Determinants for Membrane Association of the Hepatitis C Virus RNA-dependent RNA Polymerase*

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The hepatitis C virus (HCV) RNA-dependent RNA polymerase (RdRp), represented by nonstructural protein 5B (NS5B), is believed to form a membrane-associated RNA replication complex together with other nonstructural proteins and as yet unidentified host components. However, the determinants for membrane association of this essential viral enzyme have not been defined. By double label immunofluorescence analyses, NS5B was found in the endoplasmic reticulum (ER) or an ER-like modified compartment both when expressed alone or in the context of the entire HCV polyprotein. The carboxy-terminal 21 amino acid residues were necessary and sufficient to target NS5B or a heterologous protein to the cytosolic side of the ER membrane. This hydrophobic domain is highly conserved among 269 HCV isolates analyzed and predicted to form a transmembrane α-helix. Association of NS5B with the ER membrane occurred by a posttranslational mechanism that was ATP-independent. These features define the HCV RdRp as a new member of the tail-anchored protein family, a class of integral membrane proteins that are membrane-targeted posttranslationally via a carboxyl-terminal insertion sequence. Formation of the HCV replication complex, therefore, involves specific determinants for membrane association that represent potential targets for antiviral intervention.

With an estimated 170 million chronically infected individuals the hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide (1). A protective vaccine does not exist to date, and therapeutic options are still limited (2). HCV has been classified in the Hepacivirus genus within the Flaviviridae family that includes the classical flaviviruses, such as yellow fever virus, and the animal pestiviruses (3). HCV contains a single-stranded RNA genome of positive polarity and ~9600 nucleotides (nt) in length that encodes a polypeptide precursor of about 3000 amino acids (aa) (see Refs. 4 and 5 for recent reviews) (Fig. 1A).

The polyprotein precursor is co- and posttranslationally processed by cellular and viral proteases to yield the mature structural and nonstructural proteins. HCV replication proceeds via synthesis of a complementary minus strand RNA using the genome as a template and the subsequent synthesis of genomic plus strand RNA from this minus strand RNA template. The key enzyme responsible for both of these steps is the RNA-dependent RNA polymerase (RdRp), represented by nonstructural protein 5B (NS5B).

The HCV RdRp has been shown to be essential for viral replication in vitro (6) and in vivo (7). It has recently been characterized both biochemically (8–12) and with respect to its three-dimensional structure (13–15). The HCV NS5B protein contains motifs shared by all RdRPs and possesses the classical fingers, palm, and thumb subdomains. As a unique feature of the HCV RdRp extensive interactions between the fingers and thumb subdomains result in a completely encircled active site. Interestingly, deletion of the highly hydrophobic carboxyl-terminal domain of NS5B has been found to increase solubility of the protein in Escherichia coli (10, 11) and to alter the subcellular localization in mammalian cells (10). Three-dimensional structures of NS5B reported thus far lack this carboxyl-terminal domain.

Compared with the detailed knowledge of its biochemical and structural features, much less is known about the characteristics of NS5B in a cellular context. This is due in part to the lack of an efficient cell culture system permissive for HCV infection and replication and the difficulty to reliably detect viral proteins in naturally infected liver tissues. In a preliminary study, NS5B was found to be present as fine speckles in the cytoplasm of transiently transfected COS 7 cells, with accumulation in the perinuclear region. The subcellular localization of this protein was not further defined, however (16). Membrane flotation analyses from recombinant baculovirus-infected insect cells revealed NS5B in both membrane and cytosolic fractions, and several NS5B species with slightly different electrophoretic mobility were detected by immunoblot using sera from patients with chronic hepatitis C as a source of primary antibody (16). In a more recent study, a green fluorescent protein (GFP)-NS5B fusion protein was found to be distributed throughout the cytoplasm in a “mesh-like pattern” (17).

Here, we used monoclonal antibodies (mAbs) and continuous human cell lines inducibly expressing NS5B either alone or in...
In the context of the entire HCV polyprotein to define the subcellular localization of the HCV RdRp. In addition, a comprehensive set of deletion mutants and GFP fusion constructs as well as an in vitro transcription-translation (IVTT) system were employed to examine the mechanism of NS5B membrane association.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—A fragment comprising nt 7602–9377 (aa 2421–3011) of a functional HCV H strain consensus cDNA (genotype 1a) was amplified by PCR from pBRTM/HCV-3011con (18) using primers NS5Bfwd and NS5Brev (Table I). The amplification product was digested with BamHI and XbaI and cloned into the BamHI-XbaI sites of pcDNA3.1 (Invitrogen, San Diego, CA) and pGEM-11Zf(+) (Promega, Madison, WI) to yield plasmids pCMVNS5Bcon and pGEM-11BNX5Bcon, respectively. pCMVNS5Bcon allows both eukaryotic expression from a cytomegalovirus promoter and in vitro transcription from a T7 RNA polymerase promoter. The EcoRI–EcoRI fragment of pGEM-11BNX5Bcon comprising HCV nt 7602–8205 and the EcoRI–XbaI fragment comprising nt 8206–9377 were ligated together into the EcoRI–XbaI sites of pHUD10-3 (19) to yield plasmid pHUDNS5Bcon. This construct allows expression of NS5B under the transcriptional control of a tetracycline-controlled transactivator (tTA)-dependent promoter.

Plasmid pHDEGFP was constructed by ligation of the EcoRI–XbaI fragment of pEGFP-C1 (CLONTECH, Palo Alto, CA), coding for an enhanced GFP, into the EcoRI–XbaI sites of pHUD10-3. NS5B fragments with carboxyl-terminal deletions were PCR-amplified from pBRTM/HCV1-3011con using forward primer NS5Bfwd and reverse primers NS5B566–591rev, NS5B571–591rev, and NS5B571–591rev, respectively (Table I), yielding plasmids pCMVNS5BconC21, pCMVNS5BconC26, and pCMVNS5BconC63, respectively (Fig. 2A).

The NheI–EcoRI fragment of pEGFP-C1 (CLONTECH, Palo Alto, CA), comprising the coding region for an enhanced GFP, was subcloned into the NheI–EcoRI sites of pHDF1con. pCMVGFP. Plasmids pCMVGFPNS5BconC12, pCMVGFPNS5BconC16, pCMVGFPNS5BconC21, and pCMVGFPNS5BconC26 (Fig. 3A) were constructed by ligation of the preannealed primer pairs NS5B529fwd and NS5B591rev, followed by digestion of the amplification product with NheI and cloned into the NheI–EcoRI sites of pCMVGFP. All expression constructs were verified by sequencing.

Plasmid pTM1-ppl, which allows in vitro transcription of bovine preprolactin (ppl) from a T7 RNA polymerase promoter, was kindly provided by Frauke Fehrmann and Hans-Georg Krausslich, Heinrich Pette Institute, Hamburg, Germany.

Plasmids pSPUTKVam1 (20) and pSPCytb5 (20), which allow expression of vesicle-associated membrane protein 1 (Vamp1) and cytochrome b5 (C5b), respectively, under the control of an SP6 RNA polymerase promoter, were kindly provided by David W. Andrews, Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada.

**Tetracycline-regulated Cell Lines**—Tetracycline-regulated cell lines were generated as described previously (21–24). In brief, the constitutively tTA-expressing, U-2 OS human osteosarcoma (ATCC HTB-96)-
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derived founder cell line UTA-6 (25) was co-transfected with pUHDNSBcon, pUHHDHV/Hicon, or pUHDGEFP, respectively, and Babepuro (26). G418 and puromycin double-resistant clones were isolated and screened for tightly regulated HCV protein or GFP expression, respectively, by immunofluorescence microscopy and immunoblot analysis.

Stable transfections were performed by a modified calcium phosphate precipitation protocol (27). Transient transfections were performed with a 22-kDa linear polyethyleneimine derivative (ExGen 500, MBI Fermentas, Vilnius, Lithuania).

Antibodies—The NS5B-specific mAbs 5B-3B1 and 5B-12B7 will be described elsewhere.2 Briefly, mAb 5B-3B1 recognizes a linear epitope at the palm-thumb subdomain boundary of the HCV RdRp and functions well in immunoblot applications, whereas mAb 5B-12B7 recognizes a conformational epitope and functions well in immunofluorescence and immunoprecipitation analyses. A polyclonal rabbit antisera against protein disulfide isomerase was obtained from StressGen (Victoria, British Columbia, Canada). The mAb G193 against human ER-GIC-53 (25) was kindly provided by Hans-Peter Hauri, University of Basel, Switzerland. A polyclonal rabbit antisera to mannosidase II (29) was kindly provided by Kelley Moremen, University of Georgia, Athens, GA. The mAbs JL-8 against GFP and C25 (MS-3) against nucleolin were obtained from CLONTECH (Palo Alto, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Intracellular and subcellular fractionation—Immunofluorescence microscopy—Indirect immunofluorescence microscopy was performed as described previously (21, 22). 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. Bound primary antibody was revealed with fluorescein isothiocyanate (FITC)-conjugated goat F(ab')2 fragment to mouse IgG F(ab')2 (Cappel, Durham, NC) or sheep F(ab')2 fragment to rabbit IgG (Roche Molecular Biochemicals). For co-localization experiments, Texas Red (TXR)-conjugated sheep F(ab')2 to mouse IgG or goat antibody to rabbit IgG (ICN/Cappel, Aurora, OH) was used as secondary antibody. For co-localization experiments involving two mAbs of murine origin, the mAb 5B-12B7 was biotinylated using the Fluoreporter Biotin-XX labeling kit and revealed with TXR-conjugated streptavidin (both from Molecular Probes, Eugene, OR). Coverslips were mounted in SlowFade (Molecular Probes, Eugene, OR) and examined with a Zeiss Axiosvert photomicroscope equipped with an epifluorescence attachment. Confocal laser scanning microscopy was performed using a Zeiss LSM 410 microscope, and images were processed with the Adobe Photoshop 3.0.5 program.

Western Blot Analysis—Western blot analysis was performed as described previously (21, 22).

Subcellular Fractionation—Subcellular fractionation was performed essentially as described previously (21). In brief, 5 × 10⁶ cells were homogenized in a hypotonic buffer containing 10 mM Tris-HCl, pH 7.5, and 0.7 M sucrose, and a mitochondrial pellet was obtained by centrifugation at 100,000 × g for 10 min. 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.

In Vitro Transcription-Translation (IVTT)—The TNT TT- and SP6-coupled reticulocyte lysate systems (Promega, Madison, WI) were used essentially following the manufacturer’s recommendations. IVTT was routinely performed for 90 min at 30 °C in the presence of 0.8 μg/ml [35S]methionine (Amersham Pharmacia Biotech) in a volume of 25 μl. Where indicated, 1.5 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 used for membrane sedimentation analyses, 15 μl of NTE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) were 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 FLA1000 PhosphorImager and analyzed using the Fuji MacBAS version 2.4 software.

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

For membrane extraction experiments, microsomal membranes were posttranslationally 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, 6 or 10 μl urea, or 1% Triton X-100 and incubated for 20 min at 4 °C. Finally, membrane sedimentation analyses were performed and fractions analyzed by SDS-PAGE.

For protease protection assays, nuclease-free Pronase from Streptomyces griseus (Roche Molecular Biochemicals) was added to a final concentration of 0.7 μg/ml to IVTT reactions performed in the presence of microsomal membranes. Triton X-100 at a final concentration of 0.5% was added to some of the reactions to disrupt microsomal membranes. After 15 min of incubation at 35 °C, proteolysis was terminated by the addition of protease inhibitors (Complete Protease Inhibitor Mixture, Roche Molecular Biochemicals), followed by SDS-PAGE of the samples.

ATP was depleted from IVTT reactions after the addition of puromycin by incubation with 10 units/ml apyrase (Sigma) for 15 min at 30 °C.

Sequence Analyses and Structure Predictions—The NS5B aa 561–591 sequence of the HCV H strain consensus cDNA (18) (GenBank™ accession number AF009606) was used to retrieve all reported isolates from the EMBL data base using the FASTA homology search program (30). Incomplete sequences were removed from the list of matching sequences.

Incomplete sequences of all genotypes was analyzed to construct Fig. 8, A and B. Multiple sequence alignments and the consensus sequence determination were carried out with the ClustalW program (31). All analyses were made using the IBCP HCV data base website facilities (hepatitis.ibcp.fr). Visualization of sequence alignments and plotting of the most frequently represented aa residues at each position were done with the MFSA program (32). At each aa sequence position, the residue types and their respective frequencies were computed using a program developed at the IBCP.3 Various methods were combined for the prediction of transmembrane sequences as follows: PHEdhtm (33) (www.embl-heidelberg.de/predictprotein/), TMHMM (34) (www.cbs.dtu.dk/services/TMHMM-1.0/), DAS (35) (www.sbc.su.se/~-milxios/DAS/) and TopPred2 (36) (bioweb.pasteur.fr/seqanal/interfaces/toppred.html). Sequences homologous to the NS5B aa 561–591 segment were searched in the Protein Data Bank of three-dimensional structures with SSEARCH program (37) using IBCP website facilities (npas-phil.ibcp.fr/).

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 NS5B either alone (UNSSBcon) or in the context of the entire HCV polyprotein (UHCVcon). In addition, cell lines inducibly expressing GFP (UFGP) were generated as a control for the subcellular fractionation experiments. Screening of 50 antibiotic double-resistant clones each resulting from transfections of the TTA-expressing founder cell line UTA-6 with the constructs pUHDNS5Bcon, pUHHDHV/Hicon, and pUHDGEFP allowed the isolation of several tightly regulated UNSSBcon, UHCVcon, and UFGP cell lines, respectively. Detailed characteristics of the cell lines are available from the authors upon request. In the following, data obtained with the cell lines UNSSBcon-5, UHCVcon-57.3, and UFGP-9.22 will be presented. In addition, all results were confirmed in at least one independent cell clone. These cell lines were maintained in continuous culture for more than 12 months and over 50 passages with stable characteristics and without loss of tightly regulated protein expression.

NS5B Is Localized in the ER—The subcellular localization of NS5B was determined by indirect immunofluorescence microscopy. A representative analysis of UNSSBcon-5 cells is shown in Fig. 1B. Virtually no immunoreactivity was detected when the cells were cultured in the presence of tetracycline. NS5B expression became clearly detectable 6 h following tetracycline withdrawal (data not shown) and increased to reach a steady-

2 D. Moradpour, et al., manuscript in preparation.

3 C. Combet, unpublished data.
state level after 24 h. At this time point, the mAb 5B-12B7 revealed a reticular staining pattern, which surrounded the nucleus, extended through the cytoplasm, and appeared to include the nuclear membrane. No nuclear or plasma membrane staining was observed. In UHCVcon-57.3 cells, which inducibly express NS5B in the context of the entire HCV polyprotein, the cytoplasmic reticular staining pattern was very similar to that observed in UNS5Bcon-5 cells (Fig. 1B).

Taken together, the NS5B staining pattern observed in these cell lines was typical of a membrane-associated protein and highly suggestive of a localization of the protein in the ER. Co-expression of other HCV structural and nonstructural proteins in the context of the entire polyprotein did not appreciably alter the subcellular localization of NS5B. Finally, control UGFP-9.22 cells showed the typical diffuse cytoplasmic and nuclear GFP fluorescence upon tetracycline withdrawal (Fig. 1B).

Subcellular fractionation experiments were performed to confirm the membrane association of NS5B suggested by the immunofluorescence data. For this purpose, cells were lysed in a hypotonic buffer and separated roughly into nuclear, mitochondrial, microsomal, and cytosolic fractions by differential centrifugation (Fig. 1C). When equal amounts of protein from each fraction were analyzed by immunoblot using the mAb 5B-3B1 against NS5B or the mAb JL-8 against GFP, respectively. nuc, nuclear; mit, mitochondrial; mic, microsomal; cyt, cytosolic fraction.

The staining pattern and the subcellular fractionation data were highly suggestive of an association of NS5B with the ER. To explore further the subcellular localization of NS5B, double label immunofluorescence experiments with antibodies to cellular marker proteins were performed. As shown in Fig. 1D,
NS5B co-localized perfectly with protein disulfide isomerase, a marker for the ER. The NS5B staining pattern observed in these cells was different, however, from that revealed by antibodies directed against ERGIC-53, a marker of the ER-to-Golgi intermediate compartment (data not illustrated), and mannosidase II, a marker of the Golgi apparatus (Fig. 1D). A minor association of NS5B with these related compartments cannot be completely excluded by this technique. Taken together, however, these results clearly demonstrate that the major localization of NS5B is the ER or an ER-like modified compartment.

As previously shown by us (21–24) and others (38) using the tetracycline-regulated gene expression system, there was some heterogeneity in expression levels among individual cells of a given monoclonal cell line. This feature inherent to the expression system explains the observation that not all cells stained with antibodies against marker proteins also stained for NS5B in the double immunolabeling experiments.

The Carboxyl-terminal 21 aa of NS5B Serve as a Membrane Anchor—Evidence obtained in E. coli (10, 11) and mammalian cells (10) suggests that the highly hydrophobic carboxyl-terminal domain of NS5B serves as a membrane anchor. To explore systematically the role of this domain in determining the subcellular localization of NS5B, we generated a panel of carboxy-terminal deletion constructs shown in Fig. 2A. The NS5BAC12 construct encodes at its 3′ end the four leucine residues that are conserved in all HCV genotypes and were found in E. coli to be an important determinant of protein solubility (11). NS5BAC16 lacks these four leucine residues, NS5BAC21 the entire highly hydrophobic carboxyl-terminal domain, and NS5BAC26 the absolutely conserved positively charged aa residues flanking the hydrophobic domain. Finally, the NS5BAC63 construct represents the minimal domain required for polymerase activity of NS5B (11). These constructs were transiently transfected into U-2 OS human osteosarcoma and HuH-7 human hepatocellular carcinoma cells (39), followed by immunofluorescence analyses using the NS5B-specific mAb 5B-12B7. Representative data obtained in U-2 OS cells are shown in Fig. 2B. Identical results were found in HuH-7 cells (data not illustrated). Interestingly, deletion of the carboxy-terminal 12 aa of NS5B (NS5BAC12) abolished the typical ER staining pattern, resulting in a diffuse cytoplasmic and nuclear staining. Deletion of 16 aa (NS5BAC16) led in addition to a concentration in nuclear globular structures, corresponding to the nucleoli (Fig. 2C), as well as occasional large cytoplasmic dots (particularly in cells expressing high levels of the truncated protein). The staining pattern of NS5BAC21 and NS5BAC26 was very similar to NS5BAC16. NS5BAC63, however, did not accumulate in the nucleoli but rather appeared to spare these. A shared structural feature of nucleolar proteins is the presence of an RNA recognition motif that binds either ribosomal RNAs synthesized in the nucleolus or small nucleolar RNAs (40). Our observation, therefore, suggests the presence of an RNA binding domain located between aa positions 529 and 566 of NS5B. Indeed, based on modeling of the HCV RdRp with the template and primer RNA (15), aa residues 558–563 are probably involved in RNA contact. Alternatively, Arg-531, Lys-533, and Lys-535 (see Fig. 2A) are basic residues with intrinsic nucleic acid-binding properties and thus could also play a role in nucleolar localization of NS5BAC16, NS5BAC21, and NS5BAC26.

Confocal laser scanning microscopy was performed to confirm the findings obtained by conventional immunofluorescence analyses. As represented shown for the full-length NS5B and the NS5BAC21 constructs (Fig. 2C), deletion of the carboxyl-terminal 21 aa led to nuclear redistribution of the protein with accumulation in the nucleoli. A double label immunofluorescence analysis demonstrating co-localization of NS5BAC21 with the nucleolar marker protein nucleolin is shown in the inset.

The Carboxyl-terminal 21 aa of NS5B Are Necessary and Sufficient to Target a Heterologous Protein to the ER Membrane—To assess whether the membrane anchor of NS5B can target a heterologous protein to the ER, we generated a panel of fusion constructs. As shown in Fig. 3A, the last 12, 16, 21, 26, or 63 aa residues of NS5B were fused in frame to the carboxyl terminus of GFP. These constructs were transiently transfected into U-2 OS cells and examined by fluorescence microscopy. GFP as well as the GFP-C12 and GFP-C16 fusion constructs were diffusely distributed in the cytoplasm and nucleus. Interestingly, fusion of the 21 carboxyl-terminal aa of NS5B to the carboxyl terminus of GFP led to a dramatic change in the subcellular distribution. In this case, the GFP-C21 fusion construct showed the same staining pattern as NS5B with a fine reticular network involving the nuclear membrane and extending into the cytoplasm. GFP-C26 and GFP-C63 were very similar to GFP-C21. Identical results were obtained in transiently transfected HuH-7 cells (data not shown).

These fluorescence microscopy results were confirmed by subcellular fractionation of transiently transfected U-2 OS cells. As shown in Fig. 3C, the amount of cytosolic GFP dramatically decreased with addition of 21 or 26 carboxy-terminal aa of NS5B, whereas addition of 16 aa had no significant effect.

Since a lysine residue located at position –24 of the GFP-C21 fusion construct (. . . . .ELYKSG | WFWFCLLLAAGYGViYLLPNR) could functionally substitute the conserved arginine residues flanking the hydrophobic carboxyl-terminal domain of NS5B (. . . . .SHAYPR | WFWFCLLLAAGYGiYLLPNR), we mutated this lysine to a serine. However, the subcellular localization of the resulting GFP-C21-K24S construct (. . . . .ELYSSG | WFWFCLLLAAGYGViYLLPNR) was identical to that of the original GFP-C21 construct (data not illustrated), confirming that the carboxyl-terminal 21 aa of NS5B are sufficient to target a heterologous protein to the ER membrane. Nevertheless, in the subcellular fractionation experiments shown in Fig. 3C, the amount of cytosolic GFP was lower for GFP-C26 as compared with GFP-C21, suggesting that membrane association may be stabilized by the positively charged aa residues amino-terminal to the hydrophobic core sequence.

Taken together, these experiments unequivocally demonstrate that the carboxyl-terminal 21 aa of NS5B are necessary and sufficient to target a heterologous protein to the ER membrane.

Membrane Association of NS5B Occurs Post-translationally—IVTT and membrane sedimentation analyses were performed to characterize further the membrane association of NS5B. NS5B 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 NS5B was quantified in both fractions. To elucidate the mechanism of membrane association, we first examined whether membrane targeting of NS5B 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). 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 co-translationally. NS5B, however, would be expected to be inserted into membranes posttranslationally because the membrane anchor will be buried within the translating ribosome when the termination codon is
reached. To distinguish between these two possibilities, microsomal membranes were added to the reaction either during or after completion of IVTT. Puromycin was added to the reaction mixture in the posttranslational setting to stop translation and to ensure that polypeptides were released from ribosomes. As represented in Fig. 4, when NS5B was translated in the ab-
Microsomal membranes association of NS5B occurs very efficiently also in vitro.

Interestingly, 89% of NS5B was found in the pellet when the

FIG. 3. The carboxyl-terminal 21 aa of NS5B are necessary and sufficient to target a heterologous protein to the ER membrane. A, schematic representation of carboxyl-terminal GFP fusion constructs. The aa sequence of the HCV H consensus clone is shown at the top. B, U-2 OS cells were transiently transfected with pCMVGFP (GFP), pCMVGFPNS5BconC12 (GFPC12), pCMVGFPNS5BconC16 (GFPC16), pCMVGFPNS5BconC21 (GFPC21), pCMVGFPNS5BconC26 (GFPC26), or pCMVGFPNS5BconC63 (GFPC63), as indicated by the captions. Cells were subsequently viewed by fluorescence microscopy. C, subcellular fractionation. U-2 OS cells were transiently transfected with pCMVGFP (GFP), pCMVGFPNS5BconC16 (GFPC16), pCMVGFPNS5BconC21 (GFPC21), or pCMVGFPNS5BconC26 (GFPC26) and subsequently subjected to subcellular fractionation as described under Experimental Procedures. In this case, the 9000 × g centrifugation step was omitted, and the combined mitochondrial and microsomal pellet resulted from a 100,000 × g centrifugation. Per lane 15 μg of protein was separated by 12% SDS-PAGE and analyzed by immunoblot using the mAb JL-8 against GFP. nuc, nuclear; cyt, cytosolic; mit/mic, mitochondrial and microsomal fraction.
membranes were added postranslationally. This demonstrates that membrane association of NS5B can occur by a postransla-
tional mechanism.

**NS5B Is a Cytoplasmically Oriented Integral ER Membrane Protein**—The presence of a carboxyl-terminal membrane anchor mediating postranslational membrane association de-

defines the HCV RdRp as a member of the so-called tail-anchored protein family. As such, NS5B would be expected to behave as a cytoplasmically oriented integral membrane protein. This possibility was explored by membrane extraction and protease protection experiments. NS5B was translated in vitro and postr-

translationally incubated with microsomal membranes, fol-

lowed by differential extraction of the pellet. High salt extrac-
tion (1 M NaCl) shields charges and weakens ionic interactions 
that bind peripheral proteins to membranes either directly or 
indirectly through other membrane proteins (41). Treatment 
with 100 mM sodium carbonate, pH 11.5, releases peripheral proteins by transforming microsomes into membrane sheets (42). As shown in Fig. 5, NS5B remained predominantly asso-
ciated with microsomal membranes under both conditions. In 
addition, membrane pellets were extracted with 2, 4, or 6 M 
urea. As shown in Fig. 5, about 80% of the in vitro translated 
protein remained in the pellet fraction following extraction 
with 4 M urea. Finally, membranes were disrupted with 1% 
Triton X-100, resulting in the release of NS5B into the super-
natant fraction. Taken together, these results demonstrate 
that NS5B behaves as an integral membrane protein and thus 
fulfills the criteria of a typical tail-anchored protein. In this 
respect, the extraction profile paralleled that of Ch5 and 
Vamp1 (data not illustrated).

Protease protection experiments were performed to deter-
mine the orientation of NS5B in the ER membrane. As shown 
in Fig. 6, Pronase treatment of IVTT reactions performed in 
the presence of microsomal membranes resulted in the complete 
disappearance of the NS5B signal. This protease sensitivity of 
NS5B indicates that the majority of the protein is localized on 
the cytoplasmic side of the ER membrane. In these experi-
ments, ppl was used as control for the integrity of microsomal 
membranes. Ppl is directed to the ER membrane by interaction 
of its signal sequence with the SRP. Signal sequence cleavage 
is performed by the signal peptidase located at the luminal side 
of the ER membrane, followed by release of prolactin into the 
ER lumen (43). As expected, prolactin was protected from Pro-

nase digestion in the presence of microsomal membranes. It 
became accessible to proteolysis only after disruption of the 
membranes by detergent. Most importantly, addition of the 
NS5B insertion sequence to the carboxyl terminus of GFP 
targeted the fusion protein to the cytosolic side of microsomal 
membranes, as shown for GFPp26 in Fig. 6.

**Posttranslational Membrane Association of NS5B Occurs by 
an ATP-independent Mechanism**—Tail-anchored proteins fall 
to two major categories, proteins whose membrane targeting 
depends on ATP (exemplified by the VAMPs) and those whose 
membrane targeting occurs by an ATP-independent mech-
nism (exemplified by Ch5). Therefore, we next examined the 
ATP dependence using these proteins as controls. ATP was 
depleted from IVTT reactions by the adenosine 5′-tri- 

-phosphatase activity of apyrase. As shown in Fig. 7, postrans-

lational targeting of NS5B to microsomal membranes in vitro 
was not affected by ATP depletion, indicating that it occurs by 
an ATP-independent mechanism.

**Sequence Comparisons and Structure Predictions**—The data 
shown above define the carboxyl-terminal domain of NS5B as 
a new membrane insertion sequence. 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. 
Analysis of the conservation of this sequence among 269 HCV 
isolates of various genotypes revealed a high degree of aa 
sequence similarity (Fig. 8A). Fifty percent of residues are fully 
conserved, and most of the positions showing apparent vari-

ability are in fact occupied by aa residues with similar hydro-

pathic character (Fig. 8B). Overall, despite the presence of conserved neutral residues at positions 580, 582, and 584, the 
carboxyl-terminal sequence appears as a highly hydrophobic 
core (segment 572–589) that is predicted to be a transmem-
brane segment by all analyses performed (PHDhtm, TMHMM, 
DAS, and TopPred2, see under “Experimental Procedures”). 
The length of this transmembrane segment (18 aa) is consist-
ent with the typical length of transmembrane α-helices. The 
transmembrane segment is flanked by two (or three) positively 
charged residues on the amino-terminal side (aa positions 566, 
568 and 570) and a positively charged arginine residue at the 
very carboxyl terminus (aa position 591). Arg-568 and Arg-570 
were found to be absolutely conserved, indicating that they are 
essential. Position 569 is always occupied by a small residue 
(Pro, Thr or Ser), as well as position 567 (Ala or Val). Hence, 
the 566–570 segment appears flexible, positively charged, and 
is probably involved in membrane surface binding via electro-

static interactions with the polar head of phospholipids. Posi-
tion 571 is clearly variable but either occupied by a hydrophobic 
residue or histidine, i.e. residues that are likely to be located at 
the membrane interface. Positions 572–579 (except 575) as well 
as positions 585–588 are occupied by large hydrophobic resi-
dues that indicate an α-helical folding of this region. By con-
trast, the connecting segment 580–584 (SVGVG) between 
these two hydrophobic stretches exhibits flexible properties, in 
particular at the level of the fully conserved Gly-582 and Gly-
584. Because glycine residues are known to act as helix break-
ers, one can wonder whether this connecting segment can adopt 
an α-helical fold. To address this issue, we searched for 
sequence homologies between the NS5B aa 561–591 segment 
and proteins of known three-dimensional structure. Interest-
ingly, 40.9% aa identity was found between the NS5B aa 571– 
588 segment and the first transmembrane α-helix of bacterior-
hodopsin (Fig. 8C). In addition, 41.2% identity was found 
between the NS5B aa 571–587 segment and the photosynthetic 
reaction center, chain M, whose structure has been identified.
Fig. 5. NS5B is an integral membrane protein. Microsomal membranes were posttranslationally added to IVTT reactions of pCMVNS5Bcon. Subsequently, reaction mixtures were centrifuged for 15 min at 12,000 × g to sediment microsomal membranes containing associated NS5B protein. The supernatants were removed, and the pellets were resuspended in NTE buffer, 1 M NaCl, 100 mM sodium carbonate, pH 11.5, 2, 4, or 6 M urea, or 1% Triton X-100, and incubated for 20 min at 4 °C. Subsequently, membrane sedimentation analyses were performed as described under “Experimental Procedures.” Supernatant (S) and pellet (P) fractions were applied in equivalent amounts and separated by 12% SDS-PAGE. [35S]Methionine-labeled translation products were detected by autoradiography. Quantitation was performed as described under “Experimental Procedures,” and values expressed in % are given at the bottom and depicted as bars. Light gray bars represent supernatant and dark gray bars pellet fractions.

Fig. 6. NS5B is cytoplasmically oriented on the ER membrane. IVTT reactions of pCMVNS5Bcon, pCMVGFPNS5BconC26, and pTM1-ppl were performed in the absence or presence of microsomal membranes (MM), followed by digestion with 0.7 mg/ml Pronase (P) for 15 min at 35 °C in the absence or presence of 0.5% Triton X-100 (TX-100) as indicated at the top. Aliquots of each reaction were analyzed by 12% SDS-PAGE and visualized by autoradiography. Molecular mass standards in kDa are indicated on the right.

Fig. 7. Posttranslational membrane association of NS5B occurs by an ATP-independent mechanism. IVTT reactions of pCMVNS5Bcon, pSPCytb5, and pSPUTKVampI were performed in the absence or presence of microsomal membranes, followed by the addition of puromycin to 1.25 mM. ATP was depleted from one set of reactions by the addition of apyrase as described under “Experimental Procedures.” Subsequently, microsomal membranes (MM) were added for 1 h as indicated at the top. Finally, membrane sedimentation analyses were performed as described under “Experimental Procedures.” Supernatant (S) and pellet fractions (P) were applied in equivalent amounts and separated by 12% SDS-PAGE in the case of NS5B and 15% SDS-PAGE in the case of Cb5 and Vamp1. [35S]Methionine-labeled translation products were detected by autoradiography. Quantitation was performed as described under “Experimental Procedures,” and values expressed in % are given at the bottom. Molecular mass standards in kDa are indicated on the left.

Discussion

Formation of a membrane-associated replication complex is a characteristic feature of positive-strand RNA viruses (44–49). In this context, physical interactions between NS4A and a NS4B-5A cleavage substrate on the one hand (50) and between NS5B and NS3 as well as NS4A on the other hand have been described (51). The mechanisms of membrane association and the protein–protein interactions involved in formation of the HCV replication complex, however, are poorly understood. A highly complex and subtly regulated scenario is likely, not the least in view of recent data on membrane targeting of the HCV NS3-4A complex by the NS4A polypeptide (23) and the conformational changes of this complex predicted for cis- and trans-processing events (52).

The best characterized mechanism of membrane insertion in mammalian cells is the SRP-mediated pathway (53). Here, membrane targeting is initiated co-translationally by a signal sequence encoded near the amino terminus of the nascent peptide. The signal sequence in the ribosome-bound nascent chain interacts with the SRP that then docks at the ER. For these proteins, integration occurs via a complex multistep process ending with release of the polypeptide into the lipid membrane coincident with the completion of protein synthesis. Another small but rapidly growing class of membrane proteins lacks an amino-terminal signal sequence and instead is targeted via a carboxyl-terminal hydrophobic domain termed insertion sequence (reviewed in Ref. 54). The prototype of this class of integral membrane proteins, termed tail-anchored proteins, is Cb5. Other examples include members of the soluble class of integral membrane proteins, termed tail-anchored proteins, as a transmembrane α-helix as well (Protein Data Bank entry code 6PRM; data not shown). The presence of similar GLG and SASVG segments in these known α-helices confirms that GVG in NS5B can adopt an α-helical fold in a membrane environment. Finally, residues at positions 589 and 590 are characteristic of the carboxyl-terminal end of an α-helix (proline is a helix breaker and asparagine is often involved in carboxyl-terminal helix capping). In conclusion, the carboxyl-terminal helix breaker and asparagine is often involved in carboxyl-terminal capping. In conclusion, the carboxyl-terminal helix projection of the NS5B aa 571–588 segment is formed as described under “Experimental Procedures.” Supernatant (S) and pellet (P) fractions were applied in equivalent amounts and separated by 12% SDS-PAGE as indicated at the top. Aliquots of each reaction were analyzed by 12% SDS-PAGE and visualized by autoradiography. Molecular mass standards in kDa are indicated on the right.
N-ethylmaleimide-sensitive factor attachment protein receptor proteins, such as the VAMPs (20, 55, 56), Bcl-2 (57), polyoma virus middle T antigen (58, 59), vaccinia virus H3L envelope protein (60), and pseudorabies virus Us9 protein (61). The carboxyl-terminal location of insertion sequences implies that these proteins are targeted to and integrated into the bilayer of membranes posttranslationally. Therefore, neither SRP nor SRP receptor is involved in the membrane association of these proteins.

The features described here, namely posttranslational membrane association via a carboxyl-terminal insertion sequence, behavior as an integral membrane protein, and cytosolic orientation, define the HCV RdRp as a new member of the tail-anchored protein family. NS5B represents the first polymerase anchoring protein (60), and pseudorabies virus Us9 protein (61). The carboxyl-terminal location of insertion sequences implies that these proteins are targeted to and integrated into the bilayer of membranes posttranslationally. Therefore, neither SRP nor SRP receptor is involved in the membrane association of these proteins.

The mechanism of membrane association of NS5B is by current knowledge unique among HCV proteins. In this context, membrane targeting of the structural proteins appears to be mediated by the classical SRP-dependent pathway (66, 67), whereas NS3 has been shown to be targeted to the ER or an ER-like modified compartment via interaction with its cofactor NS4A (20), and NS4B is co-translationally targeted to the ER membrane (69). Therefore, Cb5 insertion sequence-mediated membrane integration and the mechanisms that regulate membrane selectivity (56, 68). Membrane integration of Cb5 in vitro is promiscuous, spontaneous, and independent of membrane proteins (20). Nevertheless, when expressed in cells, both Cb5 and fusion proteins containing the Cb5 insertion sequence associate specifically with the ER membrane (68). Therefore, Cb5 insertion sequence-mediated subcellular localization appears to be regulated by targeting of the molecule to the ER membrane. After correct targeting, membrane integration probably occurs spontaneously (20).Membrane binding of VAMPs, on the other hand, requires ATP and a trypsin-sensitive component of the ER membrane (20,
Thus, there are at least two different mechanisms for correct membrane integration of proteins with insertion sequences, one mediated primarily by targeting and one relying on putative receptors in the target membrane to mediate selective integration (20). Here, we showed that posttranslational membrane association of NS5B occurs by an ATP-independent mechanism. Further studies will be aimed at identifying the determinants for membrane selectivity and the mechanism of membrane insertion. In this context, it will be interesting to systematically mutate conserved aa residues within the NS5B insertion sequence and to analyze the phenotype of these mutants in vitro and in transfected cells. The most complete three-dimensional structure of NS5B comprises the structure up to aa residue 563 (15). Ultimately, therefore, resolution of the three-dimensional structure of the very hydrophobic carboxy-terminal domain of the HCV RdRp will provide a framework for a molecular understanding of the insertion mechanism.

The Saccharomyces cerevisiae ubiquitin-conjugating enzyme UBC6 is a tail-anchored protein found in the ER. Interestingly, lengthening of the insertion sequence from 17 to 21 aa resulted in retargeting to the Golgi complex and a further increase in length to 26 aa allowed the modified protein to traverse the secretory pathway and gain expression at the plasma membrane (70). Similar observations were made with the yeast ER t-soluble N-ethylmaleimide-sensitive factor attachment protein receptor Ufe1p, where lengthening of the transmembrane domain allows transport along the secretory pathway (71) or, in mammalian cells, in the case of Cb5, where targeting to the ER membrane was found to be defined by the length of the insertion sequence (72, 73). In the case of NS5B, however, extension of the insertion sequence by 4 or 8 hydrophobic residues did not alter the subcellular localization of the protein.4

Membrane association of NS5B was independent of the expression of other HCV proteins. In this context, co-transfection experiments with the NS5BΔC21 construct and the NS3-4A complex, NS4B, or NS5A, which by themselves are membrane-associated (23, 24),5 did not alter the subcellular localization of the carboxy-terminally truncated NS5B protein. Protein-protein interactions within the presumed HCV replication complex, therefore, do not seem to be sufficient to target NS5B to the ER membrane. By contrast, poliovirus three-dimensional polymerase expressed by itself, for example, is not membrane-associated. In this case, membrane association is mediated by interactions with other components of the poliovirus replication complex, possibly the viral protein 3AB (74, 75). With respect to related members of the Flaviviridae family, analyses of GB virus sequences by various transmembrane prediction methods clearly indicate that the carboxy terminus of GBV-B NS5B contains a putative transmembrane domain similar to that observed for HCV (data not shown). By contrast, in the case of GBV-A and GBV-C/HGV NS5B the presence of a carboxy-terminal membrane anchor is ambiguous and should be investigated experimentally. Interestingly, no transmembrane domain was predicted for flavi- and pestiviruses NS5 and NS5B proteins, respectively. This is a major difference when compared with hepacviruses, suggesting that the RdRps of flavi- and pestiviruses are membrane-targeted by different mechanisms, if at all.

In the context of the HCV polyprotein, membrane anchoring by the carboxy-terminal end, presumably occurring rapidly after release from the ribosome, could represent a strategy to hold together the components of the replication complex during polyprotein processing. Proteolytic cleavage by the NS3-4A complex has been shown both in heterologous expression systems as well as in cell lines harboring subgenomic HCV replicons to occur rapidly between NSSA and NS5B, resulting in a rather stable NS4A-4B-5A precursor that is processed slowly into the individual products (76). If NS5B has a loose association with other replicate components anchoring of NS5B to the ER membrane in a well defined orientation could facilitate low affinity interactions with the other ER-associated replicate components. Such a loose association of NS5B with other replicate components may be important for its multiple roles in initiation of minus and plus strand RNA synthesis and chain elongation.

Finally, identification of the carboxy-terminal 21 aa of NS5B as a signal for targeting and insertion of heterologous proteins to the cytosolic side of the ER membrane may have a number of interesting applications. For example, this insertion sequence may be used to target antiviral effector molecules to the HCV replication complex.

In conclusion, the results presented here define the HCV RdRp as a new member of the tail-anchored protein family. Elucidation of the determinants for membrane selectivity and the membrane insertion mechanism of the HCV RdRp as well as its involvement in formation of the membrane-associated replication complex may lead to new insights into fundamental cellular processes and define novel targets for antiviral intervention.

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