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Structure and dynamics of the N-terminal half of hepatitis C virus core protein: An intrinsically unstructured protein

Jean-Baptiste Duvignaud [a,b], Christian Savard [b], Rémi Fromentin [b], Nathalie Majeau [b], Denis Leclerc [b,*], Stéphane M. Gagné [a,*]

[a] CREFSIP and Department of Biochemistry and Microbiology, Université Laval, 1030 Avenue de la Médecine, Local 3255, Québec, QC, Canada G1V 0A6
[b] Infectious Diseases Research Centre, CHUL, Université Laval, 2705 Boul. Laurier, Québec, QC, Canada G1V 4G2

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ABSTRACT

Hepatitis C virus core protein plays an important role in the assembly and packaging of the viral genome. We have studied the structure of the N-terminal half of the core protein (C82) which was shown to be sufficient for the formation of nucleocapsid-like particle (NLP) *in vitro* and in yeast. Structural bioinformatics analysis of C82 suggests that it is mostly unstructured. Circular dichroism and structural NMR data indicate that C82 lacks secondary structure. Moreover, NMR relaxation data shows that C82 is highly disordered. These results indicate that the N-terminal half of the HCV core protein belongs to the growing family of intrinsically unstructured proteins (IUP). This explains the tendency of the hepatitis C virus core protein to interact with several host proteins, a well-documented characteristic of IUPs.

Introduction

Hepatitis C virus (HCV) is the only member representing the *hepacivirus* genus of the *flaviviridae*. Flaviviruses have a positive-sense, single-stranded RNA genome of 9.6 kDa [1] composed of one large open reading frame coding for a 3010 amino acid polyprotein [2]. HCV core protein (HCV-C) is located at the N-terminus of the polyprotein [1,3] which is cleaved by host-encoded proteinases to generate an immature and a mature core protein of 191 and 179 amino acids, respectively [4]. The core protein of HCV is larger than other *flaviviridae* which are approximately 100 amino acids long. The N-terminal half of HCV-C was shown to be sufficient for assembly in nucleocapsid-like-particles (NLPs) in presence of structured RNA [5]. Structures of core proteins from two others flaviviruses, the dengue and West Nile viruses, have been characterised by cryomicroscopy, NMR and X-ray crystallography [6–8]. These studies revealed that the structure of these proteins is mostly α-helical, although the N-terminal 20 amino acids of dengue and West Nile viruses were unstructured as shown by protease digestion, X-ray and NMR [6,7]. In contrast with the core of other *flaviviridae*, HCV-C is predicted to contain only few structural elements [9]. Although the three-dimensional structure of HCV-C is still unknown, recent studies have provided insights into the structure of HCV-C [10,11]. Boulant et al. [10] have shown that the whole protein (HCV-C 2-169) adopts an α-helical conformation for nearly 50% of the protein in presence of detergents or lipids. In this study, we have investigated the structure and dynamics of the 82-residues N-terminal half of the HCV-C (C82) in solution. C82 was shown to be sufficient for the formation of nucleocapsid-like particle (NLP) *in vitro* and in yeast [5]. We bring experimental evidence that C82 is in a highly disordered form in aqueous solution. The possibility that the core or the N-terminal half of the protein belongs to the family of intrinsically unstructured proteins (IUP) [12,13] will be discussed.

Materials and methods

Sequence analysis and secondary structure predictions. Amino acid composition of C82 was studied with the ProtParam Tool (www.expasy.org). The mean amino acid composition of 356 194 proteins was obtained from the Swiss-Prot database (UniProtKB/Swiss-Prot protein knowledgebase release 55.0 statistics). We used five algorithms to predict the secondary structure of the HCV-C. The PONDR software was used to predict disorder [14].

* Corresponding authors. Fax: +1 418 656 7176 (S.M. Gagné); fax: +1 418 654 2715 (D. Leclerc).
E-mail addresses: Denis.Leclerc@crchul.ulaval.ca (D. Leclerc), Stephane.Gagne@rsvs.ulaval.ca (S.M. Gagné).

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Expression and purification of C82. The C82 clone was optimized with the most representative codon for translation in *Escherichia coli* and fused to a C-terminal His_6-tag. This optimized C82 was cloned in a pET3d expression vector and overexpressed in the *E. coli* strains BL21 (DE3) Star (Invitrogen) as described previously [5]. The harvested cells were resuspended in a 30 mL ice cold lysis buffer of 50 mM phosphate, 300 mM NaCl at pH 12 with 1× cocktail of protease inhibitors (Roche Diagnostics GmbH), then lysed by sonication. The lysate was centrifuged at 27,000*g* for 30 min. The filtered supernatant was added to Ni–NTA resin (QIAGEN). After 90 min at 4 °C, the beads were washed with 50 mL of three buffers with increasing concentrations of imidazol (up to 20 mM) and NaCl (up to 750 mM). C82 was eluted in either an assembly buffer [5] or an elution buffer (50 mM phosphate, pH 8.0, 300 mM NaCl, 500 mM imidazol). A reversed phase HPLC method was used as the second purification step using a VYDAC C4 column. A step gradient was used as follows: 1 min, 10% acetonitrile; 1–15 min, 20% acetonitrile; 15–35 min, 30% acetonitrile; 35–45 min, 100% acetonitrile. C82 eluted between 15–35 min and was lyophilised. The purity of the protein was estimated by SDS–PAGE and confirmed by Western blot.

Trypsin digestion. Trypsin digestion of pure C82 was as previously described [15]. Trypsin digestion was done at 4 °C with a sample concentrated at ~200 μM. At each interval of the reaction, 10 μl were collected, 1× cocktail of protease inhibitors and SDS were added to stop the reaction, and the sample was heated at 95 °C for 5 min.

In vitro assembly reactions, density gradient centrifugation and electron microscopy. All experiments were carried as previously described [5].

CD spectrum experiments. C82 was lyophilysed and resuspended in the experimental buffer (10 mM phosphate, pH 7.2). CD measurements were carried on an Jasco Model J-710 instrument at room temperature, using 0.1 cm quartz cuvettes. Data were collected and processed using the Jasco software. Deconvolution was achieved with DICHROWEB [16].

NMR Samples. Uniform labeling of C82 was done in either U–^{15}N or U–^{15}N–^{13}C BioExpress Cell Growth Media (CIL, Andover, MA). Lyophilised C82 was resuspended in the NMR buffer (25 mM phosphate pH 6.6, 50 mM NaCl, 0.2 mM DSS, 0.1% NaN_3, and 1× cocktail of protease inhibitors). NMR experiments were carried at a protein concentration of 0.4 mM.

NMR data collection and data processing. All experiments were carried out at 278 K on a 600MHz VARIAN INOVA spectrometer equipped with *Z*-axis pulsed field gradient and triple resonance cryogenic probe. For protein assignment, 2D ^{15}N-HSQC, 3D CBCA (CO)NNH, 3D HNCACB and 3D HNCO were recorded. For relaxation studies, ^{15}N–R_1, ^{15}N–R_2 and {^{1}H}–^{15}N NOE were recorded (details in suppl. mat.). Chemical shift referencing is based on IUPAC recommendations using DSS [17]. All NMR data were processed using NMRPipe [18].

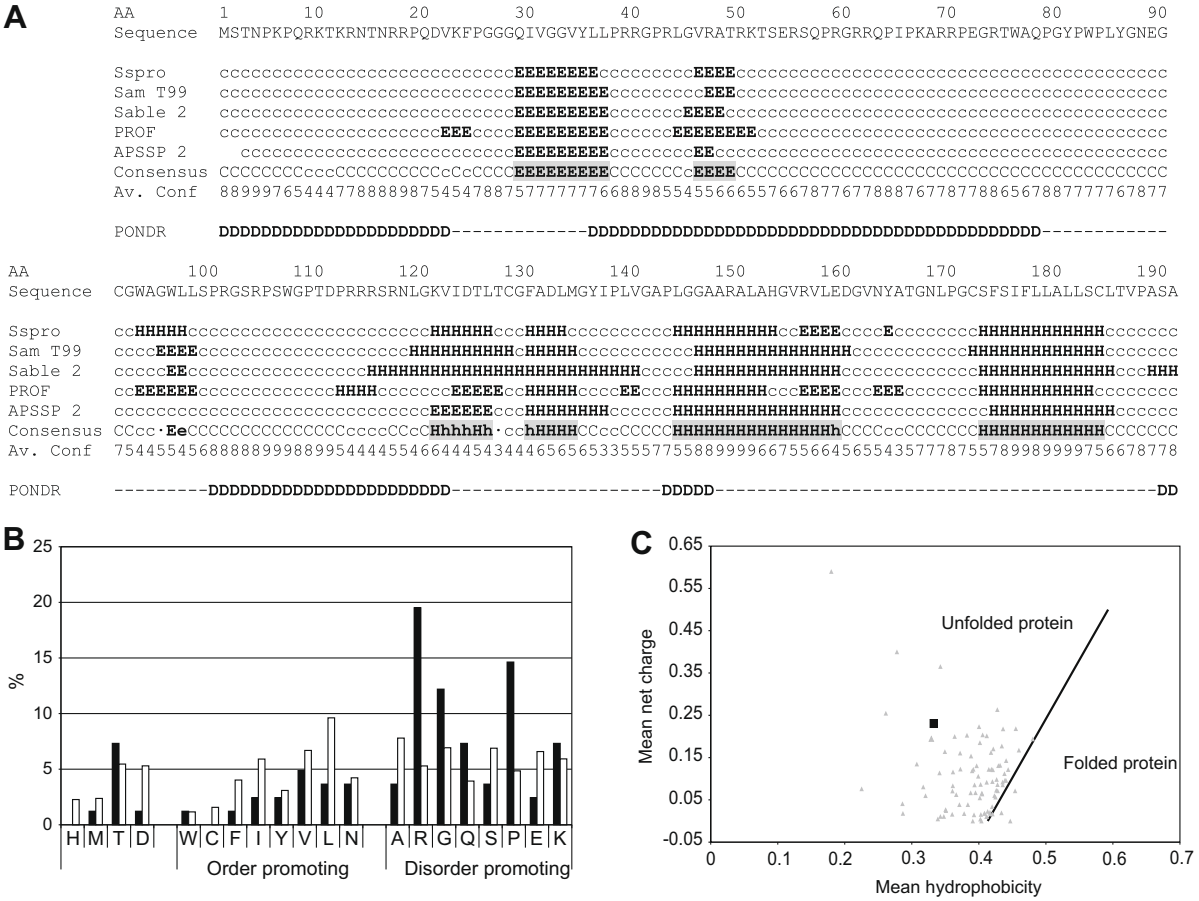

Fig. 1. (A) Secondary structure predictions for C82. Predicted β-strand, α-helix and coil/loop conformations are indicated by the letters E, H and C, respectively. For the consensus predictions, uppercase letters indicate high confidence, lowercase letters indicate lower confidence, and dots (.) indicate ambiguity. Predicted disordered residue from PONDR is indicated by a D [14]. (B) Comparison of the amino acid composition of C82 (black bars) with the average values obtained from the SWISS-Prot database (white bars). (C) Mean net charge/mean hydrophobicity plot. The black line corresponds to the equation: $H = (R + 1.151)/2.785$. C82 is represented by a black square. The gray triangle corresponds to the Uversky bank of IUPs.

Backbone assignment and relaxation analysis. NMR spectra were analyzed with NMRView [19] and assignments (BMRB entry 15767) were done using Smartnotebook [20]. Determination of R_1 and R_2 relaxation rates was done using CURVEFIT (A.G. Palmer, Columbia University, New York, NY). $\{^1H\}$–^{15}N NOE values were obtained directly from the HetNOE analysis function in NMRView. Analysis of relaxation data was performed using the extended model-free formalism [21], using the statistical approach of Mandel et al. [22]. Values for the ^{15}N gyromagnetic ratio, H-N bond length, and chemical shift anisotropy were -2.712×10^7 rad T^{-1} s^{-1}, 1.02 Å, and −172 ppm, respectively. Residues with significant overlap or poor signal-to-noise ratio were discarded, therefore allowing for the characterization of 58 residues.

Results and discussion

Secondary structure prediction and sequence analysis of C82

The first half of the protein is predicted to be quasi-depleted of secondary structure elements. In order to assess whether the lack of predicted secondary structure corresponds to disorder, we used the disorder predictor PONDR. The combination of PONDR and secondary structure predictions strongly suggest that the N-terminal region of HCV-C is largely unstructured and highly disordered (Fig. 1A). Moreover, it is known that amino acid composition in proteins differs in disordered compared to ordered regions [23]. We therefore analyzed the amino acid composition of C82 and compared it to the mean composition of proteins obtained from Swiss-Prot database to verify if it fits this model (Fig. 1B). Amino acids promoting disorder are overrepresented at 71% in C82 versus 48.6% in the databank. As suggested by Uversky et al. [24], a charge-hydrophobicity plot is a useful tool to predict whether a protein is unstructured. The calculated mean net charge of C82 is 0.23 and its mean hydrophobicity is 0.33. The comparison of C82 values with the data bank of known unstructured proteins clearly shows that C82 is in the region of unstructured proteins (Fig. 1C). Moreover, unfolded proteins have a tendency to extreme pI (<5 and >9) [24]; C82 has a pI of 12.2. All predictions strongly suggest that C82 is unstructured, but we nevertheless need experimental validation.

Expression, purification and structural characterization of C82 by CD and NMR spectroscopy

To improve the expression yield in *E. coli*, we optimized the codons of C82, and overproduction was done in the *E. coli* strain BL21 (DE3) Star (Invitrogen) (Fig. 2A, lanes 1 and 2). We used a simple and efficient two-step purification protocol. The quality of the purified samples was adequate for structural analysis by CD spectroscopy and NMR (Fig. 2A, lane 4). To confirm our final product, we performed an SDS–PAGE electrophoresis (Fig. 2A) and an *in vitro* assembly assay showing that the C82 was able to assemble into NLPs in presence of tRNA as seen by electron microscopy (Fig. 2B). A trypsin digestion test indicates that C82 is significantly sensitive to degradation (Fig. 2C)

The C82 CD spectrum shows a minimum near 200 nm indicating that this protein is a random coil that lacks apparent secondary

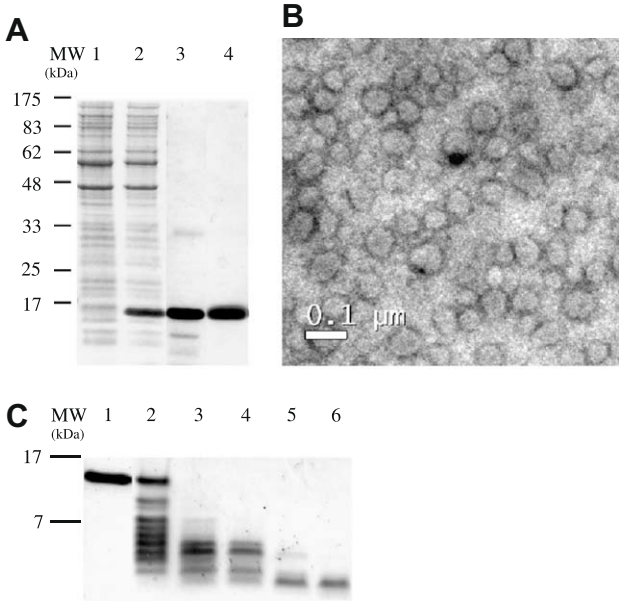

Fig. 2. (A) Purification profile of C82 on a 10% SDS–PAGE. MW, molecular weight; 1, before induction; 2, after 3 h induction; 3, first elution after IMAC; and 4, elution after reverse phase HPLC column. It should be noted that C82 migrates at an abnormal molecular weight of ~15 kDa instead of the expected ~10 kDa. (B) Electron micrograph of nucleocapsid like particles (NLPs) after *in vitro* assembly with the 5' IRES (nt 1–372) of the HCV genome. (C) Trypsin digestion of C82 on a 10% SDS–PAGE. Left to right is the reaction time: $T = 0$, 0.5, 1, 5, 10, and 20 min.

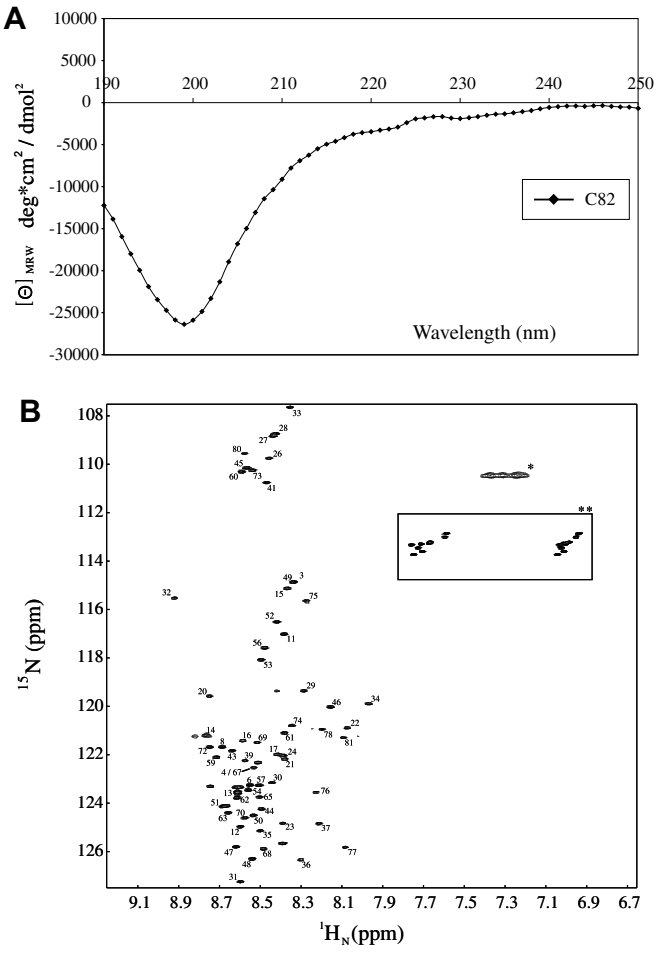

Fig. 3. (A) CD spectra of C82. The mean residue ellipsicity is calculated as follows: $[\Theta]MRW = (\delta \times MRW)/(10 \times c \times l)$, where δ is the ellipticity in degrees, MRW is the mean residue weight, c is the concentration of the sample in g/ml, and l is the path length in cm. (B) Two-dimensional ^1H–^{15}N HSQC spectra of C82 with assignments (large spectra in suppl.mat.). Not shown in this expansion is the NH of the side chain of the tryptophan residue at 10.2 ppm (H_N) and 129.66 ppm (N_H). *Correlations of Arg side chain residues. **Correlations for the side chain of Gln and Asn amino acids.

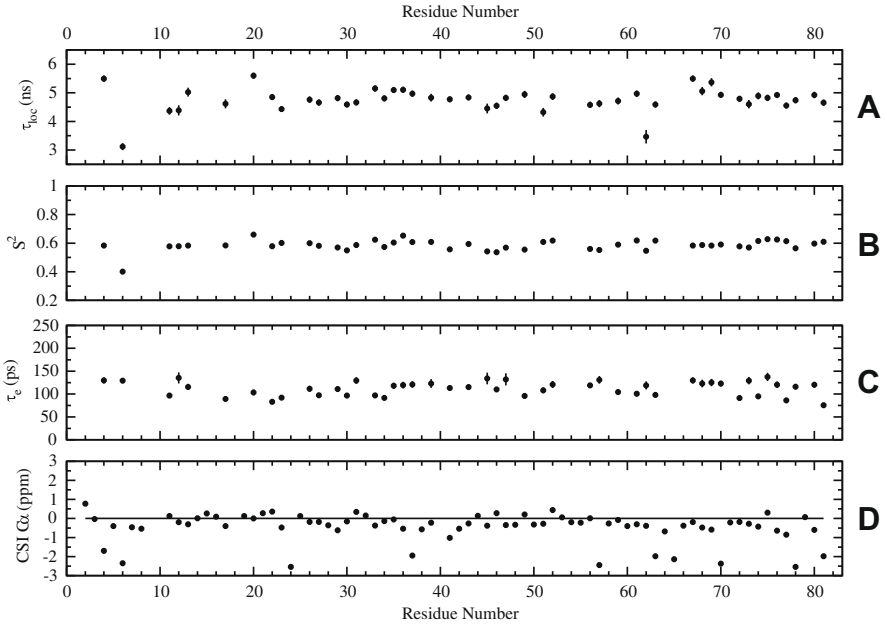

Fig. 4. Sequential NMR dynamic data of C82. (A) Local correlation time (τ_{loc}), (B) order parameters (S^2) obtained with modelfree, (C) effective correlation time for internal motions (τ_e), and (D) chemical shift index plot for C_α.

structure (Fig. 3A). However, C82 is able to organize itself *in vitro* into NLPs in the presence of structured RNA. The encapsidation phenomenon most likely induces a structural change in C82 enabling it to adopt a stable structure. The RNA/C82 mixture created turbidity, which is an indication of the encapsidation phenomenon [25] but these samples cannot be used neither for CD nor NMR spectroscopy. We therefore tested several conditions that could induce a structural change; salt concentration, detergent, and pH. Unfortunately, none of these conditions affected the CD signal (data not shown).

Two dimensional ^{15}N-HSQC (Fig. 3B) and 2D ^{15}N-NOESY-HSQC spectra (suppl. mat.) were recorded. Although there is a poor ^1H_N chemical shift distribution in the HSQC of C82 (0.95 ppm), which is typical of proteins that possess little tertiary structure and are disordered, we observed 63 of the 69 expected peaks. Moreover, the NOESY spectra of C82 contain few NOE correlations which is indicative of the absence of secondary structure.

Backbone assignments of ^1H_N, ^{13}C, and ^{15}N resonances resulted in 93% of non-proline backbone ^1H_N and ^{15}N, 95% of all $^{13}C_\alpha$, 94% of all $^{13}C_\beta$, and 93% of all ^{13}C' assignments being assigned.

The chemical shift index (CSI) [26] was used to assess secondary structure information. As shown in Figure 4D, CSI C_α of C82 is oscillating around zero, suggesting that C82 exists in an unstructured conformation.

Backbone dynamics of C82 by NMR

Relaxation data allow us to determine whether C82 is ordered without regular secondary structure or is simply disordered, as both situations are potentially compatible with the CD data.

Taking into account its molecular weight, C82 has R_1 and R_2 data that are quite typical of an unstructured protein with average values of 1.67 ± 0.06 s^{-1} and 4.96 ± 0.54 s^{-1}, respectively. The NOE values, with an average of 0.19 ± 0.10, are characteristic of an unfolded protein with unrestricted fast dynamics. This was confirmed by a comparison of the measured NOE with other well ordered proteins in the BMRB chemical shift databank (http://www.bmrb.wisc.edu) (suppl. mat.). The model-free approach was used to characterize the global and internal motions of the two samples. Acceptable fit could not be extracted with the use a global correlation time (τ_m) and motional parameters were extracted with a local correlation time τ_{loc} for each residue. 47 residues were better fitted with model 2, the others did not fit with either models 1 and 3, and models 4 and 5 were not considered as they implicate calculations with four variables and only three experimental values are available. The mean τ_{loc} is 4.76 ± 0.54 ns (from 3.1 to 5.6 ns) (Fig. 4A). The optimized dynamics parameters are summarized in Figure 4B and C. If the previous results were suggesting that C82 is unstructured, the order parameters (S^2) unambiguously confirm that C82 is highly disordered. It is interesting to note that the dynamical parameters (S^2 and τ_e, Fig. 4B and C) obtained are exactly in the range of values observed for other unstructured proteins [27]. The dynamical study of the unfolded form of the propeptide of subtilisin results in a S^2 of 0.57 ± 0.06 and a τ_e of 74 ± 11 ps; corresponding values for C82 are 0.59 ± 0.04 and 112 ± 16 ps.

HCV core protein: an IUP

In agreement with predicted data on whole HCV core, experimental data of isolated C82 indicate that the N-terminal half of HCV core protein is disordered in aqueous solution. We therefore conclude that C82, and hence the N-terminal half of whole HCV core, is a member of the IUP family.

IUPs are an important family of proteins that may represent 30% of the proteins of the human genome [28]. IUPs are known to interact with different partners and the disorder and flexibility of these proteins are important to their function. IUPs are generally involved in binding nucleic acids, other proteins, membranes or a large range of small ligands. More than 30 functions have been assigned to IUPs. They can be chaperones, regulatory proteins, proteins involved in cell signalling and, consequently, can be involved in the pathogenesis of cancers [13,28–31].

HCV-C binds to structured RNA and assembles into NLPs. Moreover HCV-C was shown in several reports to interact with several host proteins: the C-terminus of p53 [32], the intracellular domain of lymphotoxin β receptor [33], the DEAD-box protein (DDX3, CAP-Rf) [34], the 14-3-3 protein [35], and the p21Waf1/Cip1/Sid1 [36].

In addition, Cristofari et al. [37] has proposed that the core protein and its truncated forms at the C-terminus are potent nucleic acid chaperones. Consistently, Tompa & Csermely [30] have suggested that structural disorder is present in RNA and protein chaperones. It may be that HCV-C, through its unstructured domain at the N-terminus, induces a defined structure to the viral RNA for packaging of the genomic RNA. Or inversely, the binding of RNA may confers structure to the N-terminal half of the core protein permitting interaction with RNA and homotopic interactions as suggested by Kim et al. [38]. Recently, Ivanyi-Nagy et al. [39] have reported that a few other core proteins of the flavivirus family have this ability to be chaperone, and interestingly, all of these have predicted disordered regions. The dengue and West Nile viruses core proteins were shown to be structured and rich in α-helices with only short sequence (10–20 amino acids) shown to be unstructured [6,7]. Other core proteins of flavivirus family have large unstructured extremities like HCV-C or can be totally unstructured as predicted for BVDV core protein. This is the first experimental report of structural and dynamical studies that shows that a flavivirus nucleocapsid is an IUP.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.10.141.

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