

Structure–Function Analysis of Coxsackie B3 Virus Protein 2B

FRANK J. M. VAN KUPPEVELD,^{*1} WILLEM J. G. MELCHERS,^{*} KARLA KIRKEGAARD,^{†2} and JOHN R. DOEDENS[†]

^{*}Department of Medical Microbiology, University of Nijmegen, The Netherlands; and [†]Department of Molecular, Cellular and Developmental Biology, Howard Hughes Medical Institute, University of Colorado, Boulder, Colorado 80309

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Expression of poliovirus protein 2B in mammalian cells inhibits protein secretion and increases the susceptibility of the cells to hygromycin B, consistent with the increase in plasma membrane permeability seen during poliovirus infection (J. R. Doedens and K. Kirkegaard, *EMBO J.* 14, 894–907, 1995). We report here that expression of protein 2B of the closely related coxsackie B3 virus (CBV3) leads to the same biochemical alterations. Analysis of several mutant CBV3 2B proteins that contain mutations in a predicted cationic amphipathic α -helix (F. J. M. van Kuppeveld, J. M. D. Galama, J. Zoll, P. J. J. C. van den Hurk, and W. J. G. Melchers, *J. Virol.* 70, 3876–3886, 1996) demonstrated that the integrity of this domain is crucial for both biochemical functions of 2B. Mutations in a second hydrophobic domain (F. J. M. van Kuppeveld, J. M. D. Galama, J. Zoll, and W. J. G. Melchers, *J. Virol.* 69, 7782–7790, 1995), on the other hand, are more disruptive to the ability of CBV3 2B to inhibit protein secretion than to increase membrane permeability. Therefore, inhibition of protein secretion is not merely a consequence of the membrane changes that increase uptake of hygromycin B. The existence of mutations that interfere with virus growth but do not impair the ability of 2B to inhibit protein secretion or increase membrane permeability argues for additional functions of protein 2B. © 1997 Academic Press

INTRODUCTION

The genus Enterovirus consists of the polioviruses, coxsackieviruses, echoviruses, and several enterovirus serotypes. Enteroviruses, like other picornaviruses, are small nonenveloped viruses which contain a 7.5-kb single-stranded RNA molecule of positive polarity that is translated into a large polyprotein (Jang *et al.*, 1989; Pelletier and Sonenberg, 1988). This polyprotein is processed by virally encoded proteinases to the P1 region proteins, which form the viral capsid, and the P2 and P3 region proteins, most of which are required for viral RNA (vRNA) replication (reviewed in Wimmer *et al.*, 1993). The P2 region proteins have also been implicated in the structural organization of viral replication complexes and in the induction of several of the morphological and biochemical alterations that occur during infection. Protein 2A is a proteinase that, in addition to cleaving the viral polyprotein, induces the cleavage of the 220-kDa component of initiation factor eIF-4F (Krausslich *et al.*, 1987), implicated in the inhibition of translation of cellular mRNAs (Ehrenfeld, 1982; Etchison *et al.*, 1982). Protein 2BC expression can induce the formation of membrane vesicles (Barco and Carrasco, 1995; Bienz *et al.*, 1983) and therefore may be the viral protein responsible for

the generation of the small membrane vesicles on which vRNA replication takes place (Bienz *et al.*, 1983, 1987, 1990, 1994). Protein 2C is a small NTPase with RNA binding properties (Mirzayan and Wimmer, 1994; Rodriguez and Carrasco, 1993), located at the cytoplasmic surface of the virus-induced membrane vesicles where it may be involved in attaching the vRNA to the membranous replication complex (Bienz *et al.*, 1987, 1990, 1994).

Recently it was found that the expression of poliovirus protein 2B or 2BC results in two of the major biochemical alterations that occur during enterovirus infection: the inhibition of protein secretion (Doedens and Kirkegaard, 1995) and the permeabilization of the plasma membrane (Benedetto *et al.*, 1980; Doedens and Kirkegaard, 1995). The relevance of these activities to the viral life cycle remains to be elucidated. The required residues and the mechanism of induction of these alterations are also unknown. The finding that protein 2B permeabilizes cellular membranes in both mammalian cells and *Escherichia coli* (Lama and Carrasco, 1992) suggests that it may function as an ionophore or otherwise disrupt membrane function. Proteins that display such properties often contain cationic amphipathic α -helical motifs (Bernheimer and Rudy, 1986; Eisenberg *et al.*, 1984; Segrest *et al.*, 1990). Enterovirus 2B proteins all contain two hydrophobic domains, the more NH₂-terminal of which is predicted to form a cationic amphipathic α -helix (Fig. 1). Mutational analysis of coxsackie B3 virus (CBV3) protein 2B argued that the cationic and amphipathic character of the predicted α -helix was indeed required for the function of 2B in vRNA replication and virus growth (van Kuppeveld *et*

¹ To whom correspondence and reprint requests should be addressed. Fax: 31 24 3540216. E-mail: W.MELCHERS@MMB.AZN.NL.

² Current address: Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305-5402.

al., 1996a). Mutational analysis of the second hydrophobic domain of CBV3 protein 2B demonstrated that mutations that caused severe increases or decreases in the hydrophobic character of the domain also impaired the function of protein 2B in the viral replicative cycle (van Kuppeveld *et al.*, 1995).

Here we report that CBV3 protein 2B, like poliovirus protein 2B, is able to inhibit transport through the cellular secretory pathway and to modify the susceptibility of cells to hygromycin B, a translation inhibitor that normally enters cells poorly, in the absence of other viral proteins. Analysis of the activities of several mutant CBV3 2B proteins (Fig. 1) in protein secretion inhibition and increased susceptibility to hygromycin B suggests that these two activities are separate functions of protein 2B rather than that one of these effects is the consequence of the other. Furthermore, the results indicate that the predicted cationic amphipathic α -helix is involved in both the inhibition of protein secretion and the permeabilizing activity, whereas mutation of the second hydrophobic domain has a greater effect on the secretion inhibition function.

MATERIALS AND METHODS

Cells, antibodies, and reagents

COS-1 cells were grown as described (Doedens and Kirkegaard, 1995). Affinity-purified rabbit polyclonal antibody to alpha-1 protease inhibitor (A1PI) was obtained from Oswald Pfenniger and Jerry Brown (University of Colorado Health Sciences Center). Hygromycin B was from Boehringer Mannheim. Fixed *Staphylococcus aureus* cells for collecting immune complexes were obtained from Gibco-BRL.

Construction of plasmids

The dicistronic plasmid p2BNC α 6 containing the poliovirus 2B coding region as the first cistron and the A1PI coding region as the second cistron has been described previously (Doedens and Kirkegaard, 1995). pC2B α 6, containing the CBV3 2B sequence upstream of A1PI, was constructed by PCR amplification of the CBV3 2B coding sequence from pCB3/T7 (Klump *et al.*, 1990). The oligonucleotide primers, 5'-GCAATGTCG-ACCATGGGAGTGAAGGACTATGTG-3' and 5'-AAG-CCACCCGGGCTATTGGCGTTCAGCCATAGG-3', introduced a *SalI* site and an initiation codon upstream of the CBV3 2B coding region and a stop codon and a *SmaI* site downstream of the 2B coding region. The 327-bp amplified fragment was cut with *SalI* and *SmaI* and cloned into pLINK α 6, a plasmid containing *SalI* and *SmaI* sites upstream of the poliovirus 5'-noncoding region and the A1PI coding region (Doedens and Kirkegaard, 1995). The 2B coding region of the mutant pCB3/T7 plasmids carrying mutations in either the hydrophobic domain (van Kuppeveld *et al.*, 1995) or the

amphipathic α -helix (van Kuppeveld *et al.*, 1996a) of protein 2B was amplified with the same primers. Amplified products were cut with *SpeI* and *SmaI* and cloned into pC2B α 6 from which the corresponding fragment was deleted. The 2B coding sequence of all constructs was confirmed by sequence analysis.

Secretion assays

COS-1 cells growing on 60-mm dishes were transfected using a standard calcium phosphate method (Ausubel *et al.*, 1990). All labelings were performed 2 days posttransfection. To radiolabel cells with [³⁵S]methionine, transfected COS-1 cells were washed with phosphate-buffered salt solution (PBS) and incubated in 1 ml of methionine-free DMEM supplemented with 50 μ Ci of [³⁵S]methionine (Expre³⁵S³⁵S protein labeling mix, New England Nuclear) for 30 min. Following labeling, the cells were washed with PBS and placed in 1 ml fresh medium containing 0.23 mM unlabeled methionine for 2 hr. At the end of the chase period, the culture medium was removed and saved for further analysis. Cells were harvested by scraping into 0.5 ml PBS at 4°. The cells were pelleted by centrifugation at 300 *g* for 5 min and resuspended in 200 μ l of PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride at 4°. After 30 min on ice, detergent-insoluble material was pelleted in a microcentrifuge, and the supernatants were transferred to fresh tubes.

Immunoprecipitation of A1PI

Rabbit polyclonal antibody directed against A1PI was diluted in PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.5% SDS, and 1% bovine serum albumin. An equal volume of antibody dilution was added to each lysate sample to be precipitated. Two hundred microliters of antibody dilution was added to each 1-ml sample of culture supernatant. Immune precipitations were then incubated for 2 hr on ice, and antibody-antigen complexes were collected by incubation with fixed *S. aureus* cells. The cells and bound immune complexes were washed three times in ice-cold PBS containing 1% Triton X-100, 0.5% deoxycholate, and 1% SDS. Immunoprecipitated A1PI from cell lysates and culture supernatants was then displayed by SDS-PAGE and quantified on a phosphorimager (Molecular Dynamics). Relative amounts of A1PI secretion were determined from the ratio of A1PI detected in the supernatant to that detected in the cell extract. This value was adjusted to 1.0 for cells expressing A1PI alone with no 2B protein, and the ratios for cells transfected with wild-type and mutant 2B proteins were normalized.

Hygromycin B sensitivity of transfected cells

Sensitivity of transfected COS-1 cells to hygromycin B was determined as described previously (Doedens and Kirkegaard, 1995) except that the labeling period was

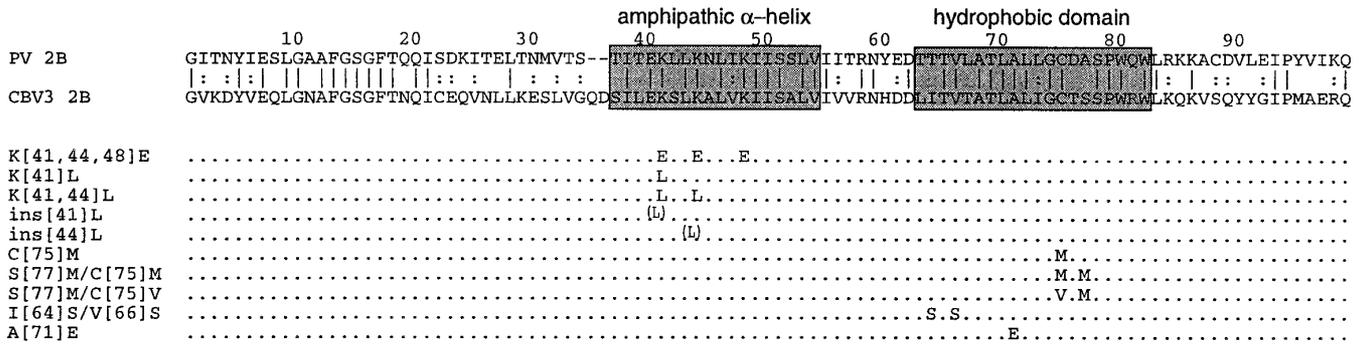


FIG. 1. Sequences of wild-type and mutant 2B proteins used in this study. Alignment of the complete amino acid sequences of poliovirus type 1 Mahoney and coxsackie B3 virus (CBV3) is shown in the top panel. Numbering is with respect to CBV3 2B protein. Solid lines indicate amino acid identity, colons indicate similarity, dashes indicate gaps in the alignment. Periods indicate unchanged residues, and parentheses indicate locations in which amino acids have been inserted, not substituted.

increased to 1 hr. Preparation of lysates and immunoprecipitation of A1PI was as described above. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad). A1PI activities were calculated as A1PI phosphorimager counts/ μ g protein input into immunoprecipitation and the ratios of A1PI synthesized in the presence and absence of hygromycin B were determined. When indicated, this value was adjusted to 1.0 for cells expressing A1PI alone with no 2B protein, and the ratios for cells transfected with wild-type and mutant 2B proteins were normalized.

RESULTS

The finding that poliovirus 2B protein inhibited transport through the cellular secretory pathway and increased plasma membrane permeability to hygromycin B in the absence of other viral proteins (Doedens and Kirkegaard, 1995) led us to ask (i) whether this function is conserved among other enteroviral 2B proteins and (ii) which structural protein domains are involved in these activities. We chose to test CBV3 protein 2B because the phenotypes of viruses containing a number of different mutations in each of two hydrophobic domains of this protein (Fig. 1) have been recently characterized (van Kuppeveld *et al.*, 1995, 1996a).

Inhibition of protein secretion by coxsackie B3 virus protein 2B

The CBV3 2B coding region was inserted into a dicistronic plasmid designed to express both a single viral protein and human A1PI, a secreted protein. The predicted mRNA from the dicistronic plasmids is diagrammed in Fig. 2A. Because A1PI is not expressed in untransfected COS-1 cells, secretion of A1PI produced from dicistronic mRNAs can be used to measure effects of the coexpressed viral proteins on secretory pathway function (Doedens and Kirkegaard, 1995). COS-1 cells were transfected with plasmids that encoded poliovirus protein 2B,

CBV3 protein 2B, or no viral protein in the first cistron, and secretion of the A1PI encoded in the second cistron was assayed in a pulse-chase experiment (Fig. 2B).

In cells that expressed only A1PI, A1PI secretion can be observed both from the observation that most of the labeled protein was found in the medium after a 2-hr

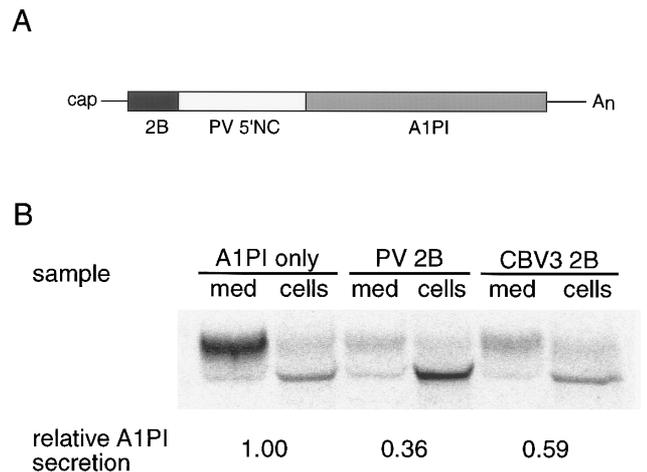


FIG. 2. Effect of expression of CBV3 2B protein on secretion of A1PI. (A) Predicted dicistronic mRNA for coexpression of 2B proteins and A1PI. Translation of A1PI in the second cistron is driven by the internal ribosome entry site (IRES) element within the poliovirus 5'-noncoding region (PV5'NC). (B) COS-1 cells were transfected with dicistronic plasmids containing the indicated 2B coding region as the first cistron and A1PI sequence as the second. Two days posttransfection, the cells were radiolabeled with [35 S]methionine for 30 min and chased with unlabeled methionine for 2 hr. At the end of the chase period, culture supernatants and cell lysates were harvested and A1PI in each sample was quantified by immunoprecipitation followed by SDS-PAGE and phosphorimager analysis. The phosphorimage shows radiolabeled A1PI immunoprecipitated from culture medium (med) and cell lysates (cells) derived from cells transfected with the indicated constructs. The relative fraction of labeled A1PI secreted into the medium within the chase period is indicated below each pair of lanes; this normalization eliminates any differences in transfection efficiency between experiments. The difference in migration between the intracellular and secreted forms of A1PI reflects incomplete glycosylation of the intracellular material (Doedens and Kirkegaard, 1995).

chase and from the shift in electrophoretic mobility that resulted from modification of N-linked oligosaccharides on A1PI within the secretory pathway. In cells transfected with the plasmid that expresses poliovirus protein 2B as well as A1PI, only 36% of the amount of radiolabeled A1PI was released into the culture medium in the 2-hr chase period. Similarly, the expression of CBV3 protein 2B reduced secretion of A1PI to 59% of the control (Fig. 2B). The lower level of inhibition of A1PI secretion by CBV3 protein 2B may reflect either a lesser ability of this protein to inhibit secretory pathway function or a lower level of expression. Nevertheless, the ability of CBV3 protein 2B to inhibit A1PI secretion indicates that this activity is conserved among these two enteroviruses.

Coxsackie B3 virus protein 2B increases the sensitivity of cellular translation to hygromycin B

Hygromycin B is an inhibitor of translation to which mammalian cells are ordinarily relatively insensitive, because the compound enters cells poorly. Increased sensitivity of translation to hygromycin B has been demonstrated in cells infected with poliovirus and several other viruses (Benedetto *et al.*, 1980; Munoz and Carrasco, 1981, 1983; Munoz *et al.*, 1985). In poliovirus-infected cells, this increased sensitivity correlates with increased efflux of ^{86}Rb (Lopez-Rivas *et al.*, 1987) and is thus thought to reflect increased permeability of the cellular plasma membrane to the drug. In addition, *Escherichia coli* that express poliovirus protein 2B show increased uptake of ONPG, increased efflux of $[^3\text{H}]$ uridine, and increased sensitivity to hygromycin B (Lama and Carrasco, 1992), further suggesting that this protein can significantly alter membrane permeability and that these changes can be detected as increased sensitivity of translation to hygromycin B.

To determine whether expression of CBV3 protein 2B also increases sensitivity to hygromycin B, we measured the effect of hygromycin B on A1PI expression in transfected COS-1 cells (Fig. 3). In cells expressing no viral protein, hygromycin B had very little effect on A1PI accumulation in a 1-hr labeling period, indicating that the cellular membranes were relatively impermeable to this inhibitor. In contrast, expression of either poliovirus or CBV3 protein 2B increased sensitivity to hygromycin B considerably. A1PI synthesis was reduced approximately threefold by hygromycin B in cells expressing either 2B protein, arguing that both proteins are capable of modifying membrane permeability.

Effects of mutations in the amphipathic helix motif on secretion inhibition and hygromycin B sensitivity

To investigate the contribution of the putative cationic amphipathic α -helix motif that comprises residues 37 to 54 of CBV3 protein 2B to secretion inhibition and hygromycin B sensitivity, several mutant alleles of CBV3 2B

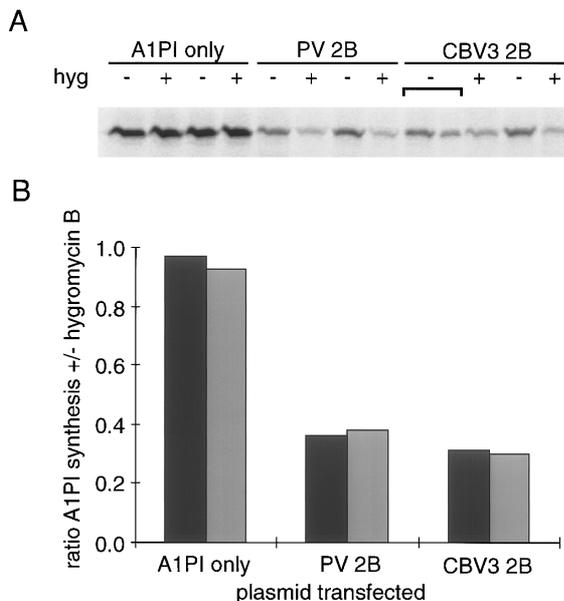


FIG. 3. Effect of expression of CBV3 protein on hygromycin B permeability. COS-1 cells were transfected with dicistronic plasmids containing the indicated 2B coding region as the first cistron and A1PI sequence as the second cistron. Two days posttransfection, hygromycin B sensitivity was assayed. (A) Phosphorimage of immunoprecipitated A1PI synthesized in the presence and absence of 0.5 mg/ml hygromycin B as indicated; results from duplicate plates are shown. The bracketed pair of lanes for the CBV3 2B samples represents a single sample inadvertently divided between two lanes of the gel. (B) Quantitation of relative hygromycin B sensitivity for samples shown in (A); black and grey bars represent duplicate samples. A1PI-specific phosphorimager counts from (A) were normalized to the total protein input into the immunoprecipitation reactions. The ratio of protein accumulation in the presence and absence of hygromycin B is shown.

with amino acid changes within this domain (van Kuppeveld *et al.*, 1996a) were cloned into the dicistronic A1PI expression plasmid. The amino acid changes of five mutations within this domain are shown in Fig. 1. Plasmids that encoded wild-type and mutant CBV3 2B proteins were transfected into COS-1 cells and assayed for the ability of the expressed mutant 2B proteins to inhibit A1PI secretion and to increase sensitivity to hygromycin B (Fig. 4).

All mutant 2B proteins could still inhibit protein secretion and increase the permeability of cells to hygromycin B. However, the ability of the mutant proteins to induce these alterations varied for the different mutations. The relative effects on virus viability (Fig. 4) and on the two different biochemical activities correlated well for the individual mutant proteins. Each of the mutations K[41, 44, 48]E, ins[41]L, and ins[44]L, which eliminated viral viability, reduced the ability to inhibit protein secretion and modify plasma membrane permeability to a greater extent than did mutations K[41]L and K[41, 44]L, which gave rise to viable, but slow-growing, viruses. Thus, mutant proteins that were the most defective in the virus were the least effective in secretion inhibition and also the

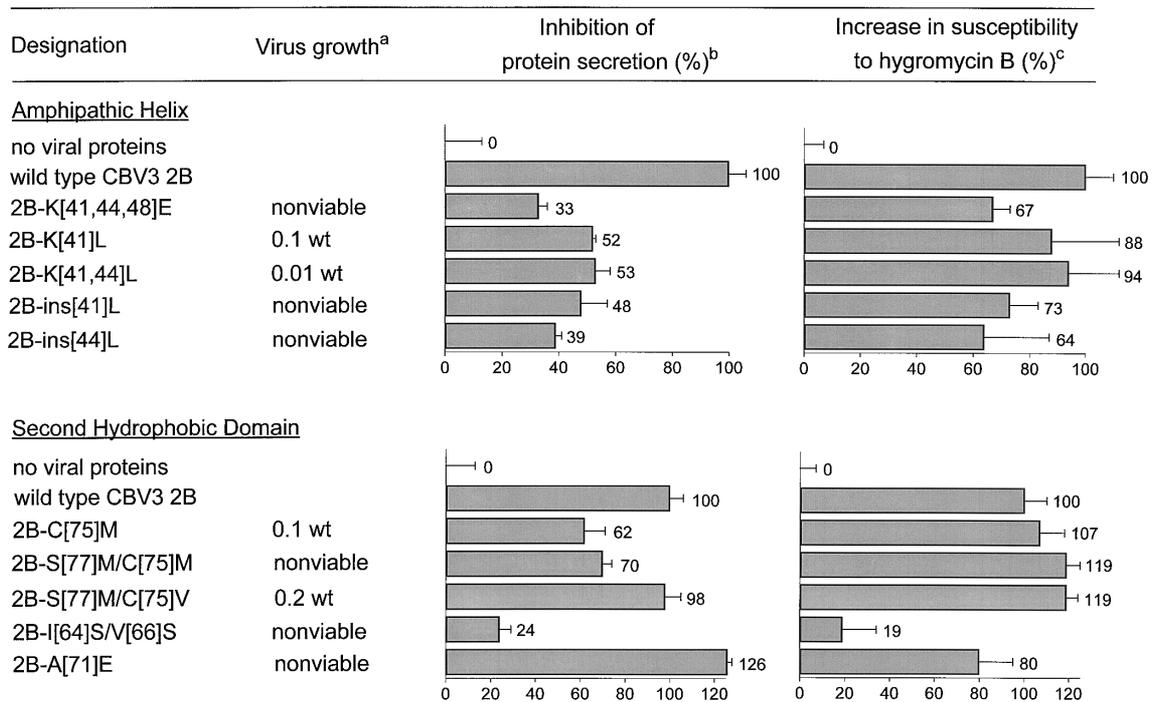


FIG. 4. The effects of mutations in the putative cationic amphipathic α -helix and the second hydrophobic domain of CBV3 protein 2B on viral growth in a single-cycle infection, inhibition of protein secretion, and increase in susceptibility to hygromycin B. ^aViral titer after 8 hr growth (van Kuppeveld *et al.*, 1995, 1996a). ^bAbility of mutant CBV3 2B proteins to inhibit protein secretion, expressed as the percentage of the effect of wild-type 2B protein. COS-1 cells were transfected with dicistronic plasmids encoding the indicated viral protein as the first cistron and A1PI secretion. Values calculated for cells transfected with wild-type CBV3 2B were normalized to 100% inhibition. Values for cells expressing A1PI alone with no 2B protein were adjusted to 0% inhibition. Error bars represent standard deviations of measured values. ^cAbility of mutant CBV3 2B proteins to increase hygromycin B sensitivity, expressed as the percentage of the effect of wild-type 2B protein. COS-1 cells were transfected with dicistronic plasmids encoding the indicated viral protein as the first cistron and A1PI as the second cistron. At 2 days posttransfection, transfected cells were assayed for hygromycin B sensitivity. Values calculated for cells transfected with wild-type CBV3 2B were normalized to 100% increase in hygromycin B sensitivity. Values calculated for cells expressing A1PI alone with no 2B protein were adjusted to 0% increase. Error bars represent standard deviations of measured values.

least effective in increasing cell sensitivity to hygromycin B. Furthermore, mutations in this domain showed that the integrity of this domain is important for both the inhibition of protein secretion and the increase in membrane permeability caused by CBV3 protein 2B.

Effects of mutations in the second hydrophobic domain on secretion inhibition and hygromycin B sensitivity

We next tested the effects of mutations in the second hydrophobic domain of CBV3 protein 2B on the two activities (Fig. 1; van Kuppeveld *et al.*, 1995). Little correlation between the relative effects of these mutations on the inhibition of protein secretion and the increase in membrane permeability was observed (Fig. 4). With the exception of mutation I[64]S/V[66]S, little effect on the ability of CBV3 2B to increase hygromycin B susceptibility was observed for any of the mutations in the second hydrophobic domain. Nevertheless, mutations C[75]M and S[77]M/C[75]M impaired the ability of protein 2B to inhibit protein secretion. Thus, the observed inhibition of protein secretion by CBV3 protein 2B is probably not caused by the increase in membrane permeability.

Virus containing mutations S[77]M/C[75]V arose due to a reversion mutation in RNAs carrying mutations S[77]M/C[75]M, which destroy viral viability. Mutant 2B protein that contained these S[77]M/C[75]V mutations exhibited wild-type activity in both biochemical assays, although virus carrying the mutant 2B protein showed reduced growth compared to wild-type virus (Fig. 4). Similarly, virus that contained mutation A[71]E was nonviable, although the 2B protein carrying this mutation showed nearly wild-type ability both to inhibit protein secretion and to increase the susceptibility of cells to hygromycin B. These observations argue that protein 2B has other functions in the viral infectious cycle in addition to those we have assayed here.

DISCUSSION

Mutations in the 2B proteins of both poliovirus and coxsackievirus give rise to viruses with primary defects in RNA synthesis (Johnson and Sarnow, 1991; Li and Baltimore, 1988; van Kuppeveld *et al.*, 1995, 1996a). However, no biochemical activity directly tied to viral genome replication has yet been demonstrated for 2B. The only activities identi-

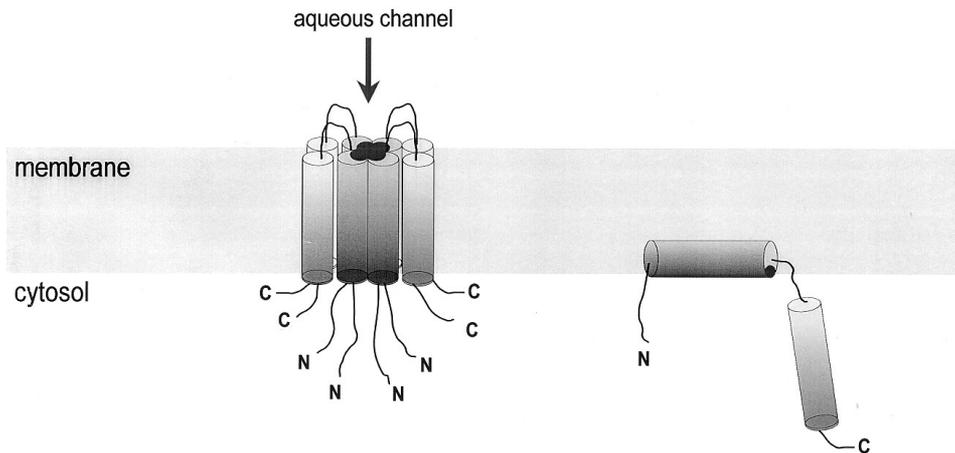


FIG. 5. Potential topologies of protein 2B. Putative multimers of 2B that allow the formation of an aqueous channel are shown on the left. Helices representing the membrane-integral amphipathic α -helix (nearer the NH_2 -terminus) and the second hydrophobic domain (nearer the COOH -terminus) are shown. The hydrophilic face of the cationic α -helix is darkly shaded. An alternative model is shown on the right, in which the hydrophobic face of the α -helix perturbs the membrane while lying parallel to it.

fied for protein 2B in eukaryotic cells are the two tested here: the inhibition of protein secretion and modification of the plasma membrane permeability. In this study, we have shown that coxsackie B3 virus protein 2B displays these two activities (Figs. 2 and 3), as previously demonstrated for poliovirus protein 2B (Doedens and Kirkegaard, 1995).

It was possible either that secretion inhibition and plasma membrane modification were two separate functions of protein 2B or that one of these effects was the consequence of the other. For example, permeabilization of the plasma membrane by 2B could cause the inhibition of protein secretion by altering the intracellular ionic milieu; increased intracellular calcium levels and changes in concentrations of monovalent cations have been documented in poliovirus-infected cells (Carrasco *et al.*, 1993; Irurzun *et al.*, 1995a; Lopez-Rivas *et al.*, 1987). Such changes might affect a number of cellular processes, including secretory transport. Alternatively, if the direct effect of 2B were on protein transport, the protein and lipid composition of the plasma membrane could be altered, resulting in altered permeability.

Analysis of mutations in CBV3 protein 2B has helped to clarify the relationship between secretion inhibition and membrane permeabilization. Experiments examining mutations in the second hydrophobic domain of CBV3 2B (Fig. 4) suggest that the two activities are separable. In particular, mutations C[75]M and S[77]M/C[75]M showed effects on membrane permeabilization similar to wild-type CBV3 2B, but less effective inhibition of protein secretion than the wild-type protein. Therefore, plasma membrane permeabilization, as measured by hygromycin B sensitivity, is insufficient to explain the secretion inhibition displayed by wild-type 2B. Other mutations in the second hydrophobic domain and those in the amphipathic helix motif affected permeability to hygromycin B and secretion inhibition to a similar extent (Fig. 4). The correlation between the two assays

may result from losses in activity stemming from general disruption of 2B structure, a role for the cationic amphipathic helix in both functions, or both.

Does either the ability to inhibit protein secretion or the ability to modify plasma membrane permeability correlate well with the phenotype displayed by CBV3 2B mutant viruses (Fig. 4)? The data are inconclusive on this point. Protein 2B carrying mutations S[77]M/C[75]M, which rendered the virus nonviable, showed a wild-type ability to increase hygromycin B sensitivity but an impaired ability to inhibit protein secretion. A revertant of this mutant, which contained a Met to Val substitution to yield 2B protein S[77]M/C[75]V, also showed wild-type ability to increase hygromycin B sensitivity but increased the ability of the mutant 2B protein to inhibit protein secretion. Thus, a reversion from nonviability to viability correlated with an increase in ability to inhibit protein secretion. Although 2B protein carrying reversion mutation S[77]M/C[75]V showed wild-type abilities in inhibition of protein secretion and increasing hygromycin B sensitivity, viruses carrying this protein exhibited an impaired virus growth. Mutation A[71]E, which rendered 2B able to inhibit protein secretion comparably to wild-type CBV3 2B and which caused only a slight reduction in the ability to induce membrane permeabilization, even completely abrogated vRNA replication and virus growth (Fig. 4). These observations point to roles for viral protein 2B in the viral replicative cycle other than the interactions with host cell membranes studies here. Consistent with this, mutant poliovirus 2B proteins defective in vRNA synthesis due to mutations outside either the cationic amphipathic α -helical domain or the second hydrophobic domain (mutations 2B201 and 2B204 at residue 29; Johnson and Sarnow, 1991) exhibited wild-type activities in inhibiting cellular protein secretion (Doedens, 1996).

The organization and structure of the cationic amphipathic

pathic α -helix in enterovirus protein 2B is similar to that of "lytic" polypeptides, a group of cationic amphipathic α -helical peptides that exert cytolytic effects on membranes (Segrest *et al.*, 1990). Two models of action have been proposed to explain the cytolytic action of these peptides. In one model, the cationic peptides form aqueous channels by traversing the membrane and forming multimers that expose their hydrophobic sides to the lipid bilayer and their hydrophilic faces to the aqueous pore. In a second model, the peptides perturb the membrane by lying parallel to the membrane, with their hydrophobic side inserted in the lipid bilayer, thereby making the phospholipids more susceptible to the action of phospholipases (Bernheimer and Rudy, 1986). Two putative structural models of 2B that are consistent with the need for processing at the 2A/2B and 2B/2C cleavage sites by protein 3C^{pro}, a cytosolic protein, are shown in Fig. 5.

The increase in sensitivity to hygromycin B caused by expression of CBV3 protein 2B is sensitive to mutations in the predicted cationic amphipathic α -helix. Cationic amphipathic α -helical peptides can form voltage gated and cation-selective channels in lipid bilayers (Agawa *et al.*, 1991; Argiolas and Pisano, 1985; Ide *et al.*, 1989; Tosteston *et al.*, 1989). It is tempting to speculate that channels formed by multimeric 2B proteins are responsible for the influx of the sodium ions, the efflux of potassium ions, and the alterations in calcium levels that are observed from the third hour postinfection by poliovirus (Carrasco *et al.*, 1993; Irurzun *et al.*, 1995a). Alterations in ionic milieu have been implicated in the shut-off of host cell translation, as high concentrations of sodium ions are inhibitory to host cell but not to viral translation (Carrasco and Smith, 1976), and the cleavage of the p220 component of initiation factor eIF-4F may not be sufficient for complete inhibition of host cell protein synthesis (Bonneau and Sonenberg, 1987; Irurzun *et al.*, 1995b; Pérez and Carrasco, 1992). Consistent with this, coxsackieviruses that produce reduced levels of 2B protein due to the presence of poorly processed 2B/2C cleavage sites, failed to completely inhibit host cell protein synthesis (van Kuppeveld *et al.*, 1996b). Modifications in membrane permeability may also be required for cell lysis, release of progeny virus, or both.

Inhibition of cellular protein secretion by viral protein 2B requires both the cationic amphipathic helix and the hydrophobic domain, and is not a direct result of increased membrane permeability. The inhibition of protein secretion during enterovirus infection is likely to result from alteration or sequestration of membranes or proteins required for secretory transport. This could simply be a consequence of RNA replication complex assembly, or it may play an additional role in viral amplification such as blocking host antiviral responses (Doedens and Kirkegaard, 1995). The exact functions of protein 2B in both membrane permeabilization and inhibition of protein secretion await further investigation.

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