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Chemistry

Discovery of biologically active peptides in random libraries: Solution-phase testing after staged orthogonal release from resin beads

(peptide library screening)

SYDNEY E. SALMON*†, KIT S. LAM*, MICHAL LEBL‡, ANITHA KANDOLA*, PARTH S. KHATTRI*, SHELLY WADE‡, MARCEL PÁTEK‡, PETR KOCIS‡, VIKTOR KRCHNÁK‡, DAVID THORPE‡, AND STEPHEN FELDER‡

*Arizona Cancer Center and Department of Medicine, University of Arizona College of Medicine, Tucson, AZ 85724; and ‡Selectide Corporation, 1580 East Hanley Boulevard, Oro Valley, AZ 85737

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ABSTRACT **To speed drug discovery, we developed an approach for identification of individual peptides with a desired biological activity from a library containing millions of peptides. The approach uses sequential orthogonal release of chemically synthesized peptides from insoluble beads, followed by testing in solution. In this system, each bead within a library of beads has one peptide sequence, but peptide molecules are attached to the bead with three types of chemical linkers, including two linkers cleavable at different pH optima. An uncleavable linker keeps some peptide attached to the bead for sequencing positives from the solution assay. Applicability of this discovery technique was documented by identifying ligands for a monoclonal antibody and for the human platelet fibrinogen receptor, glycoprotein IIb/IIIa.**

In vitro screening of chemical compounds and fermentation broths is used to identify initial leads for drug discovery. Inhibition of receptor–ligand interactions, signal transduction, and enzyme action or growth of microorganisms or tumor cells can be readily examined. High-volume screening is essential to such discovery efforts, as the number of "hits," found in screening is extremely low. Several groups have developed peptide libraries for the purpose of discovering ligands when the ligand is bound to a surface (1–6). We have recently developed a system for production and screening of large libraries of peptides produced by solid-phase chemical synthesis (5, 7), wherein any one solid-phase bead expresses only one peptide moiety. The initial screening employs binding of tagged acceptor molecules (e.g., antibodies, receptors) to individual peptide beads within a large library; the tagged bead is then recovered, and the peptide which it displays is identified by microsequencing. However, many of the assays used for drug discovery require solution-phase assay. Two groups have reported methods of producing peptides in solution for high-volume screening. Geysen and colleagues (8–10) have synthesized peptides of known amino acid sequence on plastic pegs, then released the unique peptide on each peg into an individual assay well within a 96-well microplate by cleavage of the linker at neutral pH with formation of a diketopiperazine or by cleavage of an ester bond (8–10). Houghten, *et al.* (11) have prepared sets of random peptide mixtures in packets with fixed known positions in the peptide chain, with each packet containing a mixture of 104,976 individual peptide species. After biological testing, the most active peptide mixture with known fixed positions was used for building another set of libraries with additional fixed positions, followed by further iterative testing, etc.

We developed an approach for large-scale screening in solution, extending the "one-bead, one-peptide" concept (5) to solution-phase screening, with the use of multiple release linkers (12, 13). By linkage system design, one-third of the peptide on each bead is released at neutral pH, and one-third at high pH, while the final third remains on the bead for sequencing. This allows for direct screening in solution of a large random libraries. Following synthesis, the complete library (up to several million peptides) is screened in two stages. In the first stage, peptides released from hundreds to a few thousand beads per well are tested in a microassay plate. Subsequently, the beads from the "active" well are redistributed into individual wells, and additional peptide from each bead is released at high pH and screened for activity. The corresponding peptide bead remaining on the active well is then microsequenced. We validated this approach by identifying ligands for two acceptor molecules which bind to known peptide epitopes: a monoclonal antibody against β-endorphin which binds the peptide Tyr-Gly-Gly-Phe-Leu (YGGFL) and the platelet-derived glycoprotein (gp) IIb/IIIa receptor, which binds the Arg-Gly-Asp (RGD) sequence.

MATERIALS AND METHODS

Synthesis of Peptide Libraries. *Materials.* Commercial-grade solvents were used. Protected amino acids were obtained from Bachem, Advanced ChemTech, or Propeptide (Vert-Le-Petit, France).

General procedures. Libraries of peptides were constructed on polyoxyethylene-grafted polystyrene resin beads (TentaGel, Rapp Polymere, Tubingen, Germany, 120 μm, 0.23 mmol/g) containing double cleavable linkers (12, 13) as described (5, 7, 14). N^{α}-[(9-fluorenylmethyl)oxy]carbonyl (Fmoc) chemistry was used. The resin beads were distributed into different reaction vessels for each amino acid at each coupling step; pooled, washed, and thoroughly mixed for randomization; deprotected, and redistributed into the various vessels again for the next coupling step, etc. (14). Fmoc protecting groups were cleaved with 20% piperidine in dimethylformamide for 10 min. *tert*-Butyloxycarbonyl (Boc) groups were cleaved with 30% trifluoroacetic acid in dichloromethane containing 3% anisole for 20 min; the N^{α}-{[2-(3,5-

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Abbreviations: Boc, *tert*-butyloxycarbonyl; Ddz, [2-(3,5-dimethoxyphenyl)prop-2-yl]oxycarbonyl; Fmoc, (9-fluorenylmethyl)oxycarbonyl; BSA, bovine serum albumin; gpIIb/IIIa, glycoprotein IIb/IIIa.

†To whom reprint requests should be addressed.

dimethoxyphenyl)prop-2-yl]oxy}carbonyl (Ddz) group was cleaved with 3% trifluoroacetic acid in dichloromethane for 30 min. A solution of 5% diisopropylethylamine in dichloromethane was used for neutralization after Boc and Ddz cleavage. A 1:1:1 (eq/eq) mixture of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate, *N*-hydroxybenzotriazole, and diisopropylethylamine in dimethylfurmamide was used to activate amino acids, and a 3-fold molar excess of activated amino acids was used for coupling. The completeness of each reaction (1.5–40 hr) was checked with ninhydrin (or with chloranil test when coupling to secondary amino groups). The synthetic cycle included washing six to eight times with dimethylformamide between coupling and deprotection and between deprotection and coupling. Side chains were deprotected with a mixture of 82.5% trifluoroacetic acid, 5% *p*-cresole, 5% thioanisole, 5% water, and 2.5% ethanedithiol for 2 hr.

Synthesis of tetrapeptide library. TentaGel (5 g, 0.23 mmol/g) was preswollen in dimethylformamide (swollen volume, 25 ml), Boc-Lys(Fmoc) was coupled, the Fmoc group was removed, and Fmoc-Gly-βAla-HMB (HMB, *p*-hydroxymethylbenzoic acid) was coupled. Then the Boc group was cleaved, Fmoc-Lys(Boc) was coupled, Boc was removed, and Boc-Lys(Fmoc)-Pro-HMB was coupled. After removal of Fmoc groups, the library was built. The resin was divided into 19 portions, and 19 amino acids were coupled (Cys was not used). The randomization was repeated four times, after which the Fmoc group was removed, side chains were deprotected, and the library was washed with trifluoroacetic acid (3×), dichloromethane (5×), dimethylformamide (5×), dimethylformamide/0.1% HCl, 1:1 (3×), and 0.02% HCl (3×).

Synthesis of cyclic library. TentaGel (5 g, 0.23 mmol/g) was preswollen in dimethylformamide (swollen volume, 25 ml), Fmoc-Lys(Boc) was coupled, Boc was removed, and the first part of the cleavable linker Boc-Glu-Pro-O-$(CH_2)_3$-NH←Gly←Ddz (12, 13) was coupled. Then the Fmoc group was cleaved; and the second part of the linker, Fmoc-Glu-O-$(CH_2)_3$-NH←Gly←Fmoc, was coupled. After removal of the Ddz group and both Fmoc groups, the library was built. Fmoc-Cys(Trt) (Trt, trityl) was coupled, Fmoc was removed, the resin was divided into 37 portions, and 18 L and 18 D amino acids and Gly were coupled (Cys was not used). The randomization was repeated twice, then Fmoc-Cys(Trt) was coupled, Fmoc was removed, side chains were deprotected, and the library was washed with trifluoroacetic acid, cyclized with dimethyl sulfoxide/anisole/trifluoroacetic acid (10:5:85) overnight, and washed as described for the tetrapeptide library.

Synthesis of Biotinyl-K(YGGFL-βAla)-NH_2. Biotinyl-K-(YGGFL-βAla)-NH_2 was synthesized by Fmoc chemistry. Lys(Fmoc) (400 mg) was mixed with 394 mg of biotin *p*-nitrophenyl ester (Sigma). pH was adjusted to 9–10 and the reaction was conducted for 24 hr at room temperature. The final product, biotinyl-Lys(Fmoc), was purified on a silica gel column, followed by a C_{18} reverse-phase HPLC column. For synthesis of the peptide component of the probe, a 4-fold excess of the biotinyl-Lys(Fmoc) was initially coupled to the Rink amide AM resin (Aminotech, Nepean, Canada) with the addition of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate, *N*-hydroxybenzotriazole, and diisopropylethylamine. Completeness of coupling was monitored with ninhydrin. After the synthesis of the peptide was finished, side-chain protecting groups were removed and the peptide was cleaved from the resin with trifluoroacetic acid/phenol, 95:5 (vol/vol). The biotinylated peptide was then precipitated in ether, washed, and purified on a C_{18} reverse-phase HPLC column.

Two-Stage Release for Peptide Library. For the first release, library beads were transferred into pH 4.5 buffer containing 1.0% carboxymethylcellulose, shaken, and rapidly pipetted into the upper chambers of a 96-well filtration manifold (model 09601, Millipore). In a typical experiment, ≈500 beads were deposited in each filtration well, so that each plate contained ≈48,000 unique peptides. The filtration plates served as "master" plates for retaining subsets of peptide beads in unique locations. After vacuum filtration of transfer buffer, first-stage peptide release was accomplished by dispensing the appropriate buffer or tissue culture medium (neutral pH) into each well; the plates were then incubated overnight to cleave the peptide by diketopiperazine formation, and the peptides were vacuum filtered into microassay plates, used for bioassay. After first-stage assay, "positive" wells were identified, and the beads were recovered from the corresponding well(s) of the filtration master plate by using a low-power stereomicroscope. Recovered beads were transferred individually to single microwells in 96-well filtration plates. Cleavage of the ester linker was accomplished by addition of 0.2% NaOH and overnight incubation followed by pH adjustment or, alternatively, by overnight incubation in ammonia vapors. Thereafter, the peptide in buffer was filtered for bioassay. The individual peptide bead for each positive well in second-stage assay was then recovered for microsequencing (5, 7).

Inhibition ELISA for Anti-β-Endorphin Monoclonal Antibody. For the inhibition ELISA, 96-well Immulon plates (Dynatech) were used. Each well was initially coated with 50 μl of streptavidin (20 μg/ml) in bicarbonate buffer (pH 9.4) (Pierce) overnight, followed by three washes with phosphate-buffered saline (PBS: 137 nM NaCl/3 mM KCl/8 mM Na_2HPO_4/1 mM KH_2PO_4, pH 7.4). Wells were treated with 200 μl of bovine serum albumin (BSA, 3 mg/ml) in PBS to prevent nonspecific adsorption and washed three times with PBS/0.1% (vol/vol) Tween 20, and 50 μl of biotinylated YGGFL (10 ng/ml) was added. After 1 hr, plates were washed with PBS/0.1% Tween, and 25 μl of unknown peptide (or YGGFL, positive control) was added, followed by 25 μl of anti-β-endorphin (40 ng/ml, clone 3-E7, Boehringer Mannheim). After 1 hr of incubation at room temperature, plates were washed with PBS/0.1% Tween, and 50 μl of a 1:1000 dilution of goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad) was added. One hour later, the plates were washed, and 30 μl of 30% H_2O_2 in 10 ml of 2,2′-azidobis(3-ethylbenzthiazolinesulfonic acid) substrate in citrate buffer (pH 4.2) was added. Fifteen minutes later, the ELISA plates were read at 405 nm.

Inhibition ELISA for gpIIb/IIIa. Peptides from the cyclized library were screened for ligands to gpIIb/IIIa. A competitive binding assay was performed (15). The wells of Immulon microassay plates, coated with fibrinogen at 10 μg/ml, were blocked with BSA. Assays were conducted in 100 μl with gpIIb/IIIa at 52 μg/ml in 20 mM Tris, pH 7.5/120 mM NaCl/2 mM $CaCl_2$/0.5% NaN_3/0.05% Tween 20/0.5% BSA (TNCNT/BSA) and incubated for 1 hr. Wells were washed with PBS/0.1% Tween, and 100 μl of 10 nM 4B12 monoclonal antibody to gpIIb/IIIa was added to each well in ELISA buffer (PBS/0.5% BSA/0.05% Tween 20/0.01% Thimerosal). After the plates were washed with PBS/0.1% Tween, 100 μl of a 1:5000 dilution of goat anti-mouse horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories) was added. Wells were washed, and 100 μl of substrate was added, containing *o*-phenylenediamine dihydrochloride (0.67 mg/ml) and 0.0003% H_2O_2 in 50 mM Na_2HPO_4/citrate, pH 5. Color development was stopped by addition of 50 μl of 1 M H_2SO_4. Relative amount of gpIIb/IIIa bound to fibrinogen was determined by OD_{492}. A soluble competitor of fibrinogen would reduce gpIIb/IIIa binding and absorbance. For first-stage release, 200 beads per well were delivered, washed, incubated for 1 hr with 50 μl of TNCNT/BSA, and filtered into the fibrinogen-coated assay plate, and 50 μl of

TNCNT/BSA containing gpIIb/IIIa was added and the binding assay performed. For second-stage release, 1 bead per well was delivered, washed, and incubated for 30 min with 20 μl of 50 mM NaOH. Ten microliters of neutralizing buffer (0.1 M HCl in 2.5× TNCNT/BSA) was added and filtered into assay plates. Each release well was washed with 20 μl of TNCNT/BSA and filtered into the assay well for a total volume of 50 μl. Receptor in 50 μl of TNCNT/BSA was added and the assay was performed. Significant inhibition of gpIIb/IIIa binding was defined as a decrease in optical density of 3 SD below average optical density of the plate.

RESULTS

Chemistry of Releasable Linkers. With split synthesis, each bead should contain only a single peptide species, as any one bead can only be in a single reaction vessel and exposed to only one amino acid at any one coupling step. Beads are 120 μm in diameter and a single bead with three linkers contains about 300 pmol of its peptide species. The two releasable linkers used for peptide release were a thermodynamically driven formation of six-membered diketopiperazine ring, and hydrolytically or ammonolytically cleaved ester bonds, respectively. In the original reports of peptide release (9, 10), the product formed by the cleavage reaction (i.e., of a diketopiperazine structure) remained a part of the released peptide. We have redesigned the linker to permit an "exclusive release," wherein the released peptides are free of linker and identical after both release steps (Fig. 1). The first-stage release leads to formation of diketopiperazine, and the peptide XXXXX-NH-$(CH_2)_3$-OH is released. The second release is achieved either by adding 0.2% NaOH or by exposure to ammonia vapors. Kinetics of the two-stage release are shown in Fig. 2. The peptide remaining on the bead on the third arm remains available for sequence determination by Edman degradation. Our two-stage approach to bioassay exploits the orthogonal sequential release of peptide by assaying for activity in 96-well plates in two stages, the first with 500–2000 beads per well, and the second with 1 or 2 beads per well (Fig. 3).

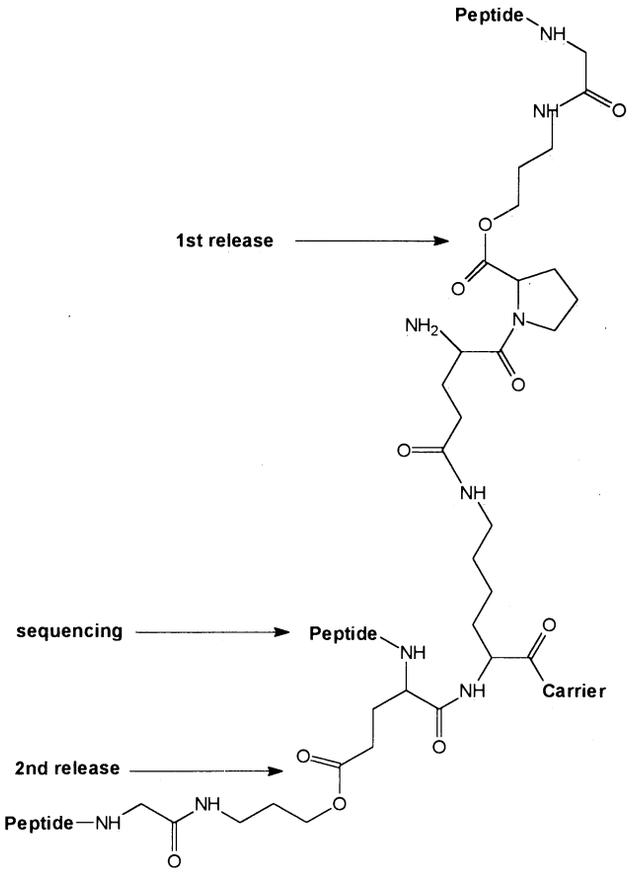

FIG. 1. Chemical structure of an orthogonal linker with the "exclusive release" structure as described by Lebl *et al.* (13).

Ligand for Anti-β-Endorphin. We devised an ELISA to detect soluble ligands within a library which inhibited the binding of leucine enkephalin (YGGFL-OH) to anti-β-endorphin monoclonal antibody in solution-phase assay. This antibody binds to its natural epitope with high affinity (K_i 17.5 nM) (5). We chemically synthesized biotinyl-K(YGGFL-βAla)-NH_2, a derivative of YGGFL in which the α-amino group of the carboxyl-terminal lysine was biotinylated. After biotinylated YGGFL was immobilized by binding to streptavidin-coated plates, we could determine the amount of YGGFL or its analog in solution by competition for binding of anti-β-endorphin to immobilized YGGFL. Bound anti-β-endorphin was then quantified by ELISA.

First release of YGGFL from a single bead completely inhibited the ELISA. This system was employed in the two-stage bioassay. We synthesized a doubly releasable pentapeptide library by using the natural amino acids (except for cysteine) in which the first four positions were randomized and coupled to glycine connected to the bead by the orthogonal linkers. The random incorporation of 19 amino acids into the four positions can produce up to 130,321 (19^4) peptides of different sequence. The frequency that a particular sequence will be present in a library is predicted by the Poisson distribution. Accordingly, for 99% certainty that all tetrapeptides in this library are present, synthesis and screening of a 16-fold excess of the library would be necessary.

From 10^6 peptides screened for anti-β-endorphin ligands, 12 positive wells were identified in first release. Beads from 4 wells underwent second-stage testing. Three relevant ligands were identified: YGGFG (2 beads), YGVFG, and YGAFG. We have resynthesized these peptides with β-alanine at the carboxyl terminus and determined their inhibition profiles and IC_{50} values (Fig. 4). This experiment established that the two-stage release assay can identify monoclonal antibody ligands.

Ligand for gpIIb/IIIa. The release assay was next applied to a receptor in the integrin family; gpIIb/IIIa. This receptor on platelets binds fibrinogen after platelet activation (16).

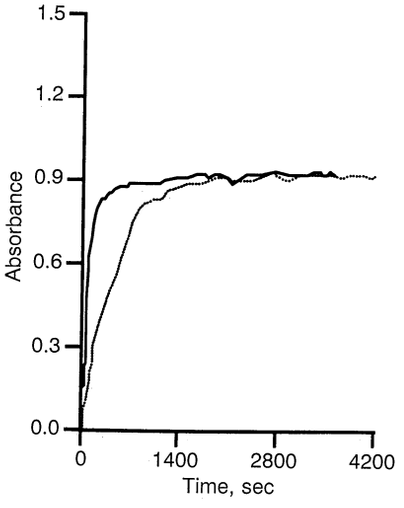

FIG. 2. Peptide release kinetics for two orthogonal linkers followed by UV spectroscopy (280 nm). Diketopiperazine formation (pH 8.3 buffer) (—) and ester hydrolysis (0.2% NaOH) (···) are shown.

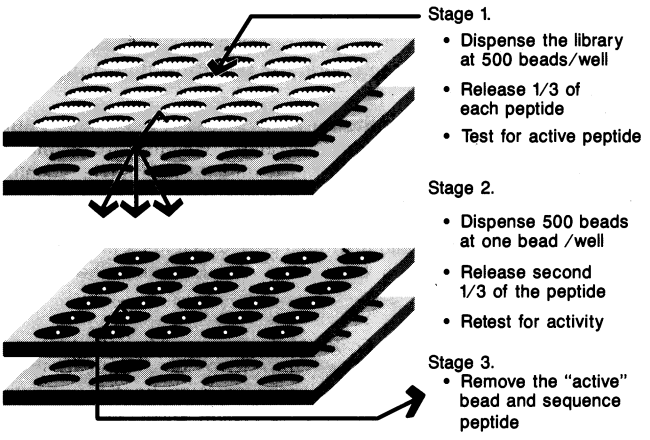

FIG. 3. Schema for two-stage release assay for peptide library testing in 96-well microassay plates.

gpIIb/IIIa recognizes the tripeptide motif RGD. Cyclic peptides incorporating this motif inhibit fibrinogen binding (15). We screened a releasable cyclic peptide library for peptides that competed with fibrinogen for binding to gpIIb/IIIa by ELISA (15). The probability of finding a cyclic peptide in this library with the L amino acid sequence RGD is 1:50,000.

Screening of 10^5 releasable cyclic peptides identified 2 beads with the RGD motif. In the first-stage release, 2 wells inhibited the binding of gpIIb/IIIa to immobilized fibrinogen. The beads from these wells were distributed at 1 bead per well in microfiltration plates, and the second-stage linker was cleaved. Following neutralization and filtration into the fibrinogen-coated plates, the competitive binding assay revealed that 3 out of a total of 406 wells had reduced OD_{492}. The two most inhibitory beads both contained CRGDC; the third, weaker bead contained CARYC.

As both L and D amino acids were used in the cyclic peptide library, both RGD and ARY cyclic peptides were synthesized with L and D amino acids in all possible combinations (except for the flanking L-cysteines). Cyclic CRGDC had an IC_{50} of 1 μM and was the active stereoisomer identified. Of the other three stereoisomers, CrGDC, CRGdC, and CrGdC (D amino acids shown in lowercase; glycine has no chirality), only CRGdC showed activity, with an IC_{50} of 100 μM. The cyclic

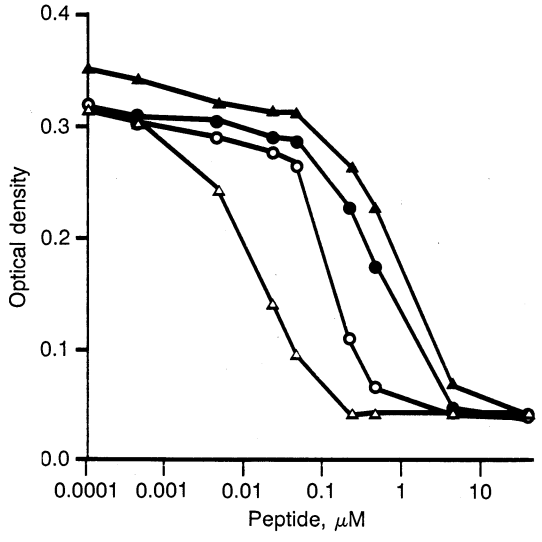

FIG. 4. Inhibition ELISA profile of a series of peptides identified as binding to anti-β-endorphin. The IC_{50} values for the native epitope YGGFL and the three isolated ligands with the β-alanine carboxyl terminus were as follows: YGGFL (△, 30 nM), YGGFG-β-Ala (○, 200 nM), YGAFG-β-Ala (▲, 700 nM), and YGVFG-β-Ala (●, 1000 nM).

ARY peptide was a false positive "hit." Generally, the frequency of false positives is less than the frequency suggested by this experiment, 1/406 wells. The two RGD "hits" inhibited binding to the degree expected, about 50%. Under the conditions of this assay, a released peptide has a final concentration of about 1 μM, which coincides with the IC_{50} for cyclic RGD. This test confirmed that true gpIIb/IIIa ligands can be identified from 50,000 random peptides.

DISCUSSION

The technology described provides high-volume screening in solution by use of orthogonal, staged release of peptides and retains the advantages of the one-bead, one-peptide synthetic peptide methodology (5). Natural amino acids, as well as unnatural amino acids and other building blocks offering diversity of composition can be used for direct screening of peptidomimetics (17–19). The peptides can have various structural constraints or motifs incorporated during library design. However, only several million peptides can be screened in one assay. Our approach is very different from that of Houghten *et al.* (11), wherein a limited number of randomized positions can be used at any one step and tested in an iterative fashion. While we can generate long random peptides (e.g., pentadecapeptides) we cannot generate complete libraries of these, due to limitations imposed by quantities and cost of chemicals and resin volume required. We can produce randomized libraries of pentapeptides of 20 amino acids and test a very high percentage of all structures in the library, or we can generate libraries of longer peptides which have far fewer than the possible number of structures. When relatively short peptides are used initially, we can fix the sequence of initial hits and extend the peptide by randomizing another three to five positions beyond the initial hit sequence. With both long and short peptides, we have observed sequence homologies between hits for any given acceptor molecule. Because we can obtain multiple hits with differing sequences for a given target, our approach to lead discovery provides opportunity for parallel development of multiple leads initially, whereas the convergent iterative approach of Houghten *et al.* (11) does not. The phage approach to peptide library synthesis (1–3) permits generation and screening of far larger libraries (e.g., 10^9 peptides) than we can achieve with chemical synthesis. However, the phage approach is limited to L amino acids, linear sequences, or simple disulfide structures and does not permit solution-phase assay.

Extension of the one-bead, one peptide approach to solution assay provides a wider range of biological targets, which need not be purified or composed of well-characterized components. The assays can be binding assays, as presented here, or functional assays.

In this paper we have delineated discovery of peptide mimotopes to anti-β-endorphin. This same antibody has been probed with the phage technique (2) and by ligand binding on the surface of individual beads (5, 20). With our methodology, one must study a sufficient number of beads to gain statistical assurance that rare events are likely to be detected. As we have conducted limited screening with the release libraries for anti-β endorphin, and because the concentration of any one peptide is limited to 1 μM, it is not surprising that we did not discover all active peptides that have been identified previously to interact with anti-β-endorphin.

We are applying the release assay system to a variety of targets of pharmacologic interest, including discovery of enzyme inhibitors, as well as in assays to detect growth-inhibitory activity against human cell lines in culture. With current solid-phase synthesis methods, 100 pmol of peptide can be released from each bead at each cleavage step. Accordingly, with a 100-μl well volume, the maximum con-

centration of any one peptide in solution is 1 μM. Modified 96-well assay plates for volumes of 10–20 μl are available and may, therefore, increase peptide concentration to the range of 10 μM. Further concentration increases will require higher degrees of peptide substitution or development of larger beads.

We believe that our releasable library approach will have broad applicability, including the recognition of molecules which interact with various acceptors which can be assayed functionally, even though they may not have been specifically purified prior to the assay. For some targets, the bead binding and release technologies can be used sequentially with the same beads. Identification of reactivity of the same peptide in these two assays with the same target confirms specificity for the molecular acceptor before the bead is sequenced. In such an experiment with anti-β-endorphin as the acceptor, we identified a positive well (containing $\approx$1000 beads) in first-stage release assay, and beads from that well were recovered. These 1000 beads were then screened by immunohistochemical staining as described (5). A single bead stained with alkaline phosphatase-linked anti-β-endorphin, and its amino acid sequence was YGGFG.

For enzyme inhibitors, one could first identify wells containing functionally active beads with first release and then identify the specific bead expressing the inhibitor by its ability to bind the enzyme using the immunohistochemical approach. When a highly purified enzyme is available, this procedure can potentially be reversed, with initial screening used to identify all reactive beads that bind the enzyme. Subsequently, the release assay can identify beads that not only bind the enzyme but also inhibit its catalytic function. The two-stage release assay is inherently slower than the bead binding assay and has no advantage if purified acceptors are available or if a functional assay is not initially required. Many of the compounds released may be insoluble and not tested. In contrast, with bead binding, structures may be tested even if they are insoluble. Initially we focused technology development on peptides for three reasons: (*i*) peptides are small but important structural and functional molecules in nature, (*ii*) large combinatorial peptide libraries can readily be synthesized by standard solid-phase synthesis with resin-splitting techniques (5, 7), and (*iii*) structural determination of a peptide from a single resin bead can be readily accomplished by microsequencing.

However, many peptides have properties which limit their utility as drugs. In contrast, small organic molecules are often more useful as drugs because of their stability, resistance to degradation, superior pharmacokinetics, and cellular uptake. The major factor limiting the application of combinatorial chemistry to nonpeptide molecules has been difficulty in determining structure from picomolar quantities. This problem has been reduced with the development of molecular encoding schemes using either nucleic acid (21) or peptide codes (18, 19). We find peptide-encoding to be compatible with solid-phase synthesis of libraries of nonpeptides (19). Many thousands of nonpeptide chemical subunits (including heterocyclic and hydrophobic structures) are available that may be sequentially added to a central molecular "scaffold." We recently combined our release technology with peptide encoding for organic molecules and synthesized libraries of releasable compounds. Organic molecules are released into solution for testing, while the relevant structure-encoding peptide is retained on the beads for structure determination.

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