by peptides may be common among anti-DNA and anti-carbohydrate Abs. With a panel of synthetic oligosaccharides, Harris *et al.* [16••] mapped the minimal epitope on the cell-wall polysaccharide of Group A *Streptococcus* for five, closely-related MAbs. They showed that all five MAbs recognize the same minimal structure, and so probably bind the same epitope on the cell-wall polysaccharide. Yet, when

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used to screen a panel of different peptide libraries, the MAbs selected different consensus sequences, and each MAb only bound the phage that it selected. This indicates that the mechanism of binding to peptide ligands differs from that of binding to the cell-wall polysaccharide, as all MAbs bind the same epitope on the cell-wall polysaccharide, yet each MAb would only bind the peptides that it selected. A study by Gaynor *et al.* [17•] also showed this type of fine-specificity recognition exhibited by peptides. They screened a 10-mer library with an anti-DNA MAb that causes immune-complex-mediated glomerular nephritis when injected into mice, and thus serves as a good model of this type of disease. Although one of the MAb-binding peptides isolated was able to block the deposition of MAb–DNA complexes *in vivo*, the peptide was shown to be MAb specific, being unable to bind another, closely related, anti-DNA MAb. These results indicate that the peptide is unlikely to be a structural mimic of DNA, and, consequently, will not function to suppress the polyclonal anti-DNA Ab response involved in this type of immune-complex disease.

Contrasting this are studies suggesting that peptides can act as structural mimics of carbohydrate epitopes. As opposed to the functional mimics described above, structural mimics offer the great advantage of being able to replace the target antigen in functions such as diagnostic assays and as inhibitors for a range of enzymes (so long as all of the proteins to be tested bind the target antigen by the same mechanism). Kieber-Emmons *et al.* [18••] immunized mice with three different peptides bearing the Aro–Aro–X–Aro (where Aro denotes an aromatic amino acid) motifs identified for three different carbohydrates (alpha-methyl mannopyranoside, Lewis Y and the major polysaccharide of *N. meningitidis* C). The resulting sera cross-reacted at low titers with human breast cancer and melanoma cells, and in another study [19•] with glycosylated HIV-1 envelope proteins, but not non-glycosylated ones. The far-ranging immunogenic mimicry observed in these studies suggests a role for this peptide-sequence motif as a ‘universal’ structural mimic of a variety of carbohydrate targets. Two other recent studies suggest a role for structural mimicry in peptide-carbohydrate cross-reactivity. In two separate screenings of an X_{15} library, Taki *et al.* [20•] isolated peptides that bound to another MAb that is specific for its isomer; a consensus-sequence motif that is common to both MAbs was identified from these two sets of peptides. Synthetic peptides bearing this motif bound the lectin from *Ricinus communis* and also affected the activity of Jack Bean β-galactosidase; one of the two glycosphingolipid isomers is the native ligand of both proteins. Thus, the MAbs, lectin and enzyme may recognize peptides bearing this sequence motif via a shared binding mechanism.

Several interesting studies on the capsular polysaccharide of *Cryptococcus neoformans* have revealed that cross-reactive peptides are recognized by the sera of multiple individuals, and that the mechanism of cross-reaction is not necessarily structural mimicry. Zhang *et al.* [21••] showed broad cross-reactivity of phage bearing the consensus sequence QT(G/T)(L/D); these clones were isolated by a human MAb against the Cryptococcal capsular polysaccharide. Phage bearing a representative consensus peptide were used in a competition assay to block binding of Abs from sera of people with HIV-1 infections and without such infections to the Cryptococcal polysaccharide. HlV-negative sera, but not HIV-positive sera, were partially inhibited from binding the polysaccharide (with inhibition levels of 27–41%), indicating that the peptide recognizes a common Ab species among non-

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infected individuals. These findings arc understandable, given the highly-restricted nature of anti-carbohydrate responses in most individuals, (in which limited numbers of V_H and V_L genes are usually expressed in response to a given carbohydrate antigen), and the diversity of Ab production caused by the polyclonal activation of B-cell responses in HIV-1 infected individuals (in which a large variety of V_H and V_L genes are expressed in the absence of specific immunization with antigen). That partial inhibition occurred with multiple sera indicates that this peptide-specific sub-species of Ab is produced by most people in response to the Cryptococcal capsule, and, thus, suggests the application of peptides in diagnostics; not as carbohydrate mimics, but as indicators of specific Ab sub-species in the Ab response against the Cryptococcal capsule.

In a related study, Valadon *et al.* [22] isolated decapeptides bearing the consensus sequence TPXW(M/L)(M/L) with a murine MAb against the Cryptococcal capsular polysaccharide. A synthetic peptide bearing this motif was able to elicit Abs having the correct idiotype, but that cross-reacted only weakly with the capsular polysaccharide. Interestingly, these Abs bore the same light-chain sequence as the screening MAb, but several different heavy chains. The crystal structute of the peptide bound to the MAb that selected it [23••] revealed that the peptide associates mostly with the light chain, whereas binding to the Cryptococcal antigen depends upon the sequence of the third hypervariable loop of V_H (H3). This indicates that the peptide most probably binds by a different mechanism from that of the capsular polysaccharide. Thus, the mechanism of cross-reactivity between peptide-mimics and their target antigens may largely depend upon the Ab used to select the peptide, with the selection of structural versus functional mimics being a matter of the peptides having the best fit with the Ab in question. These studies also emphasize the necessity of structural studies in determining the mechanism of cross-reactivity. So far, there is no direct evidence, from crystallographic or other physical studies, of structural mimicry being a mechanism of cross-reactivity of immunogenic mimicry between peptides and non-proteinaceous epitopes. Understanding of the basis of cross-reactivity is essential for applications of peptides in diagnostics, therapeutics and vaccines. Using structural information as a guide, one can decide whether a given peptide should be used in a vaccine or diagnostic application as a structural mimic of the target epitope (as probably occurs for some peptide mimics of linear epitopes on folded proteins; see below) or as an Ab-specific reagent (as appears to be the case for many non-proteinaceous epitopes).

Peptide agonists and antagonists

Peptide ligands have recently been found for receptors (thrombopoietin, melanocortin receptor, CD80, and a hantaviral receptor), receptor ligands (angiogenin, α-bungarotoxin [24••,25]), and folded domains from within larger proteins (SH2 [26•], SH3 and WW domains [27••]). In their second discovery of peptide agonists that are active in the dimeric form, the Affymax group [28••] isolated linear peptides that bind to the thrombopoietin receptor and compete with the natural ligand for binding. An optimzed peptide was covalently dimerized, and in this form was equipotent to thrombopoietin *in vitro*, and stimulated platelet production *in vivo*. In several cases, receptor ligands have been isolated by novel methods. The melanocortin (MC)1 receptor is a G-protein-coupled receptor that is activated by a peptide hormone, which also activates other MC receptor subtypes.

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Szardenings *et al.* [29••] constructed a 'sublibrary' of the hormone-core sequence, flanked by randomized residues, and screened it on whole insect cells that overexpress the MCI receptor. Peptides were isolated having moderately-high affinity (as compared to the native hormone) and very high (3–4 log) selectivity for the MC1 receptor. Thus, peptide libraries can be used to find highly selective receptor ligands by screening on whole cells. Fukumoto *et al* [30••] used a novel approach to identify a receptor-ligand using a MAb against the CTLA4 signalling protein on T cells; this MAb mimics the CD80 counter-receptor on antigen-presenting cells. They identified from an X_{15} library two candidate peptides that bind this MAb, one of which binds to CD80 and, interestingly, potentiates T-cell proliferative responses. Yet another novel screening approach is described by Heiskanen *et al.* [31••], who screened a CX_7C peptide library on whole Puumala hantavirus using 'competitive elution' with neutralizing, antiviral MAbs. They identified peptides that neutralize viral infection *in vitro* at nanomolar concentrations, which is similar to the neutralizing activity of the MAbs. Gho *et al.* [32••] identified a cyclic octapeptide that binds the factor angiogenin (by competitive elution with actin), and showed that this peptide could block angiogenin activity in a neo-vascularization assay. Furthermore, the peptide blocked angiogenesis induced by a tumor cell line that secretes angiogenin. Following a nearly-identical protocol, Choi *et al.* [33•] identified 12-mer peptides bearing a different angiogenin-binding sequence from that described by Gho *et al.* [32••], yet both groups' peptides bind to a similar or identical site on angiogenin, because they were both obtained by competitive elution with actin.

Phage that home to tumor-associated blood vessels were characterized by Arap *et al.* [34••], who used mice bearing tumors for the *in vivo* screening of peptide libraries. Phage bearing two types of cell-adhesion sequence motif (RGD and NGR) were isolated from tumor tissues after intravenous injection of cyclic-peptide libraries that was followed by perfusion to remove unbound phage from the vascular tree. Peptides bearing homing sequences were covalently coupled to the anti-cancer drug doxorubicin, injected into mice beating human breast cancer tumors and shown to cause tumor regression [34••]. Earlier work by the same group [35•] showed that phage displaying the RGD sequence home to a variety of tumors via the alpha-v integrins present in tumor-associated blood vessels.

Several other previously-identified receptor-binding peptides have now demonstrated biological activity, and others have been improved by rational design. In previous work, Bottger *et al.* [36] identified a peptide ligand for the site on the MdM2 protein that binds the p53 tumor suppressor. As described in a follow-up paper [37••], this group engineered thioredoxin to display a loop bearing the peptide, and showed that the presence of this fusion within cells causes the accumulation of p53. Similarly, Stauffer *et al.* [38••] improved the selectivity of previously-identified peptides for the SH3 domain of the Lyn tyrosine kinase, and showed that they inhibit Lyn function when electro-porated into mast-cell-like leukemia cells. Acyclic, 20-merpeptide, which, in the dimeric form, binds and acts as an agonist of the erythropoietin receptor [39,40], was further characterized with regard to its critical-binding residues, and was minimized to 13 residues [41•]; its potency was also improved by covalent dimerization [42•].

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Substrates and inhibitors have also been found for several proteases. Whereas several new substrate peptides have been identified using the novel 'substrate phage' approach of Matthews and Wells [43], inhibitors have been identified by screening phage libraries directly. O'Boyle *et al.* [44••] constructed three different substrate phage peptide libraries and screened them with the protease of Type 1-herpes simplex virus. An optimized 8-mer peptide, having a different amino acid sequence from the native site, was defined and shown to be cleaved with the same efficiency as the native site. Ke *et al.* [45•,46•] isolated peptides that are specific substrates for the tissue-type and urokinase-type plasminogen activators, or t-PA and u-PA, respectively. Ploug *et al.* [47•] further characterized a 15-mer peptide that inhibits the urokinase-type plasminogen activator. In contrast to studies identifying substrate sequences, two papers from Sollazzo's group describe inhibitors of the hepatitis C virus NS3 protease, which were identified from three different 'scaffold libraries' based on a camelized V_H domain [48••], a serine-protease inhibitor and a 'minibody' [49•].

Increasing the range of receptors that cross-react with peptides

Several problems are associated with failed screens in which low-affinity or non-binding phage are isolated. The affinity of weakly-binding phage can sometimes be improved by constructing 'sub-libtanes' of two types. If a consensus sequence is observed but the peptides bind with low affinity, a new library can be constructed in which the consensus residues are fixed, and the residues flanking them randomized. Alternatively, when there is only a single weakly-binding peptide, a library can be constructed in which the nucleotide sequence encoding a binding peptide is doped (during oligonucleotide synthesis) with lesser amounts of the remaining three bases at each nucleotide in the sequence, to yield variability at every amino acid residue. Such libraries can be re-screened under more stringent selection conditions for tighter binders. Another means of improving the apparent affinity (rather than the instrinsic affinity) of a weakly-binding peptide is to make it multivalent. Terskikh *et al.* [50•] devised the 'peptabody' which displays peptides in a pentavalent array. Alternatively, highly-multivalent phage libraries can be screened. Similar to the 'organic landscape' phage libraries of Petrenko *et al.* [51], Iannolo *et al,* [52•] constructed an octapeptide library in which the peptides were fused to every copy of the pVIII major coat protein. (Filamentous bacteriophage are composed of thousands of copies of pVIII, with the number of molecules per virion varying with the length of the genome; one pVIII molecule for every 2.3 nucleotides in the single stranded genome.) Thus, this library of high-density peptides was screened with an organic dye to find dye-binding peptides. Such multivalent systems have particular application for targets that are themselves multivalent; furthermore, the phage, being relatively large filaments, can also be used as a cheap means to a selective adsorbant.

As for the problem of negative screens, in which no binding phage are found, it is probable that the existing peptide libraries do not cover enough sequence space to include tight-binding peptides for targets that require more than 5–6 critical binding residues (these are residues that form high-energy contacts, and are usually few in number). In some cases, 'gene-fragment' libraries may provide a better way of identifying epitope sequences for protein-binding molecules. In a comparative study, Fack *et al.* [53••] mapped the epitopes for a panel of four MAbs using two random-peptide libraries (X_6 and X_{15}) versus a gene-fragment library constructed from fragments of cDNA encoding the target antigen. The

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MAbs always selected cross-reactive phage from the gene-fragment library, but not the random-peptide ones. This approach has limited application as a new library must be made for each screening target, and the target has to be a cloned protein. Alternatively, ribosome-display technology [54,55], which can accommodate libraries of 10^{13}–10^{14} sequences, may allow the screening of a much larger sequence space in the future.

Whereas the approach to epitope mapping of Fack *et al.* [53••] is recommended for immunoblot-reactive Abs that presumably recognize linear epitopes, there is still the problem of identifying discontinuous epitopes, whose structures cannot be mimicked by simple peptides. This is in part due to the gross topology of Ab combining sites, which can be related to the types of epitope that Abs can recognize. Anti-peptide Abs bear grooved sites having particular dimensions, whereas Abs against discontinuous epitopes arc larger and flatter [56,57]. Craig *et al.* [58••] showed, from screening a panel of peptide libraries with polyclonal Ab responses against two folded proteins, that peptide mimics of linear epitopes predominate in these screens, even though Abs against linear epitopes constitute only a small fraction of the total Ab response against folded proteins [59]. Linear-epitope-mimic peptides are much more abundant in peptide libaries than discontinuous-epitope mimics, as the number of different clones isolated for a given linear epitope is relatively large compared to the number of clones isolated for a discontinuous epitope. Unpublished work from our lab with a panel of MAbs known to bind discontinuous epitopes has shown that 60–70% of these types of MAbs isolate rare clones from the same panel of libraries, and these ‘discontinuous-epitope mimics’ often bind their MAbs relatively tightly. Thus, in comparison to linear-epitope mimics, peptides that cross-react with discontinuous protein epitopes are rare or altogether absent from standard peptide libraries. Jespers *et al.* [60••] have circumvented the problem of mapping discontinuous epitopes with peptides by a strategy in which a library of mutants of a protein antigen is produced, and then ‘negatively selected’ on an a MAb against the discontinuous epitope of interest. Phage that do not bind the MAb are then ‘positively selected’ for global folding by binding to a ligand for the antigen or MAb against a separately-located, discontinuous epitope on the antigen. This approach was extended to mapping the major epitopes recognized by a set of patients in their polyclonal Ab response against a protein antigen [61•].

The view that peptides only sporadically cross-react with discontinuous epitopes on protein antigens can be extended to include many other types of receptor-binding sites, especially those on protein ligands. With the intention of better mimicking discontinuous epitopes, several groups [62–64] have produced libraries that form a surface on a folded-protein scaffold. Such scaffolds are expected to form a more constrained framework for displaying the randomized (X) residues in a library, and, when displayed on separate adjacent strands of a folded protein, the X residues can assemble into a library of discontinuous epitopes. Such libraries are reminiscent of site-directed, protein-mutant libraries, such as protease-inhibitor [65,66] and zinc-finger libraries [67,68], which are targeted to specific classes of ligands, such as proteases and DNA, respectively. Two new libraries have been constructed based on different protein scaffolds [69••,70•]; both groups based their design on very stably-folding scaffolds that would allow modeling of the ligands isolated from them. Nord *et al.* [69••] designed a three-helix bundle to display 13 randomized (X) positions on its

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surface. From this library, they isolated ligands for three very different proteins: a polymerase, insulin and an apolipoprotein. Similarly, Smith *et al.* [70•] constructed a library using the knottin-like, cellulose-binding domain and identified ligands for cellulose and alkaline phosphatase, but not for α-amylase or β-glucuronidase.

Although the chances are slim that a single 'universal' constrained-scaffold library will contain ligands for most receptors and Abs, it is quite possible that a panel of different libraries will.

It is clear from several studies [9,28••,39] that the screening of a variety of peptide libraries with a given receptor or Ab increases the chances of identifying ligands for it. Besides the number and types of library screened, the design of the selection experiment is crucial to successful ligand identification; however, few studies exist in which different screening methods have been compared side-by-side. Levitan [71•] has devised a model of phage-library screening to clarify the effects of several parameters in the probability of selecting phage bearing ligand peptides. For instance, we routinely perform screenings on about 16 different peptide libraries that are mixed into seven or 11 pools. Following the general principles outlined by Levatin [71•], we firstly remove high-background phage by pre-adsorbing our libraries on plastic plates before each round of selection, secondly, perform the first round of selection by 'biopanning' [2,3] with solid-phase target [9] to ensure the capture of all target-binding phage, thirdly, introduce high-stringency, biopanning selections, [3,72] in later rounds of screeing, by capturing page-target-molecule complexes out of solution and using target-molecule concentration to control selection stringency, and finally, using phage yield (i.e. the yield of binding phage over a control phage) during the selection experiment and a functional assay (usually ELISAs) to choose phage pools from which clones will be selected for further evaluation. As the phage yield and ELISA show the enrichment of target-binding phage for a given round of selection, selection stringency can be increased by dropping target concentration (usually by 1–2 orders of magnitude) and repeating that round of selection.

Conclusions

As new libraries are constructed their value would be mote clearly demonstrated were they tested side-by-side along with several other different libraries in screenings against a variety of receptors, enzymes and Abs. Futhermore, optimization of screening methods should be performed for each target molecule screened to ensure the highest probability of identifying ligand peptides from a given library. As it stands now, most researchers only test a single library at a time using screening methods that arc usually not optimized. The results of side-by-side comparisons between different libraries would clarify the types of libraries that most effectively produce ligands for a given type of receptor or Ab, and whether there are any general rules that govern the types of libraries that contain ligands for a particular class of receptor or Ab. In the longer view, the combination of these types of functional studies with structural studies that determine the mechanisms of peptide binding (i.e. functional versus structural mimicry) would be of great benefit in determining the rules governing protein cross-reactivity.

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Abbreviations

Ab	antibody
LPS	lipopolysaccharide
M	monoclonal
MC	melanocortin
V_H	heavy chain variable region
V_L	light chain variable region

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11. Jelinek R, Terry TD, Gesell JJ, Malik P, Perham RN, Opella SJ. NMR structure of the principal neutralizing determinant of HIV-1 displayed in filamentous bacteriophage coat protein. J Mol Biol. 1997; 266:649–655. [PubMed: 9102458] The authors undertook the monumental task of deconvoluting the NMR structure of the hexapeptide GPGRAF as displayed on pVIII of the virion surface. The peptide was found to have a more persistent structure on the phage surface than its counterpart does in solution. The peptide was found to have a similar double-turn structure on the phage coat as an antibody-bound structure previously determined by X-ray crystallography (JB Ghiara *et al., Science* 1994, **264**:82–85).
12. Mennuni C, Santini C, Dotta F, Farilla L, Di Mario U, Fierabracci A, Bottazzo G, Cortese R, Luzzago A. Selection of phage-displayed peptides mimicking type 1 diabetes-specific epitopes. J Auto immun. 1996; 9:431–436.
13. Mennuni C, Santini C, Lazzaro D, Dotta F, Farilla L, Fierabracci A, Bottazzo GF, Di Mario U, Cortese R, Luzzago A. Identification of a novel type 1 diabetes-specific epitope by screening phage libraries with sera from pre-diabetic patients. J Mol Biol. 1997; 268:599–606. [PubMed: 9171283] Multiple serum IgGs from pre-diabetic subjects were previously used to identify a number of peptides that were recognized by insulin-dependent diabetes mellitus (IDDM)-related sera [12]. One peptide-bearing phage clone was selected with high frequency of reactivity with sera from IDDM patients as compared to controls. Immunization with this phage clone elicited islet Beta-cell-binding Abs in rabbits.
14. Phalipon A, Folgori A, Arondel J, Sgaramella G, Fortugno P, Cortese R, Sansonetti PJ, Felici F. Induction of anti-carbohydrate antibodies by phage library-selected peptide mimics. Eur J Immunol. 1997; 27:2620–2625. [PubMed: 9368618] Two similar IgA MAbs raised against LPS were used to screen two nonapeptide libraries, one linear and the other disulfide-constrained. One of these MAbs was previously shown to select a peptide from a zinc-finger library (E Bianchi *et al., J Mol Biol* 1995, **247**:154–160). Nineteen sequences were identified, only two of which induced anti-LPS Abs (titer 1:100) when mice were immunized with peptide-bearing phage. One of the two immunogenic peptides was recognized by both screening MAbs.
15. Sibille P, Ternynck T, Nato F, Buttin G, Strosberg D, Avrameas A. Mimotopes of polyreactive anti-DNA antibodies identified using phage-display peptide libraries. Eur J Immunol. 1997; 27:1221–1228. [PubMed: 9174614] Three polyreactive murine anti-DNA MAbs were used 1o screen a peptide library displayed on pVIII (sequence: $X_4CX_6CX_4$). Two MAbs identified unique consensus sequences, whereas the third did not. Phage displaying peptides from either of the selected consensus groups were not recognized by the non-selecting Ab. Mouse immunizations with a few selected clones resulted in sera that showed up to four times higher anti-dsDNA ELISA signals than sera from control phage (1:100 serum dilution), and the Abs elicited were largely lgG3.
16. Harris SL, Craig L, Mehroke JS, Rashed M, Zwick MB, Kenar K, Toone EJ, Greenspan N, Auzanneau FI, Marino-Albernas JR, et al. Exploring the basis of peptide-carbohydrate cross reactivity: evidence for discrimination by peptides between closely related anti-carbohydrate antibodies. Proc Natl Acad Sci USA. 1997; 94:2454–2459. [PubMed: 9122216] A panel of peptide libraries was screened with five, closely-related MAbs against a similar or identical polysaccharide epitope on the cell-wall of group A *Streptococcus*. All of the MAbs isolated peptides, and many competed with carbohydrate antigen for MAb binding. Yet, each of the peptides bound only to the MAb that isolated it, indicating that the peptides distinguish between these MAbs, and that the mechanism of binding 1o the peptides differed from that to the carbohydrate antigen.
17. Gaynor B, Putterman C, Valadon P, Spatz L, Scharff MD, Diamond B. Peptide inhibition of glomerular deposition of an anti-DNA antibody. Proc Natl Acad Sci USA. 1997; 94:1955–1960. [PubMed: 9050886] Two closely-related and pathogenic anti-DNA MAbs from mice isolated several decapeptide-bearing phage clones. The peptides appeared MAb-specific, because peptides selected by each of the two anti-DNA MAbs cross-react poorly, or not at all, with the non-

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selecting MAb. Both D- and L-forms of one minimized synthetic peptide were shown to inhibit deposition of MAb–DNA immune complexes in the glomerulus (50% inhibition at ~200 μM).

18. Kieber-Emmons T, Luo P, Qiu J, Agadjanyan M, Carey L, Hutchins W, Westerink MA, Steplewski Z. Peptide mimicry of adenocarcinoma-associated carbohydrate antigens. Hybridoma. 1997; 16:3–10. [PubMed: 9085121] Peptide-mimics of the three different carbohydrates, alpha-methyl mannopyranoside [72] (KR Oldenburg *et al., Proc Natl Acad Sci USA* 1992, **89**:5393–5398), Lewis Y (R Hoess *et al., Gene* 1993, **128**:43–49), and the major C polysaccharide alpha(2–9) sialic acid of *N. meningitidis* (MA Westerilink *et al., Proc Natl Acad Sci USA* 1995, **92**:4021–4025), were compared and used to derive putative carbohydrate-peptide-mimicking motifs that were immunized into mice. The mouse sera bound breast cancer cells and human melanoma cells, but not normal breast cells or a murine fibroblast cell line. The cross-reactive sera induced complement-mediated lysis of a breast cancer cell line at low titers (1:5, 1:10). The authors propose that the peptides “retain carbohydrate-like conformations inducing anti-carbohydrate immune responses”.
19. Agadjanyan M, Luo P, Westerink MA, Carey LA, Hutchins W, Steplewski Z, Weiner DB, Kieber-Emmons T. Peptide mimicry of carbohydrate epitopes on human immunodeficiency virus. Nat Bio technol. 1997; 15:547–551. The same murine sera used in [18••] were shown to bind glycosylated HIV-1 envelope proteins at low titers (1:2, 1:5), but not non-glycosyla1ed ones. The sera also inhibited syncytia formation at higher titers (up to 1:64), as compared to normal human sera.
20. Taki T, Ishikawa D, Hamasaki H, Handa S. Preparation of peptides which mimic glycosphingolipids by using phage peptide library and their modulation on beta-galactosidase activity. FEBS Lett. 1997; 418:219–223. [PubMed: 9414130] A 15mer library displayed on plll was screened with two MAbs, one against lactotetrosylceramide (Lc4Cer) and another against its isomer neoLc4Cer. A total of five clones were sequenced, but one motif was found in pools from both MAbs. Nonamer peptides were synthesized that contained the shared motif, and some were found to bind a lectin from *Ricinus communis*. Curiously, some of the peptides showed both inhibition and activation of activity of Jack Bean β-galactosidase whose natural substrate is neoLc4Cer.
21. Zhang H, Zhong Z, Pirofski LA. Peptide epitopes recognized by a human anti-cryptococcal glucuronoxylomannan antibody. Infect Immun. 1997; 65:1158–1164. [PubMed: 9119446] The human MAb 2E9 (lgM) binds to the glucuronoxylomannan capsule on *C. neoformans*. This MAb was used to screen a decapeptide library displayed on plll. One peptide (amino acid sequence GMDGTQLDRW), was synthesized and found to inhibit GXM binding of naturally occurring serum antibodies from HIV-negative, but not HIV-positive patients.
22. Valadon P, Nussbaum G, Boyd LF, Margulies DH, Scharff MD. Peptide libraries define the fine specificity of anti-polysaccharide antibodies to *Cryptococcus neoformans*. J Mol Biol. 1996; 261:11–22. [PubMed: 8760499]
23. Young AC, Valadon P, Casadevall A, Scharff MD, Sacchettini JC. The three-dimensional structures of a polysaccharide binding antibody to Cryptococcus neoformans and its complex with a peptide from a phage display library: implications for the identification of peptide mimotopes. J Mol Biol. 1997; 274:622–634. [PubMed: 9417940] This paper shows the crystal structure of peptide in complex with Fab that was raised against the capsular polysaccharide of *Cryptococcus neoformans*. Earlier work [22] showed that this peptide was recognized by a subset of MAbs against this polysaccharide. Importantly, the peptide associated mostly with the V_L, whereas indirect evidence indicated that binding to the carbohydrate antigen involves V_H mainly. From immunizations, this peptide was stated to elicit only a small anti-carbohydrate response, although no titering data was given.
24. Balass M, Katchalski-Katzir E, Fuchs S. The alpha-bungarotoxin binding site on the nicotinic acetylcholine receptor: analysis using a phage-epitope library. Proc Natl Acad Sci USA. 1997; 94:6054–6058. [PubMed: 9177167] A random 15mer plll-displayed peptide library was screened with α-bungarotoxin a potent snake neurotoxin with specificity for the nicotinic acetylcholine receptor. A consensus motif, YYXSSL, was identified that had similarities to a region within some of the acetylcholine receptors that bind α-bungarotoxin, Competition binding studies of different receptor-derived peptides revealed 1he requirement of two adjacent aromatic residues in the target receptor for α-bungarotoxin recognition. An accompanying article [25] shows the solution structure of one of the library-derived 13mer peptides in complex with α-bungarotoxin as solved

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by 2D proton NMR spectroscopy. The peptide assumes a globular conformation that occupies the binding pocket of α-bungarotoxin.

25. Scherf T, Balass M, Fuchs S, Katchalski-Katzir E, Anglister J. Three-dimensional solution structure of the complex of alpha-bungarotoxin with a library-derived peptide. Proc Natl Acad Sci USA. 1997; 94:6059–6064. [PubMed: 9177168]
26. Gram H, Schmitz R, Zuber JF, Baumann G. Identification of phosphopeptide ligands for the Src-homology 2 (SH2) domain of Grb2 by phage display. Eur J Bio chem. 1997; 246:633–637. A pill-displayed library, $ELEX_3YX_4A$, was first phosphorylated *in vitro* at the invariant tyrosine, and then screened with the SH2 domain of the adapter protein Grb2. Several clones were identified that bind to Grb2, only when previously phosphorylated.
27. Linn H, Ermekova KS, Rentschler S, Sparks AB, Kay BK, Sudol M. Using molecular repertoires to identify high-affinity peptide ligands of the WW domain of human and mouse YAP. Biol Chem. 1997; 378:531–537. [PubMed: 9224934] A parallel approach of using a synthetic peptide library and a plll-displayed library (SSX_6PPX_6SR) to determine the ligand preferences of the WW-domain of the human Yes-associated protein (YAP) converged on a core sequence PPPPYP. This core sequence is found within the p53 binding protein-2, a proposed binding partner for YAP.
28. Cwirla SE, Balasubramanian P, Duffin DJ, Wagstrom CR, Gates CM, Singer SC, Davis AM, Tansik RL, Mattheakis LC, Boytos CM, et al. Peptide agonist of the thrombopoietin receptor as potent as the natural cytokine. Science. 1997; 276:1696–1699. [PubMed: 9180079] Several recombinant peptide libraries including a panel of phage display libraries on pVIII were used in this study to ultimately identify a potent agonist of the thrombopoietin receptor. Two families of peptides were identified and none of the sequences were found in the primary sequence of thrombopoietin. In two stages of optimization, sublibraries were successively constructed and affinity-selected using the headpiece dimer (CM Gates *et al.*, *J Mol Biol* 1996, **255**:373–386) and polysome [54] display systems. Finally, an optimal 14 amino acid peptide was synthesized as a covalently-dimerized compound that was found to have a median effective concentration equivalent to that of thrombopoietin (100 pM) in a cell-based assay. This compound was also shown to increase platelet counts in mice.
29. Szardenings M, Tornroth S, Mutulis F, Muceniece R, Keinanen K, Kuusinen A, Wikberg JE. Phage display selection on whole cells yields a peptide specific for melanocortin receptor 1. J Biol Chem. 1997; 272:27943–27948. [PubMed: 9346944] A melanocortin receptor (MC1) was expressed on insect cells, and the cells were used to pan a 10^5-member, plll-displayed, SX_4FRWX_5 peptide library that was based on the melanocyte stimulating hormone (MSH)-core sequence. One peptide was found that shares little homology with the native α-MSH sequence, but which bound the MC1 receptor with only 30-fold lower affinity. This peptide was selective for the MC1 receptor, as it bound this receptor with nanomolar affinity, whereas it bound the other MC receptor subtypes (MC3, MC4, MC5) with high micromolar affinity. Thus, the peptide discriminated by ≥3–4 logs between α-MSH-specific, G-coupled protein receptors.
30. Fukumoto T, Torigoe N, Kawabata S, Murakami M, Uede T, Nishi T, Ito Y, Sugimura K. Peptide mimics of the CTLA4-binding domain stimulate T-cell proliferation. Nat Biotechnol. 1998; 16:267–270. [PubMed: 9528007] A 15-mer peptide library was screened with a MAb against CTLA4 (CD28), which is expressed on T cells and, when bound by CD80 or CD86 on antigen-presenting cells, generates the ‘second signal’ required for T-cell activation. One peptide was isolated that bound to CD80 and stimulated T-ceil proliferation, whereas another peptide stimulated T-cell proliferation but did not bind CD80 or CD86, The mechanism of the T-cell proliferative activity of the latter peptide was not determined.
31. Heiskanen T, Lundkvist A, Vaheri A, Lankinen H. Phage-displayed peptide targeting on the Puumala hantavirus neutralization site. J Virol. 1997; 71:3879–3885. [PubMed: 9094664] A phage-displayed peptide was identified that binds hantavirus and prevents virus binding and cellular infection *in vitro* at nanomolar concentrations. This peptide was one of several isolated from a phage-displayed CX_7C peptide library by ‘competitive elution’ with two hantavirus-neutralizing MAbs. Both the protective peptide and the MAb corresponding to it neutralized the virus *in vitro* at a similar effective concentration.
32. Gho YS, Lee JE, Oh KS, Bae DG, Chae CB. Development of antiangiogenin peptide using a phage-displayed peptide library. Cancer Res. 1997; 57:3733–3740. [PubMed: 9288781] Human angiogenin is a protein that promotes the growth of blood vessels (angiogenesis) in tumors.

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Angiogenin was used to screen a pill-displayed $X_2CX_4CX_2$ library, using actin to 'competitively elude' binding phage, A peptide was isolated that inhibits the activity of angiogenin in two different cell-culture assays.

33. Choi SJ, Ahn M, Lee JS, Jung WJ. Selection of a high affinity angiogenin-binding peptide from a peptide library displayed on phage coat protein. Mol Cells. 1997; 7:575–581. [PubMed: 9387141] A $CX_{12}C$ peptide library displayed on pill was screened with angiogenin, followed by the 'competitive elution' of binding phage with actin. Some peptides were transferred to the carboxy-terminus of MBP. and the best angiogenin-binder (Kd = 60 nM by ELISA) was sequenced.
34. Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science. 1998; 279:377–380. [PubMed: 9430587] Tumor-homing peptides isolated from phage-displayed peptide libraries were covalently crosslinked to the anti-tumor drug doxorubicin, and thereby enhanced the activity of the drug when administered to mice bearing human tumor xenografts. One peptide, CDCRGDCFC, was previously characterized [35•], and another, CNGRCVSGCAGRC, was selected from a $CX_3CX_3CX_3C$ library and contains a known cell adhesion motif, NGR. The authors suggest that the NGR and RGD peptides bind to different receptors in tumors. Significantly, the peptides also reduced the toxicity of doxorubicin.
35. Pasqualini R, Koivunen E, Ruoslahti E. Alpha v integrins as receptors for tumor targeting by circulating ligands. Nat Biotechnol. 1997; 15:542–546. [PubMed: 9181576] A phage bearing a cyclic, RGD-containing nonapeptide (CDCRGDCFC) was previously isolated (E Koivunen *et al. Bio/Technology* 1995, **13**:265–270) with specificity for the αv integrins that are known to be molecular markers in angiogenic endothelium. Phage displaying CDCRGDCFC were injected (intravenously) into mice bearing murine and human-derived tumours. Tissue was harvested 2–4 minutes after injection and saline perfusion, and phage bearing the peptide were found in the tumor at higher titers than control phage. Phage bearing the peptide were also found at higher titers in the tumor than in the brain and kidney tissue. Immunohistochemical staining using anti-M13 and polydonal anti-αv integrin Abs revealed tumor-specific phage was confined to the tumor blood vessels. After 24 hours, these phage were still detectable in the tumor, whereas 90% of the phage was eliminated from circulation probably by the reticuloendothelial system.
36. Bottger V, Bottger A, Howard SF, Picksley SM, Chene P, Garcia-Echeverria C, Hochkeppel HK, Lane DP. Identification of novel mdm2 binding peptides by phage display. Oncogene. 1996; 13:2141–2147. [PubMed: 8950981]
37. Bottger A, Bottger V, Sparks A, Liu WL, Howard SF, Lane DP. Design of a synthetic Mdm2-binding mini protein that activates the p53 response *in vivo*. Curr Biol. 1997; 7:860–869. [PubMed: 9382809] The Mdm2/Hdm2 protein binds the tumor suppressor protein p53, and causes it to be degraded. Peptides that bind to Mdm2 were isolated by screening 12-mer and 15-mer peptide libraries with Mdm2 (V Bottger *et al., Oncogene* 1996, **13**:2141–2147). These peptides were futher characterized and found to be similar to the Mdm2-binding she of p53 (A Bottger *et al., Curr Biol* 1997, **7**:860–869), In this paper, a 12-mer peptide that binds to Mdm2 was engineered into a loop of thioredoxin. When this recombinant protein was either transiently expressed in mammalian cells or microinjected directly into them, an accumulation of p53 resulted. This indicates that the peptide can bind to Mdm2 *in vivo* and block the degradation of p53, by blocking the IV1dm2-p53 interaction.
38. Stauffer TP, Martenson CH, Rider JE, Kay BK, Meyer T. Inhibition of Lyn function in mast cell activation by SH3 domain binding peptides. Biochemistry. 1997; 36:9388–9394. [PubMed: 9235982] The Lyn tyrosine kinase has been implicated in IgE-receptor-mediated mast cell activation. Peptides that bind to SH3 domains were previously isolated from phage-displayed peptide libraries (AB Sparks *et al., J Biol Chem* 1994, **269**:23853–23856; RJ Rickles *et al., Proc Natl Acad Sci USA* 1995, **92**:10909–10913). In this study, peptides with restricted binding activity toward the Lyn SH3 domain were also isolated by screening a phage-displayed peptide library, A leukemic mast-cell line was electroporated with these Lyn-specific peptides, and the resulting cells were shown to have blocked calcium signalling in response to anti Fc_ε receptor Ab and reduced Lyn function, as compared to cells that were electroporated with control peptides.
39. Wrighton NC, Farrell F, Chang R, Kashyap AK, Barbone FP, Mulcahy LS, Johnson DL, Barrett RW, Jolliffe LK, Dowen WJ. Small peptides as potent mimetics of the protein hormone erythropoietin. Science. 1996; 273:458–463. [PubMed: 8662529]

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40. Livnah O, Stura EA, Johnson DL, Middleton SA, Mulcahy LS, Wrighton NC, Dower WJ, Jolliffe LK, Wilson IA. Functional mimicry of a protein hormone by a peptide agonist: the EPO receptor complex at 2.8 Å. Science. 1996; 273:464–471. [PubMed: 8662530]

41. Johnson DL, Farrell FX, Barbone FP, McMahon FJ, Tullai J, Hoey K, Livnah O, Wrighton NC, Middleton SA, Loughney DA, et al. Identification of a 13 amino acid peptide mimetic of erythropoietin and description of amino acids critical for the mimetic activity of EMP1. Biochemistry. 1998; 37:3699–3710. [PubMed: 9521688] The authors characterize in detail a minimized, 13 aa peptide mimetic of erythropoietin that was derived from the 20-aa sequences previously identified [39].

42. Wrighton NC, Balasubramanian P, Barbone FP, Kashyap AK, Farrell FX, Jolliffe LK, Barrett RW, Dower WJ. Increased potency of an erythropoietin peptide mimetic through covalent dimerization. Nat Biotechnol. 1997; 15:1261–1265. [PubMed: 9359108] A 20-mer erythropoietin peptide mimetic [39] has a propensity 1o dimerize noncovalently. In this study, the peptide was chemically synthesized as a covalent dimer resulting in a 100-fold increase in affinity for the erythropoietin receptor and a similar increase in the stimulation of red blood cell production *in vivo*.

43. Matthews DJ, Wells JA. Substrate phage: selection of protease substrates by monovalent phage display. Science. 1993; 260:1113–1117. [PubMed: 8493554]

44. O'Boyle DR, Pokornowski KA, McCann P Jr, Weinheimer SP. Identification of a novel peptide substrate of HSV-1 protease using substrate phage display. Virology. 1997; 236:338–347. [PubMed: 9325241] Using the 'substrate phage' approach [43], the herpes simplex virus 1 protease was used to screen a random 6-mer library, and a library containing four fixed amino acids and four randomized (X) positions. The peptide libraries were engineered such that bovine pancreatic trypsin inhibitor was fused to the mature, amino-terminus of plll, followed by the peptide library and the coat protein sequence. The phage, bound to beads to which trypsin had been covalently attached, were then incubated with Type I-herpes simplex virus protease, and cleaved phage were recovered. Based on the consensus of the selected sequences, an optimized 8mer peptide (LVLASSSF) was synthesized that was cleaved slightly more efficiently than a 20-mer peptide containing the natural cleavage site.

45. Ke SH, Coombs GS, Tachias K, Navre M, Corey DR, Madison EL. Distinguishing the specificities of closely related proteases. Role of P3 in substrate and inhibitor discrimination between tissue-type plasminogen activator and urokinase. J Biol Chem. 1997; 272:16603–16609. [PubMed: 9195973] A 6-mer peptide library which bore a MAb-binding 'epitope tag' was subjected to three rounds of standard substrate-phage selection with t-PA using the immobilized MAb to retain uncleaved phage; this produced a cleaved phage pool that was enriched for clones bearing peptides that were cleaved by t-PA. The phage pool was amplified, thus restoring the peptides and epitope tags on plll. A closely-related enzyme to t-PA, u-PA, was added and phage clones bearing intact peptide were isolated with tag-specific MAb. Thus, t-PA-selected clones bearing peptides that were also cleaved by u-PA were 'subtracted' from the pool, and only clones specific for t-PA were isolated. A minimized and optimized peptide was shown to be 78 times more selective for t-PA than u-PA. Based on the substrate phage results, a natural inhibitor of bo1h t-PA and u-PA was altered at two positions by site-directed mutagenesis to be 600 times more selective for t-PA than u-PA.

46. Ke SH, Coombs GS, Tachias K, Corey DR, Madison EL. Optimal subsite occupancy and design of a selective inhibitor of urokinase. J Biol Chem. 1997; 272:20456–20462. [PubMed: 9252355] A hexapeptide phage substrate library was used to identify an optimal peptide for u-PA. A peptide is isolated that is cleaved 5,300 times more efficiently than a peptide containing the natural target sequence of the enzyme, and the peptide is cleaved 120 times more efficiently by u-PA than by t-PA. Based on these results, site-directed mutagenesis on a natural inhibitor resulted in a variant that inhibited u-PA 69 times more rapidly than it inhibited t-PA.

47. Ploug M, Stergaard S, Laurenborg Hansen LB, Holm A, Dan K. Photoaffinity labeling of the human receptor for urokinase-type plasminogen activator using a decapeptide antagonist. Evidence for a composite ligand-binding site and a short interdomain separation. Biochemistry. 1998; 37:3612–3622. [PubMed: 9521680] Previously, a 15-mer antagonist to the u-PA receptor was isolated (RJ Goodson *et al., Proc Natl Acad Sci USA* 1994, **91**:7129–7133). In this paper, alanine replacement and BIAcore analysis were used to identify a minimal 10-mer sequence. Photochemically active phenylalanine residues were placed within the peptide sequence, and

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photoaffinity labelling experiments showed that the peptide binds an assembly of domain I with either II or III of the receptor, as does u-PA.

48. Martin F, Volpari C, Steinkuhler C, Dimasi N, Brunetti M, Biasiol G, Altamura S, Cortese R, De Francesco R, Sollazzo M. Affinity selection of a camelized V(H) domain antibody inhibitor of hepatitis C virus NS3 protease. Protein Eng. 1997; 10:607–614. [PubMed: 9215580] A 'camelized' V_H domain is one in which three residues on the V_L interface of the human V_H domain are modified to mimick camel heavy chains, thus improving solubility (J Davies *et al., FEBS Lett* 1994, **339**:285–290). The result is the smallest known immunoglobin-based recognition unit. A phage display library of camelized antibodies, with CDR3 regions varied in amino acid sequence and in length (5–12 amino acids), was affinity selected with a 20 kDa fragment of the hepatitis C virus NS3 protease. Some selected clones were subcloned for expression in the periplasmic space of *Escherichia coli*. One clone was able to inhibit the protease activity of the 20 kDa fragment (IC50 ~300 nM), and the entire NS3 gene product (70 kDa, IC50 ~1 μM).
49. Dimasi N, Martin F, Volpari C, Brunetti M, Biasiol G, Altamura S, Cortese R, De Francesco R, Steinkuhler C, Sollazzo M. Characterization of engineered hepatitis C virus NS3 protease inhibitors affinity selected from human pancreatic secretory trypsin inhibitor and minibody repertoires. J Virol. 1997; 71:7461–7469. [PubMed: 9311825] Two inhibitors of the NS3 protease were isolated and characterized. One was a noncompetitive inhibitor isolated from a repertoire of mutagenized minibodies (F Martin *et al., EMBO J* 1994, **13**:5303–5309), and the other was a competitive inhibitor from a library made from the human pancreatic secretory trypsin inhibitor with a Ki = 360 nM (P Rottgen *et al. Gene* 1995, **164**:243–250).
50. Terskikh AV, Le Doussal JM, Crameri R, Fisch I, Mach JP, Kajava AV. “Peptabody”: a new type of high avidity binding protein. Proc Natl Acad Sci USA. 1997; 94:1663–1668. [PubMed: 9050835] A novel structure, the 'peptabody’, consists of five polypeptides that associate via a modification of the cartilage oligomeric matrix protein assembly domain. A phage library-derived, hexamer amino-acid sequence was fused to the amino-terminus of the assembly domain via an extended linker. An oligohistidine 'tag’ was fused to the carboxy-terminus of the polypeptide for rapid purification on a nickel column. The multivalent display of the peptide on the peptabody resulted in a dramatic increase in the apparent affinity for the cognate Ab due to an avidity boost. Two of the three peptide sequences tested were produced at high levels as peptabodies by *E. coli* cells.
51. Petrenko VA, Smith GP, Gong X, Quinn T. A library of organic landscapes on filamentous phage. Protein Eng. 1996; 9:797–801. [PubMed: 8888146]
52. lannolo G, Minenkova O, Gonfloni S, Castagnoli L, Cesareni G. Construction, exploitation and evolution of a new peptide library displayed at high density by fusion to the major coat protein of filamentous phage. Biol Chem. 1997; 378:517–521. [PubMed: 9224932] An octapeptide library displayed on all copies of pVIII was constructed and panned on three mAbs; short consensus sequences were identified that were also found in the protein antigens that the mAbs recognise. Phage were also selected that bind an organic dye Cibocron blue. It was rot determined whether the monovalent peptide would bind the dye, or if the binding was dependent on assembled peptide–pVIN fusions on the phage coat and, thus, an 'emergent property'.
53. Fack F, Hugle-Dorr B, Song D, Queitsch I, Petersen G, Bautz EK. Epitope mapping by phage display: random versus gene-fragment libraries. J Immunol Methods. 1997; 206:43–52. [PubMed: 9328567] A gene-fragment library is typically constructed by cloning random DNasel-digested gene fragments into the 5' end of the gene encoding the phage coat protein, In this study, a comparison was made in which MAbs against four different targets were used to screen gene-fragment, and random 6-mer and 15-mer peptide libraries. The gene fragment library was successful in every case, whereas the random peptide libraries were for only two MAbs, presumably because the minimal epitopes for the other MAbs were longer than that which could be practically covered in a random peptide library. Authors claim that any MAb that can detect its target in an immunoblot can be successful in isolating a sequence from a gene-fragment library.
54. Mattheakis LC, Bhatt RR, Dower WJ. An *in vitro* polysome display system for identifying ligands from very large peptide libraries. Proc Natl Acad Sci USA. 1994; 91:9022–9026. [PubMed: 7522328]
55. Hanes J, Plückthun A. *In vitro* selection and evolution of functional proteins by using ribosome display. Proc Natl Acad Sci USA. 1997; 94:4937–4942. [PubMed: 9144168]

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56. Vargas-Madrazo E, Lara-Ochoa F, Almagro JC. Canonical structure repertoire of the antigen-binding site of immunoglobulins suggests strong geometrical restrictions associated to the mechanism of immune recognition. J Mol Biol. 1995; 254:497–504. [PubMed: 7490765]
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