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Research Article

Identification of Small Peptides That Interact Specifically With a Small Organic Dye

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ABSTRACT Using a "one-bead one-peptide" combinatorial peptide library method, we have been able to identify peptide ligands that interact specifically with various macromolecular targets such as monoclonal antibodies, streptavidin, avidin, MHC-Class I molecules, proteases, growth factor receptors, and gpIIb/IIIa integrin. In this paper, we test the hypothesis that small peptides that interact specifically with a small organic molecule can also be identified using this combinatorial peptide method. Using a small organic dye molecule, indigo carmine, as a color probe to screen a random L-heptapeptide and two D-hexa and octapeptide libraries, we were able to identify a specific peptide binding motif. Potential applications of this technology are described.

Key Words: carmine, split synthesis, peptides

INTRODUCTION

Enormous progress has been made on combinatorial library methods in the past five years. It is a powerful tool for facilitating the drug discovery process as well as providing important information for the fundamental understanding of molecular recognition [Jung and Beck-Sickinger, 1992; Pavia et al., 1993]. In general, three main combinatorial peptide library methods for generating huge peptide-libraries are available: (1) biological peptide libraries with filamentous phage [Scott and Smith, 1990; Cwirla et al., 1990; Devlin et al., 1990; Felici et al., 1991] or plasmid [Cull et al., 1992]; (2) the combinatorial peptide library method, with an iterative process [Geysen et al., 1986, 1987; Houghten et al., 1991; Owens et al., 1991; Blake and Litzi-Davis, 1992] or positional scanning [Dooley and Houghten, 1993]; and (3) peptide library methods based on a "one-bead one-peptide" concept, or the "Selectide process" [Lam et al., 1991; Lam and Lebl, 1992; Salmon et al., 1993; Lam et al. 1993a,b]. These various methods have both advantages and disadvantages. Over the last few years, we have successfully applied the "Selectide process" in the identification of peptide ligands (L- or D-amino acid oligomers) that interact specifically with various targets such as monoclonal antibodies [Lam et al. 1991; Lam et al. 1993a; Lam et al. 1993b], streptavidin [Lam et al., 1991; Lam and Lebl, 1992], avidin [Lam and Lebl, 1992], gpIIb/IIIa integrin [Salmon et al., 1993], MHC-class I molecules, proteases, and growth factor receptors (Lam et al, unpublished results). However, all of these targets are protein macromolecules. Recently, using related methods, peptide ligands specific for an "artificial receptor"

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[Borchardt and Still, 1994] or metal ion [Kramer et al., 1993] were reported. In this paper we report the identification of both L- and D-amino acid containing peptide ligands that interact specifically with a small organic dye, indigo carmine. Since the probe itself is an intense indigo color, no further reporting group is needed, and the screening process is extremely simple and rapid.

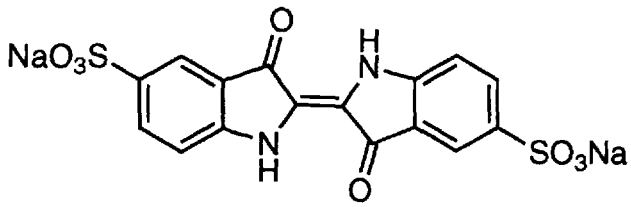

Figure 1. Chemical structure of indigo carmine.

PEPTIDE LIBRARY SYNTHESIS

Peptide library synthesis was accomplished by the "split synthesis" method [Lam et al., 1991; Furka et al., 1991] as described. TentaGel S (Rapp, Germany) and Fmoc chemistry [Atherton and Sheppard, 1988] were used in the synthesis of the random peptide libraries. TentaGel S already has a polyoxyethylene linker and therefore no further linker is needed in the synthesis of the library, although one glycine was coupled to the resin prior to synthesis of the D-hexamer- or octamer-libraries. The resins were first divided into 19 equal aliquots and four-fold excess of each Fmoc-protected amino acid was added to each aliquot (all 19 amino eukaryotic amino acids except for cysteine; in some experiments, D-amino acids were used). The coupling reaction was initiated by the addition of benzotriazolyl-oxy-trisdimethylamino-phosphonium hexafluorophosphate (BOP) and N-hydroxybenzotriazole (HOBt). Coupling was allowed to proceed for an hour. A ninhydrin test [Kaiser et al., 1969] was used to evaluate the completion of the coupling reaction. On rare occasion, double coupling may be needed to ensure completion of the reaction. The resins were then mixed, washed, and the Fmoc group removed with 20% piperidine (v/v), washed, and divided into 19 aliquots again. The coupling process was repeated until a library of desired peptide length was completed. The side chain protecting groups were then removed with a mixture of trifluoroacetic acid-phenol-anisole-ethanedithiol (94:2:2:2; v/w/v/v) as described [Lam et al., 1991; Lam and Lebl, 1992].

PEPTIDE LIBRARY SCREENING

After deprotection, the peptide library was washed extensively with doubly-distilled water to remove the DMF prior to screening. The beads were washed using PBS with 0.1% Tween 20. An aliquot of the library (approximately 300,000 beads) was then mixed with indigo carmine (5 μg/ml) in 2 × PBS with 0.1% Tween 20. Within 2 h, some deep indigo/blue color beads were readily identified. It was not necessary to remove the free dye in the incubating mixture as the background color was extremely faint. The majority of the beads in the library were colorless. The darkly colored beads were then physically isolated with a micropipette, washed with doubly distilled water, and subjected to microsequencing as described [Lam et al., 1991; Lam and Lebl, 1992].

RESULTS AND DISCUSSION

Indigo carmine was chosen as the model small molecule target because it is (1) a relatively small organic molecule (MW = 466.56), (2) water soluble, and (3) intrinsically colored. Therefore no further reporter group is needed for detection. The molecular structure is shown in Figure 1. It consists of a relatively hydrophobic planar structure with two negatively charged sulfonic acid groups, thus providing multiple potential interaction sites for a small peptide. Table 1 showed the ligands identified from an all L-amino acid hepta- and two all D-amino acid hexa- and octa-peptide libraries. It is immediately apparent that a specific motif "+000+" is readily identifiable (where + = lysine or arginine, and 0 = V, I, L, Y, F, or M). The two positively charged amino acids (with boldface in Table 1) were usually separated by three intervening residues, at least two of which were hydrophobic. These two positively charged residues probably interact with the two sulfonic acid groups, whereas the hydrophobic residues in between the lysine or arginine probably interact with the ring structure of the indigo molecule. Not surprisingly, similar motif was identified for both L- and D-amino acid peptide libraries since the indigo molecule is planar and symmetrical.

In order to understand this interaction further and in an attempt to isolate peptide ligands with higher affinities, a secondary library with the motif "X+000+X" (where X = 15% none, and 85% all 19 L-amino acids except cysteine; + = 50% K and 50% R; 0 = 15% each of V, I, L, M, F, Y, and 10% G) was synthesized and screened. Interestingly, less than 10% of the library was stained, despite the fact that every single bead has two positively charged amino acids. This indicates that a positively charged peptide alone is insufficient for interaction with the indigo dye

TABLE 1. Peptide Ligands Identified From the Primary Screen With Indigo Carmine

L-library	D-libraries
7-mer	*8-mer*
YKVVYKL	ikivyrfr
VTKIIFK	akwkwvyr
LTKLVLK	ykvvyris
	vkkmvikf
	6-mer
	klilkf
	wlikmk

TABLE 2. Peptide Ligands Identified From a Secondary Screen With Indigo Carmine

Secondary peptide library	X + 000 + X where X = 15% none, 85% all 19 amino acids except cysteine + = 50% K, and 50% R 0 = 15% each of V, I, L, M, F, Y, and 10% G
Ligands isolated	KKVVIKV IKYIIKW IKVVYKF WKLVVKR

molecule, especially under the current condition of screening where high salt (0.26 M NaCl) was used to minimize non-specific ionic interactions. Non-specific hydrophobic interactions, on the other hand, were suppressed by incorporating 0.1% Triton X-100 (a non-ionic detergent) in the screening medium. Table 2 shows the results of the secondary screening. Only four strongly positive beads were sequenced. All of them have a preference for lysine over arginine.

Besides this hepta-peptide library, we have also screened a random all L-amino acid tetra- and penta-peptide library. However, only extremely faint color beads were identified, suggesting that a tetra- or penta-peptide may be too short to allow any significant and specific interaction with the indigo molecule. We have also done similar studies on several other organic dye molecules. Many of them either did not stain any bead to a significant extent or the staining was so non-specific that approximately 10 to 40% of all beads in a random heptapeptide library were stained. In fact, indigo carmine is rather unusual in that only a very small portion of the random library was strongly stained with this organic dye. We have also resynthesized the positive ligands for indigo carmine on beads and stained the peptide-beads under various conditions. Interestingly, strong staining was detected under neutral, or acidic aqueous conditions (0.1 M HCl), and even in organic solvents such as dimethylsulfoxide or dimethylformamide (data not shown). Work is currently underway to determine the affinities of these peptide ligands to this organic dye molecule.

CONCLUSIONS

This paper illustrates that specific peptide ligands which interact with a small organic molecule can be readily identified with the "Selectide process." Potentially, this method can be used to develop binders for small molecules such as drugs. These binders, if specific and with high affinity, could potentially be useful as diagnostic reagents, biosensors, antidotes for drug overdose, or perhaps as binders for detoxifying environmental contaminants in water. Furthermore, information gained from such studies will undoubtedly help us in the fundamental understanding of molecular recognition.

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