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Protein antigen–monoclonal antibody contact sites investigated by limited proteolysis of monoclonal antibody-bound antigen: Protein "footprinting"

(measles virus/hemagglutinin/fusion protein/epitope mapping/conformational changes)

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ABSTRACT **This study describes the use of limited proteolysis of monoclonal antibody (mAb)-bound antigens in the analysis of the two measles virus surface glycoproteins. This approach is dubbed protein "footprinting" in analogy with DNA "footprinting." Protein footprinting was superior to competitive-binding assays and as good as *in vitro* mAb-selected variant analysis in differentiating among mAbs with various specificities to a given protein. Proteolytic digestion of the antigen prior to mAb binding drastically reduced mAb binding resulting in poor differentiation among mAbs. In contrast, protein footprinting showed that some mAbs retained the ability to immunoprecipitate such fragments. Thus footprinting could be used for localization of mAb epitopes on a protein and proved also to be an effective means of distinguishing among mAb-selected variants differing in single epitopes. Conformational changes caused by heat-denaturation or the binding of anti-antibody to an antigen–antibody complex could also be detected by footprinting.**

Monoclonal antibodies (mAbs) and the epitopes to which they bind conventionally have been differentiated with competitive-binding assays and, if applicable, *in vitro* mAb-selected variant analysis. More detailed mapping of continuous epitopes has been achieved with mAb binding to peptides derived from the antigen. Although these approaches are well established, they have inherent shortcomings. *In vitro*-selected variant analysis, commonly used in the study of viral proteins, is restricted to surface components, since the mAbs must be neutralizing antibodies to be studied by this technique. Due to the various mechanisms by which mAbs may inhibit the binding of other mAbs, competitive-binding assay data are most informative when reciprocal noncompetitive binding is observed. As most mAbs appear to be directed against conformational (continuous or discontinuous) epitopes, these mAbs bind poorly or not at all to peptides derived from the antigen.

It has been shown that polyvalent antibodies can protect antigenic determinants from proteolytic attack (1–3). Based on this rationale, Eisenberg *et al.* (4) showed that some mAbs could be differentiated by the peptides generated by limited proteolysis of mAb-bound antigen. Seventeen mAbs to the herpes simplex virus gD glycoprotein were divided into four groups by proteolytic peptide analysis and into eight groups by radioimmunoprecipitation and neutralization assays that used herpes simplex virus 1 and 2. mAbs were not studied by competitive-binding assay or *in vitro*-selected variant analysis.

This study describes modifications and various applications of limited proteolysis of mAb-bound protein antigen. We call this analytical approach protein "footprinting" in analogy to DNA "footprinting" (see ref. 5). For our model, we have chosen a panel of mAbs directed against the measles virus surface glycoprotein hemagglutinin (H protein). Protein footprinting is compared to competitive-binding assay, to *in vitro* H-protein variant analyses reported elsewhere (6), and to proteolytic digestion of the antigen prior to mAb binding. We also describe its use in the detection of conformational and small epitopic changes in the antigen.

MATERIALS AND METHODS

Virus and mAbs. The LEC-KI strain and the LEC-WI strain variants of measles virus (6) were used. Unless otherwise stated the LEC-KI strain was used. The competitive-binding assay and *in vitro* mAb-selected variant characterizations of the nine anti-measles virus H-protein mAbs are described elsewhere (6), and the data are summarized in Table 1 to enable comparisons with results reported here. The anti-measles virus F-protein mAbs have been described (7, 8).

Preparation of Antigens. The buffers employed were as follows: radioimmunoprecipitation (RIPA) buffer A [2% (vol/vol) Triton X-100, 0.6 M KCl, 0.15 M NaCl, 5 mM EDTA, 3 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 2.5 mM iodoacetamide, and 0.01 M Tris·HCl (pH 7.8)], RIPA buffer B [1.2% (vol/vol) Triton X-100, 0.15 M NaCl, and 0.01 M Tris·HCl (pH 8)], RIPA washing buffer [RIPA buffer A without phenylmethylsulfonyl fluoride and Trasylol], TBS [0.15 M NaCl/0.01 M Tris·HCl, pH 8], TTS [1% Triton X-100/0.1% $NaDodSO_4$/TBS], and $NaDodSO_4$/PAGE buffers (7).

Measles virus antigen metabolically labeled with [^{35}S]methionine or [1,6-^{3}H]glucosamine (New England Nuclear) as described (7) was purified from radiolabeled virus-infected cells from a 175-cm^2 confluent Vero cell monolayer that was resuspended in 10 ml of RIPA buffer A or B. Antigens prepared in RIPA buffer A were used in all experiments except for digestion prior to mAb binding that used antigens prepared in RIPA buffer B. Cell lysates were kept on ice for 1 hr and centrifuged at 60,000 × *g* for 1 hr. The supernatants were collected and stored at −20°C.

Protein Footprinting. *Basic protein footprinting technique.* Antigen (250 μl) was mixed with 500 μl of RIPA buffer A and with 2 μl of mAb ascites fluid. Antigen and mAb were incubated on ice for at least 2 hr, and the antigen–mAb complexes were precipitated by addition of 100 μl of a slurry of *Staphylococcus aureus* protein A-Sepharose CL-4B beads (Pharmacia) in TBS, 1:1 (vol/vol). The bead-bound antigen–antibody complexes were washed three times with RIPA washing buffer and twice with TTS. Excess buffer was removed from the sedimented beads, and 30 μl of stock

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Abbreviation: mAb, monoclonal antibody.

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Table 1. mAb binding site delineations by *in vitro* mAb-selected variant analysis and by competitive binding assay

mAb		Binding sites, no.	
No.	IgG subclass*	Variant analysis	CBA
I-12	2a,1	1†	1
I-29	2a,1		
V-17	2a	2	
7-AG11	1	3	
16-CD11	2b,1	4	2
I-44	2a,1	5	(4)‡
16-DE6	2b,1	6§	3
B2	2a		
I-41	2a,1	7	

CBA, competitive-binding assay.

*mAbs B2 and V-17 were cloned using a nonproducer myeloma cell line, whereas the remaining mAbs were cloned using a γ1,k producer myeloma and thus exhibit hybrid antibody molecules.

†Seventy percent of mAb I-29-selected variants do not react with mAb I-12.

‡Due to the presence of some nonreciprocal competition, it is not clear whether mAb I-44 defines a distinct competitive-binding site or the boundary between competitive-binding sites 2 and 3.

§Eighty-five percent of mAb 16-DE6-selected variants do not react with mAb B2.

enzyme solution was added per sample. Stock enzyme (Sigma) solutions were as follows: trypsin or *S. aureus* protease V8 solution was 1 mg/ml in water, and chymotrypsin was 4 mg/ml in water. The samples were kept at 37°C with occasional agitation for the time periods indicated. At the end of the digestion, samples were placed on ice and washed once with RIPA buffer A containing 2% (wt/vol) bovine serum albumin, twice with RIPA washing buffer, and once with TBS. Excess buffer was removed, and the beads were dried at 37°C before addition of 100 μl of PAGE sample buffer (7). Samples were heated to 100°C for 3 min prior to analysis by PAGE.

Protein footprinting with anti-antibody addition. Undiluted rabbit anti-mouse immunoglobulin (20 μl) (Dako, Santa Barbara, CA) was added either before or after digestion. (*i*) When the anti-antibody was added before digestion, protein A-Sepharose–mAb–antigen complexes were washed three times with RIPA washing buffer, and the excess buffer was removed from the sedimented beads. The rabbit anti-mouse immunoglobulin was added and the beads were agitated occasionally over 45 min and washed twice with TTS prior to addition of the proteolytic enzyme. (*ii*) When the anti-antibody was added after digestion, upon completion of proteolytic digestion, samples were placed on ice, and the rabbit anti-mouse immunoglobulin plus 1 ml of RIPA buffer A with 2% (wt/vol) bovine serum albumin was added per sample. Samples were agitated occasionally for 45 min, washed twice with RIPA washing buffer and once with TBS, and prepared for PAGE analysis.

Protein footprinting without washing. The basic procedure was followed through to the enzyme digestion, then 20 μl of 5× PAGE sample buffer was added to each sample, and the samples were heated to 100°C for 3 min. Samples were analyzed by PAGE without further treatment.

Protein footprinting with heat-denaturation but without washing. The same procedure was followed except that prior to enzyme digestion, the samples were heated to 100°C for 3 min and cooled to 37°C, and then the enzymes were added.

Digestion Prior to mAb Binding. Radiolabeled antigen in RIPA buffer B was treated with 0.1% $NaDodSO_4$. The antigen preparation (3.5 ml) was digested at 37°C for 5 hr. For the trypsin digestion, the antigen was treated with 5 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) before 1 ml of trypsin stock solution was added. After digestion, the mixture was treated with 5 mM *N*-α-tosyllysine chloromethyl ketone (TLCK; 1-chloro-3-tosylamido-7-amino-2-heptanone hydrochloride)/5 mM phenylmethylsulfonyl fluoride/1% Trasylol/10 mM EDTA, and the pH was adjusted to 8.5. For the chymotrypsin digestion, the procedure was the same except that the order of TPCK and TLCK addition was reversed and that chymotrypsin stock solution was used. For the V8 protease digestion, the antigen was treated with 5 mM TLCK/5 mM TPCK/5 mM phenylmethylsulfonyl fluoride (Sigma)/10 mM EDTA, the pH was adjusted to 7.8, and 1 ml of V8 stock solution was added. After digestion the mixture was treated with 1% Trasylol, and the pH was adjusted to 8.5. Bovine serum albumin was added to all enzyme-treated antigens prior to immunoprecipitation. Immunoprecipitates were washed five times with RIPA washing buffer and once with TBS.

Electrophoresis. Discontinuous $NaDodSO_4$/PAGE with separation gels consisting of 10–20% (wt/vol) polyacrylamide gradients [acrylamide/bisacrylamide, 37:1 (wt/wt)] and fluorography were as described (6, 7). The reference ^{14}C-methylated protein molecular mass standards (New England Nuclear) were phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), cytochrome *c* (12.3 kDa), and insulin (5.8 kDa).

RESULTS

Differentiation Between mAbs by Using Protein Footprinting. Table 1 shows a summary of the competitive-binding assay and *in vitro* mAb-selected variant analyses of the nine anti-H-protein mAbs (data from ref. 6). The competitive-binding assay showed a high degree of mAb competition and indicated that the mAbs were directed against three or four binding sites. In contrast, variant analysis clearly indicated that the nine mAbs delineated seven independent variable antigen sites. The antigenic binding of these nine anti-H mAbs was analyzed by protein footprinting with trypsin, chymotrypsin, and V8 protease. The results (exemplified Fig. 1) showed that footprinting was a very effective way of differentiating between all nine mAbs, and mAbs that recognized closely linked epitopes by competitive-binding assay and variant analysis could be easily differentiated by footprinting (for example, mAbs I-12 and I-29 in Table 1 and Fig. 1 *B* and *C*).

Antigen preparations digested with different proteases prior to immunoprecipitation with the mAbs were also analyzed (Fig. 2). Under the conditions used, V8 and chymotrypsin digests are partial (peptides with $M_r \leq 50$ kDa), whereas trypsin digests are more complete (peptides with $M_r \leq 8$ kDa). Surprisingly, all nine mAbs precipitated essentially the same fragments from a given enzyme digest. Furthermore, although equivalent amounts of radiolabeled antigen were used for each sample in the footprinting (Fig. 1) and the pre-mAb-binding digestion (Fig. 2), comparison of the two indicates that immunoprecipitation of peptides from the pre-mAb-binding digestion was much less efficient than from footprinting. For gels in Fig. 2 *A* and *B* film exposure was 5 times longer than for gels in Fig. 1, and for the gel in Fig. 2*C* exposure was 25 times longer. This poor immunoprecipitation was particularly pronounced with the low molecular weight peptides (≤10 kDa) from the trypsin digestion (Fig. 2*C*). The possibility of low methionine content in these peptides has been excluded since similar low molecular weight peptides were very strongly immunoprecipitated by some of the mAbs when tested by footprinting. It was, therefore, concluded that the weak immunoprecipitation was caused by a drastic reduction in the strength of the mAb–antigen interaction.

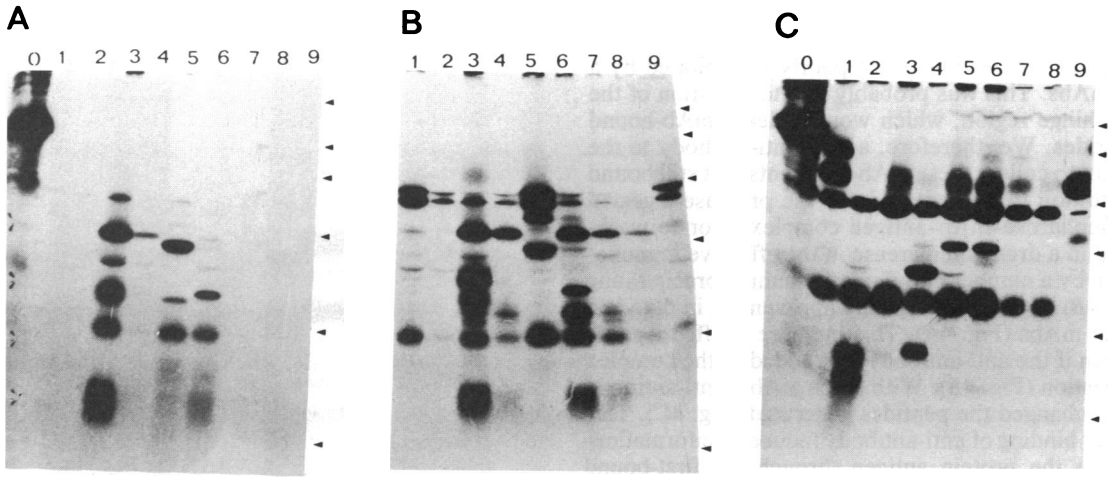

FIG. 1. Analysis of anti-H-protein mAbs by protein footprinting (with anti-antibody added after digestion) by using [^{35}S]methionine-labeled antigen. Proteases used were trypsin (*A*), chymotrypsin (*B*), and V8 protease (*C*). Digestion was for 2.5 hr. Lanes: 1, mAb I-12; 2, mAb I-29; 3, mAb I-41; 4, mAb I-44; 5, mAb 7-AG11; 6, mAb 16-CD11; 7, mAb 16-DE6; 8, mAb B2; 9, mAb V17. Control, undigested H protein immunoprecipitated with mAb I-44 is in lanes 0. Arrowheads, positions of molecular mass markers.

Epitope Localization by Using Footprinting. The fragments immunoprecipitated by a mAb either bear the epitope or are linked to the epitope-bearing regions by disulfide bonds. The H protein of measles virus is a glycoprotein that contains a number of disulfide bonds. By simultaneously footprinting [^{35}S]methionine- and [^{3}H]glucosamine-radiolabeled antigens and by PAGE analysis under both reducing and nonreducing conditions, the epitopes of some of the mAbs could be approximately localized using the H-protein sequence data (9). The data for mAb I-41 are shown in Fig. 3. The epitope is localized to peptide x (M_r, 41 kDa), because its immunoprecipitation is not indirect through a disulfide bonded peptide (Fig. 3*A*, cf. Fig. 3*B*). By taking the average molecular weight of amino acids as 110, it can be calculated that peptide x is composed of ≈372 residues. Peptide x is not glycosylated. Because there are 239 residues between the amino terminus and the glycosylated site farthest from the amino terminus (site E, Fig. 3*C*), peptide x must be derived from the region between the glycosylated region and the carboxyl terminus. The carboxyl-terminal boundary can be delineated further by considering peptide y, a 14-kDa peptide of ≈127 residues that contains the I-41 epitope and is glycosylated. Of the five potential glycosylation sites (sites A–E, Fig. 3*C*), a minimum of three sites are used (10). If only sites A, B, and C are glycosylated, then the I-41 epitope must be located between site C (residue 201) and the carboxyl terminus. If peptide y is glycosylated only at a single site, if this is site E (residue 239, Fig. 3*C*), and if site E is postulated to be at the amino terminus of peptide y, then the peptide would be derived from the region between residues 239 and 366. Thus the boundaries of the region that could contain the I-41 epitope should be residues 202 and 366. By the same approach, the epitopes for mAbs I-41, 7-AG11, I-29, and I-44 were located between residues 200 and 370.

Effect of Anti-Antibody Binding. Footprinting was originally performed by digesting the protein A-Sepharose–mAb–

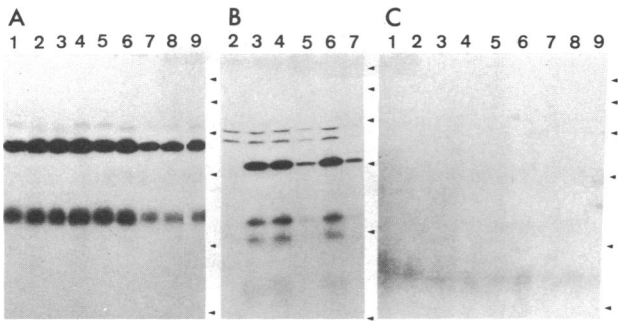

FIG. 2. mAb reactivity with peptides derived from the antigen. [^{35}S]methionine-labeled whole-cell lysates were digested for 2.5 hr at 37°C with V8 protease (*A*), chymotrypsin (*B*), or trypsin (*C*) prior to immunoprecipitation with the mAbs. Lanes: 1, mAb I-12; 2, mAb I-29; 3, mAb I-41; 4, mAb I-44; 5, mAb 7-AG11; 6, mAb 16-CD11; 7, mAb 16-DE6; 8, mAb B2; 9, mAb V17. Gel in *C* was exposed to x-ray film five times longer than the gels in *A* and *B*.

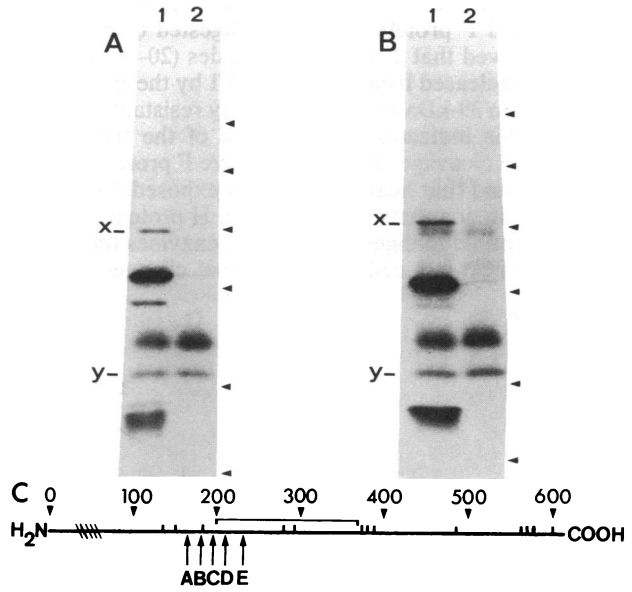

FIG. 3. Localization of the mAb I-41 epitope. (*A* and *B*) Footprinting (with anti-antibody added before digestion) with mAb I-41 and [^{35}S]methionine- and [^{3}H]glucosamine-labeled antigen (lanes 1 and 2, respectively). Protease used was trypsin, and digestion was for 2.5 hr. Samples were analyzed by PAGE under reducing (*A*) or nonreducing (*B*) conditions. (*C*) Schematic representation of the H protein based on its predicted amino acid sequence (9). Locations of the potential glycosylation sites (arrows A–E), the cysteine residues (short upright bars), and the predicted transmembrane anchor region (hatched) on the 617-residue protein are shown (amino acid numbers are indicated). Epitope of mAb I-41 is located between residues 200 and 370 (square bracket).

radioactive antigen complex followed by a washing step to remove unbound peptides. However, peptides from trypsin and chymotrypsin digestions were poorly precipitated by a number of mAbs. This was probably due to digestion of the mAb at the hinge region, which would release mAb-bound antigen peptides. We, therefore, added anti-antibody to the assay mixture to collect these mAb fragments that still bound antigen. Addition of anti-antibody to the protease-digested protein A-Sepharose–mAb–antigen complex prior to washing, resulted in a dramatic increase in the effective immunoprecipitation by a number of the poorly immunoprecipitating mAbs (Fig. 4*B*) with only marginal improvement in the yield for the other mAbs (Fig. 4*A*). This increase in effectiveness was also seen if the anti-antibody was added to the complex prior to digestion (Fig. 4*B*). With some mAbs, anti-antibody binding also changed the peptides generated (Fig. 4*C*). This indicates that binding of anti-antibody induces conformational changes in the protein antigen through the first-bound antibody. This effect was through the first antibody and was not due to steric hindrance by the second antibody, because rabbit anti-mouse IgG Fc instead of rabbit anti-mouse immunoglobulin also affected the peptides generated in the presence of some mAbs.

Comparison of mAb-Selected Variants. Ten H-protein *in vitro* mAb-selected variants (6) exhibiting differences in single epitopes were used to assess the ability of footprinting to detect small changes within a given protein. Footprinting was performed with various proteases and a mAb that retained its ability to bind to all the variants. Eight of the variants could be distinguished from each other. Results for two variants are shown in Fig. 5. Note that some variants could be differentiated with one protease but not another.

Detection of Conformational Changes in the Antigen. Detection of heat-induced conformational changes was most strikingly seen in studies on the F protein, the other measles virus glycoprotein. Non-heat-denatured ("native") F protein was poorly digested by trypsin and chymotrypsin, whereas heat-denatured F protein was easily digested (Fig. 6). The data also showed that 2- to 5-kDa peptides (20–50 residues) were rapidly released from the mature F1 by these proteases leaving a 36- to 39-kDa F1 that was highly resistant to further digestion. This indicates that the bulk of the trypsin and chymotrypsin cleavage sites on the native F protein must be inaccessible and that heat denaturation exposed these sites. Thus F protein contrasts markedly with H protein, which is highly susceptible to digestion by these enzymes (cf. Figs. 1 and 2). The heat-induced conformational changes in the F protein also drastically affected its antigenic characteristics as shown by the near total loss of reactivity of the heat-denatured F protein with all 11 anti-measles F-protein mAbs.

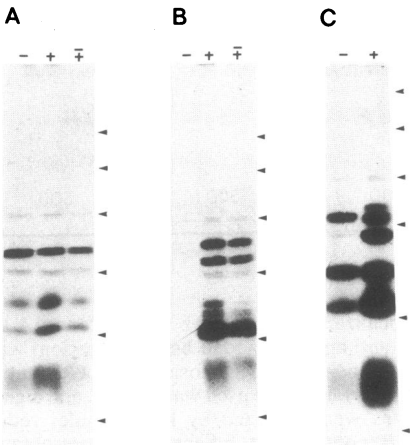

FIG. 4. Effect of anti-antibody binding. Footprinting was performed with mAbs I-41 (*A*), 7-AG11 (*B*), or 16-CD11 (*C*) and [^{35}S]methionine-labeled antigen. Digestion was with trypsin for 2.5 hr. Rabbit anti-mouse immunoglobulin was absent (−), added before digestion (+), or added after digestion (±).

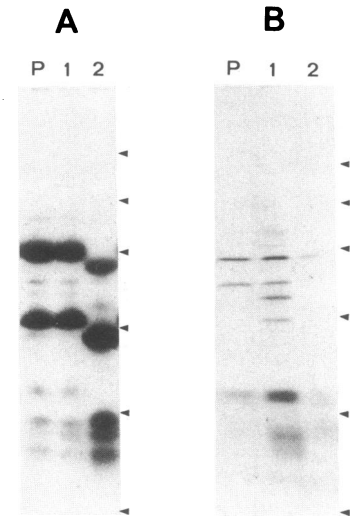

FIG. 5. Differentiation among mAb-selected H-protein variants differing in a single epitope. Variants (lanes 1 and 2) were selected from the parent virus (lane P) using two mAbs. Footprinting (with anti-antibody added before digestion) was performed with [^{35}S]methionine-labeled antigen and a mAb that retained reactivity with all three virus strains. Proteases used were V8 protease (*A*; digestion time, 2.5 hr) or trypsin (*B*; digestion time, 1 hr).

DISCUSSION

The present study describes applications of limited proteolysis of monoclonal antibody-bound protein antigens. The approach was termed protein footprinting, since various mAbs to a given protein differentially affected the peptides derived from limited proteolysis of that antigen. Measles virus mAbs directed against the H protein were chosen not only because the system was well characterized but also

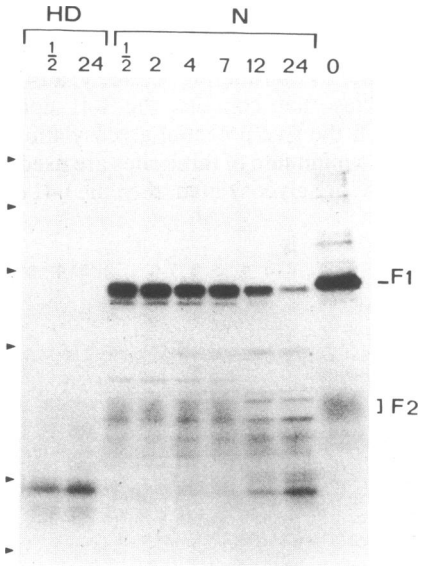

FIG. 6. Effect of heat-denaturation on the F protein. Footprinting without washing was performed with [^{35}S]methionine-labeled antigen, an anti-F mAb, and chymotrypsin. Prior to protease digestion, the immunocomplexes were either heat-denatured (HD) or untreated (N). Digestion times in hours are indicated above the respective lanes. Lane 0, control undigested F protein immunoprecipitated.

because these mAbs were difficult to distinguish by the laborious competitive-binding assay, as they exhibited a high degree of cross-competition. By *in vitro* mAb-selected variant analysis all nine mAbs were found to be directed against different epitopes, with two pairs of mAbs showing closely linked epitopes. By using three proteases, all nine mAbs could be differentiated from each other. Footprinting was, therefore, as sensitive as variant analysis. But unlike variant analysis, footprinting does not enable mapping of epitopes relative to each other, although it has some other advantages. (*i*) Differentiation between closely linked mAb epitopes requires selection of a large number of variants. (*ii*) Variant selection is not only time-consuming but also restricted to neutralizing mAbs. mAbs to internal viral proteins and nonviral proteins, therefore, cannot be studied. Only one other study (4) has attempted to differentiate among mAbs by analyzing the peptides produced by proteolysis of mAb-bound antigen. However, no comparisons with the competitive-binding assay and *in vitro*-selected variants were made in that study. Whereas the 17 herpes simplex virus mAbs examined could be separated into eight groups by conventional serological techniques, they could only be ordered into four groups by proteolysis patterns. This relative lack of success compared to the present study is probably technical, because only one proteolytic enzyme was used and anti-antibodies were not added to improve the yield of peptides collected. Anti-antibody treatment, as shown here, greatly enhances the amount of peptides obtained, which aids in differentiating among mAbs.

In sharp contrast to footprinting, if the antigen was partially digested prior to mAb binding, no major differences were seen in the pattern of peptides immunoprecipitated by the various mAbs. This traditional order of digestion was not only ineffective in distinguishing the mAbs but also resulted in drastic reduction in mAb reactivity, particularly with the low molecular weight antigen peptides. Numerous studies have indicated that mAbs are commonly directed against conformational (continuous or discontinuous) determinants, as protein fragments fail to bind effectively to the mAbs (see ref. 11). This highlights an important use of footprinting. Since some mAbs do efficiently immunoprecipitate low M_r antigen peptides, footprinting could be used as a method for localization of such conformational epitopes on a peptide, which in turn could be localized on the protein if its sequence is known. In such a study the isolation and amino acid sequence analysis of the peptides could be based on the methods described by Schwyzer *et al.* (3) and by Aebersold *et al.* (12). In this study we show how footprinting by itself can be used to localize the mAb epitopes, provided some other structural features of the protein are known. We used the glycosylation sites on the H protein as a reference point and were able to localize epitopes of four mAbs between residues 200 and 370.

Footprinting was found to be an excellent way of distinguishing mAb-selected variants. Eight of ten H-protein mAb-selected variants differing from each other in single epitopes, could be distinguished from each other by footprinting. Footprinting is superior in several ways to conventional limited proteolysis using protein bands cut from polyacrylamide gels (13). The latter approach is tedious, technically limited to proteins that can be obtained in relatively large amounts, and expected to be ineffective in detecting many conformational changes in the protein antigen because of the denaturing conditions used in electrophoresis (for example, the effect of heat denaturation on the F protein). The footprinting data indicate that the bulk of the trypsin and chymotrypsin cleavage sites on the native F protein are inaccessible to the proteases, but are accessible after the protein is heat-denatured. In contrast, footprinting data shows that the native H protein is highly susceptible to digestion by these enzymes. The proteolytic enzyme sensitivities of the two surface glycoproteins of measles virus are compatible with their post-translational modifications. The H protein does not appear to undergo any major proteolytic cleavage modifications, whereas the F protein is synthesized as a fusion-inactive precursor (F0) that is cleaved by cellular trypsin/chymotrypsin-like enzymes to yield two fragments F1 and F2. The disulfide-bonded F1 and F2 peptides form the active F protein (see ref. 14). The poor susceptibility of the native F1–F2 to further digestion by trypsin and chymotrypsin is, therefore, a suitable conformationally imposed feature of the activated F protein.

Footprinting also enabled detection of another category of conformational changes. In studies on the H protein, it was demonstrated that binding of anti-antibody to some of the mAbs during footprinting altered the peptides obtained. These data indicate that anti-antibody binding induces conformational changes in the protein through the first-bound antibody. To our knowledge, this is the first analytical demonstration of anti-antibody-induced conformational change in an antigen.

In conclusion, footprinting and its many modifications, some of which are described in this study, are all rapid, sensitive, versatile, inexpensive, and highly informative. Since the basic RIPA procedure involved is simple, it can easily be applied by investigators desiring to classify mAbs, detect amino acid sequence variation, or detect protein conformational changes. Protein footprinting should, therefore, find general application in many specific antibody–protein antigen systems.

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1. Mihalyi, E. (1978) *Application of Proteolytic Enzymes to Protein Structure Studies* (CRC, Boca Raton, FL).
2. Moelling, K., Scott, A., Dittman, K. E. J. & Owada, M. (1980) *J. Virol.* **33**, 680–688.
3. Schwyzer, M., Well, R., Frank, G. & Zuber, G. (1980) *J. Biol. Chem.* **255**, 5627–5634.
4. Eisenberg, R. J., Long, D., Periera, L., Hampar, B., Zweig, M. & Cohen, G. H. (1982) *J. Virol.* **41**, 478–488.
5. Galas, D. J. & Schmitz, A. (1978) *Nucleic Acids Res.* **5**, 3157–3170.
6. Sheshberadaran, H. & Norrby, E. (1986) *Virology* **152**, 58–65.
7. Sheshberadaran, H., Chen, S. N. & Norrby, E. (1983) *Virology* **128**, 341–353.
8. Sheshberadaran, H., Norrby, E. & Rammonhan, K. (1985) *Arch. Virol.* **83**, 251–268.
9. Alkhatib, G. & Breidis, D. J. (1986) *Virology* **150**, 479–490.
10. Bellini, W. J., Silver, G. D. & McFarlin, D. E. (1983) *Arch. Virol.* **75**, 87–101.
11. Moudallal, Z. A., Briand, J. P. & van Regenmortel, M. H. V. (1985) *EMBO J.* **4**, 1231–1235.
12. Aebersold, R. H., Teplow, D. B., Hood, L. E. & Kent, S. B. H. (1986) *J. Biol. Chem.* **261**, 4229–4238.
13. Cleveland, D. W., Fisher, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1101–1106.
14. Rima, B. K. (1983) *J. Gen. Virol.* **64**, 1205–1219.