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Selection of Phage-Displayed Peptides That Bind to a Particular Ligand-Bound Antibody

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Abstract

Phage-displayed peptides that selectively bind to aldolase catalytic antibody 93F3 when bound to a particular 1,3-diketone hapten derivative have been developed using designed selection strategies with libraries containing 7 to 12 randomized amino acid residues. These phage-displayed peptides discriminated the particular 93F3-diketone complex from ligand-free 93F3 and from 93F3 bound to other 1,3-diketone hapten derivatives. By altering the selection procedures, phage-displayed peptides that bind to antibody 93F3 in the absence of 1,3-diketone hapten derivatives have also been developed. With using these phage-displayed peptides, ligand-bound states of the antibody were distinguished from each other. A docking model of one of the peptides bound to the antibody 93F3-diketone complex was created using a sequential divide-and-conquer peptide docking strategy; the model suggests that the peptide interacts with both the antibody and the ligand through a delicate hydrogen bonding network.

Keywords

antibody; ligand; peptide; phage library

Introduction

Peptides that selectively bind a particular ligand-bound form of a protein are useful for detecting the ligand-bound protein; when the peptides bind to a particular ligand-bound protein but not to the same protein bound to other similar ligands or to the ligand-free protein, the peptides can be used for detecting the ligand-bound states of the protein.^{1,2} With this type of peptide, conditional binding is possible: Binding of the peptide to the protein can be controlled by the presence or absence of the ligand.^{3,4,5,6} Here we report the development of a strategy for the generation of phage-displayed peptides that selectively bind to a particular ligand-protein

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complex but that do not bind to the ligand-free form or to the protein complexed with other similar ligands (Figure 1).

Generation of peptides and phage-displayed peptides that bind active sites of particular proteins and other biomolecules, such as receptors and enzymes, has been reported and the use of these peptides and phage-displayed peptides have been demonstrated.^{7,8,9,10,11} However, strategies for the generation of peptides and phage-displayed peptides that selectively bind to a particular ligand-bound state have not been explored. Although phage-displayed peptides that bind ligand-bound proteins and that do not bind ligand-free proteins have been reported, they typically discriminate between conformations of the ligand-bound protein and of its unliganded form; they do not distinguish the ligands in the ligand-bound forms.¹²

Here we have developed phage-displayed peptides that selectively bind an antibody-ligand (or antibody-hapten) complex; these phage-displayed peptides not only discriminated the ligand-bound form of the antibody from its ligand-free form, but also discriminated the particular ligand-bound antibody from the antibody bound to other ligand derivatives. To complement these phage-displayed peptides, we have also generated phage-displayed peptides that bind to a ligand-free antibody; binding of these phage-displayed peptides is inhibited by the addition of the ligand of the antibody. We used combinatorial phage-displayed peptide libraries and designed selection strategies to obtain phage-displayed peptides with desired binding features. We used aldolase antibody 93F3 generated with a 1,3-diketone derivative¹³ as a target because the crystal structure of this antibody has been solved.¹⁴ In order to further our understanding of the interactions between the phage-displayed peptide, antibody 93F3, and the ligand in the selective binding, a computational docking model of one of the selected peptides with the 93F3-ligand complex was obtained based on a new sequential peptide docking strategy and by using the crystal structure of the antibody.¹⁴

Results

Selection of phage-displayed peptides that selectively bind to ligand-free antibody 93F3

In order to select phage-displayed peptides that bind to ligand-free antibody 93F3 and that do not bind to antibody 93F3-diketone complexes, we performed binding selection of phage-displayed peptides against the antibody in the absence of diketone and then the bound phage were eluted with a diketone. Phage-displayed peptide libraries that contained seven randomized amino acids constrained by disulfide bonds between a pair of cysteine residues (CX₇C, X = any of the natural 20 amino acids), linear seven-mer peptides, and linear 12-mer peptides fused via a short spacer to the N-terminus of a minor coat protein (pIII) of the filamentous bacteriophage M13 phage were used for the screening. Phage-displayed peptide libraries were added to the antibody 93F3-coated wells, unbound phage were washed away, and the bound phage were eluted with diketone **1** (10 μ M) (Chart 1). This 10 μ M concentration of **1** was chosen based on the dissociation constant of mouse-human chimeric Fab of 93F3 to 6-phenylhexane-2,4-dione (K_d 1 μ M).¹⁴ Use of a concentration 10-fold higher than the K_d should insure that the most of the antibody combining sites of antibody 93F3 are bound to the diketone during the elution. After multiple rounds of selection, culture supernatants containing phage-displayed peptide from individual 35 clones were analyzed by ELISA in the presence and absence of diketone **1** (10 μ M). Of these 35 clones, 33 clones bound to antibody 93F3 in the absence of the diketone and the binding of these 33 clones to this antibody was inhibited in the presence of **1** (10 μ M).

The sequences of the library region of 22 of these 33 clones were determined and the results are shown in Table 1. Only three sequences were found in the 22 clones, CPWHLLPFC (**A1**), WYLPDNPSTWHT (**A2**), and QVPWSYLANGLL (**A3**), indicating convergence of the selection. Phage-displayed peptides possessing each of these sequences were purified and

ELISA was performed in the absence and presence of diketones **1** and **2** (Figure 2). As observed in the ELISA of culture supernatant containing the phage-displayed peptide, these phage-displayed peptides bound antibody 93F3 in the absence of the diketones and both diketones **1** and **2** inhibited the binding of the phage-displayed peptides to antibody 93F3. In contrast, ELISA in the presence of diketone **3**, which binds less well (with slower binding) to 93F3 than diketone **1** or diketone **2**, moderately inhibited (<30% inhibition) the binding of these phage-displayed peptides to 93F3 (Table 1 and for **A3**, see Figure 2). These results indicate that the phage-displayed peptides **A1**, **A2**, and **A3** bind in the antigen-combining site of 93F3 or at the entrance to the antigen-combining site.

Phage-displayed peptides that bind to antibody 93F3 in the presence of particular diketone

The same libraries described above were used for the selection of phage-displayed peptides that bound to the 93F3-diketone liganded forms. In order to remove phage-displayed peptides that bound to ligand-free antibody 93F3, firstly phage-displayed peptides were added to antibody 93F3-coated wells. Then unbound phage were transferred to wells that were coated with antibody 93F3 with one of the diketones **1**, **2** or **3** and the phage-displayed peptides were incubated in the presence of the same diketone (10 μ M). Unbound phage and phage bound to the free compound (diketone not bound to 93F3) were washed away and the phage bound to the antibody-diketone complex-coated wells were eluted with acid. After four rounds of the selection, ELISA of individual clones were analyzed using culture supernatant containing the phage-displayed peptide in the absence and presence of the diketone (10 μ M) used for the selection.

From the libraries selected against 93F3-diketone **1**, 11 out of 20 clones tested bound 93F3-diketone **1** and these clones did not bind 93F3 in the absence of the diketones. These 11 clones did not bind 93F3-diketone **2** nor 93F3-diketone **3**. The sequences of seven of the 11 clones were analyzed and the six of them were identical (Table 1). Purified phage carrying CTPFTHWLC (**B1**) and CSPLTHALC (**B2**) also selectively bound the complex of 93F3 with diketone **1**, but did not bind ligand-free 93F3, 93F3-diketone **2**, nor 93F3-diketone **3** (Figure 3). Although diketones **1** and **2** share the same 6-phenylhexane-2,4-dione moiety, phage carrying peptide **B1** or **B2** selectively bound 93F3-diketone **1** and discriminated 93F3-diketone **1** from 93F3-diketone **2**.

Twenty individual clones from the libraries selected against 93F3-diketone **2** were analyzed by ELISA; however, no clone bound 93F3-diketone **2**. Libraries selected against 93F3-diketone **3** afforded phage carrying CPWWFPTQC (**C1**), which selectively bound the complex of 93F3-diketone **3**.

Binding profiles of the selected phage-displayed peptides

Binding profiles of the purified phage carrying QVPWSYLANGLL (**A3**), CTPFTHWLC (**B1**), CSPLTHALC (**B2**), and CPWWFPTQC (**C1**) were examined by ELISA in the absence and presence of diketones **1**, **2**, and **3**. The results are shown in Figure 3. As expected from the ELISA using culture supernatant containing the phage, purified phage carrying CTPFTHWLC (**B1**) and CSPLTHALC (**B2**), which were selected against 93F3-diketone **1**, bound to 93F3-diketone **1**, but did not bind to 93F3 alone, to 93F3-diketone **2**, nor to 93F3-diketone **3**. Purified phage carrying CPWWFPTQC (**C1**) selectively bound to 93F3-diketone **3**. These phage-displayed peptides distinguished the diketones bound to antibody 93F3. If the phage-displayed peptides recognize the conformer of 93F3 bound to diketone **1**, they should also bind the 93F3-diketone **2** complex because it is likely that conformations of diketone **1**-bound 93F3 and of **2**-bound 93F3 are similar.^{15,16} The results of selective binding of the phage-displayed peptides to the 93F3-particular diketone complex suggest that phage carrying peptides **B1**, **B2**, and **C1** probably interact with both antibody and the diketone bound to the antibody. These phage

carrying peptides of only 7 to 12 amino acid residues discriminated not only the ligand-bound antibody from ligand-free antibody but also the ligand bound to the antibody. Different selection strategies have provided phage-displayed peptides with different binding features.

Modeling and docking of peptide CTPFTHWLC (**B1**) to antibody 93F3-diketone **1**

In order to understand the interaction between phage-displayed peptide **B1** and antibody 93F3-diketone **1**, a theoretical cyclic peptide **B1** model was docked to the antibody-diketone **1** structure. Biological polymers such as peptides are known to be flexible and a great number of low energy conformational states are accessible even at room temperature. A systematic approach to search such an astronomical conformational space is not computationally feasible. Therefore we adopted a divide-and-conquer approach by separating the search space into several blocks.

Firstly, we divided peptide conformational space into side chain and backbone. The conformations of the peptides were minimized and equilibrated at the room temperature and 1000 snapshots taken every 2 ps were collected from a long molecular dynamics that reasonably captures most of peptide conformations. This guaranteed that all the conformations were statistically independent from each other. All the snapshots were then clustered against the rmsd of their backbone atoms. As a result, there were 240 clusters for peptide **A1** and 252 clusters for peptide **B1** with a 4 Å cutoff.

Secondly, each representative of all clusters of peptide **B1** was docked, using AutoDock,¹⁷ to the antibody 93F3-diketone **1** with the peptide backbone fixed. In this way there were no more than two torsions for each amino acid chain which guarantees more reliable search for the best peptide binding mode. All conformations were then ranked based on the magnitude of the estimated free energies of binding. AutoDock was previously shown to give excellent results for docking between small ligands and proteins including 2-mer peptide-protein docking.¹⁸

Among the 252 clusters of peptide **B1** conformations, 44% of the total docking runs showed negative binding energies. The best predicted binding free energy of peptide **B1** to the antibody 93F3-diketone **1** was -12 kcal/mol. The peptide-antibody complex after minimization with molecular dynamics gave the same estimated binding free energy by AutoDock, which indicated that docking against the rigid receptor 93F3-diketone **1** captured the nature of the peptide-antibody interaction. The combining site of the antibody complex after minimization is shown in Figure 4. A complicated hydrogen bonding network exists among diketone moiety, antibody, and water solvent. The diketone amide carbonyl forms two hydrogen bonds with the peptide His6 side chain at 2.8 Å and with Trp7 main chain amide at 3.1 Å. The diketone amide nitrogen also forms a hydrogen bond with antibody Ser^{L91} main chain at 2.8 Å. These hydrogen bonds were preserved after minimization by molecular dynamics. Peptide Thr5 main chain carbonyl forms a hydrogen bond with Tyr^{H97} at 2.7 Å. There is also an internal peptide backbone hydrogen bond between His6 and Leu8 at 2.8 Å. At the other side of antibody combining site, Asp^{H54} forms three hydrogen bonds with Cys1 and Thr2 at 2.6, 2.7 and 3.1 Å, respectively. The peptide main chain oxygen or nitrogen atoms in Cys1, Pro3, Thr5, Trp7, Leu8 and Cys9 are all very well solvated by water molecules at hydrogen bond distances (pictures not shown). Hydrogen bonds between peptide His6 and the diketone amide carbonyl and between peptide Trp7 and the diketone amide carbonyl, and other interactions between peptide and the antibody, ultimately anchor peptide **B1** onto the 93F3-diketone complex.

In order to estimate the accuracy of our docking strategy, we attempted to dock peptide **A1**, which bound antibody 93F3 but did not bind 93F3-diketone **1**, to 93F3-diketone **1** using the same method described above for peptide **B1**. In agreement with the ELISA results, docking experiments predicted less favored interactions between peptide **A1** and 93F3-diketone **1**. The best estimated binding free energy of peptide **A1** to 93F3-diketone **1** was smaller than that of

peptide **B1** by up to 2 kcal/mol. These results validate our docking strategy and the model of peptide **B1** bound to 93F3-diketone **1**.

Discussion

We have developed phage-displayed peptides that selectively bind to a particular 1,3-diketone derivative-bound antibody 93F3 but that do not bind to the ligand-free antibody or to the antibody bound to other related 1,3-diketone derivatives. These phage-displayed peptides were selected from libraries with 7 to 12 randomized amino acid residues using designed selection strategies. By the use of altered selection procedures, we have also generated phage-displayed peptides that bind to the ligand-free form of antibody 93F3 but that do not bind to the antibody when bound to 1,3-diketone derivatives. Using the selected phage-displayed peptides, ligand-bound states of the antibody were distinguished from each other. The binding of the phage-displayed peptides to the antibody was controlled in the presence or absence of a hapten-derived small molecule.

Phage-displayed peptide **B1** selectively bound to 93F3-diketone **1** complex. The amide functionality of diketone **1** was essential for this binding. Phage-displayed peptide **B1** did not bind diketone **2**- or **3**-bound 93F3; neither of these diketones possesses the amide moiety. In addition, phage-displayed peptide **B1** did not bind to ligand-free 93F3, indicating that the interactions between phage-displayed peptide **B1** and 93F3 were not strong enough to maintain the binding without the interactions between this peptide and diketone **1**. Therefore, the binding of phage-displayed peptide **B1** to both the antibody and diketone portions of the 93F3-diketone **1** complex was important for the selective binding to the 93F3-diketone **1** complex. A computational model of peptide **B1** bound to 93F3-diketone **1** suggests that a hydrogen bonding network among peptide, diketone, and antibody results in the observed selectivity of binding and that interactions between diketone amide moiety and peptide **B1** is especially important.

Conclusion

We have demonstrated that phage-displayed peptides that selectively bind a protein when the protein binds a particular small molecule can be developed using combinatorial libraries and designed selection strategies. It should also be noted that our experimental strategy for the generation of phage-displayed peptides specific to a particular ligand-bound form of a protein did not require structural information about the protein or its ligand-bound forms. Our strategy for the generation of phage-displayed peptides that distinguish the ligand-bound states of an antibody should be useful for the development of peptides that bind not only to antibodies but also to other proteins in the presence of their particular ligand molecules.

Experimental

Selection of peptides

Ph.D.-C7C, Ph.D.-7, and Ph.D.-12 phage peptide libraries (New England Biolabs, NEB) were used for the selection. **Selection method A.** Wells of a microtiter plate (Costar 3690) were coated with antibody 93F3 (1 μ g/25 μ L of PBS per well) at 4 $^{\circ}$ C overnight, washed with H₂O two times, and blocked with 3% BSA/PBS (170 μ L per well) at 37 $^{\circ}$ C for 1 h (PBS = 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Blocking solution was removed and the library phage were added. The plate was incubated at 37 $^{\circ}$ C for 1 h. The wells were washed 10 times with 0.5% Tween 20/PBS (PBST) to remove unbound phage. To elute the bound phage, 0.1 N HCl (100 μ L per well) was added to the wells and the plate was incubated at 37 $^{\circ}$ C for 30 min. The eluted phage solutions were neutralized by the addition of 2 M Tris (6 μ L/100 μ L of elution) and were added to *E. coli* 2537 cells in LB broth, and grown using the procedures recommended by NEB. After the first round of selection using each phage

peptide library separately, panned libraries were combined. For the additional three rounds, the bound phage in the antigen-combining site were eluted by incubation with a solution of diketone **1** (10 μ M in 0.5% DMSO/PBS, 100 μ L per well) at 37 °C for 1 h. **Selection method B.** Wells of a microtiter plate were coated with antibody 93F3 (1 μ g/25 μ L of PBS per well) in the presence of diketone **1** (final concentration 10 μ M) at 4 °C overnight, washed with H₂O two times, and blocked with 3% BSA/PBS (170 μ L per well) at 37 °C for 1 h. In another microtiter plate, wells were coated with antibody 93F3 (1 μ g/25 μ L of PBS per well) and blocked using the procedure described above. Blocking solution was removed and the library phage were added to the antibody 93F3-coated wells. The plate was incubated at 37 °C for 30 min, then the phage were transferred to wells coated with 93F3-diketone **1** and diketone **1** (final concentration 10 μ M) was added. The plate was incubated at 37 °C for 1 h. The wells were washed 10 times with PBST to remove unbound phage. To elute the bound phage, 0.1 N HCl (100 μ L per well) was added to the wells and the plate was incubated at 37 °C for 30 min. The eluted phage solutions were neutralized by the addition of 2 M Tris (6 μ L/100 μ L of elution) and were amplified as described in selection method A. An additional three rounds of selection were performed using the same procedures. **Selection method C.** Selection was performed as described in selection method B using diketone **3** instead of diketone **1**.

ELISA of phage-displayed peptides

Microtiter plates (Costar 3690) were coated with antibody 93F3 (1 μ g/25 μ L of PBS per well), incubated at 37 °C 1 h, washed two times with H₂O, and blocked with 3% BSA/PBS (150 μ L per well) at 37 °C for 1 h. Blocking solution was removed, the culture supernatant containing phage-displayed peptide (25 μ L per well) and a solution of diketone (20 μ M in 1% DMSO/PBS, 25 μ L per well) were added (final concentration of diketone 10 μ M). For the ELISA in the absence of diketone, the same volume of PBS was added. The plate was incubated at 37 °C for 1 h. The wells were washed 10 times with H₂O and the bound phage-displayed peptide was detected using anti-M13 antibody-horseradish peroxidase conjugate and the peroxidase substrates 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide. The resulting color was measured at 405 nm. For the ELISA using purified phage, phage were precipitated with PEG-NaCl and resuspended into PBS.^{19,20}

Computational procedures

Peptide conformational search—Peptides **A1** and **B1** were constructed by leap in Amber 8.²¹ In both cases two terminal cysteines were connected by a disulfide bond to form a loop based on experimental observations. In the first stage of the two-step minimization, a weak restraint was applied to the peptide and only water molecules were allowed to move freely with 500 steps of steepest descent and 500 steps of conjugate gradient minimization. Then the whole system was minimized (MAXCYC=2500, NCYC=1000). A two-stage 22 ns molecular dynamics at 300 K in periodic condition was performed with water molecules equilibrated first. Langevin dynamics (NTT=3) with collision frequency (GAMMA_LN=1.0) was adopted at a time step of 2 fs. SHAKE (NTC=2) was added to constrain bonds involving hydrogen. The constant pressure periodic boundary conditions were used (NTB=2, PRES0=1.0, NTP=1) with the reference pressure at one bar. After 2 ns equilibrium, a snapshot was collected every 2 ps and there were a total of 1000 snapshots for each peptide **A1** and **B1** at the end of the simulations. The snapshots were then clustered against the backbone heavy atoms with a 4 Å rmsd cutoff. At the end, the total number of snapshots selected for the docking analysis was 240 and 252 for peptide **A1** and peptide **B1**, respectively.

Peptide docking with 93F3-diketone **1**—Firstly a structure of the enaminone formed from diketone **1** and methylamine was generated and optimized with Gaussian 03.²² Then the methylamine moiety of the enaminone was superimposed with ϵ -amino group of the active site lysine LysL89 of the crystal structure of antibody 93F3.¹⁴ During the docking studies, the

diketone and the lysine were treated with full flexibility. A virtual screening of each of the 240 peptide **A1** and the 252 peptide **B1** conformations was conducted with the 93F3 crystal structure¹⁴ and with the 93F3-diketone **1** structure using AutoDock 3.0.¹⁷ All the peptide backbones were rigid with the side chains fully flexible in the docking. The number of side chain rotations for peptide **A1** and **B1** were ten and twelve torsions, respectively. The population size was 200 with 200 million energy evaluations in a total of ten dockings for each docking run. Two independent docking experiments were conducted for each snapshot. The best peptide binding mode was chosen according to the estimated free energy of binding in each case. The best predicted peptide-antibody conformation was then minimized by Amber as described in the previous section. The antibody-peptide pictures (Figure 4) were prepared using Pmv.²³

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Figure 1.

A schematic representation of selective binding of phage-displayed peptide to a liganded form of a protein.

Figure 2.

Inhibition of the binding of phage-displayed peptides **A1**, **A2**, and **A3** to antibody 93F3 by the addition of diketones **1** or **2**. The ELISA was performed using antibody 93F3-coated wells in the absence and in the presence of diketones (10 μ M).

Figure 3.

Binding profile of the phage-displayed peptides **A3**, **B1**, **B2**, and **C1**. The ELISA was performed using antibody 93F3-coated wells in the absence and in the presence of diketones **1**, **2**, and **3** (10 μ M).

Figure 4.

Two views of the docking model of peptide **B1** to antibody 93F3-diketone **1**. (a) Antibody 93F3 is shown as surface. The carbon atoms of diketone **1** are displayed in cyan. Nitrogens, oxygens, and sulfurs of peptide **1** are colored in blue, red, and yellow, respectively. (b) The hydrogen bonding network between peptide **B1** and antibody 93F3-diketone **1** is shown. DIK = diketone **1**. For clarity, the hydrogens are not shown.

Chart 1.

Table 1
Amino acid sequences of the selected phage-displayed peptides.

selection method ^a	sequence (name)	frequency ^b	relative signal in ELISA ^c		
			no additive	with 1	with 2
A	CPWHLLPFC (A1)	11/22 (33/35)	1.0	0.1	0.2
A	WYLPDNPSTWHT (A2)	8/22 (33/35)	1.0	0.1	0.1
A	QVPWSYLANGLL (A3)	3/22 (33/35)	1.0	0.1	0.2
B	CTPETHWLC (B1)	6/7 (11/20)	0.1	1.0	0.1
B	CSPLTHALC (B2)	1/7 (11/20)	0.2	1.0	0.2
C	CPWWFPTQC (C1)	1/1 (1/10)	0.2	0.1	0.1

^a Selection methods: (A) Selection for binding to unliganded antibody 93F3. Bound phage-displayed peptides were eluted with diketone **1**. (B) Selection for binding to 93F3-diketone **1**. (C) Selection for binding to 93F3-diketone **3**.

^b Frequency of the peptide sequence/the number of sequenced clones. The number of clones that showed desired ELISA results based on the selection strategy/the number of clones analyzed by ELISA is given in parentheses.

^c Signal in ELISA relative to the highest signal for each phage-displayed peptide. ELISA was performed against 93F3 in the absence of compound and in the presence of **1**, **2**, or **3** (10 μ M). ELISA signals were compared without background correction. Relative signal of backgrounds in ELISA was typically ~0.1.