Heterogeneity of the Genome-Linked Protein of Foot-and-Mouth Disease Virus

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The genome-linked protein of foot-and-mouth disease virus was examined by electrofocusing in polyacrylamide gels. Two proteins of different charge and amino acid composition were found. The tryptic peptide maps of the proteins were dissimilar. The possible relationship between the two proteins is discussed.

The picornaviruses contain a single-stranded RNA molecule that can act directly as an mRNA. The virus RNA is similar to other mRNA's in having a polyadenylic acid tract at the 3' end (14), but differs from most cellular and viral mRNA's in not having a capping group at the 5' end (5). Instead, picornavirus RNAs are blocked at the 5' end by a small covalently linked protein, VPg (6, 9, 16, 18). Most of the work characterizing VPg has been done with poliovirus. It has been estimated that poliovirus VPg has a molecular weight of about 5,000 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (9), although a molecular weight of about 12,000 was obtained by gel filtration (1). The protein is hydrophobic and basic and is linked to the 5'-terminal uridylic acid of the RNA through the sole tyrosine residue (1, 15). Although all the RNA molecules synthesized in poliovirus-infected cells have a protein linked to the 5' end (6, 10), the mRNA extracted from polysomes terminates with 5' pUp (11, 13). There must, therefore, be a mechanism for removing VPg from the RNA, and an enzymatic activity capable of removing VPg has been found in cell-free systems prepared from both infected and uninfected cells (2).

There is less information concerning the properties of the VPg on foot-and-mouth disease virus (FMDV) RNA. It is known that this protein is small (molecular weight 5,000 by SDS-gel electrophoresis) and, unlike poliovirus VPg, contains methionine (18). The protein is not needed for the infectivity of the RNA (18) nor for the efficient translation of the RNA in vitro (17), and at least a proportion of the mRNA lacks VPg (D. V. Sangar, unpublished data).

In this paper we have examined some other characteristics of the VPg from FMDV. VPg was resolved into two components by electrofocusing (EF) in polyacrylamide gels demonstrating charge heterogeneity in FMDV VPg. The possible relationship between these two components was studied by differential labeling and tryptic peptide mapping.

MATERIALS AND METHODS

Viruses and plaque purification. FMDV type A10 (strain A⁶¹) and type O (Pacheco) and poliovirus type 1 (Mahoney) were used. For plaque purification, virus was titrated in BHK-21 cells in glass petri dishes. After allowing 20 min for adsorption of virus, cells were overlaid with 0.9% agar in Eagle medium containing 10% ox serum and 10% tryptose phosphate broth. The cells were incubated at 37°C for 24 h and then stained with neutral red in phosphate-buffered saline (1:20,000) in 0.9% agar for 1 h at 37°C. Plaques were taken from petri dishes containing a maximum of two plaques. The cloning procedure was repeated twice, and the virus was grown to a working stock by one further passage in BHK cells. This stock was then used to prepare labeled virus.

Preparation of radioactively labeled viruses. Monolayers of 10⁶ BHK cells in Roux bottles were infected at a multiplicity of infection of ≈ 10 . Virus was allowed to adsorb for 30 min. The cell sheet was then washed in either phosphate-free Earle saline or Eagle medium lacking particular amino acids. Virus RNA was labeled with ³²P as described previously (7), using a total of 20 mCi per experiment. Protein was labeled by adding 1 to 10 mCi of [³H]leucine, [³H]tyrosine, or [³⁵S]methionine at 1.5 h postinfection using 1 mCi per Roux bottle. Isotopes were purchased from the Radiochemical Centre, Amersham, U.K.

Purification of virus RNA. BHK cell monolayers infected with virus at a high multiplicity of infection are destroyed by the virus and leave the glass at about 3 h postinfection. Cellular debris was removed from infected cell supernatants by centrifugation for 10 min at 10,000 $\times g$, and the virus and virus RNA were purified by sucrose gradient centrifugation as described (7, 16).

Iodination of VPg. Purified FMDV RNA (100 μ g) was labeled with ¹²⁵I using the Bolton and Hunter reagent. The procedure was as described (15), except that *n*-lauryl sarcosine was used instead of lithium dodecyl sulfate.

Enzymatic digestion of virus RNA. Viral RNA, in the presence of 25 μ g of *Escherichia coli* tRNA (BDH), was digested completely with a mixture of 700 U of RNase A, 500 U of RNase T₁, and 33 U of RNase T₂ per ml in 25 μ l of 0.05 M ammonium acetate (pH 5.2) by incubating for 16 h at 37°C. RNA was digested with RNase T₁ alone at an enzyme substrate ratio of 1:20 in 25 μ l of 10 mM Tris-hydrochloride-1 mM

EDTA (pH 7.4) by incubating at 37°C for 1 h.

EF. To 25 μ l of VPg were added 25 μ g of urea (Aristar, BDH), 1.9 μ l of Nonidet P-40, and 1.9 μ l of 2-mercaptoethanol. Structural proteins of FMDV, type O Pacheco (5 μ g), were added as a marker, and the mixture was electrofocused on 4% polyacrylamide gels containing 9 M urea and LKB Ampholine ampholytes. EF towards the anode was done on gels containing Ampholine at 1% (vol/vol; pH 2.5 to 4), 1.5% (vol/vol; pH 5 to 7), and 2.5% (vol/vol; pH 3.5 to 10). The upper (cathodic) reservoir contained 0.2 M KOH, and the lower (anodic) reservoir contained 0.1 M H₃PO₄. Voltage was applied for 1 h at 200 V, followed by 5 h at 400 V. EF towards the cathode was done on gels containing 5% (vol/vol) Ampholine (pH 3.5–10) with the polarity and reservoir solutions reversed and with 2.5 μ l of 0.1 M mercaptoethylammonium chloride added to the sample. EF was for 1 h at 200 V and 4 h at 400 V. Complete equilibrium is not attained in either procedure, enabling highly acidic or basic polypeptides to be focused before reaching their isoelectric points. This approach was first described (12) under the name "nonequilibrium pH gradient electrophoresis." Here, we have used the simpler term "electrofocusing." Markers were located by staining the gels for 20 min in 30% 2-methoxyethanol containing 15 μ g of Kenacid blue per ml, and the gels were fractionated into 2-mm slices. Radioactivity was measured as described previously (19).

Peptide mapping. VPg was prepared by precipitating virus RNA with 2 volumes of acetone in the presence of carrier bovine serum albumin. Carboxymethylation and trypsin digestion were done before complete digestion of the RNA with RNase. The digest was evaporated to dryness under N₂, and the peptides were analyzed by ion-exchange chromatography (4).

The two fractions obtained by EF, VPgA and VPgB, were eluted from EF gels by incubating at 37°C for 16 h in 0.5 ml of 0.1 M Tris-hydrochloride (pH 8.1), 0.1% mercaptoethanol, and 1% SDS with 5 mg of bovine serum albumin per ml. The proteins were detected by counting 10- μ l samples, and the proteins were carboxymethylated as described previously (3). Reagents were removed by precipitating the proteins with 4 volumes of acetone at -20°C for 48 h, since VPg is only partially precipitated by 15% trichloroacetic acid but is completely precipitated by 80% acetone. The precipitate was washed twice with acetone and digested with trypsin, and the peptides were analyzed by ion-exchange chromatography (4).

Polyacrylamide gel electrophoresis. VPgA and VPgB were eluted from EF gels, described as above, except that the concentration of bovine serum albumin was reduced to 25 μ g/ml. Each acetone precipitate was dissolved in 100 μ l of 0.125 M Tris-hydrochloride (pH 6.8)–2% SDS–2% mercaptoethanol–10% glycerol, boiled for 1 min, and then analyzed on 17.5% polyacrylamide gels in tubes (10 by 0.8 cm) using the Laemmli buffer system (8). Tris-borate–6 M urea gels were run as described (18).

RESULTS

EF of FMDV-VPg. FMDV RNA that had been labeled in VPg with [³H]tyrosine was di-

gested with a mixture of RNases. This treatment should result in the liberation of VPg attached to the 5'-nucleotide diphosphate, i.e., VPg-pNp. EF of this material gave two peaks, A and B (Fig. 1a); the major component (A) is more acidic than the minor component (B).

There are a number of trivial explanations for the production of more than one peak, and it was important to eliminate these before proceeding. The most likely possibilities were considered to be the following.

(i) **Incomplete digestion of the nucleic acid.** The digestion conditions used had been chosen to ensure complete hydrolysis of the RNA. Nevertheless, as a further check, material that had been treated once with RNases A, T₁, and T₂ was boiled, a fresh mixture of RNases was added, and incubation was continued for a further 18 h at 37°C. EF of this material revealed the same two components (Table 1).

(ii) **One of the peaks is a contaminant and is not linked to nucleic acid.** This possibility was eliminated by two experiments. First, RNA was digested with RNase T₁ and analyzed by EF. This treatment also gave two peaks with the

FIG. 1. EF of [³H]tyrosine-labeled VPg. (a) FMDV RNA digested for 18 h with a mixture of RNases A, T₁, and T₂ to yield VPg-pUp. (b) FMDV RNA digested for 1 h with RNase T₁ to yield VPg-pUpUpGp. Conditions for EF were as described in the text.

TABLE 1. Ratios of VPgA to VPgB obtained by recovery from EF gels

Radiolabel	Virus serotype and RNase treatment						Mean $\pm$ SD ^b
	Type A	Type A	Type A	Type A	Type A	Type O	
	A, T ₁ , T ₂ ^a	2 $\times$ A, T ₁ , T ₂	Type A (6 h) A, T ₁ , T ₂	Type A (cloned) A, T ₁ , T ₂	T ₁	A, T ₁ , T ₂	
[³² P]orthophosphate	2.3 (3) ^c						2.3 $\pm$ 0.1
[³ H]tyrosine	1.9 (4)		1.9	2.3 (2)	2.0	2.0	2.0 $\pm$ 0.3
[³ H]leucine	4.0 (2)	2.8		3.7	4.8		3.9 $\pm$ 0.7
[³⁵ S]methionine	0.09						0.09
¹²⁵ I-labeled Bolton and Hunter reagent	1.6 (2)				1.6		1.6 $\pm$ 0.03

^a A, T₁, T₂ = RNase A, RNase T₁, and RNase T₂, respectively.

^b Differences between mean ratios for [³H]Tyr and [³H]Leu and for [³H]Tyr and ¹²⁵I are statistically significant ($P < 0.01$) by Student's *t* test; [³H]Tyr - ³²P difference is not significant by the same test. SD, standard deviation.

^c The numbers in parentheses refer to the number of determinations made with different RNA preparations.

same ratio of A to B as material that had been completely digested; however, the position of the peaks was shifted towards a more acidic region of the gel (Fig. 1b). This result suggests that both forms of VPg are linked to the 5'-terminal T₁-resistant oligonucleotide (6a) and at the same time eliminates the possibility of incomplete digestion of the RNA.

Second, virus RNA labeled with ³²P and completely digested with the RNase mixture also gave two peaks of radioactivity when analyzed on EF gels. These were in the same position in the gels as the peaks labeled with tyrosine (Fig. 1a) and had the same ratio of counts in A and B (see Fig. 3b and Table 1), showing that both proteins are attached to the RNA and are thus genome-linked proteins.

(iii) **A and B arise from different virus populations.** To eliminate this possibility, the stock virus (A61) was plaque purified three times. Two separate plaques from the third cloning were selected and grown in the presence of [³H]tyrosine, and their VPg's were examined by EF. Both gave profiles that could not be distinguished from that obtained with VPg from the parent virus (Table 1). Furthermore, a different serotype of FMDV (O Pacheco), which had been plaque purified several times, gave a profile identical to that obtained with A61. Therefore, it is most unlikely that the two peaks obtained by EF are due to a mixed population of virus.

Differential labeling of VPg. We reported previously that when VPg-pUpUpGp labeled with [³⁵S]methionine was examined by electrophoresis on Tris-borate-buffered polyacrylamide gels at pH 8.3 in 6 M urea, only one peak was detected (18). In view of the fact that two peaks were obtained when [³H]tyrosine-labeled VPg-pUpUpGp was examined by EF, it was important to examine similarly labeled material on Tris-borate-6 M urea gels. Figure 2 shows that two peaks were obtained with the same

FIG. 2. Separation of [³H]tyrosine-labeled VPg on Tris-borate-polyacrylamide gels at pH 8.3. The viral RNA was digested with RNase T₁ to give VPg-pUpUpGp and analyzed as described in the text. XC shows the position of the xylene cyanol dye marker.

distribution of label between the two peaks as found in the EF gels (Fig. 1b). Since SDS-gel electrophoresis revealed no difference in the molecular weight of the two fractions (see below), their separation on Tris-borate gels is probably due to the difference in their charge.

The single peak of [³⁵S]methionine VPg that we observed previously on Tris-borate gels suggested that only one component of the mixture contained methionine. This was confirmed by EF of [³⁵S]methionine-labeled VPg-pUp. Figure 3a shows that over 90% of the label is found in peak B (see also Table 1).

This result prompted us to examine VPg labeled with another amino acid by EF. [³H]leucine was chosen because it was known from previous work that this amino acid was readily

FIG. 3. Analysis of VPg-pUp by EF using the following radioactive labels: (a) [^{35}S]methionine (●); (b) [^3H]tyrosine (●) and ^{32}P (○); (c) [^3H]leucine (●) and ^{32}P (○); (d) ^{125}I introduced by Bolton and Hunter reagent modification of amino groups.

incorporated into VPg (T. R. Doel and D. V. Sangar, unpublished data). The distribution of this amino acid in FMDV VPg is compared to that of [^3H]tyrosine in Fig. 3b and c. To eliminate errors due to variations in the recovery of radioactivity, VPg-pUp labeled with ^{32}P was incorporated as an internal marker in each gel. Examination of Fig. 3b and c shows that, although the ratio of ^{32}P in peaks A and B is the same as for [^3H]tyrosine, there is an even greater amount of leucine in peak A than in peak B (see also Table 1).

We also examined the distribution of label in the two peaks after in vitro labeling of VPg. Attempts to label tyrosine residues in VPg with ^{125}I by the chloramine-T procedure were unsuccessful. However, FMDV RNA could be labeled in VPg with ^{125}I using the Bolton and Hunter reagent specific for amino groups. Examination of this material by EF after complete digestion of the RNA gave two peaks (Fig. 3d), both of which were shifted towards the acidic end of the gel. This charge shift was expected, since modification by the Bolton and Hunter reagent neutralizes the positively charged amino groups. The magnitude of this shift was such that ^{125}I -labeled peak B of VPg-pUp focused at the same pH as unmodified peak A. That this is not a coincidence is suggested by the additional finding (not shown) that exactly the same relationship exists between the modified and unmodified forms of VPg-pUpUpGp, i.e., VPg linked to the 5'-terminal T_1 RNase-resistant oligonucleotide. These results are consistent with the possibilities that (i) peak A and peak B differ from each other by a single unit of charge, and (ii) Bolton and Hunter reagent modifies, at most, a single amino group per VPg molecule.

The ratio of counts in peaks A and B for the five different radiolabels studied is summarized in Table 1. Neither the source of the VPg nor its subsequent treatment exerted any apparent influence on the proportions of the two VPg components. In contrast, the ratio of peak A to peak B was dependent on the radiolabel used. Compared with [^{32}P]orthophosphate and [^3H]tyrosine, for which A:B was approximately 2, labeling with [^3H]leucine resulted in consistently higher ratios (mean 3.9), and ^{125}I -labeled Bolton and Hunter reagent gave lower ratios (mean 1.6); both these differences were significant ($P < 0.01$).

Tryptic peptide maps of VPg. The relationship between peaks A and B obtained by EF was investigated by comparing their tryptic peptide maps with that of unfractionated VPg labeled with [^3H]leucine (Fig. 4). Although it is clear that A and B have different maps, the single leucine-containing peptide found in peak B is

also present in peak A, suggesting that the two proteins have some sequence in common, although the extent of this similarity is unknown. The map of unfractionated VPg resembles that of VPgA except that the amount of the peptide shared by A and B is greater.

Molecular weight of VPg. Although we have previously reported that the molecular weight of FMDV-VPg is approximately 5,000, this result was obtained using [^{35}S]methionine-labeled VPg, an amino acid found only in peak B. We therefore examined the molecular weights of the separated polypeptides. Peaks A and B, labeled with [^3H]tyrosine, were isolated from EF gels and run on parallel 17.5% SDS-polyacrylamide gels (results not shown). Peaks A and B migrated as broad bands to the same place in the gel relative to the front, indicating that the two proteins have a similar molecular weight.

DISCUSSION

Nature of the linkage between the protein and the RNA. Both polypeptide fractions of VPg are linked to the genome and contain tyrosine in a form inaccessible to iodination using chloramine-T. It is probable, therefore, that each VPg molecule contains only a single tyrosine residue, the hydroxyl group of which is blocked by its linkage to RNA. Furthermore, it has been shown that the final nucleotide sequence at the 5' end of the RNA is pUpUpGp (6a). It is likely, therefore, that the VPg-RNA linkage in FMDV is the same as that found in poliovirus, namely, a tyrosine phosphodiester linkage to uridine (1, 15).

Differences in amino acid composition between VPgA and VPgB. If there is only a single tyrosine residue per mole of VPg, the distribution of [^3H]tyrosine between VPgA and VPgB provides a measure of their molar ratio. This should also be true for ^{32}P -labeled VPg. As Table 1 shows, the ratio of the two peaks was not significantly different for these two radiolabels, from which we conclude that preparations of VPg consist of approximately 2 mol of VPgA per mol of VPgB.

The three other radiolabels studied show that VPgA and VPgB have quite different amino acid compositions. Thus, VPgB contains more than 20 times as many methionine residues per mole as VPgA. Indeed, it is possible that VPgA contains no methionine and that the small number of counts detected in this region represents modification of a small percentage of the lysine residues with the consequent alteration in charge during the preparation of the sample. In contrast to this, VPgA contains approximately twice as many leucine residues per mole as VPgB. Sig-

FIG. 4. Analysis of ion-exchange chromatography of the [^3H]leucine-containing tryptic peptides of (a) unfractionated VPg; (b) VPg peak B; (c) VPg peak A.

nificantly less ^{125}I was incorporated into VPgA than into VPgB, indicating that the more basic fraction (VPgB) is a better substrate for Bolton and Hunter reagent, presumably because of the presence of an extra lysine residue.

The size of VPg was estimated from its mobility in SDS-polyacrylamide gels to correspond to a peptide of 40 to 50 amino acid residues. The present work supports the conclusion that VPg is a small protein because (i) VPg contains only a single tyrosine residue, (ii) most, if not all, molecules of VPgA are devoid of methionine, and (iii) comparison of tryptic peptide maps suggests that VPgB has only a single leucine residue.

Variations in charge among different forms of VPg. The variations in charge seen in this study arise from three independent causes, as follows. (i) The isoelectric point of VPg de-

pends on the number of nucleotides that remain attached to the polypeptide after digestion of the RNA. Digestion with a mixture of RNases A, T_1 , and T_2 yields VPg-pUp, whereas treatment with RNase T_1 produces the more acidic derivative, VPg-pUpUpGp. (ii) [^{125}I]VPg, produced by modification with Bolton and Hunter reagent, is more acidic than unmodified VPg. (iii) The separation of VPg into acidic (A) and basic (B) fractions was due to differences in amino acid composition, the two forms of VPg being similar in size.

These three kinds of charge-shift can be compared quantitatively. VPg-pUpUpGp carries two more negative charges than VPg-pUp. The resulting shift in EF position was identical to the combined effect of the other two kinds of charge-shift. Thus, the B peak of VPg-pUpUpGp (Fig. 1b) focused at the same pH as the A peak of

[¹²⁵I]VPg-pUp (Fig. 3d). This confirms our earlier conclusions that there is a net difference of one charge between VPgA and VPgB, and that the Bolton and Hunter reagent neutralizes a single positive charge.

Are VPgA and VPgB related? The same two species of VPg, in the same proportions, were found in two different serotypes of FMDV. One of the functions of VPg may be to act as a primer in RNA synthesis (6, 11), although the mechanism involved is not known. However, the high degree of charge conservation suggests that the heterogeneity in VPg may be functionally important. In a preliminary examination (not shown), [³H]tyrosine-labeled VPg from poliovirus type 1 (Mahoney) exhibited a similar heterogeneity. The major, acidic component of poliovirus VPg, corresponding to peak A, electrofocused at the same pH as VPgB of FMDV.

There are two explanations for the heterogeneity; either the different forms of VPg are unrelated in sequence and are encoded independently, or they are generated by cleavage from a common precursor. The fact that one component is deficient in methionine and the other in leucine rules out a simple precursor-product relationship between VPgA and VPgB. Moreover, the tryptic peptide maps are different. Caution must be used in the interpretation of these maps, since only the leucine-containing tryptic peptides have been analyzed. Experiments to examine the lysine- and arginine-containing peptides, which should be representative of the entire protein, have so far been unsuccessful owing to the relatively poor labeling of VPg with these amino acids.

Despite the obvious differences discussed above, the two forms of VPg have several features in common, which suggests that they may be related: (i) VPgA and VPgB are related by a single charge-shift, (ii) one leucine-containing peptide is apparently shared by both forms of VPg, (iii) they have a similar molecular weight, (iv) they each have a single genome-linked tyrosine residue. In addition, since only a single VPg molecule exists per genome, it is difficult to explain why two completely unrelated forms of VPg should be required.

If VPgA and VPgB are derived from a common precursor, then alternative cleavages must occur on either side of the genome linkage, one end of the precursor polypeptide being rich in leucine and the other containing methionine. Such cleavages take place before encapsidation of the RNA, since incubation of mature virus particles with cell harvest, although causing some degradation of the virus RNA, had no effect on the proportions of the two forms of

VPg (Table 1). Work to isolate the putative precursor of VPg is in progress and should help us to understand the role of this protein.

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