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The Three Dimensional Structure and Interaction Studies of HCV p7 in DHPC by Solution NMR

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Abstract

Hepatitis C Virus (HCV) protein p7 plays an important role in the assembly and release of mature virus particles. This small 63-residue membrane protein has been shown to induce channel activity, which may contribute to its functions. p7 is highly conserved throughout the entire range of HCV genotypes, which contributes to making p7 a potential target for anti-viral drugs. The secondary structure of p7 from the J4 genotype and the tilt angles of the helices within bilayers have been previously characterized by NMR. Here we describe the three-dimensional structure of p7 in short chain phospholipid (DHPC) micelles, which provide a reasonably effective membrane-mimicking environment that is compatible with solution NMR experiments. Using a combination of chemical shifts and residual dipolar couplings we determined the structure of p7 using an implicit membrane potential combining both CS-Rosetta decoys and Xplor-NIH refinement. The final set of structures has a backbone RMSD of 2.18 Å. Molecular dynamic simulations in NAMD indicate that several side chain interactions might be taking place, and that these could affect the dynamics of the protein. In addition to probing the dynamics of p7, several drug-protein and protein-protein interactions were evaluated. Established channel-blocking compounds such as amantadine, hexamethylene amiloride (HMA), and long alkyl-chain iminosugar derivatives inhibit the ion channel activity of p7. It has also been shown that the protein interacts with the HCV non-structural protein 2 (NS2) at the endoplasmic reticulum, and that this interaction may be important for the infectivity of the virus. Changes in the chemical shift frequencies of solution NMR spectra identify the residues taking part in these interactions.

Keywords

Hepatitis C Virus; HCV; p7; NS2; solution NMR spectroscopy; membrane protein structure; micelles

INTRODUCTION

Hepatitis C virus (HCV) infects over 150 million humans, and causes over 350,000 deaths per year (World Health Organization 2012, www.who.int). It is a member of the *Hepacivirus* genus within the Flaviviridae family[1]. Currently, no vaccines exist for HCV and the treatment regime of interferon-α and ribavirin is poorly tolerated, costly, and only effective in approximately 50% of patients. Recent trials of drugs that inhibit viral enzymes have been disappointing because of the rapid development of resistance by the virus[2-4]. Due to the high variability in HCV genotypes, new treatments in all likelihood will require combinations drugs targeted to multiple viral proteins in order to minimize the development

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of resistance. The membrane protein described here, p7, provides a novel target that may function in such a setting.

HCV is an enveloped virus with a positive single-strand of RNA genome of approximately 9.6 kb, which is translated into a 3000-amino acid polyprotein that is cleaved by intracellular and viral proteases to yield 10 mature proteins[5, 6]. These proteins are divided into two classes, structural and nonstructural (NS) proteins. The structural proteins core and envelope glycoproteins, E1 and E2, comprise the virion. The nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B modulate host metabolism and replication of viral RNA. Notably, the nucleotide sequence for a small 63-amino acid hydrophobic transmembrane protein, p7, is located between the sequences that code for the structural and the nonstructural proteins[5].

p7 has previously been shown to be essential for efficient virus particle assembly and release, but not required for RNA replication[6]. p7 is localized to the endoplasmic reticulum. Under the conditions used for electron microscopy the protein appears to oligomerize as heptamers or hexamers[7, 8]. However, SDS gels and narrow NMR linewidths are consistent with the protein being a monomer in micelles. The oligomers may account for the apparent channel activity of p7, which has been demonstrated in phospholipid bilayers[9, 10]. However, the role of the ion channel activity in the viral lifecycle remains uncertain, it can be blocked by a variety of compounds including amantadine, hexamethylene amiloride (HMA), and a number of iminosugar derivatives demonstrating the oligomeric form is a potential drug target[9-13].

The overall structure of p7 consists of two trans-membrane (TM) segments, TM1 and TM2, connected by a short, conserved inter-helical loop[14, 15]. However, the structure is more complex than this. As indicated in Figure 1C, residual dipolar couplings (RDCs) have identified seven distinct structural elements in the protein including four helical regions that constitute the two trans-membrane segments[16]. The first segment (TM1) spans from residue 6 to residue 26, and the second segment (TM2) from residue 38 to residue 58. The protein's termini face the lumen of the ER with the inter-helical loop region protruding into the cytosol[14, 15]. Previous NMR studies have also shown that the helices are tilted about 10° and 25° from the bilayer normal[17]. The magnitudes of the RDCs also revealed the presence of substantial backbone motions in the first and third helical regions of the protein. In particular, tryptophan 48 and proline 49 appear to be highly dynamic. Interestingly this tryptophan residue is strictly conserved throughout the genotypes of p7.

Recent results suggest that p7 is critical for functions in virus assembly unrelated to its channel activity[18]. Furthermore, p7 may act in unison with the HCV proteins E1, E2 and NS2, suggesting that its biological functions and possibly its ion channel activity are regulated by specific protein-protein interactions[19]. A goal of our structural studies is to determine how specific protein-protein interactions, such as between p7 and NS2, impact channel activity, virion assembly, and the release of viral progeny.

It is important to acknowledge the uncertainties associated with studying membrane proteins in any membrane-mimicking environment other than phospholipid bilayers. However, we have extensive experience with these types of samples[20], and have found that the short chain phospholipid, DHPC, is a generally good choice as a minimally perturbing detergent to solubilize p7 for solution NMR spectroscopy. Except for the length of its hydrocarbon chains, its chemical properties are identical to those of the phospholipids that assemble into bilayers, in particular it has two hydrophobic chains connected to a phosphocholine headgroup.

RDCs are highly reliable sources of structural information about helical membrane proteins because they report on the regular patterns of secondary structure as well as the backbone dynamics of the protein. Moreover, RDCs are important because there are major limitations in measuring 'long-range' nuclear Overhauser enhancements (NOEs) among hydrogens in helical membrane proteins in micelles[21]. Tryptophan 48 was investigated not only due to its anomalous location, tryptophan residues are typically located at the interface between headgroups and hydrophobic residues of phospholipids, but also to follow-up on some preliminary calculation-based evidence that the dynamics of the third helical region was attenuated by mutating this to a non-aromatic residue. Additionally, other interactions of this residue were investigated. Specifically, the interactions of tryptophan 48 with the proximal tyrosine residues 31, 42 and 45 were examined.

Additional structural characterization of p7 was obtained through the observation of paramagnetic relaxation effects (PRE) from manganese ions in solution. This allowed for the identification of, with some degree of certainty, the residues that are accessible to the aqueous solution. The experiments also provided information about the effect of binding by the channel blocking compounds amantadine, iminosugar derivative *N*-nonyl-deoxynojirimycin (*N*N-DNJ), and 5-(N,N-Hexamethylene)amiloride (HMA). Previously, Griffin et al. demonstrated that amantadine could block p7 ion channel activity *in vitro*[11]. Steinmann et al. discovered that the effects of amantadine *in vivo* were associated with specific genotypes. They also showed that deoxynojirimycin (DNJ)-containing iminosugars, such as *N*N-DNJ, had inhibitory affects *in vitro*[3]. These effects not only interfered with HCV entry into host cells but also significantly inhibited virus assembly and release. Premkumar et al. also demonstrated the ability of HMA to block p7 ion channel activity[12]. By observing the chemical shifts of individual residues it is possible to identify which residues in a protein are involved in binding, possibly assisting the design of future compounds that exploit these known interactions.

The non-structural protein 2 (NS2) interacts with several other HCV proteins including p7[18,22]. The topology of the first TM of NS2 and location of these interactions were investigated using a combination of isotopically labeled and unlabeled trans-membrane domains of both p7 and NS2. This method provides a way of looking at the effect on each of the proteins without complicating the spectra. These experiments provide a starting point for examining the interactions of the full-length versions of both proteins.

EXPERIMENTAL METHODS

Protein Expression and Purification (p7, p7W48A and NS2TM1)

Samples of p7 from genotype J4 were prepared as previously described[23]. The mutant W48A was prepared using a Quikchange Lightning Site-Directed Mutagenesis Kit (Agilent, www.agilent.com) and primers synthesized (Allele) to replace the tryptophan at position 48 with alanine. The two primers used were GCTGCTTACGCTTTCTACGGTGTTGCGCCGCTGCTGCTGCTGCTGC and GCAGCAGCAGCAGCAGCGGCGCAACACCGTAGAAAGCGTAAGCAGC. The codons in the mutation site are underlined. The DNA was transformed into XL10 Gold Competent cells (Agilent) and plated on LB agar. The mutation was confirmed by sequence analysis (Eton Biosciences, www.etonbio.com) of individual colonies. The DNA was purified from an overnight LB growth with the cells containing the correct sequence and transformed into BL21(DE3) (Agilent) cells for protein expression. The protein was expressed and purified the same way as the wild-type protein using nickel affinity chromatography, chemical cleavage from the fusion protein and reverse-phase HPLC (Waters, www.waters.com).

The same expression vector and purification protocol as used for p7 were applied to a construct corresponding to the first trans-membrane helix of the non-structural protein NS2 of HCV. NS2 TM1 was cloned using genotype J4, the same genotype that was used for p7. Oligonucleotides were purchased from Allele (www.allelebiotech.com) to clone the amino acid sequence LDREMAAASSGGAVLVGLVFLTLSPYYK. Forward primer CGGGGAAGCTTATGCTTGACCGTGAAGCTGCTGCTTCTTCTGGTGGTGCTGTTCT GGTT GGTCTGGTTTTCCTGACCCTGTCTCCG and reverse primer CCCCGGATCCTCATCATTTTTTTTTTTTTTTTTTGTAGTACGGAGACAGGGTCAGG AAAAC CAGACCAACCAGAACAGCACCACC were elongated to obtain the insert product. The DNA was cut and ligated into the pHLV TrpLE vector using the restriction enzymes HindIII and BamHI (New England Bioloabs, www.neb.com). The insert included a codon for a methionine at the N-terminus of the protein for chemical cleavage and an additional 5 lysines at the C-terminus to assist with the solubility of the protein during purification and sample preparation. Milligram amounts of the labeled polypeptide were prepared for NMR experiments.

Sample preparation

Samples for solution NMR experiments were prepared as described previously[23]. Briefly, the purified protein, dried following HPLC purification, was dissolved in 400 mM DHPC (Avanti Polar Lipids Inc., www.avantilipids.com) and diluted to an aqueous solution with a final concentration of 125 mM DHPC. D_2O was added to 10% to provide a signal for the spectrometer lock, and the final protein concentration was 0.5 mM. The pH was adjusted to 4.0 for all samples to ensure NMR spectra were well resolved and directly comparable to those analyzed previously[23]. The protein sample was transferred to a standard 5mm tube for the NMR experiments.

For RDC measurements, the isotropic sample was transferred to a Shigemi (Shigemi Inc., www.shigeminmr.com) tube containing a dried 6% neutral polyacrylamide gel that was originally cast in a 3 mm NMR tube and cut to 3 cm in length. The plunger of the NMR tube was set to a length of 2.1 cm to allow the gel to absorb the isotropic protein-containing sample in 125 mM DHPC. The compressed gel resulted in weak alignment of the protein in the NMR sample.

The samples for PRE measurements were prepared by adding chelated manganese to unaligned samples of p7-containing DHPC in aqueous solution. The chelated metal was prepared by adding manganese sulfate to ethylenediaminetetraacetic acid (EDTA) (0.5 M) at pH 8.0 and allowed sufficient time for a chelated precipitate to form. The precipitate was centrifuged and the pellet was washed twice with methanol and once with ethanol. The washed complex was dried by lyophilization and a stock solution of 200 mM was prepared in 1M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. The solution was added to the NMR sample at a final Mn^{2+}-EDTA concentration of 10 mM. To make sure that the addition of the buffer did not affect the isotropic signals, a sample with just the buffer added was used as a control.

NMR Experimental Measurements

The NMR experiments were performed on a 600 MHz Bruker spectrometer (www.bruker-biospin.com) using a triple resonance cryoprobe (^1H, ^{13}C, and ^{15}N) with three-axis pulsed gradients. The chemical shift frequencies were referenced to the proton resonance of water set to 4.70 ppm. The experiments were performed at 50°C. Most of the ^1H-^{15}N correlation experiments were performed using the fast-HSQC pulse sequence [24].Typical experiments involved the acquisition of 1024 t2 points for 256 t1 increments.

The backbone amide J couplings were measured using a modified ^{1}H-^{15}N HSQC experiment based on the in-phase/anti-phase (IPAP) measurement of JD-splitting. The RDCs were measured by taking the difference in JD coupling between an isotropic and a weakly aligned sample of p7-containing DHPC micelles.

The NMR data were processed using NMRPipe[25], and the figures were prepared using Sparky (T.D. Goddard and D.G. Kneller, SPARKY 3, University of California, San Francisco). RDCs were analyzed using Matlab (Mathworks, www.mathworks.com). A sliding window method was used to fit sinusoidal waves of a periodicity of 3.6 residues to the data to predict helical regions, as described previously[16].

Structure Calculations

A two-stage combined protocol[26, 27] was employed to calculate the structure of p7 in DHPC micelles. The determination of the structure is illustrated in Figure 2. In the first stage, 10,000 initial structures were generated in CS-Rosetta[28]. Fragment candidates were based on 58 HN, 61 N and 63 Cα chemical shift frequencies. All of the structures were relaxed under the influence of the of NH RDC data using Rosetta. In both steps, the implicit membrane potential flag was on in order to mimic a membrane-like environment. After relaxation, the 1,000 lowest energy structures were chosen from the most populated cluster, which was calculated from 10,000 structures. Forty percent of the 10,000 relaxed structures fell into the most populated cluster, while the second most favored cluster contained 22% of the structures. The lowest energy structure identified in the subset of 1,000 selected structures was used as the initial structural model of p7. Average dihedral angles and their deviations were calculated from the group of 1,000 selected low energy structures for further refinement using Xplor-NIH.

In the second stage, structure refinement was performed using a simulated annealing protocol with Xplor-NIH torsion angle molecular dynamics and the experimental restraints. The temperature was reduced from the initial value of 1000K to 50K in steps of 12.5 K. k_{ta} = 200 kcal·mol^{-1}·rad^{-2}, k_{rdc} was gradually increased from 2 kcal·mol^{-1}·rad^{-2} to 5 kcal·mol^{-1}·rad^{-2}. The calculation also includes the Xplor-NIH potential for knowledge-based torsion angles with ramped force constants of 0.002 kcal·mol^{-1}·rad^{-2} to 1 kcal·mol^{-1}·rad^{-2}. k_{vdw} and s_{vdw} were geometrically increased from 0.004 kcal·mol^{-1}·Å^{-4} to 4 kcal·mol^{-1}·Å^{-4}, and 0.9 kcal·mol^{-1}·Å^{-4} to 0.8 kcal·mol^{-1}·Å^{-4}, respectively. A total of 100 structures were calculated, and the 10 lowest energy structures were accepted for analysis.

Molecular Dynamics Simulations

1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) bilayer generated and solvated with TIP3P waters in VMD[29] was equilibrated for 1 ns. The lowest energy structure from the Xplor-NIH refinement was selected. After embedding p7 into the *de novo* bilayer, the overlapped water and lipid molecules were removed. This resulted in 80 lipid molecules and 12669 water molecules. A 50 ns simulation was performed after 10000 steps of minimization at 310 K and a constant pressure in NAMD[30] with CHARMM27 force field.

Drug Binding Studies

Drug binding studies were performed using amantadine (Sigma Aldrich, www.sigmaaldrich.com), hexylmethylamiloride (HMA, Sigma Aldrich) and N-nonyl-deoxynojirimycin (*N*N-DNJ, Toronto Chemical Company, www.trc-canada.com). Experiments were performed by adding the appropriate compound, either in water or DMSO, at a concentration that was 20× greater (10mM) than that of the protein (0.5 mM). The pH of the sample was measured to ensure that the sample conditions were consistent with the control experiment. A ^{1}H-^{15}N HSQC experiment was performed using the same

parameters as the control. The chemical shifts of the protein resonances were measured. Difference plots were made that utilized both ^{1}H and ^{15}N shifts using the equation $[(\Delta\delta H)^2+(\Delta\delta N/5)^2]^{1/2}$.

Results

Chemical shifts and residual dipolar couplings

The amide proton and nitrogen shifts were measured from the two-dimensional $^{1}H/^{15}N$ HSQC spectra, while the alpha carbon chemical shifts were measured from the three-dimensional HNCA spectra that were used to assign the protein resonances. In this experiment the magnetization is transferred from the amide proton to the amide nitrogen, and then to the alpha carbons of the same residue and the preceding residue. The magnetization pathway provides the chemical shifts of the carbon nuclei and assigns the backbone resonances. The dispersion of resonances in the HSQC spectra demonstrated that the protein is folded in the DHPC micelles, and a plot of the Cα chemical shifts as a function of residue number gives a preliminary indication of the secondary structure being composed of mostly α-helices based on the upfield chemical shifts from random coil values.

As described under “Experimental Methods” and in previous publications, p7 could be weakly aligned in a compressed polyacrylamide gel for RDC measurements[31]. The residual dipolar couplings were measured for all of the backbone amide sites using an IPAP ^{1}H-^{15}N HSQC experiment on an isotropic sample and a weakly aligned gel sample (Figure 1A and B). The differences in the measured splittings, which ranged from +4 Hz to −8 Hz, were plotted as a function of residue number (Figure 1C). This was possible because all of the resonances have been assigned to specific backbone sites in the protein[16]. Sinusoidal waves were fit to the data using a periodicity of 3.6 residues per turn, appropriate for an α-helix, to identify those residues in helical regions of the protein[32]. Four separate waves resulted from the fitting procedure, as shown in Figure 1C. The amplitudes of the waves suggest that the helical segments labeled ‘a’ and ‘c’ have internal motions due to generally low values of residual dipolar couplings. Helices ‘b’ and ‘d’ appear to be fully structured on the NMR timescales. This result is in general agreement with the previous analysis of secondary structure of p7[31].

Structure Calculations

For the structure calculations of p7, the chemical shift associated Molecular Fragment Replacement (MFR) method was used to obtain a starting structure that could be refined using RDC data as constraints. The initial set of structures calculated from Rosetta (Figure 2A) utilized the primary sequence of p7. The set shows convergence of the polypeptide backbone structure. An interesting feature, confirmed by the structure calculations, is that W48, a residue that is normally at the interface of the polar headgroups and the hydrophobic sidechains of the lipids, is buried within the lipid bilayer in p7. When the RDC data were added as constraints to the calculation, the largest change was observed in the orientation of helical segment ‘a’ (Figure 2B). Consequently, the relative orientation of the two transmembrane segments changed. The refinement of these structures in Xplor-NIH using the same constraints caused the structures to converge with a RMSD of the backbone atoms of 2.18 Å (Figure 2C). An average structure from the cluster of 10 lowest energy structures was calculated (Figure 2D).

The average structure is shown in Figure 3 from three different perspectives. From these views the orientation of the four helices can be seen. Helix ‘a’ has a different orientation than that of helix ‘b’ and resides almost perpendicular to the membrane spanning segments. Helices ‘c’ and ‘d’ are clearly separated by several residues that include Trp48 and Pro49.

Manganese data compare with structure

The PRE data[16] were plotted in the form of measured peak intensity as a function of residue number (Figure 4A). Resonance intensities that were reduced by at least 50% are highlighted on the structure of p7 (Figure 4B). The residues that are exposed to the aqueous solvent are strongly affected by the addition of the EDTA chelated paramagnetic metal. The intensities of the region between the first and second helical segments, 'a' and 'b' (residues 15-18), are reduced, suggesting that these residues are accessible to the chelated manganese in solution. This also suggests that the first helical segment is somewhat shielded from the effects of the chelated manganese within the polar head groups of the lipids. As illustrated in Figure 4, the loop region (residues 28-36) and the C-termini (residues 58-63) are also exposed to aqueous solution.

Simulations

To examine the dynamics of p7 in a lipid environment, the refined structure of p7 was placed in a *de novo* membrane made of POPC, and allowed to relax over a period of 50 ns. Due to the chain length difference of the lipids used for NMR experiments (6 carbons) and the simulations (16 and 18) some differences in the overall structure are anticipated. These differences do not necessarily affect the dynamics or the intramolecular interactions that take place within the protein. The simulations are intended to provide confirmation of the experimental findings described in this article, and to set the stage for more detailed study of these characteristics of the protein in phospholipid environments. During the simulation several side chain interactions were observed. The most interesting of these were among tyrosine residues near the interhelical loop, as illustrated in Figure 5. Simulations suggest that hydrogen bonding between tyrosine 31 of the first trans-membrane helix and tyrosines 42 and 45 of the second trans-membrane helix occurs. During the simulation, a switch of the bond from one tyrosine to the other was observed. This specific switch may be associated with the intramolecular motions observed in helical segment 'c'.

Drug Binding

Several known channel-blocking compounds have been shown to alter the ion channel activity of p7.. These commercially available compounds were added directly to the NMR samples. As shown in Figure 6, when the ^{1}H and ^{15}N chemical shift changes are plotted as a function of residue number, there is evidence of site-specific interactions between the drugs and residues on the protein. It is apparent that the three compounds, amantadine, *N*N-DNJ and HMA, interact with the protein differently. Amantadine and *N*N-DNJ have a larger effect on the chemical shifts of the terminal regions, while HMA alters the chemical shifts of residues in the loop region. Caution is required in this interpretation because of differences in their hydrophobicity and hence access to the residues in the protein that are within the hydrocarbon core of the micelles.

NS2TM1 expression and purification

NS2 and p7 have been shown to co-localize in the ER membrane, and are believed to be involved in virus assembly[18], and may interact with each other within the membrane. In order to determine possible interactions between the two proteins the NS2 construct derived from the same genotype, J4, as p7, was expressed and purified. To simplify the identification of involved residues and accelerate the research a construct corresponding to the first TM of NS2, thought to be the crucial segment for these interactions, was used[33]. NS2TM1 was successfully expressed as a fusion protein, identical to the p7 construct, and was directed to the inclusion bodies of the cells. Using a similar purification protocol to p7, the inclusion bodies were isolated during lysis, cleaved and HPLC purified. Milligram amounts of isotopically labeled ^{15}N protein that could be incorporated into DHPC micelles

were obtained. These samples appeared to be well behaved in the same conditions that the p7 samples were measured in.

The resonances in the two-dimensional HSQC spectrum of the NS2 construct (Figure 7A) are well dispersed, and 85% of the backbone signals were assigned using a single two-dimensional ^{1}H-^{15}N HSQC NOESY experiment. HSQC spectra of the trans-membrane domain of NS2 in DHPC micelles were measured before and after the addition of unlabeled p7 to the sample. Several of the NS2 resonances were obviously shifted in the spectra, indicating that p7 was interacting with specific residues (Figure 7B and C). A plot of the chemical shift changes as a function of residue number upon the addition of p7 shows that the residues affected the most are those that are at the center of the trans-membrane helix, indicating that these interactions are taking place within the membrane (Figure 7D). Ala12 and Val15, the two residues that are most affected, are three residues apart, which is consistent with their residing on the same face of the helix.

The effect of addition of the NS2 construct on p7 spectra was also measured. When unlabeled NS2 construct was added to a sample of uniformly ^{15}N-labeled p7 protein spectral changes are observed. Interestingly, the residue that displays the largest change in chemical shift frequencies is Trp48. This residue is also predicted to be in the center of the membrane, as discussed above.

Discussion

The schematic drawing of p7 and NS2 in Figure 8A shows anti-parallel orientations of the transmembrane segments within the bilayer. The sequence alignment of the two proteins (Figure 8B) shows that the Trp48 residue of p7 and Ala12 and Val15 of NS2 are aligned near the center of the membrane when boundaries of the trans-membrane helices are aligned. Since significant chemical shift changes were limited to one residue of p7 and two residues of NS2, the helices are likely to have different tilt angles within the bilayer. The tilt of the second trans-membrane helix of p7 was previously found to be approximately 25° relative to the bilayer normal in a DMPC bilayer.

Along with the protein-protein interactions, drug-protein interactions were examined using several compounds with p7. Unlike the NS2 studies, in which both components were incorporated into the lipid environment, the drug compounds were added to the solution. This limited the observable interactions to the loops and terminal regions of the protein since the drugs, with the exception of amantadine, are not hydrophobic. Although specific amino acids could be identified as interacting with the drugs, the results highlight the need for performing these studies in a bilayer environment where the protein can form the oligomers that have been observed in cryo-EM studies.

The simulation studies presented here provide an initial attempt to understand the reason p7 is so dynamic. The switching of the hydrogen bond at the tyrosine side chains during the simulations is an interesting finding that requires close analysis of other residues at the interface of the trans-membrane segments. It is clear that the mobile section of TM2 ends at the position of Trp48-Pro49. The tryptophan appears to result in steric hindrance that maintains a separation of the two segments. In order to examine this hypothesis a point mutation was made in p7 at the tryptophan residue. The mutant protein, p7 W48A, was successfully expressed and purified. The HSQC spectrum of the mutant protein (Figure 9A) indicates that it is well behaved in the same sample conditions as the wild type protein. All of the signals could be assigned by comparison with the spectra of the wild-type protein. A plot of the chemical shift changes as a function of residue number indicates that the structural changes to the backbone are not limited to TM2 in the region where the mutation

was made (Figure 9B). Although the changes appear to be localized to several short regions of the protein, suggesting that the global structure of p7 remains the same, one of these regions is in the middle of TM1. The residues most affected are phenylalanines 22, 25 and 26. This indicates the mutation has an interhelical effect. A plot of the relative intensity indicates that the third helical region is less dynamic as a result of the residue change which may suggest that the placement of a less bulky alanine at site 48 allows the two helices of the protein to move closer together leading to a possible stacking of the tyrosine rings and a more stable structure. Another region where the chemical shifts have changed is between the loop and helix ‘c’. This might indicate that this is a hinge point that is responsible for accommodating the movement of TM2 to a position that is closer to TM1.

p7 is a remarkably complex protein considering that it has only 63 residues and its secondary structure is dominated by two hydrophobic transmembrane helices. Another NMR study of p7 in DPC micelles in solution suggests that it exists as a hexamer, and the resulting channel is more likely to be a dominant mechanism of biological activity[8]. In contrast, the structural and dynamic features identified with our NMR experiments on monomers of p7 in DHPC micelles in solution indicate that its biological roles may be more numerous and multifaceted than simply acting as a channel. Indeed, its channel activity may be secondary to its principal biological activities, a notion reinforced by the position of its gene between those for the non-structural and structural proteins of HCV. At least part of its functions are likely to involve interactions with other proteins, such as those shown in the example of p7 interacting with NS2 in Figure 8. Protein-protein interactions, as well as possible channel functions, are likely to be affected by its structure and changes in it in response to the lifecycle of the virus and the addition of drugs.

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ABBREVIATIONS

HCV	Hepatitis C Virus
NS	non-structural proteins
HMA	5-(N,N-Hexamethylene)amiloride
NN-DNJ	N-nonyl-deoxynojirimycin
RDC	Residual Dipolar Coupling
TM1	transmembrane 1
TM	transmembrane 2
E1	envelope protein 1
E2	envelope protein 2
NOE	nuclear Overhauser effect
DHPC	1,2-Dihexanoyl-*sn*-Glycero-3-Phosphocholine
PRE	paramagnetic relaxation enhancement
D2O	deuterium oxide

EDTA	Ethylenediaminetetraacetic acid
IPAP	in-phase/anti-phase
HSQC	Heteronuclear single quantum coherence spectroscopy
POPC	1-Palmitoyl-2-oleoylphosphatidylcholine

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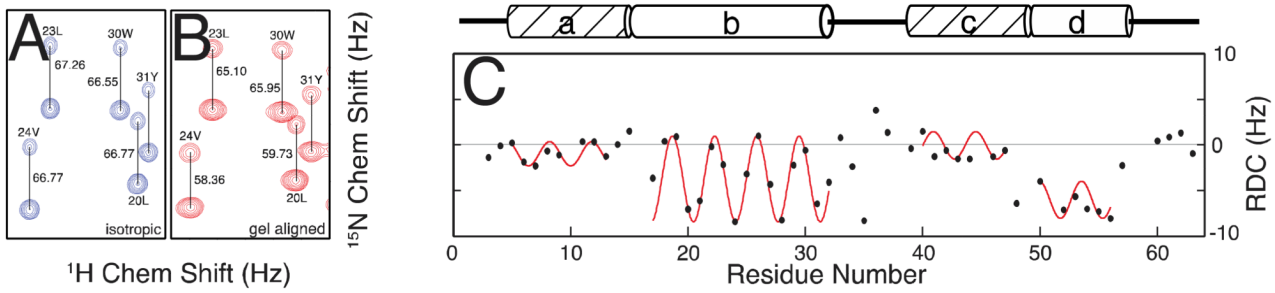

Figure 1.

Measurement and analysis of RDCs carried was performed on p7 in DHPC and aligned in a polyacrylamide gel. A. and B. are spectra showing examples of measurements of RDCs using an in-phase/anti-phase (IPAP) HSQC experiment on an isotropic sample (A) and a sample that is weakly aligned in a 6% charged and compressed polyacrylamide gel (B). C. The measured RDCs are plotted as a function of residue number. Dipolar waves were fit to the 4 helical regions shown in the plot (‘a’ through ‘d’). RDCs for helices ‘a’ and ‘c’ are near 0 while ‘b’ and ‘d’ vary drastically, suggesting that the aforementioned is more dynamic.

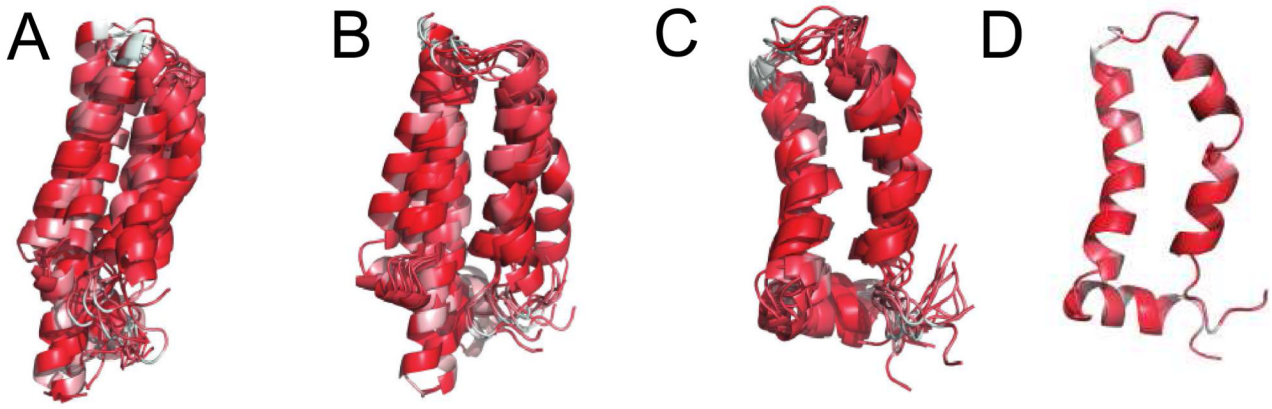

Figure 2.

Characterization of the stepwise method for determining the structure of p7 from the measured NMR data is shown. A. The chemical shifts (Cα, HN, and N) were used as the sole constraint in CS-Rosetta calculation. The cluster shown consists of the 10 lowest energy structures from 10,000 decoys that were generated. B. The ten lowest energy structures of 10,000 relaxed initial structures in presence of residual dipolar couplings as angular constraints. This set of structures converges over a larger portion of the backbone, including an overlay of helix ‘a’, which is a result of including orientation information provided by residual dipolar couplings. C. Ten lowest energy structures calculated using Xplor-NIH refinement protocol using the representative structures from the Rosetta calculation. The backbone RMSD for this ensemble is 2.18 Å. Although the RMSD is relatively low, there is still variation in the mobile region, particularly the start of helix ‘c’. (D) A single average structure from the Xplor-NIH refinement cluster was calculated.

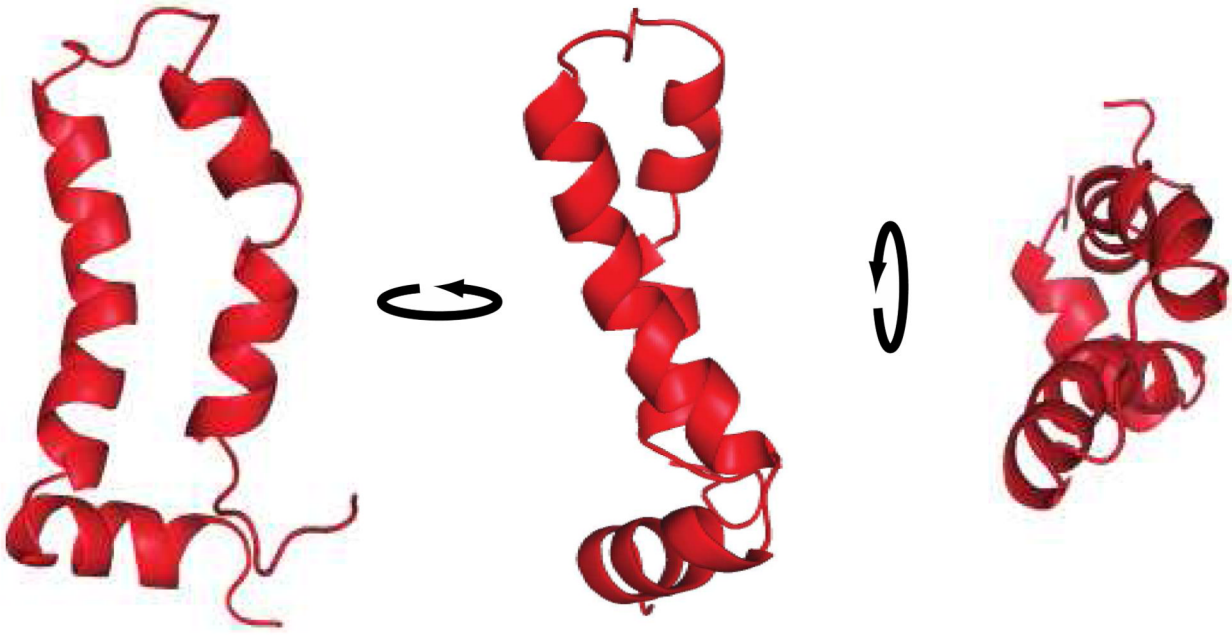

Figure 3.
The average calculated structure of p7 from the Xplor-NIH refinement is illustrated. The structure is the average structure from the lowest-energy cluster. The three views show the orientation of the four helices.

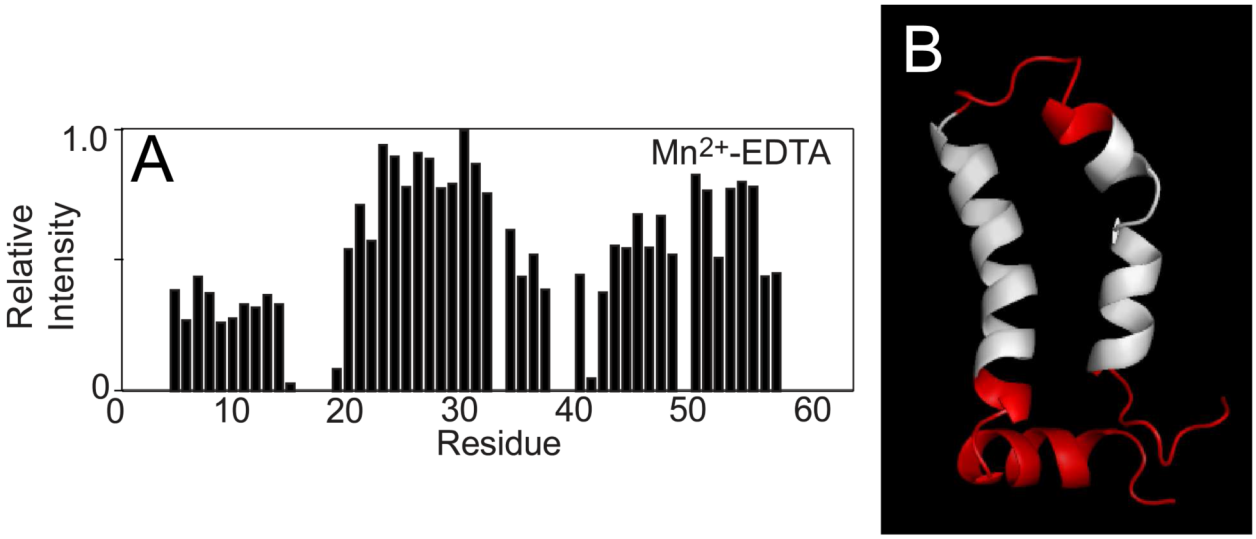

Figure 4.

The paramagnetic relaxation effect of manganese on p7 was characterized using differential signal intensities measured from ^{1}H-^{15}N HSQC spectra. A. Spectra of p7 were measured before and after the addition of Mn^{2+}-EDTA. The relative intensity, or ratio of the intensities, is plotted versus residue number. The signals with significantly reduced intensity indicate that the associated residues are solvent exposed. B. The residues with more than 50% reduction in signal are highlighted in red on the structure. From this representation it is obvious which regions of the protein are buried within the hydrophobic region of the DHPC micelle.

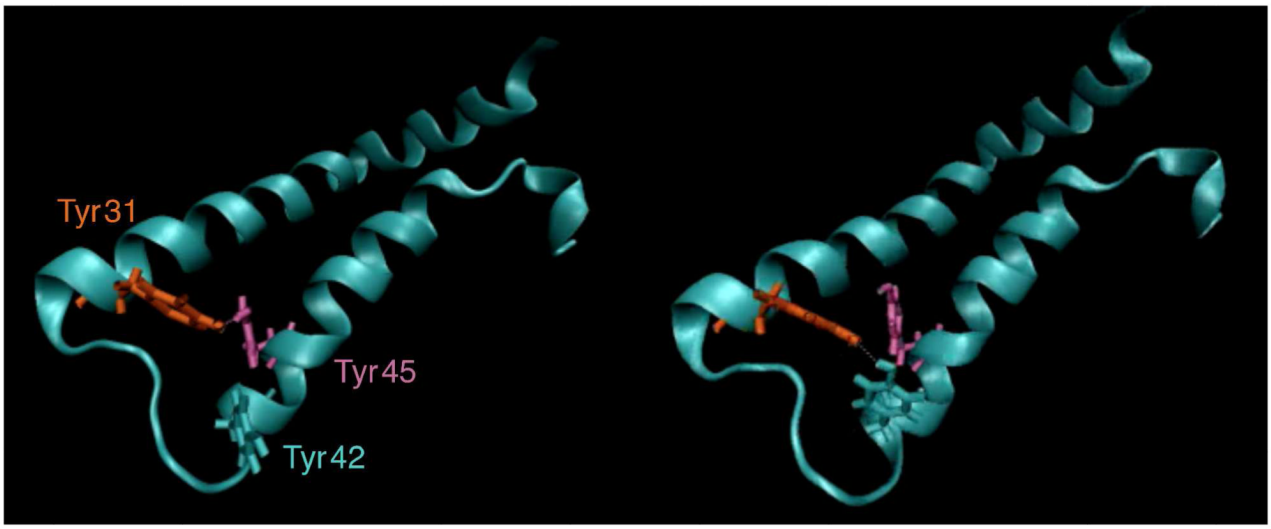

Figure 5.

Simulations show evidence of side chain interactions of p7 in an implicit POPC bilayer. Two ‘snap-shots’ were taken from a 50 ns MD simulation. The figure shows the hydrogen bonding that takes place between the side chains of tyrosine residues at the interface of the trans-membrane segments, Y31 from TM1 and Y42 and Y45 from TM2. The structure on the left shows hydrogen bonding between Y31 and Y45 and the right structure shows that hydrogen bonding has switched from Y31 to Y42.

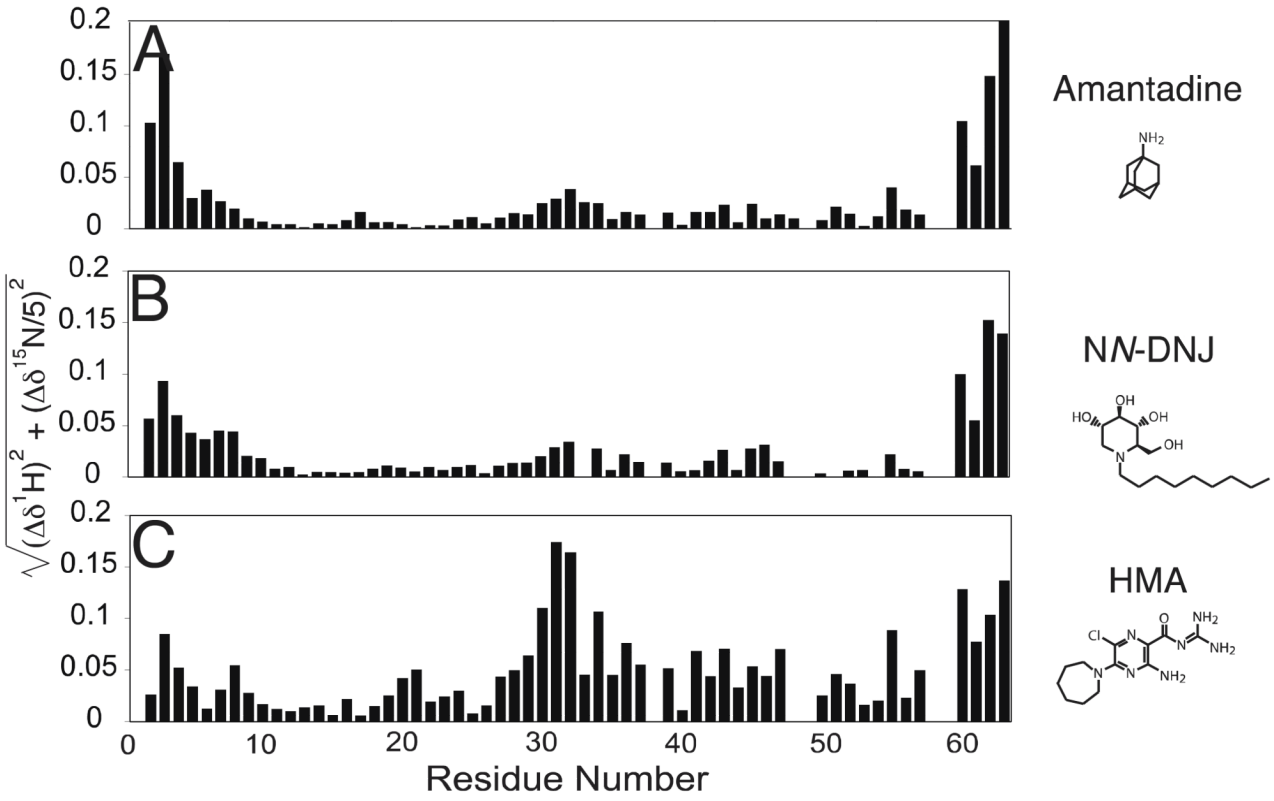

Figure 6.

Chemical shift differences plotted as a function of residue number show the changes induced when different channel-blocking compounds are added to the NMR sample of p7. The chemical shifts of the backbone amide proton and nitrogen are taken into account and plotted versus the primary sequence. A. Amantadine and the iminosugar derivative. B. *N*N-DNJ appears to interact at the terminal regions while C. Hexylmethylamiloride has a larger affect on the loop region. These three plots show how compounds interact with p7 at different regions of the protein.

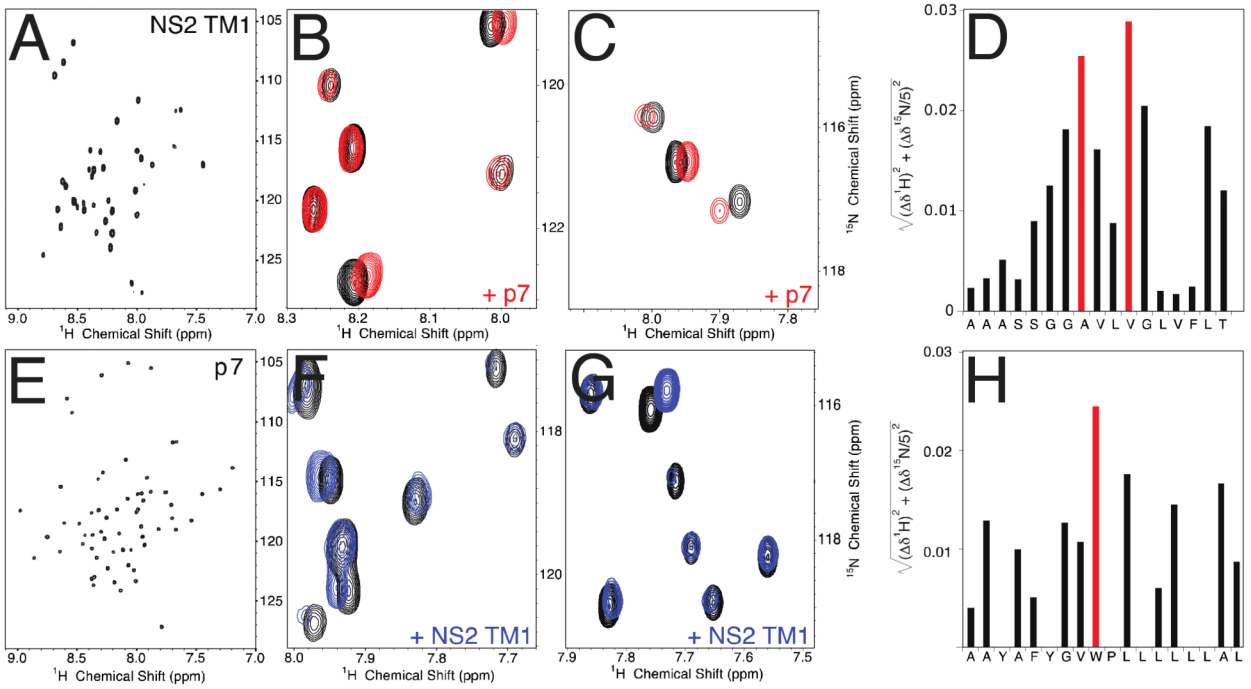

Figure 7.

Protein-protein interactions between J4 p7 and NS2 TM1 in DHPC were measured using chemical shift changes. (A. and E.) The baseline ^{1}H-^{15}N HSQC spectra of NS2 TM1 and p7, are shown respectively. (B. and C.) Regions from the NS2 TM1 HSQC spectra measured after the addition of unlabeled p7 (red) to labeled NS2 (black) is shown to highlight some of the chemical shift perturbations to the NS2 TM1 protein. (F. and G.) Similar portions of the p7 HSQC spectra measured after the addition of unlabeled NS2 (blue) to labeled p7 (black) are shown. The proteins were added at a 1:1 concentration ratio. These spectra clearly show chemical shift changes indicating structural perturbations and interactions in both proteins. (D. and H.) A plot of the chemical shift changes as a function of residue show that the interactions are likely taking place within the transmembrane regions of both proteins.

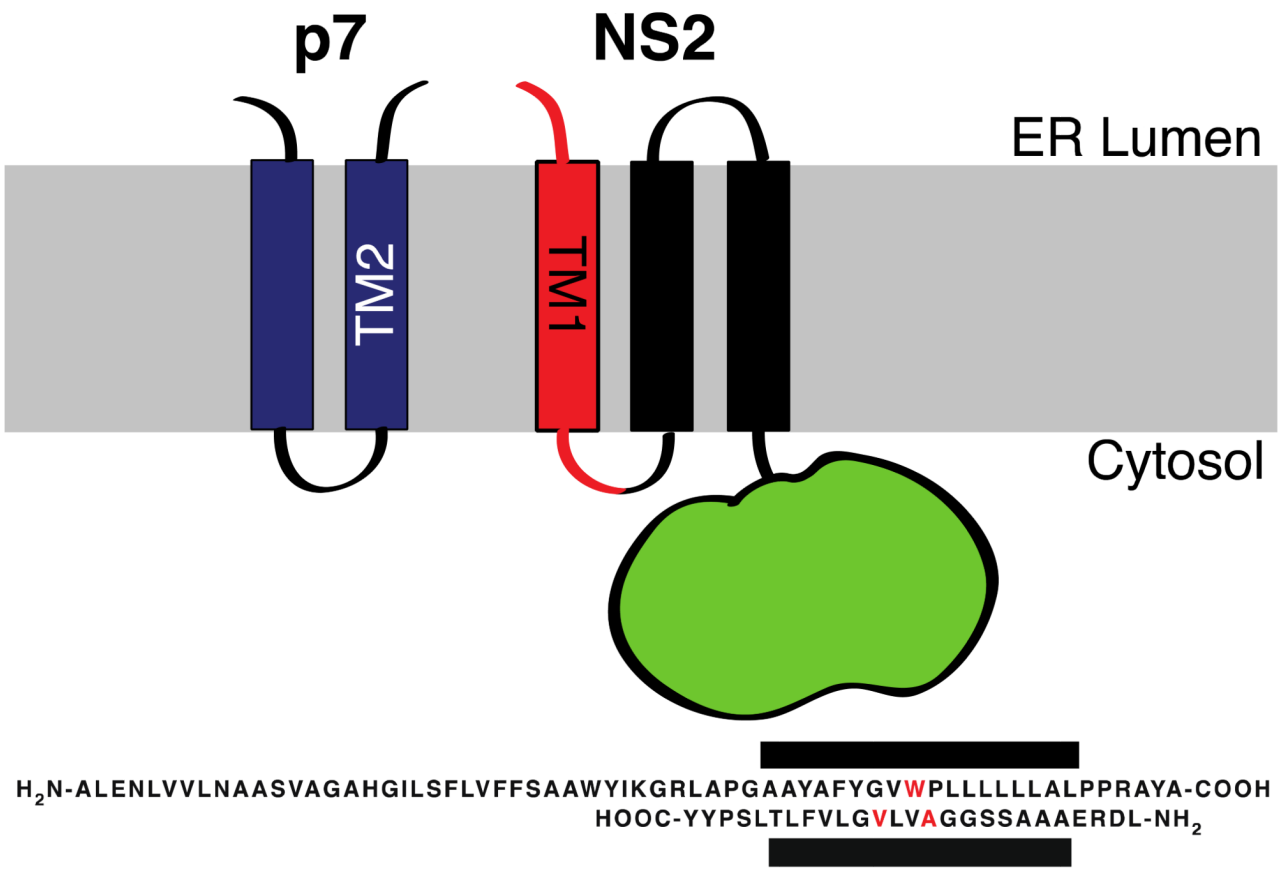

Figure 8.

A cartoon illustration represents p7 and NS2 in the membrane of the endoplasmic reticulum. The anti-parallel arrangement of TM2 of p7 and TM1 of NS2 is shown in the diagram. Below the diagram is an alignment of the respective p7 and NS2 TM1 sequences with a black rectangle corresponding to the TM regions of the proteins. The residues that have the largest chemical shift changes in Figure 6 are highlighted in red in the sequence.

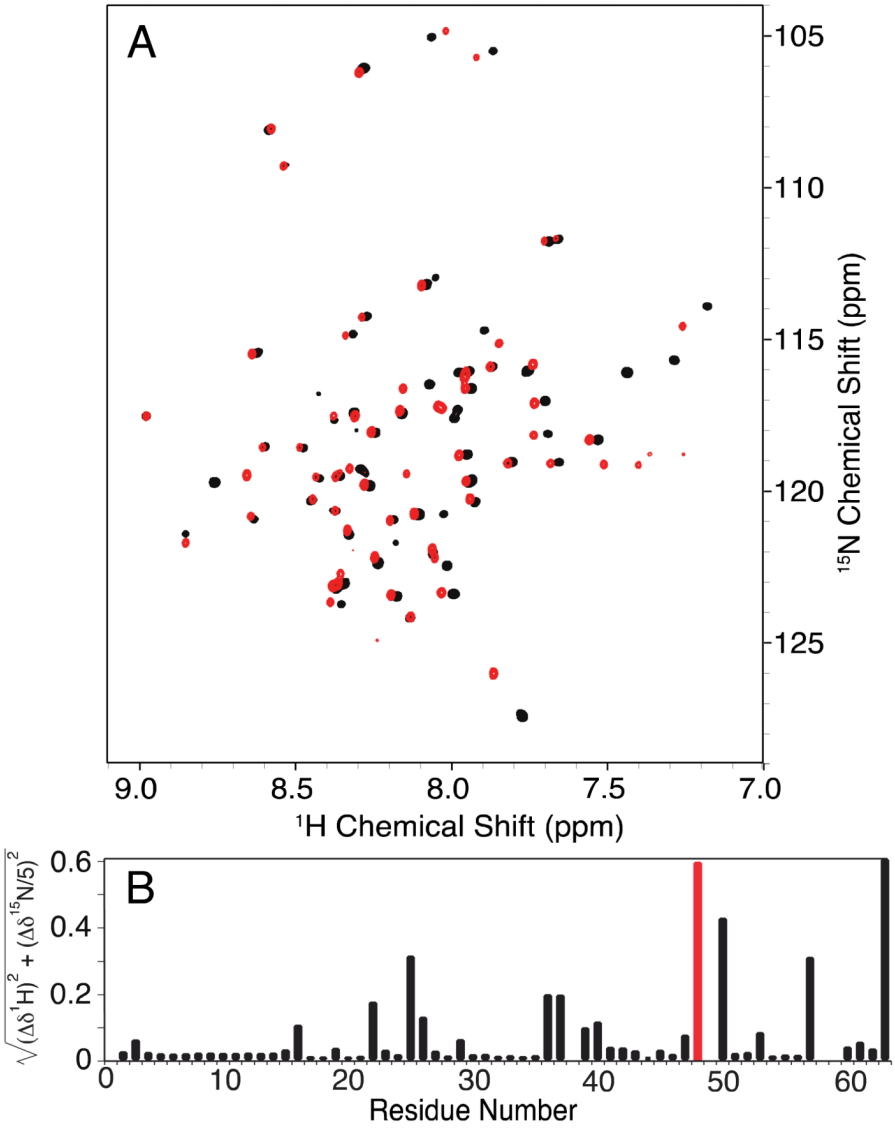

Figure 9.

An overlay of HSQC spectra of wild-type p7 (black) with that of the mutant p7 W48A (red) shows chemical shift differences. A. The HSQC spectra of p7 WT and W48A were recorded at 600 MHz at 50°C with pH 4.0 in 125 mM DHPC micelles. (In methods section.) B. Chemical shift changes are clearly evident in the second TM region where the mutation was made. Several residues directly across from residue 48 also show a degree of shift change, including three phenylalanine residues Phe22, Phe25 and Phe26.