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, PO Box 9101, 6500 HB Nijmegen, The Netherlands

The 2B proteins of coxsackievirus and poliovirus (PV) share significant structural similarity 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-thirds of their sequence, of which one has the potential to form a cationic amphipathic α-helix. 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 gene or hybrid proteins in which specific segments of CBV3 2B were substituted by their corresponding PV counterparts. In vitro synthesis and processing of the chimeric polyproteins showed no abnormalities.

Introduction

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 (CBV), 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 (Wimmer et al., 1993). 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 (Melnick, 1996). 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 (Agol et al., 1996; Racaniello, 1990).

The enteroviruses can be divided into seven clusters, based on properties of genotype and function (Hyypia et al., 1997). CBV serotypes 1–6 and PV serotypes 1–3 fall into different clusters. The nonstructural proteins encoded by the P2 and P3 regions of the genomes of CBV and PV share 50 to 65 % amino acid identity. 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 virus reproduction. Studies on 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 3Cpro, a proteinase that is responsible for the majority of the processing events that give rise to the production of the nonstructural proteins, demonstrated correct processing of the PV P2 and P3 region proteins (Dewalt et al.,

CBV3 genomes carrying the entire PV 2B gene failed to replicate. 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 2B protein consisting of the amino-terminal one-third of CBV3 and the remainder of PV failed to drive replication. These data imply that a sequence-specific interaction with another viral protein is required to drive RNA replication and suggest that the proposed sites of contact reside in the carboxy-terminal two-thirds 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 are discussed.
amplification (Johnson & Sarnow, 1991; Li & Baltimore, 1988; a role of protein 2B, or possibly its precursor 2BC, in vRNA which vRNA replication takes place (Bienz
is localized at the outer surface of the membrane vesicles on (vRNA) replication, gave rise to viable viruses (Lu
a multifunctional protein that acts as a proteinase, a trans
Eco
clone containing the Mutagenesis was performed with a subgenomic pALTER phagemid introduced, either alone or in combination, by site-directed mutagenesis.
(3743) and 5′ TTCACATAGCTCCTTAAGGCCGTTCATTGATC 3′ (Stul site at nt 3743) and 5′ TTCTTTAAGCCGTTTTACCTGGCC- TTCAGCCAT 3′ (HpaI site at nt 4042). Nucleotide sequences of the oligonucleotides used for the introduction of unique restriction sites at 2B amino acids (aa) 57–59 are: 5′ TAGGTTGCAGTCACAGTATTCAAGGCTGGTCCACATCCCAATTACACTAGTGGTCGACACACGAGCAGTATGTGGCAGTCACAGTGAT- TAGTGTGGCAGTCACAGTATTCAAGGCTGGTCCACATCCCAATTACACTAGTGGTCGACACACGAGCAGTATGTGGCAGTCACAGTGAT-
and inhibited by protein 2B proteins carrying mutations in these regions (van Kuppeveld et al., 1997a, b).
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 (van Kuppeveld et al., 1996a) and one (which will further be referred to as the second hydrophobic domain) shows characteristics typical of multimeric transmembrane helices (van Kuppeveld et al., 1995). The relevance of these domains for the ability of CBV3 2B to increase membrane permeability and inhibit protein secretion has been confirmed by individual expression of 2B proteins carrying mutations in these regions (van Kuppeveld et al., 1997a, b).

The aim of this study was to gain more insight into the structural requirements of enterovirus protein 2B for its function in virus 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. Polypeptide 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.

Methods

Construction of uniqueendonuclease restriction sites in pCB3/T7. For the introduction of PV sequences into plasmid pCB3/T7 (Klump et al., 1990), 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) 918–6177) using the Altered Sites in vitro mutagenesis system according to the manufacturer’s instructions (Promega). The nucleotide sequence of the antisense synthetic oligonucleotides (Isogen Bioscience, The Netherlands) used for the introduction of unique restriction sites (underlined) immediately upstream or downstream of the 2B-encoding sequence are; 5′ TTCACATAGCTCCTTAAGGCCGTTCATTGATC 3′ (Stul site at nt 3743) and 5′ TTCTTTAAGCCGTTTTACCTGGCC- TTCAGCCAT 3′ (HpaI site at nt 4042). Nucleotide sequences of the oligonucleotides used for the introduction of unique restriction sites at 2B amino acids (aa) 57–59 are: 5′ TAGGTTGCAGTCACAGTATTCAAGGCTGGTCCACATCCCAATTACACTAGTGGTCGACACACGAGCAGTATGTGGCAGTCACAGTGAT-

Construction of chimeric cDNAs. Chimeric cDNAs were constructed by PCR amplification and cloning of 2B-encoding segments into pCB3/T7 plasmids from which the corresponding segment was deleted. Plasmid pPV3A (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 four forward primers (f1–4) and five reverse primers (r1–5) containing restriction sites at their 3′ end (underlined) were used for amplifying PV sequences. PCR was performed using SuperTaq DNA polymerase (HT Biotechnology) according to the manufacturer’s instructions. 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 cDNAs is briefly summarized:

PV1-99: a DNA fragment containing the coding sequence of PV aa 1–99 was generated by PCR using primers f1 and r3 (5′ ATCGCA 3′) and 5′ ACCAATTACACTAGTGGTCGACACACGAGCAGTATGTGGCAGTCACAGTGAT-

PV1-60: a DNA fragment containing the coding sequence of PV aa 60–99 was generated by PCR using primers f2 and r1 (5′ ACCAATTACACTAGTGGTCGACACACGAGCAGTATGTGGCAGTCACAGTGAT-

PV1-30: a DNA fragment containing the coding sequence of PV aa 31–60 was generated by PCR using primers f1 and r3 (5′ CCAACTAAATTGTCGCGATTTGATATCTCCAGAAC- ATCCGCA 3′) and r4 (5′ TCCACGAGACATCGAATCTTGGGACAGCATGACATGATC 3′) and cloned in pCB3/T7-Stul(3743)/Hpal(4042) from which the Stul–Hpal fragment was deleted.

PV1-19: a DNA fragment containing the coding sequence of PV aa 1–19 was generated by PCR using primers f1 and r5 (5′ CCAACTAAATTGTCGCGATTTGATATCTCCAGAAC- ATCCGCA 3′) and r6 (5′ TCCACGAGACATCGAATCTTGGGACAGCATGACATGATC 3′) and cloned in pCB3/T7-Stul(3743)/Hpal(4042) from which the Stul–Hpal fragment was deleted.

PV1-59: a DNA fragment containing the coding sequence of PV aa 1–59 was generated by PCR using primers f1 and r5 (5′ CCAACTAAATTGTCGCGATTTGATATCTCCAGAAC- ATCCGCA 3′) and cloned in pCB3/T7-Stul(3743)/Hpal(4042) from which the Stul–Hpal fragment was deleted.

PV1-99: a DNA fragment containing the coding sequence of PV aa 1–99 was generated by PCR using primers f1 and r3 (5′ CCAACTAAATTGTCGCGATTTGATATCTCCAGAAC- ATCCGCA 3′) and cloned in pCB3/T7-Stul(3743)/Hpal(4042) from which the Stul–Hpal fragment was deleted.

PV1-30: a DNA fragment containing the coding sequence of PV aa 31–60 was generated by PCR using primers f1 and r3 (5′ CCAACTAAATTGTCGCGATTTGATATCTCCAGAAC- ATCCGCA 3′) and cloned in pCB3/T7-Stul(3743)/Hpal(4042) from which the Stul–Hpal fragment was deleted.

PV1-19: a DNA fragment containing the coding sequence of PV aa 1–19 was generated by PCR using primers f1 and r5 (5′ CCAACTAAATTGTCGCGATTTGATATCTCCAGAAC- ATCCGCA 3′) and cloned in pCB3/T7-Stul(3743)/Hpal(4042) from which the Stul–Hpal fragment was deleted.
Accll and SpeI, and cloned in pCB3/T7-Accll(3913)/HpaI(4042)
from which the Accll–HpaI fragment was deleted.

2B/PV60-94; a DNA fragment containing the coding sequence of PV
aa 60–94 was generated by PCR using primers f2 and r2, digested with
Accll and SpeI, and cloned in pCB3/T7-Accll(3913)/HpaI(4042) from
which the Accll–HpaI fragment was deleted.

2B/PV34-59; a DNA fragment containing the coding sequence of PV
aa 34–59 was generated by PCR using primers f3 (5′ AAAAT-
GACTAGCGATCAGCTACGTGAA 3′; Nhel site) and r4, digested with
Nhel and Stul, and cloned in pCB3/T7-HpaI(3915) from which the
SpeI (3837)–HpaI fragment was deleted. Due to the occurrence of a
SpeI site in the amplicon, a Nhel site (compatible ends with SpeI) was
built in the 5′ end of primer f3. As result of this, valine 33 is altered to
alanine.

2B/PV34-99; a DNA fragment containing the coding sequence of PV
aa 34–99 was generated by PCR using primers f3 and r1, digested with
Nhel and SpeI, and cloned in pCB3/T7-HpaI(4042) from which the SpeI
(3837)–HpaI fragment was deleted. Due to the occurrence of a Nhel site
rather than a SpeI site in primer f3 (see above), valine 33 is changed to
alanine.

2B/PV34-94; a DNA fragment containing the coding sequence of PV
aa 34–94 was generated by PCR using primers f3 and r2, digested with
Nhel and SpeI, and cloned in pCB3/T7-HpaI(4042) from which the SpeI
(3837)–HpaI fragment was deleted. Due to the occurrence of a Nhel site
rather than a SpeI site in primer f3 (see above), valine 33 is changed to
alanine.

2B/PV37-54; a DNA fragment containing the coding sequence of PV
aa 37–54 was generated by PCR using primers f4 (5′ GAG-
TTGCTACTAGGGCCAGACACATCATGAAAGACTCT-
TAAG-3′; SpeI site) and r5 (5′ TTCTAAGCCCTCAACAT-
AACTAGGGAGATGATCTT 3′; Stul site), digested with SpeI and
Stul, and cloned in pCB3/T7-HpaI(3915) from which the SpeI
(3837)–HpaI fragment was deleted. Primer r5 was designed such that
the SpeI 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 Sall 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 (van Kuppeveld et al., 1995).
After transfection, cells were fed with minimal essential medium (MEM)
containing 10% foetal bovine serum (FBS) and incubated at either 33 or
36 °C. If virus growth was observed, cultures were incubated until
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 30–35
amino acids), the amphipathic α-helix, or the second hydro-
phobic domain were replaced, either alone or in combination,
by their corresponding PV counterparts. Because the borders
of the amphipathic α-helix are not known exactly, two different
constructs (2B/PV34-59 and 2B/PV37-54) were generated.

Constructions 2B/PV1-99, 2B/PV60-99 and 2B/PV34-99 con-
tained at the P4 position of the 2B/2C cleavage site a valine

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 30–35
amino acids), the amphipathic α-helix, or the second hydro-
phobic domain were replaced, either alone or in combination,
by their corresponding PV counterparts. Because the borders
of the amphipathic α-helix are not known exactly, two different
constructs (2B/PV34-59 and 2B/PV37-54) were generated.

Constructions 2B/PV1-99, 2B/PV60-99 and 2B/PV34-99 con-
tained at the P4 position of the 2B/2C cleavage site a valine

In vitro translation reactions. Copy RNA transcripts were
synthesized and translated in a single reaction using T7 TnsT rabbit
reticulocyte lysate (Promega) supplemented with 20% (v/v) HeLa cell
initiation factors (kindly provided by J. Flanagan, University of Florida).
The translation reactions (20 µl) contained 0.5 µg of circular plasmid
dNA and 20 µCi of TranS-label (a mixture of [35]methionine and
[38]S-lysine; ICN) and were incubated for 3 h at 30 °C. Translation
products were analysed on an SDS–12.5% polyacrylamide gel (Laemmli,
1970). Gels were fixed, fluorographed and exposed to Kodak XAR film at
between –80 °C.

Sequence verification of virus. RNA extraction, synthesis of
cDNA, amplification of the 2B coding region by PCR using primers 5′-
TTGGTGCACTTCCATGGACCATGGG 3′ (nt 3648–3677) and
5′-TTGGATGGCGCCGCTCTGC 3′ (nt 4231–4251), purification
of the PCR products, and sequence analysis with reverse primer 5′-
CCATTCAGTATTTCTG 3′ (nt 4117–4134) were performed as
described previously (van Kuppeveld et al., 1996a).

Virus titrations. Virus titres were determined by endpoint titration
as described previously (van Kuppeveld et al., 1995) and expressed in
50% tissue culture infective doses (TCID₅₀) according to the method of
Reed & Muench (1938).

Single-cycle growth analysis. Confluent BGM cell monolayers
grown in 25 cm² flasks (5 × 10⁶ cells) were infected with virus at an m.o.i.
of 1 TCID₅₀ 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 post-infection, cells were disrupted by three cycles of freezing and
thawing, and the virus titres were determined.

Analysis of viral protein synthesis in vivo. Confluent monolayers
of BGM cells were infected with virus at an m.o.i. of 25 for 25 min
at room temperature. After infection, cells were fed with MEM containing
3% FBS and grown at 36 °C. At various times post-infection, the cells
were washed with PBS and incubated in methionine- and serum-free
MEM (Gibco) containing 10 µCi of TranS-label for 30 min. Lysis of cells
and analysis of the [35]methionine-labelled proteins by SDS–PAGE
were performed as previously described (van Kuppeveld et al., 1995).
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. If 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.

Transfection of cells with RNA transcripts derived from the wild-type plasmid gave complete CPE within 2 days. RNA transcripts from construct 2B/PV1-30 produced CPE in all transfected cell cultures at 5 days post-transfection. 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 apparent at 6 h post-infection, when mutant virus production was only 1% of that of wild-type virus. 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
CBV3 genomes that express hybrid CBV3–PV 2B proteins

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 viral RNAs, copy RNAs of the mutant constructs were synthesized and translated in a reticulocyte lysate. Fig. 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 3Cpro 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 m.o.i. and pulse-labelled with [35S]methionine at various times post-infection. 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 post-infection. The amount of viral proteins produced at this time-point was similar to that observed in wild-type virus-infected cells at 5 h post-infection, when wild-type protein synthesis 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 (Alexander et al., 1994; Rohll et al., 1994; Xiang et al., 1995) and nonstructural protein 2Apro (Lu et al., 1995). 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

due to a defect in vRNA replication, a dot blot hybridization was performed. BGM cells were transfected with in vitro synthesized copy RNA transcripts and at various times post-transfection total cellular RNA was isolated, denatured, immobilized on nylon membranes and hybridized with a [α-32P]dATP-labelled CBV3 cDNA probe. Fig. 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.

Fig. 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 TCID50 per cell and incubated at 36°C. At the indicated times post-infection, the cells were disrupted by three cycles of freezing and thawing and virus production was determined by endpoint titration.

Fig. 3. Analysis of wild-type and mutant viral RNA replication. BGM cell monolayers were transfected with equal amounts of genomic transcripts derived from the indicated plasmids linearized by SalI. At the indicated times post-transfection, total RNA was isolated, denatured, bound to nylon membrane and hybridized to an α-32P-labelled cDNA probe.
Fig. 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 [35S]methionine-labelled translation products were analysed on an SDS–12.5% polyacrylamide gel. An extract from wild-type virus-infected cells, labelled with [35S]methionine at 4 h post-infection, was used as marker (lane ‘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 TCID₅₀ per cell and grown at 36 °C. At the indicated times post-infection, cells were radioactively labelled in 30 min pulses with [35S]methionine. Cellular extracts were prepared and analysed on an SDS–12.5% polyacrylamide gel.

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 (Doedens & Kirkegaard, 1995) and CBV3 (van Kuppeveld et al., 1997a, b) 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 E. coli cells (Lama & Carrasco, 1992; F. J. M. van Kuppeveld & W. J. G. Melchers, unpublished results). The
exchangeability of the $2A^{pro}$ and $3C^{pro}$ proteins of PV with those of CBVs (Dewalt et al., 1989; Lu et al., 1995) and the overall similarity in structure of the nonstructural enterovirus proteins are further indicative of a conservation of the functions of these proteins. Alternative explanations for the non-exchangeability 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 (Barco & Carrasco, 1995; Bienz et al., 1983). Impaired functioning of a heterologous precursor protein was also observed with PV 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 PV capsid proteins (Dewalt et al., 1989). 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 ability 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 permeability or inhibit protein secretion (van Kuppeveld et al., 1997a).

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 non-functional. 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 correct functioning of protein 2B/PV1-30. The explanation that the carboxy-terminal two-thirds 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 non-functioning of PV 2B in vRNA replication, as discussed above.

Hybrid genomes that contained the cationic amphipathic $\alpha$-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-bombil, a chimeric CBV3 genome that produced a 2B protein in which the cationic amphipathic helix was replaced with a similar motif of the lytic peptide bombolin II (van Kuppeveld et al., 1996a). The amphipathic helix motif in CBV3 2B is a major determinant for the ability to modify membrane permeability and inhibit protein secretion (van Kuppeveld et al., 1997a, b). 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 (Bernheimer & Rudy, 1986; Shai, 1995). According to the first model, the amphipathic helix lies collateral to the membrane and inserts 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 pores by spanning the membrane and forming oligomers, exposing their hydrophobic faces to the lipid bilayer and their hydrophilic faces forming the aqueous interior. The non-exchangeability of the amphipathic helix argues against an independent role (i.e. the first model) of this domain in the structure and function of 2B. The reason for the non-functioning of these hybrid 2B proteins is unknown. A possible explanation is that the amphipathic helix and the second hydrophobic domain of CBV3 2B are cooperatively involved in the formation of membrane-integral pores. 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/PV60-99, 2B/PV60-94). Alternatively, the non-functioning of these hybrid proteins may be due to general disruption of 2B structures.

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-thirds 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 (Johnson & Sarnow, 1991), but did not interfere with the ability of protein 2B to inhibit protein secretion (van Kuppeveld et al., 1997a). Recently, it has been shown that deletion of the first 30 amino acids of protein 2B abolished the ability of PV protein 2BC to induce membrane proliferation and to interfere with the exocytic pathway in yeast cells (Barco & Carrasco, 1995). The importance of this region for a function of protein 2B, or 2BC, in mammalian cells awaits further investigation.

This research was partly supported by a grant from the European Community (INTAS/RFBR no. 01365.i96)

References


