Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 4020–4024, April 1996
Chemistry

Mapping protein–protein interactions by affinity-directed mass spectrometry

(epitope/antibody/protein ladder)

YINGMING ZHAO*, TOM W. MUIR†, STEPHEN B. H. KENT†, ED TISCHER‡, JAN MARIAN SCARDINA‡, AND BRIAN T. CHAIT*§

*The Rockefeller University, 1230 York Avenue, New York, NY 10021; †The Scripps Research Institute, La Jolla, CA 92037; and ‡Scios Nova Inc., 2450 Bayshore Parkway, Mountain View, CA 94043

Communicated by K. Barry Sharpless, The Scripps Research Institute, La Jolla, CA, December 28, 1995 (received for review August 18, 1995)

ABSTRACT **A precise and rapid method for identifying sites of interaction between proteins was demonstrated; the basis of the method is direct mass spectrometric readout from the complex to determine the specific components of the proteins that interact—a method termed affinity-directed mass spectrometry. The strategy was used to define the region of interaction of a protein growth factor with a monoclonal antibody. A combination of proteolytic digestion and affinity-directed mass spectrometry was used to rapidly determine the approximate location of a continuous binding epitope within the growth factor. The precise boundaries of the binding epitope were determined by affinity-directed mass spectrometric analysis of sets of synthetic peptide ladders that span the approximate binding region. In addition to the mapping of such linear epitopes, affinity-directed mass spectrometry can be applied to the mapping of other types of molecule–molecule contacts, including ligand–receptor and protein–oligonucleotide interactions.**

Highly specific interactions between proteins, often within complex arrays, are critical to many cellular processes such as signal transduction and gene regulation (1, 2). A comprehensive understanding of these processes requires detailed information about such interactions at the molecular level—in particular, precise identification of the sites of molecular recognition on the protein protagonists.

Here, we report a precise and rapid method for identifying specific interaction sites in proteins. As an example, we examine the interaction of a monoclonal antibody (mAb) with a protein antigen. The basis of the method is the use of direct mass spectrometric readout from the immune complex to determine the specific component of the protein antigen that interacts with the antibody (3, 4). The strategy is shown in Fig. 1. In the first step, a set of peptide fragments is produced by enzymatic digestion of the intact protein. Proteases with known specificity are used so that the sites of cleavage can be predicted and the resulting peptides readily identified by accurate mass measurement. This step provides a facile method for generating a set of peptide fragments that span the sequence of the protein. Parallel digestion of the protein by two or more proteolytic enzymes with different specificity enables rapid production of overlapping sets of protein-spanning peptides. In the second step, the component peptides that contain the binding region of interest are affinity selected by the immobilized antibody (epitope-containing peptides bind to the antibody and are retained, whereas the remainder are washed away). In the third step, the masses of the affinity-selected peptide fragments are accurately determined—directly from the immune complex—by matrix-assisted laser desorption mass spectrometry (5–7). This technique allows the accurate (0.1–0.01%), rapid (<1 min), and sensitive (<1 pmol) determination of the molecular masses of the individual components of complex mixtures of peptides, without prior separation of the components. Peptides that are specifically bound to the antibody are identified from their accurately measured molecular masses and a knowledge of the potential digestion sites in the protein antigen. The region of the protein involved in antibody binding is deduced from the sequences of the peptides that are affinity selected by the antibody. Comparison of these sequences identifies a region of common sequence that contains a dominant component of the binding epitope. The method uses affinity purification in combination with mass spectrometry (8–12) and is termed affinity-directed mass spectrometry. The steps outlined above provide low resolution definition of the binding epitope. The precise boundaries of the binding epitope are determined by affinity-directed mass spectrometric analysis of sets of synthetic peptide ladders that span the binding region.

We have used the strategy shown in Fig. 1 to map a dominant component of the binding epitope of human basic fibroblast growth factor (bFGF) for the mouse mAb 11.1, using immunoprecipitation as the affinity-selection step.

MATERIALS AND METHODS

Protein Antigen. The experiments were performed with a recombinant form of human bFGF expressed in *Escherichia coli*. The full amino acid sequence of the predicted 155-aa translation product of human bFGF (13, 14) is: MAAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVREKSDPHIKLQLQAEERGVVSIKGVCANRYLAMKEDGRLLASKCVTDECFFERLESNNYNTYRSRKYTSWYVALKRTGQYKLGSKTGPGQKAILFLPMSAKS. The purified protein used in the present study is a mixture of two sequences: bFGF-(2–155) (i.e., protein that does not contain Met^1) and bFGF-(3–155) (i.e., protein that does not contain Met^1 and Ala^2).

mAb. mAb 11.1 is a murine IgG1 mAb raised against human recombinant bFGF (2–155). BALB/c mice were immunized with bFGF conjugated to bovine thyroglobulin via glutaraldehyde and carbodiimide. Spleen cells were fused with NS1 myeloma cells (15) and hybridomas were grown in hypoxanthine/aminopterin/thymidine selection medium. mAb 11.1 was shown to bind bFGF by Western blot analysis (data not shown), consistent with the recognition of a linear epitope. mAb 11.1 was purified by protein A chromatography (Pharmacia); for use in panning, a portion was conjugated with a biotin succinimide ester (16).

Enzymatic Proteolysis of bFGF. A 1:30 ratio (wt/wt) of protease and heat-denatured protein were dissolved in an

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Abbreviations: mAb, monoclonal antibody; bFGF, basic fibroblast growth factor.

§To whom reprint requests should be addressed.

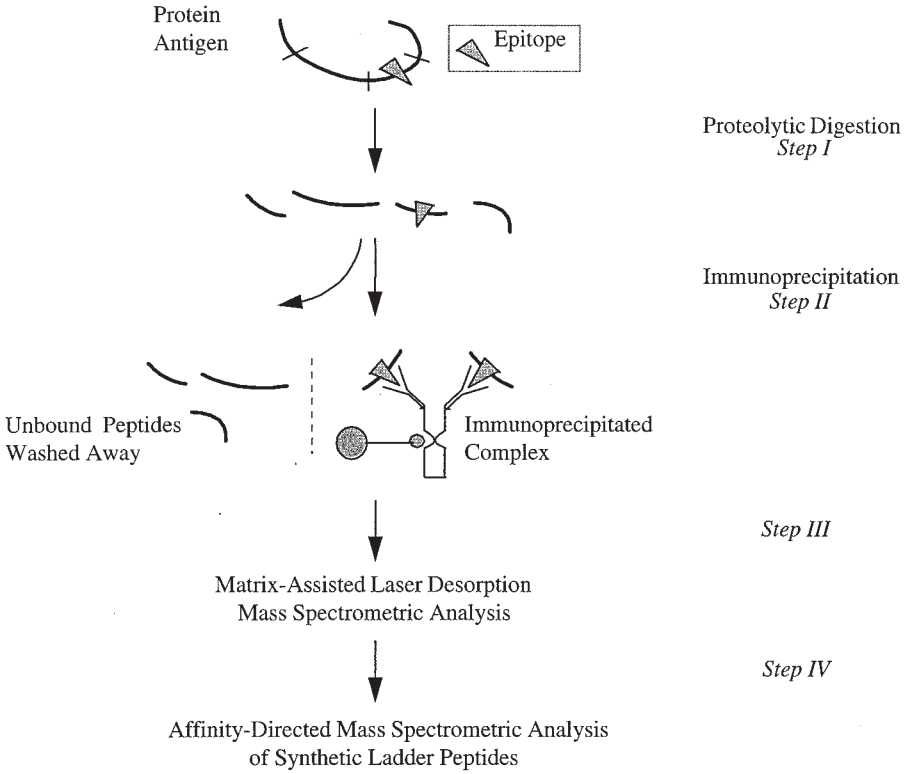

FIG. 1. Strategy for defining binding sites in a protein that interacts specifically with a mAb (see text for details). The antibody is represented by the Y-shaped symbol, and protein G/protein A agarose is represented by two circles connected by a line. The molecular mass of the peptide in the immune complex is determined by matrix-assisted laser desorption time-of-flight mass spectrometry (5–7). Under conditions of the mass spectrometric analysis, the epitope-containing peptide dissociates from the antibody and protein G/protein A agarose and is measured directly as the isolated peptide.

appropriate buffer [50 mM sodium phosphate/10 mM $CaCl_2$ (pH 8.0 for endoprotease Asp-N)/50 mM Tris·HCl/1 mM EDTA (pH 8.0 for endoprotease Lys-C)] with protein concentration between 10 and 20 μM. The resulting solution was incubated at 37°C for 2 hr. The digestions were terminated by addition of a one tenth volume of 10 mM Pefablock Sc (Boehringer Mannheim) solution (25°C for 10 min), followed by heating at 90°C for 15 min.

Immunoprecipitation. The immunoprecipitation procedure was modified from the conventional protocol (17). First, *N*-octyl glucoside replaced SDS or Triton X-100 as a detergent to reduce nonspecific molecule–molecule interactions (10). This proved important for successful coupling between immunopurification and matrix-assisted laser desorption mass spectrometry because SDS and Triton X-100 severely suppress the mass spectrometric ion response. Second, a lower amount of protein G/protein A agarose (1–3 μl) is used because only 1 μl of sample can be loaded onto the mass spectrometer probe for each analysis. mAb (2–10 μg; i.e., 13–70 pmol) and the mixture of peptides produced by the proteolytic digestion (about 20 pmol) were mixed in 60 μl of TSO solution (75 mM Tris·HCl/200 mM NaCl/0.5% *N*-octyl glucoside, pH 8.0). After 2-hr incubation at 4°C with gentle stirring, 1–3 μl of protein G/protein A agarose was added to the solution and incubated for another 0.5 hr at 4°C. The agarose beads were collected by carefully aspirating the supernatant after centrifugation of the solution for 1 min at 16,000 × *g*. The beads were washed three times with 200 μl of TSO buffer and then three times with 200 μl of TSMK buffer (10 mM Tris·HCl/200 mM NaCl/5 mM 2-mercaptoethanol, pH 8.0). The laser desorption matrix solution {4 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid (18) in 1% aqueous trifluoroacetic acid/acetonitrile [1:1 (vol/vol)]} was added to the washed beads. Finally, 0.5–1 μl of the matrix/agarose bead mixture containing the bound peptides was loaded onto the mass spectrometer probe and dried at room temperature with a stream of air for mass spectrometric analysis.

Mass Spectrometry. Mass spectrometric analysis was carried out on a laser desorption time-of-flight instrument constructed at The Rockefeller University (5). To identify peptides bound to the antibody, the entire complex consisting of protein G/protein A agarose, antibody, and the bound peptide was subjected to mass spectrometric analysis. The mass spectra were collected by adding individual spectra obtained from 200 laser shots and were mass calibrated using dynorphin A-(1–13), oxidized insulin B-chain, and glucagon-like peptide-(17–37).

Peptide Synthesis. Solid phase peptide synthesis (19) was carried out manually as described (20). To synthesize the N-terminal peptide ladder pool, an equal portion of peptide resin was removed from the reaction vessel after the addition of each amino acid residue. The 15 resulting peptide resin samples were mixed, deprotected, and then subjected to HF cleavage. The resulting peptides were used for analysis without further purification. To synthesize the C-terminal ladder peptides, an equal portion of resin containing the leader peptide was added after each cycle of the synthesis. The mixed peptide-resin product contained peptides of all possible lengths from the C-terminal amino acid residue and was deprotected and HF cleaved to produce the C-terminal peptide ladder pool.

Construction of the Phage Library. The phage display library was constructed with the M13mp19 vector. By *in vitro* mutagenesis, *Kpn* I and *Eag* I restriction sites were introduced at the end of the gene 3 signal sequence. The *Kpn* I site in the polylinker was previously deleted. To construct the phage library with random hexapeptides, two synthetic oligonucleotides were used: sense 5′-CT TTC TAT TCT CAC TCC GCT

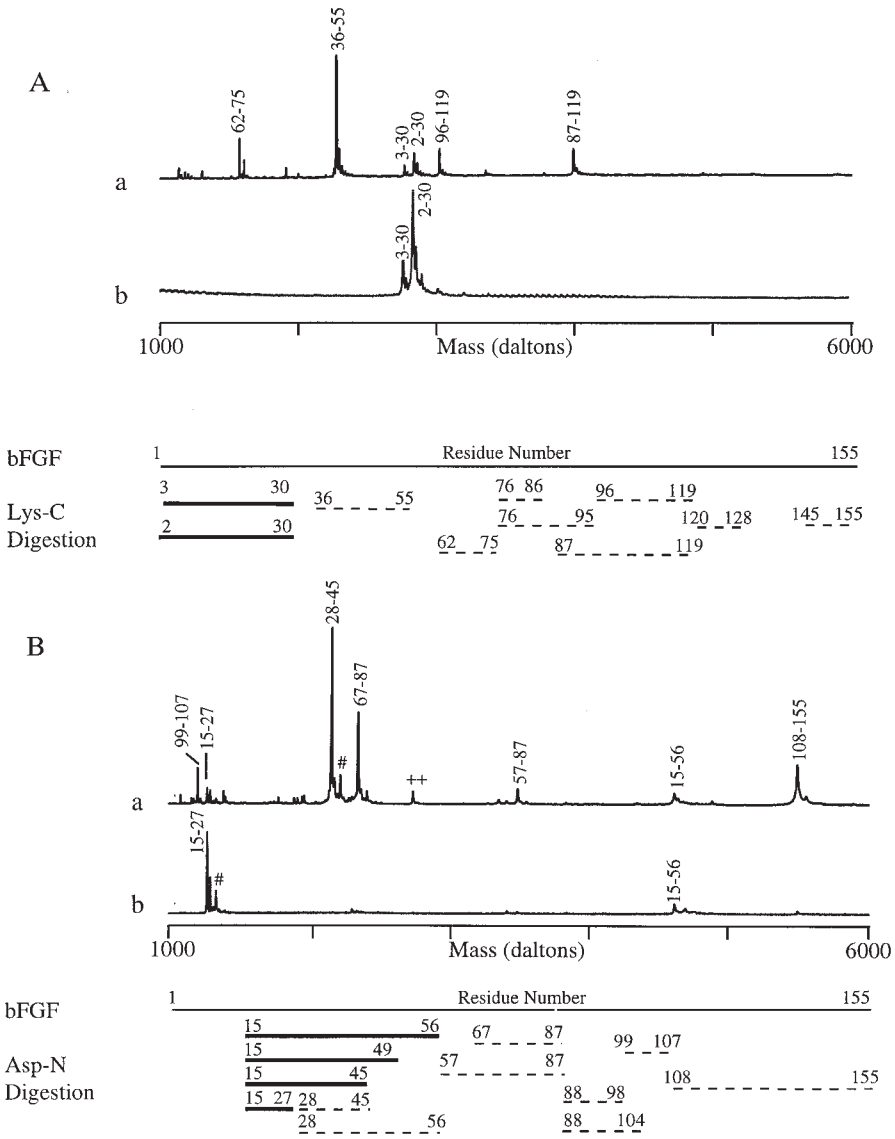

FIG. 2. Matrix-assisted laser desorption mass spectrometric readout of peptide fragments produced by enzymatic digestion of bFGF before and after immunoprecipitation with anti-bFGF mAb 11.1. (*A*) Peptide fragments produced by endoproteinase Lys-C enzymatic digestion of bFGF before (spectrum a) and after (spectrum b) immunoprecipitation with anti-bFGF mAb 11.1. (*A*) (*Lower*) Diagrammatic representation of the observed peptide binding by anti-bFGF mAb 11.1. The 155-aa residue protein bFGF is represented by a thin line. The thick lines represent peptide fragments that are bound by the antibody and dashed lines those that are not bound by the antibody. The numbers above the lines identify the sequence positions of the bFGF fragments. (*B*) Results of a parallel experiment using endoproteinase Asp-N. #, Peaks from adventitious Cu adduction (18); ++, peaks from a doubly charged peak. Given below are the experimentally determined molecular masses of peptide fragments produced by proteolytic digestion of bFGF together with the deviations from the molecular masses calculated for the fragments (units = Da). Endoprotease Lys-C: 120–128 (1130.0, −0.3), 145–155 (1177.1, −0.4), 76–86 (1225.1, −0.4), 62–75 (1569.9, +0.1), 76–95 (2196.0, +0.4), 36–55 (2270.4, +0.8), 2–30 (2836.8, +0.6), 3–30 (2765.6, +0.5), 96–119 (3021.3, −1.0), 87–119 (3991.8, −0.7). Endoprotease Asp-N: 88–98 (1161.9, −0.5), 99–107 (1205.2, −0.2), 15–27 (1272.9, −0.5), 88–104 (1950.9, −0.4), 28–45 (2161.7, +0.1), 67–87 (2352.6, −0.2), 28–56 (3360.7, −0.2), 15–45 (3417.4, +0.5), 57–87 (3497.0, −0.1), 15–49 (3843.9, −0.5), 15–56 (4615.6, −0.6), 108–155 (5490.6, −0.7).

GAA NNG/T NNG/T NNG/T NNG/T NNG/T NNG/T CCG CCG CC and antisense 5′-GGC CGG CGG CGG III III III III III III TTC AGC GGA GTG AGA ATA GAA AGG TAC (where I is deoxyinosine). These oligonucleotides maintain the signal sequence, keep the Ala and Glu at positions +1 and +2 of the wild-type mature gene 3, and introduce six random amino acids followed by three prolines. The two oligonucleotides were annealed and then ligated into the *Kpn* I/*Eag* I cut phage vector. Ligated DNA was electroporated into MC 1061 *E. coli* using 0.56-mm cuvettes for 10 msec at 600 volts. The bacteria were grown in 1 liter of medium for 5 hr at 37°C and the phage were again amplified in JM103 for an additional 5 hr at 37°C. The library was titered at 10^7 phage per μg DNA and contained a total of 1.5×10^{15} phage particles. Sequences of single phage isolates indicated that 90% of the phage had inserts.

Screening of the Phage Display Library. An aliquot of 10^{11} phage was mixed with 2 μg of purified biotinylated antibody in 100 μl of TBS and incubated overnight at 4°C. The phage were diluted to 1 ml in TBS/0.05% Tween 20 before panning on streptavidin-coated 60-mm dishes for 30 min at room temperature as described (21). After removing the phage, dishes were washed 10 times with TBS/0.05% Tween 20. Bound phage were eluted with 0.1 M HCl·glycine (pH 2.2)/1 mg of bovine serum albumin per ml and were immediately neutralized with 2 M Tris base. Eluted phage were amplified in *E. coli* JM 103 grown in 25 ml of J broth for 5 hr and were carried through two additional rounds of panning. Phage were then plated for single plaques and 46 isolates were sequenced.

RESULTS

Affinity-Directed Mass Spectrometric Analysis of Digested bFGF. Fig. 2*A* (spectrum a) shows the mass spectrum of a set of peptides produced by partial digestion of bFGF with endoproteinase Lys-C. Each peak corresponds to a peptide fragment, with only the more abundant peaks labeled. The identification of the peptides is based on their accurately determined molecular masses, the protein sequence, and the known cleavage specificity of endoproteinase Lys-C (C-terminal to lysine residues). The principal components of the digest were assigned to 10 different bFGF peptide fragments indicated on the linear representation of the sequence (Fig. 2*A Lower*).

After immunoprecipitation of the peptide mixture with anti-bFGF mAb 11.1, the entire complex consisting of protein G/protein A agarose, antibody, and the bound peptides was mixed with matrix solution and subjected to mass spectrometric analysis (Fig. 2*A*, spectrum b). Peaks corresponding to peptides that had been bound to the antibody were directly observed in the mass spectrum because the matrix-assisted laser desorption/ionization conditions used in the present experiments cause dissociation of the peptide–antibody complex. This dissociation may occur in the acidified matrix and/or during the desorption/ionization process (10). Only two peptides were identified in the immunoprecipitated complex by matrix-assisted laser desorption mass spectrometry (Fig. 2*A*, spectrum b). Examination of the binding and nonbinding peptides suggests that a binding region of bFGF for mAb 11.1 is located within peptide fragment 3–30 (Fig. 2*A Lower*).

In a parallel experiment, bFGF was digested by endoproteinase Asp-N, which cleaves amino-terminal to aspartic acid residues. Twelve peptide fragments were identified in the mass spectrum of the resulting mixture (Fig. 2*B*, spectrum a). After immunoprecipitation with mAb 11.1, the unbound peptides present in the original mixture were washed away, and four peptides (bFGF 15–27, 15–45, 15–49, and 15–56) were identified in the immunoprecipitated complex (Fig. 2*B*, spectrum b). This pattern of peptide attachment suggests that the binding region of bFGF for mAb 11.1 can be further localized to residues 15–27 (Fig. 2*B Lower*), within the 3–30 region identified above. Thus, the sequence bFGF 15–27 contains a principal component of the binding site for the mAb 11.1.

Affinity-Directed Mass Spectrometric Analysis of Synthetic Peptide Ladders. To more precisely define the binding epitope within residues 15–27, we prepared two peptide ladders by stepwise solid phase peptide synthesis (20). The design of these two peptide ladders is shown in Fig. 3. The N-terminal ladder consists of a nested set of peptides with all possible truncations of residues 15–27 at the N-terminal (Fig. 3 *Upper*) and the C-terminal ladder, a corresponding set of peptides with all possible truncations at the C-terminal (Fig. 3 *Lower*). A highly basic leader peptide (ε-NH_2-caproyl-RLKLKAR) was incorporated at the C-terminal of each peptide to ensure intensive and uniform mass spectrometric response for the different components of the peptide ladders. Two additional residues (D^{28} and P^{29}) of bFGF and the spacer residue ε-NH_2 caproic acid were included in the ladders to prevent possible ambiguities that could arise at the junction between the epitope and the leader sequence. The mass differences between consecutive peaks each correspond to an amino acid residue, and their order of occurrence in the data set defines their sequence in the peptide chain (Fig. 3, traces a and d) (22).

After immunoprecipitation of the N- and C-terminal ladder peptides with mAb 11.1, only some specific components of the ladder mixtures were observed to bind to the antibody (Fig. 3, traces b and c). This pattern of binding provides a direct readout of the epitope because only peptide components long enough to contain the full epitope bind to the antibody. Inspection of the spectral traces shows that residues

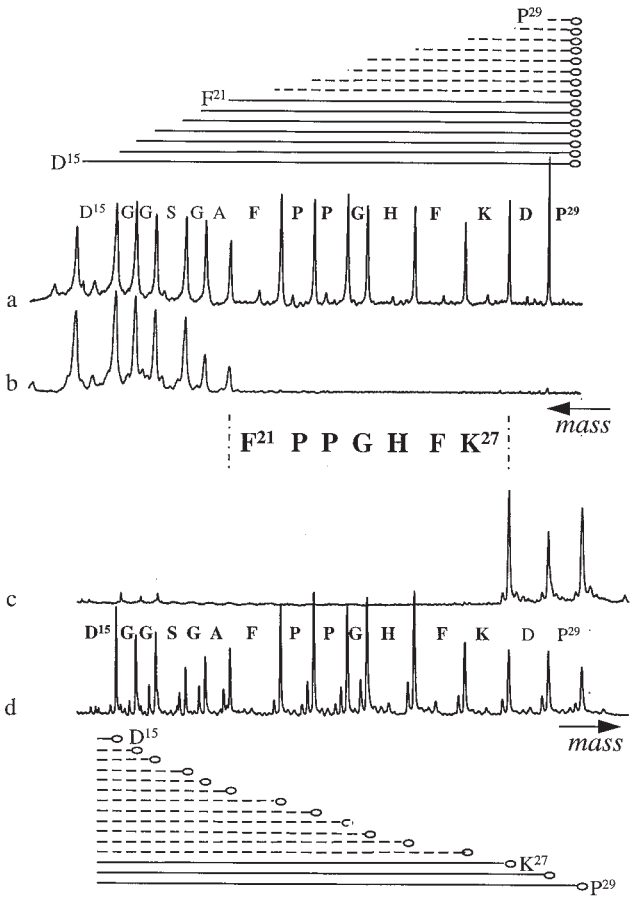

FIG. 3. Mass spectrometric readout of ladder peptides before (*a* and *d*) and after (*b* and *c*) immunoprecipitation. The length of the line is proportional to the number of amino acid residues in the peptide. The elliptical symbol designates the leader peptide, ε-NH_2-caproyl-RLKLKAR. The bold lines designate peptides that bind to the antibody and dashed lines peptides that do not bind to the antibody. The small satellite peaks close to the main peptide peaks arise from Na or Cu adduction to the peptides (18) or dehydration side-reactions produced during synthesis. Epitope-defining residues are indicated by boldface letters at the center of the diagram.

$F^{21}PPGHFK^{27}$ are required for tight binding to mAb 11.1 and therefore constitute the dominant component of the binding site on bFGF.

Epitope Mapping Using the Phage Display Library. It is of interest to compare the present result with a determination that uses the phage display peptide library approach (23–25). The results obtained by screening a random hexapeptide library composed of 1.5×10^{15} phage particles with mAb 11.1 are summarized in Table 1. All the sequences that were selected by binding to mAb 11.1 included glycine in the third position and lysine in the sixth position. Half of the 46 individual clones were sequenced as PPGXXK, aligning with the bFGF sequence $P^{22}PGHFK^{27}$. This binding sequence coincides with six of the seven residues determined with the affinity-directed mass spectrometric approach ($F^{21}PPGHFK^{27}$). The main difference in the epitope deduced from these two approaches relates to F^{21}. The sequence FPPGXX was not observed in the phage display experiment. This can be explained by noting that the phage display experiment used a hexapeptide library; if K^{27} adds considerably more to the binding strength of the epitope than does F^{21}, then the hexapeptides starting FPP. . . will not be selected. We tested this hypothesis by titration of the peptide ladder mixtures against the mAb 11.1, using the affinity-directed mass spectrometric readout (data not shown). The results of this analysis demonstrated that K^{27} indeed contributes

Table 1. Peptide sequences of phage clones affinity purified by biotinylated anti-bFGF mAb 11.1

Isolates	Frequency	Peptide sequences
1	16	P-P-G-f-m-K
2	7	P-P-G-m-h-K
3	7	P-v-G-f-F-K
4	5	P-P-G-f-F-K
5	3	P-a-G-l-w-K
6	2	P-v-G-H-F-K
7	2	P-P-G-l-m-K
8	1	P-f-G-l-m-K
9	1	l-P-G-f-y-K
10	1	P-a-G-H-s-K
11	1	P-P-G-i-F-K
bFGF sequence 22–27		**P-P-G-H-F-K**

Deduced binding epitope in bFGF is given in boldface type. Uppercase letters represent positions that match the bFGF sequence 22–27. Lowercase letters represent amino acids that vary from this sequence.

more strongly to the binding to the antibody than does F^{21} and that the method can readily resolve peptides with different binding affinities.

DISCUSSION

Our results show that proteolytic digestion and affinity-directed mass spectrometry can be used to rapidly determine the approximate location of a continuous component of a binding epitope within a protein ligand. If it is desired to explore the binding of several antibodies against a single protein, the immunoprecipitation step can be conveniently carried out in parallel with the set of antibodies. The rapidity of the mass spectrometric analysis (typically only a few minutes per spectrum) allows the approximate location to be determined for several such epitopes in a single day. Synthesis and affinity-directed mass spectrometric analysis of peptide ladders containing up to 20 aa can be achieved in little more time than is required to make a single peptide (1–2 days). Hence, the present approach allows the precise definition of a linear binding epitope for a specific antibody to a short stretch of protein (typically, 6–20 residues, depending on the available proteolytic digestion sites) in a single day and more accurate definition within one week. For appropriate applications, the procedure should be faster than, or competitive with, the current approaches (26–30). It differs from library-based approaches in that (*i*) only the natural sequence is explored in the search for a binding epitope and (*ii*) long epitopes can be investigated with little additional effort compared with short epitopes. We have successfully applied affinity-directed mass spectrometry for epitope mapping to three other antibodies [anti-melittin mAb 83144, anti-glucagon like peptide-1 7–37 mAb 26.1 (10), and anti-adenovirus-2 mAb Dav-1 (data not shown)] with binding affinities in the range 10^{-6}–10^{-9} M, indicating that the present method should have quite general applicability to the definition of linear epitopes.

In addition to the mapping of linear epitopes, affinity-directed mass spectrometry can be applied to the mapping of other types of specific molecule–molecule contacts, including ligand–receptor and protein–oligonucleotide interactions. For these studies, the protein under investigation is either partially digested (to explore domain interactions) or extensively digested (to explore linear sequence interactions). In a manner analogous to the above-described mAb example, immobilized ligand or oligonucleotide can be used to affinity purify the binding protein fragments. The sequences of the affinity-selected domains or peptide fragments are then identified by the known digestion sites in the protein and the accurately measured molecule masses. Alternatively, where appropriate, the enzymatic digestion can be carried out on the protein in the bound complex (31).

It has been predicted that the human genome will have been sequenced in large part within the next decade. The resulting wealth of sequence information will leave a monumental amount of protein chemistry to be sorted out, with particularly pressing needs in the definition of protein–protein and protein–ligand interactions. The high specificity and speed of affinity-directed mass spectrometry make it a promising tool for the elucidation of such interactions.

This work was supported in part by grants from the National Institutes of Health (RR00862 and GM38724).

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