Structure and Function of the Membrane Anchor Domain of Hepatitis C Virus Nonstructural Protein 5A

François Penin,1* Volker Brass,2# Nicole Appel,3# Stephanie Ramboarina,1# Roland Montserret,1 Damien Ficheux,1 Hubert E. Blum,2 Ralf Bartenschlager,3 and Darius Moradpour2*

1Institut de Biologie et Chimie des Protéines, CNRS-UMR 5086, IFR128 BioSciences Lyon-Gerland, F-69367 Lyon Cedex 07, France, 2Department of Medicine II, University of Freiburg, D-79106 Freiburg, and 3Department of Molecular Virology, University of Heidelberg, D-69120 Heidelberg, Germany

Running title: HCV NS5A membrane anchor domain

#V.B., N.A. and S.R. contributed equally to this work.

*Corresponding authors:
Darius Moradpour, M.D. Department of Medicine II University of Freiburg Hugstetter Strasse 55 D-79106 Freiburg Germany Tel. + 49 761 270 3510 Fax + 49 761 270 3610 E-mail: Darius.Moradpour@uni-freiburg.de
Francois Penin, Ph.D. IBCP CNRS-UMR 5086 IFR128 BioSciences Lyon-Gerland F-69367 Lyon Cedex 07 France Tel. + 33 4 7272 2648 Fax + 33 4 7272 2604 E-mail: f.penin@ibcp.fr

Copyright 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
Financial Support: This work was supported by grants Mo 799/1-3 (D.M. and H.E.B.) and SFB 638/Teilprojekt A5 (R.B.) from the Deutsche Forschungsgemeinschaft, QLK2-CT1999-00356 and QLK2-CT2002-01329 from the European Commission (F.P., R.B., D.M. and H.E.B.), 01 KI 9951 from the Bundesministerium für Bildung und Forschung (D.M. and H.E.B.), the French Centre National de la Recherche Scientifique (F.P.), the Lucie Bolte Foundation (D.M.), and the Bristol-Myers-Squibb Foundation (R.B.).

Accession codes: The coordinates of the average structures and the NMR restraints of NS5A[1-31] have been deposited in the Brookhaven Protein Data Bank under the accession codes 1R7C, 1R7E, and 1R7G for the peptide in 50% TFE, 100 mM SDS, and 100 mM DPC, respectively. The accession codes 1R7D and 1R7F correspond to the sets of 51 and 43 calculated structures for the peptide in 50% TFE and 100 mM SDS, respectively. The $^1$H and $^{13}$C chemical shifts of the assigned residues in 50% TFE, 100 SDS, and 100 mM DPC have been deposited in the BioMagResBank (BMRB) under the accession number 5978.

Abbreviations: 3D, three-dimensional; aa, amino acid; DPC, dodecylphosphocholine; ER, endoplasmic reticulum; HCV, hepatitis C virus; IRES, internal ribosome entry site; mAb, monoclonal antibody; NOESY, nuclear Overhauser effect spectroscopy; NS5A, nonstructural protein 5A; NS5A[1-31], synthetic peptide representing aa 1-31 of NS5A; PE, phosphatidylethanolamine; RMSD, root mean square deviation; SDS, sodium dodecyl sulfate; TOCSY, total correlation spectroscopy; TFE, trifluoroethanol.
Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) is a membrane-associated, essential component of the viral replication complex. Here, we report the three-dimensional structure of the membrane anchor domain of NS5A as determined by NMR spectroscopy. An $\alpha$-helix extending from amino acid residue 5 to 25 was observed in the presence of different membrane mimetic media. This helix exhibited a hydrophobic, Trp-rich side embedded in detergent micelles, while the polar, charged side was exposed to the solvent. Thus, the NS5A membrane anchor domain forms an in-plane amphipathic $\alpha$-helix embedded in the cytosolic leaflet of the membrane bilayer. Interestingly, mutations affecting the positioning of fully conserved residues located at the cytosolic surface of the helix impaired HCV RNA replication without interfering with the membrane association of NS5A. In conclusion, the NS5A membrane anchor domain constitutes a unique platform that is likely involved in specific interactions essential for the assembly of the HCV replication complex and that may represent a novel target for antiviral intervention.
INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide (1). HCV contains a 9.6 kb single-stranded RNA genome of positive polarity that encodes a polyprotein precursor of about 3000 amino acids (aa) (reviewed in refs. 2-4). The polyprotein precursor is co- and posttranslationally processed by cellular and viral proteases to yield the mature structural and nonstructural proteins. The structural proteins include the core protein and the envelope glycoproteins E1 and E2. The nonstructural proteins include the NS2-3 autoprotease and the NS3 serine protease, an RNA helicase located in the C-terminal region of NS3, the NS4A polypeptide, the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase. As in all positive-strand RNA viruses, the nonstructural proteins form a membrane-associated replication complex together with replicating viral RNA, altered membranes, and additional as yet unidentified host cell components (5-8). Determinants for membrane association of the HCV nonstructural proteins have been mapped (reviewed in ref. 9), but the protein-protein interactions involved in formation of a functional HCV replication complex are poorly understood.

HCV NS5A is a phosphoprotein of unknown structure and function (10, 11). It is found in a basally phosphorylated form of 56 kDa and in a hyperphosphorylated form of 58 kDa. NS5A has attracted considerable interest because of its potential role in modulating the interferon response (reviewed in ref. 12), and numerous additional functions have recently been ascribed to this protein (reviewed in ref. 13). Interestingly, cell culture-adaptive changes cluster in the central portion of NS5A in the context of selectable subgenomic HCV replicons (14, 15), indicating that NS5A is involved – either directly or by interaction with cellular proteins – in the viral
replication process. This observation, together with the modulation of NS5A hyperphosphorylation by the nonstructural proteins 3, 4A, and 4B (16, 17) and physical interactions described with other nonstructural proteins (18, 19), strongly support the notion of NS5A being an essential component of the HCV replication complex.

We have recently shown that the N-terminal 30 aa residues serve as a membrane anchor for NS5A (20). This domain was necessary and sufficient to target NS5A or a heterologous fusion protein to the endoplasmic reticulum (ER) or an ER-derived modified compartment by a post-translational mechanism, resulting in integral membrane association. Structure predictions and circular dichroism analyses indicated that this domain contains an amphipathic $\alpha$-helix.

Here, we describe the three-dimensional (3D) structure of a synthetic peptide corresponding to the NS5A membrane anchor domain, NS5A[1-31], as determined by NMR in the presence of different membrane mimetic media. We report that the NS5A membrane anchor forms an in-plane amphipathic $\alpha$-helix embedded in the cytosolic leaflet of the membrane bilayer. Moreover, based on targeted mutagenesis and RNA replication analyses we propose that the polar residues at the membrane surface define a unique platform that is involved in specific protein-protein interactions essential for the assembly of a functional HCV replication complex.
EXPERIMENTAL PROCEDURES

Peptide synthesis. The NS5A[1-31] peptide, representing aa 1-31 of NS5A of the HCV H strain (GenBank accession number AF009606), was synthesized by the stepwise solid-phase method of Merrifield and employing Fmoc chemistry. The peptide was highly purified by RP-HPLC and exhibited the expected molecular mass of 3764 Da.

NMR spectroscopy. For NMR samples in 50% trifluoroethanol (TFE)-$d_2$ (>99% isotopic enrichment), the NS5A[1-31] peptide was dissolved at a concentration of 1.2 mM at pH 4.5. For NMR samples in sodium dodecyl sulfate (SDS)-$d_{25}$ (>98%) or dodecylphosphocholine (DPC)-$d_{38}$ (>98%), the peptide was dissolved in a 95:5 mixture of H$_2$O:D$_2$O to a final concentration of 1-2 mM in 100 mM detergent containing 10 mM DTT-$d_{10}$ (>99.9%), and the pH was adjusted to 6.0. DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid) was used as an internal $^1$H and $^{13}$C chemical shift reference in all NMR samples. For Mn$^{++}$ experiments, 0.05 or 0.5 mM MnCl$_2$ was added to 1 mM NS5A[1-31] peptide in 100 mM SDS-$d_{25}$.

NMR experiments in H$_2$O/TFE-$d_2$ were performed at 20°C while those in SDS-$d_{25}$ and DPC-$d_{38}$ were recorded at 40°C. Multidimensional NMR experiments were carried out at 500 MHz on a Varian Unity-plus spectrometer equipped with a triple resonance 5 mm probe with a self-shielded z gradient coil. Double quantum filtered correlation spectroscopy, nuclear Overhauser effect spectroscopy (NOESY), 2D homonuclear total correlation spectroscopy (TOCSY), and $^1$H-$^{13}$C heteronuclear single-quantum correlation experiments using conventional optimized pulse sequences as well as data collection, processing and spectra analyses for spin
systems identification and sequential assignments were performed as previously described (21-23 and references therein).

**NMR derived constraints and structure calculations.** NOE intensities used as input for the structure calculations were obtained from the NOESY spectrum recorded with 100 or 150 ms mixing time. NOEs were partitioned into three categories of intensities that corresponded to distances ranging from a common lower limit of 1.8 Å to upper limits of 2.6 Å, 3.8 Å and 5 Å, for strong, medium and weak intensities, respectively. The cross-peak intensities of the \( H^\delta - H^\epsilon \) protons of Phe19 were used as reference distance (2.45 Å). No hydrogen bond or dihedral angle restraints were introduced. Protons without stereospecific assignments were treated as pseudoatoms, and the correction factors were added to the upper and lower distance constraints.

Three-dimensional structures were generated from NOE distances by the restrained dynamical simulated annealing protocol (24) with X-PLOR 3.85 using standard force fields and default parameter sets. Sets of 100 structures were calculated to widely sample the conformational space, and structures with restraint violations < 0.5 Å and angle violations < 0.5° were retained. The selected structures were compared by pairwise root mean square deviation (RMSD) over the backbone atom coordinates (N, C\( \alpha \) and C'). Local analogies were analyzed by calculating the local RMSD of a tripeptide window slid along the sequence. Statistical and structural analyses were performed using AQUA and PROCHECK-NMR software (25). Superimposition of structures, 3D graphic display and manipulations were carried out using MOLSCRIPT (26) (Fig. 3A) and RASMOL 2.5 (27).
**Plasmids.** CMV and T7 promotor-driven expression constructs harboring mutations within the NS5A membrane anchor domain were prepared by hybridization of the oligonucleotide pairs listed in Table 1 of the Supplemental Data and ligation into the EcoRI-BplI sites of pCMVNS5Acon (20), which allows expression of NS5A derived from a functional HCV genotype 1a consensus cDNA (28), to yield plasmids pCMVNS5Acon-R6A/D7A, -W9A, -C13A, -8+A, -11+A, and -Δ5-11, respectively. Plasmid pCMVNS5Acon45-448, lacking the N-terminal 44 aa residues of NS5A, has been described (20).

Replicon constructs harboring mutations within the NS5A membrane anchor domain of NS5A were prepared by PCR-based site-directed mutagenesis using oligonucleotide primers carrying the desired modifications (Table 1 of the Supplemental Data). DNA fragments were inserted into replicon construct pFKnt341-sp-Pl-lucEI3420-9605/E1202G+T1280I+K1846T (15). The SfiI fragment of the reporter replicons was introduced into pFK1-389neoEI3420-9605 (29) to yield selectable subgenomic replicons.

**Antibodies.** Monoclonal antibodies (mAbs) 11H against NS5A (20) (kindly provided by Jan Albert Hellings, bioMérieux, Boxtel, The Netherlands) and G1/296 against p63 (30) (kindly provided by Hans-Peter Hauri, University of Basel, Switzerland) have been described.

**In vitro transcription, electroporation and replication assays.** In vitro transcription, transfection of Huh-7 human hepatoma cells by RNA electroporation and RNA replication assays using luciferase reporter replicons as well as selectable HCV replicons were performed as described (31, 32).
Confocal laser scanning microscopy. Immunofluorescence staining was performed as described previously (33). Cover slips were mounted in SlowFade (Molecular Probes, Eugene, OR) and examined with a Zeiss LSM 510 laser scanning system. Images were processed with Zeiss Image 3.1.0.99 and Adobe Photoshop 7.0 software.

Membrane flotation assays. Membrane flotation assays were performed as described (34) with minor modifications. In brief, $2 \times 10^7$ transiently transfected U-2 OS cells were dounce homogenized in a hypotonic buffer (10 mM Tris·HCl, pH 7.5, 2 mM MgCl$_2$), followed by centrifugation at $1000 \times g$ for 5 min to pellet nuclei, unlysed cells, and large debris. Nycodenz (Axis Shield, Oslo, Norway) was added to postnuclear supernatants to a final concentration of 37.5% (wt/vol) in PBS. Four hundred µl lysate was placed at the bottom of a 1.5 ml thick-walled ultracentrifugation tube and overlayed with 900 µl 35% and 100 µl 5% Nycodenz in PBS. Equilibrium centrifugation was carried out at 100,000 $x$ g for 20 h at 4 °C in a Beckman TLS 55 rotor. Subsequently, 8 fractions of 175 µl each were colleted from the top and equal volumes were subjected to immunoblot as described previously (33).
RESULTS

NMR structure of NS5A[1-31]. Previous circular dichroism analyses of the NS5A[1-31] peptide performed in the presence of SDS or DPC micelles and in TFE-water mixtures indicated an \( \alpha \)-helix content of about 80\% (20). Here, the conformational behaviour of NS5A[1-31] in these commonly used membrane mimetic media (35) was further investigated by NMR to gain insights into peptide folding at the atomic level. The 2D homo- and hetero-nuclear NMR analyses of NS5A[1-31] dissolved in 100 mM SDS-\( d_{25} \), 100 mM DPC-\( d_{38} \) or 50\% TFE-\( d_2 \) yielded well resolved spectra, as illustrated in Fig. 1A by an extract of the NOESY spectrum in 100 mM SDS. Sequential attribution of all spin systems was completed in the three membrane mimetic environments.

An overview of the sequential and medium range NOE connectivities for the peptide in SDS-\( d_{25} \) is shown in Fig. 1B. NOE connectivities observed in TFE-\( d_2 \) and in DPC-\( d_{38} \) are available in Fig. 1 of the Supplemental Data. Despite the lack of numerous connectivities due to overlapping cross-peaks in SDS and DPC (indicated by asterisks), the NOE connectivity patterns are similar in the three membrane mimetic environments. The main body of the peptide (Leu5-Ala25) displays typical characteristics of an \( \alpha \)-helix, including strong dNN(\( i,i+1 \)) and medium d\( \alpha N(i,i+1) \) sequential connectivities, weak d\( \alpha N(i,i+2) \), medium d\( \alpha N(i,i+3) \), medium or strong d\( \alpha \beta(i,i+3) \), and weak d\( \alpha N(i,i+4) \) medium range connectivities. In contrast, the residues flanking the N- and C-terminal ends of the Leu5-Ala25 helix (i.e., aa 4 and 26-28) remain more flexible with fewer medium range connectivities as a sign of fraying helix ends. When compared to those observed in TFE, several NOE connectivities are missing between Val15 and Phe19 in SDS and DPC. Cross-peaks
in this region display a lower intensity compared to the other residues, indicating differences in the dynamic behaviour in the Val15-Phe19 region (see below). The N- and C-terminal ends of the peptide (i.e., aa 1-3 and 29-31) are devoid of medium range NOEs and remain unstructured in all three media.

Differences of $^1$H$\alpha$ and $^{13}$C$\alpha$ chemical shifts from those found in a random coil conformation are additional indicators of secondary structure (36). Chemical shift differences for $^1$H$\alpha$ and $^{13}$C$\alpha$ are shown in Fig. 2 (panel B) and Fig. 2 of the Supplemental Data, respectively. The long series of negative $^1$H$\alpha$ ($\Delta^1$H$\alpha \leq -0.1$ ppm) and positive $^{13}$C$\alpha$ chemical shift differences ($\Delta^{13}$C$\alpha \geq 0.7$ ppm) are typical of an $\alpha$-helical conformation. However, the weaker $\Delta^1$H$\alpha$ observed around Ile8 and Asp18 suggest some flexibility of the $\alpha$-helix around these residues. These data are in close agreement with the NOE connectivities described above. Taken together, these results clearly show that the peptide conformation is very similar in the three membrane mimetic media and that an $\alpha$-helix extends from Leu5 to Ala25.

**NMR structure model of NS5A[1-31].** The number of NOE-derived interproton distance constraints used for the structure calculations are reported in Table 2 of the Supplemental Data. From the initial 100 structures calculated with X-PLOR, final sets of 51, 43 and 27 low energy structures were retained for the peptide in TFE, SDS and DPC, respectively. Structure selection was based on NOE violations $\leq 0.5$ Å, and all three sets of structures fully satisfied the experimental NMR data. The final statistics are listed in Table 2 of the Supplemental Data. All structures show a regular $\alpha$-helical conformation extending from residues 4 to 27 in 50% TFE, 5 to 25 in SDS, and 4 to 25 in DPC, but the best superimposition of calculated structures includes residues 5
to 25. A superimposition of the calculated structures obtained in SDS is shown as an example in Fig. 2A.

The α-helix is well defined between Leu5 and Ala25, as reflected by the low global RMSD for the backbone atoms (C', Cα, and N) of 1.11 and 1.12 Å for each set of structures in TFE and SDS, respectively (Table 2 of the Supplemental Data). In contrast, a higher RMSD of 2.17 Å for the set of structures of the peptide in DPC reflects a significant conformational heterogeneity even though all 27 structures fully satisfied the experimental NMR data. Superimposition of the 27 structures shows some conformational fluctuations of the backbone around Val15, while the N- and C-terminal portions of the helix are more stable, as reflected by a global backbone RMSD of 0.88 and 0.84 Å for segments 5-15 and 15-25, respectively (data not shown). The local fluctuation affecting the 15-17 region is illustrated by the high local backbone RMSDs of Val15, Leu16 and Ser17 (Fig. 2B). This dynamical behaviour is less pronounced in the TFE and SDS structures (Fig. 2B), but it is supported by the lower chemical shift differences of ΔHα (Fig. 2B) and ΔCα (Fig. 2 of the Supplemental Data) observed in the three media when compared to the surrounding residues that are clearly involved in stable helical segments (e.g., residues 12-14 and 20-23). Although direct evidence of a dynamic behaviour remains to be provided, these results suggest that the Val15-Ser17 segment is an intrinsic flexible helical element of the NS5A[1-31] membrane anchor. This flexibility could lead to a variable orientation of the ends of the α-helix when NS5A[1-31] is bound to phospholipids (see below). Finally, the apparent structural instabilities observed on both edges of the Leu5-Ala25 α-helix can be explained by the absence of stabilizing interactions with the rest of NS5A and/or membrane phospholipids in the isolated NS5A[1-31] peptide context.
The comparison of the average conformations of NS5A[1-31] obtained in TFE, SDS and DPC shown in Fig. 3A highlights the close overlap in the folding of the Leu5-Ala25 helices but also shows differences in the slight bending of the helices. This bending is mainly due to the lack of long range distance restraints when calculating the structure of an isolated \(\alpha\)-helix. Therefore, the relevance of such bending for membrane association and/or the structure of NS5A cannot be determined. As illustrated in Fig. 3B for the structure determined in SDS, charged and polar residues are exposed on the hydrophilic side, with a remarkable asymmetric distribution of positively and negatively charged residues along both edges of this side of the helix. On the other hand, aromatic residues Trp4, Trp9, Trp11, Phe19 and Trp22 are well positioned on the hydrophobic side, suggesting an essential role in membrane association of the helix.

**Positioning of NS5A[1-31] in detergent micelles.** The positioning of NS5A[1-31] in the phospholipid bilayer was investigated by three different NMR strategies: (1) NOEs between residue side chains and detergent hydrophobic tails, (2) measurement of amide proton exchange with deuterated water, and (3) accessibility of residues to water by examining the broadening of proton signals due to the proximity of paramagnetic Mn\(^{++}\) ions. The data obtained by these three strategies are summarized in Fig. 4A.

NOESY spectra were recorded at a 1:9 ratio of protiated DPC:DPC-\(d_{38}\) to investigate direct contacts between detergent and NS5A[1-31] (22, 37). Significant intermolecular NOE cross-peaks were observed between the hydrophobic peptide residues and various CH\(_2\) groups of the hydrophobic tail of DPC. The hydrophobic region of the spectra was too crowded to yield unambiguous data. In contrast, the
aromatic region provided NOE cross-peaks of DPC with side chains of Trp and Phe (data not illustrated). These spectra indicate that Trp9, Phe19 and Trp 22 are mainly buried in the DPC micelles while Trp4 and Trp11 appear to be located at the hydrophobic-hydrophilic micelle interface.

Proton exchange analyses showed that all amide protons are exchanged after less than 24 h in TFE-\(d_2\) while some were still retained in SDS-\(d_{25}\) (Trp9, Trp11, Ile12, Val15, Leu23 and Lys24 as highlighted in Fig. 1B). In DPC-\(d_{38}\) only the amide proton of Ile12 appears up to 24 h. This indicates that the corresponding residues are likely buried in the micelle hydrophobic core.

At low concentrations, Mn\(^{++}\) primarily affects resonances of water-accessible residues at the surface of an SDS micelle (38). The paramagnetic broadening effect of Mn\(^{++}\) was studied by comparing TOCSY spectra in the presence or absence of 0.05 or 0.5 mM MnCl\(_2\). Residues for which cross-peaks disappeared in the presence of 0.05 mM MnCl\(_2\) were considered as water-accessible (e.g., Asp10 and Glu14, Fig. 4A). Conversely, residues for which cross-peaks were always visible in the presence of 0.5 mM MnCl\(_2\) were considered as buried. The unambiguous data obtained by this approach are summarized in Fig. 4A (white arrows). The combined results show that most of the hydrophobic residues of the helical region are buried in the detergent micelles. Some of the acidic residues are clearly surface exposed (Asp10, Glu14) while the side chains of basic residues Arg and Lys appeared mainly buried. Interestingly, two distinct regions connected by the Val15-Ser17 flexible helical element emerge from these analyses. The Trp-rich, N-terminal portion of the helix (aa 5-12) seems to be located more closely to the micelle interface, while the Lys-rich, C-terminal portion (aa 19-26) appears to be buried in the hydrophobic core of the detergent micelle. This is in line with the presence of short, negatively charged
residues in the first portion and Lys side chains, which permit a deeper embedding of
the helix in the hydrophobic core, in the second. From these data, it is possible to
approximately position the average NS5A[1-31] structure in a model phospholipid
bilayer (Fig. 4B). It can be deduced that the aa 5-25 helix is located at the membrane
interface, forming an in-plane amphiphatic \( \alpha \)-helix embedded in the cytosolic leaflet
of the membrane. Assuming that the charged aa interact with the polar head of
phospholipids, and taking into account the length of their side chains together with
that of the phospholipid polar head and hydrophobic tails, it appears that the whole
helix could not be deeply buried in the hydrophobic core of the membrane. This
positioning is quite similar to that deduced from the NMR analysis of the membrane-
proximal region of the HIV glycoprotein gp41 in DPC micelles where the Trp residues
were proposed to be located within the membrane-water interface of the phospholipid
bilayer (37). The putative flexible helical segment Val15-Ser17 could play a role in
such positioning, allowing either a lateral and/or a vertical bending of the helix with
respect to the plane of the membrane. The polar side of the helix is likely accessible
at the surface of the membrane, as tentatively illustrated in Fig. 4C.

**Subcellular localization of NS5A membrane anchor mutants.** The 3D structure
information was used to design a panel of NS5A mutants with aa substitutions,
insertions, or deletions in the N-terminal \( \alpha \)-helix as illustrated in Fig. 5A. The
mutations were carefully designed to preserve the overall fold of the amphipathic \( \alpha \)-
helix and taking into consideration the conservation of residues among different HCV
isolates (20). Mutation of two charged residues at position 6 and 7 to Ala resulted in
construct R6A/D7A. The absolutely conserved Trp9 and Cys13 were replaced by Ala,
yielding constructs W9A and C13A, respectively. To perturb the asymmetric charge
distribution on the hydrophilic side of the helix Ala residues were inserted at position 8 or 11, yielding constructs 8+A and 11+A, respectively. These insertions twist the helix by 110°, as illustrated in Fig. 5B and C. Finally, construct Δ5-11 was obtained by the deletion of two α-helix turns within the membrane anchor domain.

Subcellular localization of the NS5A mutants was analyzed by confocal laser scanning microscopy following transient expression in U-2 OS cells. As shown in Fig. 6, all membrane anchor mutants were found in a reticular staining pattern, which surrounded the nucleus, extended through the cytoplasm, and included the nuclear membrane. No nuclear or plasma membrane staining was observed. The staining pattern was indistinguishable from that of wild-type NS5A, which is associated with membranes of the ER or an ER-derived modified compartment (20). By contrast, an expression construct lacking the N-terminal domain of NS5A (NS5A45-448) showed a diffuse staining pattern with accumulation in the nucleus as previously reported (20). Thus, the carefully designed membrane anchor mutants retained their proper subcellular localization and apparent membrane association.

RNA replication of NS5A membrane anchor mutants. The above mutations were introduced into a reporter replicon carrying two cell culture-adaptive changes in NS3 and one in NS4B to analyze their effect on HCV RNA replication (Fig. 7A). In these replicons, translation of the firefly luciferase gene is directed by the internal ribosome entry site (IRES) of poliovirus, whereas the IRES of encephalomyocarditis virus governs translation of the HCV nonstructural proteins. RNA replication was analyzed by monitoring luciferase activity at various time points after transfection into Huh-7 cells. The parental replicon and a replication-deficient RNA (GND) served as positive and negative controls, respectively. A representative result is shown in Fig. 7B.
Single and double Ala substitutions for conserved aa located on the cytosolic side of the helix (R6A/D7A and C13A) had no influence on replication efficiency. The Ala substitution for the fully conserved Trp9 decreased luciferase activity by about 10-fold. Insertion of Ala residues at aa position 8 or 11 (8+A and 11+A, respectively) abolished HCV RNA replication. The effect of these mutations was as dramatic as that observed for a deletion of two helix turns (Δ5-11).

In some experiments, luciferase activities obtained with the mutants 8+A, 11+A and Δ5-11 were slightly higher than that of the GND negative control (Fig. 7B). Although this difference was within the range of assay variability, these mutants were reanalyzed by a more sensitive replication assay using selectable replicons in which the luciferase reporter gene was replaced by the gene encoding for neomycin phosphotransferase. Upon transfection into Huh-7 cells and G418 selection, the number of G418-resistant colonies reflects the replication efficiency of a given replicon. As shown in Fig. 7C, no colonies were obtained with these mutants under these conditions, corroborating that the introduced alterations completely block RNA replication. In conclusion, these results indicate that insertions or deletions, leading to a twist of the helix or altering its size, have a dramatic impact on the function of NS5A in HCV RNA replication.

**Membrane association of NS5A membrane anchor mutants.** Membrane flotation analyses were performed to confirm membrane association of the replication-defective mutants 8+A, 11+A and Δ5-11. Mutants were expressed transiently in U-2 OS cells, followed by hypotonic cell lysis and equilibrium centrifugation in a Nycodenz gradient. Under these conditions, membrane proteins float to the upper, low density fractions, as indicated by the behaviour of p63, an integral ER membrane protein
(Fig. 8). As expected, wild-type NS5A floated to the membrane-containing low
density fractions. Disruption of the membranes by 1% Triton X-100 results in
sedimentation of NS5A and p63 into high density fractions. As shown in Fig. 8, the
mutants 8+A, 11+A and Δ5-11 floated to the low density fractions as wild-type NS5A,
confirming the unimpaired membrane association of these constructs.

More detailed analyses of 8+A, 11+A and Δ5-11 by differential membrane
extraction revealed that deletion of 2 helix turns (Δ5-11) and, to a lesser degree,
distortion of the helix by Ala insertion attenuated the strength of membrane
association, as reflected by an increased proportion of protein extracted into the
supernatant fraction following alkali or 4 M urea treatment (Fig. 3 of the Supplemental
Data). However, the 8+A and 11+A mutants fulfilled the criteria of integral membrane
proteins. These results suggest that the dramatic effect of these mutations on HCV
RNA replication is not primarily due to an impaired membrane association of NS5A
but rather due to impairment of additional functions of the N-terminal α-helix.
DISCUSSION

Three-dimensional NMR structure analyses of the NS5A[1-31] peptide performed in different membrane mimetic media revealed that the N-terminal membrane anchor domain of NS5A includes a long amphipathic α-helix (aa 5-25) which seems to be divided into two portions separated by a putative flexible region located in the center of the helix (aa 15-17). By three different NMR analyses, the hydrophobic aa were found to be mainly buried in the hydrophobic core of the detergent-peptide micelles while the polar and charged aa were mainly accessible at their surface. One can thus conclude that the NS5A membrane anchor domain is embedded in-plane in the cytosolic leaflet of the ER membrane, with a hydrophobic side buried in the membrane and a polar/charged side accessible from the cytosol. The Trp-rich, N-terminal portion of the helix (aa 5-12) seems to be located more closely to the membrane interface, while the Lys-rich, C-terminal portion (aa 19-26) appears to be slightly more deeply buried into the hydrophobic core of the membrane.

The location of the Trp residues at the interface between the hydrophilic and hydrophobic sides of the amphipathic helix is a very typical feature. Indeed, in membrane proteins Trp residues are often located at the lipid bilayer interface, but rarely within the hydrophobic core of the membrane (39, 40). For example, in the membrane-proximal Trp-rich region of HIV glycoprotein gp41 four out of five Trp residues and one Tyr residue form a collar of aromatic residues along the axial length of the amphipathic helix, resulting in a velcro-like interaction on the outer viral membrane (37). The full conservation of Trp residues in the NS5A amphipathic α-helix among the various HCV genotypes (20) suggests that in addition to ensure membrane association they may also participate in specific protein-protein
interactions at the level of the membrane interface, possibly with other components of the HCV replication complex. The polar side of the $\alpha$-helix exhibits a striking asymmetry in charge distribution with positively charged residues (Arg6, Lys20, and Lys24) placed in-line along one border and negatively charged residues (Asp7, Asp10, Glu14, and Asp18) along the other. This particular arrangement of these remarkably conserved charged residues (20) suggest that the polar side of the helix forms a specific interaction site. Hence, the helix appears as a multi-interaction platform with both the hydrophilic side and the borders of the hydrophobic side likely involved in specific protein-protein interactions.

No dimerization or oligomerization of the NS5A[1-31] peptide was observed, indicating that evolution towards an oligomeric transmembrane structure is unlikely and that the NS5A amphipathic helix remains a monomeric structural element located at the surface of the ER membrane. This is in line with a lack of glycosylation, i.e., lack of ER luminal translocation, of artificial glycosylation acceptor sites engineered to the N-terminus of NS5A (V.B. and D.M., unpublished data).

Based on the above structural features and the observation that NS5A expressed alone behaves as an integral membrane protein (20), we propose a scenario where the rest of NS5A folds onto the polar side of the N-terminal amphipathic $\alpha$-helix membrane anchor. The 3D structures of two other monotopic membrane proteins with a similar organization have been described, namely prostaglandin H synthase and squalene cyclase. In both cases, integral membrane association is mediated by in-plane amphipathic $\alpha$-helices. Prostaglandin H synthase isoforms 1 and 2 bind to the ER membrane via four short in-plane amphipathic $\alpha$-helices which form horseshoe-shaped non-polar protrusions and interact with one leaflet of the phospholipid bilayer (41-43). The membrane anchoring region of squalene cyclase
forms a flat non-polar plateau consisting of one amphipathic α-helix, one loop, and one segment between two helices (44). Thus, in both cases there are a few additional contacts between the protein and the membrane besides the amphipathic helix. In line with this notion, the hydrophobic and basic residues following the NS5A amphipathic α-helix could participate in membrane association. In addition, we recently found that a construct comprising the first 30 aa of NS5A fused to the N-terminus of GFP associates with ER membranes, but is more easily extracted from these membranes when compared to the full-length NS5A protein (V.B. and D.M., unpublished data). This suggests that some other residues or segments located downstream of aa 1-31 interact with the membrane. Hence, although the N-terminal amphipathic α-helix is the main structural element anchoring NS5A to the membrane, the membrane binding region is likely more extended to ensure stable membrane association. In both prostaglandin H synthase and squalene cyclase, interactions between the membrane anchor and the rest of the protein are mainly ensured by charged and polar residues which define interaction sites on both part of these proteins. A similar arrangement likely occurs in NS5A, as suggested by the platform of hydrophilic residues and the asymmetric distribution of charged residues on the cytosolic side of the N-terminal α-helix.

It was recently reported that disruption of the amphipathic nature of the N-terminal helix of NS5A by introduction of several charged residues into the hydrophobic side abolished membrane association of NS5A and, by consequence, HCV RNA replication (45). The more subtle mutational analysis reported here revealed that our NS5A membrane anchor mutants, including the 8+A and 11+A mutants defective in RNA replication, show proper subcellular localization and membrane association, with only slightly reduced strength of membrane binding.
Moreover, polyprotein processing and incorporation of the mutants into the dot-like structures, which represent membranous webs harboring HCV replication complexes (5, 6), were not affected when they were expressed in the context of a NS3-5B polyprotein (data not illustrated). However, structure-function analyses of these mutants, which for HCV have become possible only recently through the development of the replicon system (14, 29), demonstrated that perturbation of the relative positioning of conserved residues by Ala insertion at position 8 or 11, leading to a twist of the $\alpha$-helix by 110° (see Fig. 5 B and C), completely abrogated RNA replication without significantly affecting membrane association. The discrepancy between RNA replication and membrane association phenotypes of these mutants suggests that the N-terminal region of NS5A has functions beyond serving as a membrane anchor and supports the idea of an essential interaction platform formed by the polar side of the amphipathic $\alpha$-helix. In contrast, Ala replacement of selected residues (R6A/D7A, W9A or C13A) had no or only moderate effect on HCV RNA replication. The absolutely conserved Cys13 could be either involved in a disulfide bond or form a link to a lipid. In both cases, replacement by Ala should result in only a limited destabilization of protein folding and/or protein-protein or protein-membrane interactions. Similarly, replacement of either Arg6 and Asp7 or Trp9 by Ala may be too subtle to destabilize essential interactions and may be compensated by the remaining residues involved in these interactions. Alternatively, the unimpaired replication of the R6A/D7A, W9A and even C13A mutants raises the possibility that the N-terminal portion of the helix may not be required for an intramolecular interaction, as discussed above, but may be involved in intermolecular interactions that are not essential for replicon activity.
Interestingly, when the NS5A membrane anchor mutants were expressed in the context of a NS3-5B polyprotein and analyzed with respect to their phosphorylation state, it was found that the replication-defective mutants 8+A, 11+A and ∆5-11 were not hyperphosphorylated in contrast to the mutants R6A/D7A, W9A and C13A (N.A. and R.B., unpublished data). This concordance between preserved hyperphosphorylation and RNA replication suggests that proper conformation of NS5A and/or the positioning of NS5A within the replication complex is altered in the replication-defective mutants, which in turn might interfere with kinase binding or accessibility of phosphoacceptor sites. Clearly, resolution of the 3D structure of the entire NS5A protein will shed further light on these mechanisms.

A synthetic peptide representing the N-terminal amphipathic α-helix of NS5A was recently found to block membrane association of NS5A in vitro in a dose-dependent and sequence-specific fashion (45). The mechanism of inhibition remains to be determined, but this observation raises the interesting possibility of interfering with membrane association of NS5A as a novel therapeutic strategy. The detailed structure-function analyses reported here suggest the presence of distinct sub-sites (e.g., aa 5-15 and 17-25 segments) that will be interesting to pursue as antiviral targets.

Predicted amphipathic α-helices mediating membrane association have been described in other plus-strand RNA viruses. For example, an amphipathic helix was predicted in the N-terminal region of poliovirus and hepatitis A virus 2C proteins (46-49). However, no structural data has been reported for these picornaviral proteins. Investigation of Semliki Forest virus mRNA-capping enzyme nsp1 by NMR spectroscopy revealed a short amphipathic α-helix mediating monotopic membrane association (50). Sequence alignments suggested that a similar amphipathic α-helix
is present in nsp1 of other alphaviruses. By analogy to HCV NS5A, it is reasonable to speculate that these amphipathic helices constitute the main structural determinants of membrane association and are essential for replication of the corresponding viruses. Hence, as for NS5A, these amphipathic helices constitute putative targets for the development of specific antiviral drugs.

In conclusion, the NS5A N-terminal in-plane amphipathic α-helix is not only a structural determinant for ER membrane targeting and binding via protein-phospholipid interactions, but is also a multi-interaction binding platform thought to ensure the stabilization of NS5A folding and/or its positioning in the HCV replication complex via intermolecular interactions. These findings have implications for the functional architecture of the HCV replication complex and may define novel antiviral targets.
ACKNOWLEDGMENTS

The authors gratefully acknowledge Rainer Gosert for assistance with confocal laser scanning microscopy, David J. Miller for sharing membrane flotation protocols, Christophe Geourjon for statistics calculations, Charles M. Rice for plasmid pBRTM/HCV1-3011con, Jan Albert Hellings for mAb 11H, and Hans-Peter Hauri for mAb G1/296.
REFERENCES


FIGURE LEGENDS

FIG. 1. NMR analysis of NS5A[1-31]. (A) Extract of the amide-aliphatic region of the NOESY spectrum of NS5A[1-31] in 100 mM SDS recorded at 40°C with a mixing time of 150 ms. Intra- and inter-residue cross-peaks with the corresponding amide proton are labeled by residue number. (B) Summary of sequential (i,i+1) and medium range (i, i+2 to i+4) NOEs. Intensities of NOEs are indicated by the height of the bars. Asterisks indicate that the presence of a NOE is not confirmed because of overlapping resonances. Amide protons that remained observable in a TOCSY spectrum after 24 h in D₂O are indicated by filled circles (slow-exchangable protons).

FIG. 2. Structural characterization of NS5A[1-31]. (A) Superimposition of the backbone atoms (C’, Cα, and N) of the 43 final structures in 100 mM SDS. The structures were aligned for best overlap of residues 5 to 25. Average positions of the Trp and Phe aromatic side chains are displayed. (B) Histogram of the three residues averaged RMSD values (i.e., local RMSD, black bars) for the backbone atoms of the final set of calculated structures in 100 mM SDS, 100 mM DPC, or 50% TFE, and ¹Hα chemical shift differences (ΔHα in ppm) versus residue number (grey bars). ¹Hα chemical shift differences were calculated by subtraction of the experimental values from the random coil conformation values reported by Merutka et al. (51). The dotted line indicates the standard threshold value for an α-helix (-0.1 ppm).

FIG. 3. Structure of the NS5A aa 5-25 amphiphatic α-helix. (A) Superimposition of calculated average structures obtained in the presence of 100 mM SDS (red), 100 mM DPC (green) and 50% TFE (light grey). The structures were aligned for best
overlap of residues 5 to 25. (B) Amino acid sequence of NS5A[1-31] including helix 5-25 (white box) and space-filling representation of the polar and hydrophobic sides of this helix (model of average structure in 100 mM SDS). Residues are color coded according to their physico-chemical properties. Hydrophobic and polar residues are grey and yellow, respectively. Positive and negative charged groups of basic (K, R) and acidic (D, E) residues are blue and red, respectively. The SH group of Cys is green.

**FIG. 4. Expected location and orientation of the NS5A amphipathic α-helix in the membrane.** (A) NMR analyses of NS5A[1-31] location in detergent micelles. Black arrows, aromatic residues exhibiting NOE cross-peaks between their side chains and DPC hydrophobic tails. Grey arrows, slow amide proton exchange with deuterated water in SDS-peptide micelles (reported from Fig. 1B). White arrows, residues buried in the SDS micelles for which TOCSY cross-peaks (Hα-HN, side chains) remain observable in spite of the presence of 0.5 mM MnCl₂. Black diamonds, residues accessible to water for which TOCSY cross-peaks totally disappear in the presence of 0.05 mM MnCl₂. Unlabeled residues correspond either to a partial broadening of their proton signal at 0.05 mM MnCl₂, to the lack of information due to the proximity of their Hα chemical shift with that of water, or to poorly resolved signals in crowded regions. The thick line indicates the helical segment. Residues corresponding to the putative flexible helical region are underlined. (B) Tentative positioning of the α-helix (ribbon diagram) at the interface between polar heads and hydrophobic tails of phospholipids. As the average structure of NS5A[1-31] was represented here (PDB entry 1R7E), the position of side-chain residues is only indicative (note for example that W4 seems to be buried in
the membrane while it was found to be poorly accessible to the hydrophobic tail of DPC, see panel A above). The phospholipid bilayer was drawn using the phosphatidylethanolamine (PE) models reported in Protein Data Bank entry 1BCC. A PE molecule colored according to atom types (blue, N; red, O; yellow, P; grey, C, H) is given on the right to illustrate the polar head/hydrophobic tail interface. (C) Top view of the peptide embedded at the interface of a model phospholipid membrane.

FIG. 5. NS5A membrane anchor mutants. (A) Amino acid sequences of NS5A membrane anchor mutants. The aa conservation among different HCV isolates (20) is indicated by an asterisk (*), a colon (:), and a dot (.) for fully conserved, conserved, and similar residues, respectively. (B, C) Space-filling representation of theoretical helices for mutants 8+A and 11+A. Ala insertion (shown in magenta) twists the helix by 110°. The C-terminal side of the helix is shown in the same orientation as in Fig. 3B to highlight the distortion of charged residues on the N-terminal side. Residues are colored as in Fig. 3B. These models were constructed by using the NMR average structure of NS5A[1-31] observed in 100 mM SDS as template and SwissPdb Viewer program (http://www.expasy.ch/swissmod/).

FIG. 6. Subcellular localization of NS5A membrane anchor mutants. U-2 OS cells were transiently transfected with pCMVNS5Acon, -R6A/D7A, -W9A, -C13A, -8+A, -11+A, -Δ5-11, and -45-448, as indicated by the captions. Cells were subsequently processed for immunofluorescence staining using mAb 11H against NS5A. Bound primary antibody was revealed with a FITC-conjugated secondary antibody. Slides were analyzed by confocal laser scanning microscopy as described
in the Experimental Procedures section. Horizontal sections taken through the center of the nuclei are shown.

**FIG. 7. RNA replication of NS5A membrane anchor mutants.** (A) Structure of the basic replicon construct carrying the firefly luciferase gene (*luc*). Adaptive mutations in NS3 and NS4B (E1202G, T1280I, K1846T) are indicated by black dots. GND indicates the position of the aa substitution in NS5B that blocks RNA-dependent RNA polymerase activity. (B) Representative results of a transient replication assay. Huh-7 cells were transfected with the replicon RNAs specified below each bar and luciferase activities were determined in cell lysates. Values refer to the percent ratio of the values measured 48 h and 4 h after transfection. The 4 h value was used to correct for different transfection efficiencies. Ref, parental replicon; GND, replication-deficient RNA. (C) Huh-7 cells were transfected with 1 µg of G418-selectable replicons specified below each plate. After 3 weeks cells were fixed and stained with Coomassie blue.

**FIG. 8. Membrane association of NS5A mutants.** Hypotonic lysates of U2-OS cells transiently transfected with pCMVNS5Acon, -8+A, -11+A, and -Δ5-11 were analyzed by centrifugation through Nycodenz gradients as described in the Experimental Procedures section. Fractions were collected from the top and analyzed by immunoblot using mAbs 11H against NS5A and G1/296 against p63.
Figure 2
Figure 3

A

In DPC 100 mM

In TFE 50%

In SDS 100 mM

B

1 5 10 15 20 25 30

SGM

KD1WNLCEVLSDKFRWLKAKLMQP
Figure 5

A

<table>
<thead>
<tr>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

NS5A

SGSWL<sub>R</sub>NDWIC<sub>E</sub>VLSDFKTLKALMPQLPG

R6A/D7A

SGSWLAN<sub>A</sub>WDWIC<sub>E</sub>VLSDFKTLKAKLMPQLPG

W9A

SGSWL<sub>R</sub>DAWIC<sub>E</sub>VLSDFKTLKAKLMPQLPG

C13A

SGSWLR<sub>D</sub>WDWIAEVLSDKTFKLKAKLMPQLPG

8+A

SGSWLR<sub>R</sub>DWIC<sub>E</sub>VLSDFKTLKAKLMPQLPG

11+A

SGSWLR<sub>R</sub>WDWIC<sub>E</sub>VLSDFKTLKAKLMPQLPG

Δ5-11

SGSW_______CEVLSDKTFKLKAKLMPQLPG

B

110°

C

110°
Figure 6
Figure 7
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Top</th>
<th>Bottom</th>
</tr>
</thead>
<tbody>
<tr>
<td>p63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p63 T X-100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS5A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS5A T X-100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8+A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11+A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ5-11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 8
**Supplemental Data**

**Table 1. Primer sequences.**

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Restriction Enzyme</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6A/D7A-fwd</td>
<td>EcoRI</td>
<td>5’ AATTCAACATGGCCGGTTCTGGCTAGCTATCTGGGACTGGATATGCGAGGTGC 3’</td>
<td>Blp</td>
</tr>
<tr>
<td>R6A/D7A-rev</td>
<td>Blp</td>
<td>5’ TCAGACATCCATATCCAGTGGCACTGGGACTGGATATGCGAGGTGC 3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td>W9A-fwd</td>
<td>EcoRI</td>
<td>5’ AATTCAACATGGCCGGTTCTGGCTAAGGGACATCGCTGACTGGGACTGGGACATGGTG 3’</td>
<td>Blp</td>
</tr>
<tr>
<td>W9A-rev</td>
<td>Blp</td>
<td>5’ TCAGACATCCATATCCAGTGGCACTGGGACTGGATATGCGAGGTGC 3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td>C13A-fwd</td>
<td>EcoRI</td>
<td>5’ AATTCAACATGGCCGGTTCTGGCTAAGGGACATCGCTGACTGGGACTGGGACATGGTG 3’</td>
<td>Blp</td>
</tr>
<tr>
<td>C13A-rev</td>
<td>Blp</td>
<td>5’ TCAGACATCCATATCCAGTGGCACTGGGACTGGGACATGGTG 3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td>8+A-fwd</td>
<td>EcoRI</td>
<td>5’ AATTCAACATGGCCGGTTCTGGCTAAGGGACATCGCTTGGGACTGGGACATGGTG 3’</td>
<td>Blp</td>
</tr>
<tr>
<td>8+A-rev</td>
<td>Blp</td>
<td>5’ TCAGACATCCATATCCAGTGGCACTGGGACTGGGACATGGTG 3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td>11+A-fwd</td>
<td>EcoRI</td>
<td>5’ AATTCAACATGGCCGGTTCTGGCTAAGGGACATCGCTGACTGGGACTGGGACATGGTG 3’</td>
<td>Blp</td>
</tr>
<tr>
<td>11+A-rev</td>
<td>Blp</td>
<td>5’ TCAGACATCCATATCCAGTGGCACTGGGACTGGGACATGGTG 3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td>∆5-11-fwd</td>
<td>EcoRI</td>
<td>5’ AATTCAACATGGCCGGTTCTGGCTAAGGGACATCGCTGACTGGGACTGGGACATGGTG 3’</td>
<td>Blp</td>
</tr>
<tr>
<td>∆5-11-rev</td>
<td>Blp</td>
<td>5’ TCAGACATCCATATCCAGTGGCACTGGGACTGGGACATGGTG 3’</td>
<td>EcoRI</td>
</tr>
</tbody>
</table>

**S R1978A+D1979A**

5’ GCTCCGGCTGGGCTAGCTATCTGGGACTGGGACATGGTG 3’

**A R1978A+D1979A**

5’ GCAATTCACCTCTGCCTAGCTATCTGGGACTGGGACATGGTG 3’

**S W1981A**

5’ CTAAGAGATTTGGCGGATTGGATATGCTACG 3’

**A W1981A**

5’ CTGCAATTCACCTCTGCCTAGCTATCTGGGACTGGGACATGGTG 3’

**S C1985A**

5’ GTTTGGGATTGGATAGCAGTGTTGACTG 3’

**A C1985A**

5’ CAGTCAACACCGTGCTATCCACCTACGACGAGTG 3’

**S 1980 Ins Ala**

5’ CTAAGAGATTTGGCGGATTGGATATGCTACG 3’

**A 1980 Ins Ala**

5’ CATATCCACCTCAGGCAACTCTCTCTG 3’

**S 1983 Ins Ala**

5’ GTTTGGGATTGGATAGCAGTGTTGACTG 3’

**A 1983 Ins Ala**

5’ CACCGTGCTATCCACCTACGACGAGTG 3’

**S △1977-83**

5’ CCATGCTCGCGTGATATGCTACGACGAGTG 3’

**A △1977-83**

5’ GTCAACACGGCAGTGCTATCCACCTACGACGAGTG 3’

Primers R6A/D6A-fwd through ∆5-11-rev were used for CMV promotor-driven expression constructs derived from the HCV H consensus cDNA clone (Kolykhalov, A.A. et al. (1997) Science 277, 570-574). Primers S R1978A+D1979A through A △1977-83 were used for mutagenesis of replicon constructs derived from the HCV Con1 cDNA clone (Lohmann, V. et al. (1999) Science 285, 110-113). Restriction enzyme recognition sites are underlined; start codons are bold.
**Table 2.** Statistics of the simulated annealing structures of NS5A[1-31] in TFE, SDS and DPC.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>TFE 50%</th>
<th>SDS 100 mM</th>
<th>DPC 100 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Constraints used</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance restraints</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-residue</td>
<td>134</td>
<td>196</td>
<td>182</td>
</tr>
<tr>
<td>Sequential</td>
<td>95</td>
<td>95</td>
<td>87</td>
</tr>
<tr>
<td>Medium range</td>
<td>97</td>
<td>64</td>
<td>57</td>
</tr>
<tr>
<td>Total distance restraints</td>
<td>326</td>
<td>355</td>
<td>326</td>
</tr>
<tr>
<td>B. Statistics for the final X-PLOR structures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of structures in the final set</td>
<td>51</td>
<td>43</td>
<td>27</td>
</tr>
<tr>
<td>X-PLOR energy (kcal.mol⁻¹)</td>
<td>-139±10</td>
<td>-112±11</td>
<td>-99±12</td>
</tr>
<tr>
<td>NOE violations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number &gt; 0.5 Å</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>R.m.s. deviation (Å)</td>
<td>0.05±0.003</td>
<td>0.04±0.009</td>
<td>0.04±0.006</td>
</tr>
<tr>
<td>Deviation from idealized covalent geometry</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angles (deg.)</td>
<td>0.5±0.01</td>
<td>0.53±0.01</td>
<td>0.5±0.02</td>
</tr>
<tr>
<td>Improper (deg.)</td>
<td>0.35±0.02</td>
<td>0.34±0.01</td>
<td>0.33±0.01</td>
</tr>
<tr>
<td>Bonds (Å)</td>
<td>0.003±0.0001</td>
<td>0.003±0.0001</td>
<td>0.004±0.0001</td>
</tr>
<tr>
<td>R.m.s. deviation (Å)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Backbone (C', Ca, N) helix (5-25)</td>
<td>1.11</td>
<td>1.12</td>
<td>2.17</td>
</tr>
<tr>
<td>all residues</td>
<td>3.39</td>
<td>3.75</td>
<td>4.58</td>
</tr>
<tr>
<td>All heavy atoms helix (5-25)</td>
<td>1.94</td>
<td>2.22</td>
<td>3.46</td>
</tr>
<tr>
<td>all residues</td>
<td>4.20</td>
<td>4.66</td>
<td>5.55</td>
</tr>
<tr>
<td>Ramachandran data (5-25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residues in mostfavoured regions (%)</td>
<td>99.7</td>
<td>98.6</td>
<td>93.1</td>
</tr>
<tr>
<td>Residues in allowed regions (%)</td>
<td>0.3</td>
<td>1.4</td>
<td>6.2</td>
</tr>
<tr>
<td>Residues in generously allowed regions (%)</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Residues in disallowed regions (%)</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>Ramachandran data</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residues in mostfavoured regions (%)</td>
<td>90.3</td>
<td>85.5</td>
<td>83.0</td>
</tr>
<tr>
<td>Residues in allowed regions (%)</td>
<td>8.4</td>
<td>11.8</td>
<td>13.9</td>
</tr>
<tr>
<td>Residues in generously allowed regions (%)</td>
<td>0.5</td>
<td>1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Residues in disallowed regions (%)</td>
<td>0.8</td>
<td>1.2</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Figure 1. Summary of sequential (i,i+1) and medium range (i, i+2 to i+4) NOEs in (A) 50% TFE, pH 4.5, 20°C, and (B) 100 mM DPC, pH 6.0, 40°C. Intensities of NOEs are indicated by the height of the bars. Asterisks indicate that the presence of a NOE is not confirmed because of overlapping resonances.
Figure 2. $^{13}$C$_\alpha$ chemical shift differences (in ppm) versus residue number for NS5A[1-31] in 100 mM SDS or 50% TFE. $^{13}$C$_\alpha$ chemical shift differences were calculated by subtraction of the experimental values from random coil conformation values. The dotted line indicates the standard threshold value for an $\alpha$-helix (0.7 ppm).
Figure 3. Differential membrane extraction. Microsomal fractions isolated from U-2 OS cells transiently transfected with pCMVNS5Acon, pCMVNS5Acon-Δ5-11, -8+A, and -11+A were resuspended in NTE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA), 1 M NaCl, 100 mM sodium carbonate, pH 11.5, 4 M urea, or 1% Triton X-100 as described (Brass, V. et al. (2002) J. Biol. Chem. 277, 8130-8139). Membrane sedimentations were performed by centrifugation at 16,000 x g for 15 min at 4°C. Supernatant (S) and pellet (P) fractions were applied in equivalent amounts, separated by 12% SDS-PAGE, and analyzed by immunoblot using mAb 11H against NS5A. Equivalent aliquots of the pellet (P-100) and supernatant (S-100) fractions from the initial 100,000 x g centrifugation step are loaded for comparison. Immunoblots were quantified using NIH image 1.59 software.
Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A
Francois Penin, Volker Brass, Nicole Appel, Stephanie Ramboarina, Roland Montserret, Damien Ficheux, Hubert E. Blum, Ralf Bartenschlager and Darius Moradpour

J. Biol. Chem. published online July 7, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404761200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/07/21/M404761200.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2004/07/07/jbc.M404761200.citation.full.html#ref-list-1