Anal. Chem. **1994**, *66*, 3723–3726

Protein Epitope Mapping By Mass Spectrometry

Yingming Zhao and Brian T. Chait*
The Rockefeller University, 1230 York Avenue, New York, New York 10021

A mass spectrometric method is described for the rapid mapping of linear epitopes in proteins that are bound by monoclonal antibodies. The method consists of three steps. In the first step, an antigen protein is digested by a proteolytic enzyme to produce an appropriate set of peptide fragments. In the second step, peptide fragments containing the linear epitope are selected and separated from the pool of peptide fragments by immunoprecipitation with the monoclonal antibody. In the final step, the immunoprecipitated peptides are identified by matrix-assisted laser desorption mass spectrometry. The method allows the rapid determination of antigenic sites without tedious peptide synthesis or protein mutagenesis. The approach is demonstrated through the mapping of epitopes in two peptides (melittin and glucagon-like peptide-1 7–37) against which monoclonal antibodies were raised. In addition to epitope mapping, the successful coupling between matrix-assisted laser desorption mass spectrometry and immunoprecipitation provides a potentially powerful tool for determining binding sites between proteins.

A monoclonal antibody (mab) raised against a protein binds only to a specific region of the protein, which is called the antigenic site or epitope. An antibody can bind either a linear (continuous) epitope or a nonlinear (conformational) epitope.[1-3] A linear epitope contains a stretch of contiguous amino acids (typically 5–10 amino acid residues) in the antigen, whereas a nonlinear epitope is composed of residues that can be distant in the primary sequence but close in space in the folded protein. A knowledge of the binding site of an antigen protein to its antibody can improve the utility of the antibody.

Techniques that have been used for antigenic site mapping of linear epitopes include binding assays of sets of synthetic peptides that span the protein[4,5] and constructs produced by recombinant gene expression.[6,7] More recently, epitope localization has been achieved through the use of bacteriophage peptide libraries.[9-11] Although effective, these methods can be costly and time-consuming. A different approach to antigenic site mapping has been reported by Przybylski and co-workers,[8] who compared the pattern of proteolytic digestion of free peptide antigen with the pattern produced from the antigen bound to an antibody. Alternatively, these workers subjected the peptide to proteolytic digestion and identified products that bound to the immobilized antibody. In both cases, the peptides of interest were identified by ^{252}Cf plasma desorption mass spectrometry.

Matrix-assisted laser desorption mass spectrometry (MALDI-MS) is a recently developed method for measuring the molecular weights of peptides and proteins.[12-14] The technique allows the accurate (better than 0.1%), rapid (<1 min), and sensitive (<1 pmol) determination of the molecular weights of components of complex mixtures of peptides. MALDI-MS is finding wide use for the rapid identification of proteins and the elucidation of their primary structures (in particular, the definition of posttranslational modifications.)

Here, we describe a method for the rapid mapping of linear protein epitopes that are bound by monoclonal antibodies. The method, which takes advantage of the purification power of immunoprecipitation and the high specificity and speed of MALDI-MS, consists of three steps. In the first step, an antigen protein is digested by a proteolytic enzyme to produce an appropriate set of peptide fragments. In the second step, peptide fragments containing the linear epitope are selected from the pool of peptide fragments by immunoprecipitation with the monoclonal antibody. In the final step, the immunoprecipitated peptides are identified by MALDI-MS and the antibody-binding peptide region is determined.

EXPERIMENTAL SECTION

Materials. Melittin was purchased from the Sigma Chemical Co. (St. Louis, MO) and used without further purification. Sequence-grade chymotrypsin, endoprotease Lys-C, and serine protease inhibitor Pefabloc Sc were purchased from Boehringer Mannheim Biochemical (Indianapolis, IN). Protein G/protein A agarose was obtained from Oncogene Science (Uniondale, NY). Antimelittin monoclonal antibody No. 83144 is mouse IgG1 subtype antibody generously provided by Dr. T. P. King of the Rockefeller University. Glucagon-like peptide-1 7–37 (GLP-1 7–37) and antiGLP-1 7–37 No. 26.1 mouse monoclonal antibody were kindly provided by Dr. Douglas Buckley of Scios Nova (Mountain View, CA).

Digestion of Peptides. A 1:100 ratio (w/w) of protease and peptide was dissolved in an appropriate buffer (100 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.0 for chymotrypsin; 50 mM Tris-HCl, 1 mM EDTA, pH 8.0 for endoprotease Lys-C) with peptide concentration between 10 and 20 μM. To obtain a partial digest of the peptide, the mixture was maintained

(1) Jin, L.; Fendly, B. M.; Wells, J. A. *J. Mol. Biol.* **1992**, *226*, 851–865.
(2) Lane, D. P.; Stephen, C. W. *Curr. Opin. Immunol.* **1993**, *5*, 268–271.
(3) Harlow, E.; Lane D. *Antibodies, a laboratory manual*; Cold Spring Laboratory: Cold Spring Harbor, NY, 1988; pp 23–35.
(4) Wang, Z.; Laursen, R. A. *Pept. Res.* **1992**, *5*, 275–280.
(5) Geysen, H. M.; Meloen, R. H.; Barteling, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3998–4002.
(6) Dias, P.; Parham, D. M.; Shapiro, D. N.; Tapscott, S. J.; Houghton, P. J. *Cancer Res.* **1992**, *52*, 6431–6439.
(7) Chen, J.; Marechal, V.; Levine, A. J. *Mol. Cell. Biol.* **1993**, *13*, 4107–4114.
(8) Suckau, D.; Kohl, J.; Karwath, G.; Schneider, K., Casaretto, M.; Bitter-Suermann, D.; Przybylski, M. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9848–9852.
(9) Devlin, J. J.; Panganiban, L. C.; Devlin, P. E. *Science* **1990**, *249*, 404–406.
(10) Scott, J. K.; Smith, G. P. *Science* **1990**, *249*, 386–390.
(11) Cwirl, S. E.; Peters, E. A.; Barrett, R. W.; Dower, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 6378–6382.
(12) Hillenkamp, F.; Karas, M.; Beavis, R. C.; Chait, B. T. *Anal. Chem.* **1991**, *63*, 1193–1203.
(13) Beavis, R. C.; Chait, B. T. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 6873–6877.
(14) Chait, B. T.; Kent, S. B. H. Science **1992**, *257*, 1885–1894.

0003-2700/94/0366-3723$04.50/0

at 37 °C for 5–10 min. Alternatively, to obtain a complete digest of the peptide, the peptide was mixed with the appropriate protease in a 1:30 ratio (w/w) and digested at 37 °C for 2 h. The digestions were terminated by addition of a one-tenth volume of 10 mM Pefablock Sc solution (25 °C; 10 min), followed by heating at 90 °C for 15 min.

Immunoprecipitation.[15] Monoclonal antibody (2–10 μg) and the mixture of peptides produced by the proteolytic digestion (10–50 pmol) were mixed in 60 μL of 75 mM Tris-HCl, 200 mM NaCl, 0.1–0.5% *n*-octyl glucoside, pH 8.0 (TSO) solution. After a 2 h incubation at 4 °C with gentle stirring, 2–3 μL of protein G/protein A agarose was added to the solution and incubated for another 0.5 h at 4 °C. The agarose beads were collected by carefully aspirating the supernatant after centrifugation of the solution for 1 min at 16000*g*. The beads were washed three times with 200 μL of TSO buffer and then three times with 200 μL 10 mM Tris-HCl, 200 mM NaCl, 5 mM β-mercaptoethanol, pH 8.0 (TSM). The laser desorption matrix (4 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid in 1% aqueous TFA/ACN (2:1, (v/v)) and a suitable amount of internal standard peptide (dynorphin A 1–13) were mixed with the washed beads. Finally, 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 MALDI-MS analysis.

Mass Spectrometry. MALDI-MS analysis was carried out on a laser desorption time-of-flight instrument constructed at the Rockefeller University and described elsewhere.[16] The entire triple complex consisting of proteinG/protein A agarose, antibody, and the bound peptide was subjected to mass spectrometric analysis. Intense peaks corresponding to the peptides that were bound to the antibodies were observed in the mass spectra because the MALDI-MS conditions used in the present experiments cause the peptide to dissociate from the antibody. This dissociation may occur in the acidified matrix and/or during the MALDI process. The mass spectra were collected by adding individual spectra obtained from 200 laser shots to improve the statistics of the measurement. The spectra were initially calibrated using dynorphin A 1-13 and oxidized insulin B-chain. Subsequent calibrations were made using either the undigested parent peptide and dynorphin A 1-13 or an external calibrant.

RESULTS AND DISCUSSION

Bee venom melittin and GLP-1 7–37 were selected as model systems for this study because monoclonal antibodies were available that bind known linear regions in these peptides. Antimelittin monoclonal antibody No. 83144 was previously determined to bind to an epitope located at residues 20–26 of melittin,[17,18] and antiGLP-1 7–37 monoclonal antibody No. 26.1 was previously determined (by an investigation of the binding of a series of synthetic peptide analogs) to bind a region that included the first three N-terminal residues of GLP-1 7–37 (personal communication from Dr. Douglas Buckley, Scios Nova, Mountain View, CA).

(15) Harlow, E.; Lane D. *Antibodies, a laboratory manual*; Cold Spring Laboratory: Cold Spring Harbor, NY, 1988; pp 421–470.
(16) Beavis, R. C.; Chait, B. T. *Anal. Chem.* **1990**, *62*, 1836–1840.
(17) King, T. P.; Kochoumian, L.; Joslyn, A. *J. Immunol.* **1984**, *133*, 2668–2673.
(18) Fehlner, P. F.; Berg, R. H.; Tam, J. P.; King, T. P. *J. Immunol.* **1991**, *146*, 799–803.

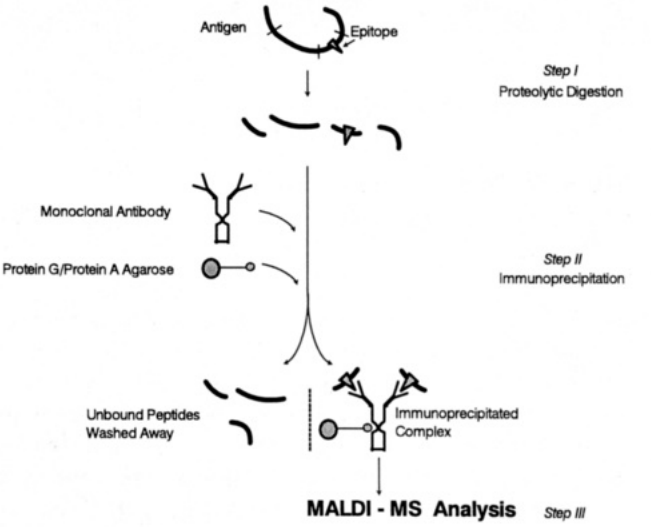

Figure 1. Three-step strategy for linear epitope mapping.

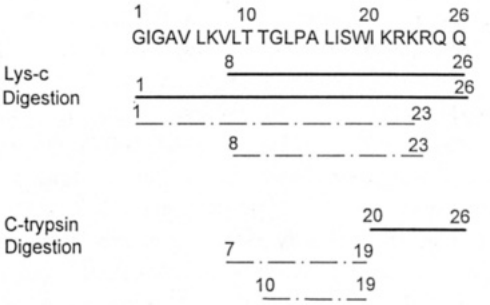

Figure 2. Amino acid sequence of melittin. The peptide fragments produced by endoprotease Lys-C and chymotrypsin digestion are shown by lines. The solid lines represent those peptide fragments that were bound by the antimelittin mab No. 83144 and the dashed lines those that were not bound by mab No. 83144.

Epitope mapping is carried out in three steps (Figure 1). *Step 1*: The antigen peptide is digested by a highly specific protease to produce a mixture of component peptide fragments. The identities of these peptide fragments can be determined by accurate measurement of their molecular weights using MALDI-MS. *Step 2*: Peptide fragments that contain the antigenic epitope are purified from the peptide fragment mixture by immunoprecipitation with the monoclonal antibody against the antigen peptide. In this step, peptides that contain the epitope, form complexes with the antibody–proteinG/protein A agarose. Other peptides fragments in the mixture that do not bind to the antibody remain in solution and can be removed by washing. *Step 3*: The molecular weights of the peptides that are bound by the antibody are determined by MALDI-MS. The identity of the antibody-binding peptides are readily assigned by their molecular weights and the known digestion sites of the specific protease.

Epitope Mapping of Melittin. Melittin is a 26 amino acid residue peptide isolated from bee venom (Figure 2). Partial digestion of melittin by endoprotease Lys-C yielded four peptide fragments that gave intense mass spectral peaks. The molecular weights of these peptides were found to correspond (within <1 Da; see Table 1) to the molecular weights predicted for melittin fragments 8–23, 8–26, 1–23, and 1–26 (Figure 3A). The other two peaks in the spectrum (designated i_1 and i_2 in Figure 3A) arise from unidentified impurities that were present in the melittin sample. After immunoprecipitation

Table 1. Measured and Calculated Values of the Molecular Weights of Peptide Fragments Produced by Proteolytic Digestion of Melittin

peptide fragment	calcd MW	measd MW	Δ mass (Da)
	Endoprotease Lys-C Digestion		
8–23	1796.2	1796.1	–0.1
8–26	2208.7	2208.0	–0.7
1–23	2435.1	2435.0	–0.1
	Chymotrypsin Digestion		
20–26	956.2	956.0	–0.2
10–19	1058.3	1058.2	–0.1
7–19	1398.7	1398.9	+0.2

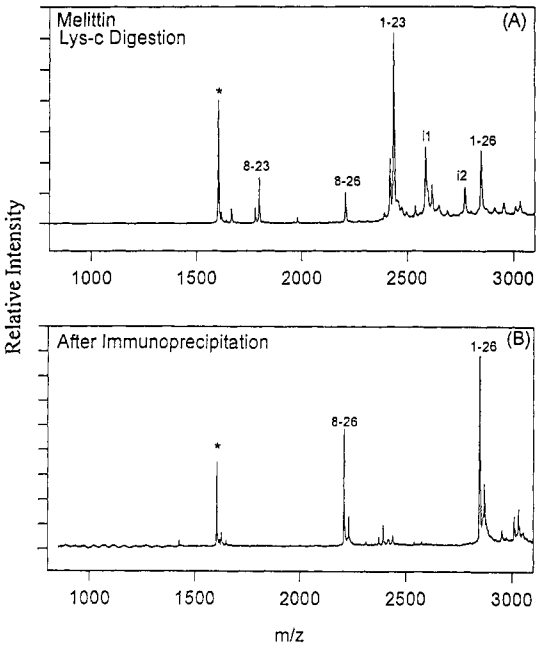

Figure 3. Matrix-assisted laser desorption mass spectra of (A) peptide fragments produced by endoprotease Lys-C digestion of melittin and (B) peptide fragments isolated by immunoprecipitation with mab No. 83144. The peak labeled with an asterisk corresponds to the internally added calibrant dynorphin A 1–13, and I_1 and I_2 refer to unidentified impurities.

with antimelittin mab No. 83144, the two impurities and peptide fragments 1–23 and 8–23 were washed away, while fragments 1–26 and 8–26 were identified in the immunoprecipitation complex (Figure 3B). As a control, we incubated the set of melittin peptide fragments with an unrelated anti-human basic fibroblast growth factor monoclonal antibody and treated the resulting solution in a manner identical to that described above. No significant peptide peaks was found in the mass spectrum of this antibody complex (data not shown). The experiment demonstrated that peptide selection is specified by the antibody used in the immunoprecipitation.

The results obtained in Figure 3 (summarized diagramatically in the upper portion of Figure 2) indicate that residues 1–7 are not required for antibody binding but that one or more of residues 24–26 is required. To further define the antigenic site, the melittin sample was digested by chymotrypsin. Three peptides were produced, which correspond to melittin fragments 20–26, 7–19, and 10–19 (Table 1 and Figure 4A), as well as an unidentified impurity (Figure 4A). The peptide fragment 1–7 was not observed in the mass spectrum. In this regard, we note that MALDI-MS sometimes does not produce a detectable response for small peptides.

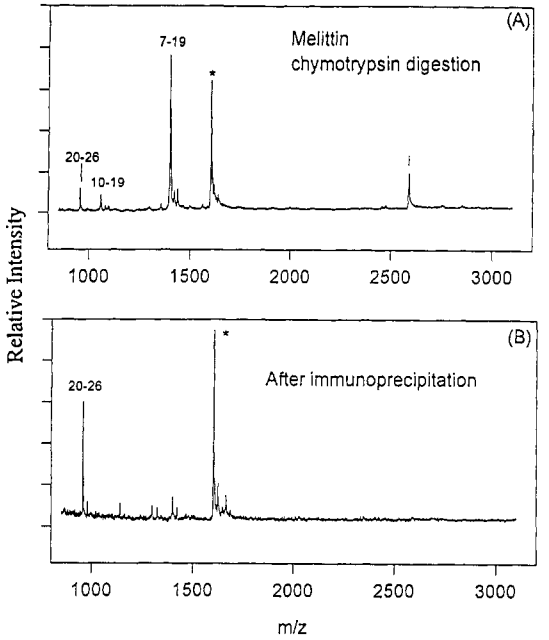

Figure 4. Matrix-assisted laser desorption mass spectra of (A) peptide fragments produced by chymotrypsin digestion of melittin and (B) peptide fragments isolated by immunoprecipitation with antimelittin mab No. 83144. The peak labeled with an asterisk corresponds to the internally added calibrant dynorphin A 1–13, and I refers to an unidentified impurity.

Upon immunoprecipitation with mab No. 83144, fragment 20–26 was selected by the antibody (Figure 4B) while fragments 7–19 and 10–19 were almost completely washed away (i.e., the intensity of fragment 7–19 relative to fragment 20–26 in Figure 4B was reduced by a factor of 34 compared to the ratio of peak intensities observed in the original peptide mixture (Figure 4A)). These findings are summarized in the lower portion of Figure 2.

Inspection of the combined data (Figure 2) obtained from the experiments shown in Figures 3 and 4 demonstrates that the region encompassing residues 20–26 is sufficient for mab No. 83144 binding. The present result is in agreement with a previous determination of the binding epitope that used a competitive binding assay of chromatographically separated peptic and tryptic fragments of melittin.[17,18] In this earlier study, the binding affinity of mab No. 83144 was determined to be approximately 10^{-6} M,[17,18] which is weaker than that of most commonly used antibodies (typical range 10^{-7}–10^{-10} M). Thus the method should be applicable to the mapping of linear epitopes for most antibodies.

Epitope Mapping of GLP-1 7–37. As a further test of the present methodology, we mapped the antigenic site of GLP-1 7–37 for antiGLP-1 7–37 mab No. 26.1. The binding affinity of this antibody is much higher than that of mab No. 83144 described above. GLP-1 7–37 was subjected to partial digestion by chymotrypsin. Five major peaks were identified in the mass spectrum, which correspond to GLP-1 7–37 fragments 1–13, 1–22, 1–25, 1–31, and 14–22 (Figure 5A). After immunoprecipitation with antiGLP-1 7–37 mab No. 26.1, fragment 14–22 and the impurity were washed away, while fragments 1–13, 1–22, 1–25, and 1–31 remained bound to the antibody (Figure 5B). A summary of the binding patterns of the chymotryptic peptides (Figure 5C) shows that the antigenic epitope resides within residues 1–13 of GLP-1

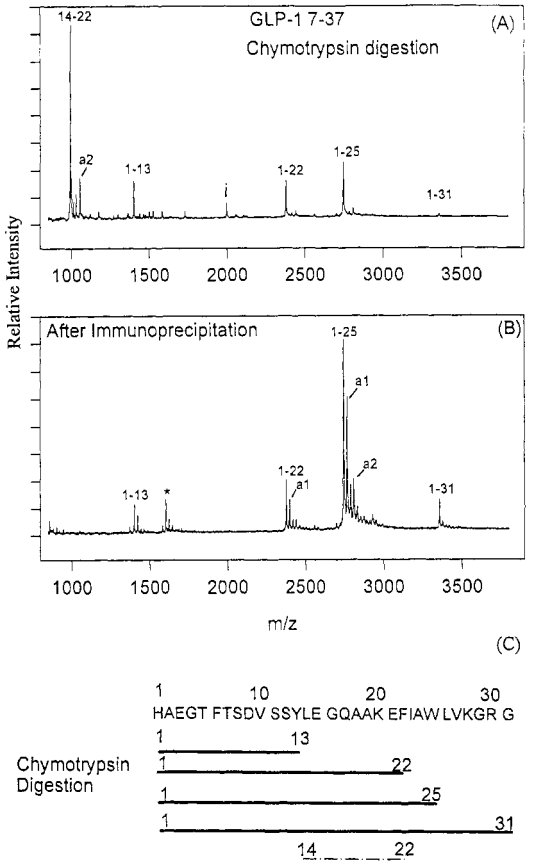

Figure 5. Matrix-assisted laser desorption mass spectra of (A) peptide fragments produced by chymotrypsin digestion of GLP-1 7–37 and (B) peptide fragments isolated by immunoprecipitation with antiGLP-1 7–37 mab No. 26.1. The peak labeled with an asterisk corresponds to the internally added calibrant dynorphin A 1–13, and i refers to an unidentified impurity. The satellite peaks labeled a_1 and a_2 arise by adventitious Na and Cu adduction to the peptide.[22] (C). Amino acid sequence of GLP-1 7–37. The peptide fragments produced by chymotrypsin digestion are shown by lines. The solid lines represent those peptide fragments that were bound by the antiGLP-1 7–37 mab No. 26.1, and the dashed lines those that were not bound by mab No. 26.1.

7–37. The present result is consistent with the previous finding (personal communication from Douglas Buckley, Scios Nova, Mountain View, CA) that the epitope includes the first three N-terminal residues of GLP-1 7–37.

CONCLUSION

The present results demonstrate the feasibility of a method for rapidly mapping linear protein epitopes that combines immunoprecipitation purification of antibody-binding peptide fragments with accurate determination of their molecular weights by mass spectrometry. Selection and analysis of antibody-binding peptides provide the basis for localizing the antigenic sites. The analysis relies on a determination of the ratio of the relative intensities of the various peptide fragments prior to and after immunoprecipitation. The method was successfully demonstrated for an antibody with a relatively low binding affinity (10^{-6} M) and therefore should be applicable to most antibodies that bind linear epitopes. On occasion, weak peaks were observed in the mass spectra that correspond to peptides that bind nonspecifically to protein A/protein G agarose or antibody. These nonspecifically binding peptides could be readily excluded by using harsher washing conditions for removing them or by comparing the relative intensities of the peptide peaks in the mass spectra prior to and after immunoprecipitation.

The present procedure differs from that of Przybylski and co-workers[8] in three respects. First, MALDI-MS is considerably more sensitive and less prone to discrimination against certain peptide components of proteolytic digests than ^{252}Cf plasma desorption MS. Thus, much less antigen and antibody is necessary in the present experiment. Second, the detergent octyl glucoside[19] is used during immunoprecipitation, which appears to reduce potentially confusing nonspecific binding of peptide to antibody. Third, the antibody–antigen complex (on the agarose beads) is loaded together with the laser desorption matrix onto the mass spectrometer probe, without separation of the bound peptides.[20] The present procedure also avoids the addition of high concentrations of salt for eluting the peptides.[8]

The present approach to linear epitope mapping is considerably more rapid than the widely used conventional approaches.[4,5,10] The antigen protein can be digested by a variety of specific proteases, which can rapidly produce a large number of peptide fragments. Thus the digestion step is comparable to fast peptide synthesis or recombinant gene expression of deletion mutants. The digestion and immunoprecipitation steps take approximately 6 h and the molecular weight determination of each sample takes 1 min. Thus, the epitope for a specific antibody can be located in a short stretch of the protein (typically 6–15 amino acids, depending on the available digestion sites in the antigen) in a single day. If more detailed localization of the epitope is required, this information can be obtained by the synthesis of a small set of peptides that span the region of interest determined by the present method.[21]

The present method represents a successful marriage between a powerful purification technique (immunoprecipitation) and an effective characterization technique (MALDI-MS). We believe that it will be useful for a range of other biomedical studies such as antigenic site mapping for polyclonal antibodies and determination of the sites of protein–protein or protein–ligand interactions.

ACKNOWLEDGMENT

We thank Dr. T. P. King of The Rockefeller University and Dr. Douglas Buckley of Scios Nova Inc., Mountain View, CA, for supplying us with monoclonal antibody and peptide used in this study and Drs. T. P. King and Peter Model for helpful discussions. Y. Z. also thanks Drs. Wenzhu Zhang, Rong Wang, Klaus Schneider, Steven Cohen, James Zhou, David Fenyo, and Jun Qin for technical help and Zhong Zhong for useful suggestions. Support for this work from the National Institutes for Health (Grants RR00862 and GM38274) is gratefully acknowledged.

Received for review April 26, 1994. Accepted August 9, 1994.®

® Abstract published in *Advance ACS Abstracts*, September 15, 1994.

(19) Vorm, O.; Chait, B. T.; Roepstorff, P. In *Proceeding 41st ASMS Conference on Mass Spectrometry and Allied Topics*, 1993; p 621.
(20) Hutchens, T. W.; Yip, T.-T. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 576–580.
(21) Manuscript in preparation.
(22) Beavis, R. C.; Chaudary, T.; Chait, B. T. *Org. Mass Spectrom.* **1992**, *27*, 156–158.