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Testing the modularity of the N-terminal amphipathic helix conserved in picornavirus 2C proteins and hepatitis C NS5A protein

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Abstract

The N-terminal region of the picornaviral 2C protein is predicted to fold into an amphipathic α -helix that is responsible for the protein's association with membranes in the viral RNA replication complex. We have identified a similar sequence in the N-terminal region of NS5A of hepaciviruses that was recently shown to form an amphipathic α -helix. The conservation of the N-terminal region in two apparently unrelated proteins of two different RNA virus families suggested that this helix might represent an independent module. To test this hypothesis, we constructed chimeric poliovirus (PV) genomes in which the sequence encoding the N-terminal 2C amphipathic helix was replaced by orthologous sequences from other picornaviral genomes or a similar sequence from NS5A of HCV. Effects of the mutations were assessed by measuring the accumulation of viable virus and viral RNA in HeLa cells after transfection, examining membrane morphology in cells expressing chimeric proteins and by *in vitro* analysis of RNA translation, protein processing and negative strand RNA synthesis in HeLa cell extracts. The chimeras manifested a wide range of growth and RNA synthesis phenotypes. The results are compatible with our hypothesis, although they demonstrate that helix exchangeability may be restricted due to requirements for interactions with other viral components involved in virus replication.

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Introduction

Replication of positive-stranded RNA viral genomes depends upon the rearrangement of host intracellular membranes to provide a scaffold for the various components of the RNA replication complex and to compartmentalize those viral and cellular components needed for viral RNA synthesis (Miller et al., 2003 and references therein). Membrane reorganization is induced by interactions of one or more viral proteins with targeted membranes of a specific subcellular organelle, although little is known about the molecular details of these interactions or the process by which the virus-induced replication structures form. Indeed, different viruses utilize

different organelle membranes (e.g., endoplasmic reticulum (ER), Golgi, endosomes, mitochondria) and generate different morphological structures on which the replication complexes assemble.

For example, several coronavirus-encoded integral membrane proteins associate with the replication complex, which morphologically consists of loose aggregates of double membrane vesicles (Gosert et al., 2002; Pedersen et al., 1999; Shi et al., 1999; van der Meer et al., 1999). In the alphavirus genus of the Togaviridae family, multifunctional gene products play a key role in organizing the RNA replication complex assembly on membranes (Chen and Ahlquist, 2000; Prod'homme et al., 2003). For Kunjin virus, a member of the Flaviviridae family, two membrane structures, vesicular packets and convoluted membranes, are reported to harbor the replication-relevant non-structural proteins (Mackenzie and Westaway, 2001; Westaway et al., 1997, 1999). In

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some flaviviruses, the vesicular packets were found to represent the replication complex (Uchil and Satchidanandam, 2003; Westaway et al., 1997, 1999); however, it is not yet determined which viral protein(s) induce formation of these structures. In contrast, for hepatitis C virus (HCV), comprising a separate genus in the Flaviviridae family, the NS4B protein (Egger et al., 2002; Konan et al., 2003) is able to trigger the induction of a structure termed “membranous web” which is the site of viral RNA replication (Gosert et al., 2003) and harbors all structural and non-structural proteins (Egger et al., 2002; El-Hage and Luo, 2003; Mottola et al., 2002). Determinants for the membrane association of HCV proteins (reviewed in Moradpour et al., 2003) include transmembrane hydrophobic segments (NS4B and NS5B) and an amphipathic helix (NS5A).

Among the Picornaviridae family members, RNA replication complexes from PV-infected HeLa cells have been the best studied. Viral protein sequences in 2B, 2C and 3A and the larger proteins that contain these sequences all have inherent membrane binding properties (Datta and Dasgupta, 1994; Doedens et al., 1997; Echeverri and Dasgupta, 1995; Teterina et al., 1997b; Towner et al., 1996; van Kuppeveld et al., 1997a). 2B and 2C sequence-containing proteins appear to target to the ER when expressed individually in HeLa cells, although a specific ER-targeting sequence is not known. Vesicles with an appearance indistinguishable from those associated with viral RNA replication complexes are generated in cells expressing all of the viral non-structural proteins (Teterina et al., 2001); and 2BC + 3A or 2BC alone can induce the formation of morphologically similar vesicles (Aldabe and Carrasco, 1995; Cho et al., 1994; Suhy et al., 2000). However, these pre-formed vesicles are not utilized by subsequently infecting virus to support RNA synthesis, suggesting that translation, vesicle formation and RNA synthesis may all be linked in infected cells (Egger et al., 2000).

Protein 2C has been implicated in multiple processes during virus replication, from induction of host cell membrane rearrangements and virus uncoating to RNA replication and encapsidation. Based on results of bioinformatics analysis, it was suggested that 2C is composed of three structural domains that may accommodate multiple activities (Teterina et al., 1997b). The central domain is highly conserved among picornaviruses and other small RNA and DNA viruses. It contains nucleoside triphosphate-binding and helicase motifs (Gorbalenya et al., 1990), and ATPase activity was demonstrated for purified recombinant 2C protein (Pfister and Wimmer, 1999). RNA binding activity has been mapped to both the amino- and carboxy-terminal regions of the protein (Rodriguez and Carrasco, 1995). Although the separately expressed C-terminal portion of 2C was able to interact with membranes (Teterina et al., 1997a), the major membrane-targeting determinant of 2C has been attributed to the amino-terminal region (Echeverri and Dasgupta, 1995; Echeverri et al., 1998; Teterina et al., 1997b). This region was predicted to form an amphipathic helix (Paul et al., 1994) which was proposed to be responsible for its membrane binding property.

The capacity to form an amphipathic helix is conserved among all picornaviruses examined. Some mutations predicted to disrupt the amphipathic helix fold were shown to impair viral RNA synthesis (Paul et al., 1994).

Construction of chimeric viruses has been successfully exploited previously to define independent functional domains and to dissect genetic compatibilities. Exchange of both non-coding and coding regions among picornaviral genomes has been performed, generating both viable and non-viable chimeras. For example, internal ribosome entry sites (IRESes) can be readily transferred, en bloc, from one picornavirus to another and even among viruses that belong to different virus families (Alexander et al., 1994; Gromeier et al., 1996; Jia et al., 1996; Johnson and Semler, 1988; Lu and Wimmer, 1996; Zhao et al., 1999, 2000). Exchanges of the 5' end cloverleaf structure or the 3' non-coding regions also have produced viable viruses (Rohll et al., 1995; Xiang et al., 1995). A hydrophobic domain within polypeptide 3AB of PV was interchangeable with the orthologous sequences from human rhinovirus 14 (HRV14) (Towner et al., 2003), although some domain substitutions caused RNA replication defects. Replacement of PV 3CD sequences with those from another enterovirus, coxsackie virus B3 (CVB3), produced a replication competent, albeit temperature-sensitive virus; however, replacement of only 3D coding sequences (Bell et al., 1999) or subdomains of 3D (Cornell et al., 2004) was lethal. Studies of chimeric CVB3 viruses containing PV 2B or segments of 2B suggested that the hydrophobic domains present in the C-terminal two-thirds of the protein contain residues involved in virus-specific protein contacts required for viral RNA replication (van Kuppeveld et al., 1997b).

In this report, we describe the properties of viruses containing substitutions of the proposed amphipathic helix in PV protein 2C with the corresponding regions from several other picornaviruses, and with the amphipathic helical region in hepatitis C virus protein NS5A that we found to be similar to the 2C helix. We analyzed these 2C chimeras for viability and other properties that are essential for the formation and functioning of replication complexes in vivo and in vitro.

Results

Conservation of the amphipathic helix in picornavirus 2C and HCV NS5A proteins

The 2C sequence represents the most highly conserved protein among all picornaviruses (Argos et al., 1984). Although its three-dimensional structure has not been resolved, it was predicted to be a three-domain protein (Kusov et al., 1998; Teterina et al., 1997b) with amphipathic α -helices at both the N- and C-termini that mediate a peripheral association with membranes (Kusov et al., 1998; Paul et al., 1994; Teterina et al., 1997b). We performed a BLAST engine search for other proteins with similarities to the N-terminal portion of PV 2C, and identified the hepatitis C virus (HCV) NS5A protein, with scores comparable to

those of the cardio- or aphthovirus 2C homologs (Fig. 1A, data not shown.). Recently, this HCV sequence was proven to be an amphipathic helix that mediates membrane association of NS5A (Brass et al., 2002; Penin et al., 2004). The similarities between NS5A and 2C extend slightly downstream of the α -helix to include three amino acids that may be part of a junction region (not shown). The N-terminal border of the conservation between NS5A and 2C is less well defined but it coincides with the predicted N-terminus of the 2C helix and is five residues downstream from the established N-terminus of the NS5A α -helix (Fig. 1A). Although comparably positioned in all proteins, the most conserved 18 amino acid region of the α -helix is separated from the protein's N-terminus by 16 to 18 residues in picornaviruses and eight residues in HCV (Fig. 1A).

Fig. 1B shows the amphipathic helix represented as a "helical wheel". As was initially observed by Paul et al. (1994), the pattern of distribution of hydrophobic and charged amino acids in the helix is well conserved. Among the residues of the predicted hydrophobic face are two absolutely conserved

Trp residues that are separated by 11 amino acids in the primary structure. The sequence from HCV protein NS5A has the hydrophobic pattern characteristic of the 2C α -helix, including the two invariant Trp residues. The similarities between the NS5A and 2C wheels lend strong support to the prediction that the N-terminus of picornaviral 2C proteins does form an amphipathic helix.

Conservation of the capacity to form a characteristic amphipathic helix near the N-terminus of all picornaviral 2C proteins, as well as in the otherwise unrelated HCV NS5A protein, suggested that this helix might form an independent, transferable module compatible with different partners. To test this hypothesis, we chose the approach of replacing the predicted amphipathic helix in PV 2C with its counterparts from other viral proteins. In each construct, we substituted the 18 amino acid sequence aligned with the predicted 2C amphipathic helix that we consider the helix "core", as defined in Fig. 1A. We analyzed the effects of this PV mutagenesis on virus viability, RNA synthesis, protein production and membrane biogenesis.

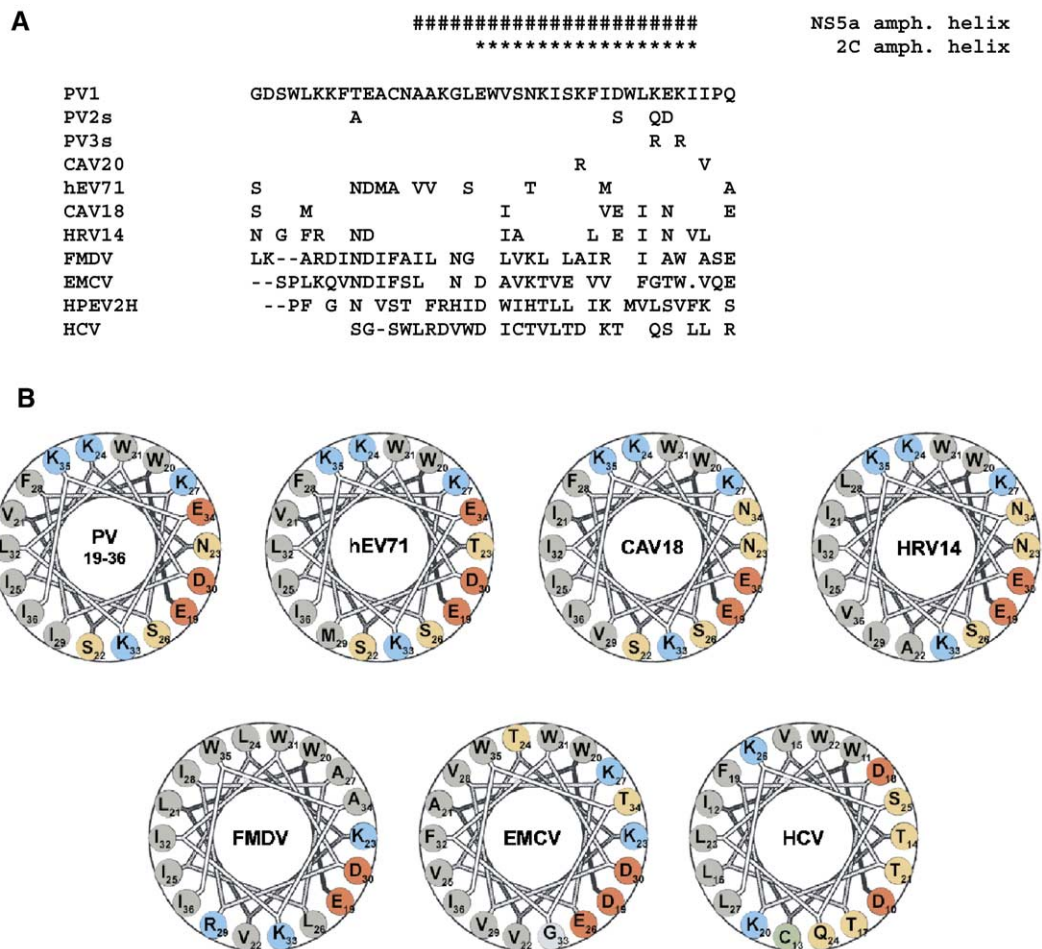


Fig. 1. Predicted N-terminal amphipathic helix in picornavirus 2C proteins and HCV protein NS5A. (A) Alignment of the N-terminal sequences of picornavirus proteins 2C and HCV protein NS5A. Amino acids forming the amphipathic helix in HCV NS5A are indicated by # and amino acids predicted to form amphipathic helices in 2C proteins are marked by * above the aligned sequences. GenBank accession numbers of the aligned sequences are given in Materials and methods. (B) Schematic helical wheel diagrams of the putative amphipathic helices. Residues are color-coded according to their physico-chemical properties: hydrophobic residues are gray; polar residues are yellow; positively charged basic amino acids are blue; negatively charged acidic residues are red and Cys is green. For the HCV NS5A helix, only the portion utilized for substitution in the PV chimera is depicted.

Viability of PV chimeras carrying substituted amphipathic helices in 2C

To determine whether substituting the 2C N-terminal amphipathic helix in PV with the helices containing different amino acid sequences from other viral proteins could generate infectious viruses, we constructed full-length PV genomes encoding chimeric 2C proteins. Cloning was facilitated by introducing two silent point mutations that created two *Xho*I restriction sites at positions 4096 and 4242 of the wild-type PV1 cDNA to produce pPVxx. These silent mutations had no effect on PV growth and replication in HeLa cells (see below). Fig. 2A shows a schematic map of the constructs

used in these experiments and the aligned sequences of the homologous predicted amphipathic helices from the various viruses used to generate chimeric 2C sequences in the Mahoney strain of poliovirus type 1 cDNA. The sequence from human enterovirus 71 (hEV71) is only slightly diverged from that of PV1 (2 amino acids, N23T and I29M), whereas coxsackie A virus 18 (CAV 18) has 5 differences among the 18 that comprise the core of the predicted helix. Both of those viruses are classified as enteroviruses, in the same genus as poliovirus. HRV14, from a different but closely related genus, shows seven changes from PV1, four of which are the same as those which appear in the CAV 18 sequence. Foot-and-mouth disease virus (FMDV) and encephalomyocarditis virus

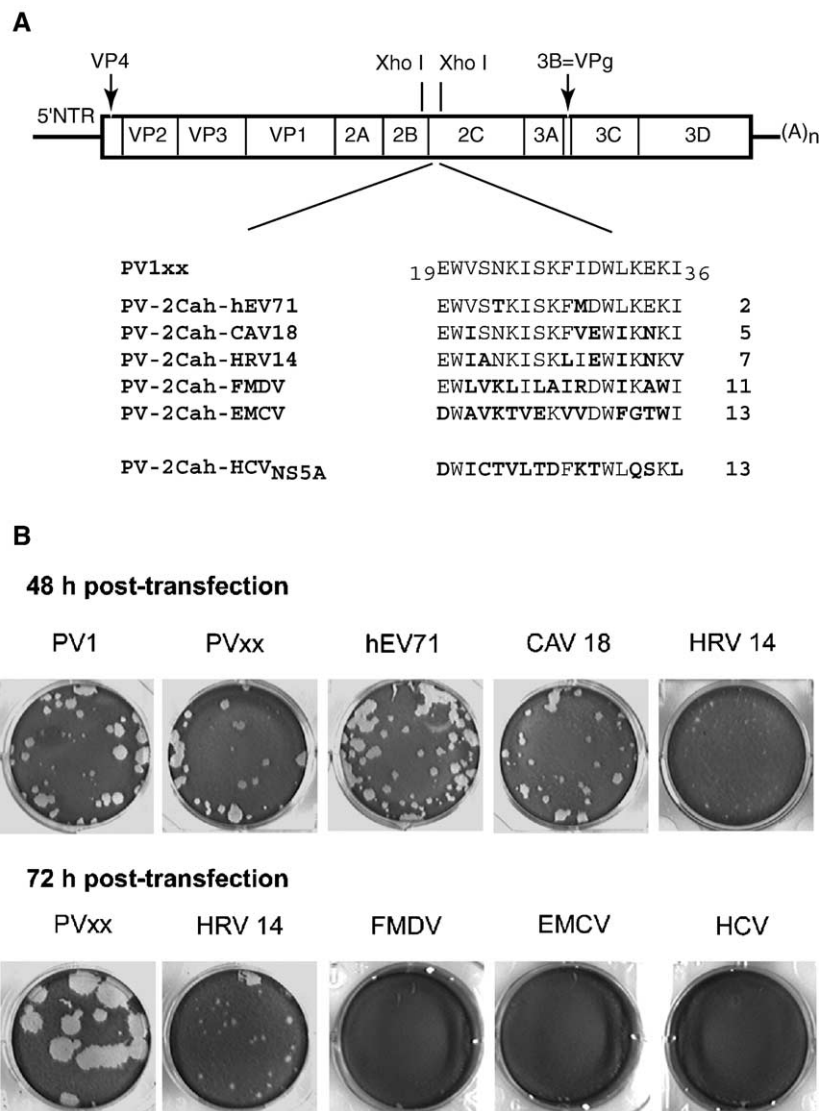


Fig. 2. Infectivity of chimeric viruses. (A) Schematic diagram of chimeric full-length PV genomes used in this study. The positions of two *Xho*I sites engineered to facilitate sequence replacements are indicated. The recombinant chimeric genomes are listed in the left column. The names indicate the origin of the amphipathic helix replacing the one in protein 2C of PV (hEV71—human enterovirus 71; CAV 18—coxsackie virus 18; HRV14—human rhinovirus 14; FMDV—foot-and-mouth disease virus; EMCV—encephalomyocarditis virus; HCV_{NS5A}—hepatitis C virus protein NS5A). The corresponding amino acid sequences of the substituting amphipathic helices are shown in the right column. Amino acids different from those present in PV are shown in bold type, and the total number of amino acid substitutions relative to PV1 is indicated on the right. PV1xx designates PV with two silent mutations introduced to create *Xho*I sites. (B) Plaque morphology of chimeric viruses. HeLa cell monolayers were transfected with serial dilutions of wild-type or mutant RNA transcripts. Plaques were visualized at 48 or 72 h as indicated, by staining with crystal violet. Specific infectivities were similar for all viable viruses, in the range of 10^5 pfu/ μ g RNA. The transfected recombinant chimeric RNAs are designated by the 2C amphipathic helix they contain.

(EMCV), each representing different genera, display 11 and 13 amino acid substitutions out of the 18, respectively, three of which are the same in these two sequences, but different from all of the others at that position. Among the picornavirus sequences, amino acid identity with the PV 2C amphipathic helix varies from 89% for hEV71 to less than 30% for EMCV. Finally, the N-terminal amphipathic helix in hepatitis C virus NS5A protein differs from the PV1 sequence at 13 of 18 residues mostly at the positions that tolerate replacements in other picornaviruses.

RNAs corresponding to wild-type PV1, PVxx and each chimeric genome were transcribed, and HeLa cell monolayers were transfected separately with serial dilutions of each RNA at 37 °C. For each chimeric genome, RNA was prepared and transfected from two independent cDNA clones, and the plates were examined for plaque number and size after 48 h and 72 h (Fig. 2B). RNAs encoding the amphipathic helix from the 2C protein of hEV71 or CAV18 manifested specific infectivities similar to that of wild-type RNA, and the plaques generated by these chimeras [PV-2Cah-hEV71 and PV-2Cah-CAV18] were similar in size to those produced by wild-type PV. For each chimera two individual virus plaques were isolated and used to generate passage 1 virus stocks. Viral RNAs isolated from HeLa cells infected with these viruses were sequenced (nts 3850–5200) to confirm that all mutations introduced to code for the 2C amphipathic helix from hEV71 and CAV 18 were present. Thus, this region from hEV71 or CAV18 can functionally substitute for the corresponding element of PV protein 2C.

Transfection of RNA PV-2Cah-HRV14 also yielded viable virus, indicating that the HRV14 amphipathic helix in 2C can functionally substitute for its counterpart in PV 2C. In this case, however, the plaques generated by this chimeric RNA were of minute size at 48 h after transfection and longer incubation of plates was required to visualize and count plaques. Similar results were observed when this PV chimeric RNA containing the HRV14 2C amphipathic helix was transfected at 33 °C (data not shown), indicating that the impaired growth of PV-2Cah-HRV14 was not due to temperature sensitivity of the chimeric virus. RNAs encoding the amphipathic helix from 2C of FMDV or EMCV, or protein NS5A of HCV did not produce any viral plaques after transfection even at high RNA concentrations (Fig. 2B). Similarly, no CPE was observed when HeLa cell monolayers transfected with these RNAs were incubated in liquid medium or during subsequent passage of these transfected cells. These results indicate that PV chimeras containing the amphipathic α -helices from FMDV or EMCV 2C protein or from HCV NS5A protein were defective in replication.

RNA synthesis directed by PV chimeras in vivo and in vitro

To examine the effect of amphipathic helix substitutions in protein 2C on RNA synthesis during the first round of virus replication, we analyzed the accumulation of virus-specific RNAs in HeLa cells during the first 12 h post-

transfection with RNA transcripts. Total RNA from transfected cells was isolated at different times post-transfection and the accumulation of positive-strand PV RNA was measured by a one-cycle RNase protection assay (Figs. 3A–B). Accumulation of increasing amounts of positive strand RNA for PVxx was readily detectable by 7.5 h post-transfection and continued with kinetics indistinguishable from that observed with wild-type PV1 RNA transcript (not shown). In good correlation with the observed plaque phenotype, replication of PV-2Cah-hEV71 and PV-2Cah-CAV18 RNA was very similar to that of PVxx RNA. A slight delay in replication of the chimera encoding the CAV18 amphipathic helix in 2C was reproduced in two independent experiments. During the first 12.5 h after transfection, chimeric genome PV-2Cah-HRV14 accumulated with significantly slower kinetics than did that of PVxx, confirming that the small plaque phenotype of virus produced by this genome results from inefficient RNA synthesis. However, when accumulation of this chimeric virus-specific RNA was followed longer (Figs. 3C–D), we observed a significant increase in positive strand RNA between 12 and 23 h post-transfection. The slight decrease in the amount of control PVxx RNA at 23 h compared with what was detected at 12.5 h was likely due to a loss of cells following cytopathic effects observed for PVxx around 20 h post-transfection. No RNA replication was detected in cells transfected with PV-2Cah-FMDV (Figs. 3A–B) or with PV-2Cah-EMCV or PV-2Cah-HCV (data not shown).

The accumulation of negative-strand RNA in cells after transfection could not readily be compared for the various chimeras, since the quantities of negative strands synthesized from transfected input templates are below the limits of detection (Teterina et al., 2001). In addition, measurement of negative-strand RNAs synthesized following the amplification of plus strand RNA would show a reduced yield of negative strands even if the only defect was in plus strand synthesis, thus complicating interpretation of the results. Because of these considerations, we examined negative strand synthesis *in vitro*, using T7 RNA transcripts to program the translation/replication reaction in extracts prepared from HeLa cells (Barton and Flanagan, 1993; Molla et al., 1991). These RNA transcripts contained two non-PV guanylate residues at the 5' terminus, which have been shown previously to inhibit synthesis of positive, but not negative strand RNA (Barton et al., 1999; Herold and Andino, 2000; Teterina et al., 2003). Fig. 4 shows similar albeit slightly decreased levels of RNA synthesized in reactions programmed with chimeric genomes encoding 2Cah-hEV71, 2Cah-CAV18 and 2Cah-HRV14. However, no negative strand RNA was produced in reactions programmed with RNAs encoding 2Cah-FMDV, 2Cah-EMCV or 2Cah-HCV. Thus, it appears that all substitutions of the amphipathic helix in protein 2C that support negative strand RNA synthesis *in vitro*, also support positive strand RNA synthesis *in vivo*. It remains unknown whether substitution of the amphipathic helix in protein 2C causes a differential effect on positive vs. negative strand synthesis.

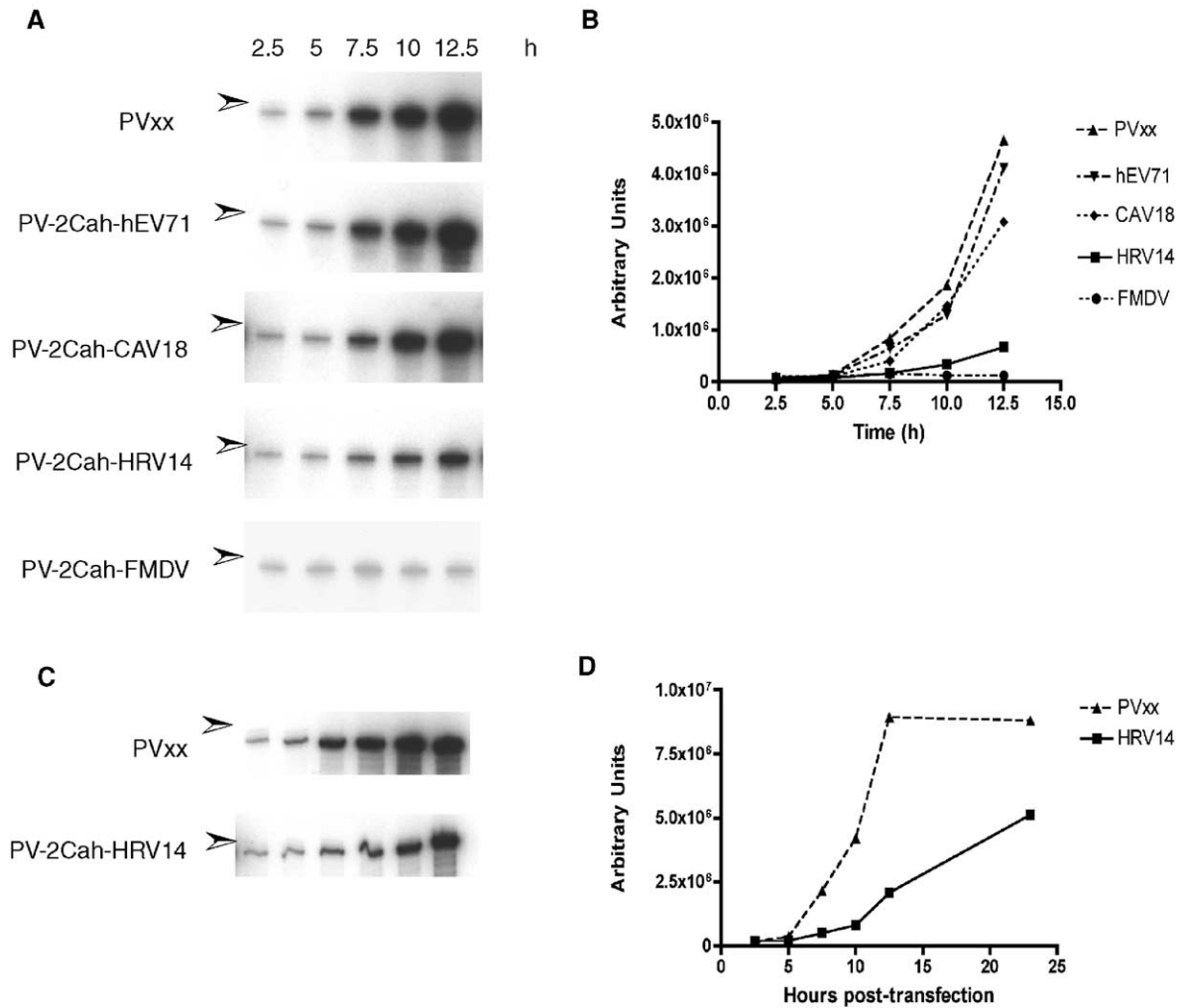


Fig. 3. Accumulation of chimeric PV RNAs in transfected cells. (A and C) HeLa cell monolayers were transfected with 1 μ g of RNA transcripts. Cells were harvested at the indicated time after transfection. Total cytoplasmic RNA isolated from approximately 5×10^4 cells were used to hybridize with a 32 P-labeled riboprobe complementary to nt 220 to 460 of PV RNA and subjected to digestion with RNases A and T1. Protected fragments were analyzed by electrophoresis on a 6% polyacrylamide-urea gel. (B and D) Quantitative presentation of data obtained by analysis with a Typhoon 8600 Imager (Amersham Biosciences) and ImageQuant software.

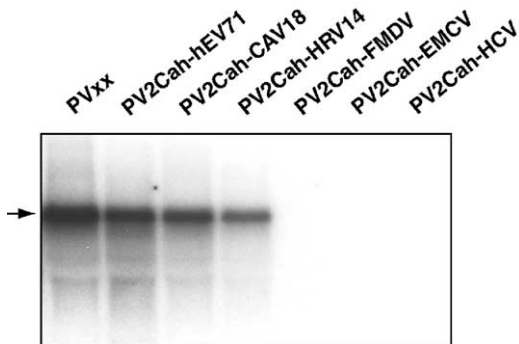


Fig. 4. Negative-strand RNA synthesis from chimeric RNA templates using the coupled *in vitro* translation/replication assay. RNAs synthesized in HeLa cell extracts from the indicated templates were labeled by incorporating [α - 32 P]CTP, denatured by glyoxylation and resolved in a 1% agarose gel. The position of marker virion RNA is indicated by the arrow. The specificity of the labeled band was demonstrated in a separate experiment when PV1 RNA was replicated in the presence of 2 mM guanidine HCl, which completely inhibited production of labeled RNA (data not shown).

Polyprotein synthesis and proteolytic processing directed by PV chimeras in vitro

Viral RNA synthesis is dependent upon viral polyprotein synthesis and proteolytic processing, since all of the viral non-structural proteins and their precursors are involved in different aspects of replication of the viral genome. It was shown previously (Paul et al., 1994) that the lethal phenotype of some mutations introduced in the region encoding the amphipathic helix of protein 2C of PV was likely due to abnormalities in processing of the viral polyprotein. To determine if the substitutions of the amphipathic helix of 2C used in this study interfered with protein synthesis or processing, we examined the products of translation of the wild-type and chimeric RNA transcripts in cell-free extracts prepared from HeLa cells. Fig. 5 shows that all mutant RNAs were translated with equal efficiency and all produced the cleavage products expected from an intact open reading frame. No differences between

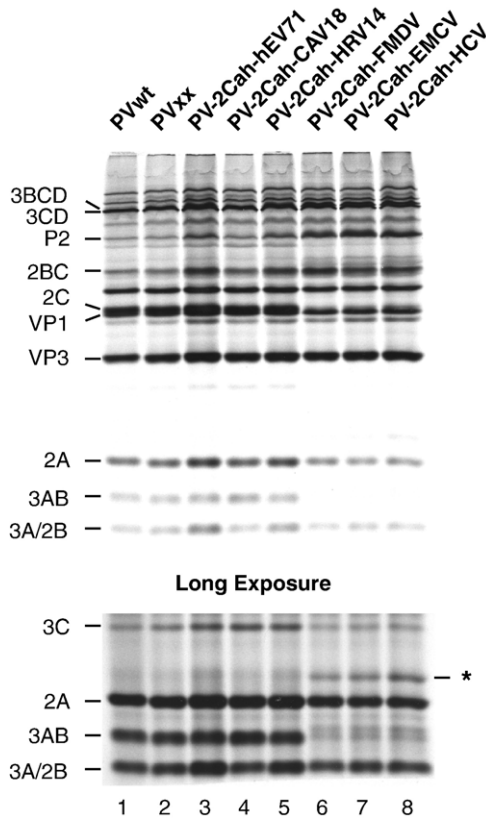


Fig. 5. Translation and protein processing profiles of chimeric RNAs. HeLa cell extracts were programmed with the indicated full-length RNA transcripts. Synthesis and processing of viral proteins were measured in reactions carried out in the presence of [35 S]methionine and 2 mM guanidine-HCl, and labeled proteins were subjected to electrophoresis in a 12.5% polyacrylamide-SDS gel. A long exposure of the bottom part of the gel is shown in the bottom panel to show products of abnormal processing in the 3AB region in lanes 6–8. *—unidentified product of abnormal processing.

wild-type and chimeras were observed in the cleavage of the P1 precursor of capsid proteins, catalyzed by 3CD^{pro}, which can be monitored by the appearance of the VP3 band. Some abnormalities, however, were detected in the processing of products from the P2/P3 region. A subtle effect of the 2C substitution on P3 processing, indicated by increased production of 3BCD protein, was detected in the viable chimeras: for wild-type PV, 3BCD is a minor band; a slightly increased accumulation of this protein was seen in the products of translation of PV-2Cah-hEV71 and PV-2Cah-HRV14 chimeras, whereas in the PV-2Cah-CAV18 chimera, this band was absent. However, the ratios of precursor/product that are derived from the P2–P3 region were affected more severely in the non-viable chimeric constructs PV-2Cah-FMDV, EMCV and HCV. These mutants produced increased amounts of P2 and 2BC, but little 2C. From the P3 region, there was an increase in 3BCD; the 3C band was significantly reduced and 3AB was virtually undetectable. An unidentified band migrating slightly more slowly than 2A was also detected. A longer exposure of the gel revealed low amounts of a doublet band migrating near the position of 3AB, perhaps indicating an alternative cleavage product (Fig. 5, bottom). These abnormalities of polyprotein processing in the P2 and P3 region are similar to those

observed previously by Paul et al. (1994) with other mutants in the 2C amphipathic helix.

Induction of membrane reorganization by chimeric PV proteins

PV proteins 2C and 2BC both have been implicated in the major intracellular membrane remodeling that occurs after PV infection (Aldabe and Carrasco, 1995; Cho et al., 1994). The predicted N-terminal amphipathic helix in 2C is a major determinant of membrane binding, which is likely essential for induction of membrane reorganization. Membrane rearrangements appear to be essential for formation of the RNA replication complexes that carry out viral RNA synthesis. To gain some insight regarding the specificity of the amphipathic helix sequences in the 2C protein, we attempted to analyze the intracellular membrane morphology in HeLa cells expressing PV proteins from the chimeric 2C constructs. To ensure comparable levels of protein expression from replicating and non-replicating genomes, we transiently expressed all PV non-structural proteins using recombinant vaccinia virus vTF7-3. This expression system generates viral proteins from mRNA transcribed by T7 RNA polymerase, independently of viral RNA replication, in the infected/transfected cells. We have shown previously that the overall cell morphology as well as the appearance of induced membrane vesicles is indistinguishable from that seen in PV-infected cells (Teterina et al., 2001). To this end, we placed sequences encoding chimeric 2C proteins in the background of plasmid pE5PV Δ P1 (Teterina et al., 2001), which contains cDNA encoding all PV non-structural proteins (P2 and P3 regions) downstream of the EMCV IRES. RNA transcripts from each chimeric plasmid were first translated in HeLa cell extracts to analyze protein production and processing. As was the case for chimeras in full-length PV genomes, all mutant RNAs directed synthesis of products expected from the intact open reading frame of the polyprotein. The same defects of protein processing were observed for E5PV Δ P1 RNAs encoding chimeric 2C amphipathic helices as were observed with full-length genomes (data not shown; refer to Fig. 5).

Transfection of the various chimeric pE5PV Δ P1 plasmids in HeLa cells infected with vTF7-3, followed by anti-2C immunofluorescence microscopy to monitor viral protein expression, revealed similar expression levels in transfected cells, although transfection efficiencies sometimes varied for the different plasmids (not shown). Examination of the intracellular membrane morphologies after 7.5 h of transfection was performed by electron microscopy (Fig. 6). As reported previously (Teterina et al., 2001), numerous clusters of vesicles characteristic of those induced by PV infection were observed in cells transfected with pE5PV Δ P1 (expressing a complete set of P2 and P3 proteins), and in cells transfected with pE5PVxx Δ P1 (Fig. 6, panel A). The appearance of vesicles in cells transfected with the pE5PVxx Δ P1 parental plasmid encoding chimeric 2C proteins containing the amphipathic helix from hEV71 or CAV18 was also indistinguishable from pE5PVxx Δ P1 containing wild-type PV sequences (Figs. 6B and C). In cells transfected with

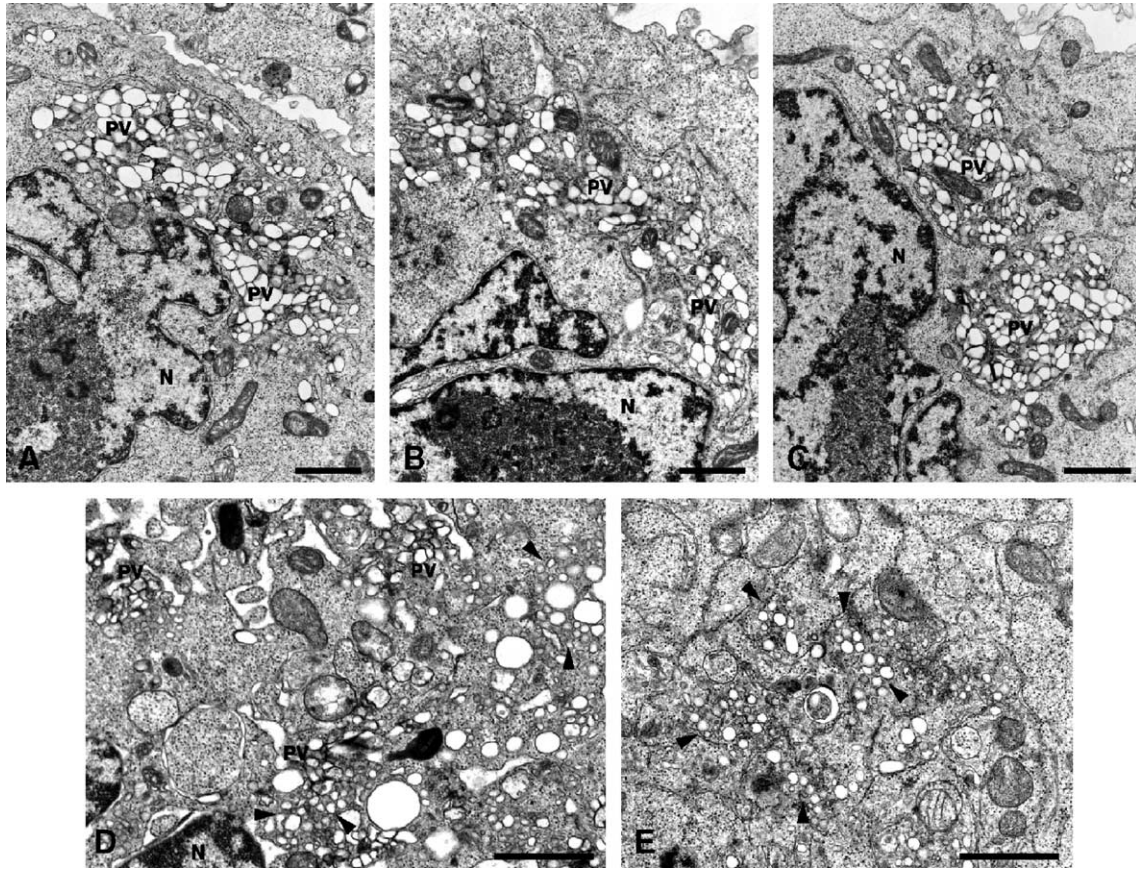


Fig. 6. Electron micrographs of HeLa cells 7.5 h after transfection with plasmid pE5PV Δ P1 containing the 2Cah of PVxx (A), hEV71 (B), CAV18 (C), HRV14 (D) or HCV (E). Panels A–D show canonical PV vesicles (PV); panels D and E show vesicles that differ morphologically from PV vesicles (arrowheads). N, nucleus. All scale bars are 1 μ m.

the same parental plasmid expressing the HRV14 chimeric 2C protein, however, vesicles were more dispersed, present only in very small clusters and were often smaller (Fig. 6D). Many vesicles displayed double membranes. There was a large variation in the appearance of vesicles in sections from different individual cells. Expression from the plasmids containing the FMDV, EMCV and HCV chimeric 2C proteins resulted in some membrane alterations including formation of loosely associated vesicles (Fig. 6E and not shown). The vesicles, however, were not typical for PV in array, size, structure or overall aspect and thus did not display the characteristic appearance of polio-induced vesicles. Thus, morphological aspects of the membrane alterations induced by PV proteins containing amphipathic helical sequences from FMDV or EMCV 2C proteins or from HCV NS5A protein were strikingly different from those formed by proteins from the chimeric viruses that were viable, suggesting that the former chimeric proteins displayed aberrant membrane interactions.

However, the non-viable chimeras also demonstrated protein processing defects that resulted in a marked reduction of 2C proteins (see Fig. 5), although large amounts of 2BC were present. To distinguish whether individual chimeric proteins were unable to associate with membranes or to function in a critical reaction, cells were transfected with plasmids encoding the individual 2C or 2BC wild-type or

FMDV-chimeric proteins, and their membrane associations and induced rearrangements were observed by electron microscopy. Consistent with our previous reports, expression of PV 2C protein alone induced formation of swirled membranes (data not shown), while 2BC induced vesicles (Fig. 7A). Cells transfected with plasmids encoding chimeric 2C or 2BC containing the amphipathic helix from the N-terminal portion of FMDV 2C, showed the same morphological changes as those transfected with the corresponding wt protein (Fig. 7B and data not shown) suggesting that the chimeric 2C and 2BC proteins have similar membrane reorganization properties as wild-type. Thus, it appears that the chimeric pE5 constructs failed to induce the characteristic membrane alterations typical of polio proteins because there was a deficiency in or altered amounts of membrane-binding proteins such as 3AB and/or 2C, caused by defective processing of the chimeric polyprotein.

Nucleotide sequences of virus isolates with improved growth properties that evolved from PV-2Cah-HRV14

Polioviruses in which the N-terminal amphipathic helix of protein 2C had been replaced with the corresponding region from HRV14 were able to replicate, albeit very poorly. However, transfection of HeLa cells with PV-2Cah-HRV14 gave rise to plaques of heterogeneous size; and accumulation of viral RNA

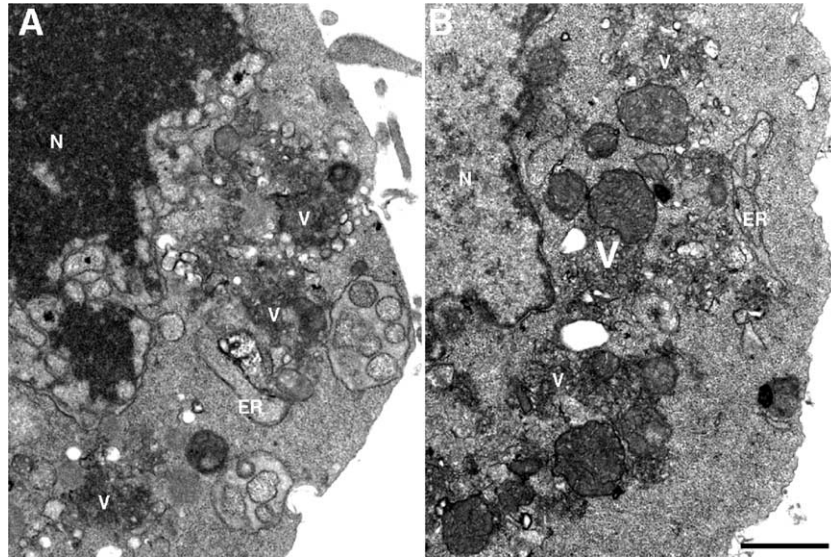


Fig. 7. Electron micrographs of HeLa cells transfected with pTM-2BCah-PV (A) or pTM-2BCah-FMDV (B). In both panels, membrane rearrangements (V) are of the same morphology. N, nucleus; ER, endoplasmic reticulum. Both panels show the same magnification; scale bar is 1 μ m.

in transfected cells was extremely slow during early rounds of replication, but showed marked increases at later times (Fig. 3). Such phenotype usually indicates that the original viral genome supports a low level of replication, and that some spontaneous mutation(s) that improved virus growth were selected during virus amplification. To identify these mutations, HeLa cells were transfected with PV-2Cah-HRV14 RNA transcripts and overlaid with semisolid medium. At 72 h after transfection, 10 individual plaques of relatively large size were selected for further analysis. Viruses recovered from each plaque were used to generate passage 1 (P1) virus stocks. Although only well isolated plaques were picked to produce virus stocks, many of these stocks still displayed mixed plaque size phenotypes (data not shown). HeLa cell monolayers were infected with P1 virus stock and total RNA was isolated 4.5–5 h post-infection, when the onset of CPE was visible for all virus isolates. Viral RNA recovered from the infected cells was sequenced within the 2C coding region using RT-PCR followed by direct sequencing of the cDNA fragments. Among 10 P1 stock virus isolates, only one showed mutation in the region encoding the 2C protein (Table 1; PV-2Cah-HRV14-R1). This virus had undergone a single nucleotide transversion (A4207-C) resulting in the reversion of HRV14-specific leucine at position 28 of 2C to the PV-specific phenylalanine. EM analysis of cells infected with PV-2Cah-HRV14-R1 revealed that this virus induced in cells morphological changes indistinguishable from those observed with wt PV (data not shown). The

other nine P1 stocks generated virus with the same nucleotide sequence within the entire 2C coding region that was present in the HRV14 2C amphipathic helix chimera initially used for transfection. Each of these P1 virus stocks was passaged twice more in HeLa cells and subjected to additional rounds of plaque purification from which 2–4 plaques were selected for generation of passage 3 (P3) virus stocks. The nucleotide sequence of the region encoding protein 2C was determined for 30 individual P3 virus stocks. Four viruses generated from two different P1 stocks contained single nucleotide mutations either A4207C or A4207T that generated the same 2C-L28F substitution (designated R1 in Table 1) as described above. Additionally, one virus (PV-2Cah-HRV14-R2) acquired mutation T4206C that produced an alternative substitution of Leu 28 by Ser. One pair of P3 virus isolates (progeny of the same P1 virus) possessed a single point mutation G4187T that encodes reversion of HRV14-specific 2C-Ala-22 to Ser, present in PV 2C (R3). Thus, despite a difference of seven out of 18 amino acids in the core sequence of the amphipathic helix between HRV14 and PV 2C proteins, reversion of only a single amino acid residue at either of two different positions in the helix (L28F and A22S) was sufficient to improve the “fit” of the rhinovirus chimeric 2C protein in the PV genome.

In addition to these three reversions that had replaced one of the HRV14-specific amino acids in 2Cah, we selected two other types of genomes with different mutations in the 2C coding sequence, outside of the region encoding the amphipathic helix. The only mutations found in the 2C region of PV-2Cah-HRV14-R4 and PV-2CahHRV14-R5 were physico-chemically similar substitutions either immediately downstream of the amphipathic helix sequence (2C-K49R) or near the C-terminus of 2C (2C-S318T). Each of these genomes was found in two separate P3 virus isolates that arose from progeny of independent P1 stocks that generated larger plaques and manifested increased growth rates. Finally, 18 analyzed virus stocks contained no sequence changes in the 2C coding region.

Table 1
Point mutations in 2C coding region found in PV-2Cah-HRV14 chimeric viruses with improved growth

Revertant	Passage 1	Passage 3	Mutation	Substitution
PV-2Cah-HRV14-R1	1	4	A4207C or A4207T	2C-L28F
PV-2Cah-HRV14-R2	–	1	T4206C	2C-L28S
PV-2Cah-HRV14-R3	–	2	G4187T	2C-A22S
PV-2CahHRV14-R4	–	2	A4269G	2C-K49R
PV-2Cah-HRV14-R5	–	2	T5075A	2C-S318T

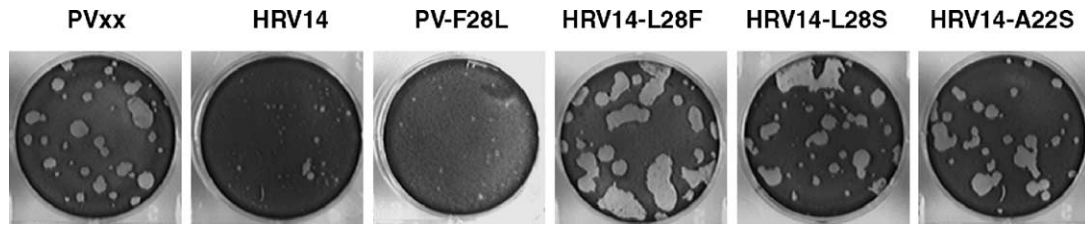


Fig. 8. Plaque morphology of PV-2Cah-HRV14 with single amino acid substitutions. HeLa cell monolayers were transfected with serial dilutions of the indicated mutant RNA transcripts. Plaques were visualized 48 h after transfection.

These viruses represent second site revertants that will be described elsewhere.

Single PV-specific amino acid substitutions in the HRV14 amphipathic helix improve replication of PV-2Cah-HRV14

To confirm that the individual amino acid reversions identified within the HRV14 amphipathic helix were responsible for the improved amplification of the mutant chimeric genomes, the mutations encoding these three changes at 2C amino acid positions 28 and 22 were individually introduced into the PV-2Cah-HRV14 cDNA and transcribed RNAs were used to transfect HeLa cells. The plaques observed after transfection with RNA coding for the PV-specific Phe at residue 28 (PV-2Cah-HRV14-L28F) showed improved growth of the chimeric virus to the wild-type level (Fig. 8). Similarly, the L28S mutation that was identified in the revertant designated R2 and the A22S mutation found in the R3 revertant also improved virus growth although the resulting plaques were slightly smaller than those formed by PVwt or PV-2Cah-HRV14-L28F. In vitro translation experiments showed that these single point mutations corrected the previously observed changes in polyprotein processing in the P3 region (data not shown).

Amino acid 28 in the amphipathic helix of PV 2C is important for virus growth

The most frequently found mutation that improved the growth properties of PV-2Cah-HRV14 chimeric genomes resulted in substitution of amino acid 28 from Leu in the HRV14 sequence to either Phe, the amino acid present in the PV sequence or to Ser (Table 1). This deleterious Leu substitution for Phe is generally considered a rather conservative change that may not affect the overall presentation of the hydrophobic surface of the proposed amphipathic helix. This suggests that the aromatic ring of the Phe residue in this position might be important for some specific interactions of protein 2C (or 2BC). Indeed, when we constructed the single 2C-F28L mutation in the PVxx genome, transfection of HeLa cells with PVxx-2C-F28L RNA yielded plaques of minute size (Fig. 8), indicating that Phe28 of 2C plays an important role in some aspect of virus growth. In view of this important role of residue Phe28 in the amphipathic helix of 2C, we attempted to see whether substitution of the PV-specific Phe28 residue for the Ile at the corresponding position in the FMDV amphipathic helix would be sufficient to rescue the replication of PV-2Cah-

FMDV. However, chimeric genome PV-2C-FMDV-I28F failed to produce any virus upon transfection of HeLa cells (data not shown).

Compensatory mutations in 2C outside of the amphipathic helix improve growth of PV-2Cah-HRV14

Large plaque virus isolates designated as R4 and R5 in Table 1 maintained sequences encoding all HRV14-specific amino acids in the amphipathic helix of 2C but acquired mutations coding for amino acid changes in 2C outside of this region; e.g., 2C-K49R or 2C-S318T. To confirm that each of these mutations is sufficient to compensate for the poor growth of PV-2Cah-HRV14, we introduced these mutations by site-directed mutagenesis into the PV-2Cah-HRV14 chimeric genome and in the parental PVxx genome. Neither of these mutations had any effect on the growth of PVxx virus after transfection of HeLa cells with RNA transcripts. However, each of these mutations resulted in significant increases in plaque size compared with those produced by PV-2Cah-HRV14 RNA after transfection (Table 2). The K49R mutation, just downstream of the amphipathic helix region, restored plaques to almost the same size as PVxx RNA, as did the S318T mutant near the C-terminus of 2C. These results confirm that mutations 2C-K49R or 2C-S318T outside of the amphipathic helix in the 2C protein compensate for defects caused by the replacement of the PV amphipathic helix with that from HRV14.

Discussion

In this study, we explored the relationships among phylogenetically diverse amphipathic helices that were identified in the N-terminal regions of replication proteins of RNA viruses. Bioinformatics analyses led to the observation of an unexpected local sequence similarity between the N-terminal amphipathic helices in the 2C protein of the Picornaviridae

Table 2
Plaque size observed 48 h after RNA transfection

RNA	Plaque size (mm)
PVxx	3–4
PV2C-K49R	3–4
PV2C-S318T	3–6
PV2Cah-HRV14	0.5–1.5
PV2Cah-HRV14-K49R	2–3.1
PV2Cah-HRV14-S318T	1.5–4

and the NS5A protein of the Flaviviridae. These proteins occupy different positions in the genomes of two virus families that are only distantly related. The 2C and NS5A helices may have originated from a common ancestor, implying that their evolution involved helix relocation in one or both lineages. Alternatively, the N-terminal helices of the 2C and NS5A proteins may have evolved independently. Regardless of the origin of these helices, our bioinformatics analysis revealed particularly strong similarity between helices from PV 2C and HCV NS5A of two different lineages, indicative of a selective pressure common for the replication cycles of these viruses.

Based on bioinformatics data, we hypothesized that this helix may form an independent module in picornaviral protein 2C and HCV protein NS5A. We tested the functional exchangeability of the amphipathic helix in PV protein 2C with orthologous elements from other picornaviruses and demonstrated the viability of poliovirus chimeras that carried an alien helix from other picornaviruses of the enterovirus and rhinovirus genera, but not from more distantly related cardiovirus and aphthovirus or HCV. Substitution of the poliovirus 2C amphipathic helix with the orthologous sequences from hEV71, CAV 18 and HRV14 resulted in the production of viable viruses, although the chimera with the amphipathic helix from HRV14, the most distant of these three viruses, manifests a significant RNA replication defect. The fact that not every chimera proved to be viable might be (partially) attributed to the somewhat arbitrary selection of the borders of sequences selected for substitution. For instance, the very N-terminal five residues of the HCV NS5A α -helix, which contains a conserved Trp residue (Penin et al., 2004), were not included in the analysis, as its counterpart in 2C of picornaviruses was not confidently identified.

Interestingly, although the amino acid sequence in the amphipathic helix of HRV 14 differs from that in PV at seven positions, only two of those residues, amino acids 22 and 28, differ from those present in the CAV18 sequence, which generated a chimera that replicated almost as well as wild-type PV. The importance of amino acids 22 and 28 of protein 2C is further supported by the analysis of faster growing viruses isolated after passage of PV2Cah-HRV14: the most frequently acquired mutations changed amino acid 28 of 2C, and another rapidly growing revertant virus substituted amino acid 22 with the one normally present in PV. Although we have not sequenced the entire genomes of these virus isolates, reconstruction of these single point mutations encoding changes of amino acid 22 or 28 in the PV-2Cah-HRV14 genome was sufficient to produce virus with significantly improved growth. In addition, when a single point mutation that substituted Phe28 in PV 2C with the HRV-14-specific Leu was introduced in the wild-type PV genome, a marked RNA replication defect resulted. These data imply that amino acid residues 22 and 28 play a critical role in functionally important interactions of the amphipathic helix with other (protein and/or RNA) partners. Both these residues are predicted to be located close to the membrane surface (Fig. 1B) that may be essential for these interactions. Amino acids at other positions of the helix may

also exert control over PV replication, as the PV-2Cah-HCV chimera that contains Phe at the 28 position and Cys (physicochemically most similar to Ser) at the 22 position failed to replicate. In addition, introduction of Phe28 in the amphipathic helix from FMDV in PV-2Cah-FMDV did not rescue this chimera.

Substitution of the amphipathic helix in protein 2C by similar elements from the more distantly related FMDV, EMCV and HCV was lethal for virus growth and no revertant virus was recovered after transfection of HeLa cells with the chimeric RNA transcripts. Neither negative- nor positive-strand RNA synthesis could be detected in cells transfected with these chimeric RNAs or in extracts translating and replicating viral RNAs. Analysis of the translation products revealed polyprotein processing defects in the P2 and P3 regions: the most prominent was abnormal accumulation of P2, 2BC and 3BCD precursors, with nearly complete absence of 2C and 3AB. It is possible that abnormal polyprotein proteolytic processing is the primary defect responsible for the dead phenotype of these chimeras. We are currently unable to uncouple polyprotein processing from the formation of active replication complexes to verify this interpretation.

It is surprising that rather conservative substitutions in the 2C protein located quite far from the cleavage site between 2B and 2C affect the processing at this site and even more significantly at distant sites in the P3 region. Similar defects in polyprotein processing were reported by Paul et al. (1994) for mutations in the 2C helix that changed the predicted amphipathic character of the helix. Interestingly, some previously reported mutations in PV protein 3AB (Giachetti et al., 1992) and substitutions in the hydrophobic domain of 3AB protein by corresponding sequences from HRV14 (Towner et al., 2003) also caused defective polyprotein processing with increased production of unprocessed P2 and 3BCD proteins. It was suggested previously (Lawson and Semler, 1992) that PV utilizes two processing cascades in the production of viral proteins. One is rapid, occurs in the membrane-bound fraction and is thought to operate in cis; the second is a slower, trans-cleavage pathway observed in the soluble fraction. It is not known how these pathways are regulated during the course of infection. Our results indicate that the N-terminal helix of 2C is an essential part of the network of interactions that determine polyprotein processing.

All positive-sense, single-strand RNA viruses appear to induce remodeling of intracellular membrane structures to provide a scaffold and compartmentalized protein location for viral RNA synthesis, although the details of replication complex morphologies and specific membrane involvements vary considerably. The amphipathic helical element presumably serves as a determinant of membrane binding for 2C-containing proteins that are part of the replication machinery in PV. We examined the effects of the N-terminal amphipathic helical substitutions on the morphology of the induced membrane structures that form in the cytoplasm of the infected host cell. The appearance of the replication complex-associated vesicles formed by proteins including hEV71 or CAV18

chimeric 2C proteins was indistinguishable from those formed by wt PV proteins, as visualized by electron microscopy. Viruses containing HRV14 chimeric 2C proteins, which grew poorly, induced vesicles that appeared different in several aspects. The chimeras with FMDV, EMCV or HCV NS5A amphipathic helix substitutions failed to show any RNA replication at all, and, accordingly, produced no typical vesicle clusters. Expression of individual chimeric 2C or 2BC proteins containing the FMDV helix, however, induced membrane alterations that appeared indistinguishable from the corresponding wild-type PV proteins. Thus, the observed defects in membrane biogenesis by chimeras may be linked to the altered relative amounts of protein precursors and products resulting from polyprotein processing changes rather than to the effects of the alien helix on the 2C or 2BC proteins per se.

Recent studies of the HCV NS5A amphipathic helix (Penin et al., 2004) showed that amino acid insertions negatively affecting this element also were detrimental for RNA replication. Apparently, they did not interfere with subcellular localization and membrane association of this protein, suggesting that the N-terminal helix performs some additional functions or participates in molecular interactions other than membrane anchoring. The results of our experiments imply that the PV 2C N-terminal amphipathic helix may also contribute to functions other than membrane anchoring. On the other hand, the N-terminal region of nodavirus Flock House Virus (FHV) protein A, which normally targets the protein to mitochondrial membranes (Miller et al., 2001), can be readily substituted by viral or cellular ER-targeting sequences without affecting the ability of the virus to replicate. Thus, details of the assembly, higher-order interactions and architectural determinants of the replication complexes may differ dramatically in different RNA viruses.

Materials and methods

Bioinformatics analysis

GenBank searches with 2C and NS5A sequences as queries were performed using the original BLAST program (Altschul et al., 1990). All hits with scores that were comparable to those detected among related picornavirus or flavivirus sequences were further analyzed. Sequence alignments were generated using the ClustalX 1.64 and 1.81, a Windows-based interface of the ClustalW program (Thompson et al., 1997, 1994), and the Dialign2 program (Morgenstern, 1999), both assisted by the Blossum scoring matrices of the amino acid similarities (Henikoff and Henikoff, 1994). Alignments presented in this report are part of all inclusive polyprotein alignments of the Picornaviridae and the Flaviviridae families (Johansson et al., 2002; Lackner et al., 2004; Tellinghuisen et al., 2004; Gorbalenya, unpublished observations). GenBank accession numbers are: PV1—AY184219; PV2s—AY184220; PV3s—AY184221; CAV20—AF465514; hEV71—AY465356; CAV18—AF465513; HRV14—NC001490; FMDV—AY593752; EMCV—M22457; HPEV2H—AF055846 and HCV—HPCJ8G.

Construction of plasmids and DNA manipulation

Standard recombinant DNA technology was used to construct and purify all plasmid DNAs. The sequences of oligonucleotides used for construction of chimeric genomes may be obtained upon request. All mutations were confirmed by automated DNA sequencing with an ABI 3100 Gene Analyzer (Applied Biosystems). The BigDye Terminator version 3.1 sequencing kit was used for sequencing according to the manufacturer's instructions. Plasmid DNAs were prepared from large-scale bacterial cultures and purified by use of a Qiagen (Valencia, Calif) Maxi-prep kit. All nucleotide numbers refer to the PV1 genome and all amino acid numbers refer to the 2C protein of PV.

The plasmid pT7PV1 (Haller and Semler, 1995) contains a full-length infectious cDNA of PV1. The modified full-length plasmid pT7PVxx has two genetically engineered *XhoI* sites at PV nt 4096 and 4242. These *XhoI* sites were created by site-directed mutagenesis performed with the QuickChange mutagenesis kit (Stratagene), which introduced two silent point mutations at nucleotides 4099 and 4244.

To create chimeric genomes with substitutions of the 2C N-terminal amphipathic region, we used a synthetic mutagenesis cassette. The 148 nt double stranded fragments representing regions 4096–4244 of PV cDNA were combined by annealing of six synthetic oligonucleotides: three common for all constructs and three specific for each chimera. Annealed oligonucleotides were ligated with T4 DNA ligase (New England Biolabs) at 16 °C for 4 h and 1 µl of product from the ligation reaction was amplified by PCR. Amplified PCR fragments were digested with *XhoI*, purified from agarose gels and ligated with the large fragment of pPVxx digested with the same enzyme. The final constructs were verified by sequencing. For simplification of the nomenclature, the name of each chimeric plasmid indicates the source of the amphipathic helix in 2C(2Cah). The same strategy was used to create genomes with single amino acid substitutions in the PV-2Cah-HRV14 amphipathic helix and to produce pPVxx-2C-F28L. Plasmids harboring mutations encoding substitutions of 2C-K49R and 2C-S318T in PVxx or PV-2Cah-HRV14 genomes were generated by site-directed mutagenesis performed with the QuickChange mutagenesis kit (Stratagene) and standard recombinant DNA technologies.

Plasmid pE5PVΔP1 contains cDNA of a PV genome encoding all non-structural proteins and the 3'NCR under control of the EMCV internal ribosome entry site (Teterina et al., 2001). To create pE5PVΔP1 derivative plasmids encoding chimeric 2C-amphipathic helices, full-length plasmids described above were digested with *SpeI* and *BglII* and the 1618 bp fragments were isolated from the gel and ligated with pE5PVΔP1 vector digested with the same enzymes.

Plasmids pTM-PV2C and pTM-PV2BC have been described previously (Cho et al., 1994). To introduce sequences encoding the 2C amphipathic helix from FMDV in these plasmids, a DNA fragment carrying this chimeric sequence was generated by PCR amplification from pPV-2Cah-FMDV using a positive strand primer representing nt 3795–3812 of PV and

negative strand primer complementary to nt 4649–4670 of PV. The PCR fragment was digested with *Bam*HI and either *Spe*I or *Sph*I; appropriate fragments were isolated from an agarose gel and used to replace the corresponding fragments in plasmid pTM-PV2BC digested with *Bam*HI and *Spe*I or in plasmid pTM-PV2C digested with *Bam*HI and *Sph*I.

RNA transcription

RNA transcriptions were performed essentially as described previously (Teterina et al., 2003) with the following change: after transcription reactions were terminated by treatment with DNase I for 15 min at 37 °C, 375 µl of sodium dodecyl sulfate buffer (0.5% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris–HCl [pH 7.5], 1 mM EDTA) containing 100 µg of proteinase K (Promega) was added and incubated an additional 30 min at 37 °C. The mixture was extracted with phenol-chloroform and RNA was precipitated with ethanol. RNA pellets were washed with 70% ethanol and dissolved in RNase-free water. For *in vitro* translation–replication reactions, RNAs were additionally purified by passage on ChromaSpin-1000 columns (Clontech). The concentration was determined from the absorbance at 260 nm.

RNA transfection, plaque assays and preparation of virus stocks

RNA transfections were performed either using DEAE-dextran as described previously (Teterina et al., 1995) or using TransIT-mRNA Transfection Kit (Mirus) according to the manufacturer's protocol. For plaque assays, HeLa cell monolayers in 6-well plates were transfected with serial dilutions of RNA transcripts and incubated for 30 min in transfection media. The medium was then replaced with minimal essential medium containing 0.4% agarose. Plates were incubated at 37 °C for the indicated times and stained with crystal violet. Plaque purification was performed in 60-mm diameter dishes. Virus from an individual plaque was released in 200 µl of minimal essential media and used to infect a fresh HeLa cell monolayer. After development of complete CPE, the material was collected, subjected to 3 cycles of freeze-thawing and designated as passage 1 (P1) virus. Subsequent passages were produced by plaque purification and infection of fresh HeLa monolayers with virus, isolated from an individual plaque.

Sequencing of recovered viruses

HeLa cells in 35-mm diameter dishes were infected with P1 or P3 virus stocks at MOI = 20 pfu/cell and incubated at 37 °C for 4–5 h when first signs of CPE became apparent. Total cytoplasmic RNA was harvested from infected cells using an RNeasy kit (Qiagen) as described previously (Teterina et al., 2001). The region of the PV genome between nt 3795 and 5360 was amplified by RT-PCR using Access RT-PCR kit (Promega) and primers annealing to these regions of PV positive- and negative-sense RNA. Homogeneity of PCR fragments was analyzed by electrophoresis in agarose gels;

PCR fragments were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced directly using different internal primers without further purification.

Analysis of RNA accumulation in HeLa cells

HeLa cells in 35-mm diameter dishes were transfected with 1 µg of RNA transcripts as described above. Total cytoplasmic RNA was harvested at different times post-transfection. RNase protection assays were performed using an RPA III kit (Ambion). Riboprobe for detection of PV-specific positive-strand RNA was transcribed with SP6 polymerase from plasmid pT220–460 (gift from B. Semler; Dildine and Semler, 1992) linearized with *Bam*HI, and contains antisense RNA corresponding to PV nt 220–460.

In vitro translation/replication assays

Assays were performed as described previously (Teterina et al., 2003).

DNA transfection and EM

For protein expression, HeLa cells were infected with vaccinia virus vTF7-3 (Moss et al., 1990) and transfected with plasmid DNA as described previously (Teterina et al., 1997b). For EM, cell cultures were trypsinized at the indicated times, fixed with 2.5% glutaraldehyde and 2% OsO₄ and embedded in Epon 812 according to standard procedures. Sections were viewed in a FEI-CM100 or in a Philips CM 100 electron microscope.

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