Elucidation of the Core Residues of an Epitope Using Membrane-based Combinatorial Peptide Libraries

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Combinatorial peptide libraries have proved to be a valuable tool for the study of the interaction of a functional protein with its ligand. Here, the epitope for a monoclonal antibody 201/9, raised against β-factor XIIa, has been identified with a two-step approach using peptide libraries attached to a polymer (polyvinylidene difluoride) membrane. First, the octapeptide libraries with two amino acids defined at position 2 and 4, represented by the formula X-O2-X-O4-X-X-X, were synthesized on a sheet of polymer membrane in which X represents a mixture of all the natural L-amino acids except cysteine, while O2 and O4 each represent a single amino acid. The libraries were probed with the antibody 201/9, and the bound antibody was detected with a sensitive chemiluminescent method. In the first cycle, the peptide mixtures X-Phe-X-Gln-X-X-X showed the strongest signal development. In the second cycle Phe and Gln were incorporated into new libraries consisting of sequences O1-Phe-X-Gln-X-X-X, X-Phe-O2-Gln-X-X-X, X-Phe-X-Gln-O2-X-X-X, X-Phe-X-Gln-O4-X-X-X, and X-Phe-X-Gln-X-X-O4. After probing these new peptides, the residues representing the core sequence of the epitope for monoclonal antibody 201/9 were elucidated. The sequence Ser-Phe-Leu-Gln-Glu-Asn, identified as the immunodominant epitope, correlates well with the sequence Ser-Phe-Leu-Gln-Glu-Ala previously identified (Gao, B., and Esnouf, M. P. (1996) J. Immunol. 157, 183–188) in a scan of overlapping peptides based on the sequence of human β-factor XIIa.

Elucidation of the interactive residues of a particular protein ligand with a functional molecule provides important information for understanding and manipulation of the biological processes in which they are involved. Combinatorial peptide libraries (2) are powerful tools for identifying these interactive sequences. The libraries normally consist of 10⁶–10¹² peptides and offer a fundamental practical advance in the elucidation of the residues involved in the interaction between antigens and their antibodies or proteins and their receptors.

Two general strategies for constructing peptide libraries have been universally adopted. One is expression of randomized peptides using phage display vectors M13 or phage fd derivatives, which allows fusion of the foreign peptide sequences to the amino terminus of phage coat proteins (3) and provides a way of generating novel sequences with biological activity for many applications. The second strategy is based on solid phase peptide synthesis. The randomized peptides are synthesized on a solid support and used while still attached to the support (3), or the peptides are cleaved from the support before use (4). The combinatorial libraries have been used successfully to study antigen-antibody interactions (5), elucidate the SH2 binding sequence (6, 7), identify the motif of the substrate for protein kinase (7), select new DNA-binding proteins (7), and develop a new enzyme inhibitor (8). However, generating libraries either by phage display or by the synthetic approach is still relatively time-consuming and expensive. This report describes a procedure for the construction of peptide libraries more easily and less expensively. The peptide libraries were synthesized on a piece of polymer membrane, and the peptides to which the antibody bound were identified by a sensitive immunoblotting assay. This strategy has been used successfully to elucidate the core residues responsible for the binding of the monoclonal antibody 201/9 to its antigen, β-factor XIIa. This procedure should be useful for the identification of those residues involved in the interaction of a functional protein with its ligand, without any prior knowledge of the sequence of either molecule.

MATERIALS AND METHODS

Isolation of Factor XII—Factor XII was isolated from fresh frozen human plasma (9). The protein migrated as a single band on SDS-gel electrophoresis (M, 80,000). β-Factor XIIa (Hageman factor fragment) was isolated from a trypsin digest of factor XII (10). The purified protein migrated as three closely spaced bands (arising from the three trypsin cleavage sites) on SDS-gel electrophoresis (M, 30,000). The absorption coefficient (A₅₃₂) of β-factor XIIa was taken as 15.3 (10).

Production of Monoclonal Antibody (mAb) 201/9—The murine mAb 201/9 to human β-factor XIIa was prepared by the general method of Kohler and Milstein (11) against β-factor XIIa conjugated with thyroglobulin. The clones were selected on the basis of their production of a mAb which exhibited minimal cross-reactivity with factor XII. The selected mAb was purified from an ascitic fluid by precipitation of the immunoglobulin fraction obtained by the addition of an equal volume of saturated ammonium sulfate at 4 °C. The selected antibody was purified further by chromatography on a Mono-Q anion exchange column (Pharmacia Biotech Ltd., St. Albans UK).

Antibodies—All the other antibodies and their conjugates were obtained from Dako, High Wycombe, Bucks, UK.

Membrane Preparation—A sheet (10 × 12 cm) of Immobilon AV-1 membrane (Millipore, Watford, UK) was immersed in 25 ml of 20% (v/v) ethylenediamine (Aldrich, Gillingham, UK) in acetonitrile with 200 μl of triethylamine (Aldrich) and reacted at room temperature overnight (15 h). The membrane was washed three times with 5 ml each with 20 ml of acetonitrile and then three times with 20 ml of methanol and dried in a stream of cold air. 18.6 mg of Fmoc-β-Ala (0.3 mm, Novabiochem, Nottingham, UK), 12 mg of N-hydroxybenzotriazole (0.45 mM, Fluka, Gillingham, UK) and 9 μl of DMAP-diisopropycarbodiimide (0.36 mm, Aldrich) dissolved in 200 μl of 1-methyl-2-pyrrolidone (Fluka) and incubated at room temperature for 30 min to activate the amino acid, and then 0.5 μl of the mixture was spotted at 4-mm intervals on the

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The abbreviations used are: Fmoc, N-(9-fluorenylethoxycarbonyl); mAb, monoclonal antibody.

24634
membrane. After 20 min when the coupling was complete, the membrane was washed three times with 20 ml of amine-free N,N-dimethylformamide (Fluka), and the unreacted amino groups were blocked by acetylation with 4% (v/v) acetic anhydride in N,N-dimethylformamide for 10 min. The protecting Fmoc group was then removed from the β-lactam with 20 ml of 20% (v/v) piperidine (Fluka) in N,N-dimethylformamide for 5 min at room temperature, after which the membrane was washed 5 times with 20 ml N,N-dimethylformamide. To monitor the efficiency of amino acid coupling, the membrane was stained with 0.01% (w/v) bromphenol blue solution in N,N-dimethylformamide for 5 min before the addition of the activated amino acid. The stained membrane was washed three times with 20 ml of methanol and finally dried under a stream of cool air. During the formation of the peptide bond the color of the spot changed from blue to yellow-green. Free amine was removed from the solvents used for peptide bond formation by storing them over a molecular sieve type 4Å (Merck Ltd., Poole, UK).

**Synthesis of the Peptide Libraries**—The octapeptide libraries were synthesized on sheets of the derivatized membrane using a modified coupling cycle (12) as follows. 1) For amino acid coupling, each of the Fmoc-amino acids (Novabiochem, UK) was dissolved in 200 μl of 1-methyl-2-pyrrolidone containing 0.45 mM N-hydroxybenzotriazole and 0.36 mM N,N′-disopropylcarbodiimide. The final concentration of the activated amino acid was 0.3 mM and 0.5 μl of each activated amino acid, or a mixture of all the amino acids (except cysteine) in equimolar proportions was added with a micropipette to each spot on the membrane. After 30 min at room temperature, the membrane was washed three times with 20 ml of N,N-dimethylformamide. 2) For acetylation of the N terminus, after each coupling cycle any free amino groups were acetylated by soaking the membrane in 4% (v/v) acetic anhydride in N,N-dimethylformamide for 15 min, and the excess acetic anhydride was decanted. The membrane was washed with N,N-dimethylformamide (20 ml, three times), followed by dichloromethane (20 ml, three times), Merck Ltd.). 3) For Fmoc deprotection, the membrane was agitated for 5 min with 20 ml of 20% (w/v) piperidine in N,N-dimethylformamide, after which the piperidine was removed, and the membrane was washed with N,N-dimethylformamide (20 ml, six times). After the last amino acid had been coupled, the Fmoc protection was removed, and the free amino groups were acetylated. To deprotect the side chains, the membrane was incubated in 20 ml of 50% (v/v) trifluoroacetic acid (Fluka) and 2.5% (v/v) trisobutylsilane (Aldrich) in dichloromethane. After 1 h at room temperature, the trifluoroacetic acid solution was decanted, and the membrane was washed three times with 20 ml of dichloromethane and then with N,N-dimethylformamide (20 ml, three times) followed by methanol (20 ml, three times) and after drying was stored at 4°C.

**Immunoblotting**—Before probing the peptides with the antibody, the membrane was wetted with 20 ml of methanol and transferred into 20 ml of 0.01 m phosphate-buffered saline with 0.02% (w/v) Tween 20, pH 7.4, for 3 min. The membrane was blocked in 20 ml of 10% (v/v) newborn calf serum in 0.01 m phosphate-buffered saline with 0.02% (w/v) Tween 20, pH 7.4, for 2 h. The membrane was washed with 0.01 m phosphate-buffered saline containing 0.02% (w/v) Tween 20, pH 7.4 (20 ml, three times). The appropriate concentration of ascites fluids or purified IgG (13), containing the murine monoclonal antibody 2019 against human β-factor XIIa, was incubated with the membrane for 2 h at room temperature. The membrane was washed and finally incubated with optimized anti-mouse IgG-peroxidase conjugate in 20 ml of 0.01 m phosphate-buffered saline with 0.02% (w/v) Tween 20, pH 7.4, for 1 h at room temperature. After extensive washing with 0.01 m phosphate-buffered saline with 0.02% (w/v) Tween 20, pH 7.4, and then buffer without Tween 20 (20 ml, three times each), the membrane was subjected to signal development. The membrane was first blotted on tissue paper and rinsed in an enhanced chemiluminescent solution (0.05 mM horseradish peroxidase, pH 8.5, containing 0.4 mM 4-iodophenol (Fluka), 1.25 mM luminol (Sigma), and 2.7 mM H2O2 (Sigma) for 30 s. The membrane was wrapped with cling-film and exposed to a photographic film, Hyperfilm (Amersham, Little Charton, UK) in a light-enhancing cassette for different times, depending on signal intensity. The films were developed in Denville-74 (Kodak, Hemel Hempstead, UK) for 2.5 min at room temperature and 5 min in a fixer solution (Kodak). The film was washed with water and dried in the air. The film with the image of the spots was scanned with an Agfa Arcus Plus scanner using the Photoshop program on a Macintosh Quadra 950 computer, and the intensity of the spots was read with NIH Image.

**Results**

To identify the residues of β-factor XIIa, the protease domain of activated coagulation factor XII (9), involved in the binding of the monoclonal antibody 2019, randomized octapeptide libraries were constructed on a piece of polymer membrane without recourse to the known sequence of β-factor XIIa. The peptide mixtures were synthesized on each of a series of spots (19 × 19) on a piece of polymer membrane (Fig. 1a). The peptide libraries were constructed with residues 2 and 4 selected as the defined amino acids instead of two adjacent residues as described by others (4). The sequence of each peptide can be represented by the formula X-O2-X-O2-XX-XX (Fig. 1a). The peptides in each column were synthesized with position 4 (O4) and in each row position 2 (O2) containing one of the 19 natural amino acids (cysteine omitted) on each spot, while a mixture of all the 19 amino acids, in equimolar proportions, was used at the other positions. Thus the membrane contained approximately 1.69 × 1010 peptides in total and in each spot there were 4.7 × 107 peptides. The membrane with the coupled peptide libraries was then incubated with monoclonal antibody 2019. The bound antibody was next reacted with an anti-mouse IgG-peroxidase conjugate and this was detected using an enhanced chemiluminescent assay. The results show (Fig. 1b) that the spots with the strongest recognition contain Phe and Gln or Ile and Ile in positions 2 and 4, respectively. The sequences of the peptides and the strength of recognition is
Elucidation of an Epitope with Peptide Libraries

The strength of binding of the monoclonal antibody 201/9 for the peptide mixtures with defined amino acids at positions 2 and 4 (Fig. 1b) was determined by computer-generated gray scale readouts. The intensity of binding is presented as a gray scale (Fig. 2).

To identify all the residues involved in the binding of the antibody, a further series of iterative experiments is required. For this, it is necessary to incorporate each of the 19 amino acids at the six remaining six positions in the octapeptide sequence. To reduce the number of iterative syntheses a positional scanning technique introduced by Houghten and colleagues (14) was adopted and modified. The two amino acids, identified in the first screening library, were incorporated into the new libraries together with a defined amino acid in a third position. The two best recognized peptide mixtures (Fig. 2) were chosen for further analysis. These contained the defined positions 2 and 4 either Phe and Gln in the first peptide series or Ile at both positions in the second. To identify the remaining residues of the epitope, two sets of six positional peptide libraries, differing only in the location of the defined position, were synthesized on two sheets of derivatized membrane. On the first membrane, the peptides were synthesized in six rows containing 19 spots each, and except for Phe at position 2 and Gln at position 4, the amino acid in each of the six remaining positions was changed in successive rows. Thus, in row one, a total of 19 peptide mixtures were synthesized, and in each of the mixtures, position one was defined as one of 19 amino acids, positions 2 and 4 contained Phe and Gln, respectively, and all the other positions contained a mixture of the 19 amino acids. In row two position 3 was a single amino acid, and as in row one positions 2 and 4 contained Phe and Gln, and a mixture of 19 amino acids was used in the remaining positions. The process was repeated until all the single amino acids had been introduced into the six available positions. The peptides on the first membrane are represented as O₁-Phe-X-Gln-X-X-X-X, X-Phe-O₂-Gln-X-X-X, X-Phe-X-Gln-O₃-X-X, X-Phe-X-Gln-X-O₄-X, X-Phe-X-Gln-X-X-O₅, and X-Phe-X-Gln-X-X-X-O₆. Thus all the remaining permutations were achieved in 114 spots. Similarly, on the second membrane another 114 peptide mixtures were synthesized as follows: O₁-Ile-X-Ile-X-X-X-X, X-Ile-O₂-Ile-X-X-X-X, X-Ile-X-Ile-O₃-X-X-X; X-Ile-X-Ile-X-O₄-X, X-Ile-X-Ile-X-O₅-X, and X-Ile-X-Ile-X-X-O₆, respectively.

The libraries on the membrane were probed with the antibody 201/9 and although the antibody bound strongly to the peptides which contained Ile at positions 2 and 4 there was little or no effect when the residues in the other positions were changed (data not shown). This suggests that there was some interaction between these Ile residues and the antibody, which was independent of the amino acid sequence. In contrast, antibody binding to the peptide libraries containing Phe at position 2 and Gln at position 4 was dependent on the amino acids in the other positions (Fig. 3a–f). The peptides which contained Ala or Ser in the first position bound the antibody more strongly than those with Gly or His (Fig. 3a), as judged by the densities of the spots on the film. Peptide mixtures which contained Leu in the third position (Fig. 3b) showed the strongest antibody binding, although peptides with Val or Ile in this position also bound the antibody well. The antibody bound most strongly to the peptides with Glu, Thr, or Val in the fifth position (Fig. 3c) and to those with Phe or Asn in position six (Fig. 3d). The requirement for a specific amino acid at positions seven and eight was less than at the other positions. Although peptides with Gln or Asn at position seven (Fig. 3e) and Pro at position eight (Fig. 3f) bound the antibody most strongly, however, these three residues could be replaced by other neutral or hydrophobic amino acids without significantly altering the binding of the antibody. This suggests that either at position seven and eight the amino acid requirement is less specific than at the other positions, or more likely, that the epitope does not include these residues and that the immunodominant epitope for the monoclonal antibody 201/9 is the sequence Ser-Phe-Leu-Gln-Glu-Asn.

DISCUSSION

The membrane-based peptide libraries provide a rapid and convenient technique to study the molecular interaction of a functional protein with its ligand.
involved in the interaction can be defined without any knowledge of the primary structure. The peptide libraries were constructed on a piece of polymer membrane by an easy manual synthetic process. Typically, only 3–4 days are required for the synthesis of the octapeptide libraries by an operator with little experience of peptide chemistry. The total number of peptides required in the first scan is 361 and in the second scan 114, and since all the peptides in each scan are present on one piece of membrane, only a small amount of ligand (antibody) is required for screening. Furthermore, the libraries on the membrane could be regenerated at least 20 times without a noticeable decrease in sensitivity, thus enabling the same peptides to be probed by many different ligands.

A “one-step” positional scanning approach to identify a peptide ligand has been described (15). In this technique, the libraries were made up of peptide mixtures each with an amino acid defined at one position and the other positions containing a mixture of 19 amino acids. Only those peptides with the correct amino acid in each position gave the strongest recognition signal. However, in this procedure, since only one residue in the sequence of the octapeptide library was defined, there could be as many as 198 peptides. Thus, only one correct sequence in 197 (1 in 8.9 × 108) peptides. Thus from 5 mg of a peptide library a method is required to detect as little as 5.6 pmol peptides. Thus from 5 mg of a peptide library a method is required for screening. Furthermore, the libraries on the membrane, only a small amount of ligand (antibody) is required for screening. Furthermore, the libraries on the membrane could be regenerated at least 20 times without a noticeable decrease in sensitivity, thus enabling the same peptides to be probed by many different ligands.

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In addition to the immunodominant peptide Phe-Leu-Gln-Ala-Gln identified in a scan of overlapping peptides derived from the sequence of β-factor XIIa, there were several other peptides (Fig. 4), which were recognized less well by the monoclonal antibody 201/9. These other peptides may represent other portions of the β-factor XIIa, which also form part of the epitope. Unfortunately there are no crystallographic data to support this idea.

It is evident that the peptides identified from the scan of the sequence of β-factor XIIa and the sequence defined by the combinatorial library scan that the library has identified an immunodominant sequence. However, combinatorial libraries may not be suitable for identifying less well recognized sequences in a discontinuous epitope, either because of the very high sensitivity required or in the difficulty in interpreting very complicated patterns of recognized sequences.

The fact that the antibody does not recognize factor XII, carboxymethylated or trypsin-digested β-factor XIIa supports the hypothesis that the antibody recognizes a conformationally dependent epitope. Because trypsin cleaves the polypeptide chain (Fig. 4) between the different recognition sites and in one case inside a recognized region (peptides 90–92), this may

\[ \text{mAb (A16) which has a 50-fold higher affinity for a linear heptapeptide, based on the sequence of glycoprotein D from herpes simplex virus, in which a Met residue in the native sequence was replaced by either Phe or Tyr.} \]

A further point of similarity between the results of the library and from the scan of the overlapping peptides is that they both show that the two C-terminal residues can be replaced by a range of amino acids without compromising the recognition by the antibody (Fig. 3, e and f) and by alanine in the sequence scan (1). The peptide spots which were recognized most strongly by the antibody (Fig. 4) in the scan of the overlapping peptide sequences, were peptide numbers 144, 145, and 146, which contained the sequences Ala-Ser-Phe-Leu-Gln-Glu-Ala-Gln, Ser-Phe-Leu-Gln-Glu-Ala-Gln-Val, and Phe-Leu-Gln-Glu-Ala-Gln-Val-Pro, respectively. These peptides all shared the sequence Phe-Leu-Gln-Glu-Ala-Gln, which suggested that the epitope was determined by these six residues. Further analysis of this sequence by alanine substitution and with peptides of different sizes suggested that the core residues for the epitope was the sequence Phe-Leu-Gln.
explain why the antibody does not bind to the digested protein. It is possible that in reduced and carboxymethylated \( \beta \)-factor XIIa the immunodominant epitope may be buried or that the relative positions of the other recognized sequences are changed so that the antibody does not bind to the modified protein.

It is interesting to note that the sequence FLQEA is conserved in human, bovine (17), and in guinea pig (18) \( \beta \)-factor XIIa, but the antibody recognizes only the human protein.  

However, other parts of the sequence recognized by the antibody are not conserved (1), and there are probable differences in the secondary structure which may prevent the interaction with the antibody.

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