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A One-Bead One-Peptide Combinatorial Library Method for B-Cell Epitope Mapping

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The one-bead one-peptide combinatorial library method represents a powerful approach to the discovery of binding peptides for various macromolecular targets. It involves the synthesis of millions of peptides on beads such that each bead displays only one peptide entity. The peptide–beads that interact with a specific macromolecular target are then isolated for structure determination. We have applied this method to discovering peptide ligands for several murine monoclonal antibodies: (i) anti-β-endorphin (continuous epitope), (ii) anti-vmos peptide, (iii) anti-human insulin (discontinuous epitope), and (iv) surface immunoglobulins ($\mu\kappa$) of two murine B-cell lymphoma cell lines (antigen unknown).

Combinatorial libraries of compounds can now be generated using chemical or biological methods and probed for functional or molecular interactions with target macromolecules. This new field has been expanding rapidly in many areas of biomedical research in the last few years. This very important emerging technology has attracted enormous interest from academia and industry alike. Several reviews on the subject have been written recently (1–5). Currently, there are four general approaches for preparing and screening huge random combinatorial peptide libraries (i.e., 10^6–10^8 entities): (i) the biologic peptide library method using filamentous phage (6–9), plasmids (10), or polysomes (11); (ii) the combinatorial library methods requiring deconvolution: an iterative approach (12–16), positional scanning (17), or orthogonal approach (17a); (iii) affinity column selection method (18); and (iv) the combinatorial library method based on the "one-bead one-peptide" concept, or the "Selectide process" (19–36). This latter method will be the main subject of this paper.

We recognized that by using a "split synthesis" method on solid-phase peptide synthesis, each individual peptide–bead from the bead library will display only one peptide entity (19, 20). With an appropriate detection scheme, the peptide–bead that interacts with a specific macromolecular target can be identified and isolated, and the peptide structure can be determined by microsequencing (19, 20). In the last few years, we have successfully applied this combinatorial library method in the identification of ligands for various targets: streptavidin (20, 21), avidin (21), monoclonal antibodies (19, 20, 22, 23), gp IIb/IIIa integrin (24), MHC-class I molecules (32), protein kinases (31, 35), proteases (unpublished result), and even a small organic dye molecule (30). In this paper, we shall focus on the methodology, the underlying principle, and the application of this method in the study of B-cell epitopes.

METHODS

Materials

TentaGel S (Rapp Polymere, Tubingen, Germany) (37) or Pepsyn gel (Milligen Inc., San Leandro, CA) were used for the synthesis of the peptide–bead library. Fmoc amino acids, with standard side-chain protecting groups, were obtained from Bachem (Torrance, CA), Advanced ChemTech (Louisville, KY), or Propeptide (Vert-le-Petit, France). Benzotriazolyl-oxy-trisdimethylamino-phosphonium hexafluorophosphate (BOP), diisopropylethylamine (DIEA), diisopropyl carbodiimide (DIC), *N*-hydrobenzotriazole (HOBt), and piperidine were obtained from Advanced ChemTech. 2-Bromo-3-chloro-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were obtained from Ameresco (Solon, OH). Anti-β-endorphin monoclonal

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antibody (MAb) (Clone 3-E7) was obtained from Boehringer Mannheim (Indianapolis, IN). Dimethylsulfoxide (DMSO) was purchased from Sigma Chemical Co. (St. Louis, MO).

Peptide–Bead Library Synthesis

As mentioned earlier, a "split synthesis" method (19, 20, 38) was used for the preparation of the peptide–bead library. The basic scheme of the split synthesis method is shown in Fig. 1. Standard Fmoc chemistry was used in the solid-phase peptide synthesis (39, 40). The resins used were polyethyleneglycol-grafted polystyrene resins (TentaGel S) or polydimethylacrylamide resin (Pepsyn gel). Other resins that are compatible with peptide synthesis (organic solvents) and screening under aqueous conditions may also be used. Since TentaGel S already has a polyethyleneglycol linker, an additional linker may not be needed. In some experiments, an additional linker with β-alanine-ϵ-aminocaproic acid-β-alanine-ϵ-aminocaproic acid was added using Fmoc chemistry prior to the synthesis of the peptide library. As for the polydimethylacrylamide resin, the resin was first mixed with ethylenediamine overnight. The resin was then thoroughly washed, and ϵ-aminocaproic acid followed by β-alanine was coupled to the resin using Fmoc chemistry. The linkers in both cases were noncleavable. In our standard peptide library synthesis, the resins were distributed evenly into 19 polypropylene vials. Nineteen Fmoc-protected eukaryotic amino acids (all but cysteine) were then added separately into each of the resin aliquots. Cysteine was omitted to avoid inter- or intra-molecular crosslinking. Threefold excess of amino acids and threefold excess of each of BOP, DIEA, and HOBt were added to initiate the coupling reaction. In some experiments, HOBt and DIC were used instead. In this case, a trace amount of bromophenol blue was added into the reaction mixture to monitor the progress of the coupling reaction (41). The vials were tightly capped and mixed gently for 30 min to 1 h at room temperature until all beads turned from blue to colorless. The ninhydrin test (42) was then used to confirm the completion of the coupling reaction. For the vials in which coupling reactions were incomplete, the supernatant was gently removed, fresh Fmoc amino acid, followed by BOP, DIEA and HOBt, was added, and the reaction was allowed to proceed for another hour. In general, double coupling was needed only on rare occasions. All the resin aliquots were then transferred to a siliconized cylindrical glass vessel fitted with a frit at the bottom. Dried N_2 was bubbled through the frit to mix the resin. After washing 8× with dimethylformamide (DMF), 20% piperidine (in DMF) was added. Ten minutes later, piperidine was removed and the resins were washed 10 times with DMF. The resins were then divided into 19 aliquots for another cycle of coupling. Although we have synthe-

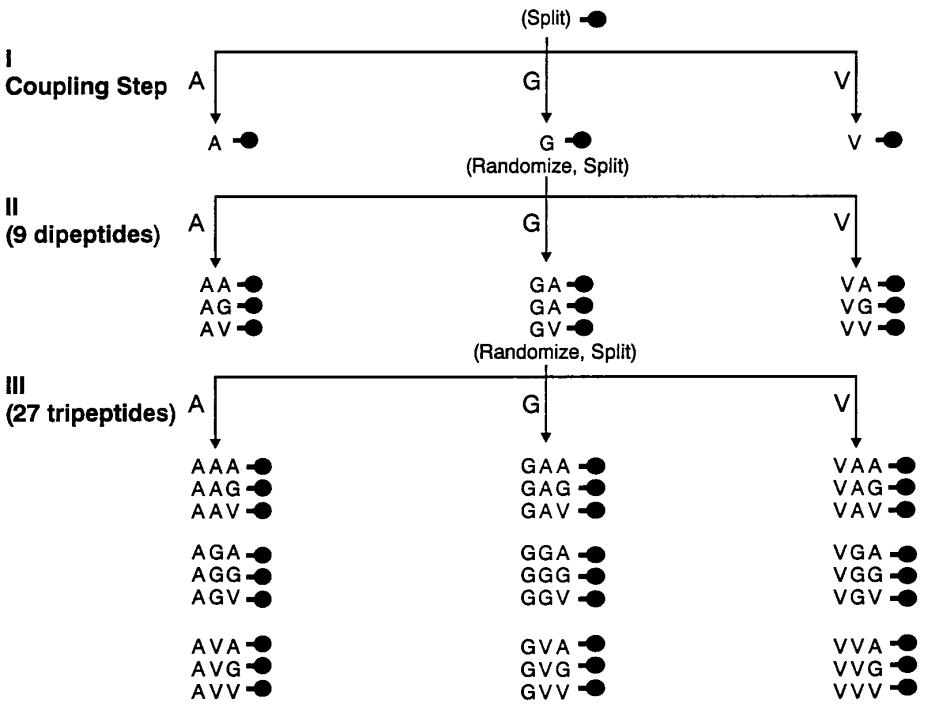

FIG. 1. Basic scheme of the "split synthesis" method. (Adapted with permission from *Nature* 354, 82. Copyright 1991 MacMillan Magazines Limited.)

sized and screened peptide libraries up to 18 amino acids long, most of our peptide libraries were less than 10 amino acids long. After all the randomization steps were completed, the *N*-α-Fmoc group was removed with 20% piperidine, and the side-chain protecting groups were removed with reagent K (TFA:phenol:water:thiophenol:ethanedithiol, 82:5:5:5:2.5, v/w/v/w/v) (43) or a mixture of TFA:phenol:anisole:ethanedithiol (94:2:2:2, v/w/v/v). The deprotected peptide–resin was then washed thoroughly with DMF, neutralized with 10% DIEA (in DMF), thoroughly washed again, and stored in 0.03% HCl in H_2O at 4°C. In some experiments, cysteine was added to the amino and carboxyl termini of the random peptide library. Cyclization of these cysteine-containing peptides via disulfide bond formation was accomplished by incubating the deprotected library with TFA:DMSO:anisole (85:10:5, v/v/v) at room temperature with gentle mixing for 24 h.

Library Screening

Using the split synthesis method described above, each individual resin–bead displays only one peptide entity: the one-bead one-peptide concept (see below). There are several methods to detect individual positive beads that interact with specific target macromolecules. The target macromolecule can be directly tagged with an enzyme (e.g., alkaline phosphatase or horseradish peroxidase), a fluorescent probe (e.g., rhodamine or fluorescein), or a radionuclide (e.g., ^{125}I or ^{32}P). Alternatively, a two-step process using the biotin/streptavidin or primary/secondary antibody system could be incorporated into the detection scheme. We have found that the enzyme-linked colorimetric detection scheme is the most convenient method, is quick, and does not require any complicated instruments such as the fluorescent activated cell sorter. Details of the method for enzyme-linked detection have been detailed elsewhere (27) and are outlined below.

The peptide–bead library was thoroughly washed with double-distilled water to remove all the DMF. Gelatin (0.1% w/v) was then used to block any non-specific binding. In some experiments, bovine serum albumin was used as a blocking agent. The beads were then mixed with the antibody–alkaline phosphatase complex in PBS with 0.1% gelatin and 0.1% Tween 20 (Buffer A) with gentle mixing. After 1 to 24 h, the beads were thoroughly washed with PBS and 0.1% Tween 20 (Buffer B), followed by TBS (Tris-buffered saline: 8 g NaCl, 0.2 g KCl, and 3 g Tris–base in 1 liter water, pH 8.0). BCIP, a standard substrate in alkaline phosphatase buffer (5.85 g NaCl, 12.1 g Tris–base, 0.476 g $MgCl_2$ in 1 liter water, pH 8.5), was then added. The bead suspension was then transferred to 10–20 polystyrene petri dishes (100 × 20 mm). No further mixing was needed. The reaction was allowed to proceed for up to 2 h. The reacting beads turned turquoise, while the majority of the beads in the library remained colorless.

Under a dissecting microscope, the positive turquoise beads were isolated with a micropipette, washed with double-distilled water, immersed in 8 M guanidine hydrochloride, pH 2.0, for 20 min, washed with double-distilled water again, and destained with DMF. In some experiments, beads were recycled and restained with the above protocol but in the presence of a competing antigen. Colorless beads were selected, treated with guanidine hydrochloride, and restained in the absence of competing ligand. Stained beads (true positive) were then isolated for microsequencing.

Structure Determination of Isolated Peptide

Positive peptide–beads were isolated, placed on a glass filter, and inserted into the sample cartridge of the protein sequencer for sequence analysis by Edman degradation. We have used the ABI 477A protein sequencer (Applied Biosystems (Perkin–Elmer), Foster City, CA) and the Porton PI 3010 instrument (Porton Instruments (Beckman), Tarzana, CA), both with satisfactory results.

Determination of Binding Affinities

Peptide ligand binding affinities for the anti-β-endorphin monoclonal antibody were determined in solution phase using peptide samples produced by standard solid-phase synthesis. The anti-β-endorphin binding assay measured peptide ligand inhibition of 5.0 nM [^{3}H][Leu^5]enkephalin, YGGFL (specific activity: 39.0 Ci/mmol, New England Nuclear, Boston, MA), binding to 125–200 ng/ml anti-β-endorphin MAb in 1.0 ml of 40 mM Tris–HCl, 150 mM NaCl, pH 7.4, buffer containing 1.0 mg/ml bovine serum albumin, 0.1% (v/v) Tween 20, and 0.05% (w/v) sodium azide. Specific binding was defined as the difference between binding measured in the presence or in the absence of 1.0 μM unlabeled [Leu^5]enkephalin. Bound radioligand was precipitated by the addition of a 10-fold excess of protein-G–Sepharose (Pharmacia) followed by an overnight incubation (23–24°C). The protein-G–Sepharose was collected by centrifugation (13,000*g* for 5 min), and the pellets were suspended in 250 μl 5% (v/v) acetic acid before transfer to vials for liquid scintillation counting. Binding data for saturation and inhibition studies were analyzed by weighted nonlinear regression methods using appropriate one-site models reported by Knapp *et al.* (44). K_i values for inhibition binding constants were calculated using the method of Cheng and Prusoff (45).

Determination of peptide ligand affinities for the anti-vmos monoclonal antibody was performed with general methodology as described above. The anti-vmos binding assay measured competitive ligand inhibition of 20 nM [^{3}H]*N*-acetyl-vmos peptide (see below) binding to 10 μg/ml anti-vmos monoclonal antibody in 1.0 ml

of phosphate-buffered saline containing 0.1% (w/v) gelatin, 0.1% (v/v) Tween 20, and 0.05% sodium azide. Nonspecific binding was measured in the presence of 100 μM unlabeled vmos peptide. [^{3}H]*N*-acetyl-vmos peptide was prepared by N-terminal acetylation of vmos peptide with [^{3}H]sodium acetate (specific activity: 2.52 Ci/mmol, New England Nuclear) and BOP and purified by reverse-phase high-performance liquid chromatography. The product coeluted with authentic acetyl-vmos peptide and was found to have a specific activity of 2.50 Ci/mmol by measurement of the ratio of UV absorbance (OD_{280}) to the amount of tritium incorporated. The binding affinity of the radioligand was determined by saturation analysis using five concentrations of [^{3}H]*N*-acetyl-vmos peptide that gave an average kDa value of 0.848 $\pm$ 0.156 μM for five measurements.

RESULTS

Anti-β-endorphin MAb

Table 1 summarizes the ligands isolated from four pentapeptide libraries using an anti-β-endorphin MAb (Clone 3-E7) as a probe (20, 22, 23). This antibody recognizes the amino terminus of β-endorphin, a 4-amino-acid linear epitope YGGF. Almost all the ligands isolated from an all L-amino acid pentapeptide library have a motif related to YGGF. Of the 45 ligands isolated, 41 have an N-terminal tyrosine. Only 4 ligands have a tyrosine in the second position. An aromatic residue (phenylalanine or tryptophan) is preferred in either position 4 or position 5. When an LDLDL-amino acid pentapeptide library was screened, two related motifs emerged: YG_f_ and Y_ _F. In addition, two related discrete sequences were isolated: YGFGL and IyGGF. A third motif, Ya(N/Q)aW, was also apparent. This latter motif is interesting as it is different from those isolated from an all L-amino acid pentapeptide library. A DLDLD-amino acid library was also screened and the identified motif was yGGF, again very similar to YGGF except that the N-terminal tyrosine is a D-enantiomer. When an all D-amino acid pentapeptide library was screened, only one ligand was identified: wtGGy. This is interesting because it appears that it is a retro-inverso of YGGTW, a plausible L-ligand for anti-β-endorphin MAb. The binding affinities of some of these ligands were determined. The YGGF motif has the highest binding affinity and is in the low nanomolar range (20). The binding affinity of many of the remaining ligands, on the other hand, is at least one to two orders of magnitude weaker than that of the YGGF motif (23).

Anti-vmos Peptide MAb/Library with Deleted Amino Acids

In a second set of experiments another antibody system was used wherein the epitope was located in the middle of the peptide chain rather than at its N-terminus (as in the case of the β-endorphin). The antibody used was an anti-vmos peptide MAb. A hybridoma line

TABLE 1

Peptide Ligands for Anti-β-endorphin MAb (Clone 3-E7)

Library	Motif	Ligands
XXXXX	YGGF_	YGGFL, YGGFI, YGGFQ, YGGFT, YGGFA
	YGG_ _	YGGMV, YGGLS
	YGAF_	YGAFM, YGAFQ, YGAFF, YGAFT
	YGA_ _	YGALQ, YGALT, YGAWD
	YG_F_	YGNFF, YGVFA, YGVFI, YGQFV, YGVFQ, YGVFE, YGFFQ, YGWF, YGWFH, YGWFQ
	YG_W_	YGWWM, YGYWQ, YGYWQ
	YG_ _F	YGEAF, YGHAF, YGGGF, YGMGF, YGLGF, YGPGF
	Y_GGF	YQGGF, YLGGF, YKGGF
	_YGGF	LYGGF, NYGGF, MYGGF,
		RYGLL
XxXxX	YG_f_	YGGfM, YGAfW, YGAfF
	Y_ _ _F	YqGGF, YGAaF, YGFGF, YGGGF
	Ya(N/Q)aW	YaNaW, YaQaW
		YGFGL
		IyGGF
xXxXx	yGGF_	yGGFa, yGGFv
xxxxx		wtGGy

Note. X, all 18 L-amino acids plus glycine, no cysteine. x, all 18 D-amino acids plus glycine, no cysteine.

was used as a source from which anti-vmos MAb was purified (No. 165-28E7, SCRF 354, Lot No. 165-119 was obtained from Microbiological Associates, Bethesda, MD). The MAb was produced by immunizing mice with the 12-amino-acid peptide LGSGGFGSVYKA, which corresponds to residues 100 to 111 of the vmos oncogene product. The peptide was conjugated to a carrier protein prior to immunization. In ELISA testing, the anti-vmos MAb detects homologous sequences of vmos, MOS, neu/HER-1, and HER-2 gene products. The epitope within the 12-amino-acid vmos peptide recognized by the anti-vmos MAb was mapped by using a commercially available multipin epitope mapping kit first described by Geysen *et al.* (46) (Cambridge Research, Biochemical, Boston). Overlapping peptide sets (tetrapeptides, pentapeptides, and hexapeptides) were used in this analysis. Using this method, the pentapeptide sequence FGSVY (i.e., residues 6–10 of the vmos dodecapeptide) was determined to be the epitope.

In our experiment, a restricted random library was used in which the amino acids valine and serine in the vmos epitope were purposely omitted. This restricted random library has the following composition: GXXXXX-β-alanine-aminocaproic acid-ethylenediamine-Pepsyn gel, wherein X is E, P, N, F, H, T, K, L, G, Y, A, M, R, or W. These 14 amino acids were carefully chosen so that despite the absence of valine and serine, all the chemically different side-chain functionalities found in proteins were included: (i) asparagine was selected but not glutamine; (ii) glutamic acid was selected but not aspartic acid; (iii) threonine was selected but not serine; (iv) leucine was selected but not isolencine or valine; and (v) methionine was selected but not cysteine. Since 14 amino acids were available for each of the five random coupling steps, the maximum number of peptides synthesized was 537,824 (14^5).

Approximately 230,000 beads were screened with an anti-vmos alkaline phosphatase conjugate (i.e., less than 43% of the possible permutations were examined).

TABLE 2

Peptide Ligands for Anti-vmos MAb, Isolated from an Incomplete Library

GRRGME	GRYAKH
GRRPYG	GRYMPK
GRRAYE	GFRHMA
GRREGP	GFRYHN
GRKTYY	GHRYFH
	GWREKE

Note. The general formula for the peptide library used in this study is GXXXXX-β-ϵ-ethylene diamine-Pepsyn gel, where X is E, P, N, F, H, T, K, L, G, Y, A, M, R, or N; β is β-alanine; and ϵ is ϵ-aminocaproic acid.

After incubation with the substrate (in this case BCIP plus NBT), 50 of the beads stained intensely blue. Twenty-four of these beads were physically removed, and the amino acid sequences for 11 of them were determined.

The anti-vmos ligand sequencing results are shown in Table 2. Since both valine and serine were purposely excluded in this peptide library, it is not surprising that none of the 11 peptide ligand sequences identified resemble the native epitope (FGSVY). Although there were no repeats in the 11 peptide ligands, their sequences were nonrandom. Arginine and tyrosine occur frequently in these sequences. Furthermore, at least one and sometimes two arginines were present at the second and/or third position of each of these ligands.

Some of the positive ligands were synthesized, and their affinity for the anti-vmos MAb were determined with solution-phase binding studies (Table 3). β-alanine amide was added to the C-terminus of some of the ligands to simulate the ligand to which the antibody binds on the bead. The affinity of the best anti-vmos peptide ligand identified was 2.5-fold less than that of the native peptide (Table 3). Although none of the peptide ligands tested have a K_i value as low as that of the native vmos ligand, the results clearly demonstrate that by using a random library lacking some of the amino acids present in the native epitope, a series of structurally quite different peptide ligands of various affinity for the anti-vmos MAb can still be identified.

Anti-insulin MAb (Discontinuous Epitope)

Table 4 summarizes the data obtained from screening four linear all L-amino acid peptide libraries using anti-insulin MAb (clone AE906) as a probe. This mu-

TABLE 3

Binding Affinity of Peptide Ligands for Anti-vmos MAb

Peptide	K_i, μM
A	
LGSGGFGSVYKA-NH_2 (vmos peptide)	3.2 ± 0.4
GFGSVY-NH_2	246–337
GFGSVY-OH	>1000
GFGSVY-β-NH_2	409–442
GFGSVY-β-OH	529–770
B	
GRRAYE-OH	6.79 ± 2.31
GRRAYE-NH_2	24.70 ± 7.00
GRRAYE-β-OH	15.10 ± 0.50
GRRAYE-β-NH_2	9.02–20.40
GRRGME-OH	>100
GRRGME-β-NH_2	24.00 ± 8.40
GRREGP-β-NH_2	26.90 ± 6.20
GRRPYG-OH	>1000
GRRPYG-β-NH_2	20.50 ± 4.50

rine antibody has an IgG_1 isotype, and it recognizes a discontinuous epitope on human or porcine insulin, with a binding constant of approximately 0.01 μM. The general formula for the libraries used in these experiments was $(X)_n$-β-ϵ-β-ϵ-TentaGel S. The peptide was attached to the resin via a β-alanine-ϵ-aminocaproic acid-β-alanine-ϵ-aminocaproic acid linker (β-ϵ-β-ϵ). When a pentapeptide library was screened, two distinct motifs emerged: FDW_ _ (or FNW_ _) and _QDPR (or _QNPR). When a hexapeptide library was screened, an additional motif was identified: _W_ _GF. In addition, a discrete sequence SQHGIW was detected. Similar to the hexapeptide library, the predominant motifs identified from both the octapeptide and the decapeptide libraries were _ _ _W_ _GF and _ _ _ _ _W_ _GF, respectively.

In addition to linear peptide libraries, libraries with secondary structures were designed and screened. The results are shown in Tables 5 and 6. The peptide libraries shown in Table 5 are cyclic peptide libraries having the following general formula: C-$(X)_n$-C-β-ϵ-β-ϵ-TentaGel S. Again, the peptide was attached to the TentaGel via a β-ϵ-β-ϵ linker. The peptide was cyclized by oxidation of the flanking cysteines to form a disulfide bond. The three distinct motifs isolated are highlighted in Table 5: CWD_GFG_C from the hepta-, C_ _ _ _HGVQC from the octa-, and CQDI_Y_ _ _ _C from the nonapeptide libraries. Interestingly, except for the CWD_GFG_C motif, which is related to the _W_ _GF motif identified from the linear hexapeptide library, both the C_ _ _ _HGVQC and the CQDI_Y_ _ _ _C motifs are new and were not isolated from screening linear peptide libraries. In addition there are several other discrete sequences identified from these screens. One sequence, CKFDWMAGGC, isolated from the octapeptide library, is interesting as it is very similar to the FDW_ _ motif identified from a linear pentapeptide library.

Table 6 summarizes the data obtained from screening other constrained peptide libraries, namely, the "turn libraries." In this case, a D-proline is added in the middle of the peptide to promote a turn. In one case, the i + 2 position has a random D- instead of an L-amino acid. The predominant motif identified from these screens was _FQp_ _PG, whether the i + 2 position is a D- or an L-enantiomer. In addition, two discrete sequences that do not fit this motif were isolated.

Table 7 shows the results of screening two all D-amino acid hexa- and octapeptide libraries. Both of these libraries used glycine, instead of β-alanine-ϵ-aminocaproic acid-β-alanine-ϵ-aminocaproic acid, as the linker. All the ligands identified from the hexapeptide library line up perfectly with the following motif: _q_Gs(t)G. Threonine is very much preferred in the sixth position. Similarly, the ligands identified from an octapeptide library have the same motif: q_Gs_G. However, the alignment of the amino acids of these octapeptides is not perfect.

Unlike some of the ligands identified from the anti-β-endorphin MAb screen, the binding affinity of the ligands identified from the primary screen against anti-insulin MAb is relatively weak ($\sim$10 μM). Perhaps this is because the anti-insulin MAb recognizes a discontinuous epitope. In order to improve the binding affinities of the ligands to the antibody, a sequential screening approach using secondary and tertiary li-

TABLE 4

Peptide Ligands for Anti-insulin MAb

5-mer[a]	6-mer	8-mer	10-mer
FNWAI	KWGSGF	GSWWAQGF	TGQDFWAMGF
FNWKR	NWGHGF		
FNWER	DWGYGF		EQGAWWEVGF
YFNWA	RWDLGF	EQAWHIGF	EDQLIWLTGF
	RWAHGF	DELWGQGF	IIGHEWRYGF
FDWKR		FHKWESGF	GDWELWSKGF
FDWAH	FDWSQC	HQNWGSGF	
NFDWK	QFDWYQ	MNYWKEGF	PQQSNYYSGF
		NLVWSMGF	GSAVQFTHGF
KQNPR	FNWAVG		
FQDPR		GNGDDQGF	
RQDPR	KKQDPR	FEGYQYGF	
PQDPR		QGQYTFGF	
	SQHGIW		
		FDWSNGGG	
		KQTHLSWM	
		TNEWFAGK	
		RQDYARVM	
		NGQTWATG	

[a] The general formula for the peptide libraries used in this study is $(X)_n$-β-ϵ-β-ϵ-TentaGel S, where X is all 18 L-amino acids plus glycine, no cysteine.

TABLE 5

Cyclic Peptide Ligands for Anti-insulin MAb

CXXXXXXXC[a]	CXXXXXXXXC	CXXXXXXXXXC
CWDKGFGYC	CFLIMHGVQC	CQDISYQSNLC
CWDMGFGIC	CVANQHGVQC	CQDIAYENKMC
	CGWARHGVQC	
CWGQGFSAC	CHENEHGVQC	CVSQHDFPLQ
	CKFDWMAGGC	CRQSTWGSTPC
CQVGNHWNC		CAHQQDYKYGC
	CMYFFQRGEC	CYDTGFGHHKC
	CTFQWGTGGC	
	CLYHRHGIQC	
	CAQTERGNEC	

[a] The general formula for the peptide libraries used in this study is C$(X)_n$C-β-ϵ-β-ϵ-TentaGel S, where C is cysteine and X is all 18 L-amino acids plus glycine, no cysteine.

braries was designed and tested. The results are shown in Fig. 2. As indicated earlier, a _W__GF motif was identified during the primary screen of a hexapeptide library. Based on this primary motif, a secondary library with the structure XXXWXXGF was synthesized and screened under a higher stringency. Two motifs were readily identified: Q_IWG_GF and ___WKYGF. Based on these two motifs, two tertiary libraries were synthesized and screened under an even higher stringency: XXQXIWGXGF and XXXXWKYGF. The final motifs identified from these two tertiary screens were S(R/K)Q_IWG_GF and NH__WKYGF. The binding affinities of these final ligands are approximately one to two orders of magnitude better than those identified from the initial primary screen (47).

Idiotype-Specific Peptides for B-Cell Lymphoma

We have recently reported the application of the Selectide process to identify peptide ligands for the surface immunoglobulins (idiotypes, μ, κ) of two murine lymphoma cell lines (WEHI-279 and WEHI-231) (36). The result is shown in Table 8. Both L- and D-amino acid peptide libraries were screened. For the WEHI-279 idiotypes, only one predominant motif was identified from the L-amino acid peptide library screen: RW(I/F)D_. On the other hand, at least two distinct motifs (Gr___w__ and _t_Gm_k_) and four other discrete sequences were identified when an all D-amino acid octapeptide library was screened. Similarly, for the WEHI-231 idiotype, one predominant motif (_WY_) was isolated from the L-amino acid peptide libraries, and three distinct motifs were identified from the D-amino acid octapeptide library screen (lw__pew(i/v), kwrGp_w, and wGey(i/v)_v).

DISCUSSION

One-Bead One-Peptide Combinatorial Library

As illustrated in this paper, the one-bead one-peptide combinatorial library method has been proven to be a valuable tool for B-cell epitope mapping. During the split synthesis, the resin–beads are first divided into several aliquots, and individual amino acids are then added separately into each of the resin aliquots and the reaction is driven to completion with excess amino acids. The resins are then mixed, washed, Nα-Fmoc deprotected, washed, and divided into several aliquots again for the next coupling. We recognized that with this synthetic strategy, millions of peptide–beads can be synthesized rapidly, and at the end of the library synthesis, each bead displays only one peptide entity. Since each 120-μm bead contains approximately 100 pmol peptide (or $>10^{13}$ copies of the same peptide), there is more than enough peptide in each bead for structure determination. Typically, a pentapeptide library of 10 million beads can be synthesized and deprotected in 2–3 days. Screening usually takes 1–2 days. The rate-limiting step of this process is microsequencing, as only 3–4 peptides (6- to 8-mers) can be microsequenced each day. Alternatively, using a capping process after each cycle of synthesis, it is possible to determine the entire peptide sequence with mass spectrometry at a faster rate (48, 49). Since the rate-limiting step is microsequencing, it is extremely important to eliminate any false positive beads. This could be accomplished by using the screening method described in this paper or by using a dual-color screening approach (34).

The Selectide process is a parallel approach in which millions of peptides are screened concurrently, and often multiple distinct motifs can be identified with a one-step screening process. This is clearly shown in the anti-insulin MAb and lymphoma surface idiotype results. In contrast, the iterative approach as pioneered by Geysen (12, 13) and Houghten (14) is a convergent approach in which multiple steps of synthesis and anal-

TABLE 6

Peptide Ligands with "Turn Structure" for Anti-insulin MAb

XXXpXXXXG[a]	XXXXXpXXXXG	XXXpxXXXG
GFQpMVPEG	NNSFQpQRPVG	RFQpqRPG
RFQpFAEPG	HDRFQpGRPRG	VFQppIPM
RFQpNIPDG		YFQpsRPL
	GFASHpVQFSG	
	RNDYWpMWDTG	

[a] The general formula for the peptide libraries used in this study is $(X)_n p(X)_m$G-TentaGel S, where X is all 18 amino acids plus glycine, no cysteine; p is D-proline; and G is glycine.

TABLE 7

D-Amino Acid Peptide Ligands for Anti-insulin MAb

xxxxxxG[a]	xxxxxxxxG
pqrGstG	dqlGslGGG
pqiGstG	GWqpGsmG
pqGGstG	asqaGsfG
yqeGstG	fkiqGGsqG
vqeGstG	mnrqfGssG
vqmGstG	psqqGGstG
vqGGstG	mqfqmGssG
rqlGstG	tsyqaGstG
rqlGsvG	rlfqmGttG
aqmGsiG	
Motif: _q_Gs(t)G	Motif: _q_Gs_G

[a] The general formula for the peptide libraries used in this study is $(x)_n$G-TentaGel S, where x is all 18 D-amino acids plus glycine, no cysteine.

ysis are required for the arrival of a single solution (or motif). The positional scanning approach developed by Houghten *et al.* (17, 18) is useful but only when there is one predominant solution. If there are several distinct motifs, as in the case of the D-amino acid peptide libraries for the lymphoma idiotypes (Table 8), the positional scanning approach will likely yield uninterpretable results.

Solution-Phase Releasable Assay

In addition to the on-bead binding assay as described in detail in this article, we have screened the peptide–bead library with a solution-phase releasable assay (50). This assay involves a two-stage sequential release and testing system using 96-well microtiter plates. Each peptide–bead has three linkers; one is noncleavable (reserved for subsequent microsequencing) and the remaining two can be cleaved orthogonally and sequentially under very mild conditions. In the first-stage release and testing, about 500 peptide–beads are pipetted into each well (so that each microplate contains approximately 48,000 unique peptide–beads). Upon neutralization, the first linker is cleaved (diketopiperazine formation), and peptide is released for solution-phase assays such as competitive ELISA or competitive RIA. Approximately 100 pmol peptide is released, and in an assay volume of 100 μl, the final concentration of each peptide is about 1 μM. For the second-stage release and testing, positive wells from the first-stage biological testing are identified, and their beads are recovered for use in the second-stage testing after redistribution of the beads with just one bead per well. For peptide distribution in both stages, beads are transferred into the upper chambers of a vacuum control 96-well filtration manifold (Model 09601, Millipore Corp., South San Francisco, CA), and the peptide filtrates are collected in a 96-well plate underneath the manifold. Cleavage of the second linker (alkylester bond) is accomplished by addition of 0.2 M NaOH followed by neutralization, or alternatively, the second-stage release can be achieved by overnight incubation in ammonia vapors. After the second-stage bioassay, positive wells from the test plate are identified and referenced back to the individual bead of origin, which is then recovered from the master plate for sequencing. We have successfully used this method to identify pep-

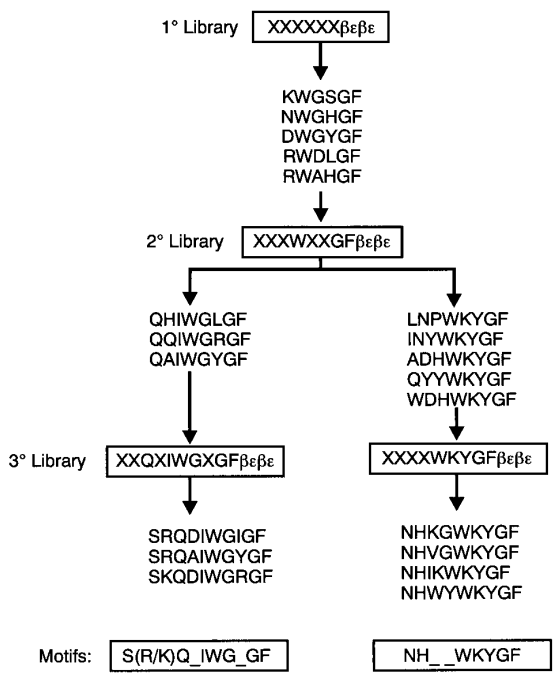

FIG. 2. Sequential screening approach with secondary and tertiary peptide libraries to improve the binding affinities of the primary leads for anti-insulin MAb.

TABLE 8

Peptide Motifs for Surface Immunoglobulins ($\mu\kappa$) of Two Murine Lymphoma Cell Lines

Library[a]	WEHI-279	WEHI-231
A		
L-amino acid peptide ligands		
7-mer	RWIDSAM	FWYDEPK
	RWIDQVT	DLWYDAV
	RWIDPQP	
	RWFDALP	
	RWFDEEH	
9-mer	RWFDTMDIA	DMWYDDPYL
	RWFDKKMEG	DGWYYPRQH
	RWFDAVTQV	YWYDDKDES
		RNLGMWYTP
	IRWFESPSA	
11-mer	RWFDAVPLGQD	RYAHLSEWYDD
	RWFDLMAAGQR	TRHNDWYTPDD
	RWFDMQLGPEI	TGWYVPKSIDN
	LRWFESSKGDF	DLRGWYTPIVE
		NWFQDEWYIPD
		DDAGWYVDYKK
B		
D-amino acid peptide ligands		
8-mer	iGrfaswl	lwqypewi
	aGrfGswm	lwkmpewv
	Grlmdweq	
	nltaGmtk	dkwrGpiw
	etwGmwkr	kwrGpawG
	alGmGmvr	
	yfvnwpdl	wGeyidvk
	fsmrqhdt	wGeyvmvn
	lftweke	

[a] The general formula for the peptide libraries used in this study is: $(X)_n$-TentaGel S and $(x)_8$G-TentaGel S for A and B, respectively.

tide ligands for the anti-β-endorphin monoclonal antibody and gpIIb/IIIa integrin (50).

Anti-β-endorphin MAb

Our initial work on the anti-β-endorphin MAb was straightforward, as it recognizes a short linear motif YGGF. High-affinity ligands (K_d at low nanomolar range) were readily identified in our initial screen. Since the antibody used for screening was bivalent, even low-affinity ligands were detected (Table 1). Many of the low-affinity ligands isolated show considerable similarity to the parent epitope. However, for high-affinity binding, YGGF is required. The all D-amino acid ligand, wtGGY ($K_d > 100\ \mu M$), is interesting as it resembles a retro-inverso structure of YGGTW. In general, we determine the structure of the isolated ligands by microsequencing one individual peptide–bead. However, for the anti-β-endorphin ligands, in addition to microsequencing individual single beads, we have microsequenced up to approximately 90 beads concurrently and nevertheless were able to extract the binding motif of YG_F (27). This is feasible because there is only one predominant motif. However, if there were multiple motifs (e.g., the pentapeptide library screen for anti-insulin MAb; Table 4), the data will be ambiguous and often uninterpretable when multiple beads are sequenced concurrently.

Anti-vmos Peptide MAb

In the case of the anti-vmos MAb, a peptide library omitting two of the amino acids in the natural epitope was used. We nevertheless discovered a linear epitope of totally different sequences with affinity 50-fold greater than the hexapeptide epitope and approximately equal in affinity to the full length 12-amino-acid peptide antigen. The binding affinity of the anti-vmos peptide was dependent on (i) the presence or absence of a β-alanine linker at the carboxyl termini and (ii) whether it is in a carboxylamide or carboxylic acid form. For example, the binding affinity of the carboxylic acid form of GRRAYE is 4-fold better than that of its carboxylamide form. In contrast, the carboxylic acid forms of GRRGME and GRRPYG was detrimental to the binding affinities of these two peptides (Table 3). This clearly demonstrates the complexity as well as the versatility of macromolecular peptide interaction.

Anti-insulin MAb

We have extensively tested the one-bead one-peptide combinatorial library method using anti-insulin MAb as a model system. This antibody was chosen because it is readily available and it recognizes a discontinuous epitope. The result of these studies is shown in Tables 4 to 7 and Fig. 2. Excess insulin can compete for the binding of all the peptide ligands to anti-insulin MAb, suggesting that all these ligands bind to the antigen combining site. Unlike the anti-β-endorphin MAb system, in which only one predominant motif was identified, the anti-insulin MAb system generated multiple distinct motifs depending on the length of the libraries, whether it was an all D-amino acid or L-amino acid peptide library, and whether there was a built-in secondary structure. For example, the _W__GF motif was not identified in a 5-mer library and was only identified in a $\geqslant$6-mer library, and C_ _ _ _HGVQC was identified in a $C(X)_8C$, but not a $C(X)_7C$ library.

Although there are multiple motifs for anti-insulin MAb identified from the L-amino acid peptide library, only one predominant motif was isolated from the D-amino acid library: _q_Gs(t)G. The fact that there was always at least one amino acid at the amino-terminal side of q_Gs(t)G suggests that this first residue, although variable, is extremely important.

As indicated earlier, the binding affinity of ligands isolated from the primary screen against anti-insulin MAb was weak. Perhaps this is because this antibody recognizes a discontinuous epitope that occupies a larger space than a short peptide does. However, with the sequential screening approach using secondary and tertiary libraries, we have been successful in identifying higher affinity ligands for anti-insulin MAb (Fig. 2). Another approach for optimization of initial leads, particularly if no clear-cut motif can be identified from the primary screen, is to screen "homolog secondary library." In a "homolog library," no specific amino acid is fixed and every position is randomized, but the synthetic scheme is biased toward a specific sequence or sets of sequences of the initial leads (47). This approach is particularly valuable for rapid optimization of all the residues of the initial lead.

Lymphoma Cell Surface Idiotypes

The antigens for the surface idiotypes of the two lymphoma cell lines are unknown. Using the combinatorial peptide library method, we succeeded in isolating several peptide ligands for these surface idiotypes. These ligands are specific for the cell line from which the idiotype was isolated. Upon binding to an intact cell, these peptides, in tetrameric form, were able to trigger signal transduction, resulting in elevated protein tyrosine phosphorylation (36). We plan to use these peptides as carriers for targeted therapy of B-cell lymphoma (36, 51). From the therapeutic standpoint, the D-amino acid ligands are particularly attractive, as they are likely to be more stable to proteolysis *in vivo*.

As indicated earlier, for the anti-insulin MAb, only one predominant motif was identified from D-amino acid peptide libraries, whereas multiple motifs were identified when L-amino acid peptide libraries were screened. In contrast, when the lymphoma idiotypes (both WEHI-231 and WEHI-279) were used, multiple

motifs were identified from the D-amino acid peptide libraries and only one predominant motif was identified from the L-amino acid peptide libraries. Although the native antigens for the two lymphoma idiotypes are unknown, it is conceivable that both antibodies recognize discontinuous epitopes, as multiple motifs have been identified from the library screen.

Mimotope Concept

There is a general belief that by employing combinatorial peptide library methods to map B-cell epitopes, one may be able to design small peptides for use as vaccines (52). Mario Geysen first coined the term mimotopes, which he defined as "a molecule able to bind to the antigen combining site of an antibody molecule, not necessarily identical with the epitope inducing the antibody, but an acceptable mimic of the essential features of the epitope" (12). Experimentally, it was defined by screening combinatorial peptide libraries (Geysen's multipin iterative approach) with an antibody. However, there is no guarantee that the mimotope can elicit an immune response against the native epitope. In order to be a "true mimotope," it has to fulfill the following two criteria: (i) it mimics the antigenicity by binding to the antibody that recognizes the native epitope, and (ii) it mimics the immunogenicity by its ability to elicit a humoral immune response against the native epitope that it mimics. This second criteria is crucial if one plans to use the mimotopes as vaccines. Based on the data reported in this paper, the mimotope concept certainly applies to antibodies that recognize a small continuous epitope (e.g., anti-β-endorphin MAb) and perhaps may even be true for a small compact discontinuous epitope although there are little data to support the latter. In case of the anti-β-endorphin MAb, almost all the ligands isolated from a truly random library have close resemblance to the native epitope, YGGF. It is likely that immunogens based on any of these mimotopes (including the D-amino acid containing ligands) will elicit antibodies that cross-react with the native epitope. Perhaps one of the few exceptions may be the Ya(N/Q)a W motif, of which the only common features with the native epitope are an N-terminal tyrosine and an aromatic residue at the C-terminus. Work is currently underway in our laboratory to immunize animals with this peptide to see if antibodies against YGGF can be elicited.

In the case of anti-insulin MAb that recognizes a discontinuous epitope, multiple distinct motifs and several discrete sequences were identified (Tables 4–7). None of these sequences have any sequence homology with the primary sequence of human or porcine insulin. The fact that only one predominant motif was identified for anti-β-endorphin MAb and yet multiple distinct motifs were identified for anti-insulin MAb suggests that either (i) the antigen combining site for anti-β-endorphin is small and only peptides that closely resemble YGGF can interact or (ii) there are other potential interaction sites created by the various CDR loops but these ligands were not isolated because their binding affinities are significantly weaker than that of YGGF. The former explanation is favored because very low-affinity ligands were indeed isolated but they all resemble YGGF (Table 1). It is conceivable that the multiple different ligands identified from anti-insulin MAb interact with different CDR loops of the antibody molecule. Even though native insulin can compete for the binding of all these peptides (including the D-ligands) to anti-insulin MAb, many of them probably have little resemblance to the structural feature of native insulin. That is, they may not be "true mimotopes" and may not be used as an immunogen for eliciting anti-insulin response. This concept is illustrated in Fig. 3. Assuming that there are seven potential interaction sites (formed by the various CDR loops) in the anti-insulin MAb, that native insulin interacts with only three of these sites (sites 2, 4, and 6), and that if we screen enough libraries and with enough diversities, we can discover ligands for each of these seven potential interaction sites. Although insulin can compete for ligands identified for sites 2–6 (but not 1 and 7), ligands for sites 3 and 5 may bear no resemblance to the structural feature of native insulin and certainly will not be able to generate anti-insulin antibody when used as an immunogen. On the other hand, ligands for sites 2, 4, or 6 may mimic some structural features of the native insulin molecule and may be able to elicit anti-insulin response. Work is underway in our laboratory to test whether the identified anti-insulin MAb peptides (Tables 4–7) are "true mimotopes" or not.

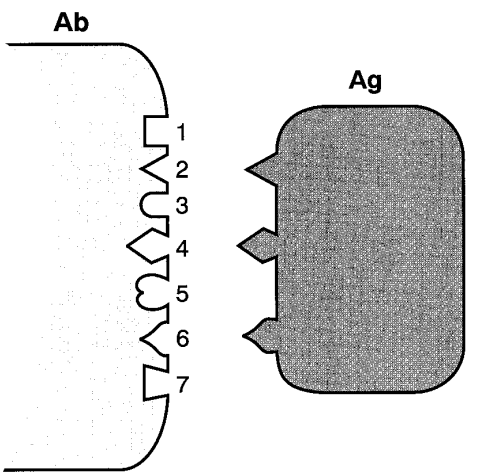

FIG. 3. Diagrammatic representation of an antibody recognizing a discontinuous epitope (e.g., anti-insulin MAb). The native antigen interacts only with sites 2, 4, and 6. Peptide ligands for sites 3 and 5 are not "true mimotopes."

CONCLUSION

The one-bead one-compound combinatorial library method is a valuable tool to study molecular recognition and for drug discovery. Since synthetic chemistry is used in the synthesis of the libraries, we can apply the same principle to peptides with unnatural amino acids, glycopeptides, peptides with reduced peptide bonds, and even small organic molecules. This latter development (3, 25, 53–56) has attracted enormous attention in the last 2 years, as it provides a new and efficient approach to drug discovery. In this paper, we have demonstrated the use of the combinatorial peptide library method for MAb epitope mapping. The following general conclusions can be drawn from the results of the five antibody systems (anti-β-endorphin, anti-insulin, anti-vmos, and two B-cell lymphoma idiotypes) reported in this paper: (i) for the antibodies that recognize a short linear continuous epitope (e.g., anti-β-endorphin MAb), high-affinity ligands, identical or very similar to the native epitope, can easily be isolated with the combinatorial peptide library method; (ii) for the antibody that recognizes a discontinuous epitope (e.g., anti-insulin MAb), multiple different motifs can be isolated. Often, the binding affinities for the initial leads are weak, but they could be improved by sequential screening with secondary and tertiary libraries; (iii) it is impossible to predict the number of different motifs one can identify from a particular screen (it is antibody- as well as library-dependent); (iv) using the combinatorial peptide library method, peptide ligands can be isolated for antibodies that have no known antigens (e.g., surface idiotypes of WEHI-231 and WEHI-279 cell lines); (v) antibodies do bind to D-amino acid ligands; and (vi) peptide ligands isolated from a combinatorial peptide library method may not be a "true mimotope" especially for those antibodies that recognize a discontinuous epitope (e.g. anti-insulin MAb).

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