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Analysis of novel streptavidin-binding peptides, identified using a phage display library, shows that amino acids external to a perfectly conserved consensus sequence and to the presented peptides contribute to binding

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Summary

Streptavidin-binding peptides containing the consensus amino acid sequence motif EPDW were identified using a phage display library. Phage presenting peptides containing these sequences bound streptavidin in a biotin-sensitive fashion and could be eluted with biotin. The previously identified 'streptag' peptide sequence (AWRHPQGG) competed with phage presenting the EPDW consensus sequence for streptavidin binding. Furthermore, the EPDW sequence has two amino acids in common with yet another previously identified streptavidin-binding sequence, GDWVFI, which has similar biochemical properties. Binding inhibition studies revealed that residues flanking EPDW, as well as residues of the modified phage pIII product to which displayed peptides are fused, contributed to streptavidin binding. The derivation of small molecules based on the structure of peptides selected using display methods is a potentially important application of phage display technology. The relevance of the observations made here for that application are discussed. Finally, a group of 'nuisance' peptides of the consensus sequence WHWWXW, whose binding specificity has not been fully elucidated, but which have been isolated in a number of biopanning experiments, including those that do not utilize streptavidin, are also described.

Introduction

Bacteriophage display libraries of peptides have been used to identify peptidic ligands of a variety of biological molecules, including monoclonal antibodies (initially described in Ref. 1, and reviewed in Ref. 2), integrins [3,4], enzymes [5] and small-molecule binding proteins such as concanavalin A [6,7] and streptavidin [8–10]. In the case of antibodies and other proteins which recognize proteins, the consensus peptide sequences derived from display libraries can identify the epitope or structure that is normally recognized by the target protein under investigation. In other cases, the identified peptides themselves can be useful as affinity tags for protein purification [11], or as inhibitors or 'leads' to small-molecule inhibitors of medically important proteins. This latter application of information derived from peptide display experiments is of potentially great interest to the pharmaceutical industry. A prerequisite for effective modelling from peptides is clear knowledge of those peptidic amino acid residues and structures involved in binding to the target protein. In most peptide display studies, the issue of which residues contribute to target protein binding is scantly addressed, and that information which is presented is often derived from comparison of the sequences of peptides identified after affinity selection. While instructive, consensus comparison cannot elucidate all nuances of peptide–target-protein interaction [12], nor can it identify amino acid residues of the fusion protein (i.e., pIII residues), that, though external to the presented peptide, nonetheless contribute to target protein binding.

This study uses biochemical methods to identify amino

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acids both within and external to a peptide identified from a phage display library that contribute to binding a target protein (streptavidin). Using binding inhibition experiments with synthetic peptides, amino acids outside the identified consensus (and external to the presented random peptide) that contribute to the binding of one of the peptides to streptavidin were identified.

Materials and Methods

Peptide display library

A peptide library, presented on fd-tet, similar to that of Cwirla [13], and encoding 10 random amino acids fused to the phage pIII, was produced as described in Ref. 14. Codons encoding the 10-amino-acid random peptides were synthesized with an equimolar mixture of all four deoxynucleotides at the first two positions and an equimolar mixture of dGTP and dTTP at the third position. Following molecular cloning of oligonucleotides specifying the peptides of the library into the fd-tet phage, sequence analysis was performed using the Genesis 2000 DNA analysis system [15].

Affinity selections

The phage library was subjected to three consecutive rounds of affinity selection, referred to as 'biopanning' [16]. Aliquots of phage containing 10^{12} transducing units (TU) in the first round of biopanning, and 10^{11} TU in subsequent rounds, were exposed to streptavidin-coated (Pierce, Rockford, IL) petri plates (prepared as in Ref. 4) for 1 h at room temperature. Plates were then washed 10 times, 10 min each with TBST (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5% Tween-20). Phage particles were eluted by treatment with 1 mg/ml BSA in 0.1 M HCl (adjusted to pH 2.2 with glycine) for 10 min. Following neutralization with 1 M Tris-HCl (pH unadjusted), phage particles were amplified in *Escherichia coli* strain K-91 [16] in preparation for the next round of biopanning.

Characterization of streptavidin binding by EPDW phage

Following the third round of biopanning, phage were characterized by DNA sequencing. The ability of individual phage species to bind streptavidin was confirmed by single-round biopannings in which the percentage of clonal phage harvested from streptavidin-coated petri plates (yield) was compared to the yield of a phage that had been isolated at random from the library (and which had not been subjected to affinity selection with streptavidin). About 10^9 TU of streptavidin-binding or control phage were added to streptavidin-coated plates. To test whether phage binding of streptavidin was inhibited by biotin, some of the plates were pretreated with biotin (10 pg/ml), incubated for 1 h at room temperature, and washed 10 times with TBST prior to addition of phage particles. Yields of phage from single-round biopannings using plates so treated were compared to yields from plates not treated with biotin. To determine whether EPDW phage could be eluted from streptavidin using biotin, 10^9 TU of phage were subjected to a single-round biopanning as described above. Prior to elution with glycine-buffered HCl, plates were treated for 1 min at room temperature with 1 ml of an aqueous solution of biotin (1 ng/ml). The biotin solution was harvested and titered. Percentage phage yield of the input phage in the biotin solution was determined and compared to the percentage obtained from a subsequent glycine-buffered HCl elution of the same plates.

Peptide synthesis and purification

Peptides were either synthesized in-house or obtained commercially from Genosys Biotechnologies, Inc. (The Woodlands, TX). Peptides synthesized in-house were assembled with an Applied Biosystems, Inc. Model 430A automated peptide synthesizer, using standard Boc-amino acid coupling protocols [17]. The peptide resin was treated with 90% HF/anisole for 60 min at 0 °C. Purification on reverse-phase HPLC gave 98% pure material, the composition of which was verified by amino acid analysis and fast-atom-bombardment mass spectroscopy.

Peptide inhibition of phage binding

Polystyrene 96-well microtiter plates (Elkay Lab Systems, Inc., San Diego, CA) were coated overnight at 4 °C with 2 μg of streptavidin in 40 μl 0.1 M $NaHCO_3$, blocked with 5 mg/ml BSA for 1 h at room temperature and washed three times with TBST. Peptides were dissolved in water and diluted to 1 mM. Of the stocks, after dilution in TBST, 40 μl was incubated on the streptavidin-coated

TABLE 1
EPDW PHAGE BIND STREPTAVIDIN IN A BIOTIN-SENSITIVE MANNER

Plate treatment	Phage harvested (% of input)[a]		
	CD9999	CD99911	Lib7[b]
BSA-blocked	Not determined	1.0×10^{-3}	1.0×10^{-3}
BSA-blocked, streptavidin-coated	1.5	1.0	1.0×10^{-3}
BSA-blocked, streptavidin-coated, biotin-treated[c]	1.6×10^{-4}	1.0×10^{-4}	1.0×10^{-4}

[a] Percentage of input phage harvested following elution with glycine-buffered HCl is shown.
[b] Lib7 is a phage chosen at random from the library prior to any affinity selection, and presents the peptide VSSLPLDGGL.
[c] This set of streptavidin-coated plates was flushed with biotin prior to addition of phage particles (see Materials and Methods).

TABLE 2
EPDW PHAGE CAN BE ELUTED FROM STREPTAVIDIN USING BIOTIN

Phage	Phage harvested (% of input)[a]	
	Biotin	Glycine-buffered HCl
CD9999	0.35	0.04
CD99911	1.06	0.20

[a] Percentage of input phage harvested from streptavidin-coated plates in response to sequential elution with biotin and glycine-buffered HCl (see Material and Methods).

wells for 2 h. About 10^9 TU of clonal phage were then added to each well, and incubated for 90 min. Wells were washed 10 times with TBST, and phage were eluted with glycine-buffered acid, as described above. The extent of binding inhibition by the peptides was determined from titers of the eluted phage in *E. coli* K-91.

Results

A large, diverse library of peptide-presenting phage was achieved. The library was derived from about 10^8 individual *E. coli* transformants, and sequence analysis of 54 randomly chosen library phages revealed that all codons specifying amino acids of the random peptides occurred at frequencies within two-fold of predicted values (data not shown).

The yield of phage increased from biopanning rounds one to three, suggesting that affinity selection of the phage population had occurred. In the first round of biopanning, 6×10^{-5}%, in the second round, 9×10^{-3}% and finally, in the third round, 3% of the phage input were harvested by elution with glycine-buffered HCl from streptavidin-coated plates. Sequence analysis of randomly chosen phage from biopanning round three revealed the phage population primarily to encode two peptide sequences that shared the identical amino acid sequence motif EPDW: SPEPDWFVEL (phage CD99911, 30/43 sequenced isolates) and REPDWYEYVK (phage CD9999, 11/43 sequenced isolates). Two other species were also identified at lower frequency (see below).

Table 1 shows that phages CD99911 and CD9999 recognized streptavidin, whereas a control phage did not. Recognition of streptavidin by EPDW phage would seem to be specific as binding was reduced to background levels if the plate was pretreated with biotin. Furthermore, Table 2 shows that CD99911 and CD9999 could be eluted from the streptavidin-coated plates with 1 ng/ml biotin.

Figure 1 shows the results of binding inhibition experiments which characterize the binding specificities of the streptavidin binding phage CD99911. The data indicate that the peptide presented by CD99911 inhibited phage binding to streptavidin in a dose-dependent fashion. Furthermore, the previously identified, but chemically distinct streptag peptide [1] also inhibited CD99911 binding to streptavidin. Schmidt and Skerra [1] showed that the presence of the C-terminal GG sequence of streptag significantly enhanced the binding of the sequence to streptavidin. Interestingly, GG residues are present immediately carboxy-terminal to the peptides presented in EPDW phage (see Discussion). Peptides containing two carboxy-terminal glycines appended to the CD99911 peptide sequence inhibited CD99911 phage binding more efficiently over a range of concentrations than did the peptide sequence lacking the terminal addition.

Figure 2 shows that peptide sequences in addition to the perfectly conserved motif (EPDW) contributed to streptavidin binding. Consistent with the data of Fig. 1, the unmodified peptide identified from CD99911 (SPEPDWFVELGG) inhibited the binding of CD99911 phage to streptavidin. However, neither the sequence EPDW nor EPDWGG detectably diminished streptavidin binding by the phage. Binding inhibition experiments using synthetic peptides, identical to that encoded by CD99911 phage, with the exception that various amino acid positions were substituted with alanine, were used to identify amino acids that contributed to the binding of streptavidin. Peptides substituted at positions at which the amino acid side chain contributed to binding would be expected to inhibit CD99911 phage binding of streptavidin less strongly than

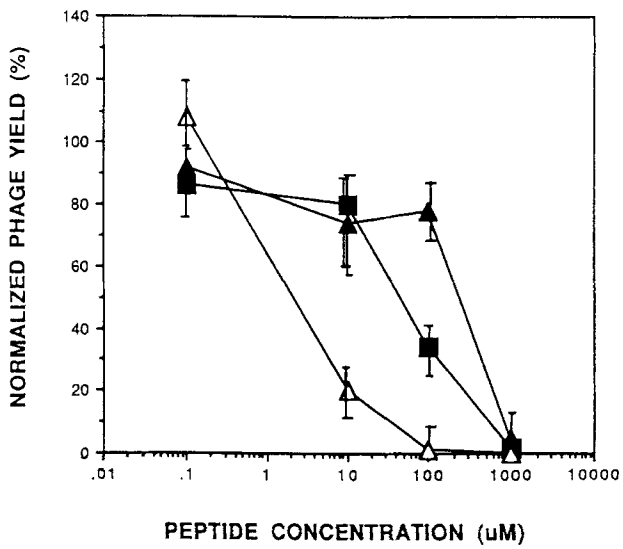

Fig. 1. Dose-dependent inhibition of CD99911 phage binding of streptavidin by synthetic peptides. Streptavidin-coated plates were treated with various synthetic peptides (concentrations shown on the horizontal axis) prior to addition of CD99911 phage (see Materials and Methods). Phage yields (normalized to the no-peptide control biopanning, in which no peptide was added to the streptavidin-coated plate prior to phage addition) are presented along the vertical axis. Average results of three replicates of the experiments are presented and error bars are shown. Binding inhibition experiments with the peptide SPEPDWVEL are indicated by solid squares (■); those with the peptide SPEPDWFVELGG are indicated by open triangles (Δ); and those with the peptide AWRHPQFGG (Streptag, see Ref. 11) are indicated by solid triangles (▲).

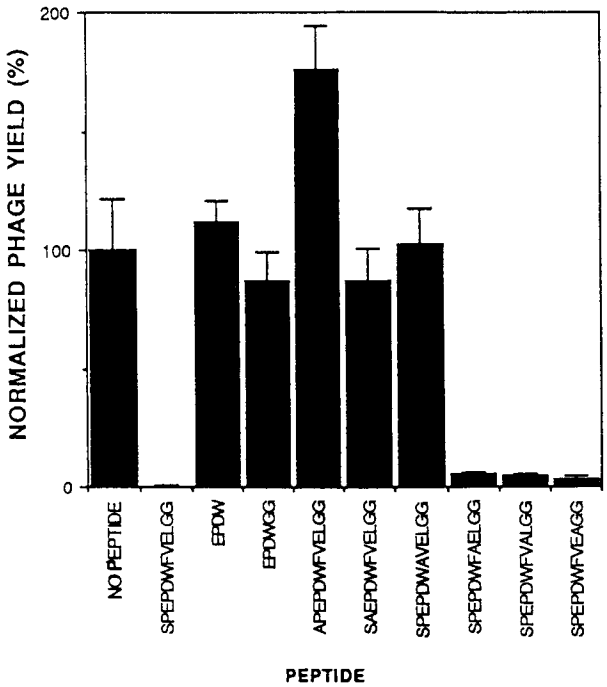

Fig. 2. Identification of phage-presented residues that contributed to streptavidin binding. Streptavidin-coated plates were treated with various synthetic peptides (1 mM) prior to addition of CD99911 phage (see Materials and Methods). Phage yields (normalized to the no-peptide control biopanning, in which no peptide was added to the streptavidin-coated plate prior to phage addition) are presented along the vertical axis. Identities of the peptides used are shown on the horizontal axis. Average results of three replicates of the experiments are presented and error bars are shown.

would the unmodified CD99911 peptide. Phage binding in the presence of peptides containing alanine substitutions for the Val, Glu, or Leu amino acid residues carboxy-terminal to the EPDW sequence of the peptide presented by CD99911 peptide was inhibited only slightly less than in the presence of the unmodified peptide. The results do not suggest that a profound contribution to streptavidin binding was made by the Val, Glu, and Leu residues individually.

In contrast, binding of streptavidin was not detectably diminished, relative to peptide-minus controls, by peptides substituting alanines for the three residues flanking the consensus EPDW amino acid sequence (Ser, Pro and Phe), suggesting that these residues contributed significantly to the ability of the peptide in CD99911 to recognize streptavidin. In toto, the data indicated that at least the residues SPEPDWF at the amino end of the presented peptide of CD99911 and the GG residues beyond the carboxy end of the random peptide contributed to the selection of CD99911 in streptavidin biopanning.

In addition to phages CD9999 and CD99911, phages encoding the peptide sequences WHRWPWLVSG and WHWWYWALDR were also isolated from streptavidin biopanning, each at a frequency of 1 of 43 phages sequenced. Isolates of this family of phage encode highly hydrophobic, tryptophan-rich peptides with histidines uniformly present at the second amino acid position, and exhibit the consensus WHWWXW (determined from a total of nine independent peptide sequences of members of this group of phage, identified in several biopanning experiments, see below). Despite their superficial similarity to the previously identified XWXWL streptavidin-binding consensus sequence [8], these phage probably do not specifically recognize streptavidin. This notion is supported by several observations. For instance, WHWWXW phage bound streptavidin/BSA-coated plates only marginally better than BSA-coated plates (with a ten-fold or less difference in phage yield between the two types of plates; data not shown), and unlike EPDW phage, the efficiency of binding was insensitive to pretreatment with biotin (data not shown). Most importantly, WHWWXW phage have been isolated repeatedly in biopannings in which no streptavidin was used. In these experiments, unbiotinylated target protein was directly deposited on the plates, and the plates were then blocked with BSA.

Discussion

This report and others contribute to the conclusion that distinct peptidic sequences can interact with a given ligand, and exhibit broadly similar binding/elution properties [8–11,18]. The consensus sequence EPDW was identified after biopanning a phage display library using streptavidin as the target protein. Given the codon frequencies inherent in the library design, the chance of independent isolation of two phage containing the perfect sequence repeat EPDW is remote (less than one chance in 10^{10}). Thus, it seems likely that the EPDW sequence contributed to the selection of CD9999 and CD99911 in biopanning.

The presented peptide of CD99911 has two amino acids (DW) in common with a previously identified streptavidin-binding sequence, GDWVFI [2,8]. Both peptides are biochemically similar in that they cross-compete with the streptag peptide sequence (AWRHPQGG, Ref. 1) for streptavidin binding, and biotin interferes with streptavidin binding by both peptides. These similarities notwithstanding, it is uncertain whether the EPDW consensus sequences, GDWVFI, or any other streptavidin-binding peptide contact the same target protein amino acids, in the absence of structural data. It is therefore uncertain whether the peptides of CD9999 and CD99911 are examples of the GDWVFI peptide family [2,8], and the sequence similarities between the two groups of peptides might be coincidental.

Use of peptide libraries to identify leads to small-molecule inhibitors of medically important macromolecules is a potentially valuable application of display technology. Unfortunately, this approach is challenging for a number of reasons. For example, complexes of HPQ-consensus

peptides with streptavidin contain a water molecule intercalated between the peptide and streptavidin [12]. While this observation would be critical for designing a small molecule modeled on the peptide, it could not be inferred from inspection of sequences isolated from biopanning a library against streptavidin. This report highlights two additional complexities inherent in use of peptide sequences derived from phage display to model small molecules: that residues outside the conserved consensus sequence can contribute to binding to the target protein, and that residues of the protein to which peptides are fused for presentation can contribute to target binding.

The results of binding-inhibition studies (Fig. 2) illustrate the necessity for structural or functional data to identify those residues, not perfectly conserved among related peptides isolated in a biopanning experiment, that contribute to peptide binding. The data suggest that at least three residues of the CD99911 peptide (Ser, Pro and Phe) outside of the consensus sequence contributed significantly to streptavidin binding. The Phe residue of the CD99911 peptide is chemically similar to the Tyr residue carboxy-terminal to the consensus EPDW sequence of CD9999 to the extent that both residues are hydrophobic and therefore may play an analogous role in streptavidin binding. However, the similarity in function, if any, between the Ser and Pro residues amino-terminal to the EPDW sequence of CD99911 and the Arg residue amino-terminal to the consensus sequence in CD9999 is less obvious. Some features of the CD9999 peptide might eliminate the necessity for either (or both) the Ser and Pro residues that apparently contribute (Fig. 2) to streptavidin binding by CD99911. Given that biopannings of target proteins generally yield a broad diversity of sequences comprised of families of more or less similar peptide sequences, the observation that peptidic residues outside the perfect consensus contribute to target protein binding is not surprising. However, unequivocal identification of such amino acid residues must be made empirically and presumably would be important in designing small-molecule inhibitors from peptides.

The data in Fig. 1 suggest a second complexity inherent in the use of peptides derived from fusion proteins to provide leads to small molecules. The presence of two Gly residues carboxy-terminal to the peptide of CD99911 increases the ability of the peptide to bind streptavidin over a range of concentrations. These residues are present appended to all peptides in the library from which the EPDW phage were derived, owing to library design: two Gly residues lie immediately carboxy-terminal to the presented random peptides (see Refs. 13 and 14), and therefore might have contributed to EPDW phage isolation. These considerations suggest the unexpected conclusion that sequences external to the presented peptide can contribute to binding to the target protein. Furthermore, the data presented here do not test the possibility that pIII amino acid residues carboxy-terminal to the two glycines, or other residues/structural features of phage particles might contribute to peptide binding of target proteins. This information presumably would also be important to modelers of peptides, but again would not be obvious without functional or structural data.

The contribution to target binding made by phage amino acid residues external to random peptides might also account for instances in which the affinity properties identified by display library approaches prove to be poorly transferable into new protein contexts. In fact, fusions of the sequence SPEPDWFVEL to the *E. coli* alkaline phosphatase gene exhibit little detectable binding to streptavidin (data not shown).

Monovalent, rather than pentavalent, presentation of peptides and proteins is often used by investigators attempting to identify high-affinity ligands of target proteins. Monovalent presentation avoids a 'chelate' effect whereby phage presenting multiple copies of peptides that have individually low affinity for target proteins exhibit high overall avidity as the result of their multivalency [19]. The contribution to target protein binding of phage residues/structures outside of the presented peptide might also result in a disparity in the affinities of phage-presented and free peptides that could prove more difficult to address than the chelate effect. Presumably, such an effect could be operative regardless of valency.

The WHWWXWW consensus sequence is described here to alert others that peptides with similar consensus sequences might not specifically recognize the expected target protein. The binding specificity of these peptides has not been fully elucidated, though they have been isolated from a number of biopannings, involving a variety of proteins, but not necessarily involving streptavidin. These peptides may recognize BSA, plastic or, in view of their hydrophobicity, the exposed cores of denatured proteins. In any event, they constitute 'nuisance' peptides that are routinely ignored when isolated.

Conclusions

Streptavidin-binding peptides containing the consensus sequence EPDW were identified from a phage display library. Binding inhibition experiments revealed that amino acids of the random peptide external to the consensus sequence, as well as amino acids outside the random peptide, contributed to the binding of the peptide to streptavidin. Analogous phenomena could complicate modelling of small molecules based on peptides isolated using display methods, and might also explain cases in which peptides exhibit diminished affinity for their target proteins when not in the context of the presentation vehicle. 'Nuisance' peptides of the consensus WHWWXW were also isolated in streptavidin biopanning experiments, but their binding did not require streptavidin.

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