Identification of Receptor Ligands with Phage Display Peptide Libraries

Erkki Koivunen, Wadih Arap, Daniel Rajotte, Johanna Lahdenranta and Renata Pasqualini

Department of Biosciences, Division of Biochemistry, University of Helsinki, Viikinkaari, Finland; The Burnham Institute, La Jolla, California

With the development and maturation of the technology of displaying peptides on bacteriophage, it has become possible to isolate peptide ligands to various targets. In the phage display strategy, up to 10^9 peptides of different permutations are expressed on the surface of filamentous phage. Thus, peptides capable of binding target molecules in vitro and even target tissues in vivo can be identified. In recent years, a series of libraries that display degenerate peptides of different lengths have been constructed, and specific ligands to cell surface receptors, such as integrins, have been isolated. In the in vivo biopanning, peptides targeting distinct organs or tumors have been rescued after intravenous administration of phage libraries into mice. In one application, the isolated peptide ligands have been used to direct a cytotoxic drug to tumor vasculature in mice. Further applications in radioimaging and radiotherapy are being investigated.

Key Words: phage display; peptide libraries; integrins; targeting; biopanning; receptors; ligands

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The principle that exogenous peptides can be displayed on the surface of filamentous bacteriophage was first described by Smith in the mid 1980s (*1*). After introducing exogenous peptide sequences into the gene of the phage capsid proteins, the peptides were displayed on the phage surface as fusions with the proteins pIII or pVIII. The last decade has seen formidable progress in the construction of phage-displayed peptide libraries and in the formulation of screening methods with the libraries to isolate peptide ligands (*2,3*). Peptide libraries offer the possibility to characterize peptide binding specificity of important proteins, such as antibodies involved in inflammatory reactions (*3,4*) or integrins that mediate cellular adherence (*5*). Furthermore, it has become possible to find peptide ligands that could be potential lead compounds to develop novel peptidomimetic drugs. Phage libraries have found wide application, not only for display of small peptides but also for larger protein domains, such as single-chain antibodies (*6,7*).

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For correspondence or reprints contact: Renata Pasqualini, PhD, The Burnham Institute, 10901 North Torrey Pines Rd., La Jolla, CA 92037.

Filamentous phage are propagated in pilus-positive bacteria that are not lysed by the phage but secrete multiple copies of phage displaying a particular insert. Phage that bind to a target molecule can be eluted and then amplified by growing in bacteria (Fig. 1). This so-called "biopanning" can be repeated several times until a population of best binders is enriched. The sequence of a binding peptide is determined by sequencing the part of the phage genome that encodes the peptide. Finally, the insert can be reproduced as a recombinant or synthetic peptide. By this methodology, specific and selective ligands to target receptors can be found.

In most display applications, peptides are fused in or near the amino terminus of the minor capsid protein pIII. Another fusion partner that has been used, though to a lesser extent, is the major capsid protein pVIII. The advantage of pIII is that quite large peptide and protein inserts can be incorporated into it without lost of phage infectivity. In each phage particle, there are only about five copies of pIII which are believed to be anchored to one tip of the particle. We have been using a phage display vector called fUSE 5, in which each copy of pIII carries an insert (*8*). This results in a multivalent display of a peptide. The multivalency is not necessarily a disadvantage, as we have found several useful peptide ligands to different targets during the past 5 y using this vector for in vitro and in vivo applications. Several other vectors have been described that enable the display of an insert in one copy of the phage capsid protein pIII or pVIII (*2,9*).

Phage particles stand surprisingly harsh conditions, such as pH 2.2 and 4–6 mol/L urea without losing their ability to infect bacteria. This feature has been used to dissociate the bound phage from a target. It is also notable that the bound phage do not necessarily need to be eluted from a microtiter well or a complex mouse tissue, but infection somehow can proceed after addition of bacteria directly to the well or to the homogenized tissue (*10,11*). More specific methods can also be used for elution, if details of the biochemistry of ligand-receptor interaction are known. For example, both an arginine-glycine-aspartic acid (RGD)-containing peptide and cation chelators such as ethylenediamine tetraacetic acid (EDTA) can dissociate the phage bound to the $\alpha_5\beta_1$ integrin (*10*).

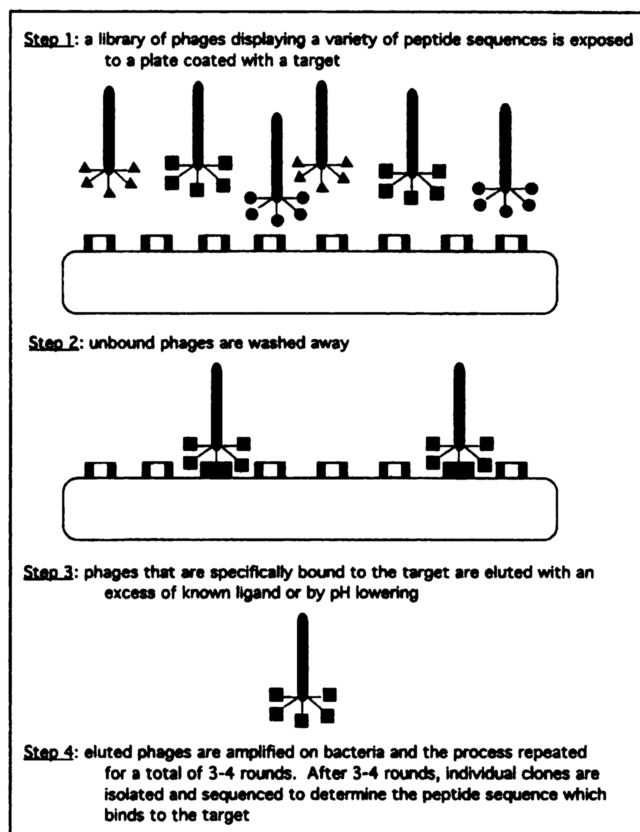

FIGURE 1. Biopanning consists of four steps: (1) screening of phage display library on specific target, (2) elution of bound phages from target, (3) amplification of eluted phages and (4) sequencing of deoxyribonucleic acid insert.

PROTEINS AND RECEPTORS THAT RECOGNIZE SHORT PEPTIDE SEQUENCES

Once a protein of interest is available in a purified form or can be expressed at the cell surface after transfection, peptide libraries provide an opportunity to characterize the ligand binding specificity of that protein. Protein-protein interactions in a living cell are often mediated by remarkably large surface areas, but there are cases where a short peptide is involved in binding (Table 1). Phage-displayed peptide libraries are particularly suitable for study of such peptide-directed interactions. A major application of phage-displayed peptide libraries has been to determine the epitope of an antibody. Antibodies recognize small peptide motifs based on only three or four conserved residues. Based on the epitope motif revealed by phage display, it is possible to delineate the region of a protein recognized by the antibody (*8,12*). Characterization of the epitopes of antibodies involved in autoimmune disorders may yield important information about the immunogenic mechanisms of disease (*13,14*).

Phage particles displaying a peptide can also be harnessed in the reverse context to produce an antibody against the phage-displayed peptide. Filamentous phage are strong immunogens, and it has been possible to isolate the antibodies to a particular phage-displayed peptide (*13,15,16*).

There are also other molecules in the immune system that recognize short peptide sequences. The function of major histocompatibility (MHC) molecules is to display short peptides from both endogenous and exogenous proteins to the host in the cell surface. Based on peptidic sequences bound to the MHC molecules, the immune system can recognize when a cell becomes abnormal or has been invaded by microorganisms harmful to the host. Phage display libraries have been used to delineate the peptide structures recognized by individual MHC molecules (*17,18*).

A family of cell surface integrins recognize the tripeptide RGD (*19*). These integrins mediate cell attachment to many types of extracellular matrix proteins that contain the RGD motif, such as fibronectin, vitronectin and fibrinogen (*5,20*). Screening with phage display libraries has shown that each of the integrins $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ has a slightly different peptide binding specificity and favors a distinct cyclic disulfide-bonded RGD-containing peptide (*21,22*). This may reflect the preference of each integrin for a different extracellular matrix protein. We found that peptides selected for binding to $\alpha_5\beta_1$ contained the sequence CRGDGWC, whereas those selected for $\alpha_v\beta_5$ had a more complex structure CDCRGDCFC (termed RGD-4C) (*22*). This α_v integrin-binding peptide contains four cysteines that can pair to make a mixture of different disulfide bridges. The synthetic double-cyclic peptide prepared first by random oxidation of the cysteines was active enough to block cell adhesion, but later experiments have indicated that one disulfide-bond arrangement gives much more active peptide than other cysteine pairings (unpublished data). Therefore, extra effort by peptide chemistry is sometimes needed to reproduce the active structure of a phage-displayed motif.

Novel, unexpected peptide ligands are often revealed using a phage library displaying degenerate peptides. Biopanning on the $\alpha_5\beta_1$ integrin yielded the cyclic peptide CRRETAWAC, which turned out to be a specific ligand for

TABLE 1
Examples of Proteins that Bind Short Peptide Motifs Derived from Phage Display Peptide Libraries

Proteins	Binding motifs	References
antibodies	Various	*2–4,8,12–14*
MHC class II	Various	*17,18*
$\alpha_5\beta_1$ integrin	RGD, NGR, CRRETAWAC	*10,22,23*
$\alpha_{IIb}\beta_3$ integrin	RGD, KGD	*21,22*
$\alpha_v\beta_3/\beta_5$ integrins	RGD, RGD-4C, NGR	*22,24*
$\alpha_6\beta_1$ integrin	VSWFSHRYSPFAVS	*25*
$\alpha_M\beta_2$ integrin	GYRDGYAGPILYN	*26*
SH2 domains	XXXY*XXX, Y*$^{E}/_{M}$NW	*30–32*
SH3 domains	RPLPPLP, APPLPPR	*27–29*

Single letter abbreviations of amino acids: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; W = Trp; X = variable; Y = Tyr; Y* = phospho-Tyr.

that integrin, showing no binding to other members of the integrin family (*23*). The peptide prevents $\alpha_5\beta_1$-mediated cell adhesion to fibronectin. Another integrin-binding motif is the sequence NGR, almost the reverse of the RGD motif (*10,22–24*). The NGR sequence occurs in many proteins, such as fibronectin, but the motif has a rather low affinity for integrins. The role of NGR in the interaction of matrix proteins with integrins is still unclear. The NGR motif may have been selected from random peptide libraries because it is an RGD mimetic in a pseudosymmetrical orientation.

Phage display libraries have also been used to select ligands to integrins which do not recognize the RGD or NGR sequence but recognize other matrix protein structures. Such integrins are $\alpha_6\beta_1$, a laminin receptor (*25*) and $\alpha_M\beta_2$, a leukocyte receptor for immunoglobulin-like adhesive proteins (ICAMs) (*26*).

The Src homology 3 (SH3) domain, to which ligands can bind in one of the two pseudosymmetrical orientations, class I and class II, have also been studied by phage display. The SH3 domain is present in many protein kinases and binds proline-rich domains of signaling molecules. The class I consensus sequence is RPLPPLP; the consensus for the class II is an opposite orientation peptide, APPLPPR (*27–29*). The proline-rich sequences occur in proteins that interact with kinases and are involved in cell signaling. Peptide libraries have also been used to find ligands to the SH2 domain, which is another common domain in protein kinases and signaling proteins. The SH2 domain recognizes peptides containing a phosphorylated tyrosine residue (*30–31*). In one study, the SH2 domain was shown to bind an unphosphorylated tyrosine when the tyrosine was presented in a phage-displayed peptide constrained by a disulfide bridge (*32*).

NOVEL PEPTIDE MIMOTOPES AND MINIMIZED PROTEINS

An antibody is not always directed against a linear amino acid sequence in a protein but can recognize a discontinuous epitope, which is a distinct conformation of a folded protein. Phage library screening with such antibodies has yielded peptides that mimic the structure of a folded protein. These ligands are called mimotopes (*2–4*). Libraries with cyclic peptides favor the isolation of mimotopes. Peptides have been isolated with activities dependent on a cyclized structure; if the cysteine residues are blocked, the peptide is inactivated, presumably because it is not folded in the appropriate conformation.

Besides antibodies, there are several other proteins for which mimotopes or structural mimetics of interacting molecules have been found by peptide phage display (Table 2). One of the first examples was a peptide motif that bound to the lectin concanavalin A (*33,34*). The peptide was found to be a functional analog of a polysaccharide recognized by the lectin. Therefore, the peptide competed with the carbohydrate in binding to the lectin. The fact that a peptide can exhibit a structure resembling a carbohydrate was further illustrated by work on antibodies binding to carbohydrates. Thus, a peptide that mimics the natural polysaccharide recognized by an antibody can be isolated (*35,36*). Peptides that bind to E-selectin, which recognizes the sialyl Lewis X carbohydrate, have also been found (37). However, these ligands to E-selectin are not acting as glycomimetics, as their binding to E-selectin is not completed by the carbohydrate.

A small peptide exhibiting an appropriate conformation can even substitute the function of a naturally occurring protein. After the isolation of the integrin-binding RGD motif from phage display libraries, we did the reverse panning to isolate peptides that bind to the RGD sequence of fibronectin. The cyclic CWDD$^L/_G$WLC peptide binds the RGD sequence and behaves in functional assays like an integrin (*38*). The cyclic peptide appears to be a minimized counterpart of an integrin and, interestingly, the peptide bears similarity to a sequence present on integrin β chains. It is notable that the same peptide motif has been discovered by biopanning on a human adenovirus capsid protein that contains a functional RGD motif (*39*). A peptide resembling an integrin sequence has also been identified by screening phage peptide libraries for binders to complement protein C1q, which is a potential ligand to the $\alpha_M\beta_2$ integrin (*40*). A similar screen using ICAM-1, the major ligand for the β_2

TABLE 2
Short Peptides that Mimic Other Molecules Structurally or Functionally

Target protein	Interacting peptide	Molecular mimicry	References
Antiprotein antibodies	Various	Discontinuous epitopes	*2–4,13,14*
Anticarbohydrate antibodies	Various	Polysaccharides	*2–4,35,36*
Concanavalin A lecitin	DVFYPYPYASGS, MYWYPY	Methyl mannopyranoside	*33,34*
E-selectin	DITWDQLWDLMK	E-selectin ligands	*37*
Type III FN repeat (RGD)	CWDD$^G/_L$WLC	β_1/β_3 integrins	*38*
ICAM-1	EWCEYLGGYLRCYA	β_2 integrins, rhinoviruses	*41*
Erythropoietin receptor	YXCXXGPXTWXCXP	Erythropoietin	*9,42*
Thrombopoietin receptor	IEGPTLRQWLAARA	Thrombopoietin	*43*

FN = fibronectin; ICAM-1 = intercellular cell adhesion molecule-1; RGD = Arg-Gly-Asp.
See Table 1 for single letter abbreviations of amino acids.

integrins, yielded a peptide that blocked the interaction between the ligand and the integrin (*41*).

Random phage display peptide libraries, in combination with other combinatorial methods, have offered an excellent approach to search for potent mimetics of proteins and minimal peptide sequences retaining activity. This has been accomplished with the polypeptide hormones erythropoietin (*42*) and thrombopoietin (*43*); the small analogs of which could have therapeutic utility in human diseases. A dimer of the 14-amino acid cyclic thrombopoietin mimetic was found to be as active as the parent polypeptide composed of 332 amino acids (*43*). A minimal agonist peptide which possesses mimetic properties of the 162-residue erythropoietin consists of only 13 amino acids (*44*).

PEPTIDE SELECTED FOR BINDING TO COMPLEX BIOLOGICAL SYSTEMS

During recent years, phage peptide libraries have been found useful in selecting peptides to more complex targets, such as human viruses, living cells and mouse tissues and tumors (Table 3). When using such complex targets, guided phage selection procedures are necessary to enhance specific peptide-mediated binding above the background phage adherence. In one of the first approaches, cells transfected with the gene for the urokinase receptor were used to isolate peptides ligands to the urokinase receptor (*45*). Selection was focused to the urokinase receptor-binding peptides by using two transfected cell lines from different species, one from a mouse and one from an insect. Thus, the urokinase receptor was the only common denominator between the two cell lines. The isolated peptide motifs did not resemble the urokinase sequence known to bind to the receptor. A selection system based on a heterologously expressed receptor on the cell surface has also been exploited to select peptides to the melanocortin receptor 1 (*46*).

TABLE 3
Peptides Isolated from Phage Display Libraries by Biopanning on Complex Biological Systems In Vitro and In Vivo

Screening system	Motifs recovered	References
Cell surface receptors		
Urokinase receptor	LWXXY/W/F/H, XFXXYLW	*45*
Melanocortin receptor	SSIISHFRWGLCD	*46*
Thrombin receptor	MSRPACPPNDKYE	*47*
Viruses		
Adenovirus*	Various	*39,48*
Echovirus	CLRSGRGC	*49*
Puumala hantavirus	CHWMFSPWC	*50*
Human immunodeficiency virus†	WXXF	*51*
Tissue culture		
Cell line monolayers	Various	*52,53*
Mouse endothelia		
Brain	CSSRLDAC	*11*
Kidney	CLPVASC	*11*
Lung	CGFECVRQCPERC	*54*
Skin	CVALCREACGEGC	*54*
Pancreas	SWCEPGWCR	*54*
Intestine	YSGKWGW	*54*
Uterus	GLSGGRS	*54*
Adrenal gland	LMLPRAD	*54*
Retina	CSCFRDVCC, CRDVVSVIC	*54*
Tumor endothelium		
Human tumor xenografts‡	RGD-4C, CNGRC, GSL	*55,56*

*Capsids and virus neutralizing antibodies.
†Vpr protein.
‡Carcinomas, sarcomas and melanomas.
See Table 1 for single letter abbreviations of amino acids.

Another way to focus the selection is to elute the bound phage specifically with reagents known to disrupt the interaction, that is, with specific antibodies or peptides. Human platelets have been used as the target to make a peptide antagonist of the thrombin receptor (*47*). The bound phage from platelets were eluted with a known agonist peptide of the receptor derived from thrombin.

Antibody elution has been used to identify peptides binding to different capsid proteins of human adenovirus (*39*). Characterization of peptide interaction with a virus may give clues to which proteins could bind the virus and be potential receptors for the virus in the cell surface. Some of the isolated peptide sequences bear similarity to known adenovirus receptors, one of which is an integrin (*39, 48*). The potential utility of virus-binding peptides is that they could interfere with infectivity and prevent the binding and/or internalization of the virus. One such peptide, CLRSGRGC, was derived by screening phage libraries on human echovirus (*49*). The cyclic peptide partially prevented the infectivity of the virus on human cells. It is notable that purified phage particles displaying a virus-targeting peptide can also prevent virus infectivity. Phage selected for binding to hantaviruses inhibited the infectivity of this virus in cell culture (*50*). Recently, an HIV-1-targeting peptide motif was obtained by screening phage libraries with Vpr, one of the retroviral proteins packaged into the virus particles (*51*). A test protein into which the peptide motif was engineered was able to bind to HIV-1 virions by interacting with Vpr, suggesting a novel strategy to guide antiviral agents into the virions.

Peptides binding to the cell surface were derived from cell-based phage display panning. In this approach, the target receptors are unknown, and peptides capable of recognizing specific cells can be isolated (*52,53*).

A step toward more complexity is in vivo biopanning, where peptides localizing to an organ of a mouse can be discovered after intravenous injection of phage libraries (*11,54*). Endothelium-targeting phage are recovered when phage are allowed to circulate only a few minutes and vasculature is perfused with phosphate buffered saline. Peptides that confer selective phage homing to the vasculature of brain, kidney, lung, skin, pancreas and several other organs have been isolated (*11,54*). Recently, the receptor for a lung targeting peptide (*54*) was identified (Rajotte and Ruoslahti, unpublished data). With this procedure, it has

been possible to discover important peptides that localize to the tumor vasculature after intravenous injection in mice bearing human tumor xenografts (*55,56*). Two tumor-targeting peptides turned out to be the integrin-binding motifs RGD-4C and CNGRC (*56*). Inhibition studies of phage homing with the two peptides suggest that the peptides do not function equally, and that CNGRC may also have targets other than the integrins.

UTILITY OF LIBRARY-DERIVED PEPTIDES: INTEGRINS AS TARGETS

To verify that phage-displayed peptides bind to their target, the peptides need to be produced as synthetic peptides or alternatively as fusion proteins, for example, in E. coli. Usually, the best synthetic peptides are obtained from those phage sequences that are enriched among the clones sequenced and show highest avidity to the target. The integrin-binding peptide ligands derived from phage libraries inhibit cell adhesion to extracellular matrix proteins and can be used to study the function of the specific integrin with which they interact (*5*). Moreover, when the synthetic peptide is coated as a substratum, cells will adhere to it, and this could be exploited in the manufacturing of artificial tissues or transplants.

Another avenue of utilization for the integrin-binding peptides became evident after finding homing of the RGD and NGR-containing peptides on the tumor vasculature (*55,56*). The receptors for the RGD-containing peptides are the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins expressed on new blood vessels (*57,58*). The formation of new vasculature, angiogenesis, is a requirement for malignant tumor growth and metastasis (*59*). The $\alpha_v\beta_3$ integrin has been found to be important in angiogenesis, as antibodies and peptide antagonists to $\alpha_v\beta_3$ promote tumor regression by inducing apoptosis of angiogenic vessels (*60–62*). Furthermore, agents targeting the α_v integrins in particular provide a means for imaging of angiogenic vasculature. The phage displaying the α_v-integrin-targeting peptide RGD-4C accumulate on the tumor vasculature after intravenous injection in mice, and can be detected in the tumor vasculature even after 24 h, when no signs of phage are seen in the kidney or liver (*55*). Recently, the $\alpha_v\beta_3$ specific antibody LM609 displayed on liposomes has been used to visualize tumor vasculature by MRI (*63*).

The integrin-binding peptides are not only useful for tumor imaging purposes. Peptides binding to a tumor vascular "address" may guide a cytotoxic drug more accurately to tumors, thus saving the healthy tissues from harmful effects (*56*). Thus, conjugates of the RGD-4C and CNGRC peptides with doxorubicin were far less toxic to the animals than doxorubicin alone, and the peptide carriers significantly improved the effectiveness of doxorubicin in suppressing the growth of human tumor xenografts. These results show that it may be possible to develop targeted chemotherapy strategies with these ligands (*62*). The challenge for the effective delivery of cancer drugs is that blood vessels within the tumors are often heterogenous and leaky, causing cancer drugs to be unevenly distributed (*64*). Solid tumors also exhibit interstitial hypertension further preventing adequate chemotherapy delivery (*64*). Because of their small size, the peptides may have less trouble to penetrate tumor tissue as compared to other agents tried in cancer therapy such as liposomes or viral particles. Therefore, an attractive option would be to use the peptides to deliver radioisotopes to tumor vasculature. Radioimmunotherapy with specific targeting antibodies has been examined in several animal models, but radiation-mediated damage to normal tissue still continues to cause problems (*65,66*). The hope is that with new targeting peptides, such as the ones we have developed, we may be able to image and treat tumors and their metastases more accurately than hitherto possible.

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