Coxsackie B3 Virus Protein 2B Contains a Cationic Amphipathic Helix That Is Required for Viral RNA Replication

FRANK J. M. VAN KUPPEVELD,* JOCHEM M. D. GALAMA, JAN ZOLL, PATRICK J. J. C. VAN DEN HURK, AND WILLEM J. G. MELCHERS

Department of Medical Microbiology, University of Nijmegen, 6500 HB Nijmegen, The Netherlands

Received 18 September 1995/Accepted 12 March 1996

Enteroviruses contain a single-stranded RNA genome of positive polarity with a genetically encoded poly(A) tail at their 3' end. The viral RNA (vRNA) directs the synthesis of a single polyprotein which is processed by the virally encoded proteases 2Apro, 3Cpro, and 3CDpro. The P1 region of the genome encodes the structural capsid proteins. The proteins encoded by the P2 and P3 regions have been implicated in viral replication (reviewed in reference 47). Replication starts by the formation of a complementary negative-stranded RNA molecule which serves as the template for the synthesis of progeny viral positive-stranded RNAs (5). The proteins encoded by the P3 region are physically involved in the process of positive-strand RNA synthesis (3, 22, 42, 43), which occurs in replication complexes on virus-induced cytoplasmic membrane vesicles (8). The proteins encoded by the P2 region are involved in the induction of some of the structural and metabolic alterations that occur in the infected cell. Protein 2Apro induces the cleavage of the 220-kDa component of the eucaryotic initiation factor eIF-4F, which results in an inhibition of cap-dependent host cell translation (18, 20, 29). Translation of viral RNA is not affected, because it is initiated via a cap-independent binding of ribosomes to secondary structures in the 5' nontranslated region (26, 35). Precursor protein 2BC has been implicated in the rearrangement of membranes and the generation of the membrane vesicles at which positive-strand RNA synthesis occurs (7-10). The observation that protein 2C alone is also capable of inducing this rearrangement (2, 14) is hampered by the use of a recombinant vaccinia virus, which modifies vesicular traffic significantly. Protein 2C is a nucleoside triphosphatase (33, 39) with RNA binding capacities (39) that is localized at the outer surface of the virus-induced membrane vesicles (7, 9, 10), which suggests that protein 2C may be involved in the structural organization of the viral replication complex by attaching the vRNA to the vesicular membranes.

Two other metabolic alterations that occur during an enterovirus infection are the inhibition of cellular protein secretion (16) and the permeabilization of the plasma membrane (12). Upon expression of individual poliovirus proteins, Dodens and Kirkegaard found that protein 2B, as well as its precursor 2BC, was capable of inducing both alterations (16). The significance of these alterations, which become evident from the third-hour postinfection, for virus replication is unclear. The block in protein secretion may be involved in the accumulation of cytoplasmic vesicles for vRNA synthesis. Apart from this, the inhibition of cellular protein secretion may interfere with host cell responses such as interferon secretion and antigen presentation. Modification of membrane permeability occurs in the late phase of infection of most cytolytic viruses and requires viral gene expression (reviewed by Carrasco et al. in reference 13). The modification is such that gradients of monovalent cations are gradually destroyed and compounds that normally do not pass the membrane leak out of the cell or leak into the cytoplasm. Carrasco et al. (13) have suggested that the resulting influx of sodium ions may be involved in the virus-induced shutdown of host cell translation, because the cleavage of the 220-kDa component of eIF-4F may not be sufficient to completely shut off host cell translation (11, 36). High concentrations of sodium are inhibitory to host mRNA translation, whereas viral RNA is optimally translated under these altered conditions by the use of specialized structures in the 5' nontranslated region (13). Another function of the membrane modification may be the induction of the cell lysis needed to liberate newly formed virus particles.

* Corresponding author. Mailing address: Department of Medical Microbiology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Phone: 31 24 3614356. Fax: 31 24 3540216. Electronic mail address: KUNAZAG3@CAOS.KUN.NL.
Enterovirus protein 2B and its precursor 2BC are localized exclusively at the outer surface of the virus-induced membrane vesicles at which positive-strand synthesis occurs (7, 8). Genetic evidence suggests that protein 2B, or possibly its precursor 2BC, is required for a cis-acting function in vRNA replication (27, 46). The expression of protein 2B in both bacteria and eucaryotic cells led to an increase in cell membrane permeability, which suggests that protein 2B possesses membranespanning or ionophoric properties. Both functions require amphipathic helix motifs with high helical hydrophobic moments (19). We have identified a well-conserved amphipathic helix motif in enterovirus protein 2B with a narrow hydrophobic face and an arrangement of cationic residues that is typical for the so-called lytic polypeptides. The conserved nature of this structural domain is indicative of an important role in the functioning of protein 2B. To examine the structural and functional roles of this putative amphipathic α-helix, we have constructed mutant coxsackie B3 virus cDNAs that contained substitutions of the charged residues in the hydrophobic face or insertions of hydrophobic leucine residues that disturb the amphipathic character of the helix. The effects of the mutations on virus viability, RNA synthesis, viral protein synthesis, and polyprotein processing were examined. Our results indicate that a cationic amphipathic α-helix is indeed required for a function of protein 2B, and possibly also its precursor 2BC, in vRNA synthesis but not for viral protein synthesis or polyprotein processing. The potential role of the amphipathic α-helix in the permeabilization of the plasma membrane is discussed.

MATERIALS AND METHODS

Cells and viruses. Virus propagations, endpoint titrations, and RNA transfections 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 bovine serum. After infection, the cells were fed with MEM supplemented with 3% fetal bovine serum. After transfection, the cells were fed with MEM containing 10% fetal bovine serum.

Oligonucleotide-directed site-specific mutagenesis. In vitro mutagenesis was performed with a subgenomic pALTM phagemid clone containing nucleotides (nt) 4176 to 4187 of coxsackie B3 virus (CBV3) (45), using the Altered Sites in vitro mutagenesis system (Promega) according to the instructions of the manufacturer. The nucleotide sequences of the antisense synthetic oligonucleotides (Promega Biosciences, The Netherlands) were used for construction of the mutants shown in parenthesis. The oligonucleotide 5'-CCATTCAATGATTTCTG-3' (nt 4117 to 4134) was used for construction of the mutants shown in parenthesis.

Figure 1 shows that amino acids 37 to 54 form the segment with the highest amphipathic potential. The high hydrophobic moment and hydrophobicity of this putative amphipathic α-helix were determined as described above.

Analysis of RNA synthesis. To study the effect of the mutations on RNA synthesis, the SpeI-to-BssHII fragments of the mutant plasmids were cloned in the chimeric subgenomic replicon pCB3/T7-LUC (46), which contains the firefly luciferase gene in place of the capped coding region. BGM cell monolayers grown in 25-cm2 flask grown to a confluency of 70 to 80% were transfected with 0.5 μg of T7 RNA polymerase-generated RNA transcripts derived from Sau3A-linearized pCB3/T7-LUC constructs. The cells were grown at 36°C, and at the indicated times posttransfection they were washed three times with phosphate-buffered saline (PBS) and lysed in 400 μl of lysis buffer. The luciferase activity was measured in a liquid scintillation counter using the Luciferase Assay System (Promega).

In vitro transcription reactions. Copy RNA transcripts were synthesized and translated in T7 TITR rabbit reticulocyte lysate (Promega) supplemented with 10% (vol/vol) HeLa cell initiation factors (kindly provided by J. Flanagan, University of Florida). The translation reaction mixtures (20 μl) contained 0.5 μg of circular plasmid DNA and 20 μl of Tran35S-label (a mixture of [35S]methionine and [35S]cysteine; specific activity 1.8×10⁶ cpm per μl) in 50 mM HEPES, pH 7.8, 150 mM NaCl, 0.1 mM phenylmethylsulfanyl fluoride, 1% Nonidet P-40, and 0.05% SDS. Labeled translation products were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis as described above.

RESULTS

Identification and classification of a putative amphipathic helix in enterovirus protein 2B. We have searched for the presence of amphipathic helix motifs in CBV3 protein 2B by calculating the mean hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 40 form the segment with the highest hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19).
and Edmundson (40) shows that this segment forms a putative amphipathic α-helix with a narrow hydrophilic face on one side of the helix (Fig. 2A). The distribution of charged residues (three lysines and one glutamic acid) on the hydrophilic face is well conserved among all enteroviruses, except that bovine enterovirus protein 2B contains arginines rather than lysines at positions 44 and 48 (Fig. 2A). The hydrophilic face of poliovirus protein 2B contains arginines rather than lysines at positions 44 and 48 (Fig. 2A). The hydrophilic face of poliovirus protein 2B is somewhat larger because of the occurrence of polar asparagine and serine residues at aa 43 and 50, respectively. On the basis of their physical-chemical and structural properties, amphipathic helices are grouped into seven classes (41). The arrangement of the charged residues in the putative amphipathic helix of CBV3 protein 2B is typical for the class of so-called lytic polypeptides. These lytic polypeptides form a group of small cationic amphipathic α-helical peptides that are characterized by narrow polar faces with intermediate charge densities, the occurrence of four times as many positively charged residues as negatively charged residues, and the occurrence of mainly lysine residues in the hydrophilic face (mean lysine/arginine ratio = 30). Figure 2B shows examples of helical wheel diagrams of two lytic polypeptides: bombolin II (4) and mastoparan (23).

**Construction of mutations in the putative amphipathic helix of CBV3 protein 2B.** The function and the structural requirements of the putative amphipathic helix in CBV3 protein 2B were examined by a genetic analysis. A total of nine mutants, containing either substitution or insertion mutations, were generated by site-directed mutagenesis. The mutations were verified by sequence analysis and introduced in the infectious cDNA clone pCBB3/T7. The genotype of the mutant plasmids and the effects of the mutations on mean hydrophobic moment and hydrophilicity are shown in Fig. 3A. Two mutants were constructed to examine the requirement for lysine residues at aa 41, 44, and 48. In these mutants, the lysine residues were replaced with either arginine residues (2B-K[41,44,48]R) or negatively charged glutamic acid residues (2B-K[41,44,48]E). Two mutant plasmids were constructed to examine the minimal number of lysine residues required for the role of this domain in the function of protein 2B. In mutant 2B-K[41], lysine 41 is replaced with a leucine residue. In mutant 2B-K[41,44,48], both lysine 41 and lysine 44 are replaced with leucine residues. In the remaining two substitution mutants, the importance of the negatively charged glutamic acid 40 was examined. In these mutants, glutamic acid 40 was replaced with either a lysine (2B-E[40]K) or an aspartic acid (2B-E[40]D) residue. In the three insertion mutants (2B-ins[41]L, 2B-ins[44]L, and 2B-ins[48]L), hydrophobic leucine residues were introduced at aa 41, 44, and 48, respectively. The insertion of
FIG. 2. (A) Helical wheel diagrams of the putative amphipathic helices found in the 2B proteins of several enteroviruses according to the method of Schiller and Edmundson (40). Positively charged (R and K) and negatively charged (D and E) residues are underlined. Hydrophobic amino acids are boxed. Abbreviations: CBV, coxsackie B virus; CAV, coxsackie A virus; ECHO, echovirus; SVDV, swine vesicular disease virus; PV, poliovirus; BEV, bovine enterovirus. (B) Helical wheel diagrams of lytic polypeptides bombolin II, a heptadecapeptide isolated from the venom of bumblebee _M. pennsylvanicus_ (4), and mastoparan, a tetradecapeptide isolated from the venom of the wasp _Vespula levisit_ (23).

The effect of the mutations on virus growth. The effect of the mutations on virus viability was studied by transfection of BGM cells with RNA transcripts. For each mutant, eight transfections were performed. After transfection, the cells were incubated at either 33 or 36°C. In case no virus growth was observed after 5 days, the cells were subjected to three cycles of freezing and thawing and 250 μl was passaged to fresh BGM cell monolayers, which were incubated for another three
plasmid amino acid sequence                  

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**Fig. 3.** (A) Partial amino acid sequence (residues 37 to 54) and the hydrophobic moment and hydrophobicity values of the 2B proteins encoded by wild-type pCB3/T7 and the pCB3/T7-derived mutant plasmids. The effects of the mutations on the hydrophobic moment (mom.) and hydrophobicity (hyd.) of both the entire 18-aa amphipathic helix and the most-amphipathic 11-aa segment (aa 40 to 50) are shown. Furthermore, the effects of the mutations on virus growth are summarized. (B) Helical wheel diagrams of the insertion mutants 2B-ins[41]L, 2B-ins[44]L, and 2B-ins[48]L, containing leucine insertions at residue positions 41, 44, and 48, respectively.

Upon transfection with RNA transcripts of mutant 2B-E[40]K, virus growth was observed in only two of the eight transfected cultures. In one cell culture, virus growth was observed on the second day posttransfection. Sequence analysis (36°C) or five (33°C) days. Viruses were obtained consistently with RNA transcripts carrying mutations K[41,44,48]R, K[41]L, K[41,44]L, and E[40]D. Plaque assays were performed, and four individual plaques were isolated from each mutant virus. Sequence analysis of the 2B coding region of these viruses showed that the mutations introduced by site-directed mutagenesis were retained in the viral RNA and that no other amino acid replacements had occurred. These viruses were designated vCB3-2B-K[41,44,48]R, vCB3-2B-K[41]L, vCB3-2B-K[41,44]L, and vCB3-2B-E[40]D, respectively. No virus growth was observed upon transfection of BGM cells with RNA transcripts containing mutation K[41,44,48]E, ins[41]L, ins[44]L, or ins[48]L. To ensure that the nonviability of these mutations was not due to additional mutations in the plasmid DNA, new constructs carrying these mutations were made and RNA transcripts were assayed for their viability. Consistent with our initial results, no virus growth was observed following transfection.

Upon transfection with RNA transcripts of mutant 2B-E[40]K, virus growth was observed in only two of the eight transfected cultures. In one cell culture, virus growth was observed on the second day posttransfection. Sequence analysis

**Fig. 4.** Single-cycle growth curves of wild-type and mutant viruses vCB3-2B-K[41,44,48]R, vCB3-2B-K[41]L, vCB3-2B-K[41,44]L, and vCB3-2B-E[40]D, respectively. Viruses were released from the transfected cells by three cycles of freezing and thawing, and the virus titers were determined by titration on BGM cells at 36°C. TCID$_{50}$, 50% tissue culture infective dose.
of the 2B coding region of these viruses showed that the introduced lysine 40 (AAG) reverted to the original glutamic acid (GAG) by a single point mutation. No cytotoxic effect was observed in the remaining seven transfected cell cultures. Upon passage of these cultures, however, virus growth became visible in one of the cultures grown at 33°C. Sequence analysis of the 2B coding region of these viruses showed that the introduced lysine 40 (AAG) was retained in the viral RNA and that no other mutations had occurred. This finding was rather unexpected, because the lack of virus growth in the other cultures suggested that a reversion of the introduced mutation to a more favorable residue or a second-site compensating mutation in protein 2B had occurred. To investigate whether mutation E[40]K indeed allows virus growth and to exclude the possibility that the phenotype of the original mutation was influenced by an additional mutation elsewhere in the plasmid DNA, which might have arisen during plasmid construction, virus nt 3837 to 4238 (which were found to be identical to those of plasmid 2B-E[40]K) were amplified, cut with DNA, which might have arisen during plasmid construction, and cloned into wild-type pCB3/T7. BGM cells were transfected with RNA transcripts of several independently reconstructed plasmids. However, no virus growth was observed in any of the transfected cell cultures. These data strongly suggest that mutation E[40]K is nonviable and that the viability and phenotype of the isolated virus are due to a second-site mutation outside 2B. For this reason, the virus was not included in further characterization studies.

Viral growth characteristics were examined by plaque assay and single-cycle growth analysis (at 33, 36, and 39°C). Mutant viruses vCB3-2B-K[41,44,48]R and vCB3-2B-E[40]D exhibited a wild-type growth at all temperatures. Mutant virus vCB3-2B-K[41]L displayed a small-plaque phenotype. In single-cycle infections, this virus produced 20% of the wild-type virus yield at 8 h postinfection, irrespective of the temperature. Virus vCB3-2B-K[41,44]L exhibited a minute-plaque phenotype. This virus appeared to be slightly cold sensitive, as the virus yield was 1% of that of the wild-type virus at 36 and 39°C but only 0.05% of that of the wild-type virus at 33°C. Figure 4 shows the viral growth curves of the mutant viruses at 36°C. The effects of the mutations on virus growth are summarized in Fig. 3.

**Effect of the mutations on RNA replication.** In a previous study, we reported the construction and use of chimeric subgenomic replicon pCB3/T7-LUC, which carries the luciferase gene in place of the PI protein coding region, to study RNA replication (46). Upon transfection of BGM cells with RNA transcripts of pCB3/T7-LUC, a triphasic pattern of luciferase accumulation was observed; luciferase activity increased as a result of translation of the input RNA (phase I), remained constant until the fifth hour posttransfection (phase II), and showed a second increase as a result of the translation of newly synthesized chimeric RNA strands (phase III). That luciferase accumulation indeed reflected viral RNA replication was demonstrated by measuring replicon RNA levels by dot blot hybridization (data not shown). To study the effect of the mutations on RNA replication, the Spel-to-BsaHIII fragments of the mutant plasmids were cloned in replicon pCB3/T7-LUC. BGM cells were transfected with RNA transcripts of the mutant replicons, and luciferase activity was determined at 1, 4, 6, 8, and 10 h posttransfection. The effects of the viable mutations on RNA replication are shown in Fig. 5A. The introduction of mutation K[41]L resulted in a reduced level of RNA replication, as the increase in luciferase activity was only fourfold, whereas the increase in luciferase accumulation was 10- to 20-fold in the case of mutations K[41,44,48]R and E[40]D and wild-type pCB3/T7-LUC. Mutation K[41,44]L caused a more drastic reduction in RNA synthesis, as demonstrated by the
Figure 7. Reactions with VCB3 wild-type and VCB3-2B-K[414]t.
labeled proteins translated from the mutant pCB3/T7 RNAs are shown in Fig. 6A. The results demonstrate that none of the mutations caused abnormalities in polyprotein processing. All cleavage products migrated with a correct mobility, and the translation patterns of all mutants were similar to that of wild-type pCB3/T7. Although it cannot be excluded that minor differences in the levels of the replication proteins contribute to the viral phenotypes, these results suggest that it is unlikely that the defects in vRNA synthesis caused by the nonviable mutations K[41,44]E, E[40]K, ins[41]L, ins[48]L and the reductions in vRNA synthesis caused by the viable mutations K[41]L and K[41,44]L are due to defects in polyprotein processing.

**Time course of protein synthesis in infected cells.** The possibility that the reductions in virus yield and vRNA synthesis caused by mutations K[41]L and K[41,44]L are due to a reduced level of viral protein synthesis was considered. To examine this possibility, we studied viral protein synthesis in vivo. BGM cells were infected with either mutant or wild-type virus at a MOI of 25 and labeled with [35S]methionine for 30 min at various times postinfection. Figure 7 shows the electrophoretic analysis of the labeled proteins. From this figure, it can be seen that all viruses cause a similar shutoff of host protein synthesis but that viral protein synthesis is delayed in cells infected with the mutant viruses. Viral protein synthesis in wild-type-virus-infected cells peaks at 4 to 5 h postinfection and is inhibited from the sixth hour. In cells infected with the small-plaque virus vCB3-2B-K[41]L, viral protein synthesis peaks at 5 to 6 h postinfection, whereas in cells infected with the minute-plaque mutant vCB3-2B-K[41,44]L, viral protein synthesis is even delayed up to 6 to 7 h postinfection. These differences probably reflect a delayed growth of the mutant viruses and, as a result, a delay in cytopathic effect. Although viral protein synthesis is delayed in the mutant-infected cells, the level of viral protein synthesis is similar to that in wild-type-virus-infected cells, suggesting that it is unlikely that the reductions in growth and vRNA synthesis of these viruses are due to an impaired level of viral protein production.

**Replacement of the amphipathic helix with lytic peptide bombolitin II.** After having demonstrated that a cationic amphipathic α-helix is required for a function of protein 2B, we tested whether the α-helix could be functionally replaced by the cationic lytic polypeptide bombolitin II. Bombolitin II is a heptadecapeptide (Ser-Lys-Ile-Thr-Asp-Ile-Leu-Lys-Leu-Gly-Lys-Val-Leu-ala-His-Val) identified in the venom of the bumblebee Megabombus pensylvanicus (4). A helical wheel representation of this peptide shows that it can form an amphipathic α-helix with a narrow polar face of six amino acids containing three lysine residues, one negatively charged aspartic acid, and two polar residues (Fig. 2B). Alignment of the amino acid sequences of both helices showed that bombolitin II resembles aa 40 to 56 of CBV3 protein 2B (Fig. 8A). We have replaced the nucleotide sequence encoding aa 40 to 54 with a sequence encoding the first 15 aa of bombolitin II. The nucleotide sequence of the introduced PCR product was verified, and the resulting plasmid was designated 2B-bomII. A helical wheel diagram of aa 37 to 54 of this chimeric plasmid is shown in Fig. 8B. In vitro translation of RNA from this plasmid showed a processing pattern that was similar to that produced by wild-type pCB3/T7 RNA (Fig. 6B). To study the viability of this chimeric construct, BGM cells were transfected with T7 RNA polymerase-generated RNA transcripts. A total of 10 transfections, including passage of the transfected cell cultures to fresh cells, was performed. In none of the transfected cell cultures, however, was virus growth observed. The SpeI-to-BsuHII fragment of 2B-bomII was introduced in subgenomic replicon pCB3/T7-LUC to study RNA replication. Analysis of luciferase accumulation following the transfection of BGM cells with copy RNA transcripts demonstrated a defect in replication of the replicon RNA (Fig. 5C). This finding suggests that despite the structural similarities between the amphipathic helix motifs, the cationic α-helix of bombolitin II cannot fulfill
the function of the cationic amphipathic α-helix of CBV3 protein 2B in vRNA synthesis.

**DISCUSSION**

Membrane permeabilization is a phenomenon that occurs during the late phase of the replication cycle of most cytolytic viruses. This modification of the membrane is dependent on viral gene expression. A number of viral proteins involved in modifying membrane permeability have been identified in recent years, and a general term for this kind of proteins has been introduced: viroporins (13). A general feature of these viroporins is the presence of amphipathic helix motifs, which are thought to destabilize the membrane integrity by interacting with the lipid bilayer. Until recently, the identity of an enterovirus viroporin was unknown. Upon expression of the poliovirus nonstructural proteins in *Escherichia coli*, it was found that both proteins 2B and 3A modified the bacterial cell membrane (31). Very recently, it has been shown that poliovirus protein 2B, but not protein 3A, permeabilizes the plasma membrane in eucaryotic cells (16), suggesting that protein 2B is most probable the enterovirus viroporin. Consistent with this idea, we have identified an amphipathic helix motif in protein 2B that is conserved among all enteroviruses (Fig. 2A). The arrangement of charged residues in a narrow hydrophilic face and the predominant occurrence of cationic lysine residues are typical of amphipathic helices formed by members of the class of lytic polypeptides.

In this study, we have examined the functional and structural roles of the putative amphipathic helix in CBV3 protein 2B. Nine mutant cDNAs were constructed by site-directed mutagenesis. The results obtained with the mutants carrying substitutions of the lysine residues (aa 41, 44, and 48) suggest that cationic residues are required for the role of the amphipathic helix in the function of protein 2B. Replacement of the lysine residues with arginine residues (K[41,44,48]R) did not affect virus growth, whereas substitution of the lysine residues with negatively charged glutamic acid residues (K[41,44,48]E) was nonviable because of defects in vRNA replication. Substitution of lysine 41 by a leucine residue (K[41]L) yielded a small-plaque virus that produced 20% of the wild-type virus yield in a single-cycle infection, because of a reduction in vRNA synthesis. Substitution of both lysine 41 and lysine 44 by leucine residues (K[41,44]L) yielded a minute-plaque virus that produced only 1% of the wild-type virus yield in a single-cycle infection, because of a more severe defect in vRNA synthesis. These results suggest that the presence of cationic residues is a major determinant of the function of the amphipathic helix in protein 2B. The introduction of negatively charged residues in the hydrophilic face of the helix disrupts the function of protein 2B, although the amphipathic helix containing mutation K[41,44,48]E displays a hydrophobic moment that is equal to or even larger than the moments of the helices containing mutation K[41]L or K[41,44]L, respectively (Fig. 3A).

Two substitution mutations were introduced to investigate the importance of the negatively charged glutamic acid 40 in the function of the amphipathic helix. The introduction of a lysine residue (E[40]K) was highly detrimental for virus growth, although this mutation has virtually no effect on the amphipathism of the α-helix (Fig. 3A). Viruses containing this mutation were isolated on only one occasion. The failure to recover viruses from simultaneously transfected cell cultures suggested that a second-site compensating mutation might have occurred. No such mutation was found in protein 2B. Reconstruction of the mutation into wild-type cDNA followed by transfection of cells with RNA transcripts of the resulting clones did not give rise to virus growth. These data are indicative of the occurrence of an additional second-site mutation outside 2B. Experiments to test this possibility are currently in progress. The wild-type growth characteristics of viruses carrying a replacement of glutamic acid 40 with a negatively charged aspartic acid (E[40]D) demonstrate that a negatively charged residue is preferred at this position. The reason for the preference for a negatively charged residue in the hydrophilic face of this cationic amphipathic α-helix is unknown. None of the substitution mutations interfered with viral protein synthesis and polyprotein processing, indicating that the effects of these mutations on virus growth are primarily attributable to defects in vRNA replication.

The importance of the amphipathic character of the helix for the function of protein 2B was demonstrated by characterization of the three insertion mutants. Leucine residues were inserted between the charged residues (ins[41]L, ins[44]L, and ins[48]L), such that the amphiphility of the α-helix was severely disturbed whereas its mean hydrophobicity was unaffected. These mutations caused severe defects in vRNA synthesis and were all nonviable. The hydrophobic moments of these helices were similar to that containing mutation K[41,44]L (Fig. 3A). A main difference, however, is that because of the insertion mutations, the charged residues are dispersed and even located in the hydrophilic part of the helix, which probably interferes with the hydrophobic interaction with the membrane, whereas the narrow hydrophilic face and the hydrophobic part of the α-helix are maintained by mutation K[41,44]L. None of the insertion mutations affected the synthesis and processing of the viral protein, indicating that they primarily affected vRNA synthesis. Altogether, the results obtained with both the substitution and insertion mutants suggest that an amphipathic α-helix containing predominantly cationic lysine residues in a narrow hydrophilic face is a major structural determinant involved in a function of protein 2B, and possibly its precursor 2BC, in vRNA replication.

The structure of the cationic amphipathic α-helices found in enterovirus protein 2B shows close similarities to that of the cationic amphipathic α-helical peptides that form the class of lytic polypeptides (41). Most of these short cationic lytic polypeptides have been identified in the venom of insects or the skin of amphibia and are involved in an antibacterial defense system. Two models of action have emerged from the structural and functional studies on these peptides (reviewed in reference 6). In one model, the cationic helices form aqueous channels by spanning the membrane and forming oligomers, exposing their hydrophobic sides to the lipid bilayer and their hydrophilic faces to the aqueous pores. Although anion selectivity may be predicted, membrane currency measurements have demonstrated that cationic peptides can form either anion-selective (15, 17, 45) or cation-selective ion channels (1, 24, 44). These channels produce ionic imbalances which will ultimately result in colloid osmotic lysis. A second model proposes that these peptides produce lysis by disrupting the phospholipid structure of the membrane by lying parallel to the membrane and perturbing the membrane, making the membrane phospholipids more susceptible to enzymatic degradation by phospholipasises (6, 41). The resulting lesions in the membrane would allow a flux of ions, causing the ionic imbalances that are responsible for lysis of the cell.

The modification of the plasma membrane that occurs during an enterovirus infection is such that both gradients of sodium and potassium are disturbed and small nonpermeative translation inhibitors like hygromycin B and edeine can enter the virus-infected cells (13). Later in infection, the cellular membrane becomes more severely disrupted, and enzymes and
sugars can also leak out of the infected cells (13). The initial permeability of the plasma membrane for both monovalent cations and small translation inhibitors suggests that pores or membrane lesions, rather than a specific ion pump, are formed. By analogy with the lytic polypeptides, protein 2B may form transmembrane cation-selective pores or lie collateral to the membrane, making the phospholipids more accessible to the action of phospholipases. Both mechanisms would account for the observed ion fluxes across the membrane, causing the ionic imbalances that may be responsible for a further destabilization of the membrane. Many cytolytic viruses have been reported to increase phospholipase activity. In poliovirus-infected cells, choline and phosphorylcholine are released in the culture medium and the levels of diacylglycerol and inositol triphosphate in the cytoplasm are increased from the third hour after infection (21). Although this finding suggests that the membrane modification may be caused by phospholipases, the possibilities that protein 2B still forms transmembrane pores and that the integration of these pores is responsible for disturbing the integrity of the membrane and the activation of the phospholipases cannot be excluded.

Although protein 2B has a profound effect on the permeability of the plasma membrane, it should be emphasized that there is no experimental proof for an interaction of protein 2B with the plasma membrane. Electron microscopic studies using immunocytochemistry have shown that proteins 2B, 2C, and 2BC of poliovirus are contained exclusively at the endoplasmic reticulum membrane or the surface of the endoplasmic reticulum-derived membrane vesicles that surround the viral replication complexes (8). Intracellular localization of individual expressed poliovirus protein 2B in eucaryotic cells showed a disseminated distribution throughout the cytoplasm (2). These findings suggest that protein 2B interacts with intracellular membranes rather than with the plasma membrane. On the basis of these observations, an alternative mechanism for the induction of the plasma membrane modification may be proposed. Because of its ionophoric or membrane-perturbing properties, protein 2B may permeabilize endoplasmic reticulum membranes and cause the leakage of stored calcium ions into the cytosol. The consequences of a permanent increase in cytosolic calcium concentration for cellular metabolism and virus reproduction are unknown. However, as calcium is a regulator of a broad spectrum of physiological processes, it is likely that an increased calcium level is highly toxic for an array of cellular structures and processes. Increased cytosolic calcium levels in rotavirus (32), Semliki Forest virus (37), cytomegalovirus (34), and recently also in poliovirus-infected cells (25) have been demonstrated. The possible role of the alterations in calcium ion concentrations in viral replication, membrane permeabilization, and cell lysis, however, remains to be elucidated.

Mutations that disrupt the cationic amphipathic alpha-helix in protein 2B cause primary defects in vRNA synthesis, suggesting that the initial consequence of the membrane modification (i.e., the efflux of potassium ions and the influx of sodium ions or, alternatively, the influx of calcium) is a prerequisite for vRNA replication. However, the possibility that the cationic amphipathic helix is also required for the second function that has been attributed to protein 2B, i.e., the inhibition of cellular protein secretion, cannot be excluded. It has been proposed that this inhibition is involved in the accumulation of membrane vesicles on which positive-strand RNA synthesis occurs (16). Permeabilization of membranes may be required for this function, by causing either an altered ionic environment that may be inhibitory to protein transport or a nonspecific leakiness of intracellular organelle membranes and the loss of molecules crucial for transport. A disruption of the cationic amphipathic helix may interfere with the accumulation of the virus-induced membrane vesicles that are required for the formation of the viral replication complexes and thereby may be responsible for the defects in vRNA replication.

Although the results presented in this paper indicate that the cationic amphipathic alpha-helix in protein 2B is an important requirement for its function in vRNA synthesis, replacement of this structural motif with the cationic amphipathic helix of lytic peptide bombolin II resulted in a nonfunctional protein 2B. Both amphipathic helices contain a small hydrophilic face formed by three lysines, one negatively charged residue, and two polar residues (2). The nonviability of this chimeric construct (2B-bomlII) suggests that the position of the charged residues in the helix may be crucial either for the formation of a cation-specific pore or for the membrane-perturbing capacity. Alternatively, alterations in the amino acid sequence of this domain may interfere with a possible interaction of the putative amphipathic helix with another membrane-interacting domain of CBV3 protein 2B. Potential candidates for such an interaction are the N-terminal 16 aa, which also have considerable potential to form an amphipathic alpha-helix (Fig. 1), and the hydrophobic domain, formed by aa 63 to 80, which contains an amino acid composition and hydrophobicity features that are characteristic of multimeric transmembrane regions (40). Either of these domains may, together with the cationic amphipathic alpha-helix, cooperatively be involved in the formation of a cation-specific pore. Additional mutational analysis and analysis of the effects of individually expressed mutant 2B proteins on membrane permeability and protein secretion are required for a better understanding of the involvement of protein 2B in membrane modification and virus replication.

REFERENCES


