The Genome-linked Proteins of Aphthoviruses: Specific Immunoprecipitation of the Three Species Detected on Virus RNA and Identification of Possible Precursors

By D. V. SANGAR, R. P. CLARK, D. J. ROWLANDS, J. L. BITTLE AND R. A. HOUGHTEN

1Wellcome Biotechnology Ltd, Ash Road, Pirbright GU24 0NQ, U.K. and 2Scripps Clinic and Research Institute, La Jolla, California 92037, U.S.A.

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SUMMARY

Synthetic peptides have been made corresponding to the C-terminal portion of each of the three presumptive genome-linked proteins (VPgs) of foot-and-mouth disease virus type A10. Antisera against each of these peptides efficiently precipitated only the homologous VPg, and the reactions were inhibited by prior absorption with homologous, but not heterologous synthetic peptide. The peptide antisera precipitated a number of proteins from infected cell extracts with mol. wt. of 100, 84, 56, 36, 27, 25 and 20, all × 10^3; all these reactions were inhibited by absorption with homologous peptide, indicating that they were probable precursors of VPg. The relationship between these proteins is at present unclear.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) is a member of the family Picornaviridae (genus aphthovirus) with a single-stranded, positive-sense RNA genome of approximately 8.5 kb. The RNA is similar to the genome of other picornaviruses in being polyadenylated at the 3' end and blocked at the 5' end by a small genome-linked virus protein designated VPg (Sangar, 1979).

The replication of picornavirus RNA has been extensively studied with poliovirus, where it has been shown that two proteins are required for transcription of the RNA in vitro. One of these is the RNA-dependent RNA polymerase (Flanegan & Baltimore, 1977) while the other is the so-called host factor (Dasgupta et al., 1980). The role of VPg in replication is somewhat controversial. Originally, it was suggested that it could act as a primer for transcription of new RNA strands (Baron & Baltimore, 1982). However, recent work has suggested that the priming event for transcription of positive-strand template RNA may involve a terminal uridylate transferase activity associated with the host factor which elongates the poly(A) with a tract of uridylate residues. It is proposed that the U tract then acts as a covalently attached primer which hybridizes to the poly(A) to initiate transcription. VPg appears to be linked to the daughter strand by a mechanism involving chain scission at the poly(A)–poly(U) junction (Young et al., 1985; Andrews et al., 1985).

Antibodies against chemically synthesized VPg, or portions of the molecule, have been used to detect possible precursors of VPg in poliovirus-infected cells (Semler et al., 1982; Baron & Baltimore, 1982). The direct precursor of VPg appears to be a protein, P3-9 (for nomenclature, see Rueckert & Wimmer, 1984) which is membrane-bound, and it has been suggested that P3-9 may be the donor of VPg to RNA chains in the membrane-bound replication complex (Semler et al., 1982).

The region of the genome that codes for VPg in poliovirus is 66 nucleotides in length and thus codes for a single protein 22 amino acids long (Kitamura et al., 1980). The situation with the aphthoviruses is, however, more complicated since isoelectric focusing (IEF) experiments
resolved aphthovirus VPg into two distinct species (King et al., 1980). Since the ratio of $^{32}$P (donated by the RNA) in these two species was 2:1 it was suggested that the VPg detected on virion RNA was a heterogeneous population of three different proteins (King et al., 1980). Sequence data revealed a region of the genome corresponding to the region known to code for VPg in poliovirus, which had a coding potential for three VPgs (Forss et al., 1984; Carroll et al., 1984; Robertson et al., 1985).

We have used the sequence information available for FMDV serotype A$_{10}$ to chemically synthesize peptides corresponding to the C-terminal 15 amino acids of each protein. These peptides were coupled to keyhole limpet haemocyanin (KLH) and used to raise antiserum. In this paper we show that the antiserum reacts not only with the peptide used to raise it but also with the equivalent native VPg and also with a number of possible precursors found in infected cells.

**METHODS**

*Synthesis of peptides and coupling to carrier protein.* Peptides were synthesized using the solid-phase method as described by Bittle et al. (1982). The peptides were coupled to a protein carrier (KLH) through the cysteine of the peptide using N-maleimidobenzoyl-N-hydroxysuccinimide ester (Liu et al., 1979). Rabbits were injected with approximately 50 µg of peptide in Freund's incomplete adjuvant, and were boosted at monthly intervals with the same material.

*ELISA assay.* The indirect ELISA test described by Abu-Elzein & Crowther (1978) was used. Fifty µl of uncoupled peptide solution (10 µg/ml) was absorbed onto a microtitre plate, and, after washing, reacted with 50 µl of antiserum at a dilution of 1/50 to 1/1600. Goat anti-rabbit IgG-conjugated horseradish peroxidase (50 µl) at a dilution of 1/1000 was added and, after washing, the reaction was developed using o-phenylenediamine and hydrogen peroxide; the colour was read at 492 nm.

*Preparation of virus RNA.* BHK cell monolayers were infected with a high multiplicity of virus (type A$_{10}$ strain A61). After approximately 3 h the infected cells were harvested and the virus RNA was purified by sucrose gradient centrifugation (Rowlands et al., 1978).

*Iodination of VPg.* Purified FMDV RNA (100 µg) was labelled with 200 µCi $^{125}$I using the Bolton & Hunter reagent (Rothberg et al., 1978).

*Enzymic digestion of virus RNA.* Virus RNA, in the presence of 25 µg *Escherichia coli* tRNA, was digested to completion with 25 µl of a mixture of 700 units RNase A, 500 units RNase T1 and 33 units RNase T2 in 0.05 M-ammonium acetate pH 5.1.

*Labelling of virus-specific proteins in infected cells.* Monolayers of BHK cells in 4 oz bottles were infected at high multiplicity (approx. 100). Cells were labelled for 10 min at 2.5 h post-infection with 100 µCi $^{35}$S-methionine. In chase experiments Eagle's medium supplemented with 1 mm-methionine was added after the labelling period. Cytoplasmic extracts for immunoprecipitation were prepared by lysing the cells in 1 ml phosphate-buffered saline containing 1% NP40 and 0.5% sodium deoxycholate. After 15 min at 0 °C the nuclei were pelleted and the supernatant was removed and stored at −70 °C.

*Translation of virus RNA in vitro.* The micrococcal nuclease-treated rabbit reticulocyte lysate of Pelham & Jackson (1976) was purchased from Amersham. The lysate was supplemented with 100 mM-KCl before use. RNA (2 µg) was added to 10 µl lysate containing 1 µl $^{35}$S-methionine (10 µCi/ml) and the mixture was incubated at 30 °C for 3 h.

*Immunoprecipitation.* Cytoplasmic extracts were used without any further preparation. Reticulocyte lysates were diluted 1:10 with NTE (0.15 M-NaCl, 50 mM-Tris–HCl pH 7.4, 5 mM-EDTA) containing 0.1% NP40. To 300 µl of antigen 15 µl of undiluted antiserum was added and the mixture was left 17 h at 4 °C. Precipitation with 1% Protein A (Pansorbin, Calbiochem) and washing of the pellets were performed as described by Harris et al. (1981).

*Antiserum was mixed with dilutions of peptide and reacted for 2 h at 4 °C. Labelled antigen was then added and the antibodies and immune complexes were precipitated with Protein A.*

*Immunoprecipitation of $^{125}$I-labelled VPg was as described above, and the antigen was prepared for IEF in the following way.* The pelleted VPg-antibody–Protein A complex was suspended in 25 µl distilled H$_2$O; 25 µg urea, 1-9 µl NP40 and 1-9 µl 2-mercaptoethanol were added and the mixture incubated for 30 min at 37 °C. The mixture was centrifuged for 2 min in a microfuge and the supernatant removed for analysis.

*IEF.* This was as described by King et al. (1980), using 5% (v/v) Ampholine (pH 3.5 to 10). Both tube gels and slab gels were used. Gels were exposed to Fuji RX film without fixing or drying.

*Polyacrylamide gel electrophoresis.* Samples were analysed on 10% polyacrylamide slab gels in SDS using the discontinuous system of Laemmli (1970). After electrophoresis the gels were treated with Autofluor (National Diagnostic Ltd), dried and exposed to Fuji RX film.
**Sequences of VPgs and synthetic peptides**

**VPg A**

\[
\text{Gly-Pro-Tyr-Ser} \underline{\text{Gly-Pro-Leu-Glu-Arg-Gln-Lys-Pro-Leu-Lys-Val-Arg-Ala-}} \\
\text{Lys-Leu-Pro-Gln-Gln-Glu-Cys}
\]

**VPg B**

\[
\text{Gly-Pro-Tyr-Ala} \underline{\text{Gly-Pro-Met-Glu-Arg-Gln-Lys-Pro-Leu-Lys-Val-}} \\
\text{Lys-Val-Lys-Ala-Pro-Val-Val-Lys-Glu-Cys}
\]

**VPg C**

\[
\text{Gly-Pro-Tyr-Glu} \underline{\text{Gly-Pro-Val-Lys-Lys-Pro-Val-Ala-Leu-Lys-Val-}} \\
\text{Lys-Ala-Arg-Asn-Leu-Leu-Val-Thr-Glu-Ser-Cys}
\]

Fig. 1. Sequences of the three presumptive VPgs. The portions underlined represent the peptides synthesized. The C-terminal cysteine is an additional amino acid added to enable the peptide to be linked to KLH by the method of Liu et al. (1979).

**Table 1. Reactions of antisera with homologous and heterologous peptides***

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Peptide A</th>
<th>Peptide B</th>
<th>Peptide C</th>
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<tbody>
<tr>
<td>A</td>
<td>1/1600</td>
<td>1/200</td>
<td>1/400</td>
</tr>
<tr>
<td>B</td>
<td>1/400</td>
<td>1/800</td>
<td>1/200</td>
</tr>
<tr>
<td>C</td>
<td>1/800</td>
<td>1/400</td>
<td>1/3200</td>
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* Assayed by means of indirect ELISA using 0.5 μg peptide/well and twofold serial dilutions of antiserum.

**RESULTS**

**Nomenclature of peptides and antipeptide antisera**

In the following Results and Discussion sections the peptides and antiserum are designated A, B, C. A is the peptide the coding sequence for which is located nearest the 5' end of the genome, B is the central peptide and C is the peptide located nearest the 3' end of the genome. The sequences of the three presumptive VPgs and the regions synthesized are shown in Fig. 1.

**Reaction of antiserum with synthetic peptides**

The ELISA test was used to determine the activity of each antiserum against its homologous synthetic peptide. All pre-bleeds were found to be negative (< 1/50) whereas all antisera became positive after one inoculation of peptide, the titre being elevated considerably after a subsequent boost with peptide. The anti-peptide activity of the serum increased after a second boost but further inoculations did not increase the antibody titre.

Antisera showing good activity against homologous peptide were used to determine the extent of the reaction with heterologous peptides (Table 1). The extent of cross-reaction varied from peptide to peptide but in all cases the reaction with the homologous peptide was strongest.

**Reaction of antiserum with native VPg**

Radiolabelled VPg was produced by iodinating the protein using Bolton & Hunter reagent. After iodination, the RNA was degraded with RNase (see Methods) and the VPg-pUp produced was used in immunoprecipitation experiments. The material that was immunoprecipitated was analysed by IEF on either tube or slab gels (Fig. 2). As described previously, VPg was
resolved into two major species (King et al., 1980). After immunoprecipitation with antisera raised to peptides A, B or C the majority of the radioactivity was located in only one of these bands dependent on the peptide antiserum used. Thus, it was shown that antiserum raised to peptide A or C precipitated proteins with identical acidic isoelectric points and antiserum B precipitated the more basic protein (Fig. 2). It is known from previous work (King et al., 1980) that only the more basic VPg can be labelled with [35S]methionine; examination of the protein sequences of the three VPgs revealed that only peptide B contained methionine. This information is therefore entirely consistent with the basic VPg being precipitated with antiserum raised to peptide B. This result was confirmed by the finding that prior absorption with peptide B eliminated the ability of the antiserum to precipitate any protein whereas absorption with peptides A and C had no effect.

The acidic VPg was precipitated by both antiserum raised to peptide A and antiserum raised to peptide C. Prior absorption with homologous peptide, i.e. antiserum A absorbed with peptide A or antiserum C absorbed with peptide C, prevented precipitation. However, prior absorption with heterologous peptide had no effect (Table 2). These results showed, therefore, that the common band precipitated by antisera A or C was composed of two species of VPg proteins.

The more basic protein was also resolved into two bands by IEF; the precipitation of both these proteins was inhibited by the homologous peptide, indicating that the two bands represented minor variations of a single protein. A similar result has been reported with the single VPg of poliovirus (Richards et al., 1984).

**Immunoprecipitation of VPg precursors from infected BHK cells**

In an attempt to detect and identify precursors of VPg, infected cells were labelled for a short period (10 min) with [35S]methionine. Cell lysates were immunoprecipitated using antiserum A, B or C. Four proteins with mol. wt. of 100000 (100K), 84K, 56K and 36K were precipitated with all antisera. In addition, proteins were precipitated in the mol. wt. range of 24K to 30K which
Fig. 3. PAGE of the polypeptides in infected BHK cells. The lysates were precipitated with: lane 1, antiserum raised to peptide A (rabbit 1); lane 2, antiserum raised to peptide B; lane 3, antiserum raised to peptide C; lane 4, hyperimmune antiserum (obtained from an infected guinea-pig); lane 5, antiserum raised to peptide A (rabbit 2).

Table 2. *Inhibition by prior absorption with peptide of the precipitation of $^{125}$I-VPg–pUp by anti-peptide sera*

<table>
<thead>
<tr>
<th>Anti-peptide serum used for precipitation</th>
<th>Pre-absorption with peptide*</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
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<tr>
<td>C</td>
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<td>B</td>
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* +, Pre-absorption with peptide inhibits precipitation by more than 80%; −, pre-absorption with peptide inhibits precipitation by less than 10%.

were specific for the particular antiserum (Fig. 3). To ascertain the specificity of the immunoprecipitation, anti-B serum was absorbed with both homologous and heterologous peptides prior to reaction with infected cell lysate (Fig. 4). The immunoprecipitation was efficiently blocked by 1 μg of homologous peptide, but no inhibition was observed with peptide...
Fig. 4. PAGE of the virus-induced polypeptides in BHK lysates precipitated with antiserum raised to peptide B after absorption with homologous and heterologous peptides. Lanes 1 to 3, absorption with 100 μg, 10 μg and 1 μg of peptide B; lanes 4 to 6, absorption with 100 μg, 10 μg and 1 μg of peptide C; lanes 7 to 9, absorption with 100 μg, 10 μg and 1 μg of peptide A.

A at 1 μg or 10 μg; however, partial blocking was observed with 100 μg of this peptide. Using peptide C, blocking was not seen even with 100 μg. The specificity of these reactions correlated with the cross-reactions observed in the ELISA test (Table 1).

This work was extended by examining the precipitation of virus-induced proteins with each of the three antisera A, B and C and the extent of inhibition obtained by pre-absorption with 10 μg of either the homologous or heterologous peptides. The precipitation was greatly inhibited in each case by the homologous peptide but only slightly, or not at all, by the heterologous peptides (Fig. 5).

**Immunoprecipitation of VPg precursors after translation in vitro**

Virus RNA was incubated for 3 h in a rabbit reticulocyte lysate *in vitro* translation system and the polypeptides synthesized were immunoprecipitated with antiserum A, B or C. In a few experiments the proteins precipitated were similar to those precipitated from infected BHK cells. In most experiments, however, the only proteins precipitated from the lysate had mol. wt. of 100K and 84K. The precipitation of these proteins could be inhibited by prior absorption with the homologous peptide but not by heterologous peptide (Fig. 6).
DISCUSSION

We have confirmed by immunoprecipitation experiments that the genome of FMDV codes for three separate VPg molecules. Antisera raised to chemically synthesized peptides corresponding to the carboxy-terminal portions of each of the VPg molecules reacted in the ELISA test with the peptide. Although the reaction was greatest with the homologous peptide there were varying degrees of cross-reaction with the other peptides. The antisera also reacted with 'native' VPg, i.e. the protein isolated from genome RNA. This reaction was more specific than that observed in ELISA tests with the synthetic peptides. Each anti-peptide serum reacted well with the homologous natural VPg and limited cross-reaction was observed with the heterologous VPgs. The heterologous reactions were qualitatively similar to the cross-reactions observed in the ELISA tests with synthetic antigens. The reaction of an antiserum with its homologous VPg could be blocked by prior absorption with the corresponding synthetic peptide but there was no apparent blocking when the antiserum was absorbed with 10 μg of either of the two heterologous peptides.

Immunoprecipitation of infected BHK cell extracts consistently precipitated four virus-induced proteins with mol. wt. of 100K, 84K, 56K and 35K. In addition to these, a number of proteins with mol. wt. approx. 20K to 30K were precipitated. The proteins precipitated depended on the peptide used to raise the antiserum. Antiserum A precipitated a protein of mol. wt. 20K which was not detected using the other antisera (Fig. 3). This protein could represent VPg A linked to the N-terminal region of P3; i.e. X–VPg A. Using antiserum B or C a protein was detected with mol. wt. of 27K; this could represent the protein X–VPg A–B–C. However, no such band was detected using antiserum A which argues against this explanation. Similarly it is difficult to explain the protein of mol. wt. 25K detected using antisera A or C but not present using antiserum B. Thus, the actual identity of these proteins must remain a matter of conjecture. In many experiments P84 also split into two bands; these may represent the C...
terminal portion of P3 linked to one or more of the VPg sequences. The precipitation of all these proteins could be prevented by absorbing the antiserum with homologous peptide, indicating that they may all be precursors of VPg. However, it is possible that some of these proteins are precipitated not because they contain VPg sequences, but because they are present as a complex with a VPg-containing protein. The addition of 0.1% SDS to the precipitation and washing buffers did not affect the pattern of polypeptides precipitated. The proteins that were immunoprecipitated from an in vitro translation extract were variable. In some experiments the same proteins as those detected in an in vivo extract were observed, whereas in other experiments only P3 (P100) and P84 were present in significant amounts. The explanation for this variability may be the unstable nature of the VPg precursors, this being demonstrated by incorporating a 10 min chase after the 10 min in vivo pulse-labelling. This resulted in a reduction of at least 90% in the amount of radiolabel precipitated with the anti-VPg peptide sera; furthermore, only P3 (P100) and P84 could be detected at this time (data not shown).

It is of interest to compare the above results with those obtained for poliovirus. A number of precursors to VPg are also precipitated from poliovirus-infected cells. One of these, P3-9, is stable and is thought to be the donor of VPg during RNA synthesis (Semler et al., 1982). We can find no evidence for a protein with similar properties in FMDV-infected cells, all the FMDV VPg precursor proteins being apparently unstable. It has also been suggested that the hydrophobic region of P3-9 is important for anchoring the protein in the membrane (Semler et al., 1982). FMDV does not have a hydrophobic region at the N terminus of P3 (Carroll et al., 1984). Furthermore, the detection of a protein with a mol. wt. of 84K containing VPg sequences presumably indicates that the N-terminal portion of P3 can be readily cleaved away.
We have not been able to detect uridine attached to any of the presumptive VPg precursors (results not shown). However, this would be the expected result if VPg is not a primer for RNA synthesis but is added post-transcriptionally following strand cleavage as recent work suggests (Andrews et al., 1985).

The finding of three VPgs in FMDV-infected cells raises a number of questions pertaining to function. It has been shown that in poliovirus only about 4% of the VPg synthesized is required for RNA replication (Semler et al., 1982). It therefore appears unlikely that multiple VPgs increase the efficiency of RNA replication. The acquisition of three VPgs apparently occurred early in the evolution of FMDV since all viruses so far examined, including southern African types, possess this feature (S. A. Orlepp, personal communication).

It has also been shown that the three VPgs are highly conserved from virus to virus. For example there is more similarity between VPg 'A' of virus serotypes A, O and C than between VPg A, B and C of a given virus isolate (Forss et al., 1984). These similarities between types may indicate different functions for each protein; however, the three VPgs are found in equimolar amounts on encapsidated virus RNA (King et al., 1980).

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REFERENCES


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