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Characterization of Nonstructural Protein Membrane Anchor Deletion Mutants Expressed in the Context of the Hepatitis C Virus Polyprotein

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Protein-protein interactions involved in formation of the membrane-associated hepatitis C virus (HCV) replication complex are poorly understood. Here, we investigated nonstructural proteins with deletions in their membrane anchor domains when expressed in the context of the entire HCV polyprotein. Interactions among cytosolic domains of HCV nonstructural proteins were found not to be sufficiently strong to rescue such mutants to the membrane. Thus, the membrane anchor domains of nonstructural proteins are essential for incorporation of these proteins into the HCV replication complex while interactions among the cytosolic domains appear to be relatively weak. This feature may provide the nonstructural proteins with a certain flexibility to perform their multiple functions during HCV replication.

Hepatitis C virus (HCV) replicates its genome in a membrane-associated complex composed of viral nonstructural proteins, replicating RNA, and altered host cell membranes (6, 11, 20, 31). A specific membrane alteration, designated the membranous web, was recently found to harbor the HCV replication complex (6, 11). Determinants for membrane association of HCV nonstructural proteins have been mapped (reviewed in references 5 and 24). However, the protein-protein interactions involved in formation of a functional replication complex are poorly understood.

HCV nonstructural protein 3 (NS3) is a multifunctional protein with a serine protease domain located in the N-terminal one-third and an NTPase/RNA helicase domain in the C-terminal two-thirds. NS3 by itself has no membrane anchor, but it forms a noncovalent complex with the central domain of the cofactor polypeptide NS4A (18), an interaction that also serves to stabilize NS3 (33, 35). The NS3-4A complex is targeted to membranes by a predicted α-helix in the N-terminal domain of NS4A (35). NS5A is a phosphoprotein of unknown function that associates with the cytosolic leaflet of the endoplasmic reticulum membrane by a N-terminal amphipathic α-helix (2, 7, 26). The NS5B RNA-dependent RNA polymerase (RdRp) is a member of the tail-anchored protein family (30). In this case, the C-terminal 21 amino acid (aa) residues, which are dispensable for polymerase activity in vitro (9, 36), traverse the phospholipid bilayer as a transmembrane segment and anchor the RdRp to the cytosolic side of the endoplasmic reticulum membrane (16, 22, 30). Deletion of the membrane anchor domains of NS3-4A, NS5A, or NS5B results in loss of membrane association and diffuse distribution with nuclear accumulation when such truncated proteins are expressed alone (2, 30, 35).

Physical interactions among HCV nonstructural proteins have been described using glutathione *S*-transferase pulldown assays, coimmunoprecipitation, far Western blot analyses, and conventional yeast two-hybrid screens (4, 10, 27, 32). However, such analyses are notoriously difficult to perform with membrane proteins. Here, we took advantage of the different subcellular localization of membrane-associated and membrane anchor domain deletion-containing HCV nonstructural proteins to investigate whether interactions among the cytosolic domains are sufficiently strong to rescue such truncated proteins to the membrane when expressed in *cis* in the context of the entire HCV polyprotein.

The subcellular localization of NS3-4A, NS5A, and NS5B proteins with deletions in their membrane anchor domains was investigated first by expression of these proteins alone. An NS3-4A mutant with a deletion of aa 2 to 19 in the N-terminal domain of NS4A (NS3-4AΔN2-19) was prepared by gene splicing by overlap extension (15). Briefly, two DNA fragments were generated by PCR using primer pairs 1-2 and 3-4 and pUHDNS3-4A (35) as template. The two DNA fragments were subsequently joined in a PCR containing primer pair 1-4. The amplification product was digested with NsiI and XbaI, followed by ligation into the NsiI-XbaI sites of pUHDNS3-4A to yield pUHDNS3-4AΔN2-19. This construct allows expression of NS3-4AΔN2-19 from a tetracycline-controlled transactivator (tTA)-dependent promoter (12). An NS5A mutant with a deletion of aa 2 to 21 in the N-terminal amphipathic α-helix (NS5AΔN2-21) was prepared by PCR using primer pair 6-7 and pUHDNS5Acon as template (2). The amplification product was digested with EcoRI and XbaI, followed by ligation into the EcoRI-XbaI sites of pUHD10-3 (12) to yield pUHDNS5AΔN2-21. The NS5B membrane anchor deletion

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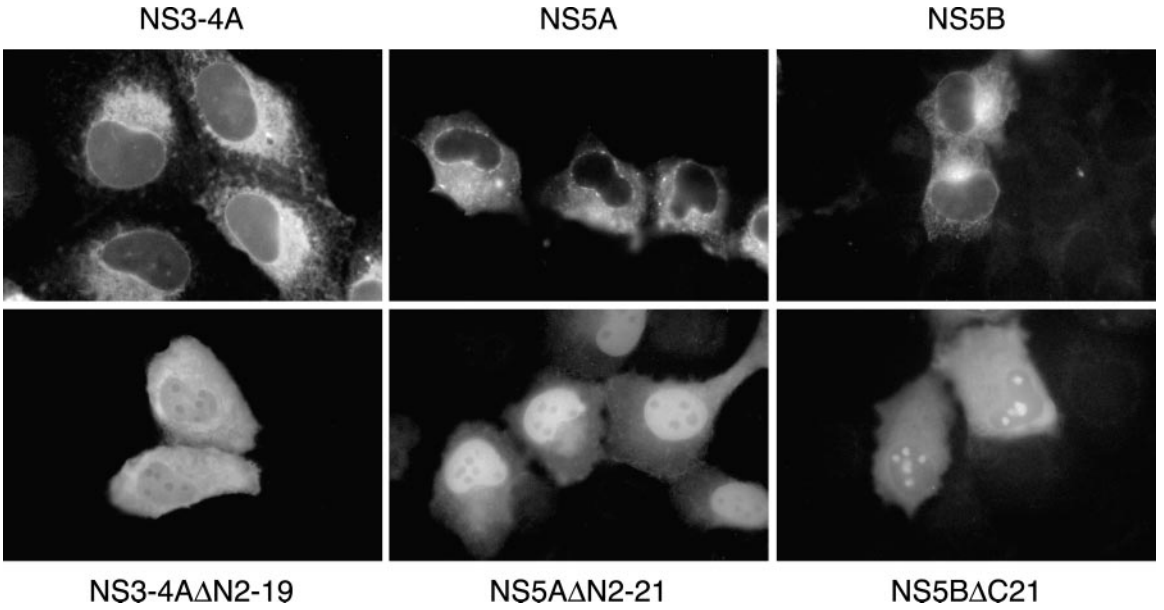

FIG. 1. Subcellular localization of NS3-4A, NS5A, and NS5B membrane anchor deletion mutants. UTA-6 cells cultured in the absence of tetracycline were transfected with pUHDNS3-4A, pUHDNS5Acon, pCMVNS5Bcon, pUHDNS3-4AΔN2-19, pUHDNS5AΔN2-21, or pCMVNS5BconΔC21, as indicated. At 48 h posttransfection, NS3, NS5A, and NS5B were detected by IF analysis using MAbs 1B6, 11H, and 5B-12B7, respectively, and a fluorescein isothiocyanate-labeled goat anti-mouse secondary antibody.

mutant pCMVNS5BconΔC21, lacking the C-terminal 21 aa residues, has been described (30). The U-2 OS human osteosarcoma-derived founder cell line UTA-6 (8), which constitutively expresses the tTA, was transiently transfected with constructs coding for wild-type NS3-4A, NS5A, and NS5B or the corresponding membrane anchor domain deletion mutants, followed by immunofluorescence (IF) analyses using monoclonal antibody (MAb) 1B6 against NS3 (35), 11H against NS5A (2) (kindly provided by Jan Albert Hellings, bioMérieux, Boxtel, The Netherlands), or 5B-12B7 against NS5B (21). Fixation with 4% paraformaldehyde, permeabilization, and immunostaining of cells were performed as previously described (11).

As shown in Fig. 1 (upper panel), NS3-4A, NS5A, and NS5B were found in the cytoplasm in a reticular staining pattern with accumulation in the perinuclear region and staining of the nuclear membrane. In sharp contrast, the membrane anchor deletion mutants lost membrane association and were diffusely distributed throughout the cells (Fig. 1, lower panel). As previously described for similar constructs (2, 30, 35), NS3-4AΔN2-19 and NS5AΔN2-21 were also found in the nucleus with sparing of the nucleoli whereas NS5BΔC21 accumulated in nucleoli, possibly due to the RNA binding properties of the RdRp. Thus, the membrane anchor deletion mutants used in this study showed the expected subcellular distribution when expressed alone.

In the next step, deletions of the membrane anchor domains of NS3-4A, NS5A, and NS5B were generated in the context of the HCV polyprotein (Fig. 2). Deletions were carefully designed to preserve functional polyprotein cleavage sites. The NS3-4A mutant was generated by PCR using primer pairs 1-2 and 3-5 (Table 1) and pBRTM/HCV1-3011con (17) (kindly provided by Charles M. Rice, The Rockefeller University, New York, NY) as template. The two DNA fragments were subsequently joined in a PCR containing primer pair 1-5. The amplification product was digested with NsiI and SnaBI, followed by ligation into the same sites of pUHDHCV(H)con (30) to yield pUHDHCV4AΔN2-19. This construct allows expression of the NS3-4A complex with a deletion of aa 2 to 19 in the N-terminal domain of NS4A in the context of the entire polyprotein derived from a functional HCV H strain consensus clone. To delete the membrane anchor of NS5A, two PCR

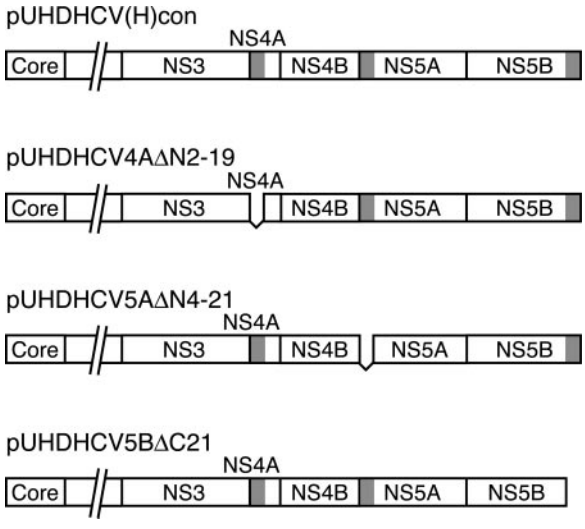

FIG. 2. HCV nonstructural protein membrane anchor deletion constructs. The HCV polyprotein, encoded by pUHDHCV(H)con, is shown at the top. The membrane anchor domains of NS4A, NS5A, and NS5B are highlighted in gray. The membrane anchor deletion constructs pUHDHCV4AΔN2-19, pUHDHCV5AΔN4-21, and pUHDHCV5BΔC21 are schematically illustrated.

FIG. 3. Polyprotein processing of membrane anchor deletion constructs. UTA-6 cells cultured in the absence of tetracycline were transfected with pUHDHCV(H)con, pUHDHCV4AΔN2-19, pUHDHCV5AΔN4-21, or pUHDHCV5BΔC21, as indicated. At 48 h posttransfection, cell lysates were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblot using MAbs C7-50 against core (23) (upper left panel), 1B6 against NS3 (upper right panel), 11H against NS5A (lower left panel), and 5B-3B1 (21) (lower right panel). Nontransfected cells served as controls. Molecular mass markers are shown on the left of each panel.

products amplified from pBRTM/HCV1-3011con using primer pairs 8-9 and 10-5 were joined in a PCR containing primer pair 8-5. The amplification product was digested with Bsu36I and SnaBI, followed by ligation into the Bsu36I-SnaBI sites of pUHDHCV(H)con to yield pUHDHCV5AΔN4-21. Finally, the EcoRI-XbaI fragment of pCMVNS5BconΔC21 was ligated together with the 7,873-bp EcoRI-EcoRI fragment of pBRTM/HCV1-3011con, encompassing the HCV polyprotein coding region from the start codon to the first one-third of NS5B, into the EcoRI-XbaI sites of pUHD10-3 to yield pUHDHCV5BΔC21.

Polyprotein processing of the different full-length constructs was examined by Western blot following transient transfection into UTA-6 cells cultured in the absence of tetracycline. This strategy was chosen because previous experience indicated that the entire HCV polyprotein can be efficiently expressed from a tTA-dependent promoter (25). As shown in Fig. 3, correctly processed core, NS3, NS5A, and NS5B proteins of the expected molecular masses of 21, 70, 56 to 58, and 68 kDa, respectively, could be detected in lysates of cells transfected with pUHDHCV(H)con, indicating robust expression and cor-

TABLE 1. PCR primers used in this study[a]

Primer no.	Sequence (5′–3′)	Plasmid(s) generated
1	AGATGTGGAAGTGTT	pUHDNS3-4AΔN2-19/ pUHDHCV4AΔN2-19
2	CACGCAGCCTGTGCTCGTGACGACCTCCAG	pUHDNS3-4AΔN2-19/ pUHDHCV4AΔN2-19
3	CTGGAGGTCGTCACGAGCACAGGCTGCGTG	pUHDNS3-4AΔN2-19/ pUHDHCV4AΔN2-19
4	GCTGTCTAGATTAGCACTCTTCCATCTCATCGAACTCCTTGG	pUHDNS3-4AΔN2-19
5	ATTCGGGCGGATGGGATC	pUHDHCV4AΔN2-19/ pUHDHCV5AΔN4-21
6	AAGAATTCCAACATGTCCTGGCTGAAAGCCAAGCTCATGCCAC	pUHDNS5AΔN2-21
7	CTGTCTAGACTAGCAGCACACGACATCTTCCGTGTCGGC	pUHDNS5AΔN2-21
8	ATACTCCAGCAGCCTC	pUHDHCV5AΔN4-21
9	CTTGGCTTTCAGCCAGGAACCGGAGCATGG	pUHDHCV5AΔN4-21
10	CCATGCTCCGGTTCCTGGCTGAAAGCCAAG	pUHDHCV5AΔN4-21
11	AAGAATTCCACCATGTCCGGTTCCTGGCTGAAAGCCAAGCTCAT	pUHDNS5AΔN2-21HA
12	GCTCTAGACAGCATAGTCAGGTACGTCATATGGATACGATCCGCAGCACACGACATCTTCCGTGTCGGCCCCACTACTGAC	pUHDNS5AΔN2-21HA
13	AAGAATTCCACCATGTCAATGTCTTATTCCTGGAC	pUHDNS5BΔC21HA
14	GCTCTAGACAGCATAGTCAGGTACGTCATATGGATACGATCCGCGGGGCCGGGCATGAGACACGCTG	pUHDNS5BΔC21HA
15	AAGAATTCCACCATGTATCCATATGACGTACCTGACTATGCTGTCTCGGGATCAATGTCTTATTCCTGGAC	pUHDHAN5BΔC21
16	GCTGTCTAGATTAGCGGGGCCGGGCATGAGACACGCTG	pUHDHAN5BΔC21

[a] See text for cloning strategies.

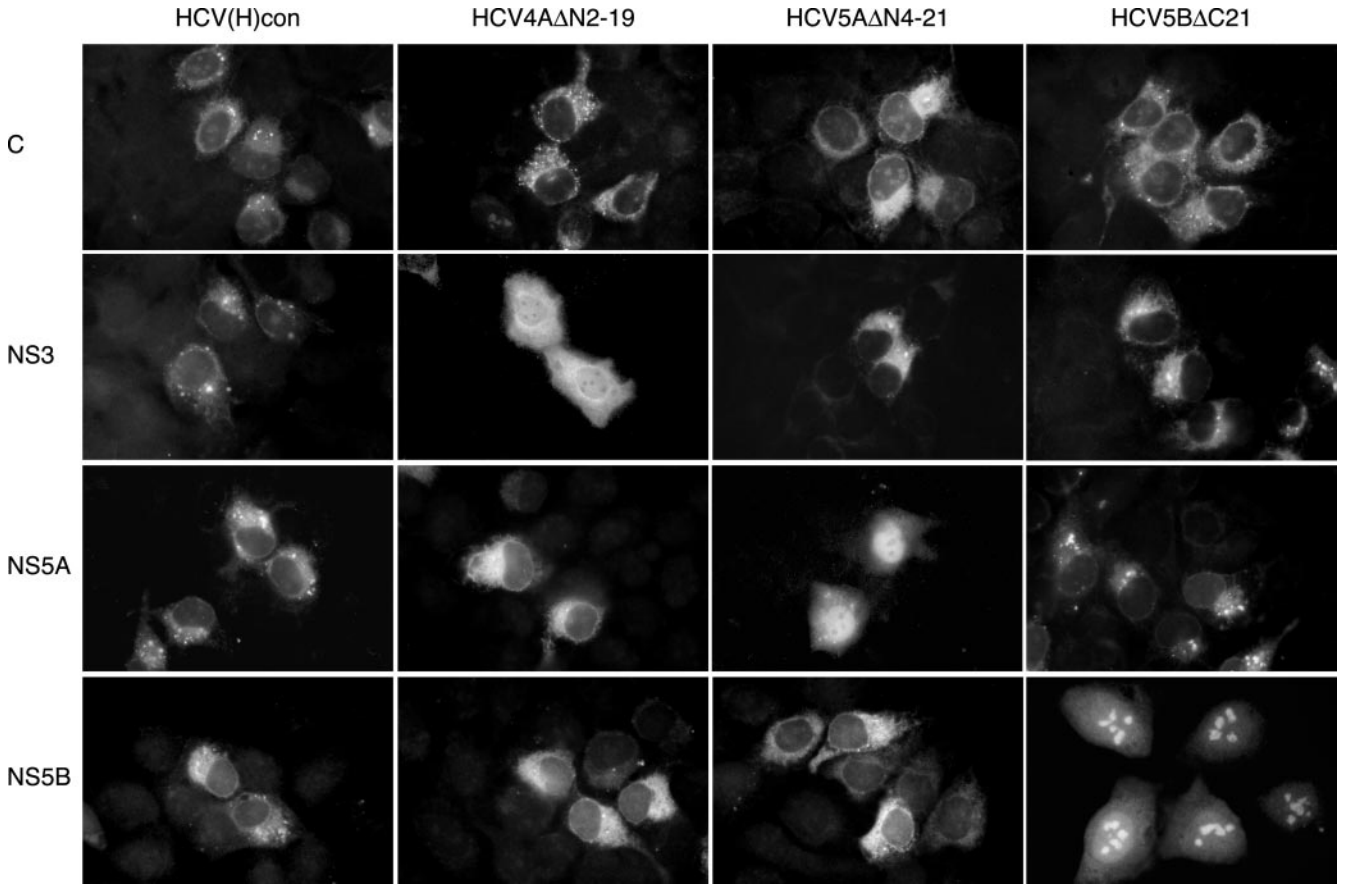

FIG. 4. Subcellular localization of NS3-4A, NS5A, and NS5B membrane anchor deletion mutants expressed in the context of the HCV polyprotein. UTA-6 cells cultured in the absence of tetracycline were transfected with pUHDHCV(H)con, pUHDHCV4AΔN2-19, pUHDHCV5AΔN4-21, or pUHDHCV5BΔC21, as indicated. At 48 h posttransfection, core (C), NS3, NS5A, and NS5B or the respective membrane anchor deletion mutants were examined by IF analysis using MAbs C7-50, 1B6, 11H, and 5B-12B7, respectively.

rect processing of the entire HCV polyprotein in this system. Similarly, transient expression of HCV5AΔN4-21 and HCV5BΔC21 yielded correctly processed proteins of the expected molecular masses. As expected for the HCV4AΔN2-19 mutant, NS3 was detectable only at low levels (Fig. 3, right upper panel), which is consistent with a reduced stability of NS3 expressed without complete NS4A (33, 35). This mutant showed a processing defect with incomplete cleavage of NS5A, presumably at the NS4A-dependent NS4B/NS5A site, and the production of an aberrant smaller-size species. However, the generation of mature NS5A clearly indicates that NS3 was active in the presence of N-terminally truncated NS4A. As anticipated, host cell signal peptidase-mediated processing in the structural region and NS4A-independent cleavage at the NS5A/NS5B site were unaffected, yielding core and NS5B proteins of the expected molecular masses.

IF analysis was performed next to investigate whether interactions among the cytosolic domains could rescue the membrane association of nonstructural proteins with deletions of their membrane anchor domains. As shown in Fig. 4 (left panel), core, NS3, NS5A, and NS5B expressed from the wild-type HCV polyprotein were found in the cytoplasm in a reticular staining pattern with accumulation in dot-like structures which, as shown previously (6, 11), represent membranous webs. It is important to note that HCV protein expression is sufficient to induce the formation of membranous webs which in the context of active HCV RNA replication harbor viral replication complexes (6, 11). Consistent with earlier observations (6), a minor fraction of core was also found in the nucleus with accumulation in nucleoli. The distribution of core, NS5A, and NS5B was similar in the case of HCV4AΔN2-19. Possibly due to the processing defect observed by immunoblotting (Fig. 3), the number and size of dot-like structures revealed by the NS5A MAb were reduced compared to the wild-type construct. More importantly, the membrane anchor deletion-containing NS3-4A complex was found in a diffuse staining pattern with even distribution between the cytoplasm and nucleus and sparing of the nucleoli (Fig. 4). This staining pattern was indistinguishable from that of NS3-4AΔN2-19 expressed alone (compare with Fig. 1, lower left panel). Similarly, the membrane anchor deletion-containing NS5A and NS5B proteins expressed by HCV5AΔN4-21 and HCV5BΔC21, respectively, were diffusely distributed and showed the same staining pattern as the corresponding mutants expressed alone (compare Fig. 4 with Fig. 1, lower middle and lower right panels). Membrane association of core, NS3, and wild-type NS5A or NS5B, respectively, as well as the formation of dot-like structures, representing membranous webs, was not significantly affected

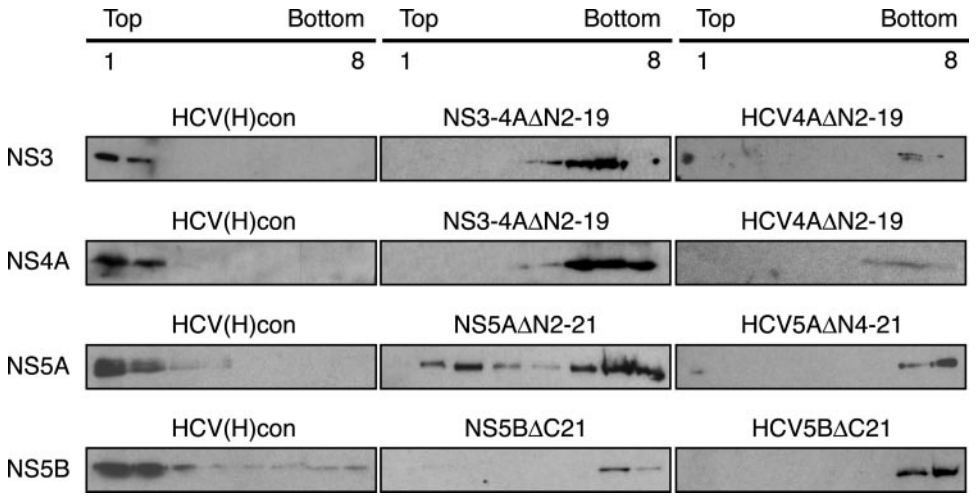

FIG. 5. Flotation analyses of membrane anchor deletion mutants. Lysates of UHCVcon-57.3 cells, which inducibly express the wild-type HCV(H)con polyprotein (30), or UTA-6 cells transfected with pUHDNS3-4AΔN2-19, pUHDNS5AΔN2-21, pCMVNS5BconΔC21, pUHDHCV4AΔN2-19, pUHDHCV5AΔN4-21, or pUHDHCV5BΔC21 were subjected to equilibrium centrifugation through Nycodenz gradients. Fractions were collected from the top and analyzed by immunoblotting using MAbs 1B6, 8N, 11H, and 5B-3B1 against NS3, NS4A, NS5A, and NS5B, respectively.

in these constructs. In conclusion, these results demonstrate that membrane association of the membrane anchor deletion mutants is not rescued by their expression in the context of the HCV polyprotein.

Membrane flotation analyses were performed to confirm these observations. To this end, cells transfected with the different expression constructs were subjected to hypotonic lysis, followed by equilibrium centrifugation of cell lysates through Nycodenz gradients, as described previously (22, 26). Under these conditions, membrane proteins float to the upper, low-density fractions, while soluble proteins remain in the lower, high-density fractions. As expected, NS3-4A, NS5A, and NS5B proteins expressed from the wild-type polyprotein construct floated to the membrane-containing low-density fractions (Fig. 5, left panels). By contrast, the membrane anchor deletion mutants remained in the high-density fractions, regardless of whether they were expressed alone (middle panels) or in the context of the HCV polyprotein (right panels). Of note, a minor proportion of the NS5A mutant was found in the membrane fractions under both conditions. This is consistent with the idea that one or more additional regions outside of the N-terminal amphipathic α-helix of NS5A may contribute to its membrane association (26).

Taken together, the IF and membrane flotation analyses shown above demonstrate that interactions of the cytosolic domains of NS3-4A, NS5A, and NS5B with the remaining HCV proteins are not sufficiently strong to rescue membrane anchor deletion mutants to the membrane. These results are in good agreement and further extend similar observations made with a membrane association-deficient NS5A mutant expressed in the context of the HCV nonstructural proteins (7). Thus, membrane association of each nonstructural protein is essential for its incorporation into the replication complex. However, formation of this complex, at least at the resolution of light microscopy, does not depend on the presence of all membrane anchor domains. By contrast, and in agreement with a central role of NS4B in formation of the membranous web (6), we have recently found that deletion of NS4B in the context of the HCV polyprotein abrogates formation of dot-like structures (Valérie Castet and D. Moradpour, unpublished data).

Recent evidence suggests that the HCV RdRp, similar to poliovirus 3D polymerase (13, 19), may form multimers and thereby gain cooperative activity (28, 34). In addition, homotypic interactions have been reported for NS5A (4). This prompted us to investigate whether protein-protein interactions between membrane anchor deletion-containing NS5A and NS5B with their corresponding wild-type counterparts may rescue membrane association of the truncated proteins. To this end, membrane anchor deletion mutants with an engineered hemagglutinin (HA) epitope tag were coexpressed in *trans* with the HCV polyprotein and their subcellular localization analyzed by IF. In brief, an NS5A fragment with a deletion of aa 2 to 21 in the N-terminal domain and a C-terminal HA tag was amplified from pBRTM/HCV1-3011con by PCR using primer pair 11-12 (Table 1). The amplification product was digested with EcoRI and XbaI, followed by ligation into the EcoRI-XbaI sites of pUHD10-3 to yield pUHDNS5AΔN2-21HA. A C-terminal HA tag was fused to NS5BΔC21 by PCR using primer pair 13-14 and pBRTM/HCV1-3011con as a template, followed by digestion with EcoRI and XbaI and ligation into the EcoRI and XbaI sites of pUHD10-3 to yield pUHDNS5BΔC21HA. To exclude the possibility that the position of the HA tag influences the subcellular localization of NS5BΔC21, a similar construct, pUHDHANS5BΔC21, with an N-terminal HA tag was prepared using primer pair 15-16.

The tagged mutants were transfected into UHCVcon-57.3 cells, which inducibly express the entire HCV polyprotein upon tetracycline withdrawal (30). Double-label IF analysis was subsequently performed to determine the subcellular distribution of the tagged mutants and of NS3 as a marker for a membrane-associated protein. As shown in Fig. 6, NS3 expressed from the

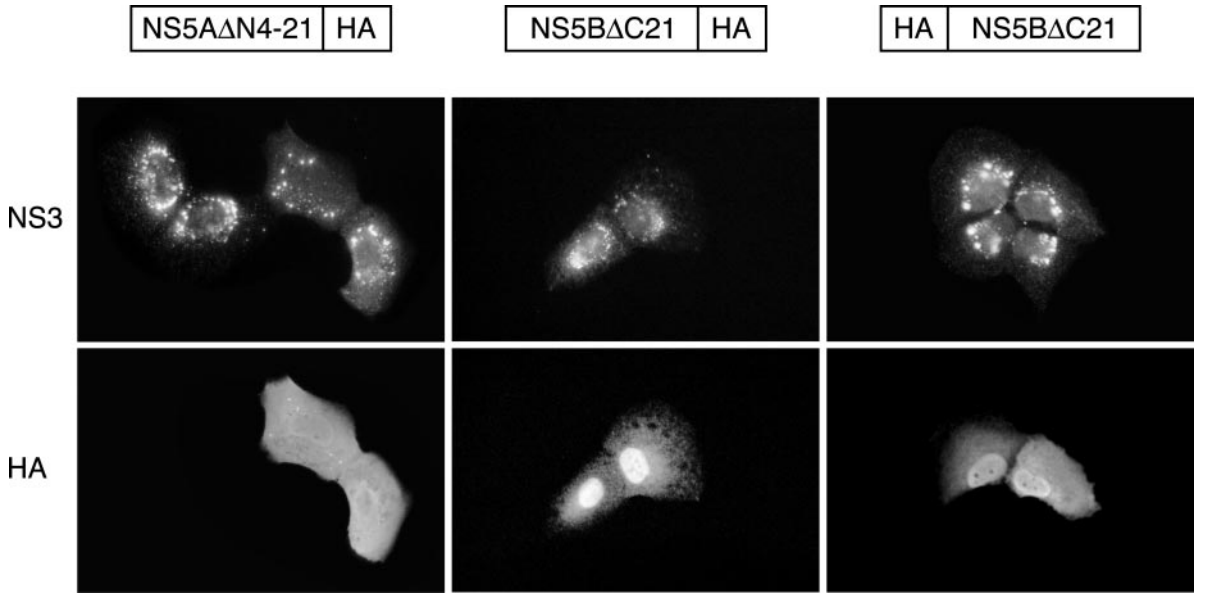

FIG. 6. Subcellular distribution of HA-tagged membrane anchor deletion mutants in the presence of the entire wild-type polyprotein. UHCVcon-57.3 cells were derepressed by tetracycline withdrawal and at the same time transiently transfected with pUHDNS5AΔN2-21HA, pUHDHANS5BΔC21, or pUHDNS5BΔC21HA, as indicated. At 48 h posttransfection, the membrane anchor deletion mutants were visualized by a polyclonal anti-HA antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and a Cy3-conjugated goat anti-rabbit secondary antibody. NS3 was detected with MAb 1B6 and an Alexa 488-conjugated goat anti-mouse secondary antibody.

inducible transgene appeared in the typical reticular staining pattern and in cytoplasmic dot-like structures irrespective of the coexpressed mutant (upper panel). In contrast, HA-tagged NS5AΔN2-21 was found in a diffuse staining pattern distributed throughout the cell. Similarly, NS5BΔC21 was diffusely distributed and accumulated in the nucleus, irrespective of the position of the HA tag. Interestingly, the HA tag seemed to prevent nucleolar accumulation of this mutant. Taken together, these results indicate that homotypic interactions of NS5A or NS5B coexpressed in *trans* are relatively weak. Identical results were obtained when the tagged mutants were cotransfected with their wild-type counterparts expressed alone or in the context of the HCV polyprotein (data not shown).

Taken together, our data suggest that interactions among cytosolic domains of NS3-4A, NS5A, and NS5B are relatively weak compared to their membrane association. Membrane association of the individual nonstructural proteins is essential for incorporation into the HCV replication complex. The absence of strong interactions between the viral nonstructural proteins may provide them with a certain flexibility to fulfill their multiple functions during viral replication, e.g., synthesis of positive- and negative-strand RNAs by the RdRp or serine protease and NTPase/RNA helicase activity of the NS3-4A complex (37). These observations, together with the identification of absolutely conserved residues in the membrane anchor domains of NS5A and NS5B that are essential for viral RNA replication, but not for membrane association per se (22, 26), suggest that the membrane anchor domains are central organizing elements in the replication complex and that important protein-protein interactions between these domains may occur within the membrane.

Two points need to be considered, however. First, it is possible that replicating viral RNA mediates interactions that we could not detect in our assays. However, interactions that have thus far been reported have also been found in the absence of a genuine viral RNA. Second, we cannot rule out conformational alterations of nonstructural proteins lacking their membrane anchor domains or, in the case of the experiments shown in Fig. 6, fused to an epitope tag. However, biochemical and structural studies have shown correct folding and functionality of NS3-4A with only the central portion of NS4A present (1, 18, 37). Accordingly, protease activity of NS3-4AΔN2-19 was partially impaired only at the NS4B/NS5A cleavage site. In addition, it was shown that the C-terminal 21 aa of NS5B are dispensable for polymerase activity in vitro (9, 36). No functional or structural information is available for NS5A. Thus, at least the NS3-4A and NS5B membrane anchor deletion mutants should have folded properly.

It is interesting that interactions among cytosolic domains of nonstructural proteins from other positive-strand RNA viruses are essential for incorporation of these proteins into membrane-associated replication complexes; e.g., membrane association of poliovirus 3D polymerase is mediated by protein precursor 3AB (14). Similar to poliovirus 3D, the RdRp of mouse hepatitis virus is directed to the membrane by an as yet unknown viral or virus-induced factor (3). Furthermore, membrane association of the nonstructural polyprotein of Semliki Forest virus is mediated by nsP1 (29). In these instances, membrane association of essential replicase components is ensured by interactions among cytosolic domains of nonstructural proteins. The observation that replication complex incorporation of each nonstructural protein depends on its own membrane anchor domain appears characteristic for HCV. Studies on the determinants and mechanisms of membrane association of nonstructural proteins from pestivirus, GB virus, and flavivirus

are in progress to explore whether this feature is shared among different members of the *Flaviviridae* family.

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REFERENCES

1. **Bartenschlager, R., V. Lohmann, T. Wilkinson, and J. O. Koch.** 1995. Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation. J. Virol. **69:**7519–7528.
2. **Brass, V., E. Bieck, R. Montserret, B. Wölk, J. A. Hellings, H. E. Blum, F. Penin, and D. Moradpour.** 2002. An aminoterminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. J. Biol. Chem. **277:**8130–8139.
3. **Brockway, S. M., C. T. Clay, X. T. Lu, and M. R. Denison.** 2003. Characterization of the expression, intracellular localization, and replication complex association of the putative mouse hepatitis virus RNA-dependent RNA polymerase. J. Virol. **77:**10515–10527.
4. **Dimitrova, M., I. Imbert, M. P. Kieny, and C. Schuster.** 2003. Protein-protein interactions between hepatitis C virus nonstructural proteins. J. Virol. **77:**5401–5414.
5. **Dubuisson, J., F. Penin, and D. Moradpour.** 2002. Interaction of hepatitis C virus proteins with host cell membranes and lipids. Trends Cell Biol. **12:**517–523.
6. **Egger, D., B. Wölk, R. Gosert, L. Bianchi, H. E. Blum, D. Moradpour, and K. Bienz.** 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J. Virol. **76:**5974–5984.
7. **Elazar, M., K. H. Cheong, P. Liu, H. B. Greenberg, C. M. Rice, and J. S. Glenn.** 2003. Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. J. Virol. **77:**6055–6061.
8. **Englert, C., X. Hou, S. Maheswaran, P. Bennett, C. Ngwu, G. G. Re, A. J. Garvin, M. R. Rosner, and D. A. Haber.** 1995. WT1 suppresses synthesis of the epidermal growth factor receptor and induces apoptosis. EMBO J. **14:**4662–4675.
9. **Ferrari, E., J. Wright-Minogue, J. W. Fang, B. M. Baroudy, J. Y. Lau, and Z. Hong.** 1999. Characterization of soluble hepatitis C virus RNA-dependent RNA polymerase expressed in *Escherichia coli*. J. Virol. **73:**1649–1654.
10. **Flajolet, M., G. Rotondo, L. Daviet, F. Bergametti, G. Inchauspé, P. Tiollais, C. Transy, and P. Legrain.** 2000. A genomic approach of the hepatitis C virus generates a protein interaction map. Gene **242:**369–379.
11. **Gosert, R., D. Egger, V. Lohmann, R. Bartenschlager, H. E. Blum, K. Bienz, and D. Moradpour.** 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J. Virol. **77:**5487–5492.
12. **Gossen, M., and H. Bujard.** 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA **89:**5547–5551.
13. **Hobson, S. D., E. S. Rosenblum, O. C. Richards, K. Richmond, K. Kirkegaard, and S. C. Schultz.** 2001. Oligomeric structures of poliovirus polymerase are important for function. EMBO J. **20:**1153–1163.
14. **Hope, D. A., S. E. Diamond, and K. Kirkegaard.** 1997. Genetic dissection of interaction between poliovirus 3D polymerase and viral protein 3AB. J. Virol. **71:**9490–9498.
15. **Horton, R. M., Z. L. Cai, S. N. Ho, and L. R. Pease.** 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. BioTechniques **8:**528–535.
16. **Ivashkina, N., B. Wölk, V. Lohmann, R. Bartenschlager, H. E. Blum, F. Penin, and D. Moradpour.** 2002. The hepatitis C virus RNA-dependent RNA polymerase membrane insertion sequence is a transmembrane segment. J. Virol. **76:**13088–13093.
17. **Kolykhalov, A. A., E. V. Agapov, K. J. Blight, K. Mihalik, S. M. Feinstone, and C. M. Rice.** 1997. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science **277:**570–574.
18. **Lin, C., J. A. Thomson, and C. M. Rice.** 1995. A central region in the hepatitis C virus NS4A protein allows formation of an active NS3-NS4A serine proteinase complex in vivo and in vitro. J. Virol. **69:**4373–4380.
19. **Lyle, J. M., E. Bullitt, K. Bienz, and K. Kirkegaard.** 2002. Visualization and functional analysis of RNA-dependent RNA polymerase lattices. Science **296:**2218–2222.
20. **Miyanari, Y., M. Hijikata, M. Yamaji, M. Hosaka, H. Takahashi, and K. Shimotohno.** 2003. Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral RNA replication. J. Biol. Chem. **278:**50301–50308.
21. **Moradpour, D., E. Bieck, T. Hügle, W. Wels, J. Z. Wu, Z. Hong, H. E. Blum, and R. Bartenschlager.** 2002. Functional properties of a monoclonal antibody inhibiting the hepatitis C virus RNA-dependent RNA polymerase. J. Biol. Chem. **277:**593–601.
22. **Moradpour, D., V. Brass, E. Bieck, P. Friebe, R. Gosert, H. E. Blum, R. Bartenschlager, F. Penin, and V. Lohmann.** 2004. Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication. J. Virol. **78:**13278–13284.
23. **Moradpour, D., C. Englert, T. Wakita, and J. R. Wands.** 1996. Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. Virology **222:**51–63.
24. **Moradpour, D., R. Gosert, D. Egger, F. Penin, H. E. Blum, and K. Bienz.** 2003. Membrane association of hepatitis C virus nonstructural proteins and identification of the membrane alteration that harbors the viral replication complex. Antiviral Res. **60:**103–109.
25. **Moradpour, D., P. Kary, C. M. Rice, and H. E. Blum.** 1998. Continuous human cell lines inducibly expressing hepatitis C virus structural and nonstructural proteins. Hepatology **28:**192–201.
26. **Penin, F., V. Brass, N. Appel, S. Ramboarina, R. Montserret, D. Ficheux, H. E. Blum, R. Bartenschlager, and D. Moradpour.** 2004. Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. J. Biol. Chem. **279:**40835–40843.
27. **Piccininni, S., A. Varaklioti, M. Nardelli, B. Dave, K. D. Raney, and J. E. McCarthy.** 2002. Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. J. Biol. Chem. **277:**45670–45679.
28. **Qin, W., H. Luo, T. Nomura, N. Hayashi, T. Yamashita, and S. Murakami.** 2002. Oligomeric interaction of hepatitis C virus NS5B is critical for catalytic activity of RNA-dependent RNA polymerase. J. Biol. Chem. **277:**2132–2137.
29. **Salonen, A., L. Vasiljeva, A. Merits, J. Magden, E. Jokitalo, and L. Kaariainen.** 2003. Properly folded nonstructural polyprotein directs the Semliki Forest virus replication complex to the endosomal compartment. J. Virol. **77:**1691–1702.
30. **Schmidt-Mende, J., E. Bieck, T. Hügle, F. Penin, C. M. Rice, H. E. Blum, and D. Moradpour.** 2001. Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. J. Biol. Chem. **276:**44052–44063.
31. **Shi, S. T., K. J. Lee, H. Aizaki, S. B. Hwang, and M. M. Lai.** 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J. Virol. **77:**4160–4168.
32. **Shirota, Y., H. Luo, W. Qin, S. Kaneko, T. Yamashita, K. Kobayashi, and S. Murakami.** 2002. Hepatitis C virus NS5A binds RNA-dependent RNA polymerase NS5B and modulates RNA-dependent RNA polymerase activity. J. Biol. Chem. **277:**11149–11155.
33. **Tanji, Y., M. Hijikata, S. Satoh, T. Kaneko, and K. Shimotohno.** 1995. Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. J. Virol. **69:**1575–1581.
34. **Wang, Q. M., M. A. Hockman, K. Staschke, R. B. Johnson, K. A. Case, J. Lu, S. Parsons, F. Zhang, R. Rathnachalam, K. Kirkegaard, and J. M. Colacino.** 2002. Oligomerization and cooperative RNA synthesis activity of hepatitis C virus RNA-dependent RNA polymerase. J. Virol. **76:**3865–3872.
35. **Wölk, B., D. Sansonno, H.-G. Kräusslich, F. Dammacco, C. M. Rice, H. E. Blum, and D. Moradpour.** 2000. Subcellular localization, stability and *trans*-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. J. Virol. **74:**2293–2304.
36. **Yamashita, T., S. Kaneko, Y. Shirota, W. Qin, T. Nomura, K. Kobayashi, and S. Murakami.** 1998. RNA-dependent RNA polymerase activity of the soluble recombinant hepatitis C virus NS5B protein truncated at the C-terminal region. J. Biol. Chem. **273:**15479–15486.
37. **Yao, N., P. Reichert, S. S. Taremi, W. W. Prosise, and P. C. Weber.** 1999. Molecular views of viral polyprotein processing revealed by the crystal structure of the hepatitis C virus bifunctional protease-helicase. Struct. Fold. Dev. **7:**1353–1363.