Protein Engineering vol.13 no.8 pp.589–592, 2000

Phages from landscape libraries as substitute antibodies

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In 'landscape' phage, as in traditional phage-display constructs, foreign peptides or proteins are fused to coat proteins on the surface of a filamentous phage particle. Unlike conventional constructs, however, each virion displays thousands of copies of the peptide in a repeating pattern, subtending a major fraction of the viral surface. The phage body serves as an interacting scaffold to constrain the peptide into a particular conformation, creating a defined organic surface structure ('landscape') that varies from one phage clone to the next. By testing landscape libraries with three representative antigens (streptavidin from the bacterium *Streptomyces avidinii*, avidin from chicken egg white and β-galactosidase from *Escherichia coli*) we have shown that landscape phages may be used as a new type of substitute antibodies—filaments that can bind protein and glycoprotein antigens with nanomolar affinities and high specificity. In many ways these substitute antibodies are more convenient than their natural immunoglobulin counterparts.

Keywords: avidin/β-galactosidase/landscape library/nanotechnology/phage display/streptavidin/substitute antibodies

Introduction

The repertoire of antibodies in a vertebrate animal can be considered as a huge library of molecular landscapes, containing binding sites for almost any antigen. Each binding site comprises a set of highly variable peptide loops—the complementarity-determining regions (CDRs)—contained within and conformationally constrained by a structurally constant framework. The CDRs differ in sequence and conformation from one antibody to another and largely determine the antigen-binding specificity of the antibodies. The immune system has evolved cellular machinery for selecting those binding specificities that are needed to meet the challenge of an invading antigen, specifically amplifying those antibodies until they become prevalent in the total antibody population.

Antibodies have proven to be extremely adaptable and effective biomaterials in a great variety of biomedical and technical applications, but there are limits to their use. Polyclonal antibodies are relatively cheap to produce but heterogeneous, while monoclonal antibodies are homogeneous but expensive. Some target ligands, such as metals and toxic substances, cannot be used to elicit specific antibodies. Both polyclonal and monoclonal antibodies easily lose their binding properties under unfavorable environmental conditions.

To some extent, these limitations have been addressed by phage antibodies, an artificial, *in vitro* immune system in which antibodies (more accurately, antigen-binding domains of antibodies) are displayed on the surface of filamentous phage carriers whose genomes contain the antibody coding sequence (Hoogenboom *et al.*, 1998). Phage-displayed antibodies can be propagated and cloned simply by infecting the phages into fresh bacterial host cells and can be selected for binding to a particular target antigen using simple microbiological procedures. Despite its promise, however, phage antibody technology is not without difficulties. The last step in particular—expressing the selected antibody genes to make usable quantities of antibody—has proven troublesome, differing idiosyncratically from one antibody to another. These considerations and others have led researchers to cast about for non-immunoglobulin frameworks that might serve as the scaffold for artificial antibodies that would be easier to work with (Ku and Schultz, 1995; McConnell and Hoess, 1995; Nord *et al.*, 1995; Martin *et al.*, 1996; Nygren and Uhlen, 1997; Gao *et al.*, 1999). This paper focuses on a particularly simple type of substitute antibody, in which the phage filament itself serves as the framework for random-peptide 'CDRs' fused to the N-terminus of every copy of the major phage coat protein. We call such particles 'landscape' phages, since the random peptide subtends a major fraction of the organic landscape on the phage surface; a large mixture of such phages, displaying up to a billion different guest peptides, is called a 'landscape library.' In some landscape phages, the guest peptide is conformationally constrained by interaction with the surrounding phage body, resulting in a well-defined, repeating surface structure (Kishchenko *et al.*, 1994); in others, the peptide is not so constrained (Malik *et al.*, 1996). We reported previously that a small organic hapten can select hapten-binding phages from a very large landscape library (Petrenko *et al.*, 1996). Here we report that three different protein antigens likewise select antigen-binding phages from the same landscape library. Taken together, this work validates the concept of landscape phages as substitute antibodies.

Materials and methods

Library and general procedures

The landscape library that served as the source of substitute antibodies has been described (Petrenko *et al.*, 1996). Each phage in the library displays the peptide AX_8 at the N-terminus of the mature form of all 4000 major coat protein subunits; the Xs in the peptide sequence are the randomized amino acids encoded by degenerate codons in the recombinant form of the coat protein gene VIII. *Escherichia coli* and phage strains and general phage-display methods have been described (Smith and Scott, 1993; Yu and Smith, 1996) and are explained in greater detail on our website (http://www.biosci.missouri.edu/SmithGP/index.html). The concentration of phage particles was calculated assuming than an absorbance of 1 at 269 nm corresponds to 6.7×10^{12} virions/ml for the 9198-base landscape phage (Day and Berkowitz, 1977). Peptides were synthesized and characterized by analytical HPLC (>95%

purity) and mass spectrometry at the Biomolecular Research Facility, Health Sciences Center of University of Virginia.

Affinity selection of antigen-binding phages from the landscape library

Small polystyrene Petri dishes (diameter 35 mm) were coated with 400 µl of 10 µg/ml antigen in 0.1 M $NaHCO_3$ (streptavidin and neutravidin) or TBS (50 mM Tris–HCl, pH 7.5; 150 mM NaCl) (β-galactosidase) for 16 h at 4°C. After washing the plates six times with TBS–Tween [TBS containing 0.5% (v/v) Tween], 400 µl of TBS–Tween and 2×10^{11} landscape virions were pipetted into each dish, which was then rocked 16 h at 4°C. Unbound phages were removed by washing the dishes 10 times with TBS–Tween and bound phages eluted in 400 µl of elution buffer (0.1 M HCl, pH adjusted to 2.2 with glycine; 1 mg/ml BSA; 0.1 mg/ml phenol red) for 10 min. Eluates were transferred to microtubes containing 75 µl of 1 M Tris–HCl (pH 9.1), concentrated to 100 µl on Centricon 100 KDa centrifugal ultrafiltration devices (Amicon) and amplified in 20 ml cultures as described (Yu and Smith, 1996). Virions were partially purified from the culture supernatants by polyethylene glycol precipitation (Yu and Smith, 1996) and dissolved in 200 µl of TBS. Half of each of these amplified eluates (~5×10^{10} infectious units) was used as input to a second round of affinity selection carried out in the same way as the first; 100 µl portions of unconcentrated eluates were amplified as before. Half of each amplified eluate was used as input to a third round of affinity selection, again carried out as before. Serial dilutions of the third eluates were mixed with starved cells and spread on to NZY–agar plates containing 40 µg/ml tetracycline to obtain individual clones of infected cells. Viral DNAs from 40 individual clones selected with each antigen were sequenced to determine the amino acid sequences of the displayed peptides (Haas and Smith, 1993).

Partial denaturation of phages with chloroform

Phages at a concentration of $(1–3)\times10^{13}$ virions/ml in 2/3×TBS were vortex mixed in a 1.5 ml microtube with an equal volume of chloroform–isoamyl alcohol (24:1) for 1 min at maximum speed at 20°C. The mixture was microfuged for 1 min and the aqueous (upper) phase was transferred to a new microtube. Recovery of phages was quantified spectrophotometrically, loss of infectivity was confirmed by titering and conversion from filaments to spheroids was confirmed by whole-virion electrophoresis (Nelson *et al.*, 1981) in 0.8% agarose gel in 50 mM NaH_2PO_4, pH adjusted to 7.5 with NaOH, 1 mM $MgCl_2$.

Enzyme-linked immunosorbent assay (ELISA) with β-galactosidase

In direct ELISA, phages at 5×10^{11} virions/ml in 40 µl of TBS were absorbed in wells of 96-well polystyrene ELISA dishes for 16 h at 4°C. The wells were washed five times with TBS–Tween on a plate washer and filled with 45 µl of Z-buffer (0.1 M sodium phosphate, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) containing β-galactosidase at graded concentrations. After 30 min at room temperature, the wells were washed thoroughly 10 times with TBS–Tween and filled with 90 µl of 0.67 mg/ml *o*-nitrophenyl-β-D-galactoside (ONPG) in Z-buffer. The difference between the optical density (OD) at 405 and 490 nm was read at 3 min intervals over a 60 min period on a kinetic plate reader in order to obtain a slope (mOD/min) for each well as described (Yu and Smith, 1996).

In inhibition ELISA, wells were coated with phages as above, washed and filled with a mixture of 58 nM β-galactosidase or 12 nM biotinylated β-galactosidase and graded concentrations of competitive inhibitor (phage, peptide or IPTG). After 30 min at room temperature, the wells were washed 10 times as described above. When non-biotinylated β-galactosidase was used as antigen, the dish was developed with ONPG as described above. When the biotinylated β-galactosidase was used as antigen, the wells were filled with 1 µg/ml alkaline phosphatase-conjugated streptavidin (AP-SA) in AP-SA diluent (50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20 and 1 mg/ml BSA), incubated for 45 min at room temperature, washed 10 times with TBS–Tween and filled with 90 µl of 0.5 mg/ml *p*-nitrophenyl phosphate (NPP) in 1 mM $MgCl_2$, 1 M diethanolamine–HCl, pH 9.8; the ELISA signal was measured on a plate reader at 3 min intervals as described above.

ELISA with streptavidin and neutravidin

In direct ELISA, the dishes were coated with phages and washed as described above and the wells were filled with 40 µl of AP-SA diluent containing AP-SA or alkaline phosphatase-conjugated neutravidin AP-NA (Pierce) at graded concentrations. After 45 min at room temperature, the dishes were washed and developed with NPP as described above. For competitive inhibition ELISAs, wells were coated with phages and washed as before, filled with 40 µl of a mixture of 1 µg/ml AP-SA or 4 µg/ml AP-NA and competitive inhibitors at graded concentrations, incubated for 45 min at room temperature, washed 10 times with TBS–Tween and developed with NPP as described above.

Results and discussion

Selection and characterization of antigen-binding landscape phages

The model protein antigens in this work were streptavidin (from the bacterium *Streptomyces avidinii*), avidin from chicken egg white and β-galactosidase from *Escherichia coli*. Streptavidin is a slightly acid tetrameric protein composed of four identical chains, each of 159 amino acid residues (Green, 1990); it binds biotin with exceedingly high affinity. Avidin—another biotin binder—is a tetrameric glycoprotein with 128-residue subunits having 33% sequence homology with streptavidin (Green, 1990). The architecture of the biotin-binding pockets of streptavidin and avidin are almost identical (Livnah *et al.*, 1993). The form of avidin used in our work was 'neutravidin', a chemically modified form of the protein with greatly reduced positive charge. The β-galactosidase protein is a tetramer of identical 1023-residue polypeptides, with molecular dimensions of 17.5×13.5×9 nm (Jacobson *et al.*, 1994).

Each antigen was absorbed to the surface of a 35 mm polystyrene Petri dish and the dish was reacted with the landscape library. Unbound phages were washed away and bound phages eluted with acid buffer and amplified by infecting fresh bacterial host cells. After three rounds of selection, individual phage clones were propagated and sequenced partly to determine the amino acid sequence of the displayed peptide.

Binding of the selected phages to their respective antigens was characterized by ELISA in which the phages were immobilized on the plastic surface of the ELISA wells and reacted with their antigens in solution phase (Goldberg and Djavadi-Ohaniance, 1993; Yu and Smith, 1996). Figure 1 plots results of an ELISA in which the antigens (alkaline phosphatase-labeled

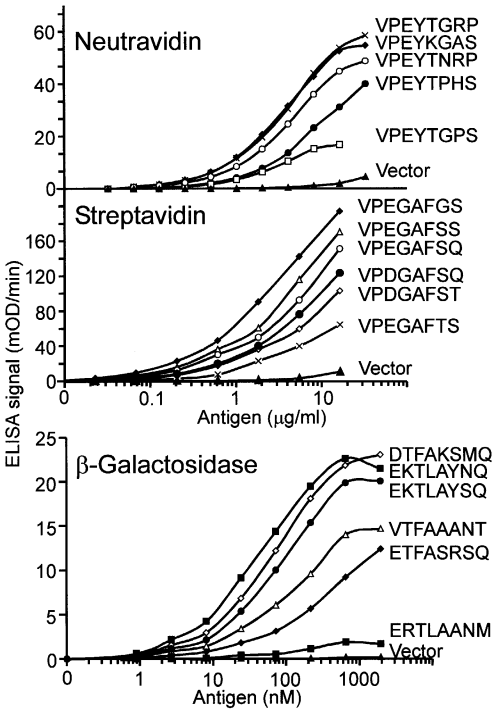

Fig. 1. Binding of antigens to immobilized phages as measured by direct ELISA (see Materials and methods). Phages displaying the indicated peptides were immobilized in the wells of microtiter dishes and reacted with graded concentrations of antigen–β-galactosidase, streptavidin labeled with alkaline phosphatase (AP-SA) or neutravidin labeled with alkaline phosphatase (AP-NA). Antigen remaining bound after washing was quantified as described in Materials and methods.

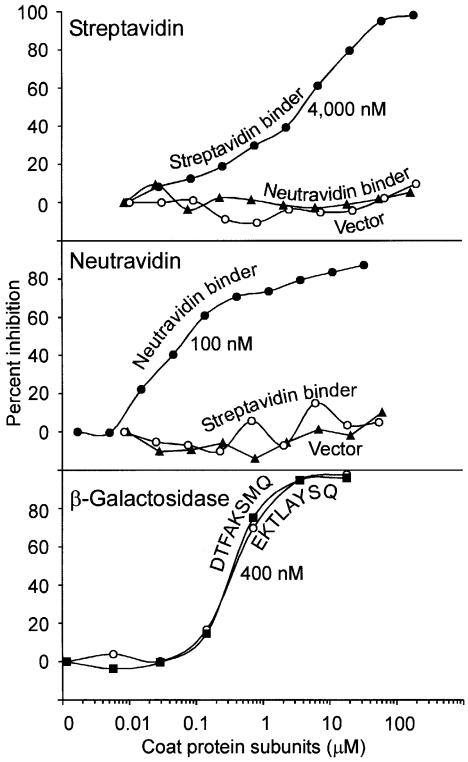

Fig. 2. Binding of antigens to soluble phage inhibitors as measured by inhibition ELISA (see Materials and methods). Phages displaying antigen-binding peptides—VPEGAFSS for streptavidin, VPEYKGAS for neutravidin, DTFAKSMQ for β-galactosidase—were immobilized in the wells of ELISA dishes. Meanwhile, antigen–streptavidin labeled with alkaline phosphatase (AP-SA), neutravidin labeled with AP (AP-NA) or β-galactosidase at fixed concentration was pre-incubated with graded concentrations of soluble competitor phages (either native or chloroform-denatured) displaying the indicated peptide. The antigen–competitor mixtures were reacted with the phage-coated wells and the antigen remaining bound after washing was quantified as described in Materials and methods. Binding of antigen to the soluble competitor decreases binding to immobilized phages, thus depressing the ELISA signal. The concentrations of competitors required to depress the ELISA signal by ~50% are indicated and correspond roughly to dissociation equilibrium constants (Goldberg and Djavadi-Ohaniance, 1993).

neutravidin and streptavidin and unlabeled β-galactosidase) were reacted directly with immobilized, peptide-bearing phages; the data demonstrate specific, dose-dependent binding of each antigen to the peptides it has selected. The inhibition ELISA presented in Figure 2 verifies that non-immobilized peptide-bearing phages compete with immobilized phages for binding to their respective antigens. The nominal dissociation equilibrium constants in Figure 2 lie in the range 0.1–4 μM. However, these nominal constants may diverge from the actual monovalent constants because of the dense crowding of the peptides on the virion surface.

The antigen-binding power of a phage might reside in the peptide alone; alternatively, it may be an emergent property of the peptide interacting with the surrounding phage body. To address this question, we compared the binding of native phages, partially denatured phages and (in some cases) synthetic peptide. Partial denaturation was achieved by shaking a solution of phages with chloroform, which transforms the particle from an infectious filament to a non-infective hollow sphere (Griffith *et al.*, 1981; Lopez and Webster, 1982); the surface architecture of the phage is radically altered by this treatment, the α-helix content of pVIII decreasing from 90 to 50–60% (Griffith *et al.*, 1981; Roberts and Dunker, 1993) and the particle becoming sensitive to detergents, 5 M urea, proteases, heating, 40% formamide and salt (Griffith *et al.*, 1981). Each of these structures was used at various concentrations to inhibit competitively the binding of soluble antigen to immobilized phage. The results of these inhibition ELISAs will be discussed in the context of individual antigens below.

Table I. Antigen-binding phages

β-Galactosidase		Neutravidin	Streptavidin
DTFAKMAQ		**VPEY**SRPS	**VPEGAF**GS
	EKTLAYNQ	**VPEY**KGAS	**VPEGAF**SS (7)
DTFAKSMQ (3)		**VPEY**TGRP (3)	**VPEGAF**SQ
ETFAKMSQ		**VPEY**VNTK	**VPDGAF**SQ (2)
	EKTLAYSQ (2)	**VPEY**TNRP	**VPEGAF**ST
ETFAKMTQ		**VPEY**TRT	**VPESAF**AQ
V**TFA**AANT (2)		**VPEY**TPHS	**VPDGAF**ST

Streptavidin

The streptavidin-binding phage displayed peptides with the sequence motif VP(E/D)(G/S)AFXX, where 60% of X is S or T (Table I). This peptide motif has no noticeable similarity with the HPQ, GDWVFI or PWPWLG streptavidin-binding peptides selected in other phage-display experiments (reviewed by Smith and Petrenko, 1997). Nevertheless, our streptavidin-binding phage resembles the previously reported ones in that biotin inhibits the binding (data not shown), indicating that in all cases the binding site on streptavidin is located close to its

biotin-binding pocket. Partially denatured phage is as potent as native phage in inhibition ELISA (data not shown); the synthetic peptide AVPEGAFSSDPAKAC-NH_2, corresponding to the N-terminal part of one of the streptavidin-binding phage, also inhibits binding, although with 10 times lower affinity than native and denatured phages (data not shown). Binding of the synthetic peptide to streptavidin could also be demonstrated by direct ELISA (data not shown). Taken together, these results show that the phage body serves only as a carrier for the streptavidin-binding peptides.

Neutravidin

The peptides displayed on neutravidin-binding phages have the sequence motif VPE(F/Y)XXXX, where the positions marked X are occupied with an unusually high proportion (34%) of S and T. This motif is strikingly similar to the motif on the streptavidin-binding phage discussed above and in this case too biotin blocks binding to the antigen (data not shown). However, it is evident that the phage-borne peptides are not true mimetics of biotin, since their binding is species specific: phages selected with streptavidin do not bind neutravidin and *vice versa* (Figure 2). There is another very important difference in the behavior of streptavidin- and neutravidin-binding phages. As was mentioned above, binding of the streptavidin-binding phage is retained in the spherical form of the phage produced by chloroform treatment. In contrast, chloroform treatment reduced the apparent affinity of neutravidin binding about 100-fold in inhibition ELISA (data not shown). Evidently binding of these phages to their antigen is strongly abetted by interaction between the displayed peptide and neighboring amino acids on the phage body. An alternative explanation of the reduced binding power of chloroform-treated phages is that chloroform itself blocks the phage–neutravidin interaction; however, in view of the very low concentration of chloroform in the aqueous phase after phage treatment, this possibility seems unlikely.

β-Galactosidase

Two families of guest peptides were displayed on β-galactosidase-binding phage, with consensus motifs EKTLAYXQ and (D/E)TFA(K/R/x)XXX; the position marked (K/R/x) has >50% basic K and R residues. Phages from the two families compete with each other for binding to β-galactosidase and therefore probably bind overlapping sites on the antigen (Figure 2). IPTG, a competitive inhibitor of the enzyme with a K_i of 70 μM (Ring and Huber, 1990), failed to inhibit binding of either phage, even at a concentration of 100 mM (data not shown). That suggests that neither phage interacts with the active site of the enzyme. Chloroform treatment destroys the antigen-binding capacity of both families of phages (data not shown), indicating that antigen binding is an emergent property of the guest peptide in complex with the surrounding phage body (assuming, as argued in the previous subsection, that the concentration of chloroform is too low to influence binding directly).

Conclusions and prospects

In this work, we have shown that phages selected from a landscape library can functionally mimic antibodies in their ability to bind protein antigens. Because it is the affinity-selected phages themselves that serve as the 'antibodies', this technology bypasses one of the most troublesome steps of phage–antibody technology: re-engineering of the selected antibody genes to express them at a high level. Indeed, a culture of cells secreting filamentous phages is an efficient, convenient protein production system. Most landscape phages behave like wild-type, non-peptide-bearing phages, regardless of the peptide they display. They are secreted from the cell nearly free of intracellular components in yields of about 20 mg/ml and can be purified by simple, routinizable procedures that do not differ idiosyncratically from one clone to another. The surface density of the phage particle is 300–400 m^2/g, comparable to the best known absorbents and catalysts. The randomized amino acids that form the 'active site' of a landscape phage comprise up to 25% by weight of the particle and subtend up to 50% of its surface area—an extraordinarily high fraction compared with natural proteins, including antibodies. In many applications, the extreme multivalency of landscape phages—thousands of binding sites/particles—may be a great advantage. The phage structure is extraordinarily robust, being resistant to heat (up to 70°C), many organic solvents (e.g. acetonitrile) (Olofsson *et al.*, 1998), urea (up to 6 M), acid, alkali and other stresses. Purified phages can be stored indefinitely at moderate temperatures without losing infectivity. These characteristics commend landscape phages as substitute antibodies in many applications, such as biological detectors, affinity sorbents, hemostatics, etc.

Acknowledgements

We thank Natalia I.Petrenko for excellent technical assistance. This work was supported by US Army Research Office Grant No. DAAG55-98-1-0258 to V.A.P. and NIH grant DHHS 5 R21 RR12647-02 to V.A.P.

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Received March 16, 2000; revised May 24, 2000; accepted May 24, 2000