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Poliovirus 2C Protein Determinants of Membrane Binding and Rearrangements in Mammalian Cells

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Poliovirus protein 2C is a 329-amino acid-protein that is essential for viral RNA synthesis and may perform multiple functions. In infected cells, it is associated with virus-specific membrane vesicles. Recombinant 2C protein expressed in transfected cells has been shown to associate with and induce rearrangement of the intracellular membrane network. This study was designed to map the determinants of membrane binding and rearrangement in the 2C protein. Computer-assisted analysis of the protein sequence led to a prediction that the protein folds into a structure composed of three domains. Expression plasmids that encode each or combinations of these predicted domains were used to examine the abilities of the partial protein sequences to associate with intracellular membranes and to induce rearrangement of these membranes in HeLa cells. Biochemical fractionation procedures suggested that the N-terminal region of the protein was required for membrane association. Electron microscopic and immunoelectron microscopic observation showed that both the N- and C-terminal regions, but not the central portion, of 2C protein interact with intracellular membranes and induce major changes in their morphology. The central portion, when fused to the N-terminal region, altered the specific membrane architecture induced by the N-terminal region, giving rise to vesicles resembling those observed during poliovirus infection.

Replication of poliovirus in an infected cell requires approximately a dozen viral proteins, all of which are produced by proteolytic cleavage from a single polyprotein precursor. The central portion of the polyprotein generates a protein designated 2BC, as well as its cleavage products, 2B and 2C, which all appear to play essential roles in viral RNA replication. Despite genetic evidence implicating both 2B and 2C in viral RNA replication, and demonstration of physical association of both 2B and 2C sequences with viral RNA replication complexes in infected cells, the precise biochemical functions of these proteins in this aspect of virus replication have not been elucidated.

The 2C protein is one of the most highly conserved viral proteins among all picornaviruses, and 2C-like proteins have been identified in a number of other animal and plant RNA viruses (21). The poliovirus protein is a 37.5-kDa protein comprised of 329 amino acids. The central portion of the protein contains motifs characteristic of nucleoside triphosphate (NTP) binding proteins (13, 23), including motif A with consensus sequence GXXXXGKS/T (in poliovirus ^{129}GSPGT GKS^{136}), which is thought to participate in binding the phosphate moiety of NTP, and motif B, consisting of two aspartate residues (DD^{177} in poliovirus 2C) preceded by a β-strand consisting of hydrophobic residues, which may chelate the Mg^{2+} involved in NTP hydrolysis (reviewed in references 48 and 63). ATP and GTP binding and hydrolytic activities have been demonstrated for 2C or a maltose binding protein fusion derivative of 2C (38, 44), and engineered mutations in the conserved residues in the nucleotide binding motifs abrogated NTP hydrolysis. Introduction of these mutations into full-length viral cDNAs abolished viral RNA replication and infectivity of the resulting RNA transcripts (37, 56), indicating the importance of these residues and possibly of this function in the virus growth cycle.

It has been proposed that additional sequence motifs in 2C-like proteins of different virus families may serve to couple NTP binding and hydrolysis to different biochemical activities, such as RNA helicase activity, membrane reorganization, or virus encapsidation (24, 38, 44). RNA helicase activity has been demonstrated for cylindrical inclusion protein of plant potyviruses (31, 32) and NS3 protein of hepatitis C virus (60), very distant relatives of 2C (19, 53), but efforts to confirm helicase activity in the poliovirus 2C (PV 2C) protein *in vitro* have not been successful (44). Both 2C and its precursor 2BC appear to be associated with viral RNA in infected cells (9), and sequences present at both the N- and C-terminal portions of the protein have been shown to promote nonspecific RNA binding *in vitro* (44, 45). Although RNA binding has been speculated to contribute to several possible functions of the 2C protein, no biochemical evidence has been obtained to link RNA binding to any particular step in the virus growth cycle, such as viral RNA replication, RNA encapsidation, or trafficking of viral RNA through the vesicular system.

In virus-infected cells, 2C (and 2BC) protein is found associated with membranous vesicles that surround the viral RNA replication complexes (7, 9). Subcellular fractionation studies confirmed that 2C colocalized with membrane fractions (26, 55). It has been proposed that 2C may be involved in the formation or maintenance of the vesicle-associated replication complex (9). The absence of a strong hydrophobic sequence element with transmembrane properties in 2C led to the suggestion that 2C associated with membranes via protein-protein interactions with another viral protein, 3AB (30, 54). It has

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been shown recently, however, that cells expressing plasmid-encoded PV 2C or 2BC protein, in the absence of other viral proteins, displayed extensive rearrangement of intracellular membranes, to form vesicles as well as other organized membrane structures by virtue of an interaction of 2C and 2BC with intracellular membranes (2, 10). In addition, 2C protein translated *in vitro* in rabbit reticulocyte lysates binds tightly to exogenous microsomal membranes (15). In these *in vitro* binding studies, the N-terminal region encompassing amino acids 21 to 54 was required for membrane binding. This region correlates with the presence of a conserved, predicted amphipathic helix between residues 18 and 35 (41). Mutations that disrupted either the hydrophobicity or the hydrophilicity of corresponding faces of the predicted amphipathic helix interfered with virus replication, although both polyprotein processing and RNA replication appeared to be affected, and the precise cause of the growth defect caused by these alterations in 2C structure was not dissected.

Both genetic and biochemical evidence implicate 2C in processes important for viral RNA replication (62). The phenotypes displayed by mutants with genetic changes at different loci in the 2C-coding region, however, are quite different. Some mutations appear to impose a *cis*-acting (noncomplementable) defect in the initiation step of RNA synthesis (29), while others affect a *trans*-acting (complementable) function required continuously for RNA synthesis (34). In addition, a different function for 2C has been suggested by the observation of a cold-sensitive 2C mutant with a virion-uncoating defect (35), suggesting that 2C may be involved in virus assembly. It thus appears that 2C, like many picornaviral proteins, may be a protein with multiple functions (42, 62).

Little is known about the structure or physicochemical properties of PV 2C. Genetic evidence has been presented that 2C functions as an oligomer (57). Efforts to purify protein expressed from recombinant cDNAs in *Escherichia coli*, insect, or mammalian cells have proven difficult, although recent analyses of fusion proteins produced in *E. coli* (44) or small amounts of 2C obtained from Sf9 cells infected with recombinant baculoviruses (38) have yielded useful biochemical information. A significant result of the biochemical studies is that mutations that inactivate one activity of the protein often have no effect on other functions. For example, membrane binding and induction of membrane rearrangements occur even when NTPase activity is abrogated (10). Similarly, deletion of the central portion of 2C that includes the NTP binding motif did not affect RNA binding (45), and a truncated protein lacking C-terminal sequence failed to bind RNA but retained NTPase activity (44). These observations prompted us to consider that the multiple activities of 2C might be determined by different regions of the molecule that function as independent structural domains.

In this study we performed an analysis of the protein sequence, the results of which led to a prediction that the protein folds into a structure composed of three domains, connected by small conserved loops or disordered regions. We constructed expression plasmids that encode each or combinations of these predicted domains and examined the abilities of the expressed partial protein sequences to associate with intracellular membranes and to induce reorganization of these membranes in HeLa cells. Surprisingly, sequences from both the N-terminal and the C-terminal portions of the protein, but not the middle region, interact with intracellular membranes.

MATERIALS AND METHODS

Sequences and comparative sequence analysis. The amino acid sequences of picornavirus 2C proteins and their relatives in other RNA viruses were extracted from the EMBL/GenBank and Swissprot databases, respectively. Database searches were performed with the help of the Blitz program (52) and a family of the Blast programs (3) through the EMBL and NCBI network servers, respectively. Global multiple sequence alignments were produced with the CLUSTAL V program (28). Local similarity searches in groups of amino acid sequences were assisted by the MACAW workbench program (50). Pairwise sequence comparisons were performed in the dot plot fashion, using the high-resolution DotHelix program (33). The programs were run in conjunction with the different amino acid scoring tables of the PAM (12) and Blossum (27) families. Secondary structure predictions were produced with the help of the PHD program (46) through the EMBL network server.

Construction of plasmids. Construction of plasmids pTM-PV2C and pTM-PV2BC for transient expression of 2C or 2BC protein in recombinant vaccinia virus-infected mammalian cells has been described previously (10). For expression of predicted 2C protein domains or combinations thereof, a set of plasmids was constructed by insertion of sequences coding for the corresponding regions in expression vector pTM-*Nde* I (10). Corresponding cDNAs were produced from pTM-PV2C or pTM-PV2BC by PCR using synthetic primers with extensions designed to create an *Nde*I site at the 5′ end of the coding sequence and a stop codon followed by a *Pst*I site at the 3′ end. The following primers were used in different combinations (for 5′-end primers, the *Nde*I sequence is underlined; for 3′-end primers, the *Pst*I sequence is underlined): 5′-2B, 5′-TTACGACCCGGGCATATGGGCATCACCAATTACATAGAG-3′; 5′-2C, 5′-CCTTATGTGATCATATGGGTGACAGTTGGTTGAAG-3′; 5′-2C-88, 5′-GGTTATCCATCCATATGAAGAGGTTTGCCCCTC-3′; 5′-2C-252, 5′-CATTCAGCATATGAATGAGTATTCTAGAGATGGG-3′ 3′-2C-122, 5′-GCCATGTACCTGCAGACATCATGGTTCAATACGGTG-3′; 3′-2C-274, 5′-CAGCATCTCTGCAGTTTGCTCATTGGTGACAGTTCTTACAC-3′; and 3′-2C, 5′-AAGTCTTAGATCTGCAGTGGTCATTGAAACAAAGCCTCC-3′.

Amplification was done with 20 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min in the presence of Vent DNA polymerase (New England Biolabs). The resulting DNA fragments were cut with restriction endonucleases *Nde*I and *Pst*I and inserted between the corresponding sites in plasmid pTM-*Nde*I (10) to produce plasmids pTM-PV2C(1-274), pTM-PV2C(1-122), pTM-PV2C(88-274), pTM-PV2C(88-329), pTM-PV2C(252-329), pTM-PV2B2C(1-274), and pTM-2B2C(1-122). Sequence analysis of the coding region of each plasmid demonstrated that no mutations were introduced during PCR amplification.

Expression of PV 2C-related proteins in transfected HeLa cells. The transient gene expression system of Fuerst et al. (17) was used with modifications. Monolayer HeLa cells were seeded in 35-mm-diameter tissue culture dishes 10 to 24 h prior to transfection. Cells (90% confluent) were transfected with supercoiled plasmid DNA containing 2C sequences under T7 promoter control and simultaneously infected with vaccinia virus vTF7-3. Transfection was mediated by Lipofectin reagent (Gibco/BRL). Typically, 3 to 10 μg of plasmid DNA in 50 μl of H_2O was mixed with 10 μg of Lipofectin in 100 μl of H_2O and incubated for 20 min at room temperature. vTF7-3 (5 to 10 PFU per cell) diluted in 0.5 ml of minimal essential medium (MEM) was added to the DNA-Lipofectin solutions, and the mixture was applied to cells. After 45 min, 1 to 2 ml of MEM was added to cells. After 3 h, the medium was changed to 2 ml of MEM with 3% fetal bovine serum. Cells were incubated at 37°C and harvested for further analysis at 16 to 20 h after transfection.

Subcellular fractionation. Cells (3×10^6) were disrupted in a Dounce homogenizer in hypotonic buffer containing 10 mM Tris-Cl (pH 7.5), 10 mM NaCl, and 1.5 mM $MgCl_2$ followed by centrifugation at 1,000 × *g* for 5 min to separate the nuclear pellet. The nuclear pellet was washed with the same buffer, and the combined supernatants were centrifuged in an SW55 rotor for 20 min at 15,000 rpm (27,000 × *g*) to produce a microsomal pellet (P-15) and cytosolic supernatant (S-15). P-15 was resuspended in 100 μl of hypotonic buffer. Proteins were extracted from the P-15 fraction by addition of sodium deoxycholate to a final concentration of 0.5%, and after 15 min incubation at room temperature, samples were centrifuged in the minicentrifuge at 13,000 × *g* to produce pellet and supernatant fractions.

Western blot analysis. Immunoblotting was performed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples according to a standard protocol (47). Briefly, proteins were electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell), which was blocked in 3% nonfat dry milk and incubated with rabbit anti-PV 2C serum (10) for 1 h. The serum was diluted 1:100 in Tris-buffered saline including 3% nonfat dry milk. Alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Promega) was used in a second incubation step.

EM and IEM. Conventional electron microscopy (EM) and EM immunocytochemistry (IEM) analyses were performed as previously described (10). Briefly, for conventional EM, cells were fixed in glutaraldehyde -osmium tetroxide and embedded in Poly/Bed 812 (Polysciences, Warrington, Pa.). For IEM, cells were fixed in 2% paraformaldehyde followed by a fixation in 0.2% uranyl carbonate and embedded in LRGold (London Resin Company) at −20°C (6). For immunocytochemical labeling, a monoclonal antibody (MAb) against PV 2C (40) or 2B (16) was used and was detected on the sections with goat anti-mouse antibody coupled to 10-nm gold (GAM-G10; Amersham International, Amersham, United Kingdom). Since the 2C MAb recognizes an epitope between amino acids 122 and 274, 2C domains not containing this part of the molecule were detected

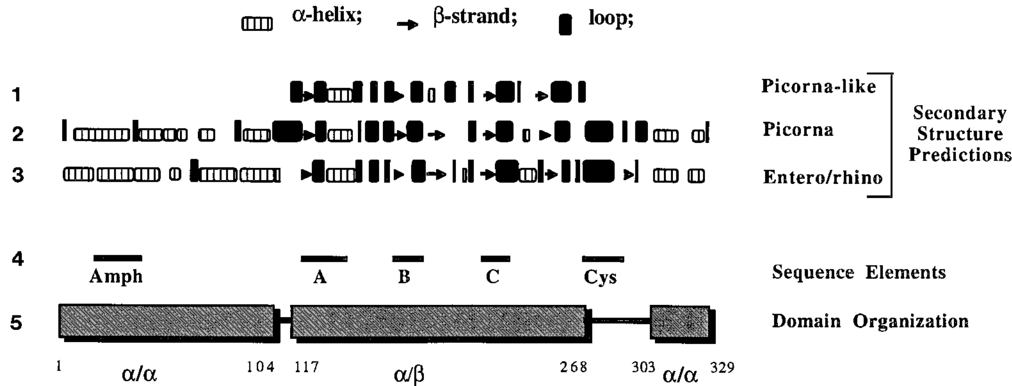

FIG. 1. Domain organization of 2C protein. Three multiple amino acid sequence alignments of 2C and related proteins encoded by enteroviruses and rhinoviruses, picornaviruses, and viruses of the picornavirus-like supergroup, respectively, were generated. The entero/rhino set includes poliovirus serotype 1 Sabin strain (Polio1s), echovirus 11 Gregory strain, human enterovirus 70, coxsackievirus (Cox) types A9, B1, B3, and B4, bovine enterovirus, swine disease virus strains U and H, and human rhinovirus 2 strain (HRV2), HRV14, and HRV89; the Picorna set includes Polio1s, CoxA9, HRV1B, HRV14, EMCV, Theiler's murine encephalomyelitis virus strain BeAn (TMEVB), foot-and-mouth disease virus serotype O (FMDVO), echovirus type 22 (Echo22), and hepatitis A virus strain L (HPAVL); the Picorna-like set includes Polio1s, CoxA9, HRV1B, HRV14, EMCV, TMEVB, FMDVO, Echo22, HPAVL, parsnip yellow fleck virus, cowpea mosaic virus, cowpea severe mosaic virus, red clover mottle virus, grapevine chrome mosaic virus, grapevine funleaf virus, tomato black ring virus strain S, tomato ringspot virus, rice tungro spherical virus, Norwalk virus, Southampton virus serotype 3, human enteric calicivirus, rabbit hemorrhagic disease virus, and feline calicivirus strain F9. Predicted secondary structure was drawn only for a limited number of residues where reliability of the predictions was estimated to be 86% or higher (46). Positions of sequence elements or motifs, which were recognized previously in the PV 2C protein, are shown. Amph, amphipathic helix (41); A and B, two motifs of the NTP binding pattern (13, 21); C, motif specific for the helicase superfamily III (24); Cys, cysteine-rich region (partly) conserved in enteroviruses and rhinoviruses (reference 41 and unpublished data). A three-domain organization of 2C derived from the results of secondary structure predictions is drawn. The domains are represented as α/α or α/β, indicating the major predicted structural elements that comprise them. Each pair of the ordered domains is connected by a disordered region which is depicted by a thick line. Amino acid residues at the domain boundaries are indicated for PV 2C protein.

with the rabbit anti-PV 2C serum. This antibody was visualized with GAR-G10 (Amersham).

RESULTS

Predicted domain organization of PV 2C protein. Previous analyses of picornaviral 2C protein sequences identified a conserved NTP binding pattern (motifs A and B) (13, 20), as well as a sequence (motif C) which is characteristic of proteins of the helicase superfamily III (24). This highly conserved region is located in the central portion of the PV 2C protein and is flanked by two less well conserved regions at the N- and C-terminal ends of the protein. This pattern of sequence organization, as well as the suggestion of multiple biochemical activities, such as membrane binding and RNA binding, in addition to NTP binding and hydrolysis prompted us to examine the 2C protein structure for potential organization into structural domains.

Initially, previous observations on the relationships among picornaviral 2C proteins and between these proteins and other viral proteins (22, 24, 64) were extended for sequence analysis. Three multiple amino acid sequence alignments were generated (Fig. 1). In each case, the 2C protein of poliovirus was compared with either (i) 2C or 2C-like proteins of selected picornaviruses, caliciviruses, and a range of plant viruses similar to picornaviruses (Picorna-like set), (ii) 2C proteins of picornaviruses (Picorna set), or (iii) 2C proteins of enteroviruses and rhinoviruses (Entero/rhino set). These alignments were submitted as input for the PHD program (46), and secondary structure predictions were obtained (Fig. 1, top three lines). The three secondary structure profiles were similar, although minor differences appeared in some places. In these places, the reliability of the predictions was set in the order Picorna-like > Picorna > Entero/rhino, according to the diversity of proteins in the respective alignments (46).

Inspection of the sequence alignments confirmed that conserved residues are unequally scattered over the PV 2C polypeptide chain. Alignment between the whole set of 2C and 2C-like sequences used for the analysis was produced only for the central region of 2C where several absolutely conserved residues were identified (not shown). This region encompasses amino acid residue 117 to approximately 268 of the PV 2C sequence and includes the NTP binding pattern (motifs A, B, and C) (Fig. 1, line 4). In agreement with other NTP binding pattern-containing proteins (51), several β-strands were strongly predicted in the central region of 2C, likely as parts of a recurrent β-strand–loop–α-helix supersecondary structure. Based on these observations, it was postulated that the central most conserved region of 2C adopts an α/β fold (Fig. 1).

The remaining, less conserved N- and C-terminal regions of 2C were aligned in the sets of enterovirus/rhinovirus and picornaviruses sequences. The N-terminal 104 amino acids were strongly predicted to adopt an α/α fold (39), although a number of helices and their borders were differently predicted for the two alignments (Fig. 1, lines 2 and 3). In the C-terminal region, two structurally different subregions were identified; residues 269 to 302, enriched with Cys residues conserved in enteroviruses and rhinoviruses (41), appeared to be mostly disordered, and residues 303 to 329 may adopt an α/α conformation (Fig. 1). A predicted N-terminal amphipathic helix (41) and several C-terminal α-helices (Fig. 1) appear to be broadly conserved.

The results of the theoretical analysis of 2C sequences implied that the PV 2C protein may consist of three structurally different domains (Fig. 1, line 5). The N-terminal α/α and central α/β domains are connected by a loop region which includes highly conserved residues. The central α/β and C-terminal α/α domains are connected by the mostly disordered Cys-rich region. The different patterns of conservation revealed in the domains of 2C indicate that they, as well as the connecting sequences, may possess independent structures consistent with independent functions, although cooperation between these structures remains possible.

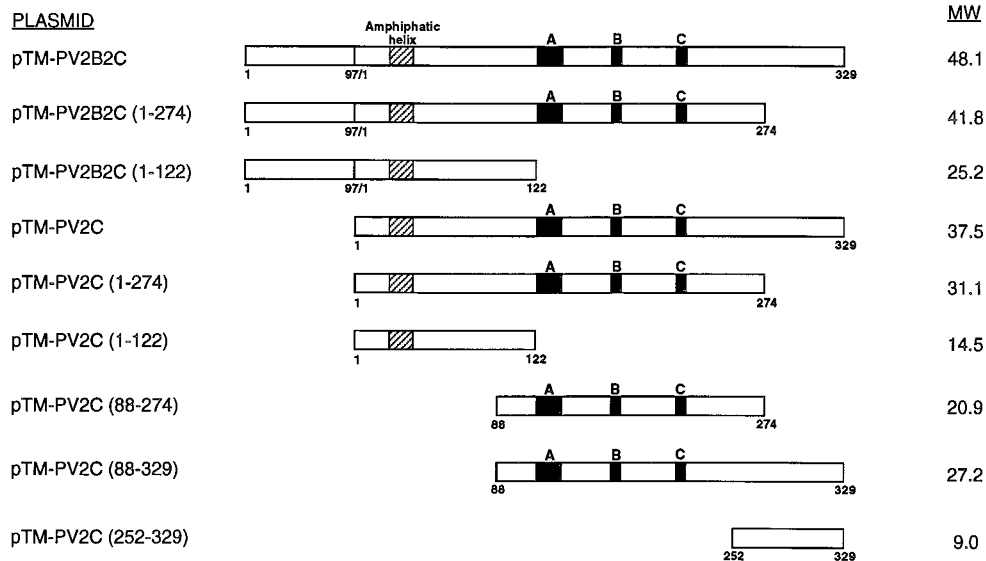

FIG. 2. Schematic diagram of PV 2C-related proteins. The corresponding pTM expression plasmids are indicated on the left. Open boxes denote expressed protein sequences. Numbers correspond to the amino acid residues in PV 2C sequence. Black boxes denote positions of the NTP binding motifs A and B and motif C. The striped box denotes the position of an amphipathic helix. Calculated molecular masses (MW) are indicated in kilodaltons on the right.

Expression of 2C protein-derived domains in HeLa cells. The multidomain model for the structural organization of the 2C protein was used to design experiments to map the determinants of membrane binding and rearrangement, which were shown to be associated with this protein. We constructed a series of plasmids that coded for different domains or combinations thereof, as shown in Fig. 2. In the constructed plasmids the desired portions of 2C protein were placed in expression vector pTM-*Nde*I under control of the T7 transcriptional promoter and the translational internal ribosome entry site from encephalomyocarditis virus (EMCV). Boundaries were selected based on the predicted boundaries of the structurally different domains (Fig. 1), with short extensions at the ends. It should be emphasized that no experimental confirmation of independent folding patterns of individual domains is yet available. Since at least some 2C-mediated functions appear to involve a form of the protein fused at its N terminus to 2B protein sequences, we also constructed several 2B-2C domain expression plasmids. Proteins were expressed in HeLa cells by simultaneous plasmid transfection and infection with recombinant vaccinia virus vTF7-3 (17) to provide T7 RNA polymerase. Immunofluorescence analysis of cells probed with anti-2C serum (data not shown) showed that 15 to 20% of cells expressed 2C antigen for pTM-PV2BC, pTM-PV2C, pTM-PV2C(1-274), pTM-PV2C(1-122), pTM-PV2C(88-274), and pTM-PV2C(88-329). For pTM-2B2C(1-274) and pTM-2B2C(1-122), the number of immunofluorescent cells was significantly lower. Cells transfected with these constructs showed degenerative changes and lysed earlier than cells expressing other proteins, suggesting that these proteins, containing 2B and 2C sequences lacking the C-terminal predicted domain, are cytotoxic.

Transfected cells were subjected to SDS-PAGE and immunoblot analysis to detect the polypeptide products expressed from these constructs. Figure 3 shows that all constructs except pTM-PV2C(252-329) direct the synthesis of anti-2C-reactive proteins with electrophoretic mobilities corresponding approximately to the calculated molecular masses. Cells expressing 2B2C(1-274) and 2B2C(1-122) are not shown in Fig. 3. Consistent with the immunofluorescence analysis described above, these proteins were detected only when significantly more cellular extract was loaded on the gel. All cell lysates usually contained a background of one or two cellular proteins with molecular masses of ~68 and 34 kDa, although these bands were almost undetectable in the control lane of vTF7-3 infected cells in the experiment shown in Fig. 3 (lane 8). For 2BC, 2C, and 2C(1-274), smaller immunoreacting proteins were also detected (Fig. 3, lanes 1 to 3). The appearance of these products during synthesis of 2C and 2BC was described previously and explained by initiation of translation from the

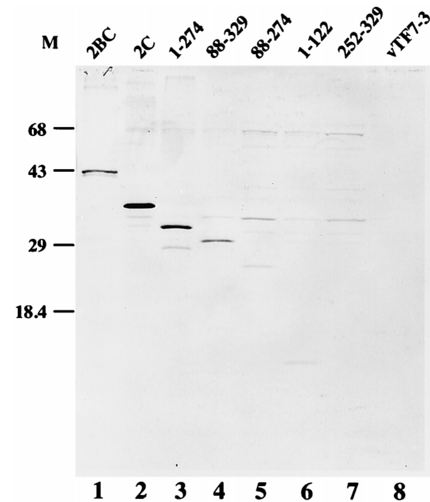

FIG. 3. Expression of PV 2C-related proteins. Extracts of cells transfected with different expression plasmids and infected with recombinant vaccinia virus vTF7-3 were subjected to SDS-PAGE before immunoblot analysis with anti-PV 2C serum. Details of the expression procedures and Western blot analysis are described in Materials and Methods. M, molecular mass markers (indicated in kilodaltons). Lanes 1 to 7, cells transfected with pTM-PV2BC, pTM-PV2C, pTM-PV2C(1-274), pTM-PV2C(88-329), pTM-PV2C(88-274), pTM-PV2C(1-122), and pTM-PV2C(252-329), respectively; lane 8, mock-transfected cells.

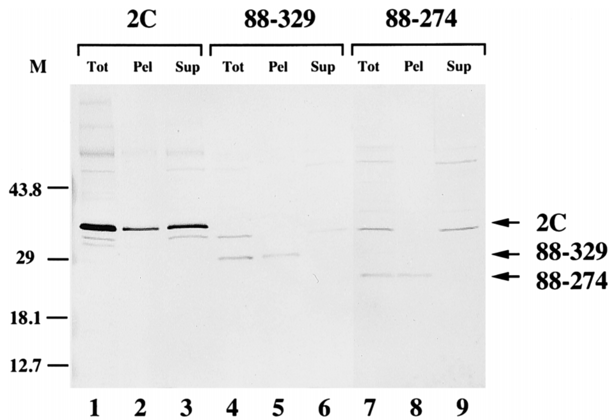

FIG. 4. Detergent extraction of PV 2C proteins. Crude pellet fractions from cells expressing 2C-related proteins were extracted with 0.5% sodium deoxycholate and analyzed by SDS-PAGE and Western immunoblotting. Lanes: 1, 4, and 7, 2C proteins present in pellet fractions; 3, 6, and 9, proteins solubilized by incubation with 0.5% sodium deoxycholate; 2, 5, and 8, proteins remaining in pellet fraction after extraction with 0.5% sodium deoxycholate. Positions of molecular mass marker proteins (lane M) and their molecular masses (in kilodaltons) are indicated to the left; the positions of proteins 2C, 2C(88-329), and 2C(88-274) are indicated to the right.

second AUG, located downstream of the protein start codon (10). Consistent with this explanation, such smaller immunoreacting proteins were detected in proteins 2C(1-274) and 2B2C(1-274) (not shown) but not in 2C(88-274) or 2C(88-329). The absence of a second immunoreactive protein for 2C(1-122) and 2B2C(1-122) is most probably due to the reduced immunoreactivity or high mobility in the gel of such potential products. The apparent reduction in expression of the shorter variants, such as 2C(88-274) and 2C(1-122) (Fig. 3), likely results from the loss of some epitopes in the truncated proteins, causing reduced reactivity with the polyclonal 2C antiserum. The polyclonal antiserum used in this study apparently does not recognize any epitopes in the C-terminal portion of 2C(252-329); no expression of this protein was detected either by immunofluorescence or by Western immunoblotting.

Biochemical analysis of membrane association of 2C domains. Cells expressing several of the engineered proteins depicted in Fig. 2 were fractionated and analyzed for protein association with membrane fractions. Initially, crude pellet fractions were prepared from cytoplasmic extracts of cells transfected with pTM-PV2C, encoding the intact 2C protein. This fraction contained mitochondria, intracellular membrane aggregates, and some insoluble protein material that was not associated with membranes. All of the expressed 2C protein sedimented with this fraction (Fig. 4, lane 1). The pellet fractions were treated with 0.5% sodium deoxycholate and resedimented, generating detergent pellet and supernatant fractions. The majority of the 2C protein was extracted by this treatment from the initial pellet, indicating association of this protein with membranes (Fig. 4; compare lanes 2 and 3). When the crude pellets containing 2C protein were treated with 4 M urea, 1 M NaCl, or sodium carbonate (pH 11.5) and resedimented as described above, the 2C protein localized to the pellet fraction after each of these treatments (data not shown). A tight association of 2C protein with membranes resistant to treatment with 2 M urea was observed earlier in poliovirus-infected cells (55). The presence of 2C in the pellet fraction after sodium carbonate treatment was more surprising, as usually only integral membrane proteins fail to extract at pH 11.5, whereas intralumenal and peripheral membrane proteins fractionate into the supernatant (18). Previous analyses have suggested a peripheral association of 2C with membranes (15, 55). When cytoplasmic extracts of cells containing 2C membranes were subjected to digestion with trypsin (5 ng/μl) for 30 min at 30°C, no detectable portion of 2C was protected from digestion (data not shown), consistent with the previous conclusion that 2C is peripherally associated with the membranes.

Similar extracts were prepared from cells transfected with pTM-PV2C(88-329) or pTM-PV2C(88-274). These plasmids encode portions of 2C protein lacking the N-terminal or both terminal domains. The expressed proteins 2C(88-274) and 2C(88-329) also sedimented with the crude pellet fraction (Fig. 4, lanes 4 and 7); however, these proteins were not extracted by treatment with detergent (Fig. 4, lanes 4 to 9), suggesting that though not soluble in the cell, these proteins were not associated with membranes or remained insoluble even after detergent treatment of the membranes. The different biochemical properties exhibited by different regions of the 2C protein in cell extracts prompted us to examine the localization and morphologic association of these proteins.

Membrane reorganization in cells expressing 2C lacking the C-terminal domain. In a previous study, we showed that expression of PV 2C or 2BC in transfected HeLa cells induced a major rearrangement of intracellular membranes into vesicles of various sizes (10). Cells producing 2C also formed extensive myelin-like tubular membrane structures, formed from reorganization of endoplasmic reticulum (ER) bilayers. IEM confirmed that 2C and 2BC were associated with the induced vesicles and tubule structures. To examine whether the determinants for membrane association and induction of membrane rearrangement were present in various subdomains of the 2C or 2BC protein, similar EM and IEM analyses were performed on cells transfected with plasmids coding for partial 2C proteins, shown in Fig. 2 and 3. We initially examined 2C(1-274), in which the C-terminal domain had been deleted. Figure 5A shows that cells expressing this truncated 2C protein accumulate aggregates of smooth membrane vesicles, approximately 100 to 400 nm in diameter. The vesicles showed intense labeling with anti-2C MAbs (inset). As in 2C-expressing cells, Golgi stacks were absent, and most of the intracellular membrane network was usurped to form vesicle clusters. Cells infected only with vTF7-3, but not transfected with pTM-PV2C(1-274), exhibited typical aspects of vaccinia virus infection, but Golgi stacks remain well developed, and no detectable membrane alterations were observed (10). To address concerns that vaccinia virus infection may influence the induction of membrane reorganization induced by poliovirus proteins, we infected cells with vTF7-3 for 15 h and then challenged with 30 PFU of poliovirus for another 4.5 h. These cells manifested typical poliovirus-induced vesiculation which was indistinguishable from that observed in cells infected with poliovirus alone (Fig. 5B) (8, 10).

The myelin-like swirls of membrane protruding into the lumen of the ER that were observed in cells producing the complete 2C protein were not observed in cells expressing 2C(1-274); however, the amount of 2C(1-274) protein accumulated in transfected cells always appeared to be somewhat less than the amount of intact 2C protein, as judged from the Western blot analysis. These results demonstrate that determinants for membrane association and induction of vesicles are located within the N-terminal two-thirds of the 2C protein and do not require the presence of the C-terminal sequences. Identical changes were observed after transfection of cells with pTM-PV2C(1-274KS), a plasmid encoding the same partial 2C sequences except for a Lys-to-Ser substitution at amino acid residue 135 in motif A of the nucleotide binding site (data not

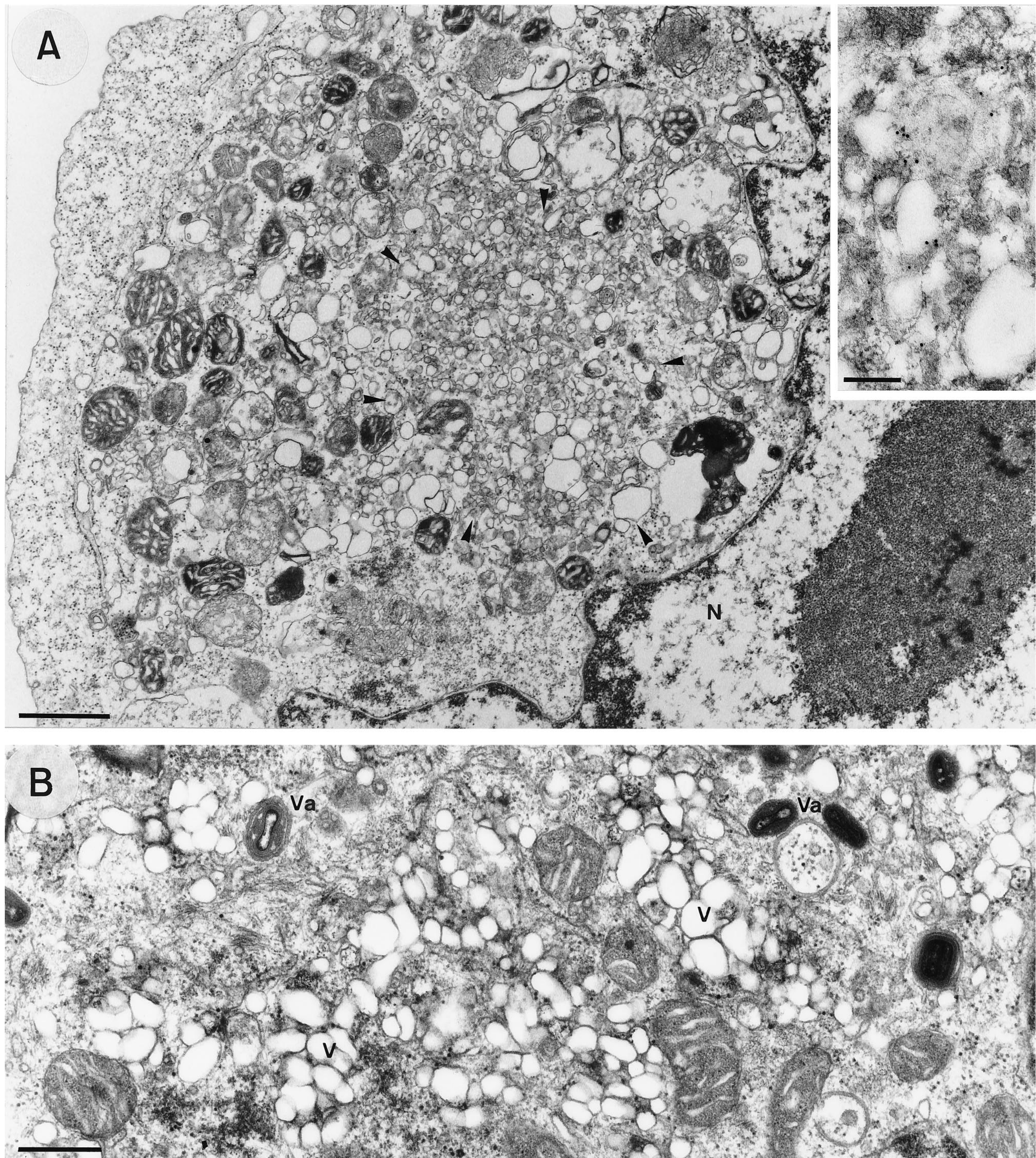

FIG. 5. EM analysis of intracellular membrane structure. (A) Expression of polypeptide 2C(1-274) in HeLa cells induces the formation of numerous smooth vesicles in a juxtanuclear area delineated by arrowheads. N, nucleus. Bar, 1,000 nm. Inset, vesicular membranes immunolabeled with a MAb against protein 2C detected by an anti-mouse–10-nm gold conjugate. Bar, 200 nm. (B) HeLa cells infected with vTF7-3 15 h before being infected with poliovirus for 4.5 h. Clusters of poliovirus-induced vesicles are apparent as in cells infected with poliovirus alone. Va, vaccinia virus; V, poliovirus-induced vesicles. Bar, 500 nm.

shown). This mutation was previously shown to eliminate the infectivity of full-length poliovirus RNA due to loss of viral RNA replication activity (56). Thus, the functional NTP binding motif is not required for induction of vesicles in cells expressing 2C sequences (see also reference 10).

Efforts to examine intracellular membrane structure in cells transfected with pTM-PV2B2C(1-274) were unsuccessful. Expression of this protein appeared to be highly toxic, and cells became fragile and died within a few hours after transfection.

Membrane reorganization in cells expressing N-terminal or central domain of 2C. To further localize the determinants for membrane association and rearrangement activity, the se-

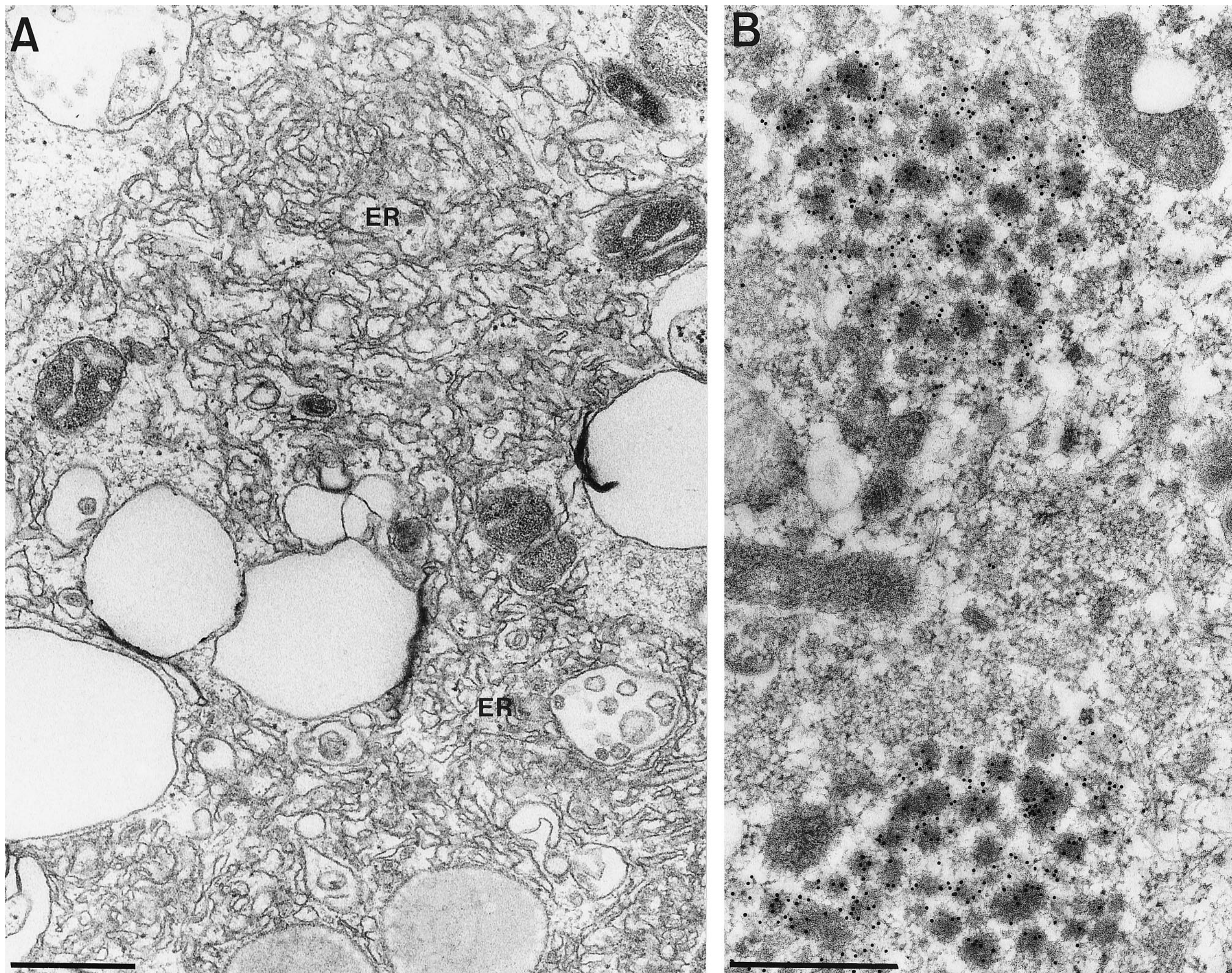

FIG. 6. Membrane structure in cells expressing the 2C N-terminal or central domain. (A) Expression of polypeptide 2C(1-122) in HeLa cells leads to the formation of tightly packed anastomotic ER. Bar, 500 nm. (B) Expression of the central domain of protein 2C, polypeptide 2C(88-274), induces the formation of electron-dense patches immunoreactive with an MAb against 2C. Immunocytochemical staining is as in Fig. 5A, inset. Bar, 500 nm.

quences encoded in pTM-PV2C(1-274) were further subdivided, according to the domain organization predicted in Fig. 1. Plasmids pTM-PV2C(1-122) and pTM-PV2C(88-274) were introduced into HeLa cells, simultaneous with vTF7-3 infection. Expression of the first 122 residues of 2C induced major membrane rearrangements, forming disordered, anastomotic, and fragmented ER, accompanied by the loss of Golgi stacks and normal ER networks (Fig. 6A). IEM analysis showed the presence of 2C sequences associated with the induced membrane aggregates (not shown). Again, addition of 2B sequences N-terminal to 2C(1-122) generated a highly toxic protein that induced cell death.

In contrast, expression of the central portion of 2C (amino acids 88 to 274) produced no membrane alterations. Instead, large protein aggregates forming patches of insoluble protein, not surrounded by or associated with membrane bilayers, were visualized (Fig. 6B). These patches reacted heavily with anti-2C antibody (Fig. 6B) and, curiously, were observed in both the cytoplasm and in the nuclei of transfected cells. No disruption of Golgi stacks or other intracellular membrane structures was observed. Thus, determinants for membrane association and reorganization activity are localized in the N-terminal domain of protein 2C and can form independently of the presence of the central or C-terminal portion of the protein.

Effect of 2C C-terminal domain on intracellular membrane structure. To examine the effects of the presence of the C-terminal 2C sequences on the intracellular membranes, HeLa cells were transfected with pTM-PV2C(88-329) or pTM-PV2C(252-329) and analyzed by EM and IEM. Figure 7A shows that protein containing both the central and C-terminal domains formed patches of insoluble material similar to those formed by protein from pTM-PV2C(88-274), encoding the central domain alone. These patches were also observed in both cytoplasm and nucleus, and they were not associated with or surrounded by membrane. In addition, however, the presence of the C-terminal domain on the protein from pTM-PV2C(88-329) caused the appearance of new membrane structures, visualized as disordered and anastomotic ER, some of which seem fragmented into heterogeneously sized vesicles. IEM analysis with the anti-2C MAb indicated the presence of 2C protein sequences in the membrane aggregates, as well as intense labeling of the patches, as expected. Although it is not possible to accurately quantitate the relative distribution of 2C(88-329) protein between patches and membranes from ex-

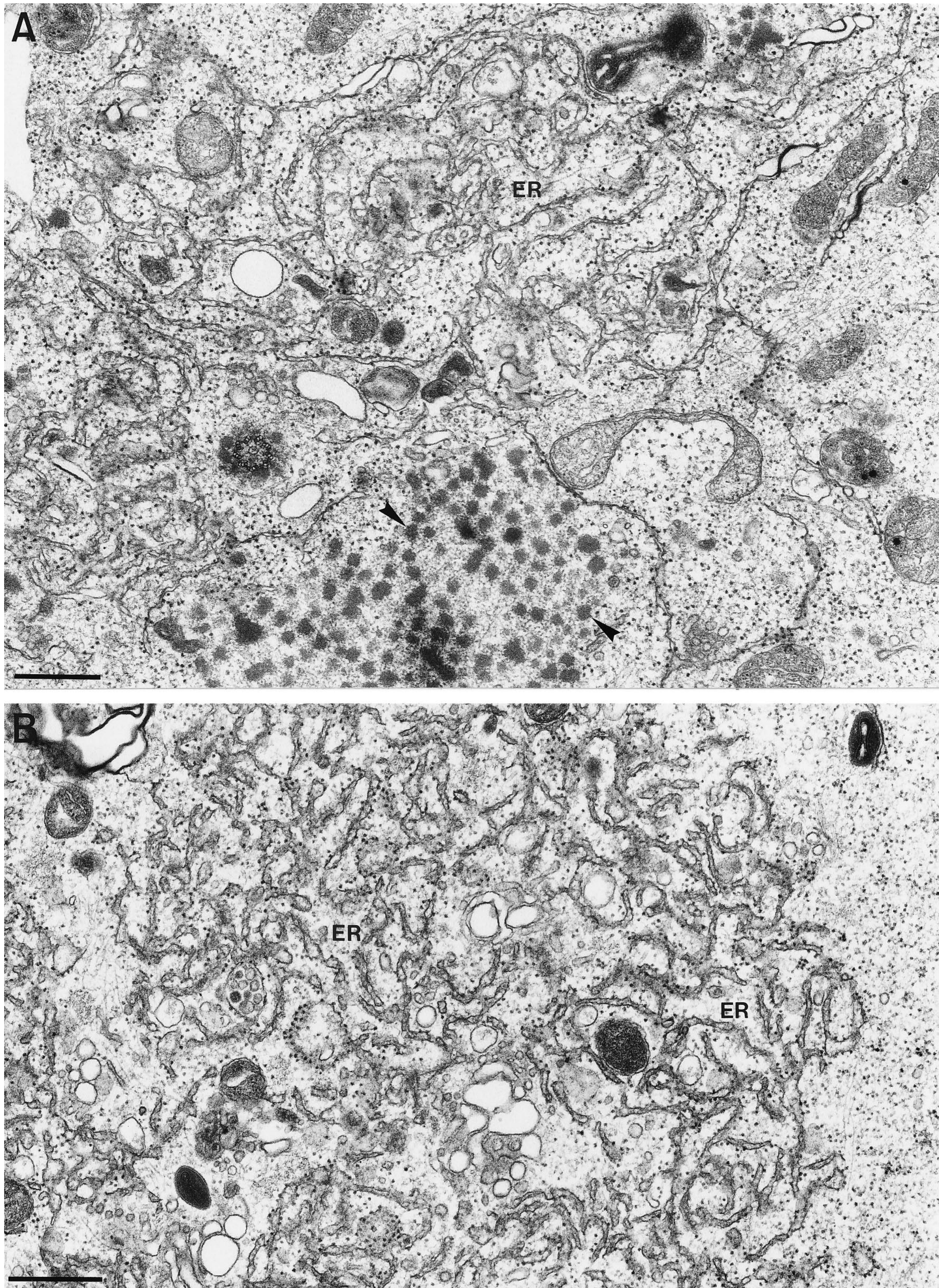

FIG. 7. Membrane structure in cells expressing the 2C C-terminal domain. (A) The N-terminally truncated protein 2C (residues 88 to 329) induces the same patches (arrowheads) as the central domain (Fig. 6B) as well as formation of anastomotic ER. Bar, 500 nm. (B) The C-terminal part of protein 2C (residues 252 to 329) induces anastomotic ER comparable to that found after transfection with the N-terminal part (1 to 122) (compare with Fig. 6A). Va, vaccinia virus. Bar, 500 nm.

aminations of thin sections, it appears that the majority of protein formed the insoluble patches dictated by the properties of the central domain, while a smaller portion of the protein exhibited affinity for membrane structures, causing some rearrangement of preexisting membrane structures. This estimate of the distribution of protein that is or is not associated with membrane is consistent with the results of the biochemical analysis shown in Fig. 4, which suggested that the majority of 2C(88-329) protein was not solubilized from the pellet fraction by extraction with detergent.

The observation of some interaction with membranes by 2C(88-329) protein prompted us to examine cells transfected with a plasmid designed to express the C-terminal sequences alone, pTM-PV2C(252-329) (Fig. 7B). As suspected, expression of 2C C-terminal sequences caused the appearance of altered membranes in the cell, with no evidence of insoluble protein patches. The cells are characterized by the appearance of fragmented ER, some small vesicles, and a loss of Golgi stacks. Overall, the aspect of these cells is very similar to that of cells transfected with pTM-PV2C(1-122). It was not possible to demonstrate the presence of C-terminal peptide sequences associated with the induced membrane structures by IEM, because this portion of the protein did not react with our anti-2C antibodies. It is unlikely, however, that membrane arrangements would occur if the protein did not interact with the bilayer.

Table 1 summarizes the main features of membrane changes induced by protein 2C or domains thereof. Only the central part of protein 2C, encoded in pTM2C88-274, does not induce membrane changes, while all other proteins induce a loss of the Golgi apparatus and larger or smaller amounts of vesicles. These vesicles are predominant in cells expressing 2C(1-274) and 2C(1-274KS), but with all other proteins, mainly a rearrangement of the rough ER is observed.

DISCUSSION

Interaction of viral proteins with intracellular membranes. In poliovirus-infected cells, clusters of membranous vesicles accumulate in the cytoplasm, where they serve as a matrix for viral RNA synthesis. Many viral proteins, including all of those known to be involved in viral RNA replication (2B, 2BC, 2C, 3A, 3AB, 3CD, and 3D), are associated with these membranous replication complexes. During poliovirus replication, one or more viral protein(s) may associate with the host's organellar membranes to induce vesicle formation, with subsequent attachment of other proteins and viral RNA; alternatively, multiple proteins (and RNA) may interact simultaneously to form the replication complex.

Several cellular and viral proteins, when expressed individually in cells, have been shown to localize to intracellular membranes and, in doing so, to induce the formation of new membrane structures and alter the morphology of preexisting components of the cell's protein secretory organelles. This was found with PV 2C and 2BC (2, 10) and 3A and 3AB (references 11 and 14 and unpublished observations) as well as an overexpressed 6-kDa protein of tobacco etch potyvirus (43). Cellular proteins with reported membrane-altering properties include cytochrome B5 (4) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (5, 25) as well as a 180-kDa ribosome receptor in yeast (59) and ATP synthase and fumarate reductase in *E. coli* (58, 61). The morphologies of the membrane alterations observed in the reports cited above are quite diverse.

Effect of expression of 2C and 2C domains on membrane rearrangement. It has been shown previously that viral protein 2C, expressed via a recombinant vaccinia virus vector in cells, associates with intracellular membranes and induces a striking membrane reorganization to generate vesicles and swirls of stacked membranes (2, 10). To dissect determinants of membrane association and reorganization in the 2C protein, a tentative model of the domain organization of this protein has been developed. It features a three-domain structure of 2C. All available experimental data are compatible with the model (see below), although no direct experimental testing of the model has been conducted. A recently published X-ray structure of a bacterial helicase that may be distantly related to 2C indirectly supports the model; it features an α/β organization of domains comprising conserved motifs associated with NTPase and helicase activities and describes an α/α organization for two auxiliary domains (53). Based on the predicted model, we designed experiments to determine whether the individual predicted domains of 2C protein would exhibit membrane binding and alterations. The results showed that both N- and C-terminal regions, but not the central portion, of 2C interacted with intracellular membranes and induced changes in their morphology, although the specific structural architecture was different.

The N-terminal portion of 2C (residues 1 to 122) has been predicted in this study to be composed of a number of α-helices, and it likely folds in a separate domain. One of these helices has already been recognized to be a conserved amphipathic helix (41) and has been shown previously to be essential for association of the protein with membranes in an *in vitro* assay (15). Mutations in the N-terminal sequence predicted to disrupt the amphipathic helix were lethal (41), suggesting that membrane interaction of this region is important for 2C function. The C-terminal portion of 2C (252 to 329) is predicted to include two helices, with the N-terminal one being a conserved amphipathic helix (Fig. 1 and unpublished observations). This helix is smaller than its counterpart in the N-terminal portion of 2C and is positioned differently from that previously predicted (41). We predict that the narrow hydrophobic edge of the C-terminal amphipathic helix is responsible for the observed membrane-related activities of this portion of 2C. It is likely, because of the conservation of this structure, that other picornaviral 2C proteins have similar properties.

The central portion of 2C (residues 88 to 274), which includes NTP binding motifs, has no predicted or observed determinants for membrane binding. Expression of this portion of 2C forms insoluble protein aggregates that are not solubilized with detergent. No alterations in the morphology or structural integrity of intracellular organelles were observed. Curiously, some of the insoluble protein aggregates appeared in the nucleus. When the central 2C sequences were fused to the membrane-binding N-terminal domain, the resulting polypeptide (1 to 274) localized with the membranes and no aggregates were found. When it was attached to the C-terminal sequences (88 to 329), both insoluble protein patches and some membrane association were detected.

Although there is currently no experimental evidence to support the predicted model of a three-domain organization of 2C structure, the data presented here and in previous experiments by Rodriguez and Carrasco (45) suggest that truncated proteins from both the N and C termini of 2C can function to provide both membrane binding and RNA binding. In both cases, both ends of the protein may be implicated in each of the two activities, consistent with the suggestion that 2C may resemble a forceps, with the helical domains interacting or engaged in common functions.

Among the different membrane alterations induced by the 2C domains investigated in this study (Table 1), those brought

TABLE 1. Summary of membrane changes induced by domains of 2C

Protein	Main ultrastructural feature
2C(1-329)	Tubular swirls of membranes; vesicles
2C(1-122)	Disordered, anastomotic, and fragmented ER
2C(1-274)	Vesicles
2C(1-274KS)	Vesicles
2C(88-274)	Insoluble protein patches; no membrane changes
2C(88-329)	Protein patches; disordered and anastomotic ER
2C(252-329)	Disordered, anastomotic, and fragmented ER

about by polypeptide 2C(1-274) (Fig. 5A) seem most similar to the smooth vesicles that proliferate in poliovirus-infected cells. Very similar alterations were observed after expression of protein 2BC (2, 10). It may be premature, however, to assign the membrane reorganization activity of the virus to any given viral protein based on the appearance of the structures formed, because final membrane architecture may depend strongly on levels of expression, as well as subtle characteristics of the protein's association with lipid and viral and cellular proteins. Under the conditions used in this study, a common denominator for peptides inducing vesicles which are morphologically similar to those observed in poliovirus-infected cells appears to be the N-terminal amphipathic helix fused to the central region of protein 2C, yielding peptide 2C(1-274). Since the wild-type 2C(1-274) sequence as well as the 2C(1-274KS) mutant induce identical membrane alteration, it can be assumed that NTP binding activity of the central domain is not required for vesicle induction. In addition, although the central domain by itself showed no interaction with membrane structures, its presence when fused to the N-terminal domain produced profound changes in the specific membrane architecture, generating formation of numerous smooth vesicles.

Source of membranes in the induced vesicles. A striking morphological feature of both poliovirus-infected cells and cells expressing 2C protein sequences is the loss of organized Golgi stacks. This suggests that the controlled balance of membrane input and output in the Golgi apparatus is disturbed (36) and thus that Golgi membranes may contribute to the reorganized membrane structures. EM observations indicate that the newly formed vesicles derive from ER. Thus, it appears that several different cellular organelles may be involved in the rearrangement of membranes in 2C-expressing cells. Recently, Schlegel et al. (49) analyzed poliovirus-induced membranes that were immunoisolated by reactivity with a MAb against 2C for the presence of several organelle-specific cellular proteins. All markers were present in the immunoisolated membranes, including those from lysosomes, *trans*-Golgi stacks and network, and ER, demonstrating that in poliovirus-infected cells, virus-induced membranes were derived from several compartments throughout the protein-secretory apparatus of the cell. By IEM, no association of 2C protein was detected with the nuclear or plasma membranes. Recently, however, it has been shown that 2B and 2BC, but not 2C, altered the permeability of the plasma membrane (1, 14), although no 2BC associated with this membrane was detected, suggesting that the induction of new vesicles inside the cell may secondarily affect the permeability barrier of the plasma membrane.

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