A Protein, VPg, Covalently Linked to 36S Calicivirus RNA

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SUMMARY

Proteins associated with 36S virus RNA from Vero cells infected with San Miguel sea lion virus, type 2 (SMSV-2), were labelled with $^{32}$P. One protein, VPg, remained linked to RNA when subjected to deproteinization techniques. VPg labelled with $^{32}$P was observed on 36S RNA from purified virions; the quantity of label was compatible with two phosphates per genome. The estimated mol. wt. of SMSV-2 VPg was 15,000.

The caliciviruses, although similar to picornaviruses in a number of respects, differ significantly in others, principally structural detail, a single major capsid polypeptide and a subgenomic messenger RNA (Burroughs & Brown, 1974; Schaffer & Soergel, 1976; Ehresmann & Schaffer, 1977; Black et al. 1978; Fretz & Schaffer, 1978). The finding that caliciviruses lack a methylated cap at the 5' end of the RNA (Ehresmann, 1978; Ehresmann & Schaffer, 1979), led us to seek a protein covalently linked to the RNA (VPg), as found with picornaviruses (Flanegan et al. 1977; Lee et al. 1977; Nomoto et al. 1977a, b; Sangar et al. 1977; Ambros & Baltimore, 1978; Golini et al. 1978; Hruby & Roberts, 1978). Independent studies at the Animal Virus Research Institute, Pirbright revealed the lack of a 5' cap and the presence of VPg on the RNA of a calicivirus, vesicular exanthema of swine virus (Black et al. 1978; Burroughs & Brown, 1978). Our study, using another calicivirus, San Miguel sea lion virus, type 2 (SMSV-2), and different approaches to the preparation and labelling of RNA, confirms the presence of calicivirus VPg.

The 36S RNA fraction labelled with $^3$H-uridine, was obtained from Vero cells 7 h p.i. with SMSV-2 by in situ solubilization and glycerol density gradient sedimentation as previously described (Ehresmann & Schaffer, 1977), except that proteinase K was omitted and treatment with sodium dodecyl sulphate (SDS) was performed rapidly at 0 to 4 °C to minimize degradation of virus RNA. The 36S RNA was subjected to a chloramine-T procedure for protein labelling with $^{125}$I (Schaffer & Soergel, 1976), and once again sedimented in a glycerol density gradient to re-isolate the 36S fraction. Portions were then subjected to various treatments, followed by polyacrylamide slab gel electrophoresis (Fig. 1a). Two major (X and Y) and numerous minor bands were seen in the portions treated with ribonuclease T2 (lane 1). The protein nature of detectable $^{125}$I-labelled material was shown by its susceptibility to proteinase K (lane 2). The more rapidly migrating major band X was absent in the untreated RNA (lane 3), indicating that this component, the presumptive VPg, was still bound to unhydrolysed RNA, whereas protein Y, mol. wt. about 19,000, was separated from RNA by electrophoresis. Its identity is not known but it may represent a minor polypeptide of calicivirions (Burroughs & Brown, 1974). In view of the difficulty in obtaining undegraded calicivirion RNA (Schaffer & Soergel, 1973; Ehresmann & Schaffer, 1977; Burroughs & Brown, 1978) and similar problems with foot-and-mouth disease virus (FMDV) RNA attributed to a virion-associated endonuclease (Denoya et al. 1978), protein Y might be an endonuclease bound by non-covalent forces to the RNA. Quantitatively more $^{131}$I was in component Y than in component X in the preparation shown (Fig. 1a); in other preparations less Y was observed.
Fig. 1. Electropherograms showing SMSV-2 VPg. (a) Slab gel electrophoresis of $^{125}$I-labelled proteins associated with intracellular 36S RNA after various treatments: lane 1, treated with RNase T2; lane 2, treated with RNase T2 followed by proteinase K; lane 3, untreated (the input radioactivity in lane 1 was three times that of lanes 2 and 3). (b) Tube gel electrophoresis of $^{32}$P-labelled virion 36S RNA following treatment with RNases A, T1 and T2. (c) Same as (b) plus treatment with proteinase K. All resolving gels contained 15% acrylamide and Laemmli discontinuous buffer system as described by Fretz & Schaffer (1978). In (a) slab gels and autoradiography were as described by Fretz & Schaffer (1978). In (b) and (c) fractionation of tube gels was as described by Schaffer & Soergel (1976); points are means of quadruplicate 10 min counts. Arrow indicates position of $^{125}$I-Yersinia pestis marker polypeptide; D indicates tracking dye. Positions of stained marker proteins in separate lanes of the slab gel are indicated in (a). Relative positions of radioactive, pigmented and stained marker proteins used to establish the mol. wt. of Y. pestis polypeptide (15 000) in separate gels are indicated in (b). Markers and their mol. wt. were: B, bovine serum albumin 68 000; H, IgG heavy chain 55 000; O, ovalbumin 43 000; L, IgG light chain 23 500; T, trypsin 23 300; P, phycocyanin 16 300 (courtesy of Dr Brian Gray); R, RNase A 13 700; C, cytochrome c 11 700; I, insulin 33 000 and 2400.

Several higher mol. wt. proteins present in trace amounts in Fig. 1(a) appeared to be contaminants. A faster migrating component (mol. wt. approx. 12 000) is barely discernible in lane 1. This component, varying in quantity from virtually undetectable to $^{125}$I equal to that of X, was present in other preparations, even after deproteinization of the RNA. Its identity was not established, but it may represent a proteolytic cleavage product of X.

Difficulties were encountered when evidence was sought for VPg on extracellular virion RNA; however, we were successful with virions from cells harvested when c.p.e. was advanced but half or more of the virus remained cell-associated. (It should be noted that at 7 h p.i., as in Fig. 1(a) much of the total 36S RNA was from unreleased virions.) A rapid procedure for cell disruption, virion purification and extraction of RNA under protein-denaturing conditions yielded 36S RNA. Fig. 1(b and c) show the results of an experiment with RNA from cell-associated virions. SMSV-2 was propagated in a monolayer of Vero cells in a 75 cm² plastic flask in the presence of 5 mCi $^{32}$P-orthophosphate in low phosphate medium and in the absence of actinomycin D. When c.p.e. was advanced, detached cells and cell debris were collected by centrifugation. Attached cells were scraped into 0.8 ml
PBS and the scraped and pelleted cells pooled with the addition of 0.7 ml more PBS. The cells plus cell debris were then treated with a mixture of phospholipase C, pancreatic DNase and RNase A (final concentrations 68 µg/ml, 34 µg/ml, 340 µg/ml, respectively) for 1 h at 37 °C on a rotary shaker. The extracts were clarified (10000 g, 5 min), layered over three gradients of 10 to 32% glycerol (w/w in PBS with 0.1% bovine serum albumin) and sedimented (40 min, 45000 rev/min, 4 °C, Spinco SW50.1 rotor). Peaks of radioactivity at about 180S were pooled, layered over a discontinuous CsCl gradient (0-9 ml of 1.25 g/ml and 0.6 ml of 1.58 g/ml, both in PBS with 0.1% bovine serum albumin) and sedimented (100 min, 35000 rev/min, 4 °C, SW50.1 rotor). Radioactive peak fractions at about 1.37 g/ml were pooled (vol. approx. 0.19 ml), mixed with 0.6 ml 4 M-potassium trichloroacetate and kept at room temperature for 30 min to denature proteins and liberate RNA from the virions (cf. Burke et al. 1978). The mixture was diluted with 2 ml of TEN buffer (10 mM-tris, 1 mM-EDTA, 0.1 M-NaCl, pH 7.4) and RNA precipitated with ethanol. The precipitate (20000 g, 20 min, -20 °C), dissolved in TEN buffer was sedimented in a glycerol gradient [10 to 30% (w/w) in TEN buffer, 50000 rev/min for 2 h at 4 °C, SW50.1 rotor] to give a sharp 36S radioactive peak which was then precipitated with ethanol. Throughout this procedure the highly radioactive fractions, usually in 1.5 ml polycarbonate microcentrifuge tubes, were assayed by counting secondary radiation from 32P in a gamma counter. To hydrolyse the RNA, the precipitate was suspended in 20 µl RNase A (70 µg in 50 mM-NH4Ac, 1 mM-EDTA, pH 5), heated for 2 min in a boiling water bath and chilled on ice. Twenty µl additional RNase A and 12 µl of a mixture of RNase T1, RNase T2, bovine serum albumin and NaN3 (57 units, 6 units, 5µg, 5 µg, respectively, in NH4Ac-EDTA buffer, pH 5) were then added and kept overnight at room temperature. The RNase digest was diluted with 160 µl electrophoresis sample preparation buffer (2.3% SDS, 10% glycerol, 0.001% bromophenol blue, 62.5 mM-tris, pH 6.8) and 1.6 µl 10% bovine serum albumin. Half of the digest was treated with proteinase K (0.2 mg/ml) for 2 h at room temperature, then heated for 2 min in a boiling water bath, followed by the addition of 80 µg bovine serum albumin. Both portions, with added Yersinia pestis fraction IB (Baker et al. 1952) labelled with 125I, were subjected to electrophoresis (Fig. 1b, c). Most of the radioactivity migrated ahead of the tracking dye and some slightly slower migrating material, perhaps incompletely digested oligonucleotides, was present in both gels. The important finding was a peak (corresponding to component X of Fig. 1a) that co-migrated with the Y. pestis marker in the portion treated with nucleases only and was absent in the portion treated with nucleases and protease.

Additional experiments provide further evidence that component X is covalently linked to RNA and is, by definition, VPg. Separation of unbound protein from the RNA was accomplished by sedimentation into an aqueous gradient of caesium trichloroacetate (CsTCA), a procedure used for the demonstration of poliovirus VPg (Lee et al. 1977) and of tobacco ringspot virus VPg (Mayo et al. 1979). Single-stranded RNA and any covalently-linked proteins band at a buoyant density of about 1.74 g/ml in CsTCA; free proteins are found at a lower density (Burke et al. 1978). Trichloroacetate salts are very effective reagents for dissociation of nucleoprotein complexes (Scott & Kuhns, 1972; Burke & Bauer, 1977; Burke et al. 1978) and no evidence is available for non-covalent proteins remaining bound to nucleic acids under these denaturing conditions, although such a possibility cannot be ruled out (R. L. Burke, personal communication). A 125I-labelled preparation (same preparation as used in Fig. 1a) showed that a sharp peak of 125I-labelled material banded with the 3H-labelled RNA at approx. 1.7 g/ml, while the majority of the 125I (unbound protein) remained at a lower density (Fig. 2). RNase treatment and electrophoresis revealed component X, but not Y. Treatment of other 125I-labelled 36S RNA preparations with SDS–phenol–chlooroform (Yogo & Wimmer, 1973) was also effective in removal of com-
Fig. 2. Separation of $^{125}$I-labelled fractions associated with SMSV-2 intracellular 36S RNA by CsTCA equilibrium density ultracentrifugation. The technique was essentially as described by Burke et al. (1978). Fractions of four drops (approx. 0.13 ml) each were assayed for total $^{125}$I (---); 2 µl samples of fractions 1 to 20 were assayed for $^{3}$H (----) by liquid scintillation counting (correcting for contribution of $^{125}$I); essentially all the input $^{3}$H was accounted for in fractions 14 to 20. Densities (●—●) were estimated by weighing 20 µl portions in capillary pipettes.

ponent Y, but not component X. Thus, CsTCA and SDS-phenol-chloroform were comparable in removing non-covalent proteins; however, when the two methods were applied to the same preparation, the yield of RNA and component X by banding in CsTCA was greater.

The apparent mol. wt. of SMSV-2 VPg is 15000 as indicated by marker proteins in slab gels (Fig. 1a) and by co-electrophoresis with Y. pestis polypeptide (Fig. 1c). We have also observed a 15000 mol. wt. VPg for another serotype of SMSV, SMSV-4. The VPg of each of these two SMSV serotypes appears to be larger than the 10000 apparent mol. wt. of another calicivirus (Burroughs & Brown, 1978), but the difference may not be real, considering uncertainties in mol. wt. estimations of small proteins.

Nucleoside diphosphates are linked to poliovirus and encephalomyocarditis virus VPgs obtained by exhaustive nuclease digestion (Lee et al. 1977; Ambros & Baltimore, 1978; Golini et al. 1978). This amounts to two of the approx. 7600 mol. wt. phosphates in the RNA genome; however, recoveries of $^{32}$P may not correspond to this theoretical quantity because of unequal labelling of nucleotides and losses of VPg due to adsorption to surfaces (Flanagan et al. 1977; Nomoto et al. 1977a). The quantity of $^{32}$P in the SMSV-2 VPg peak (difference in Fig. 1b and c) amounted to one labelled phosphate for each 6000 $^{32}$P atoms of the digest which, considering uncertainties of losses and unequal specific activities, is consistent with a nucleoside diphosphate linkage of calicivirus VPg to a 7600 to 8000 nucleotide RNA.

The mode of replication of the caliciviruses differs from that of the picornaviruses (Ehresmann & Schaffer, 1977; Black et al. 1978; Fretz & Schaffer, 1978). Both the caliciviruses and picornaviruses are in the minority of RNA viruses, having a genome-linked protein, VPg, instead of a 5' methylated cap. However, they appear to differ from each other in that calicivirus VPg is required for infectivity (Burroughs & Brown, 1978) as is a protease-sensitive structure on nepovirus RNA (Harrison & Barker, 1978), whereas picornavirus VPg is not required for infectivity (Nomoto et al. 1977b). We have preliminary evidence for the presence of VPg on calicivirus double-strand RNA, suggesting some similarity to picornavirus (Nomoto et al. 1977a) in RNA synthesis. By polyacrylamide gel
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electrophoresis the size of calicivirus VPg, 10000 to 15000 mol. wt., appears to be larger than the 4000 to 6000 mol. wt. VPg of poliovirus (Lee et al. 1977; Golini et al. 1978) or FMDV (Sangar et al. 1977) and may be somewhat larger than the 8000 to 10000 mol. wt. encephalomyocarditis virus VPg (Golini et al. 1978; Hruby & Roberts, 1978). However, in a recent study, Ambros & Baltimore (1978) obtained an estimate of 12000 for poliovirus VPg by gel filtration chromatography; this study also showed tyrosine to be involved in the poliovirus protein–RNA linkage. In spite of some similarities between these virus groups, differences are of a magnitude to justify their classification in separate families, as originally suggested by Burroughs & Brown (1974).

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