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Streptavidin and Avidin Recognize Peptide Ligands with Different Motifs

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Using streptavidin as a molecular target for synthetic peptide library screening, ligands with consensus sequences of HPQ and HPM were rapidly identified. Here, we report the discovery of several non-HPQ sequences that were found when a pentapeptide library that lacked histidine was used in the screen. Although the high-affinity natural ligand for both streptavidin and avidin is biotin, neither HPQ nor HPM sequences were discovered when avidin was used in the screen. Instead, a new motif, HP(Y/F)P, was identified. Data obtained from such peptide library screens will help us to better understand molecular recognition. © 1992 Academic Press, Inc.

A major component of drug discovery is based on high-volume screening of numerous synthetic compounds and natural products. After a drug lead has been identified, subsequent detailed serial structure–function studies remain the principal method of drug design. Over the past few years, several attempts have been made to speed up the process by using various peptide- or polynucleotide-library approaches.

Geysen *et al.* (1, 2) devised a system for synthesizing peptides on polyethylene pins which could then be tested in multiwell plates in an effort to identify ligands that bound to a specific monoclonal antibody. Fodor *et al.* (3) reported the development of a "light-directed, spatially addressable parallel chemical synthesis" technique that uses complex instrumentation and photochemistry techniques to synthesize an array of 1024 peptides simultaneously on a glass microscope slide. The interaction of slide-bound peptides with a monoclonal antibody was detected with fluorescence microscopy.

Furka *et al.* (4, 5) reported the use of "split synthesis" to generate an equimolar mixture of peptides in solution, and proposed the concept of using such a library for drug discovery. Houghten *et al.* (6) reported a combinatorial library approach in which mixtures of random peptides with certain fixed residues were screened in solution phase, and along with an iterative selection and synthesis scheme, specific peptide ligands were identified.

Smith and co-workers envisioned the concept of using recombinant bacteriophage incorporating random nucleic acid sequences to produce large libraries of phage that display random peptides (7). Subsequently, three groups (8–10) have reported the insertion of randomly generated DNA fragments into gene III of a filamentous bacteriophage for generating peptide libraries. From such large peptide libraries they were able to identify and sequence ligands specific to two anti-myohemerythrin monoclonal antibodies (8), an anti-β-endorphin monoclonal antibody (9), and streptavidin (10). More recently, peptide ligands specific for concanavalin A, a carbohydrate-binding protein, have been identified (11, 12).

In addition to using peptide libraries, several groups have described the use of synthetic oligonucleotide libraries in screening and applied the PCR technique to amplification and subsequent sequence determination (13–16).

We (17–19) recently reported the synthesis of a very large peptide library (1 to 10 million) such that each resin bead expresses a specific peptide species—the

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"one-bead one-peptide" concept. Using an enzyme-linked immunoassay system, we were able to rapidly screen a pentapeptide library and identify peptides that bind specifically to (i) a monoclonal antibody, anti-β-endorphin, and (ii) streptavidin. In the case of the streptavidin, 28 peptides were sequenced, many of which have a consensus sequence of HPQ and a few that have a sequence of HPM (18). This result is similar to those obtained by Devlin *et al.* (10) using the filamentous phage system.

In this paper, we report the discovery of peptide ligands that are specific for avidin. Furthermore, we have also taken advantage of the versatility of our methodology and purposely omitted histidine in our library synthesis. This led to the discovery of specific non-HPQ sequences that interact with streptavidin.

METHODS

Synthesis of Peptide Library

Central to the simplicity and success of our new technology is the "one-bead one-peptide" concept. Based on the use of a split synthesis methodology (4, 5, 17–19) each bead was exposed only to a single activated amino acid at each coupling cycle. A large pentapeptide library with the structure XXXXX–β-Ala–ε-aminocaproic acid–ethylenediamine resin was prepared wherein $X = 19$ of the 20 eukaryotic amino acids (all but cysteine) that were utilized at each coupling step. In some experiments, histidine in addition to cysteine was purposely omitted from the library synthesis. The peptide library was synthesized using $N\alpha$-Fmoc strategy with fourfold excesses of the amino acids at each coupling step.

Polydimethylacrylamide resin beads (Milligen Inc., San Leandro, CA) (50–300 μm in diameter with 0.2–0.5 mmol/g substitution) were used in synthesizing the peptide libraries. The chemistry and the methodology of peptide synthesis using this resin were carried out according to Sheppard and Atherton (20). Three grams of resin (approximately 2 million beads) was mixed gently with ethylenediamine overnight. After a thorough washing, ε-aminocaproic acid, followed by β-alanine, was coupled to the resin using Fmoc chemistry, but without a cleavable linker.

Randomization was carried out in the next five coupling steps, and all 19 Fmoc–amino acid–OPfp except cysteine were used separately during each coupling step. In some experiments, BOP and HOBt were used as the coupling reagents. After the five randomization steps were completed, the Fmoc group was remov with 20% piperidine (v/v) in dimethylformam (DMF), and the side-chain protecting groups were moved from the peptide–linker–resin bead with a m ture of trifluoroacetic acid–phenol–anisole–ethane thiol (94:2:2:2; v/w/v/v). The resin was then wash and neutralized with 10% diisopropylethylamine DMF) and stored in DMF at 4°C.

Specific Identification and Selection of Peptide Ligands from the Library

Immunohistochemical techniques were used for detection of streptavidin binding beads. The rand library of peptide beads was gently mixed with inc mentally increasing double-distilled water to rem the DMF. Subsequently, the beads were washed th oughly with PBS, and gelatin (0.1% w/v) was used block any nonspecific binding. A 1:200,000 dilution streptavidin–alkaline phosphatase at 2 mg/ml (Pier Rockford, IL) was then added to the beads with gen mixing for 1 h. The beads were then thoroughly wash and the standard substrates 5-bromo-4-chloro-3- dolyl phosphate and nitroblue tetrazolium were add The beads in substrate solution were transferred i 15 polystyrene petri dishes (100 × 20 mm), and reaction was carried out for up to 2 h. The beads w bound streptavidin–alkaline phosphatase turned di blue on their surfaces, while the majority of the be in the library remained colorless.

Peptide Sequencing

The positive beads were physically picked up wit micropipet. After treatment with 8 M guanidine drochloride, pH 2.0, and washing with double-distil water, individual beads were placed on a glass fi and inserted in a microsequencer (Model 477A, Appl Biosystems) as described (18).

RESULTS

In our previous report, streptavidin–alkaline ph phatase at a concentration of 10 μg/ml was used screen the peptide library, and numerous pentapepti with HPQ and some with HPM sequence motifs w isolated. However, when we screened a pentapep library lacking histidine and cysteine using the sa concentration of streptavidin–alkaline phosphat conjugate, no positive beads were detected. The sa library was then washed and rescreened with a 10-

higher concentration of streptavidin–alkaline phosphatase conjugate. This time, approximately 200 beads were isolated and 14 were sequenced. The result is shown in Table 1. These 14 sequences could be divided into 4 groups. Group I had a general motif of WPA_ or W_A_. Group II had a general motif of RMDLY. This sequence was detected two times. Group III had a general motif of YME_W. The two peptides that did not fit into any of these three motifs were put into group IV. Peptides WPAIG, WPANL, RMDLY, YMEFM, and QYWQS were resynthesized on the resin, and restaining with streptavidin–alkaline phosphatase was reconfirmed. Furthermore, staining of these beads was completely abolished with the addition of 1 μM biotin during the incubation step with streptavidin–alkaline phosphatase, suggesting that these peptide ligands all bind to the biotin-binding pocket on streptavidin.

A pentapeptide library (all 19 amino acids, except cysteine) was screened with avidin–alkaline phosphatase. The result is shown in Table 2. The ligands could be divided into four groups with the following consensus: (I) HPYP_; (II) HPF_ _; (III) HP_P_; and (IV) HK_ _ _. The first three groups were related but group IV was definitely distinct.

Resynthesis of some of these ligands on resin reconfirmed specific staining by avidin–alkaline phosphatase, and the staining was again inhibited with the addition of 1 μM biotin during the incubation step with avidin–alkaline phosphatase.

Since both streptavidin and avidin bind strongly to biotin, we examined the cross-reactivity of the isolated ligand with the two biotin-binding proteins. The result is qualitatively shown in Table 3. Streptavidin interacted strongly with LHPQF but not with HPYPP,

TABLE 1

Amino Acid Sequences of Individual Pentapeptide Beads That Interacted with Streptavidin When a Pentapeptide Library Lacking Histidine and Cysteine Was Used in the Screen

Group I	Group II	Group III	Group IV
WPAIG[a]	RMDLY(2)[a,b]	YMEYW(2)[b]	PPWPY
WPANL[a]		YMETW	QYWQS[a]
WAARG		YMEFW[a]	

[a] These peptides were resynthesized on resin for retesting (see text).
[b] The RMDLY and YMEYW peptides were each detected on two beads.

TABLE 2

Amino Acid Sequences of Individual Pentapeptide Beads That Interact with Avidin

Group I	Group II	Group III	Group IV
HPYPI(3)	HPFPP(2)	HPIPN	HKFPP
HPYPS(3)	HPFPS	HPAPP	HKTHG
HPYPP(2)	HPFYG		HKDHK
HPYPM			
HPYPG			
HPYPK			

Note. The number in parentheses denotes the number of times these sequences were detected.

whereas the reverse was true for avidin. Again, the binding of these ligands to their corresponding biotin-binding protein was inhibited by biotin. As a positive control, the biotinylated beads were stained by both streptavidin– and avidin–alkaline phosphatase and their staining was completely inhibited by 1 μM biotin.

DISCUSSION

We have previously reported pentapeptide sequences with an HPQ and HPM motif isolated from a random pentapeptide library using streptavidin–alkaline phosphatase. During that screen, from a library of 2 million, 70 strongly reactive beads were isolated, and 28 were sequenced. Most of those 28 ligands had a consensus of HPQ and a few had the HPM motif. Since then, we

TABLE 3

Relative Color Intensities of the Stained Beads

	Ligand[a]		
Receptor	LHPQF	HPYPP	Biotin
Streptavidin–AP	++++	—	++++
Streptavidin–AP + biotin	—	—	—
Avidin–AP	—	+++	++++
Avidin–AP + biotin	—	—	—

[a] LHPQF and HPYPP sequences were resynthesized on polydimethylacrylamide resin and probed with either streptavidin or avidin–alkaline phosphatase in the presence or absence of biotin. Biotinylated bead with the same linker was used as a positive control ("—" denotes no color reaction; "++++" denotes strong color reaction).

have sequenced additional streptavidin-binding beads selected from various libraries (linear tetra- to decalibraries and cyclic libraries), all of which showed similar results. The dominant sequences were always HPQ and HPM. To select for non-HPQ or non-HPM sequences that are specific for streptavidin, we purposely omitted histidine and cysteine in our pentapeptide library. Positive beads with specific motifs (Table 1) were detected only when higher concentrations of streptavidin–alkaline phosphatase were used, indicating that these ligands probably have lower affinity to streptavidin than HPQ- or HPM-containing peptides have. However, their binding is specific since biotin inhibited their binding to streptavidin.

Generation of these non-HPQ motifs, despite their lower binding affinities, will aid us in better understanding ligand–receptor interactions at the molecular level. Currently, we are using computer modeling and various biophysical techniques to study their interactions with streptavidin.

Although both streptavidin and avidin bind strongly to biotin (dissociation constant, $K_d \cong 10^{-14}$ to 10^{-15} M), it is interesting to discover that the peptide ligands isolated from the same library by these two proteins are somewhat different: HPQ and HPM for streptavidin, HP(Y/F)P for avidin. Surprisingly, despite the consensus of HP_ _ for these two groups of peptides, they do not cross-react significantly. It would be extremely interesting to study the structural relationship between biotin, HPQ, and HP(Y/F)P and try to understand at the molecular level how they interact with the biotin-binding pockets.

The fact that we can easily select or omit specific amino acids in our library exemplifies the versatility of our methodology (18) compared to the biologic system (8–12). Likewise, D-amino acids and unnatural amino acids such as N-methylated amino acids and glycosylated amino acids can easily be included in the synthesis of our libraries. Furthermore, secondary structures such as α-helix, β-sheet, or cyclic structure can be incorporated into the design of our libraries. One important feature of our library screen is that we use a "parallel approach" in which every entity in the library is screened concurrently, and often peptide ligands with different motifs emerge at the end of the screen (see Tables 1 and 2). This differs from the screening methods of Houghten *et al.* (6) and of Geysen *et al.* (1, 2) in which a "convergent approach" is used and after several iterative steps of library synthesis and screenings, one peptide motif finally emerges.

Both the synthesis and the screening of our peptide library can be accomplished within a week and therefore are relatively fast. The rate-limiting step o method, however, is peptide sequencing; only 3–4 tides can be sequenced per day per microseque Currently, we are attempting to speed up the seq ing process by using mass spectrometry. Unlik filamentous phage technique, the practical limit of our peptide library is that we have to limit th of our peptide to smaller than 20-mers, and scre more than 10 million beads per person per day.

In addition to screening the peptide library w binding assay as described in this paper, we hav cently developed a method of sequential orthogon lease of peptides from the beads, followed by sol phase testing. The bead-of-origin that tests po can then be recovered and the residual peptide o bead analyzed by a microsequencer (Salmon manuscript in preparation). The advantage of method is that purified target is no longer nece and we can adapt our screening to many existin logical assays.

As illustrated in this paper, the rapid develop of various library screening techniques in the pas years will undoubtedly facilitate not only drug di ery, but also a better understanding of molecula ognition.

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REFERENCES

1. Geysen, H. M., Rodda, S. J., and Mason, T. J. (1986) *M munol.* **23**, 709–715.
2. Geysen, H. M., Rodda, S. J., Mason, T. J., Tribbick, Schoofs, P. G. (1987) *J. Immunol. Methods* **102**, 259–2
3. Fodor, S. P., *et al.* (1991) *Science* **251**, 767–773.
4. Furka, A., *et al.* (1988) 14th International Congress of Bi istry, FR:013.
5. Furka, A., Sebestyen, F., Asgedom, M., and Dibo, G. (19 *J. Peptide Protein Res.* **37**, 487–493.
6. Houghten, R. A., Pinilla, C., Blondelle, S. E., Appel, J. R., C. T., and Cuervo, J. H. (1991) *Nature* **354**, 84–86.
7. Parmley, S. F., and Smith, G. P. (1988) *Gene* **73**, 305–3
8. Scott, J. K., and Smith, G. P. (1990) *Science* **249**, 386–

9. Cwirla, S. E., Peters, E. A., Barrett, R. W., and Dower, W. J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6378–6382.
10. Devlin, J. J., Panganiban, L. C., and Devlin, P. E. (1990) *Science* **249**, 404–406.
11. Scott, J. F., Loganathan, D., Easley, R. B., Gong, X., and Goldstein, I. J. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5398–5402.
12. Oldenburg, K. R., Loganathan, D., Goldstein, I. J., Schultz, P. G., and Gallop, M. A. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5393–5397.
13. Turk, C., and Gold, L. (1990) *Science* **249**, 505–510.
14. Green, R., Ellington, A. D., and Szostak, J. W. (1990) *Nature* **347**, 406–408.
15. Robertson, D. L., and Joyce, G. F. (1990) *Nature* **344**, 467–468.
16. Blackwell, T. K., and Weintraub, H. (1990) *Science* **250**, 1104–1110.
17. Lam, K. S., *et al.* (1992) *in* Peptides: Chemistry, Structure and Biology (Proceedings of the Twelfth American Peptide Symposium) (River, J. E., and Smith, J., Eds.), pp. 492–495, Escom, Leiden.
18. Lam, K. S., Salmon, S. E., Hersh, E. M., *et al.* (1991) *Nature* **354**, 82–84.
19. Lam, K. S., Hruby, V. J., Lebl, M., Knapp, R. J., Kazmierski, W. M., Hersh, E. M., and Salmon, S. E. (1992) *Bioorg. Med. Chem. Lett.* in press.
20. Atherton, E., and Sheppard, R. C. (1988) Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford.