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Genetic Analysis of a Hydrophobic Domain of Coxsackie B3 Virus Protein 2B: a Moderate Degree of Hydrophobicity Is Required for a cis-Acting Function in Viral RNA Synthesis

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Coxsackie B virus protein 2B contains near its C terminus a hydrophobic domain with an amino acid composition that is characteristic for transmembrane regions. A molecular genetic approach was followed to define the role of this domain in virus reproduction and to study the structural and hydrophobic requirements of the domain. Nine substitution mutations were introduced in an infectious cDNA clone of coxsackie B3 virus. The effects of the mutations were studied in vivo by transfection of Buffalo green monkey cells with copy RNA transcripts. The results reported here suggest that a critical degree of hydrophobicity of the domain is essential for virus growth. The mutations S77M, C75M, I64S, and V66S, which caused a small increase or decrease in mean hydrophobicity, yielded viable viruses. The double mutations S77M/C75M and I64S/V66S, which caused a more pronounced increase or decrease in hydrophobicity, were nonviable. Negatively charged residues (mutations A71E, I73E, and A71E/I73E) abolished virus growth. The mutations had no effect on the synthesis and processing of the viral polyprotein. Replication and complementation were studied by using a subgenomic coxsackievirus replicon containing the luciferase gene in place of the capsid coding region. Analysis of luciferase accumulation demonstrated that the mutations cause primary defects in viral RNA synthesis that cannot be complemented by wild-type protein 2B provided in trans. The hydrophobic domain is predicted by computer analysis to form a multimeric transmembrane helix. The proposed interaction with the membrane and the implications of the mutations on this interaction are discussed.

The genus Enterovirus, a subgroup of the Picornaviridae, consists of the polioviruses, coxsackieviruses, echoviruses, and enterovirus serotypes 68 to 71. The structures, genomic organization, and molecular biology of these viruses are relatively well known from the extensive studies of poliovirus. Enteroviruses are positive-strand RNA viruses with a single-stranded genome of approximately 7,500 nucleotides (nt). A small viral 5' nontranslated RNA, and a genetically encoded poly(A) tail is present at the 3' end (see Fig. 2A). The viral genome acts as mRNA to direct the synthesis of a polyprotein. Translation occurs via a CAP-independent binding of ribosomes to the 3' nontranslated region (19, 32). The viral polyprotein is proteolytically processed by the virus-encoded proteases 2Apro, 3Cpro, and 3CDpro to functional cleavage intermediates and the individual proteins. The proteins encoded by the P1 region of the genome are capsid proteins. The nonstructural proteins encoded by the P2 and P3 regions are involved in viral RNA (vRNA) replication. For more details on the genomic structure of enteroviruses, we refer to a recent review of Wimmer et al. of the genetics of poliovirus (40).

Replication of the enterovirus RNA genome starts with the synthesis of a complementary negative strand RNA in the cytoplasm of the host cell by the RNA-dependent RNA polymerase 3DPol (3). Viral positive RNA strand synthesis takes place in replication complexes, which are specialized structures composed of partially double-stranded replicative intermediates plus the replication proteins (17). Genetic and biochemical studies have shown that the proteins encoded by the P3 region are involved in vRNA synthesis within these complexes (40). The replication complexes are closely associated with virus-induced membrane vesicles (6). Both the initiation of vRNA synthesis and the release of vRNA from the complexes are dependent on these vesicles (7, 37, 38). The inhibition of poliovirus RNA replication by Brefeldin A, an antibiotic that blocks membrane traffic between the endoplasmic reticulum (ER) and the Golgi apparatus and between the cis and trans Golgi apparatus, further indicates that intact secretory pathway traffic is required for vRNA replication (14, 18, 28).

Enterovirus proteins 2B, 2C, and 2BC seem to be involved in the functional and structural organization of the replication complexes. These proteins are rough ER (rER) associated soon after their synthesis and are exclusively localized at the cytoplasmic surface of the ER-derived membrane vesicles that surround the viral replication complex (5, 6, 8). Electron microscopy and biochemical studies have shown that protein 2C or its precursor 2BC is involved in the attachment of vRNA to the vesicular membranes (7, 9, 35). Furthermore, it has been shown that poliovirus protein 2C is an NTPase with both ATPase and GTPase activities (29, 35). The transient expression of poliovirus protein 2C or its precursor 2BC in eucaryotic cells resulted in the proliferation of cellular membranes and accumulation of membrane vesicles in a way similar to that seen during virus infection, suggesting that protein 2C belongs to the family of small GTPases involved in the regulation of membrane traffic (1, 10). Enterovirus protein 2B is a small hydrophobic protein which has profound effects on cellular membranes. Mutations mapping to the N terminus of poliovirus protein 2B exhibited primary defects in RNA synthesis (20, 27). These 2B mutants displayed a dosage-dependent dominance over wild-type virus, which led to the suggestion that protein 2B plays a structural role in the viral replication complexes, possibly by interacting with a limiting membrane at-
attachment site (20). The expression of both poliovirus and coxsackievirus B3 virus (CBV3) protein 2B in Escherichia coli resulted in a modified membrane permeability (26, 39a). Recently, it has been shown that the expression of poliovirus protein 2B in eucaryotic cells leads to permeabilization of the cell membrane and inhibition of secretory pathway function (13). These observations suggest that enterovirus protein 2B is a membrane-active protein which may be involved in the accumulation of membrane vesicles for RNA replication.

The regions of protein 2B that are involved in the interaction with the ER membrane are not yet known. Membrane anchors are typically composed of mainly hydrophobic amino acids and a significant portion of serine and threonine residues (15). About 18 amino acid (aa) residues are required to span the ER membrane (31). Analysis of the hydropathy of CBV3 protein 2B according to the method described by Kyte and Doolittle (24) demonstrates the existence of two hydrophobic domains (Fig. 1A). The hydrophobicity of these domains is well conserved in all enteroviruses, despite extensive differences in amino acid sequence among members of the CBV-like and poliovirus-like subgroups (Fig. 1B). The hydrophobic domain near the C terminus of CBV3 protein 2B is composed of a stretch of 18 aa (aa 63 to 80) which is devoid of charged residues. The amino acid composition of this hydrophobic domain is characteristic for transmembrane domains, since it contains 12 hydrophobic aa, 4 threonine residues, and 2 serine residues. The importance of this domain is reflected in the conservation of the hydrophobic features among all enteroviruses. The limited intratypic sequence heterogeneity found in five CBV1 isolates containing divergent 2A coding regions (41) further strengthens the importance of conservation in this region of protein 2B (Fig. 1B).

![Hydropathy of CBV3 protein 2B](image_url)

**FIG. 1.** (A) Hydropathy plot of CBV3 protein 2B according to Kyte and Doolittle (24). The hydropathy window size used for this plot is 9 residues. Positive hydropathy indicates a hydrophobic nature. Residue numbers of amino acid residues are indicated on the ordinate. (B) Alignment of the amino acid sequences of the 2B proteins of enteroviruses for which sequences are known. The enteroviruses are divided into a CBV-like subgroup and a poliovirus-like subgroup. Enteroviruses with distinct amino acid sequences are shown apart. In this alignment, we have included the 2B amino acid sequences of five nonrelated CBV1 isolates with highly divergent 2AP'° regions (41). Virus propagation, RNA extraction, reverse transcription, amplification by PCR, and sequence analysis of the 2B coding regions of these five CBV1 isolates were performed as described elsewhere (41). Abbreviations: CAV, coxsackie A virus; ECHO, echovirus; SVDV, swine vesicular disease virus; CBV1Tuc, myocardic strain Tucson; CBV1Jte, strain isolated 23 July 1976 from an immigrant child from Israel; CBV1Net, strain isolated in 1992 from a patient in The Netherlands; CBV1Isr, strain isolated 1 April 1986 from an immigrant child from Colombia; CBV1Sur, strain isolated 6 July 1981 from surface water in The Netherlands; PV1(M), poliovirus type 1 strain Mahoney; PV2(L), poliovirus type 2 strain Lansing; PV3(L), poliovirus type 3 strain Leone; EV70, enterovirus type 70; BEV1, bovine enterovirus type 1.
To examine the structural and hydrophobic requirements of this domain and to define its role in viral reproduction, we have constructed nine CBV3 protein 2B mutants. The effect of the mutations on virus viability was assayed by transfection of Buffalo green monkey (BGM) cells with copy RNA transcripts. Translation and processing of the virus polypeptide were examined both in vitro and in vivo. Replication and complementation of the mutants were tested by introduction and analysis of the mutations in a subgenomic replica in which the capsid-coding region had been replaced by the luciferase gene. The results reported here indicate that a moderate degree of hydrophobicity of the domain is essential for the function of protein 2B. Mutations which caused a major increase or decrease in hydrophobicity as well as the introduction of negatively charged residues interfered with virus growth. These mutations caused primary defects in vRNA synthesis that could not be complemented in trans by wild-type protein 2B. The proposed interaction of this hydrophobic domain with the membrane and the implications of the mutations on this interaction are discussed.

MATERIALS AND METHODS

Cells and viruses. Virus propagations and RNA transfections were performed with BGM cells. Plaque assays were performed with Vero cells. The cells were grown in minimal essential medium (MEM) (GIBCO) supplemented with 10% fetal bovine serum (GIBCO). After infection, cells were fed with MEM containing 3% fetal bovine serum. After transfection, MEM containing 10% fetal bovine serum was added to the cells. Virus yields were determined by endpoint titration. Serial 10-fold dilutions were tested in 8 replicate wells of 96-well plates containing BGM cell monolayers (36). Viral titers were calculated according to the method described by Reed and Muench and were expressed in 50% tissue culture infective dose (TCID50) values (34).

Oligonucleotide-directed site-specific mutagenesis. Plasmid pCB3/T7 was kindly provided by R. Kandolf (University of Tübingen, Tübingen, Germany). This plasmid contains a full-length copy DNA of CBV3 (strain Nancy) cloned in pCB3/T7-APl to generate pALTCB3/2080-4947. Synthetic oligonucleotides were used to introduce site-specific mutations. The nucleotide sequence of the mutant was confirmed by dideoxy chain termination sequence analysis of the PCR products (24).

Transcription and transfection of cells with copy RNA transcripts. Plasmids were linearized by digestion with SnaBI, extracted with phenol-chloroform, and precipitated. Copy RNA transcripts were generated in a 100-µl reaction mixture containing 2 µg of linearized template DNA, 40 mM Tris-HCl (pH 7.5), 10 mM NaCl, 6 mM MgCl2, 2 mM spermidine, 2.5 mM each nucleoside triphosphate, 100 U of RNAsin (Promega), and 30 U of T7 RNA polymerase (Promega). Small portions (2 µl) were analyzed by agarose gel electrophoresis. Twenty microliters of the transcription mixture (2 to 4 µg of DNA) was used for transfection of BGM cells by the DEAE-dextran method (39) with some modifications. Briefly, 80 µl of a N2-hydroxyethylpiperazine-N2-ethanesulfonic acid (HEPES)-buffered salt solution (HBS) (20 mM HEPES [pH 7.0], 137 mM NaCl, 5 mM KCl, 1 mM NaHPO4, 3.5 mM glucose) was added to a 20-µl transcription mixture and mixed with 100 µl of a DEAE-dextran solution (1 mg/ml in HBS), and the solution was incubated on ice for 30 min. BGM monolayer cells grown in 25-cm2 flasks to a confluency of 75% were washed three times with phosphate-buffered saline (PBS) and incubated with the RNA/DEAE-dextran mixture for 30 min at room temperature. The cells were washed three times with PBS, overlaid with cell culture medium, and incubated at either 33 or 36°C for the times indicated.

Sequence analysis of the 2B coding region of mutant viruses. RNA was isolated from transfected BGM cells. RNA was extracted from virus stock by a single extraction procedure with guanidinium thiocyanate-phenol-chloroform according to the method described by Chomczynski and Sacchi (11). Reverse transcription of RNA and amplification of cDNA by PCR with forward primer 5’-GCAATTGAAGCGGAGATGCGC-3’ (nt 735-763) and reverse primer 5’-TTGGGATGGCATCTGCGCTGCTC-3’ (nt 4231 to 4251) were performed as described previously (42). The resulting 519-bp PCR products were purified by low-melting-point agarose gel electrophoresis. Sequence analysis of the PCR products was performed as described above.

Plaque assays. Plaque assays were carried out with 100% confluent monolayers of Vero cells on 10-cm2 dishes in 6-well plates. Virus dilutions were made in MEM containing 3% fetal bovine serum. Vero cells were infected with different virus dilutions and incubated without 30 min at room temperature. After removal of the inoculum, the cells were overlaid with 5 ml of overlay medium containing 1% agarose (Sorbo) and 22 mM MgCl2. The cells were grown at 33, 36, and 39°C, and plaque phenotypes were analyzed at 3 to 5 days postinfection.

Single-cycle growth analysis. BGM cell monolayers grown in 25-cm2 flasks (5 X 10^6 cells/well) were infected with virus. Infected cell cultures were analyzed both in vitro and in vivo. Replication and complementation of the mutants were tested by introduction and analysis of the mutations in a subgenomic replica in which the capsid-coding region had been replaced by the luciferase gene. The results reported here indicate that a moderate degree of hydrophobicity of the domain is essential for the function of protein 2B. Mutations which caused a major increase or decrease in hydrophobicity as well as the introduction of negatively charged residues interfered with virus growth. These mutations caused primary defects in vRNA synthesis that could not be complemented in trans by wild-type protein 2B. The proposed interaction of this hydrophobic domain with the membrane and the implications of the mutations on this interaction are discussed.

Construction of subgenomic replicon pCB3/T7-LUC. For the substitution of the capsid-coding region by the luciferase coding sequence, the PI capsid-coding region was deleted from pCB3/T7 by two subsequent PCR product subcloning steps by using the unique restriction sites for SfiI (nt 250), BstII (nt 2024), and BclII (nt 4238). PCRs were performed with UltraTma DNA polymerase (Perkin-Elmer), a DNA polymerase containing proofreading activity. First, nt 243 to 728 were amplified with primers 5’-AAC TAC TTC GAA AAA CCT AGT AAC -3’ (complementary to nt 3733 to 3765) and reverse primer 5’-TTGG GATGGC TCAC ACATTG CAG ACTACG AATTTGC TGT GTA TTC AAC TTA-3’ (complementary to nt 4117 to 4134) with the Ampli Cycle sequencing kit according to the manufacturer’s instructions (Perkin-Elmer). From these clones, the full-length cDNA region of the plasmid containing BGM cell monolayers (36) was amplified with primers 5’-GCC GAAC TCC TCT GCT ACG ACG ACT TGC CCG CTC CTT CTC-3’ (complementary to nt 4321 to 4251). This PCR product was cut with SfiI and BclII and cloned in pCB3/T7 cut with the same enzymes. In the resulting plasmid, pCB3/T7-1746/2024, the N-terminal half of the PI region is deleted. Second, nt 2080 to 4251 were amplified with primers 5’-GGGA AGA CAT CTT GCC GCT GC GCT CAC TCT TCT GCT CTC CTC-3’ (complementary to nt 3280 to 3300 plus restriction sites for BglII and HphI) and 5’-CTG GAA TGC GGC GCC CAT TCT TTC GGC AAT AAA ACTA-3’ (complementary to nt 4900 to 4880 plus restriction sites for BglII and HphI) according to the recommendations of the manufacturer (Promega). From these clones, the 519-bp PCR product was verified by dideoxy chain termination sequence analysis of the PCR products.

Analysis of viral positive RNA synthesis. BGM cell monolayers grown in 25-cm2 flasks (5 X 10^6 cells/well) were infected with virus. Infected cell cultures were analyzed both in vitro and in vivo. Replication and complementation of the mutants were tested by introduction and analysis of the mutations in a subgenomic replica in which the capsid-coding region had been replaced by the luciferase coding sequence in plasmid pCB3/T7-LUC. The resulting construct (pCB3/T7-LUC) contains the following recombinant plasmid DNA region: 5’-CCATTCAATGAATTTCTG-3’ (complementary to nt 4238 to 4251). The resulting construct was linearized by digestion with SfiI and BglII and introduced into BGM cells. The cells were infected with different virus dilutions and incubated without 30 min at room temperature. After removal of the inoculum, the cells were overlaid with 5 ml of overlay medium containing 1% agarose (Sorbo) and 22 mM MgCl2. The cells were grown at 33, 36, or 39°C for 2, 4, or 6 h. Viral RNAs were released by three successive cycles of freezing and thawing. Viral titers were determined by titration on BGM cell monolayers in 96-well plates.

Analysis of viral protein synthesis in vivo. BGM monolayer cells (2 X 10^5) were infected with different virus dilutions and incubated with a MOI of 50. After virus adsorption for 30 min at room temperature, medium was added and the cells were incubated at 36°C. At various times postinfection, the cells were washed three times with PBS and lysed in 400 µl of lysis buffer, and the luciferase activity was measured in a liquid scintillation counter with the luciferase assay system according to the recommendations of the manufacturer (Promega).

In vitro translation reactions. Copy RNA transcripts were synthesized and translated in T7 TNT rabbit reticulocyte lysate (Promega) according to the manufacturer’s recommendations. The translation reaction mixture (20 µl) was programmed with 0.5 µg of Sall-linearized plasmid DNA and supplemented with 20% (vol/vol) HeLa cell initiation factors (kindly provided by J. Flanagan, University of Florida). In vitro-synthesized proteins were labeled with [35S]methionine (specific activity, >1,000 Ci/mmol; Amersham) for 3 h at 30°C. After this incubation, RNA was degraded by treatment with RNase T1 (200 U), 150 µM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 15% Nonidet P-40, and 0.05% SDS. Labeled proteins were analyzed by polyacrylamide gel electrophoresis as described above.

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RESULTS

Introduction of mutations in a hydrophobic domain of CBV3 protein 2B. Nine CBV3 mutants containing amino acid substitutions in a hydrophobic 18-residue domain of protein 2B were constructed by site-directed mutagenesis. The mutations were verified by sequence analysis and introduced in the infectious cDNA clone pCB3/T7. The integrity of the resulting plasmids was checked by restriction enzyme analysis. Figure 2 shows the genotypes of the mutants and the predicted effects of the mutations on hydrophobicity. On the basis of the effects of the mutations on the mean hydrophobicity and net charge of this region, the pCB3/T7 2B mutants can be divided into three groups as follows, (i) In mutants 2B-S77M, 2B-C75M, and 2B-S77M/C75M, serine 77 (TCA) and cysteine 75 (TGT) were replaced by more-hydrophobic methionine (ATG) residues, (ii) In mutants 2B-I64S, 2B-V66S, and 2B-I64S/V66S, the mean hydrophobicity is decreased by substitution of hydrophobic residues isoleucine 64 (ATC) and valine 66 (GTG) by polar serine residues (codons AGT and TCG, respectively), which contain aliphatic hydroxyl side chains. (iii) In mutants 2B-A71E, 2B-I73E, and 2B-A71E/I73E, the mean hydrophobicity is decreased by the replacement of alanine 71 (GCA) and isoleucine 73 (ATC) by negatively charged glutamic acid (GAG) residues.

Effect of the mutations on virus growth. The effect of the mutations on virus viability was studied by transfection of BGM cells with copy RNA transcripts. For each mutant, eight transfections were performed. Four transfected cell cultures were subjected to posttransfection. No CPE was observed up to 5 days posttransfection. Complete CPE was not observed before 72 to 96 h postinfection, respectively (data not shown). All mutant viruses showed similar reproductive capacities at 33 and 39°C, which indicates that none of the mutations caused temperature-sensitive growth defects. The results of the transfections and the characterization of the viable mutants are summarized in Fig. 2B.

Isolation of two revertant viruses. Passage of cells transfected with copy RNA of mutant 2B-S77M/C75M revealed virus growth in one of the cultures grown at 33°C. Sequence analysis demonstrated that these viruses still contained the methionine at position 77 but a reversion of the introduced virus growth revertant methionine (AUG) at position 75 to a valine (GUG) residue. This partial revertant virus, vCB3-2B-S77M/C75M, exhibited a small-plaque phenotype (Fig. 3). In single-cycle infections, this virus produced 3 and 5% of the wild-type virus yield at 6 and 8 h postinfection, respectively (data not shown). All mutant viruses showed similar reproductive capacities at 33, 36, and 39°C, which indicates that no other amino acid replacements had occurred. Viral growth characteristics were examined by plaque assays and single-cycle growth analysis. Viruses vCB3-2B-S77M, -I64S, and -V66S exhibited wild-type growth. Virus vCB3-2B-C75M exhibited a small-plaque phenotype (Fig. 3). In single-cycle infections, this virus produced 3 and 5% of the wild-type virus yield at 6 and 8 h postinfection, respectively (data not shown). All mutant viruses showed similar reproductive capacities at 33, 36, and 39°C, which indicates that none of the mutations caused temperature-sensitive growth defects. The results of the transfections and the characterization of the viable mutants are summarized in Fig. 2B.

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small plaques were picked from the plates, and the viruses were grown on BGM cells. Sequence analysis demonstrated that the viruses isolated from the small plaques had retained the introduced methionine (AUG) residue. However, the viruses isolated from the three large plaques contained a valine (GUG) residue at amino acid position 17 instead of the introduced methionine and were named vCB3-2B-C75V. No other mutations in the 2B coding sequence were observed. This revertant, vCB3-2B-C75V, exhibited a wild-type plaque size at all temperatures.

To investigate if the reversions in the mutants vCB3-2B-C75V and vCB3-2B-S77M/C-75V were solely responsible for the observed phenotypes, we introduced these mutations into the wild-type pCB3/T7 clone. For this purpose, the 519-bp PCR products (nt 3733 to 4251) that were used for the sequence analysis of the 2B coding region (nt 3746 to 4041) were cut with SpeI (nt 3837) and BssHII (nt 4238), purified, and cloned in pCB3/T7. nt 4042 to 4238 were sequenced to verify that no additional mutations had occurred in this region. BGM cells were transfected with copy RNA generated from the resulting plasmid clones 2B-C75V and 2B-S77M/C75V. Sequence analysis of the 2B coding regions of the resulting viruses demonstrated that the introduced mutations were retained in the vRNA. These viruses exhibited the same plaque phenotype that the revertants obtained after transfection of cells with copy RNA of mutants 2B-C75M and 2B-S77M/C75M, respectively, exhibited, which indicates that the reversions were sufficient to confer the observed phenotypes (data not shown).

Analysis of vRNA replication with subgenomic replicon pCB3/T7-LUC. The observation that the capsid-coding P1 region could be deleted from poliovirus RNA without any effect on the efficiency of genome replication (21) has led to the construction of chimeric subgenomic poliovirus replicons in which the P1 region was replaced by reporter genes (2, 33). Andino et al. (2) have described a subgenomic poliovirus replicon (RLuc-31) in which the P1 region was replaced by the luciferase gene and have shown that the activity of the reporter protein following transfection correlated with the levels of accumulated RNA (2).

To study vRNA replication of the 2B mutants, chimeric subgenomic replicon pCB3/T7-LUC was constructed (Fig. 4A). In this replicon, the P1 region of pCB3/T7 is substituted by the firefly luciferase gene. The suitability of this replicon to study vRNA replication was examined by transfection of BGM cells with copy RNA of pCB3/T7-LUC or the replication-defective control pCB3/T7-LUCΔ3D. This plasmid contains a large in-frame deletion in the 3Dpol region by deletion of the BbrP1 fragment (nt 6117 to 7152). At several times posttransfection, the cells were lysed and the levels of luciferase activity were measured. Figure 4B demonstrates that the kinetics of luciferase production by pCB3/T7-LUC copy RNA can be divided into three phases, which are similar to those described for RLuc-31 (2). In phase I (1 to 3 h posttransfection), luciferase activity increased as a result of translation of the input RNA. Phase II (3 to 5 h posttransfection) is characterized by a steady state in luciferase activity. Phase III (5 to 12 h posttransfection) demonstrated a second increase (10- to 50-fold) in luciferase activity as a result of the translation of newly synthesized chimeric RNA strands. In contrast, phase III of RLuc-31 is already detectable from the 3rd h posttransfection, and replication results in a 100- to 500-fold increase in luciferase activity (2). This difference is probably due to the low infectivity of pCB3/T7-derived copy RNA, which is only 14 to 30 PFU/μg of copy RNA (22). Upon transfection of pCB3/T7-LUC RNA, luciferase activity is generated in all transfected cells, but only in a few of these cells will an infectious cycle be initiated. The exponential increase in luciferase activity as a result of vRNA replication will, therefore, be less evident and cannot be detected before 6 h posttransfection. However, the absence of phase III in cells transfected with copy RNA of pCB3/T7-LUC-3D (Fig. 4B) demonstrates the suitability of replicon pCB3/T7-LUC to study vRNA replication.

Growth-defective 2B mutants display defects in vRNA synthesis. vRNA synthesis of the nine originally constructed mutants was examined after introduction of the SpeI-BssHII fragments in replicon pCB3/T7-LUC. BGM cells were transfected with copy RNA of pCB3/T7-LUC replicons containing the 2B mutations and grown at 36°C, and the luciferase activities were determined at several times posttransfection. Replicons carrying the nonviable 2B mutations (S77M/C75M, I64S/V66S, A71E, I73E, and A71E/I73E) displayed defects in RNA replication (Fig. 4C). These replicons exhibited phases I and II of the luciferase production, which are the result of translation of the input RNA, but not the phase III increase that reflects translation of newly synthesized RNA strands. Replicons carrying the viable mutations S77M, I64S, and V66S, which produced viruses with wild-type growth characteristics, displayed the same kinetics of luciferase production that wild-type pCB3/T7-LUC did (Fig. 4D). Mutation C75M, which yielded virus with a small-plaque phenotype, caused a severe reduction in RNA synthesis. The absence of an increase in luciferase activity above the translation level, which might have been expected from a low level of replication, is most likely due to the low level of infectivity of the copy RNA transcripts.

Figures 4C and D show that the luciferase activity generated by the replication-defective replicons remained constant up to 10 h posttransfection. Because luciferase is an unstable enzyme in vivo (2), the constant level must represent an equilibrium between translation of the input RNA and degradation of luciferase. The constant level of luciferase activity suggests that none of the 2B mutations affected the translation ability or stability of the chimeric input RNA.

In vitro protein synthesis and processing of RNAs containing mutations in protein 2B. In vitro translation reactions were performed to investigate whether the mutations affect translation and processing of the polyprotein. Figure 5 shows that the patterns of [35S]methionine-labeled proteins obtained with the 2B mutant plasmids were similar to that produced by wild-type plasmid, which indicates that none of the mutations caused defects in synthesis and processing of the viral polyprotein. The positions of proteins 2BC and 2C were identified by in vitro
translation of copy RNAs carrying the 2BC or 2C coding sequence behind the encephalomyocarditis virus 5' nontranslated region (data not shown). In vitro-translated protein 2B (±11 kDa) could not be visualized because of the presence of only two cysteines in protein 2B and the interference of the excess of globin in the reticulocyte lysate with a correct migration of proteins with molecular masses of less than 15 kDa. However, the production of protein 2C at levels equal to that produced by wild-type RNA demonstrates that none of the mutants displayed processing defects at the 2B-2C junction.

Analysis of the viral protein synthesis in vivo. The rates of viral protein synthesis in cells infected with small-plaque viruses vCB3-2B-C75M and vCB3-2B-S77M/C75V were compared with that in wild-type virus-infected cells to examine whether the defects in RNA synthesis are a consequence of an impaired level of viral protein synthesis in vivo. BGM cells were infected with either vCB3-2B-C75M, vCB3-2B-S77M/C75V, or wild-type virus at a MOI of 50, and proteins were labeled with [35S]methionine at different times postinfection. Figure 6 shows that the shutoff of host cell translation after infection with these two mutant viruses was similar to the shutoff induced by wild-type virus. At 4 and 5 h postinfection, the level of viral protein synthesis in the mutant virus-infected cells was slightly below the level in wild-type-infected cells. At 6 h postinfection, the rates of viral protein synthesis in the mutant-infected and wild-type-infected cells were similar, which indicates that it is unlikely that the mutations cause primary defects in the rate of synthesis of the viral polyprotein. In the previous paragraphs, it has been shown that mutations in protein 2B cause defects in vRNA synthesis. The slightly decreased level of viral protein synthesis in cells infected with vCB3-2B-C75M and vCB3-2B-S77M/C75V at 4 and 5 h postinfection is probably a reflection of the defects in RNA synthesis
and, as a consequence, the reduced amount of available viral positive RNA strands. The reduction in vRNA synthesis is probably also responsible for the delay in CPE and, as result, for the prolonged synthesis of viral proteins up to 7 h postinfection, when viral protein synthesis in wild-type virus-infected cells is already severely decreased.

**Defects in vRNA synthesis caused by mutations in a hydrophobic domain of protein 2B cannot be rescued in trans.** To study whether the defects in vRNA synthesis could be rescued in vivo by wild-type protein 2B provided in trans, genetic complementation experiments were performed. We studied complementation of the defects caused by mutation C75M, which yields small-plaque viruses, and the nonviable mutations S77M/C75M, I64S/V66S, A71E, I73E, and A71E/I73E. To assay for complementation, BGM cells were transfected with copy RNAs of the mutant pCB3/T7-LUC replicons, were incubated at 36°C, and at 90 min posttransfection were either mock or virus infected with wild-type virus at a MOI of 50. At 4 and 8 h posttransfection, the cells were lysed and the luciferase activity was measured. Wild-type pCB3/T7-LUC copy RNA transcripts were tested to exclude the possibility that the replication of wild-type virus interfered with the luciferase accumulation. The results shown in Table 1 demonstrate that virus growth did not adversely affect replication and translation of the pCB3/T7-LUC RNA strands. Complementation tests with the mutant pCB3/T7-LUC replicons showed similar levels of luciferase activity at 8 h posttransfection in the mock- and virus-infected cells (Table 1). The luciferase activities measured at 8 h posttransfection in both mock- and virus-infected cells were comparable to the activities measured at 4 h posttransfection, a time point at which the level of luciferase activity reflects the translation of the input copy RNA. The absence of major increases in luciferase activity in the virus-infected cells suggests that the defects in RNA synthesis caused by mutations in a hydrophobic domain of protein 2B could not be efficiently complemented by the function of wild-type protein 2B provided in trans. However, the possibility that low levels of complementation have occurred cannot be excluded. Low levels of replication are difficult to detect because of the low infectivity of the copy RNA transcripts (see above) and the small variations in transfection efficiency and luciferase assays that were observed.

**DISCUSSION**

Enterovirus protein 2B is a small hydrophobic protein that is localized at the outer surfaces of the virus-induced membrane vesicles on which vRNA synthesis takes place. The nature of the interaction of protein 2B with the membrane is yet unknown. Examination of the amino acid sequence of protein 2B of CVB3 revealed an 18-aa hydrophobic domain (aa 63 to 80) with a composition that is characteristic of integral transmembrane regions. In this study, we examined the effects of amino acid substitutions in this hydrophobic domain of CVB3 protein 2B on virus growth, vRNA replication, and viral polypeptide processing to gain more insight in the role of this domain in the function of protein 2B. The mutations that were introduced can be divided into three groups on the basis of their effects on hydrophobicity and net charge of the domain (Fig. 2). In the first group, serine 77 and cysteine 75 were replaced by more hydrophobic methionine residues. Mutation S77M yielded virus with wild-type growth characteristics. Mutation C75M yielded mutant virus with a small-plaque phenotype because of a reduction in vRNA synthesis. Large-plaque revertants of this mutant were isolated and found to contain a reverse of methionine 75 to a valine residue (vCB3-2B-C75V). The double mutation S77M/C75M caused a defect in vRNA synthesis and was nonviable. However, the isolation of small-plaque revertant virus vCB3-2B-S77M/C75V demonstrates that mutation S77M/C75M did not completely abolish vRNA replication. In the second group, hydrophobic amino acids with aliphatic side chains (isoleucine 64 and valine 66) were replaced with polar serine residues with aliphatic hydroxyl side chains. Mutations I64S and V66S produced mutant viruses with wild-type plaque phenotypes. However, the double mutation I64S/V66S disturbed vRNA synthesis and was nonviable. In the third group, alanine 71 and valine 73 were replaced with negatively charged glutamic acid residues. Mutations A71E, I73E, and A71E/I73E disturbed vRNA replication and were all nonviable. RNA synthesis seemed to be completely abrogated, since no revertant viruses could be isolated. In vitro translation reactions and analysis of the viral protein synthesis in infected cells demonstrated that none of the mutations interfered with the synthesis or processing of the viral polypeptide, indicating that the mutations in the hydrophobic domain primarily affected vRNA synthesis.

The results presented here suggest that the hydrophobic character of the domain is crucial for the function of protein 2B in vRNA replication. Furthermore, the data suggest that for efficient functioning of the domain, the hydrophobicity (<H>)

![Graph or figure](image-url)

**FIG. 6.** Protein synthesis in BGM cells infected with wild-type virus, vCB3-2B-C75M, and vCB3-2B-S77M/C75V. BGM cells were infected at an MOI of 50 and were grown at 36°C. At the indicated times, the cells were incubated with methionine- and serum-free MEM containing 10 µCi of Tran35SLabel (a mixture of [35S]methionine and [35S]cysteine). After a 1-h labeling period, the cells were washed and lysed. The labeled proteins were analyzed on an SDS-12.5% polyacrylamide minigel.
is restricted by both lower and upper hydrophobicity limits. The single amino acid substitutions that caused only minor alterations in hydrophobicity (S77M [<H>, 0.57], I64S [<H>, 0.44], V66S [<H>, 0.45], and C75M [<H>, 0.54]) all yielded viable viruses. These structural alterations and the concomitant increases or decreases in mean hydrophobicity had no effect or only little effect (small-plaque mutation C75M) on the function of protein 2B in vRNA synthesis. However, both double mutations S77M/C75M (<H>, 0.59) and I64S/V66S (<H>, 0.37) were nonviable. Since each of the single substitutions did not severely affect the structure of the domain, the disturbance of vRNA synthesis caused by the double mutations is most likely due to the more profound increase or decrease in the overall hydrophobicity of the domain. The suggestion that the hydrophobicity of the domain is restricted by an upper limit is further supported by the observation that revertant vCB3-2B-S77M/C75V (<H>, 0.61) exhibited a small-plaque phenotype, whereas viruses containing each of the single mutations displayed wild-type growth. The disturbance of the hydrophobic character of the domain by the nonviable mutations A71E (<H>, 0.45), I73E (<H>, 0.40), and A71E/I73E (<H>, 0.33) may be due to the introduction of the negative charges rather than to the decrease in the overall hydrophobicity of the domain, since the decrease caused by mutation A71E is similar to those caused by mutations I64S and V66S. Another observation which cannot simply be explained by means of hydrophobicity is the finding that mutation C75M (<H>, 0.54) yielded a small-plaque virus, whereas a revertant containing a rever­
sion of the introduced methionine 75 to a valine residue (<H>, 0.57) displayed a wild-type growth. It is difficult to explain the adverse effect of mutation C75M. The wild-type growth of revertant vCB3-2B-C75V makes it unlikely that the sulphydryl group of cysteine 75 is involved in the formation of disulfide bridges. The defect in vRNA synthesis caused by mutation C75M may be caused by the relatively long side chain of the methionine residue, which may interfere with the structure of the domain or the interaction of the domain with its target.

The exact function of protein 2B in vRNA replication is not yet known. The expression of poliovirus protein 2B led to an increase in plasma membrane permeability and the inhibition of protein secretion (13), suggesting that protein 2B has profound effects on cellular membranes. Protein 2B has a high affinity for membranes and becomes associated with the rER membrane immediately after its synthesis (5). It is likely that the hydrophobic domain is involved in this association and that major alterations in the hydrophobic domain interfere with the localization of protein 2B at the vesicular membrane. The displacement of protein 2B from the membrane may hinder a functional role of protein 2B in vRNA synthesis in the replication complex. Alternatively, the disturbance of the interaction with the membrane may interfere with a structural role of protein 2B in the organization of the viral replication complex (20). The interaction of protein 2B with the membrane may be a prerequisite for the inhibition of protein secretion. Mutations that disturb the interaction of protein 2B with the membrane may interfere with the accumulation of membrane vesicles for vRNA synthesis and thereby account for the defects in the synthesis of viral positive RNA strands. However, it cannot be excluded on the other hand that this hydrophobic domain is involved in a specific interaction with another viral or cellular protein required for vRNA synthesis. Alterations in the hydrophobicity of the domain would affect these protein-protein interactions and in this way account for the observed defects in vRNA synthesis. The possible disposition of protein 2B in the membrane was analyzed by a theoretical method. Eisenberg and coworkers have developed an algorithm to identify membrane-associated helices in membrane proteins and to distinguish them from helices in globular proteins (15). Candidate membrane-associated helices are identified by calculating the hydrophobicity of 21-residue windows run over the entire protein. Candidate helices must have a mean <H> value of at least 0.42. Furthermore, there must be either one highly hydrophobic helix with an <H> value of at least 0.68 or several shorter hydrophobic helices, which may be provided either intramolecularly, with a summation value of at least 1.10. Candidate helices are then classified as either a monomeric transmembrane helix, a multimeric transmembrane helix, or a surface-seeking helix with the aid of the hydrophobic moment plot, on which the hydrophobic moment of the helix is plotted as a function of its hydrophobicity (15). We have identified candidate membrane-associated helices in enterovirus protein 2B using 18- to 21-residue windows, since it has been reported that 18 aa are appropriate for spanning the ER membrane (31). In members of the CBV-like subgroup, the most hydrophobic segment is formed by aa 63 to 80 (<H> = 0.52 to 0.60). In members of the poliovirus-like subgroup, the segment with the highest <H> value is formed by residues 64 to 81 (<H> = 0.49 to 0.55). The mean hydrophobicity of these segments is probably too low for the integration as a monomeric transmembrane helix but may be sufficient to form a multimeric transmen­
brane helix. Classification of these segments on the hydropho­bic moment plot also suggests that they form multimeric transmen­
brane helices, i.e., protein segments that are cooperatively associated within the lipid bilayer and stabilized by either an intermolecular (e.g., in the form of multimers) or an intramo­
lecular linkage with other transmembrane a-helices.

At present, there is no biochemical evidence for the predic­
tion that the hydrophobic domain forms an integral transmem­
brane helix that is associated with other transmembrane helices. However, the suggestion that the hydrophobic domain forms a multimeric rather than a monomeric transmembrane helix is supported by several observations made in this study. (i) The mean hydrophobicity values of the mutants with wild­
type phenotypes varied between 0.44 and 0.57. These values are too low for monomeric transmembrane proteins to effi­
ciently penetrate the membrane and maintain an energetically stable configuration (15). (ii) The substitution of cysteine 75 with a hydrophobic methionine residue interfered with vRNA synthesis, which makes it unlikely that this residue is involved in a hydrophobic interaction with the lipid bilayer. (iii) The mutations which caused the greatest increase in the hydropho­bicity of the domain were either lethal for virus growth (S77M/ C75M [<H> = 0.59]) or resulted in a small-plaque virus (S77M/C75V [<H> = 0.61]). This suggests that there may exist an upper hydrophobicity limit for the domain and that by exceeding this limit, the domain may become too hydrophobic to efficiently interact with other transmembrane helices.

A complementation analysis was performed to examine whether the defects in vRNA synthesis caused by the mutations in the hydrophobic domain could be rescued by wild-type proteins provided in trans. Mutations in the N-terminal part of poliovirus protein 2B have been reported to cause noncom­ple­mentable defects in vRNA synthesis (4, 12, 20, 27). However, complementation experiments with protein 3A mutants have shown that even within one protein, cis-acting and trans-acting functional regions can be discriminated (4, 16). We found that none of the mutations in the hydrophobic domain that caused phenotypes in vRNA synthesis could be efficiently comple­mented by wild-type protein 2B. Both the N-terminal region and the hydrophobic domain of protein 2B appear to be in­
volved in a cis-acting function in vRNA replication. It is not clear whether the effects that are observed upon expression of poliovirus protein 2B (i.e., permeabilization of the plasma membrane and inhibition of protein secretion) (13) reflect different functions of protein 2B or whether one of these effec-
tives is the consequence of the other. If permeabilizing the plasma membrane were the primary function of the hydropho-
bic domain and if the inhibition of protein secretion were only a side effect of this function, then a certain degree of trans complementation should have been expected. The lack of complementability does not exclude the possibility that the hydrophobic domain is involved in permeabilization of the plasma membrane but suggests that the hydrophobic domain is also involved in another function of protein 2B in vRNA syn-
thesis.

Several explanations may account for the cis-acting function of protein 2B. First, protein 2B may interact directly with the ER membrane to function in the accumulation or structural organization of virus-induced membranous structures around the same positive RNA strand from which it is synthesized. This interaction would ensure a tight linkage between translation and replication and prevent RNAs other than viral posi-
tive RNA strands from being replicated by 3Dpol, which lacks template specificity (20). A second possibility is that wild-type 2B proteins cannot reach the mutant templates. Replication of the vRNA takes places in close conjunction with membranes. This membranous environment may interfere with the func-
tional interchange of mutant and wild-type protein 2B. A third possibility is that the mutant 2B proteins interact with a limiting target and that wild-type protein 2B, which is provided later, cannot efficiently compete with them. This possibility was raised by Johnson and Sarnow (20), who found that poliovirus 2B mutants exhibited a dosage-dependent dominance over wild-type protein 2B. The interaction of the mutant CBV3 2B proteins with this limiting target may interfere with the action of wild-type protein 2B and thereby account for the lack of complementation.

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