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Research Article

Highly Selective Cyclic Peptide Ligands for NeutrAvidin and Avidin Identified by Phage Display

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Screening combinatorial libraries of conformationally constrained peptides against macromolecular targets is utilized in identifying novel drug leads and in developing new reagents for chemical biology. In methods such as phage-display selections, biotinylated macromolecular targets are often immobilized on avidin- and streptavidin-functionalized supports. Thus, the characterization of peptides that bind avidin and streptavidin is necessary for accurate interpretation of screening and selection results. Toward this goal, we panned a phage-displayed cyclic peptide library against NeutrAvidin, a chemically deglycosylated version of avidin. The selection produced a highly homologous consensus motif (Asp-Arg/Leu-Ala-Ser/Thr-Pro-Tyr/Trp). Two of these cyclic peptides, CDRATPYC and CDRASPYC, bound both NeutrAvidin and avidin with low-micromolar dissociation constants, whereas their acyclic counterparts had negligible affinity (<80-fold). Moreover, these cyclic peptides were very specific for their targets and did not bind the structurally and functionally similar protein, streptavidin. Thus, we have identified a new class of cyclic peptides, distinct from the much-studied streptavidin-binding His-Pro-Gln peptide motif. These results will not only allow for discriminating between desired and background cyclic peptide motifs in selections and screens but also provide a new protein/peptide model system and a useful reagent in chemical biology that can have utility in protein immobilization, purification, and chemical tagging.

Key words: avidin, 4′-hydroxyazobenzene-2-carboxylic acid, *in vitro* selection, NeutrAvidin, peptide, phage display, streptavidin

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Drug discovery efforts often begin with peptide ligands as lead compounds (1–5). These peptides, in turn, are discovered in many ways, including elucidation of native ligands (6), combinatorial library screening (7), and *in vitro* selection methodologies (8). Selection methodologies, such as phage display (9,10), ribosome display (11), and mRNA display (12), rely on immobilization of target proteins on solid surfaces that are amenable to panning procedures. One immobilization method of choice is the use of known biological interactions. For example, the glycoprotein avidin has an affinity for the small-molecule biotin that is one of the strongest non-covalent interactions known, with a K_d of 10^{-15} M (13). As such, avidin, as well as the related protein streptavidin, is routinely used with biotin for immobilization in combinatorial library screenings and *in vitro* selections (14). The (strept)avidin–biotin interactions allow for very specific immobilization, generally with low backgrounds, even from complex biological mixtures (15).

One of the important methodologies to which biotin-binding proteins have been applied is phage display (9). In phage display, avidin and streptavidin have been used for both direct immobilization and solution-phase capture of targets (16). A number of groups have also used streptavidin, not for immobilization, but rather as a target itself (7,17–22). One of the earliest phage-display selections was carried out by Devlin *et al.* (17) and targeted streptavidin. While this target provided a convenient demonstration of the phage-display methodology, the authors also recognized the importance of identifying peptides that bound streptavidin. Knowing streptavidin-binding motifs allows for the identification of background sequences in screenings and selections that can be easily identified as off-target binders. Similar studies have been performed to characterize peptides that demonstrate other off-target interactions, such as plastic-binding peptides (23).

Streptavidin and avidin have also been used as model receptors in library screenings and drug discovery. As streptavidin is so well studied and accessible, it has been used as a target to demonstrate drug discovery methodologies, such as phage display (17), peptide library screening (7), and ligand–receptor interaction analysis (24). The differences in avidin and streptavidin also give insight into ligand specificity of receptors, as both proteins bind biotin with very high affinity yet share only 33% sequence identity (13). It has been shown that peptides containing the ubiquitous consensus motif for streptavidin [His-Pro-Gln (HPQ)] do not bind avidin in its native or deglycosylated state (18). Furthermore, streptavidin and avidin differ in their affinities for the biotin-competitive dye, 4′-hydroxyazobenzene-2-carboxylic acid (HABA), by more than an order of magnitude (streptavidin $K_d = 100$ μM, avidin $K_d = 7$ μM at pH 7) (25). Studying the different modes of peptide binding between two similar receptors (streptavidin and avidin) that share a common ligand (biotin) provides useful model systems and allows for new insight into

other systems displaying high homology, such as protein kinases (26) and closely related G protein-coupled receptor (GPCR) families (4).

Streptavidin has gained wider use than avidin both as a model receptor and in other biological applications despite the abundance of avidin. This is because avidin, which can be readily isolated from hen egg white (27), has the unfavorable characteristic of diminished specificity due to its high isoelectric point (pI = 10) and its glycosylated native state (13). The oligosaccharide of glycosylated avidin has been shown to interact with lectin-like molecules and its positive charge at neutral pH facilitates electrostatic interactions with negatively charged species (28). On the other hand, streptavidin is not glycosylated and has a relatively neutral isoelectric point (pI = 5–6) (13). However, streptavidin is not entirely free of non-specific interactions, exemplified by its motif Arg-Tyr-Asp, similar to Arg-Gly-Asp, the universal recognition site in fibronectin and other adhesion molecules (29). Apart from this biotin-independent binding, streptavidin has the additional disadvantage of being more expensive to produce than avidin. In an effort to address these concerns, useful commercial variants of avidin, including a chemically deglycosylated form of the protein, called NeutrAvidin (Pierce, Rockford, IL, USA), have been produced. Chemical modifications also reduce the isoelectric point of NeutrAvidin to a more neutral pH (pI = 6.3). These modifications reduce non-specific interactions for NeutrAvidin (30), while maintaining its biotin-binding ability (31), thereby providing an alternative to streptavidin for drug discovery and biological applications.

To our knowledge, a monovalent peptide selection against avidin or NeutrAvidin has not been reported to date (20). Thus, consensus motifs obtained from phage-display selection against NeutrAvidin will help map the off-target sequences for combinatorial peptide library screens and *in vitro* selections like phage display. Additionally, peptides that bind NeutrAvidin can be used in bioconjugation applications similar to those of the streptavidin-binding peptide, Strep-tag (32). Small peptides such as the Strep-tag can easily be expressed as fusions with larger proteins for use in purification (33) or other conjugation applications (32). The availability of labeled streptavidin, as well as streptavidin immobilized on solid supports, has made these peptides extremely useful. The ability to use NeutrAvidin in similar situations will provide valuable new tools for drug discovery and biological applications.

With the goals of (i) providing known background motifs for *in vitro* selections and screenings, (ii) developing new reagents for NeutrAvidin technology, and (iii) studying ligand differentiation in model receptors, we present the results of an *in vitro* selection using a phage-displayed six-residue disulfide-constrained cyclic peptide library against NeutrAvidin. The resulting peptides' affinities for NeutrAvidin were characterized via a competition assay with the biotin-competitive dye, HABA, and the specificities of the peptides for NeutrAvidin were explored by analogous assays with avidin and streptavidin.

Materials and Methods

M13KO7 helper phage and all enzymes were purchased from New England Biolabs (Beverly, MA, USA). NeutrAvidin, avidin and streptavidin were obtained from Pierce. Peptide synthesis reagents and resin were purchased from Novabiochem (San Diego, CA, USA). All other reagents, unless otherwise noted, were obtained from Sigma (St Louis, MO, USA).

Library construction

The six-residue disulfide-constrained cyclic peptide library was constructed N-terminal to a peptide linker to the gene III fusion protein encoded by the phagemid vector pCANTAB-5E (Amersham Biosciences, Princeton, NJ, USA). A gene encoding a peptide linker and containing an internal *Pst*I restriction site had been previously cloned into pCANTAB-5E between *Sfi*I and *Not*I restriction sites in our laboratory to produce pCANTAB-Fos (data not shown). After transfection into *Escherichia coli* and subsequent isolation of the amplified pCANTAB-Fos, a gene encoding the cyclic peptide library was cloned into the *Sfi*I and *Pst*I sites of the vector, as previously described (34,35). The gene was constructed using overlapping primers. The synthesized oligonucleotide library contained the NNS mixed codon set for randomized positions, where N corresponds to G, C, A, or T; and S corresponds to G or C. The primers were obtained from IDT (Integrated DNA Technologies, Coralville, IL, USA).

LibFwd1: cgatgcggcccagccggccatgggttgcnnsnnsnnsnnsnnsnnstgcggtggaggc

LibRev1: gcaagcgctgcagcaccgcctccaccgca

The primers were extended to the full duplex DNA by mutually primed synthesis with the Klenow fragment of *E. coli* DNA polymerase I. The insert was purified and digested with *Sfi*I and *Pst*I and subsequently ligated into digested pCANTAB-Fos (see Supplementary Material for a complete library sequence). The library was then transformed into XL1-Blue *E. coli* cells (Stratagene, La Jolla, CA, USA) via electroporation. The library size was estimated by titration of the transformation mixture on ampicillin- and glucose-containing LB agar plates, and was found to be 1.1×10^9 CFU. The phagemid DNA from the transformation mixtures was isolated after amplification in *E. coli* and was retransformed into XL1-Blue cells, which were grown overnight with ampicillin and tetracycline selection in the presence of glucose. The library containing *E. coli* was stored in glycerol (20%) at −78 °C.

Phage-displayed peptide selection against NeutrAvidin

XL1-Blue *E. coli* containing the phagemid library vector were grown from glycerol stocks in 5 mL of 2x YT media with glucose and ampicillin selection at 37 °C. Titered M13KO7 helper phage (5×10^9 PFU) was added when the culture reached an OD_{600} of 0.8 and was incubated for 1 h. The culture was then pelleted via centrifugation, the cells were resuspended in 2x YT with ampicillin and kanamycin, and allowed to grow overnight. After 10 h of incubation, the culture was again pelleted by centrifugation and the supernatant was filtered through a 0.45-μm sterile filter to remove trace *E. coli*. The phage was isolated from the supernatant by polyethylene glycol (PEG) precipitation. One milliliters of 20% PEG in 2.5 M NaCl was added to the 5 mL of filtered media. The resulting precipitate was isolated by centrifugation at 18 000 g. The phage pellet was resuspended in 5 mL of Tris Buffer A (20 mM Tris–HCl, 150 mM

NaCl, and 0.05% Tween 20 at pH 7.4). The phage was then re-precipitated with 1 mL of PEG/NaCl, isolated via centrifugation, and re-suspended in 1 mL of Tris Buffer A.

Phage solution (100 μL) was then exposed to a well of a NeutrAvidin-coated polystyrene plate (Pierce) that had been previously rinsed with Tris Buffer A. After 1 h of incubation, the phage solution was discarded and the well was washed six times for 10 min each with 200 μL of Tris Buffer A. Bound phage was eluted with 200 μL of 0.2 M glycine (pH 2.0) by incubation for 10 min, followed by neutralization with 40 μL of 2 M Tris base (pH 11). The input phage and eluted phage were then used to infect two 5-mL tetracycline-selected cultures of XL1-Blue *E. coli* ($OD_{600} = 0.8$). After 1 h, the cells were pelleted and resuspended in 5 mL of 2xYT with ampicillin and glucose. To estimate the number of input and output phages, 20 μL of 10-fold serial dilutions of each culture was plated on LB agar plates that contained ampicillin. The rest of the output culture was grown overnight, at which point 1 mL was used to start the next round of selection, while the other 4 mL was stored in glycerol (20%) at −78 °C. DNA from colonies from the LB agar plates was isolated for DNA sequencing.

Solid-phase peptide synthesis and peptide cyclization

The selected NeutrAvidin-binding peptides were synthesized via standard Fmoc solid-phase peptide synthesis strategy on Rink-Amide-AM resin. All peptides were synthesized with a C-terminal glycine and two cysteines flanking the consensus sequences (i.e. CXXXXXXCG). Cleavage from RinkAmide resin with trifluoroacetic acid (TFA) left an amide bond on the C-terminal carbonyl of the peptide. After cleavage and global deprotection with 94% TFA, 2.5% water, 2.5% ethanedithiol and 1% triisopropylsilane, the peptides were purified essentially as described previously (36). Briefly, the peptides were precipitated three times in chilled ether, and the dried peptides were further purified by HPLC in 0.1% TFA with a gradient of 10–20% acetonitrile in water. The peptides were lyophilized and either stored at −20 °C for direct use as a linear peptide, or were cyclized before characterization.

Peptide cyclization was carried out by oxidation of the two cysteines to form an intramolecular disulfide bond. The peptides (500 μM) were shaken in phosphate-buffered saline (PBS; pH 7.4) with 10% dimethyl sulfoxide (DMSO) for 8 h at 37 °C. Extent of the disulfide bond formation was monitored as a loss of free thiol using Ellman's reagent, as we reported previously (37). Reflective-phase Matrix-assisted laser desorption (MALDI) mass spectrometry confirmed the peptides' molecular masses, as well as their cyclization states. Results for the cyclized peptides are as follows: CDRASPYCG, expected: 968.0 g/mol, found: 968.4 *m/z*; CDLASPWCG, expected: 948.0 g/mol, found: 948.0 *m/z*; CDRATPYCG, expected: 982.1 g/mol, found: 981.8 *m/z*. Amino acid analysis was also carried out on the cyclized peptides (W.M. Keck Facility, Yale University, New Haven, CT, USA).

HABA-competitive binding determination

For the competition assays between the NeutrAvidin-selected peptides and HABA, increasing amounts of peptide were titrated into an equimolar complex of HABA and NeutrAvidin, avidin, or streptavidin (50 μM final concentrations) in PBS. After reaching equilibrium (60 min), the absorbance of the complex was monitored at 500 nm. To calculate the IC_{50}s of the selected peptides, the average of three separate trials was fitted to the Hill equation:

$$LR = [L]_B + \frac{[L]_F - [L]_B}{1 + \left(\frac{IC_{50}}{[L]}\right)^{nH}} \quad (1)$$

where LR is the fraction of bound ligand [L] is the total ligand concentration, $[L]_F$ is free ligand concentration, $[L]_B$ is bound ligand concentration and nH is the Hill coefficient (38). Only the NeutrAvidin and avidin data could be fit to eqn 1 (Figure 1). The dissociation constants of the peptides were then determined using:

$$K_d = K_{L_2} = \frac{IC_{50}}{1 + \frac{[L_1]}{K_{L_1}}} \quad (2)$$

where $[L_1]$ is the HABA concentration, K_{L_1} is the dissociation constant of the complex of HABA and the biotin-binding protein, and K_{L_2} is the dissociation constant of the selected peptide for the biotin-binding protein (39). We used our calculated value of 15 μM for the dissociation constant of the HABA–NeutrAvidin complex (see Supplementary Material), and the literature value of 7 μM for the HABA–avidin complex (25). Best-fit equations were calculated using KaleidaGraph (Synergy Software, Reading, PA, USA).

Results and Discussion

Biotin-binding proteins are used frequently as immobilization and bioconjugation tools in biotechnology; thus, it is important to identify the peptide epitopes that they recognize. In this regard, the most thoroughly studied biotin-binding protein is streptavidin. The early work by Devlin *et al.* (17) found a unique consensus motif, HPQ, which has been confirmed in several later studies (7,18–22). Although most of the studies reported HPQ as a major consensus motif, the flanking sequences varied widely. In the studies performed with cyclic peptide libraries, cyclization of the selected peptides led to an increase in binding affinity (40,41). Given the availability and stability of immobilized streptavidin, streptavidin-binding peptides have been used as purification tags (33,42) and in other applications (32). In an effort to reduce the non-specific binding of avidin and streptavidin in such applications, a chemically deglycosylated form of avidin, called NeutrAvidin, was developed. The low cost and low non-specific binding of NeutrAvidin make it an excellent choice for use in immobilization for various applications, especially *in vitro* selection. However, before selections are carried out, it is prudent to characterize the background binding of the immobilization matrix.

Phage-display selection and synthesis of NeutrAvidin-binding peptides

While performing a selection against a separate target, we discovered an interesting NeutrAvidin-binding peptide epitope in our control selections (data not shown). The rapid and complete convergence to this novel motif encouraged us to repeat the selec-

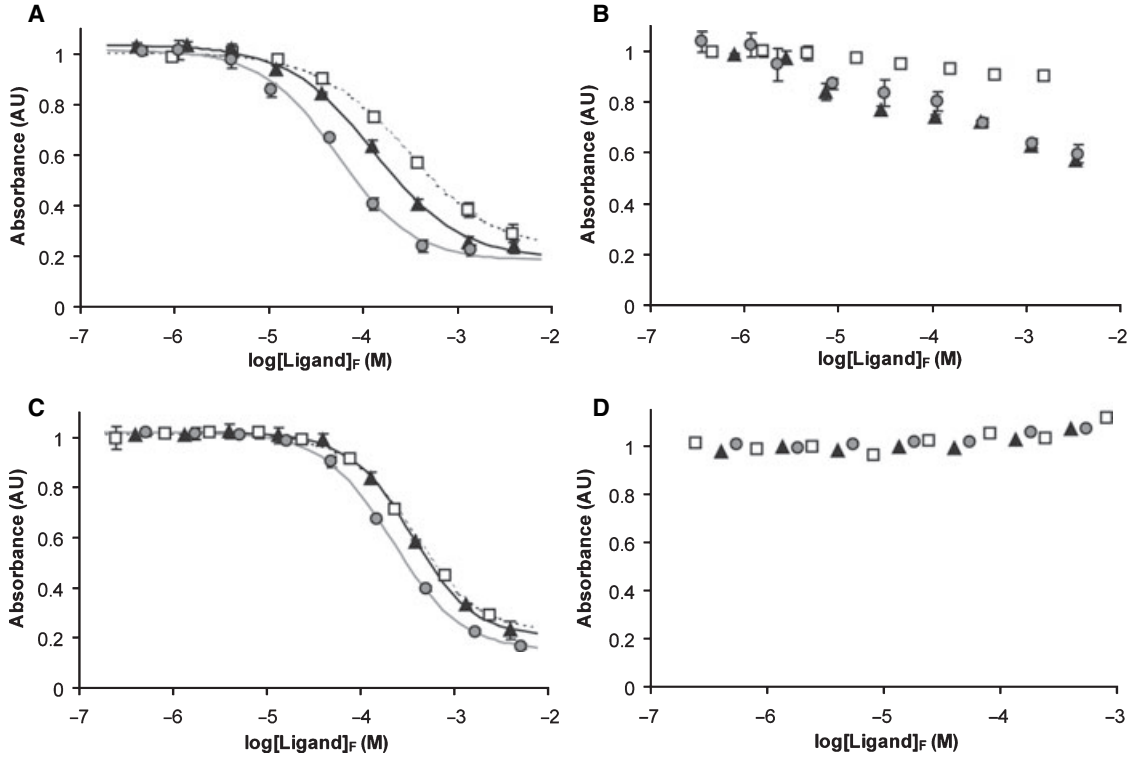

Figure 1: Competitive ligand-binding analysis of CDRASPYCG (black triangles), CDRATPYCG (gray circles) and CDLASPWCG (empty squares) for (A) cyclized peptides with the 4′-hydroxyazobenzene-2-carboxylic acid (HABA)–NeutrAvidin complex, (B) uncyclized peptides with the HABA–NeutrAvidin complex, (C) cyclized peptides with the avidin–HABA complex and (D) cyclized peptides with the streptavidin–HABA complex. Protein–HABA complexes were 50 μM in concentration for (A)–(D). The absorbance was measured at 500 nm and normalized to the 50 μM HABA–protein complex. Error bars indicate the standard deviation of three separate assays. In cases where no error bars are seen, they are smaller than the symbols used in the figure. The best-fit lines to eqn 2 in (A) and (C) are shown for CDRASPYCG (solid black), CDRATPYCG (solid gray), and CDLASPWCG (dashed black).

tion against NeutrAvidin and characterize the selected peptides more fully. The selection utilized a six-residue cyclic peptide library constrained with a disulfide bond from two conserved cysteines. The library size was chosen to enable complete coverage (1.1×10^9 unique nucleotide sequences encoding for 6.4×10^7 unique peptides) and a cyclic architecture was chosen because of the increase in affinity that cyclization provides relative to linear peptides (40,41). The library was expressed as a fusion to the gene III protein of M13 filamentous bacteriophage, C-terminal to the periplasmic signaling sequence and N-terminal to a peptide linker and the rest of the gene III protein (see Supplementary Material). Phage-display selection rounds were carried out against NeutrAvidin-coated polystyrene plates without further preparation. After only three rounds of selection, a striking motif was discovered (Table 1). The motif is of the general form $DX_aAX_bPX_c$, where X_a = R or L; X_b = S or T; and X_c = Y or W. After two more rounds, the selection slightly favored the peptide DRASPY. The consensus sequences all have Asp in the first position, Ala in the third position, and Pro in the fifth position. It is worth noting that even in the positions of variability, close consensus was maintained. For instance, the fourth position strictly requires a hydroxyl containing residue (Ser or Thr) and the sixth position requires an aromatic amino acid (Tyr or Trp). The second position allows the most drastic change within the motif, with Arg as the favored residue, but Leu being tolerated.

Table 1: Selected cyclic peptide phage-display results

Round 3	%[a]	Round 4	%[b]	Round 5	%[c]
CDRASPYC	27	CDRASPYC	46	CDRASPYC	49
CDLASPWC	27	CDRATPYC	27	CDLASPWC	18
CDRATPYC	20	CDLASPWC	12	CDRATPYC	15
CDRASPWC	20	CDRASPWC	8	CDRASPWC	5

[a]15 clones sequenced.
[b]26 clones sequenced.
[c]40 clones sequenced.

Many previous studies targeting streptavidin have found consensus sequences with the motif HPQ (17). Other reported streptavidin-binding motifs include: GDF/WXF, PWXWL, EPDWF/Y, and DVEAWL/I (43). A NeutrAvidin-binding epitope selected by Petrenko and Smith in a multivalent context, VPEY (20), was not detected in our monovalent selections. Lam and Lebl have reported an *in vitro* combinatorial screen of a linear pentapeptide library against NeutrAvidin's glycosylated parent, avidin, which produced a histidine-containing motif HP(Y/F/I/A)P (44) that shares a modicum of similarity with our consensus motif, that is, a proline followed by an aromatic residue. Thus, the novelty of our selected epitopes,

along with their relatively early appearance, led us to further investigate the binding of these peptides to NeutrAvidin and explore their selectivity. In light of the fact that all of the selected peptides fell into the consensus motif of $DX_aAX_bPX_c$, we decided to synthesize the three most frequently observed peptides for further characterization, namely DRASPY, DLASPW, and DRATPY.

The peptides were synthesized via standard Fmoc strategies and consisted of a C-terminal glycine, the consensus sequence, and two flanking cysteines ($CDX_aAX_bPX_cCG$). All of the consensus peptides readily cyclized upon overnight shaking in PBS with 10% DMSO. The MALDI mass spectrometry confirmed both the monomer status of the peptides, as well as their cyclization state. The composition and concentration of the peptides were confirmed via amino acid analysis (data not shown).

Competition between HABA and the selected peptides for NeutrAvidin binding

A common method for quantifying biotin in solution is a competition assay with the dye HABA, which was developed by Green (45). The binding of HABA to avidin causes a significant increase in absorbance of light at 500 nm. As HABA binds avidin in a biotin-competitive manner, it is possible to quantify the amount of biotin in a solution based on the loss of absorbance at 500 nm of the HABA/avidin complex (25). As Green noted, the extent to which HABA can be out-competed by a biotin analog is dependent upon the binding affinity of the competitor (45). Therefore, we examined NeutrAvidin's HABA-binding ability with the goal of characterizing the selected peptides through a competition assay (see Supplementary Material).

As the phage-display selections against NeutrAvidin did not have any bias toward the biotin-binding site of the protein, it was far from certain that the selected peptides would compete with HABA. However, as a previously discovered HPQ-containing peptide was shown to bind in the biotin/HABA pocket of streptavidin (46), we felt that it was likely that the peptides from our selection might bind the analogous site in NeutrAvidin. Therefore, we titrated increasing amounts of our selected peptides into a complex of HABA and NeutrAvidin and monitored the decrease in absorbance at 500 nm (Figure 1). The decrease in absorbance at 500 nm observed upon addition of the peptide ligands indicates that they bind in a HABA-competitive fashion. As HABA and biotin are known to bind to the same pocket (47), it is likely that the selected peptides also bind in the same manner.

It is interesting to note that all of the selected peptides assayed for HABA-competitive binding to NeutrAvidin showed affinity, although not in the order of consensus (Table 2). The tightest binder according to the competition assay, DRATPY, was not the major consensus sequence from the selection. These results suggest either (i) the selection does not discriminate peptides within a fivefold affinity variation or (ii) the extent of cyclization is inconsistent between the peptides on the surface of the phage during the selection.

Our assumption, based on literature precedence, was that peptides displayed on the surface of phage with two conserved cysteines would spontaneously cyclize under the phage preparation conditions (16). To test the necessity of cyclization for the selected peptides to bind NeutrAvidin, a competition assay was carried out with the uncyclized peptides (Figure 1). All three peptides that were assayed showed a marked decrease of binding to NeutrAvidin in their uncyclized states. The increase of affinity between receptors and peptides in the cyclized form has been well documented (40,41).

Table 2: Cyclic peptide-binding constants[a]

Peptide	NeutrAvidin	Avidin	Streptavidin
CDRASPYCG	31.5 ± 4.4	44.9 ± 2.3	>5000
CDRATPYCG	12.5 ± 0.7	28.1 ± 0.9	>5000
CDLASPWCG	62.8 ± 10.8	46.2 ± 3.7	>5000

[a] K_d values are in units of μM.

Selectivity of the selected peptides in HABA-competitive binding

Having established that the selected peptides indeed bound NeutrAvidin, we wanted to investigate whether or not they could bind avidin (the glycosylated parent of NeutrAvidin) or streptavidin. Therefore, streptavidin and avidin were assayed for peptide binding using the same conditions as the HABA-competitive NeutrAvidin assay (Figure 1, Table 2). The similarity of the biotin-binding pockets of avidin and streptavidin would seem to indicate that peptides binding these sites would not discriminate well between streptavidin and avidin. However, our results show that the Neutr Avidin-selected peptides do not bind streptavidin in a HABA-competitive manner with any measurable affinity (Figure 1). Indeed, it has been shown that some HPQ-containing peptides that bound streptavidin tightly did not bind avidin (18,44), indicating that mutually exclusive recognition may be a common theme for these proteins. Avidin, on the other hand, does show significant binding to the NeutrAvidin-selected peptides (Table 2). This indicates that the chemical modifications carried out on avidin to produce NeutrAvidin are outweighed in this system by the similarity in primary structure of the two proteins. The similarity in binding constants (Table 2) implies that the binding of the selected peptides is generally independent of the glycosylation state of avidin.

The demonstrated selectivity for avidin and NeutrAvidin is remarkable for the selected peptides, although the $DX_aAX_bPX_c$ motif (where X_a = R or L; X_b = S or T; and X_c = Y or W) is itself interesting in a number of ways. First, the Pro that is absolutely conserved at position 5 suggests that the peptides assume a turn motif. Interestingly, the variations seen in positions 4 and 6 are logical substitutions of similar amino acid residues. The appearance of Ser and Thr at position 4 indicates that this residue might be involved in a hydrogen bond, although it is not tightly packed because either residue enables binding. Likewise, the allowance of both Trp and Tyr at position 6 indicates some potential π–π interaction. For the absolutely conserved Asp at position 1, it is interesting that no Glu was seen. This might indicate that Asp forms an essential interaction that is very size or distance dependent. The conserved Asp residue might also be the source of specificity for NeutrAvidin versus streptavidin.

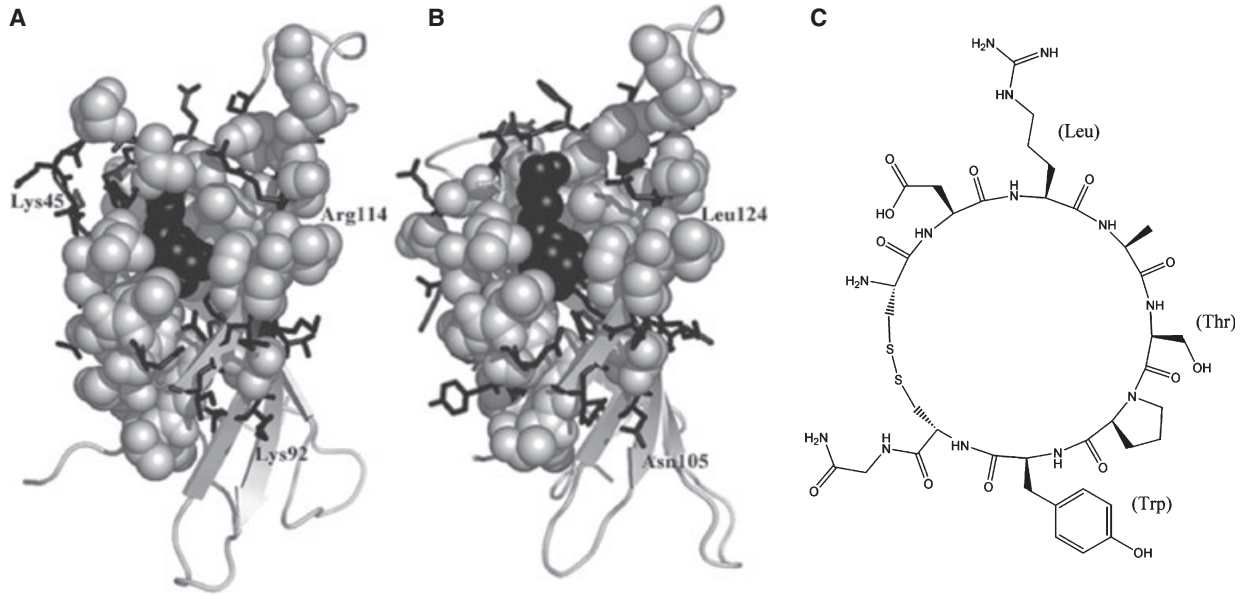

Figure 2: The three-dimensional structure of (A) avidin (PDB ID: 1AVD) (48) and (B) streptavidin (PDB ID: 1STP) (49) bound to biotin (illustrated as black spheres within the interior of each protein). Amino acids represented as light spheres are conserved residues within 12 Å of the biotin molecule. Amino acids represented as dark sticks are divergent residues within the same area. The positively charged Arg114, Lys92, and Lys45 are labeled in (A) (Arg100 is not visible). The analogous residues from streptavidin, Leu124 and Asn105 (Leu56 and Thr111 are not visible), are labeled in (B). The chemical structure of the selected peptide CDRASPYCG is shown in (C). Alternate residues found in the consensus motif are indicated in parentheses.

If one compares the aligned structures of avidin and streptavidin (Figure 2) (48,49), the similarity of the binding pocket is striking, considering that the two complete proteins only share 33% identity. However, there are two lysines (Lys45 and Lys92) and two arginine (Arg114 and Arg100) residues near the binding pocket of avidin (within 12 Å of biotin) that are not present in streptavidin. These lysines or arginines could form a salt bridge with the conserved Asp in position 1 of the selected epitopes.

Conclusions and Future Directions

We have discovered a new NeutrAvidin/avidin binding cyclic peptide motif that was reproducibly selected by phage display. Members of this motif, $DX_aAX_bPX_c$ (where X_a = R or L; X_b = S or T; and X_c = Y or W), were characterized by a competition with the biotin-competitive dye HABA and found to have binding constants between 12 and 63 μM for both NeutrAvidin and avidin. Furthermore, the cyclic peptides were shown to be 1000-fold more selective for NeutrAvidin/avidin versus streptavidin. Interestingly, previous studies showed that streptavidin-selected HPQ epitopes do not bind avidin (44) or NeutrAvidin (18). This specificity suggests that the two classes of peptides could be used in mixed systems where orthogonal recognition of NeutrAvidin and streptavidin could be beneficial.

Besides being an interesting off-target consensus motif for NeutrAvidin-immobilized screenings and *in vitro* selections, this epitope presents an alternative to the HPQ sequences used in a variety of applications. For instance, the $DX_aAX_bPX_c$ motif could be used as an immobilization tag for protein purification (33) or for immunoassays and blots (32). NeutrAvidin could also complement streptavidin as a model receptor for novel *in vitro* selections and in new screening strategies (7,8,14,22).

In summary, we have discovered, through cyclic peptide phage display, a novel avidin/NeutrAvidin-specific motif that binds to these proteins in a HABA-competitive manner. The newly identified epitopes will not only help in the identification of false positives from *in vitro* selections, but they will also serve as new reagents for drug discovery and biotechnology.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1: The sequence of the peptide library.

Figure S2: HABA saturation of NeutrAvidin.

Table S1: Sequences from the cyclic peptide phage display against NeutrAvidin

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