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Mutational Analysis of Different Regions in the Coxsackievirus 2B Protein

REQUIREMENTS FOR HOMO-MULTIMERIZATION, MEMBRANE PERMEABILIZATION, SUBCELLULAR LOCALIZATION, AND VIRUS REPLICATION*

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The coxsackievirus 2B protein is a small hydrophobic protein (99 amino acids) that increases host cell membrane permeability, possibly by forming homo-multimers that build membrane-integral pores. Previously, we defined the functional role of the two hydrophobic regions HR1 and HR2. Here, we investigated the importance of regions outside HR1 and HR2 for multimerization, increasing membrane permeability, subcellular localization, and virus replication through analysis of linker insertion and substitution mutants. From these studies, the following conclusions could be drawn. (*i*) The hydrophilic region (^{58}RNHDD^{62}) between HR1 and HR2 is critical for multimerization and increasing membrane permeability. Substitution analysis of Asn^{61} and Asn^{62} demonstrated the preference for short polar side chains (Asp, Asn), residues that are often present in turns, over long polar side chains (Glu, Gln). This finding supports the idea that the hydrophilic region is involved in pore formation by facilitating a turn between HR1 and HR2 to reverse chain direction. (*ii*) Studies undertaken to define the downstream boundary of HR2 demonstrated that the aromatic residues Trp^{80} and Trp^{82}, but not the positively charged residues Arg^{81}, Lys^{84}, and Lys^{86} are important for increasing membrane permeability. (*iii*) The N terminus is not required for multimerization but does contribute to the membrane-active character of 2B. (*iv*) The subcellular localization of 2B does not rely on regions outside HR1 and HR2 and does not require multimerization. (*v*) Virus replication requires both the membrane-active character and an additional function of 2B that is not connected to this activity.

Enteroviruses (*e.g.* poliovirus, coxsackievirus, echovirus) belong to the family of Picornaviridae, a large family of nonenveloped, cytolytic viruses. The enterovirus genome consists of a 7.5-kb RNA molecule of positive polarity that is translated into one single 220-kDa polyprotein in a cap-independent manner. Proteolytic processing of the polyprotein by virus-encoded proteases yields the P1, P2, and P3 region proteins. The P1 region is further processed to generate the capsid proteins; the P2 and P3 regions are cleaved to yield the replication proteins 2A^{pro}, 2B, 2C, 3A, 3B, 3C^{pro}, and 3D^{pol} and the more stable cleavage intermediates 2BC, 3AB, and 3CD^{pro}, which have functions distinct from their cleavage products (reviewed in Refs. 1, 2).

Enteroviruses induce a number of profound functional and morphological alterations in host cell membranes. An important membrane modification that can be observed in infected cells is the increase in membrane permeability, which affects both intracellular membranes and the plasma membrane (3–5). The gradual modification of membrane permeability starts with the release of calcium from intracellular stores, followed (or accompanied) by the disruption of ionic gradients over the plasma membrane. Later in infection, low molecular weight compounds like hygromycin B (hygB)[1], a small non-permeative inhibitor of translation, can also efficiently pass the plasma membrane. At the end of the virus replication cycle, cells are lysed to release virus progeny. Of the morphological modifications, the massive accumulation of small, cytoplasmic membrane vesicles with which the viral replication complexes are associated is the most prominent (6, 7). Both biochemical and microscopical evidence indicates that these vesicles are derived from the secretory pathway (7), and that the 2BC precursor protein plays an important role in the accumulation of these vesicles (8–11).

The enterovirus 2B protein plays a major role in these virus-induced membrane modifications. Expression of the 2B protein or its precursor 2BC is sufficient to release calcium from intracellular stores (*i.e.* ER and Golgi; Ref. 4) and to increase plasma membrane permeability to calcium and low molecular weight compounds (4, 12–16). How the 2B protein modifies membrane permeability is at present largely unknown. In infected cells, the 2B protein is present at the secretory pathway-derived membranes of the replication complex (7, 10, 11). Upon individual expression, the 2B protein localizes at membranes of the ER and Golgi complex (17, 18). This localization pattern suggests that the 2B protein increases membrane permeability of ER and Golgi membranes directly, whereas the permeability of the plasma membrane is increased indirectly, by means of an as yet unknown mechanism. Recently, we demonstrated that mutations that disrupt the ability of 2B to increase ER and Golgi membrane permeability also disrupt the 2B-induced in-

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[1] The abbreviations used are: hygB, hygromycin B; BGM, Buffalo green monkey; ER, endoplasmic reticulum; CLSM, confocal laser scanning microscopy.

TABLE I
Mutagenesis oligonucleotides

Nucleotide sequences of the (antisense) oligonucleotides used for site-directed mutagenesis. The triplets coding for the altered amino acids are underlined and bold.

Mutant	Primer sequence
D61A/D62A	5′-ggcagtcacagtgatgag**tgctgc**gtggttcctcaccac
D61V/D62V	5′-agtcacagtgatcag**caccac**gtggttcctcaccac
D61N/D62N	5′-ggcagtcacagtgatcag**gttatt**gtggtttcgaaccacaattactaaggctga
D61E/D62E	5′-ggcagtcacagtgatgag**ctcttc**gtggttcctcaccac
D61Q/D62Q	5′-ggcagtcacagtgatcag**ctgatg**gtggttcctcaccacaat
D61A	5′-agtcacagtgatcagatc**tgc**gtggttcctcaccac
D61V	5′-agtcacagtgatcaggtc**gac**gtggttcctcaccacaat
D61N	5′-agtcacagtgatcaggtc**gtt**gtggttcctcaccacaat
D61E	5′-agtgatcaggtc**ttc**gtggttcct
D61Q	5′-agtcacagtgatcaggtc**ctg**gtggttcctcaccac
D62A	5′-agtcacagtgatca**gggc**gtcgtggttcctcaccac
D61V	5′-agtcacagtgatcag**gac**gtcgtggttcctcaccac
D62N	5′-ggcagtcacagtgatcag**gtt**atcgtggtttcgaaccacaattactaaggctga
D62E	5′-agtcacagtgatgag**ctc**atcgtggtt
D62Q	5′-ggcagtcacagtgatcag**ctg**atcgtggttcctcaccac
W80A/W82A	5′-tgacaccttctgtttgag**cgc**tcgagccggggacgaggtacaacc
W80Y/W82Y	5′-tgacaccttctgtttgag**ata**ccg**gta**cggggacgaggtacaacc
W80A	5′-ctgtttgagccaccg**cgc**cggcgacgaggtacaaccgat
W82A	5′-caccttctgtttgag**ggc**ccgccacggggacga
R81A/K84A/K86A	5′-gtaatattgtgacac**cgc**ct**gtgcg**agccaggcccacggggacgaggt
R81A	5′-cttctgtttgagcac**ggc**ccacggggacgaggt
K84A	5′-ttgtgacaccttctg**cgc**aagccaccgccacgggga
K86A	5′-gtaatattgtgacac**ggc**ctgtttgagccaccg

crease in plasma membrane permeability, suggesting that these two phenomena are coupled (56). Mutations in 2B that affect its ability to increase membrane permeability interfere with an early step in viral RNA replication (15). It is possible that the membrane-active character of 2B is involved in the accumulation of replication vesicles by the 2BC protein. Virus replication is also affected by mutations in 2B that do not affect its ability to increase membrane permeability (15), arguing for an additional role of 2B in RNA replication.

The enterovirus 2B protein is a small, hydrophobic protein (97–99 amino acids) that was recently classified as a viroporin, a group of virus proteins that modify host cell membrane permeability and share a number of common features (reviewed in Ref. 20). The 2B protein contains two hydrophobic regions, designated HR1 (amino acids 37–54) and HR2 (amino acids 63–80), of which the first is predicted to form a cationic amphipathic α-helix (21, 22). The amphipathic α-helix displays characteristics typical for the group of lytic polypeptides, which may build membrane-integral pores upon the formation of multimeric transmembrane complexes (23–25). Accordingly, the 2B protein was suggested to form multimeric pores in which both HR1 and HR2 span the phospholipid bilayer. Several lines of evidence support this hypothesis. First, homo-multimerization reactions of the 2B protein were demonstrated *in vivo* by yeast and mammalian two-hybrid analysis (26, 27) and fluorescence resonance energy transfer microscopy (28), as well as *in vitro* using a biochemical approach (29). Second, mutations in each of the hydrophobic regions interfere with the ability of the 2B protein to homo-multimerize and to increase membrane permeability (15, 27, 30). Finally, the 2B protein was demonstrated to be an integral membrane protein, and the presence of each of the hydrophobic regions was shown to be required for this integral membrane association and the correct subcellular localization (18).

The structure-function relationship and the membrane architecture of the 2B protein are as yet poorly understood. Our current view is mainly based on the phenotypic characterization of mutations in HR1 and HR2. The functional role of domains upstream, between and downstream of HR1 and HR2, however, has not yet been characterized. Here, we describe the effects of linker insertion and substitution mutations in different regions of the 2B protein on homo-multimerization, increasing membrane permeability, subcellular localization, and virus replication.

EXPERIMENTAL PROCEDURES

Cells and Media—Buffalo green monkey (BGM) cells were grown in minimal essential medium (Invitrogen), supplemented with 10% fetal bovine serum, 100 units penicillin per ml, and 25 μg streptomycin per ml. COS-1 cells were grown in Dulbecco's modified minimal essential medium (Invitrogen), supplemented with 10% fetal bovine serum, 100 units penicillin per ml, and 25 μg streptomycin per ml. Cells were grown at 37 °C in a 5% CO_2 incubator.

Construction of Mutants—Oligonucleotide-directed site-specific mutagenesis was performed using the subgenomic phagemid pALTCB3/2080–4947 (21) with the Altered Sites *in vitro* mutagenesis system, according to the instructions of the manufacturer (Promega). The (antisense) oligonucleotides that were used are depicted in Table I, in which the triplets containing mutations are *underlined* and depicted in *bold*. The mutated fragments were cloned in the infectious cDNA clone pCB3/T7 using SpeI (nt 3837) and BssHII (nt 4238) restriction enzymes. The mutations were confirmed by sequence analysis.

For the linker scanning analysis, mutations were introduced by PCR, and the mutated fragments were cloned in pCB3/T7 (31). In the sequences of the oligonucleotides described below, the restriction sites are shown in italics and the sequence encoding the 9-amino acids linker (a hemagglutinin epitope tag, YPYDVPDYA) is underlined. pCB3/T7–2B(ins (5)linker) was generated using a forward primer that contained an SmaI restriction site (italics) and the linker sequence (underlined) (5′-ggggggg*cccggg*tcaaagattatccgtacgacgtccccgattatgccggagtgaaggactatgtggaa). The reverse primer (5′-cagagatttctcgagctaggagtcttggcccactag, nt 3837) was chosen such that the PCR product contained the unique SpeI restriction site. The PCR product was digested with SmaI and SpeI and cloned in pCB3/T7-StuI(3743) (32) that was digested with StuI and SpeI. pCB3/T7–2B(ins (34)linker) was generated with a forward primer that contained the SpeI restriction site and introduced the linker sequence (5′-ggggg*actagt*gtatccgtacgacgtccccgattatgccggtcaagactccatcttagag) and a reverse primer that contained the *BssH*II restriction site (nt 4246) (5′-ttgggat*ggcgcgc*tctgctc, nt 4230). The PCR product was cloned in pCB3/T7 using SpeI and BssHII. pCB3/T7–2B(ins (61)linker) was generated with a forward primer that contained an SpeI restriction site (5′-gtcaaccggatccaagaatc*actagt*gggtcaa, nt 3844) and a reverse primer that contained an StuI restriction site and the linker sequence (5′-ggggggg*aggcct*ggcataatcggggacgtcgtcggatacaccacaattactaaggctga). The PCR product was digested with SpeI and StuI and cloned in pCB3/T7-HpaI(3915) (32) that was digested with SpeI and HpaI. pCB3/T7–2B(ins (5)linker) was generated with a forward primer that contained an SpeI restriction site (5′-gtcaaccggatccaagaatc*actagt*gggtcaa, nt 3844)

and a reverse primer that contained an ScaI restriction site and the linker sequence (5′-ggggggagtactttggcgttcagccatagggcataatcggggacgtcgtacggataagggattccgtaatattgtga). The PCR product was digested with SpeI and ScaI and cloned in pCB3/T7-HpaI(4042), which was digested with SpeI and HpaI. By consequence, the sequence of the 2B/2C junction is altered from AERQ ↓ N to AERQ ↓ S; however, this sequence is efficiently processed by $3C^{pro}$, and virus replication is not affected by the presence of this sequence at the 2B/2C junction (33). All sequences that were amplified by PCR were verified by sequence analysis.

RNA Transfections and Virus Replication—The effect of the mutations on virus viability was examined by transfection of BGM cells, with RNA transcripts generated from the mutated infectious pCB3/T7 cDNA clones, as described previously (21). After transfection, cells were incubated at 36 °C until cytopathic effect was observed. In case no cytopathic effect was observed after 6 days, the cell cultures were subjected to three cycles of freezing and thawing, passaging to fresh monolayers of BGM cells, and incubation for another 6 days. If no virus replication was observed at 6 days after passage, it was concluded that the mutated proteins did not support virus replication. All viruses that were obtained were subjected to sequence analysis. To ensure that the observed phenotypes were not due to additional mutations in the plasmid DNA, all mutants were generated from two individual mutagenesis reactions and analyzed separately. Single-cycle growth curves were performed as described previously (21). Briefly, monolayers of BGM cells were infected at a multiplicity of infection of 1, incubated at 36 °C, and harvested at 2, 4, 6, and 8 h postinfection. Viruses were released by three cycles of freezing and thawing, and virus titers were determined by titration on BGM cells at 36 °C as described (34). *In vitro* translation reactions demonstrated that defects in virus replication were not caused by alterations in polyprotein processing (data not shown).

Mammalian Two-hybrid Analysis—Homo-multimerization reactions were studied using plasmids pACT, pBIND, and pG5luc of the Checkmate™ Mammalian two-hybrid system (Promega) (27). From the mutant pCB3/T7 plasmids, the NsiI-to-BssHII fragments were cloned into pSP72–2BC (27) and digested with the same enzymes. From the resulting mutant pSP72–2BC constructs, the BamHI-to-SmaI fragments were cloned into pACT-2BC and pBIND-2BC (27) and digested with BamHI and EcoRV. Analysis was performed using pACT-2BC and pBIND-2BC fusion proteins, because the 2BC multimerization reactions always yielded higher luciferase signals, and all the 2B mutants behaved identically in the 2BC and 2B multimerization reactions (27). COS-1 cells in 24-well plates were transfected with the indicated constructs and lysed at 48 h posttransfection, using the FuGENE transfection reagent (Roche Applied Science), as described previously (27). The firefly luciferase and Renilla luciferase enzyme activities were measured from the same cell lysate sample with the Dual-Luciferase® reporter assay system, according to the instructions of the manufacturer (Promega). Luciferase activities were measured in a luminometer (Bio-Orbit 1251). Measurement of the Renilla luciferase production revealed only small differences among different samples from the same experiment. Because these small differences merely reflected variations in the luciferase measurement, we did not normalize for transfection efficiency. Protein expression was confirmed on Western blots stained with anti-Gal 4 DNA-binding domain (data not shown).

Hygromycin B Assay—The ability of 2B to increase plasma membrane permeability to hygB was studied using 2B-EGFP fusion proteins, as described previously (18). Mutant p2B-EGFP plasmids were constructed by amplification of the 2B coding sequences of the mutant pCB3/T7 plasmids. A forward primer was used that contained an SaII restriction site (italics) and a start codon (underlined), preceded by a Kozak sequence (5′-ctcctgtggctggctagc*gtcgac*gccaccatgggagtgaaggactatgtggaa-3′), and a reverse primer that introduced a BamHI restriction site (5′-ccagct*ggatcc*ttggcgttcagccatagg-3′). The PCR product was cloned into pEGFP-N3 (Clontech) and digested with SalI and BamHI to yield mutant p2B-EGFP. All sequences that were amplified by PCR were verified by sequence analysis. BGM cells grown in 6-well plates were transfected in duplicate with plasmids encoding wild-type or mutant 2B-EGFP fusion proteins, using the FuGENE transfection reagent (Roche Applied Science), as described previously (27). At 40 h posttransfection, cells were starved of methionine for 15 min in the presence or absence of 500 μg/ml hygB, pulse-labeled for 1 h with [^{35}S]methionine (50 μCi/well) in the presence or absence of hygB, washed with ice-cold phosphate-buffered saline, and lysed in 1 ml of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 M phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.05% SDS). Protein synthesis was analyzed by SDS-PAGE of immuno-precipitated EGFP fusion proteins. The amount of radiolabeled anti-EGFP-precipitated protein was quantified by using a PhosphorImager (Bio-Rad Multi-Analyst version 1.0.1), and the ratios of the amount of protein synthesized in the presence and absence of hygB was determined. Protein expression was checked on Western blots stained with anti-EGFP (not shown).

Immunofluorescence and Confocal Laser Scanning Microscopy—The subcellular localization of wild-type and mutant 2B proteins was determined as described previously (18). Briefly, BGM cells were grown on coverslips in 24-well plates and transfected with the indicated plasmids using the FuGene transfection reagent, according to the instructions of the manufacturer (Roche Applied Science). At 24 h posttransfection, cells were fixed, stained, and analyzed by confocal laser scanning microscopy (CLSM) using a Leica TCS NT (Leica Lasertechnik GmbH, Heidelberg, Germany). Mouse monoclonal anti-c-Myc (clone 9E10) was obtained from Sigma-Aldrich. Rabbit polyclonal anti-calreticulin was obtained from Affinity Bioreagents, Inc. Fluorescein isothiocyanate-conjugated goat-anti-rabbit polyclonal antibody, Texas Red-conjugated goat-anti-mouse polyclonal antibody, and Texas Red-conjugated goat-anti-rabbit polyclonal antibody were obtained from Jackson ImmunoResearch Laboratories. Primary antibodies were diluted 1:200 (anti-c-Myc) or 1:150 (anti-calreticulin). Conjugates were diluted 1:75.

Statistical Analysis—Data are presented as mean values ± S.D. Differences were tested for significance by means of the Student's *t* test.

RESULTS

Identification of Functional Regions in the 2B Protein—Previously, we demonstrated that both HR1 and HR2 are important determinants for multimerization and increasing membrane permeability (4, 15, 27). Here, we analyzed the functional role of domains upstream, between, and downstream HR1 and HR2 using a linker scanning analysis (Fig. 1*A*). A nine-amino acid linker was introduced in the N terminus of the protein (linker insertion at position 5, ins (5)linker), immediately upstream HR1 (ins (34)linker), in the hydrophilic sequence between HR1 and HR2 (ins (61)linker), and in the C terminus of the protein (ins (94)linker). In mutant ins (5)linker, the first four amino acids of the 2B protein were duplicated to retain the correct environment for the 3C protease to cleave the 2A/2B junction in the viral polyprotein expressed by the infectious cDNA clone. Alternative codons were used for the duplicated amino acid sequences to minimize the chance of recombination events in the viral RNA.

The ability of 2B mutants to form homo-multimers was analyzed using a mammalian two-hybrid system. BGM cells were transfected with fusion proteins containing the activation domain of herpes simplex virus type 1 VP16 or the DNA-binding domain of the yeast GAL4 gene product, and synthesis of the firefly luciferase reporter protein was determined to measure protein-protein interactions. The ability of mutant 2B proteins to increase membrane permeability was studied by determining the entry of hygB, a small inhibitor of translation that, under physiological conditions, poorly passes the plasma membrane. HygB entry was determined by analyzing protein synthesis in cells expressing mutant 2B-EGFP fusion proteins in the absence and presence of the drug.

The ability of the 2B mutant containing a linker between HR1 and HR2 (ins (61)linker) to multimerize and increase hygB entry was drastically reduced (Fig. 1, *B* and *C*). In contrast, the regions upstream of HR1 or downstream of HR2 were found to be of less importance. Mutants carrying linkers immediately upstream HR1 (ins (34)linker) or in the C terminus of the 2B protein (ins (94)linker) showed wild-type multimerization and hygB entry. The presence of the linker in the N terminus of the 2B protein (ins (5)linker) did not interfere with multimerization, yet reduced the ability to increase hygB entry, indicating that the N terminus somehow contributes to the membrane-active character of 2B.

We demonstrated previously that the 2B protein is present predominantly in the Golgi complex and that its Golgi localization is an important prerequisite for its ability to increase plasma membrane permeability to hygB (18). Therefore, it

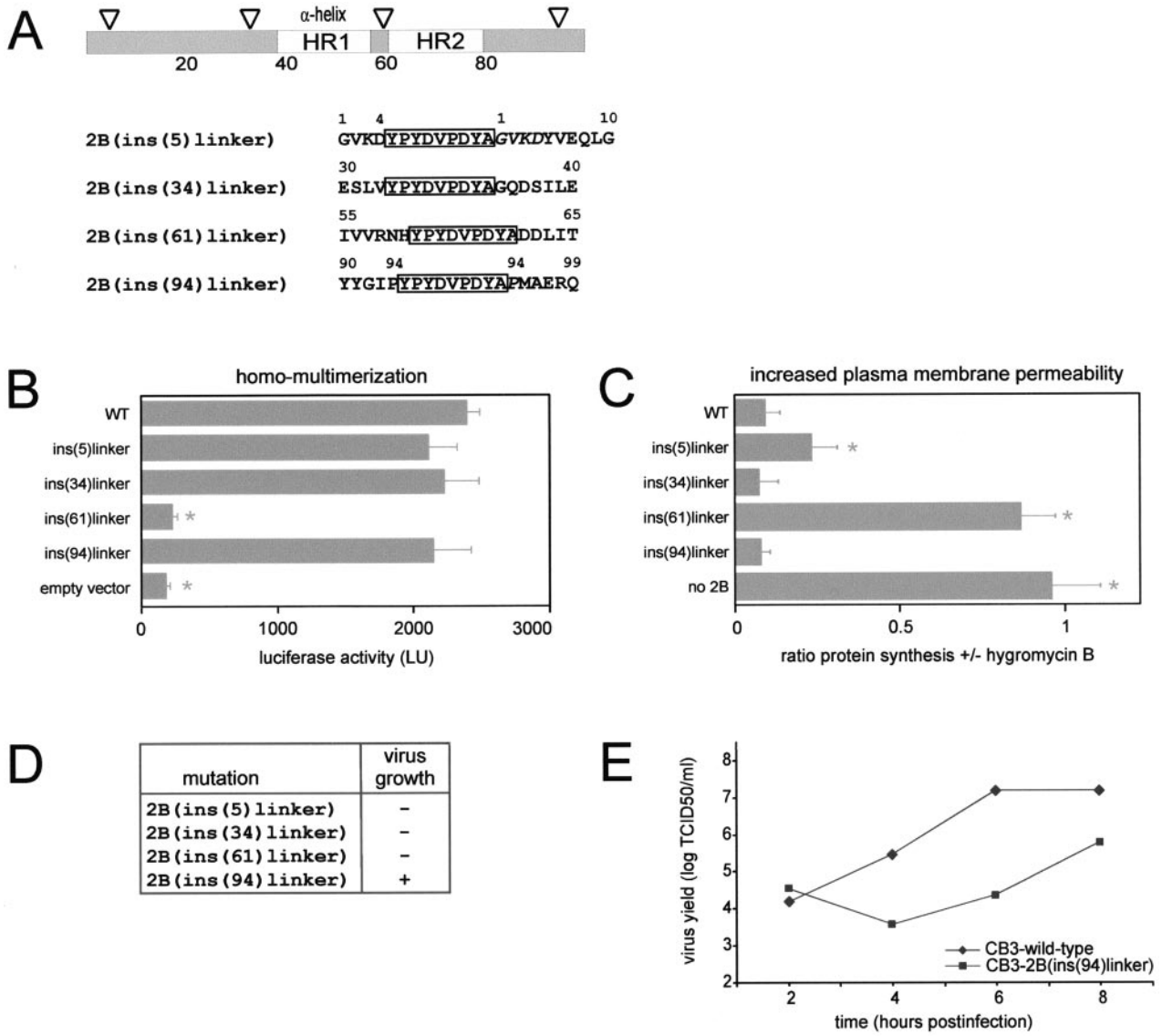


FIG. 1. **Functional characterization of linker insertion mutants.** *A*, schematic representation of the 2B protein of coxsackievirus, the inserted linker (*boxes*), and the amino acid sequences flanking the linker. Amino acids that were duplicated upon insertion of the linker are depicted in *italics*. *Triangles* indicate the positions at which the linker was inserted. *B*, analysis of the effects of mutations on homo-multimerization. Multimerization reactions were analyzed in a mammalian two-hybrid analysis, in which wild-type and mutant 2BC proteins were fused to the yeast GAL4 DNA-binding domain and to the HSV VP16 activation domain. COS-1 cells were transfected with the indicated constructs and assayed for luciferase production at 48 h posttransfection. *LU*, light units. *C*, analysis of the effects of mutations on the membrane-active character of the 2B protein. The ability to increase plasma membrane permeability to hygB was analyzed. BGM cells were transfected with plasmids encoding wild-type or mutant 2B-EGFP. At 40 h posttransfection, cells were pulse-labeled in the absence or presence of hygB, and the ratio of protein synthesis in the absence and presence of hygB was determined by phosphorimaging of the anti-EGFP precipitated proteins. *B* and *C*, values represent means ± S.D. of measurements of three independent experiments. *, $p < 0.005$. *D*, summary of the effects of mutations on virus viability. BGM cells were transfected with wild-type or mutant pCB3/T7-derived RNA transcripts and analyzed for the appearance of cytopathic effect. *E*, single-cycle growth curve of mutant viruses. BGM cells were infected at a multiplicity of infection of 1, incubated at 36 °C, and harvested at 2, 4, 6, and 8 h postinfection. Virus titers were determined by titration on BGM at 36 °C. One representative of three independently performed experiments is shown. *TCID50*, 50% tissue culture infective dose.

should be considered that the failure of 2B mutants to increase hygB entry may be due to an altered subcellular localization. We determined the subcellular localization of 2B mutants by confocal microscopy using markers for the Golgi and the ER. The linker insertions upstream (see Fig. 5), between, and downstream of the hydrophobic regions had no effect on the subcellular localization of 2B; ins (5)linker, ins (34)linker, ins (61)linker, and ins (94)linker all showed clear colocalization with the EGFP-Golgi marker, similar to wild-type 2B. Thus, the failure of the ins (5)linker and ins (61)linker mutants to increase plasma membrane permeability to hygB is not due to an altered localization.

The ability of mutant 2B proteins to support virus replication was investigated by transfection of cells with RNA transcripts derived from the infectious cDNA clone pCB3/T7. Consistent with previous observations that 2B mutants which have lost the ability to increase membrane permeability fail to replicate (15), both the ins (61)linker mutant and the ins (5)linker mutant were found to be nonviable (Fig. 1*D*). Mutations ins (34)linker and ins (94)linker, which had no effect on multimerization and the membrane-active character of 2B, caused defects in virus replication. The ins (34)linker mutation completely disrupted viral replication, whereas the ins (94)linker mutation gave rise to a slower growing virus in which the inserted linker was retained, and no additional mutations were present in the 2B protein (Fig. 1*E*). These latter results argue

that the region immediately upstream from HR1 and the C terminus of 2B are required for a (yet unknown) function of 2B in virus replication that is not connected to its membrane-active character (see Fig. 4*C* for a summary of the results of the linker insertion mutations).

Molecular Requirements of the Hydrophilic Region between HR1 and HR2—The linker insertion mutation in the hydrophilic region between HR1 and HR2 (^{58}RNHDD^{62}) demonstrated the importance of this region for multimerization and the membrane-active character of 2B. The pore-forming model suggests that this region is required to facilitate a reverse turn between the two putative transmembrane domains. The molecular requirements of this sequence were investigated by introducing specific point mutations (Fig. 2*A*). In this study, we focused on the negatively charged amino acid residues, which are conserved among the enteroviruses and rhinoviruses. First, both negatively charged residues (Asp^{61} and Asp^{62}) were replaced by neutral Ala and hydrophobic Val residues. Both the D61A/D62A and the D61V/D62V double mutations abolished multimerization and severely reduced the ability to increase hygB entry (Fig. 2, *B* and *C*), suggesting a preference for hydrophilic residues at these positions. Analysis of the subcellular localization of the D61A/D62A mutant (see Fig. 5*F*) and the D61V/D62V mutant (data not shown) demonstrated that these mutations had no effect on the Golgi localization of the 2B protein. Analysis of the effects of the mutations on virus growth showed that both D61A/D62A and D61V/D62V double mutations abolished the ability of the 2B protein to support virus replication (Fig. 2*D*).

To investigate the charge and length requirement of the amino acid side chains at positions 61 and 62 in more detail, Asp^{61} and Asp^{62} were replaced by residues that have a short, uncharged side chain (Asn, D61N/D62N), residues that have a long, negatively charged side chain (Glu, D61E/D62E), or residues that have a long, uncharged side chain (Gln, D61Q/D62Q) (Fig. 2*A*). Introduction of residues with a long side chain (mutations D61E/D62E and D61Q/D62Q) caused severe defects in multimerization and the ability to increase hygB entry. Mutation D61N/D62N, on the other hand, had little effect on the membrane-active character of 2B, although the ability to multimerize was reduced but not completely abolished (Fig. 2, *B* and *C*). Analysis of the subcellular localization of the D61E/D62E, D61N/D62N, and D61Q/D62Q mutants revealed that these mutations had no effect on the Golgi localization of the 2B protein (not shown). Analysis of the effects of the mutations on virus growth showed that mutations D61E/D62E and D61Q/D62Q caused a dramatic effect on virus replication. Mutation D61Q/D62Q completely abolished virus replication, whereas mutation D61E/D62E caused a quasi-infectious phenotype (which means that the mutation disrupts replication to such an extent that reversions can arise but that no virus progeny can be observed carrying the introduced mutation), yielding virus in which Gln^{62} was changed into the original Asp^{62} (Fig. 2*D*). This revertant virus (containing the D61E genotype) displayed wild-type growth kinetics (Fig. 2*F*). Mutation D61N/D62N did not abolish virus replication and gave rise to a slow growing virus in which the introduced mutations were retained and no additional second-site suppression mutations were present in the 2B protein (Fig. 2*E*). Together, these data indicate that the presence of a shorter side chain at positions 61 and 62 is of greater importance than the presence of a negative charge.

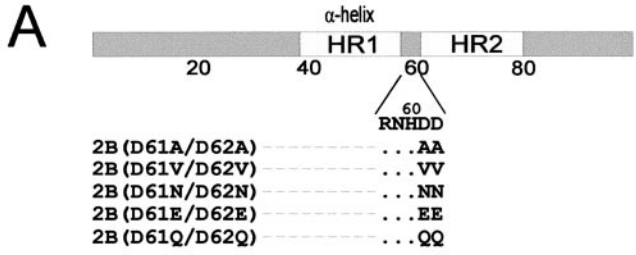
To investigate the functional importance of Asp^{61} and Asp^{62} individually, single mutants D61A and D62A were characterized. Fig. 2, *B* and *C* show that the D62A but not the D61A mutation severely affected multimerization and the membrane-active character of 2B. Consistent with this, the D62A mutation abolished virus growth, whereas the D61A mutation gave rise to viruses with wild-type growth kinetics (Fig. 2, *F* and *G*). These results suggest that the identity of the amino acid at position 62 is of greater importance for the function(s) of 2B than that of the amino acid at position 61. Consistently, other Asp^{62} mutations (D62V, D62N, D62E, and D62Q) caused a more dramatic effect on virus replication than the corresponding Asp^{61} mutations (D61V, D61N, D61E, and D61Q; see Fig. 2, *F–H*). The observation that Asp$^{61/62}$ double mutations caused more severe defects in virus replication than the corresponding Asp^{62} single mutations suggests that Asp^{61} is of importance and argues that there is a synergistic role of Asp^{61} and Asp^{62} in the correct structure/behavior of the hydrophilic region between HR1 and HR2 (see Fig. 4*C* for a summary of the results of the mutations in the hydrophilic loop).

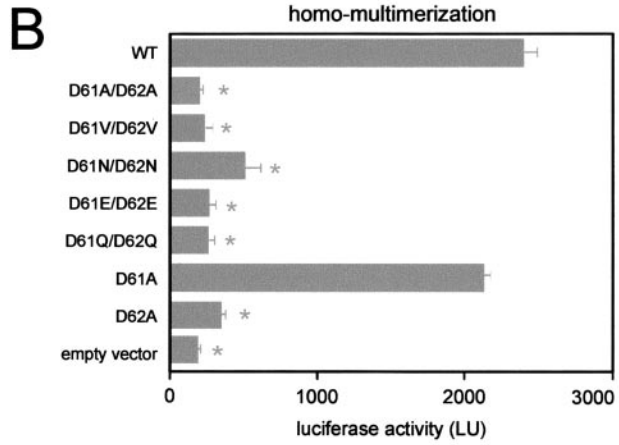
Defining the Boundary of HR2—The second hydrophobic region of 2B was previously defined to be composed of the amino acids 63–80 (21) based upon the method described by Kyte and Doolittle (35). Its C terminus and the region immediately downstream contain a number of aromatic and positively charged amino acids that are well conserved among enterovirus and rhinovirus 2B proteins. In the 2B protein of coxsackievirus B3, this region (^{80}WRWLKQK^{86}) contains (*i*) two tryptophan residues spaced by one amino acid, and (*ii*) three positively charged residues (Fig. 3*A*). The spatial distance between the positively charged residues varies between the different viruses. Tryptophans as well as positively charged residues have been implicated in membrane anchoring of transmembrane peptides (36–38). The presence of aromatic and positively charged residues flanking transmembrane domains is a common feature in the group of viroporins (20), suggesting that these residues are of importance for their membrane-active character. Specific point mutations were introduced at these positions to investigate whether these amino acids contribute to the membrane-active character of 2B and, thus, should be considered to be part of HR2.

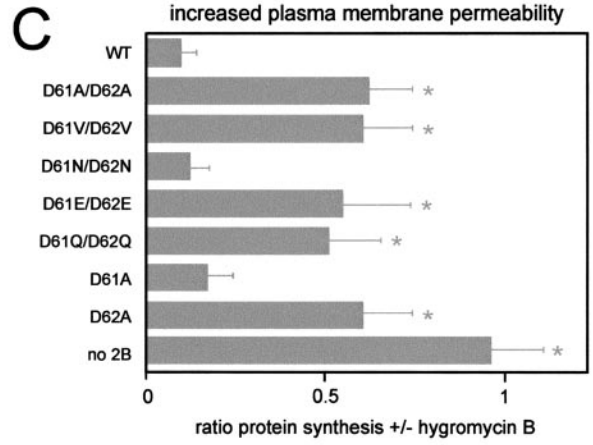
First, Trp^{80} and Trp^{82} were replaced by neutral Ala residues (W80A/W82A). Multimerization was not affected by this mutation; however, the ability of the W80A/W82A mutant to increase plasma membrane permeability to hygB was reduced (Fig. 3, *B* and *C*). This reduction was not due to alterations in the subcellular localization, as the W80A/W82A mutant was present predominantly in the Golgi complex (see Fig. 5*G*). The W80A/W82A mutant failed to support virus replication (Fig. 3*D*). Substitution of both Trp residues by aromatic Tyr residues (W80Y/W82Y) had no effect on multimerization and the ability to increase hygB entry (data not shown), which supports the idea that there is a preference for aromatic residues at positions 80 and 82.

Trp^{80} and Trp^{82} were mutated individually into Ala (W80A/W82A) to investigate the functional importance of the individual amino acids. Both W80A and W82A single mutations had no effect on the ability to increase hygB entry (Fig. 3*C*). Surprisingly, the W80A mutation abolished virus growth, whereas the W82A mutation gave rise to a virus with wild-type growth characteristics (Figs. 3, *D* and *E*). The observation that the W80A/W82A double mutation affects the ability to increase hygB entry, whereas the single mutations have no effect, argues for a synergistic role of the Trp residues in the membrane-active character of 2B.

Substitution of the three positively charged residues by neutral Ala residues (R81A/K84A/K86A) did not affect multimerization or the ability to increase hygB entry (Fig. 3, *B* and *C*). In agreement with this, the mutant 2B protein was localized predominantly in the Golgi complex (see Fig. 5*H*). However, virus replication was abolished by this mutation (Fig. 3*D*),

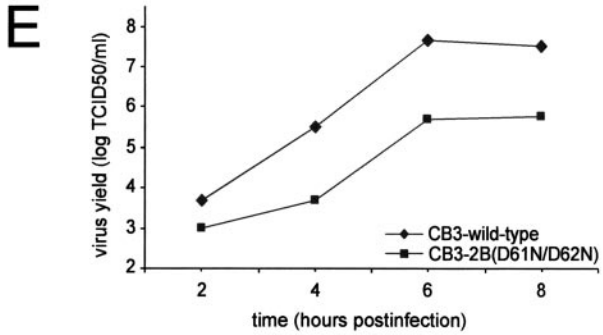






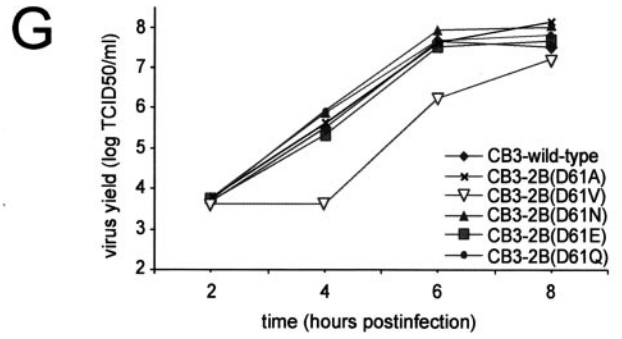
D

mutation	virus growth	revertant
2B (D61A/D62A)	–	
2B (D61V/D62V)	–	
2B (D61N/D62N)	+	
2B (D61E/D62E)	qi	D61E
2B (D61Q/D62Q)	–	



F

mutation	virus growth	mutation	virus growth
2B (D61A)	+	2B (D62A)	–
2B (D61V)	+	2B (D62V)	–
2B (D61N)	+	2B (D62N)	+
2B (D61E)	+	2B (D62E)	+
2B (D61Q)	+	2B (D62Q)	+



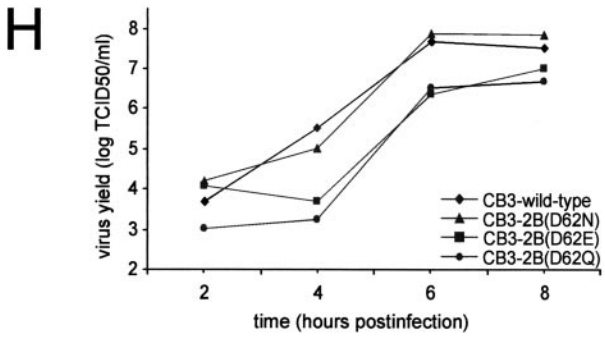


FIG. 2. **Functional characterization of mutants of the hydrophilic region between HR1 and HR2.** *A*, schematic representation of the 2B protein of coxsackievirus. The amino acids present in the region between HR1 and HR2 and the introduced mutations are depicted. *B*, analysis of the effects of the mutations on homo-multimerization of the 2B protein. *C*, analysis of the effects of mutations on the membrane-active character of the 2B protein. *B* and *C*, values represent means ± S.D. of measurements of three independent experiments. *, $p < 0.005$. *D* and *F*, summary of the effects of double mutations (*D*) and single mutations (*F*) on virus viability. *E*, *G*, *H*, single-cycle growth curve of viruses containing double mutations (*E*) or a single mutant (*G*, *H*). One representative of three independently performed experiments is shown. For details, see the legend of Fig. 1.

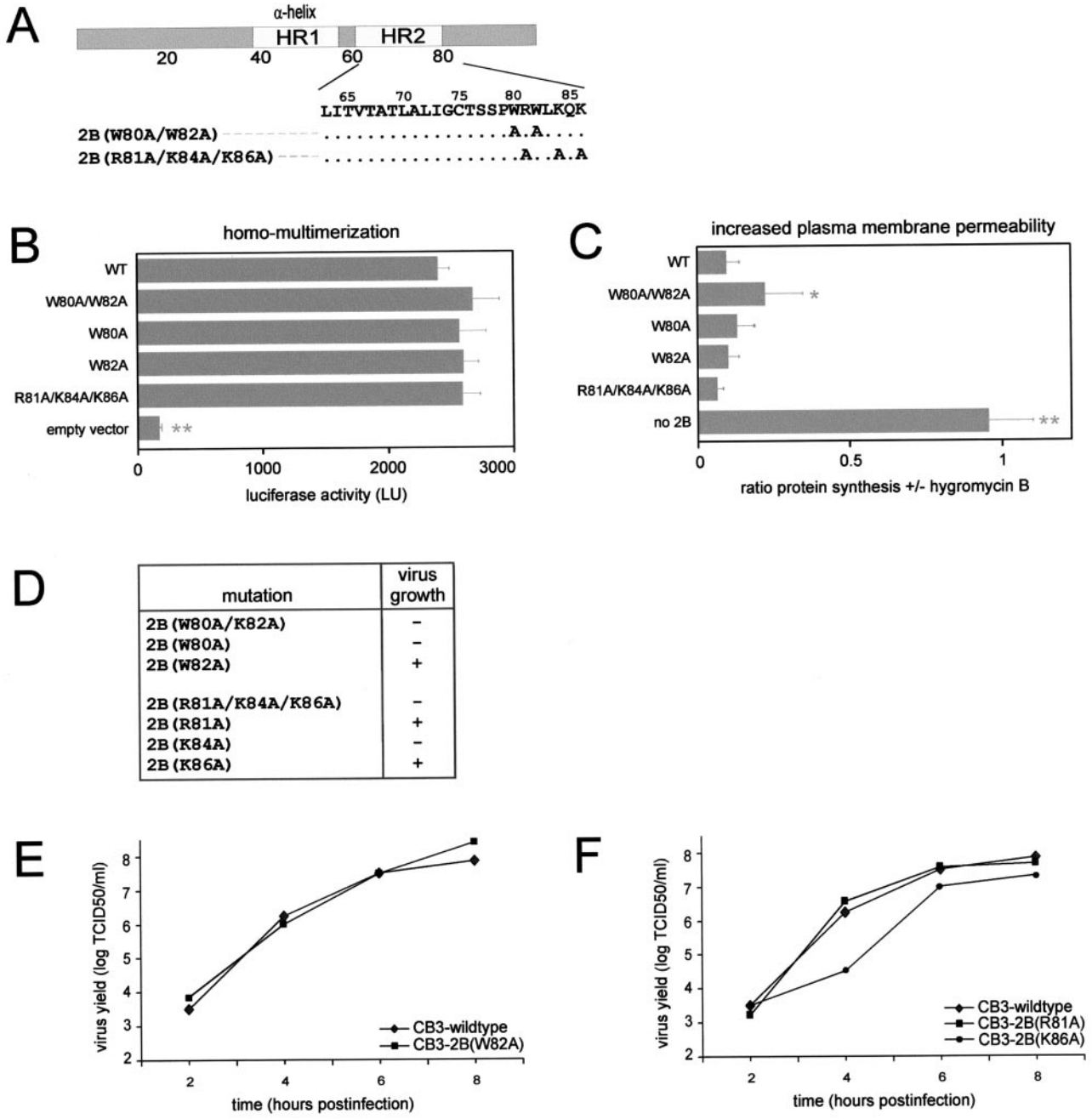
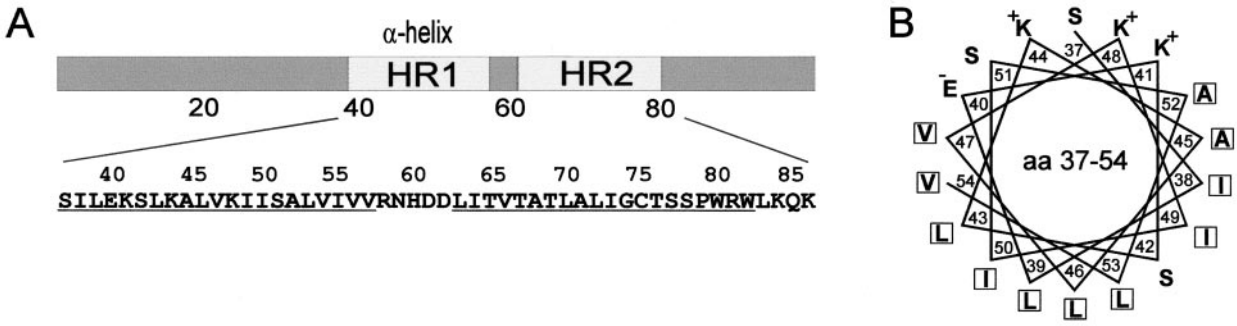


FIG. 3. **Functional characterization of mutants at the boundary of HR2.** *A*, schematic representation of the 2B protein of coxsackievirus. The amino acids present in and downstream of HR2 and the introduced mutations are depicted. *B*, analysis of the effects of the mutations on homo-multimerization of the 2B protein. *C*, analysis of the effects of the mutations on the membrane-active character of the 2B protein. *B* and *C*, values represent means ± S.D. of measurements of three independent experiments. *, $p < 0.01$; **, $p < 0.005$. *D*, summary of the effects of mutations on virus viability. *E* and *F*, single-cycle growth curve of mutant viruses. One representative of three independently performed experiments is shown. For details, see the legend of Fig. 1.

suggesting that these amino acids are involved in a function of 2B in virus replication not connected to its membrane-active character. Analysis of the effects of the individual mutations of Arg^{81}, Lys^{84}, and Lys^{86} on virus replication identified Lys^{84} as the most important of these conserved residues (Fig. 3, *D* and *F*).

Together, these data suggest that Trp^{80} and Trp^{82} are cooperatively involved in the membrane-active character of 2B, possibly through the correct membrane anchoring of HR2, whereas Arg^{81}, Lys^{84}, or Lys^{86} do not contribute to this function. The results of the mutations at the boundary of HR2 are summarized in Fig. 4*C*.

Effects of HR1 and HR2 Mutations on Subcellular Localization of 2B—None of the mutations upstream from HR1, between HR1 and HR2, or downstream from HR2 had any effect on the subcellular localization of the 2B protein, suggesting that the major localization determinants reside in HR1 and HR2. Consistent with this, we previously observed that deletion of either HR1 or HR2 affected the correct localization of 2B in the Golgi complex (18). The specific requirements in HR1 and HR2 for the Golgi localization of 2B, however, have not yet been determined. Here, we studied the localization of a number of previously described HR1 and HR2 mutants (see Fig. 4*C*) to better understand the structural requirements in HR1 and HR2 for Golgi localization. This analysis will also provide us important insight into whether alterations in subcellular localization may be responsible for the failure of these HR1 and



C

	homo-multimerization[a]	increased plasma membrane permeability[b]	subcellular localization	virus replication[c]
Wild-type	++	++	Golgi	++
Linker insertion mutations				
ins(5)linker	++	+	Golgi	–
ins(34)linker	++	++	Golgi	–
ins(61)linker	–	–	Golgi	–
ins(94)linker	++	++	Golgi	+
Mutations in hydrophilic loop				
D61A/D62A	–	–/+	Golgi	–
D61V/D62V	–	–/+	Golgi[f]	–
D61N/D62N	–/+	++	Golgi[f]	+
D61E/D62E	–	–/+	Golgi[f]	–
D61Q/D62Q	–	–/+	Golgi[f]	–
Mutations at boundary of HR2				
W80A/W82A	++	+	Golgi	–
R81A/K84A/K86A	++	++	Golgi	–
Mutations in HR1				
K41E/K44E/K48E	++[d]	–/+[e]	Golgi + ER	–[g]
K41L/K44L/K48L	++[d]	–/+[f]	ER	–[d]
ins(41)L	++[d]	–/+[e]	ER	–[g]
L46N/V47N/I49N/I50N	–[d]	–/+[f]	not Golgi, not ER	–[d]
Mutations in HR2				
C75M/S77M	++[d]	++[e]	Golgi	*qi*[h]
I64S/V66S	–[d]	–[e]	Golgi	–[h]
A71E/I73E	–[d]	–/+[f]	Golgi + ER	–[h]

FIG. 4. **Overview of 2B mutants.** *A*, schematic representation of the 2B protein of coxsackievirus. Amino acid residues 37 to 86 are shown (HR1 and HR2 are underlined). *B*, helical wheel diagram of the cationic amphipathic α-helix formed by HR1. The hydrophobic residues (*boxes*) are positioned at one face of the α-helix; the polar and charged residues are positioned at the other face of the α-helix. *C*, summary of 2B mutants. The mutant genotypes and the corresponding phenotypes are depicted. [a], homo-multimerization is scored ++ when 75–100% luciferase was produced compared with the wild-type; + when luciferase production was 50–75%; −/+ when luciferase production was 25–50%; and − when luciferase was 0–25%. [b], the membrane-active character is scored ++ when protein synthesis in the presence of hygB was reduced by 80–100% compared with that in the absence of hygB; + when protein synthesis was reduced by 50–80%; −/+ when protein synthesis was reduced by 20–50%; and − when protein synthesis was reduced by less than 20%. [c], virus replication is scored ++ when the mutant virus displayed wild-type growth kinetics; + when the mutant virus displayed delayed growth kinetics; and − when virus replication was abolished. *qi*, quasi infectious. [d], previously published, Ref. 27. [e], previously published, Ref. 15. [f], this study (data not shown). [g], previously published, Ref. 22). [h], previously published, Ref. 21.

HR2 mutants to increase plasma membrane permeability to hygB.

The HR1 mutants contain alterations that disrupt the cationic or the amphipathic character of the α-helix. In mutants K41L/K44L/K48L and K41E/K44E/K48E, the three positively charged Lys residues in the small hydrophilic face of the helix (Fig. 4*B*), were replaced by negatively charged Leu residues or hydrophobic Glu residues, respectively. These mutations did

not affect multimerization, yet they inhibited the ability to increase hygB entry (Fig. 4*C*) and severely altered the subcellular localization. The localization of the K41L/K44L/K48L mutant closely resembled that of the 2B(ΔHR1)-Myc protein (18): the mutant protein was virtually absent from the Golgi complex (Fig. 5*I*) but was present predominantly in a reticular pattern that was identified as the ER (Fig. 5*J*). The K41E/K44E/K48E mutant was only partially present in the Golgi complex and also showed staining of the ER (Fig. 5*K*). These data suggest that the failure of these mutants to increase plasma membrane permeability to hygB may indeed be due (partly) to an altered localization. Irrespective of its localization, substitution of the three Leu residues may also result in the formation of nonfunctional multimers that have lost the ability to permeabilize membranes. Mutant ins(41)L contains an insertion of a Leu residue at position 41, which results in the dispersion of the charged residues over the α-helix and thereby a reduced amphipathy (22). This mutation, which had no effect on multimerization yet reduced the ability to increase hygB entry, did not affect its subcellular localization (Fig. 5*L*). In mutant L46N/V47N/I49N/I50N, four hydrophobic residues were replaced by hydrophilic Asn residues, thereby affecting the hydrophobic backbone of HR1. This mutation abolished multimerization and the ability to increase hygB entry, and drastically altered the subcellular localization of 2B. The L46N/V47N/I49N/I50N mutant was absent from both the Golgi complex (Fig. 5*M*) and the ER (Fig. 5*N*). Together, these results point to the importance of both the cationic residues and the overall hydrophobic character of HR1 for the Golgi localization of 2B.

In the HR2 mutants, the overall hydrophobic character of this region is altered. Mutation C75M/S77M, which increases the overall hydrophobicity of HR2 through the substitution of polar residues by more hydrophobic Met residues (22), did not affect multimerization and the ability to increase hygB entry. The C75M/S77M mutant was present predominantly in the Golgi complex (Fig. 5*O*). Mutation I64S/V66S, which reduces the overall hydrophobicity of HR2 through the introduction of polar Ser residues (22), abolished multimerization and the ability to increase hygB entry. The I64S/V66S mutant protein was present predominantly in the Golgi complex (Fig. 5*P*), suggesting that its failure to increase hygB entry is not related to its subcellular localization. Moreover, this results indicates that multimerization of 2B is not required for its Golgi localization. Mutation A71E/I73E, which more severely disturbs the overall hydrophobicity of HR2 through the introduction of negatively charged Glu residues (22), also abolished multimerization and the ability to increase hygB entry. The subcellular localization of the A71E/I73E mutant was altered and closely resembled that of the 2B(ΔHR2)-Myc protein (18): the protein was present in the Golgi complex, but also showed significant staining of the ER network (Fig. 5*Q*). The altered subcellular localization of the A71E/I73E mutant may be (partly) responsible for its failure to increase hygB entry. Moreover, this finding indicates that, in addition to determinants present in the cationic amphipathic α-helix, the overall hydrophobic character of HR2 is required for the subcellular localization of 2B.

DISCUSSION

The enterovirus 2B protein was recently classified as a viroporin, which is a group of virus proteins that modify membrane permeability, and is the first viroporin of a non-enveloped virus (reviewed in Ref. 20). Expression of the enterovirus 2B protein, a small hydrophobic protein that localizes at ER and Golgi membranes, results in an increased permeability of the ER and Golgi membranes, and (most likely as a downstream consequence) an increased permeability of the plasma membrane to ions and low molecular weight compounds like hygB. The molecular mechanism used by the 2B protein to increase membrane permeability is as yet poorly understood. Growing evidence suggests that multimers of 2B build transmembrane pores, in which each of the hydrophobic regions, HR1 and HR2, span the phospholipid bilayer (26–29). According to the hypothesized pore model, the short hydrophilic sequence between the transmembrane domains is required to facilitate a turn to reverse chain direction, a common structural motif in membrane-permeating structures (39, 40). Reverse turns are mostly composed of polar residues (41) and display a preference for small polar amino acids (42, 43). The studies presented here show that the negatively charged Asp residues in the short hydrophilic sequence between HR1 and HR2 are indeed an important determinant for the membrane-active character of the 2B protein. Substitution of both Asp^{61} and Asp^{62} residues by neutral Ala residues or hydrophobic Val residues abolished multimerization and the membrane-active character of 2B, suggesting the requirement for hydrophilic residues. Further analysis demonstrated the preference for the small polar Asp and Asn residues at amino acid positions 61 and 62 over the larger Glu and Gln residues. Both Asp and Asn residues, and not Glu and Gln, have been implicated in the formation of reverse turns, possibly because these amino acids easily form hydrogen bonds with the peptide backbone (44, 45). Together, these data indicate that the hydrophilic region between HR1 and HR2 indeed facilitates a reverse turn, and they suggest that 2B adopts a helix-loop-helix structure, a commonly found structural feature in membrane-active proteins (29, 46).

A number of aromatic and positively charged amino acid residues are present at the C terminus of HR2. These residues are highly conserved among all enterovirus and rhinovirus 2B proteins. The presence of aromatic and positively charged residues immediately downstream from hydrophobic domains is a common feature of viroporins (20), suggesting that these residues may constitute an important functional membrane-active determinant. These amino acid residues are preferably present at the lipid-water interface and are suggested to play a role in the precise location of transmembrane segments in the lipid bilayer, to stabilize proteins in the lipid bilayer, and to play a role in proper functioning of membrane proteins (36, 38, 47–50). In our studies of the putative importance of these residues for multimerization and the membrane-active character of 2B, we found no evidence for a role of the positively charged residues Arg^{81}, Lys^{84}, and Lys^{86}, suggesting that these residues do not play a role in the interaction of 2B with membranes. In contrast, we did observe a cooperative role for the aromatic Trp^{80} and Trp^{82} residues. These residues were found to contribute to the membrane-active character of 2B, without being required for multimerization. This finding suggests that the interaction of these residues with the phospholipid bilayer is involved in correct positioning of HR2 in the membrane, thereby contributing to the overall pore architecture of 2B. This finding defines the Trp residues as the downstream boundary of HR2.

The possible contribution of the regions upstream of HR1 and downstream of HR2 in pore formation is at present poorly understood. We found that the linker insertion in the C-terminal region of 2B had no effect on multimerization and the ability to increase hygB entry, which is consistent with the previous observation that the deletion of the C-terminal 15 amino acids had no adverse effect on the membrane-active character of 2B (18). Linker insertion at position 34 had no effect on multimerization and the membrane-active character of 2B, suggesting that the region immediately upstream of HR1 is not involved in formation and functioning of the pore. Inter-

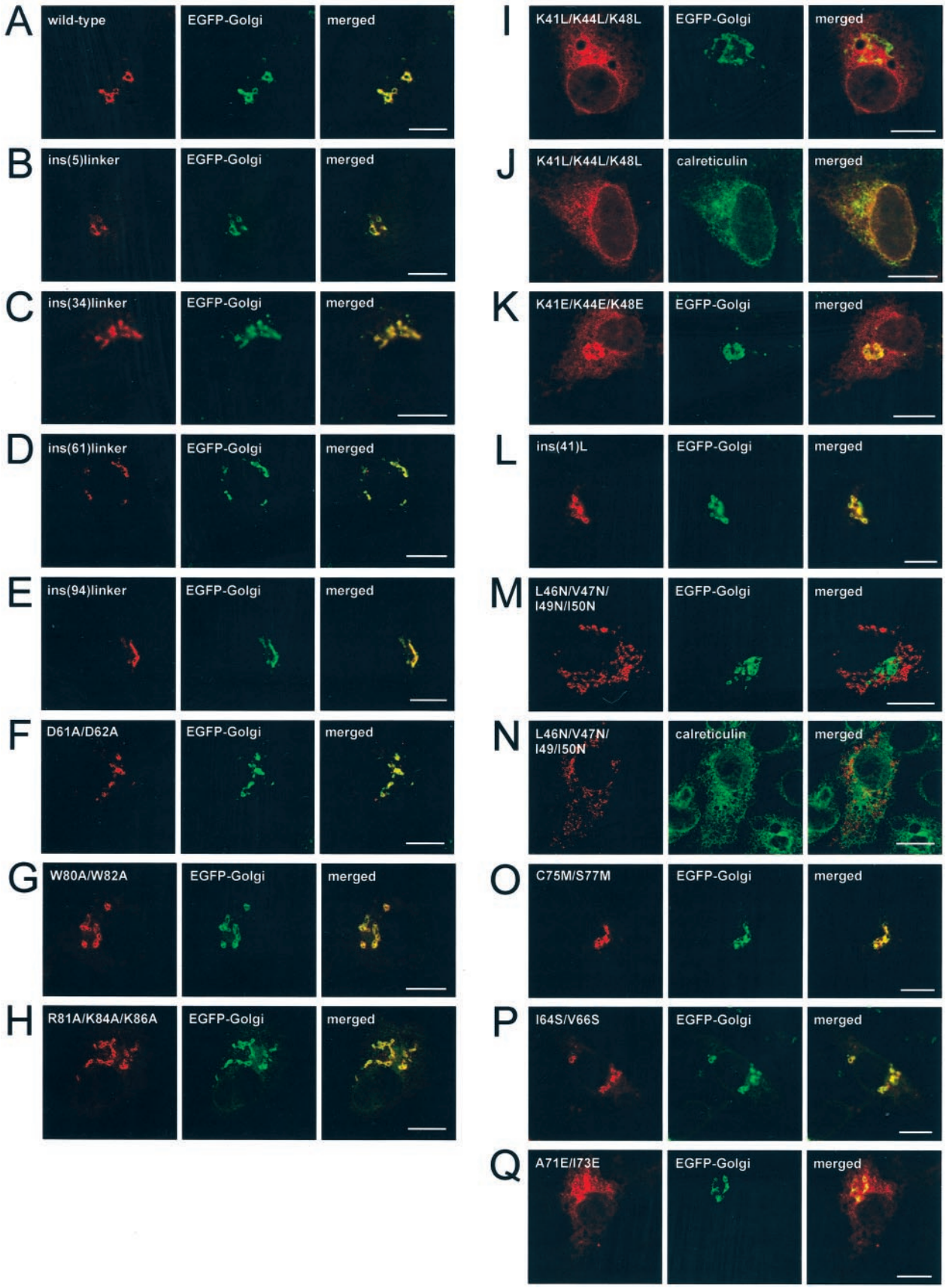


FIG. 5. **Subcellular localization of 2B mutants.** *A–I*, *K–M*, *O–Q*, cells were co-transfected with the indicated mutant 2B-Myc construct and EGFP-Golgi, fixed at 24 h posttransfection, and stained with the anti-c-Myc antiserum. *J* and *N*, cells were transfected with the indicated mutant 2B-Myc construct, fixed at 24 h posttransfection, and stained with the anti-c-Myc and anti-calreticulin antisera. Cells were analyzed by CLSM. *Bar*, 10 μm.

estingly, we did observe a role for the extreme N terminus in the membrane-active character of 2B. Insertion of a linker at position 5 reduced the membrane-active character of 2B without affecting multimerization, similar to that observed for an N-terminal deletion mutant of 2B (18, 27). This finding is the first indication that regions other than HR1 and HR2 may be involved in pore function. How the N terminus contributes to the membrane-active character of 2B, a feature that is difficult to explain in light of the pore formation by HR1 and HR2, requires further investigation.

Analysis of the subcellular localization of the different linker insertion and substitution mutants showed that the Golgi localization of the 2B protein does not rely on regions other than HR1 and HR2, yet depends on specific determinants within these hydrophobic regions. Some 2B mutants carrying alterations in HR2 (I64S/V66S) or the hydrophilic region between HR1 and HR2 (D61A/D62A) showed a wild-type Golgi localization, although these mutations interfered with multimerization. Multimerization is suggested to be involved in the subcellular localization of a number of Golgi resident proteins (51, 52). Our results argue that multimerization of 2B is not required for its targeting and retention in the Golgi complex. The importance of the Golgi localization for the function of 2B (18) is demonstrated by the observation that all mutants that increased plasma membrane permeability to hygB were indeed localized in the Golgi complex. How the 2B protein is able to modify plasma membrane permeability while being localized in the Golgi complex is as yet unknown.

How does the formation of pores by the 2B protein contribute to the virus life cycle? The data presented here suggest that pore formation is required for efficient replication. Based on their effect on the membrane-active character of 2B, mutations that caused a lethal phenotype can be divided into two groups. The largest group consists of mutations that abolished the ability to multimerize and increase membrane permeability. In addition, this group contains the ins (5)linker and W80A/W82A mutations, which did not affect multimerization, yet reduced (but not abolished) the membrane-active character of 2B. Our results demonstrate that all mutations that interfered with the membrane-active character of 2B impaired virus replication. Previous reports (21, 22, 53) demonstrated that the reduction in virus replication of 2B mutants was caused by defects early in RNA replication, suggesting that the membrane-active character of 2B is required for an early step in virus replication, presumably for creating the correct environment required for viral RNA replication. One possibility is that the alterations in ER and Golgi membrane permeability (4, 56) contribute to the 2BC-induced accumulation of the anterograde membrane vesicles at which viral RNA replication takes place (8, 11). The accumulation of these transport vesicles may be the result of inhibition of membrane fusion. It is tempting to speculate that 2B-induced alterations in organelle Ca^{2+}-homeostasis are involved in this process, possibly by interfering with Ca^{2+}-dependent membrane fusion events (54).

The second group of mutations, containing ins (34)linker, ins (94)linker, and R81A/K84A/K86A, did not affect multimerization and the ability to increase membrane permeability, yet inhibited virus replication. These data suggest that the 2B protein is involved in more than one function in virus genome replication, possibly through the interaction with other viral or host cell proteins. In this view, it is interesting to note that it was demonstrated recently (55) that severe replication defects of poliovirus genomes containing a poliovirus/human rhinovirus chimeric 3AB protein could be rescued by a compensating Thr^{33}→Ile mutation in the 2B protein. This observation suggests that a functional interaction between 2B(C) and 3A(B) may exist, and that this interaction is involved in some step of viral replication. Whether the 2B(C)-3A(B) interaction is involved in the accumulation of membrane vesicles (19) or in a different function in replication requires further investigation.

The studies presented here provide new insights into the molecular determinants for the membrane-active character of the 2B protein and lend support to the hypothesis that 2B multimers form transmembrane pores by adopting a helix-loop-helix conformation. Further investigations are required to understand how the membrane-active character of 2B is involved in viral RNA replication, and to gain further insight into the additional function of 2B in viral replication that is not connected to its membrane-active character.

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REFERENCES

1. Wimmer, E., Hellen, C. U., and Cao, X. (1993) *Annu. Rev. Genet.* **27,** 353–436
2. Porter, A. G. (1993) *J. Virol.* **67,** 6917–6921
3. Carrasco, L., Otero, M. J., and Castrillo, J. L. (1989) *Pharmacol. Ther.* **40,** 171–212
4. Van Kuppeveld, F. J. M., Hoenderop, J. G. J., Smeets, R. L. L., Willems, P. H. G. M., Dijkman, H. B. P. M., Galama, J. M. D., and Melchers, W. J. G. (1997) *EMBO J.* **16,** 3519–3532
5. Carrasco, L. (1995) *Adv. Virus Res.* **45,** 61–112
6. Bienz, K., Egger, D., and Pfister, T. (1994) *Arch. Virol. Suppl.* **9,** 147–157
7. Schlegel, A., Giddings, T. H., Ladinsky, M. S., and Kirkegaard, K. (1996) *J. Virol.* **70,** 6576–6588
8. Cho, M. W., Teterina, N., Egger, D., Bienz, K., and Ehrenfeld, E. (1994) *Virology* **202,** 129–145
9. Aldabe, R., and Carrasco, L. (1995) *Biochem. Biophys. Res. Commun.* **206,** 64–76
10. Bienz, K., Egger, D., and Pasamontes, L. (1987) *Virology* **160,** 220–226
11. Rust, R. C., Landmann, L., Gosert, R., Tang, B. L., Hong, W., Hauri, H. P., Egger, D., and Bienz, K. (2001) *J. Virol.* **75,** 9808–9818
12. Lama, J., and Carrasco, L. (1992) *J. Biol. Chem.* **267,** 15932–15937
13. Doedens, J. R., and Kirkegaard, K. (1995) *EMBO J.* **14,** 894–907
14. Aldabe, R., Barco, A., and Carrasco, L. (1996) *J. Biol. Chem.* **271,** 23134–23137
15. Van Kuppeveld, F. J. M., Melchers, W. J. G., Kirkegaard, K., and Doedens, J. R. (1997) *Virology* **227,** 111–118
16. Aldabe, R., Irurzun, A., and Carrasco, L. (1997) *J. Virol.* **71,** 6214–6217
17. Sandoval, I. V., and Carrasco, L. (1997) *J. Virol.* **71,** 4679–4693
18. De Jong, A. S., Wessels, E., Dijkman, H. B., Galama, J. M., Melchers, W. J., Willems, P. H., and Van Kuppeveld, F. J. (2003) *J. Biol. Chem.* **278,** 1012–1021
19. Suhy, D. A., Giddings, T. H., and Kirkegaard, K. (2000) *J. Virol.* **74,** 8953–8965
20. Gonzalez, M. E., and Carrasco, L. (2003) *FEBS Lett.* **552,** 28–34
21. Van Kuppeveld, F. J. M., Galama, J. M. D., Zoll, J., and Melchers, W. J. G. (1995) *J. Virol.* **69,** 7782–7790
22. Van Kuppeveld, F. J. M., Galama, J. M. D., Zoll, J., van den Hurk, P. J. J. C., and Melchers, W. J. G. (1996) *J. Virol.* **70,** 3876–3886
23. Segrest, J. P., De-Loof, H., Dohlman, J. G., Brouillette, C. G., and Anantharamaiah, G. M. (1990) *Proteins* **8,** 103–117
24. Shai, Y. (1995) *Trends. Biochem. Sci.* **20,** 460–464
25. Shai, Y. (1999) *Biochim. Biophys. Acta* **1462,** 55–70
26. Cuconati, A., Xiang, W., Lahser, F., Pfister, T., and Wimmer, E. (1998) *J. Virol.* **72,** 1297–1307
27. De Jong, A. S., Schrama, I. W. J., Willems, P. H. G. M., Galama, J. M. D., Melchers, W. J. G., and Van Kuppeveld, F. J. M. (2002) *J. Gen. Virol.* **83,** 783–793
28. Van Kuppeveld, F. J. M., Melchers, W. J. G., Willems, P. H. G. M., and Gadella, T. W. J. (2002) *J. Virol.* **76,** 9446–9456
29. Agirre, A., Barco, A., Carrasco, L., and Nieva, J. L. (2002) *J. Biol. Chem.* **277,** 40434–40441
30. Barco, A., and Carrasco, L. (1998) *J. Virol.* **72,** 3560–3570
31. Klump, W. M., Bergmann, I., Muller, B. C., Ameis, D., and Kandolf, R. (1990) *J. Virol.* **64,** 1573–1583
32. Van Kuppeveld, F. J. M., van den Hurk, P. J. J. C., van der Vliet, W., Galama, J. M. D., and Melchers, W. J. G. (1997) *J. Gen. Virol.* **78,** 1833–1840
33. Van Kuppeveld, F. J. M., van den Hurk, P. J. J. C., Zoll, J., Galama, J. M. D., and Melchers, W. J. G. (1996) *J. Virol.* **70,** 7632–7640
34. Reed, L. J., and Muench, H. (1938) *Am. J. Hyg.* **27,** 493–497
35. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132
36. Persson, S., Killian, J. A., and Lindblom, G. (1998) *Biophys. J.* **75,** 1365–1371
37. de Planque, M. R., Bonev, B. B., Demmers, J. A., Greathouse, D. V., Koeppe, R. E., Separovic, F., Watts, A., and Killian, J. A. (2003) *Biochemistry* **42,** 5341–5348
38. Matsuzaki, K. (1999) *Biochim. Biophys. Acta* **1462,** 1–10
39. Fu, D., Libson, A., Miercke, L. J., Weitzman, C., Nollert, P., Krucinski, J., and Stroud, R. M. (2000) *Science* **290,** 481–486
40. Nieva, J. L., Agirre, A., Nir, S., and Carrasco, L. (2003) *FEBS Lett.* **552,** 68–73

41. Rose, G. D. (1978) *Nature* **272,** 586–590
42. Chou, P. Y., and Fasman, G. D. (1974) *Biochemistry* **13,** 222–245
43. Gunasekaran, K., Ramakrishnan, C., and Balaram, P. (1997) *Protein Eng.* **10,** 1131–1141
44. Vijayakumar, M., Qian, H., and Zhou, H. X. (1999) *Proteins* **34,** 497–507
45. Northey, J. G., Maxwell, K. L., and Davidson, A. R. (2002) *J. Mol. Biol.* **320,** 389–402
46. Ibrahim, H. R., Thomas, U., and Pellegrini, A. (2001) *J. Biol. Chem.* **276,** 43767–43774
47. Jacobs, R. E., and White, S. H. (1989) *Biochemistry* **28,** 3421–3437
48. de Planque, M. R., Boots, J. W., Rijkers, D. T., Liskamp, R. M., Greathouse, D. V., and Killian, J. A. (2002) *Biochemistry* **41,** 8396–8404
49. Strandberg, E., and Killian, J. A. (2003) *FEBS Lett.* **544,** 69–73
50. Binder, H., and Lindblom, G. (2003) *Biophys. J.* **85,** 982–995
51. Nilsson, T., Slusarewicz, P., Hoe, M. H., and Warren, G. (1993) *FEBS Lett.* **330,** 1–4
52. Nilsson, T., Rabouille, C., Hui, N., Watson, R., and Warren, G. (1996) *J. Cell Sci.* **109,** Pt 7, 1975–1989
53. Johnson, K. L., and Sarnow, P. (1991) *J. Virol.* **65,** 4341–4349
54. Burgoyne, R. D., and Clague, M. J. (2003) *Biochim. Biophys. Acta* **1641,** 137–143
55. Towner, J. S., Brown, D. M., Nguyen, J. H., and Semler, B. L. (2003) *Virology* **314,** 432–442
56. Campanella, M., de Jong, A. S., Lanke, K. W. H., Melchers, W. J. G., Willems, P. H. G. M., Pinton, P., Rizzuto, R., and Van Kuppeveld, F. J. M. (2004) *J. Biol. Chem.* **279,** 18440–18450