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Characterization of the Sendai Virus V Protein with an Anti-Peptide Antiserum

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The Sendai virus V protein, which is a fusion of the P and V ORFs of the P gene, was characterized with antisera to a portion of the V ORF and compared to the P protein. The only property found in common with P is that V is also highly phosphorylated, and this is so even when these proteins are expressed independently of the other viral proteins. Otherwise, V was not found in virions, was not strongly associated with viral nucleocapsids like P, and anti-V had no effect on viral RNA synthesis in vitro under conditions where anti-P was highly inhibitory. The available evidence suggests that V may play a role in RNA synthesis, but it is not an essential one like that of the P protein.

Paramyxovirions contain a helical nucleocapsid (NC) core composed of the minus-strand RNA genome (15-16 kb) and ca. 2000 copies of the nucleocapsid protein NP. In addition, ca. 200 copies of the phosphoprotein P and 50 copies of the large protein L are bound to this structure. The genome is expressed via complementary monocistronic mRNAs and is replicated via a full-length complementary strand, the antigenome. Reconstitution experiments with Newcastle disease virus (NDV) have shown that polymerase activity in vitro requires both the P and the L proteins as well as the RNA-NP core (Hamaguchi et al., 1983). The P and L proteins are also found as a complex, both on and off the NC core (Hamaguchi et al., 1983; Portner et al., 1988). By analogy to the rhabdovirus VSV, whose L gene and that of the paramyxoviruses are thought to share a common ancestor (Pochet et al., 1989), L is the actual polymerase, and capping and poly(A) formation of mRNAs are thought to map to this protein as well (Banerjee, 1987). The VSV counterpart to the paramyxovirus P protein is NS, which has recently been referred to as P (Herman, 1986; Bilser et al., 1990), as NS (nonstructural) is a misnomer. The paramyxovirus and rhabdovirus P proteins are not related by sequence homology or size, but are organized similarly. Both are acidic proteins, whose N terminal halves are also highly phosphorylated (Bell and Prevec, 1985; Vidal et al., 1988) and poorly conserved among related viruses. The C-termini are better conserved and contain the site(s) by which this protein binds to the RNA-NP core (Emerson and Schubert, 1987; Gill et al., 1986; Kolakofsky et al., 1990; Ryan and Portner, 1990).

The paramyxovirus P genes are, however, more complex. Those of the morbilliviruses measles (Bellini et al., 1985) and CDV (Barrett et al., 1985), and the parainfluenza viruses SEN (Giorgi et al., 1983; Shiode et al., 1983) and parainfluenza virus 3 (PIV3) (Galinski et al., 1986; Luk et al., 1986; Sakai et al., 1987; Spriggs and Collins, 1986), which are 568-602 residues long, also contain a 200 aa ORF (called C) overlapping the N-terminus of P. The P and C ORFs are expressed by ribosomal choice (Curran and Kolakofsky, 1988). Those of the other parainfluenza viruses (SV5 (Thomas et al., 1988), murine virus (Elliot et al., 1990; Paterson and Lamb, 1990; Takeuchi et al., 1990), NDV (Sato et al., 1987; McGiness et al., 1988), PIV2 (Ohgimoto et al., 1990; Southern et al., 1990), and PIV4 (Kondo et al., 1990)) are only 392-398 aa in length and lack the C ORF. However, all paramyxovirus P genes except respiratory syncitial virus (RSV) and PIV1 (see Discussion) also contain an ORF called V and are transcribed into at least two slightly different mRNAs, one for the P and one for the V protein (Fig. 1). In all cases one mRNA is an exact copy of the gene, whereas the other is modified by the addition of either one or two Gs, within a short run of Gs (Thomas et al., 1988; Cattaneo et al., 1989; Vidal et al., 1990a). The G additions are thought to take place by the viral polymerase reiteratively copying a template C residue during mRNA synthesis, by a stuttering mechanism (Vidal et al., 1990b; Pelet et al., 1991). These insertions alter the reading frame in the middle of the gene and allow ribosomes to translate the alternate ORF downstream (Fig. 1).

The V ORF is the most highly conserved of all of the sequences of this gene (Thomas et al., 1988) and is a Cys-rich domain similar to other transcriptional cofactors such as zinc-finger proteins (Klug and Rhoads,
SENDAI VIRUS V PROTEIN 109

1987) and the HIV tat protein (Frankel et al., 1988). It seems plausible then that the SEN V protein, which shares the first 317 residues with P, also acts in RNA synthesis. Actually, the SEN V protein has not yet been detected; in most strains it is expected to comigrate with IN on SDS gels and could be hidden by the more abundant NP. Only the inserted mRNA has been found, which is half as abundant as that of P (Vidal et al., 1990a). To learn more about this protein, we have prepared an antibody to a V peptide (anti-V).

**MATERIALS AND METHODS**

**Cell cultures and virus**

BHK cells were subcultured in minimal essential medium (MEM) containing 5% fetal bovine serum. Cells were infected with Sendai virus (20–50 PFU per cell) or vaccinia viruses (5–10 PFU per cell). Infections were maintained in serum free MEM at 33°.

SEN was purified from infected cell medium by first pelleting out cellular debris (5 min at 10,000 g) and then loading the supernatant onto a 25% glycerol–TNE (50 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA) cushion. Virus was pelleted by centrifugation at 150,000 g for 1 hr in either an SW41 or an SW26 rotor.

**Metabolic labeling and immunoprecipitation**

Cellular proteins were pulse labeled with [35S]cysteine essentially as outlined previously (Curran and Kolakofsky, 1988, 1989). When labeling with [32P]PO₄, cell monolayers on 9-cm dishes were incubated for 3 hr in phosphate free medium prior to addition of 2 mM of [32P]PO₄, at 5 hr p.i. Infections were harvested at 24 hr p.i. by directly solubilizing the monolayer in 500 µl of RIPA buffer (150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris, pH 8.3) containing 2% SDS, 1% 2-mercaptoethanol, 1% aprotinin, and 6 mM PMSF. Chromosomal DNA was sheared by sonication.

For immunoprecipitation (Curran and Kolakofsky, 1989), cell extracts were diluted 20-fold in RIPA buffer prior to addition of the antibody and incubated for 2–4 hr at 4° before addition of 50 µl of a 50% suspension of Protein A–Sepharose 4B (Pharmacia) prepared in 20 mM Tris, pH 8.0. Incubation at 4° was then continued for a further 2 hr. The Protein A beads were then washed twice in 150 mM NaCl, 50 mM Tris, pH 7.8, 5 mM EDTA, 0.5% NP-40 and once in 0.5 M LiCl, 1% 2-mercaptoethanol, 0.1 M Tris, pH 7.8, before being mixed with 30 µl of 2× protein sample buffer (6% SDS, 4% 2-mercaptoethanol, 125 mM Tris, pH 6.8, 20% glycerol, and 0.2% bromophenol blue). For [32P]PO₄-labeled cell extracts, an additional wash in PBS containing 5 µg/ml DNase and 5 µg/ml RNase A at 37° for 20 min was carried out.

**Preparation of V polyclonal antibody**

The peptide HRREHIIYERDGYIVDESWC, representing amino acids 2–21 in the V ORF (or residues 319–338 of the V protein), was coupled to keyhole limpet haemocyanin via m-maleimidobenzoyl N-hydroxysuccinimide ester. The coupled peptide was purified on Sephadex G-25. Rabbits were twice injected subcutaneously at a 1-month interval with the equivalent of 200 µg of peptide and boosted 2 months later with the same amount. Blood was then taken 2 and 3 weeks after the boost. Animals responding to the peptide (as assayed by ELISA against the peptide coupled to BSA) were further boosted at 6-week intervals and blood was taken 2 weeks later.

**Immunofluorescence**

BHK cells seeded at low density on Lab-Tek 8-chamber glass slides (Miles Scientific) were infected with Sendai virus at a multiplicity of 10–20 PFU/cell. At 18 hr postinfection, mock and infected cells were washed with PBS (without Ca²⁺, Mg²⁺) and fixed with 3% formaldehyde in PBS. After fixation, cells were permeabilized with acetone for 3 min at −20° and then blocked with 1% BSA, 0.1% Triton X-100 in PBS for 1 hr at 4°. The primary anti-P monoclonal antibody was diluted 1/100 in blocking solution and incubated for 1 hr at 4°. The cells were then washed twice in PBS.
before incubation with the second antibody, goat anti-
mouse conjugated to fluorescein (diluted 1/50 in
blocking buffer: Nordic, Switzerland), for 1 hr at 4°.
After two washes with PBS, an anti-V rabbit antibody
diluted in blocking solution (1/100) was applied for 1 hr
at 4°. The cells were then washed two times in PBS
before incubation for 1 hr at 4° with a 1/50 dilution of
the secondary antibody, goat anti-rabbit coupled to
rhodamine (Nordic, Switzerland). Chambers were then
washed two times in PBS before viewing in a Nikon
Optiphot microscope.

The specificity of both the primary and the second-
ary antibodies was carefully controlled. The anti-P and
anti-V primary antibodies produced no detectable sig-
nals on mock-infected cells and no cross-reactivity
was observed between the anti-mouse and anti-rabbit
secondary antibodies.

Isolation of Sendai virus RNP

Infected cell extracts were prepared by three
methods.

1. Lysolecithin extracts were prepared as described
by Carlson et al. (1985). Cells were disrupted either in
TN buffer (30 mM NaCl, 10 mM Tris, pH 7.6) or in RSB
(10 mM KCl, 10 mM Tris, pH 7.5, 1.5 mM MgCl₂).

2. Cells were scraped into cold PBS, pelleted, and
resuspended in 150 mM NaCl, 50 mM Tris, pH 7.4,
0.6% NP-40. Nuclei were pelleted from the extract (5
min at 10,000 g), and the supernatant was adjusted to
6 mM EDTA and stored on ice.

3. Cell pellets were resuspended in ice-cold RSB
buffer, and the cell membrane was broken by 10
strokes in a Dounce homogenizer. Nuclei and mem-
branes were pelleted by centrifugation.

Viral RNP was pelleted through 50% (v/v) glycerol–
TN buffer onto a 500-µl cushion composed of 68%
w/w sucrose in D₂O–TN buffer (43,000 rpm for 90 min
at 20°) in an SW60 rotater. Gradient fractions (0.5 ml)
were collected and analyzed either by Western ELISA
when the cell extracts were unlabeled or by immuno-
precipitation when labeled with [35S]cysteine.

Sendai virus RNA synthesis in vitro

Purified SEN and DI-H4 (19 µl) were incubated in a
reaction mix (100 µl) as described previously (Carlsson
et al., 1985), but with 20 µM [32P]CTP instead of [3H]–
UTP and with the addition of RNasin (1 U/ml) and 10%
glycerol. The soluble protein fraction was prepared by
sedimenting the extract at 50,000 rpm for 80 min at 4°
in the SW25 rotor. The samples were incubated in the
absence or presence of various Protein A–Sepharose
CL-4B (Pharmacia)-purified antibodies, as indicated in
the text, for 30 min at 4° and then for 2 hr at 30°. The

RNA products were isolated and analyzed by electro-
phoresis on acid-urea–1.5% agarose gels.

RESULTS

Sendai virus V protein is nonstructural

To more easily detect the V protein, we used the Z
strain of SEN, in which NP migrates slightly faster on
gels than in the other strains, and radiolabeled the in-
fections with [35S]Cys. When the cell extracts were sep-
arated on SDS gels (lane 2, Fig. 2) and compared to the
uninfected control (lane 1), three closely spaced virus-
specific bands of 55–65 kDa are seen. The top and
bottom bands were found to represent Fo and NP, re-
spectively, using monoclonal antibodies (mAbs) (not
shown). The middle band could be specifically immuno-
precipitated with anti-V from infected (lane 5) but not
uninfected cells (lane 7), as well as from cells infected
with a recombinant vaccinia virus (w) carrying the P
gene with the precise 1G insertion (w–SEN–V, lane 6),
but not from cells infected with nonrecombinant w
(lane 8). The V proteins made in vivo also comigrated
with V made in vitro from a plasmid-generated mRNA
(lane 9). The V band in lane 2 appears twice as strong
as that of P, and this is consistent with the relative Cys
content of these proteins and the abundance of their mRNAs (V contains 10 Cys and P contains 3 Cys, but there are twice as many P as V mRNAs).

Virions purified from the culture medium, however, did not contain detectable amounts of V when examined directly (lane 3). Neither could this protein be immunoprecipitated from these samples (lane 4), even though eight times more material was used than was analyzed directly in lane 3. V is clearly underrepresented in virions relative to infected cells, in contrast to structural proteins such as P. We find less V in virions than the C proteins (not shown) which are considered as nonstructural proteins. By this criterion, V is then also a nonstructural protein. This is consistent with the description of the SV5 (Peluso et al., 1977) and mumps virus (Herrler and Compans, 1982) V proteins as nonstructural proteins. However, a recent study has found considerable amounts of V in mumps virions (Takeuchi et al., 1990). This variability may be due to the difficulty in purifying mumps virus, which grows to very low titers.

**Intracellular location of the V protein**

To examine whether V was associated with NCs, cytoplasmic extracts were centrifuged on sucrose gradients, and equal amounts of each fraction were immunoprecipitated with a mixture of anti-V and a mAb to the C-terminus of P. As shown in Fig. 3, the majority of the P protein, as expected, was found in the bottom fraction where the NCs have sedimented. The selection of the L protein as well from this fraction is presumably due to a P-L complex resistant to the detergent conditions used. The V protein, in contrast, was found only in the top two fractions. In this experiment the extract was prepared with the nonionic detergent NP-40 in normal salt, but identical results were obtained with the lyssolecithin protocol, or when the extract was prepared by dounce homogenization without detergent, and in low salt. Moreover, similar results were obtained when the gradients were examined by Western blotting rather than precipitation (not shown). This latter result appears to rule out the possibility that the absence of V in the NC fraction was due to the masking of the anti-V epitope on the NC with another protein. When fraction 1 of Fig. 3 was examined for RNA synthesis, it was found to be as active as a comparable amount of purified virions (not shown). It remains possible that V is weakly associated with NCs, but is removed during the centrifugation, even though the gradient solutions contained only 30 mM NaCl. Nevertheless, it is clear that the SEN V protein is not associated with NCs in a stable manner as is P.

The intracellular location of V relative to P was also examined by double label immunofluorescence studies, using a mAb to P (1180, which reacts with the C-terminus of P) and the anti-V peptide antiserum (Materials and Methods). We chose cells which give a punctate anti-P staining in the cytoplasm, with relatively large patches as well (Fig. 4), as found previously (Kristensson and Oervell, 1983). The same pattern is obtained with an anti-NP mAb (not shown), and these are probably areas of NC concentration. These large patches are mostly near the nucleus and to one side of it (Fig. 4). The anti-V fluorescence is generally also found in the areas where P is most intense, but in 1/4 of the cells examined, there were clearly areas where V was found in the relative absence of P (arrow, Fig. 4). In addition, there is often a ring of anti-V fluorescence around the nucleus which is not seen with anti-P, and the anti-V fluorescence tends to be more granular and evenly distributed in the cytoplasm than that of P. In contrast, we were unable to find any differences in P and NP localization in similar experiments (not shown). Uninfected cells treated identically on adjacent chambers of the slide showed no fluorescence under these conditions (Fig. 4). Thus, whereas the vast majority of P colocalizes with NCs, there appears to be a significant frac-

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**Fig. 3. Analysis of viral proteins which cosediment with RNP.** An NP-40 cytoplasmic extract of SEN-infected cells labeled with [³⁵S]-cysteine (10 μCi/ml) at 16-20 hr p.i. was layered onto a step gradient composed of a 500-μl cushion of 68% (w/v) sucrose in D₂O-TN and 2.5 ml of 50% glycerol-TN formed in an SW60 centrifuge tube. The gradient was spun at 43,000 rpm for 90 min at 20°C. Samples of each fraction were immunoprecipitated with anti-V and a mAb to P, and the proteins resolved on a 12.5% SDS-polyacrylamide gel. Lane A: [³⁵S]-cysteine-labeled infected cell extract. Lanes 1-6: fractions from step gradient.
Effect of anti-V on RNA synthesis

A possible role for the V protein in viral RNA synthesis in vitro was next examined. For these studies, we used a mixture of purified SEN and a DI particle (H4, Kolakofsky, 1976) which, when supplemented with a soluble supernatant from uninfected cells and either no antibodies (left lane) or 2 or 4 µl of anti-P, anti-V from two separate rabbits (V. and Va), or rabbit anti-rabbit poxvirus as an unrelated control (RPV). Total RNA was isolated and analyzed by acid-urea-agarose gel electrophoresis. The various bands are indicated on the left side. DI indicates the doublet of (+) and (-) strands which separate in this system; P, NP, and M, refer to these viral mRNAs.

The phosphorylation of the V protein

Purified virions have long been known to contain kinase activity, which phosphorylates P and NP (Roux and Kolakofsky, 1974; Lamb, 1975). Unexpectedly, the kinase is tightly associated with the NC core and not the envelope (Vidal et al., 1988) and prefers the P and NP proteins to model kinase substrates such as histones and protamine (unpublished). Moreover, Einberger et al. (1990) have recently suggested that the L protein is in fact the P and NP kinase. The P protein is phosphorylated at sites contained within the N-terminal half of the protein, a region that is shared with V.

To further study this phosphorylation, cells infected with either the natural Z strain (Fig. 6A) or vv-recombinants expressing the V or P proteins (Fig. 6B) were labeled with 32PO4, and cell extracts were precipitated with specific antibodies. We found that V was indeed phosphorylated, and apparently to the same extent as P during the natural infection. Moreover, when these proteins were expressed in isolation of the other SEN proteins via the vv-recombinants, they were phosphorylated to the same relative extent as during the natural infection.

The kinase responsible for the majority of the phosphorylation of P and V is then unlikely to be the L protein.
Fig. 6. Detection of viral phosphoproteins. BHK cells infected with either SEN (A) or vv-recombinants expressing either the SEN P or V proteins (B) were labeled with \(^{32}\)P\(_4\). Samples were immunoprecipitated with either anti-V or mAbs to the P and NP proteins as indicated below. Proteins were analyzed on a 12.5% SDS-polyacrylamide gel. (A) lanes 1–3, precipitations of uninfected cell extracts; lanes 4–6, precipitations of SEN-infected cell extracts. (B) lanes 1 and 2, wild-type vv-infected cell extracts; lane 3, extract prepared from a vv-recombinant expressing the V protein; lanes 4 and 5, extracts prepared from vv-recombinants expressing the P protein.

**DISCUSSION**

Attention has recently been focused on the paramyxovirus V proteins because of the unusual mechanism involved in generating their mRNAs. Moreover, the C-termini of these proteins are cysteine rich and highly conserved even among very different paramyxoviruses, in contrast to the P proteins which are poorly conserved. The conserved Cys residues of V are reminiscent of other transcription factors such as the HIV tat protein and zinc-finger proteins, which interact with nucleic acids and modify transcription. Since P and V share N-terminal sequences, the obvious assumption has been that V would also play a role in viral RNA synthesis.

The work presented here, however, suggests that the situation is not a simple one. In contrast to P, we have been unable to find V in virions or intracellular NCs that are transcriptionally active. This is consistent with previous data. Ryan et al. (1991) have found that only two blocks of P, both in the C-terminal half, are necessary and sufficient for P binding to NCs in vitro. The absence of both of these binding sites in V would explain the lack of a stable association of V with NCs and why it is not selectively packaged in virions. There are, however, as many as 200 copies of P per NC, and our data cannot exclude that a few copies of V are present. The reason for so many P proteins on each NC is enigmatic, and it remains possible that unlike P, only very small amounts of V are required for RNA synthesis. However, the only anti-V-specific antibody available so far has no effect on RNA synthesis in vitro. We have thus been unable to provide evidence that, like P, V is required for RNA synthesis. In this respect, the recently determined sequence of the PIV1 P gene (Matsuoka et al., 1991) is germane.

PIV1 and SEN are the most closely related pair of paramyxoviruses (Matsuoka et al., 1991), except for bovine and human PIV3. This is also true of their P proteins, although there is only 52% sequence identity
between them. Until PIV1 P was determined, the next closest relative to SEN P was bPIV3 (only 24% identity), making structural comparisons ambiguous. The SEN/PIV1 alignment, in contrast, is unambiguous and shows that the protein is divided into N- and C-terminal parts, with a hypervariable region in between (Fig. 1). One measure of the relatedness of PIV1 and SEN is that PIV1 can replicate a SEN DI genome (Curran and Kolakofsky, 1991). Remarkably, whereas the P and C proteins of SEN and PIV1 are well conserved (residues 75–204 of C are 88% identical), the V ORF in PIV1 is interrupted by nine stop codons, and there is no semblance of an editing site in the vicinity. Barring the possibility that the V function is expressed from an area of the PIV1 genome which has not as yet been elucidated, this would indicate that V is not required for RNA synthesis.

However, even though PIV1 (and RSV) do not appear to code for a V protein, all the other paramyxoviruses do so within the P gene, and the V-specific sequences are the most highly conserved of the three P gene ORFs among these viruses. This conservation, when V is expressed, suggests that it does play an important role in virus replication. We have recently developed an in vivo system in which DI genome replication is dependent on cloned viral genes expressed from plasmids (manuscript in preparation). In this system, P expression is essential for RNA synthesis, whereas V expression is not. Moreover, coexpression of V with P inhibits RNA synthesis in a dose-dependent manner. These studies suggest that V is a negative regulator of RNA synthesis. If so, one can more easily reconcile the absence of V in PIV1.

In the particular case of measles virus (Ballart et al., 1990), it will be of interest to determine what effect abrogation of V function has in this system. It has generally been assumed that all gene products of RNA viruses represent essential functions, because of the limited size of RNA genomes. However, Schwarz et al. (1990) have recently found that a nonstructural protein (NS2) is not essential for coronavirus replication in cell culture, and Huang et al., (1990) have shown that the nonstructural proteins of influenza virus (NS1 and NS2) are not essential for the replication of an artificial genome.

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