Hepatitis C virus (HCV) nonstructural protein 5A (NS5A), a phosphoprotein of unknown function, is believed to be a component of a membrane-associated viral replication complex. The determinants for membrane association of NS5A, however, have not been defined. By double label immunofluorescence analyses, NS5A was found to be associated with the endoplasmic reticulum (ER) or an ER-derived modified compartment both when expressed alone or in the context of the entire HCV polyprotein. Systematic deletion and green fluorescent protein fusion analyses allowed us to map the membrane anchor to the amino-terminal 30 amino acid residues of NS5A. Membrane association occurred by a posttranslational mechanism and resulted in properties of an integral membrane protein. Circular dichroism structural studies of a synthetic peptide corresponding to the NS5A membrane anchor, designated NS5A(1–31), demonstrated the presence of an amphipathic α-helix that was found to be highly conserved among 280 HCV isolates of various genotypes. The detergent-binding properties of this helical peptide together with the nature and location of its amino acids suggest a mechanism of membrane insertion via the helix hydrophobic side, yielding a topology parallel to the lipid bilayer in the cytoplasmic leaflet of the ER membrane. These findings have important implications for the structural and functional organization of the HCV replication complex and may define novel targets for antiviral intervention.

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide. A protective vaccine does not exist to date, and therapeutic options are still limited. HCV has been classified in the Hepacivirus genus within the Flaviviridae family which includes the classical flaviviruses, such as yellow fever virus, and the animal pestiviruses, such as bovine viral diarrhea virus (BVDV). The structure and replication cycle of HCV are incompletely understood due to the low viral titers found in sera and livers of HCV-infected individuals and the lack of an efficient cell culture system or small animal model permissive for HCV infection. Nevertheless, considerable progress has been made using heterologous expression systems, functional cDNA clones, and more recently, selectable subgenomic replicons (see Refs. 5 and 6 for recent reviews).

HCV contains a single-stranded RNA genome of positive polarity and ~9600 nucleotides (nt) length that encodes a polyprotein precursor of about 3000 amino acids (aa) (Fig. 1A). 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, which forms the viral nucleocapsid, and the envelope glycoproteins E1 and E2. The non-structural proteins NS2 through NS5B include the NS2-3 autoproteinase and the NS3 serine protease, an RNA helicase located in the carboxyl-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 viral nonstructural proteins are believed to form a membrane-associated replication complex together with as yet unidentified host cell components. However, the determinants for membrane association and the protein-protein interactions involved in formation of the HCV replication complex are poorly understood.

The aim of this study was to investigate the subcellular localization and mechanism of membrane association of the HCV NS5A protein. NS5A is a phosphoprotein of unknown function (7, 8). It is found in a basally phosphorylated form of 56 kDa and in a hyperphosphorylated form of 58 kDa. NS5A of HCV and BVDV as well as NS5 of yellow fever virus are phosphorylated by as yet unidentified serine/threonine kinases, suggesting that these proteins share a common function related to their phosphorylation state (9). NS5A has attracted considerable interest because of its potential role in modulating the interferon response (reviewed in Ref. 10). Numerous additional potential functions have been described recently in heterologous expression systems (11–13). However, the relevance of these observations for the natural course and pathogenesis of hepatitis C remains to be established. Interestingly, adaptive mutations have been found in NS5A in the context of selectable subgenomic HCV replicons, suggesting that NS5A is involved, either directly or by interaction with cellular proteins and pathways, in the viral replication process. This observation,
together with the modulation of NS5A hyperphosphorylation by the nonstructural proteins 3, 4A, and 4B (14, 15), strongly support the notion of NS5A being an essential component of the HCV replication complex. In line with this notion, NS5A has recently been shown to be essential for flavivirus Kunjin and BVDV RNA replication (16, 17).

Although HCV NS5A has recently attracted much attention, the subcellular localization and the mechanism of membrane association have not been defined. NS5A or related fusion proteins were found to be localized in the cytoplasm, but the subcellular compartment was not further explored (7, 18). Of note, evidence for a determinant for cytoplasmic localization in subcellular compartment was not further explored (7, 18). Of note, evidence for a determinant for cytoplasmic localization in

| Primer sequences               | Restriction enzyme recognition sites |
|-------------------------------|--------------------------------------|
| NSSAfwd                        | EcoRI                                 |
| NSSA-fwd-2                    | EcoRI/BspEI                           |
| NSSA45fwd                     | EcoRI                                |
| NSSArev                       | XbaI                                 |
| NSSA30rev                     | StuI                                 |
| NSSA30rev-2                   | XbaI                                 |
| NSSA360rev                    | XbaI                                 |
| EGFPAATGfwd                   | PmlI                                 |
| EGFPrev                       | XbaI                                 |
| EcoRI                        | 5′-GACGACATTCGACATTCGATTCACTGATAC-3′  |
| EcoRI                            | 5′-GACGATTTATCCGATGCTCGTCTGGAGACATC-3′ |
| EcoRI                        | 5′-GACGATTTCATCGGAGGCGAGGACATGTATG-3′ |
| StuI                         | 5′-CTGTCTGATTGCTGGACATCTTCTGGGGCAAGCAAC-3′ |
| XbaI                         | 5′-CTGTCTGATTGCTGGACATCTTCTGGGGCAAGCAAC-3′ |
| XbaI                         | 5′-CTGTCTGATTGCTGGACATCTTCTGGGGCAAGCAAC-3′ |

Membrane Association of HCV NS5A

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**TABLE I**

Restriction enzyme recognition sites are underlined and start and stop codons are in boldface.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs—**A fragment comprising nucleotides 6258–7603 (aa 1973–2420) of a functional HCV strain consensus cDNA (genotype 1a) was amplified by PCR from pBRTM/HCVI-3011con (21) (kindly provided by Charles M. Rice, Rockefeller University, New York, NY) using primers NSSAfwd and NSSArev (Table I). The amplification product was digested with EcoRI and XbaI and cloned into the EcoRI-XbaI sites of pcDNA3.1 (Invitrogen) and pUHD10-3 (22) to yield plasmids pCMVNSSAcon and pUHDNS5Acon, respectively. pCMVNSSAcon allows both eukaryotic expression from a cytomegalovirus promoter and in *vitro* transcription from a T7 RNA polymerase promoter. pUHDNS5Acon allows expression of NS5A under the transcriptional control of a tetracycline-controlled transactivator (T7A)-dependent promoter. NS5A fragments with amino- and carboxyl-terminal deletions were PCR-amplified from pBRTM/HCVI-3011con using primer pairs NSSA45fwd-NSSArev and NSSA45fwd-NSSA360rev (Table I) and cloned into the EcoRI-XbaI sites of pcDNA3.1 to yield plasmids pCMVNSSAcon45-448 and pCMVNSSAcon1-360, respectively (Fig. 24). A fragment representing the amino-terminal 30 aa of NS5A with an engineered start codon was amplified from pBRTM/HCVI-3011con using primers NSSAfwd and NSSA30rev (Table I), followed by digestion with EcoRI and StuI. A fragment representing an enhanced GFP without the ATG initiation codon was amplified from pEGFP-N1 (CLONTECH, Palo Alto, CA) using primers EGFPAGATGfwd and EGFPRev (Table I), followed by digestion with PmlI and XbaI. Subsequently, both fragments were ligated together into the EcoRI-XbaI sites of pcDNA3.1 to yield plasmid pCMVNSSAcon1-30GFP. This construct allows expression of the amino-terminal 30 aa of NS5A fused in frame with the amino-terminal 30 aa of NS5A. To construct plasmid pCMVGFPNS5Acon1-30, a fragment representing the amino-terminal 30 aa of NS5A was amplified from pBRTM/HCVI-3011con using primers NS5Afwd-2 and NS5A30rev-2 (Table I), digested with BspEI and XbaI, and cloned into the BspEI-XbaI sites of pCMVGFP (23). This construct allows expression of GFP with the amino-terminal 30 aa of NS5A fused to its carboxyl terminus (Fig. 2C). All expression constructs were verified by sequencing.

**Tetracycline-regulated Cell Lines—**Tetracycline-regulated cell lines were generated as described previously (24, 25). In brief, the constitutively tTA-expressing, U-2 OS human osteosarcoma (ATCC HTB-96)-derived founder cell line UTA-6 (26) was cotransfected with pUHDNS5Acon and pBabepeuro (27), followed by selection with G418 and puromycin. Antibiotic double-resistant clones were isolated and screened for tightly regulated NS5A expression by immunofluorescence microscopy and immunoblot analyses. UHCVcon-57.3 cells, which inducibly express the entire polyprotein derived from a functional HCV strain consensus cDNA (21), have been described previously (23).

Stable transfections were performed by a modified calcium phosphate precipitation protocol (28). Transient transfections were performed with a 22-kDa linear polyethyleneimine derivative (Excen 500, MBI Fermentas, Vilnius, Lithuania). Antibodies—NS5A-specific murine mAbs were generated by hybridoma fusion using an E. coli expressed recombinant protein comprising HCV aa 1948–2501 as antigen.

A polyclonal rabbit antiserum against protein-disulfide isomerase was obtained from StressGen (Victoria, British Columbia, Canada). A polyclonal rabbit antiserum to mannosidase II (29) was kindly provided by Kelley Moremen, University of Georgia, Athens, GA.

**Indirect Immunofluorescence and Confocal Laser Scanning Microscopy—**Indirect immunofluorescence microscopy was performed as described previously (24, 25). In brief, cells grown as monolayers on glass coverslips were fixed with 2% paraformaldehyde, permeabilized with 0.05% saponin, and incubated with primary antibodies in phosphate-buffered saline containing 3% bovine serum albumin and 0.05% saponin. Non-specific antibody was blocked with fluorescein isothiocyanate (FITC)-conjugated goat F(ab′)2 fragment to mouse IgG F(ab′)2 (ICN/Cappel, Aurora, OH), Texas Red (TXR)-conjugated sheep F(ab′)2 fragment to rabbit IgG (Roche Molecular Biochemicals). Coverslips were mounted in SlowFade (Molecular Probes, Eugene, OR) and examined in a Zeiss Axiom microscope equipped with an epifluorescence attachment. Confocal laser scanning microscopy was performed using a Zeiss LSM 410 microscope, and images were processed with Adobe Photoshop 3.0.5.

**Indirect Immunofluorescence and Confocal Laser Scanning Microscopy—**Indirect immunofluorescence microscopy was performed as described previously (24, 25).

**Subcellular Fractionation—**Subcellular fractionation was performed essentially as described previously (23, 24). In brief, 5 × 10⁶ cells were homogenized in a hypotonic buffer containing 10 mm Tris-HCl, pH 7.5, and 2 mm MgCl₂, followed by centrifugation at 1000 × g for 5 min to yield a nuclear pellet. The supernatant fraction was adjusted to 0.25 M sucrose, and a mitochondrial pellet was obtained by centrifugation at 9,000 × g for 10 min. Finally, a microsomal pellet was separated from the cytosolic supernatant by centrifugation at 100,000 × g for 40 min. All procedures were carried out at 4 °C.

**In Vitro Transcription-Translation (IVTT)—**The TNT T7-coupled reticulocyte lysate system (Promega, Madison, WI) was used essentially following the manufacturer’s recommendations. IVTT was performed for 90 min at 30 °C in the presence of 0.8 μCi/ml [35S]methionine (Amersham Biosciences) in a volume of 25 μl. Where indicated, 2.0 μl of...
canine pancreatic microsomes (kindly provided by Martin Spiess, Biozentrum, University of Basel, Switzerland, and Matthias Müller, Department of Biochemistry, University of Freiburg, Germany) were added. Our initial experiments revealed that a proportion of the in vitro translated NS5A protein aggregated and precipitated spontaneously in the absence of the artificial membrane. Careful optimization of the IVTT conditions showed that this tendency could be improved without significant reduction of specific membrane association by the addition of the mild detergent digitonin at a concentration of 0.02%. Therefore, all IVTT reactions contained 0.02% digitonin.

For membrane sedimentation analyses, 15 μl of NTE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) was added after completion of the IVTT reaction, followed by centrifugation at 12,000 g for 15 min. Supernatants were collected, and pellets were resuspended in 40 μl of NTE buffer. Subsequently, pellet and supernatant fractions were analyzed by SDS-PAGE, followed by autoradiography. Gels were scanned on a Fuji BAS1000 PhosphorImager and analyzed using the Fuji MacBAS version 2.4 software.

For analyses of co- and posttranslational membrane association, microsomal membranes were added to the reaction either during or for 45 min at 30 °C after completion of IVTT. In the latter setting translation was stopped by 1.25 mM puromycin prior to the addition of microsomal membranes.

For membrane extraction experiments, microsomal membranes were posttranslationalally added to IVTT reactions, followed by sedimentation of membrane-associated material as described above. Subsequently, membrane pellets were resuspended in NTE buffer, 1 mM NaCl, 100 mM sodium carbonate, pH 11.5, 2, 4, or 6 mM, or 1% Triton X-100 and incubated for 20 min at 4 °C. Finally, membrane sedimentation analyses were performed and fractions were analyzed by SDS-PAGE.

Sequence Analyses and Structure Predictions—All analyses were performed using the IBCP HCV data base website facilities (HCVDB, hepatitis.ibcp.fr) which contains all HCV sequences available from the EMBL data base. The amino-terminal NS5A sequence of the HCV H strain consensus cDNA (21) (GenBank™ accession number AF009606) was used to retrieve all reported isolates from the EMBL data base using the FASTA homology search program (30). A final set of 280 sequences of various genotypes was analyzed to construct Fig. 5. Multiple sequence alignment was carried out with the ClustalW program (31). Visualization of sequence alignments and plotting of the most frequently represented aa residues at each position was performed with the MPSA program (32). At each aa sequence position, the residue types and their respective frequencies were computed using a program developed at IBCP. The secondary structure of NS5A from various genotypes was predicted using a large set of methods available at the NPSA website (33), including DSC, HNNC, SPMAP96, SOPM, plpk, PHD, and Predator (see npsa-phil.ibcp.fr/NPSA and references therein).

Peptide Synthesis, Circular Dichroism, and Gel Filtration—The NS5A(1–31) peptide was synthesized using the stepwise solid-phase method of Merrifield employing Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry and purified to homogeneity by reverse-phase-high pressure liquid chromatography. CD spectra were recorded on a CD6 dichrograph from John Yvon calibrated with (1S)-(+)-10-camphorsulfonic acid. Measurements were carried out at room temperature using a 0.1-cm path length quartz cuvette, with peptide concentrations ranging from 15 to 40 μM at pH 6.5. Spectra were recorded in the 190–250 nm wavelength range with a 0.2-nm increment and a 2-s integration time. The spectra were processed, with peptide concentrations determined by measurements of UV light absorption of tryptophan at 280 nm (molar extinction coefficient of 5990 cm⁻¹ mol⁻¹ cm⁻¹). The α-helix content was estimated at 222 nm using the empirical equation of Chen et al. (34) as detailed previously (35, 36) with a theoretical molar ellipticity of −36.10° deg cm² mol⁻¹ for 100% helical conformation of the NS5A(1–31) peptide in the various media. Gel filtration chromatography was performed on a Protein PAK 200SW column (0.8 × 30 cm) from Waters Associates at an isocratic flow rate of 0.5 ml/min and using 0.1 M sodium phosphate buffer, pH 6.5, containing 0.1 mM dithiothreitol (DTT). For assays in the presence of detergent (3% Delipid), the peptide was previously dissolved either in 250 mM n-dodecyl β-n-maltoside (DM) or 400 mM dodecyl phosphocholine (DPC) to a final concentration of 0.5 mM. Note that no detergent was present in the chromatographic buffer. Calibration of the column was performed with various molecular weight markers under the same conditions (see legend to Fig. 6).

RESULTS

Tetracycline-regulated Cell Lines—A tetracycline-regulated gene expression system was used to establish U-2 OS human osteosarcoma-derived cell lines inducibly expressing NS5A. Screening of 48 G418- and puromycin-resistant clones resulting from the transfection of the tTA-expressing founder cell line UTA-6 with the construct pUHDNS5Acon allowed the isolation of 13 tightly regulated UNSS5Acon cell lines. In the following, data obtained with cell lines UNSS5Acon-6 (high expression) and UNSS5Acon-37.2 (medium expression) will be presented. These cell lines were maintained in continuous culture for more than 12 months and over 50 passages with stable characteristics.

Monoclonal Antibodies—Recombinant NS5A protein expressed in Escherichia coli was used as an antigen to generate murine mAbs. A mAb of IgG1s isotype, designated 11H, was selected from a large panel of NS5A-specific mAbs. This mAb functioned well in indirect immunofluorescence and immunoblot applications, indicating that it recognizes a linear epitope on NS5A. This epitope was mapped to residues 2221–2232 using a panel of overlapping synthetic peptides (Fig. 2A).

NS5A Is Associated with the ER or an ER-derived Modified Compartment—The subcellular localization of NS5A was determined by indirect immunofluorescence microscopy. A representative analysis of UNSS5Acon-37.2 cells is shown in Fig. 1B. No immunoreactivity was detected when the cells were cultured in the absence of tetracycline. NS5A expression became clearly detectable 6 h following tetracycline withdrawal (data not shown) and increased to reach a steady-state level after 24 h. At this time point mAb 11H revealed a staining pattern that included the nuclear membrane, was emphasized in the perinuclear region, and extended in a reticular fashion through the cytoplasm. No staining of the nuclear or plasma membrane was observed. In UHCVcon-57.3 cells, which inducibly express NS5A in the context of the entire HCV polypeptide (23), the cytoplasmic reticular staining pattern was similar to that observed in UNSS5Acon-37.2 cells (Fig. 1B). In these cells, NS5A tended to accumulate in additional cytoplasmic dots, suggesting the formation of a distinct subcellular compartment harboring NS5A expressed in the context of the entire HCV polypeptide.

Subcellular fractionation by differential centrifugation revealed NS5A only in membrane-containing fractions, but not in the cytosolic S-100 fraction (data not shown). Thus, the staining pattern and the subcellular fractionation data were highly suggestive of an association of NS5A with membranes of the ER. To explore further the subcellular localization of NS5A, double label confocal laser scanning microscopy with antibodies to cellular marker proteins was performed. As shown in Fig. 1C, NS5A colocalized with protein-disulfide isomerase, a marker for the ER in UHCVcon-57.3 and UHCVcon-57.3 cells. However, the NS5A staining pattern observed in these cells was different from that revealed by antibodies directed against a marker of the ER-to-Golgi intermediate compartment (data not shown) and mannosidase II, a marker of the ER-to-Golgi intermediate compartment (data not shown). Association of a minor proportion of NS5A with these reticulated compartments cannot be excluded by this technique. Taken together, however, these results clearly demonstrate that the major localization of NS5A, independently of the coexpression of other HCV proteins, is the ER or an ER-derived modified compartment.

The Amino-terminal 30 aa of NS5A Serve as a Membrane Anchor—The data shown above suggest that NS5A possesses an intrinsic determinant for subcellular targeting and membrane association. To explore this domain systematically, we

C. Combet, unpublished data.
D. Ficheux, unpublished results.
generated a set of deletion constructs as shown in Fig. 2A. Construct NS5A45–448 lacks the amino-terminal 44 aa and construct NS5A1–360 the carboxy-terminal 88 aa. Both constructs retained the 11H mAb epitope and the putative nuclear localization signal identified by Ide et al. (37). These constructs were transiently transfected into U-2 OS human osteosarcoma cells, followed by immunofluorescence analyses using mAb 11H. As shown in Fig. 2A, deletion of the amino-terminal 44 aa abolished the typical ER staining pattern and resulted in a diffuse cytoplasmic staining with accumulation of the protein in the nuclei. By contrast, the distribution of the NS5A1–360 construct was virtually identical to that of full-length NS5A. These results indicate the presence of a membrane anchor within the amino-terminal 44 aa of NS5A. Interestingly, the cryptic nuclear localization signal (see above) becomes dominant once the membrane targeting domain is deleted and results in nuclear accumulation of NS5A45–448. Of note, the nucleoli were spared by this construct, suggesting indirectly that NS5A does not possess intrinsic RNA-binding properties. This is in contrast to carboxy-terminally truncated HCV core and NS5B proteins which accumulate in the nucleoli (23, 24).

Based on sequence analyses and structure predictions for the amino-terminal domain of NS5A (see below, Fig. 5), we predicted the membrane anchor to be localized within the amino-terminal 30 aa. To assess whether this segment is sufficient to target a heterologous protein to the ER membrane, we generated the GFP fusion constructs shown in Fig. 2B, where the amino-terminal 30 aa residues of NS5A were fused directly in frame either to the amino terminus or to the carboxy terminus of GFP. These constructs were transiently transfected into U-2 OS cells. Because of the very bright fluorescence of GFP, confocal laser scanning microscopy was employed to demonstrate unequivocally the subcellular distribution of these constructs. As expected, GFP was diffusely distributed in the cytoplasm and nucleus. By contrast, the NS5A1–30GFP fusion construct showed the same staining pattern as NS5A with a fine reticular network involving the nuclear membrane, surrounding the nucleus, and extending through the cytoplasm. Interestingly, fusion of the amino-terminal 30 aa of NS5A to the carboxy terminus of GFP resulted in the same staining pattern, indicating that this segment does not function as a classical signal sequence but may mediate posttranslational targeting to the ER membrane. Taken together, these results demonstrate that the amino-terminal 30 aa of NS5A serve as a membrane anchor.

**Membrane Association of NS5A Can Occur by a Posttranslational Mechanism—IVTT and membrane sedimentation analyses were performed to characterize further the membrane association of NS5A. NS5A was translated in a coupled rabbit reticulocyte lysate system in the presence or absence of microsomal membranes. Subsequently, membrane-associated material was separated by centrifugation and NS5A was quantified in both fractions. To elucidate the mechanism of membrane association, we first examined whether membrane targeting of NS5A occurs co- or posttranslationally. In eukaryotic cells, ER transport of membrane proteins is generally mediated by a signal sequence that is recognized by the signal recognition particle (SRP) (38). The SRP interacts with the signal sequence...**
of nascent polypeptide chains during translation and directs the translation complex to the ER membrane. SRP-mediated ER transport, therefore, occurs only cotranslationally. However, based on the results obtained above, the membrane anchor of NS5A would be expected to mediate posttranslational membrane association because in the case of GFPNS5A1–30 it is buried within the translating ribosome when the termination codon is reached. Thus, to explore these two possibilities, microsomal membranes were added to the reaction either during or after completion of IVTT. Puromycin was added prior to the addition of microsomal membranes in the posttranslational setting to stop translation and to ensure that polypeptides were released from ribosomes. As representatively shown in Fig. 3, when NS5A was translated in the absence of microsomal membranes only 20% was subsequently found in the pellet fraction. By contrast, 65% of the protein was pelleted when translation was performed in the presence of microsomal membranes. These results demonstrate that membrane association of NS5A occurs efficiently also in vitro. Interestingly, 71% of NS5A was found in the pellet fraction when the membranes were added posttranslationally. These results demonstrate that membrane association of NS5A can occur by a posttranslational mechanism.

NS5A Behaves as an Integral Membrane Protein—Membrane extraction experiments were performed to characterize the nature of the association of NS5A with the ER membrane. To this end, NS5A was translated in vitro in the presence of microsomal membranes, and the pellet fraction was subsequently subjected to differential extraction methods. High salt extraction (1 M NaCl) shields charges and weakens ionic interactions that bind peripheral proteins to membranes either directly or indirectly through other membrane proteins (39). Treatment with 100 mM sodium carbonate, pH 11.5, releases peripheral proteins by transforming microsomes into membrane sheets (40). In addition, urea extraction (8 M urea) is a strong chaotropic agent that extracts peripheral membrane proteins. As shown in Fig. 4A, NS5A remained associated with microsomal membranes under all conditions examined. NS5A, therefore, is tightly associated with the ER membrane and behaves as an integral membrane protein. As a control, membranes were disrupted with 1% Triton X-100, resulting in the release of NS5A into the supernatant fraction. To extend these in vitro observations into a cellular setting, membrane extraction experiments were performed with microsomal fractions isolated from U87 cells and HCV+ cells. In this case, supernatant and pellet fractions were analyzed by immunoblotting using the NS5A-specific mAb 11H. As shown in Fig. 4B, the extraction profile of cellularly expressed NS5A reflected the behavior of the in vitro translated protein and confirmed the notion of NS5A having properties of an integral membrane protein. Interestingly, when one compares the proportion of NS5A in the supernatant fraction following alkali and urea extraction, NS5A appears to be more tightly membrane associated when expressed in UHCVC-57.3 cells.
in the context of the entire HCV polyprotein as compared with UNS5Acon-6 cells where the protein is expressed alone.

**Sequence Comparisons and Structure Predictions**—The experiments described above allowed the identification of a membrane anchor within the amino-terminal 30 aa of NS5A that can mediate posttranslational membrane association and insertion as an integral membrane protein. Such sequences are rather unique and have been described primarily at the carboxyl terminus of a class of proteins termed “tail-anchored proteins” (41). Sequence comparisons were performed to assess the degree of conservation of this sequence among different HCV isolates and to identify motifs potentially involved in membrane targeting and insertion. The repertoire of aa deduced from the analysis of 280 HCV isolates of various genotypes revealed that aa are strictly conserved in 50% of sequence positions (Fig. 5A). Moreover, most of the positions showing apparent variability are occupied by residues with similar hydrophatic character, as illustrated in Fig. 5B, where residues were classified into 3 classes according to their hydrophobic character (see legend to Fig. 5). A letter-coded motif summarizing the NS5A(1–33) hydrophatic pattern is shown in Fig. 5C. Typically, most of NS5A(1–33) positions bear exclusively hydrophobic (o), neutral (n), or hydrophilic (i) residues, whereas a few positions bear two classes of residues, and only one position (residue 21) bears 3 classes of residues (named v for “variable position”). Overall, despite the apparent variability of aa at some positions, the full conservation of specific and charged residues at most positions and the hydrophatic character in the other positions reveal a strict conservation of the NS5A(1–33) sequence and of its interaction properties among the various HCV genotypes. Secondary structure predictions by various methods were very informative because in all genotypes a consensus α-helix is predicted in the aa 26–32 segment by all methods used, as shown in Fig. 5C. In contrast, the short segment 29–32 containing the strictly conserved Pro-29 is predicted to adopt a turned conformation (Fig. 5C), whereas segment 35–48 is predicted as extended (data not illustrated). An ideal α-helix projection of the aa 4–25 segment clearly revealed an amphipathic helix with a very hydrophobic side, rich in Trp, residues, and a hydrophilic, charged side (Fig. 5D).

**Structure and Lipid-binding Properties of the NS5A Membrane Anchor**—To study the structural features and lipid-binding properties of the NS5A membrane anchor, a peptide identical to the NS5A aa 1–31 segment was chemically synthesized, highly purified by reverse phase-high pressure liquid chromatography, and analyzed by CD and gel filtration in the presence of membrane mimetic media. This peptide, designated NS5A(1–31), was designed to represent the entire α-helix residues 4–26 predicted by the above analyses. The NS5A(1–31) peptide appeared to be quite soluble in water and exhibited a CD spectrum typical of α-helix conformation with a maximum around 192 nm and two minima around 208 and 222 nm (Fig. 6A). Addition of trifluoroethanol (TFE) up to 50% had almost no effect on the shape and the amplitude of the CD spectra, indi-
cating that the NS5A(1–31) peptide reaches its maximum α-helical fold in aqueous buffer. Indeed, TFE is well known to induce α-helical folding of peptidic sequences presenting an intrinsic propensity to fold into α-helix (42, 43). Moreover, TFE is also known to break hydrophobic interactions, thereby preventing peptide aggregation or leading to dissociation of oligomeric or aggregated states of peptides. When dissolved in aqueous buffer, the NS5A(1–31) peptide yielded poorly resolved proton NMR spectra exhibiting broad resonance signals (data not shown). This contrasts with the well resolved NMR spectra obtained after addition of moderate amounts of TFE (25–30%), indicating that in the absence of TFE the NS5A(1–31) peptide likely forms oligomers interacting by hydrophobic interactions. By CD analyses, a helical content of 80% was estimated at 222 nm, indicating that about 25 of the 31 aa adopt an α-helical fold. This is perfectly in line with the above secondary structure predictions and supports the presence of an α-helix core from aa residue 4 to 26 with possibly one or two additional helical residues on one or both ends of the helix.

In the presence of various detergents used here to mimic the membrane environment, CD spectra showed roughly the same shape and amplitude (Fig. 6B). This demonstrates that the NS5A(1–31) peptide conserved its helical fold with an α-helix content varying from 78 to 83%. However, the slight differences in the shape of CD spectra recorded in the presence of detergents as compared with those obtained in aqueous buffer or 50% TFE indicate a modulation of peptide conformation that is likely due to its interaction with detergent micelles. Interaction of the NS5A(1–31) peptide with detergents was therefore further explored by gel filtration chromatography, as shown in Fig. 6C. When previously dissolved in DPC or DM, the NS5A(1–31) peptide is eluted at a molecular weight corresponding roughly to the molecular weight of detergent micelles (i.e. about 66 and 35 kDa for mixed micelles of peptide-DPC and peptide-DM, respectively). In contrast, in the absence of detergent the peptide was eluted with a poor yield (about 20%) at 43 min (solid line). This is much more than expected (about 26 min) based on its low molecular weight (3766 Da). These data indicate that the peptide interacts with the chromatographic media, probably by nonspecific hydrophobic interactions, and likely forms aggregates. Indeed, detergents were mandatory to elute efficiently all the peptide nonspecifically bound to the gel filtration column. This behavior is in agreement with a strong propensity of the peptide to bind both to neutral or phospholipid-mimicking detergents (DM and t-o-lysophosphatidylcholine, respectively). In summary, these data demonstrate the high propensity of the NS5A(1–31) peptide to bind to lipids.

**DISCUSSION**

Formation of a membrane-associated replication complex is a characteristic feature of positive-strand RNA viruses (44–49). In line with this notion, physical (50) and functional interactions (14, 15, 51, 52) among HCV nonstructural proteins suggest that these, together with as yet unidentified host cell components, assemble into a membrane-bound replication complex. The mechanisms of membrane association, however, and the protein-protein interactions involved in formation of the HCV replication complex are poorly understood.

Here we show that HCV NS5A, a phosphoprotein of unknown function, is targeted to membranes of the ER or an ER-derived modified compartment both when expressed alone or in the context of the entire HCV polyprotein. By systematic deletion and GFP fusion analyses, the membrane anchor was mapped to the amino-terminal 30 aa residues of NS5A. IVTT
and membrane sedimentation analyses performed in the presence or absence of microsomal membranes indicated that NS5A can associate with membranes by a posttranslational mechanism and behaves as an integral membrane protein. Finally, a synthetic peptide corresponding to the NS5A membrane anchor, designated NS5A(1–31), formed an amphipathic α-helix with intrinsic lipid-binding properties, as shown by CD and gel filtration analyses. Thus, an amino-terminal amphipathic helix can mediate posttranslational membrane association of NS5A as an integral membrane protein.

The best characterized mechanism of membrane insertion in mammalian cells is the SRP-mediated pathway where membrane targeting is initiated cotranslationally by a signal sequence close to the amino terminus of the nascent polypeptide (38). The signal sequence interacts with the SRP that then docks at the ER. Subsequently, integration occurs via a multistep process ending with release of the polypeptide into the lipid membrane coincident with the completion of protein synthesis. In this context, membrane targeting of the HCV structural proteins appears to be mediated by the SRP-dependent pathway (53, 54), whereas NS3 has been shown to be targeted to the ER or an ER-like modified compartment via interaction with its cofactor NS4A (55).

Another small but rapidly growing class of membrane proteins, termed tail-anchored proteins, lacks an amino-terminal signal sequence and instead is membrane-targeted posttranslationally via a carboxyl-terminal insertion sequence (41, 56). We have recently shown that the HCV NS5B RNA-dependent RNA polymerase fulfills the criteria of a classical tail-anchored protein (23).

The results reported in this paper suggest that HCV NS5A belongs to a new class of membrane proteins that can be targeted posttranslationally (and, presumably, via an SRP-independent pathway) to the ER via a rather unique amino-terminal membrane anchor. In analogy to the tail-anchored proteins, this sort of membrane protein could be termed “tip-anchored protein.”

The CD structural studies of the amino terminus of NS5A based on the synthetic peptide NS5A(1–31) clearly demonstrate the existence of an α-helix including segment 4–26. Helix projection of this segment highlights the amphipathic nature of this helix which is highly conserved among 280 HCV isolates of various genotypes analyzed. Its hydrophilic side contains numerous strictly conserved charged residues suggesting a role in specific protein-protein interactions. The hydrophobic side contains large hydrophobic residues with strictly conserved Trp residues at the interface between the hydrophilic and hydrophobic sides of the 4–26 amphipathic helix. The solubility of NS5A(1–31) peptide in water as oligomer strongly suggests that multiple peptide molecules can interact via the hydrophobic helix side, whereas the hydrophilic side surrounding the resulting oligomer ensures its solubility. In addition, we show that the NS5A(1–31) peptide exhibits a strong propensity to bind to detergents that mimic membrane lipids. Such interactions do not induce substantial changes in the α-helical folding, suggesting that the hydrophobic core created by oligomerization of the NS5A(1–31) peptide in water is efficiently substituted by the hydrophobic core of detergent micelles. The monomeric peptide is thus supposed to interact at the surface of the detergent micelles with hydrophobic residues buried within the hydrophobic core of the micelle. This is supported by the location of strictly conserved Trp residues at the interface between the hydrophilic and hydrophobic sides of the 4–26 amphipathic helix. Indeed, in membrane proteins, Trp residues are often located at the lipid bilayer interface but rarely within the hydrophobic core of the membrane (57, 58). Taken together, these data suggest that

![Fig. 6. Structural analysis of the NS5A(1–31) peptide and interaction with lipids. A and B, far UV CD spectra of NS5A(1–31) in the presence of 10 μM DTT at pH 6.5. A, spectra of the peptide in water solution (solid line) and in 50:50 TFE/water solution (dotted line). B, spectra of the peptide in the presence of detergents: 0.5% L-lysophosphatidylcholine (LPC, dashed line), 100 mM dodecyl phosphocholine (DPC, dotted line), and 200 mM SDS (solid line). A similar spectrum was obtained with 100 mM dodecyl β-maltoside (DM) but was not presented for the clarity of the figure. C, gel filtration of the NS5A(1–31) peptide in 100 mM phosphate buffer, pH 6.5, and 0.1 mM DTT. Solid line, peptide alone (22 μg loaded); dashed line, peptide dissolved in DM (9 μg of peptide loaded); dotted line, peptide dissolved in DPC (9 μg of peptide loaded). Note that no detergent was added to the chromatographic buffer. Arrows indicate the position of molecular mass markers: 1, blue dextran (2,000 kDa); 2, bovine serum albumin (66 kDa); 3, β-lactoglobulin (35 kDa); 4, myoglobin (17.8 kDa); and 5, DTT (154 Da).]()}
membrane insertion of the NS5A amino-terminal domain occurs via the helix hydrophobic side with residues Trp, Val, Leu, Ile, and Phe intercalating the leaflet of the membrane, whereas hydrophilic charged residues remain accessible at the surface of the membrane. It is thus rational to assume that in NS5A the amphipathic helix 4–26 is parallel with the lipid bilayer and embedded in the cytoplasmic leaflet of the ER membrane. This topology, termed monotopic (59), was shown for various amphipathic α-helices (60, 61).

The amphipathic helix is a structural motif found in many proteins that bind to membranes. For example, a conserved amino-terminal amphipathic α-helix is required for plasma membrane targeting of “regulators of G protein signaling” proteins (62). Moreover, prostaglandin H2 synthase-1 (63), CTP:phosphocholine cytidyltransferase (64), and annexin I (65) are membrane-associated by amphipathic helices. Among the positive-strand RNA viruses, Semliki Forest virus nonstructural protein 1 associates with the cytoplasmic side of the plasma membrane and to some extent with endolysosomal membranes via an amphipathic α-helix in the middle of the protein (66).

Based on sequence comparisons, this feature was predicted to be conserved among all alphaviruses. Interestingly, amphipathic helices can modulate membrane bilayer properties and can be stabilizing, pore-forming, or lytic to membranes (67). In this context, it was discussed that enterovirus protein 2B, which contains an amphipathic helix essential for viral replication, may function as a viroporin (68).

Interestingly, membrane association of NS5A expressed in the IVTT system as well as in mammalian cells was tight enough for the protein to behave as an integral membrane protein. It is uncertain whether the number of lipid-amino acid hydrophobic interactions would be sufficient to confer such a strong membrane association in the case of a simple in-plane membrane anchorage. One can thus speculate that the in-plane membrane anchorage represents only a transient topology that could evolve to a more complex assembly via protein–protein interactions, maybe within the cytoplasmic leaflet of the membrane bilayer or as a transmembrane anchor.

Recently, we have shown that NS4B, which precedes NS5A in the HCV polyprotein, associates with the ER membrane in a cotranslational fashion, resulting in a tightly membrane-associated, integral membrane protein (69). In the context of HCV polyprotein expression and processing, the translating ribosome is presumably already located at the ER membrane when NS5A is synthesized, and membrane association could occur cotranslationally. However, in view of the unique structure of the NS5A membrane anchor, alternative scenarios could be evoked as discussed above. In this context, it is interesting to note that in the BVDV system defects in the NS5A genetic unit can be complemented in trans (17). Future work will aim at defining the topology and possible protein–protein interactions of the NS5A membrane anchor within the ER membrane. Finally, resolution of its three-dimensional structure by NMR spectroscopy should lead to new insights into this unique mechanism of membrane association.

Membrane association of NS5A was independent of the expression of other HCV proteins. In this context, cotransfection of the NS5A45–448 construct with the NS3–4A complex, NS4B, or NS5B, which by themselves are membrane-associated (23, 55, 69), did not alter the subcellular localization of the amino-terminally truncated NS5A protein.4 This observation suggests that, at least when expressed in trans, protein–protein interactions between cytosolic domains within the HCV replication complex are relatively weak as compared with the membrane association of these proteins. This may provide a certain flexibility to these proteins to fulfill their multiple functions during HCV polyprotein processing and RNA replication. Alternatively, interactions among these proteins may be mediated by their membrane domains within the membrane bilayer. Studies are in progress to investigate further these possibilities.

Ultimately, elucidation of the determinants for membrane association of the HCV nonstructural proteins and of their involvement in formation of the viral replication complex may define novel targets for specific antiviral intervention.

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