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Molecular and Structural Characterization of the Domain 2 of Hepatitis C Virus Non-structural Protein 5A

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Hepatitis C virus (HCV) non-structural protein 5A protein (NS5A), which consists of three functional domains, is involved in regulating viral replication, interferon resistance, and apoptosis. Recently, the three-dimensional structure of the domain 1 was determined. However, currently the molecular basis for the domains 2 and 3 of HCV NS5A is yet to be defined. Toward this end, we expressed, purified the domain 2 of the NS5A (NS5A-D2), and then performed biochemical and structural studies. The purified domain 2 was active and was able to bind NS5B and PKR, biological partners of NS5A. The results from gel filtration, CD analysis, 1D 1H NMR and 2D 1H-15N heteronuclear single quantum correlation (HSQC) spectroscopy indicate that the domain 2 of NS5A appears to be flexible and disordered.

Keywords: Expression and Purification; Hepatitis C Virus NS5A; NMR; NS5A Domain 2; NS5B.

Introduction

As a worldwide prevalent pathogen, hepatitis C virus (HCV) is responsible for chronic hepatitis, liver cirrhosis and the development of hepatocellular carcinoma (Ohsawa et al., 1999; Okuda, 1998; Trepo et al., 1998). It belongs to the Flaviviridae

Abbreviations: HCV, hepatitis C virus; HSQC, heteronuclear single quantum correlation; NS5A, non-structural protein 5A protein.
family and is single-stranded RNA virus. HCV genomic RNA is approximately 9600 nucleotides long and its single open reading frame encodes a polyprotein precursor of 3010–3033 amino acids (Miller et al., 1990). Cellular signal peptidase and virus-encoded proteases further process the polyprotein to at least ten mature viral proteins/enzymes, in the order of core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B (Miller et al., 1990).

HCV non-structural protein 5A (NS5A) exists in two phosphorylated forms in the infected cells, p56 and p58 (Pawlotsky et al., 1999). Based upon the secondary structure analysis and protease digestion studies, HCV NS5A was predicted to contain domain 1 (amino acids 1–213), domain 2 (amino acids 250–342), and domain 3 (amino acids 356–447) (Tellinghuisen et al., 2004). The first 30 amino acids at the N-terminus of the NS5A is the membrane anchoring domain responsible for localizing the NS5A at the endoplasmic reticulum (Brass et al., 2002; Penin et al., 2002). Recent structural study revealed that the domain 1 (amino acids 36–198) is a novel zinc-coordinating motif and has a disulphide bond (Tellinghuisen et al., 2005). However, currently, little is known about the structural characteristics of the domain 2 and domain 3 of HCV NS5A.

While its functions have not been clearly elucidated, NS5A has been shown to be important in the viral replication as a part of HCV replication complex (Blight et al., 2000; Lohmann et al., 2001). NS5A is also involved in the viral resistance to interferon therapy by binding and interacting with IFN-induced double-stranded RNA-activated PKR (Gale et al., 1997). Recently the anti-apoptotic function of NS5A has been also described (Chung et al., 2003; Gale et al., 1999). In this study we focused our effort on the domain 2 of HCV NS5A (NS5A-D2). The domain 2 appears to participate in several important biological regulations but little is known about its molecular and structural basis. Previously, it was shown that the domain 2 interacts with NS5B, an RNA-dependent RNA polymerase (RdRP), which is essential in viral HCV RNA synthesis and replication (Shirota et al., 2002) and also contains the Interferon (IFN) sensitivity-determining region (ISDR, amino acids 237–276) and the PKR binding domain (amino acids 237–309) (Gale et al., 1997). In addition, a potential Bcl-2 homology region 2 (BH2) was predicted in the domain 2 (Chung et al., 2003).

In view of this, HCV NS5A appears to be multifunctional protein. Here, for a better
understanding of its biological function and molecular basis, we designed and constructed an over-expression system for the domain 2 using ubiquitin and ubiquitin-specific, carboxy-terminal protease (Huang et al., 2004). The system was efficient in over-producing the NS5A-D2 and allowed us to obtain soluble NS5A-D2. With the purified protein, we performed biochemical characterization and structural studies. Our results demonstrate that the purified NS5A-D2 was active in binding NS5B in vitro. Our NMR data appears to suggest that NS5A-D2 exists in a form of intrinsically disordered protein.

Materials and Methods

Materials Monoclonal antibody against NS5A was purchased from ID labs (Canada). Polyclonal antibody against NS5B was purchased from Santa Cruz Biotech (USA). PKR-agarose was from Upstate Biotechnology (USA). Ni$^{2+}$-NTA resin was from Qiagen (Germany). Glutathione-Sepharose 4B was from Amersham Biosciences (UK). Isopropyl-thio-β-D-galactopyranoside (IPTG) was from Promega (USA). HiPrep 26/60 Sephacryl S-200 gel filtration column was from Amersham Biosciences (UK). $^{15}$N-NH$_4$Cl was from Cambridge Isotope Laboratories (USA). NS5B peptides for NMR titration assay were obtained from GL Biochem. Ltd. (China). All the other chemicals were obtained from Sigma (USA).

Plasmid construction The DNA sequence for NS5A-D2 (amino acids 240-335) of HCV NS5A was amplified from HCV-1a cDNA (a generous gift of Dr. Ding Xiang Liu) by polymerase chain reaction using the forward primer (5′-GCGGGTACCCCGCGGTGGAAGGCAACTTGACCCGCAACCAT-3′) which includes a SacII restriction enzyme site, and the reverse primer (5′-GCGGGTACCAAGCTTCTATTAATGGTGGGATGTGGGATGTGGTTCGTAGTCAGGCTTTTACCCAGT-3′) which includes a HindIII restriction enzyme site. The PCR product was cloned into the pET-UbCHis vector (a generous gift of Dr. Craig Cameron). The resulting plasmid, pET-Ub-NS5A-D2-His, codes for the NS5A-D2 and a C-terminal (His)$_6$-tag to facilitate purification. The coding sequence was verified by DNA sequencing. NS5AΔN (a mutant of HCV NS5A lacking the N-terminal 30 amino acids) was also amplified from the HCV-1a cDNA using the forward primer (5′-GCGGGTACCCCGCGGTGGAAGGCAACTTGACCCGCAACCAT-3′) and
the reverse primer (5′- GCGGTACCAAGCTTCTATTAATGTTGATGG-
TGTTGACCAGAGGATCCGCACACGACATCCCTCCGTTGCG-3′). The PCR
product was subcloned into the SacII and HindIII sites of the pET-UbCHis vector,
resulting in the pET-Ub-NS5AΔN-His. The coding sequence was verified by DNA
sequencing.

The cDNA coding for the truncated form of HCV NS5B, which lacks the 21 amino
acids at the C-terminal (hereafter referred to as to NS5Bt), was amplified from the same
HCV-1a cDNA using the forward primer (5′-CACTCAGCATATGTCAG-
TGTCTTATTCCCTGGACAGG-3′) which includes a NdeI restriction enzyme site, and
the reverse primer (5′-GCGATCTCGAGTTACTAGCAGCCCTGCCTCTCT-
GAGACAGA-3′) which includes a XhoI restriction enzyme site. The PCR product
was cloned into the pGEX-4T-1 vector. The resulting plasmid pGEX-4T-1-NS5Bt
was used to express NS5Bt with the N-terminal GST-tag. The coding sequence was
verified by DNA sequencing.

**Expression and purification of NS5A domain 2 (NS5A-D2)** Plasmids pET-Ub-
NS5A-D2-His was co-transformed with pCG1 (generous gift of Prof. Craig Cameron)
which encodes for a yeast ubiquitin C-terminal protease into E. coli BL21 (DE3) cells
in the prescience of 30 μg/ml kanamycin and 25 μg/ml chloramphenicol. Cells were
grown overnight in 25 ml NZCYM supplemented with 30 μg/ml kanamycin, 25 μg/ml
chloramphenicol, and 0.1% dextrose at 37°C. The overnight culture was diluted 100-
fold into 1 L NZCYM with 30 μg/ml kanamycin and 25 μg/ml chloramphenicol, 0.1%
dextrose. The cells were grown at 37°C to the absorbance of 0.8−1.0 at 600 nm, and
protein was induced with the addition of 0.5 mM isopropyl-β-D-
thiogalactopyranoside (IPTG) for additional 4 h at 20°C. Approximately 5 g of the cell
pellet was resuspended in 30 ml lysis buffer (100 mM Tris-Cl, pH 7.0, 200 mM NaCl,
20 mM Imidazole, 1 mM PMSF, 5 mM 2-mercaptoethanol), and lysed by sonication
for 20 min. The disrupted cell lysate was centrifuged at 20,000 × g for 30 min. The
supernatant was loaded onto 1 ml Ni-NTA-agarose column (Qiagen, Germany) pre-
equilibrated with the lysis buffer. The Ni-NTA-agarose column was washed further
with 10 ml of the lysis buffer, followed by 20 ml washing buffer (100 mM Tris-Cl,
pH 7.0, 1 M NaCl, 50 mM Imidazole, 1 mM Phenylmethylsulphonylfluoride (PMSF),
5 mM 2- mercaptoethanol). The purified NS5A-D2 was eluted by elution buffer (100
mM Tris-Cl, pH 6.4, 200 mM NaCl, 500 mM Imidazole, 1 mM PMSF, 5 mM 2-
mercaptoethanol). The eluted protein sample was further purified by high-resolution Sephacryl S-200 gel filtration column (Amersham Biosciences). For the expression and purification of NS5A-D2 for NMR study, uniformly $^{15}$N-labeled NS5A-D2 were expressed in *E. coli* BL21 (DE3) cells grown on M9 media containing $^{15}$NH$_4$Cl, and purified using Ni$^{2+}$-NTA affinity chromatography and the Sephacryl S-200 gel filtration column chromatography. The purified proteins samples were analyzed on 15% SDS-PAGE gel and protein concentration was determined by a protein assay kit from Bio-Rad Laboratories (USA). Proteins were stored at 4°C before use.

**Expression and purification of NS5AΔN** Briefly, plasmids pET-Ub-NS5AΔN-His and pCG1 were co-transformed into *E. coli* BL21 (DE3) cells on plates with 30 μg/ml kanamycin and 25 μg/ml chloramphenicol. The cell growth and induction conditions were same as that of NS5A-D2. The purification of NS5AΔN was performed under the same condition described for NS5A-D2, except for the addition of 0.1% NP40 in all the purification buffers, which is necessary for the stability and solubility of NS5AΔN.

**Expression and purification of GST-NS5Bt** GST-fused HCV NS5Bt protein was expressed and purified as described before (Yamashita *et al*., 1998). Briefly, the plasmid pGET-4T-1-NS5Bt was transformed into *E. coli* BL21 (DE3) cells, and the transformed cells were then cultured in 10 ml LB medium with 100 μg/ml ampicillin at 30°C overnight. The overnight culture was diluted 100-fold into 1 L of LB medium with 100 μg/ml ampicillin and cultured at 30°C until the absorbance at 600 nm reached 0.6−0.7, and the cultures were then induced by 0.4 mM IPTG and incubated at 30°C overnight. The cells from 1 L were harvested by centrifugation and the pellet was resuspended in 30 ml lysis buffer [phosphate-buffered saline (PBS), pH 7.2, 1 mM dithiothreitol (DTT) and 1% Triton X-100]. The suspension was sonicated on ice for 30 min and then centrifuged at 18,000 × g. The supernatant was passed through 1 ml glutathione-Sepharose 4B beads (Amersham Biosciences) equilibrated with the lysis buffer. The beads were washed with lysis buffer and then with 50 mM Tris-HCl, pH 8.0, and 1 mM DTT. The GST-NS5Bt was eluted with 2 ml of elution buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM glutathione, 10 mM DTT, and 0.1% Triton X-100) and then eluted with 2 ml of elution buffer containing 500 mM NaCl. The eluted protein sample was further purified by high-resolution Sephacryl S-200 gel
filtration column (Amersham Biosciences). The purified proteins samples were analyzed on a 10% SDS-PAGE gel and stored at 4°C before use.

**Western blot analysis** The protein samples were separated on a 12% SDS-polyacrylamide gel (PAGE) and then transferred onto polyvinylidenedifluoride (PVDF) membranes. The blotted membranes were treated with blocking solution (TBS with 5% milk), and incubated with the primary antibody – Hepatitis C NS5A monoclonal antibody at 1:1000 dilution, for 16 h at 4°C. The membranes were then incubated with the goat anti-mouse IgG at 1:3000 diluted for 2 h at 4°C. The membranes were rinsed, treated with enhanced chemiluminescent reagent (Bio-Rad Laboratories, USA), and exposed to X-ray films for visualization.

**GST pull-down assay** The binding of NS5A-D2 (or NS5AΔN) with NS5B was examined by GST pull-down assay. Approximately 1 μg GST or GST-NS5Bt was immobilized to 10 μl of glutathione-Sepharose 4B at 4°C for 2 h. The immobilized beads were incubated with approximately 5 μg of NS5A-D2 (or NS5AΔN) in 50 μl of modified GBT buffer [10% glycerol, 50 mM Hapes-NaOH (pH 8.0), 170 mM KCl, 7.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 1% Triton X-100] containing 1% bovine serum albumin. After extensively washing with GBT buffer, the bound proteins were analyzed by Western blot using anti-HCV NS5A monoclonal and HCV NS5B polyclonal antibodies, respectively. The interaction between NS5A-D2 (or NS5AΔN) and PKR was also examined by GST pull-down assay. Approximately 5 μg of NS5A-D2 (or NS5AΔN) was incubated with 24 μl PKR-agarose (Upstate Biotechnology, USA), which binds approximately 1 μg GST-PKR protein. The mixture was incubated in 50 μl of modified GBT buffer [10% glycerol, 50 mM Hapes- NaOH (pH 8.0), 170 mM KCl, 7.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 1% Triton X-100] containing 1% bovine serum albumin. After extensively washing, the bound proteins were analyzed by Western blot using anti-HCV NS5A monoclonal antibody.

**Gel-filtration analysis** Gel-filtration analysis was performed on a Sephacryl S-200 column pre-equilibrated with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0 mM DTT and 0.01% NaN₃. The buffer flow rate is 0.5 ml/min. The molecular weight of the NS5A-D2 determined based upon the Kₘ values [Kₘ = (Vₑ–Vₒ)/ (Vₒ–Vₖ), Vₒ = 37 ml, Vₖ = 125 ml] using the standard proteins: bovine thyroglobulin, 670 kDa; bovine r-globulin 158 kDa; chicken ovalbumin 44 kDa; horse myoglobin 17 kDa; vitamin B12,
Circular dichroism (CD) spectroscopy The CD spectrum of the NS5A-D2 (100 μM) in 20 mM sodium phosphate, pH 6.5, 50 mM sodium chloride buffer was collected at 25°C in the range of 190–250 nm using a Chirascan spectropolarimeter (Applied Photophysics, UK) in continuous scanning mode. The acquisition parameters for the CD spectrum were 20 nm/min with a 4 s response and a 2-nm bandwidth. For the consistency of the experiment, the protein concentration was also determined at the end of the run.

NMR Experiments The unlabeled or uniformly ^15^N-labeled NS5A-D2 samples for NMR experiments were used at a final concentration of 0.6 mM in 20 mM NaPO_4_, pH 6.5, 50 mM NaCl, 1 mM DTT, 0.01% NaN_3_, and 90% H_2_0/10% D_2_0. 1D ^1^H NMR and 2D ^1^H-^15^N heteronuclear single quantum correlation spectroscopy (HSQC) spectra were acquired at 300 K on a Bruker Avance AV700 equipped with a cryoprobe accessory (Bruker, Switzerland). The spectra were processed and analyzed on Linux workstations using Topspin software (Bruker, Switzerland).

NMR titration to study NS5A-D2 and NS5B MK-17 peptide interaction Four regions of NS5B (residues 139–145, 149–155, 365–371, and 382–388) were reported to be important for binding to NS5A (Qin et al., 2001). For NMR study, two peptides derived from NS5B (residues 139–155: MAKNEVFCVQPEKGGRK, hereafter referred as to MK-17; residues 365–388: SCSSNVSVAHGDAGKRVYYLTRDP, hereafter referred as to SP-24) were synthesized (GL Biochem. Ltd., China). NMR experiments were performed on a Bruker 600 MHz with a cryoprobe. Uniformly ^15^N-labeled NS5A-D2 with concentration of 0.2 mM was prepared in a buffer containing 20 mM Na-PO_4_, pH 6.5, 50 mM NaCl, 0.1% NaN_3_, 1 mM DTT. ^15^N-HSQC was performed at 298 K with varying ratio of between ^15^N-labeled protein and the peptides.

Results

Expression and purification of HCV NS5A-D2 As shown in Fig. 1, NS5A-D2 is important for interacting with NS5B, PKR and also it appears to be also associated with interferon resistance and apoptosis. To further define and characterize its function, we over-expressed NS5A-D2 and purified it for biochemical and structural studies. In our study, we used the ubiquitin system to (Huang et al., 2004) over-
express NS5A-D2 protein, where an ubiquitin-specific, carboxy-terminal protease (Ubp1) is constitutively expressed from the second plasmid – pCG1 to cleave ubiquitin off from the fusion protein and released NS5A-D2 protein in cells. Our results showed that the system used in this study was effective in over-expressing the NS5A-D2 and allowed us to purify the NS5A-D2 for further studies (Fig. 2A). The purified protein was stable after storage for more than one month at 4°C. We confirmed the identity of the NS5A-D2 by Western blot analysis (Fig. 2B).

**Molecular interaction between NS5A-D2 and its biological ligands** It has been elucidated that HCV NS5A interacts with HCV NS5B directly as a component of HCV replicase complex (Shirota et al., 2002). To determine whether the NS5A-D2 still maintains the ability to bind NS5B, we performed an *in vitro* binding study between NS5A-D2 and GST-NS5Bt using the GST pull-down assays (Fig. 3C). Both NS5AΔN and NS5A-D2 were pulled down with GST-NS5Bt (Fig. 3C, lane 2 and lane 5) but not with GST (Fig. 3C, lane 3 and lane 6), indicating the specific interaction of NS5A-D2 with NS5B *in vitro*. To further prove that NS5A-D2 is in its functional form, the binding between the purified NS5A-D2 and PKR was also tested by the GST pull-down assay. The result showed that NS5A-D2 interacts with PKR (Fig. 3D, lane 5). This proves that the purified NS5A-D2 is active and functional in interacting with the NS5B and PKR, which are characterized biological partners of HCV NS5A.

**Gel-filtration analysis of NS5A-D2** The theoretical molecular weight of NS5A-D2 (amino acids 240–335) is 11.856 kDa. On the S-200 gel filtration column, the elution volume of this protein is about 74 ml (Fig. 4). Using the equation described, the determined molecular weight of the NS5A-D2 is 20.3 kDa, which is significantly larger than the theoretical one. This suggests that the NS5A-D2 may contain a high degree of unstructured regions or exist in a dimer form under the condition used in our study.

**Secondary structural analysis of NS5A-D2** We recorded CD spectra to analyze the secondary structure of NS5A-D2 (Fig. 5). The spectrum exhibits a minimum at about 205 nm. With a small negative ellipticity at about 225 nm, suggesting that some ordered secondary structures might exist in the NS5A-D2; the typical CD spectrum of α-helices is characterized by a minimum near 208 and 222 nm and the β-sheet
structures yield a minimum at 215 nm, and random coil structures are characterized by a negative peak near 200 nm (Adler et al., 1973). It was observed that the minimum is shifted from 200 to 205 nm, indicating that NS5A-D2 is not composed entirely of random coils. Additionally, the slight negative ellipticity at 225 nm points to a small but detectable contribution of α-helical structures.

**Analysis of NS5A-D2 by NMR**

NMR is a valuable tool to identify intrinsically unstructured proteins. In our study, to further define the structural characteristics of the NS5A-D2, we performed 1D $^1$H and 2D $^1$H-$^{15}$N HSQC NMR spectroscopy (Figs. 6A and 6B). The analysis of the dispersion of NMR resonance signals in the regions of methyl protons (−0.5~1.5 ppm), α-protons (3.5−6 ppm), and amide protons (6−10 ppm) is a good indicator of folded globular proteins (Page et al., 2005). Our NMR data demonstrated that the 1D $^1$H spectrum of NS5A-D2 lacks NMR signals in the region of the methyl protons and the 2D $^1$H-$^{15}$N HSQC spectrum show a narrow dispersion in the backbone amide proton resonance signals which only covers the range of 6.7−8.5 ppm. Taken together, our data suggest that the NS5A-D2 is natively disordered or dynamic and is not well structured.

Based upon the reported sequences of NS5B essential for binding to NS5A, we designed two peptides derived from residues 139−155 and 365−388 of NS5B, for NMR titration study. The NMR result showed that some perturbations in the chemical shifts were detected in the $^{15}$N-HSQC spectrum of $^{15}$N labeled NS5A-D2 protein upon the addition of NS5B MK-17 peptide (Figs. 7A and 7B), indicating that the purified NS5A-D2 shows binding to the peptide. In the meantime, the titration with the peptide SP-24 caused a heavy precipitation upon its addition (data not shown).

**Discussion**

HCV NS5A is essential in HCV life cycle but its precise function is currently not well defined. The NS5A-D2 appears to be critical in NS5A’s function. In our current study, to characterize the biochemical behavior of the NS5A and define its structural basis, we designed an over-expression system of the domain 2 of NS5A which allowed us to consistently obtain soluble and stable NS5A-D2. Our gel-filtration study, CD, 1D $^1$H NMR and 2D $^1$H-$^{15}$N HSQC analyses (Figs. 4−6), collectively, suggest
that the NS5A-D2 appears to contain high amount of flexible and unstructured regions with potentially small amount of α-helices. It has been shown that the non-structured regions of proteins are essential for protein functions (Bode et al., 1978). Recently, it is emerging that the occurrence of intrinsically disordered proteins or intrinsically unstructured proteins in functional proteins is increasing. More than 100 proteins including Bcl-2, p53, and eIF1A have been reported to have intrinsically disordered regions (Dunker et al., 2001; Tompa, 2002; Uversky, 2002). In their native states, these protein domains or regions are disordered or contain large unstructured area. It was shown that HCV NS5A has a modulating role in the HCV replication, through regulating NS5B activity (Qin et al., 2001; Shirota et al., 2002). More recent analysis addressed the molecular interaction of NS5A with NS5B during HCV RNA replication in a HCV replicon model system. The deletions of the regions within NS5A essential to the interaction with NS5B (amino acids 105–162, amino acids 277–334) can cause the subgenomic replicon to be non-functional, whereas the one with deletion having no effect on NS5B binding was replication competent (Shimakami et al., 2004). This strongly suggests that the interaction between NS5A and NS5B is critical for the HCV RNA replication. Our in vitro binding assay showed that even though NS5A-D2 is natively unstructured, it still active and maintains the ability to bind the NS5B like full length NS5A does (Fig. 3). This is consistent with the previous studies indicating that NS5A-D2 plays an important role in regulating virus replication by making complex with NS5B. Natively unstructured proteins are allowed to interact with more associating partners (Liu et al., 2002; Wright et al., 1999). NS5A-D2 contains regions which were shown to be is a part of ISDR, a potential BH2 domain homologue, and a possible binding site for PKR. With the unstructured nature of NS5A-D2 observed from this study, we performed an NMR experiment to examine whether a conformational change in NS5A could be induced upon the addition of the peptides derived from NS5B (Fig. 7). Our results indicated that the peptide can bind NS5A-D2. However, the chemical shifts perturbations detected were localized to the limited number of residues in NS5A. This result suggests that the molecular interaction between NS5A-D2 and the MK-17 peptide doesn’t appear to induce a significant conformational change in NS5A. It is possible that other regions of NS5B or other ligands might be necessary for changing the conformation of NS5A-D2. Whether NS5A-D2 natively exists in a disordered state and its disordered nature of the NS5A-D2 is important for the biological function of
NS5A remain to be further explored.

**Acknowledgments** We would like to thank Prof. Cameron for providing us with pET-UbNHis, pET-UbCHis, and pCG1. We also thank Prof. Ding Xiang Liu for HCV-1a cDNA.
References


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Fig. 1  Schematic of the HCV NS5A domain structure. Trypsin cleavage sites are indicated at residues 215 and 355. H1, N-terminal α-Helical membrane anchoring domain. BH, Bcl-2 homology region; NS5B-binding region; ISDR, interferon sensitivity-determining region; PKR, RNA-dependent protein kinase interacting region.

Fig. 2  Expression, purification and Western blot analysis of NS5A-D2. A. Protein samples were analyzed on a 15% SDSPAGE gel. Lane 1, molecular weight marker; lane 2, uninduced cells; lane 3, cells after 4 h induction; lane 4, sample loaded on Ni-NTA column; lane 5, eluted fraction from Ni-NTA column; lane 6, protein further purified by S200 gel filtration. B. Western blot analysis of the NS5A-D2. Lane 1, uninduced cells; lane 2, cells after induction; lane 3, purified NS5A-D2.

Fig. 3  In vitro binding studies of NS5A-D2 and NS5B, NS5A-D2 and PKR by GST pull-down assay. A. Purified NS5AΔN (lane 2) and NS5A-D2 (lane 3) were separated on a 12% SDSPAGE and detected by Coomassie Brilliant Blue staining. B. Purified GST-NS5Bt (lane 2) were separated on a 10% SDSPAGE and detected by Coomassie Brilliant Blue staining. C. 5 μg NS5AΔN or NS5A-D2 was mixed with 1 μg of GST-NS5Bt and pulled downed with 10 μl GST resin after preblocking by 1% bovine serum albumin. After washing with GBT buffer, each bound protein was analyzed by Western blot with anti-HCV NS5A monoclonal antibody. Lane 1, NS5AΔN input; lane 2, after loading NS5AΔN, protein bound to the GST resin immobilized with GST-NS5Bt; lane 3, after loading NS5AΔN, protein bound to the GST resin immobilized with GST; lane 4, NS5A-D2 input; lane 5, after loading NS5A-D2, protein bound to the GST resin immobilized with GST-NS5Bt; lane 6, after loading NS5A-D2, protein bound to the GST resin immobilized with GST. D. 5 μg NS5AΔN or NS5A-D2 was mixed with 24 μl PKR-agarose beads (1 μg GST-PKR bound) after preblocking by 1% bovine serum albumin. After extensive washing, each bound protein was analyzed by western blot with anti-HCV NS5A monoclonal antibody. Lane 1, NS5AΔN input; lane 2, after loading NS5AΔN, protein bound to the PKR agarose beads; lane 3, after loading NS5AΔN, protein bound
to the GST resin immobilized with GST; lane 4, NS5A-D2 input; lane 5, after loading NS5A-D2, protein bound to the PKR-agarose beads; lane 6, after loading NS5A-D2, protein bound to the GST resin immobilized with GST. E. Western blot analysis of the binding of GST-NS5Bt (1 μg) to GST resin by anti-HCV NS5B polyclonal antibody. Lane 1 and lane 4, GST-NS5Bt input; lane 2 and lane 5, protein bound to the GST resin immobilized with GST-NS5Bt; lane 3 and lane 6, protein bound to the GST resin immobilized with GST.

Fig. 4 Gel filtration analysis of NS5A-D2. The Ni-NTA-purified NS5A-D2 was loaded onto a S200 gel filtration column. The arrow indicates the elution position of NS5A-D2. The $K_{av}$ [$K_{av} = (V_e - V_o)/(V_t - V_o)$, $V_e = 74$ ml, $V_o = 37$ ml, $V_t = 125$ ml] of NS5A-D2 was fitted to the standard curve (right above the gel filtration figure). The molecular weight of NS5A-D2 was calculated by fitting its $K_{av}$ to the equation $K_{av} = -0.40907 \log$ (molecular weight) + 2.182250.

Fig. 5 Analysis of NS5A-D2 by CD. The CD spectrum was recorded using the S200-purified NS5A (0.1 mM) in 20 mM sodium phosphate, pH 6.5, 50 mM sodium chloride.

Fig. 6 Analysis of NS5A-D2 by NMR. The NMR samples contained 0.6 mM of either unlabeled or uniformly $^{15}$N-labeled NS5A-D2. A. 1D $^1$H spectrum of the NS5A-D2. B. 2D $^1$H-$^{15}$N HSQC spectrum for NS5A-D2.

Fig. 7 NS5A-D2 interacts with NS5B MK-17 peptide in a NMR-based titration assay. A. $^{15}$N-labeled NS5A-D2 was monitored on a 2D $^1$H-$^{15}$N HSQC upon the addition of the unlabeled NS5B MK-17 peptide. The spectrum of free NS5A-D2 is shown in blue. The spectrum of NS5A-D2 with the addition of NS5B MK-17 peptide is shown in red. Concentrations of $^{15}$N-labeled NS5A-D2 and NS5B MK-17 peptide was 0.2 mM and 1.6 mM, respectively. B. Section of the $^{15}$N-HSQC spectrum recorded. The chemical shift of the indicated amino acid was changed upon the addition of increasing amount of MK-17 peptide from 0 to 1.6 mM.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5