

Identification of an Exposed Region of the Immunogenic Capsid Polypeptide VP₁ on Foot-and-Mouth Disease Virus

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Received 6 October 1982/Accepted 21 December 1982

Iodination of intact foot-and-mouth disease virus results in the selective labeling of VP₁, substantiating its exposed location on the virion. A comparison of tryptic peptides revealed that a single tyrosine-containing peptide was labeled with iodine on intact or protease-cleaved virus. The labeled peptide from intact and protease-cleaved virus was characterized by molecular weight sizing and sequence analysis. Carboxypeptidase digestion of intact VP₁, limited trypsin-cleaved VP₁, and VP₁ purified from bacterially contaminated tissue cultures yielded carboxy-terminal residues of leucine, valine-arginine, and serine-alanine, respectively. The correlation of these findings with previous data on the amino acid sequence derived from nucleotide sequencing of serotypes A₁₂ and O₁ of foot-and-mouth disease virus VP₁ places the probable exposed antigenic region of VP₁ in a serotype-variable region including residues 136 through 144.

Foot-and-mouth disease virus (FMDV), an aphthovirus of the Picornaviridae, contains a positive, single-stranded RNA enclosed in a protein capsid. The capsid contains 60 copies each of four polypeptides, VP₁, VP₂, VP₃, and VP₄, and 1 to 2 copies of VP₀, an uncleaved precursor of VP₂ and VP₄ (30). Previous reports have shown that the purified VP₁ polypeptide isolated from virus or biosynthesized by *Escherichia coli* is capable of eliciting neutralizing antibody in livestock and protecting them against challenge with foot-and-mouth disease virus (2, 3, 18, 22). (The fourth FMDV capsid polypeptide translated has been designated VP₃, VP₁, or VP_{Thr} due to its variable migration in different polyacrylamide gel electrophoresis (PAGE) systems (28). This structural component will be referred to as VP₁ in this report, in keeping with the provisional unified picornaviral nomenclature as recommended at the 1982 American Society of Virology meeting at Ithaca, N.Y.)

Unlike other picornaviruses, FMDV is sensitive to controlled protease digestion of intact virions, resulting in a specific cleavage of the VP₁ molecule, suggesting that VP₁ is the most exposed polypeptide of the capsid. Two distinct protease-derived fragments of VP₁, a 16-kilodalton (kd) fragment resulting from controlled trypsin digestion (16-kd T fragment) and a 15-kd fragment isolated from virus purified from bacterially contaminated tissue cultures (15-kd S fragment), have been shown to protect animals

against FMDV when purified and injected as a vaccine (3). Amino acid sequencing of these fragments revealed that both components originated at the amino terminus of the VP₁ molecule (3; unpublished data). A 13-kd CNBr-derived fragment from the interior of the VP₁ molecule was also determined to be immunogenic and protective (4, 17). Therefore, we postulate that all three molecules should contain a common exposed immunogenic region.

Virions were prepared for PAGE as follows. FMDV (strain A₁₂119, large-plaque, ab variant [15]) was grown in BHK-21 monolayer cells in rolling bottle cultures, concentrated by polyethylene glycol precipitation (31), and purified by CsCl density gradient centrifugation (6). Intact 140S virions containing the 16-kd T fragment derived from VP₁ were prepared by adjusting the virus concentration to 2 mg/ml with 0.014 M Tris (pH 8.0). Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington Biochemicals, Freehold, N.J.) was added at an enzyme-substrate ratio of 1:200, and the virions were digested at 37°C for 1 h. Virus was either repurified through sucrose gradients before iodination or disrupted and subjected to preparative PAGE as described below. Virions containing the 15-kd S fragment derived from VP₁ were occasionally obtained from our virus production unit. This *in situ* cleavage apparently resulted from tissue cultures containing an unidentified bacterial protease. A 300-μg quantity of virus in Tris-buffered saline (0.05 M Tris, 0.15 M NaCl,

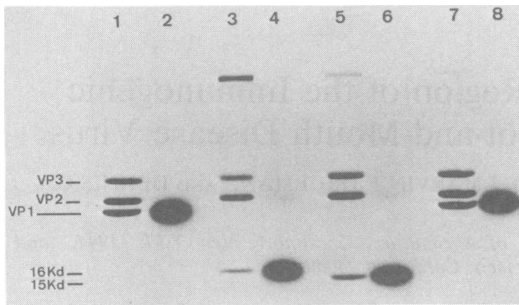


FIG. 1. Iodinated polypeptides from intact, trypsin-cleaved, and bacterially cleaved virions. Lanes: 1 and 7, Coomassie blue-stained FMDV marker polypeptides VP₁, VP₂, and VP₃ from intact virions; 2 and 8, iodinated VP₁ resulting from iodination of intact virions; 3, Coomassie blue-stained polypeptides from trypsin-treated virus (16-kd T fragment); 4, iodinated 16-kd T fragment resulting from iodination of trypsin-treated virions; 5, Coomassie blue-stained, bacterially cleaved virus (15-kd S fragment); 6, iodinated 15-kd S fragment resulting from bacterially cleaved virions. Lanes 2, 4, 6, and 8, Autoradiographs showing uptake of label for each type of particle.

pH 7.5) or 100 μg of VP₁ in 0.1% sodium dodecyl sulfate in Tris-buffered saline was reacted for 5 min with 0.5 mCi of ^{125}I (Na^{125}I , 100 mCi/ml in pH 7 to 10 NaOH; IMS.30, Amersham Corp., Arlington Heights, Ill.) over 25 μg of 1,3,4,6-tetrachloro-3,6-diphenylglycouril (Iodogen; Pierce Laboratories, Rockford, Ill.). After being labeled, the sample was transferred to a tube containing 0.2 μM NaI and 2.5 μM mercaptoethanol. Labeled virus or protein was separated from unincorporated iodine by Sephadex G-25 gel filtration. Before iodinated VP₁ was isolated from virus, the virus was repurified by sucrose gradient sedimentation to remove iodinated 12S subunits and obtain VP₁ labeled only on intact 140S virions. Unlabeled intact or protease-cleaved virus was then combined with iodinated particles and subjected to PAGE in a 12.5% polyacrylamide gel containing 8 M urea. After being stained and destained, the wet gel was exposed to Kodak BB film for 12 h at 4°C.

Although disruption of the viral capsid results in iodination of the four capsid polypeptides (23, 25, 26), iodination of intact or protease-cleaved virions resulted in the labeling of VP₁ or a VP₁-derived polypeptide (Fig. 1). Both trypsinized and bacterially cleaved virions contained accessible tyrosine groups. The VP₁ component of intact virions was the major polypeptide iodinated (lane 2), and the VP₁-derived 16-kd T (lane 4) and 15-kd S fragments (lane 6) were the major components iodinated in their respective particles.

VP₁ and its proteolytic fragments were purified by DEAE-Sephadex extraction (9) or by

PAGE on 8-mm thick 10 or 12.5% slab gels containing 8 M urea (1, 24). For preparative PAGE, the VP₁ band was visualized by cooling (1), cut out, and electroeluted into 0.01 M *N*-ethylmorpholine acetate buffer (pH 8.1) containing 0.02% sodium dodecyl sulfate with an ISCO 1750 electrophoretic concentrator. A trichloroacetic acid-precipitated and acetone-washed sample was suspended in 1 ml of 0.1 M ammonium bicarbonate (pH 8.0), and crystalline bovine serum albumin was added to 1 $\mu\text{g}/\mu\text{l}$. Trypsin was then added at an enzyme-substrate ratio of 1:4, and the sample was incubated at 37°C. After 5 h, an additional amount of enzyme was added, and digestion was allowed to proceed overnight. Digestion was terminated by the addition of a few drops of glacial acetic acid. Peptides resulting from extensive tryptic digestion were evacuated to dryness in a Savant Speed-Vac and suspended in 0.1% phosphoric acid. High-pressure separation was performed on a Hewlett-Packard 1084B liquid chromatograph with a Zorbax C18 reverse-phase column (3.9 by 300 mm; Waters Associates, Milford, Mass.). The column was eluted with 0.1% phosphoric acid or 0.1% trifluoroacetic acid, with a linear gradient of 0 to 60% acetonitrile at a flow rate of 2 ml/min. Fractions were collected every 0.4 min and counted in a Packard gamma counter.

Multiple peaks were observed in a high-pressure liquid chromatography (HPLC) reverse-phase tryptic map of denatured iodinated VP₁ (Fig. 2A). However, after iodination of intact virions and tryptic digestion of the VP₁ molecule, a single peptide was observed as the major product (Fig. 2B). The labeled tryptic peptide from iodinated, 16-kd T-containing virions had the same HPLC properties as the peptide iodinated on intact virions (data not shown). However, the iodinated tryptic peptide derived from virions containing the 15-kd S fragment migrated with different reverse-phase properties (Fig. 2C), indicating that cleavage to this fragment resulted in the exposure of a different tyrosine-containing peptide or the alteration of a property of the normally exposed peptide. When analyzed by gel filtration, the molecular weights of the iodinated tryptic peptides of denatured VP₁ ranged from 3,000 to 900, and the approximate molecular weight of the tryptic peptide labeled while associated with the virion was 900, based on extrapolation from known standards (data not shown). Similar analysis of the iodinated tryptic peptide from the 15-kd S-containing virions revealed a molecular weight of about 300, verifying that this peptide differed from the peptide iodinated on virions containing intact or trypsinized VP₁.

The iodinated tryptic peptides from intact virions and cleaved virions were collected after

HPLC separation (Fig. 2B and C). A thoroughly dried, ^{125}I -labeled sample was subjected to a modification of the manual Edman-dansyl degradation (16) in 50% aqueous pyridine buffer (pH

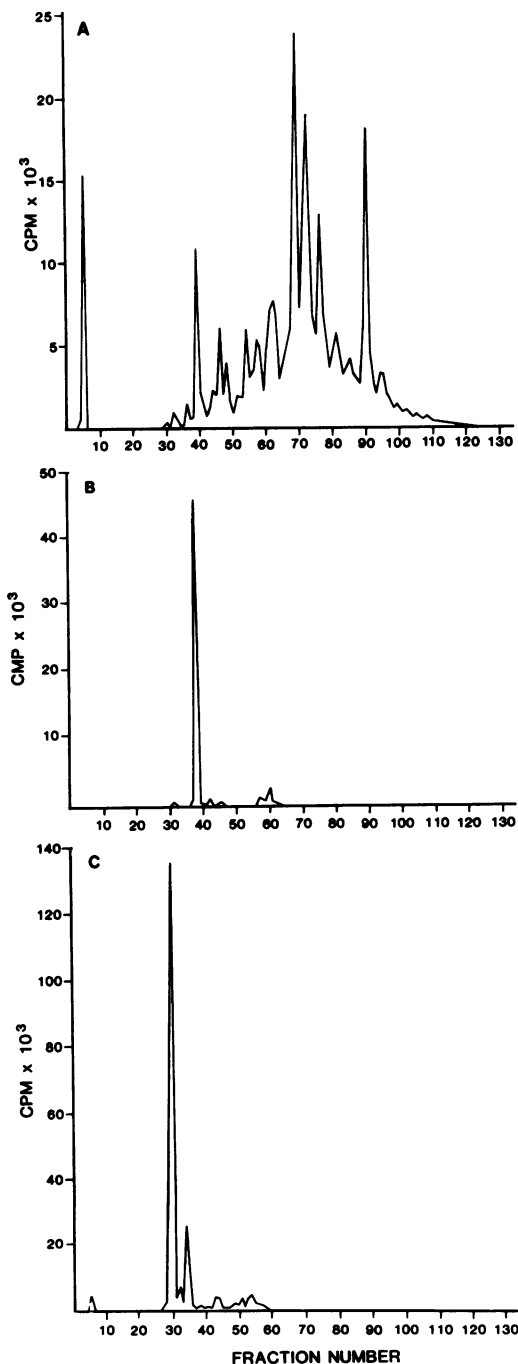


FIG. 2. Tryptic digestion of VP_1 labeled while denatured or virion associated. Tryptic peptides resulting from iodination of (A) VP_1 after purification, (B) the intact 140S capsid, and (C) 140S bacterially cleaved virions.

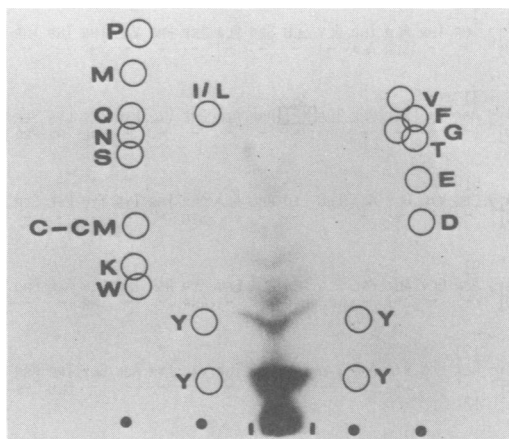


FIG. 3. Autoradiograph indicating amino-terminal residue of iodinated tryptic peptide derived from intact virions. Single-letter code for the Abbreviations: P, proline; M, methionine; Q, glutamine; N, asparagine; S, serine; C-CM, carboxy methyl-cysteine; K, lysine; W, tryptophan; V, valine; E, glutamic acid; G, glycine; T, threonine; D, aspartic acid; Y, tyrosine; I, isoleucine; L, leucine; F, phenylalanine.

8.5 to 9.0) with 5 μl of Beckman phenylisothiocyanate. After cleavage with anhydrous trifluoroacetic acid for 10 min at 50°C, the sample was dried, and the anilinothiazolinone derivative amino acids were extracted with butyl acetate. The butyl acetate extract was dried, and cyclization to the phenylthiohydantion (PTH) derivative was performed with 40% aqueous trifluoroacetic acid. After being dried, the mixture was spotted on a Chang-Chin polyamide thin-layer plate (without fluorescent indicator) with standard PTH amino acids on each side and chromatographed as described by Kulbe (19). The unlabeled standards were circled as a reference, and the plate was exposed to Kodak SB X-ray film for 2 to 3 days at -70°C . The developed film was overlaid on the circled standards to identify the position of any labeled residues.

From 50 to 80% of the radioactivity in the iodinated tryptic peptide from intact or cleaved virions was released in the first cycle. The resulting PTH derivatives were subjected to thin-layer chromatography with appropriate PTH standard amino acids, followed by autoradiography. Tyrosine was found to be the amino terminus of both the iodinated intact virion and the cleaved virion 15-kd S tryptic peptide, as shown by a representative autoradiogram (Fig. 3). The only tryptic peptide in VP_1 that contained an amino-terminal tyrosine was located between residues 136 and 144 (Fig. 4), therefore allowing us to conclude that this peptide or a fragment of it is exposed on intact and 15-kd-containing virions.

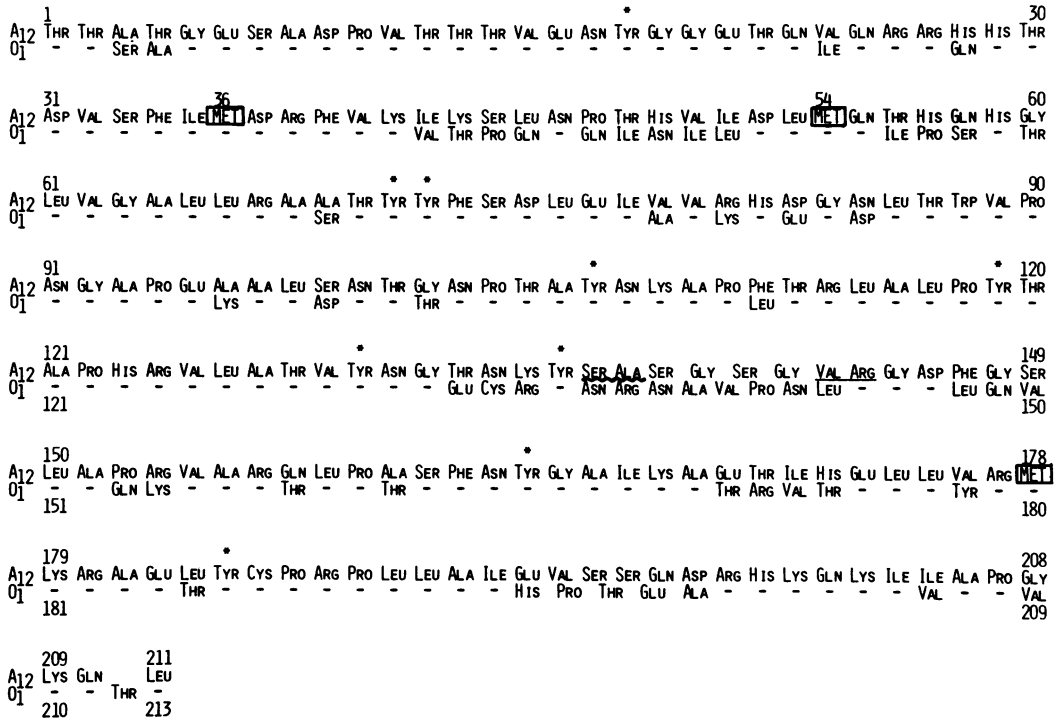


FIG. 4. Comparison of derived amino acid sequence for VP₁ of FMDV serotypes A₁₂ and O₁ Campos. Methionine residues cleaved by CNBr are indicated by boxes at positions 36, 54, and 179, and the carboxy termini of the A₁₂ 15-kd S (wavy lines) and 16-kd T (solid lines) components are designated beneath the respective amino acids. *, Tyrosine residues capable of iodination.

VP₁ and its 15-kd S and 16-kd T fragments (1 to 5 nmol of each), after being purified and acetone precipitated, were suspended in 0.2 M *N*-ethylmorpholine acetate (pH 8.2) containing 6 M urea. They were then subjected to cleavage with carboxypeptidase A (Worthington) or with carboxypeptidases A and B (Sigma Chemical Co., St. Louis, Mo.) at an enzyme-substrate ratio of 1:10 for 5 h at 37°C (11). The reaction was terminated by the addition of 1 ml of sulfonated polystyrene beads (Bio-Rad Laboratories, Richmond, Calif., no. AG50WX8), and the individual bound amino acids were eluted with 5 M aqueous ammonia. After lyophilization, the amino acids were identified with a Glenco custom modular MN-60 amino acid analyzer by fluorescence detection on a Gilson fluorimeter and quantitated with a Hewlett-Packard 3390A integrator.

Carboxypeptidase digestion of VP₁ verified the carboxy-terminal residue as leucine (Table 1). In addition, there was some material migrating in the position of serine (serine, glutamine, and asparagine comigrated in our system) after 5 h of digestion, but it was less than the quantitative release. Carboxy-terminal analysis of the 16-kd T fragment (Table 1; Fig. 4, solid line)

definitively placed the last two residues with the combination of carboxypeptidases B (a 1-h sample contained only arginine) and A. The wavy line (Fig. 4) designating the carboxy terminus of the 15-kd S fragment was determined on the basis of carboxypeptidase analysis (Table 1) and molecular weight characterization. Even at early incubation times, it was difficult to assess whether serine or alanine was released first, but on the basis of molecular weight characterization (the calculated molecular weight of Tyr-Ser-Ala is 321) and comparison with the amino acid sequence, it was concluded that alanine was the carboxy-terminal residue.

These experiments showed that tyrosine 136 (Fig. 4) was labeled with ¹²⁵I on intact virions and on particles containing the 16-kd T and 15-kd S VP₁ fragments. The information resulting from these experiments, when correlated with the derived amino acid sequence data (12, 18, 20, 21) of bacterially cloned serotype A₁₂ and O₁ VP₁'s (Fig. 4), indicates that the region including amino acids 136 through 144 is exposed to the exterior of the virion. These conclusions are based on the identification of an exposed iodinated tyrosine residue in proximity to the regions identified that were susceptible to protease

TABLE 1. Amino acids released by carboxypeptidase digestion

Enzyme ^a	Amino acids released (nmol) after 5-h digestion of (amt [nmol]):		
	VP ₁ (1.1)	16-kd T (1.8)	15-kd S (2.3)
Carboxypeptidase A	Leucine (1.0)	ND ^b	Alanine (1.5), serine (1.5)
Carboxypeptidases A and B	ND	Valine (1.4), arginine (1.4)	ND
No enzyme	None	None	None

^a Carboxypeptidase A digestion for 5 h, or carboxypeptidase B digestion for 1 h, followed by a 4-h digestion with carboxypeptidase A at 37°C.

^b ND, Not done.

cleavage. The area (residues 136 through 144, Fig. 4), which we have directly characterized as being exposed on the virion, is in the same region as one concluded by Strohmaier et al. (29) to be involved in inducing neutralizing antibodies in mice. In addition, Bittle et al. (10) recently showed that a chemically synthesized peptide spanning the analogous region of the serotype O₁ VP₁ sequence induced neutralizing antibodies against FMDV in rabbits and guinea pigs. As nothing is known of the tertiary structure of VP₁ within the capsid structure of FMDV, our information substantiates previous speculations (18, 29) that this region is an exposed region of VP₁ responsible for inducing effective immunity against FMDV.

Protease treatment does not appear to cause a major reorganization of the virus surface, at least relative to tyrosines (as detected by iodination), or the exposure of VP₂ and VP₃ to iodination or proteolysis. However, charge alterations (8, 14, 28) indicate some changes in the virion surface. This is also suggested because protease treatment drastically reduces the ability of type A virus to bind to cells (7), although the ability of protease-treated virus to induce neutralizing antibody and immunity appears to vary (2, 3, 25). Since the virion in situ protease sites are contained in an amino acid-variable region of VP₁, corresponding differences in the cleavage location might explain discrepancies in the ability of various protease-treated FMDV subtypes and serotypes to induce effective neutralizing antibodies and immunity in animals (2, 3, 14).

The 16-kd T and 15-kd S fragments of serotype A₁₂ (3), as well as the 13-kd fragment derived from CNBr digestion (4, 17) (residues 55 through 179), are immunogenic and protective, suggesting that a region included within residues 55 through 138 would effectively induce immunity. Preliminary assessment of this hypothesis with an 8.5-kd segment resulting from CNBr cleavage of the 15-kd S fragment (residues 55 through 138) revealed that this region was not effective in competing against virions for VP₁-reactive antibodies, nor did it induce virus-neutralizing antibodies in guinea pigs (D. M.

Moore and B. H. Robertson, manuscript in preparation). This information suggests that there are at least two antigenic regions in the VP₁ molecule that may be involved in inducing effective immunity against FMDV. As CNBr treatment of the 15-kd S or 16-kd T fragment would remove a serotype-variable region spanning residues 42 through 51, studies are currently underway to assess the role of this portion of the molecule in effective immunity against foot-and-mouth disease.

The helpful technical assistance of K. Axelson, J. Dean, T. Fischer, and E. Whittle is gratefully appreciated.

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