Interaction of picornavirus 2C polypeptide with the viral negative-strand RNA

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The picornavirus membrane-associated polypeptide 2C is believed to be required for viral RNA synthesis. Hepatitis A virus (HAV)- and human rhinovirus (HRV)-encoded recombinant 2C proteins have been expressed, purified and examined for their ability to interact with the terminal sequences of viral positive- and negative-strand RNAs. The results demonstrate that both the HAV- and the HRV-encoded 2C polypeptide specifically interact with the 3′-terminal sequences of the negative-strand RNA, but not with the complementary sequences at the 5′-terminus of the positive-strand RNA. This interaction was detected by both mobility gel shift and UV cross-linking assays. Furthermore, complex formation exhibited dose-dependency and competition assays confirmed specificity. These results are consistent with our previous observation using the poliovirus 2C protein. The implication of the picornavirus 2C protein binding to the 3′-terminal sequence of the negative-strand untranslated region in viral RNA synthesis is discussed.

Picornaviruses encompass a large group of medically important viruses, including those that induce poliomyelitis (polioviruses, PV), the common cold (human rhinoviruses, HRV), infectious hepatitis (hepatitis A virus, HAV) and myocarditis and encephalitis (coxsackieviruses). These viruses contain single-strand, positive-sense RNA genomes. PV has been used as the prototype of picornaviruses to understand the molecular biology of replication of these viruses. The RNA genome translates into a single large polyprotein that is proteolytically processed into mature viral structural and nonstructural polypeptides, including the polymerase (3Dpoly), proteases (2Apro and 3Cpro) and VPg-3B, the genome-linked protein (Kitamura et al., 1981). The 5′-untranslated region (UTR) of both PV and HRV type 14 (HRV-14) comprises two distinct elements that appear to act independently: a cloverleaf structure that is formed from the first 100 nucleotides and which is required for replication and the remaining nucleotides 120–640 contain the ribosome entry site (IRES). In contrast to PV and HRV-14, the 5′ 100 nucleotides of HAV 5′ UTR fold into three separate stem-loop structures called SL-I, -Ila and -Ilb, which are followed by the polypyrrimidine tract that demarcates the IRES (Brown et al., 1991; Lemon & Robertson, 1993; Kusov & Gauss-Muller, 1997). The input viral RNA replicates via synthesis of a complementary negative-strand RNA, which serves as a template for the production of progeny virion RNA. Most, if not all, of the nonstructural polypeptides appear to be involved in viral RNA replication.

The 2C protein encoded by the P2 region of the polypeptide is highly conserved among picornaviruses (Gorbaleyna et al., 1989). During PV infection, 2C and its precursor 2BC migrate to the rough endoplasmic reticulum where they induce the formation of smooth membrane vesicles that bud off and become the site of viral RNA synthesis, the replication complex (Bienz et al., 1987, 1992, 1990; Cho et al., 1994; Teterina et al., 1997). 2C is a multifunctional protein and some of these functions include ATPase and GTPase (Rodriguez & Carrasco, 1993; Mirzayan & Wimmer, 1994; Pfister & Wimmer, 1999), membrane-binding (Kusov et al., 1998; Echeverri & Dasgupta, 1995; Aldabe & Carrasco, 1995) and RNA-binding activities (Rodriguez & Carrasco, 1995).

In a previous study, we have shown that PV-encoded 2C and the precursor polypeptide 2BC specifically interact with the 3′-terminal sequences of the negative-strand RNA, but not with the corresponding 5′-terminal sequences of the positive-strand RNA (Banerjee et al., 1997, 2001). We have also demonstrated that this interaction requires a stable stem–loop structure to be present at the 3′ terminus of the negative-strand RNA. Because PV replication occurs in the cytoplasmic membrane and the 2C protein is capable of interacting with both the membrane and the viral negative-strand RNA, it was hypothesized that the 2C protein anchors the negative-strand RNA.
RNA to the membrane. This anchoring may be crucial for the synthesis of positive-strand RNA from the negative-strand RNA template. Here, we have extended our 2C–RNA interaction studies to demonstrate that the purified 2C proteins from both HAV and HRV-14 also interact specifically with the 3′-terminal sequences of the corresponding negative-strand RNAs.

To examine the RNA-binding properties of HAV- and HRV-encoded 2C protein, plasmids containing the 2C-coding sequences were expressed in *Escherichia coli*. The expressed proteins had a T7 tag at the N terminus and a six residue histidine tag at the C terminus for ease of purification. The recombinant proteins were affinity purified through a Co⁺⁺-charged resin, as reported previously (Banerjee et al., 2000). As can be seen in Fig. 1, both HAV- and HRV-encoded 2C polypeptides (molecular mass of approximately 45 kDa) were purified to near homogeneity, as judged by Coomassie staining after SDS–PAGE. Both proteins reacted with a monoclonal antibody against the sequence of the T7 tag on Western blot. However, the PV-encoded 2C protein lacking the T7 tag did not react with the antibody, thus confirming the specificity of this antibody (Fig. 1B, compare lanes 1 and 2).

The first 100 nucleotides at the 5′ end of the positive-strand RNAs of HAV and HRV-14 have a high probability of folding into secondary structures (Fig. 2A). A similar type of stem–loop structure is also predicted to form at the 3′ end of the negative-strand RNA, which is complementary to the 5′-100 nucleotides of the positive-strand RNA. In PV, the cloverleaf structure at the 5′ end of positive-strand RNA plays an important role in viral RNA synthesis (Andino et al., 1990, 1993). Two viral proteins, 3C and 3D (or the precursor polypeptide 3CD), and a ribosome-associated cellular protein (p36) bind to this structure. To investigate the interaction of HAV and HRV 2C with the predicted stem–loop structures at the 5′ end of positive-strand RNA and the 3′ end of negative-strand RNA, the corresponding labelled UTR sequences were synthesized *in vitro*. The affinity purified 2C protein was incubated with gel-eluted positive- and negative-strand RNA probes in a binding reaction and the RNA–protein complexes were examined by a gel shift assay. The typical binding reaction contained 5 mM HEPES (pH 7–9), 25 mM KCl, 2 mM MgCl₂, 5% glycerol, 20 mM DTT, 2 mM ATP, 12 µg of purified yeast t-RNA, RNasin (30 U) and 100 ng of purified 2C in a total volume of 25 µl. The free negative-strand RNA probes derived from both HAV and HRV were found to have more than one form under nondenaturing conditions, which could be due to the formation of various secondary structures (Fig. 2B, lane 3) (Banerjee et al., 1997, 2001). The positive-strand RNA probes also exhibited forms that migrated more slowly, but to a lesser extent. However, both the negative- and positive-strand RNA probes showed single bands that corresponded to full-length transcripts when analysed by denaturing

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**Fig. 1.** Expression and purification of virus-encoded 2C protein. The coding sequences of the proteins were aligned to data published previously (Cohen et al., 1987; Stanway et al., 1984). Primer pairs incorporating either a HindIII or a BamHI enzyme restriction site were designed to amplify HRV 2C (5′-TAAGCCGGATCCGAATATGGATGGTTCT 3′ and 3′-AGCGGGACTGATGTTGAGGATGATTACAGTA 5′) and 2BC (5′-CGATTAAGGATCCGGGCTGAGTGATTACATCA 3′ and the above reverse primer). To generate the HAV 2C clone, the primer pair 5′-CGCGCAAGGATCCGAGTTTTTCCAACTGGTTAA 3′ and 3′-CGAGCGCTCGAGCTGAGACCACAACTCCATGA 5′ was used. The amplified, sequentially digested and gel-purified sequences were inserted into the corresponding enzyme restriction sites of the pET expression vector (Novagen) using standard protocols. *E. coli* BL21(DE3) cells harbouring recombinant pET with the 2C/2BC-coding sequence were induced by IPTG. Cells were harvested 3–5 h post-induction and the proteins were isolated by metal-affinity chromatography. The Coomassie stain of the expressed, isolated 2C proteins corresponding to either (A) HRV-14 or (B) HAV are shown. The identity of the purified proteins was examined by Western blot analysis (chemiluminescence). (B) Either purified PV 2C (without the T7 tag, lane 1) or HAV 2C (with the intact T7 tag, lane 2) was examined by immunoblot blot analysis using an antibody against the T7 tag.

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**Fig. 2.** **A.** The first 100 nucleotides at the 5′ end of the positive-strand RNA of HAV and HRV-14 have a high probability of folding into secondary structures. **B.** A similar type of stem–loop structure is also predicted to form at the 3′ end of the negative-strand RNA, which is complementary to the 5′-100 nucleotides of the positive-strand RNA. In PV, the cloverleaf structure at the 5′ end of positive-strand RNA plays an important role in viral RNA synthesis (Andino et al., 1990, 1993). Two viral proteins, 3C and 3D (or the precursor polypeptide 3CD), and a ribosome-associated cellular protein (p36) bind to this structure. To investigate the interaction of HAV and HRV 2C with the predicted stem–loop structures at the 5′ end of positive-strand RNA and the 3′ end of negative-strand RNA, the corresponding labelled UTR sequences were synthesized *in vitro*. The affinity purified 2C protein was incubated with gel-eluted positive- and negative-strand RNA probes in a binding reaction and the RNA–protein complexes were examined by a gel shift assay. The typical binding reaction contained 5 mM HEPES (pH 7–9), 25 mM KCl, 2 mM MgCl₂, 5% glycerol, 20 mM DTT, 2 mM ATP, 12 µg of purified yeast t-RNA, RNasin (30 U) and 100 ng of purified 2C in a total volume of 25 µl. The free negative-strand RNA probes derived from both HAV and HRV were found to have more than one form under nondenaturing conditions, which could be due to the formation of various secondary structures (Fig. 2B, lane 3) (Banerjee et al., 1997, 2001). The positive-strand RNA probes also exhibited forms that migrated more slowly, but to a lesser extent. However, both the negative- and positive-strand RNA probes showed single bands that corresponded to full-length transcripts when analysed by denaturing
Fig. 2. (A) Schematic representation of the viral 5' and 3' UTRs used in the present analysis. The sequence and the predicted structure of the first 100 nucleotides of the positive-strand UTR (5' UTR$_{100}$) from the two picornaviruses are shown (adapted from Rohll et al., 1994; Brown et al., 1991; Cohen et al., 1987). The negative-strand 3' UTR$_{100}$ is predicted to form a similar structure with complementary sequences of the bases indicated. The UTR sequences were amplified from the template cDNAs using the primer pairs R-f, 5' CAGCTTGATTTCTAAACACGGATG 3', and R-r, 3' CTAACTAGCTAAAGGAAAGGTGGTTG 5', for HRV and A-f, 5' GTAGCCGAATTCTTCAAGAGGGGTCTCC 3', and A-r, 3' GCGCCGAAGCTTAAATTTACCTATAGC 5', for HAV. The UTR sequences were then ligated into the pGEM-3 vector (Promega) at the HindIII and EcoRI sites (underlined). Probes were generated using the appropriate RNA polymerase, along with [32P]UTP (0–1 PBq/mmol) (Amersham) in the transcription reaction. A filter binding assay was used to determine the counts of radioactivity incorporated. Probes used later during the binding assay were diluted to either 20000 (for gel shift analysis) or 100000 c.p.m./µl (for UV cross-linking analysis) with nuclease-free water for each reaction. The filter assay was repeated after dilution to ensure that similar counts were added to individual reactions. (B) Binding analysis of HRV and HAV 2C proteins to the cognate UTR$_{100}$ RNA probe. Gel-purified, 32P-labelled RNA probes corresponding to the 5' UTR$_{100}$ of the positive-strand RNA or the 3' terminal sequence of the negative-strand RNA were incubated for 15 min at 30 °C with purified 2C/2BC and the binding reaction components (as described in the text). The specific RNA–protein complexes formed were analysed by electrophoretic mobility gel shift assay. The positions of the free probe (labelled F) and the retarded nucleoprotein complex (labelled C) are indicated. Lanes 1 and 2 correspond to the binding reactions with the HRV positive-strand UTR$_{100}$ probe and lanes 3–6 are reactions incubated with the negative-strand RNA probe. The gel shown on the right represents reactions that contain either positive- (lanes 7 and 8) or negative-strand (lanes 9 and 10) HAV RNA probes. The unevenly numbered lanes represent control reactions with no viral protein and the evenly numbered lanes contained approximately 100 ng of either the 2C or the 2BC polypeptide, as indicated. Reactions in lanes 1–6 contained HRV 2C/2BC, whereas lanes 7–10 contained HAV 2C recombinant protein. (C) Dose-dependent interaction between the 2C protein and the negative-strand UTR$_{100}$ probe. Increasing concentrations (10, 20, 40, 60, 80 and 100 ng) of the purified 2C protein from either HRV or HAV were added to the binding reactions, as above, and incubated with the appropriate 3' UTR$_{100}$ probe. The specific nucleoprotein complex (labelled C) formed was analysed, as described in an earlier experiment. In both sets, lanes 1 represent control reactions with no protein. The RNA–protein complexes observed with HRV 2C migrating more slowly are indicated by a dot and an asterisk.

gel electrophoresis (see Fig. 3C and D, lower panel). Although of identical length, the HRV positive-strand RNA probe always migrated slightly faster than the negative-strand RNA probe (Fig. 2B, compare lanes 1 and 3). Both HAV and HRV 2C proteins formed distinct gel-retarded complexes when incubated with the corresponding negative-strand RNA probe (Fig. 2B, labelled C in lanes 4 and 10). A distinct RNA–protein complex was also detected when HRV 2BC (a precursor of 2C) was incubated with the corresponding negative-strand RNA probe (Fig. 2B, lane 6). In contrast, almost no RNA–protein
complexes could be detected when either HAV or HRV 2C was incubated with the appropriate positive-strand RNA probe (Fig. 2B, lanes 2 and 8).

The formation of the RNA–protein complex increased almost linearly with increasing concentrations of the purified 2C protein. Results presented in Fig. 2(C, lanes 1–7) show that at the highest concentration of HAV 2C (approximately 100 ng), almost 50% of the probe was converted into an RNA–protein complex. With the HRV 2C, the intensity of the major complex (Fig. 2C, indicated by a C) increased up to a certain level with increasing protein; however, at higher concentrations of 2C, minor, but larger, complexes that migrated more slowly than the major complexes were detected (Fig. 2C, indicated by an asterisk and a dot).

The specificity of RNA–protein complex formation was examined by competition gel retardation analyses. The addition of increasing quantities of unlabelled, homologous, negative-strand HAV RNA (3’ UTR<sub>390</sub>) inhibited the formation of labelled RNA–protein complexes to approximately 10% of the control, while the addition of the same concentrations of an unlabelled, heterologous RNA of a similar size did not affect the formation of RNA–protein complexes (Fig. 3 A, compare lanes 3 and 4 with lanes 5 and 6). The possibility that the heterologous RNA is less stable than the homologous competitor was ruled out by comparing its half-life with that of the homologous RNA (data not shown). These results suggest that the HAV 2C protein specifically binds to the 3’-terminal sequence of the negative-strand UTR. Similar results were observed for the HRV 2C protein (Fig. 3 B).

UV cross-linking studies were performed to confirm the results obtained by gel retardation analysis. The nucleoprotein complex that was formed between the HRV 2C protein and the 3’- or 5’-positive-strand RNA probes was subjected to UV irradiation and the resulting complex was analysed by SDS–PAGE, as detailed earlier (Banerjee et al., 2000). As can be seen in Fig. 3 (C), a distinct UV cross-linked band was detected with the 3’-negative-strand RNA probe (3’ UTR<sub>390</sub>) when the binding reaction contained the 2C protein (Fig. 3C, compare lanes 3 and 4). A very faint protein–nucleotidyl complex was detected when the protein was incubated with the 5’-positive-strand RNA probe (Fig. 3 C, lane 2). To examine the stability of the RNA probes, labelled nucleoprotein complexes formed after incubation were deproteinized and analysed by denaturing gel electrophoresis. As shown in Fig. 3 (C, lower panel), both the positive- and negative-strand RNA probes were equally stable in either the absence or the presence of 2C. UV cross-linking analysis with the purified HAV 2C protein using the viral UTR sequence from the positive- as well as the negative-strand RNA also showed that HAV 2C interacted with the 3’-negative-strand UTR more efficiently than with the 5’-positive-strand UTR (Fig. 3 D). To prove that the polypeptide bound to the 3’-negative-strand UTR probe during gel retardation analysis was indeed 2C, the HRV 2C complex was excised from the native gel (Fig. 2B) and resolved by second dimension SDS–PAGE, followed by silver-staining and simultaneous Western blot analysis using an antibody against the N-terminal T7 tag epitope. As can be seen in Fig. 3(E), the RNA–protein complex was resolved into a single polypeptide that reacted to the antibody directed against the T7 tag epitope and also migrated to the range of the expected molecular mass.

The results presented in this communication suggest strongly that, like the PV 2C protein, both HRV- and HAV-encoded 2C polypeptides are capable of forming specific complexes with the 3’-terminal negative-strand RNA. Very little, if any, complex was detected when HRV and HAV 2C protein was incubated with the complementary positive-strand RNA.

Previous results from our laboratory have shown that N-terminal sequences of PV 2C encompassing amino acids 21–54 and containing the putative amphipathic helix appear to play an important role in membrane binding both in vitro and in vivo (Echeverri & Dasgupta, 1995; Echeverri et al., 1998). The inherent capacity of this protein to bind both the membrane and the 3’ end of viral negative-strand RNA suggests that 2C may be involved in anchoring negative-strand RNA to the virus-induced membranous complex. Such an anchoring mechanism may be essential to keep the 3’ end of the negative-strand RNA immobilized so that other viral and host proteins complexed to the 5’-positive-strand cloverleaf structure (3CD–3AB–p36) may be transferred to the 3’ end of the negative-strand RNA (Andino et al., 1993; Xiang et al., 1995; Harris et al., 1994; Parsley et al., 1997). In turn, this would facilitate the initiation of positive-strand RNA synthesis from the membrane-anchored negative-strand RNA. Results published recently have shown that the HAV 2C/2BC protein induces membrane rearrangement and is capable of binding eukaryotic membranes and, also, that this membrane-binding ability resides within the N-terminal amphipathic helix of 2C (Kusov et al., 1998; Monika et al., 1988; Teterina et al., 1992, 1997). Thus, like its PV counterpart, HAV 2C is capable also of interacting with both 3’-terminal negative-strand RNA sequences (this report) and cellular membranes. These observations, therefore, reinforce our view that the 2C protein may be involved in anchoring negative-strand RNA into the intracellular membrane structures (membranous replication complexes) and aid in the initiation of positive-strand RNA synthesis. In contrast to our findings, a recent report has shown that the 2C protein from echovirus type 9, also a picornavirus, exhibits nonspecific binding when an RNA sequence (371 bases) was used in the assay (Klein et al., 2000). The precise reason for this discrepancy is not known, but it could be due to the fact that no nonspecific RNA was used in the binding reaction.

The results presented here do not rule out the possibility that the interaction of other viral or host cell proteins with the 5’- and 3’-terminal sequences of viral positive- and/or negative-strand RNA is important for viral genome replication.
Fig. 3. (A) Specificity of HAV interaction with the corresponding UTR$_{100}$ RNA probe. Increasing quantities of cold, homologous RNA representing the 3'-terminal 100 bases (of the negative-strand) and a comparably sized heterologous RNA (obtained from a standard in vitro transcription reaction) were used in the competition assay. This nonspecific RNA was derived from a clone containing a partial hepatitis C virus 5' UTR sequence (Banerjee & Dasgupta, 2001). The integrity of the cold RNAs was confirmed prior to their use. The resulting retarded nucleoprotein complex (labelled C) formed in the presence of competing specific or nonspecific RNAs was analysed, as described in earlier experiments. Lane 1 represents a reaction with no protein, whereas 100 ng of HAV 2C was added to the reactions shown in lanes 2–6. Lanes 3–5 contain 100 and 200 ng of either specific (lanes 3 and 4) or nonspecific (lanes 5 and 6) competing RNA. Lane 6 represents a reaction with no protein. HRV 32P-labelled RNA probe was used as the labelled probe in all lanes. The position of the free probe (labelled F) is indicated. (B) Specificity of HRV 2C. Lane 1 is a control reaction with no competitor RNA, while lanes 2–5 contain 150 and 250 ng of either nonspecific (lanes 2 and 3) or specific (lanes 4 and 5) unlabelled RNA. Lane 6 represents a reaction with no protein. HRV 32P-labelled RNA probe was used as the labelled probe in all lanes. The position of the free probe (labelled F) is indicated. (C, D) UV cross-linking analysis of HRV and HAV 2C interaction with the positive- and negative-strand UTR$_{100}$ probes. UV cross-linking and 32P-label transfer to purified 2C, following incubation, was carried out as described earlier (Banerjee et al., 2000). Samples were mixed with 2× SDS sample buffer (1:1 v/v) and resolved on a 14% denaturing gel. The unevenly numbered lanes represent control reactions and the evenly numbered lanes represent reactions to which HRV (C) or HAV (D) 2C has been added. Lanes 1 and 2 indicate reactions with the positive-strand probe, while lanes 3 and 4 represent reactions containing the negative-strand RNA probe. The labelled proteins in (C) were derived from the HRV RNA, while HAV RNA probes were used in (D). The migration of the [35S]methionine-labelled (in vitro translated) HRV or HAV 2C proteins (obtained from the same expression clone) is shown in the lanes marked R. The numbers on the side of the gels correspond to the migration positions of pre-stained molecular mass markers (kDa). The specific UV-induced ribonucleoprotein complex formed is indicated by the arrow. The lower gel panels in both (C) and (D) show the stability of the two RNA probes used in the presence or absence of 2C analysed on a denaturing gel (8% acrylamide:8 M urea sequencing gel). (E) Analysis of the 2C–UTR interaction. The specific complex observed between the HRV 2C protein and the UTR$_{100}$ RNA probes (as in Fig. 2) was excised and analysed for the presence of 2C. The complexes from three independent binding reactions were pooled and resolved by second dimension SDS–PAGE. A portion of the gel was silver-stained and an identical portion was probed with a monoclonal antibody against the N-terminal T7 tag, following transfer to nitrocellulose. The molecular mass marker proteins were also resolved alongside during the staining process.

In fact, interaction of HAV 3AB and 3ABC with the 5'- and 3'-termini of the HAV RNA as well as binding of host cell proteins to rhinovirus 3'-terminal sequences have been reported (Kusov et al., 1997; Todd et al., 1995; Mellits et al., 1998). Future studies will be directed towards understanding the precise role of the 2C protein in virus replication and mapping the sequence contributing towards the specificity.

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References


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