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# Searching for Peptide Ligands with an Epitope Library

JAMIE K. SCOTT AND GEORGE P. SMITH*

**Tens of millions of short peptides can be easily surveyed for tight binding to an antibody, receptor or other binding protein using an "epitope library." The library is a vast mixture of filamentous phage clones, each displaying one peptide sequence on the virion surface. The survey is accomplished by using the binding protein to affinity-purify phage that display tight-binding peptides and propagating the purified phage in *Escherichia coli*. The amino acid sequences of the peptides displayed on the phage are then determined by sequencing the corresponding coding region in the viral DNA's. Potential applications of the epitope library include investigation of the specificity of antibodies and discovery of mimetic drug candidates.**

"Fusion phage" are filamentous bacteriophage vectors in which foreign antigenic determinants are cloned into phage gene III and displayed as part of the gene III protein (pIII) at one tip of the virion. Fusion phage whose displayed determinant binds an antibody (Ab) can be selected from a vast background of nonbinding phage by affinity purification (AP) as follows (*1*). First, phage are reacted with biotinylated Ab (bio-Ab), then diluted and placed on a streptavidin-coated petri dish, thereby specifically attaching Ab-reactive phage to the plastic surface through the Ab-biotin-streptavidin bridge. Free phage are washed away, and bound phage eluted in acid and used to infect *Escherichia coli* cells. A single round of AP can enrich Ab-binding phage by as much as a factor of $10^5$ relative to unreactive phage; further enrichment is achieved by further rounds of AP after amplification on agar medium (*1*). Thus Ab serves as a powerful selective agent favoring the target clones, so that vast numbers of phage can be surveyed.

The idea of using fusion phage to develop an "epitope library" (*1, 2*) was inspired by the synthetic "mimotope" strategy of Geysen *et al.* (*3*). By synthesizing peptide mixtures on plastic pins and assessing their ability to bind an Ab against a protein antigen, these workers delineated a peptide that mimicks a discontinuous epitope—an Ab-binding determinant composed of residues distant in the primary sequence but adjacent in the folded structure. They called these peptide mimics mimotopes. In this way ligands can be discovered for an Ab whose specificity is not known in advance.

Fusion phage displaying short cloned peptides are infectious analogs of chemically synthesized mimotopes, with the key advantages of replicability and clonability. A large library of such phage—an "epitope library"—may display tens of millions of peptide epitopes. The peptides can in effect be individually surveyed for binding to an Ab or other binding protein by affinity purifying reactive phage from the library, progagating individual phage clones, and sequencing the relevant part of their DNA's to determine the amino acid sequences of their displayed peptides. A survey based on the epitope library undoubtedly would be imperfect because of bias introduced by the biology of the phage and other factors; still, it would represent a powerful new approach to the study of the specificity of Ab's and other binding proteins.

In this article we report on construction and characterization of epitope libraries. Our library is a mixture of fusion phage theoretically displaying approximately $4 \times 10^7$ different hexapeptide epitopes. Devlin *et al.* (*4*) and Cwirla *et al.* (*5*) have also described the construction and characterization of epitope libraries.

**Construction of the library.** We constructed phage fUSE5 as the vector for the epitope library (Fig. 1A); it has several advantages in common with other vectors in the fUSE series (*1*), including vector

The authors are in the Division of Biological Sciences, Tucker Hall, University of Missouri, Columbia, MO 65211; J. Scott is also in the Department of Medicine, Health Sciences Complex, University of Missouri, Columbia, MO 65212.

*To whom correspondence should be addressed.

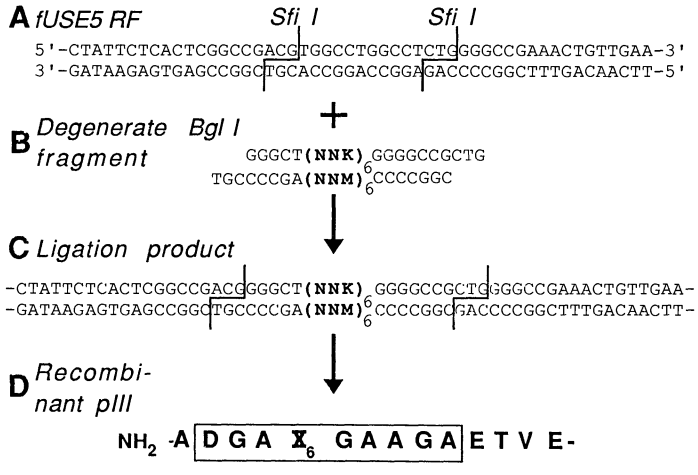


**Fig. 1.** Construction of the epitope library. (**A**) Nucleotide sequence of fUSE5 around the Sfi I cloning sites. The vector was constructed by oligonucleotide-directed mutagenesis of phage fd-tet (*1, 9*); intracellular replicative form (RF; yield 180 μg) was prepared from 2 liters of stationary-phase culture by boiling lysis and CsCl-ethidium bromide gradient centrifugation (*22*). (**B**) Sequence of degenerate 33-bp Bgl I fragment used as the insert. See text for meaning of N and K; M is an equal mixture of A and C. This insert was prepared by PCR amplification and Bgl I digestion; and ligated to fUSE5 RF that had previously been cleaved with Sfi I and freed of the 14-bp segment that lies between the two Sfi I sites (*10*). (**C**) Nucleotide sequence of ligation product. (**D**) Amino acid sequence near the beginning of the recombinant pIII on virions in the epitope library. Boxed residues, which include the hexapeptide epitope $X_6$ that differs from clone to clone, are absent from wild-type pIII.

fAFF1 (*5*). (i) Foreign inserts do not significantly debilitate pIII, either during morphogenesis, when it is incorporated into the virion from the inner membrane; or during infection, when it binds the F pilus. (ii) Since the vector bears a tetracycline (Tc) resistance gene and filamentous phage do not kill their host, a single infection event suffices to give a detectable Tc-resistant transductant clone, which can be propagated without further infection. This minimizes demand on pIII function, and allows infective phage to be quantified as transducing units (TU) rather than plaque-forming units (*1*). (iii) Noninfectious fUSE strains with severe defects in gene III can be readily propagated (*6*), allowing inclusion of a frameshift mutation at the cloning site. The gene III reading frame can only be restored by inserts of the correct length without in-frame stop codons; hence only phage-bearing gene III inserts contribute infectious particles to the library. (iv) The fUSE phage have most of the conveniences of commonly used filamentous phage cloning vectors like M13 (*7*), including uncomplicated propagation and ready isolation of single-stranded viral DNA for dideoxy sequencing (*8*) or oligonucleotide-directed mutagenesis (*9*).

The library was made by ligating a synthetic 33-bp Bgl I fragment into fUSE5 and transfecting *E. coli* cells with the ligation product by electroporation (Fig. 1) (*10*). Within the Bgl I fragment was the degenerate coding sequence $(NNK)_6$; where N stands for an equal mixture of the deoxynucleotides G, A, T, and C and K stands for an equal mixture of G and T. Thus NNK represents an equal mixture of 32 triplets, including codons for all 20 amino acids along with the amber stop codon; and the 33-bp fragment is an equal mixture of $32^6$ ($\sim 10^9$) different nucleotide sequences. An individual phage contains one of these sequences in its DNA, and displays the corresponding hexapeptide epitope on its five pIII molecules, just downstream of the signal peptidase cleavage site. Provided that only peptidase-cleaved molecules can be incorporated into the virion, and that incorporated pIII is not degraded (neither supposition has been verified experimentally), each hexapeptide is flanked on the $NH_2$-terminal side by $NH_2$-ADGA- (*11*) and on the COOH-terminal side by -GAAGA- (Fig. 1) (*11*). The flanking sequences are intended as structureless linkers to minimize the influence of pIII on epitope conformation.

The entire library contains $1.3 \times 10^{14}$ phage, representing $2 \times 10^8$ clones. A smaller library ($2.3 \times 10^7$ clones) was similarly constructed and used in the experiments below. Theoretically, 69 and 21 percent of the $6.4 \times 10^7$ possible hexapeptides are represented in the larger and smaller library, respectively. Among the missing peptides will be all those (12,000 in all) with the sequences XϕPXWθ and ϕPXWθX, since the DNA's encoding these sequences have obligatory internal Bgl I sites.

**Affinity purification of phage displaying antibody-binding peptides.** As a model system for testing the epitope library we chose two monoclonal Ab's (MAb's), A2 and M33, that are specific for the hexapeptide DFLEKI, an epitope of the protein myohemerythrin (MHr). Fieser *et al.* (*12*) had previously tested these MAb's for binding to a set of 114 peptides comprising all possible single amino acid substitutions of the MHr epitope sequence, as well as a set of peptides bearing single amino acid deletions. MAb A2 bound MHr 13 times more strongly than did M33 (dissociation constants of 50 and 640 nM, respectively); and bound only six substituted peptides with more than 50 percent the relative binding of the MHr epitope, whereas M33 bound 56 peptides. The first three residues of the hexapeptide were required for binding to both Ab's, most allowable substitutions occurring in the last three positions.

Both MAb's were biotinylated and used to select clones from a portion of the smaller ($2.3 \times 10^7$ clone) epitope library by successive rounds of AP (Table 1). In the first rounds, $10^{12}$ phage particles were incubated overnight with 1 μM bio-MAb in 10-μl reaction mixtures; the phage were diluted and reacted 10 to 20 minutes on streptavidin-coated dishes; the dishes were extensively washed; and the bound phage were eluted in acid as described (*1*). Phage eluted in the first rounds were amplified on agar medium (*1*) and subjected to second rounds of AP with the same bio-MAb's at concentrations of 10 and 0.1 nM; and phage from the second rounds using 0.1 nM bio-MAb's were amplified and subjected to third rounds with 0.1 nM bio-MAb's. The decrease in Ab concentration in the second and third rounds, and the use of excess phage in the rounds with 0.1 nM bio-MAb to introduce binding competition, were intended to select for high-affinity epitopes. As percent of input phage, the yields from the second and third rounds of AP were significantly above the background of nonspecific adsorption ($\sim 3 \times 10^{-5}$ percent) (*1*), indicating a high proportion of Ab-binding phage clones (Table 1). In these rounds, where Ab was limiting, yields relative to the number of Ab molecules were ~1 percent. This may be close to a maximum, since it is comparable to the yield of purified, high-affinity phage subjected to the AP procedure with excess Ab (*1*).

Phage in the eluates from the second and third rounds of AP were cloned and propagated, their DNA's were sequenced to determine the amino acid sequences of their peptide epitopes (*13*), and their binding to MAb's and monovalent Fab' fragments [Fab's (*14*)] were measured by enzyme-linked immunosorbent assay (ELISA). The peptide sequences were compared to DFLEKI, the sequence of the native MHr epitope; and the relative affinities of each clone for the MAb's and Fab's were compared to those of phage clone MHr, which displays the DFLEKI peptide (Table 2). Most phage from the third rounds of AP and from the second rounds with 0.1 nM bio-MAb bind the MAb's about as well as does the MHr phage; in all cases binding was strongly inhibited by a peptide (10 μM) containing the DFLEKI epitope (data not shown). Control ELISA wells with a nonbinding phage and with the starting epitope library showed no binding with either Mab (not shown). With one exception (peptide 5, discussed below), Ab-binding phage display

**Table 1.** Yields from affinity purification with MAb's M33 and A2. MAb's M33 and A2 were biotinylated (*1*) and reacted with phage in 10 μl of TBS overnight; MAb concentrations and ratios of phage to MAb are shown. In the first rounds of AP the phage were portions of the original epitope library; in subsequent rounds phage were amplified eluates from previous rounds as described in the text. The concentration of purified phage in the original epitope library was determined spectrophotometrically (*18*). All other phage concentrations were calculated from the titer of transducing units, using the ratio 0.21 TU per phage particle as determined for the original library. Reactions with streptavidin-coated plates, washing, acid elution, neutralization of the eluates, titering, and amplification on agar medium were carried out essentially as described (*1*). Yields are reported as percentage of input phage and of MAb molecules.

| Round of AP | MAb (nM) | M33 | | | A2 | | |
|---|---|---|---|---|---|---|---|
| | | Ratio of phage to MAb | Yield (%) | | Ratio of phage to MAb | Yield (%) | |
| | | | Per phage | Per MAb | | Per phage | Per MAb |
| 1 | 1000 | 0.017 | $1.8 \times 10^{-4}$ | $2.7 \times 10^{-6}$ | 0.017 | $0.8 \times 10^{-4}$ | $1.3 \times 10^{-6}$ |
| 2 | 0.1 | 667 | $1.0 \times 10^{-3}$ | 0.67 | 483 | $1.4 \times 10^{-3}$ | 0.67 |
| 3 | 0.1 | 62 | $2.4 \times 10^{-2}$ | 1.5 | 17 | $5.2 \times 10^{-2}$ | 0.86 |
| 2 | 10 | 6.7 | $7.5 \times 10^{-2}$ | 0.50 | 4.7 | $3.9 \times 10^{-2}$ | 0.18 |

peptides with striking similarity to the DFLEKI epitope—particularly in the first three residues known to be most important for Ab binding (*12*). These MHr-like sequences were isolated even though no information about MHr was incorporated in the epitope library. Thus surveying the library with successive AP's rapidly and effectively identified peptides that bind MAb's A2 and M33, without benefit of any prior knowledge of the antibodies' specificity.

Some phage from the second rounds of AP with 10 nM bio-MAb bind Ab poorly and display peptides showing little resemblance to the DFLEKI epitope (Table 2, peptides 16 and 19). This suggests that the higher Ab concentration used in these AP's reduced the stringency of selection.

**Selectivity and specificity of affinity purification.** Our studies, as well as those of Cwirla *et al.* (*5*), show that AP with MAb's selects tight binding phage. By omitting the Ab in the AP procedure, Devlin *et al.* (*4*) identified peptides that bind directly to streptavidin—apparently at or near its biotin-binding site, since binding was abolished in the presence of biotin. The exact relation between strength of binding of the peptide to the protein and the strength of selection of phage by AP is not clear, however. Antibody is bivalent, and streptavidin can bind two biotins at once; these proteins can therefore potentially bind two epitopes on a single phage particle. Bivalent binding may be essentially irreversible when the displayed peptide has a certain minimal threshold affinity for the binding protein. If so, AP may be unable to distinguish peptides with moderately high affinity from peptides with very high affinity; Cwirla *et al.* (*5*) reached a similar conclusion. Irreversible binding (whether resulting from bivalency or other causes) would provide a plausible explanation for the effectiveness of AP at Ab concentrations (0.1 nM) 500 to 6400 times lower than the dissociation constants of the Ab's for MHr. Indeed, in our ELISA's the concentration of monovalent Fab′ fragments had to be raised to 300 nM to bind effectively to phage; even at that concentration Fab failed to bind some clones that strongly bound the corresponding MAb at 1 nM (Table 2, peptides 3 and 6 with MAb M33 and peptide 11 with MAb A2). It is also not known whether competition among phage for a limiting amount of MAb—as in our second and third rounds of AP—alters the basis of AP. The use of Fab′ fragments or other monovalent binding proteins should make it possible to study the kinetics of AP without the complication of bivalency.

There are several reasons why the clones we isolated are unlikely to represent all tight-binding peptides. First, many hexapeptides—theoretically 79 percent of the 64 million possible—will be missing by chance from our test library of 23 million clones. For example, only three of the $32^6$ sequences in the degenerate insert encode the MHr epitope DFLEKI. The theoretical probability that such a peptide is absent is $\exp(-3 \times 23 \times 10^6/32^6)$ or 94 percent; our failure to find the DFLEKI peptide among the affinity-purified clones is therefore plausibly attributed simply to chance. (In our library of 200 million clones, the probability that DFLEKI is missing drops to 57 percent.) Second, high-affinity peptides may have been lost during phage amplification between rounds of AP; this possibility is consistent with the observation that most clones analyzed in the third round had identical nucleotide sequences and therefore may have derived from the same clone. Third, some bias may be imposed on the library because of small differences in the growth rates of phage bearing different epitopes. Finally, the binding affinity of some peptides might be influenced, either positively or negatively, by the pIII protein to which they are fused, or by the flanking linkers. Nevertheless, our results demonstrate the ability of the epitope library to identify some high-affinity peptides.

In our experiments, MAb's A2 and M33 were confronted with tens of millions of random peptides, very few with appreciable similarity to MHr. Despite this multiplicity of possibilities, the MAb's still preferentially selected peptides similar to the MHr epitope (Table 2). Nevertheless, one of the peptides displayed on Ab-binding phage has no recognizable similarity to the DFLEKI epitope: CRFVWC, which was affinity-purified with MAb M33 and reacted strongly with that antibody in ELISA (Table 2, peptide 5). Binding was abolished by a free peptide containing the DFLEKI epitope (as for all Ab-binding phage we isolated); it is likely, therefore, that the cross-reacting CRFVWC peptide binds at or near the same site as DFLEKI, not at an adventitious site elsewhere on the antibody surface. Significantly, this peptide did not bind MAb A2 in ELISA, even though both MAb's were elicited with the same epitope. This is not altogether surprising; an Ab elicited with one epitope could bind other epitopes with entirely different structures, and there is no compelling reason to believe that these cross-reacting ligands would be the same for all Ab's elicited by a given epitope. It has been proposed that such uncorrelated cross-reactions expand the range of binding specificities of a limited Ab repertoire without compromising the specificity of polyclonal Ab responses (*15*). This result points up a key advantage of the epitope library: because peptides are surveyed individually for binding affinity, multiple ligands can be discovered.

**Applications.** We do not expect the usefulness of the epitope library to be limited to binding proteins like MAb's M33 and A2 that recognize continuous epitopes on protein antigens. The mimotope experiments of Geysen *et al.* (*3*) show that in at least some cases discontinuous epitopes can be mimicked by short peptides. Similarly, it might be possible to mimic determinants—whether continuous

or discontinuous—on hormones or other ligands that specifically bind receptors or other proteins. The work of Devlin *et al.* (*4*), in which an epitope library was used to find peptides that bind streptavidin, fosters the prospect that an epitope library can also be used to find peptide mimics of nonproteinaceous ligands; this would substantially broaden the applicability of the epitope library.

An immediate application of this technology is epitope mapping. For example, the sequence information in Table 2 would suffice to pinpoint the region of the MHr sequence recognized by MAb's A2 and M33 if we had not known it in advance. If this result proves general, the epitope library would provide a simple, inexpensive alternative to chemical synthesis of peptides ["epitope scanning"; (*16*)] for mapping continuous epitopes. Even discontinuous epitopes might be identified on proteins of known three-dimensional structure.

The epitope library can identify ligands for component Ab's in complex polyclonal antisera, whether or not the antigen is known or available. Thus, for instance, autoimmune sera might be used to identify peptides that discriminate among autoimmune disease entities. Similarly, affinity purification with sera from patients infected with viruses, bacteria, or parasites may identify peptides that distinguish strains or serve as vaccines or immunogens [the phage themselves are immunogenic (*17*)].

By using receptors in soluble form or other binding proteins to affinity-purify phage from the epitope library, it may be possible to discover peptides that biologically mimic active determinants on hormones, cytokines, enzyme substrates, viruses, and other biomolecules. Such mimetic peptides might antagonize, agonize, or modulate the physiological action of the natural ligands, and as such would be attractive candidates for drug development.

In the library constructed by Cwirla and co-workers the peptide epitope starts immediately after the signal peptide cleavage site (*5*). Their library may be particularly useful in applications whose goal is to find free peptide ligands, since the free and phage-bound peptides have the same $NH_2$-terminal structure. In the library of Devlin *et al.* (*4*) phage display epitopes with 15 rather than 6 amino acids (the typical size of continuous epitopes) (*12*). This increases the effective size of the library, since each phage represents several short peptide determinants (up to ten hexapeptides, for instance) in various contexts of flanking residues; and may make possible the display of epitopes comprising more than six residues.

The value of the epitope library is that a large and important part of the epitope universe can be encompassed in a few microliters of solution and effectively surveyed for specific affinity for an antibody, receptor, or other binding protein by simple recombinant DNA methods.

**Table 2.** Peptide sequences and binding characteristics of affinity-purified clones. MHr is the hexapeptide epitope at positions 79 to 84 of MHr protein; a phage clone bearing this peptide was isolated from another library. A dash in the remaining peptide sequences indicates identity to the corresponding residue of MHr. For each peptide sequence, the table records binding of a representative phage clone to the MAb's in both their intact, bivalent form [immunoglobulin G (IgG)] and in the form of monovalent Fab′ fragments (*14*). The third and fourth columns show the number of clones isolated by AP with MAb M33 or A2 having the indicated peptide sequence. Phage for sequencing and ELISA were propagated in NZY-Tc medium and partially purified by two precipitations with polyethylene glycol (PEG) and one precipitation in acid (*19*). Viral DNA was prepared from 12-μl portions of the partially purified phage (~$1.2 \times 10^{11}$ virions) essentially as described (*1*), and sequenced by a simplified method requiring only two chain-termination reactions per template (*20*). Binding was measured as the optical density (OD) obtained in ELISA experiments (*21*). The IgG binding experiments were repeated for all peptides except 14 with MAb A2, and for all peptides except 14 to 19 with MAb M33. Discrepancies were considered significant if the difference between duplicate OD's was greater than 50 percent of average and also greater than 0.02. Such discrepancies were observed for the following peptides (duplicate OD's × $10^3$ in parentheses): for MAb M33, peptides 7 (77), 8 (69), and 9 (19); for A2, peptides 6 (23), 7 (76), 8 (65), 11 (144), 16 (973), and 17 (250).

| Displayed peptides | | Number of clones isolated with | | Binding (OD × $10^3$) | | | |
|---|---|---|---|---|---|---|---|
| | | | | MAb M33 | | MAb A2 | |
| No. | Sequence | MAb M33 | MAb A2 | Fab | IgG | Fab | IgG |
| MHr | DFLEKI | | | 72 | 352 | 136 | 235 |
| | *Third round with 0.1 nM MAb* | | | | | | |
| 1 | ----WL* | 16 | | 203 | 551 | 229 | 233 |
| 2 | ----ML | 1 | 2 | 92 | 558 | 269 | 236 |
| 3 | ---AWL | | 14 | 14 | 242 | 139 | 273 |
| | *Second round with 0.1 nM MAb* | | | | | | |
| 4 | ----R- | 1 | | 94 | 318 | 118 | 290 |
| 5 | CRFVWC | 2 | | 65 | 331 | −4 | −5 |
| 6 | CE---C | 1 | | 18 | 294 | −1 | 123 |
| 7 | CR---C | 1 | | 21 | 205 | 4 | 127 |
| 8 | --M-WL* | 1 | | 35 | 288 | 1 | 125 |
| 9 | ---VQL† | 1 | | 5 | 127 | 23 | 147 |
| 10 | ---AIV† | | 4 | 15 | 43 | 97 | 142 |
| 11 | ----Y- | | 2 | 67 | 157 | 9 | 253 |
| 12 | ----IL | | 1 | 100 | 431 | 159 | 308 |
| 13 | ----IV | | 1 | 37 | 168 | 195 | 293 |
| | *Second round with 10 nM MAb* | | | | | | |
| 14 | ----QL | 1 | | | | | |
| 15 | ---HF- | 1 | | 6 | 108 | −3 | −2 |
| 16 | AWERRG | 1 | | 4 | 8 | −3 | 9 |
| 17 | --F-I- | | 1 | 29 | 244 | 77 | 224 |
| 18 | ---MLV | | 1 | 3 | 18 | 17 | 50 |
| 19 | Q-VFCW | | 1 | 3 | 16 | −5 | 28 |

*Four clones with the peptide 1 sequence and one with the peptide 8 sequence were also isolated from the second round of AP with 10 nM MAb M33. †One clone each with the peptide 9 and 10 sequences were also isolated from the second round of AP with 10 nM MAb A2.


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10. The fUSE5 vector was prepared for ligation by digesting 30 μg of RF DNA with 120 units of Sfi I [Bethesda Research Laboratories (BRL)] in 120 μl, as recommended by the supplier. After extraction with phenol and chloroform (*22*), the volume was brought to 810 μl with TE [10 mM tris-HCl (pH 8), 1 mM EDTA (pH adjusted to 8 with NaOH)], and the DNA was precipitated by additions of 90 μl of sodium acetate buffer (3 M sodium acetate, pH adjusted to 6 with acetic acid) and 540 μl of isopropanol, incubating for 20 minutes at 0°C, and centrifuging (microfuging) for 30 minutes at 5°C. The pellet was washed with 70 percent (v/v) ethanol, redissolved in TE, ethanol-precipitated (*22*), and dissolved in 120 μl of TE (final yield, 17 μg). This DNA could not be self-ligated, indicating complete removal of the 14-bp "stuffer" that lies between the Sfi I sites. The insert was prepared by (i) polymerase chain reaction (PCR) amplification [K. B. Mullis and F. A. Faloona, *Methods Enzymol.* **155**, 335 (1987)] of a 70-base degenerate template (sequence shown in the top strand of Fig. 1C) with 5′-biotinylated primers (sequences corresponding to the 20 residues at the 5′ ends of both strands shown in Fig. 1A); (ii) cleavage at the two Bgl I sites; and (iii) adsorption with streptavidin-agarose to remove the biotinylated terminal fragments along with undigested and partially digested by-products. The 1-ml PCR mixture contained 1 μg of template, 5 μg of each biotinylated primer, and 25 units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) in the buffer recommended by supplier; after five temperature cycles (2.5 minutes at 95°C, 4 minutes at 42°C, 4.4 minutes at 72°C) and 5 minutes at 72°C, polymerization was stopped by the addition of 4 μl of EDTA solution (250 mM EDTA, pH adjusted to 8 with NaOH). The product was precipitated with ethanol and dissolved in 100 μl of TE. A 30-μl portion was digested with 40 μl of Bgl I (80 unit/μl; Promega) for 2 hours at 37°C in a 500-μl reaction mixture; the digestion was stopped by addition of 22 μl of EDTA solution. Streptavidin-agarose beads (BRL; 200 μl of 50 percent suspension) were

washed ten times with 0.1 M NaCl in TE (suspension and centrifugation), and suspended in the DNA digest. After 30 minutes of gentle agitation, beads were centrifuged and the supernatant was removed to another tube. Beads were washed twice with 200 μl of water, and the supernatants were pooled with the main supernatant. The final product was extracted with phenol and chloroform and evaporated to 100 μl at reduced pressure. The 2-ml ligation reaction [R. Lathe, M. P. Kieny, S. Skory, J. P. Lecocq, *DNA* **3**, 173 (1984)] contained 10 μg of Sfi I digest of fUSE5 and 36 μl of degenerate 33-bp insert. The product was extracted with phenol and chloroform, precipitated with ethanol, and dissolved in 200 μl of TE. The concentrations of both open circular and covalently closed circular ligation products were estimated from gel electrophoresis to be ~5 μg/ml. Frozen MC1061 cells (50 μl) were thawed, mixed with 2.5 μl of the ligation product, and electroporated by discharging a 25-microfarad capacitor charged to 2.5 kV across a 2-mm gap in parallel with a 400-ohm resistor [W. J. Dower, J. F. Miller, C. W. Ragsdale, *Nucleic Acids Res.* **16**, 6127 (1988)]. Immediately after each electroporation, cells were diluted in 2 ml of SOC medium (bacto-tryptone at 20 g/liter, yeast extract at 5 g/liter, NaCl at 0.58 g/liter, KCl at 0.19 g/liter, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose; the last three components were added from separately autoclaved 100× stocks) containing Tc at 0.2 μg/ml and transferred to a culture tube (17 by 100 mm); after 1 hour shaking at 37°C, Tc-resistant cells were quantified by spotting 20 μl of a $10^{-3}$ dilution on NZY-Tc agar dishes [20 μg/ml of Tc in NZY medium, which is NZYM (*22*) without $MgSO_4$]. Meanwhile, the remaining culture was spread on 250 to 300 ml of NZY-Tc agar medium in a 23 by 23 cm polystyrene dish (Nunc) which was incubated at 37°C for 36 to 48 hours, giving ~3 million colonies per dish (dishes were reused after they were rinsed and irradiated with UV). Phage from 64 dishes altogether (along with cells that secreted them) were eluted in TBS [50 mM tris-HCl (pH 7.5), 0.15 M NaCl] and purified essentially by procedure I of Lin *et al.* [T. C. Lin, R. E. Webster, W. Konigsberg, *J. Biol. Chem.* **215**, 10331 (1980)]. The reason for propagating the library on dishes rather than in liquid culture was to minimize bias resulting from competition among clones; we do not know if this precaution is necessary. The MC1061 host is $F^-$ and cannot be infected; this prevents propagation by infection, which might conceivably favor some clones because of slight differences in pIII function.

11. Single-letter abbreviations of amino acids: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, unknown; Y, Tyr; βC, β-cysteine; φ, Met, Trp, Gln, Lys, or Glu; θ, Pro, His, or Gln.
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13. Clones from the third round of AP were screened for binding to MAb's by dot-blot analysis (*1*); only clones that reacted with at least one MAb (a majority of clones) were chosen for sequencing and ELISA. All clones reported were propagated and sequenced twice with consistent results. Many affinity-purified clones proved to be contaminants from another library in which MHr-like sequences predominate [J. K. Scott, G. P. Smith, W. J. Dower, in *Advances in Gene Technology: The Molecular Biology of Immune Diseases and the Immune Response*, J. W. Streilein *et al.*, Eds. (IRL Press, Oxford, 1990), p. 224]. Clones from this library could be readily distinguished because they have a G rather than a T just before the degenerate NNK codons (Fig. 1). These contaminants are not reported here.
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19. After cells were removed by centrifugation, phage from 1.8 ml of supernatant were precipitated twice with PEG (*1*); all the supernatant was removed and the redissolved precipitate from the first precipitation was centrifuged for 1 minute in a microfuge before reprecipitation from the supernatant. Phage from the second PEG precipitation were dissolved in 210 μl of 0.15 M NaCl, microfuged for 1 minute, precipitated from 200 μl of the supernatant by adding 21.2 μl of 1 M acetic acid, and incubated for 10 minutes at room temperature (~21°C) and 10 minutes on ice [W. Wickner, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4749 (1975)]. Phage were sedimented (microfuge, 45 minutes, 4°C, with removal of all supernatant) and dissolved in 62 μl of TBS. Phage concentrations were ~$10^{10}$ particles per microliter as determined by gel electrophoresis of viral DNA (*6*).
20. Template DNA in a 1.5-ml microcentrifuge tube was dissolved in 7 μl of water and stored at −20°C. An 18-base primer (*1*) was labeled with $^{32}P$ at its 5′ end to ~1000 Ci/mmol (*22*), adsorbed to a NENSORB 20 column (New England Nuclear), desorbed with a 1:1 mixture of ethanol and water, and concentrated at reduced pressure. To each template tube was added 6.25 μl of primer-buffer mix [64 mM tris-HCl (pH 7.5), 80 mM NaCl, 24 mM D,L-isocitric acid (pH adjusted to 7 to 7.5 with NaOH), 27 nM (~0.03 μCi/μl) end-labeled primer, 8 mM $MnCl_2$]; tubes were microfuged briefly to mix contents, heated at 60° to 70°C for 10 minutes, cooled from 60° to 35°C over at least 1 hour, and microfuged briefly to drive condensation to the bottom. Two 3-μl droplets (called R and W) of each primed template were deposited on the bottom of polystyrene petri dishes (usually 20 templates per dish) [S. S. Ner, D. B. Goodin, G. J. Pielak, M. Smith, *Biotechniques* **6**, 408 (1988)]; onto the R and W droplets, respectively, were deposited 3-μl droplets of R and W termination mixes [3.2 μM ddGTP (for R) or ddTTP (W), 3.2 μM ddATP, 0.32 μM ddCTP, 80 μM all four dNTP's, 66.7 mM NaCl, 16.7 mM tris-HCl (pH 7.5), 13.3 mM dithioerythritol, nuclease-free bovine serum albumin at 100 μg/ml, 0.245 U/μl Sequenase (U.S. Biochemical; enzyme added just before dispensing)]. The dish was covered with its lid and floated 5 to 10 minutes on a water bath at 37°C. Reactions were terminated by depositing 4-μl droplets of formamide buffer [95 percent (v/v) formamide, 20 mM EDTA (pH adjusted to 8 with NaOH), bromophenol blue at 0.5 mg/ml, xylene cyanol FF at 0.5 mg/ml] onto the R and W droplets. Just before placing the samples on the sequencing gel, the dish was floated for 3.5 minutes on a water bath at 85° to 95°C with its cover off to denature the DNA and concentrate it by evaporation. Samples were run on an 8 percent acrylamide sequencing gel on a Model S2 sequencer (BRL) as recommended by the supplier. One group of up to 20 templates (40 lanes) were run until the xylene cyanol FF had migrated to 18 cm; then a second group of templates was loaded and run until the xylene cyanol FF from the first group had migrated to 31 cm. Bands were interpreted as follows: a strong band in both the R and W lanes signifies an A in the synthesized strand; a strong band in the R lane next to a blank in the W lane signifies a G; a strong band in the W lane next to a blank in the R lane signifies a T; and a weak band in both lanes signifies a C.
21. Wells of a flat-bottom 96-well assay plate (Corning) were coated with 2 μl (~2 × $10^{10}$ virions) of partially purified phage (*19*) in a total volume of 37 μl of TBS at 37°C for 6 hours; blank wells had 35 μl of TBS alone. Blocking solution [200 μl of nonfat dry milk (50 mg/ml) in TBS with 200 μg/ml $NaN_3$] was added to the wells, which were then emptied by aspiration and refilled with 350 μl of blocking solution. After at least 90 minutes at room temperature, wells were washed three times in a mixture of TBS and Tween 20 [200:1 (v/v)]. Biotinylated intact MAb's (1 nM) or Fab′ fragments (300 nM) with or without 10 μM competitor pepide (Ac-KDFLEKIGGL-βC-$NH_2$) were added to wells in 35 μl of diluent [1 mg/ml bovine serum albumin (extensively dialyzed; Sigma), 200 μg/ml $NaN_3$ in TBS] and reacted 8 to 12 hours at room temperature. Wells were washed ten times in the TBS-Tween mixture and once with TBS; filled with 85 μl of a complex of avidin and horseradish peroxidase (ABC; Vector Laboratories) in TBS containing 0.1 percent (v/v) Tween 20; incubated for 15 to 30 minutes at room temperature; washed eight times with TBS-Tween and once with TBS; developed for 2 hours at room temperature with 85 μl of ABTS solution [400 μM 2,2′-azinobis(3-ethylbenthiazoline-6-sulfonic acid), 0.03 percent $H_2O_2$ in 77.2 mM $Na_2HPO_4$, 61.4 mM citric acid]; and read in a microplate reader. Data are reported as $OD_{405}$-$OD_{490}$ for sample wells minus the average of the corresponding numbers for the blank wells.
22. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
23. We thank W. Dower for help and advice on electroporation; J. Larrick and W. Dower, among others, for pointing out the use of the library for drug discovery; H. Alexander for many discussions and for the competitive peptide; R. Samodal and R. Lerner for MAb's; G. Fieser and I. Wilson for Fab′ fragments; and R. Davis for technical assistance. Supported by NIH grant GM41478 and by the Molecular Biology Program of the University of Missouri–Columbia.

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