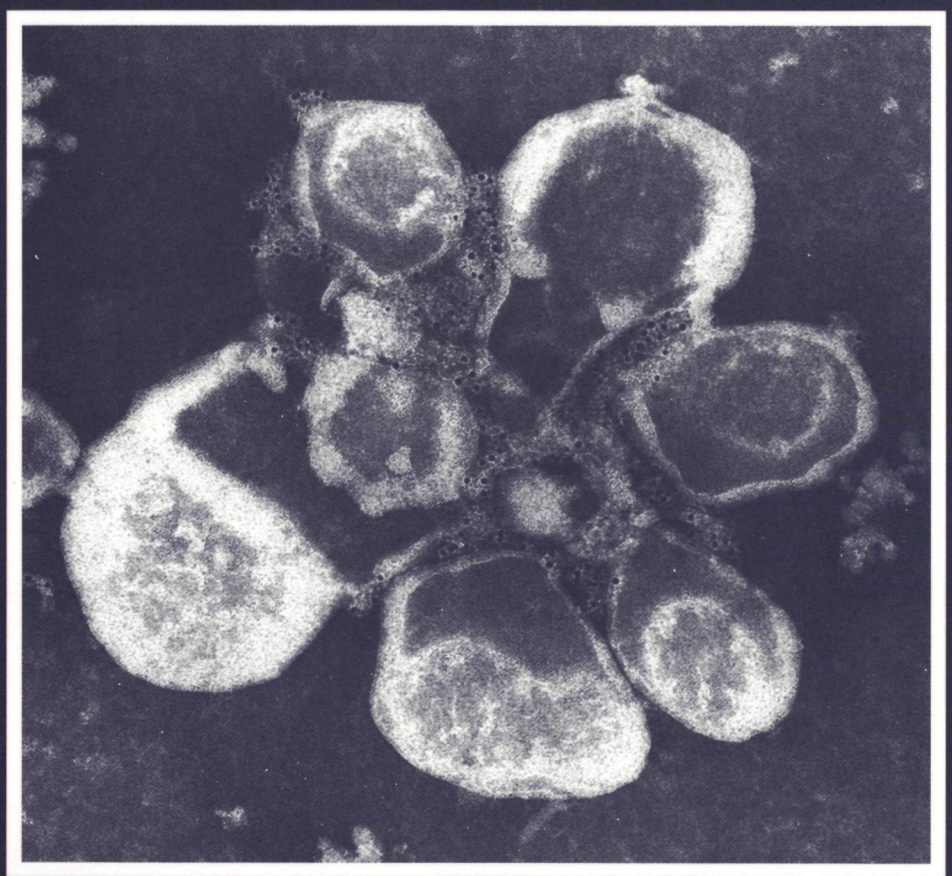
Structural and Functional Analysis of Coxsackievirus Protein 2B

a molecular genetic approach



Frank J. M. van Kuppeveld

Structural and Functional Analysis of Coxsackievirus Protein 2B

a molecular genetic approach

een wetenschappelijke proeve op het gebied
van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
donderdag 13 maart 1997
des namiddags om 1.30 uur precies

door

Franciscus Johannes Maria van Kuppeveld

geboren op 25 augustus 1965
te Oss

Promotor:	Prof. Dr. J.A.A. Hoogkamp-Korstanje
Co-promotores:	Dr. W.J.G. Melchers Dr. J.M.D. Galama
Manuscriptcommissie:	Prof. Dr. J.J.H.H.M. de Pont Prof. Dr. P.J.M. Rottier (UU) Prof. Dr. W.J.M. Spaan (RUL)

Cover illustration:
Electron microscopic photograph of the enteroviral replication complex containing protein 2B at the outer surface of the virus-induced membrane vesicles. Protein 2B was visualized by immuno-cytochemical labeling with antibodies against 2B and colloidal gold. The photograph was kindly provided by Dr. K. Bienz, University of Basel, Switserland.

The research presented in this thesis was carried out at the Department of Medical Microbiology of the University of Nijmegen, The Netherlands

CIP Data, Koninklijke Bibliotheek, Den Haag

van Kuppeveld, Franciscus Johannes Maria

Structural and functional analysis of coxsackievirus protein 2B /
Franciscus Johannes Maria van Kuppeveld. - [S.l. : s.n.]. - Ill.
Thesis Katholieke Universiteit Nijmegen. - With ref. - With summary in Dutch.
ISBN 90-9010179-9
Subject headings: enterovirus / RNA replication / virus-host interactions

1997
Offset: Ponsen & Looijen b.v.

2B or not 2B, that is the question

vrij vertaald naar William Shakespeare (1564 - 1616)

Contents

Preface

Enteroviruses are common human pathogens that usually cause an acute infection. These viruses may, however, be involved in chronic diseases such as polymyositis/dermatomyositis, idiopathic dilated cardiomyopathy, and juvenile (type I) diabetes mellitus. Two different mechanisms have been proposed to explain an involvement of enteroviruses in the etiology of these chronic diseases;

1) The virus may cause a persistent infection;
2) The virus may induce an autoimmune process in the organs or tissues affected.

Both possibilities are under investigation at the Virology Section of the Department of Medical Microbiology, University Hospital Nijmegen.

Usually, enterovirus reproduction results in cell death within a short time. This excludes a chronic course of infection. A persistent infection may therefore reflect an alteration in virus reproduction. The process of viral replication itself and the alterations in cellular functions and structures that take place during virus reproduction are still incompletely understood. This thesis was undertaken to gain more insight into these issues. As an approach, the structure and function of one of the viral proteins involved in virus reproduction was investigated.

General Introduction

During the enteroviral life cycle, a number of complex activities must be performed and coordinated These include receptor binding, cell entry, virion uncoating, synthesis and proteolytic processing of a polyprotein, replication of the plus-strand RNA via a minus-strand intermediate, virion assembly, and cell lysis During infection, enteroviruses induce a number of alterations in cellular functions and structures, most of which serve to facilitate viral replication In this chapter, an overview will be given of the genetic organization of the viral genome, the replication cycle, the virus-induced modifications of host cell functions and structures, and the functional roles of the nonstructural proteins in virus reproduction

GENETIC ORGANIZATION OF THE VIRAL GENOME

The genus *Enterovirus* belongs to the family of the *Picornaviridae*, a group of small, nonenveloped RNA viruses that contain a single-stranded RNA genome of about 7,500 to 8,000 nucleotides Other viruses that belong to this family are the rhinoviruses, cardioviruses, aphtoviruses, and hepatovirus Replication and genome organization of the enteroviruses and rhinoviruses appear to be basically similar, but genomic organization of the cardioviruses, aphtoviruses, and hepatovirus differs in a number of aspects (47)

The genus *Enterovirus* consists of polioviruses, coxsackie group A and B viruses, ECHO viruses, and several distinct enterovirus serotypes Despite their great divergence in tissue tropism and in disease syndromes they produce, these viruses share a striking similarity in virion structure, gene organization, and replication cycle Enteroviruses are possibly the best known of all viruses due to the extensive studies on poliovirus (77) Due to the advances in molecular biology much has been learnt during the last 15 years about the structure, molecular organization and expression of the enteroviral RNA genome Milestones in enterovirus genetic research were the cloning of a poliovirus cDNA and the elucidation of its genetic map (36, 62), and the finding that cDNAs and copy RNA transcripts were infectious when transfected into cultured mammalian cells (63, 75), discoveries that created the possibility to construct and test genetically manipulated RNA genomes A genetic map that is typical of all enteroviruses is shown in Fig 1 The enteroviral RNA (± 7,500 nucleotides) is of plus-strand polarity and is thereby equivalent to cellular mRNAs A viral protein VPg (virion protein, genome linked) is covalently attached to the 5' end and a genetically encoded polyadenosine tail is present at the 3' end of the RNA The large open reading frame is preceded by an unusually long 5' noncoding region and followed by a short 3' noncoding region

The 5' noncoding region contains about 750 nucleotides upstream of the AUG codon used for the initiation of translation in vivo This region is highly structured and possesses multiple noninitiating AUGs Genetic studies suggest that the higher order structures in this region are required for functions in replication, translation, and neurovirulence Mutational analysis and chemical and enzymatic probing of the first 100 nucleotides have shown that a cloverleaf secondary structure is crucial for viral replication and that such a structure is functional in plus-strand RNA synthesis (2) Furthermore, this region harbors a novel *cis*-acting genetic element called the internal ribosome entry site (IRES) This element, which is composed of multiple cloverleaf and stem-loop structures, has been identified in all picornavirus genera and drives internal initiation of translation by allowing a cap- and 5'-independent entry of ribosomes (33, 57) Mutations in this element have been found to attenuate neurovirulence, possibly due to their effect on translational efficiency (70, 71) Attenuated poliovirus strains have been successfully applied as vaccines in the fight against poliomyelitis (68)

The viral RNA is monocistronic and directs the synthesis of a polyprotein, a large polypeptide of about 250 kDa that is proteolytically processed to yield individual proteins with different functions The polyprotein encoding region can be divided into three segments The P1 region, the 5' terminal segment of the viral coding region, encodes the four structural capsid proteins (VP4, VP2, VP3, and VP1, from 5' to 3') that were identified in purified virions The P2 region, the middle segment, encodes three nonstructural proteins, 2A, 2B, and 2C The P3 region encompasses the carboxy-terminal segment of the coding region The nonstructural gene products of this region are protein 3A, 3B, 3C, and 3D For the release of these proteins, enteroviruses do not rely on cellular proteolytic activities but, instead, encode their own proteinases Synthesis of a polyprotein has the advantage that genetic elements specifying the regulation of transcription of different genetic units are unnecessary and allows the compaction of the RNA genome To optimally benefit the minimum of genetic information, most viral proteins have multiple functions The effective genetic content is further enlarged by the use of processing intermediates that have functions in RNA replication distinct from those of their cleavage products Unlike minus-strand RNA viruses, overlapping open reading frames are not exploited

The 3' noncoding region consists of approximately 70 nucleotides which, just like the 5' noncoding region, are extensively folded Several cloverleaf structures (59), a pseudoknot (29, 32), and a loop-loop (kissing) interaction (46) have been proposed The phenotypes of con-

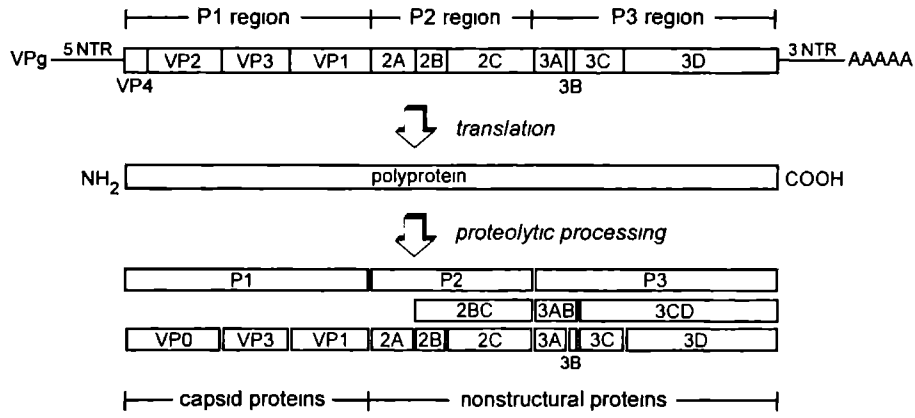


Figure 1. Schematic structure of the enteroviral genomic RNA Shown is the 7 5-kb single-stranded RNA genome with a VPg protein covalently bound at the 5 end of the nontranslated region (NTR) and the genomic encoded poly(A) tract at the end of the 3 nontranslated region The protein coding region is shown boxed Translation yields a single polyprotein that is proteolytically processed by the viral proteinases $2A^{pro}$ $3C^{pro}$, and $3CD^{pro}$ to produce the structural capsid P1 region proteins and the individual nonstructural P2 and P3 region proteins Polyprotein processing yields both the individual proteins as well as some relatively stable processing intermediates with functions in viral replication distinct from those of their cleavage products

structs bearing mutations in these domains suggest that the higher order structures serve as recognition signals for minus-strand RNA synthesis (69)

Replicative Cycle

Enteroviruses have a lytic infection cycle (Fig 2) Viruses enter the cell via specific cell surface receptors Polioviruses bind to a member of the immunoglobulin superfamily (48) Coxsackie B viruses attach to a nucleolin-related membrane protein (61) The integrin VLA-2 has been identified as a receptor for ECHO virus (6) The mechanisms of entry and uncoating are unknown, but uptake probably involves the endocytotic pathway (44) Once released in the cytoplasm, the 5'-terminal VPg is removed from the genomic RNA by a cellular unlinking enzyme (76) The viral RNA then serves as a mRNA to direct the synthesis of the polyprotein via an IRES-mediated initiation of translation This polyprotein is processed by viral proteinases to yield the products required for RNA replication Replication starts with the synthesis of a complementary minus-strand RNA in the cytoplasm by the RNA-dependent RNA polymerase $3D^{pol}$, yielding a double-stranded replicative form (4) This process has been proposed to involve (i) uridylation of the 3' poly(A) of plus-strand RNA, presumably by a host terminal transferase, (ii) hybridization of the resultant oligo(U) to the adjacent poly(A) tail, and (iii) initiation of minus-strand RNA synthesis at the 3' terminus of the oligo(U) by a template-priming mechanism (22) Plus-strand RNA synthesis takes place in replication complexes, specialized structures composed of partially double-stranded replicative intermediates and viral replication proteins The replication complex can be found in the center of a rosette formed by many virus-induced membrane vesicles (Fig 3) These vesicles are derived from the rough endoplasmic reticulum (ER) and are homologous to the intermediate or transport vesicles, which carry proteins to the Golgi apparatus (8) Both initiation of viral RNA synthesis and release of RNA from the complexes are dependent on the association of the replication complex with the vesicles (8) Newly synthesized RNA that is released from the replication complex may go into another round of translation and replication, processes that are tightly coupled, or be packaged into capsid proteins to produce infectious virus particles Encapsidation, a gradual process that occurs via a number of assembly intermediates (67), is highly specific since purified virions contain only plus-strand viral RNA and exclude VPg-lacking plus-strand viral mRNA, VPg-containing minus-strand viral RNA, as well as rRNA, tRNA, and other cytoplasmic mRNAs (77) This specificity is indicative for the occurrence of an encapsidation signal Extensive mutagenesis of VPg (i e , protein 3B) did not reveal any packaging mutants (77) It is therefore presumed that a *cis*-acting RNA segment provides packaging specificity The mechanism employed to induce cell lysis, a process required to release virus progeny, is completely unknown

Modifications of Host Cell Functions and Structures

During enterovirus infection, the host cell undergoes dramatic metabolic and morphological changes One of the most saillant effects is the inhibition of cap-depend-

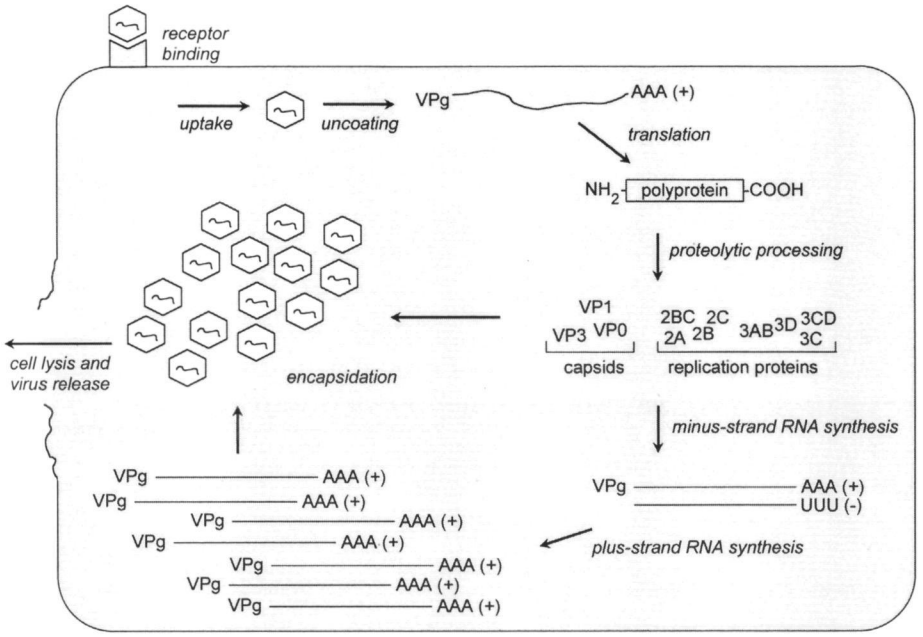


Figure 2. Overview of the enteroviral life cycle. Infection starts with binding of the virus to a specific receptor at the outer membrane of the cell. After endosomal uptake, the viral RNA is released and translated to produce a large polyprotein. Proteolytic processing of this polyprotein yields the structural capsid proteins and the nonstructural proteins implicated in RNA replication. For RNA replication, the genomic RNA is used as a template to synthesize a complementary minus-strand RNA, which, in turn, is transcribed into new molecules of virion RNA. Newly formed plus-strand RNAs are packaged into capsids to produce new viruses. Cell lysis is induced to release virus progeny.

ent translation of cellular mRNAs (20). Translation of enterovirus RNA, one of the few RNAs that can be translated in the infected cell (54) is not affected because it is initiated by a cap-independent internal binding of ribosomes to the IRES in the 5' noncoding region. Most likely the virus inhibits cellular translation to recrute all ribosomes for the translation of the viral genome. The virus inhibits cellular RNA synthesis (28) by inactivating transcription factors required for RNA polymerases I, II, and III (13, 14, 66). The plasma membrane becomes permeable for monovalent cations and small translation inhibitors that are normally nonpermeative (10). Of the morphological changes, the most prominent is the massive accumulation of small, cytoplasmic membrane vesicles with which the replication complexes are associated (8). This membrane proliferation starts with the formation of membrane protrusions at the rough ER, which then grow into membranous vesicles. The inhibition of viral replication by Brefeldin A, a fungal metabolite that blocks ER-to-Golgi traffic by preventing the formation of transport vesicles, suggests that an intact secretory pathway function is required for the generation of the vesicles (30, 45). The activities of phospholipases C and A2 are modified (25) and phospholipid synthesis is enhanced (52), presumably to account for the proliferation of the membrane vesicles. Inhibitors of lipid syn-

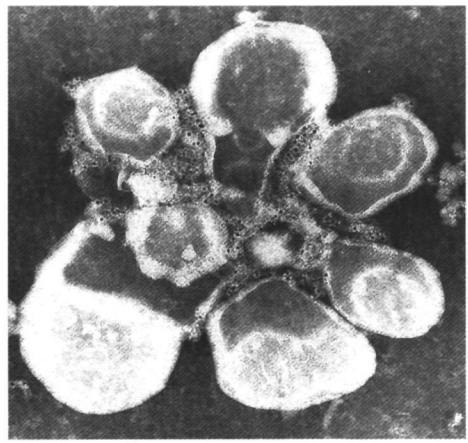


Figure 3. Electron microscopic photograph of a replication complex surrounded by and attached to a rosette of virus-induced membrane vesicles. Immunocytochemical labeling with anti-2B and colloidal gold shows that protein 2B is localized at the outer surface of the membrane vesicles. This structure was isolated from poliovirus-infected Hep-2 cells by sucrose step gradient centrifugation at 4 h postinfection and negatively stained. The photograph was kindly provided by dr K. Bienz, University of Basel.

thesis abolish viral replication, suggesting that the formation of the membrane vesicles is at least in part a result of de novo synthesis (24). Whether the virus-induced inhibition of vesicular protein transport between the ER and medial Golgi compartments (17) is responsible for the accumulation of the vesicles, or merely a consequence, is yet unknown. Other virus-induced alterations in cellular architecture are the remodeling of the intermediate filament system (41), structural alterations of cytoskeletal proteins (34), and the rounding and detachment of infected cells from the substrate. Collectively, these biochemical and structural alterations are known as the cytopathic effect.

FUNCTIONS OF NONSTRUCTURAL PROTEINS IN VIRAL REPLICATION

The individual roles of the nonstructural proteins and of their precursor products in RNA amplification and modification of cellular functions and structures are yet poorly understood or even unknown. The functions of individual viral gene products have been investigated by purification and enzymatic assays in vitro, by analysis of well defined mutants, and by expression in bacteria and eucaryotic cells. The putative action of each of the nonstructural products will be described.

Protein 2A. The 2A region codes for a protease that is autocatalytically released from the nascent polyprotein by rapid cotranslational cleavage at its own amino terminus. Cleavage is a prerequisite for further proteolytic processing of the capsid precursor region. A second function of 2A is to initiate the cleavage of the p220 subunit of the ribosomal initiation factor eIF-4F complex, leading to the inhibition of 5'- and cap-dependent translation of host cell mRNAs but not translation of uncapped viral mRNAs (21, 37). Proteolysis of p220 seems to be necessary but may not be sufficient to completely inhibit cellular translation, because substantial levels of host cell protein synthesis can take place when all p220 is degraded (9, 31, 58). Expression of protein 2A in mammalian cells led to a marked reduction in RNA polymerase II transcription and, to a lesser extent, to a reduction in DNA replication and translation (16). Although it was unclear whether these effects were direct or indirect, these results imply the existence of further activities of 2A and indicate how mechanisms other than p220 cleavage might contribute to the complete shutoff of host protein synthesis in infected cells. Another role of 2A may be as a specific enhancer of viral mRNA translation (26). Second-site compensating mutations mapping in 2A have been isolated in response to mutations in the IRES element (43). *Trans*-activation of translation may be important for efficient translation of viral mRNAs early in infection, when host mRNAs can still compete for the cellular protein synthesis machinery. In addition, recent studies suggest that protein 2A may be involved in RNA replication (51, 78).

Proteins 2B, 2C, and 2BC. The functions of the proteins coded for by the 2B and 2C regions are poorly understood. Mutations in either of these proteins led to strong defects in RNA replication (35, 42). These proteins, and their precursor 2BC, are contained exclusively at the outer surface of the virus-induced membrane vesicles at which viral plus-strand RNA synthesis takes place (8) (Fig. 3). Protein 2BC seems to be responsible for the induction of these vesicles. Electron microscopic examination of infected cells in which complete processing of the polyprotein was inhibited showed a correlation between the accumulation of vesicles and the presence of detectable levels of this protein (7). Consistent with this, it was found that expression of 2BC, but not that of 2B or 2C, either individually or in combination, induced the formation of membrane vesicles in yeast cells (5). Beside 2BC, also 2C was found to induce membrane proliferation and vesicle induction in vaccinia virus-infected cells, although the vesicles induced by protein 2BC were more similar to those that appear during virus infection (1, 12). The finding that 2C can induce vesicle formation should be taken with caution because vaccinia virus modifies the vesicular system itself, and complementation of enterovirus gene products by vaccinia virus proteins has been reported (55).

Poliovirus 2B mutants exhibited noncomplementable defects in viral RNA replication and displayed a dominance over wild-type virus, suggesting that 2B may interact with a limiting factor (e.g., another protein or a membrane attachment site) and that 2B, or 2BC, may be involved in the formation of a replication complex around the same RNA strand from which it is translated (35). Expression of protein 2B in either bacteria or mammalian cells led to an increased membrane permeability (17, 39). In addition to this modification, protein secretion was inhibited in 2B-expressing mammalian cells (17). Examination of the subcellular localization of a coexpressed secreted protein showed that 2B did not block a specific step in membrane transport. Therefore, inhibition of protein secretion may be a side effect caused by the permeabilization of the plasma membrane and/or a nonspecific leakiness of organelle membranes (17).

Protein 2C has also been implicated in the structural and functional organization of the viral replication complex. The RNA and membrane binding capacities of this protein suggest that 2C, or 2BC, may be responsible for the attachment of the viral RNA to the vesicular membranes (19, 65). Biochemical studies have shown that 2C possesses ATPase and GTPase activities (49, 64). This NTPase activity may reflect a helicase function required for viral RNA strand separation during replication. The resemblance of 2C with the small GTPases involved in regulation of membrane transport, however, suggests that 2C may participate in the traffic of the virus-induced membrane vesicles (64).

Proteins 3A, 3B, and 3AB. 3B is usually referred to as VPg, the hydrophilic peptide of 22 amino acids covalently linked via tyrosine to the 5' termini of all full-length and nascent viral plus- and minus-strand RNAs.

VPg has been proposed in the initiation of viral RNA synthesis. The current view is that an uridylylated form of VPg, VPg-pU or VPg-pU-pU, acts as a primer of plus-strand RNA synthesis (38, 72). Uridylylation is strictly dependent on the presence of intact membranes (72). The observation that mutations in 3A, a hydrophobic membrane protein (15), affected uridylylation of VPg and selectively inhibited initiation of plus-strand RNA synthesis suggests that 3AB, i.e., the precursor of VPg, may serve as an anchor and a donor for VPg in the membrane-associated RNA-synthesizing complex (23). VPg is not required for the initiation of minus-strand RNA synthesis. The covalent linkage of VPg to the minus-strand RNA has been claimed to occur by a self-catalyzed transesterification reaction which requires only the replication intermediate and VPg (73), but this has not yet been substantiated by any observation in vivo.

In addition to its role as anchor and donor for VPg, protein 3AB has been found to interact with $3CD^{pro}$ and $3D^{pol}$ and to serve as a cofactor for (i) the binding of protein $3CD^{pro}$ to the 5' and 3' terminal cloverleafs of the RNA genome (27), (ii) the polymerase activity of $3D^{pol}$ (40, 56), and (iii) the autocatalytic processing of $3CD^{pro}$ to $3C^{pro}$ and $3D^{pol}$ (50).

Protein 3A, but not 3AB, inhibits protein secretion to a similar extent as 2B when expressed in mammalian cells (17). In contrast to 2B, however, protein 3A caused the accumulation of a coexpressed secreted protein in the ER, suggesting that 3A specifically blocks transport from the ER to the Golgi apparatus.

Proteins 3C, 3D, and 3CD. The 3C region codes for a protease ($3C^{pro}$) that is responsible for the maturation cleavages between the nonstructural P2 and P3 region proteins in the precursor polyprotein (18). Proteolysis by $3C^{pro}$ occurs in an incompletely understood cascade of *cis*- and *trans*-cleavages mainly at AxxQ↓G sites (where x may be any amino acid). Cleavage of the P1 precursor protein, which is cotranslationally released by $2A^{pro}$, to the individual capsid proteins is performed in *trans* by $3CD^{pro}$. There is convincing evidence that $3C^{pro}$, or $3CD^{pro}$, is responsible for the inhibition of host cell RNA transcription by proteolytic inactivation of the TATA-binding protein subunit transcription factor IID, required for RNA polymerases I and II, and transcription factor IIIC, required for RNA polymerase III (13, 14, 66).

The 3D region codes for a template- and primer-dependent RNA polymerase ($3D^{pol}$). This polymerase accepts any polyadenylated RNA as template in vitro, suggesting that the observed specificity in vivo must be confered by a yet unknown viral or host component(s) (38). A second activity of $3D^{pol}$ is an RNA helicase activity, suggesting that the RNA duplex unwinding activity required for melting the double-stranded RNA replicative form may be an intrinsic activity of $3D^{pol}$ rather than being provided by protein 2C (11). Furthermore, $3D^{pol}$ has been shown to possess terminal adenylyl transferase activity (53, 60, 74), suggesting that $3D^{pol}$ may be responsible for the uridylylation of Vpg.

Besides being a proteinase, $3CD^{pro}$ has been shown to be involved in the formation of a ribonucleoprotein complex, together with protein 3AB, around the 5' terminal cloverleaf in the 5' noncoding region and the higher order structures in 3' noncoding region of the viral plus-strand RNA (2, 3, 27). These ribonucleoprotein complexes are most likely involved in the initiation of plus-strand and minus-strand RNA synthesis, respectively. A model for the membrane-bound initiation of plus-strand RNA synthesis in *trans* has been proposed and involves the following steps (3, 27) (Fig. 4); (i) Uridylylation of membrane-bound 3AB to yield 3AB-pU-pU. (ii) Unwinding of the 5' end of the replicative form, consisting

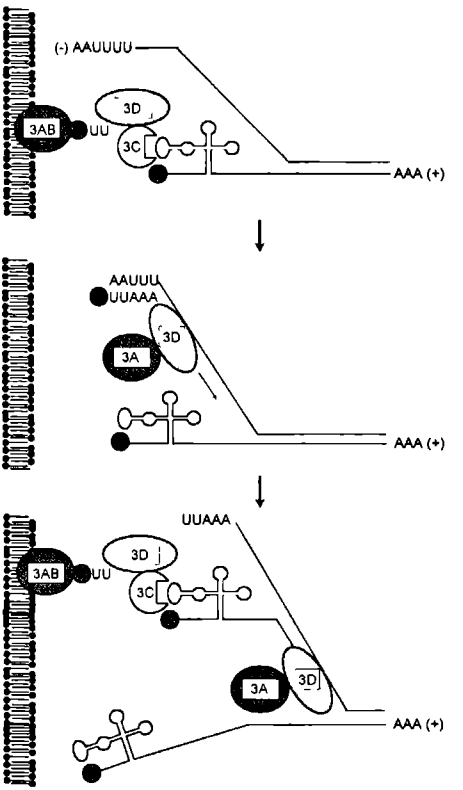


Figure 4. A model for plus-strand RNA synthesis, the *trans*-initiation mechanism (adapted from Andino et al [3] and Harris et al [27]) Three steps of initiation of RNA synthesis are shown The upper picture represents a minus-strand (-) acting as a template and a resident plus-strand (+) holding the ribonucleoprotein complex consisting of the 5' cloverleaf RNA, 3CD, and membrane-bound 3AB as donor for the primer peptide VPg (i e , 3B) The middle picture shows the progress of the reaction after VPg-pU(pU) is used as a primer for $3D^{pol}$, stimulated by 3A, to begin the elongation reaction The bottom picture shows that after $3D^{pol}$ has synthesized at least the first 100 nucleotides of a plus-strand RNA a new cloverleaf structure will form, allowing the formation of a new ribonucleoprotein complex that can then catalyze the initiation of the next plus-strand RNA For more details, the reader is directed to the text.

of the 5' end of the plus-strand and the 3' end of the minus-strand, by a helicase activity. (iii) Formation of a 3AB-3CD^{pro} complex around the plus-strand RNA cloverleaf. (iv) Cleavage of the uridilylated form of 3AB to yield VPg-pU-pU. (v) Initiation of plus-strand synthesis in *trans* by VPg-pU-pU at the 3' end of the neighboring minus-strand. (vi) Autocatalytic processing of 3CD^{pro} in *cis*, stimulated by 3AB, to activate 3D^{pol}. When plus-strand RNA synthesis is proceeding, a new 3AB-3CD^{pro} complex may form around the 5' end of the newly synthesized plus-strand and catalyze the synthesis of another plus-strand. Initiation of minus-strand RNA synthesis most likely involves a similar mechanism, except that priming seems to occur by an oligo(U) driven template-priming mechanism (see above) rather than by an uridylylated form of VPg.

AIM AND OUTLINE OF THE THESIS

In the next chapters of this thesis, experiments are described undertaken to study; (i) the functional role of enterovirus protein 2B in viral RNA replication and the modification of cellular functions and structures, and (ii) the structural determinants that are involved in these functional activities.

A detailed computer analysis suggested that 2B is a membrane protein and predicted the occcurence of two putative transmembrane domains, of which one had the potential to form a cationic amphipathic α-helix. An extensive genetic analysis of each of these domains was performed. Well defined mutations were introduced in a full-length cDNA clone of coxsackievirus and their effects on virus viability, RNA replication, polyprotein synthesis and processing, and complementability in *trans* were examined **(chapters 2 and 3)**. To examine whether coxsackievirus 2B is able to inhibit cellular protein secretion and increase the permeability of the plasma membrane similar as poliovirus 2B does, and to identify the essential domains for these activities, both wild-type and several mutant 2B proteins were expressed in mammalian cells **(chapter 4)**. A mutational analysis of the 2B/2C cleavage site was performed to study the determinants that regulate processing efficiency at this site and to examine the effects of altered levels of proteins 2B and 2C on viral replication **(chapter 5)**. The construction of chimeric coxsackievirus genomes that express hybrid coxsackievirus-poliovirus 2B proteins was used to further dissect the structural requirements of protein 2B for its role in viral reproduction **(chapter 6)**. The ability of 2B to increase membrane permeability and its localization at the outer surface of the ER-derived membrane vesicles suggested that 2B might mobilize calcium from ER stores. Therefore, the effect of CBV3 infection and protein 2B expression on intracellular calcium homeostasis was assayed **(chapter 7)**. In addition, a function of protein 2B in the virus release was investigated **(chapter 7)**. In the last chapter, the experimental data described in this thesis are summarized and discussed **(chapter 8)**.

REFERENCES

1 **Aldabe, R., and L. Carrasco.** 1995 Induction of membrane proliferation by poliovirus proteins 2C and 2BC Biochem Biophys Res Commun **206**:64-76

2 **Andino, R. G.E. Rieckhof, and D. Baltimore.** 1990 A functional ribonucleoprotein complex forms around the 5' end of poliovirus RNA Cell **63**:369-380

3 **Andino, R., G.E. Rieckhof, P.L. Achacoso, and D. Baltimore.** 1993 Poliovirus RNA synthesis utilizes an RNP complex formed around the 5' end of viral RNA EMBO Journal **12**:3587-3598

4 **Baltimore, D.** 1968 Structure of the poliovirus replicative intermediate RNA J Mol Biol **32**:359-368

5 **Barco, A., and L. Carrasco.** 1995 A human virus protein, poliovirus protein 2BC, induces membrane proliferation and blocks the exocytic pathway in the yeast *Saccharomyces cerevisiae* LMBO Journal **14**:3349-3364

6 **Bergelson, J.M., M.P. Shepley, B.M.C. Chan, M.E. Hemler, and R.W. Finberg.** 1992 Identification of the integrin VLA-2 as a receptor for echovirus 1 Science **255**:1718-1720

7 **Bienz, K., D. Egger, and L. Pasamontes.** 1987 Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography Virology **160**:220-226

8 **Bienz, K., D. Egger, and T. Pfister.** 1994 Characteristics of the poliovirus replication complex Arch Virol **suppl. 9**:147-157

9 **Bonneau, A., and N. Sonenberg.** 1987 Proteolysis of the p220 component of the Cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection J Virol **61**:986-991

10 **Carrasco, L.** 1995 Modification of membrane permeability by animal viruses Adv Virus Res **45**:61-112

11 **Cho, M.W., O.C. Richards, T.M. Dmitrieva, V. Agol, and E. Ehrenfeld.** 1993 RNA duplex unwinding activity of poliovirus RNA-dependent RNA polymerase 3D^{pol} J Virol **67**:3010-3018

12 **Cho, M.W., N. Teterina, D. Egger, K. Bienz, and E. Ehrenfeld.** 1994 Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells Virology **202**:129-145

13 **Clark, M.E., T. Hämmerle, E. Wimmer, and A. Dasgupta.** 1991 Poliovirus proteinase 3C converts an active form of transcription factor IIIC to an inactive form a mechanism for inhibition of host cell polymerase III transcription by poliovirus EMBO J **10**:2941-2947

14 **Clark, M.E., P.M. Lieberman, A.J. Berk, and A. Dasgupta.** 1993 Direct cleavage of human TATA-binding protein by poliovirus protease 3C in vivo and in vitro Mol Cell Biol **13**:1232-1237

15 **Datta, U., and A. Dasgupta.** 1994 Expression and subcellular localization of poliovirus VPg-precursor protein 3AB in eukaryotic cells evidence for glycosylation in vitro J Virol **68**:4468-4477

16 **Davies, M.V., J. Pelletier, K. Meerovitch, N. Sonenberg, and R.J. Kaufman.** 1991 The effect of poliovirus proteinase 2A^{pro} expression on cellular metabolism J Biol Chem **266**: 14714-14720

17 **Doedens, J.R., and K. Kirkegaard.** 1995 Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A EMBO Journal **14**:894-907

18 **Dougherty, W.G., and B.L. Semler.** 1993 Expression of virus-encoded proteinases functional and structural similarities with cellular enzymes Microbiol Rev **57**:781-822

19 **Echeverri, A., and A. Dasgupta.** 1995 Amino terminal regions of poliovirus 2C protein mediate membrane binding Virology **208**:540-553

20 **Ehrenfeld, E.** 1982 Poliovirus-induced inhibition of host-cell protein synthesis Cell **28**:435-436

21 **Etchison, D., S.C. Milburn, I. Edery, N. Sonenberg, and J.W.B. Hershey.** 1982 Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 222,000 Da polypeptide associated with eukaryotic initiation factor 3 and a cap binding protein complex J Biol Chem **258**:7236-7239

22 **Flanegan, J.B., G.J. Tobin, M.S. Oberste, B.J. Morasco, D.C. Young, and P.S. Collis.** 1990 Mechanism of poliovirus negative strand RNA synthesis and the self-catalyzed covalent linkage of VPg to RNA, p 55-60 *In* M A Brinton and F X Heinz (ed), New aspects of positive-strand RNA viruses American Society for Microbiology, Washington, D C

23 **Giachetti, C., and B.L. Semler.** 1991 Role of a viral membrane polypeptide in strand-specific initiation of poliovirus RNA synthesis J Virol **65**:2647-2654

24 **Guinea, R , and L. Carrasco.** 1990 Phospholipid biosynthesis and poliovirus genome replication, two coupled phenomena FMBO J **9**:2011-2016

25 **Guinea, R., A. Lopez Rivas, and L. Carrasco.** 1989 Modification of phospholipase C and phospholipase A2 activities during poliovirus infection J Biol Chem **264**:21923-21927

26 **Hambidge, S.J., and P. Sarnow.** 1992 Translational enhancement of the poliovirus 5' noncoding region mediated by virus-encoded polypeptide 2A Proc Natl Acad Sci USA **89**:10272-10276

27 **Harris, K.S., W. Xiang, L. Alexander, W.S. Lane, A.V. Paul, and E. Wimmer.** 1994 Interaction of poliovirus polypeptide $3CD^{pro}$ with the 5' and 3' termini of the poliovirus genome J Biol Chem **269**:27004-27014

28 **Holland, J.J.** 1962 Inhibition of DNA-primed RNA synthesis during poliovirus infection of human cells Biochem Biophys Res Commun **9**:556-562

29 **Iizuka, N., S. Kuge, and A. Nomoto.** 1987 Complete nucleotide sequence of the genome of coxsackievirus B1 Virology **156**:64-73

30 **Irurzun, A., L. Perez, and L. Carrasco.** 1992 Involvement of membrane traffic in the replication of poliovirus genomes effects of Brefeldin A Virology **191**:166-175

31 **Irurzun, A., S. Sanchez-Palomino, I. Novoa, and L. Carrasco.** 1995 Monensin and nigericin prevent the inhibition of host translation by poliovirus, without affecting p220 cleavage J Virol **69**:7453-7460

32 **Jacobson, S.J., D.A.M. Konings, and P. Sarnow.** 1993 Biochemical and genetic evidence for a pseudoknot structure at the 3' terminus of the poliovirus RNA genome and its role in viral RNA amplification J Virol **67**:2961-2971

33 **Jang, S.K., H.G. Krausslich, M.J H. Nicklin, G.M. Duke, A.C. Palmenberg, and E. Wimmer.** 1988 A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry during in vitro translation J Virol **62**: 2636-2643

34 **Joachims, M., and D. Etchison.** 1992 Poliovirus infection results in structural alteration of a microtubule-associated protein J Virol **66**:5797-5804

35 **Johnson, K.L., and P. Sarnow.** 1991 Three poliovirus 2B mutants exhibit noncomplementable defects in viral RNA amplification and display dosage-dependent dominance over wild-type poliovirus J Virol **65**:4341-4349

36 **Kitamura, N., B.L. Semler, P.G. Rothberg, G.R.Larsen, C.J. Adler, A.J. Dorner, E.A. Emini, R. Hanecak, J.J. Lee, S. van der Werf, C.W. Anderson, and E. Wimmer.** 1981 Primary structure gene organization and polypeptide expression of poliovirus RNA Nature (London) **291**:547-553

37 **Kräusslich, H.G., M.J.H. Nicklin, H. Toyoda, D. Etchison, and E. Wimmer.** 1987 Poliovirus proteinase 2A induces cleavage of eucaryotic initiation factor 4F polypeptide p220 J Virol **61**:2711-2718

38 **Kuhn, R.J., and E. Wimmer.** 1987 The replication of picornaviruses, p 17-51 *In* D J Rowlands, M A Mayo, and B W J Mahy (ed), The molecular biology of the positive strand RNA viruses Academic Press, Inc , London

39 **Lama, J., and L. Carrasco.** 1992 Expression of poliovirus nonstructural proteins in *Escherichia coli* cells J Biol Chem **267**:15932-15937

40 **Lama, J. A.V. Paul, K.S. Harris, and E. Wimmer.** 1994 Properties of purified recombinant poliovirus protein 3AB as substrate for viral proteinases and as co-factor for RNA polymerase $3D^{pol}$ J Biol Chem **269**:66-70

41 **Lenk, R., and S. Penman.** 1979 The cytoskeletal framework and poliovirus metabolism Cell **16**:289-301

42 **Li, J.-P., and D. Baltimore.** 1988 Isolation of poliovirus mutants defective in viral RNA synthesis J Virol **62**:4016-4021

43 **Macadam, A.J., G. Ferguson, T. Fleming, D.M. Stone, J.W. Almond, and P.M. Minor.** 1994 Role for poliovirus protease 2A in cap independent translation EMBO J **13**:924-927

44 **Marsh, M., and A. Helenius.** 1989 Virus entry into animal cells Adv Virus Res **36**:107-151

45 **Maynell, L.A., K. Kirkegaard, and M.W. Klymkowsky.** 1992 Inhibition of poliovirus RNA synthesis by brefeldin A J Virol **66**:1985-1994

46 **Melchers, W.J.G., J.G.J. Hoenderop, H.J. Bruins Slot, C.W.A. Pleij, E.V. Pilipenko, V I. Agol, and J.M.D. Galama.** 1997 Kissing of the two predominant hairpin-loops in the coxsackie B virus 3'UTR is the essential structural feature of the origin of replication required for (-) strand RNA synthesis J Virol **71**:686-696

47 **Melnick, J.L.** 1996 Enteroviruses polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses p 655-712 *In* B N Fields, D M Knipe, P M Howley, R M Chanock, J I Melnick, T P Morath, B Roizman, and S E Straus (ed), Fields Virology, 32rd ed , vol 1 Lippincott-Raven Publishers, Philadelphia

48 **Mendelsohn, C.L., E. Wimmer, and V.R. Racaniello.** 1989 Cellular receptor for poliovirus molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily Cell **56**:855-865

49 **Mirzayan, C., and E. Wimmer.** 1994 Biochemical studies on poliovirus polypeptide 2C evidence for ATPase acitivity Virology **199**:176-187

50 **Molla, A., K.S. Harris, A.V. Paul, S.H. Shin, J. Mugavero, and E. Wimmer.** 1994 Stimulation of poliovirus proteinase $3C^{pro}$-related proteolysis by the genome-linked protein VPg and its precursor 3AB J Biol Chem **269**:27015-27020

51 **Molla, A., A.V. Paul, M. Schmid, S.K. Jang, and E. Wimmer.** 1993 Studies on dicistronic polioviruses implicate viral proteinase $2A^{pro}$ in RNA replication Virology **196**:739-747

52 **Mosser, A.G., L.A. Caliguiri, and J. Tamm.** 1972 Incorporation of lipid precursors into cytoplasmic membranes of poliovirus-infected HeLa cells Virology **47**:39-47

53 **Neufeld, K.L., J.M. Galarza, O.C. Richards, D.F. Summers, and E. Ehrenfeld.** 1994 Identification of terminal adenylyl transferase activity of the poliovirus polymerase $3D^{pol}$ J Virol **68**:5811-5818

54 **Oh, S.K., and P. Sarnow.** 1993 Gene regulation translational initiation by internal ribosome binding Curr Opin Genet Dev **3**:295-300

55 **Pal-Ghosh, R., and C.D. Morrow.** 1993 A poliovirus mini-replicon containing an inactive 2A proteinase is expressed in vaccinia virus-infected cells J Virol **67**:4621-4629

56 **Paul, A.V., X. Cao, K.S. Harris, J. Lama, and E. Wimmer.** 1994 Studies with poliovirus polymerase $3D^{pol}$ J Biol Chem **269**:29173-29181

57 **Pelletier, J., and N. Sonenberg.** 1988 Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA Nature (London) **334**:320-325

58 **Pérez, L., and L. Carrasco.** 1992 Lack of direct correlation between p220 cleavage and the shut-off of host translation

after poliovirus infection Virology **189**:178-186
59 **Pilipenko, E.V., S.V. Maslova, A.N. Sinyakov, and V.I. Agol.** 1992 Towards identification of *cis*-acting elements involved in the replication of enterovirus and rhinovirus RNAs a proposal for the existence of tRNA-like terminal structures Nucleic Acids Res **20**:1739-1745
60 **Plotch, S.J., O. Palant, and Y. Gluzman.** 1989 Purification and properties of poliovirus RNA polymerase expressed in *Escherichia coli* J Virol **63**:216-225
61 **Raab de Verdugo, U., H.-C. Selinka, M. Huber, B. Kramer, J. Kellerman, P.H. Hofschneider, and R. Kandolf.** 1995 Characterization of a 100-kilodalton binding protein for the six serotypes of coxsackie B viruses J Virol **69**:6751-6757
62 **Racaniello, V.R., and D. Baltimore.** 1981 Cloned poliovirus DNA is infectious in mammalian cells Science **214**:916-919
63 **Racaniello, V.R., and D. Baltimore.** 1981 Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome Proc Natl Acad Sci USA **78**:4887-4891
64 **Rodriguez, P.L., and L. Carrasco.** 1993 Poliovirus protein 2C has ATPase and GTPase activities J Biol Chem **268**: 8105-8110
65 **Rodriguez, P.L., and L. Carrasco.** 1995 Poliovirus protein 2C contains two regions involved in RNA binding activity J Biol Chem **270**:10105-10112
66 **Rubinstein, S.J., T. Hammerle, E. Wimmer, and A. Dasgupta.** 1992 Infection of HeLa cells results in modification of a complex that binds to the rRNA promoter J Virol **66**: 3062-3068
67 **Rueckert, R.R.** 1996 *Picornaviridae* the viruses and their replication p 609-654 *In* B N Fields, D M Knipe, P M Howley, R M Chanock, J L Melnick, T P Morath, B Roizman, and S E Straus (ed) Fields Virology 32rd ed , vol 1 Lippincott-Raven Publishers, Philadelphia
68 **Sabin, A.** 1955 Characteristics and genetic potentialities of experimentally produced and naturally occurring variants of poliomyelitis virus Ann NY Acad Sci **61**:924-938
69 **Sarnow, P., H.D. Bernstein, and D. Baltimore.** 1986 A poliovirus temperature-sensitive RNA synthesis mutant located in a noncoding region of the genome Proc Natl Acad Sci USA **883**:571-575
70 **Svitkin, Y.V., S.V. Maslova, and V.I. Agol.** 1985 The genomes of attenuated and virulent poliovirus strains differ in their in vitro translation efficiencies Virology **147**:243-252
71 **Svitkin, Y.V., T.V. Pestova, S.V. Maslova, and V.I. Agol.** 1988 Point mutations modify the response of poliovirus RNA to a translation initiation factor a comparison of neurovirulent and attenuated strains Virology **166**:394-404
72 **Takeda, N., R.J. Kuhn, C.F. Yang, T. Takegami, and E. Wimmer.** 1986 Initiation of poliovirus plus-strand RNA synthesis in a membrane complex of infected HeLa cells J Virol **60**:43-53
73 **Tobin, G.J., D.C. Young, and J.B. Flanegan.** 1989 Self-catalyzed linkage of poliovirus terminal protein VPg to poliovirus RNA Cell **59**:511-519
74 **Toyoda, M. C.-F. Yang, N. Takeda, A. Nomoto, and E. Wimmer.** 1987 Analysis of RNA synthesis of type 1 poliovirus by using an in vitro molecular genetic approach J Virol **66**:2943-2951
75 **van der Werf, S., J. Bradley, E. Wimmer, F.W. Studier, and J. Dunn.** 1986 Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase Proc Natl Acad Sci USA **83**:2330-2334
76 **Wimmer, E.** 1982 Genome-linked proteins of viruses Cell **28**:199-201
77 **Wimmer, E., C.U.T. Helen, and X. Cao.** 1993 Genetics of poliovirus Annu Rev Genet **27**:353-436
78 **Yu, S., P. Benton, M. Bovee, J. Session, and R.E. Lloyd.** 1995 Defective RNA replication by poliovirus mutants deficient in 2A protease cleavage activity J Virol **69**:247-252

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

Journal of Virology 1995, 69: 7782-7790

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

FRANK J M VAN KUPPEVELD, JOCHEM M D GALAMA, JAN ZOLL, AND WILLEM J G MELCHERS

Department of Medical Microbiology University of Nijmegen The Netherlands

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 either 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 structure, genomic organization, and molecular biology of these viruses are relatively well known from the extensive studies on poliovirus Enteroviruses are positive-strand RNA viruses with a single-stranded genome of approximately 7,500 nucleotides (nt) A small virally encoded protein, VPg, is covalently attached at the 5' end of the 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 5' nontranslated region (19, 32) The viral polyprotein is proteolytically processed by the virus encoded proteases $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$ 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 RNA strand in the cytoplasm of the host cell by the RNA-dependent RNA polymerase $3D^{pol}$ (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 the vRNA to the vesicular membranes (7, 9, 35) Further-

A

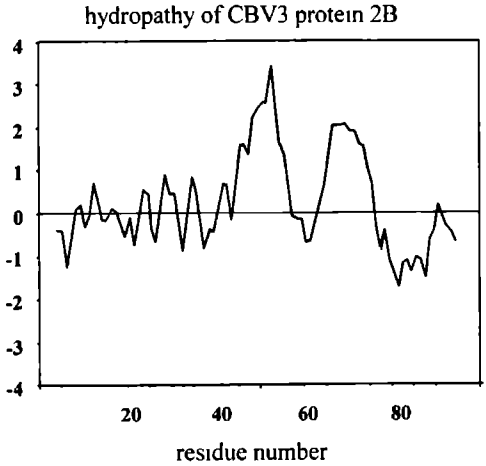


Figure 1 **(A)** Hydropathy plot of CBV3 protein 2B according to Kyte and Doolittle (24) The hydropathy window size used for this figure is 9 residues Positive hydropathy indicates a hydrophobic nature The numbers of amino acid residues are indicated on the ordinate **(B)** Alignment of the amino acid sequences of the 2B protein of enteroviruses for which sequences are known The enteroviruses are divided in a CBV-like subgroup and a poliovirus (PV)-like subgroup Enteroviruses with distinct amino acid sequences are shown apart In this alignment we have included the 2B amino acid sequence of five nonrelated CBV1 isolates with highly divergent $2A^{pro}$ regions (41) Virus propagation, RNA extraction, reverse transcription amplification by PCR and sequence analysis of the 2B coding region of these five CBV1 isolates were performed as described elsewhere (41) Abbreviations CBV coxsackie B virus, CAV coxsackie A virus, ECHO, echovirus SVDV swine vesicular disease virus CBV1Tuc, myotropic CBV1 strain Tucson CBV1Isr, strain isolated 23 July 1978 from an immigrant child from Israel CBV1Net strain isolated in 1992 from a patient in The Netherlands, CBV1Col, 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 Leon, EV70 enterovirus type 70 BFV1, bovine enterovirus type 1

B

ENTEROVIRUS	2B AMINO ACID SEQUENCE
CBV-LIKE SUBGROUP	
	10 20 30 40 50 60 70 80 90
CBV3	GVKDYVEQLG NAFGSGFTNQ ICEQVNLLKE SLVGQDSILE KSLKALVKII SALVIVVRNE DDLITVTATL ALIGCTSSPW RWLKQKVSQY YGIPMAERQ
CBV4	H
CBV5	V I
CBV1	V V
ECHO11	
ECHO12	H H
CAV9	I
SVDV	R T I V T
CBV1Tuc	I
CBV1Isr	H
CBV1Net	T Y A
CBV1Col	I I T
CBV1sur	I I T
PV-LIKE SUBGROUP	
PV1(M)	ITN I S A Q SDKITE TN MVTS--T T L N I S IT Y E TT L L DA Q RK ACDV LE YVIK
PV2(L)	SN I S A Q GNKISE TS MVTS--T T L N I S IT Y E TT L L DA Q K ACDI LE YIM
PV3(L)	ISN I S A Q GDKISE TS MVTS--T T L N I S IT Y E TT L L DV Q K ACDT LE YVI
CAV21	ITS I S T A HT S K TE TT MVTS--T T L N V Y E TT IL L DI Q K ACDL LE HVM
CAV24	ITS I S A G K SE TS MVTS-- T L T I T IS Y E TT L L DY Q I K ACDV LEL YVM
DISTINCT ENTEROVIRUSES	
CAV16	S IKG D T DA VSRE EA N H I SEGAV I N I L I SD Y MV L HG A I A TASI L I QK
EV70	IT IQN S T ET S KAKEIQN M E L L I V M I S E V L ND AF CS L YTI
BEV1	IT Q A AE ANYT Q RD M M S VV IIRS RLV Q G L EG K R C I L N

more, 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 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 the 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 attachment site (20) The expression of both poliovirus and coxsackie B3 virus (CBV3) protein 2B in *Escherichia coli* resulted in a modified membrane permeability (26, 39a) Recent-

ly, 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 vRNA 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 5 CBV1 isolates containing divergent 2A coding regions (41) further strenghtens the importance of conservation in this region of protein 2B (Fig 1B)

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 cells (BGM) with copy RNA transcripts Translation and processing of the viral polyprotein 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 replicon 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 replicative wells of 96-well plates containing BGM cell monolayers (36) Virus titers were calculated according to the method described by Reed and Muench and expressed in 50% tissue culture infective dose ($TCID_{50}$) values (34)

Oligonucleotide-directed site-specific mutagenesis. Plasmid pCB3/T7 was kindly provided by R Kandolf (University of Tubingen, Germany) This plasmid contains a full-length copy DNA of coxsackie B3 virus (strain Nancy) cloned behind a T7 RNA polymerase promoter (22) In vitro mutagenesis was performed using the Altered Sites in vitro mutagenesis system (Promega) according to the recommendations of the manufacturer For mutagenesis, the *Hind*III (nt 2080) to *λba*I (nt 4947) fragment of pCB3/T7 was introduced in phagemid pALTER-1 to generate pALTCB3/2080-4947 Synthetic oligonucleotides were used to introduce site-specific mutations The nucleotide sequence of the mutant pALTCB3 clones was verified by dideoxy chain termination sequence analysis of plasmid DNA with oligonucleotide 5-CCATTCAATGAA TTTCTG-3 (complementary to nt 4117 to 4134), using the Ampli Cycle sequencing kit according to the instructions of the manufacturer (Perkin-Elmer) From these clones, the *Spe*I (nt 3837)-to-*Bss*HII (nt 4238) fragment was cloned in pCB3/T7

Transcription and transfection of cells with copy RNA transcripts. Plasmids were linearized by digestion with *Sal*I, 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 $MgCl_2$, 2 mM spermidine, 2 5 mM each nucleoside triphosphate, 100 U of RNAsin (Promega), and 30 U of T7 RNA polymerase (Promega) Small portions (2μl) were analyzed on a 1% agarose gel Twenty microliters of the transcription mixture (2 to 4 μg RNA) was used for transfection of BGM cells by the DEAE-dextran method (39) with some modifications Briefly, 80 μl of a *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES)-buffered salt solution (HBS) (20 mM HEPES pH 7 05, 137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 5 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-cm^2 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, overlayed with cell culture medium, and incubated at either 33 or 36°C for the times indicated in the text When virus growth was observed, the cultures were incubated until cytopathic effect (CPE) was complete The cultures were then subjected to three cycles of freezing and thawing, and the viruses were aliquoted in stocks of 1 ml and stored at -80°C

Sequence analysis of the 2B coding region of mutant viruses. RNA was isolated from 100 μl of virus stock using 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'-GCAATGGAACAGGGAGTGAAGGACTATGTGGA-3' (nt 3733 to 3765) and reverse primer 5-TTGGGATGGCGCGCT CTGCTC-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-cm^2 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 for 30 min at room temperature After removal of the inoculum, the cells were overlayed with 5 ml of cell culture medium containing 1% plaque agarose (Servo) and 25 mM $MgCl_2$ 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-cm^2 flasks (5 x 10^6 cells per flask) were infected with virus at a multiplicity of infection (MOI) of 1 $TCID_{50}$ per cell After 30 min of adsorption, the cells were washed three times with MEM and 5 ml of cell culture medium was added The cells were incubated at 33, 36, or 39°C for 2, 4, 6, or 8 h Viruses were released by three succesive cycles of freezing and thawing Virus titers were determined by titration on BGM cell monolayers in 96-well plates

Construction of subgenomic replicon pCB3/T7-LUC. For the substitution of the capsid-coding region by the luciferase coding sequence, the P1 capsid-coding region was deleted from pCB3/T7 by two subsequent PCR product subcloning steps by using the unique restriction sites for *Sful* (nt 250), *Bgl*II (nt 2024), and *Bss*HII (nt 4238) PCRs were performed with UlTma DNA polymerase (Perkin-Elmer), a DNA polymerase containing proofreading activity First, nt 243 to 745 were amplified with primers 5'-AAC TAC TTC GAA AAA CCT AGT AAC ACC-3' (nt 243 to 269) and 5'-TTG CGT AGA TCT GCG GCC GCC CAT TTT GCT GTA TTC AAC TTA-3' (complementary to nt 721 to 745 plus restriction sites, which are underlined, for *Not*I and *Bgl*II) This PCR product was cut with *Sful* and *Bgl*II and cloned in pCB3/T7 cut with the same enzymes In the resulting plasmid pCB3/T7-Δ746/2024, the N-terminal half of the P1 region is deleted Second, nt 3280 to 4251 were amplified with primers 5'-ACT ACT AGG AGA TCT GTT AAC ACA ATG ACA AAT ACG GGC GCA-3 (nt 3280 to 3300 plus restriction sites for *Bgl*II and *Hpa*I) and 5'-TTG GGA TGG CGC GCT CTG CTC-3' (complementary to nt 4231 to 4251) This PCR product was cut with *Bgl*II and *Bss*HII and introduced in pCB3/T7-Δ746/2024 cut with the same enzymes In the resulting plasmid (pCB3/T7-ΔP1), nt 746 to 3279 of the P1 coding region are deleted and replaced by the sequence GGC GGC CGC AGA TCT GTT AAC (the restriction sites for *Not*I, *Bgl*II, and *Hpa*I, respectively) Except for its initiation and stopcodon, the firefly luciferase gene was amplified by PCR using plasmid pGFM-luc (Promega) as template with primers 5'-GCC CGG AGC GGC CGC GAA GAC GCC AAA AAC ATA-3' and 5'-AGT TAC GTT AAC CAA TTT GGA CTT TCC GCC-3', cut with *Not*I and *Hpa*I, and cloned in pCB3/T7-ΔP1 cut with the same enzymes The resulting plasmid was designated pCB3/T7-LUC (see Fig 4A) Because of the cloning procedure, the luciferase product contains three additional amino acids (Gly-Gly-Arg), which correspond to the *Not*I site, after the initial methionine The guanidine residue following the initiation codon was retained in order to keep the Kozak consensus sequence intact (23) Furthermore, luciferase contains seven additional residues at its C terminus The Val and Asn residues correspond to the *Hpa*I site The five C-terminal amino acids of VP1 were retained to ensure a correct proteolytic processing by $2A^{pro}$

Analysis of viral positive-strand RNA synthesis. BGM cell monolayers grown in 25-cm^2 flasks to a confluency of 75% were transfected with 0 5 to 1 0 μg of T7 RNA polymerase-generated pCB3/T7-LUC copy RNA derived from *Sal*I-linearized plasmids At the indicated times posttransfection, 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)

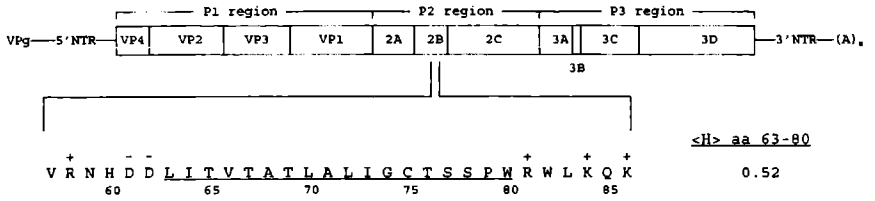
In vitro translation reactions. Copy RNA transcipts were synthesized and translated in T7 TNT rabbit reticulocyte lysate (Promega) according to the manufacturer's recommendations The translation reaction mixtures (20 μl) were programmed with 0 5 μg of *Sal*I-linearized plasmid DNA and supplemented with 20% (vol/vol) HeLa cell initiation factors (kindly provided by J Flanegan, University of Florida) In vitro-synthesized proteins were labeled with 20 μCi of [^{35}S]cysteine (specific activity >1,000 Ci/mmol, Amersham) for 3 h at 30°C After this incubation, RNA was degraded by treatment with RNase T_1 (500 U) and RNase A (5 μg) for 10 min at 30°C Translation products (5μl) were analyzed on a 12 5% polyacrylamide gel containing sodium dodecyl sulfate (SDS) (25) The gels were fixed in 30% methanol-10% acetic acid, rinsed in dimethyl sulfoxide, fluorographed with 20% 2,5-diphenyloxazole in dimethylsulfoxide, dried under vacuum, and exposed to Kodak XAR film at -80°C

Analysis of viral protein synthesis in vivo. BGM monolayer cells (2 x 10^5) were infected with either wild-type or mutant viruses at 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 for three times with PBS and pulse-labeled in methionine- and serum-free MEM (Gibco) containing 10 μCi of Tran^{35}S-label (a mixture of [^{35}S]methionine and [^{35}S]cysteine, specific activity, >1,000 Ci/mmol, ICN) After 1 h, the medium was removed and the cells were lysed in cold lysis buffer containing 500 mM Tris-HCl (pH 8 0), 150 mM NaCl, 0 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40 and 0 05% SDS Labeled proteins were analyzed by polyacrylamide gel electrophoresis as described above

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 genotype of the mutants and the predicted effects of the mutations on hydrophobicity On 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 (TCG) 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 alipathic hydroxyl side chains (iii) In mutants 2B-A71E, 2B-I73E, and 2B-

A



B

mutant	genotype	<H> 63-80	virus growth	revertant
2B-S77M	. . . . . M . . .	0.57	wild-type	
2B-C75M	. . . . . M	0.54	small-plaque	C75V
2B-S77M/C75M	. . . . . M . M . . .	0.59	-	S77M/C75V
2B-I64S	. S	0.44	wild-type	
2B-V66S	. . . S	0.45	wild-type	
2B-I64S/V66S	. S . S	0.37	-	
2B-A71E	. . . . . E	0.45	-	
2B-I73E	. . . . . E	0.40	-	
2B-A71E/I73E	. . . . . E . E	0.33	-	

Figure 2. **(A)** Schematic structure of the genome of CBV3 showing the 7.5-kb single-stranded RNA genome with the covalently bound VPg protein at the 5' end of the nontranslated region (NTR) and the genomic encoded poly(A) tract at the end of the 3' NTR. The boxed region shows the polyprotein containing the structural P1 region proteins and the nonstructural P2 and P3 region proteins. Also shown is the partial amino acid sequence of protein 2B including the hydrophobic domain formed by aa 63 to 80 (which is underlined) and the mean <H> per residue of this domain according to Eisenberg et al. (15). **(B)** aa 63 to 80 of the pCB3/T7 2B mutant plasmids, the mean <H> per residue of this hydrophobic region, and the effect of the mutations on virus growth.

A71E/I73E, the mean hydrophobicity is decreased by the replacement of alanine 71 (GCC) 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 incubated at 33°C, and four were incubated at 36°C. Cells transfected with copy RNA of mutants 2B-S77M, 2B-I64S, and 2B-V66S exhibited complete CPE at 48 to 72 h posttransfection, which is similar to that of wild-type pCB3/T7. Transfection of copy RNA of mutant 2B-C75M showed a delayed virus growth. Complete CPE was not observed before 72 to 96 h posttransfection. No CPE was observed up to 5 days posttransfection at either 33°C or 36°C after transfection with copy RNAs of mutants 2B-S77M/C75M, 2B-I64S/V66S, 2B-A71E, 2B-I73E, and 2B-A71E/I73E. These cell cultures were subjected to three cycles of freezing and thawing, and 250 μl was subsequently passaged to fresh BGM cell monolayers. CPE was observed after passage of one of the cell cultures transfected with copy RNA of mutant 2B-S77M/C75M (see the following paragraph), but not after passage of cells transfected with copy RNAs of any of the other mutants.

Sequence analysis of the 2B coding region of the viable mutant viruses showed that the original mutations introduced by site-specific mutagenesis were retained in the vRNA and 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.

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 methionine (AUG) at position 75 to a valine (GUG) residue. This partial revertant virus, vCB3-2B-S77M/C75V, exhibited a small-plaque phenotype at all temperatures (data not shown). In single-cycle infections, this mutant produced 10 and 20% of wild-type virus yield at 6 and 8 h postinfection, respectively (data not shown). Amplification and sequence analysis of the 2B coding region of virus from the primary transfected culture showed that the reversion had already

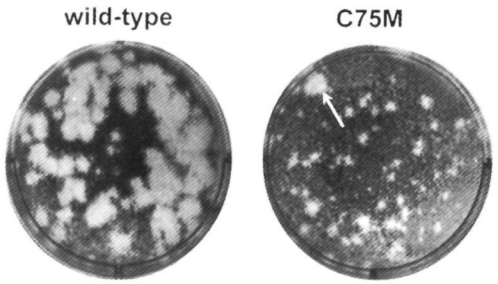


Figure 3. Plaque phenotype of vCB3-2B-C75M and wild-type virus grown at 36°C at 3 days postinfection. Plaque assays were performed on Vero cells as described in Materials and Methods. The arrow in the plate of vCB3-2B-C75M indicates the occurrence of a large-plaque revertant (vCB3-2B-C75V).

occurred in the transfected cells.

The plaque assay plates of mutant vCB3-2B-C75M contained predominantly small plaques; however, larger plaques were also observed (Fig. 3). Three large plaques and three 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 75 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/C75V 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 *Spe*I (nt 3837) and *Bss*HII (nt 4238), purified, and cloned in pCB3/T7. Nucleotides 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-LUC-Δ3D. This plasmid contains a large in-frame deletion in the $3D^{pol}$ region by deletion of the *Bbr*PI 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 result of the translation of newly synthesized chimeric RNA strands. In contrast, phase III of RLuc-31 is already detectable from the third 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 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-Δ3D (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 *Spe*I-to-*Bss*HIII 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

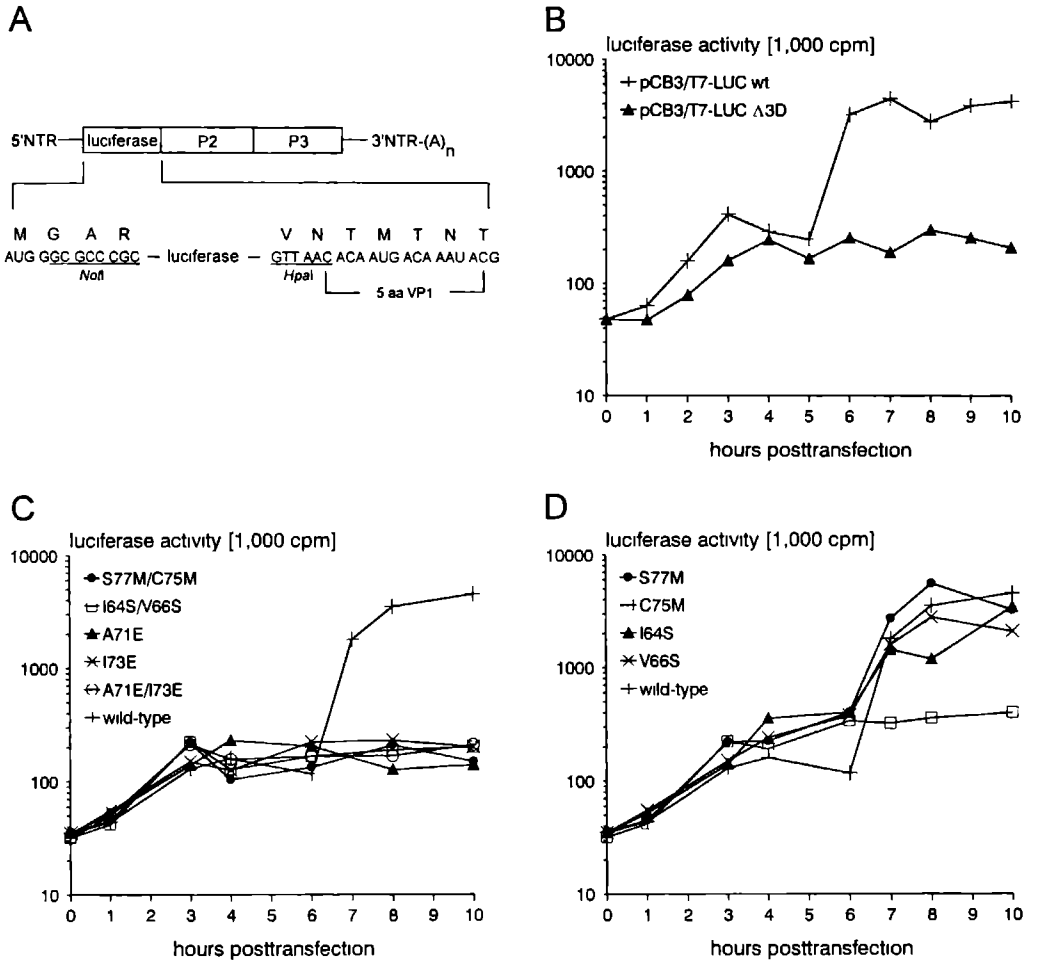


Figure 4. Analysis of vRNA replication of the 2B mutants with chimeric luciferase replicon pCB3/T7-LUC **(A)** Nucleotide and amino acid sequences of construct pCB3/T7-LUC at the N and C termini of the introduced luciferase gene The last 5 aa are conserved in this construct to ensure a correct cleavage by protease 2A Underlined are the *Not*I and *Hpa*I restriction sites used for the introduction of the luciferase gene **(B-D)** Luciferase accumulation after transfection of BGM cells with copy RNAs of wild-type pCB3/T7-LUC and pCB3/T7-LUC-Δ3D **(B)**, pCB3/T7-LUC containing the lethal 2B mutations **(C)**, and pCB3/T7-LUC containing the viable 2B mutations **(D)** Luciferase activities were measured as described in Materials and Methods and are expressed on a logarithmic scale

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 the 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 infectivity of the copy RNA transcripts.

Figures 4C and 4D 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 [^{35}S]-cysteine-labeled proteins obtained with the 2B mutant

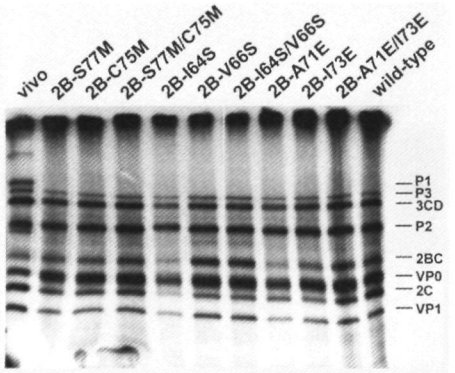


Figure 5. In vitro translation of RNA derived from pCB3/T7 wild-type and the 2B mutant plasmids. *Sal*I-linearized plasmid DNA (0.5 μg) was incubated for 3 h in TNT reticulocyte lysate, a coupled transcription-translation system, supplemented with HeLa initiation factors. The [^{35}S]cysteine-labeled translation products were analyzed on an SDS-12.5% polyacrylamide minigel. An extract from wild-type virus-infected cells, labeled with [^{35}S]methionine for 1 h at 4 h postinfection, was used as marker.

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 globine 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 an MOI of 50, and proteins were labeled with [^{35}S]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 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/

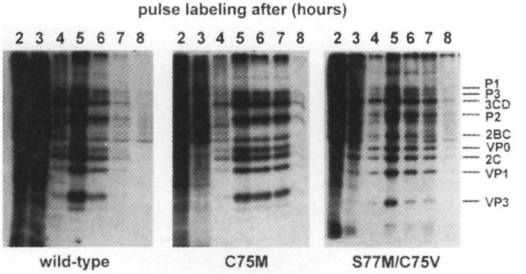


Figure 6. Protein synthesis in BGM cells infected with wild-type virus, vCB3-2B-C75M, and vCB3-2B-S77M/C75V. BGM cells were infected at a MOI of 50 and were grown at 36°C. At the indicated times, the cells were incubated with methionine- and serum-free MEM containing 10 μCi of Tran^{35}S-label (a mixture of [^{35}S]methionine and [^{35}S]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.

Table 1. Complementation tests between pCB3/T7-LUC copy RNAs containing the 2B mutations and wild-type virus[a].

Mutation or wild-type	Luciferase activity (cpm [10^3])[b]		
	4 h pt	8 h pt (mock-infected)	8 h pt (virus-infected)
C75M	142	244 (1.7)	376 (2.6)
S77M/C75M	106	181 (1.7)	137 (1.3)
I64S/V66S	354	212 (0.6)	283 (0.8)
A71E	185	326 (1.8)	152 (0.8)
I73E	198	95 (0.5)	93 (0.5)
A71E/I73E	373	405 (1.1)	370 (1.0)
wild-type	251	3,491 (13.9)	3,050 (12.1)

[a] BGM cells were transfected with 0.5 μg RNA of pCB3/T7-LUC carrying the 2B mutations and either mock or virus infected with wild-type virus at a MOI of 50 at 90 min posttransfection (pt). At 4 and 8 h posttransfection, the cells were lysed and the luciferase activities were determined.

[b] Values in parenthesis are the ratios of the luciferase activities to those measured at 4 h posttransfection.

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 CBV3 revealed an 18-aa hydrophobic domain (aa 63 to 80) with a composition that is characteristic for integral transmembrane regions. In this study, we examined the effects of amino acid substitutions in this hydrophobic domain of CBV3 protein 2B on virus growth, vRNA replication, and viral polyprotein 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 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 reversion 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 alipathic side chains (isoleucine 64 and valine 66) were replaced with polar serine residues with alipathic 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 polyprotein, 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>) 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 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 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 reversion 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 sulfhydryl 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 hydrophobicity or structure 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 two moderate hydrophobic helices, which may be provided either intra- or intermolecularly, 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 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 transmembrane helix. Classification of these segments on the hydrophobic moment plot also suggests that they form multimeric transmembrane 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 intramolecular linkage with other transmembrane α-helices.

At present, there is no biochemical evidence for the prediction that the hydrophobic domain forms an integral transmembrane 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 of the mutants with wild-type phenotypes varied between 0.44 and 0.57. These values are too low for monomeric transmembrane proteins to efficiently penetrate the membrane and maintain an energetically stable configuration (15). (ii) The substitution of cysteine 75 by a more 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 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 involved 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 effects is the consequence of the other. If permeabilizing the plasma membrane were the primary function of the hydrophobic 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 synthesis.

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 mechanism would ensure a tight linkage between translation and replication and prevent RNAs other than viral positive RNA strands from being replicated by $3D^{pol}$, which lacks template specificity (30). 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 functional 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.

REFERENCES

1 **Aldabe, R, and L. Carrasco.** 1995 Induction of membrane proliferation by poliovirus proteins 2C and 2BC Biochem Biophys Res Commun **206**:64-76

2 **Andino, R., G.E. Rieckhof, P.L. Achacoso, and D. Baltimore.** 1993 Poliovirus RNA synthesis utilizes an RNP complex formed around the 5' end of viral RNA EMBO Journal **12**:3587-3598

3 **Baltimore, D.** 1968 Structure of the poliovirus replicative intermediate RNA J Mol Biol **32**:359-368

4 **Bernstein, H.D., P. Sarnow, and D. Baltimore.** 1986 Genetic complementation among poliovirus mutants derived from an infectious cDNA clone J Virol **60**:1040-1049

5 **Bienz, K., D. Egger, and L. Pasamontes.** 1987 Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography Virology **160**:220-226

6 **Bienz, K., D. Egger, and T. Pfister.** 1994 Characteristics of the poliovirus replication complex Arch Virol **suppl. 9**:147-157

7 **Bienz, K., D. Egger, T. Pfister, and M. Troxler.** 1992 Structural and functional characterization of the poliovirus replication complex J Virol **66**:2740-2747

8 **Bienz, K., D. Egger, Y. Rasser, and W. Bossart.** 1983 Intracellular distribution of poliovirus proteins and the induction of virus-specific cytoplasmic structures Virology **131**: 39-48

9 **Bienz, K., D. Egger, M. Troxler, and L. Pasamontes.** 1990 Structural organization of poliovirus RNA replication is mediated by viral proteins of the P2 genomic region J Virol **64**:1156-1163

10 **Cho, M.W., N. Teterina, D. Egger, K. Bienz, and E. Ehrenfeld.** 1994 Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells Virology **202**:129-145

11 **Chomczynski, P., and N. Sacchi** 1987 Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction Anal Biochem **162**:156-159

12 **Collis, P.S., B.J. O'Donnel, D.J. Barton, J.A. Rogers, and J.B. Flanegan.** 1992 Replication of poliovirus RNA and subgenomic RNA transcripts in transfected cells J Virol **66**: 6480-6488

13 **Doedens, J.R., and K. Kirkegaard.** 1995 Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A EMBO Journal **14**:894-907

14 **Doedens, J., L.A. Maynell, M.W. Klymkowsky, and K. Kirkegaard.** 1994 Secretory pathway function, but not cytoskeletal integrity, is required in poliovirus infection Arch Virol **suppl. 9**:159-172

15 **Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall.** 1984 Analysis of membrane and surface protein sequences with the hydrophobic moment plot J Mol Biol **179**:125-142

16 **Giachetti, C., S.-S. Hwang, and B.L. Semler.** 1992 *cis*-Acting lesions targeted to the hydrophobic domain of a poliovirus membrane protein involved in RNA replication J Virol **66**:6045-6057

17 **Girard, M.** 1969 In vitro synthesis of poliovirus ribonucleic acid role of the replicative intermediate J Virol **3**:376-384

18 **Irurzun, A., L. Perez, and L. Carrasco.** 1992 Involvement of membrane traffic in the replication of poliovirus genomes effects of Brefeldin A Virology **191**:166-175

19 **Jang, S.K., M.V. Davies, R.J. Kaufman, and E. Wimmer.** 1989 Initiation of protein synthesis by internal entry of ribosomes into the 5' non-translated region of encephalomyocarditis RNA in vivo J Virol **63**:1651-1660

20 **Johnson, K.L., and P. Sarnow.** 1991 Three poliovirus 2B mutants exhibit noncomplementable defects in viral RNA amplification and display dosage-dependent dominance over wild-type poliovirus J Virol **65**:4341-4349

21 **Kaplan, G., and V.R. Racaniello.** 1988 Construction and characterization of poliovirus subgenomic replicons J Virol **62**:1687-1696

22 **Klump, W.M., I. Bergman, B.C. Müller, D. Ameis, and R. Kandolf.** 1990 Complete nucleotide sequence of infectious coxsackievirus B3 cDNA Two initial 5' uridine residues are regained during plus-strand RNA synthesis J Virol **64**:1573-1583

23 **Kozak, M.** 1989 The scanning model for translation an update J Cell Biol **108**:229-241

24 **Kyte, J., and R.F. Doolittle.** 1982 A simple method for displaying the hydropathic character of a protein J Mol Biol **157**:105-132
25 **Laemmli, U.K.** 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4 Nature **227**: 680-685
26 **Lama, J., and L. Carrasco.** 1992 Expression of poliovirus nonstructural proteins in *Escherichia coli* cells J Biol Chem **267** 15932-15937
27 **Li, J.-P., and D. Baltimore.** 1988 Isolation of poliovirus mutants defective in viral RNA synthesis J Virol **62**:4016-4021
28 **Maynell, L.A., K. Kirkegaard, and M.W. Klymkowsky.** 1992 Inhibition of poliovirus RNA synthesis by Brefeldin A J Virol **66**:1985-1994
29 **Mirzayan, C., and E. Wimmer.** 1994 Biochemical studies on poliovirus polypeptide 2C evidence for ATPase activity Virology **199**:176-187
30 **Oberste, M.S., and J.B. Flanegan.** 1988 Measurement of poliovirus RNA polymerase binding to poliovirion and non-viral RNAs using a filter-binding assay Nucleic Acid Res **16**: 10339-10352
31 **Pelham, H.R.B., and S. Munro.** 1993 Sorting of membrane proteins in the secretory pathway Cell **75**:603-605
32 **Pelletier, J., and N. Sonenberg.** 1988 Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA Nature **334**:320-325
33 **Percy, N., W.S. Barclay, M. Sullivan, and J.W. Almond.** 1992 A poliovirus replicon containing the chloramphenicol acetyltransferase gene can be used to study the replication and encapsidation of poliovirus RNA J Virol **66**:5040-5046
34 **Reed, L.J., and H. Muench.** 1938 A simple method of estimating fifty per cent endpoints Am J Hyg **27**:493-497
35 **Rodriguez, P.L., and L. Carrasco.** 1993 Poliovirus protein 2C has ATPase and GTPase activities J Biol Chem **268**: 8105-8110
36 **Schmidt, N.J.** 1979 Cell culture techniques for diagnostic virology, p 65-139 *In* E H Lennette, and N J Schmidt (ed), Diagnostic procedures for viral, rickettsial and chlamydial infections American Public Health Association, Washington D C
37 **Takeda, N., R.J. Kuhn, C.F. Yang, T. Takegami, and E. Wimmer.** 1986 Initiation of poliovirus plus-strand RNA synthesis in a membrane complex of infected HeLa cells J Virol **60**:43-53
38 **Takegami, T., B.L. Semler, C.W. Anderson, and E. Wimmer.** 1983 Membrane fractions active in poliovirus RNA replication contain VPg precursor polypeptides Virology **128**:33-47
39 **van der Werf, S., J. Bradley, E. Wimmer, F.W. Studier, and J.J. Dunn.** 1986 Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase Proc Natl Acad Sci USA **83**:2330-2334
39a **van Kuppeveld, F.J.M., J.M.D. Galama, G.J. Zoll, and W.J.G. Melchers** unpublished data
40 **Wimmer, E., C.U.T. Helen, and X. Cao.** 1993 Genetics of poliovirus Annu Rev Genet **27**:353-436
41 **Zoll, J., J. Galama, and W. Melchers.** 1994 Intratypic genome variability of the coxsackievirus B1 2A protease region J Gen Virol **75**:687-692
42 **Zoll, J., P. Jongen, J. Galama, F. van Kuppeveld, and W. Melchers.** 1993 coxsackievirus B1-induced murine myositis no evidence for viral persistence J Gen Virol **74**:2071-2076

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

Journal of Virology 1996, 70: 3876-3886

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

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

Department of Medical Microbiology, University of Nijmegen, The Netherlands

Enterovirus protein 2B has been shown to increase plasma membrane permeability. We have identified a conserved putative amphipathic α-helix with a narrow hydrophilic face and an arrangement of cationic residues that is typical for the so-called lytic polypeptides. To examine the functional and structural roles of this putative amphipathic α-helix, we have constructed nine coxsackie B3 virus mutants by site-directed mutagenesis of an infectious cDNA clone. Six mutants contained substitutions of the charged residues in the hydrophilic face of the α-helix. Three mutants contained insertions of leucine residues between the charged residues, causing a disturbance of the amphipathic character of the α-helix. The effect of the mutations on virus viability was assayed by transfection of cells with copy RNA transcripts. The effect on positive-strand RNA replication was examined by introduction of the mutations in a subgenomic luciferase replicon and the analysis of the luciferase accumulation following transfection of BGM cells with RNA transcripts. It is shown that both the amphipathy of the domain and the presence of cationic residues in the hydrophilic face of the α-helix are required for virus growth. Mutations that disturbed either one of these features caused defects in viral RNA synthesis. In vitro translation reactions and the analysis of viral protein synthesis in vivo demonstrated that the mutations did not affect synthesis and processing of the viral polyprotein. These results suggest that a cationic amphipathic α-helix is a major determinant for a function of protein 2B, and possibly its precursor 2BC, in viral RNA synthesis. The potential role of the amphipathic α-helix in the permeabilization of cellular membranes is discussed.

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

Two other metabolic alterations that occur during an enterovirus infection are the inhibition of cellular protein secretion (16) and the permeabilization of the plasma membrane (12). Upon expression of individual poliovirus proteins, Doedens and Kirkegaard found that protein 2B, as well as its precursor 2BC, was capable of inducing both alterations (16). The significance of these alterations, which become evident from the third hour postinfection, for virus replication is unclear. The block in protein secretion may be involved in the accumulation of cytoplasmic vesicles for vRNA synthesis. Apart from this, the inhibition of cellular protein secretion may interfere with host cell responses as interferon secretion and antigen presentation. Modification of the membrane permeability occurs in the late phase of infection of most cytolytic viruses and requires viral gene expression (reviewed by Carrasco et al. in reference 13). The modification is such that gradients of monovalent

cations are gradually destroyed and compounds that do normally not pass the membrane leak out of the cell or leak into the cytoplasm Carrasco et al (13) have suggested that the resulting influx of sodium ions may be involved in the virus-induced shutoff of host cell translation, because the cleavage of the 220-kDa component of eIF-4F may not be sufficient to completely shut off host cell translation (11, 36) High concentrations of sodium are inhibitory to host mRNA translation, whereas vRNA is optimally translated under these altered conditions by the use of specialized structures in the 5' nontranslated region (13) Another function of the membrane modification may be the induction of the cell lysis needed to liberate newly formed virus particles

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

MATERIALS AND METHODS

Cells and viruses. Virus propagations, endpoint titrations, and RNA transfections were performed with Buffalo green monkey (BGM) cells Plaque assays were performed with Vero cells The cells were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum After infection, cells were fed with MEM supplemented with 3% fetal bovine serum After transfection, cells were fed with MEM containing 10% fetal bovine serum

Oligonucleotide-directed site-specific mutagenesis. In vitro mutagenesis was performed with a subgenomic pALTER phagemid clone containing nucleotides (nt) 2080 to 4947 of coxsackie B3 virus (CBV3) (46), using the Altered Sites in vitro mutagenesis system (Promega) according to the instructions of the manufacturer The nucleotide sequence of the antisense synthetic oligonucleotides (Isogen Bioscience, The Netherlands) used for the construction of the mutants (shown in parentheses) are as follows 5'-TGATATTATCCTAAC TAAGGCTCTTAGAGATCTCTCTAAGAT-3' (2B-K[41,44, 48]R [This mutant contains K-to-R mutations at positions 41, 44, and 48]), 5'-TGATATTATCTCAACTAAGGCCTCTAA GATTCCTCTAAGAT-3' (2B-K[41,44,48]E), 5'-TAAGGCT TTTAGAGAGAGCTCTAAGATGGAGTC-3' (2B-K[41]L), 5'-TATCTTAACTAAGGCTAGCAGAGAGAGCTCTAA G ATGGAGTC-3' (2B-K[41,44]L), 5'-TTTTAGAGATTTCTTT AAAATGGAGTCTTGACC-3' (2B-E[40]K), 5'-GGCTTTT AGAGATTTGTCTAGAATGGAGTCTTGACCCAC-3' (2B-E[40]D), 5'-AACTAAGGCTTTTAGAGACTTAAGCTCTAA GATGGAGTCTTGACC-3' (2B-ins[41]L), 5'-TATTATCTT AACTAAGGCCTTAAGTAGAGATTTCTCTAAGATGGA-3' (2B-ins[44]L), 5'-TACTAAGGCTGA TATTATCTTAAGA ACTAAGGCTTTTAGAGATTT-3' (2B-ins[48]L) Each of these oligonucleotides created a novel restriction endonuclease site Mutant clones were identified by restriction enzyme analysis The nucleotide sequences of the mutant pALTER clones were verified by dideoxy chain termination sequencing of plasmid DNA with the oligonucleotide 5'-CCATTCAATG AATTTCTG-3' (nt 4117 to 4134) using the AmpliCycle sequencing kit according to the instructions of the manufacturer (Perkin-Elmer) The 402-bp *Spe*I-to-*Bss*HII fragments of the mutant clones were introduced in the unique *Spe*I (nt 3837) and *Bss*HII (nt 4238) sites of plasmid pCB3/T7 which contains a cDNA of CBV3 (strain Nancy) behind a T7 RNA polymerase promoter (28)

Construction of plasmid 2B-bomII. Nucleotides 3862 to 3906 of pCB3/T7 (coding sequence for protein 2B amino acids [aa] 40 to 55) were replaced by 45 nt encoding 15 aa of bombolitin II by using PCR with the synthetic 105-mer 5'-GAATCACTAGTGGGTCAAGACTCCATCTTAAGCAAGA TCACCGATATCCTCGCGAAGCTTGGAAAAGTACTG G CCATTGTGGTGAGGAACCACGATGACCTGATC-3' (nt 3832 to 3936, nucleotides encoding the bombolitin II amino acids are underlined) and the oligonucleotide 5'-TTGGGAT GGCGCGCTCTGCTC-3' (complementary to nt 4231 to 4251) PCRs were performed with ULTma DNA polymerase (Perkin-Elmer), a thermostable DNA polymerase with proofreading activity, and pCB3/T7 DNA as the template DNA The thermal profile consisted of 20 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 2 min The resulting PCR product was cut with *Spe*I and *Bss*HII and cloned in pCB3/T7 cut with the same enzymes The entire introduced fragment was verified by sequence analysis The resulting plasmid was designated 2B-bomII

Transfection of cells with copy RNA transcripts. Plasmids were linearized with *Sal*I and transcribed by phage T7 RNA polymerase as described previously (46) The integrity of the RNA transcripts was checked on a 1% agarose gel BGM cell monolayers grown in 25-cm^2 flasks to a confluency of 70 to 80% were transfected with 5 μg of RNA transcripts using the DEAE-dextran method as described previously (46) After transfection, the cells were incubated at either 33 or 36°C When virus growth was observed, the cultures were incubated until cytopathic effect was complete The cultures were then subjected to three cycles of freezing and thawing, and the viruses were aliquoted in stocks of 1 ml and stored at -80°C

Sequence analysis of the 2B coding region of mutant viruses. Extraction of RNA, cDNA synthesis, and amplification by PCR were performed as described previously (46),

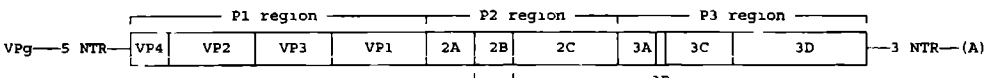
using forward primer 5'-TGGTGTCATTGGCATTGTGACC ATGGGGGG-3' (nt 3648 to 3677) and reverse primer 5'-TTGGGATGGCGCGCTCTGCTC-3' (nt 4231 to 4251) The resulting 604-bp PCR products were purified by low-melting-point agarose gel electrophoresis Sequence analysis was performed as describe above

Plaque assays and single-cycle growth analysis. Plaque assays were performed with 100% confluent Vero cell monolayers grown in 6-well plates as described previously (46) For single-cycle infections, 100% confluent BGM cell monolayers grown in 25-cm^2 flasks (5 x 10^6 cells) were infected with virus at a multiplicity of infection (MOI) of 1 50% tissue culture infective dose per cell for 30 min at room temperature The cells were grown at 33, 36, or 39°C for 2, 4, 6, or 8 h Viruses were released by three cycles of freezing and thawing Viruses were titrated on BGM cell monolayers in 96-well plates, as described (46) 50% tissue culture infective dose values were calulated according to the method of Reed and Muench (38)

Analysis of RNA synthesis. To study the effect of the mutations on RNA synthesis, the *Spe*I-to-*Bss*HII fragments of the mutant plasmids were cloned in the chimeric subgenomic replicon pCB3/T7-LUC (46), which contains the firefly luciferase gene in place of the capsid coding P1 region BGM cell monolayers in 25-cm^2 flasks grown to a confluency of 70 to 80% were transfected with 0 5 µg of T7 RNA polymerase generated RNA transcripts derived from *Sal*I-linearized pCB3/T7-LUC constructs Cells were grown at 36°C, and at the indicated times posttransfection they were washed for three times with phospate-buffered saline (PBS) and lysed in 400 µl of lysis buffer The luciferase activity was measured in a liquid scintillation counter using the Luciferase Assay System according to the recommendations of the manufacturer (Promega)

In vitro translation reactions. Copy RNA transcipts were synthesized and translated in T7 TNT rabbit reticulocyte lysate (Promega) supplemented with 20% (vol/vol) HeLa cell initiation factors (kindly provided by J Flanegan, University of Florida) The translation reactions (20 µl) contained 0 5 µg circular plasmid DNA and 20 µCi of Tran^{35}S-label (a mixture of [^{35}S]methionine and [^{35}S]cysteine, specific activity, >1,000 Ci/mmol, ICN) After 3 h of incubation at 30°C, RNA was degraded by treatment with RNase T$_1$ (500 U) and RNase A (5 µg) for 10 min at 30°C Labeled translation products were analyzed on a 12 5% polyacrylamide gel containing sodium dodecyl sulfate (SDS) (30) Gels were fixed, fluorographed, and exposed to Kodak XAR film at -80°C

Analysis of viral protein synthesis in vivo. BGM cell monolayers (2x10^5 cells) were infected with either wild-type or mutant virus at a MOI of 25 At the indicated times post-infection, the cells were washed with PBS and incubated in methionine- and serum-free MEM (Gibco) containing 10 µCi



pos	aa	mom	hyd	pos	aa	mom	hyd	pos	aa	mom	hyd
1	G			34	G	0 18	0 06	67	T	0 21 +	0 53
2	V			35	Q	0 13	0 13	68	A	0 11 +	0 74
3	K			36	D	0 15	0 06	69	T	0 05 +	0 68
4	D			37	S	0 15	0 06	70	L	0 13 +	0 59
5	Y			38	I	0 12	0 06	71	A	0 12 +	0 59
6	V	* 0 54	-0 03	39	L	0 34	-0 17	72	L	0 01	0 47
7	E	* 0 56	0 02	40	E	0 36	-0 15	73	I	0 02	0 46
8	Q	* 0 56	0 01	41	K	* 0 52	0 02	74	G	0 06	0 41
9	L	* 0 53	0 17	42	S	* 0 50	0 20	75	C	0 13	0 49
10	G	* 0 46	0 24	43	L	** 0 62 +	0 08	76	T	0 32	0 17
11	N	* 0 48	0 26	44	K	** 0 66 +	0 08	77	S	0 34	0 18
12	A	* 0 47	0 27	45	A	** 0 67 +	0 11	78	S	0 34	0 18
13	F	* 0 44	0 33	46	L	** 0 66 +	0 16	79	P	* 0 43	-0 08
14	G	0 41	0 34	47	V	* 0 51 +	0 35	80	W	* 0 52	-0 20
15	S	0 29	0 16	48	K	** 0 61 +	0 46	81	R	0 40	-0 36
16	G	0 34	0 25	49	I	* 0 58 +	0 46	82	W	* 0 47	0 26
17	F	0 29	0 34	50	I	0 33 +	0 73	83	L	* 0 47	0 26
18	T	0 39	0 22	51	S	0 35 +	0 77	84	Q	* 0 50	-0 32
19	N	0 33	0 03	52	A	0 35 +	0 77	85	K	* 0 51	-0 31
20	Q	0 37	0 09	53	L	* 0 53 +	0 44	86	Q	* 0 48	0 36
21	I	0 41	0 03	54	V	* 0 47 +	0 51	87	V	0 23	0 08
22	C	0 39	0 09	55	I	0 33	0 34	88	S	0 26	-0 03
23	E	0 40	0 07	56	V	0 31	0 14	89	Q	0 17	-0 12
24	Q	0 38	0 06	57	V	0 37	0 07	90	Y	0 24	0 07
25	V	0 38	0 05	58	R	0 41	0 11	91	Y	0 15	0 21
26	N	0 37	0 01	59	N	0 41	0 14	92	G	0 07	0 28
27	L	0 33	0 02	60	H	* 0 48	0 04	93	I	0 26	0 05
28	L	0 38	0 05	61	D	* 0 52	0 01	94	P	0 23	-0 11
29	K	0 19	0 16	62	D	* 0 43	0 09	95	M		
30	E	0 18	0 16	63	L	0 40	-0 13	96	A		
31	S	0 22	-0 02	64	I	0 18	0 09	97	E		
32	L	0 17	0 04	65	T	0 26	0 26	98	R		
33	V	0 21	0 06	66	V	0 31	0 35	99	Q		

Figure 1. Analysis of the hydrophobic moment (amphipathy) and hydrophobicity of coxsackie B3 virus protein 2B according to the method of Eisenberg et al (19) The hydrophobic moment (mom) and hydrophobicity (hyd) values for each amino acid were calculated using an 11-residue moving window A positive hydrophobicity indicates a hydrophobic nature Single and double asterisks indicate one and two standard deviations respectively above the average amphipathy of the Protein Identification Resource Database (1989) A plus sign indicates that the 11-residue window satisfies the criterion for membrane binding according to the method of Eisenberg et al (19) At the top of the figure is shown the 7 5-kb single-stranded RNA genome of CBV3 containing (from 5' to 3) the 5' nontranslated region (NTR) with protein VPg covalently bound at its 5' end the polyprotein-encoding region (boxed), which can be divided in the structural P1 protein region and the nonstructural P2 and P3 protein regions and the 3 NTR containing a polyadenylate tract at its 3 end

of Tran^{35}S-label. After 30 min, the medium was removed and the cells were lysed in cold lysis buffer containing 500 mM Tris-HCl (pH 8 0), 150 mM NaCl, 0 1 m M phenylmethylsulfanyl fluoride, 1% Nonidet P-40, and 0 05% SDS Labeled proteins were analyzed by polyacrylamide gel electrophoresis as described above

RESULTS

Identification and classification of a putative amphipathic helix in enterovirus protein 2B. We have searched for the presence of amphipathic helix motifs in CBV3 protein 2B by calculating the mean hydrophobic moment and hydrophobicity of 11-residue windows according to the method of Eisenberg et al. (19). Figure 1 shows that amino acids 37 to 54 form the segment with the highest amphipathic potential. The high hydrophobic moment and the moderate hydrophobicity of the 11-residue segment with the highest hydrophobic moment (aa 40-50; moment, 0.67; hydrophobicity, 0.11) are characteristic for surface seeking amphipathic helices (19), i.e., helices which insert their hydrophobic part in the lipid membrane and expose their hydrophilic part to the aquous face. Graphic representation on a helical wheel diagram according to Schiffer and Edmundson (40) shows that this segment forms a putative amphipathic α-

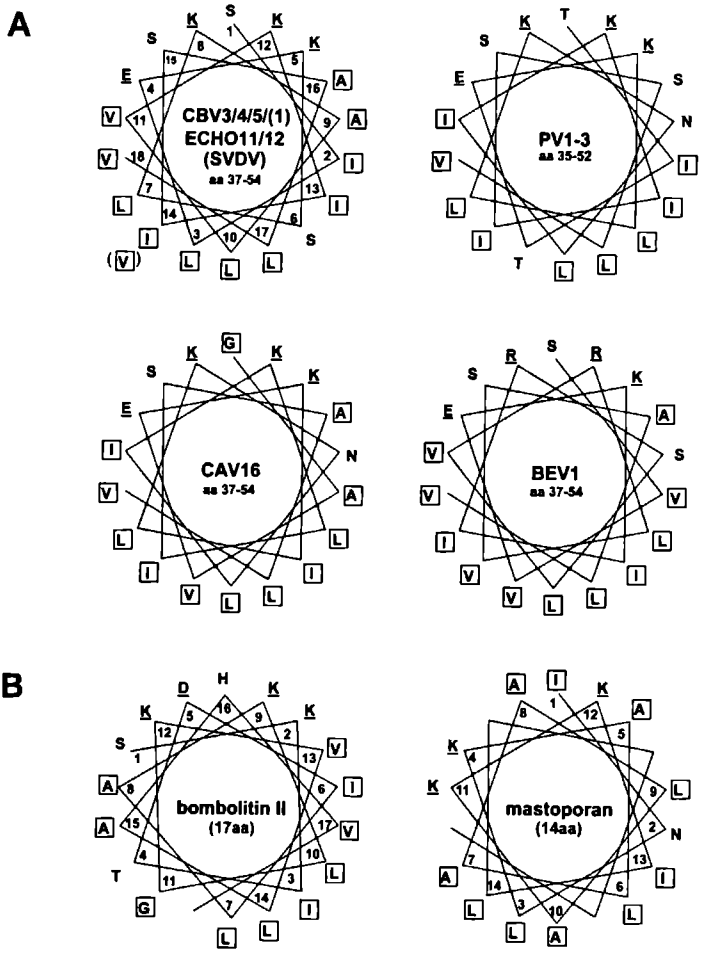


Figure 2. **(A)** Helical wheel diagrams of the putative amphipathic helices found in the 2B proteins of several enteroviruses according to the method of Schiffer and Edmundson (40) Positively charged (R and K) and negatively charged (D and E) residues are underlined Hydrophobic amino acids are boxed Abbreviations CBV, coxsackie B virus, CAV, coxsackie A virus, ECHO, echovirus, SVDV, swine vesicular disease virus, PV, poliovirus, BEV, bovine enterovirus **(B)** Helical wheel diagrams of lytic polypeptides bombolitin II, a heptadecapeptide isolated from the venom of bumblebee *M pennsylvanicus* (4), and mastoporan, a tetradecapeptide isolated from the venom of the wasp *Vespula lewisii* (23)

helix with a narrow hydrophilic face on one side of the helix (Fig. 2A). The distribution of charged residues (three lysines and one glutamic acid) on the hydrophilic face is well conserved among all enteroviruses, except that bovine enterovirus protein 2B contains arginines rather than lysines at positions 44 and 48 (Fig. 2A). The hydrophilic face of poliovirus protein 2B is somewhat larger because of the occurrence of polar asparagine and serine residues at aa 43 and 50, respectively. On basis of their physical-chemical and structural properties, amphipathic helices are grouped into seven classes (41). The arrangement of the charged residues in the putative amphipathic helix of CBV3 protein 2B is typical for the class of the so-called lytic polypeptides. These lytic polypeptides form a group of small cationic amphipathic α-helical peptides that are characterized by narrow polar faces with intermediate charge densities, the occurrence of 4 times as many positively charged residues as negatively charged residues, and the occurrence of mainly lysine residues in the hydrophilic face (mean lysine/arginine ratio = 30). Figure 2B shows examples of helical wheel diagrams of two lytic polypeptides: bombolitin II (4) and mastoporan (23).

Construction of mutations in the putative amphipathic helix of CBV3 protein 2B. The function and the structural requirements of the putative amphipathic helix in CBV3 protein 2B were examined by a genetic analysis. A total of nine mutants, containing either substitution or insertion mutations, were generated by site-directed mutagenesis. The mutations were verified by sequence analysis and introduced in the infectious cDNA clone pCB3/T7. The genotype of the mutant plasmids and the effects of the mutations on mean hydrophobic moment and hydrophobicity are shown in Fig. 3A. Two mutants were constructed to examine the requirement for lysine residues at aa 41, 44, and 48. In these mutants, the lysine residues were replaced with either arginine residues (2B-K[41,44,48]R) or negatively charged glutamic acid residues (2B-K[41,44,48]E). Two mutant plasmids were constructed to examine the minimal number of lysine residues required for the role of this domain in the function of protein 2B. In mutant 2B-K[41]L, lysine 41 is replaced with a leucine residue. In mutant 2B-K[41, 44]L, both lysine 41 and lysine 44 are replaced with leucine residues. In the remaining two substitution mutants, the importance of the negatively

A

plasmid	amino acid sequence	aa 37-54 mom.	aa 37-54 hyd.	aa 40-50 mom.	aa 40-50 hyd.	plaque phenotype
	- + + +					
pCB3/T7 wt	S I L E K S L K A L V K I I S A L V	0.54	0.33	0.67	0.11	
	40 45 50					
2B-K[41,44,48]R	. . . . R . . R . . . R	0.69	0.16	0.93	-0.18	wild-type
2B-K[41,44,48]E	. . . . E . . E . . . E	0.43	0.46	0.49	0.31	-
2B-K[41]L	. . . . L	0.43	0.48	0.48	0.34	small
2B-K[41,44]L	. . . . L . . L	0.30	0.62	0.27	0.57	minute
2B-E[40]K	. . . K	0.56	0.29	0.70	0.04	-
2B-E[40]D	. . . D	0.53	0.32	0.68	0.09	wild-type
2B-ins[41]L	. . . .L.	0.30	0.37	0.45	0.08	-
2B-ins[44]L	. . . . . .L.	0.37	0.37	0.56	0.08	-
2B-ins[48]L	. . . . . .L.	0.28	0.37	0.42	0.08	-

B

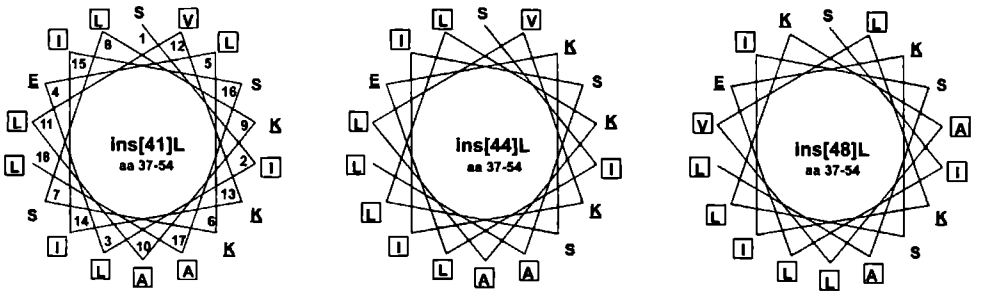


Figure 3. **(A)** Partial amino acid sequence (residues 37 to 54) and the hydrophobic moment and hydrophobicity values of the 2B proteins encoded by wild-type pCB3/T7 and the pCB3/T7-derived mutant plasmids. The effects of the mutations on the hydrophobic moment (mom.) and hydrophobicity (hyd.) of both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

charged glutamic acid 40 was examined. In these mutants, glutamic acid 40 was replaced with either a lysine (2B-E[40]K) or an aspartic acid residue (2B-E[40]D). In the three insertion mutants (2B-ins[41]L, 2B-ins[44]L, and 2B-ins[48]L), hydrophobic leucine residues were introduced at aa 41, 44, and 48, respectively. The insertion of these residues leads to the dispersion of the charged residues and, as a consequence, the disruption of the narrow polar face of the putative amphipathic helix (Fig. 3B), without disturbing the overall hydrophobicity of the domain (Fig. 3A).

Effect of the mutations on virus growth. The effect of the mutations on virus viability was studied by transfection of BGM cells with RNA transcripts. For each mutant, eight transfections were performed. After transfection, the cells were incubated at either 33 or 36°C. In case no virus growth was observed after 5 days, the cells were subjected to three cycles of freezing and thawing and 250 μl was passaged to fresh BGM cell monolayers, which were incubated for another three (36°C) or five days (33°C). Viruses were obtained consistently with RNA transcripts carrying mutations K[41,44,48]R, K[41]L, K[41,44]L, and E[40]D. Plaque assays were performed and four individual plaques were isolated from each mutant virus. Sequence analysis of the 2B coding region of these viruses showed that the mutations introduced by site-directed mutagenesis were retained in the vRNA and that no other amino acid replacements had occurred. These viruses were designated vCB3-2B-K[41,44,48]R, vCB3-2B-K[41]L, vCB3-2B-K[41,44]L, and vCB3-2B-E[40]D, respectively. No virus growth was observed upon transfection of BGM cells with RNA transcripts containing mutations K[41,44,48]E, ins[41]L, ins[44]L, or ins[48]L. To ensure that the nonviability of these mutations was not due to additional mutations in the plasmid DNA, new constructs carrying these mutations were made and RNA transcripts were assayed for their viability. Consistent with our initial results, no virus growth was observed following transfection.

Upon transfection with RNA transcripts of mutant 2B-E[40]K, virus growth was observed in only two of the eight transfected cultures. In one cell culture, virus growth was observed at the second day posttransfection. Sequence analysis of the 2B coding region of these viruses showed that the introduced lysine 40 (AAG) was reverted to the original glutamic acid (GAG) by a single point mutation. No cytopathic effect was observed in the remaining seven transfected cell cultures. Upon passage of these cultures, however, virus growth became visible in one of the cultures grown at 33°C. Sequence analysis of the 2B coding region of these viruses showed that the introduced lysine 40 (AAG) was retained in the vRNA and that no other mutations had occurred. This finding was rather unexpected, because the lack of virus growth in the other cultures suggested that a reversion of the introduced mutation to a more favorable residue or a second-site compensating mutation in protein 2B had occurred. To investigate whether mutation E[40]K indeed allows virus growth and to exclude the possibility that the phenotype of the original mutation was influenced by an additional mutation elsewhere in the plasmid DNA, which might have arisen during the plasmid construction, virus nt 3837 to 4238 (which were found to be identical to those of plasmid 2B-E[40]K) were amplified, cut with *Spe*I and *Bss*HII, and cloned into wild-type pCB3/T7. BGM cells were transfected with RNA transcripts of several independently reconstructed plasmids. However, no virus growth was observed in any the transfected cell cultures. These data strongly suggest that mutation E[40]K is nonviable, and that the viability and phenotype of the isolated virus are due to a second-site mutation outside 2B. For this reason, the virus was not included in further characterization studies.

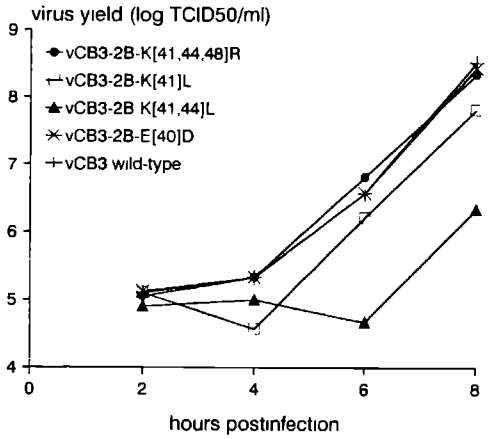
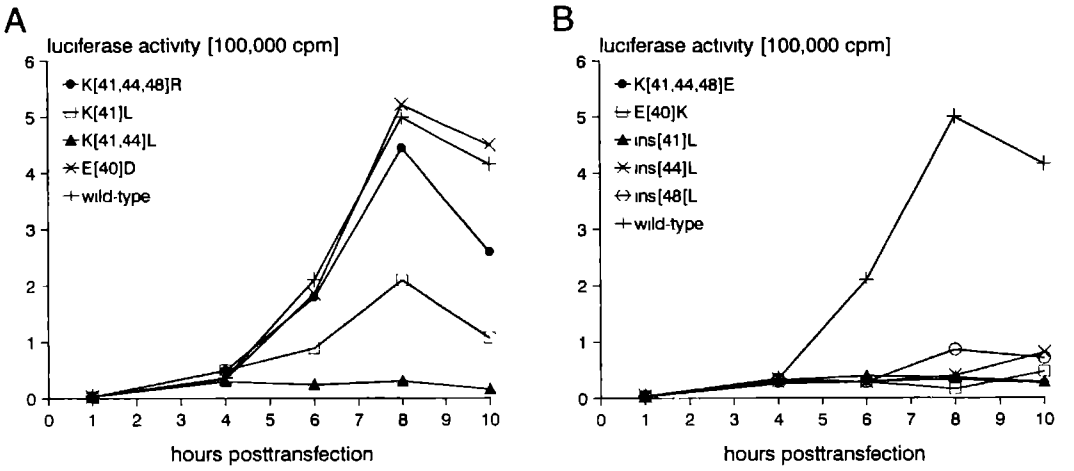


Figure 4. Single-cycle growth curves of wild-type and mutant viruses vCB3-2B-K[41,44,48]R, vCB3-2B-K[41]L, vCB3-2B-K[41,44]L, and vCB3-2B-E[40]D. BGM cells were infected at a MOI of 1, incubated at 36°C, and harvested at 2, 4, 6, and 8 h postinfection. Viruses were released from the infected cells by three cycles of freezing and thawing, and the virus titers were determined by titration on BGM cells at 36°C. $TCID_{50}$, 50% tissue culture infective dose.

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

Effect of the mutations on RNA replication. In a previous study, we reported the construction and use of



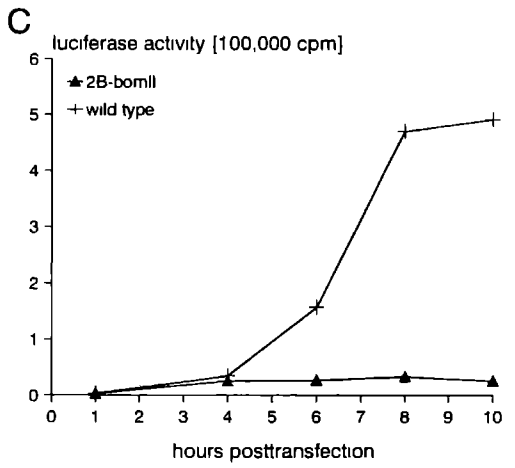


Figure 5. Effects of the viable **(A)** and nonviable **(B)** mutations on the replication of chimeric luciferase replicon pCB3/T7-LUC BGM cells were transfected with copy RNA transcripts of mutant replicons, and the luciferase activity was determined as described in Materials and Methods at the indicated times posttransfection **(C)** Luciferase accumulation after transfection of BGM cells with copy RNA of mutant replicon pCB3/T7-LUC-bomII, which contains a substitution of protein 2B aa 40 to 54 by 15 aa of lytic peptide bombolitin II.

chimeric subgenomic replicon pCB3/T7-LUC, which carries the luciferase gene in place of the P1 protein coding region, to study RNA replication (46). Upon transfection of BGM cells with RNA transcripts of pCB3/T7-LUC, a triphasic pattern of luciferase accumulation was observed; luciferase acitivity increased as a result of translation of the input RNA (phase I), remained constant until the fifth hour posttransfection (phase II), and showed a second increase as a result of the translation of newly synthesized chimeric RNA strands (phase III). That luciferase accumulation indeed reflected vRNA replication was demonstrated by measuring replicon RNA levels by dot blot hybridization (data not shown). To study the effect of the mutations on RNA replication, the *Spe*I-to-*Bss*HII fragments of the mutant plasmids were cloned in replicon pCB3/T7-LUC. BGM cells were transfected with RNA transcripts of the mutant replicons, and luciferase activity was determined at 1, 4, 6, 8, and 10 h posttransfection. The effects of the viable mutations on RNA replication are shown in Fig. 5A. The introduction of mutation K[41]L resulted in a reduced level of RNA replication, as the increase in luciferase activity was only fourfold, whereas the increase in luciferase accumulation was 10- to 20-fold in the case of mutations K[41,44,48]R and E[40]D and wild-type pCB3/T7-LUC. Mutation K[41,44]L caused a more drastic reduction in RNA synthesis, as demonstrated by the absence of a notable increase in luciferase activity. The failure to detect a low level of RNA replication is most likely due to the low infectivity of the RNA transcripts, as has been explained previously (46). The effects of the nonviable mutations on replicon replication are shown in Fig. 5B. This figure shows that each of the mutations K[41,44,48]E, E[40]K, ins[41]L, ins[44]L, and ins[48]L, disturbed RNA replication but not translation of the chimeric RNA transcripts. The stability of the RNA appeared to be unaffected by the mutations, as the activity of luciferase, which is an unstable enzyme in vivo, remained constant up to 10 h posttransfection, suggesting a balance between translation of the input RNA and luciferase degradation.

Analysis of in vitro protein synthesis and polyprotein processing. In vitro translation reactions were performed to examine whether any of the mutations affected polyprotein synthesis and processing. RNA was synthesized and translated in a single reaction mixture using T7 TNT rabbit reticulocyte lysate, a combined transcription-translation system, supplemented with HeLa cell initiation factors. The patterns of ^{35}S-labeled proteins translated from the mutant pCB3/T7 RNAs are shown in Fig. 6A. The results demonstrate that none of

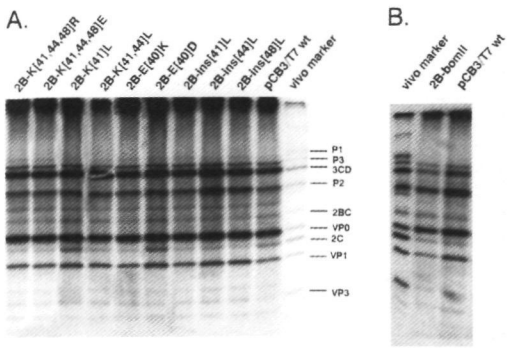


Figure 6. In vitro translation reactions of RNA transcripts derived from the plasmids carrying mutations in the putative amphipathic helix **(A)** and plasmid 2B-bomII **(B)**. Circular plasmid DNA (0.5 μg) was incubated for 3 h in TNT rabbit reticulocyte lysate, a coupled transcription-translation system, supplemented with HeLa cell initiation factors. The [^{35}S]-methionine-labeled translation products were analyzed on an SDS-12.5% polyacrylamide minigel. An extract from wild-type-virus-infected cells, labeled with [^{35}S]methionine for 30 min at 4 h postinfection, was used as a marker.

the mutations caused abnormalities in polyprotein processing. All cleavage products migrated with a correct mobility and the translation patterns of all mutants were similar to that of wild-type pCB3/T7. Although it cannot be excluded that minor differences in the levels of the replication proteins contribute to the viral phenotypes, these results suggest that it is unlikely that the defects in vRNA synthesis caused by nonviable mutations K[41, 44,48]E, E[40]K, ins[41]L, ins[44]L, and ins[48]L, and the reductions in vRNA synthesis caused by viable mutations K[41]L and K[41,44]L are due to defects in polyprotein processing.

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

Replacement of the amphipathic helix with lytic peptide bombolitin II. After having demonstrated that a cationic amphipathic α-helix is required for a function of protein 2B, we tested whether the α-helix could be functionally replaced by cationic lytic polypeptide bombolitin II. Bombolitin II is a heptadecapeptide (Ser-Lys-Ile-Thr-Asp-Ile-Leu-Ala-Lys-Leu-Gly-Lys-Val-Leu-Ala-His-Val) identified in the venom of the bumblebee *Megabombus pennsylvanicus* (4). A helical wheel repre-

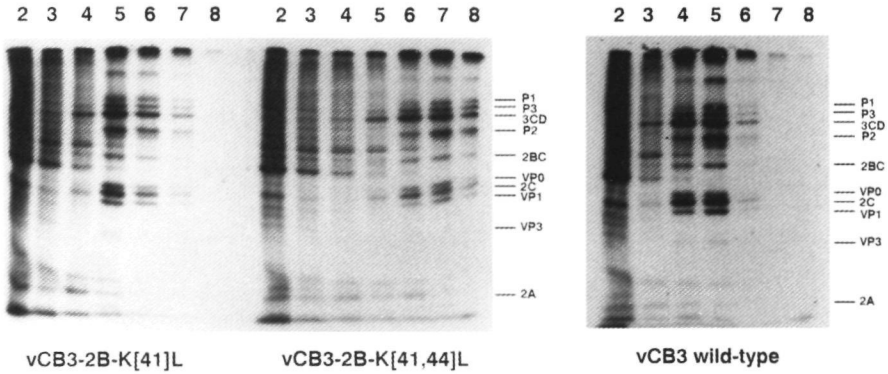


Figure 7. Protein synthesis in BGM cells infected with vCB3-2B-K[41]L, vCB3-2B-K[41,44]L, and wild-type virus. Cells were infected at a MOI of 25, grown at 36°C, and incubated with methionine- and serum-free MEM containing 10 μCi of Tran^{35}S-label (a mixture of [^{35}S]methionine and [^{35}S]cysteine) at various times postinfection (indicated [in hours] above each lane). After a 30-min labeling period, the cells were washed and lysed. The labeled proteins were analyzed on an SDS-12.5% polyacrylamide minigel.

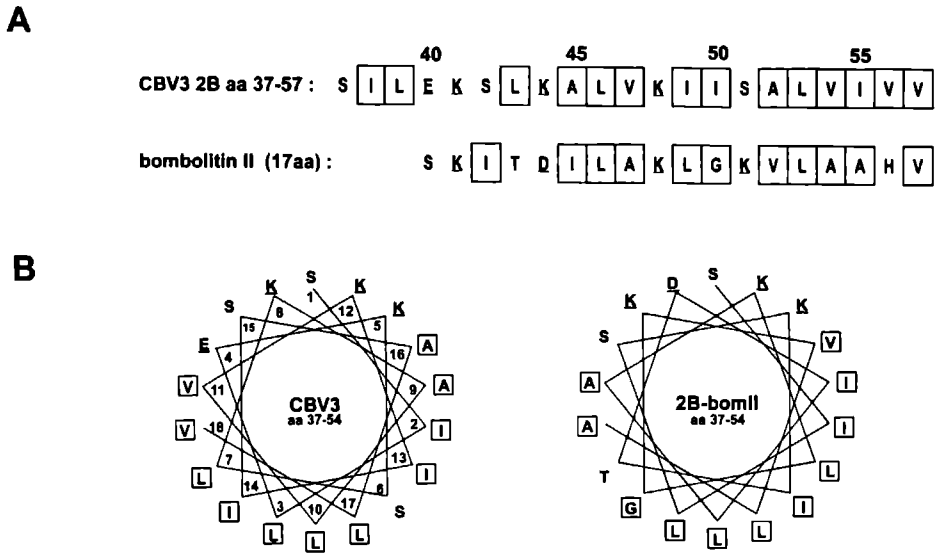


Figure 8. (A) Alignment of aa 37 to 57 of CBV3 protein 2B with the amino acid sequence of cationic α-helical lytic polypeptide bombolitin II (4) Positively charged (R and K) and negatively charged (D and E) residues are underlined Hydrophobic amino acids are boxed **(B)** Helical wheel diagrams showing the amphipathic α-helices (aa 37 to 54) of wild-type CBV3 protein 2B (as encoded in plasmid pCB3/T7) and chimeric protein 2B encoded in plasmid 2B-bomII. In plasmid 2B-bomII, nt 3862 to 3906 (coding for aa 40 to 54) are replaced with 45 nt encoding the first 15 aa of bombolitin II.

sentation of this peptide shows that if can form an amphipathic α-helix with a narrow polar face of six amino acids containing three lysine residues, one negatively charged aspartic acid, and two polar residues (Fig. 2B). Alignment of the amino acid sequences of both helices showed that bombolitin II resembles aa 40 to 56 of CBV3 protein 2B (Fig. 8A). We have replaced the nucleotide sequence encoding aa 40 to 54 with a sequence encoding the first 15 aa of bombolitin II. The nucleotide sequence of the introduced PCR product was verified, and the resulting plasmid was designated 2B-bomII. A helical wheel diagram of aa 37 to 54 of this chimeric plasmid is shown in Fig. 8B. In vitro translation of RNA from this plasmid showed a processing pattern that was similar to that produced by wild-type pCB3/T7 RNA (Fig. 6B). To study the viability of this chimeric construct, BGM cells were transfected with T7 RNA polymerase-generated RNA transcripts. A total of 10 transfections, including passage of the transfected cell cultures to fresh cells, was performed. In none of the transfected cell cultures, however, was virus growth observed. The *Spe*I-to-*Bss*HII fragment of 2B-bomII was introduced in subgenomic replicon pCB3/T7-LUC to study RNA replication. Analysis of the luciferase accumulation following the transfection of BGM cells with copy RNA transcripts demonstrated a defect in replication of the replicon RNA (Fig. 5C). This finding suggests that despite the structural similarities between the amphipathic helix motifs, the cationic α-helix of bombolitin II cannot fulfill the function of the cationic amphipathic α-helix of CBV3 protein 2B in vRNA synthesis.

DISCUSSION

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

helices formed by members of the class of lytic polypeptides

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

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

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

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

The modification of the plasma membrane that occurs during an enterovirus infection is such that both gradients of sodium and potassium are disturbed and small nonpermeative translation inhibitors like hygromycin B and edeine can enter the virus-infected cells (13)

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

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

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

Although the results presented in this paper indicate that the cationic amphipathic α-helix in protein 2B is an important requirement for its function in vRNA synthesis, replacement of this structural motif with the cationic amphipathic helix of lytic peptide bombolitin II resulted in a nonfunctional protein 2B Both amphipathic helices contain a small hydrophilic face formed by three lysines, one negatively charged residue and two polar residues (Fig 2) The nonviability of this chimeric construct (2B-bomII) suggests that the position of the charged residues in the helix may be crucial either for the formation of a cation specific pore or for the membrane-perturbing capacity Alternatively, alterations in amino acid sequence of this domain may interfere with a possible interaction of the putative amphipathic helix with another membrane-interacting domain of CBV3 protein 2B Potential candidates for such an interaction are the N-terminal 16 aa, which also have considerable potential to form an amphipathic α-helix (Fig 1), and the hydrophobic domain, formed by aa 63 to 80, which contains an amino acid composition and hydrophobicity features that are characteristic of multimeric transmembrane regions (46) Either of these domains may, together with the cationic amphipathic α-helix, cooperatively be involved in the formation of a cation-specific pore Additional mutational analysis and analysis of the effect of individually expressed mutant 2B proteins on membrane permeability and protein secretion are required for a better understanding of the involvement of protein 2B in membrane modification and virus replication

REFERENCES

1 **Agawa, Y , S Lee, S Ono, H Aoyagi, M Ohno, T Taniguchi, K Anzai, and Y Kirino** 1991 Interaction with phospholipid bilayers ion channel formation and antimicrobial activity of basic amphipathic α-helical model peptides of

various chain lengths J Biol Chem **266**:20218-20222
2 **Aldabe, R, and L. Carrasco.** 1995 Induction of membrane proliferation by poliovirus proteins 2C and 2BC Biochem Biophys Res Commun **206**:64-76
3 **Andino, R., G.E. Rieckhof, P.L. Achacoso, and D. Baltimore.** 1993 Poliovirus RNA synthesis utilizes an RNP complex formed around the 5' end of viral RNA EMBO Journal **12**:3587-3598
4 **Argiolas, A., and J.J. Pisano.** 1985 Bombolitins, a new class of mast cell degranulating peptides from the venom of bumblebee *Megabombus pennsylvanicus* J Biol Chem **260**: 1437-1444
5 **Baltimore, D.** 1968 Structure of the poliovirus replicative intermediate RNA J Mol Biol **32**:359-368
6 **Bernheimer, A.W., and B. Rudy.** 1986 Interactions between membranes and cytolytic peptides Biochem Biophys Acta **864**:123-141
7 **Bienz, K., D. Egger, and L. Pasamontes.** 1987 Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography Virology **160**:220-226
8 **Bienz, K., D. Egger, and T. Pfister.** 1994 Characteristics of the poliovirus replication complex Arch Virol **suppl. 9**.147-157
9 **Bienz, K., D. Egger, Y. Rasser, and W. Bossart.** 1983 Intracellular distribution of poliovirus proteins and induction of virus-specific cytoplasmic structures Virology **131**: 39-48
10 **Bienz, K., D. Egger, M. Troxler, and L. Pasamontes.** 1990 Structural organization of poliovirus RNA replication is mediated by viral proteins of the P2 genomic region J Virol **64**:1156-1163
11 **Bonneau, A., and N. Sonenberg.** 1987 Proteolysis of the p220 component of the Cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection J Virol **61**:986-991
12 **Carrasco, L.** 1978 Membrane leakiness after viral infection and a new approach to the development of antiviral agents Nature (London) **272**:694-699
13 **Carrasco, L., L. Pérez, A. Irurzun, J. Lama, F. Martínez-Abarca, P. Rodríguez, R. Guinea, J.L. Castrillo, M.A. Sanz and M J. Ayala.** 1993 Modification of membrane permeability by animal viruses, p 283-305 *In* L Carrasco, N Sonenberg, and E Wimmer (ed), Regulation of gene expression in animal viruses Plenum press, New York
14 **Cho, M.W., N. Teterina, D. Egger, K. Bienz, and E. Ehrenfeld.** 1994 Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells Virology **202**:129-145
15 **Christensen, B., J. Fink, R.B. Merriefield, and D. Mauzerall** 1988 Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes Proc Natl Acad Sci USA **85**:5072-5076
16 **Doedens, J.R., and K. Kirkegaard.** 1995 Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A EMBO Journal **14**:894-907
17 **Duclohier, H., G. Molle, and G. Spach.** 1989 Antimicrobial peptide magainin I from Xenopus skin forms anion-permeable channels in planar lipid bilayers Biophys J **56**:1017-1021
18 **Ehrenfeld, E.** 1982 Poliovirus-induced inhibition of host-cell protein synthesis Cell **28**:435-436
19 **Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall.** 1984 Analysis of membrane and surface protein sequences with the hydrophobic moment plot J Mol Biol **179**:125-142
20 **Etchison, D., S.C. Milburn, I. Edery, N. Sonenberg, and J.W B. Hershey.** 1982 Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 222 000 Da polypeptide associated with eukaryotic initiation factor 3 and a cap binding protein complex J Biol Chem **258**:7236-7239
21 **Guinea, R., A. Lopez Rivas, and L. Carrasco.** 1989 Modification of phospholipase C and phospholipase A2 activities during poliovirus infection J Biol Chem **264**:21923-21927
22 **Harris, K.S., W. Xiang, L. Alexander, W S. Lane, A.V. Paul, and E. Wimmer.** 1994 Interaction of poliovirus polypeptide $3CD^{pro}$ with the 5' and 3' termini of the poliovirus genome J Biol Chem **269**:27004-27014
23 **Hirai, Y., T. Yasuhara, H Yoshida, T. Nakajima, M. Fujino, and C. Kitada.** 1979 A new mast cell degranulating peptide "mastoporan" in the venom of Vespula lewisii Chem Pharm Bull **27**:1942-1944
24 **Ide, T., T. Taguchi, T. Morita, M. Sato, K. Ikenaka, S. Aimoto, T. Kondo, H Hojo, M. Kasai, and K. Mikoshiba.** 1989 Mast cell degranulating peptide forms voltage gated and cation-selective channels in lipid bilayers Biochem Biophys Res Commun **163**:155-160
25 **Irurzun, A., J. Arroyo, A. Alvarez, and L. Carrasco.** 1995 Enhanced intracellular calcium concentration during poliovirus infection J Virol **69**:5142-5146
26 **Jang, S.K., M.V. Davies, R.J. Kaufman, and E. Wimmer.** 1989 Initiation of protein synthesis by internal entry of ribosomes into the 5' non-translated region of encephalomyocarditis RNA in vivo J Virol **63**:1651-1660
27 **Johnson, K.L., and P. Sarnow.** 1991 Three poliovirus 2B mutants exhibit noncomplementable defects in viral RNA amplification and display dosage-dependent dominance over wild-type poliovirus J Virol **65**:4341-4349
28 **Klump, W.M., I. Bergman, B.C. Müller, D. Ameis, and R. Kandolf.** 1990 Complete nucleotide sequence of infectious coxsackievirus B3 cDNA Two initial 5' uridine residues are regained during plus-strand RNA synthesis J Virol **64**:1573-1583
29 **Kräusslich, H.G., M J.H. Nicklin, H. Toyoda, D Etchison, and E. Wimmer** 1987 Poliovirus proteinase 2A induces cleavage of eucaryotic initiation factor 4F polypeptide p220 J Virol **61**:2711-2718
30 **Laemmli, U.K.** 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4 Nature (London) **227**:680-685
31 **Lama, J., and L. Carrasco.** 1992 Expression of poliovirus nonstructural proteins in *Escherichia coli* cells J Biol Chem **267** 15932-15937
32 **Michelangeli, F., M.C. Ruiz, J.R. del Castillo, J.E. Ludert, and F. Liprandi.** 1991 Effect of rotavirus infection on intracellular calcium homeostasis in cultured cells Virology **181**: 520-527
33 **Mirzayan, C., and E. Wimmer.** 1994 Biochemical studies on poliovirus polypeptide 2C evidence for ATPase activity Virology **199**:176-187
34 **Nokta, M., D. Eaton, O.S. Steinsland, and T. Albrecht.** 1987 Ca^{2+} responses in cytomegalovirus-infected fibroblasts of human origin Virology **157**:259-267
35 **Pelletier, J., and N. Sonenberg.** 1988 Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA Nature (London) **334**:320-325
36 **Pérez, L., and L. Carrasco.** 1992 Lack of direct correlation between p220 cleavage and the shut-off of host translation after poliovirus infection Virology **189**:178-186
37 **Peterhans, E., Haenggeli, P Wild, and R. Wyler.** 1979 Mitochondrial calcium uptake during infection of chicken embryo with Semliki Forest virus J Virol **29**:143-152
38 **Reed, L.J., and H. Muench.** 1938 A simple method of estimating fifty per cent endpoints Am J Hyg **27**:493-497
39 **Rodriguez P.L., and L. Carrasco.** 1993 Poliovirus protein 2C has ATPase and GTPase activities J Biol Chem **268**:8105-8110
40 **Schiffer, M., and A.B. Edmundson.** 1967 Use of helical wheels to represent the structures of proteins and to identify segments with helical potential Biophys J **7**:121-135
41 **Segrest, J.P., H de Loof, J G. Dohlman, C.G. Brouillette, and G.M Anantharamaiah.** 1990 Amphipathic helix motif Classes and properties Proteins Struct Funct Genet **8**:103-117

42 **Takeda, N., R.J. Kuhn, C.F. Yang, T. Takegami, and E. Wimmer.** 1986 Initiation of poliovirus plus-strand RNA synthesis in a membrane complex of infected HeLa cells J Virol **60**:43-53

43 **Takegami, T., B.L. Semler, C.W. Anderson, and E. Wimmer.** 1983 Membrane fractions active in poliovirus RNA replication contain VPg precursor polypeptides Virology **128**:33-47

44 **Tosteson, M.T., D.S. Auld, and D.C. Tosteson.** 1989 Voltage-gated channels formed in lipid bilayers by a positively charged segment of the Na-channel polypeptide Proc Natl Acad Sci USA **86**:707-710

45 **Tosteson, M.T., and D.C. Tosteson.** 1981 The sting Mellitin forms channels in lipid bilayers Biophys J **36**:109-116

46 **van Kuppeveld, F.J.M., J.M.D. Galama, J. Zoll, and W.J.G. Melchers** 1995 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 J Virol **69**:7782-7790

47 **Wimmer E., C.U.T. Helen, and X. Cao.** 1993 Genetics of poliovirus Annu Rev Genet **27**:353-436

Structure-Function Analysis of Coxsackie B3 Virus Protein 2B

Virology 1997, 227: 111-118

Structure-Function Analysis of Coxsackie B3 Virus Protein 2B

FRANK J. M. VAN KUPPEVELD[1], WILLEM J. G. MELCHERS[1], KARLA KIRKEGAARD[2], AND JOHN R. DOEDENS[2]

Department of Medical Microbiology[1], University of Nijmegen, The Netherlands
Department of Molecular, Cellular, and Developmental Biology[2], University of Colorado, USA

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

The genus *Enterovirus* consists of the polioviruses, coxsackieviruses, echoviruses, and several enterovirus serotypes. Enteroviruses, like other picornaviruses, are small nonenveloped viruses which contain a 7.5-kb single-stranded RNA molecule of positive polarity that is translated into a large polyprotein (22, 34). This polyprotein is processed by virally encoded proteinases to the P1 region proteins, which form the viral capsid, and the P2 and P3 region proteins, most of which are required for viral RNA (vRNA) replication (reviewed in reference 42). The P2 region proteins have also been implicated in the structural organization of viral replication complexes and in the induction of several of the morphological and biochemical alterations that occur during infection. Protein 2A is a proteinase that, in addition to cleaving the viral polyprotein, induces the cleavage of the 220-kDa component of initiation factor eIF-4F (25), implicated in the inhibition of translation of cellular mRNAs (16, 18). Protein 2BC expression can induce the formation of membrane vesicles (4, 9) and therefore may be the viral protein responsible for the generation of the small membrane vesicles on which vRNA replication takes place (7-10). Protein 2C is a small NTPase with RNA binding properties (30, 36), located at the cytoplasmic surface of the virus-induced membrane vesicles where it may be involved in attaching the vRNA to the membranous replication complex (7, 8, 10).

Recently it was found that the expression of poliovirus protein 2B or 2BC results in two of the major biochemical alterations that occur during enterovirus infection: the inhibition of protein secretion (14) and the permeabilization of the plasma membrane (5, 14). The relevance of these activities to the viral life cycle remains to be elucidated. The required residues and the mechanism of induction of these alterations are also unknown. The finding that protein 2B permeabilizes cellular membranes in both mammalian cells and *Escherichia coli* (27) suggests that it may function as an ionophore or otherwise disrupt membrane function. Proteins that display such properties often contain cationic amphipathic α-helical motifs (6, 17, 37). Enterovirus 2B proteins all contain two hydrophobic domains, the more NH_2-terminal of which is predicted to form a cationic amphipathic α-helix (Fig. 1). Mutational analysis of coxsackie B3 virus (CBV3) protein 2B argued that the cationic and amphipathic character of the predicted α-helix was indeed required for the function of 2B in vRNA replication and virus growth (39). Mutational analysis of the second hydrophobic domain of CBV3 protein 2B demonstrated that mutations that caused severe increases or decreases in the hydrophobic character of the domain also impaired the function of protein 2B in the viral replicative cycle (40).

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

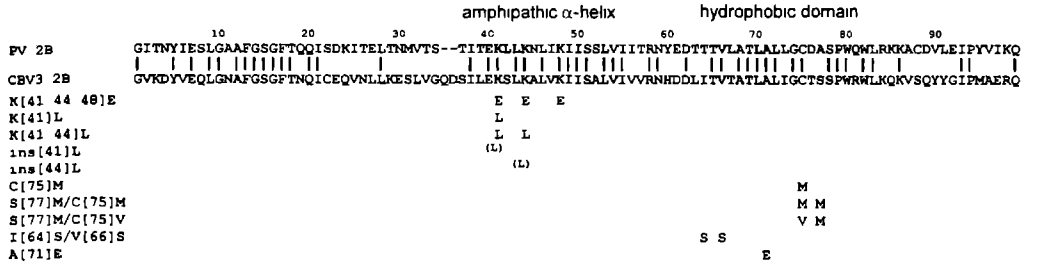


Figure 1. Sequences of wild-type and mutant 2B proteins used in this study Alignment of the complete amino acid sequences of poliovirus (PV) type 1 Mahoney and coxsackie B3 virus (CBV3) is shown in the top panel Numbering is with respect to CBV3 2B protein Solid lines indicate amino acid identity colons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

volved in both the inhibition of protein secretion and the permeabilizing activity, whereas mutation of the second hydrophobic domain has a greater effect on the secretion inhibition function

MATERIALS AND METHODS

Cells, antibodies, and reagents. COS-1 cells were grown as described (14) Affinity-purified rabbit polyclonal antibody to alpha-1 protease inhibitor (A1PI) was obtained from Oswald Pfenniger and Jerry Brown (University of Colorado Health Sciences Center) Hygromycin B was from Boehringer Mannheim Fixed *Staphylococcus aureus* cells for collecting immune complexes were obtained from Gibco-BRL

Construction of plasmids. The dicistronic plasmid p2BNCα6 containing the poliovirus 2B coding region as the first cistron and the A1PI coding region as the second cistron has been described previously (14) pC2Bα6, containing the CBV3 2B sequence upstream of A1PI, was constructed by PCR amplification of the CBV3 2B coding sequence from pCB3/T7 (24) The oligonucleotide primers, 5 -GCAATGTCG ACCATGGGAGTGAAGGACTATGTG-3' and 5'-AAGCCA CCCGGGCTATTGGCGTTCAGCCATAGG-3', introduced a *Sal*I site and an initiation codon upstream of the CBV3 2B coding region and a stop codon and a *Sma*I site downstream of the 2B coding region The 327-bp amplified fragment was cut with *Sal*I and *Sma*I and cloned into pLINKα6, a plasmid containing *Sal*I and *Sma*I sites upstream of the poliovirus 5 -noncoding region and the A1PI coding region (14) The 2B coding region of the mutant pCB3/T7 plasmids carrying mutations in either the hydrophobic domain (40) or the amphipathic α-helix (39) of protein 2B was amplified with the same primers Amplified products were cut with *Spe*I and *Sma*I and cloned into pC2Bα6 from which the corresponding fragment was deleted The 2B coding sequence of all constructs was confirmed by sequence analysis

Secretion assays. COS-1 cells growing on 60-mm dishes were transfected using a standard calcium phosphate method (3) All labelings were performed 2 days posttransfection To radiolabel cells with [^{35}S]methionine, transfected COS-1 cells were washed with phosphate-buffered salt solution (PBS) and incubated in 1 ml of methionine-free DMEM supplemented with 50 μCi of [^{35}S]methionine (Expre^{35}S^{35}S protein labeling mix, New England Nuclear) for 30 min Following labeling, the cells were washed with PBS and placed in 1 ml fresh medium containing 0 23 mM unlabeled methionine for 2 h At the end of the chase period, the culture medium was removed and saved for further analysis Cells were harvested by scraping into 0 5 ml PBS at 4°C The cells were pelleted by centrifugation at 300 x g for 5 min and resuspended in 200 μl of PBS containing 1% Triton X-100, 0 5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride at 4°C After 30 min on ice, detergent-insoluble material was pelleted in a microcentrifuge, and the supernatants were transferred to fresh tubes

Immunoprecipitation of A1PI. Rabbit polyclonal antibody directed against A1PI was diluted in PBS containing 1% Triton X-100, 0 5% sodium deoxycholate, 0 5% SDS, and 1% bovine serum albumin An equal volume of antibody dilution was added to each lysate sample to be precipitated Two hundred microliters of antibody dilution was added to each 1-ml sample of culture supernatant Immune precipitations were then incubated for 2 h on ice, and antibody-antigen complexes were collected by incubation with fixed *S aureus* cells The cells and bound immune complexes were washed three times in ice-cold PBS containing 1% Triton X-100, 0 5% deoxycholate, and 1% SDS Immunoprecipitated A1PI from cell lysates and culture supernatants was then displayed by SDS-PAGE and quantified on a phosphorimager (Molecular Dynamics) Relative amounts of A1PI secretion were determined from the ratio of A1PI detected in the supernatant to that detected in the cell extract This value was adjusted to 1 0 for cells expressing A1PI alone with no 2B protein, and the ratios for cells transfected with wild-type and mutant 2B proteins were normalized

Hygromycin B sensitivity of transfected cells. Sensitivity of transfected COS-1 cells to hygromycin B was determined as described previously (14) except that the labeling period was increased to 1 h Preparation of lysates and immunoprecipitation of A1PI was as described above Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad) A1PI activities were calculated as A1PI phosphorimager counts/μg protein input into immunoprecipitation and the ratios of A1PI synthesized in the presence and absence of hygromycin B were determined When indicated this value was adjusted to 1 0 for cells expressing A1PI alone with no 2B protein, and the ratios for cells transfected with wild-type and mutant 2B proteins were normalized

RESULTS

The finding that poliovirus 2B protein inhibited transport through the cellular secretory pathway and increased plasma membrane permeability to hygromycin

B in the absence of other viral proteins (14) led us to ask (i) whether this function is conserved among other enteroviral 2B proteins and (ii) which structural protein domains are involved in these activities. We chose to test CBV3 protein 2B because the phenotypes of viruses containing a number of different mutations in each of two hydrophobic domains of this protein (Fig. 1) have been recently characterized (39, 40).

Inhibition of protein secretion by coxsackie B3 virus protein 2B. The CBV3 2B coding region was inserted into a dicistronic plasmid designed to express both a single viral protein and human A1PI, a secreted protein. The predicted mRNA from the dicistronic plasmids is diagramed in Fig. 2A. Because A1PI is not expressed in untransfected COS-1 cells, secretion of A1PI produced from dicistronic mRNAs can be used to measure effects of the coexpressed viral proteins on secretory pathway function (14). COS-1 cells were transfected with plasmids that encoded poliovirus protein 2B, CBV3 protein 2B, or no viral protein in the first cistron, and secretion of the A1PI encoded in the second cistron was assayed in a pulse-chase experiment (Fig. 2B).

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

Coxsackie B3 virus protein 2B increases the sensitivity of cellular translation to hygromycin B. Hygromycin B is an inhibitor of translation to which mammalian cells are ordinarily relatively insensitive, because the compound enters cells poorly. Increased sensitivity of translation to hygromycin B has been demonstrated in cells infected with poliovirus and several other viruses (5, 31, 32, 33). In poliovirus-infected cells, this increased sensitivity correlates with increased efflux of ^{86}Rb (29) and is thus thought to reflect increased permeability of the cellular plasma membrane to the drug. In addition, *E coli* that express poliovirus protein 2B show increased uptake of ONPG, increased efflux of [^{3}H]uridine, and increased sensitivity to hygromycin B (27), further suggesting that this protein can significantly alter membrane permeability and that these changes can be detected as increased sensitivity of translation to hygromycin B.

To determine whether expression of CBV3 protein

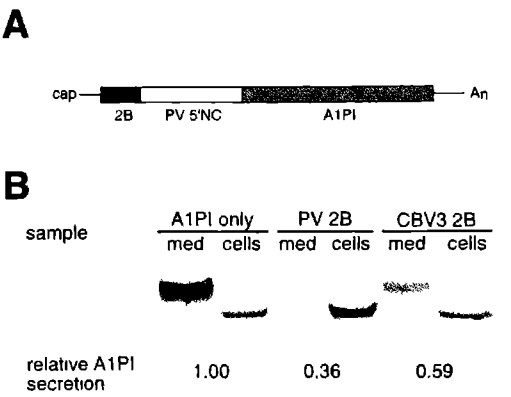


Figure 2. Effect of expression of CBV3 2B protein on secretion of A1PI **(A)** Predicted dicistronic mRNA for coexpression of 2B proteins and A1PI Translation of A1PI in the second cistron is driven by the internal ribosome entry site (IRES) element within the poliovirus 5'-noncoding region (PV5'NC) **(B)** COS-1 cells were transfected with dicistronic plasmids containing the indicated 2B coding region as the first cistron and A1PI sequence as the second Two days posttransfection, the cells were radiolabeled with [^{35}S]methionine for 30 min and chased in unlabeled methionine for 2 h At the end of the chase period, culture supernatants and cell lysates were harvested and A1PI in each sample was quantified by immunoprecipitation followed by SDS-PAGE and phosphorimager analysis The phosphorimage shows radiolabeled A1PI immunoprecipitated from culture medium (med) and cell lysates (cells) derived from cells transfected with the indicated constructs The relative fraction of labeled A1PI secreted into the medium within the chase period is indicated below each pair of lanes, this normalization eliminates any differences in transfection efficiency between experiments The difference in migration between the intracellular and secreted forms of A1PI reflects incomplete glycosylation of the intracellular material (14)

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

Effects of mutations in the amphipathic helix motif on secretion inhibition and hygromycin B sensitivity. To investigate the contribution of the putative cationic amphipathic α-helix motif that comprises residues 37 to 54 of CBV3 protein 2B to secretion inhibition and hygromycin B sensitivity, several mutant alleles of CBV3 2B with amino acid changes within this domain (39) were cloned into the dicistronic A1PI expression plasmid. The amino acid changes of five mutations within this domain are shown in Fig. 1. Plasmids that

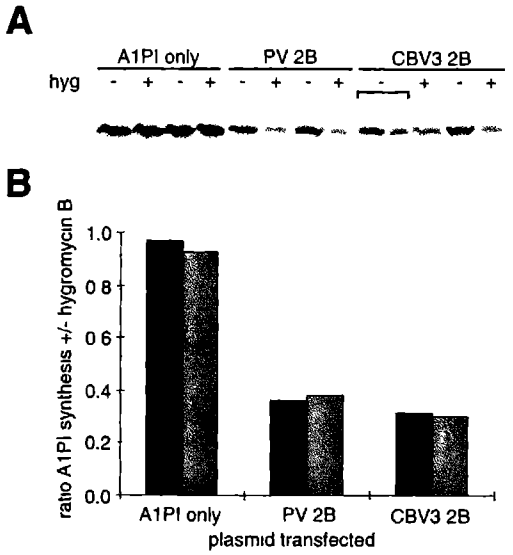


Figure 3. Effect of expression of CBV3 2B protein on hygromycin B permeability COS-1 cells were transfected with dicistronic plasmids containing the indicated 2B coding region as the first cistron and A1PI sequence as the second cistron Two days posttransfection, hygromycin B sensitivity was assayed **(A)** Phosphorimage of immunoprecipitated A1PI synthesized in the presence and absence of 0 5 mg/ml hygromycin B as indicated, results from duplicate plates are shown The bracketed pair of lanes for the CBV3 2B samples represents a single sample inadvertently divided between two lanes of the gel **(B)** Quantitation of relative hygromycin B sensitivity for samples shown in (A), black and grey bars represent duplicate samples A1PI-specific phosphorimager counts from (A) were normalized to the total protein input into the immunoprecipitation reactions The ratio of protein accumulation in the presence and absence of hygromycin B is shown

encoded wild-type and mutant CBV3 2B proteins were transfected into COS-1 cells and assayed for the ability of the expressed mutant 2B proteins to inhibit A1PI secretion and to increase sensitivity to hygromycin B (Fig. 4).

All mutant 2B proteins could still inhibit protein secretion and increase the permeability of cells to hygromycin B. However, the ability of the mutant proteins to induce these alterations varied for the different mutations. The relative effects on virus viability (Fig. 4) and on the two different biochemical activities correlated well for the individual mutant proteins. Each of the mutations K[41,44,48]E, ins[41]L, and ins[44]L, which eliminated viral viability, reduced the ability to inhibit protein secretion and modify plasma membrane permeability to a greater extent than did mutations K[41]L and K[41,44]L, which gave rise to viable, but slow-growing, viruses. Thus, mutant proteins that were the most defective in the virus were the least effective in secretion inhibition and also the least effective in increasing cell sensitivity to hygromycin B. Furthermore, mutations in this domain showed that the integrity of this domain is important for both the inhibition of protein secretion and the increase in membrane permeability caused by CBV3 protein 2B.

Effects of mutations in the second hydrophobic domain on secretion inhibition and hygromycin B sensitivity. We next tested the effects of mutations in the second hydrophobic domain of CBV3 protein 2B (40) on the two activities (Fig. 1). Little correlation between the relative effects of these mutations on the inhibition of protein secretion and the increase in membrane permeability was observed (Fig. 4). With the exception of mutation I[64]S/V[66]S, little effect on the ability of CBV3 2B to increase hygromycin B susceptibility was observed for any of the mutations in the second hydrophobic domain. Nevertheless, mutations C[75]M and S[77]M/C[75]M impaired the ability of protein 2B to inhibit protein secretion. Thus, the observed inhibition of protein secretion by CBV3 protein 2B is probably not caused by the increase in membrane permeability.

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

DISCUSSION

Mutations in the 2B proteins of both poliovirus and coxsackievirus give rise to viruses with primary defects in RNA synthesis (23, 28, 39, 40). However, no biochemical activity directly tied to viral genome replication has yet been demonstrated for 2B. The only activities identified for protein 2B in eukaryotic cells are the two tested here: the inhibition of protein secretion and modification of the plasma membrane permeability. In this study, we have shown that coxsackie B3 virus protein 2B displays these two activities (Figs. 2 and 3), as previously demonstrated for poliovirus protein 2B (14).

It was possible either that secretion inhibition and plasma membrane modification were two separate functions of protein 2B or that one of these effects was the consequence of the other. For example, permeabilization of the plasma membrane by 2B could cause the inhibition of protein secretion by altering the intracellular ionic milieu; increased intracellular calcium levels and changes in concentrations of monovalent cations have been documented in poliovirus-infected cells (13, 20,

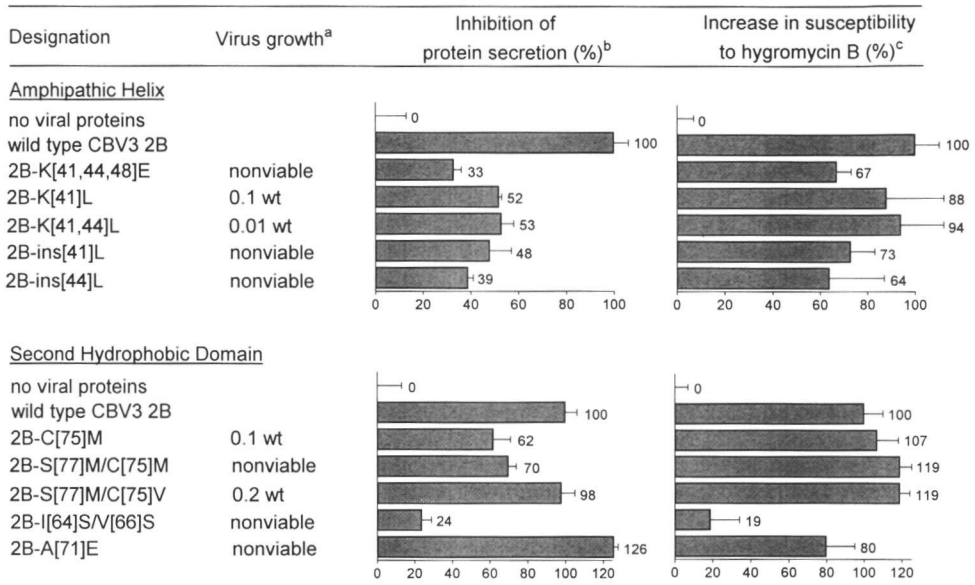


Figure 4. The effects of mutations in the putative cationic amphipathic α-helix and the second hydrophobic domain of CBV3 protein 2B on viral growth in a single-cycle infection, inhibition of protein secretion, and increase in susceptibility to hygromycin B. **(a)** Viral titer after 8 h growth (39, 40). **(b)** Ability of mutant CBV3 2B proteins to inhibit protein secretion, expressed as the percentage of the effect of wild-type 2B protein. COS-1 cells were transfected with dicistronic plasmids encoding the indicated viral protein as the first cistron and A1PI as the second cistron. At 2 days posttransfection, transfected cells were assayed for A1PI secretion. Values calculated for cells transfected with wild-type CBV3 2B were normalized to 100% inhibition. Values for cells expressing A1PI alone with no 2B protein were adjusted to 0% inhibition. Error bars represent standard deviations of measured values. **(c)** Ability of mutant CBV3 2B proteins to increase hygromycin B sensitivity, expressed as the percentage of the effect of wild-type 2B protein. COS-1 cells were transfected with dicistronic plasmids encoding the indicated viral protein as the first cistron and A1PI as the second cistron. At 2 days posttransfection, transfected cells were assayed for hygromycin B sensitivity. Values calculated for cells transfected with wild-type CBV3 2B were normalized to 100% increase in hygromycin B sensitivity. Values calculated for cells expressing A1PI alone with no 2B protein were adjusted to 0% increase. Error bars represent standard deviations of measured values.

29). Such changes might affect a number of cellular processes, including secretory transport. Alternatively, if the direct effect of 2B were on protein transport, the protein and lipid composition of the plasma membrane could be altered, resulting in altered permeability.

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

Does either the ability to inhibit protein secretion or the ability to modify plasma membrane permeability correlate well with the phenotype displayed by CBV3 2B mutant viruses (Fig. 4)? The data are inconclusive on this point. Protein 2B carrying mutations S[77]M/C[75]M, which rendered the virus nonviable, showed a wild-type ability to increase hygromycin B sensitivity but an impaired ability to inhibit protein secretion. A revertant of this mutant, which contained a Met to Val substitution to yield 2B protein S[77]M/C[75]V, also showed wild-type ability to increase hygromycin B sensitivity but increased the ability of the mutant 2B protein to inhibit protein secretion. Thus, a reversion from nonviability to viability correlated with an increase in ability to inhibit protein secretion. Although 2B protein carrying reversion mutation S[77]M/C[75]V showed wild-type abilities in inhibition of protein secretion and increasing hygromycin B sensitivity, viruses carrying this protein exhibited an impaired virus growth. Mutation A[71]E, which rendered 2B able to inhibit protein secretion comparably to wild-type CBV3 2B and which caused only a slight reduction in the ability to induce

membrane permeabilization, even completely abrogated vRNA replication and virus growth (Fig. 4). These observations point to roles for viral protein 2B in the viral replicative cycle other than the interactions with host cell membranes studies here. Consistent with this, mutant poliovirus 2B proteins defective in vRNA synthesis due to mutations outside either the cationic amphipathic α-helical domain or the second hydrophobic domain (mutations 2B201 and 2B204 at residue 29) (23) exhibited wild-type activities in inhibiting cellular protein secretion (15).

The organization and structure of the cationic amphipathic α-helix in enterovirus protein 2B is similar to that of "lytic" polypeptides, a group of cationic amphipathic α-helical peptides that exert cytolytic effects on membranes (37). Two models of action have been proposed to explain the cytolytic action of these peptides. In one model, the cationic peptides form aqueous channels by traversing the membrane and forming multimers that expose their hydrophobic sides to the lipid bilayer and their hydrophilic faces to the aqueous pore. In a second model, the peptides perturb the membrane by lying parallel to the membrane, with their hydrophobic side inserted in the lipid bilayer, thereby making the phospholipids more susceptible to the action of phospholipases (6). Two putative structural models of 2B that are consistent with the need for processing at the 2A/2B and 2B/2C cleavage sites by protein $3C^{pro}$, a cytosolic protein, are shown in Fig. 5.

The increase in sensitivity to hygromycin B caused by expression of CBV3 protein 2B is sensitive to mutations in the predicted cationic amphipathic α-helix. Cationic amphipathic α-helical peptides can form voltage-gated and cation-selective channels in lipid bilayers (1, 2, 19, 38). It is tempting to speculate that channels formed by multimeric 2B proteins are responsible for the influx of the sodium ions, the efflux of potassium ions, and the alterations in calcium levels that are observed from the third hour postinfection by poliovirus (13, 20). Alterations in ionic milieu have been implicated in the shutoff of host cell translation, as high concentrations of sodium ions are inhibitory to host cell but not to viral translation (12), and the cleavage of the p220 component of initiation factor eIF-4F may not be sufficient for complete inhibition of host cell protein synthesis (11, 21, 35). Consistent with this, coxsackieviruses that produce reduced levels of 2B protein due to the presence of poorly processed 2B/2C cleavage sites, failed to completely inhibit host cell protein synthesis (41). Modifications in membrane permeability may also be required for cell lysis, release of progeny virus, or both.

Inhibition of cellular protein secretion by viral protein 2B requires both the cationic amphipathic helix and the hydrophobic domain, and is not a direct result of increased membrane permeability. The inhibition of protein secretion during enterovirus infection is likely to result from alteration or sequestration of membranes or proteins required for secretory transport. This could simply be a consequence of RNA replication complex

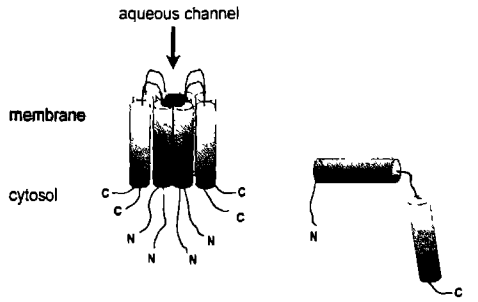


Figure 5. Potential topologies of protein 2B. Putative multimers of 2B proteins that allow the formation of an aqueous channel are shown on the left. Helices representing the membrane-integral amphipathic α-helix (nearer the NH_2-terminus) and the second hydrophobic domain (nearer the COOH-terminus)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

assembly, or it may play an additional role in viral amplification such as blocking host antiviral reponses (14). The exact functions of protein 2B in both membrane permeabilization and inhibition of protein secretion await further investigation.

ACKNOWLEDGMENTS

We thank Michelle DuBois, Peter Sarnow, Jochem Galama, and Joost Hoenderop for comments on the manuscript and Jerry Brown and Oswald Pfenniger for the provision of antibodies. J.D. was supported by a fellowship from the ARCS Foundation, and K.K. was an Assistent Investigator of the Howard Hughes Medical Institute. This work was supported by NIH grant AI-25166 to K.K.

REFERENCES

1 **Agawa, Y., S. Lee, S. Ono, H. Aoyagi, M. Ohno, T. Taniguchi, K. Anzai, and Y. Kirino.** 1991. Interaction with phospholipid bilayers, ion channel formation, and antimicrobial activity of basic amphipathic α-helical model peptides of various chain lengths. J. Biol. Chem. **266**:20218-20222.

2 **Argiolas, A., and J.J. Pisano.** 1985. Bombolitins, a new class of mast cell degranulating peptides from the venom of bumblebee *Megabombus pennsylvanicus*. J. Biol. Chem. **260**: 1437-1444.

3 **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current Protocols in Molecular Biology, vol. 1. John Wiley & Sons, New York.

4 **Barco, A., and L. Carrasco.** 1995. A human virus protein, poliovirus protein 2BC, induces membrane proliferation and blocks the exocytic pathway in the yeast *Saccharomyces cerevisiae*. EMBO J. **14**:3349-3364.

5 **Benedetto, A., G. B. Rossi, C. Amici, F. Belardelli, L. Cioe, G. Carruba, and L. Carrasco.** 1980. Inhibition of animal virus production by means of translation inhibitors unable to penetrate normal cells. Virology **106**:123-132.

6 **Bernheimer, A.W., and B. Rudy.** 1986 Interactions between membranes and cytolytic peptides Biochem Biophys Acta **864**:123-141

7 **Bienz, K., D. Egger, and L. Pasamontes.** 1987 Association of poliviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography Virology **160**:220-226

8 **Bienz, K., D. Egger, and T. Pfister.** 1994 Characteristics of the poliovirus replication complex Arch Virol **suppl. 9**:147-157

9 **Bienz, K., D. Egger, Y. Rasser, and W. Bossart.** 1983 Intracellular distribution of poliovirus proteins and the induction of virus-specific cytoplasmic structures Virology **131**: 39-48

10 **Bienz, K., D. Egger, M. Troxler, and L. Pasamontes.** 1990 Structural organization of poliovirus RNA replication is mediated by viral proteins of the P2 genomic region J Virol **64**.1156-1163

11 **Bonneau, A., and N. Sonenberg.** 1987 Proteolysis of the p220 component of the Cap-binding protein complex is not sufficient for complete inhibiton of host cell protein synthesis after poliovirus infection J Virol **61**:986-991

12 **Carrasco, L., and A.E. Smith.** 1976 Sodium ions and the shut-off of host cell protein synthesis by picornaviruses Nature (London) **264**:807-809

13 **Carrasco, L., L. Pérez, A. Irurzun, J. Lama, F. Martínez-Abarca, P. Rodríguez, R. Guinea, J.L. Castrillo, M.A. Sanz and M.J. Ayala.** 1993 Modification of membrane permeability by animal viruses, p 283-305 *In* I Carrasco, N Sonenberg and E Wimmer (ed), Regulation of gene expression in animal viruses Plenum press New York

14 **Doedens, J.R., and K. Kirkegaard.** 1995 Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A EMBO J **14**:894-907

15 **Doedens, J.R.** 1996 Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A Ph D thesis, University of Colorado, Boulder

16 **Ehrenfeld, E.** 1982 Poliovirus-induced inhibition of host-cell protein synthesis Cell **28**:435-436

17 **Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall.** 1984 Analysis of membrane and surface protein sequences with the hydrophobic moment plot J Mol Biol **179**:125-142

18 **Etchison, D., S.C. Milburn, I. Edery, N. Sonenberg, and J.W.B. Hershey.** 1982 Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 222,000 Da polypeptide associated with eukaryotic initiation factor 3 and a cap binding protein complex J Biol Chem **258**:7236-7239

19 **Ide, T., T. Taguchi, T. Morita, M. Sato, K. Ikenaka, S. Aimoto, T. Kondo, H. Hojo, M. Kasai, and K. Mikoshiba.** 1989 Mast cell degranulating peptide forms voltage gated and cation-selective channels in lipid bilayers Biochem Biophys Res Commun **163**:155-160

20 **Irurzun, A., J. Arroyo, A. Alvarez, and L. Carrasco.** 1995 Enhanced intracellular calcium concentration during poliovirus infection J Virol **69**:5142-5146

21 **Irurzun, A., S. Sanchez-Palomino, I. Novoa, and L. Carrasco.** 1995 Monensin and nigericin prevent the inhibition of host translation by poliovirus, without affecting p220 cleavage J Virol **69**:7453-7460

22 **Jang, S.K., M.V. Davies, R.J. Kaufman, and E. Wimmer.** 1989 Initiation of protein synthesis by internal entry of ribosomes into the 5' non-translated region of encephalomyocarditis RNA in vivo J Virol **63**:1651-1660

23 **Johnson, K.L., and P. Sarnow.** 1991 Three poliovirus 2B mutants exhibit noncomplementable defects in viral RNA amplification and display dosage-dependent dominance over wild-type poliovirus J Virol **65**:4341-4349

24 **Klump, W.M., I. Bergman, B.C. Müller, D. Ameis, and R. Kandolf.** 1990 Complete nucleotide sequence of infectious coxsackievirus B3 cDNA Two initial 5' uridine residues are regained during plus-strand RNA synthesis J Virol **64**:1573-1583

25 **Kräusslich, H.G., M.J.H. Nicklin, H. Toyoda, D. Etchison, and E. Wimmer.** 1987 Poliovirus proteinase 2A induces cleavage of eucaryotic initiation factor 4F polypeptide p220 J Virol **61**:2711-2718

26 **Kyte, J., and R.F. Doolittle.** 1982 A simple method for displaying the hydropathic character of a protein J Mol Biol **157**:105-132

27 **Lama, J., and L. Carrasco.** 1992 Expression of poliovirus nonstructural proteins in *Escherichia coli* cells J Biol Chem **267** 15932-15937

28 **Li, J.-P., and D. Baltimore.** 1988 Isolation of poliovirus mutants defective in viral RNA synthesis J Virol **62**:4016-4021

29 **Lopez-Rivas, A., J. L. Castrillo, and L. Carrasco.** 1987 Cation content in poliovirus-infected HeLa cells J Gen Virol **68**:335-342

30 **Mirzayan, C., and E. Wimmer.** 1994 Biochemical studies on poliovirus polypeptide 2C evidence for ATPase activity Virology **199**:176-187

31 **Munoz, A., and L. Carrasco.** 1981 Protein synthesis and membrane integrity in interferon-treated HeLa cells infected with encephalomyocarditis virus J Gen Virol **56**:153-162

32 **Munoz, A., and L. Carrasco.** 1983 Effect of interferon treatment on blockade of protein synthesis induced by poliovirus infection Eur J Biochem **137**:623-629

33 **Munoz, A., J. L. Castrillo, and L. Carrasco.** 1985 Modification of membrane permeability during Semliki Forest virus infection Virology **146**:203-212

34 **Pelletier, J., and N. Sonenberg.** 1988 Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA Nature (London) **334**:320-325

35 **Pérez, L., and L. Carrasco.** 1992 Lack of direct correlation between p220 cleavage and the shut-off of host translation after poliovirus infection Virology **189**:178-186

36 **Rodriguez, P.L., and L. Carrasco.** 1993 Poliovirus protein 2C has ATPase and GTPase activities J Biol Chem **268**: 8105-8110

37 **Segrest, J.P., H. de Loof, J.G. Dohlman, C.G. Brouillette, and G.M. Anantharamaiah.** 1990 Amphipathic helix motif Classes and properties Proteins Struct Funct Genet **8**:103-117

38 **Tosteson, M.T., D.S. Auld, and D.C. Tosteson.** 1989 Voltage-gated channels formed in lipid bilayers by a positively charged segment of the Na-channel polypeptide Proc Natl Acad Sci USA **86**:707-710

39 **van Kuppeveld, F.J.M., J.M.D. Galama, J. Zoll, P.J.J.C. van den Hurk, and W.J.G. Melchers** 1996 Coxsackie B3 virus protein 2B contains a cationic amphipathic helix that is required for viral RNA replication J Virol **70**:3876-3886

40 **van Kuppeveld, F.J.M., J.M.D. Galama, J. Zoll, and W.J.G. Melchers** 1995 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 J Virol **69**:7782-7790

41 **van Kuppeveld, F.J.M., P.J.J.C. van den Hurk, J.M.D. Galama, and W.J.G. Melchers.** 1996 Mutagenesis of the coxsackie B3 virus 2B/2C cleavage site determinants of processing efficiency and effects on viral replication J Virol **70**:7632-7640

42 **Wimmer, E., C.U.T. Helen, and X. Cao.** 1993 Genetics of poliovirus Annu Rev Genet **27**:353-436

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

Journal of Virology 1996, 70: 7632-7640

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

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

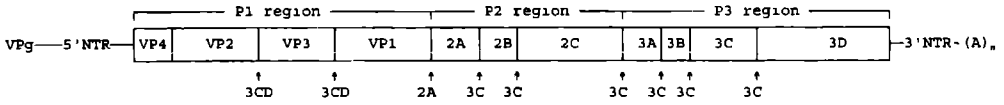
Department of Medical Microbiology, University of Nijmegen, The Netherlands

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

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

The production and stability of proteins 2BC, 3AB, and 3CD require a finely tuned regulation of the temporal processing at different cleavage sites in the polyprotein. Proteolysis may be regulated by the occurrence of less favorable recognition sequences. In PVs, all $3C^{pro}$-mediated cleavages occur between glutamine-glycine

A



B

enterovirus		amino acids at $3C^{pro}$ cleavage sites (pos P4-P3-P2-P1-P1')					
subgroup	virus	2A/2B	2B/2C	2C/3A	3A/3B	3B/3C	3C/3D
CBV-like	CBV1/3/4/5	AxxQ/G	AerQ/N	AxxQ/G	AxxQ/G	AxxQ/G	NxxQ/G
	ECHO11		AerQ/N				NxxQ/G
	ECHO12		AerQ/S				NxxQ/G
	CAV9		AerQ/N				NxxQ/G
	SVDV		AerQ/N				NxxQ/G
PV-like	PV1(S)		VtkQ/G				TxxQ/G
	PV1(M)		VikQ/G				TxxQ/G
	PV2(S)		AieQ/G				TxxQ/G
	PV2(L)		ImrQ/G				TxxQ/G
	PV3(S)/(L)		VirQ/G				TxxQ/G
	CAV21/24		VmrQ/G				TxxQ/G
distinct viruses	CAV16		AqkQ/S				NxxQ/G
	EV70		TirQ/S				TxxQ/G
	BEV1		AerQ/S		SxxQ/G	VxxQ/G	TxxQ/G

Figure 1. (A) Schematic structure of the 7.5-kb single-stranded RNA genome of CBV3 containing protein VPg at 5' end of the nontranslated region (NTR) and a polyadenylate tract at the end of the 3' nontranslated region. The polyprotein-encoding region is shown boxed. The proteinase that is responsible for cleavage at a particular cleavage site (indicated by an arrow) is shown below the polyprotein. **(B)** Alignment of amino acid sequences that occur at the P4-P3-P2-P1/P1' of the 2A/2B, 2B/2C, 2C/3A, 3A/3B, 3B/3C, and 3C/3D cleavage sites of enteroviruses for which complete sequences of the P2 and P3 regions are known. The enteroviruses are divided into a CBV-like subgroup and a PV-like subgroup. Enteroviruses with distinct amino acid sequences are shown apart. Only sequence motifs that differ from the most frequently occurring motif AxxQ/G are shown. Abberant amino acids are in boldface. Abbreviations: ECHO, echovirus; CAV, coxsackie A virus; SVDV, swine vesicular disease virus; PV1(S) and PV1(M), PV type 1 strains Sabin and Mahoney, respectively; PV2(S) and (L), PV type 2 strains Sabin and Lansing, respectively; PV3(S) and PV3(L), PV type 3 strains Sabin and Leon, respectively; EV70, enterovirus type 70; BEV1, bovine enterovirus type 1.

(Q-G) dipeptide pairs. Additional determinants in substrate recognition, however, have been proposed because the PV polyprotein contains four Q-G amino acid pairs that are not cleaved and cleavage sites other than Q-G, as identified in other enteroviruses (15, 27, 32), were also cleavable by PV protein $3C^{pro}$ (17). The amino acid at the P4 position (i.e., the fourth residue proximal to the Q-G cleavage site), which is in most cases an alanine, seems to be a major determinant of the efficiency of cleavage. Data that support the role of the P4 amino acid in substrate recognition have been obtained by mutagenesis of infectious PV cDNA clones (9). The importance of the P4 amino acid for the stability of precursor protein $3CD^{pro}$ has been demonstrated by Pallai et al. (36), who found that a synthetic peptide containing the authentic PV 3C/3D cleavage site (TxxQ↓G) was resistant to hydrolysis by $3C^{pro}$, whereas a peptide containing a substitution of the threonine by an alanine was efficiently cleaved. Other determinants that may affect substrate processing are the recognition of secondary or tertiary structures and the accessibility to a potential cleavage site. Analysis of structural data suggests that cleavage sites must be correctly displayed at the surface in a flexible turn configuration at the end of an α-helix or ß-sheet to be recognized efficiently (4, 35, 36). Processing at the 3A/3B cleavage site seems to be determined by structural rather than by sequence-specific determinants. Lama et al. found that only membrane-associated protein 3AB, not solubilized 3AB, could be cleaved by $3C^{pro}$, indicating that either the recognition or exposure of the 3A/3B cleavage site, which lies in close proximity to the membrane-binding domain of protein 3A, is dependent on a hydrophobic environment (30).

Alignment of the $3C^{pro}$ cleavage sites of the enterovirus nonstructural proteins (Fig. 1B) shows that nearly all enteroviruses contain the sequence AxxQ↓G (P4-P3-P2-P1-P1') at their 2A/2B, 2C/3A, 3A/3B, and 3B/3C junctions. The 3C/3D cleavage sites differ from this sequence by the occurrence of unfavorable residues at the P4 position, which seem to be involved in the stability of protein $3CD^{pro}$ (see above). It is remarkable that also all 2B/2C cleavage site motifs are aberrant from the sequence AxxQ↓G. Coxsackie B virus (CBV)-like viruses contain polair asparagine or serine residues at the P1' position rather than a neutral glycine residue. PV-like viruses, on the other hand, contain at the P4 position either a valine or an isoleucine, residues which are heavily branched at the ß-carbon, rather than an alanine,

which has only a small methyl group as its side chain The only virus that contains the sequence AxxQ1G at its 2B/2C junction, PV type 2 strain Sabin, differs from all other enteroviruses by the occurrence of a negatively rather than a positively charged residue at its P2 position The occurrence of aberrant sequence motifs at the 2B/2C cleavage site of all enteroviruses may indicate a regulatory role in the kinetics of 2B/2C processing and, thereby, in the stability of protein 2BC To test this possibility and to gain more insight in the determinants of the 2B/2C cleavage site specificity and processing efficiency, eight mutant coxsackie B3 virus (CBV3) cDNAs were constructed and the effects of the mutations on in vitro polyprotein processing were analyzed To understand the importance of efficient cleavage at this site for viral replication, the effects of the mutations on virus viability and growth, RNA replication, and in vivo protein synthesis were assayed

MATERIALS AND METHODS

Cells and viruses. Virus propagations and vRNA transfections were performed with Buffalo green monkey (BGM) cells Plaque assays were performed with Vero cells The cells were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum After infection, cells were fed with MEM containing 3% serum After transfection, MEM containing 10% serum was added to the cells Viruses were titrated in 96-well plates with BGM cell monolayers as described previously (46) Virus titers were calculated by the method of Reed and Muench and expressed in 50% tissue culture infective dose ($TCID_{50}$) values (40)

Site-directed mutagenesis. Oligonucleotide-directed site-specific mutagenesis was performed with subgenomic phagemid pALTCB3/2080-4947 (46), using the Altered Sites in vitro mutagenesis system (Promega) according to the manufacturer's recommendations The nucleotide sequence of the synthetic oligonucleotides (Isogen Bioscience, Maarssen, The Netherlands) are 5'-CTTAAGCCAGCTATTGCTTGGCGTT CAGCCAT-3' (mutation 2B/2C-1'G), 5'-CTTAAGCCAGCTA TTTGGTTGGCGTTCAGCCAT-3' (2B/2C-1'P), 5'-CTTAAG CCAGCTATTCGATTGGCGTTCAGCCAT-3' (2B/2C-1'S), 5'-ATTGTTTTGGCGTTCGACCATAGGGATTCCGTA-3' (2B/2C-4V), 5'-ATTGTTTTGGCGCTCATTCATAGGGATT CCGTA-3' (2B/2C-4N), 5'-CTTAAGCCAGCTATTGCCTT GGCGCTCATTCATAGGGATTCCGTA-3' (2B/2C-4N,1'G), 5'-CTTAAGCCAGCTATTGCCTTGGCGT1CGACCATAGG GATTCCGTA-3' (2B/2C-4V,1'G), 5'-CTTAAGCCAATTG CCTTGGCGGATCACCATAGGGATGATTCCGTAATA-3' (2B/2C-4V,3I,1 G) Each of these oligonucleotides created a novel endonuclease restriction site Clones carrying the desired mutation were identified by restriction enzyme analysis The nucleotide (nt) sequence around the 2B/2C junction of these clones was verified by dideoxy chain termination sequencing of plasmid DNA, using primer 5'-CCATTCAAT-GAATTTC TG-3' (nt 4117 to 4134) From the mutant clones, the *Spe*I (nt 3837)-to-*Bss*HII (nt 4238) fragment was cloned in plasmids pCB3/T7, which contains a full-length cDNA of CBV3 Nancy behind a T7 RNA polymerase promoter (28), and pCB3/T7-LUC, a pCB3/T7-derived construct that contains the luciferase gene in place of the capsid coding region (46)

In vitro translation reactions. *Sal*I-linearized plasmid DNA (0 5 μg) was transcribed and translated in a single reaction, using T7 TNT rabbit reticulocyte lysate (Promega) supplemented with HeLa cell initiation factors (kindly provided by J Flanegan, University of Florida) and Tran^{35}S-label (a mixture of [^{35}S]methionine and [^{35}S]cysteine, ICN) Labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (47)

Transfection of cells with RNA transcripts. Eighty percent confluent BGM cell monolayers were transfected with 5 μg of full-length copy RNA transcripts, generated with T7 RNA polymerase from *Sal*I-linearized cDNA templates, using the DEAE-dextran method, as described previously (46) After transfection, the cells were grown at either 33 or 36°C When virus growth was observed, the cultures were incubated until cytopathic effect was complete Otherwise, the cultures were subjected to three cycles of freezing and thawing, and 200 μl was passaged to fresh BGM monolayers, which were incubated for another five (33°C) or three days (36°C)

Sequence analysis of 2B/2C junction of mutant viruses. vRNA extraction, cDNA synthesis and amplification by PCR using forward primer 5'-GCAATGGAACAGGGAGTGAAGG ACTATGTGGA-3' (nt 3733 to 3765) and reverse primer 5'-TTGGGATGGCGCGCTCTGCTC-3' (nt 4231 to 4251) were performed as described (46) The resulting PCR products were purified from low-melting-point agarose, and the nucleotide sequence around the 2B/2C junction was determined as described above

Plaque assays and single-cycle yield analysis. Plaque assays were carried out with confluent monolayers of Vero cells on 10-cm^2 dishes in six-well plates as described previously (46) To determine the virus yield in a single replicative cycle, 100% confluent BGM cell monolayers (5 x 10^6 cells) were infected with virus at a multiplicity of infection (MOI) of 1 $TCID_{50}$ per cell and grown at either 33, 36, or 39°C At 8 h postinfection, viruses were released by three cycles of freezing and thawing and the virus titer was determined

Analysis of positive-strand RNA synthesis. BGM cell monolayers were transfected with 0 5 μg of T7 RNA polymerase generated full-length copy RNA transcripts of the mutant pCB3/T7-LUC plasmids as described above At 1, 4, 6, 8, and 10 h posttransfection, the cells were lysed and the luciferase production was measured as described previously (46)

Analysis of viral protein synthesis in vivo. BGM cell monolayers were infected with virus at an MOI of 25 for 30 min at room termperature After infection, cells were supplied with new medium and incubated at 36°C At several times postinfection, protein synthesis was monitored by labeling with 10 μCi of Tran^{35}S-label in methionine- and serum-free MEM (Gibco) After 30 min, the medium was discarded, the cells were lysed in cold lysis buffer, and labeled proteins were analyzed by SDS-PAGE as described previously (46)

In experiments in which the permeability of cells to hygromycin B was assayed, the same procedure was followed except that cells were incubated in methionine- and serum-free MEM, in the presence or absence of 500 μg of hygromycin B (Sigma) per ml, for 15 min prior to the addition of 10 μCi of Tran^{35}S-label to the medium

To determine the [^{35}S]methionine incorporation, sample lanes were cut from the gel and measured in a liquid scintilation counter following SDS-PAGE and autoradiography

RESULTS

Construction and description of CBV3 2B/2C cleavage site mutants. Substitution mutations at the 2B/2C cleavage site were generated by oligonucleotide-directed mutagenesis and introduced in infectious CBV3

cDNA clone pCB3/T7 and subgenomic replicon pCB3/T7-LUC. The mutants fall into three categories. Within the first group are four mutants that were constructed to examine the importance of the aberrant P1' or P4 residues that occur at the enteroviral 2B/2C cleavage site (Fig. 1B). For this, the P1' asparagine was replaced with a glycine (mutation 2B/2C-1'G), a proline (2B/2C-1'P), or a serine (2B/2C-1'S) or the P4 alanine was replaced with a valine (2B/2C-4V). The second group consists of two mutants that were constructed to examine the effect on 2B/2C cleavage efficiency of a P4 asparagine, as can be found at the 3C/3D cleavage site of CBV-like viruses. The P4 asparagine was introduced either alone (2B/2C-4N) or, in order to create a site that resembles the 3C/3D cleavage site, together with a P1' glycine (2B/2C-4N;1'G). The third group consists of two mutants that were engineered to examine the cross-species substrate specificity of CBV3 $3C^{pro}$. In one mutant, a valine was introduced at the P4 position and a glycine was introduced at the P1' position to create a motif with P4, P1, and P1' residues that occur at the 2B/2C junction of most PV-like viruses (2B/2C-4V;1'G). The other mutant contained an additional replacement of the P3 glutamic acid with an isoleucine (2B/2C-4V;3I;1'G) to create a P4 to P1' sequence that is identical to that found at a PV 2B/2C junction (PV type 3).

In vitro polyprotein processing of 2B/2C cleavage site mutants. The effect of the mutations on the $3C^{pro}$-mediated processing at the 2B/2C cleavage site was studied by in vitro translation of copy RNA transcripts of the mutant pCB3/T7 plasmids. Figure 2A shows the protein patterns generated after 3 h. This figure shows that some of the mutations affected the efficiency of 2B/2C processing, but not the 2B/2C cleavage site specificity, as shown by a correct migration of protein 2C. Remarkably, no accumulation of protein 2BC or any other potential precursor protein was observed. None of the mutations affected any of the other proteolytic processing events in the viral polyprotein, as shown by the normal production of other viral proteins, including protein 2A (Fig. 2C), to levels similar to those produced by wild-type RNA. Protein 2B (11 kDa) could not be visualized because it migrated in the heavily overloaded globin spot.

To determine the relative efficiency of 2B/2C cleavage, autoradiograms from three independent translation experiments were analyzed by densitometric scanning and the ratio of protein 2C/3CD was calculated (to correct for variations in total protein yield). The amount of protein 2C produced by RNA transcripts carrying mutations 2B/2C-1'G and 2B/2C-1'S was similar to that produced by wild-type pCB3/T7 RNA. Mutation 2B/2C-1'P completely abolished 2B/2C cleavage. Even after prolonged exposure of the gels, no protein 2C could be detected. Mutation 2B/2C-4V slightly impaired 2B/2C cleavage, and approximately 85% of the wild-type protein 2C amount was produced. The introduction of asparagine residues at the P4 position (single mutation 2B/2C-4N and double mutation 2B/2C-4N;1'G) reduced

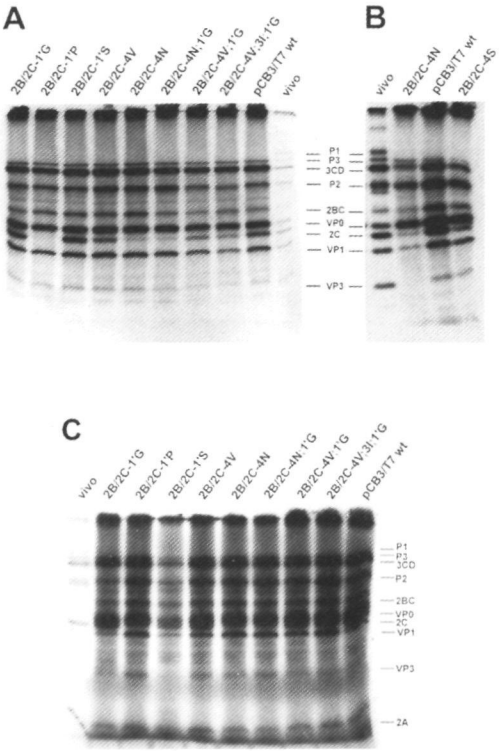


Figure 2. Effects of 2B/2C cleavage site mutations on polyprotein processing in vitro. In vitro translation reactions of RNA transcripts of the wild-type and mutant pCB3/T7 plasmids **(A and C)** and RNA transcripts carrying nonviable mutation 2B/2C-4N and reversion mutation 2B/2C-4S, which was identified at the 2B/2C junction of revertant viruses isolated from cells transfected with RNA transcripts carrying mutation 2B/2C-4N **(B)**. RNA transcripts were synthesized and translated in a single reaction using T7 TNT rabbit reticulocyte lysate. Reactions were incubated for 3 h at 30°C. [^{35}S]methionine labeled translation products were analyzed by SDS- PAGE on 12.5% (A and B) or 15% (C) polyacrylamide minigels. An extract from cells infected with wild-type virus, labeled with [^{35}S]methionine for 1 h at 4 h postinfection, was used as a marker (vivo).

2B/2C cleavage efficiency to about 10% of the wild-type level. Mutations 2B/2C-4V;1'G and 2B/2C-4V;3I;1'G reduced the cleavage efficiency to about 25% of that of the wild-type.

Effects of 2B/2C cleavage site mutations on RNA replication. To study the effects of the mutations on positive-strand RNA replication, BGM cells were transfected with copy RNA transcripts of wild-type and mutant pCB3/T7-LUC constructs, and the luciferase activity was measured at several times posttransfection. Figure 3 shows that none of the mutations interfered with the initial increase in luciferase activity (between 1 and 4 h) that reflects translation of the input RNA (46). However,

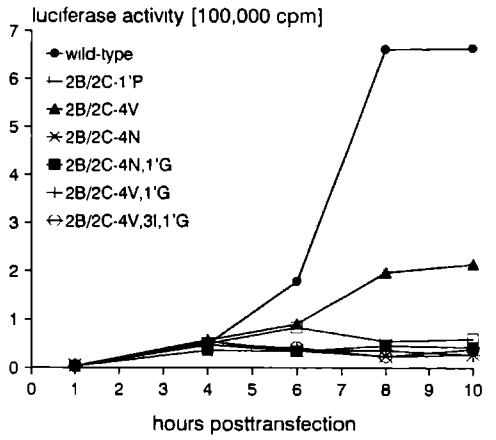


Figure 3. Effects of 2B/2C cleavage site mutations on RNA replication RNA transcripts were synthesized from both mutant and wild-type luciferase replicon pCB3/T7-LUC BGM cells were transfected with both mutant and wild-type RNA transcripts (0 5 μg), and the luciferase activity was determined at the indicated time points posttransfection as described in Materials and Methods

some mutations interfered with the second increase in luciferase activity, which occurs from the fourth hour and reflects the replication of the input RNA and subsequent translation of the newly synthesized RNA strands (46, 47). Replicons carrying mutations 2B/2C-1'G and 2B/2C-1'S, which had virtually no effect on 2B/2C cleavage, displayed an increase (about 10- to 15-fold) in luciferase activity similar to that of wild-type pCB3/T7-LUC (data not shown). Mutation 2B/2C-4V, which caused a minimal reduction in 2B/2C cleavage, reduced RNA replication and yielded an increase in luciferase accumulation that was only fourfold. Mutations that either abrogated (2B/2C-1'P) or severely reduced (2B/2C-4N, 2B/2C-4N;1'G, 2B/2C-4V;1'G, and 2B/2C-4V;3I;1'G) 2B/2C cleavage caused severe defects in RNA replication, as shown by the absence of an increase in luciferase activity above the initial translation level.

Effects of 2B/2C cleavage site mutations on virus viability and growth. The effects of the mutations on virus viability were determined by transfection of BGM cells with copy RNA transcripts from the mutant pCB3/T7 plasmids. For each mutation, two independently made constructs were tested. Four transfections were performed with RNA from each construct. After transfection, two cell cultures were incubated at 33°C and two were incubated at 36°C. Viruses were obtained consistently upon transfection of cells with RNA transcripts carrying mutations 2B/2C-1'G, 2B/2C-1'S, 2B/2C-4V, 2B/2C-1'G;4N, 2B/2C-1'G;4V and 2B/2C-1'G;3I;4V, albeit virus growth was severely delayed with RNA transcripts carrying the three latter mutations. Sequence analysis demonstrated that all viruses had retained the introduced mutation at the 2B/2C junction. The mutant viruses were named vCB3-2B/2C-1'G, vCB3-2B/2C-1'S, vCB3-2B/2C-4V, vCB3-2B/2C-4N;1'G, vCB3-2B/2C-4V;1'G, and vCB3-2B/2C-4V;3I;1'G, respectively. No virus growth was observed in any of the cell cultures transfected with RNA transcripts carrying mutation 2B/2C-1'P. Upon transfection of cells with RNA transcripts carrying mutation 2B/2C-4N, a revertant virus was isolated (see below).

Viral growth characteristics were examined both by plaque assay and measuring the virus yield at 8 h postinfection in a single-cycle infection (Table 1). vCB3-2B/2C-1'G and vCB3-2B/2C-1'S showed wild-type growth characteristics. vCB3-2B/2C-4V produced 16% of the wild-type virus yield and displayed a small-plaque phenotype. vCB3-2B/2C-4N;1'G and vCB3-2B/2C-4V;1'G produced about 2% of the wild-type virus yield and exhibited a minute-plaque phenotype. Minute-plaque virus vCB3-2B/2C-4V;3I;1'G produced only 0.3% of the wild-type virus yield. The relative virus yield of all mutants was the same at 33, 36, and 39°C.

Isolation of revertant virus vCB3-2B/2C-4S. Upon transfection of cells with RNA transcripts carrying mutation 2B/2C-4N, virus growth was observed in only one of the eight transfected cell cultures. Sequence analysis of the 2B/2C junction and the region surrounding this cleavage site showed that this virus contained a reversion of the introduced asparagine (AAU) to a serine (AGU). This mutant virus was named vCB3-2B/2C-4S. To examine whether this reversion mutation was sufficient to confer virus viability, mutation 2B/2C-4S was introduced in pCB3/T7, and BGM cells were transfected with RNA transcripts of the resulting plasmid. The sys-

Table 1. Effects of mutations on 2B/2C cleavage efficiency, plaque size, and single-cycle virus yield.

Mutation	2B/2C junction[a]	2B/2C cleavage[b]	Plaque size[c]	Single-cycle virus yield[d]	
				$\log_{10}$ $TCID_{50}$/ml	% of wt yield
None (wt)	AerQ/N	+++	wt	8 9	
2B/2C-1 G	AerQ/**G**	+++	wt	9 0	
2B/2C-1'P	AerQ/**P**	-	nonviable		
2B/2C-1'S	AerQ/**S**	+++	wt	8 8	
2B/2C-4V	VerQ/N	+++	small	8 1	15 8
2B/2C-4N	NerQ/N	++	nonviable		
2B/2C-4S (revertant)	SerQ/N	±	minute	7 2	2 0
2B/2C-4N 1 G	NerQ/**G**	±	minute	7 1	1 7
2B/2C-4V 1 G	VerQ/**G**	+	minute	7 2	2 0
2B/2C-4V,3I 1'G	VirQ/**G**	+	minute	6 4	0 3

[a] Amino acids at the P4-P3-P2-P1/P1' positions, respectively Mutated amino acids are in boldface

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

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

[d] Confluent BGM cell monolayers were infected with virus at an MOI of 1 $TCID_{50}$ per cell and grown at 36°C At 8 h postinfection, viruses were released by freezing and thawing and the virus titer was determined by endpoint titration

tematic isolation of viruses carrying this mutation indicates that the reversion of the asparagine to the serine at the P4 position of the 2B/2C cleavage site was indeed sufficient to confer virus viability. This mutant virus displayed a minute-plaque phenotype and produced about 2% of the wild-type virus yield in single-cycle infections at different temparatures.

To examine whether the viability of reversion mutation 2B/2C-4S correlated with an increased 2B/2C cleavage, the in vitro processing profiles of pCB3/T7 plasmids carrying mutations 2B/2C-4N and 2B/2C-4S were compared. Figure 2B demonstrates that the efficiency of 2B/2C cleavage was indeed increased by reversion mutation 2B/2C-4S; RNA transcripts carrying mutation 2B/2C-4S generated about 25% of the wild-type protein 2C level, whereas RNA transcripts carrying mutation 2B/2C-4N produced only 10% of the wild-type protein 2C level.

In vivo protein synthesis of mutant viruses. The time course and pattern of viral proteins synthesized in vivo was examined by infection of BGM cells with either wild-type virus or mutant viruses at an MOI of 25 and pulse-labeling with [^{35}S]methionine at several times postinfection. The production of viral proteins in cells infected with vCB3-2B/2C-1'G and vCB3-2B/2C-1'S was similar to that produced in wild-type virus-infected cells (data not shown). The protein patterns generated in cells infected with mutant viruses vCB3-2B/2C-4V, vCB3-2B/2C-4S, vCB3-2B/2C-4N;1'G, vCB3-2B/2C-4V;1'G, and vCB3-2B/2C-4V;3I;1'G are shown in Fig. 4A. This figure shows that in wild-type virus-infected cells, cellular protein synthesis was completely shut off at 4 h postinfection and that viral protein synthesis reached a maximum level at 5 h postinfection. In cells infected with small-plaque virus vCB3-2B/2C-4V, viral protein synthesis was delayed and reached a maximum level at 6 and 7 h postinfection, reflecting the reduced growth rate of this virus. Remarkably, cellular protein synthesis was reduced but not completely shut off. This feature was also observed with minute-plaque viruses vCB3-2B/2C-4S, vCB3-2B/2C-4N;1'G, and vCB3-2B/2C-4V;1'G, which produced viral proteins in a large background of cellular proteins. Even at 10 h postinfection, no complete inhibition of cellular translation was observed (data not shown). In cells infected with minute-plaque virus vCB3-2B/2C-4V;3I;1'G, virtually no shutoff of host cell translation occurred. For a comparison of the protein patterns, lysates of cells infected with the different mutant viruses were concurrently electrophoresed on a single gel (Fig. 4B).

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

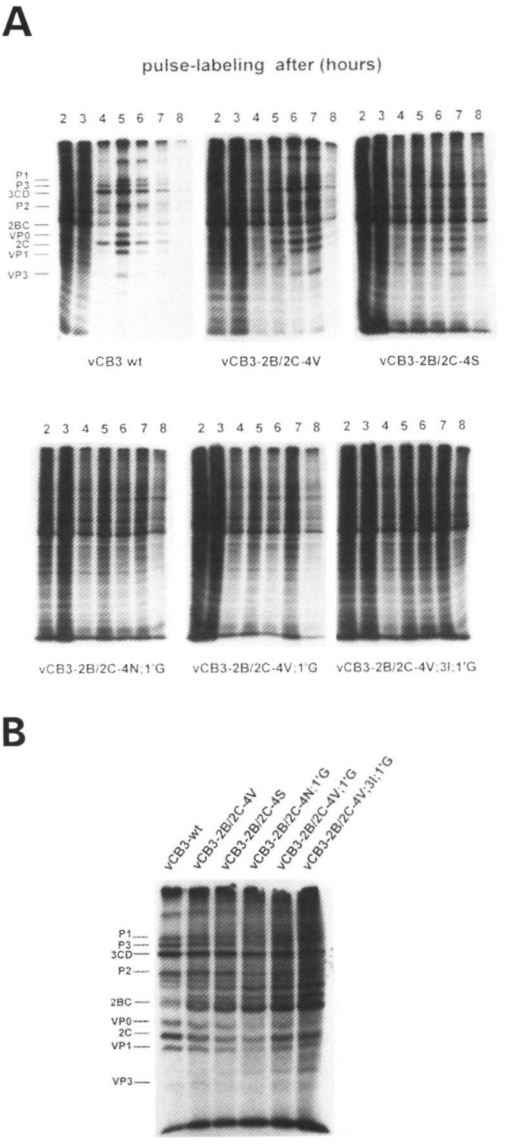


Figure 4. (A) Time course of appearance of viral proteins after infection of BGM cells with wild-type recombinant virus (vCB3 wt) and viruses carrying 2B/2C cleavage site mutations 2B/2C-4V, 2B/2C-4S, 2B/2C-4N;1'G, 2B/2C-4V;1'G, and 2B/2C-4V;3I;1'G. BGM cells (2×10^5) were infected at an equal MOI of 25 $TCID_{50}$ per cell and incubated at 36°C. At the indicated times postinfection, the cells were washed and incubated for 30 min in methionine- and serum-free medium containing 10 µCi of Tran^{35}S-label as source of [^{35}S]methionine. Labeled proteins were analyzed by SDS-PAGE on a 12.5% polyacrylamide minigel. **(B)** Comparison of labeled proteins in lysates of cells infected with the wild-type vCB3 (labeled at 5 h postinfection) and the mutant viruses (labeled at 7 h postinfection) shown in panel A.

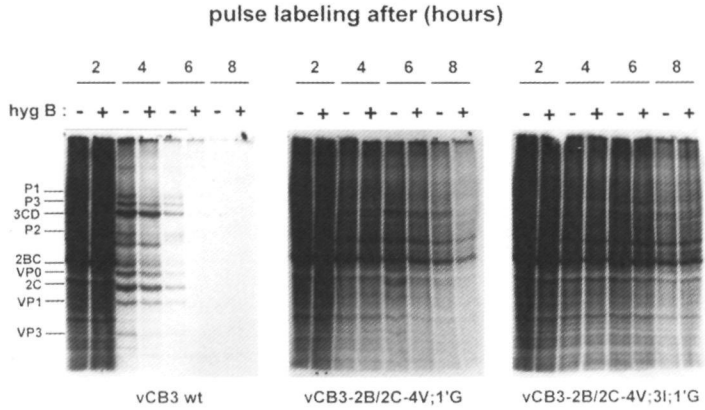


Figure 5. Ability of mutant viruses to increase the plasma membrane permeability to the unpermeant translation inhibitor hygromycin B. BGM cells were infected with either wild-type virus or mutant virus vCB3-2B/2C-4V;1'G or vCB3-2B/2C-4V;3I;1'G at an MOI of 25 $TCID_{50}$ per cell. Pulse-labeling and analysis of proteins were performed as described in the legend to Fig. 4 except that the cells were incubated in methionine- and serum-free MEM for 15 min in the presence (+) or absence (-) of 500 μg of hygromycin (hyg) B per ml prior to the addition of 10 μCi of Tran^{35}S-label to the medium.

To examine whether the failure of the mutant coxsackie B3 viruses to shut off cellular translation correlated with a decreased ability to modify membrane permeability, the sensitivity of translation to hygromycin B was assayed. Cells were infected with wild-type virus, vCB3-2B/2C-4V;1'G, or vCB3-2B/2C-4V;3I;1'G and pulse-labeled in the presence or absence of hygromycin B at 2, 4, 6, and 8 h postinfection. In wild-type virus-infected cells, hygromycin B reduced translation about 70% at both 4 and 6 h postinfection, time points at which cellular translation was indeed shut off and when only viral proteins were synthesized (Fig. 5). In contrast, hygromycin B had little effect on translation in cells infected with the mutant viruses. No inhibition of translation was observed at 4 and 6 h postinfection, when protein synthesis was suppressed to about 40% (vCB3-2B/2C-4V;1'G) and 55% (vCB3-2B/2C-4V;3I;1'G) of the level observed at 2 h postinfection. At 8 h postinfection, when translation was suppressed to about 20% (vCB3-2B/2C-4V;1'G) and 40% (vCB3-2B/2C-4V;3I;1'G), some inhibition of translation (about 40%) by hygromycin B was observed in cells infected with vCB3-2B/2C-4V;1'G but not in cells infected with vCB3-2B/2C-4V;3I;1'G.

DISCUSSION

Enterovirus protein 2BC is a proteolytic processing intermediate that exerts a specific function in vRNA replication (5, 8). Protein 2BC is a poor substrate for $3C^{pro}$ and is only very slowly processed into proteins 2B and 2C in vitro (20, 21, 34). The stability and function of this protein require a subtle regulation of processing efficiency at the 2B/2C cleavage site. One mechanism to regulate the kinetics of proteolysis is the occurrence of unfavorable residues at the scissile bond or positions surrounding this amino acid pair. The enterovirus 2B/2C cleavage site differs from the common motif AxxQ↓G by the occurrence of either polar P1' residues or large aliphatic P4 residues (Fig. 1B). We have constructed several CBV3 2B/2C cleavage site mutants (i) to examine the importance of these aberrant residues for the efficiency of 2B/2C cleavage and, thereby, the stability of protein 2BC, (ii) to gain more insight in the determinants of cleavage site specificity and efficiency of CBV3 $3C^{pro}$, and (iii) to analyse the effects of alterations in the 2B/2C cleavage efficiency, leading to altered levels of proteins 2B and 2C, on virus viability and growth, vRNA replication, and protein synthesis in vivo.

The mutants that were generated fell into three groups. The first group was designed to investigate the importance of the aberrant P1' and P4 residues. Replacement of the P1' asparagine neither with a glycine (2B/2C-1'G), which created the common AxxQ↓G motif, nor with a serine (2B/2C-1'S) affected 2B/2C cleavage, as shown by the production of wild-type levels of proteins 2BC and 2C. RNAs containing these mutant cleavage site motifs replicated efficiently and gave rise to viruses that produced viral proteins and exhibited growth similar to that of the wild-type virus. These results suggest that it is unlikely that the polar P1' residues occurring in CBV-like viruses serve to regulate 2B/2C processing efficiency and, thereby, protein 2BC stability. This is irrespective of whether all 2B and 2C proteins are produced exclusively by *trans* cleavage of protein 2BC or whether a substantial amount of these proteins are produced by successive *cis*-cleavage events from larger precursors (e.g., 2BC-P3 or 2BC-3ABC). This latter possibility should be considered given the relative ineffi-

ciency of cleavage of 2BC in *trans* (20, 21, 34) and the finding that insertion of internal ribosome entry site elements between nonstructural proteins or mutations at specific cleavage sites may interfere with processing at upstream located sites (11, 50) The efficient cleavage of $3C^{pro}$ at a Q-S dipeptide pair and the wild-type growth of these mutant viruses are consistent with the occurrence of this residue at the 2B/2C cleavage site of several other enteroviruses (Fig 1B) and also with the wild-type growth of PVs containing a Q-S dipeptide pair at their 3C/3D cleavage site (27) The relative efficient 2B/2C processing observed at sites containing a P4 valine (2B/2C-4V) argues that it is also unlikely that the large aliphatic P4 amino acids occurring in PV-like viruses are unfavorable residues that provide the stability of protein 2BC Based on these results, we propose that alternative determinants such as inefficient recognition of secondary and tertiary protein structures surrounding the cleavage site or decreased accessibility of $3C^{pro}$ to the cleavage site contribute to the stability of protein 2BC and that a conformational change is required to improve recognition or exposure of the cleavage site Such a change may be induced by an interaction with a specific target (e g , membrane or another protein) The observation of Molla et al that the addition of protein 3AB significantly enhanced proteolysis of PV protein 2BC by $3C^{pro}$ in vitro lends support to such a hypothesis (34)

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

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

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

P1' glycine is due to stabilization of the protein conformation.

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

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

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

REFERENCES

1 **Aldabe, R, and L. Carrasco.** 1995 Induction of membrane proliferation by poliovirus proteins 2C and 2BC Biochem Biophys Res Commun **206**:64-76

2 **Andino, R., G.E. Rieckhof, P.L. Achacoso, and D. Baltimore.** 1993 Poliovirus RNA synthesis utilizes an RNP complex formed around the 5' end of viral RNA EMBO J **12**:3587-3598

3 **Andino, R., G.G. Rieckhof, and D. Baltimore.** 1990 A functional ribonucleoprotein complex forms around the 5' end poliovirus RNA Cell **63**:369-380

4 **Arnold, E., M. Luo, G. Vriend, M.G. Rossmann, A.C. Palmenberg, G.D. Parks, M.J.H. Nicklin, and E. Wimmer.** 1987 Implications of the picornavirus capsid structure for polyprotein processing Proc Natl Acad Sci USA **84**:21-25

5 **Barco, A., and L. Carrasco.** 1995 A human virus protein, poliovirus protein 2BC, induces membrane proliferation and blocks the exocytic pathway in the yeast *Saccharomyces cerevisiae* EMBO J **14**:3349-3364

6 **Benedetto, A., G.B. Rossi, C. Amici, F. Belardelli, L. Cioe, G. Carruba, and L. Carrasco.** 1980 Inhibition of animal virus production by means of translation inhibitors unable to penetrate normal cells Virology **106**:123-132

7 **Bienz, K., D. Egger, and T. Pfister.** 1994 Characteristics of the poliovirus replication complex Arch Virol **suppl. 9**:147-157

8 **Bienz, K., D. Egger, Y. Rasser, and W. Bossart.** 1983 Intracellular distribution of poliovirus proteins and the induction of virus-specific cytoplasmic structures Virology **131**:39-48

9 **Blair, W.S., and B.L. Semler.** 1991 Role for the P4 amino acid residue in substrate utilization by the poliovirus 3CD proteinase J Virol **65**:6111-6123

10 **Bonneau, A., and N. Sonenberg.** 1987 Proteolysis of the p220 component of the Cap-binding protein complex is not sufficient to complete inhibition of host cell protein synthesis after poliovirus infection J Virol **61**:986-991

11 **Cao, X., and E. Wimmer.** 1996 Genetic variation of the poliovirus genome with two VPg coding units EMBO J **15**:23-33

12 **Carrasco, L., L. Pérez, A. Irurzun, J. Lama, F. Martínez-Abarca, P. Rodríguez, R. Guinea, J.L. Castrillo, M.A. Sanz and M.J. Ayala.** 1993 Modification of membrane permeability by animal viruses, p 283-305 *In* L Carrasco, N Sonenberg, and E Wimmer (ed), Regulation of gene expression in animal viruses Plenum press, New York

13 **Castrillo, J.L., A. López-Rivas, and L. Carrasco.** 1987 Effects of extracellular cations on translation in poliovirus-infected cells J Gen Virol **68**:325-333

14 **Cho, M.W., N. Teterina, D. Egger, K. Bienz, and E. Ehrenfeld.** 1994 Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells Virology **202**:129-145

15 **Dewalt, P.G., M.A. Lawson, R.J. Colonno, and B.L. Semler.** 1989 Chimeric picornavirus polyproteins demonstrate a common 3C proteinase substrate specificity J Virol **63**: 3444-3452

16 **Doedens, J.R., and K. Kirkegaard.** 1995 Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A EMBO J **14**:894-907

17 **Dougherty, W.G. and B.L. Semler.** 1993 Expression of virus-encoded proteinases functional and structural similarities with cellular enzymes Microbiol Rev **57**:781-822

18 **Ehrenfeld, E.** 1982 Poliovirus-induced inhibition of host-cell protein synthesis Cell **28**:435-436

19 **Etchison, D., S.C. Milburn, I. Edery, N. Sonenberg, and J.W.B. Hershey.** 1982 Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 222,000 Da polypeptide associated with eukaryotic initiation factor 3 and a cap binding protein complex J Biol Chem **258**:7236-7239

20 **Giachetti, C., S.-S. Hwang, and B. Semler.** 1992 *cis*-Acting lesions targeted to the hydrophobic domain of a poliovirus membrane protein involved in RNA replication J Virol **66**:6045-6057

21 **Hämmerle, T., C.U.T. Helen, and E. Wimmer.** 1991 Site-directed mutagenesis of the putative catalytic triad of poliovirus 3C proteinase J Biol Chem **266**:5412-5416

22 **Hanecak, R., B.L. Semler, C.W. Anderson, and E. Wimmer** 1982 Proteolytic processing of poliovirus polypeptides antibodies to polypeptide P3-7c inhibit cleavage at glutamine-glycine pairs Proc Natl Acad Sci USA **79**:3973-3977

23 **Harris, K.S., W. Xiang, L. Alexander, W.S. Lane, A.V. Paul, and E. Wimmer.** 1994 Interaction of poliovirus polypeptide $3CD^{pro}$ with the 5' and 3' termini of the poliovirus genome J Biol Chem **269**:27004-27014

24 **Irurzun, A., S. Sanchez-Palomino, I. Novoa, and L. Carrasco.** 1995 Monensin and nigericin prevent the inhibition of host translation by poliovirus, without affecting p220 cleavage J Virol **69**:7453-7460

25 **Jang, S.K., M.V. Davies, R J. Kaufman, and E. Wimmer.** 1989 Initiation of protein synthesis by internal entry of ribosomes into the 5' non-translated region of encephalomyocarditis RNA in vivo J Virol **63**:1651-1660

26 **Jore, J., B. de Geus, R.J. Jackson, P.H. Pouwels, and B.E. Enger-Valk.** 1988 Poliovirus protein 3CD is the active protease for processing of the precursor protein P1 in vitro J Gen Virol **69**:1627-1636

27 **Kean, K.M., N. Teterina, and M. Girard.** 1990 Cleavage specificity of the poliovirus 3C protease is not restricted to Gln-Gly at the 3C/3D junction J Gen Virol **71**:2553-2563

28 **Klump, W.M., I. Bergman, B.C. Müller, D. Ameis, and R. Kandolf.** 1990 Complete nucleotide sequence of infectious coxsackievirus B3 cDNA Two initial 5' uridine residues are regained during plus-strand RNA synthesis J Virol **64**:1573-1583

29 **Kräusslich, H.G., M.J.H. Nicklin, H. Toyoda, D. Etchison, and E. Wimmer.** 1987 Poliovirus proteinase 2A induces cleavage of eucaryotic initiation factor 4F polypeptide p220 J Virol **61**:2711-2718

30 **Lama, J., A.V. Paul, K.S. Harris, and E. Wimmer.** 1994 Properties of purified recombinant poliovirus protein 3AB as substrate for viral proteinases and as co-factor for RNA polymerase $3D^{pol}$ J Biol Chem **269**:66-70

31 **Matthews, D.A., W.W. Smith, R.A. Ferre, B. Condon, G. Budahazi, W. Sisson, J.E. Villafranca, C.A. Janson, J.E. McElroy, C.L. Gribskov, and S. Worland.** 1994 Structure of human rhinovirus 3C protease reveals a trypsin-like polypeptide fold, RNA-binding site, and means for cleaving precursor polyprotein Cell **77**:761-771

32 **Mirzayan, C., R. Ingraham, and E. Wimmer.** 1991 Specificity of the poliviral proteinase 3C towards genetically engineered cleavage sites in the viral capsid J Gen Virol **137**:1159-1163

33 **Mirzayan, C., and E. Wimmer.** 1994 Biochemical studies on poliovirus polypeptide 2C evidence for ATPase acitivity Virology **199**:176-187

34 **Molla, A., K.S. Harris, A.V. Paul, S.H. Shin, J. Mugavero, and E. Wimmer.** 1994 Stimulation of poliovirus proteinase $3C^{pro}$-related proteolysis by the genome linked protein VPg and its precursor 3AB J Biol Chem **269**:27015-27020

35 **Nicklin, M.H., K.S. Harris, P.V. Pallai, and E. Wimmer.** 1988 Poliovirus proteinase 3C Large-scale expression, purification and specific cleavage activity on natural and synthetic substrates in vitro J Virol **62**:4586-4593

36 **Pallai, P.V., F. Burkhardt, M. Skoog, K. Schreiner, P. Bax, K.A. Cohen, G. Hansen, D.E.H. Palladino, K.S. Harris, M.J. Nicklin, and E. Wimmer.** 1989 Cleavage of synthetic peptides by purified poliovirus 3C proteinase J Biol Chem **264**:9738-9741

37 **Paul, A.V., X. Cao, K.S. Harris, J. Lama, and E. Wimmer.** 1994 Studies with poliovirus polymerase $3D^{pol}$ J Biol Chem **269**:29173-29181

38 **Pelletier, J., and N. Sonenberg.** 1988 Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA Nature (London) **334**:320-325
39 **Pérez, L., and L. Carrasco.** 1992 Lack of direct correlation between p220 cleavage and the shut-off of host translation after poliovirus infection Virology **189**:178-186
40 **Reed, L.J., and H. Muench.** 1938 A simple method of estimating fifty per cent endpoints Am J Hyg **27**:493-497
41 **Rodriguez, P.L., and L. Carrasco.** 1993 Poliovirus protein 2C has ATPase and GTPase activities J Biol Chem **268**: 8105-8110
42 **Semler, B.L., C.W. Anderson, R. Hanecak, L. Dorner, and E. Wimmer.** 1982 A membrane-associated precursor to poliovirus VPg identified by immunoprecipitation with antibodies directed against a synthetic heptapeptide Cell **28**:405-412
43 **Takeda, N., R.J. Kuhn, C.F. Yang, T. Takegami, and E. Wimmer.** 1986 Initiation of poliovirus plus-strand RNA synthesis in a membrane complex of infected HeLa cells J Virol **60**:43-53
44 **Takegami, T., B.L. Semler, C.W. Anderson, and E. Wimmer.** 1983 Membrane fractions active in poliovirus RNA replication contain VPg precursor polypeptides Virology **128**: 33-47
45 **Toyoda, H., M.J.H. Nicklin, M.G. Murray, C.W. Anderson, J.J. Dunn, F.W. Studier, and E. Wimmer.** 1986 A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein Cell **45**:761-770
46 **van Kuppeveld, F.J.M., J.M.D. Galama, J. Zoll, and W.J.G. Melchers** 1995 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 J Virol **69**:7782-7790
47 **van Kuppeveld, F.J.M., J.M.D. Galama, J. Zoll, P.J.J.C. van den Hurk, and W.J.G. Melchers** 1996 Coxsackie B3 virus protein 2B contains a cationic amphipathic helix that is required for viral RNA replication J Virol **70**:3876-3886
48 **van Kuppeveld, F.J.M., and W.J.G. Melchers.** Unpublished data
49 **van Kuppeveld, F.J.M., W.J.G. Melchers, K. Kirkegaard, and J.R. Doedens.** Virology, In press
50 **Wimmer, E., C.U.T. Helen, and X. Cao.** 1993 Genetics of poliovirus Annu Rev Genet **27**:353-436
51 **Ypma-Wong, M.F., P.G. Dewalt, V.H. Johnson, J.G. Lamb, and B.L. Semler.** 1988 Protein 3CD is the major poliovirus proteinase responsible for cleavage of the P1 precursor Virology **166**:265-270

Chimeric Coxsackie B3 Virus Genomes That Express Hybrid Coxsackievirus-Poliovirus 2B Proteins: Functional Dissection of Structural Domains Involved in RNA Replication

Chimeric Coxsackie B3 Virus Genomes That Express Hybrid Coxsackievirus-Poliovirus 2B Proteins: Functional Dissection of Structural Domains Involved in RNA Replication

FRANK J. M. VAN KUPPEVELD, PATRICK J. J. C. VAN DEN HURK, WALTER VAN DER VLIET, JOCHEM M. D. GALAMA, AND WILLEM J. G. MELCHERS

Department of Medical Microbiology, University of Nijmegen, The Netherlands

The nonstructural 2B proteins of coxsackievirus and poliovirus (PV) share a significant similarity in structure and exhibit similar biochemical activities, namely inhibition of protein secretion and modification of membrane permeability. Both proteins contain two hydrophobic domains in the carboxy-terminal two-third of their sequence, of which one has the potential to form a cationic amphipathic α-helix and one displays characteristics typical for transmembrane domains. To gain more insight into the structural requirements of enterovirus protein 2B for its functioning in viral RNA replication, a chimeric cDNA approach was used. Chimeric coxsackie B3 virus (CBV3) genomes were constructed that expressed either the entire PV 2B protein or hybrid proteins in which specific segments of CBV3 protein 2B were substituted by their corresponding PV counterparts. In vitro synthesis and processing of the chimeric polyproteins showed no abnormalities. CBV3 genomes carrying the entire PV 2B gene exhibited a defect in viral RNA replication and were nonviable. A chimeric genome that expressed a hybrid 2B protein consisting of the amino-terminal one-third of PV and the remainder of CBV3 yielded viable viruses. In contrast, a hybrid genome that produced a 2B protein consisting of the amino-terminal one-third of CBV3 and the remainder of PV failed to replicate. These data argue that a sequence-specific interaction with another viral replication protein is required to drive viral RNA replication and suggest that the proposed sites of contact reside in the carboxy-terminal two-third of 2B. Hybrid genomes in which either the amphipathic α-helix or the other hydrophobic domain was replaced failed to replicate. The potential contribution of these domains to the structure and functioning of protein 2B is discussed.

The genus *Enterovirus* of the family of *Picornaviridae*, a large group of plus-strand RNA viruses, comprises polioviruses (PV), coxsackie A viruses, coxsackie B viruses, echoviruses, and several distinct enterovirus serotypes. These enteroviruses are closely related and share marked similarities with respect to virus structure, genomic organization, and replicative cycle (29). The main differences between these viruses are observed in the clinical syndromes they produce in humans, growth in particular cell lines, and the illness and pathology they produce in monkeys and suckling mice (18). The differences in tropism are predominantly based on the ability to bind to specific receptors for cell entry, reflecting differences in the structural capsid proteins encoded by the P1 region of the genome, and the occurrence of host-specific *cis*-acting translational control elements in the genomic RNA (1, 19).

The enteroviruses can be divided into two major subclasses, namely a CBV-like group and a PV-like group. The nonstructural proteins encoded by the P2 and P3 regions of the genomes of CBV-like and PV-like enteroviruses share 50 to 65% amino acid homology. Despite the differences in primary sequence, there is a high degree of conservation of important structural domains and sequence motifs, suggesting that these proteins share similar functions in viral reproduction. Studies towards the functional exchangeability of these proteins have provided experimental evidence for this suggestion. Chimeric PV genomes containing the coxsackie B3 virus (CBV3) counterpart of protein $3C^{pro}$, a proteinase that is responsible for the majority of the processing events that give rise to the production of the nonstructural proteins, demonstrated a correct processing of the PV P2 and P3 region proteins (10). In addition, PV genomes carrying CBV4 protein $2A^{pro}$, a multifunctional protein that acts as a proteinase, a *trans*-activator of viral translation, and as a component in viral RNA (vRNA) replication, gave rise to viable viruses (17).

Enterovirus protein 2B is a small hydrophobic protein that is localized at the outer surface of the membrane vesicles on which vRNA replication takes place (6). The phenotypes of 2B mutants of both PV and CBV3 have implicated a role of protein 2B, or possibly its precursor 2BC, in vRNA amplification (12, 16, 23, 24, 26). The exact function of this protein is yet unclear but seems to be related to its effects on cellular membranes. Expression of the 2B proteins of both PV and CBV3 in mammalian cells caused a modification in permeability of the plasma membrane and inhibition of vesicular protein transport (11, 27), two phenomena that

also occur during enterovirus infection (8, 11) The interaction of enterovirus protein 2B with membranes is most likely provided by the two conserved hydrophobic domains, of which one has the potential to form a cationic amphipathic α-helix (23) and one (which will further be referred to as "the second hydrophobic domain") shows characteristics typical for multimeric transmembrane helices (24) The relevance of these domains for the ability of CBV3 2B to increase membrane permeability and inhibit protein secretion has recently been confirmed by individual expression of 2B proteins carrying mutations in these regions (27)

The aim of this study was to gain more insight into the structural requirements of enterovirus protein 2B for its functioning in viral reproduction To this end, we have constructed chimeric CBV3 cDNAs that contained either the entire PV 2B protein or hybrid proteins in which specific segments of CBV3 protein 2B were substituted by the corresponding PV regions The ability of PV 2B and the hybrid 2B proteins to drive vRNA replication and virus growth was assayed by transfection of cells with copy RNA transcripts of the chimeric cDNAs Polyprotein synthesis and processing were studied by translation of RNA transcripts in a cell-free extract The potential contribution of the distinct domains to the structure and function of protein 2B are discussed

MATERIALS AND METHODS

Construction of unique endonuclease restriction sites in pCB3/T7. For the introduction of PV sequences in plasmid pCB3/T7 (13), which contains a cDNA of CBV3 (strain Nancy) behind a T7 RNA polymerase promoter, unique restriction sites were introduced, either alone or in combination, by site-directed mutagenesis Mutagenesis was performed with a subgenomic pALTER phagemid clone containing the EcoRV fragment of pCB3/T7 (nucleotide [nt] 918 to 6177) using the Altered Sites in vitro mutagenesis system according to the instructions of the manufacturer (Promega) The nucleotide sequence of the antisense synthetic oligonucleotides (Isogen Bioscience, The Netherlands) used for the introduction of unique restriction sites (which are underlined) immediately upstream or downstream of the 2B-encoding sequence are, 5'-TTCCACAAGTCCTTCAAGGCCTGTTCCATTGCATC-3' (*Stu*I site at nt 3743) and 5'-TTTCTTAAGCCAGCTGTTAACTTGGCGTTCGCCAT-3 (*Hpa*I site at nt 4042) Nucleotide sequences of the oligonucleotides used for the introduction of unique restriction sites at 2B amino acids (aa) 57 to 59 are, 5'-TAGTGTGGCAGTCACAGTGATCAGGTCGTGGTTCCGGACCACAATTACTAAGGCTGATAT-3 (*Acc*III site at nt 3913) and 5'-GATCAGGTCATCGTGGTTAACCACCACAATTACTAAGGC-3' (*Hpa*I site at nt 3915) Mutant pAlTER clones were identified by restriction enzyme analysis The nucleotide sequence of the mutant clones was verified by dideoxy chain termination sequencing of plasmid DNA using the Ampli Cycle sequencing kit according to the instructions of the manufacturer (Perkin-Elmer) The unique restriction sites were intro-duced in pCB3/T7 by cloning of the *Bgl*II (nt 2040)-to-*Bss*HII (nt 4238) fragments in plasmids from which the corresponding fragment was deleted In this way, plasmids pCB3/T7-*Stu*I(3743), pCB3/T7-*Hpa*I(4042), pCB3/T7-*Hpa*I (3915), pCB3/T7-*Stu*I(3743)/*Hpa*I(4042), pCB3/T7-*Stu*I (3743)/*Hpa*I(3915), and pCB3/T7-*Acc*III(3913)/*Hpa*I(4042) were generated

Construction of chimeric cDNAs. Chimeric cDNAs were constructed by PCR amplification and cloning of PV 2B-encoding segments into pCB3/T7 plasmids from which the corresponding segment was deleted Plasmid pXpA (generously provided by R Andino, University of California), which contains a cDNA of PV type 1, was used as template for PCR A total of 4 forward primers (f1 to f4) and 5 reverse primers (r1 to r5) containing restriction sites (which are underlined) at their 5' end were used for amplifying PV sequences PCR was performed using Super*Taq* DNA polymerase (HT Biotechnology) according to the instructions of the manufacturer The chimeric cDNAs were named according to the CBV3 amino acids that were replaced by the corresponding residues of PV The construction of each of the chimeric plasmids is briefly summarized

2B/PV1-99, A DNA fragment containing the coding sequence of PV aa 1 to 99 was generated by PCR using primers f1 (5'-ATGGAACTGCGCATCACCAATTACATA-3', *Avr*II site) and r1 (5'-CCAACTAATATTTTGCTTGATGACATAAGG-3, *Ssp*I site), digested with *Avr*II and *Ssp*I, and cloned in pCB3/T7-*Stu*I(3743)/*Hpa*I(4042) from which the *Stu*I-to-*Hpa*I fragment was deleted

2B/PV1-94, A DNA fragment containing the coding sequence of PV aa 1 to 94 was generated by PCR using primers f1 and r2 (5-CCAACTAATATTTTGGCGTTCAGCCATAGGTATCTCCAGAACATCGCA3, *Ssp*I site), digested with *Avr*II and *Ssp*I, and cloned in pCB3/T7-*Stu*I(3743)/*Hpa*I(4042) from which the *Stu*I-to-*Hpa*I fragment was deleted

2B/PV1-30, A DNA fragment containing the coding sequence of PV aa 1 to 30 was generated by PCR using primers f1 and r3 (5-GGTACTGACTAGTGAATTGGTCAACTCTGTTAT-3', *Spe*I site), digested with *Avr*II and *Spe*I, and cloned in pCB3/T7-*Stu*I(3743) from which the *Stu*I-to-*Spe*I (3837) fragment was deleted

2B/PV1-59, A DNA fragment containing the coding sequence of PV aa 1 to 59 was generated by PCR using primers f1 and r4 (5-TTCATAAGGCCTAGTTATAATAACTAG-3, *Stu*I site), digested with *Avr*II and *Stu*I, and cloned in pCB3/T7-*Stu*I(3743)/*Hpa*I(3915) from which the *Stu*I-to-*Hpa*I fragment was deleted

2B/PV60-99, A DNA fragment containing the coding sequence of PV aa 60 to 99 was generated by PCR using primers f2 (5'-ATTATAATCCGGAACTATGAAGACACC-3, *Acc*III site) and r1, digested with *Acc*III and *Ssp*I, and cloned in pCB3/T7-*Acc*III(3913)/*Hpa*I(4042) from which the *Acc*III-to-*Hpa*I fragment was deleted

2B/PV60-94, A DNA fragment containing the coding sequence of PV aa 60 to 94 was generated by PCR using primers f2 and r2, digested with *Acc*III and *Ssp*I, and cloned in pCB3/T7-*Acc*III(3913)/*Hpa*I(4042) from which the *Acc*III-to-*Hpa*I fragment was deleted

2B/PV34-59, A DNA fragment containing the coding sequence of PV aa 34 to 59 was generated by PCR using primers f3 (5-AATATGCTAGCCAGTACCATCACTGAA-3, *Nhe*I site) and r4, digested with *Nhe*I and *Stu*I, and cloned in pCB3/T7-*Hpa*I(3915) from which the *Spe*I (nt 3837)-to-*Hpa*I fragment was deleted Due to the occurrence of a *Spe*I site in the amplicon, a *Nhe*I site (compatible ends with *Spe*I) was build in the 5' end of primer f3 As a result of this, valine 33 is altered into an alanine

2B/PV34-99, A DNA fragment containing the coding sequence of PV aa 34 to 99 was generated by PCR using primers f3 and r1, digested with *Nhe*I and *Ssp*I, and cloned in pCB3/T7-*Hpa*I(4042) from which the *Spe*I (nt 3837)-to-*Hpa*I fragment was deleted Due to the occurrence of a *Nhe*I site

rather than a *Spe*I site in primer f3 (see above), valine 33 is changed into an alanine

2B/PV34-94, A DNA fragment containing the coding sequence of PV aa 34 to 94 was generated by PCR using primers f3 and r2, digested with *Nhe*I and *Ssp*I, and cloned in pCB3/T7-*Hpa*I(4042) from which the *Spe*I (nt 3837)-to-*Hpa*I fragment was deleted Due to the occurrence of a *Nhe*I site rather than a *Spe*I site in primer f3 (see above), valine 33 is changed into an alanine

2B/PV37-54, A DNA fragment containing the coding sequence of PV aa 37 to 54 was generated by PCR using primers f4 (5-GAGTTGTCACTAGTGGGTCAAGACACCA TCACTGAAAAGCTACTTAAG-3, *Spe*I site) and r5 (5-TTCATAAGGCCTCACCACAATAACTAGGGAGGATATG ATCTT-3, *Stu*I site), digested with *Spe*I and *Stu*I, and cloned in pCB3/T7-*Hpa*I(3915) from which the *Spe*I (nt 3837)-to-*Hpa*I fragment was deleted Primer r5 was designed such that the *Spe*I site occurring in the PV sequence was deleted while leaving the amino acid sequence intact

From all mutant constructs, the entire PV segment that was introduced was confirmed by sequence analysis

Transfection of cells with copy RNA transcripts. In vitro transcription of *Sal*I-linearized plasmids by phage T7 RNA polymerase and transfection of 75% confluent Buffalo green monkey (BGM) cell monolayers with RNA transcripts (5 μg) using the DEAE-dextran method were performed as described previously (24) After transfection cells were fed with minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) and incubated at either 33 or 36°C In case virus growth was observed, cultures were incubated until cytopathic effect (CPE) was complete, followed by three cycles of freezing and thawing to release intracellular viruses Viruses were aliquoted and stored at -80°C

Analysis of RNA synthesis by dot blot hybridization. BGM cell monolayers were transfected with wild-type and mutant RNA transcripts as described above At various times posttransfection, the medium was discarded and the cells were washed with phosphate-buffered saline (PBS) for three times Total RNA was isolated by a single extraction procedure with guanidium thiocyanate-phenol-chloroform as described by Chomczynski and Sacchi (9) RNAs were denatured with formamide and formaldehyde and spotted onto Hybond nylon membranes (Amersham) according to standard procedures (3) cDNA clone pCB3/T7 was labeled with [α-^{32}P]dATP by nick-translation Membranes were prehybridized, hybridized, washed, and analyzed by autoradiography according to standard procedures (3)

In vitro translation reactions. Copy RNA transcipts were synthesized and translated in a single reaction using 17 TNT rabbit reticulocyte lysate (Promega) supplemented with 20% (vol/vol) HeLa cell initiation factors (kindly provided by J Flanegan, University of Florida) The translation reactions (20 μl) contained 0 5 μg of circular plasmid DNA and 20 μCi of Tran^{35}S-label (a mixture of [^{35}S]methionine and [^{35}S]cysteine, ICN) and were incubated for 3 h at 30°C Translation products were analyzed on a sodium dodecyl sulfate (SDS) containing 12 5% polyacrylamide gel (14) Gels were fixed, fluorographed and exposed to Kodak XAR film at -80°C

Sequence verification of virus. RNA extraction, synthesis of cDNA, amplification of the 2B coding region by PCR using primers 5-TGGTGTCATTGGCATTGTGACCATGGGGGG-3 (nt 3648 to 3677) and 5-TTGGGATGGCGCGC1CTGCTC -3 (nt 4231 to 4251), purification of the PCR products, and sequence analysis with reverse primer 5-CCATTCAATGA ATTTCTG-3' (nt 4117 to 4134) were all performed as described previously (23)

Virus titrations Virus titers were determined by endpoint titration as described previously (24) and expressed in 50% tissue culture infective doses ($TCID_{50}$) according to the method of Reed and Muench (20)

Single-cycle growth analysis. Confluent BGM cell monolayers grown in 25-cm^2 flaks (5 x 10^6 cells) were infected with virus at a multiplicity of infection (MOI) of 1 $TCID_{50}$ per cell for 30 min at room temperature The cells were fed with MEM containing 3% FBS and grown at 33, 36, or 39°C At various times postinfection, cells were disrupted by three cycles of freezing and thawing, and the virus titers were determined

Analysis of viral protein synthesis in vivo. Confluent monolayers of BGM cells were infected with virus at a MOI of 25 for 30 min at room temperature After infection, cells were fed with MEM containing 3% FBS and grown at 36°C At various times postinfection, the cells were washed with PBS and incubated in methionine- and serum-free MEM (Gibco) containing 10 μCi of Tran^{35}S-label for 30 min Lysis of cells and analysis of the [^{35}S]methionine-labeled proteins by SDS-polyacrylamide gel electrophoresis were performed as described previously (24)

RESULTS

Construction of heterologous CBV3 cDNAs. To allow the construction of chimeric CBV3 genomes, unique restriction sites were introduced, either alone or in combination, at the 2A/2B and 2B/2C junctions and within the 2B coding sequence of plasmid pCB3/T7 PV sequences were amplified by PCR and cloned in these constructs PCR primers were designed such that no additional amino acid changes outside the substituted fragment were introduced A total of ten chimeric constructs, schematically diagrammed in Fig 1, was generated In construct 2B/PV1-99, the complete 2B coding region of CBV3 was replaced by that of PV In the other constructs, the amino-terminal region (i e , the first 30 to 35 amino acids), the amphipathic α-helix, or the second hydrophobic domain were replaced, either alone or in combination, by their corresponding PV counterparts Because the borders of the amphipathic α-helix were not exactly known, two different constructs (2B/PV34-59 and 2B/PV37-54) were generated Constructs 2B/PV1-99, 2B/PV60-99, and 2B/PV34-99 contained at the P4 position of the 2B/2C cleavage site a valine rather than an alanine (which occurs in wild-type CBV3) Therefore, three additional constructs (2B/PV1-94, 2B/PV60-94, and 2B/PV34-94) containing the last five amino acids of CBV3 2B were generated as well

Viability of chimeric CBV3 genomes. To examine whether PV protein 2B and the hybrid PV-CBV3 2B proteins could functionally replace the 2B protein of CBV3 in vivo, BGM cells were transfected with in vitro synthesized RNA transcripts from wild-type pCB3/T7 and the chimeric constructs Four transfections were performed for each construct Two transfected cell cultures were grown at 33°C and two were grown at 36°C In case no virus growth was observed after 5 days, the cultures were subjected to three cycles of freezing and thawing, and passaged to fresh cell cultures, which were incubated for an additional 3 days

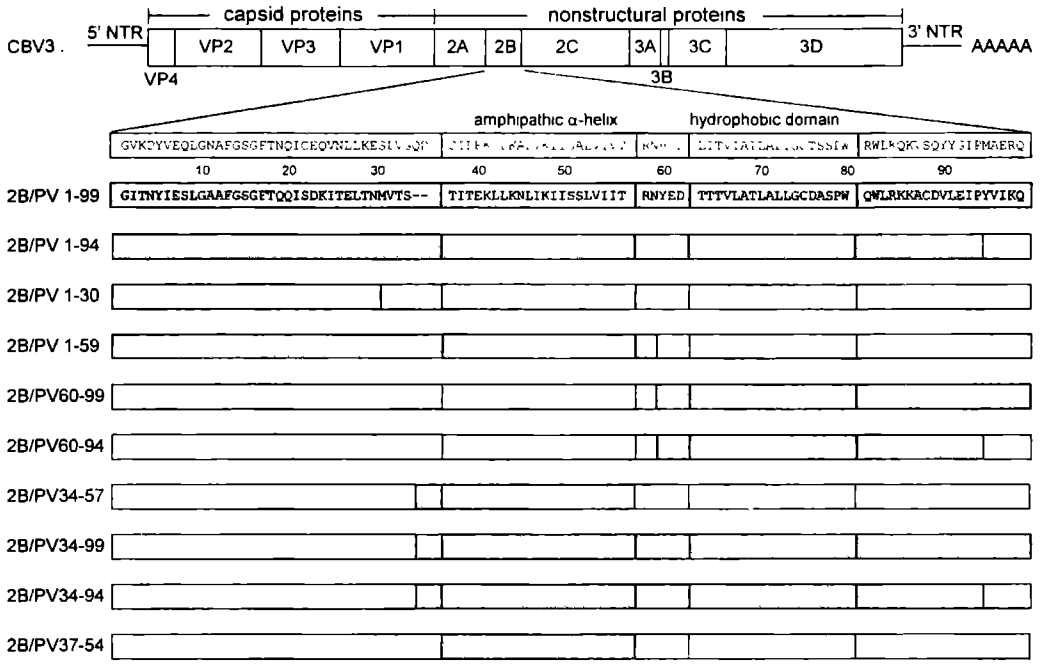


Figure 1. Schematic representation of the chimeric CBV3 genomes. At the top of the figure is shown the 7.5-kb single-stranded RNA genome of CBV3 with the polyadenylate tract at the end of the 3' nontranslated region (NTR). The boxed region shows the polyprotein and vertical lines within the box indicate proteinase cleavage sites. The complete amino acid sequence of protein 2B, containing the cationic amphipathic α-helix and the second hydrophobic domain, is enlarged. The poliovirus 2B encoding segments are shaded.

Transfection of cells with RNA transcripts derived from the wild-type plasmid gave complete CPE within two days. RNA transcripts from construct 2B/PV1-30 produced CPE in all transfected cell cultures at 5 days posttransfection. Sequence analysis of the 2B coding region of this virus (vCB3-2B/PV1-30) showed that the introduced PV segment was retained and that no other mutations had occurred. No CPE was observed in any of the cell cultures that were transfected with RNA of the other chimeras. Passage of cytoplasmic extracts to fresh BGM cell monolayers also failed to reveal virus growth.

Analysis of the virus growth of vCB3-2B/PV1-30 in a single-cycle infection showed a delay in virus production relative to wild-type virus (Fig. 2). This delay was most apparant at 6 h postinfection when mutant virus production was only 1% of that of wild-type virus. At 8 h postinfection there was only a 10-fold difference in virus yield, whereas at 12 h postinfection virus production was similar. The delay in virus growth was not temperature sensitive; the kinetics of virus reproduction of vCB3-2B/PV1-30 relative to that of wild-type virus was similar at 33, 36, and 39°C (data not shown).

Analysis of vRNA replication. To examine whether the reduced growth of vCB3-2B/PV1-30 and the nonviability of the other chimeric genomes were due to a defect in vRNA replication, a dot blot hybridization was

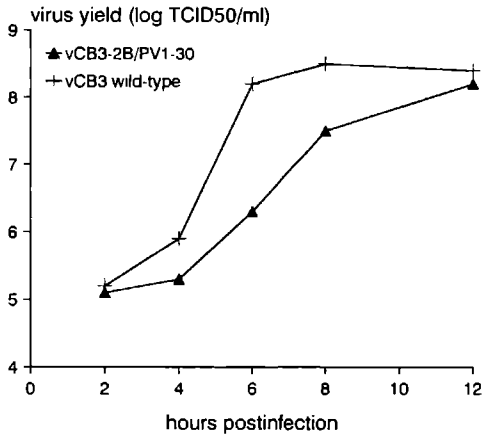


Figure 2. One-step growth curves of wild-type virus and mutant virus vCB3-2B/PV1-30. BGM cell monolayers were infected at a multiplicity of 1 $TCID_{50}$ per cell and incubated at 36°C. At the indicated times postinfection, the cells were disrupted by three cycles of freezing and thawing, and the virus production was determined by endpoint titration. $TCID_{50}$, 50% tissue culture infective dose.

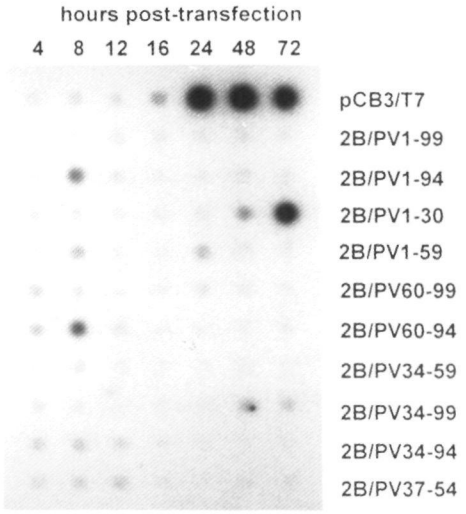


Figure 3. Analysis of wild-type and mutant vRNA replication. BGM cell monolayers were transfected with equal amounts of genomic transcripts derived from the indicated plasmids linearized by *SalI*. At the indicated times posttransfection, total RNA was isolated. The RNA was denatured, bound to nylon membrane, and hybridized to a [α-^{32}P]-labeled cDNA probe.

performed. BGM cells were transfected with in vitro synthesized copy RNA transcripts and at various times posttransfection total cellular RNA was isolated, denatured, immobilized on nylon membranes, and hybridized with a [α-^{32}P]dATP-labeled CBV3 cDNA probe. Figure 3 shows that there was no detectable vRNA synthesis in cells transfected with any of the nonviable chimeras. From the hybridization signals obtained with construct 2B/PV1-30 it is evident that vRNA synthesis is delayed.

Synthesis and processing of hybrid polyproteins. To examine whether the chimeric genomes were able to correctly generate all nonstructural and structural proteins required to replicate and assemble vRNAs, copy RNAs of the mutant constructs were synthesized and translated in a reticulocyte lysate. Figure 4 shows that all mutant RNAs produced cleavage products to levels similar to those produced by RNA derived from pCB3/T7. Protein 2B (11 kDa) could not be visualized because it migrated in the heavily overloaded globin spot. Nevertheless, the efficient production of proteins 2A and 2C indicated that none of the introduced PV segments interfered with cleavage site specificity and processing efficiency of 3C^{pro} at either the 2A/2B or 2B/2C junction. Thus, the defects in vRNA replication and virus growth of the chimeric genomes are unlikely to be due to impaired processing of the viral polyprotein.

To examine the possibility that the defect in vRNA replication of vCB3-2B/PV1-30 was due to a reduction in the rate of viral protein synthesis in vivo, we compared protein synthesis following infection of BGM cells with either wild-type or the chimeric virus. Cells were infected at an equal multiplicity of infection and pulse-labeled with [^{35}S]methionine at various times postinfection. Analysis of the cell lysates shows that the chimeric virus has retained the ability to shut off cellular protein synthesis (Fig. 5). In chimeric virus-infected cells, viral protein synthesis was maximal at 7 h postinfection. The amount of viral proteins produced at this time point was similar to that observed in wild-type virus-infected cells at 5 h postinfection, when wild-type protein synthesis

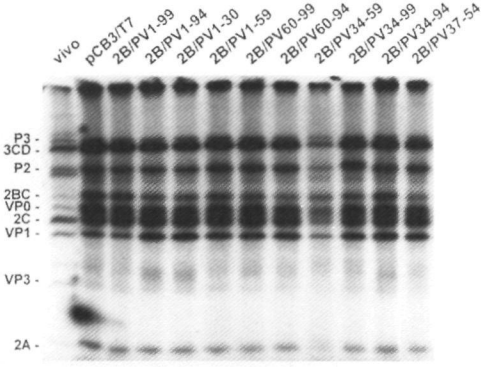


Figure 4. In vitro translation of wild-type and mutant RNAs in a cell-free extract. RNA transcripts were synthesized and translated in TNT rabbit reticulocyte lysate, a coupled transcription-translation system, supplemented with HeLa cell initiation factors. Reactions were programmed with 0.5 µg of circular plasmid DNA and incubated for 3 h at 30°C. The [^{35}S]-methionine-labeled translation products were analyzed on an SDS-12.5% polyacrylamide gel. An extract from wild-type virus-infected cells, labeled with [^{35}S]methionine at 4 h postinfection, was used as a marker (vivo).

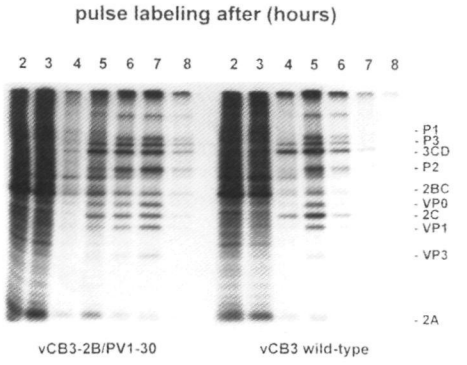


Fig. 5. Protein synthesis in wild-type virus and vCB3-2B/PV1-30-infected cells. BGM cells were infected at a multiplicity of 25 $TCID_{50}$ per cell and grown at 36°C. At the indicated times postinfection, cells were radioactively labeled in 30-min pulses with [^{35}S]methionine. Cellular extracts were prepared and analyzed on an SDS-12.5% polyacrylamide gel.

was maximal This suggests that it is unlikely that the defect in vRNA replication is due to an impaired rate of viral protein synthesis in vivo

DISCUSSION

The construction of chimeric viral genomes is a suitable approach to dissect multiple functions of specific genetic elements Hybrid picornavirus genomes have provided interesting information on the occurrence of independent functional domains essential for vRNA replication and translation in both the 5' nontranslated region (2, 21, 30) and nonstructural protein $2A^{pro}$ (17) We have employed a chimeric cDNA approach to dissect the structural requirements of enterovirus protein 2B for its functioning in vRNA replication Chimeric CBV3 genomes were constructed that expressed either the entire PV 2B protein, which is 50% homologous to the 2B protein of CBV3, or hybrid proteins in which specific structural elements of CBV3 2B were substituted by their PV counterpart All hybrid polyproteins were efficiently synthesized and correctly processed Defects in vRNA replication are therefore primarily attributable to impaired functioning of the 2B protein

A chimeric CBV3 genome that expressed the entire PV 2B protein failed to replicate That PV 2B cannot functionally replace CBV3 2B is remarkable because these proteins are endowed with the same biochemical activities Expression in mammalian cells of the 2B proteins of both PV (11) and CBV3 (27) results in modification of plasma membrane permeability and inhibition of protein secretion Modification of membrane permeability is also observed when these 2B proteins are expressed in *Escherichia coli* cells (15, 25) The exchangeability of the $2A^{pro}$ and $3C^{pro}$ proteins of PV with those of coxsackie B viruses (10, 17) and the overall similarity in structure of the nonstructural enterovirus proteins, are further indicative for a conservation of the functions of these proteins Alternative explanations for the nonexchangeability of protein 2B must therefore be considered A possible cause for the defect in vRNA replication of this chimera may be the heterologous nature of protein 2BC, a relatively stable processing intermediate that is required for the induction of the membrane vesicles on which vRNA amplification takes place (4, 7) Impaired functioning of a heterologous precursor protein was also observed with poliovirus chimeras that contained $3C^{pro}$ of either human rhinovirus 14 or CBV3 These 3C proteinases were able to cleave the nonstructural precursor polypeptides, but the hybrid $3CD^{pro}$ proteins were unable to process the poliovirus capsid proteins (10) Another possible explanation for the inability of PV 2B to drive vRNA synthesis may be a failure to recognize and contact other CBV3 replication proteins Thus, apart from its abilities to modify membrane permeability and inhibit protein secretion, protein 2B may play a direct role in vRNA replication in which interactions with other viral proteins are essential This suggestion is consistent with the existence of mutations that interfere with vRNA replication but that do not impair the ability of 2B to increase membrane permeabilty or inhibit protein secretion (27)

Only one of the hybrid 2B proteins was functional in vRNA replication This hybrid protein (2B/PV1-30) contained the amino-terminal one-third of PV 2B, i e, the region upstream of the hydrophobic domains, and the remainder of CBV3 2B Remarkably, a hybrid protein that contained the amino-terminal one-third of CBV3 2B and the remainder of PV 2B (2B/PV34-99), was nonfunctional The loss of activity of this protein may be due to a general disruption of the structure of either 2B or 2BC However, this possibility seems unlikely in view of the well functioning of protein 2B/PV1-30 The explanation that the carboxy-terminal two-third of the protein contains the sequence-specific determinants required for an intramolecular interaction with 2C (in precursor 2BC) or for intermolecular contacts with other viral replication proteins seems more plausible and is in agreement with the nonfunctioning of PV 2B in vRNA replication as discussed above

Hybrid genomes that contained the cationic amphipathic α-helix of PV 2B (2B/PV34-59, 2B/PV37-54, 2B/PV1-59) failed to replicate This is in agreement with the previously described defect in vRNA replication of 2B-bomII, a chimeric CBV3 genome that produced a 2B protein in which the cationic amphipathic helix was replaced with a similar motif of lytic peptide bombolitin II (23) The amphipathic helix motif in CBV3 2B is a major determinant for the ability to modify membrane permeability and inhibit protein secretion (27) The occurrence of such a motif is characteristic for membrane-disrupting proteins Two models of action have been proposed to explain the membrane-perturbing activities of these proteins (4, 22) According to the first model, the amphipathic helix lies colateral to the membrane and inserts a few Angstrom into it, thereby making the membrane phospholipids more susceptible to degradation by phospholipases The second model suggests that the amphipathic helices form aqueous pores by spanning the membrane and forming oligomers, exposing their hydrophobic faces to the lipid bilayer and their hydrophilic faces forming the aqueous interior That the cationic amphipathic helices of both PV 2B and bombolitin II cannot functionally replace that of CBV3 2B argues against an independent role (i e, the first model) of this domain in the structure and function of 2B A possible explanation for the nonexchangeability of the amphipathic helix is that both this helix and the second hydrophobic domain traverse the lipid bilayer and that contacts between these two domains are required for the formation of an aqueous pore The observation that individually expressed protein 2B-bomII is unable to modify plasma membrane permeability and inhibit protein secretion (28) lends support to this idea Further support for this suggestion comes from the finding that, in addition to mutations targeted to the amphipathic helix, also mutations in the second hydro-

phobic domain can disrupt the ability of 2B to modify membrane permeability and inhibit protein secretion (27) Disturbances of contacts between these two domains may also account for the nonviability of the constructs that contained the amphipathic helix of CBV3 2B but the second hydrophobic domain of PV 2B (2B/PV 60-99, 2B/PV60-94)

In conclusion, these studies have allowed us to separate the contribution of distinct domains to the function of protein 2B in vRNA replication We have shown that the amino-terminal one-third of CBV3 2B, but not the middle one-third or the carboxy-terminal one-third of the protein, neither alone nor in combination, can be functionally replaced with its PV counterpart In contrast to the carboxy-terminal two-third of the protein, the role of the amino-terminal region in the function of 2B protein is yet unclear Mutations in this region caused defects in vRNA replication (12), but did not interfere with the ability of protein 2B to inhibit protein secretion (27) Recently it has been shown that deletion of the first 30 amino acids of protein 2B abolished the ability of poliovirus protein 2BC to induce membrane proliferation and to interfere with the exocytic pathway in yeast cells (4) The importance of this region for a function of protein 2B, or 2BC, in mammalian cells awaits further investigation

REFERENCES

1 **Agol, V I., E.V. Pilipenko, and O R. Slobodskaya.** 1996 Modification of translational control elements as a new approach to design of attenuated picornavirus strains J Biotechnology **44**:119-128

2 **Alexander, L., H.H. Lu, and E. Wimmer** 1994 Polioviruses containing picornavirus type 1 and/or type 2 internal ribosome entry site elements genetic hybrids and the expression of a foreign gene Proc Natl Acad Sci USA **91**:1406-1410

3 **Ausubel, F M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J A. Smith, and K. Struhl (ed.).** 1990 Current protocols in molecular biology, vol 1 John Wiley & Sons, New York

4 **Barco, A., and L. Carrasco.** 1995 A human virus protein, poliovirus protein 2BC, includes membrane proliferation and blocks the exocytic pathway in the yeast Saccharomyces cerevisiae EMBO J **14**:3349-3364

5 **Bernheimer, A W., and B. Rudy.** 1986 Interactions between membranes and cytolytic peptides Biochem Biophys Acta **864**.123-141

6 **Bienz, K., D. Egger, and T. Pfister.** 1994 Characteristics of the poliovirus replication complex Arch Virol **suppl. 9**:147-157

7 **Bienz, K., D Egger, Y. Rasser, and W. Bossart.** 1983 Intracellular distribution of poliovirus proteins and the induction of virus-specific cytoplasmic structures Virology **131**:39-48

8 **Carrasco, L., L. Pérez, A. Irurzun, J. Lama, F. Martínez-Abarca, P. Rodríguez, R. Guinea, J L. Castrillo, M.A. Sanz and M.J. Ayala.** 1993 Modification of membrane permeability by animal viruses p 283-305 *In* L Carrasco, N Sonenberg, and E Wimmer (ed), Regulation of gene expression in animal viruses Plenum press, New York

9 **Chomczynski, P., and N. Sacchi.** 1987 Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction Anal Biochem **162**:156-159

10 **Dewalt, P G , M.A. Lawson, R J. Colonno, and B.L. Semler.** 1989 Chimeric picornavirus polyproteins demonstrate a common 3C proteinase substrate specificity J Virol **63**: 3444-3452

11 **Doedens, J.R., and K. Kirkegaard.** 1995 Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A EMBO J **14**:894-907

12 **Johnson, K.L., and P. Sarnow.** 1991 Three poliovirus 2B mutants exhibit noncomplementable defects in viral RNA amplification and display dosage-dependent dominance over wild-type poliovirus J Virol **65**:4341-4349

13 **Klump, W.M., I. Bergman, B.C. Müller, D. Ameis, and R. Kandolf.** 1990 Complete nucleotide sequence of infectious coxsackievirus B3 cDNA Two initial 5' uridine residues are regained during plus-strand RNA synthesis J Virol **64**:1573-1583

14 **Laemmli, U.K.** 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4 Nature (London) **227**:680-685

15 **Lama, J., and L. Carrasco.** 1992 Expression of poliovirus nonstructural proteins in *Escherichia coli* cells J Biol Chem **267**:15932-15937

16 **Li, J.-P., and D. Baltimore.** 1988 Isolation of poliovirus mutants defective in viral RNA synthesis J Virol **62**:4016-4021

17 **Lu, H.-H., X. Li, A. Cuconati, and E. Wimmer.** 1995 Analysis of picornavirus $2A^{pro}$ proteins separation of proteinase from translation and replication functions J Virol **69**:7445-7452

18 **Melnick, J.L.** 1996 Enteroviruses polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses p 655-712 *In* B N Fields D M Knipe, P M Howley, R M Chanock, J L Melnick T P Morath B Roizman and S E Straus (ed), Fields Virology, 3rd ed , vol 1 Lippincott-Raven Publishers, Philadelphia

19 **Racaniello, V.R.** 1990 Cell receptors for picornaviruses Curr Top Immunol Microbiol **161**:1-22

20 **Reed, L.J., and H. Muench.** 1938 A simple method of estimating fifty per cent endpoints Am J Hyg **27**:493-497

21 **Rohll, J.B., N. Percy, R. Ley, D.J. Evans, J.F. Almond, and W.S. Barclay.** 1994 The 5'-untranslated region of picornavirus RNA contain independent functional domains essential for RNA replication and translation J Virol **68**:4383-4391

22 **Shai, Y** 1995 Molecular recognition between membrane-spanning polypeptides Trends Biol Sci **20**:460-464

23 **van Kuppeveld, F.J M., J.M.D. Galama, J. Zoll, P.J.J.C. van den Hurk, and W.J.G. Melchers.** 1996 Coxsackie B3 virus protein 2B contains a cationic amphipathic helix that is required for viral replication J Virol **70**:3876-3886

24 **van Kuppeveld, F.J.M., J.M.D. Galama, J. Zoll, and W J G. Melchers** 1995 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 J Virol **69**:7782-7790

25 **van Kuppeveld, F.J.M., J M.D. Galama, J. Zoll, and W.J.G. Melchers.** Unpublished data

26 **van Kuppeveld, F.J.M., P.J J C. van den Hurk, J. Zoll, J.M.D. Galama, and W.J.G. Melchers.** 1996 Mutagenesis of the coxsackie B3 virus 2B/2C cleavage site determinants of processing efficiency and effects on viral replication J Virol **70**:7632-7640

27 **van Kuppeveld, F.J.M., W.J.G. Melchers, K. Kirkegaard, and J.R. Doedens.** 1997 Structure-function analysis of coxsackie B3 virus protein 2B Virology **227**:111-118

28 **van Kuppeveld, F.J.M., W.J.G. Melchers, K. Kirkegaard, and J R Doedens.** Unpublished data

29 **Wimmer E., C.U.T. Helen, and X. Cao.** 1993 Genetics of poliovirus Annu Rev Genet **27**.353-436

30 **Xiang, W., K.S. Harris, L. Alexander, and E. Wimmer.** 1995 Interaction between the 5'-terminal cloverleaf and $3AB/3CD^{pro}$ of poliovirus is essential for RNA replication J Virol **69**:3658-3667

Coxsackievirus Protein 2B Is a Viroporin

Coxsackievirus Protein 2B Is a Viroporin

FRANK J M VAN KUPPEVELD[1], JOOST G J HOENDEROP[1], ROLF L L SMEETS[2], PETER H G M WILLEMS[2], HENRI B P M DIJKMAN[3], JOCHEM M D GALAMA[1], AND WILLEM J G MELCHERS[1]

Departments of Medical Microbiology[1] Biochemistry[2] and Pathology[3] University of Nijmegen The Netherlands

Digital-imaging microscopy was performed to study the effect of coxsackie B3 virus infection on the cytosolic free Ca^{2+} concentration and the Ca^{2+} content of the endoplasmic reticulum (ER). During the course of infection a gradual increase in the cytosolic free Ca^{2+} concentration was observed, due to the influx of extracellular Ca^{2+}. The Ca^{2+} content of the ER decreased in time with a kinetics inversely proportional to that of viral protein synthesis. Individual expression of protein 2B was sufficient to induce the influx of extracellular Ca^{2+} and to release Ca^{2+} from ER stores. Analysis of mutant 2B proteins showed that both a cationic amphipathic α-helix and a second hydrophobic domain in 2B were required for these activities. Consistent with a presumed ability of protein 2B to increase membrane permeability, viruses carrying a mutant 2B protein exhibited a defect in virus release. We propose that 2B is a viroporin that gradually enhances membrane permeability, thereby disrupting the intracellular Ca^{2+} homeostasis and ultimately causing the membrane lesions that allow release of virus progeny

The genus *Enterovirus* of the family of *Picornaviridae* comprises polioviruses, coxsackie group A and B viruses, ECHO viruses, and several unnamed enteroviruses The molecular biology of these cytolytic animal viruses is relatively well known, mainly from the extensive studies on poliovirus Enteroviruses are nonenveloped viruses that contain a single-stranded RNA genome of positive polarity After cell entry and virion uncoating, the RNA molecule acts as a mRNA directing the synthesis of a single polyprotein This polyprotein is subsequently processed by virus-encoded proteases to produce the structural capsid proteins and the nonstructural proteins that have been implicated in viral RNA (vRNA) replication (for review see references 29 and 48) For replication, the genomic RNA is used as a template to synthesize a complementary minus-strand, which, in turn, is transcribed into new molecules of RNA Plus-strand RNA synthesis occurs at the outer surface of virus-induced, membranous vesicles that proliferate and accumulate in the cytoplasm of infected cells (6) During infection, enteroviruses induce a number of alterations in metabolic functions and morphological structures of the cell, most of which serve to facilitate viral replication These include inhibition of host cell protein and RNA synthesis (13, 17), stimulation of lipid synthesis (26), and inhibition of vesicular protein transport (12)

The molecular mechanism employed by enteroviruses to induce cell lysis and release of virion progeny is largely unknown Cell lysis is presumably the ultimate result of the increase in plasma membrane permeability that occurs from the third hour postinfection (9) This modification is such that gradients of monovalent ions are gradually destroyed and compounds that normally do not pass the membrane leak out of the cell or flow into the cytoplasm (9) It has long been thought that either the bulk of viral gene expression or the formation and accumulation of virus particles is responsible for enhancing membrane permeability and lysis of the cell However, recent data suggest that a single viral protein may be responsible for the enhancement of plasma membrane permeability individual expression of the 2B proteins of both poliovirus and coxsackievirus in mammalian cells led to an increased permeability of the plasma membrane to the nonpermeative translation inhibitor hygromycin B (12, 46) Increased membrane permeability was also observed in *Escherichia coli* cells that expressed protein 2B (20) The mechanism of this activity and its relevance to the viral life cycle remain to be established No experiments have yet directly addressed the role of 2B, or any other viral protein, in virus release or cell lysis

Enterovirus 2B is a small hydrophobic protein that has been localized at the rER membrane and the outer surface of the ER-derived membranous vesicles at which plus-strand RNA replication takes place (5, 6) Biochemical analysis of the isolated vesicles confirmed that the membranes are derived at least in part from the ER (37) The ER is the major intracellular store of Ca^{2+} ions (8) Based on its localization and proposed potential to modify membrane permeability, we hypothesized that protein 2B might release Ca^{2+} from internal stores To examine this, we measured the intracellular Ca^{2+} concentration ($[Ca^{2+}]$) and the Ca^{2+} content of the ER in coxsackie B3 virus (CBV3)-infected cells Here, we demonstrate that the Ca^{2+} gradients maintained by the plasma membrane and ER membrane are disrupted during infection Individual expression of protein 2B appeared to be sufficient to induce these effects Analysis of mutant 2B proteins showed that both a cationic amphipathic α-helix

and a second hydrophobic domain were required for these activities The ability of protein 2B to enhance membrane permeability served to facilitate virus release The increase in $[Ca^{2+}]_i$ further potentiated virus release We propose that protein 2B by forming membrane-embedded pores accounts for the disturbance of the intracellular Ca^{2+} homeostasis and, ultimately, the formation of the membrane lesions that allow virus release Based on this ability, protein 2B is recognized as a new member of the family of viroporins Protein 2B represents the first example of a viroporin of a naked virus A putative function of the release of ER-stored Ca^{2+} in the assembly of the viral replication complex is proposed

MATERIALS AND METHODS

Cells, media, virus infections and titrations. COS-1 cells were grown in Dulbecco s modified Eagle's medium (Gibco) HeLa Ohio cells were grown in minimal essential medium (MEM) (Gibco) Media were supplemented with 10% fetal bovine serum (FBS), 100 units penicillin per ml, and 100 mg streptomycin per ml Cells were grown at 37°C in a 5% CO_2 incubator

Ca^{2+}-free MEM was the same as regular MEM except that it lacked $CaCl_2$ In experiments in which the effects of extracellular Ca^{2+} were assayed, as Ca^{2+}-containing medium we used Ca^{2+}-free medium to which $CaCl_2$ was added to a final concentration of 1 8 mM Both media were adjusted to pH 7 3 To Ca^{2+}-free MEM, 0 5 mM ethylene glycol-bis(ß-amino ethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (pH 7 3) was added immediately before use

All viruses used in this study are recombinant CBV3 viruses derived from plasmid pCB3/T7 (19), which contains a full-length cDNA of CBV3 strain Nancy behind a T7 RNA polymerase promoter HeLa Ohio cell monolayers were infected with virus at the indicated multiplicity of infection (MOI) for 30 min at room temperature After this, cells were washed three times with phosphate-buffered saline (PBS), supplied with MEM (with or without Ca^{2+}) containing 3% FBS, and grown at 37°C

Virus yields were determined by endpoint titration Serial 10-fold dilutions were tested in 8 replicative wells of 96-well plates as described (44) Virus titers were calculated and expressed in 50% tissue culture infective dose ($TCID_{50}$) values (31)

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured by using the fluorescent Ca^{2+} indicator fura-2 essentially as described previously (47) Briefly, at 30 min before measurement, the cells were incubated in new medium containing 2 5 µM fura-2 in its membrane-permeant acetoxymethyl (AM) ester form (Molecular Probe, Inc , Eugene, Oreg) Cells were incubated for 30 min at 37°C and then washed three times with medium to remove extracellular probe Cells were supplied with pre-heated medium and the coverslip was introduced to a thermostated (37°C) incubation chamber on the stage of an inverted fluorescence microscope (Nikon Diaphot) Routinely, an epi-fluorescent x40 magnification oil immersion objective was used, allowing simultaneous monitoring of about 25 to 50 single cells Dynamic video imaging was carried out using the MagiCal hardware and TARDIS software (Joyce Loebl, UK) Fluorescence was measured every 2 s with the excitation wavelength being altered between 340 and 380 nm and the emission fluorescence being recorded at 492 nm (binding of Ca^{2+} shifts the absorbance spectra from 380 to 340 nm) At the end of each experiment, a region free of cells was selected and one averaged background frame was collected at each excitation wavelength The averaged background frames were subtracted from the corresponding experimental frames After this, the fluorescence ratio R, which equals F_{340}/F_{380}, where F_{340} and F_{380} are the emission intensities at 340 and 380 nm excitation, respectively, was calculated $[Ca^{2+}]_i$ was calculated using the equation $[Ca^{2+}] = K_d \times (R-R_{min})/(R_{max}-R) \times (F_{min}/F_{max})$, where R_{min} and R_{max} are the minimum and maximum fluorescence ratios, obtained in the presence of 10 mM EGTA and 1 µM ionomycin (Sigma), respectively, and F_{min} and F_{max} are the emission fluorescence values at 380 nm at these two extreme conditions The equilibrium dissociation constant (K_d) for the Ca^{2+}-fura-2 complex at 37°C was taken to be 224 nM (14)

Measurement of Ca^{2+} release from endoplasmic reticulum stores. Cells on coverslips were loaded with fura-2 as described above and placed on the stage of the microscope After recording cells in Ca^{2+}-containing medium, the medium was replaced with 200 µl of Ca^{2+}-free medium After 10 min, 800 µl of Ca^{2+}-free medium containing 1 µM (final concentration) thapsigargin (LC Services, Woburn MA, USA) was added The $[Ca^{2+}]_i$ was measured as described above

Plasmids. For the expression of both the wild-type CBV3 2B protein and the mutant 2B proteins, we made use of previously described pC2Bα6 plasmids (46) These dicistronic plasmids were constructed by cloning of the coding region of 2B with an additional methionine at its 5' end and a stopcodon at its 3 end, as the first cistron in plasmid pLINKα6 The plasmids contain a SV40 origin of replication to allow their amplification in COS-1 cells and a SV40 late promoter to drive transcription of RNA

Transfections. COS-1 cell monolayers grown to confluency of 70% were harvested by trypsinization, collected by centrifugation for 5 min at 1,500 x g, and resuspended in PBS to a density of 1 x 10^7 cells per ml 2 x 10^6 COS-1 cells were transfected with 15 µg of plasmid DNA by electroporation at 300 Volts and 125 µFarraday using the Gene Pulser (Bio-Rad) After electroporation, cells were resuspended in fresh medium and seeded onto coverslips (diameter, 2 4 cm) placed inside the wells of 6-well plates Cells were seeded at a density of 2 x 10^5 cells per well and grown at 37°C until further analysis

Transfection of HeLa cell monolayers with in vitro transcribed copy RNA of chimeric replicon pCB3/T7-LUC and measurement of the luciferase activity were performed as described (44)

Indirect immunofluorescence. Transfected COS-1 cells grown on coverslips were fixed by treatment with cold methanol, followed by treatment with cold aceton, each for 2 min The coverslips were incubated for 1 h at 37°C with rabbit polyclonal serum raised against CBV3 protein 2B (kind gift from the laboratory of R Kandolf, University of Tübingen, Germany) that was 1 40 diluted in PBS After 2 washes with PBS, the coverslips were incubated for 1 h at 37°C with fluorescein isothiocyanate-conjugated goat-anti-rabbit IgG (Cappel laboratories, Cochranville PA, USA), which was 1 40 diluted in 0 2% evans blue/PBS The coverslips were washed with PBS, dried, overlaid with a 1 1 mixture of glycerol and PBS, and viewed with a Leitz dialux 20 EB microscope The percentage of protein-expressing cells was determined *de visu* of 500 cells

Immuno-electron microscopy. CBV3-infected HeLa cells and transfected COS-1 cells were harvested by scraping in MEM without FBS and collected by centrifugation for 5 min at 1,500 x g After fixation in periodate-lysine-2% paraformaldehyde for 2 h at room temperature, cells were pelleted in 15% gelatin Gelatin pellets were cut in small blocks, cryoprotected in 2 3 M sucrose for 45 min, and snap frozen in

liquid nitrogen Cryostat sections (25 µm) were washed with PBS and incubated in 50 mM glycine (pH 7 4) for 10 min After two washes in PBS, the sections were incubated overnight at 4°C with anti-2B serum (see above) that was 1 40 diluted in 1% bovine serum albumin (BSA)/PBS Sections were washed in PBS and incubated for 1 h with goat-anti-rabbit antibodies, coupled to colloidal gold of 1 nm (Nanoprobes Immunosource, Stony Brook, NY, USA), that were 1 50 diluted in 1% BSA/PBS This was followed by a 1 h incubation in PBS and postfixation with 1% glutaraldehyde/PBS After three washes in distilled water, the gold signal was enhanced using HQ silver (Nanoprobes Immunosource) for 7 min Sections were incubated overnight in distilled water and treated with 1% OsO_4 for 30 min After this, they were dehydrated in alcohol, embedded in Epon 812, and viewed with a JEOL 1200 EX II electron microscope

Analysis of viral protein synthesis in vivo. HeLa cell monolayers were infected at a MOI of 25 $TCID_{50}$ per cell After infection, cells were supplied with MEM with or without Ca^{2+} and incubated at 37°C At various times p i , protein synthesis was monitored by pulse labeling with 10 µCi of Tran^{35}S-label (a mixture of [^{35}S]methionine and [^{35}S]cysteine, ICN) in methionine- and serum free MEM or methionine- and serum-free MFM without $CaCl_2$ for 30 min Lysis of cells and analysis of labeled proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described previously (44)

In experiments in which the permeability of cells to hygromycin B was assayed, cells were incubated in methionine- and serum-free MEM, with or without Ca^{2+} and in the presence or absence of 500 µg hygromycin B (Sigma) per ml, for 15 min prior to the addition of 10 µCi of Tran^{35}S-label to the medium

Viral growth curves. Confluent HeLa cell monolayers in T125 flasks were infected with virus at a MOI of 5 $TCID_{50}$ per cell After infection, cells were supplied with 5 ml of MEM with or without Ca^{2+} and grown at 37°C At the indicated times p i , flasks were subjected to three cycles of freezing and thawing to release intracellular viruses Viruses contained in these flasks represented the total virus population (i e , extracellular plus intracellular virus) Virus titers were determined by endpoint titration

In experiments in which the release of viruses from the cells was studied, 1 ml of medium was removed prior to freezing of the flasks This sample was microcentrifuged for 5 min at 1,500 x g to remove intact cells The titer of extracellular viruses maintained in the supernatant was determined as described above

Propidium iodide uptake. HeLa cell monolayers were infected with CBV3 at a MOI of 5 $TCID_{50}$ per cell After infection, cells were supplied with MEM with or without Ca^{2+} and grown at 37°C At the desired time p i , the medium was collected Cells remaining at the surface of the flask were harvested by incubating in 1 ml of PBS containing 50 mM EDTA and combined with the medium Cells were collected by centrifugation for 5 min at 1,500 x g and resuspended in 300 µl of medium Propidium iodide (Sigma) was added to a final concentration of 5 µg Cells were incubated for 10 min at room temperature The number of permeable cells was determined using a Coulter XL flow cytometer (Coulter corp , Hialeah FL, USA)

RESULTS

Enhanced intracellular Ca^{2+} concentration during coxsackie B3 virus infection. To monitor the $[Ca^{2+}]_i$ during CBV3 infection, we carried out digital-imaging microscopy using the fluorescent Ca^{2+} indicator fura-2 HeLa cells grown on coverslips were infected with wild-type CBV3 at a MOI of 25 $TCID_{50}$ per cell At various times postinfection (p i), the $[Ca^{2+}]_i$ in 250 individual cells was determined The basal $[Ca^{2+}]_i$ in uninfected HeLa cells was about 20 nM Figure 1 shows that the $[Ca^{2+}]_i$ in infected cells gradually increased from 2 h p i Cells that showed the highest $[Ca^{2+}]_i$, which were also those that exhibited the most severe cytopathic effects (e g , rounding and granulation), started to detach from the coverslip from 10 h p i The few cells that were still attached to the coverslip at 14 h p i all exhibited an enormous increase in $[Ca^{2+}]_i$

Two possible mechanisms may explain the rise in $[Ca^{2+}]_i$ One possibility is that the virus induces the influx of extracellular Ca^{2+} Alternatively, the virus may release Ca^{2+} from intracellular stores To examine this, the $[Ca^{2+}]_i$ in CBV3-infected HeLa cells that were supplied with medium without Ca^{2+} was measured In the absence of extracellular Ca^{2+}, no increases in $[Ca^{2+}]_i$ were observed (data not shown) Moreover, lowering of the external Ca^{2+} concentration of cells exhibiting an increase in $[Ca^{2+}]_i$ resulted in an immediate decrease in $[Ca^{2+}]_i$ (see Fig 2B, lower pannel) From this it follows that the Ca^{2+} responsible for increasing the $[Ca^{2+}]_i$ comes mainly from the external medium

Leakage of Ca^{2+} from the endoplasmic reticulum during infection. We have measured the inducible Ca^{2+} release of ER stores at various times during infection using thapsigargin, a specific inhibitor of the ER Ca^{2+}-ATPase (22) This Ca^{2+}-ATPase transports Ca^{2+} from the cytoplasma into the ER, an activity that is required to compensate for the continuous leakage of stored Ca^{2+} through channels in the ER membrane (8) When this ATPase is inhibited, Ca^{2+} that leaks from the ER is not resequestered and accumulates in the cytosol In Ca^{2+}-free medium, this accumulation is transient (minutes) because the Ca^{2+} ions that leak out of the ER are rapidly pumped out of the cell by Ca^{2+}-ATPases present in the plasma membrane (8) Under the latter condition (Ca^{2+}-free medium), the size of the thapsigargin-induced peak increase in $[Ca^{2+}]_i$ may be considered as a reflection of the Ca^{2+} content of the ER

HeI a cells grown on coverslips were infected with wild-type CBV3 at a MOI of 25 $TCID_{50}$ per cell At various times p i , first the average $[Ca^{2+}]_i$ of groups of fura-2-loaded cells was determined Then, the cells were incubated in Ca^{2+}-free medium and challenged with thapsigargin after 10 min Figure 2A shows that the average $[Ca^{2+}]_i$ gradually increased and that there is about a 2-fold increase at 4 h, a 5-fold increase at 6 h, and a 10-fold increase at 8 h p i The average Ca^{2+} content of ER was calculated relative to that of mock-infected cells (0 h p i) In these cells, the thapsigargin-induced Ca^{2+} release caused a peak increase in $[Ca^{2+}]_i$ of 130 ± 10 nM (mean ± SEM) (Fig 2B, upper panel) Representative traces measured at 4 and 8 h p i are shown in Figure 2B (middle and lower panel, respectively) The relative responses to thapsigargin measured

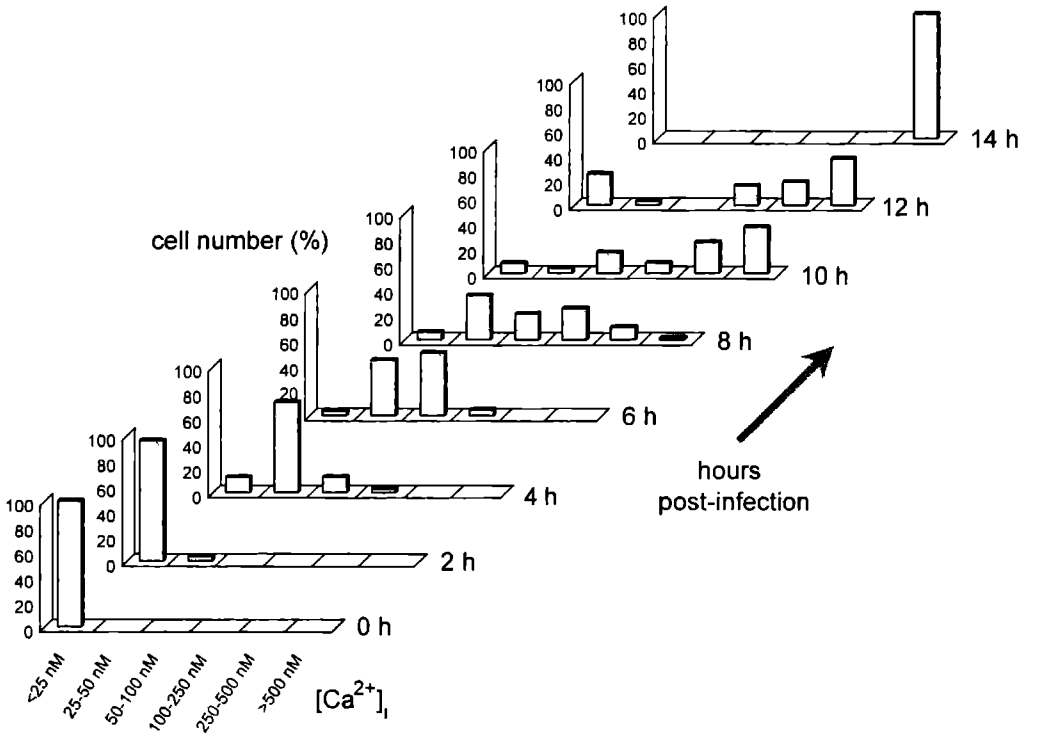


Figure 1. $[Ca^{2+}]_i$ during CBV3 infection HeLa cells grown on coverslips were infected with CBV3 at a MOI of 25 $TCID_{50}$ per cell for 30 min at room temperature and grown at 37°C The cells were loaded with the fluorescent Ca^{2+} indicator fura-2 (2 5 μM) for 30 min at 37°C, starting at 30 min before the indicated times At each time, the $[Ca^{2+}]_i$ in 250 individual cells was determined using digital-imaging microscopy, focusing on independently chosen cells lying in different areas of the coverslip The $[Ca^{2+}]_i$ was calculated from the fluorescence ratio of the emission intensities measured at 340 and 380 nm as described in Materials and Methods

hourly between 1 h and 10 h p.i. are shown in figure 2C. From this figure it can be seen that the virus-induced decrease in the ER Ca^{2+} content followed a triphasic kinetics. Infected cells showed a decrease in the amount of releasable Ca^{2+} already at 1 h p.i. (phase I). At this time, the thapsigargin-induced peak increase in $[Ca^{2+}]_i$ was only 65% of that of control cells. From 2 to 5 h p.i., a relative constant response to thapsigargin (about 40% of that of control cells) was observed (phase II). Thereafter, the inducible Ca^{2+} release rapidly declined and from 7 h p.i. virtually no response to thapsigargin was observed anymore (phase III).

The kinetics of Ca^{2+} depletion from the ER was compared with that of viral protein synthesis. Viral translation was visualized by transfection of HeLa cells with copy RNA transcripts of pCB3/T7-LUC, a chimeric CBV3 replicon that contains the luciferase gene in place of the capsid coding region (44). In transfected cells, the replicon RNA drives translation of a chimeric polyprotein that is processed to yield luciferase and all nonstructural proteins involved in RNA replication. The monocistronic nature of the RNA guarantees that luciferase accumulation faithfully represents levels of viral proteins produced. Figure 2D shows that in transfected cells a triphasic pattern of luciferase accumulation was observed; luciferase activity initially increased as a result of translation of the input RNA (phase I), remained constant between the second and fifth hour (phase II), and showed a second increase as a result of translation of newly synthesized chimeric RNA strands (phase III). The triphasic kinetics of Ca^{2+} depletion from the ER and that of viral protein synthesis are inversely proportional. This suggests that a viral protein is directly responsible for the leakage of ER-stored Ca^{2+}.

Expression of protein 2B is sufficient to increase $[Ca^{2+}]_i$. Transient expression of each nonstructural protein of poliovirus indicated that protein 2B, and also its precursor 2BC, strongly enhanced membrane permeability to the hydrophilic antibiotic hygromycin B (1, 12). This ability was found to be conserved in CBV3 protein 2B (46). To examine whether expression of CBV3 protein 2B was also sufficient to increase $[Ca^{2+}]_i$, COS cells were transfected with plasmids that allowed efficient expression of this protein or that encoded no protein. At

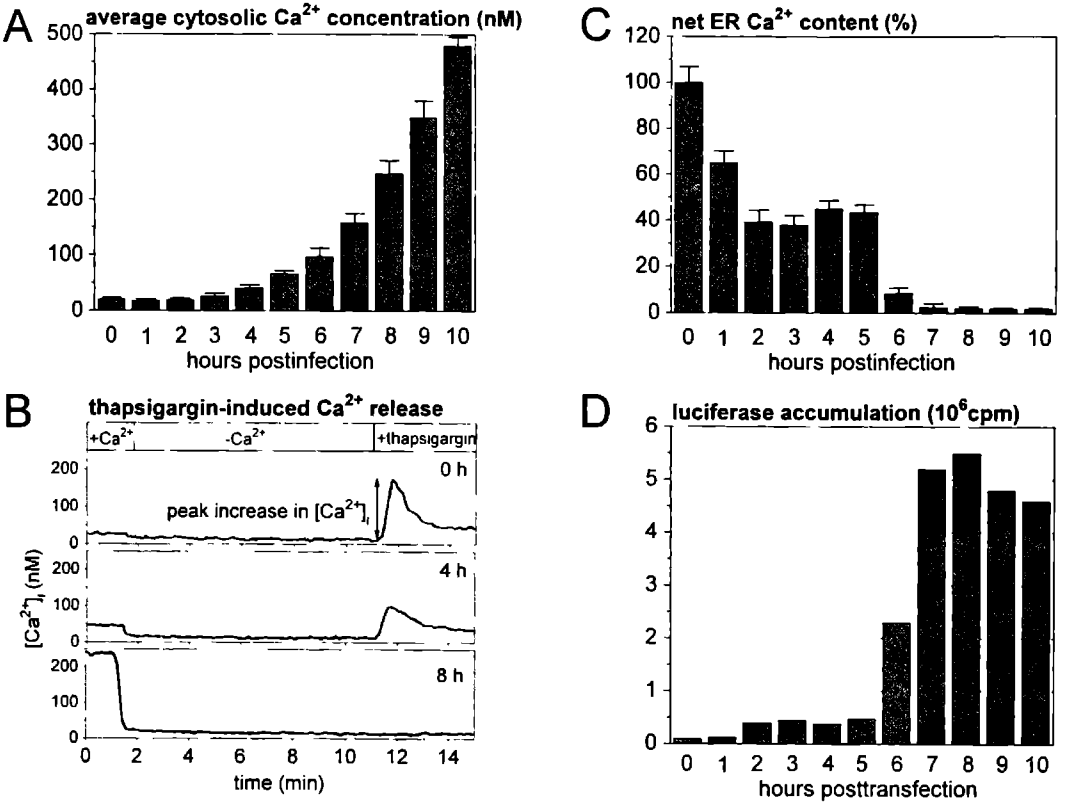


Figure 2. (**A-C**) $[Ca^{2+}]_i$ and the Ca^{2+} content of the ER in CBV3-infected cells at various times p.i. HeLa cells grown on coverslips were infected with CBV3 at a MOI of 25 $TCID_{50}$ per cell for 30 min at room temperature and grown at 37°C. At the indicated times, first the average $[Ca^{2+}]_i$ in groups of fura-2-loaded cells was determined (**A**). Then the cells were stimulated with 1 μM thapsigargin following a 10 min incubation in Ca^{2+}-free medium and the average thapsigargin-induced peak increase in $[Ca^{2+}]_i$, a reflection of the ER Ca^{2+} content, was determined. Representative traces measured at 0, 4, and 8 h p.i. are shown in (**B**). Peak increases in response to thapsigargin measured hourly between the 1 and 10 h p.i. relative to that of noninfected cells are shown in (**C**). Data in panels (A) and (C) are means ± standard errors of measurements of two independent experiments. Five groups of cells (5 to 10 cells per group, all groups lying in the same area of the coverslip) were analyzed in each experiment. (**D**) Kinetics of viral protein synthesis. HeLa cell monolayers were transfected with 1 μg of copy RNA transcripts of pCB3/T7-LUC, a subgenomic CBV3 replicon that contains the luciferase gene in place of the capsid coding region. At various times posttransfection, cells were lysed and the luciferase activity was determined.

2 days posttransfection, the $[Ca^{2+}]_i$ in 250 individual cells was determined and expression of 2B was tested by immunofluorescence microscopy.

Figure 3A shows that in the cell cultures transfected with the 2B-encoding plasmid, about 20% of the cells exhibited an increased $[Ca^{2+}]_i$ (> 500 nM). A similar percentage of cells showed reactivity with a polyclonal antiserum against 2B (Fig 3B). No immuno-reactivity was observed in cell cultures that were transfected with the plasmid that encoded no protein. These latter cells displayed a $[Ca^{2+}]_i$ of about 40 nM, which is similar to that of nontransfected COS cells. The small portion (about 3%) of the cells that displayed an elevated $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$ > 500 nM) probably represented necrotic, membrane-permeable cells, the occurrence of which is most likely due to the electroporation procedure. That protein 2B is expressed in about 20% of the cells and that a similar percentage of cells exhibited an increase in $[Ca^{2+}]_i$, strongly suggests that 2B is sufficient to increase $[Ca^{2+}]_i$. Figure 3C shows the $[Ca^{2+}]_i$ in COS cells transfected with the 2B-encoding plasmid as a function of time posttransfection.

Protein 2B causes leakage of Ca^{2+} from the endoplasmic reticulum. To examine whether protein 2B could also induce leakage of ER-stored Ca^{2+}, we compared the ER Ca^{2+} content of COS cells exhibiting an increased $[Ca^{2+}]_i$ (> 500 nM), which were considered as 2B-expressing cells, to that of cells present at the same coverslip and displaying a normal $[Ca^{2+}]_i$. The latter cells most likely represented nontransfected and non-2B-

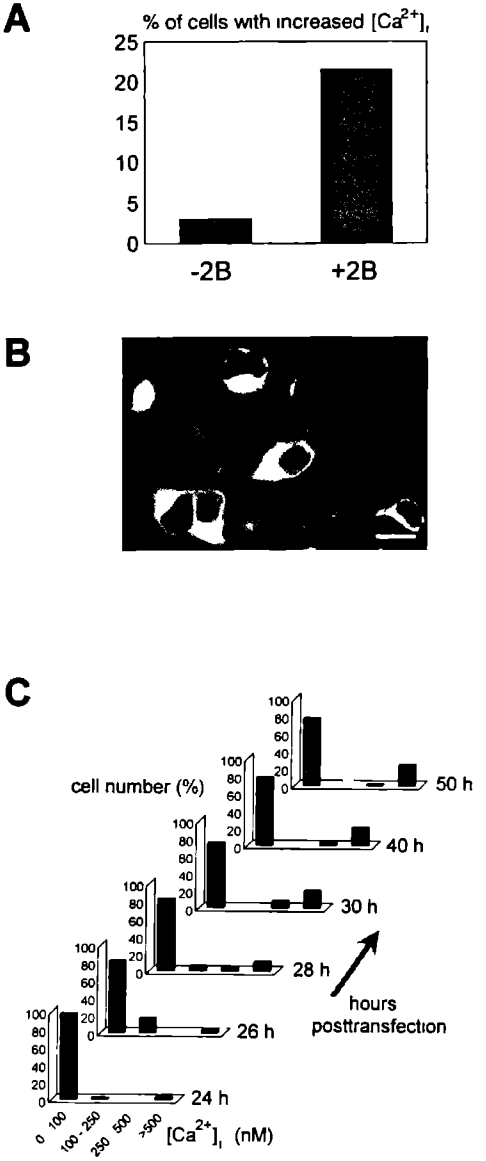


Figure 3. (**A**) Effect of CBV3 protein 2B on $[Ca^{2+}]_i$. COS cells were transfected with plasmids containing the coding sequence of 2B (plasmid pC2α6) or no viral protein (plasmid pLINKα6) and grown at 37°C. At 2 days posttransfection, the $[Ca^{2+}]_i$ in individual cells was determined. (**B**) Immunofluoresence micrograph of 2B-expressing COS cells. Protein 2B was detected using rabbit polyclonal serum raised against 2B and fluorescein isothiocyanate-conjugated goat-anti-rabbit IgG. Bar, 10 μm (magnification x1,000). (**C**) Determination of $[Ca^{2+}]_i$ in individual cells following transfection of COS cells with the CBV3 2B-protein encoding plasmid. At each time, the $[Ca^{2+}]_i$ in 250 individual fura-2-loaded cells was determined.

expressing cells and were further used as control cells. Fura-2-loaded cells were stimulated with thapsigargin following a 10 min incubation in Ca^{2+}-free medium. At the end of each experiment, the thapsigargin-induced peak increase in $[Ca^{2+}]_i$ in individual 2B-expressing cells and control cells was determined and the average peak increase was calculated. Because Ca^{2+} releases on differing $[Ca^{2+}]_i$ are not comparable, 2B-expressing cells that had not been able to lower their $[Ca^{2+}]_i$ to control levels during the 10 min incubation in Ca^{2+}-free medium were not considered. A total of 6 experiments was performed (between 32 and 48 h posttransfection). In control cells, the basal $[Ca^{2+}]_i$ in Ca^{2+}-free medium was about 20 nM and the thapsigargin-induced net change in $[Ca^{2+}]_i$ was 50 ± 15 nM (mean ± SEM). The net change in $[Ca^{2+}]_i$ in 2B-expressing cells relative to that of control cells, which was adjusted to 100% in each experiment, was on average 41 ± 13% (Fig. 4). These results provide evidence that protein 2B indeed induces leakage of Ca^{2+} from ER stores ($P < 0.02$).

A cationic amphipathic α-helix in protein 2B is required to elevate $[Ca^{2+}]_i$. CBV3 protein 2B contains two hydrophobic domains. One of these domains (aa 37 to 54) has the potential to form a cationic amphipathic α-helix with a structural arrangement typical for lytic polypeptides (43). The second domain (aa 63 to 80) displays characteristics of multimeric transmembrane domains (44). To examine the importance of these structural motifs for the ability of protein 2B to increase $[Ca^{2+}]_i$, five 2B proteins carrying mutations in these domains were tested. Three proteins contained mutations in the amphipathic α-helix (Fig. 5A). In protein 2B-K[41,44,

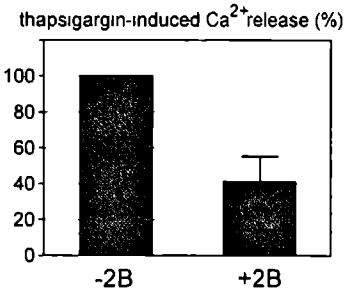


Figure 4. Protein 2B causes release of Ca^{2+} from ER stores. COS cells were transfected with a 2B-encoding plasmid and grown at 37°C. Fura-2-loaded cells were stimulated with 1 μM thapsigargin following a 10 min incubation in Ca^{2+}-free medium 25 to 50 cells were monitored. At the end of the experiment, the average thapsigargin-induced peak increase in $[Ca^{2+}]_i$, a reflection of the Ca^{2+} content of the ER, was calculated from the 2B-expressing cells that had been able to discharge their Ca^{2+} content during the 10 min incubation in Ca^{2+}-free medium (for explanation, see text) and an equal amount of control cells. The average net change in $[Ca^{2+}]_i$ in control cells was adjusted to 100% in each experiment. A total of 6 experiments was performed. The relative response of the 2B-expressing cells represents the mean ± standard error. Experiments were performed between 32 and 48 h posttransfection.

48]E, the cationic character of the helix is disturbed by the replacement of the three lysines with glutamic acid residues. In protein 2B-ins[44]L, the amphipathic character of the helix is disturbed by the insertion of a leucine. In protein 2B-K[41,44]L, the amphipathy is diminished, but not abolished, due to the substitution of two of the lysines with leucines. Two proteins contained mutations in the second hydrophobic domain. These mutations either severely increased (2B-S[77]M/C[75]M) or decreased (2B-I[64]S/V[66]S) the hydrophobicity of this domain.

COS cells were transfected with plasmids that expressed either wild-type or mutant 2B. It seemed possible that the mutant 2B proteins might increase $[Ca^{2+}]_i$ in a delayed manner. Therefore, the $[Ca^{2+}]_i$ was measured (in 250 individual cells) after both 30 h (Fig. 5B) and 50 h (Fig. 5C), the 30 h time point being the time at which the number of cells displaying a marked increase in $[Ca^{2+}]_i$ (> 500 nM) first reached its maximum (Fig 3C). The 2B proteins carrying mutations K[41,44,48]E and 2B-ins[44]L were unable to elevate $[Ca^{2+}]_i$. Protein 2B carrying mutation K[41,44]L was still able to increase $[Ca^{2+}]_i$, but its activity was considerably delayed. The 2B proteins that carried mutations in the second hydrophobic domain exhibited different abilities in increasing $[Ca^{2+}]_i$; protein 2B-I[64]S/V2[66]S was unable to elevate $[Ca^{2+}]_i$, whereas protein 2B-S[77]M/C[75]M showed a wild-type 2B activity in elevating $[Ca^{2+}]_i$. Using immunofluorescence, it was confirmed that all mutant 2B proteins were expressed in about 20% of the cells, similar as wild-type 2B (data not shown). These data demonstrate that both the cationic amphipathic α-helix and the second hydrophobic domain are required to increase $[Ca^{2+}]_i$.

Intracellular distribution of protein 2B. To gain more insight in the mechanism by which CBV3 protein 2B affects Ca^{2+} homeostasis, we examined its subcellular localization in both transfected COS cells and infected HeLa cells. Immuno-electron microscopic analysis of 2B-expressing COS cells showed intense immunolabeling of the plasma membrane and, to a lesser extent, of ER membranes (Fig. 6A-C). 2B did not have an indiscriminate affinity to all membranes, since the nuclear and mitochondrial membranes were not labeled. In CBV3-infected HeLa cells (Fig. 6D-F), protein 2B predominantly occurred in the cytoplasm, where it has been localized at the outer surface of the virus-induced, ER-derived membranous vesicles that surround the viral

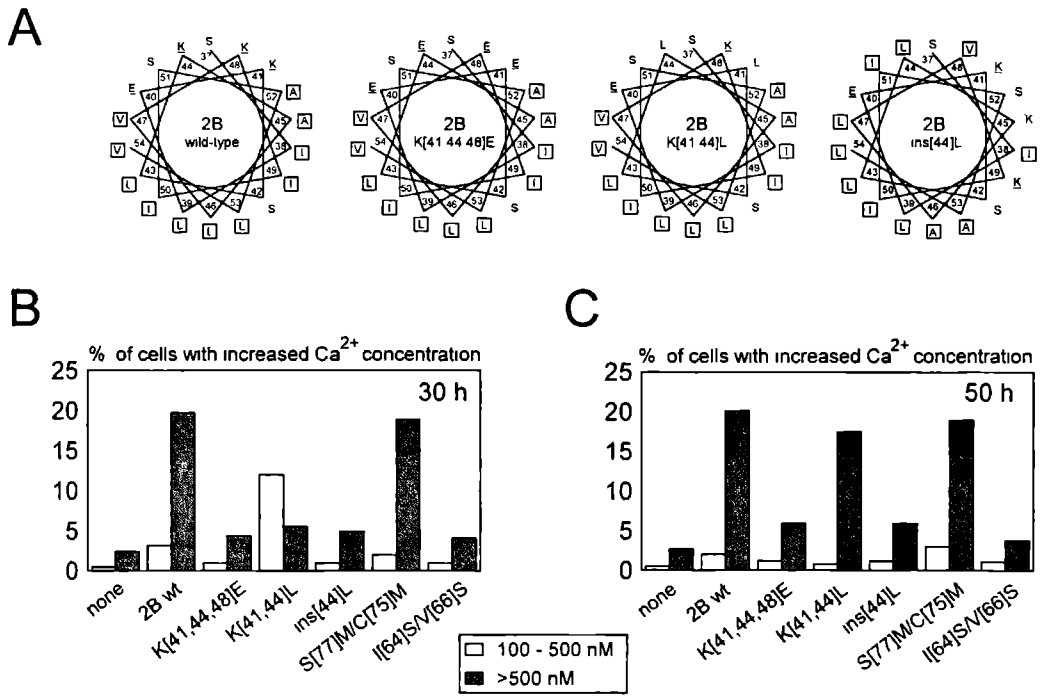


Figure 5. Ability of mutant 2B proteins to increase $[Ca^{2+}]_i$. **(A)** Helical wheel diagrams of mutants that carry alterations in the putative cationic amphipathic α-helix formed by 2B amino acids 37 to 54. Charged residues are underlined. Hydrophobic amino acids are boxed. **(B and C)** $[Ca^{2+}]_i$, measured at 30 h (B) and 50 h (C) after transfection of COS cells with plasmids that direct the synthesis of wild-type 2B or the indicated mutant 2B proteins. At each time, the $[Ca^{2+}]_i$ in 250 individual fura-2-loaded cells was determined.

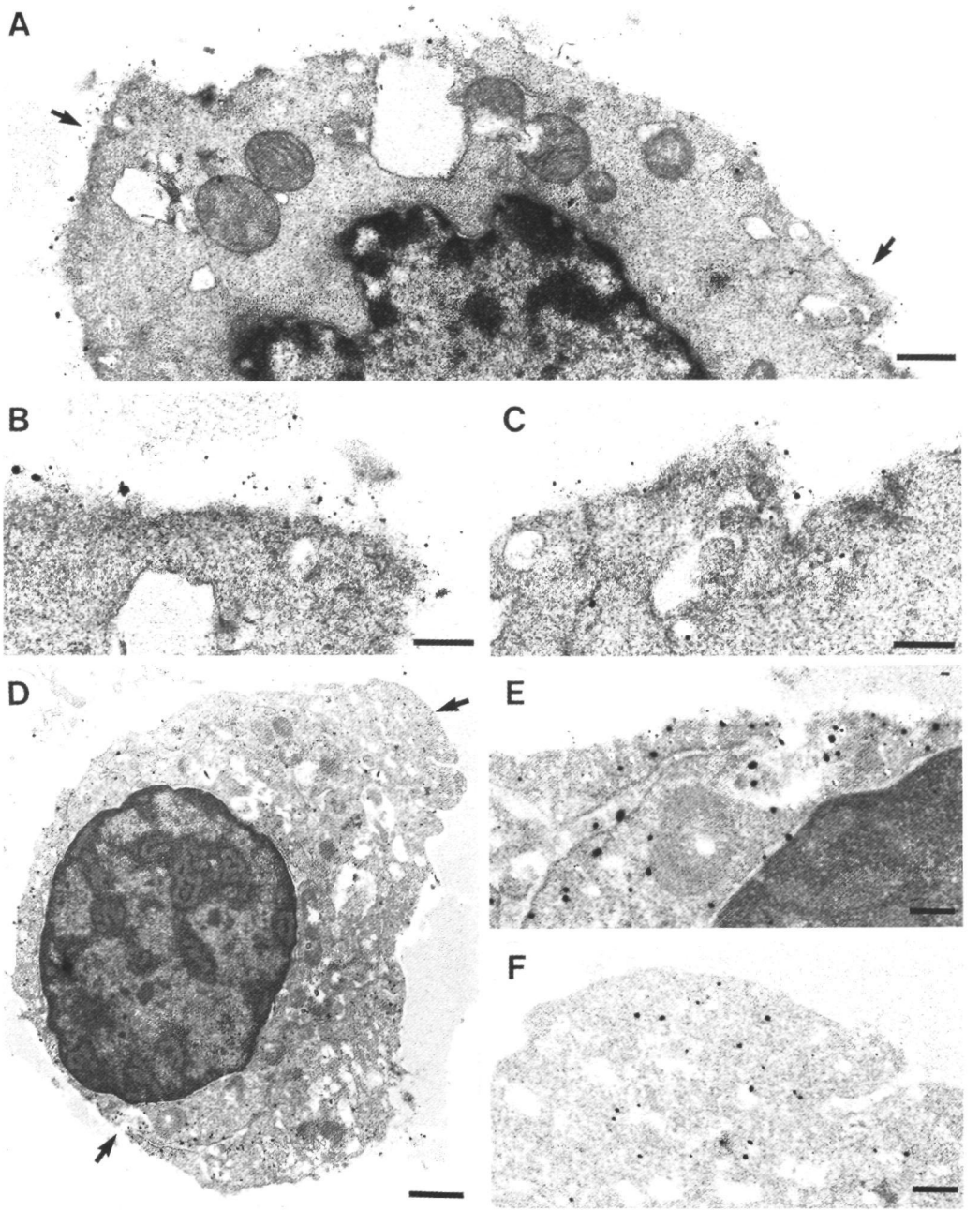


Figure 6. Immunoelectron microscopic localization of protein 2B in 2B-expressing COS cells (**A-C**) and CBV3-infected HeLa cells (**D-F**). COS cells were transfected with a CBV3 2B-encoding plasmid and grown at 37°C for 40 h. HeLa cells were infected with CBV3 at a MOI of 25 $TCID_{50}$ per cell and grown at 37°C for 5 h. Cryostat sections (25 µm) were generated and processed as described in Materials and Methods. The arrows in (A) and (D) point to the places that are shown enlarged in sections (B) and (C), and in sections (E) and (F), respectively. Bar in section (A), 0.5 µm (magnification x19,000). Bar in sections (B) and (C), 0.25 µm (magnification x40,000). Bar in section (D), 1 µm (magnification x9,100). Bar in sections (E) and (F), 0.25 µm (magnification x30,000).

replication complex (6). In addition to its cytoplasmic localization (Fig. 6F), a small fraction of the 2B proteins seemed to occur at the plasma membrane level (Fig. 6E). No immunolabeling was found in mock-infected HeLa cells or COS cells that were transfected with a plasmid that encoded no protein (data not shown).

Protein 2B promotes release of virus progeny from infected cells. Based on its observed potential to enhance membrane permeability, we reasoned that a function of protein 2B might be to release virion progeny from infected cells. To establish such a role, virus release of vCB3-2B-K[41,44]L-infected cells was examined. This virus produces 2B-K[41,44]L, i.e., the mutant 2B protein that showed a delay in increasing $[Ca^{2+}]_i$, most likely as a result of an impaired ability to increase membrane permeability.

HeLa cells were infected with either wild-type virus or vCB3-2B-K[41,44]L and the production and release of viruses was compared. In the same experiment, we also tested vCB3-3'UTR-A[7335]C, a mutant virus that exhibits a reduction in virus growth and yield due to a nucleotide substitution in its 3' untranslated region (23). This virus was included to allow a comparison of the kinetics of virus release of vCB3-2B-K[41,44]L with that of a mutant virus that produces a wild-type 2B protein and which accumulates virion progeny to considerable lesser levels. Figure 7 shows the amounts of viruses released at various times p.i. In cells infected with wild-type virus or vCB3-3'UTR-A[7335]C, nearly all virion progeny was released at 16 h p.i. At this time, roughly only 1 % of the progeny of vCB3-2B-K[41,44]L was released. With the latter mutant, virus release was not complete before 32 h p.i. These results support the idea that virus release is dependent on the membrane-disturbing action of protein 2B, rather than on the accumulation of virion particles (which in vCB3-2B-K[41,44]L-infected cells is more than 100-fold higher than in vCB3-3'UTR-A[7335]C-infected cells).

Do increased $[Ca^{2+}]_i$ levels contribute to certain stages of the viral replicative cycle? The question of whether the virus-induced increase in $[Ca^{2+}]_i$ serves to modulate a viral process or function, or is just a side-effect of the enhanced permeability of the plasma membrane, was addressed. For this, we compared the efficiency and course of several stages of the viral replicative cycle taking place in CBV3-infected cells supplied with medium with or without Ca^{2+}.

The importance of Ca^{2+} for the shutoff of cellular protein synthesis and the efficiency of viral translation was examined by pulse-labeling of infected cells at various times p.i. Figure 8A shows that extracellular Ca^{2+} is dispensable for the inhibition of host cell protein synthesis (which is evident at 3 h p.i.). In the absence of extracellular Ca^{2+}, a small delay in the onset of viral protein synthesis was observed. However, once initiated, the rate of viral translation was not affected.

The role of Ca^{2+} in virus amplification was analyzed by measuring the virus titer at several times p.i. Figure 8B shows that virus growth is largely independent of

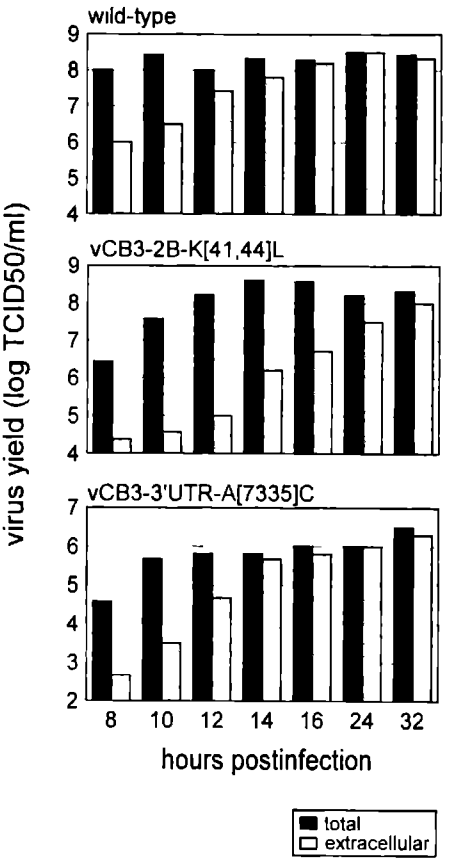


Figure 7. Release of virion progeny from cells infected with wild-type CBV3, vCB3-2B-K[41,44]L, or vCB3-3'UTR-A[7335]C HeLa cell monolayers were infected at a MOI of 5 $TCID_{50}$ per cell and grown at 37°C At the indicated times p i , a small portion of the medium was removed the flasks Cells and the remainder of the medium were subjected to three cycles of freezing and thawing to release intracellular viruses The medium sample that was kept apart was microcentrifuged for 5 min at 1,500 x g to remove intact cells The number of viruses present in the medium (containing extracellular viruses only) and the flasks (containing total virus, i.e., extracellular and intracellular viruses) was determined by endpoint titration $TCID_{50}$, 50% tissue culture infective dose One of two representative experiments is shown

$[Ca^{2+}]_i$. In the absence of extracellular Ca^{2+}, a small reduction in virus titer was observed at 6 h p.i., but viruses grew to similar titers at 8 h p.i. That viruses replicated efficiently in the absence of extracellular Ca^{2+} implies that increases in $[Ca^{2+}]_i$ are dispensable for processes like viral minus and plus-strand RNA synthesis and virion assembly.

The possibility was investigated that the elevated $[Ca^{2+}]_i$ might aid in increasing plasma membrane permeability. First, the sensitivity of translation to hygromycin

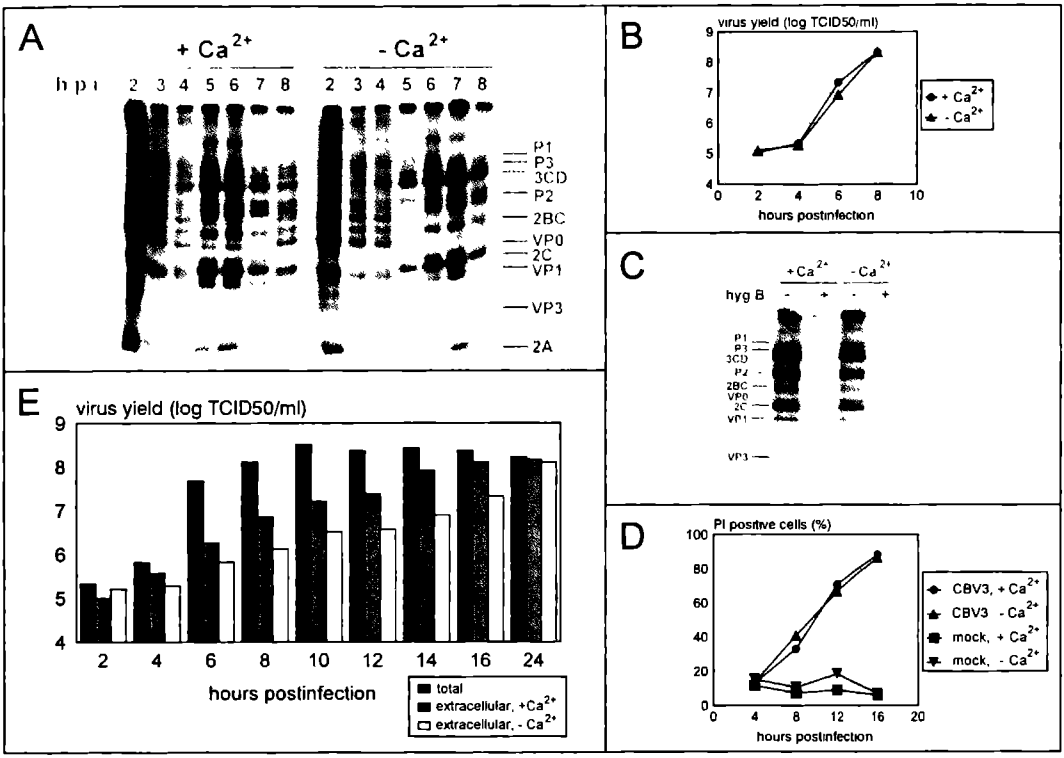


Figure 8. Importance of Ca^{2+} for several stages of the viral replicative cycle. In all panels, HeLa cell monolayers were infected with CBV3 at the indicated MOI (given in $TCID_{50}$ per cell), supplied with medium with or without Ca^{2+}, and grown at 37°C until further analysis. (**A**) Pulse-labeling of infected cells (MOI 25) at various times p.i. (indicated above each lane). At the indicated times, cells were incubated for 30 min in methionine-free MEM, with or without Ca^{2+}, in the presence of 10 µCi of Tran^{35}S-label (a mixture of [^{35}S]methionine and [^{35}S]cysteine). Labeled proteins were analyzed on an SDS-12.5% polyacrylamide gel. Viral proteins are indicated. (**B**) Single-cycle growth curves of infected cells (MOI 5). At the indicated times, cells were subjected to three cycles of freezing and thawing. Virus titers were determined by endpoint titration. (**C**) Permeability of the plasma membrane of infected cells (MOI 25) to hygromycin B. At 6 h p.i., cells were pulse-labeled with 10 µCi of Tran^{35}S-label for 30 min in methionine-free MEM, with or without Ca^{2+}, and in the presence or absence of 500 µgl hygromycin B (hyg B) per ml. (**D**) Permeability of infected cells (MOI 5) and mock-infected cells to propidium iodide (PI). At the indicated times, cells were processed for propidium iodide uptake and flow cytometric analysis as described in Materials and methods. (**E**) Release of virion progeny from infected cells (MOI 5). Titers of total and extracellular virus were determined as described in the legend of Figure 7. One of two representative experiments is shown.

B in infected HeLa cells was assayed. The effect of hygromycin B was tested at 6 h p.i., a time at which host cell translation is shut off and only viral translation takes place. Figure 8C shows that the inhibition of translation by hygromycin B occurred irrespective of the $[Ca^{2+}]_i$. We also tested the permeability of the plasma membrane at increasing times p.i.. Now, permeability was assessed by the uptake of propidium iodide, a membrane-impermeable, fluorescent DNA stain. The percentage of permeable cells was then determined by flow cytometry. Figure 8D shows that during infection the number of permeable cells rapidly increased independent of the presence of extracellular Ca^{2+}. These findings suggest that it is unlikely that the 2B-induced increase in plasma membrane permeability is facilitated by the increase in $[Ca^{2+}]_i$.

To examine whether the increase in $[Ca^{2+}]_i$ contributes to the release of virus progeny, the amount of total virus and extracellular virus produced and released in the presence or absence of extracellular Ca^{2+} was compared (Fig. 8E). The amount of total virus produced in the absence of Ca^{2+} was found to be similar to that produced in the presence of Ca^{2+} and is therefore not shown. In the presence of Ca^{2+}, it took about 16 h before all viruses were released from the cells, i.e., when the amount of total virus and extracellular virus were the same. In the absence of Ca^{2+}, however, virus release was not complete before 24 h p.i. Between 8 and 14 h p.i., the amount of virus that was released in the absence of Ca^{2+} was 5 to 10-fold lower than that released in the presence of Ca^{2+}. Thus, increases in $[Ca^{2+}]_i$ facilitate the release of virus progeny.

DISCUSSION

Protein 2B is a viroporin that facilitates virus release by modifying membrane permeability. Cytolytic animal viruses need to modify membrane permeability in order to release their progeny. This may be particularly important for nonenveloped viruses that do not leave the cells by budding but that need to lyse the membrane. The molecular mechanisms employed to disturb the membrane are yet poorly understood. It has been suggested that membrane permeability is due to the expression of a single or a limited number of virus genes rather than to the bulk of viral gene expression or the formation and accumulation of virus particles (9). Several results presented here provide ample evidence for this suggestion. First, we have shown that CBV3 infection leads to disruption of the Ca^{2+} gradients maintained by the plasma membrane and ER membrane, and that protein 2B is sufficient to induce these effects. Second, viruses carrying a mutant 2B protein exhibited an impairment in the release of virion progeny. Third, mutant viruses that showed a 100-fold reduction in accumulation of virion particles, but which produced a wild-type 2B protein, showed a wild-type efficiency of virus release. These findings strongly suggest that 2B is a protein targeted to enhance plasma membrane permeability, and that a function of this activity is to facilitate the release of virion progeny.

The recognition of virus proteins capable of enhancing membrane permeability is currently leading to the description of a new family of virus proteins; the viroporins (9). Until now, only two of such proteins have been identified; influenza virus protein M2 and togavirus 6K protein, small proteins that contain a predicted amphipathic α-helix. Both proteins are able to modify membrane permeability in the absence of other viral proteins (15, 28, 36) and deletion or blockage of the function of these proteins caused a decrease in the release of virions due to a defect in budding from the cell surface (21, 33). CBV3 protein 2B represents the first example of a viroporin of a naked virus. An understanding of the mechanism by which 2B affects membrane permeability will be of great interest in understanding how cytolytic viruses release their progeny and will contribute to our knowledge on the structure of proteins designed to lyse cellular membranes.

Destabilization of the lipid bilayer by membrane-disturbing proteins is a wide-spread strategy used in nature to lyse cells. Among these proteins are the holins, proteins from bacteriophages that lyse bacteria by forming holes in their membranes (49, 50), hemolysins, cytolytic toxins of bacterial origin (3), cecropines, peptides that occur in the venom of several insect species (38), magainins, antibiotic peptides that occur in the skin of amphibia (51), and defensins, vertebrate antimicrobial peptides (11). A general feature of these membrane-disturbing proteins is the occurrence of amphipathic α-helices, mostly containing cationic amino acids in their hydrophilic faces. Two molecular models of action have emerged from structural and functional studies on these proteins (3, 39). According to the first type of model, the helices form aqueous pores by spanning the membrane and forming oligomers, exposing their hydrophobic sides to the lipid bilayer and their hydrophilic faces forming the aqueous interior. The second model suggests that the amphipathic helix is lying parallel to the membrane plane and penetrates a few Angstroms into it, thereby making the phospholipids more susceptible to degradation by phospholipases. The distinction between these models may not be so clear because membrane-embedded pores may also activate phospholipase activity (50).

Analysis of mutations in individually expressed 2B proteins confirmed that the predicted cationic amphipathic α-helix in 2B is a major determinant for its ability to increase $[Ca^{2+}]_i$. In addition to the amphipathic helix, however, also the second hydrophobic domain appeared to be involved in this ability. This argues against an independent role of the amphipathic α-helix (i.e., the second model) in increasing membrane permeability. It should therefore be considered that multimers of 2B form membrane-embedded pores and that the formation of these pores depends on a correct positioning of the second hydrophobic domain in the membrane. When considering the mechanism by which protein 2B increases plasma membrane permeability, however, the subcellular localization of the protein should be taken into account. Studies examing the localization of poliovirus 2B showed that the protein is contained exclusively central in the cytoplasm, where is it is associated with the ER-derived, membranous vesicles that surround the viral replication complex (6). The possibility that the 2B-induced release of ER-stored Ca^{2+} is indirectly responsible for the increase in plasma membrane permeability seems unlikely, because the enhancement of plasma membrane permeability did not rely on the presence of Ca^{2+} ions. In addition, the thapsigargin-induced evacuation of ER-stored Ca^{2+} in noninfected cells had no effect on plasma membrane permeability to hygromycin B (unpublished data). Another possibility is that a small fraction of the 2B proteins directly associate with the plasma membrane. The immunocytochemical studies reported here provide a first indication for this possibility. In CBV3-infected cells, protein 2B was not only observed central in the cytoplasm, but also at the plasma membrane level. Whether these 2B proteins actually represent membrane-associated proteins or, alternatively, proteins that are associated with viral replication complexes that lie in close vicinity to the plasma membrane remains to be established. That protein 2B is able to associate with the plasma membrane, however, is strongly suggested by the plasma membrane labeling observed in 2B-expressing cells.

A second activity identified for protein 2B is the ability to interfere with protein trafficking through the vesicular system (12). It could be postulated that this activity is indirectly responsible for the increase in plasma membrane permeability by altering the protein and

lipid composition of the membrane. However, this possibility seems unlikely because of two reasons. First, expression of enterovirus protein 3A, which is an even more potent inhibitor of vesicular protein transport than 2B, does not lead to enhanced plasma membrane permeability (12). Second, analysis of the abilities of mutant CBV3 2B proteins to increase membrane permeability and inhibit protein transport suggested that these two activities are separate functions of protein 2B rather than that one of these effects is the consequence of the other (46)

It is tempting to speculate that by forming pores in both the ER membrane and the plasma membrane, 2B is responsible for the disturbance of ionic gradients and the activation of phospholipases. Increased phospholipase C activities have indeed been reported in poliovirus-infected cells (16) and it is likely that these contribute to a further disruption of the membrane, resulting in increased pore sizes. A progressive recruitment of 2B proteins may also cause widening of the pore. The idea that 2B forms pores that gradually increase in size is consistent with the phenomenology observed in poliovirus-infected cells: initially ionic gradients are disrupted in both directions, then low-molecular-weight compounds can pass the membrane, and finally enzymes leak out of the infected cells (9). It is likely that this membrane damage will ultimately allow release of newly formed viruses. The observation that polioviruses are released exclusively from the apical surface of polarized human intestinal epithelial cells (42) may be due to a focal distribution of 2B proteins at the apical plasma membrane.

Disruption of intracellular Ca^{2+} homeostasis by protein 2B. CBV3 infection gradually enhances $[Ca^{2+}]_i$, similar as recently reported in poliovirus-infected cells (18). The increase in $[Ca^{2+}]_i$ in both CBV3-infected HeLa cells and 2B-expressing COS cells is mainly due to the influx of extracellular Ca^{2+}. This influx may be explained by the increased plasma membrane permeability. However, the influx of Ca^{2+} may also be triggered by the partial emptying of the ER. Reductions in the state of filling of intracellular Ca^{2+} stores stimulate the influx of Ca^{2+} by opening specific Ca^{2+} channels in the plasma membrane, a phenomenon known as capacitative Ca^{2+} entry (reviewed in reference 4). The capacitative Ca^{2+} entry mechanism is present in many cells and can be switched on by a great variety of stimuli all of which share a common property of releasing stored Ca^{2+} (30). Capacitative Ca^{2+} entry is therefore likely to occur in both CBV3-infected HeLa cells and 2B-expressing COS cells. The relative contribution and importance of the capacitative Ca^{2+} entry in increasing the $[Ca^{2+}]_i$, however, is difficult to define because the time at which the $[Ca^{2+}]_i$ starts to increase in infected cells (i.e., from 4 h p.i.) is the same as that at which the permeability of the plasma membrane starts to increase (9). Besides, the capacitative Ca^{2+} entry channel has not been identified and specific inhibitors are yet unknown.

It seems plausible that protein 2B releases Ca^{2+} from the ER by increasing the permeability of the ER membrane. However, alternative mechanisms should be considered. The release of ER-stored Ca^{2+} could be caused by (i) the enhanced activity of phospholipases, generating increased levels of inositol-1,4,5-trisphosphate (IP_3), (ii) a direct inhibition of the ER Ca^{2+}-ATPase, or (iii) a direct stimulation of the ER IP_3 receptor. The first possibility seems unlikely because increased levels of IP_3 in poliovirus-infected cells were detected only at 3 to 4 h p.i. (16), whereas the Ca^{2+} content of the ER is already decreased at 1 h p.i. Direct interactions of protein 2B with the ER Ca^{2+}-ATPase or the ER IP_3 receptor also seem unlikely, since we have demonstrated that mutations that abrogate the membrane-disturbing potential of 2B also abolish its ability to increase $[Ca^{2+}]_i$. The possibility that membrane-embedded 2B proteins sterically hinder the functioning of these proteins, however, cannot be excluded.

Until now, only one other viral protein capable of mobilizing Ca^{2+} from the ER has been identified; rotavirus NSP4. This nonstructural ER transmembrane glycoprotein contains a predicted amphipathic α-helix and this helix has been shown to be involved in the mobilization of Ca^{2+} from the ER (40). The formation of cation channels has been proposed as a possible mechanism by which NSP4 alters the ER permeability. Thus, rotavirus NSP4 and 2B may release ER-stored Ca^{2+} by a common mechanism.

A model that may account for the alterations in Ca^{2+} homeostasis that take place in time during CBV3 infection, and the release of virus progeny from infected cells is illustrated in Fig. 9. We propose that early in infection (during the first 2 hours) the 2B protein is produced and inserted into the ER membrane, where it promotes a partial release (of about 50%) of stored Ca^{2+} by forming membrane-embedded pores. As a consequence, capacitative Ca^{2+} entry will be induced. However, the $[Ca^{2+}]_i$ remains constant during the first 3 to 4 h p.i., as a result of the action of the plasma membrane Ca^{2+}-ATPase. A small fraction of the 2B proteins is transported to the plasma membrane, where it causes the influx of Ca^{2+} (evident from 4 h p.i.). Between 2 h and 5 h p.i., the Ca^{2+} content of the ER remains constant. This steady-state phase probably reflects the time needed to replicate the plus-strand genomic RNA via a complementary minus-strand RNA intermediate. Translation of these newly synthesized plus-strand RNAs (between 5 and 6 h p.i.) gives rise to the production of large numbers of 2B protein, which cause a rapid evacuation of ER-stored Ca^{2+} by further disturbing the ER membrane. Progressive insertion of 2B proteins in the plasma membrane results in a further collapse of ionic gradients. An increased influx of Ca^{2+} ions through both capacitative Ca^{2+} entry channels and pores formed by 2B will be the result. A rapid increase in $[Ca^{2+}]_i$ occurs from 6 h p.i., most likely because from this time the Ca^{2+} influx exceeds the pumping capacity of the plasma membrane Ca^{2+}-ATPase. Progressive membrane disruption ultimately allows the release of virus progeny.

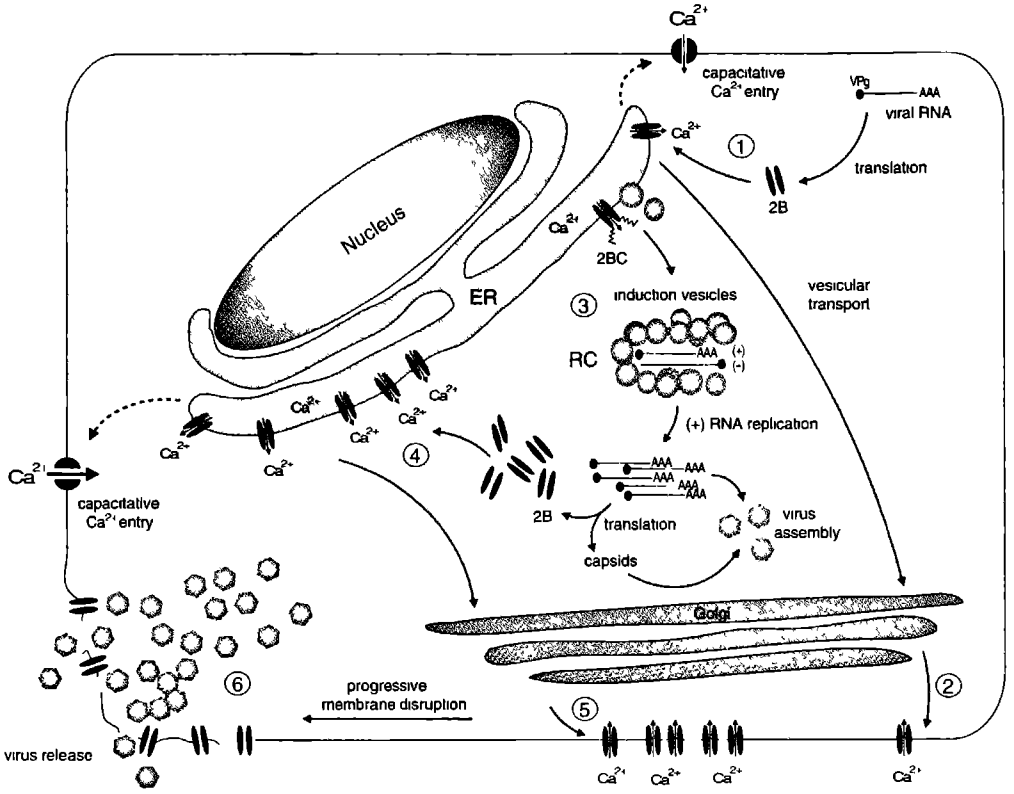


Figure 9. Model for the mobilization of ER-stored Ca^{2+}, the influx of extracellular Ca^{2+}, and the release of virus progeny by protein 2B through the formation of membrane-embedded pores. Early events (up to 4 h p.i.) in the viral replicative cycle involve **(1)** production of protein 2B and insertion in the ER membrane, leading to a release of Ca^{2+} and, as a consequence, opening of capacitative Ca^{2+} entry channels, **(2)** transport of 2B to the plasma membrane where it is inserted and causes influx of extracellular Ca^{2+}, **(3)** induction of the membranous vesicles by protein 2BC that are required for viral plus-strand RNA replication (possibly through the release of ER-stored Ca^{2+}). Late events (from 5 h p.i.) in the viral replicative cycle involve **(4)** translation of newly formed plus-strand RNAs yielding large numbers of protein 2B, insertion of these proteins in the ER membrane causes a rapid evacuation of Ca^{2+} and gives rise to an increased capacitative Ca^{2+} entry, **(5)** collapse of Ca^{2+} gradient maintained by the plasma membrane by massive insertion of 2B proteins, **(6)** progressive increase in the size of the pores formed by 2B causes disruption of the membrane and results in the membrane lesions that allow virus release. For details, the reader is directed to the text. RC, replication complex.

Studies towards the potential contribution of the enhanced $[Ca^{2+}]_i$ to different stages of the viral life cycle showed that increases in $[Ca^{2+}]_i$ were dispensable for the shutoff of cellular translation, the rate of viral translation, viral minus-strand and plus-strand RNA replication, virion assembly, and virus release. This latter process, however, appeared to be potentiated by increases in $[Ca^{2+}]_i$. The reason for this is unknown. A possible explanation is that the elevated $[Ca^{2+}]_i$ is cytotoxic and enhances cell lysis. Increases in $[Ca^{2+}]_i$ have been shown to be involved in animal virus-induced cytopathic effects and cell killing (24, 27, 35, 41). Whether the increase in $[Ca^{2+}]_i$ in CBV3-infected cells also contributes to cytopathology, and whether this contributes to virus release, remains to be established.

Putative function of the mobilization of Ca^{2+} from the ER in viral genome replication. We have shown that CBV3 protein 2B enhances membrane permeability and that this activity serves to release virus progeny. However, it seems unlikely that this is the only function of 2B. Mutations in the 2B gene that disrupt essential structural domains abolish vRNA replication and virus growth (43-45). However, if the only function of 2B were to modify plasma membrane permeability in order to release virus progeny, then genomic RNAs carrying mutations in their 2B gene would be expected to be replicated and encapsidated to produce viruses that accumulate in the cell. Additional functions of 2B connected with its ability to disturb membrane integrity should therefore be considered. A possible explanation

is that the release of ER-stored Ca^{2+} is required for vRNA replication. Viral plus-strand RNA synthesis takes place at the outer surface of virus-induced membrane vesicles that originate from the ER (6). Protein 2BC has been identified as the viral protein responsible for the induction of these vesicles (2, 7, 10). The function of 2B in precursor 2BC may involve its ability to release ER-stored Ca^{2+}. The ER contains a gel of Ca^{2+} binding proteins that is responsible for the sequestration of Ca^{2+} ions. These Ca^{2+} binding proteins form coordination complexes with Ca^{2+} ions that are bound to the negatively charged phospholipid head groups on the luminal face of the ER membrane, thereby stabilizing the underlying membrane and preventing its vesiculation (34). It has been predicted that transport vesicles derived from the ER normally bud from regions of membrane that are not stabilized by the Ca^{2+}-protein gel. The artificial evacuation of Ca^{2+} ions from the lumen of the ER that occurs when cells are treated with Ca^{2+} ionophores has been proposed to destabilize the entire Ca^{2+}-protein gel and its associated membrane, leading to a mass vesiculation (34). By analogy with the effect of the ionophores, the release of ER-stored Ca^{2+} by the 2B polypeptide in protein 2BC may be involved in the massive induction of ER-derived membrane vesicles by 2BC (Fig. 9). According to this model, mutations that disrupt the ability of 2B to enhance membrane permeability will interfere with the ability of protein 2BC to induce these membrane vesicles and thereby account for defects in vRNA replication and virus growth.

The exact mechanism by which protein 2BC induces membrane proliferation and vesicle accumulation is still puzzling. The release of ER-stored Ca^{2+} may be involved, but is clearly not sufficient for these activities, as protein 2B alone is unable to account for these structural alterations. Therefore, a function of protein 2C in precursor 2BC seems to be required as well. Protein 2C has been identified as an NTPase that shares structural similarities with the group of small GTPases involved in the regulation of vesicle transport (25, 32), but its exact function is unknown. Further investigations are required to define the contribution of proteins 2B and 2C to the ability of protein 2BC to rearrange membranes and to induce membranous vesicles.

ACKNOWLEDGMENTS

We are grateful to Drs. Karin Klingel and Reinhard Kandolf for providing us antiserum against CBV3 protein 2B, and to Cor Jacobs for advice and use of the flow cytometry facilities. The research of Dr P H G M. Willems has been made possible by a fellowship of the Royal Academy of Arts and Sciences.

REFERENCES

1 **Aldabe, A., A. Barco, and L. Carrasco.** 1996 Membrane permeabilization by poliovirus proteins 2B and 2BC J Biol Chem **271**:23134-23137

2 **Barco, A., and L. Carrasco.** 1995 A human virus protein, poliovirus protein 2BC, induces membrane proliferation and blocks the exocytic pathway in the yeast *Saccharomyces cerevisiae* EMBO J **14**:3349-3364

3 **Bernheimer, A.W., and B. Rudy.** 1986 Interactions between membranes and cytolytic peptides Biochem Biophys Acta **864**:123-141

4 **Berridge, M.J.** 1995 Capacitative calcium entry Biochem J **312**:1-11

5 **Bienz, K., D. Egger, and L. Pasamontes.** 1987 Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography Virology **160**:220-226

6 **Bienz, K., D. Egger, and T. Pfister.** 1994 Characteristics of the poliovirus replication complex Arch Virol suppl **9**:147-157

7 **Bienz, K., D. Egger, Y. Rasser, and W. Bossart.** 1983 Intracellular distribution of poliovirus proteins and the induction of virus-specific cytoplasmic structures Virology **131**: 39-48

8 **Carafoli, E.** 1987 Intracellular calcium homeostasis. Annu Rev Biochem **56**:395-433

9 **Carrasco, L.** 1995 Modification of membrane permeability by animal viruses Adv Virus Res **45**:61-112

10 **Cho, M.W., N. Teterina, D. Egger, K. Bienz, and E. Ehrenfeld.** 1994 Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells Virology **202**:129-145

11 **Cociancich, S., M. Goyffon, F. Bontems, P. Bulet, F. Bouet, A. Menez, and J. Hoffmann.** 1993 Purification and characterization of a scorpion defensin, a 4 kDa antibacterial peptide presenting structural similarities with insect defensins and scorpion toxins Biochem Biophys Res Commun **194**:17-22

12 **Doedens, J.R., and K. Kirkegaard.** 1995 Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A EMBO J **14**:894-907

13 **Ehrenfeld, E.** 1982 Poliovirus-induced inhibition of host-cell protein synthesis Cell **28**:435-436

14 **Grynkiewicz, G., M. Poenie, and R.Y. Tsien.** 1985 A new generation of Ca^{2+} indicators with greatly improved fluorescence properties J Biol Chem **260**:3440-3450

15 **Guinea, R., and L. Carrasco.** 1994 Influenza virus M2 protein modifies membrane permeability in *E. coli* cells FEBS Lett **343**:242-246

16 **Guinea, R., A. Lopez Rivas, and L. Carrasco.** 1989 Modification of phospholipase C and phospholipase A2 activities during poliovirus infection J Biol Chem **264**:21923-21927

17 **Holland, J.J.** 1962 Inhibition of DNA-primed RNA synthesis during poliovirus infection of human cells Biochem Biophys Res Commun **9**:556-562

18 **Irurzun, A., J. Arroyo, A. Alvarez, and L. Carrasco.** 1995 Enhanced intracellular calcium concentration during poliovirus infection J Virol **69**:5142-5146

19 **Klump, W.M., I. Bergman, B.C. Müller, D. Ameis, and R. Kandolf.** 1990 Complete nucleotide sequence of infectious coxsackievirus B3 cDNA Two initial 5' uridine residues are regained during plus-strand RNA synthesis J Virol **64**:1573-1583

20 **Lama, J., and L. Carrasco.** 1992 Expression of poliovirus nonstructural proteins in *Escherichia coli* cells J Biol Chem **267**:15932-15937

21 **Liljeström, P., S. Lusa, D. Huylebroeck, and H. Garoff.** 1991 In vitro mutagenesis of full-length cDNA clone of Semliki Forest virus the small 6,000-molecular weight membrane protein modulates virus release J Virol **65**:4107-4113

22 **Lytton, J., M. Westlin, and M.R. Hauley.** 1991 Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps J Biol Chem **266**:17067-17071

23 **Melchers, W.J.G., J.G.J. Hoenderop, H.J. Bruins Slot, C.W.A. Pleij, E.V. Pilipenko, V.I. Agol, and J.M.D. Gala-**

ma. 1997 Kissing of the two predominant hairpin-loops in the coxsackie B virus 3'UTR is the essential structural feature of the origin of replication required for (-) strand RNA synthesis J Virol **71**:686-696

24 **Michelangeli, F., M.C. Ruiz, J.R. del Castillo, J.E. Ludert, and F. Liprandi.** 1991 Effect of rotavirus infection on intracellular calcium homeostasis in cultured cells Virology **181**: 520-527

25 **Mirzayan, C., and E. Wimmer.** 1994 Biochemical studies on poliovirus polypeptide 2C evidence for ATPase activity Virology **199**:176-187

26 **Mosser, A.G., L.A. Caliguiri, and J. Tamm.** 1972 Incorporation of lipid precursors into cytoplasmic membranes of poliovirus-infected HeLa cells Virology **47**:39-47

27 **Nokta, M., D. Eaton, O.S. Steinsland, and T. Albrecht.** 1987 Ca^{2+} responses in cytomegalovirus-infected fibroblasts of human origin Virology **157**:259-267

28 **Pinto, L.H., L.J. Holsinger, and R.A. Lamb.** 1992 Influenza virus M2 protein has ion channel activity Cell **69**:517-528

29 **Porter, A.G.** 1993 Picornavirus nonstructural proteins emerging roles in virus replication and inhibition of host cell functions J Virol **67**:6917-6921

30 **Putney, J.W., Jr, and G.S.J. Bird.** 1994 Calcium mobilization by inositol phosphates and other intracellular messengers Trends Endocrinol Metab **5**:256-260

31 **Reed, L.J., and H. Muench.** 1938 A simple method of estimating fifty per cent endpoints Am J Hyg **27**:493-497

32 **Rodriguez, P.L., and L. Carrasco.** 1993 Poliovirus protein 2C has ATPase and GTPase activities J Biol Chem **268**: 8105-8110

33 **Ruigrok, R.W.H., E.M.A. Hirst, and A.J. Hay.** 1991 The specific inhibition of influenza A virus maturation by amantadine an electron microscopic examination J Gen Virol **72**: 191-194

34 **Sambrook, J.F.** 1990 The involvement of calcium in transport of secretory proteins from the endoplasmic reticulum Cell **61**:197-199

35 **Sanderson, C.M., J.E. Parkinson, M. Hollinshead, and G.L. Smith.** 1996 Overexpression of the vaccinia virus A38L integral membrane protein promotes Ca^{2+} influx into infected cells J Virol **70**:905-914

36 **Sanz, M.A., L. Pérez, and L. Carrasco.** 1994 Semliki Forest Virus 6K protein modifies membrane permeability after inducible expression in *Escherichia coli* cells J Biol Chem **269**:12106-12110

37 **Schlegel, A., T.H. Giddings, Jr., M.L. Ladinsky, and K. Kirkegaard.** 1996 Cellular origin and ultrastructure of membranes induced during poliovirus infection J Virol **70**:6576-6588

38 **Segrest, J.P., H. de Loof, J.G. Dohlman, C.G. Brouillette, and G.M. Anantharamaiah.** 1990 Amphipathic helix motif Classes and properties Struct Funct Genet **8**:103-117

39 **Shai, Y.** 1995 Molecular recognition between membrane-spanning polypeptides Trends Biol Sci **20**:460-464

40 **Tian, P., M.K. Estes, Y. Hu, J.M. Ball, C.Q.-Y. Zeng, and W.P. Schilling.** 1995 The rotavirus nonstructural glycoprotein NSP4 mobilizes Ca^{2+} from the endoplasmic reticulum J Virol **69**:5763-5772

41 **Tian, P., Y. Hu, W.P. Schilling, D.A. Lindsay, J. Eiden, and M.K. Estes.** 1994 The nonstructural glycoprotein of rotavirus affects intracellular calcium levels J Virol **68**:251-257

42 **Tucker, S.P., C.L. Thornton, E. Wimmer, and R.W. Compans.** 1993 Vectorial release of poliovirus from polarized human intestinal epithelial cells J Virol **67**:4274-4282

43 **van Kuppeveld, F.J.M., J.M.D. Galama, J. Zoll, P.J.J.C. van den Hurk, and W.J.G. Melchers.** 1996 Coxsackie B3 virus protein 2B contains a cationic amphipathic helix that is required for viral RNA replication J Virol **70**:3876-3886

44 **van Kuppeveld, F.J.M., J.M.D. Galama, J. Zoll, and W.J.G. Melchers.** 1995 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 J Virol **69**:7782-7790

45 **van Kuppeveld, F.J.M., P.J.J.C. van den Hurk, J. Zoll, J.M.D. Galama, and W.J.G. Melchers.** 1996 Mutagenesis of the coxsackie B3 virus 2B/2C cleavage site determinants of processing efficiency and effects on viral replication J Virol **70**:7632-7640

46 **van Kuppeveld, F.J.M., W.J.G. Melchers, K. Kirkegaard, and J.R. Doedens.** 1997 Structure-function analysis of coxsackie B3 virus protein 2B Virology **227**:111-118

47 **Willems, P.H.G.M., S.E. van Emst-de Vries, C.H. van Os, and J.J.H.H.M. de Pont.** 1993 Dose-dependent recruitment of pancreatic acinar cells during receptor-mediated calcium mobilization Cell Calcium **14**:145-159

48 **Wimmer, E., C.U.T. Helen, and X. Cao.** 1993 Genetics of poliovirus Annu Rev Genet **27**:353-436

49 **Witte, A., G. Wanner, U. Bläsi, G. Halfmann, M. Szostak, and W. Lubitz.** 1990 Endogenous transmembrane tunnel formation by phi X174 lysis protein E J Bacteriol **172**:4109-4119

50 **Young, R.** 1992 Bacteriophage lysis mechanism and regulation Microbiol Rev **56**:430-481

51 **Zasloff, M.** 1987 Magainins, a class of antimicrobial peptides from *Xenopus* skin, isolation, characterization of two active forms, and partial cDNA sequence of a precursor Proc Natl Acad Sci USA **84**:5449-5453

Summary and General Discussion

Summary and General Discussion

The genus *Enterovirus*, which belongs to the family of the *Picornaviridae*, comprises polioviruses, coxsackie A and B viruses, ECHO viruses, and a number of distinct enterovirus serotypes Enteroviruses are human pathogens that cause a broad spectrum of diseases Coxsackie B viruses usually cause acute infections that are either subclinical or so mild that they do not come to attention These viruses may, however, be involved in several chronic diseases such as idiopathic dilated cardiomyopathy, polymyositis/dermatomyositis, and juvenile (type I) diabetes mellitus A possible explanation for the involvement of coxsackie B viruses in these chronic diseases is that the virus causes a persistent infection However, coxsackievirus reproduction usually results in cell death within a short time This excludes a chronic course of infection A persistent infection may therefore reflect an alteration in viral reproduction The process of viral replication is still incompletely understood This thesis was undertaken to gain more insight in the molecular mechanism of viral replication

Enteroviruses are small cytolytic viruses that contain a single-stranded RNA genome of plus-strand polarity (15) After cell entry and virion uncoating, the genomic RNA serves as a messenger RNA to direct the synthesis of a single polyprotein Initiation of translation occurs via a cap-independent mechanism The polyprotein is processed by virally encoded proteases to yield four structural capsid proteins and ten nonstructural proteins, seven mature proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) and three cleavage intermediates (2BC, 3AB, and 3CD) (15) Replication of the viral RNA (vRNA) starts with the synthesis of a complementary minus-strand RNA which, in turn, serves as a template to transcribe new plus-strand RNA molecules Plus-strand RNA synthesis takes place in a replication complex at the outer surface of virus-induced, membranous vesicles that originate from the endoplasmic reticulum (ER) (3, 12) Newly formed plus-strand RNAs are packaged into the structural capsid proteins to produce new virions During infection, enteroviruses induce a number of alterations in cellular functions and structures, most of which serve to facilitate viral translation, RNA replication, and virus release These alterations include inhibition of host cell transcription and translation, rearrangement of intracellular membranes, inhibition of vesicular protein transport, and modification of the plasma membrane permeability (10) All of these activities must be directed by the ten nonstructural proteins The genetic organization of the viral RNA genome and the putative roles of the each of the nonstructural proteins in vRNA replication and modification of host cell functions and structures are reviewed in **chapter 1**

The investigations described in this thesis have focussed on the nonstructural 2B protein of coxsackie B3 virus (CBV3), a small protein that had been identified at the outer surface of the ER-derived membrane vesicles at which viral plus-strand RNA synthesis takes place, but whose function was completely unknown

SUMMARY OF THE MAIN FINDINGS

To gain more insight into the structure and function of protein 2B, first an extensive computer analysis was performed Alignment studies revealed no similarity with any cellular protein Analysis of the hydropathy showed the hydrophobic nature of the protein and allowed the identification of two hydrophobic domains Both domains are well conserved in the 2B proteins of all enteroviruses One of these domains (which will further be referred to as "the hydrophobic domain") contained a moderate degree of hydrophobicity, a feature that is typical of multimeric transmembrane helices The other domain displayed a high hydrophobic moment and had the potential to form a cationic amphipathic α-helix

Chapter 2 describes a molecular genetic approach to define the role of the hydrophobic domain of CBV3 2B in virus reproduction, and to examine the structural and hydrophobic requirements of the domain Defined mutations were introduced in a full-length cDNA clone of CBV3 The effects of the mutations on virus viability and growth were assayed by transfection of cells with in vitro transcribed copy RNA transcripts Furthermore, the effects of the mutations on RNA replication, polyprotein synthesis and processing, and protein synthesis in infected cells were examined The results support the idea that a moderate degree of hydrophobicity of this domain is required for the structure and function of the protein Mutations that caused a major increase or decrease in hydrophobicity disrupted vRNA replication and virus growth, but did not interfere with the synthesis and processing of the polyprotein

A detailed genetic analysis of the putative cationic amphipathic α-helix in CBV3 2B is described in **chapter 3** This amphipathic α-helix contains a narrow hydrophilic face and an arrangement of cationic residues that is characteristic for the so-called lytic polypeptides, small cationic peptides that modify the permeability and integrity of cellular membranes (2, 13, 14) Both the amphipathy of the helix and the presence of cationic residues in the hydrophilic face of the amphipathic helix in protein 2B were found to be required for vRNA replication and virus growth This supports the idea that a cationic amphipathic α-helix is formed and that this structure is involved in a function of protein 2B

In **chapter 4** it is shown that individual expression of CBV3 2B in COS cells leads to (i) inhibition of protein secretion, as shown by the impaired secretion of a co-expressed, secreted reporter protein, and (ii) an increase

in the permeability of the plasma membrane, as shown by the increased sensitivity of cellular translation to hygromycin B, an inhibitor of translation that normally enters cells poorly. Analysis of mutant CBV3 2B proteins showed that both the cationic amphipathic α-helix and the hydrophobic domain contribute to these activities. Some mutant 2B proteins exhibited an impaired ability to inhibit protein secretion but not to increase membrane permeability. This suggests that both activities represent distinct functions of 2B rather than that one is the consequence of the other.

In **chapter 5** it is shown that mutant viruses containing poorly processed 2B/2C cleavage sites, and that, as a consequence, produced reduced levels of proteins 2B and 2C, were unable to completely shut off cap-dependent cellular protein synthesis. The failure of the mutant viruses to completely inhibit host cell translation coincided with an impaired ability to increase the permeability of the plasma membrane to hygromycin B. These data are again indicative for a role or protein 2B in increasing plasma membrane permeability. Furthermore, they suggest that by modifying the plasma membrane permeability, protein 2B may participate in the shutoff of cap-dependent cellular translation.

To further dissect the structural requirements of protein 2B for its function in vRNA replication, the approach of constructing chimeric coxsackievirus-poliovirus genomes was followed in **chapter 6**. CBV3 genomes that expressed poliovirus 2B, a protein that has a similar structure and function as CBV3 2B (7) but which differs significantly in its amino acid sequence , failed to replicate. This suggests that a sequence-specific interaction between 2B and another viral protein is required for a function in vRNA replication. Only a hybrid 2B protein that contained the amino-terminal one-third of poliovirus 2B and the remainder of CBV3 2B was functional in vRNA replication and gave rise to virus growth. This argues that the proposed sequence-specific contacts reside in the carboxy-terminal two-third of 2B. The failure to drive vRNA replication of the hybrid 2B proteins that contained either the amphipathic helix or the hydrophobic domain of poliovirus 2B suggests that the functioning of 2B depends on a mutual interaction of these domains.

The ability of protein 2B to modify membrane permeability and its localization at the ER and the virus-induced, ER-derived membranous vesicles suggested that 2B might release Ca^{2+} from ER stores. In **chapter 7**, digital-imaging microscopy was performed to study the effect of CBV3 infection on the cytosolic free Ca^{2+} concentration and the Ca^{2+} content of the ER. It is shown that CBV3 infection leads to the influx of extracellular Ca^{2+} and the release of ER-stored Ca^{2+}. The individual expression of protein 2B appeared to be sufficient to induce these effects. Analysis of mutant 2B proteins showed that both the cationic amphipathic α-helix and the hydrophobic domain are required for these functions. Electron microscopy argued that protein 2B not only occurs at the ER membrane but also at the plasma membrane. A mutant virus that carried a defective 2B protein exhibited an impairment in the release of virus progeny. These findings support the idea that protein 2B increases the permeability of the cellular membranes with which it interacts, resulting in a disruption of ionic gradients and, ultimately, the membrane lesions that allow virus release.

PROPOSED STRUCTURAL MODEL OF PROTEIN 2B

A cationic amphipathic α-helix is crucial for a function of protein 2B in vRNA replication **(chapter 3)** and its abilty to increase membrane permeability **(chapters 4 and 7).** The occurrence of cationic amphipathic helical motifs is typical for membrane-perturbing proteins and peptides (13). Two mechanisms have been proposed to explain the action of these proteins (2, 14). According to the first model, the amphipathic helix perturbs the membrane by lying collateral to it and penetrating a few Angstroms into it, thereby making the membrane phospholipids more susceptible to degradation by phospholipases. The second model suggests that the amphipathic helices form aqueous channels by spanning the membrane and forming oligomers, exposing their hydrophobic faces to the lipid bilayer and their hydrophilic faces forming the aqueous interior of the pores.

Two observations argue against an independent role (i.e., the first model) of the amphipathic α-helix of CBV3 2B in increasing membrane permeability. First, this activity appeared to be sensitive to mutations in the hydrophobic domain **(chapters 4 and 7)**. Second, a chimeric 2B protein in which the α-helix was replaced with another cationic amphipathic α-helix failed to modify

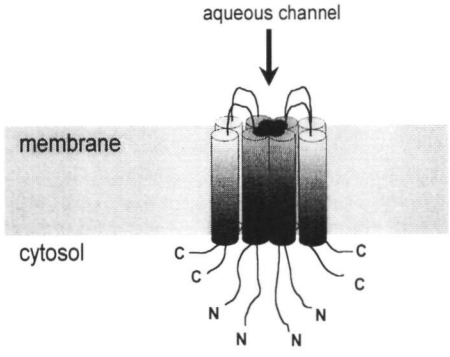


Figure 1. Proposed membrane topology of protein 2B. Helices representing the amphipathic α-helix (nearer the N-terminus) and the second hydrophobic domain (nearer the C-terminus) are depicted. An aqueous channel can be formed by multimers of 2B that expose the hydrophobic faces of their amphipathic helices to the lipid bilayer and the hydrophilic faces (darkly shaded) to the aqueous inner surface of the channel.

plasma membrane permeability **(chapter 6)** The formation of aqueous channels should therefore be considered A possible explanation for the importance of the hydrophobic domain and the nonexchangeability of the amphipathic α-helix is that both domains occur cooperatively associated within the lipid bilayer and that intimate contacts are required for the formation or stabilization of such a channel A putative structural model of the membrane-embedded pores formed by multimers of protein 2B is shown in Fig 1 Transmembrane positioning of both the amphipathic helix and the hydrophobic domain is in agreement with the lack of glycosylation of potential glycosylation sites engineered in the NH_2- and COOH-termini of 2B (van Kuppeveld et al , unpublished results), a finding that suggests that both termini reside on the cytosolic rather than on the luminal side of the membrane The cooperative association of hydrophobic domains in the membrane is compatible with the observation that a moderate degree of hydrophobicity of this domain is required for the well functioning of protein 2B **(chapter 2)**, which suggests that the domain forms a multimeric rather than a monomeric transmembrane helix

ROLE OF PROTEIN 2B IN VIRAL REPLICATION: IMPLICATIONS AND SPECULATIONS

The expression of protein 2B in COS cells leads to an increased permeability of the plasma membrane and the release of Ca^{2+} from the ER **(chapter 7)**, probably through the formation of membrane-embedded pores Another ability of protein 2B is to inhibit protein transport through the vesicular pathway **(chapter 4)** It is yet unknown whether each of these activities represent true functions that contribute to virus reproduction Until now, only one activity has been directly tied to the viral replicative cycle, the ability of protein 2B to facilitate virus release from infected cells, which is presumably a direct effect of its ability to increase the permeability of the plasma membrane It seems unlikely, however, that this is the only function of 2B Mutations in the 2B protein that disrupt essential structural domains abolish vRNA replication and virus growth **(chapters 2, 3, and 6)** If the only function of 2B were to modify plasma membrane permeability in order to release virus progeny, then genomic RNAs carrying mutations in their 2B gene would be expected to replicate and to be encapsidated to produce viruses that accumulate in the cell Additional functions of protein 2B should therefore be considered

A second function of the increase in plasma membrane permeability may be the inhibition of the host cell protein synthesis Mutant viruses that produced reduced levels of protein 2B failed to completely inhibit host cell translation and showed an impaired ability to modify membrane permeability **(chapter 5)** The mechanism by which enteroviruses shut off host cell translation is still debated According to the traditional view, the shutoff of cap-dependent translation of host mRNAs relies only on the protein $2A^{pro}$-mediated cleavage of the 220-kDa component (p220) of eucaryotic initiation factor eIF-4F (10) However, several reports have described that substantial levels of cellular protein synthesis can take place in cells where all p220 has been degraded (4, 8, 9) It has therefore been suggested that the p220 cleavage is necessary, but not sufficient, to completely inhibit host cell protein synthesis and that a second event is required to block cellular translation (5) The increase in plasma membrane permeability and the resulting influx of sodium ions have been suggested as potential second events because high concentrations of sodium ions are inhibitory to host mRNA translation but not to the translation of vRNAs (6) The observation that a poor increase in plasma membrane permeability coincided with a reduced ability to inhibit cellular translation lends support to this idea Additional studies are required to shed more light on the possible participation of 2B in the shutoff of host cell protein synthesis

The ability of protein 2B to release Ca^{2+} from the ER may be involved in the induction of the ER-derived, membranous vesicles that accumulate during infection and at which viral plus-strand RNA synthesis takes place Protein 2BC is thought to be responsible for the induction of these vesicles (1, 3) The function of 2B in precursor 2BC may involve its ability to release ER-stored Ca^{2+} The ER contains a gel of Ca^{2+} binding proteins that is responsible for the sequestration of Ca^{2+} ions These Ca^{2+} binding proteins have been proposed to form coordination complexes with Ca^{2+} ions that are bound to the negatively charged phospholipid head groups on the luminal face of the ER membrane, thereby stabilizing the underlying membrane and preventing its vesiculation (11) It is assumed that vesicles derived from the ER normally bud from regions of the membrane that are not stabilized by the Ca^{2+}-protein gel The artificial evacuation of Ca^{2+} ions from the lumen of the ER that occurs when cells are treated with Ca^{2+} ionophores has been proposed to destabilize the entire Ca^{2+}-protein gel and its associated membrane, leading to a mass vesiculation (11) By analogy with the effect of the ionophores, the release of ER-stored Ca^{2+} by the 2B polypeptide in protein 2BC may be involved in the massive induction of ER-derived membrane vesicles by 2BC Mutations that disrupt the membrane-perturbing activity of 2B may interfere with the ability of 2BC to induce vesicle proliferation and thereby account for defects in vRNA replication and virus growth

The inhibition of protein secretion by protein 2B is likely to result from alteration or sequestration of membranes or proteins required for secretory transport This inhibition may contribute to the assembly of the ER-derived membrane vesicles that surround the viral replication complexes Inhibition of the protein secretory apparatus may provide another direct advantage to viral amplification In tissue culture cells, enteroviruses inhibit protein secretion even earlier than they inhibit cellular protein synthesis (7) Crucial responses of mam-

malian host cells to viral infection, such as interferon secretion and antigen presentation in the context of MHC class I molecules, may be inhibited concomitantly with the inhibition of protein secretion. The importance of the inhibition of protein secretion for the viral replicative cycle awaits further investigation.

In conclusion, the results described in this thesis provide meaningful insight in the structure and function of coxsackievirus protein 2B. Protein 2B is a multifunctional protein that appears to be involved in (i) replication of the viral RNA, (ii) the inhibition of host cell translation, (iii) the inhibition of vesicular protein transport, and (iv) the release of virion progeny. These activities seem to be linked to the ability of 2B to increase the permeability of both the ER membrane and the plasma membrane by forming membrane-embedded pores, and thereby to account for (i) the release of Ca^{2+} from the ER, (ii) a disturbance of the ionic gradients maintained by the plasma membrane, and, ultimately, (iii) the membrane lesions that allow virus release. Based on its ability to alter membrane permeability, protein 2B is classified as a new viroporin. Protein 2B represents the first example of a viroporin of a naked virus. This new insight in the function of protein 2B in viral RNA replication and modification of cellular functions may open new avenues for research on the possible alterations in virus replication that may allow a persistent infection, and the involvement of these viruses in chronic diseases in humans.

REFERENCES

1 **Barco, A., and L. Carrasco.** 1995 A human virus protein, poliovirus protein 2BC, induces membrane proliferation and blocks the exocytic pathway in the yeast *Saccharomyces cerevisiae* EMBO J **14**:3349-3364

2 **Bernheimer, A.W., and B. Rudy.** 1986 Interactions between membranes and cytolytic peptides Biochem Biophys Acta **864**:123-141

3 **Bienz, K., D. Egger, and T. Pfister.** 1994 Characteristics of the poliovirus replication complex Arch Virol **suppl. 9**:147-157

4 **Bonneau, A., and N. Sonenberg.** 1987 Proteolysis of the p220 component of the Cap-binding protein complex is not sufficient for complete inhibiton of host cell protein synthesis after poliovirus infection J Virol **61**:986-991

5 **Carrasco, L.,** 1995 Modification of membrane permeability by animal viruses Adv Vir Res **45**:61-112

6 **Carrasco, L., and A.E. Smith.** 1976 Sodium ions and the shut-off of host cell protein synthesis by picornaviruses Nature (London) **264**:807-809

7 **Doedens, J.R., and K. Kirkegaard.** 1995 Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A EMBO J **14**:894-907

8 **Irurzun, A., S. Sanchez-Palomino, I. Novoa, and L. Carrasco.** 1995 Monensin and nigericin prevent the inhibition of host translation by poliovirus, without affecting p220 cleavage J Virol **69**:7453-7460

9 **Pérez, L., and L. Carrasco.** 1992 Lack of direct correlation between p220 cleavage and the shut-off of host translation after poliovirus infection Virology **189**:178-186

10 **Porter, A.G.** 1993 Picornavirus nonstructural proteins emerging roles in virus replication and inhibition of host cell functions J Virol **67**:6917-6921

11 **Sambrook, J.F.** 1990 The involvement of calcium in transport of secretory proteins from the endoplasmic reticulum Cell **61**:197-199

12 **Schlegel, A., T.J. Giddings, Jr., M.L. Ladinisky, and K. Kirkegaard.** 1996 Cellular origin and ultrastructure of membranes induced during poliovirus infection J Virol **70**:6576-6588

13 **Segrest, J.P., H. de Loof, J.G. Dohlman, C.G. Brouilletted, and G.M. Anantharamaiah.** 1990 Amphipathic helix motif Classes and properties Struct Funct Genet **8**:103-117

14 **Shai, Y.** 1995 Molecular recognition between membrane-spanning polypeptides Trends Biol Sci **20**:460-464

15 **Wimmer, E., C.U.T. Helen, and X. Cao.** 1993 Genetics of poliovirus Annu Rev Genet **27**:353-436

Samenvatting

Samenvatting

Het genus *Enterovirus*, dat behoort tot de familie van de *Picornaviridae*, wordt gevormd door poliovirussen, coxsackie A en B virussen, ECHO virussen, en een aantal enterovirus serotypen Enterovirussen zijn humaan pathogenen die een breed spectrum aan verschillende ziekten kunnen veroorzaken Het onderzoek dat in dit proefschrift beschreven is heeft met name betrekking op de coxsackie B virussen Coxsackie B virussen veroorzaken over het algemeen acute infecties die mild of zelfs asymptomatisch verlopen Deze virussen worden echter ook in verband gebracht met verschillende ernstige chronische aandoeningen bij de mens, zoals idiopathisch gedilateerde cardiomyopathie, polymyositis/ dermatomyositis en insuline-afhankelijke diabetes mellitus Een mogelijke verklaring voor een rol van coxsackie B virussen in deze chronische ziekten is dat zij een persisterende infectie veroorzaken Normaal resulteert replicatie van deze virussen in celdood Een persisterende infectie is mogelijk het gevolg van een verandering in virusreplicatie Het proces van virusreplicatie wordt nog altijd niet goed begrepen Het in dit proefschrift beschreven onderzoek is verricht om meer inzicht te krijgen in het moleculaire mechanisme van replicatie van coxsackievirussen

Enterovirussen zijn kleine cytolytische virussen die een enkelstrengs RNA molecuul als drager van hun genetische informatie bezitten Het virale RNA is van positieve polariteit, hetgeen betekent dat het in de cel van de gastheer wordt gebruikt als boodschapper RNA voor de productie van virale eiwitten Infectie van een cel start met binding aan een specifieke receptor op de buitenkant van de cel Na opname van het virus in de cel wordt het RNA vrijgemaakt uit zijn eiwitmantel en via een cap-onafhankelijk mechanisme getransleerd in een groot polyproteine Dit polyproteine wordt door drie viraal gecodeerde proteases (2A, 3C en 3CD) geknipt tot de verschillende functionele eiwitten Hiertoe behoren de vier structurele capside eiwitten en de tien niet-structurele eiwitten, zeven eindproducten (2A, 2B, 2C, 3A, 3B, 3C en 3D) en drie klievingsintermediairen (2BC, 3AB en 3CD) De replicatie van het virale plus-streng RNA start met de synthese van een complementaire min-streng RNA, die vervolgens dient als matrijs voor de synthese van een groot aantal plus-streng RNA moleculen De plus-streng RNA synthese vindt plaats in het replicatiecomplex, een gespecialiseerde structuur die bestaat uit het RNA en de virale replicatie eiwitten, en die wordt omgeven door een groot aantal membraanvesicles Deze vesicles zijn afkomstig van het endoplasmatisch reticulum (ER) en hun vorming wordt door het virus, waarschijnlijk door eiwit 2BC, geinduceerd De nieuw gevormde plus-streng RNA moleculen worden vervolgens ingepakt in de capside eiwitten om nieuwe virussen te vormen Voor een efficient verloop van de hierboven beschreven processen induceert het virus een aantal veranderingen in biochemische functies en morfologische structuren van de cel De belangrijkste veranderingen zijn remming van de gastheertranscriptie en gastheertranslatie, herstructurering van intracellulaire membranen, remming van de eiwitsecretie, en verhoging van de permeabiliteit van de plasmamembraan De genetische organisatie van het enterovirale RNA genoom en de mogelijke functies van de niet-structurele eiwitten in RNA replicatie en modificatie van gastheer functies en structuren zijn samengevat in **hoofdstuk 1**

In dit proefschrift is de structuur en functie onderzocht van het niet-structurele eiwit 2B, een klein eiwit dat voorkomt aan de buitenkant van de membraan vesicles waarop de plus-streng RNA synthese plaatsvindt

STRUCTUUR VAN EIWIT 2B

Om meer inzicht te verkrijgen in de structuur van eiwit 2B werd eerst een uitgebreide computeranalyse uitgevoerd De aminozuurvolgorde van 2B vertoonde geen enkele overeenkomst met cellulaire eiwitten Analyse van de hydropathie toonde aan dat het 2B eiwit twee hydrofobe domeinen bezit Beide domeinen bleken geconserveerd te zijn en ook in de 2B eiwitten van andere enterovirussen voor te komen Een van deze domeinen vertoonde een hoog hydrofoob moment en bleek potentieel in staat om een kationische amfipathische α-helix (een amfipathische helix met positief geladen aminozuren in zijn hydrofiele deel) te vormen De mate van hydrofobiciteit van het andere domein (dat verder zal worden aangeduid als "het hydrofobe domein") bleek typisch te zijn voor multimere transmembraanhelices

In **hoofdstuk 2** is een moleculair genetische analyse toegepast om na te gaan welke rol het hydrofobe domein speelt in de structuur en functie van het 2B eiwit Hiervoor werden verschillende mutaties aangebracht in een cDNA kloon van coxsackie B3 virus (CBV3) Van de cDNAs werd RNA afgeschreven en getransfecteerd in cellen om de effecten van de mutaties op de virusgroei te bestuderen Daarnaast werden de effecten van de mutaties op de replicatie van het virale RNA en de synthese en processing van het polyproteine onderzocht Uit de resultaten blijkt dat de hydrofobe eigenschappen van dit domein vereist zijn voor een functie van het 2B eiwit in de replicatie van het virale RNA en de virusgroei, maar niet voor de synthese en kliving van het polyproteine

Een genetische analyse van de voorspelde kationische amfipathische α-helix in CBV3 eiwit 2B is beschreven in **hoofdstuk 3** Zowel de amfipathie als het kationische karakter van de helix bleken vereist te zijn voor de replicatie van het virale RNA en de virusgroei Deze resultaten ondersteunen het idee dat een kationische amfipathische α-helix gevormd wordt en dat deze structuur vereist is voor een functie van het 2B eiwit in de

replicatie van het virale RNA.

De structurele vereisten van 2B voor zijn functie(s) in RNA replicatie werden verder onderzocht door het maken van chimere CBV3 cDNAs (**hoofdstuk 6**). In deze chimeren werd het complete 2B eiwit, of bepaalde structurele elementen daarvan, vervangen door het corresponderende segment van poliovirus eiwit 2B, een eiwit dat dezelfde structuur en functies bezit als CBV3 2B, maar dat aanzienlijk verschilt in zijn aminozuur volgorde. CBV3 chimeren die het poliovirus 2B eiwit bezaten bleken niet in staat tot RNA replicatie. Dit suggereert dat een seqentie-specifieke interactie tussen 2B en een ander viraal eiwit vereist is voor dit proces. Van de hybride eiwitten bleek alleen het eiwit dat bestond uit het amino-terminale een-derde van poliovirus 2B en de rest van CBV3 2B functioneel te zijn in RNA replicatie en virusgroei. Hieruit volgt dat het domein dat verantwoordelijk is voor de interactie met het andere virale eiwit waarschijnlijk gelegen is in het carboxy-terminale twee-derde van 2B. Hybride 2B eiwitten die of de amfipathische helix of het hydrofobe domein van poliovirus 2B bevatten bleken niet functioneel in RNA replicatie. Dit wijst erop dat deze twee domeinen mogelijk een directe interactie met elkaar aangaan.

FUNCTIES VAN EIWIT 2B

Om meer inzicht te krijgen in de functie van 2B werd het eiwit individueel tot expressie gebracht in COS cellen. In **hoofdstuk 4** worden twee functies van het 2B eiwit beschreven. De expressie van eiwit 2B bleek te leiden tot (i) een verhoogde permeabiliteit van de plasmamembraan voor hygromycine B, een translatieremmer die normaal niet in staat is om de membraan te passeren, en (ii) een remming van de eiwitsecretie, hetgeen bleek uit de verstoorde secretie van een reporter eiwit dat in dezelfde cellen tot expressie werd gebracht. De rol van het hydrofobe domein en de amfipathische helix in deze functies werd onderzocht door de expressie van 2B eiwitten die mutaties in deze domeinen bevatten. Uit de resultaten bleek dat beide domeinen een belangrijke rol spelen bij het verhogen van de membraanpermeabiliteit en het remmen van de eiwitsecretie. Sommige mutante 2B eiwitten bleken wel in staat om de permeabiliteit van de plasmamembraan te verhogen maar niet om de eiwitsecretie te remmen. Dit suggereert dat beide activiteiten mogelijk onafhankelijke functies van 2B zijn, in plaats van dat één van beide effecten het gevolg is van de ander.

In **hoofdstuk 5** is beschreven dat mutante virussen die minder 2B en 2C produceren, als gevolg van mutaties in de 2B/2C knipplaats, niet in staat zijn tot een volledige remming van de cap-afhankelijke eiwitsynthese van de gastheer. Daarnaast bleken deze virussen ook een defect te vertonen in het verhogen van de permeabiliteit van de plasmamembraan, waarschijnlijk als gevolg van de verminderde hoeveelheid 2B eiwit. Dat deze mutante virussen niet in staat waren om de gastheertranslatie te remmen ondersteunt de hypothese dat de klieving door het virale eiwit 2A van de eukaryotische initiatiefactor 4F, een onderdeel van het cap-bindende complex dat een rol speelt bij de cap-afhankelijke translatie van cellulaire boodschapper RNAs, niet voldoende is voor de complete remming van de gastheertranslatie en dat hiervoor een tweede proces vereist is. Dat deze mutante virussen ook niet of nauwelijks in staat bleken om de permeabiliteit van de plasma membraan te verhogen suggereert dat een verhoging van de membraanpermeabiliteit door 2B, en de veranderingen in intracellulair ionisch milieu die daarvan het gevolg zijn, vereist zijn voor een volledige remming van de gastheertranslatie.

De eigenschap van het 2B eiwit om de permeabiliteit van membranen te veranderen en zijn localisatie op de membraan vesicles die van het ER afkomstig zijn, suggereerden dat 2B mogelijk calcium vrijmaakt uit het ER, de belangrijkste intracellulaire opslagplaats van calcium. In **hoofdstuk 7** wordt aangetoond dat gedurende een CBV3 infectie de calcium concentratie in het cytosol stijgt a.g.v de influx van extracellular calcium, en dat de calcium opslagplaatsen in het ER worden leeggemaakt. De individuele expressie van het 2B eiwit bleek inderdaad voldoende te zijn om deze effecten te veroorzaken. Zowel de kationische amfipathische α-helix als het hydrofobe domein bleken hierbij een rol te spelen. Electronenmicroscopische studies toonden aan dat het 2B eiwit waarschijnlijk niet alleen voorkomt op membranen van het ER maar ook op de plasmamembraan. Tevens werd gevonden dat een virus met een mutant 2B eiwit een defect vertoonde in het vrijmaken van nieuwe virussen. Deze gegevens ondersteunen het idee dat 2B de permeabiliteit van zowel de ER membraan als de plasmamembraan verhoogt, en dat dit leidt tot de efflux van calcium uit het ER, een verstoring van de ionische gradienten die door de plasma membraan in stand worden gehouden, en, uiteindelijk, het vrijkomen van nieuw gevormde virussen.

STRUCTUUR-FUNCTIE RELATIE

In **hoofdstukken 4 en 7** is aangetoond dat een kationische amfipathische helix vereist is voor de eigenschap van 2B om membranen permeabel te maken. De aanwezigheid van kationische amfipathische helix motieven is karakteristiek voor lytische peptiden, kleine peptiden die de permeabiliteit en structuur van membranen verstoren. Analoog aan de werking van deze peptiden zijn er twee modellen mogelijk voor de verhoging van de membraanpermeabiliteit door 2B. Volgens het eerste model ligt de amfipathische helix evenwijdig aan de membraan en maakt hij de fosfolipiden in het membraan beter toegankelijk voor afbraak door fosfolipases. Het tweede model veronderstelt dat de amfipathische helices betrokken zijn bij de vorming van porieën. Zulke porieën kunnen gevormd worden door multimeren van 2B die de hydrofiele delen van de helices naar elkaar richten en de hydrofobe delen naar de lipiden van de membraan.

Twee argumenten suggereren dat het onwaarschijnlijk is dat de amfipathische helix alleen verantwoordelijk is voor het verhogen van de membraanpermeabiliteit (d.i. het eerste model). Allereerst bleek deze activiteit gevoelig voor mutaties in het hydrofobe domein (**hoofdstukken 4 en 7**). Daarnaast bleek dat de amfipathische helix niet kon worden uitgewisseld met een structureel nauw verwante amfipathische helix (**hoofdstuk 6**). Een mogelijke verklaring hiervoor is dat zowel de amfipathische helix en het hydrofobe domein betrokken zijn bij de vorming van porieën in de membraan. Een hypothetisch structuurmodel van multimeren van 2B die zo'n porie vormen door de hydrofiele delen van de amfipathische α-helix naar elkaar te richten is weergegeven in **hoofdstuk 8**.

Conclusie

De resultaten van dit proefschrift geven inzicht in de structuur en functie van het 2B eiwit. 2B is een multifunctioneel eiwit dat betrokken lijkt te zijn bij (i) de replicatie van het virale RNA, (ii) de remming van de gastheertranslatie, (iii) de remming van de eiwitsecretie, en (iv) het vrijmaken van nieuw gevormde virussen. Deze activiteiten lijken het gevolg te zijn van de eigenschap van 2B om de permeabiliteit van cellulaire membranen te verhogen, waarschijnlijk door het vormen van poricën in de membraan, hetgeen resulteert in (i) de efflux van calcium uit het ER, (ii) een verstoring van de ionische gradienten die door de plasma membraan in stand worden gehouden, en, uiteindelijk, (iii) het ontstaan van de membraan lesies waardoor nieuw gevormde virussen de cel kunnen verlaten. De rol van eiwit 2B in de replicatie van het virale RNA is mogelijk verbonden met zijn eigenschap om calcium vrij te maken uit het ER. Deze eigenschap zou een rol kunnen spelen in de inductie van de membraanvesicles waarop de plus-streng RNA synthese plaats vindt door eiwit 2BC, aangezien is aangetoond dat een efflux van calcium kan leiden tot de afsnoering van vesicles van het ER.

Op basis van zijn structurele en functionele eigenschappen kan het 2B eiwit worden ingedeeld in de familie van de viroporines, viruseiwitten die de plasma membraan permeabel maken om nieuw gevormde virussen uit de cel vrij te maken. Het 2B eiwit is het eerste geïdentificeerde viroporine van een cytolytisch virus zonder envelop.

De vindingen zoals die in dit proefschrift beschreven staan geven een beter inzicht in het proces van virusreplicatie en in de veranderingen in cellulaire functies en structuren die plaatsvinden tijdens dit proces. Hopelijk zal dit een bijdrage kunnen leveren tot een beter begrip van het mogelijke verband tussen veranderingen in virusreplicatie en het ontstaan van coxsackievirusgeassocieerde chronische aandoeningen bij de mens.

Dankwoord

Dit proefschrift is tot stand gekomen dankzij de medewerking van velen. Zeker op het laatst leek zo'n beetje mijn gehele omgeving direct of indirect betrokken bij mijn promotie. Al degenen die op enigerwijze een bijdrage hebben geleverd wil ik hiervoor van harte bedanken. Zonder iemand tekort te willen doen wil ik toch enkele personen met name noemen:

Mijn promotor Prof. Mieke Hoogkamp-Korstanje wil ik bedanken voor de gelegenheid die ze mij gegeven heeft om dit onderzoek uit te voeren. Veel dank ben ik verschuldigd aan mijn beide co-promotores en leiders van het virusonderzoek, dr. Willem Melchers en dr. Joep Galama. Willem en Joep, bedankt voor een bijzonder prettige en wetenschappelijk vruchtbare samenwerking. Tevens wil ik jullie danken voor het vertrouwen dat jullie in mij hadden. De grote mate van vrijheid die ik van jullie kreeg om zelf richting en invulling aan het onderzoek te mogen geven, gecombineerd met de altijd snelle en kritische commentaren op mijn schrijfsels, heb ik als bijzonder stimulerend ervaren. Dr. Jos van der Logt, hoofd van de ICLAS, ben ik zeer dankbaar voor de prettige samenwerking tijdens het mycoplasma-onderzoek en voor het financieren van mijn onderzoeksplaats. Jos, bedankt.

Kamergenoot Jan Zoll wil ik bedanken voor de gezellige tijd, die doorspekt was met Mr Beanachtige humor, de vele discussies en de kundige hulp bij allerlei technische kwesties op momenten dat ik met mijn handen in het haar zat. Theo, Thea en Carla van de weefselkweek, het spijt me dat ik jullie zovele jaren heb gekweld, zowel lichamelijk als geestelijk, met enorme bestellingen van cellen. Rick Brouwer, Roland Kuyper, Rémon van Aubel, en Walter van der Vliet, hebben in het kader van hun studie een belangrijke bijdrage geleverd aan het 2B-onderzoek. Mijn (ex-)collega's van de research groep (in chronologische volgorde) Esther Schoondermark, Wil Camps, Diana Willemse, Patrick van den Hurk, Johan Kissing, Nicole de Leeuw, Paul Odinot, Annet Heuvelink, en Gienke Vreugdenhil, wil ik bedanken voor de gezelligheid, hun belangstelling en bereidheid om te helpen indien dit nodig was.

Dr. Peter Willems wil ik danken voor zijn enorme hulp en betrokkenheid bij de calciummetingen en het tot stand komen van het bijbehorende artikel. Rolf Smeets, bedankt voor de hulp bij de electroporaties. Henri Dijkman, bedankt voor de prachtige elektronenmicroscopie plaatjes.

Dr. John Doedens and Dr. Karla Kirkegaard, I very much enjoyed our coorporation and our enthousiastic discussions over the phone and by fax. Prof. Kurt Bienz, thank you for your valuable comments on our electron microscopic photographs, and for providing me the photograph that is shown on the cover of this thesis.

Ook wil ik mijn beide paranimfen Patrick ("king of the lab") van den Hurk en Joost Hoenderop bedanken. Patrick, bedankt voor alle hulp bij het maken van de enorme series mutanten, de transfecties, de groeicurves en het doen van de "luckies". Voor dit laatste moest niet alleen ik, maar ook jij vaak vroeg uit bed en ik weet dat dat nou niet bepaald jouw sterkste punt is. Tevens bedankt voor het car-poolen. Ook dit was niet makkelijk voor jou als fanatiek PSV-supporter in een tijd waarin Ajax eenzaam aan de top stond.

Joost, bedankt voor je enorme hulp bij de calcium-metingen. We zijn samen ontelbare avonden, nachten en weekenden bezig geweest met die metingen, terwijl jij niet eens "mijn" student was. Zonder jouw hulp en enthousiasme had ik die proeven nooit kunnen volbrengen. Die metingen waren iederer keer weer spannend maar bovenal ook bijzonder gezellig. Dat je als HLO-er direct een AIO-plaats hebt weten te bemachtigen is een bevestiging van jouw kwaliteiten en juiste instelling en is in mijn ogen ook dik verdiend. Tevens bedankt voor je hulp bij het tot stand komen van de lay-out van dit proefschrift. Zonder jouw hulp had dit proefschrift er niet uitgezien zoals het er nu uitziet.

Verder wil ik ook mijn vrienden en familie bedanken voor hun voortdurende interesse gedurende het onderzoek en het schrijven van dit proefschrift. Pap en mam, hartelijk bedankt voor de gelegenheid die jullie mij hebben gegeven om te studeren.

Ik wil eindigen met het bedanken van de persoon die ik het meest tekort heb gedaan tijdens deze beproeving. Judith, bedankt voor alle steun en begrip dat je wist op te brengen als ik weer eens een avond, nacht of weekend moest werken en ook voor alle avonden, nachten en weekenden dat ik wel thuis was, maar waarin ik in gedachten meer bij het werk was dan bij jou. Desondanks wilde jij toch altijd weten waar ik nou precies mee bezig was en hoe dat ging. De bewondering die ik hiervoor heb kan ik niet in woorden uitdrukken.

Curriculum Vitae

Frank van Kuppeveld werd geboren op 25 augustus 1965 te Oss. In 1983 behaalde hij het Atheneum-B diploma aan het Maasland College te Oss. In datzelfde jaar begon hij zijn studie Biologie aan de Katholieke Universiteit Nijmegen. Het doctoraal examen werd behaald in november 1988, met als hoodvak Moleculaire Biologie (Prof. Dr. J.G.G. Schoenmakers) en als bijvakken Medische Microbiologie (Dr. A. van Loon) en Biofysische Chemie (bedrijfsstage op het NIZO, Ede; Dr. H. Rollema en Prof. Dr. R.N.H. Konings). Na het vervullen van de militaire dienstplicht bij de Militair Geneeskundige Dienst volgde een post-graduate bedrijfsstage bij Ciba Geigy AG te Basel (Biotechnologie Divisie; Dr. B. Chaudury). Van oktober 1990 tot oktober 1995 was hij als wetenschappelijk medewerker verbonden aan de afdeling Medische Microbiologie van het Sint Radboud Ziekenhuis te Nijmegen. De eerste 2 jaar heeft hij gewerkt aan de ontwikkeling van de moleculaire diagnostiek van mycoplasma infecties. Daarna heeft hij onderzoek verricht naar de functie en structuur van het enterovirus 2B eiwit, waarvan de resultaten in dit proefschrift zijn beschreven. Sinds juni 1996 is hij werkzaam als post-doctoraal onderzoeksmedewerker op de afdeling Moleculaire Dierfysiologie van de Katholieke Universiteit Nijmegen (Prof. Dr. G.J.M. Martens).

List of Publications

publications related to mycoplasma research :

F.J.M. van Kuppeveld, J.T.M. van der Logt, A.F. Angulo, M.J. van Zoest, W.G.V. Quint, H.G.M. Niesters, J.M.D. Galama, and W.J.G. Melchers: Genus and species specific identification of mycoplasmas by 16S rRNA amplification. *Applied and Environmental Microbiology 1992, 58: 2606-2615.*

J. Meis, **F. van Kuppeveld**, J. Kremer, J. Nijhuis, and W. Melchers: Fatal intrauterine infection associated with Mycoplasma hominis. *Clinical and Infectious Diseases 1992, 15:753-754.*

F.J.M. van Kuppeveld, W.J.G. Melchers, H.F.M. Willemse, J. Kissing, J.M.D. Galama, and J.T.M. van der Logt: Detection of *Mycoplasma pulmonis* in experimentally infected laboratory rats by 16S rRNA amplification. *Journal of Clinical Microbiology 1993, 31 524-527.*

J.T.M. van der Logt, **F.J.M. van Kuppeveld**, J. Kissing, and W.J.G. Melchers: Enzymatic amplification of *Mycoplasma pulmonis* 16S rRNA as a diagnostic tool for the identification of infected laboratory rats. In: *Welfare and science. Proceedings of the fifth symposium of the Federation of European Laboratory Animal Science Associations 1992, p. 392-394.* (Ed. J. Bunyan), Royal society of medicine press, London.

F.J.M. van Kuppeveld, K.-E. Johansson, J.M.D. Galama, J. Kissing, G. Bölske, J.T.M. van der Logt, and W.J.G. Melchers: Detection of Mycoplasma contamination in cell cultures by a mycoplasma group specific polymerase chain reaction. *Applied and Environmental Microbiology 1994, 60:149-152.*

J.H.T. Tjhie, **F.J.M. van Kuppeveld**, R. Roosendaal, W.J.G. Melchers, R. Gordijn, D.M. MacLaren, J.M.M. Walboomers, C.J.L. Meijer, and A.J.C. van den Brule: Direct PCR enables detection of *Mycoplasma pneumoniae* in patients with respiratory tract infections. *Journal of Clinical Microbiology 1994, 32.11-16.*

F.J.M. van Kuppeveld, K.-E. Johansson, J.M.D. Galama, J. Kissing, G. Bölske, E. Hjelm, J.T.M. van der Logt, and W.J.G. Melchers: 16S rRNA based polymerase chain reaction compared with culture and serology for diagnosis of *Mycoplasma pneumoniae* infection. *European Journal of Clinical Microbiology and Infectious Diseases 1994, 13.401-405.*

F.J.M. van Kuppeveld, W.J.G. Melchers, J. Kissing, J.T.M. van der Logt, and J.M.D. Galama: No evidence for mycoplasmas in peripheral blood mononuclear cell fraction of HIV-infected patients. *Infection 1994, 22.204-206.*

F. van Kuppeveld, J. van der Logt, K.-E. Johansson, and W. Melchers: Demonstration of mycoplasma contamination in cell cultures by a mycoplasma group-specific polymerase chain reaction. In: *Human cell culture protocols. Methods in molecular biology, vol 20, 1996, p 525-538* (Ed. G.E. Jones), Humana press inc., Totowa, NJ.

List of Publications

publications related to picornavirus research :

J Zoll, P Jongen J Galama, **F. van Kuppeveld**, and W Melchers Coxsackievirus B1 induced murine myositis is not associated with viral persistence *Journal of General Virology 1993, 74 2071-2076*

W Melchers, J Zoll, **F. van Kuppeveld**, C Swanink, and J Galama There is no evidence for persistent enterovirus infections in chronic medical conditions in humans *Reviews in Medical Virology 1994, 4 235-243*

J Zoll, J M D Galama, **F.J.M. van Kuppeveld**, and W J G Melchers Mengovirus leader is involved in the inhibition of host cell protein synthesis *Journal of Virology 1996, 70 4948-4952*

F.J.M. van Kuppeveld, J M D Galama, J Zoll, and W J G Melchers Genetic analysis of a hydrophobic domain in coxsackie B3 virus protein 2B, a moderate degree of hydrophobicity is required for a *cis*-acting function in viral RNA synthesis *Journal of Virology 1995, 69 7782-7790*

F.J.M. van Kuppeveld, J M D Galama, J Zoll, P J J C van den Hurk, and W J G Melchers Coxsackie B3 virus protein 2B contains a cationic amphipathic helix that is required for viral RNA replication *Journal of Virology 1996, 70 3876-3886*

F.J.M. van Kuppeveld, J M D Galama, J Zoll, P J J C van den Hurk, and W J G Melchers Mutagenesis of the coxsackie B3 virus 2B/2C cleavage site determinants of processing efficiency and effects on viral replication *Journal of Virology 1996, 70 7632-7640*

F.J.M. van Kuppeveld, W J G Melchers, K Kirkegaard, and J R Doedens Structure-function analysis of coxsackie B3 virus protein 2B *Virology 1997, 227 111-118*

F.J.M. van Kuppeveld, J M D Galama, W van der Vliet, P J J C van den Hurk, and W J G Melchers Chimeric coxsackie B3 virus genomes that express hybrid coxsackievirus-poliovirus 2B proteins functional dissection of structural domains involved in RNA replication *Submitted for publication*

F.J.M. van Kuppeveld, J G J Hoenderop, R L L Smeets, P H G M Willems, H B P M Dijkman, J M D Galama, and W J G Melchers Coxsackievirus protein 2B is a viroporin *Submitted for publication*