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Palmitoylation and Polymerization of Hepatitis C Virus NS4B Protein

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Hepatitis C Virus (HCV) NS4B protein induces a specialized membrane structure which may serve as the replication platform for HCV RNA replication. In the present study, we demonstrated that NS4B has lipid modifications (palmitoylation) on two cysteine residues (cysteines 257 and 261) at the C-terminal end. Site-specific mutagenesis of these cysteine residues on individual NS4B proteins and on an HCV subgenomic replicon showed that the lipid modifications, particularly of Cys261, are important for protein-protein interaction in the formation of the HCV RNA replication complex. We further demonstrated that NS4B can undergo polymerization. The main polymerization determinants were mapped in the N-terminal cytosolic domain of NS4B protein; however, the lipid modifications on the C terminus also facilitate the polymerization process. The lipid modification and the polymerization activity could be two properties of NS4B important for its induction of the specialized membrane structure involved in viral RNA replication.

Hepatitis C virus (HCV) has a single-stranded, positive-sense RNA genome, which encodes a polyprotein. The polyprotein is processed into at least 10 individual proteins (Core-E1-E2-p7-NS2-NS3-NS4A-NS3-NS4A-NS4B-NS5A-NS5B). Core-E1-E2-p7 proteins are structural proteins and are cleaved by cellular proteases. NS2 to NS5B are nonstructural proteins, which are cleaved by viral proteases (NS2 and NS3) (4). By using a subgenomic-replicon system (21), our laboratory has shown that HCV RNA replication occurs on a specialized membrane microdomain, or lipid raft (2, 30). NS4B and the cellular protein hVAP-33 (human homologue of the VAMP-associated 33-kDa protein) are the two main players in initiating the formation of the RNA replication complex (14). Other nonstructural proteins of HCV also reside in lipid rafts (2, 30); furthermore, a subpopulation of core proteins has been detected in detergent-resistant membranes (24). Therefore, this specialized micromembrane domain could be important in the HCV replication cycle.

NS4B protein is a relatively hydrophobic membrane protein with a protein size of 27 kDa. According to a topology study (22), NS4B has four transmembrane segments, with the N and C termini being located on the cytoplasmic side. The amino acid composition of the cytoplasmic N terminus is very hydrophobic; thus, a fifth transmembrane domain has been proposed in this region (22). However, it is still unknown whether the flipping of the fifth transmembrane domain indeed occurs. A putative amphipathic helix on amino acid residues 1 to 26 of the NS4B N terminus is crucial for virus replication (13). In addition, a nucleotide-binding motif located between the second and third transmembrane domains of NS4B (residues 129 to 135 of NS4B) has also been identified (12).

Although the exact function of NS4B in viral replication is still unclear, it is striking that the expression of NS4B alone or in the context of HCV polyprotein induces a specialized membranous web (11), which has been postulated to be the HCV RNA replication complex. In the cells harboring an HCV subgenomic replicon, NS4B plays a crucial role in maintaining the replication complex in the detergent-resistant membrane (14). The induction of the membranous web by NS4B and its central role in lipid raft targeting have implicated a structural role for NS4B in viral RNA replication, namely, the formation of the HCV RNA replication complex. In the present study, we further defined the characteristics of NS4B protein by which NS4B executes its role in HCV RNA replication.

MATERIALS AND METHODS

Cell cultures, viruses, and antibodies. Huh7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and nonessential amino acids. Huh7 cells harboring an HCV subgenomic replicon (19, 21) were maintained in DMEM containing 0.5 mg/ml of G418 (Gibco-BRL, Gaithersburg, Md.). The recombinant vaccinia virus encoding T7 RNA polymerase (VV-T7) (32) was amplified in HeLa cells. The mouse monoclonal antibody against NS5A was purchased from Biodesign (Saco, Maine). The rabbit anti-Flag antibody was purchased from Sigma (St. Louis, Mo.).

Plasmids. The full-length NS4B (from genotype 1a, isolate H77) and its truncation mutants were PCR-generated with a Flag sequence at the N terminus and a stop codon at the C terminus, and the fragments were ligated into the pcDNA3.1/V5-His expression vector (Invitrogen, Carlsbad, Calif.) by TA cloning procedures as per the manufacturer's instructions. All of the deletion mutants were PCR amplified, using appropriate primers containing Flag-tag sequences, and were cloned into the pcDNA3.1 vector by TA cloning. The vector contains a cytomegalovirus immediate-early promoter for expression in mammalian cells and a T7 promoter recognized by T7 RNA polymerase. The cysteine mutations on Flag-NS4B and replicon pUC-Rep/S1179I (19) were generated by using the Quickchange mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, Calif.). The mutation sites were confirmed by sequencing.

Metabolic labeling and immunoprecipitation. Huh7 cells were transfected with various constructs by using Fugene 6 reagent (Roche, Penzberg, Germany) and were then infected with vaccinia virus carrying the T7 polymerase gene (multiplicity of infection = 10). The infected cells were incubated in labeling medium (DMEM, 2% fetal bovine serum, nonessential amino acids, 5 mM sodium pyruvate) containing 33 μCi/ml [9,10-^{3}H(N)]palmitic acid (PerkinElmer, Boston, Mass.) at 37°C for 12 h. For protein detection, another set of infected cells was incubated in the same labeling medium containing 15μCi/ml of ^{35}S-Translabel (ICN, Costa Mesa, Calif.). After being incubated, the cells were

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washed once with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride, 1× Complete Protease Inhibitors [Roche, Mannheim, Germany]). The cell lysate was centrifuged at 1,000 × *g* for 10 min; the supernatant was collected for immunoprecipitation. Flag-tagged proteins were purified from the supernatant by using anti-FLAG M2 affinity gel (Sigma, Saint Louis, Mo.). SDS-PAGE sample buffer without dithiothreitol (DTT) was added to the washed agarose gel, and the samples were heated at 65°C for 5 min and run on tricine gel. The signals from the gel were detected by autoradiography and quantified by ImageQuant software.

PEGylation. Polyethylene glycol 5000-maleimide (PEG-mal) (Nektar Therapeutics) was used to determine the extent of lipid modification of NS4B. The method used was as previously described (26), with minor modifications. After being transfected with DNA and infected with VV-T7, the NS4B (or NS4B cysteine mutant)-overexpressing cells were lysed in 50 μl lysis buffer (100 mM Tris-HCl [pH 6.5], 100 mM NaCl, and 0.5% Triton X-100). After being centrifuged (10,000 × *g*, 5 min, 4°C), 10 μl supernatant was treated with 2.5 μl of 100 mM DTT for 5 min at room temperature to reduce disulfide bonds. Then 80 μl of 30 mM PEG-mal (dialyzed) was added to the DTT-treated lysate, and the mixture was incubated at 37°C for 1 h. The PEG-mal solution was dialyzed against phosphate buffer (pH 6.5) overnight at 4°C. The PEGylation reaction was stopped by addition of 1 μl of β-mercaptoethanol, and nine volumes of ice-cold 100% acetone were added for protein precipitation (at −80°C overnight). After being centrifuged (12,000 × *g*, 5 min, 4°C), the precipitated protein was washed with ice-cold 85% acetone at −20°C for 1 h. Then the precipitated protein was dried after briefly being centrifuged and was resuspended in Laemmli buffer for detection by Western blotting.

Coimmunoprecipitation. The Huh7 cells harboring subgenomic replicons were transfected with various constructs and infected with VV-T7 at 24 h posttransfection. The infected cells were harvested at 16 h postinfection, washed with PBS once, and scraped into PBS. After briefly being centrifuged, the cell pellet was resuspended in buffer A (10 mM HEPES-KOH [pH 7.8], 10 mM KCl, 1.5 mM $MgCl_2$, 20% glycerol) containing 1% NP-40, 1× Complete Protease Inhibitor, and 1 mM phenylmethylsulfonyl fluoride and was incubated on ice for 20 min. After being centrifuged at 1,000 × *g* at 4°C for 5 min, the supernatant was collected and mixed with an equal volume of 2× TM10 buffer (100 mM Tris-HCl [pH 7.9], 200 mM KCl, 50 mM $MgCl_2$, 2 mM EDTA, 20% glycerol, 0.2% NP-40). The cell lysate was incubated with anti-FLAG M2 affinity gel at 4°C overnight. Then the agarose gel was washed with 1× TM10 buffer three times and eluted with 3× FLAG peptide. The eluents were subjected to immunoblotting by using HCV patient serum or NS5A-specific antibody.

Colony formation assay. For colony formation assays (15), the pUC-Rep/S1179I (19) plasmid containing C257A, C261T, or a double mutation (C257A, C261T) was linearized with ScaI and used as a template for in vitro RNA transcription by using the MEGAscript kit (Ambion, Austin, Tex.). The in vitro-transcribed RNAs (40 μg) were transfected into Huh7 cells (4×10^6 cells in 400 μl serum-free DMEM) by electroporation with Gene Pulser II (Bio-Rad, Hercules, Calif.) set to 220V and 975 μF. One-fourth of the cells that had undergone electroporation were plated onto one 10-cm plate in DMEM containing 1.25% dimethyl sulfoxide mesophile. After being incubated overnight, the medium was changed to DMEM containing 500 μg/ml G418 (Gibco-BRL, Gaithersburg, Md.). The G418-resistant colonies were stained with 0.1% crystal violet (in 20% ethanol) after 4 weeks of selection.

Protein polymerization assay. For protein polymerization assays (23), cells were transfected with DNA and infected with VV-T7 and then washed with PBS once and collected by scraping into PBS. After briefly being centrifuged, the cell pellet was resuspended in hypotonic buffer (10 mM Tris-HCl [pH 7.5], 10 mM KCl, and 5 mM $MgCl_2$) and incubated on ice for 20 min. The swollen cells were passed through a 25-gauge needle 20 times to disrupt the cells, followed by centrifugation at 1,000 × *g*. The supernatant was incubated with glutaraldehyde (Sigma, Saint Louis, Mo.) at a final concentration of 0.01% at room temperature for various time periods. The reaction was stopped by adding SDS-sample buffer, and the samples were subjected to Western blotting.

RESULTS

NS4B is palmitoylated. Three common protein modifications in the lipid raft resident proteins are glycosylphosphatidylinositol modification, N-terminal myristoylation plus palmitoylation (Met-Gly-Cys motif), and palmitoylation of dual Cys residues (25). The NS4B amino acid sequence (genotype 1a) (NP_751926) contains three cysteine residues (Cys187, 257, and 261), two of which, Cys257 and Cys261, are located at the very C-terminal end (Cys261 is the last amino acid residue of NS4B).

To test whether NS4B has lipid modification, NS4B was overexpressed. The NS4B gene was amplified from an H77 isolate (genotype 1a) with a Flag-tag sequence on the forward primer, and then the amplified fragment was cloned into the pcDNA3.1 vector. The Flag-tagged protein was expressed in Huh7 cells after they were infected with the recombinant VV-T7, and the protein was purified by using Flag-tag affinity gel. As shown in Fig. 1A, Flag-NS4B, but not Flag-NS5B, was labeled with [^{3}H]palmitic acid. The palmitoylation signal was seen not only at the expected size of NS4B (around 30 kDa) but also at that of the NS4B dimer (around 60 kDa) (Fig. 1B). This result indicated that NS4B is palmitoylated. Since the reducing agent was removed from the Laemmli sample buffer used in this experiment to prevent the disruption of palmitoylation from the labeled protein, it was not surprising that some proteins remained in a partially denatured state under this condition. We have also expressed NS4B protein by transfecting Huh7 cells with a poly(A)-tailed, NS4B-encoding mRNA, which yielded a high level of NS4B in the absence of VV-T7 infection. NS4B protein could also be labeled by [^{3}H]palmitic acid under this expression condition, indicating that [^{3}H]palmitic acid labeling of NS4B was not an artifact of vaccinia virus infection (data not shown). These results indicated that NS4B is palmitoylated.

NS4B palmitoylation is insensitive to Brefeldin treatment. In mammalian cells, protein palmitoylation is performed by palmitoyl acyltransferases, which are found in subcellular fractions containing plasma membrane, Golgi compartments, and mitochondrial membranes (10). The enzymatic activity is also enriched in sphingomyelin- and cholesterol-rich membrane microdomains (9). In the cases of vesicular stomatitis virus glycoprotein and influenza virus hemagglutinin, palmitoylation occurs on the intermediate compartment between the endoplasmic reticulum (ER) and *cis*-Golgi compartments (6). Therefore, to test whether the Golgi apparatus or post-Golgi compartments are involved in NS4B palmitoylation, the Golgi cisternae disruption agent, Brefeldin A (BFA) (17), was applied in the palmitoylation-labeling experiment. As shown in Fig. 1B, BFA treatment affected neither NS4B expression ([^{35}S]methionine labeling) nor its palmitoylation, suggesting that palmitoylation of NS4B occurs on a pre-Golgi compartment.

The palmitoylation sites of NS4B are located on the C-terminal cytoplasmic domain. To identify NS4B palmitoylation sites, various NS4B deletion mutants were used. We first used the TMAP (27) and TMHMM programs (18) to predict the transmembrane domains of NS4B protein. Both programs predicted four transmembrane domains from the NS4B protein sequence (genotype 1a) with slight variations on the exact boundaries of the transmembrane regions. To map lipid modification sites, a series of NS4B deletion mutants were constructed by following the TMAP prediction map. As shown in Fig. 2A, the four predicted transmembrane segments of NS4B are amino acids 72 to 92, 101 to 121, 136 to 156, and172 to 197. The various deletion mutants were expressed separately in Huh7 cells. Among the four NS4B deletion mutants examined,

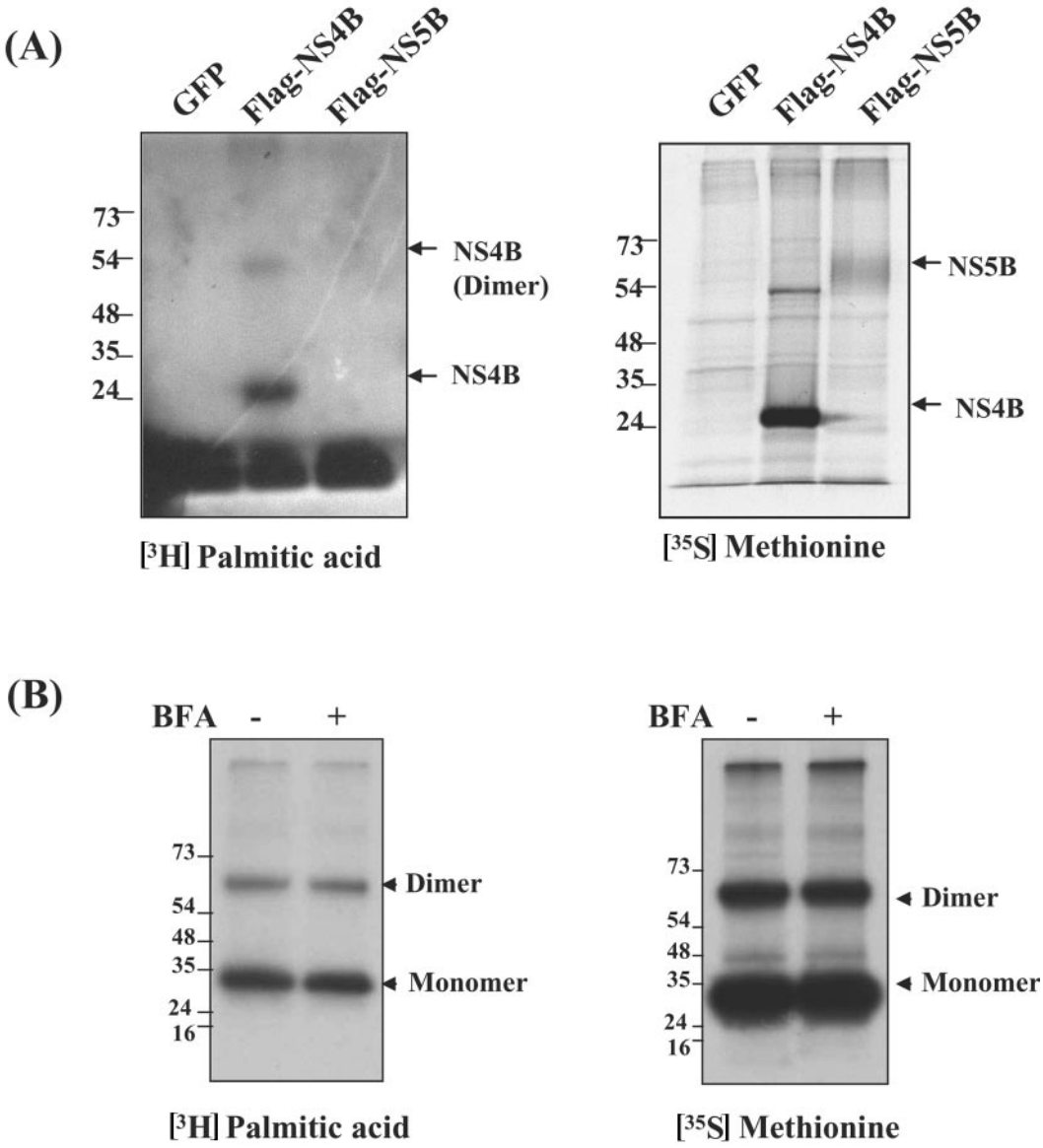
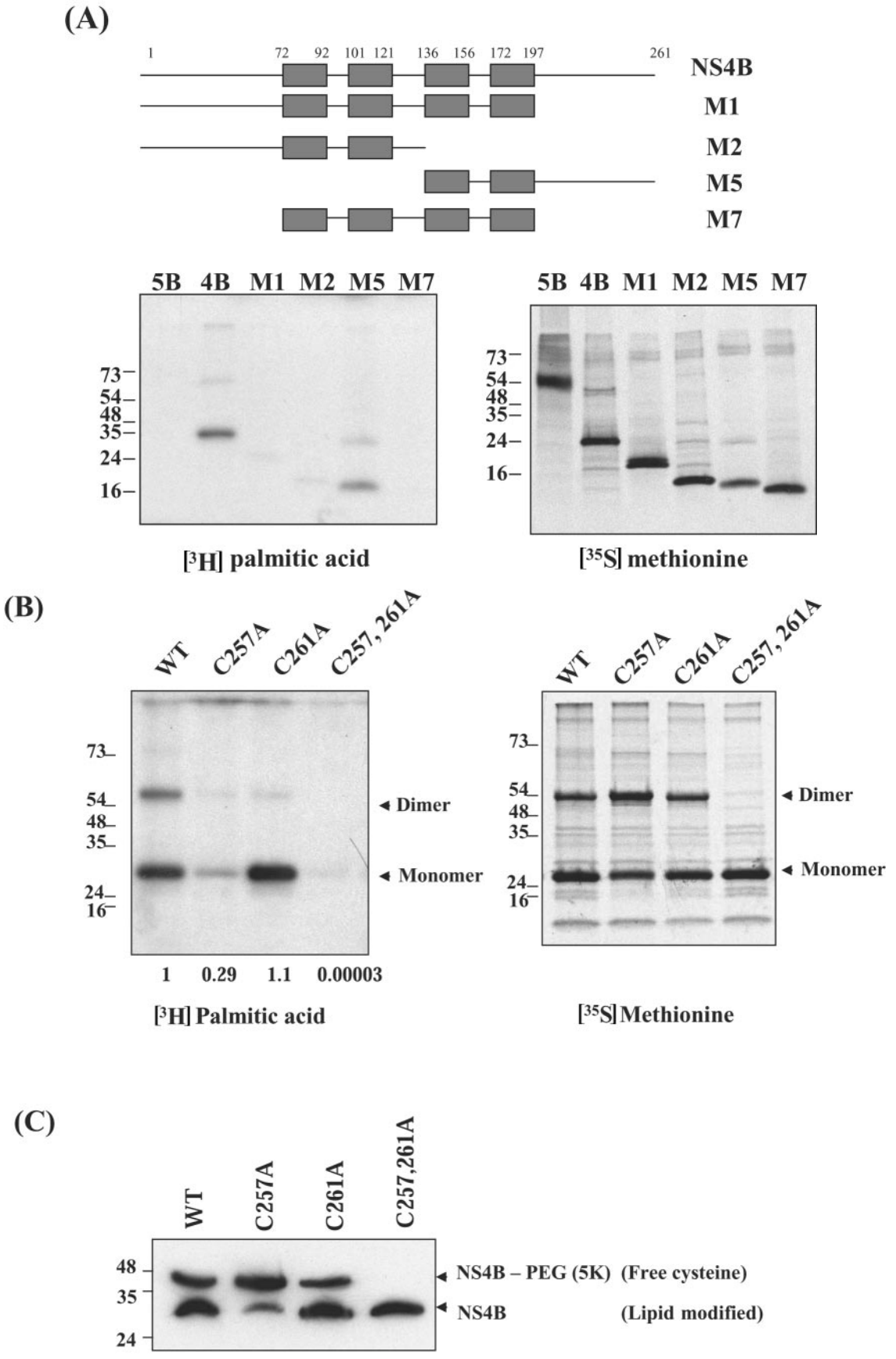


FIG. 1. NS4B was palmitoylated, and the palmitoylation was insensitive to BFA treatment. (A) Huh7 cells were transfected with various constructs. At 24 h posttransfection, the cells were infected with VV-T7. The infected cells were incubated with [9,10-^{3}H(N)]palmitic acid or [^{35}S]methionine for 12 h. The cell lysates were immunoprecipitated with anti-Flag agarose gel, and the precipitated proteins were analyzed by SDS-PAGE and autoradiography. (B) Sensitivity of the NS4B palmitoylation to BFA. Huh7 cells expressing Flag-NS4B protein were subjected to metabolic labeling with or without BFA. GFP, green fluorescent protein.

only M5 retained the palmitoylation modification signal. M5 has two transmembrane segments, which are also present in M7. Since M7 is not palmitoylated, we concluded that the main palmitoylation sites are mapped within the C-terminal cytoplasmic region (amino acids [aa] 198 to 261).

Cysteine 257 and cysteine 261 are the palmitoylation sites on NS4B. Cysteine residues are the common palmitoylation sites (5, 20, 29). There are three cysteine residues (Cys187, 257, and 261) in the NS4B amino acid sequence (genotype 1a). The M5 mutant contains all three cysteine residues. Cys187 is also present in M1, M5, and M7, but M1 and M7 were not palmitoylated. Therefore, Cys257 and Cys261, located at the very C-terminal end of the NS4B protein, are the potential palmitoylation sites. To address whether these two cysteine residues are truly the modification sites, we performed site-directed mutagenesis to replace cysteine with alanine. NS4B proteins with either a single cysteine mutation (C257A or C261A) or dual mutations (C257 and 261A) were applied for palmitic acid labeling. The signals of palmitoylation labeling from autoradiography were analyzed by ImageQuant software. Compared to the signals of wild-type NS4B (set as 1), the signals of C257A, C261A, and the dual mutant were 0.29, 1.1, and 0.00003, respectively (Fig. 2B). The C257A mutation dramatically reduced the palmitoylation signal; on the other hand, the C261A mutant retained a palmitoylation signal as strong as that of the wild-type protein. Therefore, we concluded that


(A)
1
72
92
101
121
136
156
172
197
261
NS4B
M1
M2
M5
M7
5B 4B M1 M2 M5 M7
73
54
48
35
24
16
[3H] palmitic acid
5B 4B M1 M2 M5 M7
73
54
48
35
24
16
[35S] methionine
(B)
WT
C257A
C261A
C257, 261A
73
54
48
35
24
16
Dimer
Monomer
1
0.29
1.1
0.00003
[3H] Palmitic acid
WT
C257A
C261A
C257, 261A
73
54
48
35
24
16
Dimer
Monomer
[35S] Methionine
(C)
WT
C257A
C261A
C257,261A
48
35
24
NS4B – PEG (5K) (Free cysteine)
NS4B (Lipid modified)

Cys257 is the main palmitoylation site. Nevertheless, the Cys257A mutant still retained some signals, compared with the double mutant (C257, 261A). Since the protein expression level of these mutants ([^{35}S]methionine labeling) was almost the same, these results suggested that Cys261 was also palmitoylated, though to a smaller degree than Cys257. Interestingly, the wild type and the C257A and C261A mutants formed dimers and trimers in tricine gel. In contrast, the dual-cysteine mutant did not form dimers or trimers, suggesting that lipid modification is important for the oligomerization of NS4B protein. The dimer was also palmitoylated. These results indicate that C257 is the major palmitoylation site, while C261 is also palmitoylated to a certain extent.

The next question we addressed was the extent of lipid modification in NS4B: are both cysteine residues fully modified or only partially modified by palmitic acid? To answer this question, we tried other biochemical methods to define the extent of modification of the cysteine residues in NS4B. Recently, O'Malley and Lanzinski developed a method to quantify the extent of farnesylation of a cysteine residue located at the C-terminal end of the hepatitis delta virus large delta antigen by using PEG-mal (26), based on the principle that PEG-mal reacts to the free sulfhydryl group of cysteine residues, thus increasing the molecular weight of the protein. In this assay, cell lysate was pretreated with DTT before the PEG-mal reaction, so that disulfide bonds, if there are any, will not prevent the PEG-mal reaction. Full-length NS4B and its cysteine mutations were treated with PEG-mal (5 kDa) and detected by Western blotting. Two NS4B proteins of different sizes were detected (Fig. 2C). The smaller protein corresponds to the original NS4B (representing the protein with modified cysteine residues); the larger protein corresponds to the protein with one attached PEG-5-kDa (5K-PEG) molecule (representing the protein with at least one free cysteine residue). These two proteins were present in almost equal molar amounts, suggesting that approximately half of the NS4B molecule was modified and that half of the proteins had at least one free cysteine residue. NS4B with two free cysteines (two 5K-PEG-labeled species) was not detected, suggesting either of the two possibilities that NS4B always has one of the two cysteine residues modified or that the first 5K-PEG modification affects the subsequent PEG modification on the adjacent free cysteine residue. In the C257A mutant, more PEG-modified than unmodified NS4B was detected, suggesting that most of the Cys261 residues were in the free status and were modified by PEG. In contrast, the C261A mutant had a similar modification ratio to that of the wild type. As expected, the double-cysteine mutant did not react with PEG because it did not have cysteine at the C-terminal end. Although there is another cysteine residue (Cys187) in transmembrane domain 4 in all four constructs, this cysteine residue appeared not to be accessible to the PEG-mal reagent. The ratio of the modified NS4B protein of the wild type and that of the mutants is consistent with the relative ratio determined by palmitic acid labeling (Fig. 2B).

TABLE 1. NS4B protein sequence residues 257 to 261 from different HCV genotypes

Genotype	NS4B consensus sequence (257–261)	% Conservation at position 257	% Conservation at position 261	Number of sequences counted
1a	CTTPC	77.8	100	9
1b	CSTPC	100	100	85
1c	CIAPC or CTAPC	100	100	2
2a	CPIPC	100	100	12
2b	CPVPC	100	100	13
2c	CPVPC			1
3a	YPSPC	100	100	4
4a	CSTPC			1
5a	YSTPC			1
6a	TATPC			1

Cysteine 257 and cysteine 261 are well conserved in various HCV genotypes. Since we have determined that NS4B is palmitoylated on cysteine 257 and cysteine 261, it is interesting to examine whether these two cysteine residues are conserved among different HCV genotypes. The NS4B aa residues 257 to 261 from various genotypes are listed in Table 1. Cysteine 261 is well conserved, partly because cysteine 261 is also the recognition site for NS3 protease cleavage. Cysteine 257, the main palmitoylation site, is well conserved in genotypes 1, 2, and 4. In genotypes 3, 5, and 6, aa residue 257 is tyrosine or threonine. Both tyrosine and threonine have a hydroxyl group. Other than cysteine residues, palmitoylation may occur on serine or threonine residues to form an acyloxyester (31). It remains to be determined whether NS4B from these genotypes also has lipid modification.

The NS4B lipid modifications are important for protein-protein interactions and formation of the replication complex. The next question that we addressed was whether lipid modification affects NS4B functions. HCV nonstructural proteins interact with each other and form multisubunit complexes for RNA replication (8, 14). We first examined whether the lipid modification affected the interaction between NS4B and other viral proteins. For this purpose, NS5A was coexpressed with the various Flag-tagged NS4B mutants in Huh7 cells and precipitated with Flag antibody. The precipitated NS5A was detected by NS5A antibody. As shown in Fig. 3A, single cysteine mutations (C257A or C261A) did not affect the interaction of NS4B with NS5A. However, the dual cysteine mutant (DM) coprecipitated a significantly smaller amount of NS5A. We also expressed Flag-NS4B into Huh7 cells containing a subgenomic HCV replicon, which express all of the nonstructural

FIG. 2. Cys257 and Cys261 of NS4B are palmitoylated. (A) Palmitoylation of the various Flag-tagged NS4B deletion mutants overexpressed in Huh7 cells and subjected to metabolic labeling. (B) Palmitoylation of Flag-tagged NS4B cysteine mutants analyzed as for panel A. The signal of palmitoylation was quantified by ImageQuant software. (C) To determine the fractions of NS4B that were palmitoylated, cells expressing Flag-NS4B mutants were lysed and the cell lysates were treated with PEG (5K)-maleimide, which reacts with free cysteine residues (cysteine without palmitoylation). The Flag-NS4B protein and its mutants were detected by Western blotting using anti-Flag antibody.

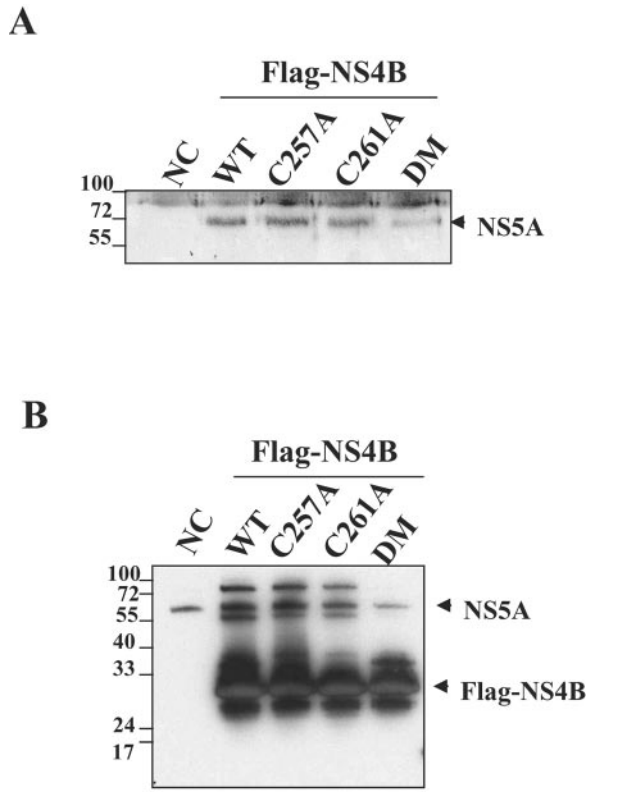


FIG. 3. Lipid modification of NS4B and protein-protein interactions. (A) The effects of palmitoylation of NS4B on the interaction between NS4B and NS5A. NS5A and Flag-tagged NS4B-cysteine mutants were expressed in Huh7 cells and immunoprecipitated with an anti-Flag antibody, and NS5A protein was detected by anti-NS5A antibody. (B) The effects of NS4B palmitoylation on the interactions of NS4B protein with other viral proteins in the HCV replicon cells. Flag-tagged NS4B mutants were expressed in replicon cells and precipitated with an anti-Flag antibody. The precipitated viral proteins were detected by an HCV patient serum. NC, negative controls (cells cotransfected with NS5A and the empty vector).

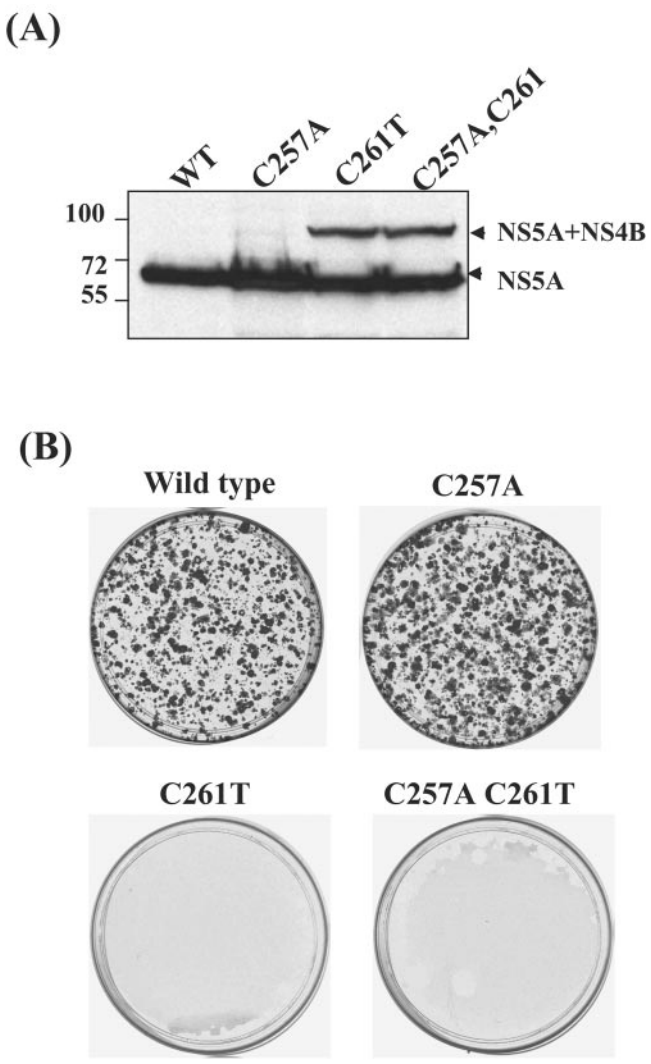


FIG. 4. NS4B mutations in HCV subgenomic replicons. (A) Cleavage efficiency of the NS4B-NS5A junction in the HCV polyprotein containing various cysteine mutations of NS4B. HCV subgenomic-replicon DNA (containing a T7 promoter) containing various NS4B mutations was transfected into Huh7 cells, and protein expression was driven by VV-T7 induction. NS5A protein in the cell lysates was detected by Western blotting using anti-NS5A antibody. (B) The viability of the cysteine mutation replicons was tested by colony formation assay. The in vitro-transcribed replicon RNAs were transfected into Huh7 cells by electroporation, and the cells were cultured under G418 selection for 4 weeks. Cell colonies were stained by crystal violet.

proteins (NS3 to NS5B) and also undergo active RNA replication. As shown in Fig. 3B, the NS4B wild type and single-cysteine mutants, but not the dual mutant, could pull down NS5A and other viral proteins. These results suggest that NS4B palmitoylation is important for protein-protein interactions.

Cys261, but not Cys257, of NS4B is indispensable for subgenomic-replicon replication. To examine the importance of NS4B lipid modification in HCV RNA replication, site-directed mutagenesis of NS4B in subgenomic replicons was performed. As in the previous design for single-protein expression, Cys257 was mutated to alanine. However, since Cys261 is the residue adjacent to the first amino acid of NS5A in the context of NS3-5B polyprotein, it is crucial for NS3 protease cleavage of the polyprotein to produce mature NS4B and NS5A (3). Other than cysteine, threonine could also be recognized by NS3 protease at this position (3). Therefore, we mutated Cys261 to threonine. We first examined the cleavage efficiency of the NS4B-NS5A junction of these replicon mutants. If the NS3 protease cleaves the NS4B-NS5A junction, mature NS5A could be detected as the 55-kDa protein by immunoblotting; otherwise, NS4B-NS5A fusion protein would be detected as an 85-kDa protein. As shown in Fig. 4A, all of the NS5A protein produced from the wild-type and C257A mutant replicons was detected as the mature form (55 kDa). In the cases of C261T and double-mutant replicons, the mature NS5A was also detected, indicating that the threonine residue at this position could be cleaved. However, the cleavage efficiency was lower, so that some intermediate (85-kDa) NS4B-NS5A was also detected.

A colony formation assay was then performed to test whether these two potential lipid modification sites are important for replication. As shown in Fig. 4B, the replicon contain-

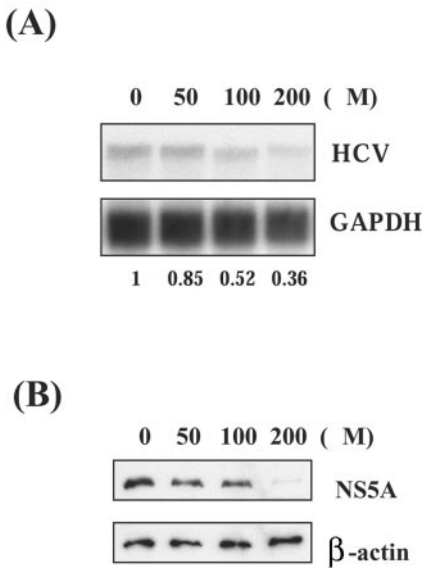


FIG. 5. The palmitoylation inhibitor 2-BP interferes with HCV RNA replication. Huh7 cells harboring the stable HCV replicon were treated with different concentrations of 2-BP for 48 h. (A) HCV RNA was detected by Northern blotting. GAPDH RNA served as an internal control. Quantification of HCV signals was normalized to that of GAPDH. (B) NS5A and β-actin in the same cells as in panel A were detected with specific antibodies.

ing the C257A mutation yielded as many colonies as the wild-type replicon. In contrast, the replicons containing the Cys261 mutation (the C261T single mutation and the C257A-C261T double mutations) did not form any colony. Since subgenomic-replicon RNA might produce adaptive mutations during RNA replication, the C257A replicon RNA was recovered from the G418-resistant colonies and examined by sequencing. The C257A mutation was still retained in the replicon of the surviving cells, suggesting that this cysteine is not crucial for RNA replication. We have confirmed this result by performing transient transfection of the HCV full-length replicon into Huh7 cells and then determining NS5A expression. The result showed that HCV NS5A protein expression was substantially reduced in the C261T and C257A-C261T double mutants (data not shown). These results are consistent with the interpretation that Cys261 is important for HCV RNA replication, whereas Cys257 is not. Since the C261T mutant could process NS4B-5A cleavage, its failure to replicate is most likely due to direct effects of the mutation on RNA replication. Nevertheless, we could not rule out the possibility that the cleavage efficiency of NS4B-5A may be too low in the C261T mutants. Alternatively, the cysteine residue at this position per se may be crucial for RNA replication.

2-Bromopalmitate inhibited HCV RNA replication. Since Cys261 is crucial for HCV RNA replication and is partially palmitoylated, we next examined whether palmitoylation is important for HCV RNA replication. We examined the effects of a specific palmitoylation inhibitor, 2-bromopalmitate (2-BP), on RNA replication in cells harboring the HCV subgenomic replicon. As shown in Fig. 5A, in the presence of the palmitoylation inhibitor, HCV RNA replication was inhibited in a dose-dependent manner. The viral protein as represented by NS5A expression was also correspondingly decreased (Fig. 5B). This result suggests an important role for palmitoylation in subgenomic RNA replication. How-

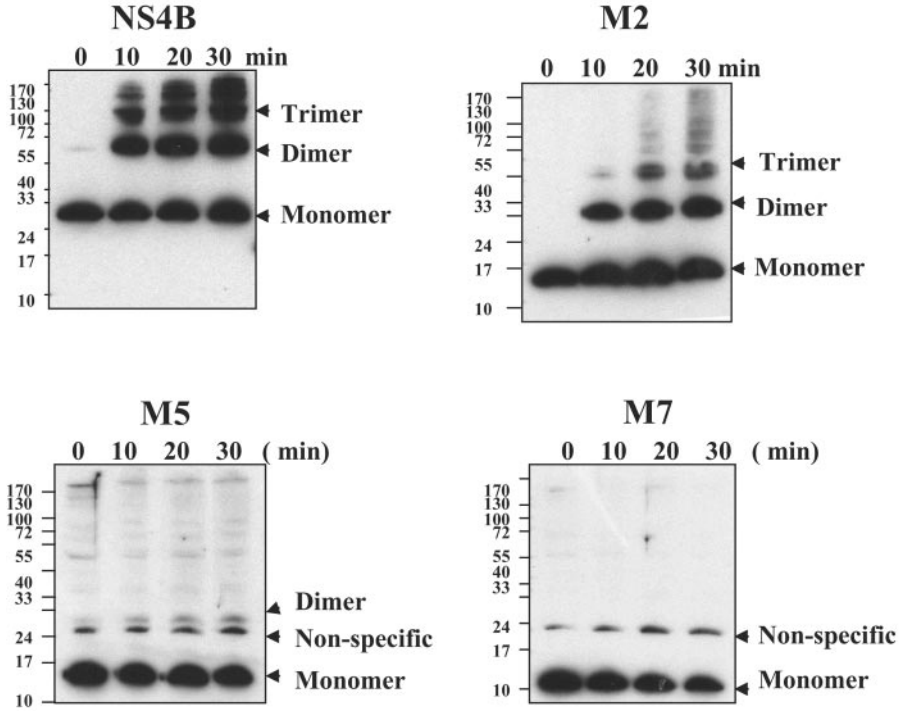


FIG. 6. Polymerization of NS4B after cross-linking. Cell lysates expressing the full-length NS4B and its deletion mutants were incubated with 0.01% glutaraldehyde for various lengths of time and analyzed by Western blotting using anti-Flag antibody.

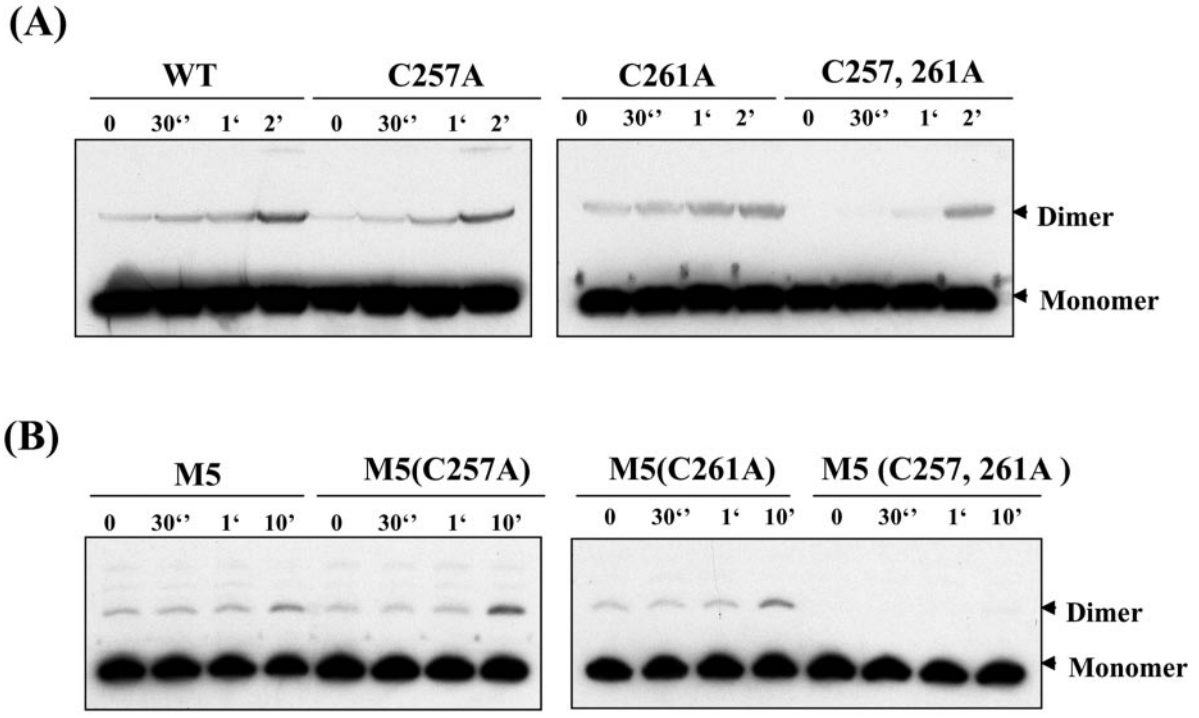


FIG. 7. NS4B cysteine residues contribute to the polymerization activity. (A) NS4B cysteine mutants were overexpressed in Huh7 cells and treated with glutaraldehyde for various periods of time. (B) The same cysteine mutants on an M5 backbone were analyzed as for panel A.

ever, the cellular GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was also slightly decreased by a high concentration (200 μM) of 2-BP; therefore, we could not rule out the possibility that the inhibitory effect of 2-BP on HCV RNA replication might partially be due to the indirect effects of 2-BP on host factors. Nevertheless, this result suggests the importance of palmitoylation in HCV RNA replication.

NS4B has polymerization activity, and the polymerization determinants are distributed on both cytoplasmic ends of NS4B. Protein polymerization is important for the function of scaffolding proteins such as caveolin-1 (7). NS4B could form dimers in nonreducing gel (Fig. 2B). Therefore, NS4B may have polymerization activity, which could contribute to the function of NS4B in membranous web formation. To test this possibility, NS4B was treated with a cross-linking reagent, glutaraldehyde (23, 33). As shown in Fig. 6, after being treated with glutaraldehyde, NS4B formed dimers, trimers, and even larger polymers. Since NS4B expression was at an extremely high level under this condition, the amount of monomer did not decrease significantly, even after extensive polymer formation. The amount of monomer was reduced when a lesser amount of the sample was used for analysis (data not shown).

To identify which structural element of NS4B was responsible for its polymerization, the various NS4B deletion mutants were treated with glutaraldehyde (Fig. 6). The M2 mutant, which contains the N-terminal half of NS4B, could form polymers as efficiently as the full-length NS4B did. The M5 mutant containing the C-terminal half of NS4B could form dimers only very weakly under the same condition. M7 could not form any dimer or other polymer. We concluded that the major determinant of NS4B polymerization resides within the 70 amino acid residues in the N terminus, while the C-terminal end of NS4B also has a weak polymerization ability.

The lipid modifications of Cys257 and Cys261 contribute to the polymerization activity of NS4B. We further examined whether lipid modifications of NS4B might affect its polymerization activities. To test this possibility, we examined the kinetics of polymerization of the various NS4B mutants after treating them with glutaraldehyde. As shown in Fig. 7A, dimer formation of the wild-type protein could be seen even at time zero, and the amount of dimer increased over time. NS4B(C257A) and NS4B(C261A) had polymerization activities similar to that of the wild type. However, NS4B(C257, 261A) did not form dimers until after treatment for 2 min. Similar mutations were created on the M5 mutant to eliminate the contributions from the N-terminal polymerization domain. M5 had a weaker polymerization activity, so that a longer incubation time with glutaraldehyde was needed for dimer formation (Fig. 7B). M5, M5(C257A), and M5(C261A) had very similar polymerization activities (Fig. 7B); in contrast, M5(C257, 261A) did not form any polymer during the 10-min incubation time. This result suggested that lipid modification contributes to the polymerization activity of NS4B.

The polymerization determinants of the NS4B N terminus are scattered. To narrow down the exact determinants of polymerization on the N-terminal end of the NS4B protein, a series of truncation mutants (M11 to M13) were constructed. As shown in Fig. 8A, M11 (residues 23 to 135 of NS4B) had comparable polymerization activity to that of M2 (residues 1 to

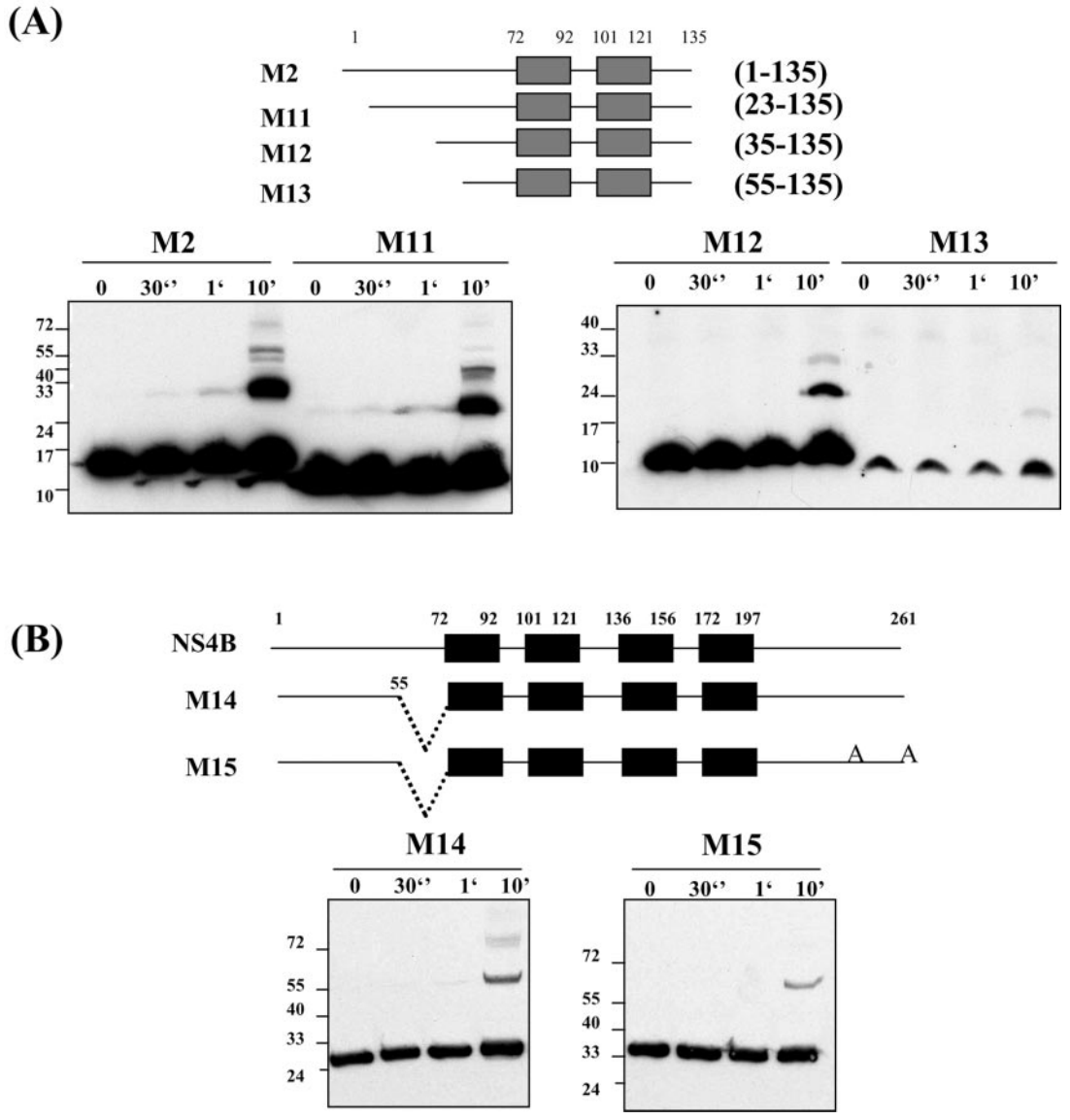


FIG. 8. Mapping of N-terminal polymerization determinants. (A) The various M2-based deletion mutants were analyzed for polymerization as for Fig. 7. (B) To determine whether the polymerization activity of NS4B resides in the 55 to 72 amino acid region, the truncation mutants M14 and M15 were tested by polymerization assay as for panel A.

135). M12 and M13 (residues 35 to 135 and 55 to 135, respectively) also retained the polymerization activity, even though the expression level of M13 was low compared to that of the other mutants (Fig. 8A). We also constructed mutants with residues 55 to 76 being deleted on both the full-length (M14) and the double-Cys mutant (M15) backgrounds. As shown in Fig. 8B, both the M14 and M15 mutants retained the polymerization activity. These results suggested that the polymerization activity of the NS4B N terminus is not limited to a small stretch but is scattered throughout this region.

DISCUSSION

In the present study, we have provided evidence that NS4B has lipid modification on two cysteine residues (cysteines 257 and 261) located at the C-terminal end. The lipid modifications on these two cysteines are important for NS4B protein-protein interaction in the formation of the HCV RNA replication complex. The polymerization activity was also found in the NS4B protein, and the main polymerization determinants are scattered in the N-terminal cytoplasmic region of NS4B protein. Moreover, lipid modification of the C-terminal cysteines also facilitates the polymerization process. Both of these properties contribute to the role of NS4B in forming the HCV RNA replication complex, since formation of the replication complex requires not only protein-membrane interaction but also interactions between viral and cellular proteins (14). Site-specific mutagenesis of the last cysteine residue (Cys261) dramatically affected HCV replication, although the inhibitory effects may have come from both direct and indirect effects of the

lipid modification. Cys257 by itself is not crucial for HCV replication; however, when combined with Cys261, Cys257 has an additive effect on HCV replication and protein-protein interactions. Furthermore, Cys257 palmitoylation may play a role in HCV pathogenesis.

Note that we have not been able to directly demonstrate the presence of NS4B palmitoylation and polymerization in the HCV replicon or the complete viral replication systems. For studying NS4B palmitoylation in cells containing an HCV replicon, the main problem we encountered was that only a small amount of NS4B could be immunoprecipitated, probably due to the hydrophobic property of NS4B, the lower expression level of NS4B, or the relative inaccessibility of NS4B in the context of the replicon system. Similarly, we also had difficulties in demonstrating NS4B polymerization in replicon cells, probably because the sensitivity of the NS4B-specific antibody was too low for NS4B detection by Western blotting. We could detect NS4B by using HCV patient serum, but after the cellular lysates were cross-linked with glutaraldehyde, a smear of high-molecular-weight proteins, instead of discrete dimers, trimers, or polymers of NS4B, was seen in the gel. It is possible that, other than homotypic interactions, NS4B also interacts with other viral or cellular proteins. Therefore, whether palmitoylation and polymerization of NS4B occur in the context of HCV polyprotein remains unresolved.

The cytoplasmic domain at the N terminus of NS4B has potent polymerization activity; thus, full-length NS4B with the double-cysteine mutation could polymerize after cross-linking with glutaraldehyde (Fig. 7A). However, in Fig. 2B, the [^{35}S]methionine-labeled cysteine double mutant could not form dimers. In the latter experiment, the immunoprecipitated proteins were analyzed by tricine gel containing 10% glycerol and SDS, and the reducing agent was removed from the Laemmli sample buffer. It is possible that the dimerization activity, particularly the weak one, was retarded under this condition.

Most plus-strand RNA viruses induce distinct membrane alterations, which provide the necessary structural scaffold for RNA replication (1). Membrane alterations have been seen in cells containing HCV subgenomic replicons and in HCV-infected liver tissue (11, 28). Furthermore, the HCV RNA replication complex is located on the detergent-resistant membranes, namely, lipid rafts (30). Almost any subdomain of NS4B, when expressed alone, was located on the detergent-resistant membrane (data not shown), suggesting that the hydrophobic properties of the peptides rather than the primary sequences of NS4B are responsible for its membrane association. However, our data reported here have shown that lipid modifications of NS4B affect protein-protein interactions, thus affecting the formation of the RNA replication complex, even though these modifications do not affect membrane association for NS4B. These results further suggest that both membrane association and protein-protein interactions are important for the formation of the RNA complex (2). The failure of the NS4B cysteine mutants to form the proper replication complex was most likely due to the failure of protein-protein interactions, not the failure of association of NS4B with detergent-resistant membranes. Similar to caveolin-1, NS4B possesses a scaffolding function in the induction of specialized membrane structures (7). Therefore, the role of caveolin-1 in caveolae formation could be a useful model to decipher how NS4B induces membranous web.

The importance of lipid biosynthesis and metabolism in HCV replication is increasingly being recognized. Previously, cholesterol and sphingomyelin biosynthesis have been shown to be required for HCV replication (2, 16, 34). Inhibitors of geranygeranylation inhibited HCV replication (16, 34). Our studies here further showed that inhibitors of palmitoylation also inhibited HCV replication. All these lipid biosyntheses may affect viral RNA replication directly or indirectly.

Among the different HCV genotypes, Cys257 is conserved in genotypes 1, 2, and 4. In genotypes 3, 5, and 6, the corresponding amino acid residue is threonine or tyrosine. Palmitoylation can occur on serine and/or threonine residues to form an acyloxyester, but in most cases, palmitate is linked to a cysteinyl residue as an acylthioester. It would be interesting to determine whether the palmitoylation happens when the amino acid residues are tyrosine or threonine. Moreover, the biological function of palmitoylation in the virus life cycle and in pathogenesis remains to be studied. NS4B has a GTPase activity (12) and induces membranous web (11); whether lipid modifications and polymerization activity of NS4B are crucial for these activities is an interesting question to address.

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