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Epitope mapping of the gastrin-releasing peptide/anti-bombesin monoclonal antibody complex by proteolysis followed by matrix-assisted laser desorption ionization mass spectrometry

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Abstract

We have developed a method to rapidly identify the antigenic determinant for an antibody using in situ proteolysis of an immobilized antigen-antibody complex followed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI/TOF). A mouse anti-bombesin monoclonal antibody was immobilized to agarose beads and then the antigen, gastrin-releasing peptide (GRP), was allowed to bind. Direct analysis of the immobilized antigen-antibody complex by MALDI/TOF is demonstrated and allows identification of ca. 1 pmol of the bound GRP. To identify the epitope, the immobilized antigen-antibody complex was subjected to proteolysis with trypsin, chymotrypsin, thermolysin, and aminopeptidase M. Following proteolysis, the part of the antigen in contact with the antibody and protected from proteolysis was identified directly by MALDI/TOF. Subsequently, the epitope was eluted from the immobilized antibody with 0.1 M glycine buffer (pH 2.3), separated by reversed-phase HPLC, and its identity confirmed by MALDI/TOF. Using this approach, the epitope for the anti-bombesin monoclonal antibody was shown to comprise the last 7-8 residues (HWAVGHLM-NH_2) of GRP.

Keywords: affinity chromatography; antibody; bombesin; epitope; mass spectrometry; proteolysis

Antigenic determinants, also called epitopes, constitute the region of the protein in intimate contact with the antigen-binding region of an antibody (Jerne, 1960). Epitopes are typically classified as being either continuous or discontinuous. Continuous epitopes are defined as being contiguous residues in series within the sequence of the antigen, whereas discontinuous epitopes are defined as being a set of residues topographically localized by the folding of the polypeptide chain that forms the antigen's tertiary structure (Atassi & Smith, 1978).

Many ingenious approaches have been devised for the identification of epitopes on proteins. X-ray crystallography of the antigen-antibody complex has provided the most detailed structural information about the interface between an antigen and antibody (Amit et al., 1986; Stanfield et al., 1990). Both continuous and discontinuous epitopes can be identified by X-ray crystallography; however, the results can take years to obtain, and large amounts of sample are generally required for crystallization. Probably the most routine method for epitope mapping relies upon several variations of the peptide scanning approach. These methods involve either the synthesis of a series of overlapping peptides derived from the antigen (Geysen et al., 1984) or the construction of peptide libraries containing random peptides (Scott & Smith, 1990). Both the synthetic and random peptides are assayed for their ability to bind to the antibody, allowing identification of the epitope. Recently, matrix-assisted laser desorption mass spectrometry has been incorporated into this approach to allow rapid sequence determination of the synthetic peptides that bind the antibody (Youngquist et al., 1994). An unfortunate drawback to these rapid techniques is that only continuous epitopes are typically identified. This is a distinct limitation because most epitopes are believed to be discontinuous based upon models of globular proteins, which show that very few stretches of linear peptide sequence are surface accessible (Van Regenmortel & de Marcillac, 1988). In agreement with this prediction, a recent study found that greater than 98% of all antibodies to the globular protein, cytochrome *c*, were discontinuous (Schwab et al., 1993). Moreover, the peptide scanning approach is not readily amenable to identifying epitopes that contain posttranslationally modified residues. Presumably, both

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phosphate groups (Black & Lee, 1988) and oligosaccharides (Abramson et al., 1989), which have been implicated as being part of epitopes, would be overlooked by this approach.

One method for mapping epitopes that has been underutilized is the protection type assay. Both chemical (Burnens et al., 1987) and enzymatic (Jemmerson & Paterson, 1986) protection assays have been used for the identification of discontinuous epitopes on cytochrome *c*. The chemical protection assay compares the relative rates of derivatization in the absence and presence of a monoclonal antibody. Correspondingly, the enzymatic protection assay compares the rates of enzymatic cleavage at specific residues on the antigen in the absence and presence of a monoclonal antibody. A slower rate of derivatization or generation and release of peptides into solution implies that those amino acid residues are protected by the antibody and therefore constitute part of the epitope. An improvement in the enzymatic protection assay was subsequently introduced by immobilizing the antibody to agarose and analyzing the HPLC fractions by plasma desorption mass spectrometry (Suckau et al., 1990). The incorporation of mass spectrometry into this approach enabled the unambiguous identification of the proteolytic fragments found in the HPLC fractions.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI/TOF) was first introduced in 1988 (Karas & Hillenkamp, 1988; Tanaka et al., 1988). In MALDI/TOF, the analyte is typically mixed with a UV light-absorbing substance called the matrix. The matrix and analyte are allowed to cocrystallize on the probe surface of the mass spectrometer. The sample is then irradiated with UV light from a laser, which is absorbed by the matrix, causing the sample and matrix to be vaporized and ionized. The generated ions are accelerated through an electric field and then allowed to drift through a field-free region until they reach the detector. This procedure has been demonstrated to be capable of detecting mid-femtomole to low picomole levels of proteins and peptides (Roepstorff, 1992). Additionally, MALDI/TOF has been shown to have a very high mass range, allowing analysis of immunoglobulins with molecular weights of 150,000 Da (Beavis & Chait, 1990a). Most notably, MALDI/TOF has demonstrated its utility in being able to analyze complex biological mixtures (Beavis & Chait, 1990b) and protein digests (Billeci & Stults, 1993) without the need for sample cleanup. Recently, MALDI/TOF has been used to analyze a protein directly attached to an affinity matrix. The investigators named this technique surface-enhanced affinity capture (SEAC) time-of-flight mass spectrometry (Hutchens & Yip, 1993). Human lactoferrin from urine was captured with immobilized single-stranded DNA and analyzed directly by MALDI/TOF (Hutchens & Yip, 1993). We presumed that the direct analysis of an affinity-bound analyte could be accomplished with an immobilized antibody as the affinity capture device. Using this approach in conjunction with the proteolytic protection assays, we believe this method offers a rapid means to map epitopes at the resolution of single amino acids.

In this paper we will demonstrate the ability to analyze an antigen directly from an antigen-antibody complex by MALDI/TOF. We chose the anti-bombesin monoclonal antibody and gastrin-releasing peptide (GRP) as our model system for epitope mapping because the location of the epitope on GRP is reasonably well defined (Cuttitta et al., 1985). Using the enzymes chymotrypsin, trypsin, aminopeptidase M, and thermolysin, we will demonstrate that the epitope on GRP is composed of the last 7-8 residues.

Results

MALDI/TOF of the immobilized antigen-antibody complex

Direct analysis of an immobilized antigen-antibody complex by matrix-assisted laser desorption mass spectrometry was undertaken to shorten the time required to identify the epitope. The binding efficiency of antibody to agarose was not determined but was assumed to be high based upon the literature accompanying the antigen-antibody immobilization kit (Pierce). The binding of the antigen to antibody was directly assayed by MALDI/TOF analysis of the effluent from the compact reaction column following antigen binding. No unbound GRP was ever observed in the effluent.

Preparation of the beads for MALDI/TOF was conducted in either of 2 ways. In the first method, a 1-μL aliquot of the beads suspended in deionized water was placed onto the target and 1 μL of matrix containing formic acid was added. In the second method, 10 μL of beads suspended in deionized water was centrifuged and the supernatant removed. To the beads was added 20 μL of matrix containing formic acid. Following mixing of the matrix with the beads, the beads were centrifuged and 0.5 μL of the supernatant was spotted onto the target. Both methods of sample preparation gave good results; however, using the supernatant produced a much stronger signal. Figure 1 shows the results from spotting beads directly onto the target. An MH^+ of 2,856.7 Da is observed (calculated $MH^+ = 2,860.4$ Da). This value was obtained using glucagon as an external mass calibrant ($MH^+ = 3,483.8$ Da; $(M+2H)^{2+} = 1,742.4$ Da). When the supernatant was analyzed by MALDI/TOF, an MH^+ of 2,859.3 Da was observed (data not shown).

Epitope elution

Another factor that had to be determined was what eluent should be used to release the bound antigen or epitope. Elution of bound GRP did not occur with 0.1 M acetic acid (pH 2.9). However, 1.0% trifluoroacetic acid (TFA) (pH 1.9) and 0.1 M glycine (pH 2.3) were found to be effective for release of the antigen. Recovery of the bound antigen was determined to be approximately 80% using 1% TFA. No additional GRP could be made to elute from the column with 1.0% TFA following an initial wash with the 0.1 M glycine buffer. The glycine buffer was decided on as the eluent of choice because the 1.0% TFA was likely to denature the antibody irreversibly.

Trypsin cleavage of the antigen-antibody complex

Prior to trypsin cleavage of the antigen-antibody complex, the immobilized antibody was subjected to cleavage under the anticipated conditions to determine the extent of cleavage and release of the antibody. Following cleavage, the immobilized antibody was washed with phosphate-buffered saline (pH 7.4). HPLC of the eluent was performed to determine if peptides from the antibody were being generated and to establish a background chromatogram. Several large peaks were observed in the chromatogram. MALDI/TOF analysis suggested that these peaks were not proteinaceous in nature. These same peaks were also observed in the chromatogram when the immobilized anti-

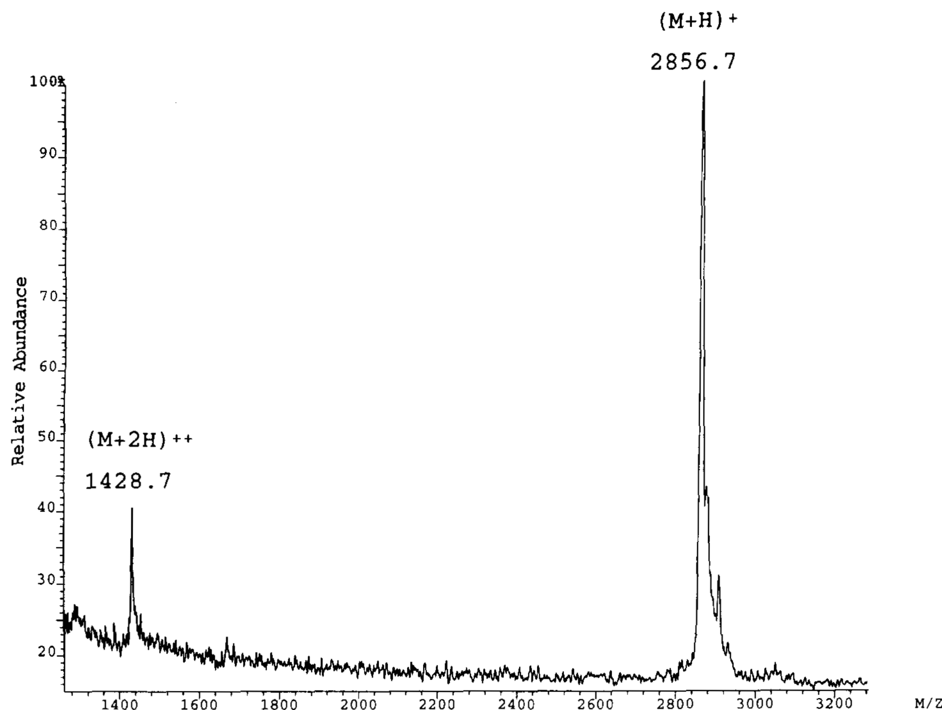

Fig. 1. Matrix-assisted laser desorption mass spectrum of 0.5 μL (ca. 1 pmol) of immobilized anti-bombesin monoclonal antibody with human GRP attached.

body was washed with phosphate-buffered saline (pH 7.4) without prior enzymatic digestion.

Next, trypsin digestion conditions identical to those used for digestion of the immobilized anti-bombesin monoclonal antibody were used to cleave GRP while in solution to define the sites accessible to trypsin cleavage. All 3 potential tryptic sites were found to be susceptible to proteolysis. No uncleaved material was found, and all 3 expected peptides (residues 1-13 [calculated MH^+ = 1,210.5 Da]; residues 14-17 [calculated MH^+ = 566.7 Da]; residues 18-27 [calculated MH^+ = 1,121.3 Da]) were observed by MALDI/TOF (Fig. 2).

Cleavage of the immobilized antigen-antibody complex with trypsin at an enzyme to substrate ratio (E/S) of 1:1 followed by direct analysis of the beads by MALDI/TOF yielded a single species at 1,120.2 Da, corresponding to residues 18-27 of GRP (Table 1). HPLC separation of the unbound tryptic peptides washed from the compact reaction column with water yielded several peaks (data not shown). One peak contained residues 1-13 and a small peak was observed for residues 14-17. No peak was observed for residues 18-27. When the 0.1 M glycine wash from the tryptic digest of the immobilized antigen-antibody complex was separated by HPLC, 1 major peak was observed (Fig. 3). This peak had the same retention time as GNHWA-VGHLM-NH_2 and, when analyzed by MALDI/TOF, showed an MH^+ of 1,120.9 Da (calculated MH^+ = 1,121.3 Da) in accordance with residues 18-27 (Table 1).

Chymotrypsin cleavage of the antigen-antibody complex

To further define the epitope on GRP, the immobilized antigen-antibody complex was cleaved with chymotrypsin. Cleavage of GRP in solution with chymotrypsin revealed that the predominant cleavage sites were at the C-terminal side of residues L^{11}

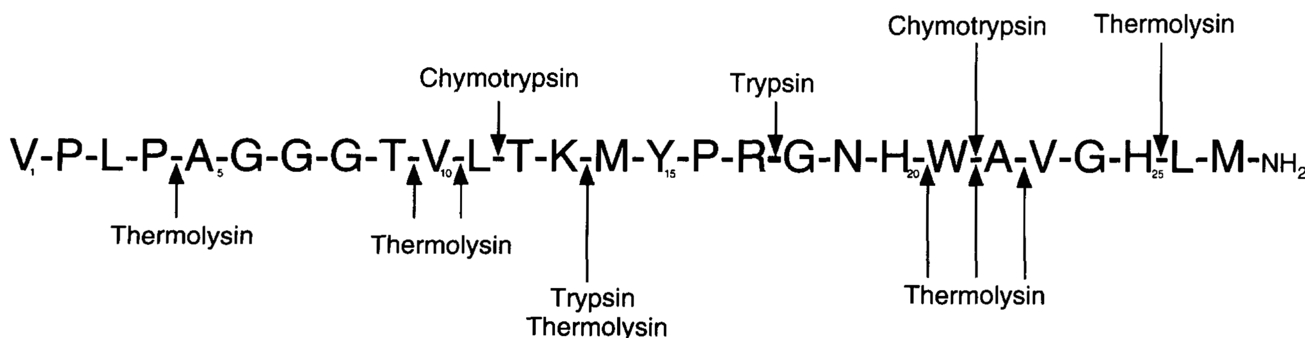

Fig. 2. Amino acid sequence of human GRP showing the sites of cleavage by trypsin, chymotrypsin, and thermolysin in the absence of the monoclonal antibody.

Table 1. *Peptides observed by MALDI/TOF of the immobilized antigen-antibody complex following enzymatic cleavage*

Enzyme	Solution cleavage sites[a]	Epitope released	
		$MH^{+}_{calculated}$	Residues
Trypsin	Arg^{17} (A); Lys^{13} (A)	1,669.0	14-27
		1,121.3	18-27
Chymotrypsin	Leu^{11} (A); Trp^{21} (B)	1,898.3	12-27
Thermolysin	Ala^{5} (A); Val^{10} (A); Leu^{11} (A)	2,110.6	10-27
	Met^{14} (A); Trp^{21} (B); Ala^{22} (B)	2,011.4	11-27
	Val^{23} (B); Leu^{26} (B)	1,669.0	14-27
Aminopeptidase M	Gly^{18} (A)	1,064.3	19-27
	Asn^{19} (A)	950.2	20-27
	His^{20} (P)	813.0	21-27

[a] (A), Site was accessible to enzymatic cleavage; (B), site was blocked to enzymatic cleavage; (P), site was partially accessible to enzymatic cleavage.

and W^{21} (Fig. 2). Cleavage was also found to occur at V^{10}, Tyr^{15}, and Arg^{17} at a slower rate.

Cleavage of the immobilized antigen-antibody complex with chymotrypsin, followed by HPLC separation of the glycine wash, revealed 1 peak. The material eluted at 35.6 min and its mass spectrum showed an MH^+ of 1,897.3 Da, corresponding to residues 12-27 (calculated MH^+ = 1,898.3) (Table 1). In the water wash containing the unbound material, a peptide was observed with an MH^+ of 980.4 Da, corresponding to residues 1-11 (calculated MH^+ = 981.2 Da).

Thermolysin cleavage of the antigen-antibody complex

Thermolysin typically cleaves on the amino-terminal side of Val, Ala, Leu, Ile, Phe, and Met (Ambler & Meadway, 1968). Due to this lack of specificity, a time course determination of the cleavage of GRP in solution was necessary to identify all the sites susceptible to thermolysin cleavage. Cleavage on the amino-terminal side of Ala^{22}, Val^{23}, and Leu^{26} was observed within 1 min with no intact GRP found (Fig. 2). By 30 min, cleavages were observed on the amino-terminal side of Ala^{5}, Val^{10}, Leu^{11}, Met^{14}, and Trp^{21} (Fig. 2).

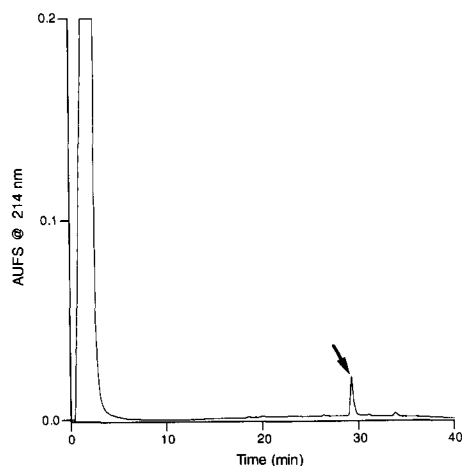

Fig. 3. Chromatogram of the epitope wash of the immobilized antigen-antibody complex following trypsin digestion. The early eluting peak is the void volume and contains salts. The peak indicated with the arrow corresponds to the peptide consisting of residues 18-27 of human GRP.

HPLC separation of the glycine wash from the thermolysin digest of the immobilized antigen-antibody complex produced a chromatogram with several small peaks (data not shown). One of the peaks corresponded to uncleaved GRP. Two additional peaks corresponded to fragments of GRP. The fragments were residues 10-27, 11-27, and 14-27 (Table 1). No fragments involving cleavage at Trp^{21}, Ala^{22}, Val^{23}, or Leu^{26} were observed (Table 1). Additionally, no fragments of GRP were observed in the unbound fraction eluted from the column. This apparent absence of fragments is probably due to complete cleavage of residues 1-9 into 3 small peptides, which could elute in the void volume of the HPLC. To ensure that cleavage could not occur at Met^{27} or Leu^{26}, a second experiment was performed at a longer incubation time and with a higher enzyme concentration. The progress of this reaction was monitored by removing an aliquot of beads at several time points followed by MALDI/TOF analysis. After 18 h of digestion at an E/S of 2:5, 4 major ions were observed in the mass spectrum. Ions for residues 14-27 (MH^+ = 1,669.0 Da), 11-27 (MH^+ = 2,011.3 Da), 10-27 (MH^+ = 2,110.3 Da), and intact GRP (MH^+ = 2,860.3 Da) were observed. After 42 h of digestion, only the 3 fragment ions were observed with no intact GRP remaining.

Aminopeptidase cleavage of the antigen-antibody complex

The peptide, GNHWAVGHLM-NH_2, remains bound to the antibody following trypsin cleavage. If 1 or more of the amino-terminal residues in this peptide are not located in the antibody-binding pocket, they may be susceptible to cleavage with an exopeptidase. Therefore, in an attempt to further delimit the amino-terminal boundary of the epitope, aminopeptidase M was used to cleave the previously trypsin-cleaved immobilized antigen-antibody complex. Aminopeptidase M is an exopepti-

dase that cleaves from the amino-terminal of peptides and proteins and recognizes all 20 of the common L-amino acids, except probably proline (Pfleiderer, 1970).

The synthetic peptide, GNHWAVGHLM, was used to determine the extent of cleavage the peptide would experience in the absence of the monoclonal antibody. We discovered that, to observe proteolytic activity, aminopeptidase M should be diluted 250-fold in ice-cold digestion buffer prior to use. If the enzyme concentration was too high, no cleavage was observed, presumably due to the inhibitory action of the 3 M ammonium sulfate present in the enzyme suspension. We were able to demonstrate the sequential removal of residues from the synthetic peptide with aminopeptidase M by performing a time course experiment from 3.5 to 30 min. The following peptide fragments were observed at the 15-min time point: NHWAVGHLM, HWAVGHLM, WAVGHLM, AVGHLM, VGHLM, and HLM. On the basis of the results from the time course experiment, we presumed that aminopeptidase M should be able to define the epitope to a distance compatible with the steric constraints of the enzyme and antibody.

Cleavage of the immobilized GRP–antibody complex was first performed with trypsin. The completeness of the trypsin digestion was monitored by removing aliquots of the beads at various time points and analyzing the beads by MALDI/TOF. After 4 h of trypsin cleavage, 3 ions were observed in the mass spectrum. The 3 ions corresponded to intact GRP ($MH^+ = 2,862.5$ Da), residues 14–27 ($MH^+ = 1,671.1$ Da), and residues 18–27 ($MH^+ = 1,122.9$ Da). Based upon the relative abundance of the 3 ions, 12% of the GRP remained uncleaved and residues 18–27 accounted for approximately 50% of the bound material. After 18 h of cleavage, the predominant ion observed was at 1,122.4 Da, residues 18–27, with a small ion signal at 1,670.6 Da, residues 14–27 (20% relative abundance). No intact GRP was observed.

Following the 18-h tryptic digest of the immobilized GRP-antibody complex, the resulting GNHWAVGHLM-NH_2-antibody complex was next cleaved with aminopeptidase M. Again the progress of digestion was monitored by frequently removing a 5-μL aliquot of beads for MALDI/TOF analysis. The reaction was allowed to proceed for 48 h to ensure aminopeptidase M had cleaved as far as sterically possible. MALDI/TOF analysis of the beads after the 48-h aminopeptidase M digest revealed ions supporting the presence of uncleaved GNHWAVGHLM-NH_2 (1,121.5 Da), NHWAVGHLM-NH_2 (1,064.3 Da), HWAVGHLM-NH_2 (950.0 Da), and a small peak at 813.4 Da, WAVGHLM-NH_2 (Fig. 4). Additionally, a peak at 1,104.6 Da was observed, which appeared to be due to a loss of ammonia (Fig. 4).

Discussion

Human GRP and the anti-bombesin monoclonal antibody were chosen as the model system to validate the viability of epitope mapping using a proteolytic protection assay followed by MALDI/TOF. The epitope is known to be contained within the carboxyl-terminal portion of GRP because both bombesin and GRP bind to the antibody (Cuttitta et al., 1985). The last 10 residues of bombesin and GRP are homologous, except His^{20} in GRP is replaced by a Gln in bombesin.

A cornerstone to the success of the proteolytic protection assay is the stability of the antibody to proteolysis. If the antibody is degraded during proteolysis, no useful information can be gained. Fortunately, antibodies have been demonstrated to be very stable to proteolysis (Parham, 1983). We chose an IgG1 antibody for our studies because they have been demonstrated to be the most stable isotype (Parham, 1983). We did not directly test the stability of our anti-bombesin monoclonal antibody to all the different enzymes used in this study. However, we did test the stability of the antibody to trypsin digestion and found no apparent cleavage. In addition, we saw no evidence to suggest that the antibody was unstable during digestion of the

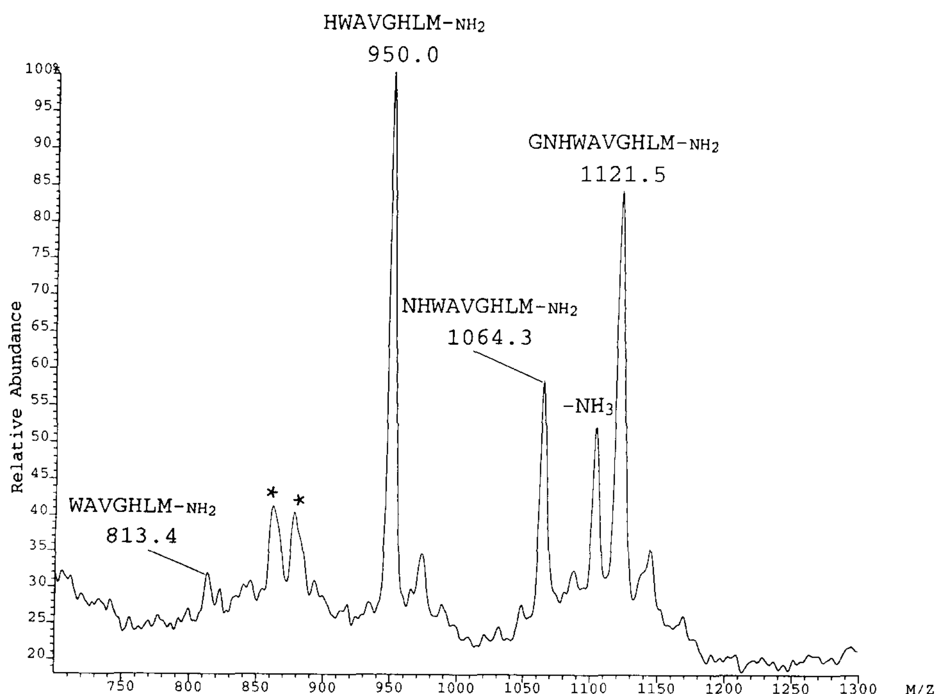

Fig. 4. Matrix-assisted laser desorption mass spectrum of 1 μL of agarose beads containing the immobilized antigen-antibody complex following aminopeptidase M digestion of the previously trypsin-digested complex. Successive losses of the amino acid residues are indicated with the corresponding sequence. The asterisks indicate background ions and the "$-NH_3$" indicates a loss of ammonia from the ion at 1,121.5 Da.

antigen–antibody complex with the other enzymes used in this study.

The ability to identify the antigen while attached to the immobilized antibody by MALDI/TOF substantially reduced the time required to identify the epitope using the proteolysis protection assay. Although the antigen could be observed in a matrix containing only ethanol/water (1:1), addition of 10% formic acid to the matrix greatly enhanced the observed signal (Fig. 1). This suggests that the antigen is released from the antibody prior to crystallization on the probe surface. An even stronger signal was observed for the antigen when supernatant from a small amount of beads mixed with the matrix containing 10% formic acid was analyzed. Nevertheless, 1 pmol of antigen applied to the target in any of these manners produced a signal-to-noise ratio of greater than 10:1.

Initially, cleavage of the immobilized anti-bombesin monoclonal antibody–GRP complex with trypsin resulted in the identification of residues 18–27 as containing the epitope. In good agreement with the anticipated site of the epitope, this first experiment confined the epitope to the last 10 residues of GRP (Table 1). Subsequently, cleavage of the antigen–antibody complex with chymotrypsin indicated that W^{21} was not accessible to the enzyme (Table 1). This implies that W^{21} is found within the paratope of the antibody or is sterically blocked by the antibody. This same site, W^{21}, was also protected from cleavage by thermolysin. Furthermore, thermolysin acted very rapidly on free GRP to cleave several other sites that were also found to be protected in the antigen–antibody complex (Table 1). Apparently, the last 2 residues, Leu^{26} and Met^{27}, were protected by the antibody and are probably within the paratope. Unfortunately, at this stage, we could not state unequivocally that Met^{27} is protected from cleavage by the antibody because cleavage at Met^{27} could not be demonstrated on GRP in the absence of the antibody. Cleavage at Met^{27} is apparently slower than cleavage at many of the other sites; therefore, partial cleavage products displaying cleavage at Met^{27} were not observed. Because thermolysin is known to cleave at methionine residues, we presume that if Met^{27} were available for cleavage, it would have been cleaved.

To eliminate our uncertainty about Met^{27} as being part of the epitope, we had GNHWAVGHLM synthesized as the free acid. We anticipated using carboxypeptidase P to determine whether or not Met^{27} was part of the antigenic domain. The free acid of the peptide GNHWAVGHLM, however, did not bind very strongly, suggesting that the amide was critical for either the correct folding of the peptide or involved in an important contact within the paratope. This observation is consistent with previous antibody-binding studies demonstrating that the free acid of bombesin was unable to inhibit $[^{125}I]Tyr^{4}$-bombesin binding to the anti-bombesin monoclonal antibody 2A11 (Cuttitta et al., 1985).

To further delimit the amino-terminal boundary of the epitope, the tryptic digest was subjected to proteolysis with aminopeptidase M. Using this secondary cleavage, the boundary of the epitope was demonstrated to be at either the His^{20} or the Trp^{21} (Table 1). Interestingly, we observed an apparent loss of ammonia from the tryptic peptide GNHWAVGHLM. This observation was unexpected because aminopeptidase M reputedly does not hydrolyze either asparagine or glutamine to release ammonia (Wachsmuth et al., 1966). We investigated this observation by incubating 2 different asparagine-containing peptides (physalaemin and neurotensin) with aminopeptidase M. Because both peptides contain amino-terminal pyroglutamic acid residues, no cleavage was expected to occur, only hydrolysis of asparagine. Indeed, no cleavage of the pyroglutamic acid residue did occur. Moreover, no loss of ammonia could be detected. The apparent loss of ammonia is probably not a result of fragmentation during ionization because the loss of ammonia is not observed for the same antigen–antibody complex that has not been exposed to aminopeptidase M. Therefore, aminopeptidase M may be hydrolyzing the asparagine to release the ammonia due to the steric restraints imposed by the monoclonal antibody.

The combined results from all of the proteolytic protection experiments indicate that the epitope consists of the last 7 or 8 residues and includes the carboxyl-terminal amide group. These findings are in good agreement with the results reported for a functional binding assay with the anti-bombesin monoclonal antibody 2A11. Cuttitta et al. (1985) found that a peptide containing residues 20–27 of porcine GRP was capable of 112% cross-reactivity with the anti-bombesin monoclonal antibody 2A11. Alytensin, which has a common C-terminal heptapeptide sequence to GRP, also maximally inhibited binding of $[^{125}I]Tyr^{4}$-bombesin in their assay (Cuttitta et al., 1985). In contrast, a peptide containing residues 22–27 of GRP was tested for cross-reactivity and none was found (Cuttitta et al., 1985).

In conclusion, this technique has allowed the rapid identification of a continuous epitope on GRP to the anti-bombesin monoclonal antibody. Our results suggest that proteolytic protection of an immobilized antigen–antibody complex followed by matrix-assisted laser desorption mass spectrometry is capable of identifying the epitope to within 1 residue of its contacts within the antibody. Suckau et al. (1990) demonstrated that trypsin could cleave an antigen to within 2 residues of the epitope. This technique should be applicable to both continuous and discontinuous epitopes. Moreover, the speed and sensitivity of this approach should allow the identification of any epitope, thereby greatly enhancing the value of the particular antibody.

Materials and methods

Materials

The mouse anti-bombesin monoclonal antibody was an IgG1 subtype that was obtained from Boehringer Mannheim (Indianapolis, Indiana) as the sodium sulfate-precipitated ascites and used without any further purification. Human GRP was acquired from Sigma Chemical Co. (St. Louis, Missouri). Sequencing-grade modified trypsin was purchased from Promega (Madison, Wisconsin). Thermolysin and aminopeptidase M were purchased from Boehringer Mannheim. The synthetic peptide Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met was prepared by standard Fmoc chemistry at the Protein Chemistry Laboratory of the University of North Carolina at Chapel Hill–National Institute of Environmental Health Sciences. The peptide was subsequently purified by reversed-phase HPLC and the molecular weight verified by MALDI/TOF.

Immobilization of antibody and attachment of antigen

Compact reaction columns (United States Biochemical, Cleveland, Ohio) were packed with 100–200 μL of aldehyde-activated agarose (Aminolink gel, Pierce Chemical, Rockford, Illinois). The anti-bombesin monoclonal antibody (50–100 μg) was at-

tached to the agarose gel via a secondary amine using the Aminolink immobilization kit #1 from Pierce. Following the blocking of the unreacted aldehyde groups with 1 M Tris-HCl (pH 7.4), the compact reaction column was washed with phosphate-buffered saline (pH 7.0). Human GRP (1-2.5 μg) was dissolved in 400 μL of phosphate-buffered saline (pH 7.0) and was added to the compact reaction column containing the immobilized antibody. Binding of the antigen was performed at room temperature while gently rotating the column at 2 rpm for 2 h. The unbound GRP was then removed by washing the column 3 times with 0.6 mL deionized water. The effluent from the column was collected and analyzed by HPLC to determine the relative binding efficiency. Following washing, a 10-μL aliquot of beads was removed for later analysis by MALDI/TOF.

Proteolysis of the antigen-antibody complex

All digestion reactions of the antigen-antibody complex were accomplished in phosphate-buffered saline (pH 7.4) at 37 °C while rotating at 2 rpm. Cleavage with modified trypsin was performed with an E/S of 1:1 for 18 h. Digestion with chymotrypsin was accomplished with an E/S of 1:7 for 4 h and with thermolysin, an E/S of 1:10 for 3 h was used. For digestion with aminopeptidase M, the trypsin-cleaved antigen-antibody complex was first washed 3 times with 0.6 mL deionized water and 3 times with phosphate-buffered saline (pH 7.4). Aminopeptidase M was then added at an E/S of 1:4 and incubated for 48 h. Following proteolysis, the compact reaction columns containing the immobilized antigen-antibody complex were drained and washed 3 times with 0.6 mL deionized water to remove all of the unbound peptides. The effluent from the washes was pooled and vacuum dried. The unbound peptides were subsequently separated by HPLC. A 10-μL aliquot of the beads was removed at this point for later analysis by MALDI/TOF.

Epitope elution

The bound GRP and the proteolytic fragments of GRP were eluted from the immobilized antibody with either 0.6 mL of 0.1 M glycine (pH 2.3) or 1.0% TFA (pH 1.9). The compact reaction columns were then washed 3 times with 0.6 mL deionized water. The washes were pooled and vacuum dried.

HPLC

Samples for separation by reversed-phase HPLC were dissolved in 0.1% TFA. Separation of the proteolytic fragments was achieved with gradient elution on a C-18 spheri-5 column (Brownlee, Foster City, California), 2.1 × 30 mm. The gradient began with the mobile phase consisting of 90% 0.1% aqueous TFA and 10% acetonitrile containing 0.084% TFA. The gradient was held initially at those conditions for 5 min and then ramped over 30 min to 45% acetonitrile containing 0.084% TFA. The flow rate was 0.2 mL/min and the effluent was monitored at 214 nm.

Matrix-assisted laser desorption mass spectrometry

Mass spectrometric analysis of samples was performed with a VG TofSpec mass spectrometer (Manchester, UK). The instrument was operated with an acceleration voltage of 24 kV and a multichannel plate detector voltage of 1,800 V. Samples were ionized with a nitrogen laser (λ = 337 nm). Most analyses were conducted following external calibration with 2 points which bracketed the mass range of interest. The external calibrants were usually either GRP (calculated MH^+ = 2,860.3 Da and $(M+2H)^{2+}$ = 1,430.7 Da) or synthetic GNHWAVGHLM (calculated MH^+ = 1,122.3 Da) and the dimer of α-cyano-4-hydroxycinnamic acid (calculated $(2M+H)^+$ = 379.4 Da). With external calibration, the mass accuracy was typically 0.13% or better. Samples (1 μL) were mixed on the target with 0.5 μL of a saturated solution of recrystallized α-cyano-4-hydroxycinnamic acid in either ethanol/water (1:1) or ethanol/water/formic acid (45:45:10). Samples were dried with cool air from a hair dryer. Approximately 50 scans were averaged to obtain the spectra.

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