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Processing of a Cellular Polypeptide by 3CD Proteinase Is Required for Poliovirus Ribonucleoprotein Complex Formation

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Poliovirus interactions with host cells were investigated by studying the formation of ribonucleoprotein complexes at the 3′ end of poliovirus negative-strand RNA which are presumed to be involved in viral RNA synthesis. It was previously shown that two host cell proteins with molecular masses of 36 and 38 kDa bind to the 3′ end of viral negative-strand RNA at approximately 3 to 4 h after infection. We tested the hypothesis that preexisting cellular proteins are modified during the course of infection and are subsequently recruited to play a role in viral replication. It was demonstrated that the 38-kDa protein, either directly or indirectly, is the product of processing by poliovirus 3CD/3C proteinase. Only the modified 38-kDa protein, not its precursor protein, has a high affinity for binding to the 3′ end of viral negative-strand RNA. This modification depends on proteolytically active proteinase, and a direct correlation between the levels of 3CD proteinase and the 38-kDa protein was demonstrated in infected tissue culture cells. The nucleotide (nt) 5–10 region (positive-strand numbers) of poliovirus negative-strand RNA is important for binding of the 38-kDa protein. Deletion of the nt 5–10 region in full-length, positive-strand RNA renders the RNA noninfectious in transfection experiments. These results suggest that poliovirus 3CD/3C proteinase processes a cellular protein which then plays an essential role during the viral life cycle.

During the poliovirus (PV) life cycle, virus-host cell interactions regulate and control key events such as viral RNA translation and formation of membranous replication complexes. Initiation and fidelity of PV RNA replication are determined by specific RNA-protein interactions. Due to the limited coding capacity of the PV genome, it is presumed that host cell proteins are recruited to play a role in the initiation of viral RNA synthesis. The formation of putative replication initiation complexes has been investigated, and we have previously shown that a 36- and a 38-kDa protein bind to the 3′ end of PV negative-strand RNA (34). Both are host cell proteins, and their binding affinities or their availability for binding to negative-strand RNA was initially low and increased dramatically at approximately 3 to 4 h after PV infection. Our data suggested that these proteins were not synthesized de novo following infection. It was hypothesized that preexisting cellular proteins were modified by a viral protein(s) during the course of infection, and the modified proteins were then recruited to play a role during replication.

Several host cell factors involved in host cell transcription and translation have been shown to be proteolytically processed by picornavirus proteinases (Table 1). This proteolytic processing of cellular proteins ultimately leads to host cell shutoff, allowing PV to utilize host cell activities for functions that its genome does not encode. PV infection leads to inhibition of all three classes of host cell RNA synthesis by approximately 3 to 5 h following infection (20). RNA polymerase III-mediated transcription is decreased by PV 3C proteinase-induced proteolysis of transcription factor IIIC (9). The TATA-binding protein, a component of transcription factor IID, is cleaved directly in vivo and in vitro by PV 3C proteinase, leading to an inhibition of RNA polymerase II-mediated transcription (10). Inhibition of RNA polymerase I-mediated rRNA synthesis in PV-infected cells is presumably due to a decrease in a specific activity that is needed for transcription initiation (36).

The inhibition of host cell transcription is preceded by the inhibition of cap-dependent host cell translation which occurs within the 2 h following PV infection. Etchison et al. have shown that the p220 subunit of eukaryotic initiation factor 4F is cleaved during PV infection (12). This cleavage prevents the binding of capped mRNAs to the ribosomal 40S preinitiation complex and leads to the shutoff of cap-dependent host cell translation. This allows PV to utilize the host cell translation machinery exclusively for translating its own RNA, which is initiated by internal ribosome entry via a cap-independent mechanism (29). Cleavage of p220 is an important event in the viral life cycle, as illustrated by experiments with a mutant PV which had a mutation in 2A proteinase (3). This mutation resulted in the loss of viral inhibition of host cell translation, and the mutant virus could not replicate in HeLa cells and yielded only low titers in CV-1 cells.

In addition to cleavage of host proteins to disrupt their normal cellular functions, PV may utilize the products of proteolytic cleavage as well as uncleaved cellular proteins for important steps of the viral life cycle, including the formation of ribonucleoprotein complexes that play a role in initiation of viral RNA replication. At present, it is not clear what proteins are required to assemble a functional replication initiation complex and on what RNA sequences these complexes assemble. Most studies utilizing in vitro systems have investigated the *cis*-acting determinants located at the 5′ and 3′ noncoding regions (NCRs) of positive-strand RNA. The role of the 3′ NCR of negative-strand RNA, possibly together with the 5′ NCR of positive-strand RNA, in mediating initiation of positive-strand RNA replication is not well understood (1, 34).

We have previously provided evidence for the involvement of a 36- and 38-kDa host cell protein in ribonucleoprotein complex formation at the 3′ end of PV negative-strand RNA. Here, we demonstrate that the 38-kDa protein, either directly

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TABLE 1. Cellular proteins cleaved by picornavirus proteinases

Proteinase[a]	Host cell substrate	Reference(s)
PV 2A	Indirectly cleaves p220 subunit of eIF-4F	3, 12, 21, 25
PV 3C	TF IIIC	9
PV 3C	TATA-binding protein	10
PV 3C	Mediates cleavage of microtubule-associated protein 4	19
FMDV 3C	Histone protein H3	13, 41
HRV 2A, CVB4 2A	p220 subunit of eIF-4F	24

[a] FMDV, foot-and-mouth disease virus; HRV, human rhinovirus; CVB4, coxsackievirus B4.

or indirectly, is the product of processing by PV 3CD or 3C proteinase. Levels of the 38-kDa protein present in extracts from PV-infected cells correlate with levels of 3CD proteinase present in infected tissue culture cells. By deletion analysis, it was shown that PV nucleotides (nt) 5 to 10 (note that positive-strand numbers are used, even though the RNA is of negative-strand polarity) are required for efficient binding of the 38-kDa protein. To correlate these in vitro results to viral replication in vivo, full-length, positive-sense RNA containing a deletion between nt 5 and 10 was transfected into tissue culture cells. This mutated RNA was noninfectious, and no viable revertants were recovered. These results suggest that the binding of the 38-kDa protein to nt 5 to 10 plays an essential role during viral replication.

MATERIALS AND METHODS

Construction of transcription templates. Plasmids pT7N66- and pT7N108- (34) were used to generate two sets of cDNA deletion constructs that were used to transcribe PV negative-strand RNA in the context of PV nt 66 to 2 and nt 108 to 2, respectively. The starting plasmids were subjected to partial digestion with *Bgl*I (PV nt 35, pGEM nt 1281) and digestion with *Eco*RI (pGEM2 nt 10). The resulting fragments were purified by gel electrophoresis. Synthetic oligonucleotides that have the PV sequence of interest deleted and have *Eco*RI and *Bgl*I half sites at their ends were incubated together with the plasmid fragments and T4 DNA ligase to generate pT7N66- D5-10, pT7N66- D12-17 pT7N66- D27-32, pT7N108- D5-10, pT7N108- D12-17, and pT7N108- D27-32. The integrity of these constructs and all constructs described below was verified by sequencing across junctions of DNA fragments and the region containing the introduced mutations, using the modified T7 DNA polymerase method.

Plasmids pT7N66- D35-45 and pT7N108- D35-45 were constructed essentially as described above except that the starting plasmids were subjected to partial digestion with *Sca*I (PV nt 50, pGEM nt 1645) instead of *Bgl*I and subsequent digestion with *Eco*RI. Analogously, the synthetic oligonucleotides that have PV nt 35 to 45 deleted have *Eco*RI and *Sca*I half sites at their ends. To generate pT7N108- D48-59, plasmid pT7N108- was subjected to partial digestion with *Bgl*I and subsequent digestion with *Kpn*I (PV nt 66). The oligonucleotides used have both *Bgl*I and *Kpn*I half sites at their ends and have PV nt 48 to 59 deleted.

To generate pT7N66- D48-59, plasmid pT7N66- was subjected to partial digestion with *Bgl*I (PV nt 35, pGEM nt 1281) and then digested with *Pvu*II (pGEM nt 98). The following two complementary oligonucleotides were used: PV+D48-59 (5′-TGGCGGTATTGCGCC**TATAGTGAGTCGTATTA**CAG-3′ [positive strand]) and PV-D48-59 (5′-CTG**TAATACGACTCACTATA**GGCGCA ATACCGCCACGT-3′ [negative strand]). The annealed oligonucleotides have both *Bgl*I and *Pvu*II half sites at their ends, have PV nt 48 to 59 deleted, and contain the T7 promoter sequence (boldface sequence).

Plasmid pT7PV1-ΔN5-10 (encoding a full-length PV cDNA which has nt 5 to 10 deleted) was essentially constructed as previously described for pT7(τ)-PV1 (8). The only modification was that the following four synthetic oligonucleotides were used: HRT3 (5′-AGGCCT**TAATACGACTCACTATA**GG-3′ [positive strand]), HRT3′ (5′-**TAGTGAGTCGTATTA**AGGCCT-3′ [negative strand]), HRT4 (5′-TTAATCTGGGGTTGTACCCACCCCAGAGGCCCACG-3′ [positive strand]), and HRT4′ (5′-GGGCCTCTGGGGTGGGTACAACCCCAGA TTAACCTA-3′ [negative strand]). The underlined sequences in HRT3 and HRT3′ represent *Stu*I recognition sites, and the T7 promoter sequence is shown in boldface. The underlined sequences in HRT4 and HRT4′ represent *Bgl*I half sites, and PV nt 5 to 10 are deleted.

Preparation of cellular extract. Cytoplasmic extracts from PV-infected and uninfected HeLa cells were prepared as described elsewhere (34, 37).

Purification of PV 3CD and 3C polypeptides. Histidine-tagged recombinant PV 3CD containing the μ10 mutation (38) or single amino acid substitutions, T142I and A172V, were expressed in *Escherichia coli* and purified as described previously (5). For purification of poliovirus 3C proteinase, the wild-type 3CD coding sequence was placed into a pET15b (Novagen) expression vector. Expression of this recombinant 3CD in *E. coli* BL21(DE3) cells allowed autocatalysis at the 3C-3D junction by the proteinase, resulting in the production of a histidine-tagged 3C polypeptide. Total bacterial lysate containing histidine-tagged 3C was subjected to nickel affinity chromatography on a Hi-Trap chelating column (Pharmacia). Nickel affinity-purified, histidine-tagged 3C was dialyzed overnight at 4°C against HD buffer (50 mM HEPES [pH 7.4], 1 mM dithiothreitol) in the presence of 25 U of thrombin (Sigma) per mg to remove the histidine-tag. Dialyzed 3C was then subjected to anion-exchange chromatography on a Mono-Q column, and 3C proteinase was collected in the flowthrough fraction.

In vitro cleavage assays. In vitro cleavage assays were performed as described previously (4). Briefly, 150 μg of extract from PV-infected or uninfected cells was incubated with 50 pmol of proteinase for 2 h at 30°C. Extracts were stored at −70°C until further use in UV cross-linking assays.

In vitro RNA transcription and UV cross-linking assays. In vitro RNA transcriptions were carried out as described previously (33). Transcription templates used for generating RNAs corresponding to the 3′ end of PV negative-strand RNA were digested with *Mse*I. Transcription templates used for generating full-length positive-strand RNA were linearized with *Eco*RI. UV cross-linking assays were performed essentially as described previously (33).

[^{35}S]methionine labeling of total cellular proteins and immunoprecipitations with anti-3C antibody. One hour prior to harvesting of infected cells, 120 μCi of [^{35}S]methionine/4 × 10^6 cells was added to the culture. Equal numbers of cells were then harvested, washed twice in phosphate-buffered saline, and resuspended in Laemmli sample buffer. Immunoprecipitations were performed as described previously (11), using the EΔ3C polyclonal antibody (26) raised against 3C proteinase.

Transfection of RNAs derived from wild-type and mutant full-length PV cDNAs. RNA transfections were performed by using either the DOTAP procedure (43) or the DEAE procedure (22), with modifications described elsewhere (17). HeLa cell monolayers (95% confluent) in 60-mm-diameter dishes were transfected with 2, 0.2, and 0.02 μg of in vitro-transcribed full-length PV RNA derived from pT7PV1-ΔN5-10 or in vitro-transcribed full-length wild-type PV RNA derived from pT7PV1 (16). Transfections for subsequent slot blot analysis of positive-strand RNA were performed by using the DEAE procedure. Per 60-mm-diameter plate, 150 μg of in vitro-transcribed full-length PV RNA derived from plasmids pT7PV1, pT7PV1-ΔN5-10, and pT7PV1-3C-Δ54 was used. After transfection, cells were overlaid with liquid medium and incubated at 37°C until they were harvested.

RNA preparation and slot blot analysis. Total RNA was prepared by using the method of Favaloro et al. (14), with modifications described elsewhere (32). RNA (10 μg per time point) was incubated in glyoxal and dimethyl sulfoxide and transferred onto a GenScreen Plus membrane by slot blotting as described elsewhere (8). The slot blot was probed with a cocktail of four [γ-^{32}P]ATP-end-labeled, PV-specific oligonucleotides (PV2B-, complementary to PV nt 3995 to 4015; PV3C-, complementary to PV nt 5480 to 5501; WB1-, complementary to PV nt 2619 to 2638; and L391*, complementary to PV nt 253 to 272). Prehybridization, hybridization, and washing conditions were described previously (8).

RESULTS

In vitro processing of cellular extracts by viral proteinases. A previous study investigating the assembly of RNA replication initiation complexes showed binding of a 36- and a 38-kDa host cell protein to the 3′ end of PV negative-strand RNA (34). In UV cross-linking assays, it was shown that binding of both proteins to the 3′ end of PV negative-strand RNA was not detectable in the first 3 h following infection, and then binding increased significantly until it reached maximal levels at 5 h after infection. Previous results suggested that the 36- and 38-kDa proteins were not synthesized de novo following PV infection, and we were interested in determining how these proteins are generated during the course of infection.

Specific cleavage of cellular proteins by picornavirus proteinases has been well documented (Table 1). Processing of these proteins results in host cell shutoff, as these proteins are mainly involved in cellular transcription and translation. Here, we investigated the possibility that virus-encoded proteinases proteolytically process cellular precursor proteins and the processed proteins are then recruited to play a role during replication. Extracts from uninfected and PV-infected cells were

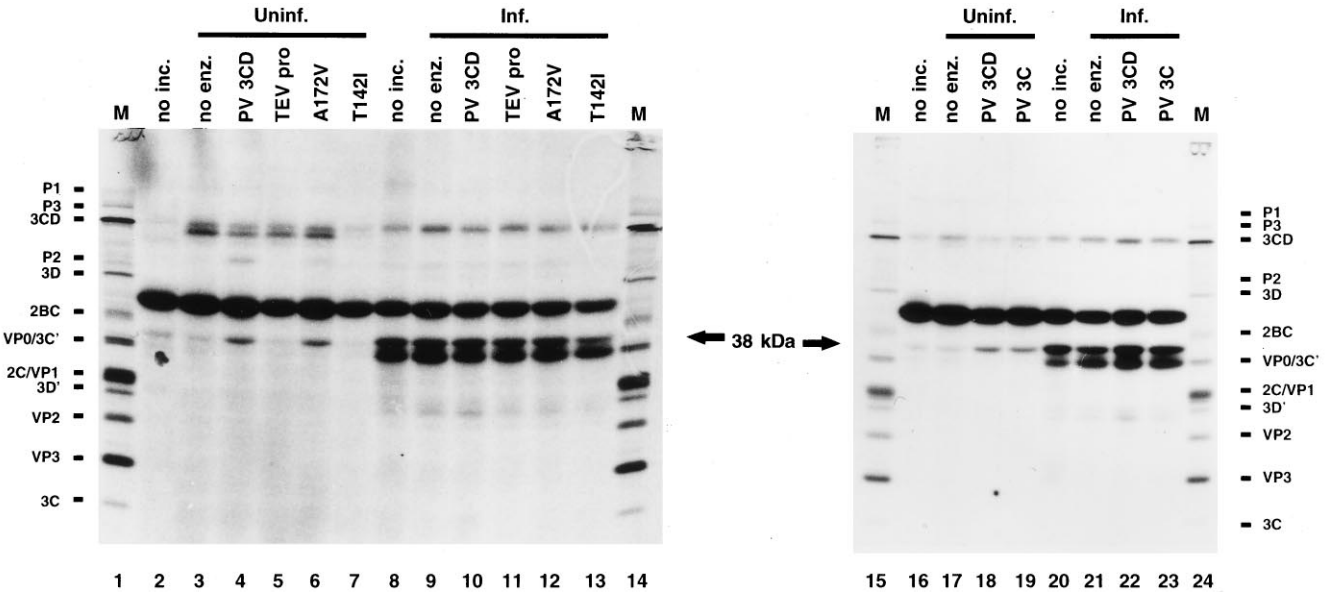

FIG. 1. UV cross-linking assays using cellular extracts preincubated with different viral proteinases. Three recombinant PV 3CD proteinases were used: (i) wild-type 3CD proteinase; (ii) mutated 3CD (A172V), which had alanine$_{172}$ substituted with valine; (iii) mutated 3CD (T142I), which had threonine$_{142}$ substituted with isoleucine; and (iv) wild type 3C proteinase. TEV NIa proteinase (27 kDa) (generously provided by William G. Dougherty) was also used. Extracts from uninfected (Uninf.) and PV-infected (Inf.) cells were incubated separately with each proteinase at 30°C for 2 h. UV cross-linking assays using equal amounts of the in vitro-processed extracts and radiolabeled 66- probe were performed. The 38-kDa protein is marked by an arrow. Lanes M, marker proteins ([^{35}S]Met labeled) from PV-infected HeLa cells. inc., incubation; enz., enzyme.

used as substrates in in vitro cleavage assays together with equimolar amounts of purified wild-type and mutated proteinases. Four recombinant, purified PV proteinases were used for the in vitro cleavage assay: (i) wild-type 3CD proteinase; (ii) mutated 3CD (A172V), which has alanine$_{172}$ substituted with valine, a mutation that only slightly affects its proteolytic activity; (iii) mutated 3CD (T142I), which has threonine$_{142}$ substituted for isoleucine, a mutation that completely abolishes its proteolytic activity (5); and (iv) wild-type 3C proteinase. Additionally, tobacco etch virus (TEV) proteinase, a 3C-like proteinase from a plant virus, was used as a control. To determine whether the 36- and 38-kDa proteins were generated, UV cross-linking assays utilizing equal amounts of the in vitro-processed extracts together with a radiolabeled transcript corresponding to the 3′-most 66 nt of PV-negative strand RNA (66-) were performed (Fig. 1). When extracts from uninfected cells (lanes 2 to 7) were incubated with wild-type PV 3CD proteinase (lane 4) or proteolytically active, mutated proteinase (A172V) (lane 6), a 38-kDa protein which bound to the 3′ end of PV negative-strand RNA was generated. Proteolytically inactive 3CD proteinase (T142I) (lane 7) or TEV proteinase (lane 5) did not generate the 38-kDa protein. The 38-kDa protein was also generated when extracts from uninfected cells were incubated with wild-type 3C proteinase (lane 19). The fact that 3C proteinase can also generate the 38-kDa protein suggests that putative substrate recognition determinants located in the 3D moiety of 3CD proteinase are dispensable for processing of the precursor of the 38-kDa protein.

When extracts from PV-infected cells were incubated with proteinases (lanes 10 to 13, 22, and 23), no further increase in binding of the 38-kDa protein to the 3′ end of PV negative-strand RNA was observed. This suggests that by 5 h after infection, all of the available precursor protein of the 38-kDa protein is already processed. The 36-kDa protein seen just below the 38-kDa protein in lanes 8 to 13 and 20 to 23 was not detected in assays using extract from uninfected cells. These results suggested that the 38-kDa protein was, either directly or indirectly, the product of proteolytic processing of a cellular precursor protein by PV 3CD/3C proteinase. The processed product, but not the precursor protein, can be detectably UV cross-linked to PV negative-strand RNA.

Correlation between PV 3CD proteinase and 38-kDa protein levels. To correlate the results of the in vitro cleavage assays with viral replication in infected HeLa cells, a temperature-sensitive virus, designated Se1-3C-31 (5), was used. The 3CD polypeptide of 3C-31 has alanine$_{172}$ substituted by valine, resulting in a virus with an RNA replication defect at the nonpermissive temperature (39°C). We investigated whether this replication defect at the nonpermissive temperature correlated to decreased levels of the 38-kDa protein, possibly as a result of decreased levels of 3CD polypeptide in infected cells.

HeLa cells were infected and incubated at 33°C for 2.5 h. Half of the cells were then shifted to the nonpermissive temperature of 39°C, and the remaining half were maintained at 33°C (Fig. 2A). Extracts were prepared at the indicated times after infection and used in UV cross-linking assays together with radiolabeled 66- RNA (Fig. 2B). Levels of the 38-kDa protein were lower in cells infected with 3C-31 than in cells infected with wild-type PV (lanes 3 and 4 compared to lanes 6 to 9). Twelve hours after infection, levels of binding of the 38-kDa protein to the 3′ end of PV negative-strand RNA were higher in extracts from cells incubated at the permissive temperature than in extracts from cells incubated at the nonpermissive temperature (lane 7 compared to lane 9). It is conceivable that the reduced appearance of the 38-kDa protein at the nonpermissive temperature accounts for the in vivo replication defect of the 3C-31 virus, suggesting a possible role for the 38-kDa protein in viral replication.

To monitor the efficiency of infection, [^{35}S]methionine labeling of total cellular proteins was performed in parallel to the experiment shown in Fig. 2B (data not shown). Following infection with 3C-31 at the permissive temperature, viral pro-

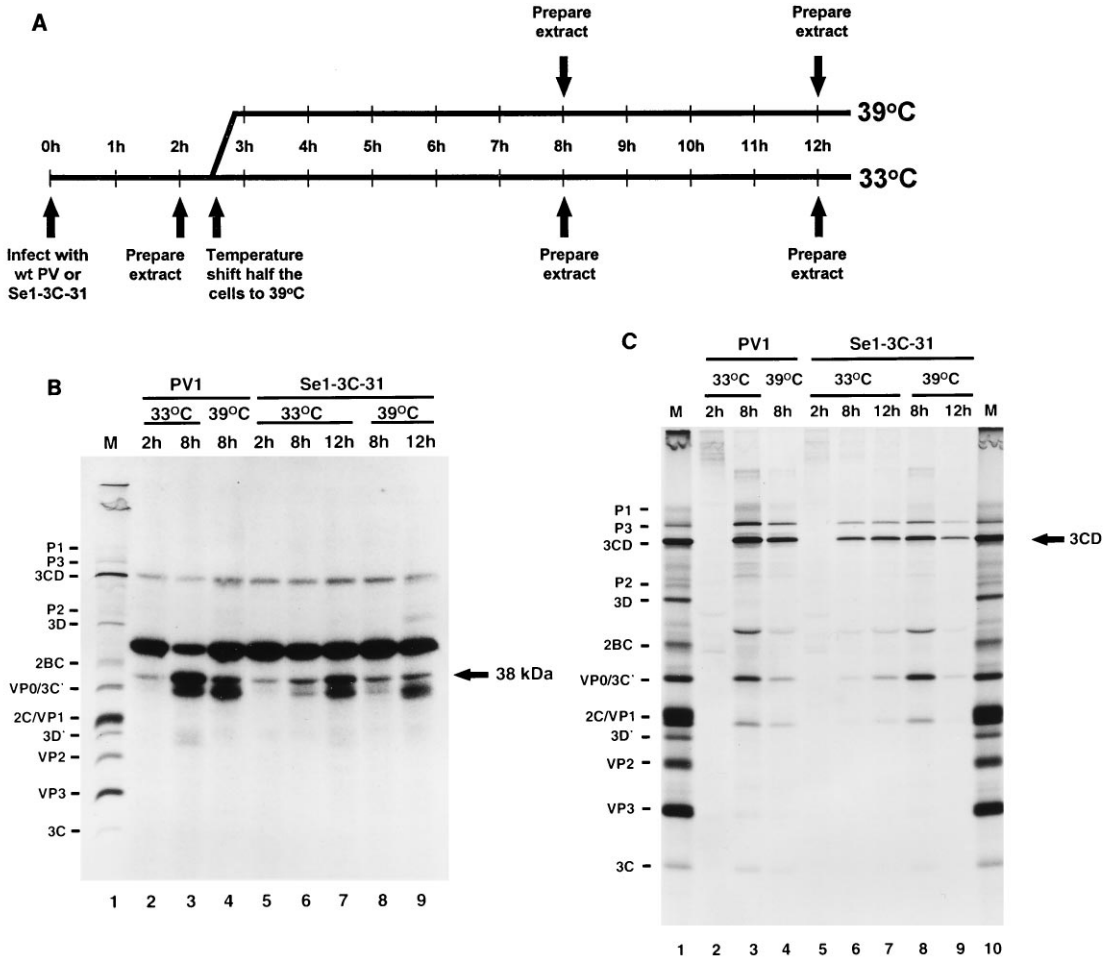

FIG. 2. Temperature shift experiment utilizing the temperature-sensitive virus Se1-3C-31. (A) Time line of the protocol used for the temperature shift experiment with wild-type (wt) PV and 3C-31. (B) HeLa cells in suspension culture were infected at a multiplicity of infection of 20 and incubated at 33°C for 2.5 h. Half of the cells in the culture were then shifted to the nonpermissive temperature of 39°C, and the remaining half of the culture was maintained at 33°C (see panel A). Extracts were prepared at the indicated times after infection and used in UV cross-linking assays together with radiolabeled 66- RNA. The 38-kDa protein is marked by an arrow. Lane M, marker proteins ([^{35}S]Met labeled) from PV-infected HeLa cells. (C) Immunoprecipitations of [^{35}S]methionine-labeled proteins with antibodies against 3C proteinase. One hour prior to harvesting of cells at the indicated times, 120 μCi of [^{35}S]methionine/4 × 10^6 cells was added to the culture. Equal numbers of cells were then harvested, washed, and resuspended in Laemmli sample buffer. Proteins were immunoprecipitated with polyclonal antibodies raised against 3C proteinase and separated on a sodium dodecyl sulfate–12.5% polyacrylamide gel. 3CD proteinase is marked by an arrow. Lane M, marker proteins ([^{35}S]Met labeled) from PV-infected HeLa cells.

tein synthesis is slightly delayed compared to infection with wild-type PV, but the total accumulation of viral proteins is comparable to levels observed during a wild-type PV infection. Following infection with 3C-31 at the nonpermissive temperature, the amount of viral proteins produced is severely decreased compared to that seen at the permissive temperature.

To determine the levels of 3CD proteinase at the indicated times after infection, immunoprecipitations of equal amounts of [^{35}S]methionine-labeled proteins with antibodies against 3C proteinase were performed (Fig. 2C). Twelve hours after infection, the relative amount of 3CD proteinase present in cells infected with 3C-31 and incubated at the permissive temperature was higher than the amount present in extract from cells incubated at the nonpermissive temperature (lane 7 compared to lane 9). This experiment was carried out four times, and the decrease in the amount of 3CD proteinase correlates with the decreased binding of the 38-kDa protein to PV negative-strand RNA shown in Fig. 2B. Taken together, these results support the hypothesis that processing by PV 3CD proteinase is necessary for the 38-kDa protein to bind to the 3′ end of PV negative-strand RNA and that decreased production of 3CD in vivo results in decreased processing of the cellular precursor protein.

Binding site characterization of the 38-kDa protein, using PV negative-strand probes. It was previously shown that the 38-kDa protein binds between nt 2 and 66 on PV negative-strand RNA. Nucleotides 20 to 25, the binding site for the 36-kDa protein, did not appear to be important for binding of the 38-kDa protein (34). Our goal was to systematically delete stem or loop regions between nt 2 and 66 and assess binding of the 38-kDa protein to the mutated RNAs. Two sets of cDNA constructs, which could serve as templates for transcription of PV negative-strand RNA, were generated (Fig. 3). One set of constructs was used to transcribe mutated PV negative-strand RNAs in the context of PV nt 66 to 2; the other was used to transcribe mutated PV negative-strand RNAs in the context of PV nt 108 to 2, the region of negative-strand RNA complementary to the so-called cloverleaf structure of genomic RNA (31, 40). The purpose of examining identical deletion muta-

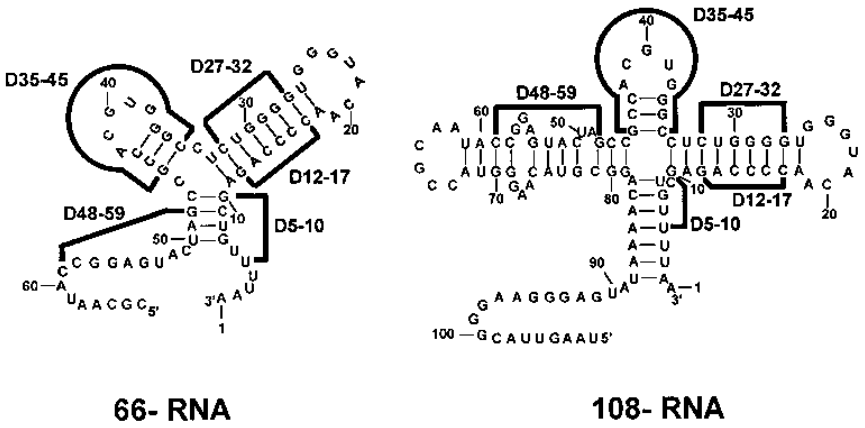

FIG. 3. Computer-predicted secondary structure of the 3′ end of PV negative-strand RNA showing regions that were deleted. Negative-strand RNA sequence complementary to either nt 1 to 66 (66- RNA) or nt 1 to 108 (108- RNA) was used in the University of Wisconsin MFOLD program (44). The sequences shown are of negative polarity, but positive-strand numbers are used. Deleted regions are bracketed by the thick lines, and the names of the resulting RNAs are shown.

tions in different backgrounds was to account for possible effects of RNA secondary structure on protein binding.

In vitro-transcribed PV negative-strand RNAs with the various deletions were used together with either 20 or 40 μg of extract from PV-infected cells in UV cross-linking assays. As seen in Fig. 4A, deleting nt 5 to 10 in the 108- D5-10 RNA probe drastically affects binding of the 38-kDa protein (lanes 4 and 5). Binding of the 36-kDa protein, whose binding site has been shown to overlap with the nt 20–25 region (34), is not affected. Binding of a 50-kDa protein is also decreased, suggesting that its binding site overlaps with the nt 5–10 region. All other mutated RNA probes bind the 38-kDa protein at levels comparable to that seen with wild-type 108- RNA, suggesting that specific stretches of RNA between nt 11 and 108 are not necessary for binding of the 38-kDa protein. Binding of the 38-kDa protein is also drastically decreased when the 66-D5-10 RNA probe is used (Fig. 4B, lanes 4 and 5). It should be noted that the 36-kDa protein binding is also decreased in the context of 66- RNA. The reason for this decrease is not known, but it is possible that in the nt 66–2 background, interaction of the 38-kDa protein with nt 5 to 10 is a prerequisite for subsequent binding of the 36-kDa protein to nt 20 to 25. Additionally, it is possible that nt 67 to 108 stabilize binding of the 36-kDa protein in the absence of the 38-kDa protein. Binding of the 38-kDa protein is decreased when nt 5 to 10 were deleted in both the nt 66–2 and nt 108–2 backgrounds. This finding suggests either that the nt 5–10 region forms an identical secondary structure in both backgrounds or that primary nucleotide sequence, rather than secondary structure, is important for efficient binding of the 38-kDa protein. Together, these results demonstrate that the nt 5–10 region is important for efficient cross-linking of the 38-kDa protein.

Characterization of the effects of nt 5–10 deletion during PV replication. To investigate the relevance of binding the 38-kDa protein to PV negative-strand RNA for viral replication, the nt 5–10 deletion was introduced into a PV cDNA clone (pT7PV1-ΔN5-10). Full-length positive-strand RNA harboring the nt 5–10 deletion was transfected into HeLa cells. Transfected cells were incubated at 33 or 37°C for up to 6 days. Transfections were carried out three times independently, and the mutated RNA was noninfectious in every experiment at both temperatures. Liquid supernatants derived from the transfections were also assayed and found to contain no virus. Therefore, it appears that PV nt 5 to 10 play an important role in the

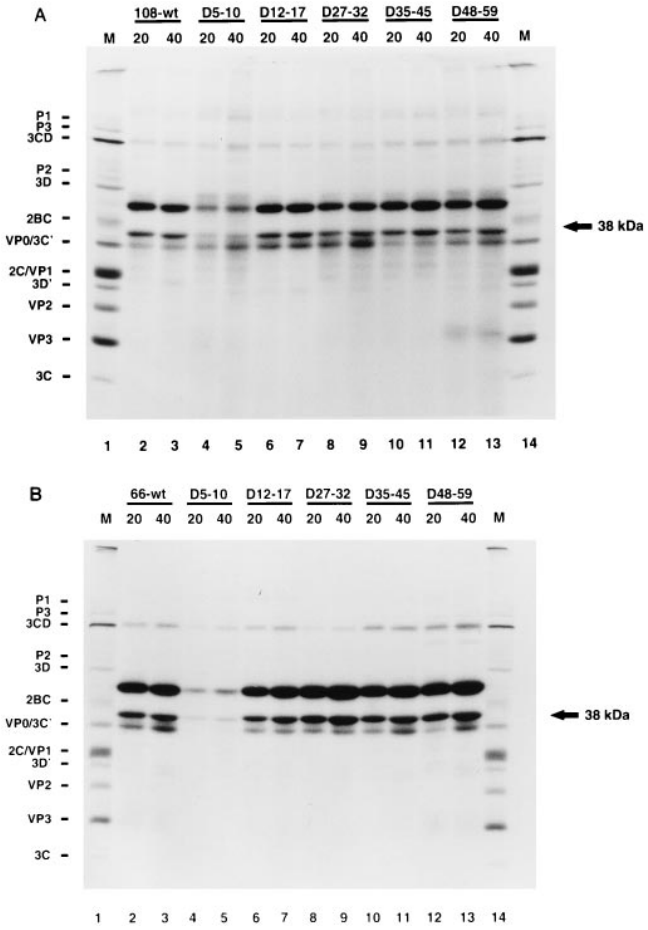

FIG. 4. UV cross-linking assay using 108- and 66- RNA deletion constructs. UV cross-linking assays were performed with 20 and 40 μg of extract from PV-infected cells as indicated above the autoradiographs. (A) Radiolabeled PV negative-strand RNAs, in the context of PV nt 108 to 2, which have the indicated regions deleted were assayed for binding of the 38-kDa protein. The 38-kDa protein is marked by an arrow. Lanes M, marker proteins ([^{35}S]Met labeled) from PV-infected HeLa cells. wt, wild type. (B) Radiolabeled PV negative-strand RNAs, in the context of PV nt 66 to 2, which have the indicated regions deleted were assayed for binding of the 38-kDa protein. The 38-kDa protein is marked by an arrow. Lanes M, marker proteins ([^{35}S]Met labeled) from PV-infected HeLa cells.

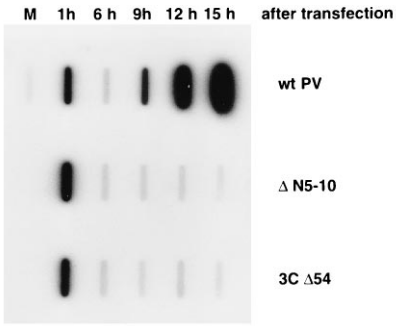

FIG. 5. Characterization of the nt 5–10 deletion during PV replication. Shown is a slot blot analysis of positive-strand RNA levels following transfection of wild-type (wt) and mutated PV positive-strand RNAs into tissue culture cells. Equal amounts of wild-type PV RNA, ΔN5-10 RNA (which has nt 5 to 10 deleted), and 3C Δ54 RNA (which has nt 5597 to 5599, which encode $valine_{54}$ of 3C proteinase, deleted) were used for transfection. Total RNA was harvested at the indicated times after transfection, transferred to a nylon membrane by slot blotting, and probed with radiolabeled synthetic oligonucleotides complementary to positive-strand RNA. M, mock transfection.

viral life cycle and that their deletion constitutes a lethal defect for viral replication in tissue culture cells.

To understand the role of the interaction of the 38-kDa protein with the PV nt 5–10 region, we wanted to examine the level of viral RNA replication in transfected cells. Three sets of in vitro-transcribed full-length positive-strand PV RNAs were used: (i) PV wild-type RNA, (ii) ΔN5-10 RNA containing the nt 5–10 deletion, and (iii) 3C Δ54 RNA, which has nt 5597 to 5599 deleted. The 3C Δ54 RNA, which served as a negative control, is unable to generate negative-strand RNA intermediates due to having residues 5597 to 5599, which encode $valine_{54}$ of 3C proteinase, deleted (11). The three sets of full-length positive-strand RNAs were used to transfect HeLa tissue culture cells, and total cellular RNA was prepared at the indicated times after transfection (Fig. 5). Equal amounts of RNA were transferred to nylon membrane by slot blotting and probed with radiolabeled synthetic oligonucleotides specific for positive-strand RNA. Wild-type positive-strand RNA was detected by 9 h after transfection, and its levels increased until 15 h after transfection. ΔN5-10 RNA transfections yielded no detectable progeny positive-strand RNA throughout the course of the experiment, demonstrating that RNA synthesis is perturbed when nt 5 to 10 are deleted.

DISCUSSION

The results presented in this study demonstrate that the activity of the PV 3CD/3C proteinase results in the modification of a cellular precursor protein, yielding a 38-kDa protein which binds to PV negative-strand RNA. This modification depends on proteolytically active PV proteinase, and a direct correlation between the levels of 3CD proteinase and the 38-kDa protein in infected cells has been established. The binding site of the 38-kDa protein overlaps with the nt 5–10 region of PV negative-strand RNA. Deleting the nt 5–10 region in full-length PV positive-strand RNA renders this RNA noninfectious in transfection experiments, indicating that this region has an important function during the viral life cycle. These results show that PV proteinase specifically processes a cellular protein, which may play an essential role during viral replication.

Previous studies showed that the main effect of processing cellular substrates by picornavirus proteinases is to shut off host cell functions (Table 1). However, there is evidence that p220 cleavage products are recruited to play a role in viral RNA translation (7). Here, we demonstrate that another product of processing by a proteinase, the 38-kDa protein, is used for assembling a ribonucleoprotein complex at the 3′ end of PV negative-strand RNA. A 36-kDa protein that binds to the 5′ end of PV positive-strand RNA and is believed to be involved in RNA replication has been identified as an N-terminal proteolytic fragment of eukaryotic elongation factor EF-1α (18). Generation of this N-terminal proteolytic fragment, however, is thought to be an artifact of protein purification rather than the result of a specific enzymatic cleavage. It is not clear if there is any relationship between the 38-kDa protein described here, the 36-kDa proteolytic fragment of EF-1α, and another, unidentified 36-kDa protein (1, 2) that has been shown to bind to the 5′ end of PV positive-strand RNA. Our ongoing studies are aimed at identifying the precursor of the 38-kDa protein to understand the nature of its interaction with PV 3CD/3C proteinase. It has not been determined whether PV 3CD/3C proteinase itself proteolytically cleaves the precursor of the 38-kDa protein or if the modification/processing occurs indirectly via one or more intermediate proteins.

UV cross-linking experiments utilizing extracts from uninfected cells that were processed in vitro by 3CD/3C proteinase show binding of a 38-kDa protein to the 3′ end of PV negative-strand RNA. The level of binding observed, however, is lower than the level of 38-kDa protein binding seen when extracts from PV-infected cells are used. It is possible that salt and/or pH conditions used in the in vitro cleavage assay do not faithfully mimic the conditions in infected cells. Alternatively, for efficient processing of the precursor of the 38-kDa protein to occur, 3CD/3C proteinase may need other viral factors that are not present in extracts from uninfected cells. The identity of the 36-kDa protein, the other cellular protein that binds to the 3′ end of PV negative-strand RNA, is not known. It is possible that its production during infection is not directly mediated by PV proteinases 3CD/3C.

Deletion analysis showed that the region between nt 5 and 10 at the 3′ end of PV negative-strand RNA appears to be important for binding of the 38-kDa protein. Our results suggest that the primary sequence between nt 5 and 10 is necessary for binding of the 38-kDa protein. It cannot be ruled out that deleting nt 5 to 10 disturbs RNA secondary structure necessary for binding of the 38-kDa protein and that its actual binding site resides elsewhere. This, however, appears unlikely because binding of the 38-kDa protein was severely decreased regardless of whether the nt 5–10 deletion was in the 66- RNA or 108- RNA background. Both 66- D5-10 and 108- D5-10 showed decreased binding not only of the 38-kDa protein but also of another prominent UV cross-linked protein, a 50-kDa protein. Previous competition experiments suggested that this protein interacted nonspecifically with a variety of different RNAs, including the 3′ end of PV negative-strand RNA (34). However, the data shown in Fig. 4 suggest that the 50-kDa protein has an affinity for the nt 5–10 region. This interaction has not been investigated further, and we do not know if the 50-kDa protein is a precursor to the 38-kDa protein.

Full-length positive-strand PV RNAs which have nt 5 to 10 deleted were shown to be noninfectious in three independent transfection experiments, suggesting that nt 5 to 10 play an important role in the viral life cycle and that their deletion constitutes a lethal defect in tissue culture cells. This finding is supported by another report which describes the generation of a temperature-sensitive virus which had a RNA replication defect when nt 10 was deleted (30), demonstrating that important *cis*-acting sequences for viral replication are located between nt 5 and 10.

It is possible that deleting nt 5 to 10 affects viral replication on more than one level. We cannot formally rule out the possibility that the nt 5–10 deletion affects initiation of negative-strand RNA synthesis. This, however, appears to be highly unlikely because *cis*-acting sequences presumed to be required for initiation of negative-strand RNA synthesis are thought to reside at the 3′ end of positive-strand RNA. It is also possible that deleting nt 5 to 10 affects initiation of viral translation, despite the fact that these sequences reside upstream of the PV internal ribosome entry site (17, 28, 29, 42). Simoes and Sarnow (39) reported that a 6-nt insertion at nt 21 results in a decrease of viral translation. We have in fact observed a decrease in the in vitro translation efficiency of ΔN5-10 RNA (three to fivefold) compared to wild-type PV RNA (35). The reason for this decrease is not known, but it is possibly due to the decreased binding of the poly(rC)-binding protein 2 (PCBP2) (23) to stem-loop I of PV positive-strand RNA (27). PCBP2 has recently been shown to bind to stem-loop IV within the PV internal ribosome entry site and is believed to play a role in the initiation of viral translation (6). It is conceivable that PCBP2 bound to stem-loop I is part of a multimeric complex that interacts with PCBP2 bound to stem-loop IV, and perturbation of this complex decreases translation efficiency. It is also possible that the replication initiation complex for positive-strand RNA synthesis assembles in vivo on partially double stranded RNA that is formed by hybridization of positive- and negative-strand RNA, as has been suggested for coronavirus replication (15). Such a mechanism could explain the importance of the 5′-most 100 nt of PV positive-strand RNA for the initiation of positive-strand RNA synthesis (1). UV cross-linking experiments using double-stranded RNA showed no increase in binding of either the 38- or the 36-kDa protein or the formation of additional complexes (35).

In our current model, we propose that binding of the 38-kDa protein to the nt 5–10 region at the 3′ end of PV negative-strand RNA is an early step in the assembly of a preinitiation complex for positive-strand RNA synthesis. Since the PV genome has a limited coding capacity, from an evolutionary point of view it was probably crucial for the virus to employ host cell proteins during its life cycle. The virus has evolved to utilize existing host cell proteins, and it is conceivable that for RNA replication, for example, a coevolution of RNA sequences at the 3′ ends of viral RNAs and host cell proteins interacting with these sequences occurred. The unmodified cellular precursor protein of the 38-kDa protein is present in the cell prior to infection, and it binds to PV negative-strand RNA very inefficiently. Its identity is presently unknown, but it is possible that this precursor protein, for example, is involved in RNA metabolism in the cell. This precursor protein might normally play a role in ribonucleoprotein complex formation, but it does not bind RNA itself or binds to an RNA sequence not present at the 3′ end of PV RNA. Only after processing of the precursor protein by 3CD/3C proteinase, the 38-kDa protein is generated, which then binds to the 3′ end of negative-strand RNA very efficiently. Binding of the 38-kDa protein to the nt 5–10 region could facilitate subsequent interaction of the 36-kDa protein with the nt 20–25 region, which may be a prerequisite for efficient binding of other cellular and viral proteins. PV 3D RNA polymerase or a precursor might then interact with this ribonucleoprotein complex to assemble a functional replication initiation complex that is competent to utilize the negative-strand RNA template for synthesis of progeny positive strands. In such a model, viral functions and host cell proteins interact in a defined, stepwise fashion to carefully orchestrate and regulate the events leading to the production of positive-strand RNAs which will eventually result in the release of progeny virions from the infected cell.

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