Characterization of the VPg–dsRNA linkage of infectious pancreatic necrosis virus

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By the use of strong denaturing agents, a genome-linked protein (VPg)–RNA complex was purified from infectious pancreatic necrosis virus. Ribonuclease treatment of 125I-labelled VPg–RNA released a 90K polypeptide identical to the minor structural polypeptide VP1 (the putative RNA polymerase), as determined by peptide mapping. The polypeptide is linked to the RNA by a serine–5' GMP phosphodiester bond. The results identify birnaviruses as the only dsRNA viruses with a VPg. The size of which is the largest of the VPgs of RNA viruses.

Many RNA and DNA viruses possess genome-linked proteins (VPgs), which are virus-encoded polypeptides covalently linked to the 5' termini of virus nucleic acids (for reviews see Wimmer, 1982; Daubert & Bruening, 1984; Vartapetian & Bogdanov, 1987). In all cases studied, the linkage is a phosphodiester bond between the α-phosphoryl group of the 5'-terminal nucleotide of the polynucleotide chain and either the β-hydroxyl group of a serine residue or the Oα-hydroxyl group of a tyrosine residue of the protein; so far, threonine has not been found in this linkage. VPgs play a fundamental role in the replication of viral genomes and might also be important during virus morphogenesis.

Hitherto, the genomes of all VPg-containing RNA viruses identified have contained positive-sense ssRNA; negative-sense ssRNA viruses and those with dsRNA genomes were not thought to possess VPgs (Vartapetian & Bogdanov, 1987). Recent reports indicate that members of the family Birnaviridae may be an exception to this. This family includes infectious pancreatic necrosis virus (IPNV), the causative agent of an acute contagious disease of young salmonid fish, infectious bursal disease virus (IBDV), which causes a highly contagious disease in chickens characterized by the destruction of B lymphocytes in the bursa of Fabricius, and drosophila X virus (DXV) of the common fruit fly (Brown, 1986). Birnaviruses possess a bisegmented dsRNA genome surrounded by a medium size (60 nm), unenveloped, single-shelled, icosahedral capsid. Genome segment A (approximately 3100 bp) encodes a 100K polypeptide (NH2-VP2-protease-VP3-COOH) which is cleaved by the protease to generate the major capsid proteins VP2 and VP3 (Duncan et al., 1987; Jagadish et al., 1988). The only gene product of genome segment B (approximately 2900 bp) is a minor internal capsid polypeptide VP1 (approximately 90K) which, based on its size low copy number in virions and association with genomic RNA (Nagy et al., 1987; Duncan et al., 1991), is the putative virion-associated RNA-dependent RNA polymerase. Virion-associated RNA polymerase activity has been reported in all three members of the Birnaviridae (Bernard, 1980; Mertens et al., 1982; Spies et al., 1987).

The first indication that birnaviruses might contain a VPg came from the work of Persson & Macdonald (1982), who isolated an RNA-protein complex from SDS-disrupted IPNV. When viewed under the electron microscope the RNA–protein complex appeared as circular structures which became linear after proteinase K treatment. When radioiodinated RNA–protein complex was treated with RNase A, the radioactive polypeptide released comigrated with VP1 on polyacrylamide gels. Müller & Nitschke (1987) have made similar observations using purified IBDV. Both groups postulated that VPg and VP1 were the same, and that both the free polypeptide and its genome-linked form can be found in the purified virus. Qualitative comparisons of VPg and VP1 were not made, nor was the covalent linkage tested or the nature of the linkage established. Furthermore, in neither study was the RNA–protein...
complex test subjected to strong denaturing agents. The latter test is especially important because some capsid proteins tend to bind strongly, albeit non-covalently, to virus genomes (Daubert & Bruening, 1984). Such proteins are known to survive SDS treatment and phenol–chloroform extraction of RNA, as has been demonstrated for cowpea mosaic virus (Zabel et al., 1982) and poliovirus (Rothberg et al., 1978). Only proteins that survive all attempts at removal can be considered candidates for VPg. Therefore, we attempted to prepare the VPg–RNA complex of IPNV using strong denaturants such as guanidinium hydrochloride, SDS, 2-mercaptoethanol (2-ME) and heat to ensure that VP1 was not binding to RNA in a tight, non-covalent manner.

IPNV (Jasper strain), grown and purified as described by Dobos & Rowe (1977), was disrupted by heating (55 °C, 15 min) in denaturation buffer [5 M-guanidinium hydrochloride, 5% sodium-3-lauryl sarcosine (SLS), 5% 2-ME in 100 mM-Tris–HCl pH 7.4, 10 mM-EDTA, 1 mM-PMSF], followed by gel exclusion chromatography (Bio-Gel A-50m) equilibrated and eluted with 4 M-urea, 0.3% SLS, 5 mM-DTT, 0.3 mM-PMSF in 0.5 × TNE buffer (1 × TNE buffer is 10 mM-Tris–HCl pH 7.9, 100 mM-NaCl, 1 mM-EDTA). Fractions were analysed for RNA content and the presence of virion polypeptides by agarose gel electrophoresis and PAGE, respectively. RNA-containing fractions were pooled, iodinated in vitro using Bolton–Hunter reagent, precipitated with ethanol, resuspended in denaturation buffer and sedimented through a 40% CsCl cushion. Virus RNA was labelled with [32P]H3PO4. The virus was purified, disrupted with SDS, digested with proteinase K, and the RNA was extracted with phenol–chloroform and analysed by agarose gel electrophoresis together with the radiiodinated VPg–RNA complex (Fig. 1a). The latter tended to aggregate and produced streaking even in the presence of SLS, leaving a substantial amount of radioactivity in the sample well; it also migrated more slowly than the deproteinized, [32P]-labelled viral RNA. Proteinase K digestion of iodine-labelled VPg–RNA complex released the viral RNA, which comigrated with the [32P]-labelled RNA and could be detected by ethidium bromide staining (data not shown).

When purified radioiodinated VPg–RNA complex was analysed by SDS-PAGE, it barely entered the resolving gel under conditions in which the stained virion capsid polypeptides were separated. Ribonuclease digestion of the complex released a labelled polypeptide that comigrated with VP1 (Fig. 1b). The minor radioactive bands in the nuclease-treated preparation gave peptide maps partially overlapping with that of VPg (data not shown), indicating that they were minor degradation products of VPg and not contaminating capsid polypeptides. This degradation occurred during storage even though PMSF was included in all preparations and in the electrophoresis sample buffer. A qualitative comparison of VP1 and the RNase-released VPg is shown in tryptic peptide maps of the two polypeptides (Fig. 1c), which were performed as described by Dobos & Rowe (1977). The peptide patterns of the two proteins show a large degree of overlap. Specifically, of the 40 spots resolved in the digest of VP1, only six are not found in that of VPg, and only two of the spots resolved in the digest of VPg are missing from that of VP1. These differences may be due to post-translational modifications (e.g. phosphorylation, acylation etc.) involving only one polypeptide, or may reflect differences in the region of VPg involved in the linkage. It may also be inferred from these results that genome segments A and B contain the same VPg.

To identify the amino acid involved in the linkage, [32P]-labelled virus was digested with proteinase K (1 mg/ml in TNE containing 0.5% SDS, at 37 °C for 16 h) to hydrolyse VPg so that only the amino acid covalently linked to the 5'-terminal nucleotide of the RNA remained. The RNA was phenol-extracted and ethanol-precipitated, digested with micrococcal nuclease [20 units (U)/ml, 37 °C, 2 h], acid-hydrolysed, and analysed by two-dimensional thin-layer electrophoresis under conditions in which the phosphoamino acids are resolved and separated from mononucleotides, ribose phosphate and free inorganic phosphate (Manai & Cozzone, 1982). The data in Fig. 2(a) show that the only phosphoamino acid detected was phosphoserine, indicating that the RNA is linked to one of the serine residues of VP1.

Identification of the VPg-linked nucleotide proved to be more difficult. Preliminary results indicated that treating [32P]-labelled VPg–RNA complex with a battery of RNases did not result in a VPg–phosphonucleotide (pN) complex. Steric hindrance by the unusually large VPg probably protected a short, 5' oligonucleotide segment of the RNA from RNase digestion; subsequent treatment with proteinase K (to hydrolyse the VPg) and snake venom phosphodiesterase yielded a mixture of all four labelled nucleotides when analysed by thin-layer electrophoresis. To overcome this problem we digested the labelled VPg–RNA complex with trypsin, and the ethanol-precipitated RNA was treated with phosphatases and RNases [RNases A (10 μg), T1 (1400 U) and T2 (10 U) and acid (10 U) and alkaline phosphatases (7 U) in a reaction mixture of 50 μl] to yield a tryptic peptide–pN which could then be separated from labelled inorganic orthophosphate by PAGE using 18% gels. The [32P]-labelled peptide–pN complex was located by auto-
radiography, cut out from the gel, eluted, desalted using an HPLC mini-column, and digested with snake venom phosphodiesterase (10 µg for 2 h at 37 °C) to release the terminal pN; using thin-layer electrophoresis (pyridine/ acetic acid/water, 5/50/945 at 500 V for 100 min) and unlabelled pN markers it was identified as pG (Fig. 2b). Since snake venom phosphodiesterase will hydrolyse a P--O but not a P--N bond (Daubert & Bruening, 1984), these results suggested that the 5'-terminal G residue is linked to a serine residue by a phosphodiester bond. Treatment of the phosphopeptide with phosphatases was necessary to eliminate labelled phosphoamino acids which may have been present as part of the peptide chain, but not involved in the linkage. This was particularly important because VP1 is thought to be the viral RNA polymerase; viral RNA polymerases have been shown to exist in phosphorylated forms (Ransone & Dasgupta, 1989).

Our results agree with those of others (Persson & Macdonald 1982; Müller & Nitschke, 1987) which show that VPg is a genome-linked form of VP1 in IPNV and IBDV, and that both forms are found in purified virus. Preliminary results indicate that the same is true for DXV. The nature of the linkage (serine-5' GMP in IPNV) has not been determined in the other members of the family. These results identify birnaviruses as the only dsRNA viruses with a VPg.

Since IPNV dsRNA can be polyadenylated at the 3' end in vitro (Duncan et al., 1987), the VPg presumably is located at the 5' end. However, because all four strands of the genome of IPNV (Jasper strain) contain a G residue at their 5' ends (Duncan & Dobos, 1986; Duncan et al., 1991) the results do not reveal whether the linkage involves the 5' ends of the plus strands or the 5' ends of the minus strands, or both. Persson & Macdonald (1982) identified circular VPg-RNA complexes, which became linear after proteinase K treatment, by electron microscopy. Since VPg tends to aggregate one might speculate that it is present at the 5' end of both plus and minus RNA strands, and that when these terminal proteins stick to each other they circularize the RNA. Our attempts to resolve this question using strand-separating virion polypeptides and RNase-treated VPg-RNA were analysed by PAGE and autoradiography. The labelled VP1 and VPg bands were cut out of the dried gel and subjected to trypsin digestion, electrophoresis (TLE) and chromatography (TLC) using thin-layer silica gels. For direct comparison, the two digests were electrophoresed in parallel on the same plate, which was then cut in half for subsequent chromatography; the autoradiogram thus shows mirror images of the two samples on the two sides of the dashed line. Samples were applied at the origin (Ori). Small arrowheads indicate labelled peptides in the digest of VP1 which are not present in that of VPg, or peptides in VPg not present in the digest of VP1. (d) Tracing of (c). Shaded spots are present in one digest only.
Short communication

Although we have identified the plus and minus strands of proteinase K-treated IPNV dsRNA previously (Nagy et al., 1987), numerous attempts to separate iodinated VPg–RNA strands in strand-separating gels failed owing to aggregation of the complex at the top of the gel, which resulted in streaking (even in the presence of SLS) and no resolution of the RNA strands. We plan to solve this problem by treating VPg–RNA with colloidal gold-labelled anti-VP1 antibody and analysing these complexes by electron microscopy. The resolution of this question is important because viral VPgs have been shown to act as primers in the replication of double-stranded viral nucleic acids (Hsieh et al., 1990).

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References


Fig. 2. (a) Phosphoamino acid analysis of the VPg–RNA linkage. Proteinase K-treated, 32P-labelled IPNV RNA was digested with micrococcal nuclease, acid-hydrolysed, and analysed by two-dimensional thin-layer electrophoresis and autoradiography. The sample was applied in the lower left corner together with marker phosphoamino acids. P-Ser (phosphoserine), P-Thr (phosphothreonine) and P-Tyr (phosphotyrosine) were located by ninhydrin staining. Pi denotes inorganic phosphate. (b) Identification of the VPg-linked nucleotide. Purified 32P-labelled VPg–RNA complex was digested with RNases, phosphatases and trypsin, and the labelled phosphopeptide was detected and recovered after PAGE. Snake venom phosphodiesterase treatment released a labelled G residue which was identified as pG after thin-layer electrophoresis and autoradiography. The arrowheads indicate the position of marker pNs located by u.v. illumination.


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