Mutagenesis of the Coxsackie B3 Virus 2B/2C Cleavage Site: Determinants of Processing Efficiency and Effects on Viral Replication

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The enterovirus 2B/2C cleavage site differs from the common cleavage site motif AxxQ ↓ G by the occurrence of either polar residues at the P1' position or large aliphatic residues at the P4 position. To study (i) the putative contribution of these aberrant residues to the stability of precursor protein 2BC, (ii) the determinants of cleavage site specificity and efficiency of 3CDpro, and (iii) the importance of efficient cleavage at this site for viral replication, a mutational analysis of the coxsackie B3 virus (CBV3) 2B/2C cleavage site (AxxQ ↓ N) was performed. Neither replacement of the P1' asparagine with a serine or a glycine nor replacement of the P4 alanine with a valine significantly affected 2B/2C cleavage efficiency, RNA replication, or virus growth. This introduction of a P4 asparagine, as can be found at the CBV3 3C/3D cleavage site, caused a severe reduction in 2B/2C cleavage and abolished virus growth. These data support the idea that a P4 asparagine is an unfavorable residue that contributes to a slow turnover of precursor protein 3CD but argue that it is unlikely that the aberrant 2B/2C cleavage site motifs serve to regulate 2B/2C processing efficiency and protein 2BC stability. The viability of a double mutant containing a P4 asparagine and a P1' glycine demonstrated that a P1' residue can compensate for the adverse effects of an unfavorable P4 residue. Poliovirus (or poliovirus-like) 2B/2C cleavage site motifs were correctly processed by CBV 3Cpro, albeit with a reduced efficiency, and yielded viable viruses. Analysis of in vivo protein synthesis showed that mutant viruses containing poorly processed 2B/2C cleavage sites were unable to completely shut off cellular protein synthesis. The failure to inhibit host translation coincided with a reduced ability to modify membrane permeability, as measured by the sensitivity to the unpermeant translation inhibitor hygromycin B. These data suggest that a critical level of protein 2B or 2C, or both, may be required to alter membrane permeability and, possibly as a consequence, to shut off host cell translation.

Enteroviruses contain a positive-strand RNA genome of 7.5 kb in length which encodes a single polyprotein. This polyprotein is processed by three virus-encoded proteinases, 2Apro, 3Cpro, and 3CDpro (Fig. 1A), into the structural P1 capsid proteins and the nonstructural P2 and P3 proteins that are involved in viral RNA (vRNA) replication (reviewed in reference 50). Proteinase 2Apro cleaves between the P1 and P2 regions cotranslationally (45). The capsid proteins are processed in trans by proteinase 3CDpro (26, 51). Processing of the nonstructural proteins by proteinase 3CDpro yields both the final cleavage products (2A, 2B, 2C, 3A, 3B, 3C, and 3D) and relatively stable processing intermediates (2BC, 3AB, and 3CD) (22). These precursor proteins have functions in vRNA replication that are distinct from those of their cleavage products. Protein 3AB is the membrane-bound precursor that delivers VPg (3B) to the membranous replication complex (42-44). Furthermore, protein 3AB stimulates 3Dpol activity and the autocatalytic processing of 3CDpro to 3Cpol and 3Dpol (30, 34, 37). Protein 3CD not only is a proteinase but also is involved in the formation of a ribonucleoprotein complex at the 5' end of the RNA, which is required for the initiation of viral positive-strand RNA synthesis (2, 3, 23). Protein 2BC has been proposed to play a role in the induction of the membrane vesicles at which viral positive-strand RNA synthesis occurs (7). Bienz et al. showed that in poliovirus (PV)-infected cells in which polyprotein processing was partially inhibited, the formation of these vesicles always coincided with the production of protein 2BC (8). Consistent with this finding, Barco and Carrasco demonstrated that the expression of PV protein 2BC, but not that of protein 2B or 2C, either individually or in combination, induced the formation of membrane vesicles in yeast cells (5). The finding that protein 2C alone can induce vesicles in human cells (1, 14) may have been hampered by the use of vaccinia viruses, which modify vesicular traffic themselves.

The production and stability of proteins 2BC, 3AB, and 3CD require a finely tuned regulation of the temporal processing at different cleavage sites in the polyprotein. Proteolysis may be regulated by the occurrence of less favorable recognition sequences. In PVs, all 3Cpol-mediated cleavages occur between glutamine-glycine (Q-G) dipeptide pairs. Additional determinants in substrate recognition, however, have been proposed because the PV polyprotein contains four Q-G amino acid pairs that are not cleaved and cleavage sites other than Q-G, as identified in other enteroviruses (15, 27, 32), were also cleavable by PV protein 3Cpol (17). The amino acid at the P4 position (i.e., the fourth residue proximal to the Q-G cleavage site), which is in most cases an alanine, seems to be a major determinant of the efficiency of cleavage. Data that support the role of the P4 amino acid in substrate recognition have been obtained by mutagenesis of infectious PV cDNA clones (9). The importance of the P4 amino acid for the stability of pro-
Coxsackie B3 virus 2B/2C cleavage site mutations

The 2B/2C cleavage site of enteroviruses is crucial for viral replication. Mutations at this site can affect the processing efficiency and stability of the viral polyprotein. The amino acid sequence at the 2B/2C cleavage site typically follows the pattern AxxQ/G, where AxxQ is usually hydrophobic and G is glycine.

Several studies have investigated the effects of 2B/2C cleavage site mutations on viral viability and replication. Pallai et al. (36) found that substitution of glycine with an alanine at the P4 position resulted in altered cleavage efficiency. This suggests that the 2B/2C cleavage site is sensitive to changes in the amino acid sequence.

Materials and Methods

**Cells and Viruses.** Virus propagation and mRNA transfection were performed with Buffalo green monkey (BGM) cells. Plaque assays were performed with vero cells. The cells were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum. After infection, cells were fed with MEM containing 10% serum. After transfection, MEM containing 10% serum was added to the cells. Viruses were titrated in 96-well plates with BGM cell monolayers as described previously (46). Virus titers were calculated by the
Site-directed mutagenesis. Oligonucleotide-directed site-specific mutagenesis was performed with subgenomic phage and pALTCB3 (28) using the Altered Sites in vitro mutagenesis system (Promega) according to the manufacturer's recommendations. The nucleotide sequences of the synthetic oligonucleotides (Isogen Biotechnicals, Maarssen, The Netherlands) are 5'-CCTAAGAGGAG ATATCCGGCTGCCCATC-3' (mutation 2B/2C-1G), 5'-CCTAAGAGG AGATATCCGGCTGCCCATC-3' (2B/2C-1A), 5'-CCTAAGAGG AGATATCCGGCTGCCCATC-3' (2B/2C-1C), 5'-CTGTTGGG ATGGCGCTCTGCTC-3' (nucleotides 3735 to 3765), and 5'-CTA AAGCATTGCGGTCCACTAGGCCATAGGGATTCCGTA-3' (2B/2C-4V). Each of these oligonucleotides created a novel endonuclease restriction site. Clones carrying the desired mutation were identified by restriction enzyme analysis. The nucleotide sequence around the 2B/2C junction of these clones was verified by dideoxy-chain termination sequencing of plasmid DNA, using primer 5'-CCATTCAATGAATTTCTG-3' (nucleotides 4117 to 4134). From the mutant clones, the Spel (position 3837)-to-BstHII (position 4238) fragment was cloned in plasmids pCB3/T7, which contains a full-length cDNA of CBV3. 

In vitro translation reactions. Self-linearized plasmid DNA (0.5 μg) was transcribed and translated in a single reaction, using TNT rabbit reticulocyte lysate (Promega) supplemented with HeLa cell initiation factors (kindly provided by J. Flanagan, University of Florida) and Trans-[ς]-label (a mixture of [35S]methionine and [35S]cysteine; ICN). Labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (47).

Sequence analysis of viral protein synthesis in vivo. BGM cell monolayers were infected with virus at an MOI of 25 for 30 min at room temperature. After infection, cells were supplied with new medium and incubated at 36°C. At several times postinfection, proteins were released by three cycles of freezing and thawing, and 200 μl was passaged to fresh BGM monolayers, which were incubated for another 3 (33°C) or 5 (39°C) days.

Sequence analysis of 2B/2C junction of mutant viruses. RNA extraction, cDNA synthesis, and amplification by PCR using forward primer 5'-GAAAC GGGATGGAAGATGTCGCACTG-3' (nucleotides 3735 to 3765) and reverse primer 5'-TCCGGCTGCCCATGAGGGATTCCGTA-3' (nucleotides 4231 to 4251) were performed as described previously (46). The resulting PCR products were purified from low-melting-point agarose, and the nucleotide sequence around the 2B/2C junction was determined as described above.

Analysis of viral protein synthesis. BGM cell monolayers were infected with virus at an MOI of 1 TCID50 per cell and grown at either 33, 36, or 39°C. At 8 h postinfection, cells were lysed by freezing and thawing, and the luciferase reaction was measured as described previously (46).

Analysis of viral protein synthesis in vitro. BGM cell monolayers were infected with virus in total of 1 TCID50 per cell and grown at either 33, 36, or 39°C. At 8 h postinfection, cells were lysed by freezing and thawing, and the luciferase reaction was measured as described previously (46).

Analysis of viral protein synthesis. BGM cell monolayers were infected with virus in total of 1 TCID50 per cell and grown at either 33, 36, or 39°C. At 8 h postinfection, cells were lysed by freezing and thawing, and the luciferase reaction was measured as described previously (46).

RESULTS

Construction and description of CBV3 2B/2C cleavage site mutants. Substitution mutations at the 2B/2C cleavage site were generated by oligonucleotide-directed mutagenesis and introduced in infectious CBV3 cDNA clone pCB3/T7 and subgenomic replicon pCB3/T7-LUC. The mutants fall into three categories. Within the first group are four mutants that were constructed to examine the importance of the aberrant P1' or P4 residues that occur at the enterovirus 2B/2C cleavage site (Fig. 1B). For this, the P1' asparagine was replaced with a glycine (mutation 2B/2C-1G), a proline (2B/2C-1P), or a serine (2B/2C-1S) or the P4 alanine was replaced with a valine (2B/2C-4V). The second group consists of two mutants that were constructed to examine the effect on 2B/2C cleavage efficiency of a P4 asparagine, as can be found at the 3C/3D cleavage site of CBV-like viruses. The P4 asparagine was introduced either alone (2B/2C-4N) or, in order to create a site that resembles the 3C/3D cleavage site, together with a P1' glycine (2B/2C-4N;1'G). The third group consists of two mutants that were engineered to examine the cross-species substrate specificity of CBV3 3C(30). In one mutant, a valine was introduced at the P4 position and a glycine was introduced at the P1' position to create a motif with P4, P1, and P1' residues that occur at the 2B/2C junction of most PV-like viruses (2B/2C-4V;1'G). The other mutant contained an additional replacement of the P3 glutamic acid with a isoleucine (2B/2C-4V;3I;1'G) to create a 4P to P1' sequence that is identical to that found at a PV 2B/2C junction (PV type 3).
In vivo studies of minicircle DNA. The use of different deoxyribonucleotide triphosphates (dCTP, dATP, dGTP) in vitro replication was possible only when dCTP was used. A 20% increase in the yield of minicircles was observed when dCTP was used instead of dATP. The results also showed that the yield of minicircles was increased when dCTP was used instead of dGTP. The use of different deoxyribonucleotide triphosphates in vitro replication was possible only when dCTP was used. A 20% increase in the yield of minicircles was observed when dCTP was used instead of dATP. The results also showed that the yield of minicircles was increased when dCTP was used instead of dGTP.
fection, reflecting the reduced growth rate of this virus. Remarkably, cellular protein synthesis was reduced but not completely shut off. This feature was also observed with minute-plaque viruses vCB3-2B/2C-4S, vCB3-2B/2C-4N;1'G, and vCB3-2B/2C-4V;1'G, which produced viral proteins in a large background of cellular proteins. Even at 10 h postinfection, no complete inhibition of cellular translation was observed (data not shown). In cells infected with minute-plaque virus vCB3-2B/2C-4V;3I;1'G, virtually no shut off of host cell translation occurred. For a comparison of the protein patterns, lysates of cells infected with the different mutant viruses were concurrently electrophoresed on a single gel (Fig. 4B).

Ability of mutant viruses to increase plasma membrane permeability. PV-infected cells show an enhanced permeability to monovalent cations and other low-molecular-weight compounds, including the nonpermeant translation inhibitor hygromycin B, from the third hour postinfection (6, 12). It has been suggested that this membrane modification and the resulting alterations in ionic milieu may be involved in the shutoff of host cell protein synthesis (12).

To examine whether the failure of the CBV3 mutants to shut off cellular translation correlated with a decreased ability to modify membrane permeability, the sensitivity of translation to hygromycin B was assayed. Cells were infected with wild-type virus, vCB3-2B/2C-4V;1'G, or vCB3-2B/2C-4V;3I;1'G and pulse-labeled in the presence or absence of hygromycin B at 2, 4, 6, and 8 h postinfection. In wild-type virus-infected cells, hygromycin B reduced translation about 70% at both 4 and 6 h postinfection, time points at which cellular translation was indeed shut off and when only viral proteins were synthesized (Fig. 5). In contrast, hygromycin B had little effect on translation in cells infected with the mutant viruses. No inhibition of translation was observed at 4 and 6 h postinfection, when protein synthesis was suppressed to about 40% (vCB3-2B/2C-4V;1'G) and 55% (vCB3-2B/2C-4V;3I;1'G) of the level observed at 2 h postinfection. At 8 h postinfection, when translation was suppressed to about 20% (vCB3-2B/2C-4V;1'G) and 40% (vCB3-2B/2C-4V;3I;1'G), some inhibition of translation (about 40%) by hygromycin B was observed in cells infected with vCB3-2B/2C-4V;1'G but not in cells infected with vCB3-2B/2C-4V;3I;1'G.

**DISCUSSION**

Enterovirus protein 2BC is a proteolytic processing intermediate that exerts a specific function in vRNA replication (5, 8). Protein 2BC is a poor substrate for 3Cpro and is only very slowly processed into proteins 2B and 2C in vitro (20, 21, 34). The stability and function of this protein require a subtle regulation of processing efficiency at the 2B/2C cleavage site. One mechanism to regulate the kinetics of proteolysis is the occurrence of unfavorable residues at the scissile bond or positions

<table>
<thead>
<tr>
<th>Mutation</th>
<th>2B/2C junction*</th>
<th>2B/2C cleavageb</th>
<th>Plaque sizec</th>
<th>Single-cycle virus yieldd</th>
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</thead>
<tbody>
<tr>
<td>None (wt)</td>
<td>AerQ/N</td>
<td>+++</td>
<td>wt</td>
<td>8.9</td>
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<tr>
<td>2B/2C-1'G</td>
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<td>+++</td>
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<td>2B/2C-1'F</td>
<td>AerQ/F</td>
<td>-</td>
<td>Nonviable</td>
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<td>2B/2C-1'S</td>
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<td>+++</td>
<td>wt</td>
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<td>VerQ/N</td>
<td>+</td>
<td>Small</td>
<td>15.8</td>
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<td>2B/2C-4N</td>
<td>VerQ/N</td>
<td>±</td>
<td>Nonviable</td>
<td>7.2</td>
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<td>2B/2C-4S (revertant)</td>
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<td>+</td>
<td>Minute</td>
<td>7.2</td>
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<tr>
<td>2B/2C-4N;1'G</td>
<td>NonQ/G</td>
<td>±</td>
<td>Minute</td>
<td>7.1</td>
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<td>2B/2C-4V;1'G</td>
<td>VerQ/G</td>
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<td>Minute</td>
<td>7.2</td>
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<tr>
<td>2B/2C-4V;3I;1'G</td>
<td>VirQ/G</td>
<td>+</td>
<td>Minute</td>
<td>6.4</td>
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* Amino acids at the P4-P3-P2-P1/P1' positions, respectively. Mutated amino acids are in boldface.

b Relative efficiency of cleavage at the 2B/2C junction based on the densitometric analysis of the cell-free cleavage assay shown in Fig. 2.

c Relative plaque size at 96 h postinfection of confluent Vero cell monolayers.

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**TABLE 1. Effects of mutations on 2B/2C cleavage efficiency, plaque size, and single-cycle virus yield**
In the determination of cleavage site specificity and efficiency of the cleavage of protein BVC, (i) to gain more insight into the determinants of cleavage site specificity, and (ii) to examine the importance of cleavage residues. We have constructed several C4B2cR2C cleavage residues. The presence of either polar or non-polar residues or large aromatic P4 residues differing from the common motif AXXG by the oc-cord. This amino acid pair. The oc-cord residues B2C cleave

postulated (shown in panel A) and the mutant viruses (labeled as 0 in panel B).}
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may be induced by an interaction with a specific target (e.g., membrane or another protein). The observation of Molla et al. that the addition of protein 3AB significantly enhanced proteolysis of PV protein 2BC by 3Cpro in vitro lends support to such a hypothesis (34).

The introduction of a P1’ proline (2B/2C-1’P) completely abolished 2B/2C cleavage. The nonviability of this mutation may be due to an adverse effect on the function of protein 2BC in vRNA replication. However, it seems more likely that efficient vRNA replication requires the production of either protein 2B or protein 2C or both. The activities that have been ascribed to protein 2B, i.e., inhibition of protein secretion and permeabilization of the plasma membrane (16), and protein 2C, which is endowed with nucleoside triphosphatase and RNA binding activities (31, 41), however, can also be fulfilled by protein 2BC (16, 41). The requirement for either mature protein 2B or 2C suggests that one of these proteins, or both, exerts a yet unidentified function in vRNA replication that cannot be fulfilled by protein 2BC. Alternatively, protein 2BC may be not be able to exert these activities when engaged with other viral proteins.

In the second group of mutants, the effect of P4 asparagine on 2B/2C cleavage and viral replication was examined. This polar residue has been identified at the P4 position of the 3C/3D cleavage site of CBV-like viruses. Introduction of this residue (2B/2C-4N) reduced the efficiency of 2B/2C cleavage to about 10% of that of the wild type. This finding provides experimental evidence for a role of this polar residue in determining a slow turnover rate of protein 3CD, analogous with the role of the unfavorable polar threonine occurring at the P4 position of the PV 3C/3D cleavage site (36). The nonviability of this mutation argues that critical levels of protein 2B or 2C, or both, are required for efficient virus growth. Alternatively, mutation 2B/2C-4N may abolish a function of protein 2B in vRNA replication. The mutation did not completely abolish vRNA synthesis, as shown by the isolation of a revertant virus carrying a P4 serine. The viability of this reversion mutation is most probably due to the increase in 2B/2C cleavage efficiency (to about 25% of that of the wild type) and, as a consequence, the production of increased levels of proteins 2B and 2C. These data fit well with the crystal structure of 3Cpro of human rhinovirus 14, a picornavirus closely related to enteroviruses (31). It was found that the S4 substrate pocket of this proteinase is small and hydrophobic and that it best accommodates small and hydrophobic amino acids. Any change in the P4 position from a small aliphatic to a polar residue results in strong energetic constraints. Our data suggest that these constraints are more severe with a polar residue containing a large side chain (asparagine) than with a small polar residue (serine). Impaired processing efficiency was also observed in PV genomes that contained a serine, threonine, or glutamic acid residue at the P4 position of the cleavage site between two genetically engineered VFG coding units (11).

In contrast to nonviable mutation 2B/2C-4N, simultaneous introduction of a P4 asparagine and a P1’ glycine (2B/2C-4N; 1’G) yielded viable viruses. That a P1’ residue (i.e., the first amino acid of protein 2C) can intramolecularly compensate for a nonviable mutation at the P4 position (i.e., amino acid 96 of protein 2B) makes it unlikely that the nonviability of mutation 2B/2C-4N was due to an impaired function of protein 2B in vRNA replication. It seems unlikely that the rescuing effect of the P1’ glycine is due to an effect on the function of protein 2C in vRNA replication, because viruses carrying a P1’ asparagine (wild type) or a P1’ glycine (2B/2C-1’G) replicated equally well. Two alternative explanations must be considered. First, the P4, P1, and P1’ residues may play a synergistic role in determining the cleavage site conformation, and the compensating effect of the P1’ glycine may be due to a more efficient 2B/2C cleavage. All enterovirus 2B/2C cleavage sites contain small amino acids at either the P4 position, the P1’ position, or both. Cleavage sites carrying mutation 2B/2C-4N contain asparagine residues at both the P4 and P1’ positions. The simultaneous occurrence of such large P4 and P1’ residues may interfere with the cleavage site conformation and, as a consequence, the recognition of the substrate or its accessibility to the active center of 3Cpro. However, no profound differences in processing efficiency at sites carrying mutations 2B/2C-4N and 2B/2C-4N;1’G were observed. Densitometric scanning of autoradiograms, however, may not be sensitive enough to detect minor quantitative differences. It therefore cannot be excluded that the additional introduction of a P1’ glycine causes a subtle enhancement of 2B/2C cleavage, leading to levels of proteins 2B and 2C that are sufficient to enable virus growth. Another possibility is that mutation 2B/2C-4N disrupts the structure and, as a consequence, the function of precursor protein 2BC in vRNA replication, and that the compensating effect of a P1’ glycine is due to stabilization of the protein conformation.

The third group consisted of two mutants that were engineered to examine the ability of CBV3 3Cpro to process PV (or PV-like) 2B/2C cleavage sites. Cleavage sites carrying P4, P1, and P1’ residues that occur at the 2B/2C cleavage site of most PV-like viruses (2B/2C-4V;1’G) or P4 to P1’ residues that are identical as those found at the 2B/2C cleavage site of PV type 3 (2B/2C-4V;3l;1’G) were correctly processed by CBV3 3Cpro, albeit with a reduced efficiency (about 25% of that of the wild type). These findings are consistent with the impaired cleavage of P2 proteins observed with a chimeric PV polyprotein containing CBV3 protein 3Cpro (15). The cross-species substrate specificity of CBV3 3Cpro confirms the existence of both conformational and sequence-specific cleavage determinants. The finding that double mutation 2B/2C-4V;1’G reduced cleavage efficiency to a much greater extent than each of the single mutations did supports the proposed synergistic action of the P4, P1, and P1’ residues in determining the cleavage site conformation (see above). The P3 residue seems to be of less importance for the cleavage site conformation, as mutations 2B/2C-4V;1’G and 2B/2C-4V;3l;1’G had similar effects on cleavage efficiency. The finding that mutation 2B/2C-4V;3l;1’G reduced virus growth to a greater degree than mutations that caused reductions in cleavage efficiency that were similar (2B/2C-4V;1’G and 2B/2C-4S) or even more severe (2B/2C-4N;1’G) suggests that the P3 glutamic acid is a determinant of the structure and function of protein 2B, or 2BC, in vRNA synthesis rather than of 2B/2C cleavage efficiency. This view is consistent with structural data for human rhinovirus 14 3Cpro, which suggested that the side chain groups of residues at the P5 and P3 positions are pointing away from the active center of 3Cpro (31).

Analysis of the viral protein synthesis in vivo showed that none of the minute-plaque viruses containing poorly processed 2B/2C cleavage sites was capable to completely inhibit cellular protein synthesis. A reduced but significant amount of cellular protein synthesis was observed in minute-plaque viruses containing poorly processed 2B/2C cleavage sites. Cleavage sites carrying mutation 2B/2C-4N;3l;1’G caused virtually no shutoff of host cell translation. The simultaneous synthesis of viral and cellular proteins late in infection is remarkable and has, to our knowledge, not been demonstrated previously. It is unlikely that this increase in cell viability is due to the reduced growth rate of these viruses, since it was not observed with other CBV3 mutants that exhibited a similar or an even more severe decrease in growth (47, 48).
which enteroviruses shut off host cell translation is still debated. According to the traditional view, the shut off of cellular translation relies only on the protein 2Aplaying-mediated cleavage of the 220-kDa component (p220) of eukaryotic initiation factor 4F (18, 19, 29). The integrity of p220 seems to be required for the translation of cellular mRNAs but not for the initiation of translation at rRNA, which occurs by internal entry of ribosomes to internal ribosome entry site elements in the 5' nontranslated region (25, 38). However, several reports have described that substantial levels (25 to 45%) of cellular protein synthesis can take place in cells in which all p220 has been degraded (10, 24, 39). It has therefore been suggested that the p220 cleavage is necessary but not sufficient to completely inhibit host cell protein synthesis and that a second event, which requires RNA replication, is required to block cellular translation. The permeabilization of the plasma membrane and the resulting influx of sodium ions have been suggested as potential second events. High concentrations of sodium ions are inhibitory to host mRNA translation but not to the translation of vRNA (12). The reversal of the shut off and the continuous synthesis of cellular proteins that occur in sodium-free medium are further indicative of a role of sodium ions in the inhibition of cellular protein synthesis (13).

Recently, it has been shown that of the PV nonstructural proteins, protein 2B has the highest intrinsic capacity to modify the plasma membrane permeability to hygromycin B in human cells (16). This capacity was found to be conserved in CBV3 protein 2B (49), which contains a cationic amphipathic α-helical motif that is required for vRNA replication and that is typical for so-called lytic polyproteins (47). In this study, we have shown that the failure of mutant coxsackieviruses containing poorly processed 2B/2C cleavage sites to completely shut off host cell translation coincided with a reduction of levels of mature proteins 2B and 2C, but not of protein 2A, and a reduced ability to modify the plasma membrane permeability. These data suggest that the permeabilization of the membrane by protein 2B may indeed be the second event required for the shut off of cellular translation. Reduced levels of protein 2B may be responsible for a poor permeabilization of the plasma membrane and, as a consequence, a reduced influx of sodium ions. The failure to increase the intracellular sodium concentration could account for the maintenance of host cell translation, the reduction in viral protein synthesis, and the reduced virus yield. However, it should be emphasized that normal levels of protein 2A were demonstrated in vitro but not yet in vivo. Furthermore, as it remains to be established that an increased membrane permeability contributes to the inhibition of cellular translation, it cannot be excluded that the accumulation of either protein 2B or 2C, or both, is required for another, yet unidentified process that is necessary to fully suppress cellular protein synthesis. In addition, the mutations introduced near the 2B/2C cleavage site may affect not only the relative amounts of 2BC, 2B, and 2C but also their functions. Additional studies on the functions of the P2 region proteins are required to shed more light on the possible participation of these proteins in the shut off of host cell protein synthesis.

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purified recombinant poliovirus protein 3AB as substrate for viral proteinases and as co-factor for RNA polymerase 3D


