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Virology 344 (2006) 453 – 467
VIROLOGY
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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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Received 8 July 2005; returned to author for revision 4 August 2005; accepted 24 August 2005
Available online 14 October 2005

**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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*Keywords:* Poliovirus; Hepatitis C virus; Chimera; Protein 2C; Amphipathic helix; Membrane anchor; Polyprotein processing; Replication complex

## 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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0042-6822/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2005.08.044

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