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A new type of synthetic peptide library for identifying ligand-binding activity

Kit S. Lam, Sydney E. Salmon, Evan M. Hersh, Victor J. Hruby*, Wieslaw M. Kazmierski† & Richard J. Knapp†

Arizona Cancer Center and Department of Internal Medicine, College of Medicine, Tucson, Arizona 85724, USA
* Department of Chemistry, Faculty of Science, University of Arizona, Tucson, Arizona, USA
† Selectide Corporation, 10900 N. Stallard Place, Tucson, Arizona 85737, USA

OUR aim was to improve techniques for drug development by facilitating the identification of small molecules that bind with high affinity to acceptor molecules (for example, cell-surface receptors, enzymes, antibodies) and so to mimic or block their interaction with the natural ligand[1,2]. Previously such small molecules have been characterized individually on a serial basis. The systematic synthesis and screening of peptide libraries of defined structure represents a new approach. For relatively small libraries, predetermined sequence variations on solid-phase supports have been used[3,4], and large libraries have been produced using a bacteriophage vector into which random oligodeoxynucleotide sequences have been introduced[5-8], but these techniques have severe limitations. Here we investigate an alternative approach to synthesis and screening of peptide libraries. Our simple methodology greatly enhances the production and rapid evaluation of random libraries of millions of peptides so that acceptor-binding ligands of high affinity can be rapidly identified and sequenced, on the basis of a 'one-bead, one-peptide' approach.

Our method involves creating a large peptide library consisting of millions of beads, with each bead containing a single peptide and with the complete collection representing the universe of possible random peptides in roughly equimolar proportion. It is clearly not enough to use a random mixture of activated amino acids in a peptide synthesis protocol, because the widely different coupling rates of different amino acids will lead to unequal representation and because each bead will contain a mixture of different peptides. Our solution was to use a 'split synthesis' approach. The first cycle consisted of distributing a pool of resin beads into separate reaction vessels each with a single amino acid, allowing the coupling reactions to go to completion, and then repooling the beads. This cycle was repeated several times to extend the peptide chain (Fig. 1*a*). In this fashion, each bead should contain only a single peptide species.

We then developed a rapid approach for screening the library to find beads containing peptides able to bind to any particular acceptor molecule. Acceptor molecules were coupled to an enzyme (alkaline phosphatase) or to fluorescein and added in soluble form to the peptide-bead library. Typically, a few beads were intensely stained and were visible to the naked eye and easily seen with a low-power dissecting microscope (bead diameter of 100-200 μm) against a background of colourless, nonreactive beads (Fig. 2). With the aid of tiny forceps coupled to a micromanipulator, the intensely staining beads could be removed for analysis (Fig. 1*b*). We washed each bead with 8M guanidine hydrochloride to remove the acceptor complex, and then determined the peptide sequence contained on the bead by placing it on a glass filter which was inserted into a peptide microsequencer (model 477A, Applied Biosystems). A library containing several million beads could be screened in 10-15 Petri dishes in an afternoon. Afterwards, it could be washed with 8M guanidine hydrochloride and subsequently reused for screening new acceptors.

Our sequencing studies established that the peptide present on any given single bead is sufficient for unambiguous sequence analysis as each bead sequenced contained 50-200 pmol of peptide (the lower limit of sensitivity of the instrument is in the range of 5 pmol). Furthermore, as measured by preview analysis[9] for several dozen individual beads from this library, the coupling reactions in the split synthesis procedure were virtually complete as most individual beads displayed peptides that were over 99% pure (range 97-100%). At least three pentapeptide beads were sequenced daily using the microsequencer.

We applied the process to produce a library of millions of pentapeptides and screen it against two well-studied acceptor molecules. Using the split synthesis approach with 19 reaction vessels, we synthesized pentapeptide libraries incorporating all the natural amino acids except for cysteine (for simplicity to eliminate disulphide crosslinking). The random incorporation of 19 amino acids into pentapeptides can produce a total of up to 2,476,099 (19^5) individual peptides of differing sequence with any one sequence represented on at least one solid-phase resin bead (of course, the number of beads of any given sequence will follow a Poisson distribution, and many multiples of this minimal number of beads are required to assure that the maximum theoretical number of possible peptide entities are approximated in the library). Libraries were readily synthesized in a few days.

We studied a monoclonal antibody against β-endorphin with high affinity (K_i = 17.5 nM) for the epitope sequence YGGFL (single-letter amino-acid code). A total of six reactive beads were retrieved from about 2 million beads screened from the pentapeptide library. One peptide ligand sequence retrieved, YGGFQ, had an affinity (K_i = 15.0 nM) nearly identical to the native epitope. Two of the other peptide ligands retrieved had K_is of less than 37 nM (Table 1). These affinities were more than 50-fold better than those obtained for the identical monoclonal antibody by Cwirla *et al.*, who used a phage library method[7].

TABLE 1 Affinity of anti-β-endorphin ligands: comparison of the natural ligand and peptide ligands identified from a large pentapeptide library

	Sequence	K_i (nM)
Native ligand	YGGFL	17.5 ± 3.2
Peptide ligand	YGGFQ	15.0 ± 1.7
	YGGFA	32.9 ± 2.0
	YGGFT	36.9 ± 7.7
	YGGLS	726 ± 134
	YGALQ	1980 ± 303
	YGGMQ	8780 ± 1500

Affinity constants for the peptide ligands determined with competitive radioligand binding assays using tritiated YGGFL as the standard.

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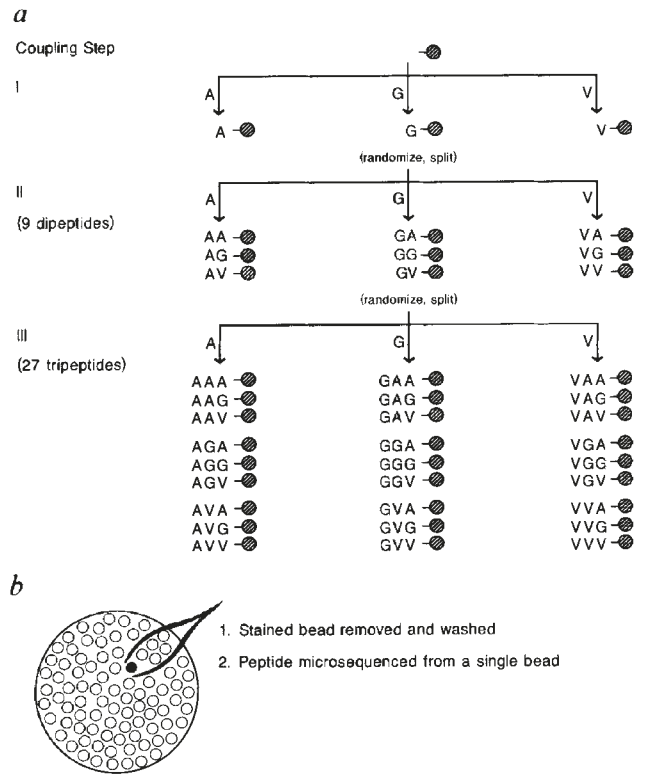

FIG. 1 Steps in peptide library synthesis and screening. *a*, Flow diagram of a simplified example of solid phase 'split synthesis' of tripeptides consisting of alanine (A), glycine (G) and valine (V) using standard solid-phase peptide synthesis methods with Fmoc or Boc chemistry. After each coupling step, the beads from each of the three reaction vessels are combined for randomization and then split again to the three vessels for the next coupling step. After three such steps the 27 possible peptide sequences (3^3) are all represented on separate beads. *b*, A single bead binding an acceptor molecule tagged with an enzyme is identified after being stained by enzymatic reaction on a dye substrate. The stained bead is physically removed from the colourless beads remaining in the library, washed free of the acceptor complex, and subjected to microsequencing. Once identified, the reactive peptide is then synthesized in larger quantities for confirmatory binding and biological studies.

We also used the same pentapeptide bead library to find peptides binding streptavidin (chosen as a receptor-like target). Of 75 reactive beads retrieved, 28 were sequenced and a triplet consensus sequence of HPQ was found in either the amino or carboxy terminus or the central portion of 23 of the recovered pentapeptides (Table 2). The five other peptide ligands sequenced contained the triplet sequence HPM in the pentapeptide. When beads containing LHPQF were synthesized, competitive binding studies established that the HPQ sequence was recognized at the same binding site on streptavidin as the native ligand biotin. These studies establish proof of concept for the process, and show that our synthetic libraries can be effectively recycled.

TABLE 2 Amino-acid sequences of individual pentapeptide beads that interacted with streptavidin

HPQFV	LHPQF	MYHPQ	WNHPM
HPQGP	FHPQG	REHPQ	WTHPM
HQPAG	GHPQN	IQHPQ	VHPMA
	THPQN	GNHPQ	MHPMA (2)
	QHPQG	TVHPQ	
	IHPQG	IGHPQ	
	GHPQG	WMHPQ	
		GAHPQ	
		PLHPQ	
		AIHPQ	
		AAHPQ	
		TPHPQ (2)	

All sequences listed above were found on single beads except for TPHPQ and MHPMA for which two beads were obtained. The first three columns list the sequences found with HPQ located in the amino terminus, central region or carboxy terminus respectively. The fourth column lists the HPM sequences retrieved.

Other investigators have attempted to develop peptide libraries for similar purposes. For example, Geysen and colleagues[3] synthesized peptides of known amino-acid sequence on plastic pegs in 96-well plates. This approach permitted the synthesis of several thousand peptides[4]. A related technique using complex instrumentation, photochemistry, and computerized inventory control reported by Fodor *et al.*[4] permitted synthesis of known arrays of at least 1,024 peptides on an individual microscope slide. Finally, Smith and colleagues pioneered the concept of using a recombinant bacteriophage incorporating random nucleic acids to produce phage displaying millions of random peptides[5]. This approach is innovative but faced with the inherent limitations of synthetic and selection biases of biological systems. Various investigators have used these techniques to identify ligands for several monoclonal antibodies and streptavidin[3-8]. On the basis of comparisons with this published information, it is clear that our process is far simpler and more rapid than any of the alternative methods and unlike these methods has identified peptide ligands with affinities virtually identical to those of the native ligands. Additionally, our approach has far greater potential for applying the richness of well-established peptide chemistry to synthesize libraries incorporating D-amino acids or unnatural amino acids as well as specific secondary structures including cyclic peptides. All of this can be accomplished without need to keep records of the synthetic products as our interest is focused just on those peptides which provide a strong interaction signal with the acceptor. On the basis of the combinatorial possibilities and chemical procedures available, it is feasible for us to develop far larger

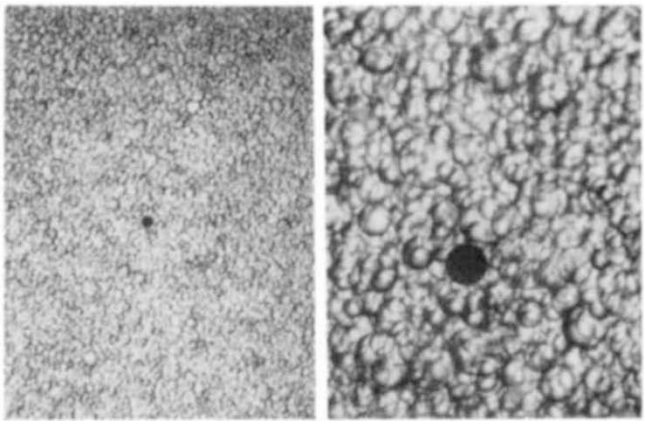

FIG. 2 Low- and high-power photomicrographs of a peptide ligand library screening in which a reactive (dark) bead stained with the alkaline phosphatase reaction can be easily identified in a background of many thousands of nonreactive (colourless) beads.

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libraries of longer or more diverse peptides should they be required for any given application.

We have expanded the applications of our peptide library approach by modifying the synthesis procedure to incorporate cleavable linkers on each bead. After exposure to the cleaving agent, such beads can then release a portion of their peptides into solution for biological assay while still retaining sufficient peptides on the beads for subsequent structure determination.

The one-bead, one-peptide concept and its applications discussed above demonstrate that this approach provides important new tools with which to search for specific ligands of potential diagnostic or therapeutic value. Such information should also enhance fundamental understanding of interactions between ligands and acceptor molecules. □

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Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery

Richard A. Houghten, Clemencia Pinilla, Sylvie E. Blondelle, Jon R. Appel, Colette T. Dooley & Julio H. Cuervo

Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, San Diego, California 92121, USA

EXISTING methods for the synthesis and screening of large numbers of peptides are limited by their inability to generate and screen the requisite number (millions) of individual peptides[1–4] and/or their inability to generate unmodified free peptides in quantities able to interact in solution[4–8]. We have circumvented these limitations by developing synthetic peptide combinatorial libraries composed of mixtures of free peptides in quantities which can be used directly in virtually all existing assay systems. The screening of these heterogeneous libraries, along with an iterative selection and synthesis process, permits the systematic identification of optimal peptide ligands. Starting with a library composed of more than 34 million hexa-peptides, we present here the precise identification of an antigenic determinant recognized by a monoclonal antibody as well as the straightforward development of new potent antimicrobial peptides.

The initial synthetic peptide combinatorial library (SPCL) prepared and used in this work consisted of six-residue peptide sequences with acetylated N terminals and amidated C terminals. The first two positions in each peptide were individually and specifically defined, whereas the last four positions consisted of equimolar mixtures of 18 of the 20 natural L-amino acids (for ease of synthesis, cysteine and tryptophan were omitted in this initial library). Such libraries can be generally represented by the sequence Ac-O_1O_2XXXX-NH_2 (where Ac represents acetyl) (see legend to Fig. 1).

Using a competitive enzyme-linked immunosorbent assay (ELISA), each of the 324 different peptide mixtures of the SPCL (Ac-O_1O_2XXXX-NH_2) was assayed to determine its ability to inhibit the interaction of a monoclonal antibody with a larger 13-residue peptide (Ac-YPYDVPDYASLRS-NH_2; single-letter amino-acid code). Of the 324 peptide mixtures examined (Fig. 1), Ac-DVXXXX-NH_2 caused the greatest inhibition of antibody binding (Table 1). Twenty new peptide mixtures were then synthesized in which the third position of the peptide mixture Ac-DVXXXX-NH_2 was defined (Ac-DVOXXX-NH_2, tryptophan now included in the X positions). Each new peptide mixture contained 6,859 (19^3) individual peptides (137,180 in total). The most effective inhibiting peptide mixture was Ac-DVPXXX-NH_2 (50% inhibitory concentration, $IC_{50} = 41\ \mu M$; Table 1*b*). The above iterative process, which reduces the number of peptide sequences by 20-fold each time it is repeated, was then carried out for the remaining three positions (Table 1, *c–e*). It should be noted that on defining the fifth position (Ac-DVPDOX-NH_2, Table 1*d*), the IC_{50} found for Ac-DVPDYX-NH_2 (0.38 μM) was at least 3,500-fold lower than any of the other 19 peptide mixtures. Also, the peptide mixtures Ac-DVPDXX-NH_2 and Ac-DVPXXX-NH_2 had IC_{50} values lower than all of the peptide mixtures with the fifth position defined, with the exception of Ac-DVPDYX-NH_2. This clearly

TABLE 1 Identification of the antigenic determinant recognized by monoclonal antibody 19B10

Peptide mixture	IC_{50} (μM)	Peptide	IC_{50} (μM)
(*a*)		(*e*)	
Ac-DVXXXX-NH_2	250	Ac-DVPDYA-NH_2	0.03
Ac-DIXXXX-NH_2	318	Ac-DVPDYS-NH_2	0.27
Ac-DMXXXX-NH_2	752	**Ac-DVPDYX-NH_2**	**0.38**
Ac-DLXXXX-NH_2	>1,400	Ac-DVPDYC-NH_2	0.90
		Ac-DVPDYV-NH_2	1.10
(*b*)		Ac-DVPDYT-NH_2	1.50
Ac-DVPXXX-NH_2	41	Ac-DVPDYG-NH_2	1.60
Ac-DVEXXX-NH_2	146	Ac-DVPDYE-NH_2	4.06
Ac-DVQXXX-NH_2	215	Ac-DVPDYI-NH_2	5.29
Ac-DVXXXX-NH_2	**250**	Ac-DVPDYM-NH_2	7.70
Ac-DVMXXX-NH_2	451	Ac-DVPDYQ-NH_2	8.18
Ac-DVRXXX-NH_2	906	Ac-DVPDYH-NH_2	8.99
Ac-DVAXXX-NH_2	1,107	Ac-DVPDYL-NH_2	9.98
Ac-DVCXXX-NH_2	>1,400	Ac-DVPDYR-NH_2	10.90
		Ac-DVPDYF-NH_2	12.02
(*c*)		Ac-DVPDYN-NH_2	15.56
Ac-DVPDXX-NH_2	4.4	Ac-DVPDYK-NH_2	17.60
Ac-DVPXXX-NH_2	**41**	Ac-DVPDYY-NH_2	22.48
Ac-DVPAXX-NH_2	>1,400	Ac-DVPDYP-NH_2	26.14
		Ac-DVPDYW-NH_2	32.14
(*d*)		Ac-DVPDYD-NH_2	48.00
Ac-DVPDYX-NH_2	0.38		
Ac-DVPDXX-NH_2	**4.4**		
Ac-DVPDAX-NH_2	>1,400		

The IC_{50}s of the most effective inhibitory peptide mixtures obtained at each iterative step are illustrated for: *a*, peptide mixtures from the initial screening of the SPCL; *b*, the third position defined (AC-DVOXXX-NH_2); *c*, the fourth position defined (Ac-DVPOXX-NH_2); *d*, the fifth position defined (Ac-DVPDOX-NH_2); and *e*, the sixth position defined (Ac-DVPDYO-NH_2). The IC_{50} of the peptide mixture derived from the previous iterative step is in bold for comparison. Peptide mixtures were assayed by competitive ELISA (see Fig. 1). The concentration of each peptide mixture necessary to inhibit 50% of the antibody binding to the control peptide on the plate was obtained by serial dilutions of the peptide mixture. The IC_{50}s were calculated using the software GRAPHPAD (ISI, San Diego). The four-step iterative screening and synthesis process takes approximately 4 weeks. This time frame will vary depending on the assay being used and the number of cases moved forward at each iterative step.

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