The Hepatitis C Virus Nonstructural Protein 4B Is an Integral Endoplasmic Reticulum Membrane Protein

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The hepatitis C virus (HCV) nonstructural protein 4B (NS4B) is a relatively hydrophobic 27-kDa protein of unknown function. A tetracycline-regulated gene expression system, a novel monoclonal antibody, and in vitro transcription–translation were employed to investigate the subcellular localization and to characterize the membrane association of this viral protein. When expressed individually or in the context of the entire HCV polyprotein, NS4B was localized in the endoplasmic reticulum (ER), as shown by subcellular fractionation, immunofluorescence analyses, and double-label confocal laser scanning microscopy. In this compartment NS4B colocalized with the other HCV nonstructural proteins. Association of NS4B with the ER membrane occurred cotranslationally, presumably via engagement of the signal recognition particle by an internal signal sequence. In membrane extraction and proteinase protection assays NS4B displayed properties of a cytoplasmically oriented integral membrane protein. Taken together, our findings suggest that NS4B is a component of a membrane-associated cytoplasmic HCV replication complex. An efficient replication system will be essential to further define the role of NS4B in the viral life cycle.

Key Words: hepatitis C virus; nonstructural proteins; endoplasmic reticulum; tetracycline-regulated gene expression system; replication complex; in vitro transcription–translation.

INTRODUCTION

The hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide (National Institutes of Health, 1997; European Association for the Study of the Liver, 1998). HCV has been classified in the genus Hepacivirus within the family Flaviviridae, which includes the classical flaviviruses, such as yellow fever virus and the animal pestiviruses (Rice, 1996; van Regenmortel et al., 2000). HCV contains a single-stranded, positive-sense RNA genome of approximately 9600 nucleotides (nt) in length that encodes a polyprotein precursor of about 3000 amino acids (aa) (see Bartenschlager and Lohmann, 2000, and Reed and Rice, 2000, for recent reviews). The polyprotein precursor is co- and posttranslationally processed by cellular and viral proteases to yield the mature structural and nonstructural proteins (Fig. 1A). The structural proteins core, E1, and E2 are released from the polyprotein precursor by the endoplasmic reticulum (ER) signal peptidase of the host cell. The nonstructural (NS) proteins include two viral proteases, a RNA helicase located in the carboxy-terminal region of NS3, the NS4A polypeptide, the NS4B and NS5A proteins, and a RNA-dependent RNA polymerase represented by NS5B. Cleavage of the polypeptide precursor at the NS2–NS3 junction is accomplished in cis by an autoprotease encoded by NS2 and the aminoterminal one-third of NS3. A distinct serine protease located in the amino-terminal one-third of NS3 is responsible for the downstream cleavages in the nonstructural region. The NS4A polypeptide serves as a cofactor for the NS3 serine protease and is incorporated as an integral component into the enzyme core. NS5A is a serine phosphoprotein of unknown function.

NS4B, a relatively hydrophobic protein of 27 kDa, is the least characterized HCV protein. The NS4B proteins of HCV, pestiviruses, and flaviviruses are similar in size, aa composition, and hydrophobic properties. No function, however, has yet been ascribed to NS4B in any of these related viruses. Interestingly, it has recently been shown that hyperphosphorylation of the HCV NS5A protein depends on the presence of the nonstructural proteins 3,
4A, and 4B (Koch and Bartenschlager, 1999; Neddermann et al., 1999). NS4B appears to be directly involved in the modulation of NS5A hyperphosphorylation, since two deletions in NS4B that do not affect polyprotein processing and basal NS5A phosphorylation abolish NS5A hyperphosphorylation (Koch and Bartenschlager, 1999). This observation, together with the demonstration of a physical interaction between NS4A (and, by consequence, also NS3) and a NS4B–5A cleavage substrate (Lin et al., 1997), suggests that NS4B associates with the other nonstructural proteins to form a replication complex. In this context, it has recently been shown in the related flavivirus Kunjin that NS4B is essential for viral replication and functions only \textit{in cis} (Khromykh et al., 2000). Intriguingly, HCV NS4B has recently been reported to transform NIH3T3 cells in cooperation with the Ha-ras oncogene (Park et al., 2000).

As a first step toward determining the function of HCV NS4B, we investigated its subcellular localization and characterized its membrane association. For this purpose, continuous human cell lines inducibly expressing NS4B either individually or in the context of the entire HCV polyprotein were established using a tetracycline-regulated gene expression system, and monoclonal antibodies (mAbs) were generated by hybridoma fusion. Subcellular fractionation and double-label immunofluorescence analyses revealed that NS4B was localized in the ER, where it colocalized with the other HCV nonstructural proteins. \textit{In vitro} transcription–translation experiments performed in the presence or absence of microsomal membranes revealed cotranslational targeting to the ER membrane, where NS4B displayed properties of a cytoplasmically oriented integral membrane protein.

**RESULTS**

**NS4B mAb and tetracycline-regulated cell lines**

A NS4B-specific mAb of the IgG1 isotype, designated 4b-52, was obtained by hybridoma fusion after immunization of BALB/c mice with recombinant NS4B protein produced in Escherichia coli. This mAb was found to function well in ELISA and indirect immunofluorescence analyses, but not in immunoblot analyses, suggesting that it recognizes a conformational epitope.

A tetracycline-regulated gene expression system was used to generate cell lines inducibly expressing NS4B. Screening of 60 antibiotic double-resistant clones resulting from the transfection of the tetracycline-controlled transactivator (tTA)-expressing founder cell line UTA-6 with the construct pUHHDNS4Bcon allowed the isolation of two tightly regulated cell lines, designated UNS4Bcon-4 (high-level expression) and UNS4Bcon-52 (medium-level expression). These cell lines were maintained in continuous culture for more than 12 months and over 50 passages with stable characteristics and without loss of the tightly regulated NS4B expression.

An immunoblot analysis of UNS4Bcon-4 cells cultured for 24 h in the presence or absence of tetracycline is shown in Fig. 1B. No NS4B reactivity was observed when the cells were cultured in the presence of tetracycline. By contrast, a single NS4B-specific band of approximately 27 kDa was observed in cells cultured in the absence of tetracycline. This illustrates the tight regulation of NS4B expression in this cell line.

Interestingly, high-level expression of NS4B affected viability and growth of UNS4Bcon-4 cells seeded at low confluence. Colony formation efficiency assays did not yield any colonies when these cells were seeded at low confluence and subsequently cultured in the absence of tetracycline. By contrast, the expected number of colonies formed when these cells were cultured in the presence of 1 μg/ml tetracycline (i.e., when NS4B expression was completely repressed) or when expression was titrated to low levels in the presence of 0.01 μg/ml tetracycline (data not shown). Consistent with the dose-dependent cytotoxic effect, colony formation was not affected in UNS4Bcon-52 cells, which produce lower amounts of NS4B.

The subcellular localization of NS4B was investigated by indirect immunofluorescence microscopy. An immunofluorescence analysis of UNS4Bcon-4 cells stained with the mAb 4b-52 is shown in Fig. 1C. No immunoreactivity was detected when the cells were cultured in the presence of tetracycline (0 h). NS4B expression became clearly detectable 6 h following tetracycline withdrawal and increased to reach a steady-state level after 24 h. At this time point, mAb 4b-52 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. An analogous staining pattern was found in UNS4Bcon-52 cells (data not illustrated). In UHCVcon-57 cells, which inducibly express NS4B in the context of the entire HCV polyprotein, overall expression levels were lower, but the cytoplasmic reticular staining was very similar to that observed in UNS4Bcon-4 and UNS4Bcon-52 cells (Fig. 3, right panel). Taken together, the NS4B 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.

Subcellular fractionation experiments were performed to confirm the membrane association of NS4B suggested by the immunofluorescence data. For this purpose, cells were lysed in a hypotonic buffer and separated into nuclear, mitochondrial, microsomal, and cytosolic fractions by differential centrifugation (Fig. 1D). When equal amounts of protein from each fraction were analyzed by immunoblot, NS4B was detected only in the membrane-containing fractions, i.e., the nuclear pellet (which contains the outer nuclear membrane (contiguous with the ER) and membranes adsorbed to the nucleus), the mito-
chondrial pellet [which contains ER membranes that in U-2 OS cells are often wrapped around mitochondria (Moradpour et al., 1996)], and the microsomal pellet. However, NS4B was not detected in the cytosolic fraction, which contains soluble proteins. By contrast, green fluorescent protein (GFP), a protein that is diffusely distributed within cells, was found in all fractions (Fig. 1D, lower panel). Taken together, these results clearly demonstrate the membrane association of NS4B.

NS4B is localized in the ER

The staining pattern and the subcellular fractionation data shown in Fig. 1 were highly suggestive of a localization of NS4B in the ER. Double-label confocal laser scanning microscopy with antibodies to cellular marker proteins was performed to explore the subcellular localization of NS4B in more detail. As shown in Fig. 2, NS4B colocalized perfectly with protein disulfide isomerase (PDI), a marker specific for the ER. The NS4B 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, and mannosidase (Man) II, a marker of the Golgi apparatus. The possibility of an association of a minor proportion of NS4B with these related compartments cannot be excluded by this technique. Taken together, how-
ever, the results clearly demonstrate that NS4B is localized predominantly in the ER.

As found previously by us (Moradpour et al., 1996, 1998a,b) and others (Precious et al., 1995) using the tetracycline-regulated gene expression system, there was some heterogeneity in expression levels among individual cells of a given monoclonal cell line. This inherent feature of the expression system explains the observation that not all cells stained with antibodies against marker proteins stained for NS4B in the double-immunolabeling experiments.

**NS4B colocalizes with the other HCV nonstructural proteins**

The nonstructural proteins NS3 through NS5B represent an independent module sufficient for the autonomous replication of subgenomic HCV RNA (Lohmann et al., 1999). To further explore the role of NS4B we compared the localization of NS4B with that of the other HCV nonstructural proteins. In a first set of experiments (Fig. 3, left panels), UNS4Bcon-4 cells cultured in the absence of tetracycline were transiently transfected with cytomegalovirus promoter-driven expression constructs coding for the HCV nonstructural proteins 3–4A, 5A, and 5B. Subsequently, cells were processed for double-label immunofluorescence with a polyclonal rabbit antiserum against NS4B and murine mAbs against NS3, NS4A, NS5A, or NS5B. In a second set of experiments (Fig. 3, right panels), double-immunolabeling was performed in UHCVcon-57 cells, which inducibly express the entire open reading frame derived from a functional HCV consensus cDNA. As shown in Fig. 3, NS4B colocalized with
the other nonstructural proteins under both experimental conditions. These observations do not necessarily imply, but raise the possibility, that NS4B is a component of the HCV replication complex. Furthermore, these experiments demonstrate that the subcellular localization of NS4B is not influenced by the coexpression of other HCV proteins. NS4B per se, therefore, contains the signals necessary for ER targeting.

We have previously shown that NS3 expressed alone was diffusely distributed in the cytoplasm and nucleus while coexpression of NS4A in cis or in trans directed NS3 to the ER membrane (Wölk et al., 2000). Moreover, a carboxy-terminal truncated NS5B protein was recently found to be redistributed from the ER to the nucleus, where it accumulated in nucleoli (Moradpour et al., unpublished data). Based on these observations, we performed cotransfection experiments of NS4B with NS3 or carboxy-terminal truncated NS5B constructs, to examine if interactions of NS4B with these nonstructural proteins may direct them to the ER. However, the subcellular distribution of these proteins was not influenced by the coexpression of NS4B (data not shown). NS4B, therefore, even if interacting in some way with NS3 and NS5B in the context of a replication complex, was unable to direct these proteins to the ER.

**Association of NS4B with microsomal membranes in vitro**

In vitro transcription–translation and membrane sedimentation analyses were performed to further characterize the membrane association of NS4B. To this end, NS4B 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 NS4B was quantified in both fractions. As a control, MIA2, which encodes the Gag, Gag-PR, and Gag-PR-Pol polyproteins of the murine intracisternal A-type particle (IAP) MIA14, was used. The IAP Gag protein contains an amino-
terminal signal sequence that directs these polyproteins specifically to the ER membrane in transfected cells (Welker et al., 1997) and in vitro (Fehrmann et al., manuscript in preparation). The results of a typical experiment are shown in Fig. 4. When NS4B was translated in the absence of microsomal membranes only 26% was subsequently found in the pellet. By contrast, 68% of the NS4B protein sedimented when translation was performed in the presence of microsomal membranes. These results demonstrate that membrane association of NS4B occurs also in vitro. Similarly, MIA2 associated efficiently with microsomal membranes (8 vs 79% pelleted in reactions performed in the absence or presence of microsomal membranes, respectively). Relatively more NS4B compared to MIA2 was found in the pellet fraction of in vitro transcription–translation reactions performed in the absence of microsomal membranes. This is likely a consequence of some aggregation due to nonspecific hydrophobic interactions. This tendency of NS4B could be reduced by the addition of 0.01% digitonin to the translation reactions (Fig. 5 and data not shown). A minor, slightly faster migrating band was observed in the NS4B translations irrespective of the presence or absence of microsomal membranes (Fig. 4). This smaller product, therefore, is likely a consequence of leaky scanning and internal translation initiation at two ATGs located 10 and 11 codons downstream from the engineered translation initiation codon.

Membrane association of NS4B occurs cotranslationally

To gain insight into the mechanism of membrane association of NS4B we examined whether membrane targeting of NS4B occurred co- or posttranslationally. In eukaryotic cells, ER transport of secreted and integral 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. By consequence, SRP-mediated ER transport occurs only cotranslationally, while SRP-independent transport and binding of hydrophobic proteins should also occur posttranslationally. To distinguish between these two possibilities, therefore, microsomal membranes were added to the reaction either during or after completion of in vitro transcription–translation. Puromycin was added to the reaction mixture in the posttranslational setting to stop translation and to ascertain that polypeptides were released from ribosomes. As shown in Fig. 5, 47% of NS4B was found in the pellet fraction in the cotranslational reaction. By contrast, only 9% was found in the pellet when the membranes were added posttranslationally. This observation was confirmed in three independent experiments with mean values of 40% of the NS4B protein pelleted in the cotranslational and 8% in the posttranslational setting. MIA2 Gag polyproteins, which are directed to the ER via the SRP (Fehrmann et al., manuscript in preparation), served as a control for cotranslational ER membrane targeting (Fig. 5, middle panel). The MIA4 construct, in which the amino-terminal signal se-

FIG. 4. Association of NS4B with microsomal membranes in vitro. In vitro transcription–translation reactions of pCMVNS4Bcon (left panel) and pTM1-MIA2 (right panel) were performed in the presence or absence of microsomal membranes as indicated. Subsequently, membrane sedimentation analyses were performed as described under Materials and Methods. Supernatant (S) and pellet fractions (P) 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 Materials and Methods and values expressed in % are given at the top. Molecular weight standards in kDa are indicated at left.

FIG. 5. Association of NS4B with the ER membrane occurs cotranslationally. In vitro transcription–translation reactions of pCMVNS4Bcon (top panel), pTM1-MIA2 (middle panel), and pTM1-MIA4 (bottom panel) were performed in the presence (co) or absence of microsomal membranes (post and −). Digitonin at a concentration of 0.01% was present in all translation reactions to reduce nonspecific aggregation and sedimentation of NS4B. After 1 h, translation was stopped by the addition of puromycin to 1.25 mM and microsomal membranes were added to a set of the reactions without membranes (post) and incubated for one additional hour. Subsequently, membrane sedimentation analyses were performed as described under Materials and Methods. Supernatant (S) and pellet fractions (P) 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 Materials and Methods and values expressed in % are given at the bottom of each panel.
sequence has been replaced by the membrane targeting signal of the Src protein, represented a control for post-translational ER targeting (Fig. 5, bottom panel). Membrane association of this construct is mediated by myristoylation of the engineered Src protein domain. Taken together, these results demonstrate that NS4B is co-translationally targeted to the ER membrane.

NS4B is an integral membrane protein

Membrane extraction experiments were performed to characterize the nature of the association of NS4B with the ER membrane. To this end, NS4B 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) is expected to shield charges and weaken ionic interactions that bind peripheral proteins to membranes either directly or indirectly through other membrane proteins (Kretzschmar et al., 1996). Treatment with 100 mM sodium carbonate, pH 11.5, should release peripheral proteins by transforming microsomes into membrane sheets (Fujiki et al., 1982). However, as shown in Fig. 6, NS4B remained associated with microsomal membranes under both conditions. Sixty percent of the protein remained membrane-associated even under strongly denaturing conditions, i.e., treatment with 4 M urea. NS4B, therefore, is tightly associated with the ER membrane and behaves as an integral membrane protein. As a control, membranes were disrupted with 0.5% Triton X-100. Under these conditions, 78% of the protein could be extracted into the supernatant fraction.

NS4B is cytoplasmically oriented in the ER membrane

Proteinase protection experiments were performed to determine the orientation of NS4B in the ER membrane. As shown in Fig. 7, treatment of in vitro transcription–translation reactions performed in the presence of microsomal membranes with proteinase K resulted in the complete disappearance of the NS4B signal. Thus, the protease sensitivity of NS4B indicates that the majority of the protein is localized on the cytoplasmic side of the ER membrane. In these experiments, preprolactin (ppl) was used as a 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 (pl) into the ER lumen. As shown in Fig. 7, pl was protected from proteinase K digestion in the presence of microsomal membranes. It became accessible to proteolysis only after disruption of the membranes by detergent treatment. Because potentially existing transmembrane or luminal fragments of NS4B may be very small and may lack methionine residues, we repeated these experiments using [14C]leucine instead of [35S]methionine (12.3% of NS4B aa are leucine vs 2.3% that are methionine) as a radioactive label, followed by analyses on high-resolution 10 to 16.5% tricine gels. However, even under these more sensitive experimental conditions we observed no protected fragments (data not shown).

FIG. 6. NS4B is an integral membrane protein. In vitro transcription–translation reactions of pCMVNS4Bcon were performed in the presence of microsomal membranes. Subsequently, reaction mixtures were centrifuged for 15 min at 12,000 g to sediment microsomal membranes containing associated NS4B protein. The supernatants were removed and the pellets were resuspended in NTE buffer, 1 M NaCl, 100 mM sodium carbonate, pH 11.5, 4 M urea, or 0.5% Triton X-100 and incubated for 20 min at 4°C. Subsequently, membrane sedimentation analyses were performed as described under Materials and Methods. Supernatant (S) and pellet fractions (P) were applied in equivalent amounts and separated by SDS-PAGE. [35S]Methionine-labeled translation products were detected by autoradiography. Quantitation was performed as described under Materials and Methods and values expressed in % are given at the bottom and depicted as bars. White bars represent supernatant, while dark gray bars represent pellet fractions.

FIG. 7. NS4B is cytoplasmically oriented on the ER membrane. In vitro transcription–translation reactions of pCMVNS4Bcon and pTM1-ppl were performed in the presence of microsomal membranes, followed by digestion with 50 μg/ml proteinase K (PK) for 1 h at 0°C in the absence or presence of 0.1% Triton X-100 as indicated at the top. Aliquots of each reaction were analyzed by 12% SDS–PAGE and visualized by autoradiography. Molecular weight standards in kDa are indicated on the left. ppl, preprolactin; pl, prolactin.
DISCUSSION

NS4B is the least understood of all HCV proteins. No function has yet been ascribed to this protein in any of the related Flaviviridae family members. In the present initial characterization, therefore, we investigated the subcellular localization and examined the membrane association of HCV NS4B. A novel mAb, designated 4b-52, and cell lines inducibly expressing NS4B were generated for this purpose. Investigation of the viral life cycle has been limited by the lack of reproducible cell culture systems permissive for HCV infection and replication. In this context, the tetracycline-regulated cell lines described here represent a well-defined and highly reproducible model system to study structural and functional properties of NS4B.

Interestingly, inducible expression of NS4B in tetracycline-regulated cell lines revealed a dose-dependent cytotoxic effect. This effect was seen only at high expression levels and under low density seeding conditions, when cells are particularly vulnerable. We previously made similar observations in U-2 OS-derived cell lines inducibly expressing the entire HCV polyprotein (Moradpour et al., 1998b) or the structural region (Moradpour et al., 1998c). However, under the same experimental conditions no cytotoxicity was observed for the core protein (Moradpour et al., 1996) or the nonstructural proteins 4A, 5A, and 5B (Moradpour et al., unpublished data). NS4B, therefore, appears to be more toxic compared to the other nonstructural HCV proteins. Intriguingly, recent work has shown that mutations in NS4B can attenuate pestivirus cytopathogenicity (Qu and Rice, submitted for publication). Clearly, the relevance of these findings for the viral life cycle and the pathogenesis of hepatitis C needs to be investigated further.

Immunofluorescence analyses using the mAb 4b-52 and subcellular fractionation experiments suggested that NS4B is associated with the ER. This was confirmed by double-label confocal laser scanning microscopy, in which NS4B was found to colocalize with the ER-resident protein PDI. Preliminary data on the subcellular localization has thus far been reported only for a GFP–NS4B fusion protein (Kim et al., 1999) and for a FLAG-tagged NS4B protein (Park et al., 2000). In both reports, the engineered proteins were localized in the cytoplasm, but the subcellular compartment targeted by these proteins was not further explored. Interestingly, NS4B of the related flavivirus Kunjin has been reported to translocate to the nucleus during viral replication (Westaway et al., 1997a). In our studies, however, we never observed a nuclear staining of HCV NS4B. Localization of NS4B in the ER was independent of the coexpression of other HCV proteins, indicating the presence of endogenous signals for ER targeting and membrane anchorage. Similarly, we recently found that NS4A, NS5A, and NS5B were targeted to the ER when these nonstructural proteins were expressed individually (Moradpour et al., unpublished data). This is in contrast to NS3, which is directed to the ER via interaction with its cofactor polypeptide NS4A (Wölk et al., 2000).

In the ER, NS4B colocalized with the other HCV nonstructural proteins. This observation suggests that NS4B is a component of the HCV replication complex. This notion is supported by recent data indicating that NS4B, together with NS3 and NS4A, is implicated in the modulation of NS5A hyperphosphorylation (Koch and Bartenschlager, 1999; Neddermann et al., 1999) and by the demonstration of a physical interaction between NS4A and a NS4B–5A cleavage substrate (Lin et al., 1997). Formation of a membrane-associated replication complex is a characteristic feature of positive-strand RNA viruses and has been characterized in some detail, e.g., in the case of poliovirus (Bienz et al., 1992; Egger et al., 1996, 2000; Suhy et al., 2000) and the flavivirus Kunjin (Westaway et al., 1997b; Mackenzie et al., 1999; Khromykh et al., 2000). The mechanisms of membrane association and the protein–protein interactions involved in the formation of the HCV replication complex, however, are poorly understood. A subtly regulated and highly complex scenario is likely, not the least in view of recent data on membrane targeting of the HCV NS3–4A complex by the NS4A polypeptide (Wölk et al., 2000) and the conformational changes of this complex predicted for cis- and trans-processing events (Yao et al., 1999).

The membrane association of NS4B was further explored by in vitro transcription–translation experiments performed in the presence or absence of microsomal membranes isolated from canine pancreas. An earlier study suggested that most HCV nonstructural proteins are more or less membrane-associated (Hijikata et al., 1993). NS4B, however, was not specifically addressed in that study. Our results demonstrate a clear membrane association also in vitro. About 40–70% of NS4B protein sedimented after in vitro transcription–translation in the presence of microsomal membranes, while only 5–30% was pelleted, presumably as a result of nonspecific hydrophobic interactions, when microsomal membranes were omitted from the reaction.

In mammalian cells, membrane and secreted proteins are directed first to the ER membrane. Protein transport to the ER is most commonly mediated by signal sequences that are cotranslationally bound by the SRP and targeted to the docking protein and the Sec61p complex on the ER membrane (Walter and Johnson, 1994; Matlack et al., 1998). This complex forms a channel through which polypeptides are translocated and released into the ER lumen as soluble proteins or inserted into the membrane as transmembrane proteins (Schatz and Dobberstein, 1996; Matlack et al., 1998). Posttranslational membrane targeting is common in yeast (Deshaiés et al., 1991; Panzner et al., 1995) and bacteria (Schatz and Beckwith, 1990; Wickner and Leonard, 1996), but few examples...
have been described for mammalian cells (Anderson et al., 1983; Müller and Zimmermann, 1987; Kutay et al., 1995). Posttranslational membrane targeting does not involve the SRP. The cotranslational membrane association of NS4B, therefore, suggests an SRP-dependent mechanism of membrane targeting. Since NS4B does not possess a classical amino-terminal signal sequence, we propose that one or more of the internal hydrophobic sequences function as signal anchor sequences. The hydrophobicity profiles of NS4B are similar among flaviviruses, with a conserved hydrophobic plateau in the center and two short hydrophobic domains at the carboxy terminus. In this context, we found that deletion of the carboxy-terminal 96 aa, including two relatively conserved highly hydrophobic domains, did not alter the subcellular distribution of NS4B (data not illustrated). It is possible, therefore, that one or multiple determinants within the long hydrophobic domain in the center of NS4B promote membrane association.

In membrane extraction and proteinase protection assays, NS4B behaved as a cytoplasmically oriented integral membrane protein. However, no protected transmembrane or luminal fragments were detected after radioactive labeling with [35S]methionine or [14C]leucine. This was somewhat unexpected since in the case of the related yellow fever virus NS4B has been shown to span the ER membrane at least once with multiple membrane-associated hydrophobic segments and cytoplasmic loops (Lin et al., 1993). Furthermore, membrane topology models predicted by PredictProtein (on the World Wide Web at URL www.embl-heidelberg.de) or TMHMM (URL: www.cbs.dtu.dk) and TopPred2 (URL: www.biokemi.su.se) featured six or four internal transmembrane domains, respectively. On the other hand, observations similar to ours, with a complete sensitivity to proteinase digestion, have been reported for NS4B of the related West Nile, Kunjin, and Dengue viruses (Wengler et al., 1991; Cauchi et al., 1991; Westaway et al., 1997a). A refined membrane topology model of HCV NS4B will be the subject of further experimentation.

In summary, we have shown that the HCV NS4B protein is tightly associated with the ER membrane, where it behaved as a cytoplastically oriented integral membrane protein and colocalized with the other nonstructural proteins. The data suggest that NS4B is a component of a membrane-associated cytoplastic HCV replication complex. An efficient replication system will be essential to further define the role of NS4B in the viral life cycle.

MATERIALS AND METHODS

Expression constructs

A fragment comprising nt 5475 to 6258 (aa 1712 to 1997) using sense primer 5’-GAGAATTCACCAGTCTCGACACTTACGTCATCGAGCAAGG-3’ (EcoRI site underlined, engineered translation initiation codon in boldface) and reverse primer 5’-GCTGTCTAGATTAAGATGATGATACACTCGAGC- TTATCC-3’ (XbaI site underlined, engineered ochre stop codon in boldface). The amplification product was cloned into the EcoRI-XbaI sites of pUHD10-3 (Gossen and Bujard, 1992) and pcDNA3.1 (Invitrogen, San Diego, CA) to yield the expression constructs pUHDNS4Bcon and pCMVNS4Bcon, respectively. pUHDNS4Bcon allows expression of NS4B under the transcriptional control of a tTA-dependent promoter (Fig. 1A). pCMVNS4Bcon allows both eukaryotic expression from a CMV promoter and in vitro transcription from a T7 RNA polymerase promoter. pCMVNS3–4A (Wölk et al., 2000), pCMVNS5Acon, and pCMVNS5Bcon (Moradpour et al., unpublished data) allow the CMV promoter-driven expression of the HCV NS3–4A complex, NS5A, or NS5B, respectively.

pTM1 (Moss et al., 1990) allows high-level in vitro expression from a T7 RNA polymerase promoter followed by the encephalomyocarditis virus internal ribosome entry site. pTM1-MIA2 contains almost the entire gag-pro-pol region (nt 594 to 4102) of the murine IAP MIA14 (Fehrmann et al., 1997; Mietz et al., 1987). Plasmids pTM1-MIA4 and pTM1-ppl will be described in detail elsewhere (Fehrmann et al., manuscript in preparation). Briefly, pTM1-MIA4 was derived from pL-MIA4 (Welker et al., 1997) and allows expression of a MIA14 gag gene of which the first 28 codons have been replaced by the membrane-targeting signal of the Src protein. pTM1-ppl contains the entire coding region of bovine ppl derived from pSPB4 (Schlenstedt et al., 1992).

Tetracycline-regulated cell lines

Tetracycline-regulated cell lines were generated as previously described (Moradpour et al., 1996, 1998b,c; Wölk et al., 2000). In brief, the constitutively tTA-expressing, U-2 OS human osteosarcoma-derived founder cell line UTA-6 (Engliert et al., 1995) was cotransfected with pUHDNS4Bcon and pBabeuro (Morgenstern and Land, 1990). G418 and puromycin double-resistant clones were isolated and screened for tightly regulated NS4B expression by indirect immunofluorescence microscopy and immunoblot. UHCVcon-57 cells, which inducibly express the entire open reading frame derived from a functional HCV H strain consensus cDNA (Kolykhalov et al., 1997), and UGFP-9.22 cells, which inducibly express an enhanced GFP derived from pEGFP-N1 (Clontech, Palo Alto, CA), will be described in detail elsewhere (Moradpour et al., manuscript in preparation).

Antibodies

NS4B derived from a genotype 1b HCV cDNA was expressed in E. coli and used as an antigen for the
production of mAbs. Spleen cells from female BALB/c mice immunized with this recombinant protein were fused with the X63-Ag8.653 myeloma cell line (American Type Culture Collection, Rockville, MD). Hybridomas were selected and supernatants were screened by ELISA essentially as described (Harlow and Lane, 1988). Hybridomas immunoreactive with recombinant NS4B protein were cloned at least twice by limiting dilution.

The polyclonal rabbit antiserum WU151 against NS4B was produced by immunization of rabbits with a synthetic peptide corresponding to the first 18 aa of genotype 1a NS4B (SQHLPY1EQGMLAEQFK; Lin et al., unpublished data). A pool of high-titer anti-HCV-positive sera from 10 patients with chronic hepatitis C was used as a source of primary antibodies in some immunoblot analyses. The NS3-specific mAb 1B6 was described previously (Wölk et al., 2000). mAbs 8N against NS4A and 11H against NS5A were kindly provided by Jan Albert Hellings and Winand Habets (Organon Teknika B.V., Boxtel, The Netherlands), mAb 5B-12B7 against NS5B will be described elsewhere (Moradpour et al., manuscript in preparation). A polyclonal rabbit antiserum against PDI was obtained from StressGen (Victoria, BC, Canada). The mAb G1/93 against human ERGIC-53 (Schweizer et al., 1988) was kindly provided by Hans-Peter Hauri (University of Basel, Switzerland). A polyclonal rabbit antiserum to Man II (Moremen, 1991) was kindly provided by Kelley Moremen (University of Georgia, Athens, GA). The mAb JL-8 against GFP was purchased from Clontech.

Indirect immunofluorescence and confocal laser scanning microscopy

Indirect immunofluorescence microscopy was performed as described previously (Moradpour et al., 1996, 1998b). 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(\(\text{ab'}\))2 fragment to mouse IgG F(\(\text{ab'}\))2 (Cappel, Durham, NC) or sheep F(\(\text{ab'}\))2 to mouse IgG (ICN/Cappel, Aurora, OH) was used as a secondary antibody to reveal bound murine mAbs. Coverslips were mounted in SlowFade (Molecular Probes, Eugene, OR) and examined with a Zeiss Axiobert 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 program, version 3.0.5.

Western blot analysis

Western blot analysis was performed as described previously (Moradpour et al., 1996, 1998b).

Subcellular fractionation

Subcellular fractionation was performed essentially as described previously (Moradpour et al., 1996). In brief, 5 × 107 HCV NS4Bcon-4 or UGFP-9.22 cells cultured for 48 h in the absence of tetracycline were dounce-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 9000 g for 10 min. Finally, a microsomal pellet was separated from the cytosolic supernatant by centrifugation at 105,000 g for 40 min.

In vitro transcription–translation

In vitro transcription–translation was performed using the TNT T7 coupled rabbit reticulocyte lysate system (Promega, Madison, WI) essentially following the manufacturer’s recommendations. Reactions were incubated for 90 min at 30°C in the presence of 0.75 mCi/ml \([\text{35}S]\)methionine (Amersham, UK) in a volume of 25 \(\mu\)l. Where indicated, 1.5 \(\mu\)l canine pancreatic microsomes (a gift from Richard Zimmermann, Medizinische Biochemie und Molekularbiologie, Universität des Saarlandes, Homburg, Germany) and digitonin at a final concentration of 0.01% were added. Initial titration experiments indicated that 1.5 \(\mu\)l microsomal membranes per 25 \(\mu\)l coupled in vitro transcription–translation reaction was optimal since larger quantities led to a strong reduction of translation efficiency.

For membrane sedimentation analyses, 15 \(\mu\)l NTE buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA) was added after completion of the in vitro transcription–translation reaction, followed by centrifugation at 12,000 g for 15 min. Supernatants were collected and pellets were resuspended in 40 \(\mu\)l NTE buffer. Subsequently, pellet and supernatant fractions were separated by SDS–PAGE and analyzed by autoradiography. Gels were scanned on a Fuji BAS1000 phosphorimager and analyzed using the Fuji MacBAS V2.4 software.

For analyses of co- and posttranslational membrane association, microsomal membranes were added to the reactions either during or for 1 h at 30°C after completion of in vitro transcription–translation. In the latter setting, translation was stopped by 1.25 mM puromycin prior to the addition of microsomal membranes.

For membrane extraction experiments, the pellets from 15 \(\mu\)l in vitro transcription–translation reactions performed in the presence of microsomal membranes were resuspended in 40 \(\mu\)l NTE buffer, 1 M NaCl, 100
mM sodium carbonate, pH 11.5, 4 M urea, or 0.5% Triton X-100 and incubated for 20 min at 4°C. Subsequently, membrane sedimentation analyses were performed as described above.

For proteinase protection assays, 1 μl proteinase K stock solution (500 μg/ml) was added to a 10-μl in vitro transcription–translation reaction containing microsomal membranes. After 30 min incubation at 4°C, proteolysis was terminated by the addition of 2 μl 12.5 mM phenylmethylsulfonyl fluoride, followed by SDS–PAGE of the samples.

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