distinct from the classical lipid rafts (20). However, the biological function of the HCV core protein localized in the DRM has not been clarified.

The HCV core protein is cleaved from a precursor polyprotein by a signal peptidase (SP) to liberate it from the envelope protein E1 and is then further processed by a signal peptide peptidase (SPP) (21). However, the biological significance of the intramembrane processing of the HCV core protein by SPP remains largely unknown. Furthermore, the C-terminal end of the mature HCV core protein expressed in insect cells has been reported to be Phe^{177} or Leu^{179} (12, 29), while that in mammalian cells has not been determined. Expression of SPP enhanced the accumulation of nonenveloped nucleocapsid and reduced that of enveloped nucleocapsid in yeast cells, suggesting that maturation of core protein is carried out after the formation of enveloped particles (17). However, the effect of SPP cleavage on viral assembly in mammalian cells has not been well characterized. Randall et al. have reported that introduction of a small interfering RNA targeted to SPP reduced the production of infectious HCV particles (36), suggesting that SPP is required for the production of HCV particles. In this study, we determined the cleavage site of the mature HCV core protein expressed in human cells and examined the biological significance of the intramembrane processing of the core protein by SPP for the localization of the core protein in the DRM and the production of infectious particles.

MATERIALS AND METHODS

Cell lines and HCV infection. HCV subgenomic RNA was removed from the replicon cell line 9-13 (16) by treatment with alpha interferon. A cell line that was highly permissive for JFH-1 infection was cloned from the resulting crude populations by the limited-dilution method and designated Huh7OK1 (32). The Huh7OK1 cell line retained the ability to produce type I interferons through the RIG-I-dependent signaling pathway upon infection with RNA viruses and exhibited a cell surface expression level of human CD81 comparable to that of the parental cell line. The detailed characteristics of this cell line will be described in a future communication. The HuhOK1 and Huh7.5.1 cell lines (the latter was kindly provided by F. Chisari) and the human embryonic kidney cell line 293T were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and nonessential amino acids (Sigma, St. Louis, MO). Huh7OK1 or Huh7.5.1 cells were infected with HCV strain JFH-1 as described by Wakita et al. (45). The plasmid carrying strain JFH-1 cDNA under the control of the pol1 promoter (19) was transfected into Huh7OK1 or Huh7.5.1 cells, and propagation of the JFH-1 virus was determined by the production of HCV core protein (as described below) and by the titration of infectious particles (39). The persistently infected Huh7OK1 cells were maintained under normal conditions after 8 passages before use. The 9-13 cell line, which possesses an HCV subgenomic replicon (16), was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1 mg/ml G418.

Plasmids. Genes encoding the N-terminally FLAG-tagged and/or C-terminally hemagglutinin (HA)-tagged core proteins derived from the HCV genotype 1b strain J1 or its mutants were introduced into plasmid vector peDNA3.1 (Invitrogen, Carlsbad, CA) as described previously (30). Each insert gene was transferred into a pCAGGS vector (28) at the PmeI site. The resulting plasmids encoded the HCV core protein (amino acid residues 1 to 191) with or without FLAG and HA tags at the N and C termini, respectively. All of the core proteins with these tags (FLAG-core-HA proteins) had a mutation of Ala^{191} to Arg in order to prevent cleavage by the SP (7). Plasmid pHH21/JFH-1, carrying a full genomic cDNA of strain JFH-1 under the control of the pol1 promoter, was used to produce the infectious JFH-1 virus (19). An adaptive mutation of Leu to Val at amino acid position 758 in the p7 region was introduced during a long-term passage of the JFH-1 virus into Huh7.5.1 cells (data not shown). To improve the replication efficiency of the JFH-1 virus, a mutation of Leu to Val was introduced into pHH21/JFH-1 by site-directed mutagenesis, and the resulting plasmid was designated pHH21/JFH-1/L758V. To generate plasmids encoding the mutant JFH-1 viruses, the following substitutions were introduced into pHH21/JFH-1/L758V: Val^{139}, Val^{140}, and Leu^{141} were replaced with Ala (JFH-1/VVL/3A); $Ile^{156}$and Phe^{157} were replaced with Ala and Leu, respectively (JFH-1/IF/AL); Ala^{190}, Ser^{185}, and Cys^{184} were replaced with Val, Leu, and Val, respectively (JFH-1/ASC/VLV); and Asp^{274} was replaced with Asn (JFH-1/GND).

Antibodies and reagents. Antisera against HCV genotype 1 or 2a core proteins were raised in rabbits by immunization with peptides corresponding to the region spanning residues 103 to 115, conserved among genotypes 1a and 1b, or to the region from residue 101 to 119 of genotype 2a (strain JFH-1). These peptides were synthesized and conjugated with keyhole limpet hemocyanin (Scrum Inc., Tokyo, Japan). Antisera were purified with an affinity column conjugated with the antigenic peptides. A monoclonal antibody to HCV NS5A (5A27) was prepared from BALB/c mice (CLEA Japan, Tokyo, Japan) immunized with the recombinant domain I of NS5A by a method described previously (31). Antibodies to caveolin-1, calreticulin, and the FLAG tag (M2) were purchased from Sigma. Antibodies to the HA tag and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Babco (Richmond, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The aspartic protease inhibitors $(Z\text{-}LL)_2$ ketone and L685,458 were purchased from the Peptide Institute (Osaka, Japan). These inhibitors were dissolved in dimethyl sulfoxide and stored at −20°C until use.

Transfection, SDS-PAGE, and Western blotting. Huh7.5.1 and 293T cells were transfected with plasmids by lipofection with *Trans* IT LT-1 (Mirus, Madison, WI) and Lipofectamine 2000 (Invitrogen), respectively, according to the manufacturers' protocols. Cells were lysed on ice in Triton lysis buffer (20 mM Tris-HCl [pH 7.4], 135 mM NaCl, 1% Triton-X 100, 10% glycerol) supplemented with a protease inhibitor mix (Nacalai Tesque, Kyoto, Japan) at 24 or 48 h after transfection and were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Tris-glycine buffer and Western blotting using appropriate antibodies as previously described (30). The stained protein bands were visualized using the SuperSignal West Femto enhanced-chemiluminescence substrate (Pierce, Rockford, IL) and an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan).

Determination of the expression of the C terminus of the mature HCV core protein in mammalian cells. Two million 293T cells cultured in a collagen-coated dish (diameter, 10 cm) were transfected with pCAGGS-FLAG-core (26) by lipofection, harvested at 20 h posttransfection with a rubber policeman after two washes with ice-cold phosphate-buffered saline (PBS), and collected by centrifugation at 1,000 × *g* for 5 min. The cells were lysed with 0.1 ml of triple-detergent lysis buffer (45 mM Tris-HCl [pH 7.4] containing 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 135 mM NaCl, and a protease inhibitor mix [Nacalai Tesque]) (24). The lysate was stored at −80°C until use. The lysate was thawed on ice and then centrifuged at 20,000 × *g* for 10 min at 4°C. The supernatant was mixed with 20 μl of 50% (vol/vol) anti-FLAG M2 affinity gel (Sigma) and then rotated at 4°C for 90 min. The gel beads were washed with the triple-detergent lysis buffer and then suspended in 30 μl of the loading buffer. The suspended gel beads were boiled for 5 min and then centrifuged at 20,000 × *g* for 5 min at room temperature. The resulting supernatant was subjected to SDS-PAGE, and the gel was stained with Sypro Ruby dye (Invitrogen). The portion of the gel including proteins with an expected molecular size of 20 kDa was excised from the stained gel, washed twice with 200 μl of 50 mM NH_4HCO_3 dissolved in 50% acetonitrile (vol/vol), and then immersed in 100 μl of 100% acetonitrile for dehydration. The dehydrated gel was incubated in 10 mM dithiothreitol and 100 mM NH_4HCO_3 at 56°C for 1 h. To prevent the digestion of Cys residues at the C termini by endoproteinase Asp-N, alkylation of the gels was carried out in 55 mM iodoacetamide and 100 mM NH_4HCO_3 at 25°C for 45 min in the dark. Finally, gel pieces were washed twice with 100 mM ammonium carbonate dissolved in acetonitrile and were dried completely before digestion. An immersed volume of endoproteinase Asp-N solution (10 μg/ml Asp-N and 50 mM NH_4HCO_3) was added to the dried gel and incubated at 37°C overnight, and the supernatant (the digested solution) after centrifugation was transferred to a new centrifuge tube. The precipitated gels were washed first with 20 μl of 20 mM NH_4HCO_3 and then with 20 μl of 50% (vol/vol) acetonitrile in 5% (vol/vol) formic acid, and the washed solutions were mixed with the digested solution and dried completely under a vacuum. The digested mixtures were applied to a ZipTip C_{18} column (Millipore, Tokyo, Japan). After a wash with 0.1% (vol/vol) trifluoroacetic acid, the peptides were eluted with 1 μl of 0.1% (vol/vol) trifluoroacetic acid dissolved in 75% (vol/vol) acetonitrile. Samples with 10 mg of 2,5-dihydroxybenzoic acid per ml of 33% acetonitrile matrix were analyzed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) using a MALDI-quadrupole-TOF tandem MS (MS-MS) QStar Pulsar *i* system (Applied Biosystems, Foster City, CA) in the linear positive-ion mode following the method of Hitachi Science Systems (Ibaraki, Japan).

Flotation assay. The flotation assay was carried out according to the method of Lecat et al. (14). Briefly, 10 million transfected or infected cells were washed with ice-cold PBS and then harvested with a rubber policeman. Collected cells were suspended in 0.6 ml of TNE buffer (25 mM Tris-HCl [pH 7.4] containing 150 mM NaCl, a protease inhibitor mix [Nacalai Tesque], and 5 mM EDTA) and then homogenated with a Dounce homogenizer or suspended with a 24-gauge needle. Each homogenate was incubated for 30 min on ice with or without 1% Triton X-100. The lysates were mixed with 0.4 ml of Optiprep (Sigma) to a final concentration of 40%. This mixture was overlaid with 1.2 ml of 30%, 1.2 ml of 25%, and 0.8 ml of 5% Optiprep and was then centrifuged at 42,000 rpm and 4°C for 5 h in an SW50 rotor (Beckman Coulter, Fullerton, CA). Each fraction was collected as 0.4 ml from the top of the centrifuging tube and was then precipitated with 4 volumes of cold acetone. The pellets were resolved in the loading buffer, boiled, and then subjected to SDS-PAGE and Western blotting. The fractions containing calreticulin, which is resident in the ER, in the absence and presence of the detergent were defined as the membrane and detergent-soluble fractions, respectively. In the presence of the detergent, the fractions with caveolin-1 were defined as the detergent-resistant fractions.

Quantitative real-time PCR. Total RNA was prepared from Huh7OK1 cells persistently infected with the JFH-1 virus or 9-13 cells by using an RNeasy minikit (Qiagen, Tokyo, Japan). The HCV genomic RNA was reverse transcribed and amplified by using a TaqMan EZ RT-PCR reagent kit (Applied Biosystems) with sense (5'-GAG TGT CGT GCA GCC TCC A-3') and antisense (5'-CAC TCG CAA GCA CCC TAT CA-3') primers corresponding to nucleotides 98 to 116 and 294 to 313, respectively. The kinetics of cDNA amplification were monitored by an ABI Prism 7000 sequence detection system (Applied Biosystems) using a reporter probe corresponding to nucleotides 238 to 267 of the 5'-conserved region for the HCV genotypes (5'-GCC CGC AAG ACT GCT AGC CGA GTA GTG TTG G-3') conjugated with 6-carboxyfluorescein and 6-carboxytetramethylrhodamine at the 5' and 3' termini, respectively. A serial dilution of the partial HCV RNA synthesized by in vitro transcription from plasmids encoding the 5'-terminal region of HCV cDNA under the control of a T7 promoter was used as the standard for HCV genomic RNA. Intracellular GAPDH mRNA was also amplified using the TaqMan Pre-Developed Assay Reagent human GAPDH (Applied Biosystems). The values for HCV genomic RNA were normalized to those for GAPDH mRNA.

Quantitative detection of HCV core protein by ELISA. HCV core protein was quantified by using an Ortho HCV antigen enzyme-linked immunosorbent assay (ELISA) (Ortho Clinical Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. Huh7.5.1 cells were transfected with pHH21/JFH-1/L758V or its mutants by lipofection. Cells and culture supernatants were harvested at 2, 4, 6, or 8 days after transfection. To determine the amounts of the intracellular core protein, cells were lysed with Triton lysis buffer on ice and subjected to the ELISA after 100- to 10,000-fold dilutions with PBS. Total protein levels were determined with a Micro BCA protein assay reagent kit (Pierce). Amounts of intracellular and extracellular core protein were normalized to total-protein amounts.

Immunofluorescent assay. Transfected Huh7.5.1 cells were fixed with a cold acetone-and-methanol mixture (50:50, vol/vol). After being blocked with 1% normal goat serum, cells were incubated with a mouse monoclonal antibody to NS5A at 4°C for 16 h, washed three times with PBS containing 0.5% Tween 20, and then incubated with an Alexa Fluor 594-conjugated antibody to mouse immunoglobulin G (Invitrogen). Cell nuclei were stained with Hoechst dye. The stained cells were washed three times with PBS containing 0.5% Tween 20 and then observed with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

RESULTS

Mutation in the HCV core protein confers resistance to SPP cleavage. Amino acid residues Ala^{180}, Ser^{183}, and Cys^{184} of the HCV core protein have been shown by others to be essential for intramembrane processing by SPP (10, 21), although our data suggested that Ile^{176} and Phe^{177}, but not Ala^{180}, Ser^{183}, and Cys^{184}, were required for the processing of the HCV core protein by SPP (30). To clarify this discrepancy, we constructed an N-terminally FLAG-tagged and C-terminally HA-tagged wild-type HCV core protein and similarly tagged mutant core proteins in which Ala^{180}, Ser^{183}, and Cys^{184} were replaced with Val, Leu, and Val, respectively (referred to below as Core ASC/VLV, or M1) (21), or Ile^{176} and Phe^{177} were replaced with Ala and Leu, respectively (referred to below as Core IF/AL, or M2) (30). We then expressed these core proteins in 293T cells (Fig. 1). Ala^{191} was replaced with Arg in these FLAG-core-HA constructs to prevent cleavage by SP (7), and only the SPP-resistant core protein was detected by an anti-HA

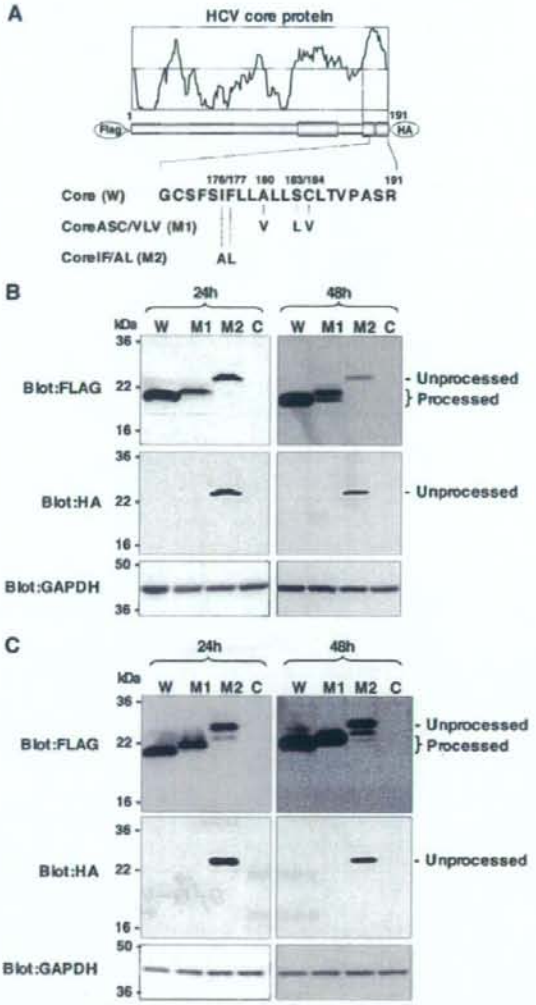
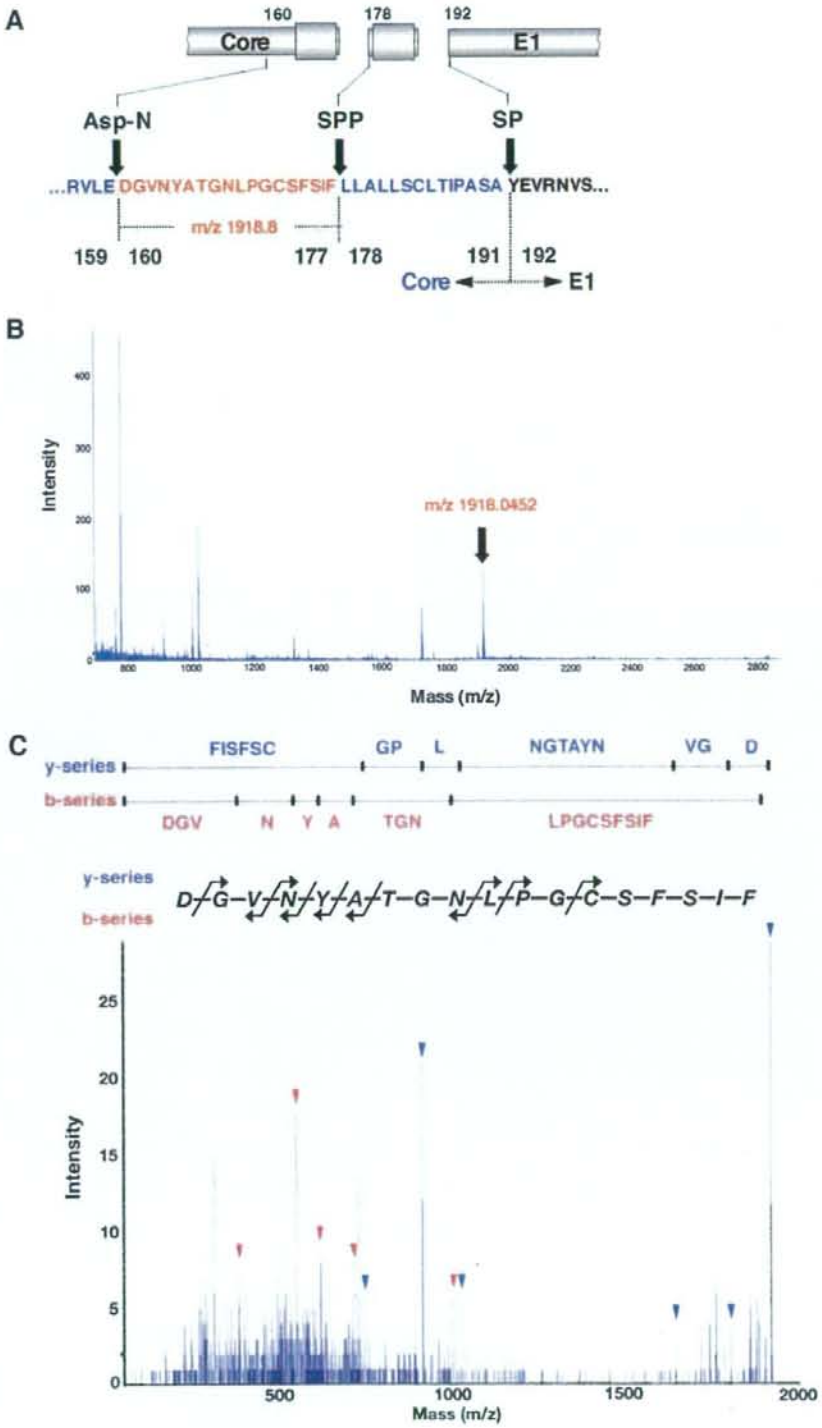


FIG. 1. Effects of mutations in the HCV core protein on cleavage by SPP. (A) cDNA constructs encoding the N-terminally FLAG- and C-terminally HA-tagged wild-type HCV core protein (W), Core ASC/VLV (M1), and Core IF/AL (M2). The Ala at amino acid residue 191 of all constructs was mutated to Arg in order to prevent the processing of an HA tag by SP. (B) Each of the core constructs or an empty vector (lane C) was transfected into 293T cells. Cell lysates harvested at 24 or 48 h posttransfection were subjected to Western blotting using antibodies against the indicated proteins. (C) Cells transfected with each of the core constructs or an empty vector were treated with 15 μM MG132 for 5 h and examined as described for panel B.



antibody in this experimental setting. Core IF/AL was detected in cells by both anti-FLAG and anti-HA antibodies at 24 h and 48 h posttransfection, whereas the wild-type core and Core ASC/VLV were detected by an anti-FLAG antibody but not by an anti-HA antibody (Fig. 1B). These results indicate that Core IF/AL is resistant to SPP cleavage, in contrast to the complete processing of the wild-type core and Core ASC/VLV. Although Core ASC/VLV exhibited a single band that was slightly larger than the wild-type core protein at 24 h posttransfection, an extra band with the same mobility as the wild-type core protein appeared at 48 h posttransfection (Fig. 1B), suggesting that the introduction of mutations in Ala^{180}, Ser^{183}, and Cys^{184} induces multiple processing in the signal sequence of the mutant core protein. To exclude the possibility that unprocessed Core ASC/VLV is degraded by a proteasome due to misfolding, each of the core constructs or the empty vector was transfected into 293T cells and treated with a proteasome inhibitor for 5 h. The unprocessed band of Core IF/AL, but not that of Core ASC/VLV, was detected by the anti-HA antibody (Fig. 1C). These results further support the notion that Core ASC/VLV is sensitive to SPP-dependent processing. Bands observed between unprocessed and processed proteins in cells expressing wild-type core or Core IF/AL in the presence of a proteasome inhibitor were not detected by the anti-HA antibody, suggesting that these products are generated by C-terminal truncation and are sensitive to proteasome degradation.

Identification of the C-terminal residue of the mature HCV core protein. Previous reports have suggested that the C terminus of the mature HCV core protein expressed in insect cells by using a baculovirus expression system is Phe^{177} (29) or Leu^{179} (12). To clarify the C-terminal amino acid residue of the mature HCV core protein expressed in human cells, a purified fragment of the HCV core protein was analyzed by MALDI-TOF MS. The FLAG-tagged HCV core protein was expressed under the control of a CAG promoter in 293T cells, purified by immunoprecipitation with beads conjugated with the anti-FLAG antibody, and then released from the beads by the addition of free FLAG peptide. The purified FLAG-tagged core protein was digested with Asp-N protease, and the final sample was subjected to MALDI-TOF MS for determination of the C-terminal residue. The N-terminal amino acid of the peptide fragment including the C terminus of the mature HCV core protein was expected to be Asp^{160} (Fig. 2A). The peptide fragment with an *m/z* of 1,918.0452, which is close to the calculated value (*m/z* 1,918.8) of the sequence DGVNYATG NLPGCSFSIF (Fig. 2A), was detected, and no larger peak was evident (Fig. 2B). MS-MS analysis showed that the fragment has the amino acid sequence DGVNYATGNLPGCSFSIF (Fig. 2C). These results indicate that the C terminus of the mature HCV core protein expressed in human cells is Phe^{177}. This is consistent with our previous observation (30) and with the data shown in Fig. 1, which indicate that the M2 mutation completely abrogated the processing of core protein by SPP. Both Ile^{176} and Phe^{177} may play crucial roles in recognition by SPP for intramembrane cleaving activity.

SPP processing is required for the localization of HCV core protein in the DRM. Based on confocal microscopy observations, Matto et al. reported that the HCV core protein associates with a DRM that is distinct from the classical raft fraction, as evidenced by the lack of colocalization of typical raft markers, including caveolin-1 and the B subunit of the cholera toxin, which binds to glycosphingolipid GM1 in the plasma membrane (20). We have previously suggested that intramembrane processing by SPP affects the intracellular localization of the HCV core protein, and the replacement of Leu^{139}, Val^{140}, and Leu^{144} with Ala in the HCV core protein (Core LVL/3A [M3]) (Fig. 3A) abrogated SPP-mediated processing and ER retention (30). In this study, we examined the effect of SPP cleavage on the DRM localization of the HCV core protein. The wild-type or mutant HCV core protein was expressed in 293T cells, solubilized at 4°C in the presence or absence of 1% Triton X-100, and subjected to sucrose gradient centrifugation. Fractions were collected after ultracentrifugation and analyzed by immunoblotting. The wild-type core protein was partially detected in fraction 3, which corresponded to the DRM fraction, and was mainly detected in the detergent-soluble fraction (Fig. 3B). However, the mutant core proteins Core LVL/3A (M3) and Core IF/AL (M2) were localized in the membrane fraction but not in the DRM fraction (Fig. 3B). Although the M2 mutant exhibits clear resistance to SPP-dependent cleavage, as shown in Fig. 1B, processed core proteins of M2 and M3 mutants were detected by flotation analyses (Fig. 3B), suggesting that the M2 and M3 mutants are cleaved by unknown mechanisms during the concentration step. These results suggest that processing by SPP is required for the DRM localization of the HCV core protein.

A dominant-negative SPP mutant inhibits the intramembrane processing and DRM localization of the HCV core protein. SPP belongs to the family of aspartic proteases, which share two Asp residues for the active sites of protease activity. Asp^{219} and Asp^{264} have been identified as active sites for the protease activity of SPP (48). Overexpression of the SPP mutant in which Asp^{219} was replaced with Ala (SPPD219A) resulted in a dominant-negative activity that prevented the intramembrane processing of the HCV core protein (30). To examine the relationship between intramembrane processing by SPP and the localization of the HCV core protein in the DRM fraction, a C-terminally HA-tagged wild-type (SPP-HA)

FIG. 2. Determination of the C termini of the mature HCV core protein. (A) Schematic representation of the junction between the core and E1 proteins. The cleavage sites for the exogenous Asp-N protease and the host SP were the N-terminal residue Asp^{160} and the C-terminal residue Ala^{191}, respectively. The cleavage site of the host SPP was determined to be the C-terminal residue Phe^{177} in this study. The expected *m/z* of the peptide fragment (spanning residues 160 to 177) processed by the Asp-N protease and SPP is indicated. (B) The FLAG-core protein was purified with an anti-FLAG antibody, digested with Asp-N, and analyzed on a 2,5-dihydroxybenzoic acid matrix by MALDI-TOF MS in the linear positive-ion mode. The peak at *m/z* 1,918.0452 corresponded to the expected fragment (*m/z* 1,918.8) derived from the Asp-N- and SPP-digested core protein, DGVNYATGNLPGCSFSIF. (C) The peak at *m/z* 1,918.0452 was subjected to MS-MS analysis with a MALDI-Qq-TOF MS-MS QStar Pulsar *i* system. The resulting spectrum was applied to MASCOT to determine the amino acid sequence. The analyzed peak at *m/z* 1,918.0452 corresponded to the sequence DGVNYATGNLPGCSFSIF.

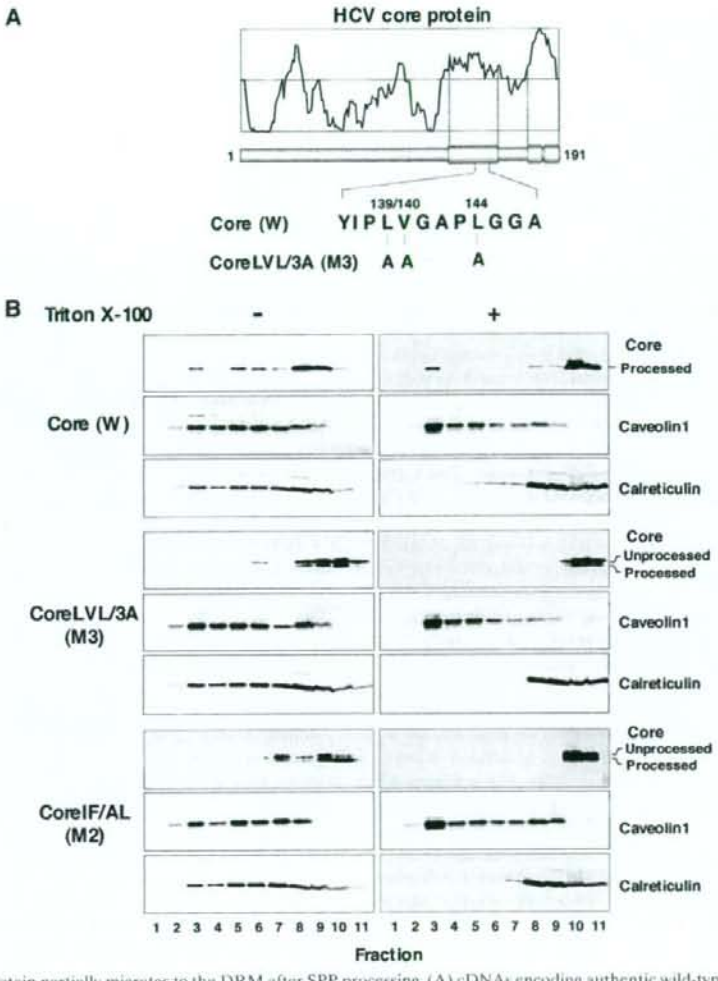


FIG. 3. HCV core protein partially migrates to the DRM after SPP processing. (A) cDNAs encoding authentic wild-type (W) and Core LVL/3A (M3) HCV core proteins. (B) Each plasmid was transfected into 293T cells, lysed with or without 1% Triton X-100, and then subjected to a flotation assay. Proteins in each fraction were concentrated with cold acetone and then subjected to Western blotting using antibodies against core protein, caveolin-1, and calreticulin. Membrane (left panels, lanes 1 to 9), DRM (right panels, lanes 1 to 7), and detergent-soluble (right panels, lanes 8 to 11) fractions were identified based on the localization of the marker proteins.

or D219A mutant (SPPD219A-HA) SPP was coexpressed with FLAG-core-HA in 293T cells (Fig. 4A). Overexpression of SPP-HA showed no effect on the localization of the HCV core protein, and the processed HCV core protein was partially localized in the DRM fraction (Fig. 4B, left). In contrast, overexpression of SPPD219A-HA inhibited the processing of the HCV core protein by endogenous SPP, and the level of unprocessed core protein, which was detected in the detergent-soluble fraction but not in the DRM fraction, was increased, whereas part of the processed core protein was localized in the DRM fraction (Fig. 4B, right). These results suggest that SPP cleavage is a prerequisite for the localization of HCV core protein in the DRM fraction. We have previously shown that the HCV core protein is degraded through proteasome pathways (26, 39, 43). To rule out the possibility of proteasome-dependent degradation of the unprocessed HCV core protein in the DRM fraction, we examined the effect of the proteasome inhibitor MG132 on the localization of HCV core protein. The processed HCV core protein, but not the unprocessed core protein, was partially localized in the DRM fraction, irrespective of treatment with MG132 (Fig. 4C). These results indicate that the failure of the unprocessed HCV core protein to localize in the DRM fraction was not due to selective degradation of the unprocessed core protein by proteasomes.

An SPP inhibitor prevents the processing of HCV core protein and its localization in the DRM. To further assess the role

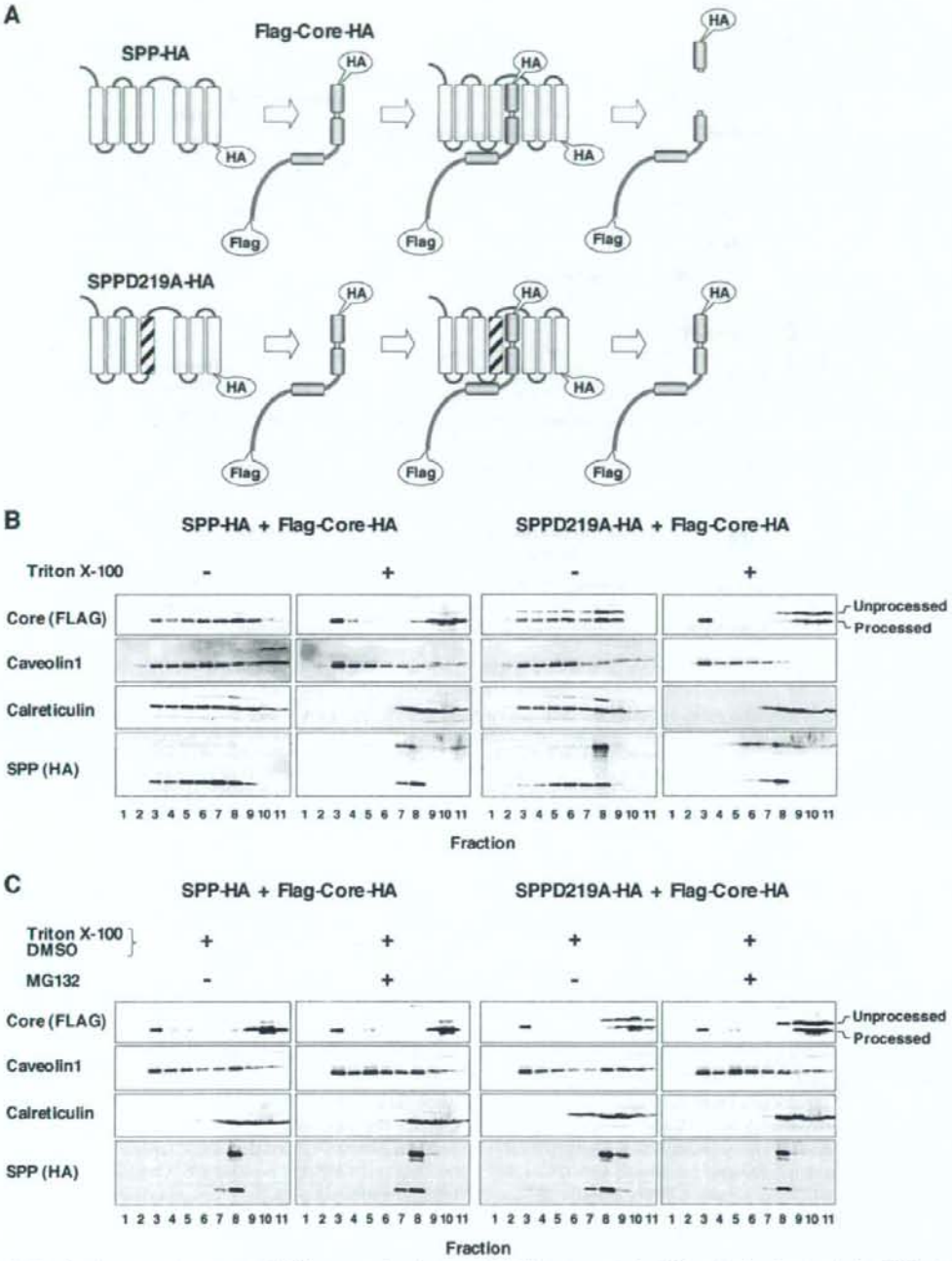


FIG. 4. The dominant-negative mutant of SPP prevents the cleavage of HCV core protein by SPP and its localization in the DRM. (A) Schematic representation of the processing of FLAG-core-HA by a wild-type SPP (SPP-HA) (top) and the dominant-negative effect of SPPD219A-HA (bottom). (B) FLAG-core-HA was coexpressed with SPP-HA or SPPD219A-HA in 293T cells, lysed in the presence or absence of detergent, and subjected to a flotation assay. (C) Effect of a proteasome inhibitor, MG132, on the DRM localization of the HCV core protein. Proteins in each fraction were concentrated with acetone and analyzed by immunoblotting using antibodies against the FLAG epitope tag, caveolin-1, calreticulin, and the HA epitope tag. The membrane (lanes 1 to 9 in the absence of Triton X-100), DRM (lanes 1 to 7 in the presence of Triton X-100), and detergent-soluble (lanes 8 to 11 in the presence of Triton X-100) fractions were identified based on the localization of the marker proteins.

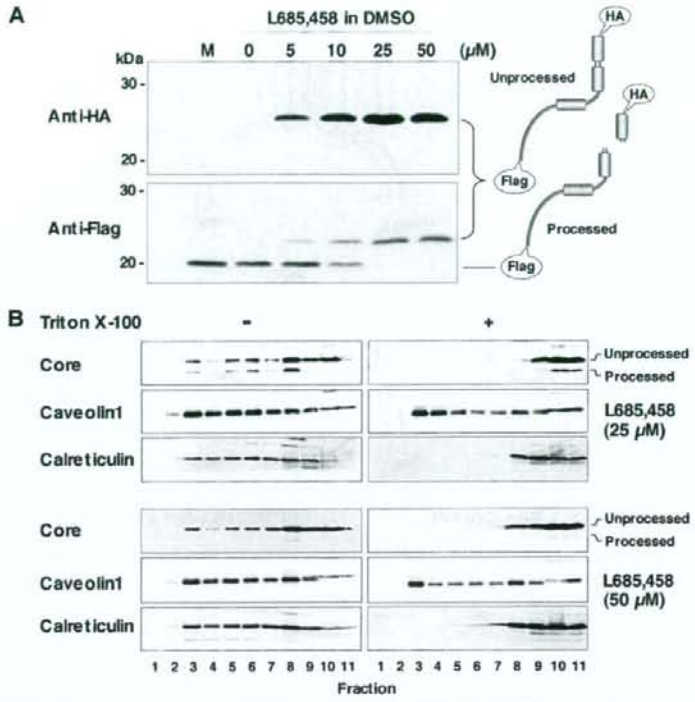


FIG. 5. Effect of an SPP inhibitor on the cleavage of HCV core protein by SPP. The HCV core protein was expressed in 293T cells, and L685,458 was added to the culture supernatant, at the indicated concentrations, at 5 h posttransfection. Cells harvested at 29 h posttransfection were lysed with 1% Triton X-100 and subjected to Western blotting (A) or a flotation assay (B). DMSO, dimethyl sulfoxide. In the flotation assay, proteins in each fraction were concentrated with acetone and analyzed by immunoblotting using antibodies against core protein, caveolin-1, and calreticulin. The membrane (left panels, lanes 1 to 9), DRM (right panels, lanes 1 to 7), and detergent-soluble (right panels, lanes 8 to 11) fractions were identified based on the localization of the marker proteins.

of intramembrane processing by SPP on the localization of HCV core protein in the DRM, we examined the effect of the SPP inhibitors $(Z\text{-}LL)_2$ ketone and L685,458 on the processing of the HCV core protein. Although $(Z\text{-}LL)_2$ ketone was insoluble at a concentration of 10 μM and was highly toxic to 293T, Huh7, and Huh7-derived cell lines (data not shown), L685,458 was capable of penetrating the plasma membrane (49) and showed no visible cytotoxicity to the cell lines examined. Treatment with L685,458 inhibited the cleavage of the HCV core protein by SPP in a dose-dependent manner (Fig. 5A). As determined by flotation analyses of 293T cells expressing HCV core protein, the processed core protein was no longer localized in the DRM fraction following treatment with 25 or 50 μM L685,458 (Fig. 5B). These results further support the notion that intramembrane processing by SPP is required for the localization of HCV core protein in the DRM.

Processing of the HCV core protein by SPP participates in viral propagation. To examine the effect of the processing of the HCV core protein by SPP on the propagation of strain JFH-1, Huh7OK1 cells persistently infected with the JFH-1 virus were treated with 25 μM L685,458, and the cells were examined for processing of the HCV core protein and replication of viral RNA. The processed core protein of strain JFH-1 was clearly detected in the DRM fraction in untreated control cells, whereas processing of the core protein was impaired by treatment with L685,458, corresponding to the decrease in the level of processed core protein in the DRM (Fig. 6A). In Huh7OK1 cells infected with strain JFH-1, intracellular viral RNA levels were reduced 30% by treatment with L685,458 at 2 days posttreatment but showed no reduction at 1 day (Fig. 6B, left), and viral RNA levels in the culture supernatant were reduced 60% to 70% by treatment with the compound at 1 and 2 days posttreatment (Fig. 6B, center). To exclude the possibility of deleterious effects of L685,458 on cellular proteins involved in viral replication, we determined the effect of L685,458 on viral RNA replication by using HCV subgenomic-replicon cells. The replication of the RNA lacking the region coding for structural proteins showed a slight enhancement rather than suppression at 1 and 2 days after treatment with L685,485 (Fig. 6B, right), suggesting that the SPP inhibitor treatment used in this study is not toxic to the cellular proteins involved in HCV RNA replication. The slight decrease in the level of intracellular HCV RNA in infected cells after treatment with L685,458 (Fig. 6B, left), but not in replicon cells, may be attributable to the ER stress induced by the accumulation of unprocessed core proteins in infected cells.

Although no effect of the inhibitor treatment on the expression of the intracellular core was observed, the secretion of core protein was slightly reduced (Fig. 6C). Furthermore, the production of infectious viral particles in the culture supernatants was clearly impaired by treatment with the SPP inhibitor (Fig. 6D).

The amino acid residues Val^{140}, Leu^{144}, Ile^{176}, Phe^{177}, Ala^{180}, Ser^{183}, and Cys^{184} were conserved within the core proteins of the genotype 1b strain J1 and the genotype 2a strain JFH-1, while the hydrophobic amino acid residues Leu and Val were found at position 139 in the core proteins of strains J1 and JFH-1, respectively. In order to examine the role of SPP-mediated cleavage of the HCV core protein on the growth of HCV strain JFH-1, mutations of Val^{139}, Val^{140}, and Leu^{144} to Ala (JFH-1/VVL/3A), of Ile^{176} and Phe^{177} to Ala and Leu (JFH-1/IF/AL), or of Ala^{180}, Ser^{183}, and Cys^{184} to Val, Leu, and Val, respectively (JFH-1/ASC/VLV), in the core protein, or mutation of the Gly-Asp-Asp motif to Gly-Asn-Asp in NS5B (JFH-1/GND) as a negative control, were introduced into cDNAs encoding strain JFH-1. The plasmid carrying each cDNA under the control of the polI promoter (19) was transfected into Huh7.5.1 cells, and the propagation of the JFH-1 viruses was determined. The expression of the core protein both in the culture medium and in cells transfected with the wild-type strain JFH-1 was increased during incubation, whereas it was severely impaired in the culture medium and cells transfected with JFH-1/VVL/3A, JFH-1/IF/AL, or the replication-deficient mutant JFH-1/GND. In contrast to JFH-1/VVL/3A and JFH-1/IF/AL, JFH-1/ASC/VLV was still capable of producing the core protein at a lower level than the wild-type strain JFH-1 (Fig. 6E). Furthermore, production of infectious particles was completely abrogated in the culture supernatants of cells transfected with JFH-1/VVL/3A, JFH-1/IF/AL, or the replication-deficient mutant JFH-1/GND, whereas JFH-1/ASC/VLV was still capable of producing infectious particles at a lower level than the wild-type strain JFH-1 (Fig. 6E, right). Expression of NS5A proteins was detected by immunofluorescent analyses in cells transfected with wild-type JFH-1, JFH-1/VVL/3A, JFH-1/IF/AL, or JFH-1/ASC/VLV but not in those transfected with JFH-1/GND, suggesting that JFH-1/VVL/3A and JFH-1/IF/AL are capable of replicating in cells but incapable of generating infectious particles (Fig. 6F). The propagation of JFH-1/ASC/VLV, bearing mutations in Ala^{180}, Ser^{183}, and Cys^{184}, residues that are suggested to be essential for the processing of the HCV core protein by SPP (10, 30), further supports our notion that mutation of these residues is unable to completely abrogate the intramembrane cleavage of the core protein (30). Collectively, these results suggest that the processing of the HCV core protein by SPP plays crucial roles in viral propagation.

DISCUSSION

A previous report has suggested that the amino acid residues Ala^{180}, Ser^{183}, and Cys^{184} in the signal sequence are essential for the intramembrane proteolysis by SPP of the core protein of the HCV genotype 1a strain Glasgow expressed in the BHK and Huh7 cell lines by using the Semliki Forest virus expression system (21). However, we have shown that Leu^{139}, Val^{140}, and Leu^{144} in the hydrophobic region and Ile^{176} and Phe^{177} in the region upstream of the cleavage site, but not Ala^{180}, Ser^{183}, or Cys^{184}, are required for the ER retention and SPP cleavage of the core proteins derived from the genotype 1b strain J1 and the genotype 1a strain H77 expressed in 293T cells by transfection of expression plasmids (30). Subsequently, Hope et al. suggested that these discrepancies were attributable to differences in the SDS-PAGE systems used to separate the processed and unprocessed core proteins, not to any difference in the HCV strains or expression systems, indicating that the core protein cleaved by SPP could be separated by a Tris/Bicine-buffered system but not by a Tris/glycine system (10). In this study, we added an HA tag at the C terminus of each core protein in order to easily distinguish between the cleaved and uncleaved HCV core proteins, and we then examined the processing of the wild-type and mutant core proteins by SDS-PAGE using Tris/glycine buffer. The resistance of Core IF/AL to SPP cleavage was consistent with the finding that Ile^{176} and Phe^{177} are located just upstream of the SPP cleavage site identified in this study. In contrast, Core ASC/VLV was not detected by the anti-HA antibody, indicating that Ala^{180}, Ser^{183}, and Cys^{184} in the signal sequence of the HCV core protein are not required for processing by SPP. A similar result was also obtained by immunoblotting using a Tris/Bicine-buffered system (data not shown). Furthermore, treatment with the SPP inhibitor L685,458 suppressed the cleavage of the core protein and abrogated both the localization of the mature core protein in the DRM and the propagation of strain JFH-1, suggesting that the intramembrane cleavage of the HCV core protein by SPP plays crucial roles in the DRM localization of the HCV core protein and the propagation of HCV. To further confirm the biological significance of the cleavage of the HCV core protein with respect to infectivity, we generated mutant viruses carrying mutations identical to each mutation of core protein described above. A JFH-1 mutant virus carrying the same mutation as Core ASC/VLV, but not other mutants, was still sufficiently viable to propagate in Huh7.5.1 cells. These findings clearly indicate that mutation of Ala^{180}, Ser^{183}, and Cys^{184} to Val, Leu, and Val, respectively, in the signal sequence of the HCV core protein is not able to completely abrogate the cleavage of the core protein by SPP.

Interestingly, the Core ASC/VLV mutant exhibited an extra band that was identical in size to the band of the wild-type core protein, in addition to a slow-migrating band, on the SDS-PAGE gel at 48 h posttransfection (Fig. 1B). Vauloup-Fellous et al. also reported that the Core ASC/VLV mutant expressed by a recombinant Semliki Forest virus in mammalian cells or by a baculovirus in insect cells exhibited bands between the mature (21 kDa) and the immature (23 kDa) core protein (44). If Core ASC/VLV was cleaved at the same site as the wild-type core protein, the processed core protein should have the same molecular size as the processed wild-type core protein, because the mutations in Core ASC/VLV were introduced into the region downstream of the cleavage site. These results suggest that Core ASC/VLV is first processed downstream of the authentic SPP cleavage site and is then further processed at the residue close to Phe^{177}. Presenilins, which are involved in the cleavage of amyloid β protein precursor (APP), belong to the same aspartic protease family as SPP, which contains two Asp residues in the enzymatic active site (48). SPP might be able to cleave a substrate at multiple sites, as observed in the

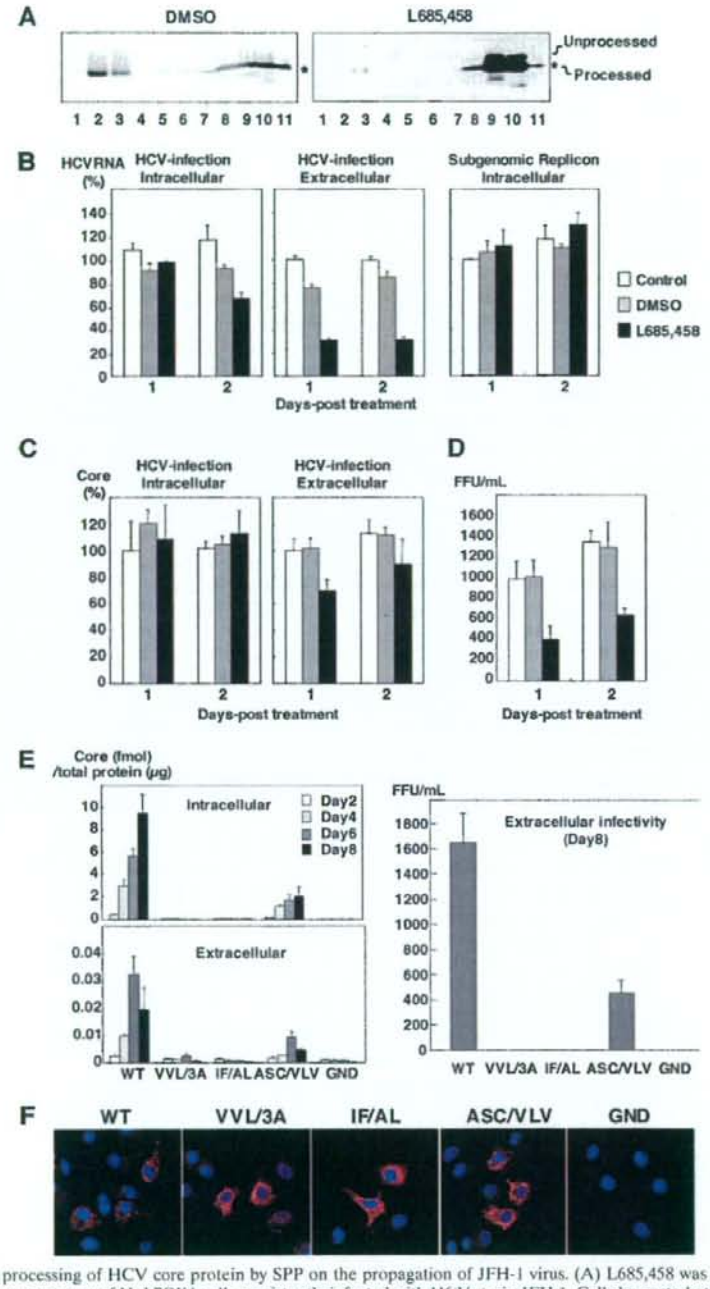


FIG. 6. Effect of the processing of HCV core protein by SPP on the propagation of JFH-1 virus. (A) L685,458 was added, at a concentration of 25 μM, to the culture supernatant of Huh7OK1 cells persistently infected with HCV strain JFH-1. Cells harvested at 24 h after treatment were lysed with 1% Triton X-100 and subjected to a flotation assay. DRM (lanes 1 to 7) and detergent-soluble (lanes 8 to 11) fractions were identified based on the localization of the marker proteins (data not shown). Asterisks indicate processed core proteins. DMSO, dimethyl sulfoxide. (B to D) Cells persistently infected with HCV strain JFH-1 were harvested at 1 or 2 days after treatment with the inhibitor. The data shown in each panel are representative of three independent experiments. (B) Total RNA was prepared from the cells (left) and the culture supernatant (center). Levels of HCV viral RNA and GAPDH mRNA were determined by real-time quantitative PCR. Values for the levels of viral DNA were normalized to that for GAPDH mRNA as described in Materials and Methods. The subgenomic-replicon cell line 9-13 was treated with the inhibitor, and total

processing of APP by presenilins (33, 37). The Core ASC/VLV mutant may exhibit a preference for cleaving at the site between Asp^{178} and Ala^{191} rather than at that between Phe^{177} and Leu^{176}. However, we still do not know whether SPP can cleave multiple sites within the C-terminal transmembrane region of the wild-type HCV core protein, because our mass spectrometry data show that there was no peptide larger than *m/z* 1,918.0452, the size corresponding to the amino acid residues from position 160 to 177 (Fig. 2).

Although the wild-type HCV core protein is known to be partially localized in the DRM fraction (20), Core LVL/3A and Core IF/AL, which are resistant to cleavage by SPP, were detected in the detergent-soluble fraction. Furthermore, overexpression of a dominant-negative SPP mutant or treatment with an SPP inhibitor increased the amount of unprocessed core protein in the detergent-soluble fraction irrespective of the presence of the proteasome inhibitor. These results suggest that processing of the HCV core protein by SPP is a prerequisite for stable localization of the mature core protein in the DRM. Indeed, the biological significance of the DRM localization of the mature HCV core protein is still unclear. In addition, we still do not know how HCV core protein migrates into the DRM fraction, and we could not exclude the possibility of involvement of other cellular and viral proteins in the DRM localization of HCV core protein. The DRM fraction is suggested to consist of various membrane microdomains that include lipid rafts, which are enriched in cholesterol and sphingolipids. The immunofluorescent analyses by Matto et al. showed that the DRM fraction containing the HCV core protein in replicon cells harboring a full genomic HCV RNA was different from the classical lipid raft, as evidenced by the lack of colocalization of the typical lipid raft markers, including caveolin-1 and the cholera toxin B subunit (20). However, Aizaki et al. suggested that the HCV replication complex was localized in a lipid-raft-like DRM fraction that included sphingolipids (2). Previous studies have indicated that the HCV core protein is localized in lipid droplets (1, 10, 20, 21, 23) and that processing by SPP is essential for the localization of the HCV core protein in lipid droplets (21). Furthermore, it was shown that the HCV core protein of strain JFH-1 recruits the replication complex to the lipid-droplet-associated membranes, and HCV particles were detected in close proximity to the lipid droplets, suggesting that the lipid droplets and the lipid-droplet-associated membranes induced by the core protein participate in the assembly of HCV particles (23). In addition, lipid droplets including the core protein surrounded by nonstructural proteins were also detected in cells expressing the nonstructural proteins of strain JFH-1 (23). Based on these observations, it might be feasible to speculate that the HCV core protein is matured through processing by the SP and SPP and is then translocated to the DRM and to the lipid droplets for viral assembly. A recent report by Aizaki et al. shows that HCV particles are enriched with cholesterol and sphingolipids (3), suggesting that the DRM is involved in viral assembly. On the other hand, some fraction of the core protein has been shown to migrate into the nucleus, where it is degraded by nuclear proteasomes (26, 41).

An alanine-scanning mutagenesis study of the HCV core protein has suggested that numerous residues within the carboxy-terminal two-thirds of the core protein are dispensable for RNA replication but essential for efficient infectious-virus production and that alanine substitution of the residues between positions 137 and 144 or 177 and 180 abrogated the extracellular release and intracellular stability of the mutant core proteins of chimeric JFH-1 viruses (27). This is consistent with the severe impairment of virus production by the JFH-1/VVL/3A mutant, in which Val^{139}, Val^{140}, and Leu^{144} are all replaced with Ala, and by the JFH-1/IF/AL mutant, in which Ile^{176} and Phe^{177} are replaced with Ala and Leu, respectively, in spite of the substantial RNA replication in the cells (Fig. 6E and F). The impairment of viral assembly by the introduction of SPP-resistant mutations in the core protein and the reduction of viral production by treatment with an SPP inhibitor, without any effect on subgenomic-RNA replication, also support the notion that SPP-dependent cleavage of the HCV core protein is required for viral assembly rather than for viral replication. Furthermore, the lack of significant effects on viral production and on the stability of the core protein in cells infected with JFH-1 mutants in which residues from 181 to 190 were replaced with Ala (27) is also consistent with the incomplete inhibition of the replication of the JFH-1/ASC/VLV mutant, in which Ala^{180}, Ser^{183}, and Cys^{184} are replaced with Val, Leu, and Val, respectively.

Increases in the levels of saturated and monounsaturated fatty acids enhance HCV RNA replication, in contrast to its suppression by polyunsaturated fatty acids (13), suggesting that enzymes associated with lipid biosynthesis are also involved in HCV replication. SREBP-1c regulates the transcription of acetyl coenzyme A carboxylase, fatty acid synthase, and stearoyl coenzyme A desaturase, leading to the production of saturated and monounsaturated fatty acids and triglycerides (11). Expression of the HCV core protein induces the production of lipid droplets composed mainly of triglycerides (4). Our recent study suggests that SREBP-1c was upregulated in the livers of transgenic mice expressing the HCV core protein through the LXRα/RXRα-dependent pathway, which leads to the development of fatty liver (25). The upregulation of SREBP-1c in the transgenic mice was required for the expression of PA28γ, an HCV core-binding host protein involved in the activation of nuclear proteasome activity (26). The HCV core protein cleaved by SPP may

RNA was prepared from the cells (right). The amount of RNA is represented as a percentage of the amount in the untreated sample at 24 h after treatment (taken as 100%). (C) The amounts of intracellular (left) and extracellular (right) core protein were quantified by a quantitative ELISA. (D) Virus production in the culture supernatants was determined by a focus-forming assay. FFU, focus-forming units. (E) Plasmids coding for the full-length of the wild-type (WT) JFH-1 virus or a mutant (VVL/3A, IF/AL, ASC/VLV, or GND) were transfected into Huh7.5.1 cells. (Left) The amounts of intracellular and extracellular core protein were quantified by a quantitative ELISA at 2, 4, 6, and 8 days posttransfection. (Right) Virus production in the culture supernatants of Huh7.5.1 cells at 8 days after transfection with each plasmid was determined by a focus-forming assay. The data in each panel are representative of three independent experiments. (F) Detection of HCV RNA replication by NS5A immunofluorescence. At 6 days after transfection, NS5A and nuclei were stained red and blue, respectively.

play a role in the formation of lipid droplets associated with the core protein, leading to an enhancement of viral assembly.

In summary, we determined the C-terminal end of the mature HCV core protein expressed in human cells and demonstrated that SPP processing is essential for the DRM localization and stability of the mature core protein. Furthermore, both mutation in the core protein resistant to cleavage by SPP and treatment with an SPP inhibitor abrogated the propagation of strain JFH-1 in the permissive cell line. These results suggest that SPP is a promising target for the development of novel antiviral drugs for the treatment of chronic hepatitis C.

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Interaction of Hepatitis C Virus Nonstructural Protein 5A with Core Protein Is Critical for the Production of Infectious Virus Particles▽

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Nonstructural protein 5A (NS5A) of the hepatitis C virus (HCV) possesses multiple and diverse functions in RNA replication, interferon resistance, and viral pathogenesis. Recent studies suggest that NS5A is involved in the assembly and maturation of infectious viral particles; however, precisely how NS5A participates in virus production has not been fully elucidated. In the present study, we demonstrate that NS5A is a prerequisite for HCV particle production as a result of its interaction with the viral capsid protein (core protein). The efficiency of virus production correlated well with the levels of interaction between NS5A and the core protein. Alanine substitutions for the C-terminal serine cluster in domain III of NS5A (amino acids 2428, 2430, and 2433) impaired NS5A basal phosphorylation, leading to a marked decrease in NS5A-core interaction, disturbance of the subcellular localization of NS5A, and disruption of virion production. Replacing the same serine cluster with glutamic acid, which mimics the presence of phosphoserines, partially preserved the NS5A-core interaction and virion production, suggesting that phosphorylation of these serine residues is important for virion production. In addition, we found that the alanine substitutions in the serine cluster suppressed the association of the core protein with viral genome RNA, possibly resulting in the inhibition of nucleocapsid assembly. These results suggest that NS5A plays a key role in regulating the early phase of HCV particle formation by interacting with core protein and that its C-terminal serine cluster is a determinant of the NS5A-core interaction.

Hepatitis C virus (HCV) infection is a major public health problem and is prevalent in about 200 million people worldwide (27, 40, 42). Current protocols for treating HCV infection fail to produce a sustained virological response in as many as half of treated individuals, and many cases progress to chronic liver disease, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (15, 31, 35, 43).

HCV is a positive-strand RNA virus classified in the *Hepacivirus* genus within the *Flaviviridae* family (55). Its approximately 9.6-kb genome is translated into a single polypeptide of about 3,000 amino acids (aa), in which the structural proteins core, E1, and E2 reside in the N-terminal region. A crucial function of core protein is assembly of the viral nucleocapsid. The amino acid sequence of this protein is well conserved among different HCV strains compared to other HCV proteins. The nonstructural (NS) proteins NS3-NS5B are considered to assemble into a membrane-associated HCV RNA replicase complex. NS3 possesses the enzymatic activities of serine protease and RNA helicase, and NS4A serves as a cofactor for NS3 protease. NS4B plays a role in the remodeling of host cell membranes, probably to generate the site for the replicase assembly. NS5B functions as the RNA-dependent RNA polymerase. NS5A is known to play an important but undefined role in viral RNA replication.

NS5A is a phosphoprotein that can be found in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms (49). Comparative sequence analyses and limited proteolysis of recombinant NS5A have demonstrated that NS5A is composed of three domains (52). Domain I is relatively conserved among HCV genotypes compared to domains II and III. Analysis of the crystal structure of the conserved domain I that immediately follows the membrane-anchoring α-helix localized at the N terminus revealed a dimeric structure (53). The interface between protein molecules is characterized by a large, basic groove, which has been proposed as a site of RNA binding. In fact, its RNA binding property has been demonstrated biochemically (17). Domains II and III of NS5A are far less understood. Domain II contains a region referred to as the interferon sensitivity determining region, and this region and its C-terminal 26 residues have been shown to be essential for interaction with the interferon-induced, double-stranded RNA-dependent protein kinase (6–10, 38, 39, 48). Domain III includes a number of potential phosphoacceptor sites and is most likely involved in basal phosphorylation. This domain tolerates insertion of large heterologous sequences such as green fluorescent protein (GFP) and is not required for function of NS5A in HCV RNA replication (1, 34). However, a study with the recently established productive HCV cell culture system using genotype 2a isolate JFH-1 (28, 56, 58) demonstrated that while insertion of GFP within the NS5A region does not affect RNA replication, it does produce marked decreases in the production of infectious virus particles (41). This suggests that the C-terminal region of NS5A may affect virus particle production independent of RNA replication. Re-

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cently, Miyanari et al. reported that the association of core protein with the NS proteins and replication complexes around lipid droplets (LDs) is critical for producing infectious viruses (33).

In the present study, we demonstrated that NS5A is a prerequisite for HCV particle production via its interaction with core protein, and we identified serine residues in the C-terminal region of NS5A that play an important role in virion production. Substitution of the serine residues with alanine residues inhibited not only the interaction of NS5A with core protein but also HCV RNA-core association and led to a decrease in HCV particle production with no effect on RNA replication.

MATERIALS AND METHODS

DNA construction. Plasmids pJFH1, which contains the full-length JFH-1 cDNA downstream of the T7 RNA promoter sequence, and pSGR-JFH1/Luc, in which the neomycin resistance gene of pSGR-JFH1 has been replaced by the firefly luciferase reporter gene, have been previously described (24, 56). To generate the fluorochrome gene-tagged full-length JFH-1 plasmid, pJFH1/NS5A-GFP, the region encompassing the RsrII site of NS5A and the BsrGI site of NS5B was amplified by PCR, the amplification product was cloned into pGEM-T Easy vector (Promega, Madison, WI), and the resultant plasmid was designated pGEM-JFH1/RsrII-BsrGI. A GFP reporter gene was amplified by PCR from pGreen Lantern-1 (Invitrogen, Carlsbad, CA) with primers containing the XhoI sequence and inserted, after restriction digestion with XhoI, into the XhoI site of pGEM-JFH1/RsrII-BsrGI. The resulting plasmid was digested by RsrII and BsrGI and ligated into pJFH1 similarly digested by RsrII and BsrGI to produce pJFH1/NS5A-GFP. For generation of the fluorochrome gene-tagged subgenomic reporter plasmid, pJFH1/NS5A-GFP was digested by RsrII and SnaBI and ligated into pSGR-JFH1/Luc similarly digested by RsrII and SnaBI. The mutations in the NS5A gene were generated by oligonucleotide-directed mutagenesis (57). To construct plasmids expressing N-terminally FLAG-tagged HCV core protein or hemagglutinin (HA)-tagged NS5A, DNA fragments encoding core protein or NS5A (wild type or mutants) were generated from the full-length JFH-1 cDNA by PCR. The core protein coding sequence, together with a FLAG sequence linked to its N terminus, was cloned into the pCAGGS vector (37). The coding sequences of NS5A, together with an HA sequence linked to their N termini, were also cloned into pCAGGS vectors. All PCR products were confirmed by automated nucleotide sequencing with an ABI Prism 3130 Avant Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

Cells and viruses. The human hepatoma cell line, Huh-7, and JFH1/4-1 cells, which are Huh-7 cells carrying a subgenomic replicon of JFH-1 (32), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with minimal essential medium nonessential amino acids (Invitrogen), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. Huh/c-p7 cells, which are Huh-7 cells stably expressing the proteins core to p7 derived from the JFH-1 strain (18), were incubated in DMEM containing 300 μg/ml of zeocin (Invitrogen). HCV particles derived from JFH-1 were produced by transient transfection of Huh-7 cells with in vitro transcribed RNA, as described previously (56, 58). Recombinant vaccinia virus strain DIs, which expresses the bacteriophage T7 RNA polymerase under the control of the vaccinia virus early/late promoter P7.5, was generated and propagated as previously described (19).

DNA transfection, immunoprecipitation (IP), and immunoblotting. For coexpression of FLAG-tagged core protein and HA-tagged NS5A, cells were seeded onto 35-mm wells of a six-well cell culture plate and cultured overnight. Plasmid DNAs (2 μg) were transfected into cells using TransIT-LT1 transfection reagent (Mirus, Madison, WI). Cells were harvested at 48 h posttransfection, washed three times with 1 ml of ice-cold phosphate-buffered saline (PBS), and suspended in 0.25 ml lysis buffer (20 mM Tris-HCl [pH 7.4] containing 135 mM NaCl, 1% Triton X-100, 0.05% sodium dodecyl sulfate [SDS], and 10% glycerol) supplemented with 50 mM NaF, 5 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were sonicated at 4°C for 5 min, incubated for 30 min at 4°C, and centrifuged at 14,000 × *g* for 5 min at 4°C. After precleaning, the supernatant was immunoprecipitated with 10 μl of anti-FLAG M2-agarose beads (Sigma, St. Louis, MO). For expression of the full-length HCV polyprotein, Huh-7 cells transfected with 10 μg of in vitro transcribed RNAs by electroporation were resuspended in 20 or 30 ml of culture medium, and 10-ml aliquots were seeded into 100-mm culture dishes. At 72 h posttransfection, the cells were incubated in 0.5 ml of lysis buffer (20 mM Tris-HCl [pH 7.4] containing 135 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 10% glycerol) supplemented with 50 mM NaF, 5 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF. After precleaning, the supernatant was immunoprecipitated with 5 μg of polyclonal anti-NS5A antibody (34a) or polyclonal anti-C/EBPβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and 20 μl of protein G-agarose beads (Invitrogen). The immunocomplex was precipitated with the beads by centrifugation at 800 × *g* for 30 s and then was washed five times with lysis buffer by centrifugation. The proteins binding to the beads were boiled in 20 μl of SDS sample buffer and then subjected to SDS–12.5% polyacrylamide gel electrophoresis (PAGE). The proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon: Millipore, Bedford, MA) and then reacted with a primary antibody and a secondary horseradish peroxidase-conjugated antibody. The immunocomplexes were visualized with an ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, United Kingdom) and detected using an LAS-3000 imaging analyzer (Fujifilm, Tokyo, Japan).

In vitro synthesis of HCV RNA and RNA transfection. Plasmid DNAs were digested with XbaI and treated with mung bean nuclease (New England Biolabs, Ipswich, MA) to remove the four terminal nucleotides, resulting in the correct 3′ end of the HCV cDNA. Digested DNAs were purified and used as templates for RNA synthesis. HCV RNA was synthesized in vitro using a MEGAscript T7 kit (Ambion, Austin, TX). Synthesized RNA was treated with DNase I (Ambion), followed by acid guanidinium thiocyanate-phenol-chloroform extraction to remove any remaining template DNA. Synthesized HCV RNAs were used for electroporation. Trypsinized Huh-7 cells were washed with Opti-MEM I reduced-serum medium (Invitrogen) and resuspended at 3×10^6 cells/ml with Cytomix buffer (54). RNA was mixed with 400 μl of cell suspension and transferred into an electroporation cuvette (Precision Universal Cuvettes; Thermo Hybaid, Middlesex, United Kingdom). Cells were then pulsed at 260 V and 950 μF using a Gene Pulser II unit (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred onto six-well culture plates or 100-mm culture dishes.

Luciferase assay. Cells were harvested at different time points posttransfection of subgenomic reporter replicons and lysed in passive lysis buffer (Promega). The luciferase activity in cells was determined using a luciferase assay system (Promega).

Quantification of HCV core protein. HCV core protein in transfected cells or cell culture supernatants was quantified using a highly sensitive enzyme immunoassay (Ortho HCV antigen ELISA Kit; Ortho Clinical Diagnostics, Tokyo, Japan). To determine intracellular core protein amounts, cell lysates were prepared as described previously (41). To determine the efficiency of core protein release, the ratio of extracellular core protein to total core protein (the sum of intra- and extracellular core protein amounts) was calculated.

Intra- and extracellular infectivity assay. Culture supernatants were harvested 72 h posttransfection, and virus titers were determined by a 50% tissue culture infectious dose ($TCID_{50}$) assay as described previously (28, 46). Virus titration was performed by seeding naive Huh-7 cells in 96-well plates at a density of 1×10^4 cells/well. Samples were serially diluted fivefold in complete growth medium and used to infect the seeded cells (six wells per dilution). At 72 h after infection, the inoculated cells were fixed and immunostained with a mouse monoclonal anti-core protein antibody (2H9) (56), followed by an Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (IgG) (Invitrogen). Wells that showed at least one core protein-expressing cell was counted as positive. Cell-associated infectivity was determined essentially as described previously (12, 47). Briefly, cells were extensively washed with PBS, scraped, and centrifuged for 3 min at 120 × *g*. Cell pellets were resuspended in 1 ml of DMEM containing 10% FBS and subjected to four cycles of freezing and thawing using dry ice and a 37°C water bath. Samples were then centrifuged at 2,400 × *g* for 10 min at 4°C to remove cell debris, and cell-associated infectivity was determined by $TCID_{50}$ assay.

Expression of HCV proteins using vaccinia viruses, metabolic labeling of cells, and radioimmunoprecipitation analysis. Metabolic labeling of cells and radioimmunoprecipitation analysis were performed as described by Huang et al. (17) with some modifications. A total of 4×10^5 Huh-7 cells were seeded onto each well of six-well cell culture plates and cultured overnight. A 2-μg amount of subgenomic replicon DNAs carrying defined NS5A mutations was transfected into cells using TransIT-LT1 transfection reagent, and at 12 h posttransfection the cells were then infected at a multiplicity of infection of 10 with recombinant vaccinia viruses expressing the T7 RNA polymerase. After 40 h of transfection, cells were incubated in methionine- and cysteine-deficient DMEM (Invitrogen) or phosphate-deficient DMEM (Invitrogen) for 2 h and labeled for 6 h with [^{35}S]methionine and [^{35}S]cysteine (200 μCi/well; GE Healthcare) or

[^{32}P]orthophosphate (250 μCi/well; GE Healthcare). The cells were then washed twice with cold PBS and lysed with SDS lysis buffer (50 mM Tris-HCl [pH 7.6], 0.5% SDS, 1 mM EDTA, 20 μg/ml of PMSF). The cell lysates were passed through a 27-gauge needle several times to shear cellular DNA. After a 10-min incubation at 75°C, the lysates were clarified by centrifugation and diluted fivefold with HNAET buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 0.67% bovine serum albumin, 1 mM EDTA, 0.33% Triton X-100). After precleaning by incubation with 20 μl of protein G-agarose beads for 1 h at 4°C, the supernatant was incubated with 2 μg of rabbit polyclonal anti-NS5A antibody overnight at 4°C. A 20-μl aliquot of protein G agarose beads was further added and incubated for 2 h at 4°C. The cell pellets were washed three times with 0.5 ml of HNAETS buffer (HNAET containing 0.5% SDS), followed by washing once with 0.5 ml of HNE buffer (50 mM HEPES [pH 7.5], 150 mM NaCl and 1 mM EDTA). After treatment with or without λ protein phosphatase (New England Biolabs), the cell pellets were suspended in 20 μl of SDS sample buffer and boiled for 10 min. The proteins were resolved on 10% SDS-polyacrylamide gels and analyzed by autoradiography.

Subcellular fractionation analysis. All steps were carried out at 4°C in the presence of a protease inhibitor cocktail (Complete; Roche, Mannheim, Germany) as described previously (20), with some modifications. Cells were suspended in four cell volumes of homogenization buffer (50 mM NaCl, 10 mM triethylamine [pH 7.4], 1 mM EDTA), snap frozen in liquid nitrogen, stored at −80°C, and thawed in a water bath at room temperature. Supernatants (0.4 ml) were layered on linear 10-ml iodixanol gradients from 2.5 to 25% and centrifuged at 37,000 rpm for 3.5 h in an SW41 rotor (Beckman, Fullerton, CA), followed by collection of 0.8-ml fractions from the top. Each fraction was concentrated by Centricon YM30 (Millipore), separated by SDS-PAGE, and immunoblotted with a rabbit polyclonal anti-calnexin antibody (Stressgen Biotechnologies, Victoria, Canada), a mouse monoclonal anti-adipose differentiation-related protein (ADRP) antibody (Progen Biotechnik, Heidelberg, Germany), or a rabbit polyclonal anti-NS5A antibody. The core protein amount in each fraction was also determined by enzyme-linked immunosorbent assay (ELISA).

IP-RT-PCR. The process of cell lysis to RNA purification was carried out essentially as described by Johnson et al. (21) with some modifications. A total of 3×10^6 Huh-7 cells were transfected with 10 μg of in vitro transcribed HCV RNAs and resuspended in 20 or 30 ml of culture medium, after which 10-ml aliquots were seeded into 100-mm culture dishes. At 72 h posttransfection, the cells were scraped and incubated in 500 μl of hypotonic buffer (10 mM HEPES [pH 7.6], 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF) per dish. The cells were passed through a 20-gauge needle several times, lysed with Nonidet P-40 at a final concentration of 1%, and incubated on ice for an additional 10 min. After centrifugation at 4,000 × *g* at 4°C for 15 min, glycerol was added to the supernatants at a final concentration of 5%. The cell lysates were incubated with 20 μl of protein G-agarose beads for 30 min at room temperature. After the cell lysates were removed from protein G-agarose beads, 5 μg of mouse monoclonal anti-core protein antibody or normal mouse IgG (Sigma) as a negative control was added, and samples were incubated for an additional 1 h at room temperature. A 20-μl aliquot of protein G-agarose beads per sample was added to the cell lysates and incubated for 1 h. After incubation, the beads were washed three times with wash buffer (10 mM Tris-HCl [pH 7.6], 100 mM KCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol) and eluted in 100 μl of elution buffer (50 mM Tris-HCl [pH 8.0], 1% SDS, and 10 mM EDTA) at 65°C for 10 min. After treatment with 100 μg of proteinase K at 37°C for 30 min, the RNAs in immunocomplexes were isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. Reverse transcriptase PCR (RT-PCR) was carried out using random hexamer and Superscript II RT (Invitrogen), followed by nested PCR with LA *Taq* DNA polymerase (TaKaRa, Shiga, Japan) and primer sets amplifying the fragments of nucleotides (nt) 129 to 2367 and nt 7267 to 9463 of the JFH-1 genome. To amplify the fragment of nt 129 to 2367, the sense primer 5′-CTGTGAGGAACTACTGTCTT-3′ and the antisense primer 5′-TCCACGATGTTCTGGTGAAG-3′ were used for first-round PCR; the sense primer 5′-CGGGAGAGCCATAGTGG-3′ and the antisense primer 5′-CATTCCGTGGTAGAGTGCA-3′ were used for second-round PCR. To amplify the fragment of nt 7267 to 9463, the sense primer 5′-GTCCAGGGTGCCCGTTCTGGACT-3′ and the antisense primer 5′-GCGGCTCACGGACCTTTCAC-3′ were used for first-round PCR; the sense primer 5′-CACCGTTGCTGGTTGTGCT-3′ and the antisense primer 5′-GTGTACCTAGTGTGTGCCGCTCTA-3′ were used for second-round PCR.

Indirect immunofluorescence analysis. Cells incubated for 3 days after transfection with JFH-1 RNAs were seeded in an eight-well chamber slide (BD Biosciences, San Jose, CA) and cultured overnight. The adherent cells were washed twice with PBS and fixed with 4% paraformaldehyde at room temperature. After a washing step with PBS, the cells were permeabilized with PBS containing 0.3% Triton X-100 and 2% FBS for 1 h at room temperature and stained with a rabbit polyclonal anti-NS5A antibody and a mouse monoclonal anti-core protein antibody. The fluorescent secondary antibodies were Alexa Fluor 488- or Alexa Fluor 555-conjugated anti-rabbit or anti-mouse IgG antibodies (Invitrogen). Analyses of JFH-1 were performed on a Zeiss confocal laser scanning microscope LSM 510 (Carl Zeiss, Oberkochen, Germany).

RESULTS

Mutations of serine residues at the NS5A C terminus impair basal phosphorylation but have little effect on viral RNA replication. As demonstrated in a previous study, insertion of GFP into the NS5A C terminus does not significantly affect viral RNA replication but reduces the generation of infectious HCV particles (41). The C-terminal region of NS5A contains highly conserved serine residues that are involved in basal phosphorylation (1, 23, 49). To examine the involvement of the serine clusters (cluster 3-A [CL3A] and cluster 3-B [CL3B]) in the C-terminal region of NS5A in HCV particle production, we created mutated HCV genomes as well as subgenomic replicons carrying alanine substitutions for the conserved serine residues at aa 2384, 2388, 2390, and 2391 (residues are numbered according to the positions within the original JFH-1 polyprotein) (CL3A/SA); at aa 2428, 2430, and 2433 (CL3B/SA); or an in-frame deletion spanning aa 2384 to 2433 (Δ2384–2433) (Fig. 1). A construct with an in-frame insertion of GFP (NS5A-GFP) was also generated as described previously for the Con1 isolate (34).

First, we analyzed the effects of the NS5A mutations on HCV RNA replication using a transient RNA replication assay using subgenomic luciferase reporter replicons (Fig. 2A) and found that the serine-to-alanine substitutions (CL3A/SA and CL3B/SA) did not affect viral RNA replication. NS5A-GFP and Δ2384–2433 slightly reduced RNA replication, indicating that the mutations of the NS5A C terminus tested in this study do not critically affect RNA replication, which is consistent with previous reports (1, 34, 51).

Next, the phosphorylation status of the mutated NS5A was analyzed as described in Materials and Methods (Fig. 2B). NS5A was isolated from radiolabeled cells by IP and analyzed either directly by SDS-PAGE or after treatment with λ protein phosphatase. Analysis of ^{32}P-radiolabeled proteins revealed that the CL3A/SA, CL3B/SA, and Δ2384–2433 mutations resulted in marked reduction of basal phosphorylation (Fig. 2B, compare lane 1 with lanes 3, 5, and 7 in the top panel). All ^{32}P-labeled NS5A proteins were sensitive to treatment with phosphatase (lanes 2, 4, 6, and 8). The possibility that loss of signal after dephosphorylation was due to contaminating proteases present in the phosphatase preparations can be ruled out because no degradation of the ^{35}S-labeled proteins was observed (Fig. 2B, bottom panel). These results suggest that mutations in the C-terminal serine cluster of NS5A impair basal phosphorylation but have no significant effect on viral RNA replication.

Effect of mutations introduced into the NS5A C terminus on the production of infectious HCV particles. To analyze HCV particle production from cells transfected with the in vitro transcribed viral genomic RNAs, we harvested supernatants and cells at 4, 24, 48, 72, and 96 h posttransfection and measured the amounts of core protein. As shown in Fig. 3A, comparable amounts of core proteins were detected in all transfected cells 4 h after transfection, reflecting unchanged

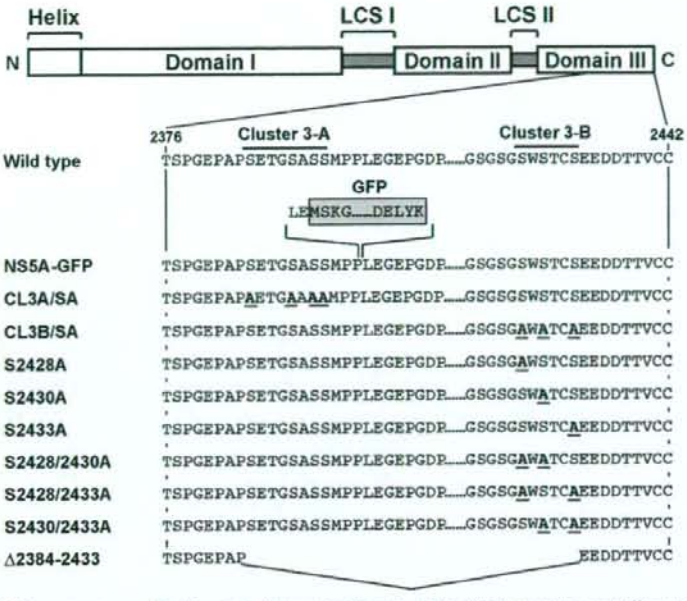

FIG. 1. Structures of HCV constructs used in this study. Schematic diagram of the NS5A structure according to Tellinghuisen et al. (52) is shown in the top panel. The three domains are indicated by white boxes and are separated by trypsin-sensitive regions with presumably low structural complexity (low-complexity sequence [LCS]). The numbers indicate amino acid residues within the original JFH-1 polyprotein. The names listed on the left represent full-length HCV constructs, subgenomic reporter replicons, or N-terminally HA-tagged NS5A constructs used in this study. NS5A-GFP carries a GFP insertion between aa 2394 and 2395 as indicated by a shaded box. CL3A/SA and CL3B/SA carry several serine-to-alanine substitutions in the NS5A C terminus constructed as described previously (1). HCV constructs from S2428A to S2430/2433A carry single or double serine-to-alanine substitutions generated by modification of the CL3B/SA construct. The Δ2384–2433 mutant possesses an in-frame deletion in the C-terminal region of NS5A. Amino acid substitutions are marked in bold and underlined. N and C represent N terminus and C terminus, respectively.

transfection efficiencies, and the kinetics of intracellular core protein levels was similar among transfectants. By contrast, core protein released from cells transfected either with the mutated genome of CL3B/SA, Δ2384–2433, or NS5A-GFP was more than 10-fold lower than that for the wild-type JFH-1 or CL3A/SA (Fig. 3B). Figure 3C shows the efficiency of core protein release from each transfectant, which is expressed as a percentage of the extracellular core protein level relative to the amount of total core protein (the sum of intra- and extracellular core protein). Core protein release efficiency with the wild type and CL3A/SA was 2 to 13% at 48 to 96 h after transfection, while only 1% or less of core protein was released in the cases of CL3B/SA, Δ2384–2433, and NS5A-GFP strains.

To further investigate production and release of infectious virus particles, naïve Huh-7 cells were infected with culture supernatants of cells harvested 72 h posttransfection, and infectious virus titers were determined by $TCID_{50}$ assay at 72 h after infection. Figure 3D shows that release of infectious virus particles from cells transfected with the genome of CL3B/SA or Δ2384–2433 mutants was markedly reduced (about 10,000-fold) compared to that from wild-type- or CL3A/SA-transfected cells (white bars). To examine whether such a decrease in infectious HCV in the culture supernatants was attributable to defective virion assembly or impaired release of virions, we determined cell-associated infectivity (Fig. 3D). Production of intracellular infectious virions in CL3B/SA- and Δ2384-2433-transfected cells was strongly impaired in comparison with that in wild-type-transfected (~1,000-fold) and CL3A/SA-transfected (~100-fold) cells. Thus, the results suggest a potential role for the serine cluster at aa 2428, 2430, and 2433 of NS5A in assembly of infectious HCV particles. Among the NS5A mutations tested, CL3B/SA is of particular interest because this mutation leads to a marked reduction in HCV production with no impact on viral RNA replication.

Serine residues at aa 2428, 2430, and 2433 are important for the interaction between NS5A and core protein. Miyanari et al. reported that the association of core protein with NS proteins is critical for infectious HCV production and that mutations of the core protein and NS5A that cause these proteins to fail to associate with each other impair the production of infectious virus (33). Based on these observations and the findings noted above, we hypothesize that NS5A plays a key role in recruiting viral RNA, which is synthesized at the viral replication complex, to nucleocapsid formation via interaction between the NS5A C-terminal region and the core protein. To prove this, we analyzed the interaction of NS5A with the core protein by coimmunoprecipitation experiments. HA-tagged NS5A constructs carrying defined mutations were generated (Fig. 1) and coexpressed with the FLAG-tagged core protein in Huh-7 cells. As shown in Fig. 4A, coimmunoprecipitation of NS5A

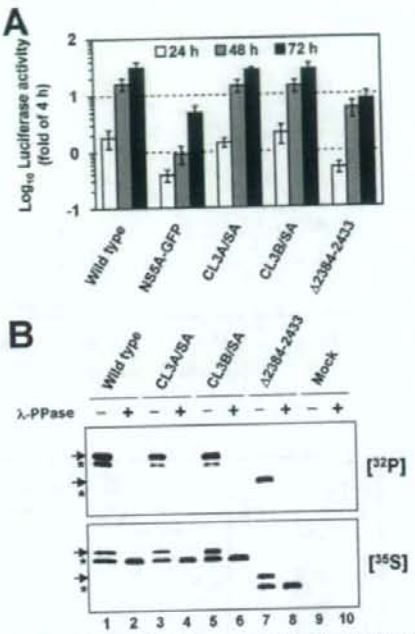

FIG. 2. Mutations at the C terminus of NS5A impair basal phosphorylation and have only a minor impact on RNA replication. (A) Replication of given mutants in transfected Huh-7 cells as determined by luciferase reporter assays performed at 24, 48, and 72 h posttransfection (white, gray, and black bars, respectively). Values given were normalized for transfection efficiency using the luciferase activity determined 4 h after transfection, which was set to 1. Mean values of quadruplicate measurements and the standard deviations are given. (B) Phosphorylation analysis of NS5A using the vaccinia virus T7 hybrid system. NS3-to-NS5B polyprotein fragments carrying the mutations specified above the lanes were transfected into Huh-7 cells, and proteins were radiolabeled with [^{32}P]orthophosphate or [^{35}S]methionine and [^{35}S]cysteine. NS5A proteins were isolated by IP and separated by SDS-PAGE (10% polyacrylamide). Mock-transfected cells served as a negative control (lanes 9 and 10). Half of the samples were treated with λ protein phosphatase (λ-PPase) (+) whereas the other half was mock treated (−) prior to SDS-PAGE. Arrows and asterisks indicate hyperphosphorylated and basally phosphorylated forms, respectively.

with the core protein was observed in cells expressing the wild-type NS5A and the CL3A/SA-mutated NS5A, but the amount of immunoprecipitated NS5A in the CL3A/SA-expressing cells was slightly lower than that in the wild-type-expressing cells. In contrast, the CL3B/SA- or the Δ2384-2433-mutated NS5A coimmunoprecipitated with the core protein only slightly or not at all.

We further examined the interaction of NS5A with core protein in cells expressing HCV genomes. At 72 h posttransfection with the wild type or CL3B/SA, cells were harvested and immunoprecipitated with an anti-NS5A antibody or an anti-C/EBPβ antibody as a negative control, followed by immunoblotting. Under these experimental conditions, the amount of extracellular core protein released from cells transfected with the CL3B/SA genome was about 10-fold lower than that for the wild type, although comparable amounts of intracellular core protein were observed in both transfectants (Fig. 4B, left panels). As shown in the right panels of Fig. 4B, the core protein was specifically coimmunoprecipitated with NS5A in cells expressing the wild-type JFH-1 genome but not with the mutated NS5A in cells expressing the CL3B/SA genome. These results demonstrate that NS5A interacts with the core protein in cells producing infectious particles and that serine residues at aa 2428, 2430, and 2433 are important to the success of this interaction.

Two serine residues among aa 2428, 2430, and 2433 are responsible for regulating the interaction of NS5A with the core protein as well as HCV particle production. To further determine the critical residues in the C-terminal serine cluster of NS5A responsible for HCV particle production, we replaced one or two serine residues in the region with alanine (Fig. 1) and investigated which serine-to-alanine substitution influenced HCV particle production. Core protein levels in cells transfected with any construct were comparable over 4 days after transfection, indicating similar efficiencies of transfection and RNA replication from each construct (data not shown). As shown in Fig. 5A, we observed a slight delay in the kinetics of core protein release from cells transfected with the single-substitution genomes, S2428A, S2430A, and S2433A, up to 48 or 72 h posttransfection. However, core protein release from these cells reached comparable levels to that for the wild type at 96 h after transfection. In the cases of the double-substitution mutants (Fig. 5B), core protein release from cells transfected with the double-substitution genomes was markedly reduced, with 10- to 30-fold decreases compared to that for wild type observed. The kinetics of core protein release were similar to that for CL3B/SA.

Interaction of NS5A carrying single or double serine-to-alanine substitutions with the core protein was investigated by coimmunoprecipitation analysis using HA-tagged NS5A constructs. NS5A mutants carrying a single substitution were coimmunoprecipitated with the core protein (Fig. 5C), while none of the double-substitution NS5A mutants or the triple-substitution mutant, CL3B/SA, coimmunoprecipitated with the core protein (Fig. 5D). These results suggest that at least two serine residues in the C-terminal serine cluster of NS5A (aa 2428, 2430, and 2433) are necessary for the interaction between NS5A and the core protein as well as for regulation of HCV particle production and that there is positive correlation between their interaction and the amount of core protein released.

Glutamic acid partially substitutes for serine phosphorylation in the interaction of NS5A with the core protein and virus production. A consequence of phosphorylation is the addition of negative charge to a protein. In some cases, phosphoserine can be mimicked by glutamic or aspartic acid (14). To determine whether the introduction of negative charges into aa 2428, 2430, and 2433 instead of phosphoserines positively regulates the interaction of NS5A with the core protein and virus production, we replaced the serine residues with glutamic acid residues and constructed the CL3B/SE and S2428/2430E mutants (Fig. 6A). Cells transfected with the double-glutamic acid substitution, S2428/2430E, exhibited similar kinetics to the wild-type-transfected cells and released ~22-fold more core protein than S2428/2430A-transfected cells by 96 h posttransfection (Fig. 6B). In contrast,

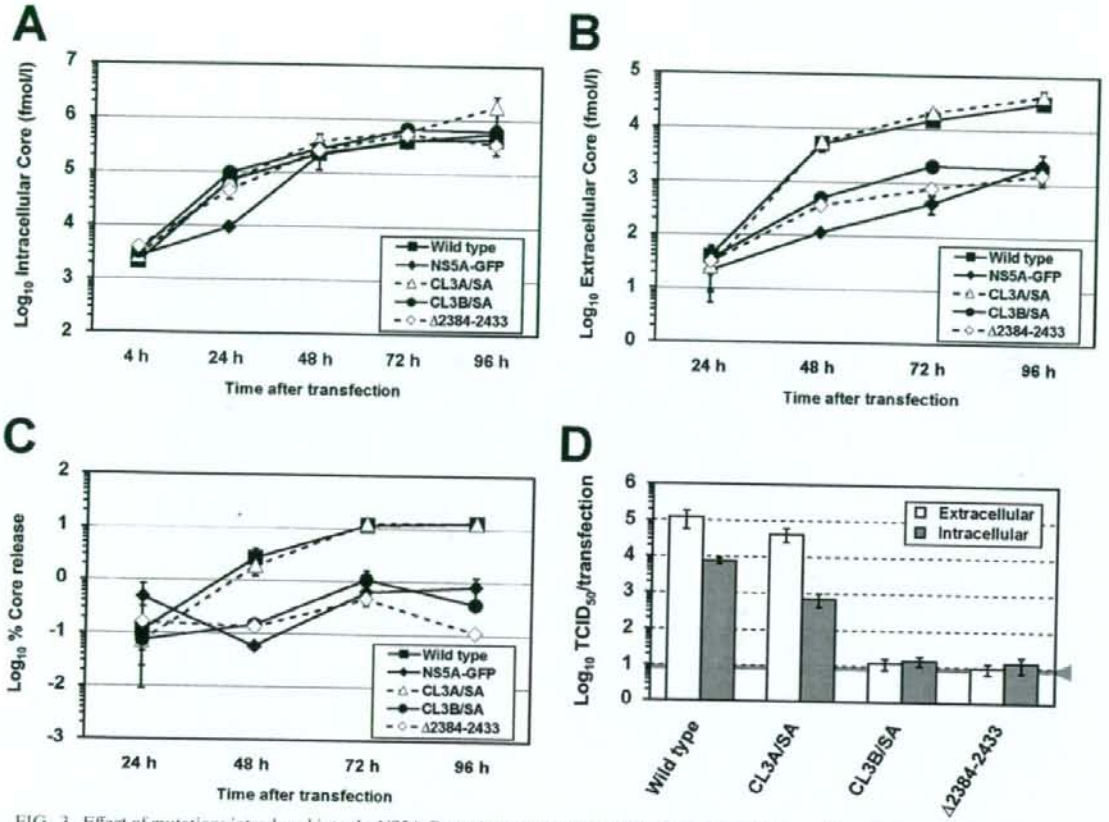

FIG. 3. Effect of mutations introduced into the NS5A C terminus on the production of infectious HCV particles. (A) Intracellular levels of core protein measured at various time points after transfection. A total of 3×10^6 Huh-7 cells were transfected with 10 μg of in vitro-transcribed HCV RNAs specified in the inset and resuspended in 10 ml of culture medium, after which 2-ml aliquots were seeded into each well of a six-well culture plate. The cells were harvested at different time points between 4 h and 96 h posttransfection, and then 500 μl of cell lysate per well was prepared. After centrifugation, supernatants were processed for a core protein-specific ELISA. (B) Release of core protein from cells transfected with the HCV genomes specified in the inset. Cell culture supernatants harvested from cells given in panel A were analyzed by a core protein ELISA. (C) Efficiency of core protein release from cells transfected with the HCV genomes specified in the inset. The percent core protein release (vertical axis) indicates the percentage of released core protein in relation to total core protein (the sum of intra- and extracellular core protein) calculated for each time point. (D) Infectivity of virus particles contained in supernatants and cells after transfection with mutants specified below the graph. Culture supernatants and cells were harvested 72 h posttransfection, and extracellular (white bars) and intracellular infectivity (gray bars) levels were determined by $TCID_{50}$ assay. The gray line and arrowhead represent the detection limit of the limiting dilution assay. Mean values and standard deviations for at least triplicates are shown in all panels.

the transfectant with the triple glutamic acid substitution, CL3B/SE, showed similar trends to that of CL3B/SA. In the coimmunoprecipitation experiments with FLAG-tagged core protein and HA-tagged NS5A constructs (Fig. 6C), S2428/2430E, but not S2428/2430A, restored the ability of NS5A to interact with the core protein up to a similar level to that of wild type. As expected, neither CL3B/SE nor CL3B/SA coimmunoprecipitated with the core protein. Taken together, these results indicate that negative charges at aa 2428 and 2430 preserve the ability of NS5A to interact with the core protein and positively regulate virus production. However, the data of the CL3B/SE mutant indicate that it is likely that negative charges alone are not sufficient to enhance either the interaction of NS5A with the core protein or virus production.

Subcellular localization of NS5A and core protein in Huh-7 cells expressing HCV genomes. The coimmunoprecipitation experiments described above indicate that the wild-type NS5A but not the CL3B/SA mutant interacts with the core protein. To evaluate the NS5A-core protein interaction in intact cells, we examined the subcellular localization of NS5A with the core protein by immunofluorescence analysis. NS5A colocalized with the core protein in cells transfected with the JFH-1 wild type (Fig. 7A), whereas their colocalization was rarely observed in cells transfected with the CL3B/SA RNA (Fig. 7B).

To further analyze the subcellular compartments for the localization of NS5A and core protein in cytoplasmic membrane structures, including the endoplasmic reticulum (ER) and LDs, we performed subcellular fractionation studies as

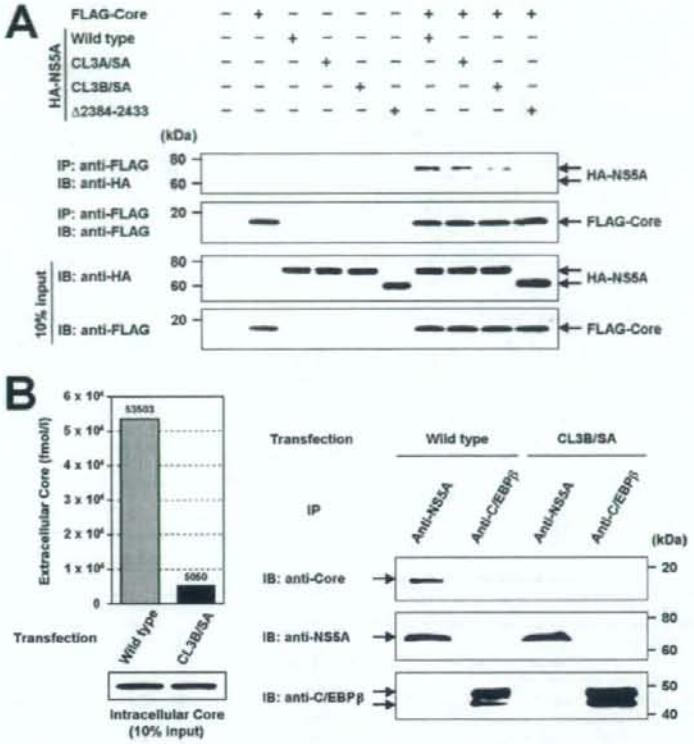

FIG. 4. aa 2428, 2430, and 2433 are essential for the interaction between NS5A and the core protein. (A) Effect of mutations at the NS5A C terminus on the interaction of NS5A with the core protein. N-terminally FLAG-tagged core protein and N-terminally HA-tagged NS5A carrying defined mutations were coexpressed in Huh-7 cells and immunoprecipitated with anti-FLAG antibody. The resulting precipitates were examined by immunoblotting using anti-HA or FLAG antibody. One-tenth of the cell lysates used in IP is shown as the 10% input. (B) Interaction between NS5A and the core protein in HCV-replicating cells. Huh-7 cells were lysed 72 h after transfection of the in vitro transcript of the HCV genome (wild type or CL3B/SA) and were immunoprecipitated with anti-NS5A antibody or anti-C/EBPβ antibody as a negative control. The resulting precipitates were examined by immunoblotting using anti-core protein, NS5A, or C/EBPβ antibody. One-tenth of cell lysates used in IP was immunoblotted with anti-core protein antibody (10% input). Cell culture supernatants harvested from transfected cells were analyzed by a core protein ELISA in parallel. IB, immunoblotting.

described in Materials and Methods. The iodixanol gradient was collected from the top to the bottom into 12 fractions (fractions 1 to 12). As shown in Fig. 7C, an ER marker, calnexin, was found in fractions 7 to 12 and was localized primarily in fractions 11 and 12. In contrast, ADRP, a cellular marker for LDs, was mainly observed in fractions 4 to 7. These two markers were equally distributed among cells analyzed (data not shown). The distribution of the wild-type NS5A was found in fractions 4 to 7, which was parallel to the fractionation profile of ADRP. The CL3B/SA-mutated NS5A was more broadly distributed and was also observed in heavier fractions than the wild-type NS5A, which was analogous to distribution of NS5A expressed in JFH1/4-1 cells bearing subgenomic replicons. The core protein in cells expressing the JFH-1 wild type, the CL3B/SA mutant, and in Huh/c-p7 cells that express JFH-1 structural proteins was distributed in a similar fashion, indicating that the distribution of core protein is not affected by NS5A mutation. The fractionation profile of the core protein, with a peak in fraction 4 or 5, was similar to that of the wild-type NS5A or ADRP but not to that of the CL3B/SA-mutated NS5A or calnexin, suggesting that core protein interacts with the wild-type NS5A in LD fractions, which is consistent with previous reports (33, 44, 45).

NS5A-core protein interaction is important for association of the core protein with the viral genomic RNA. To further address our hypothesis regarding involvement of NS5A in recruiting viral RNA to nucleocapsid formation, we analyzed the association of the core protein with HCV RNA in wild-type- or CL3B/SA-expressing cells by IP-RT-PCR (Fig. 8). Both cell lysates were immunoprecipitated with an anti-core protein antibody or a negative control, mouse IgG. Total RNA prepared from each immunoprecipitate was subjected to RT-PCR in order to detect HCV RNA. The amounts of immunoprecipitated core protein (Fig. 8, lower panel) as well as the expression of HCV RNA (Fig. 8, upper panels, Input) were comparable in both cells. In cells expressing the wild-type JFH-1 genome, the viral RNAs covering the 5′ terminal 2.2-kb as well as the 3′ terminal 2.2-kb regions were detected in immunopre-

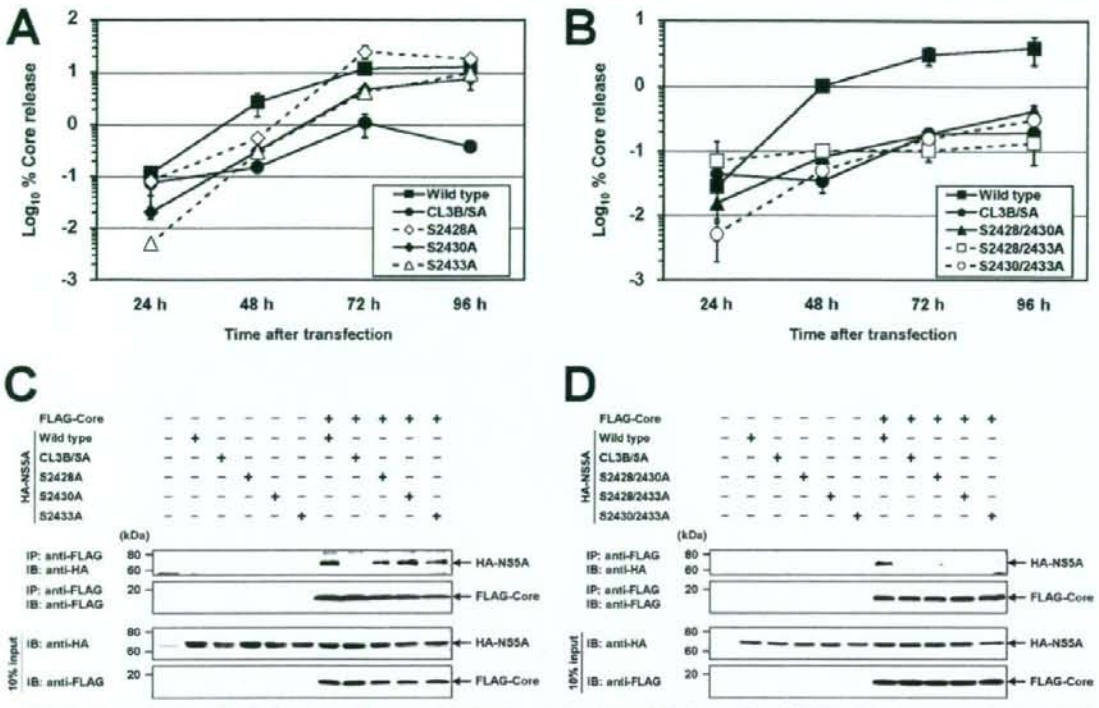

FIG. 5. Determination of critical amino acids responsible for virus production and the interaction of NS5A with the core protein. (A and B) Effect of single or double serine-to-alanine substitutions on virus production. After transfection of in vitro transcripts of the HCV genomes specified in the inset into Huh-7 cells, the cells and culture supernatants were harvested at the time points given, and the amounts of the core protein were determined by core protein-specific ELISA. Percent core protein release (vertical axis) indicates the percentage of released core protein in relation to total core protein (the sum of intra- and extracellular core protein) calculated for each time point. Mean values and standard deviations for at least triplicate experiments are shown. (C and D) Effect of single or double serine-to-alanine substitutions on the interaction between NS5A and the core protein. N-terminally FLAG-tagged core protein and N-terminally HA-tagged NS5A carrying defined mutations were coexpressed in Huh-7 cells and immunoprecipitated with anti-FLAG antibody. The resulting precipitates were examined by immunoblotting using anti-HA or FLAG antibody. One-tenth of the cell lysates used in IP is shown as the 10% input. IB, immunoblotting.

cipitates obtained with the anti-core protein antibody but not with the mouse IgG. In contrast, in cells expressing the CL3B/SA genome, HCV RNA was not detected in the immunoprecipitates with either antibody. These results demonstrate that HCV RNA associates with the core protein in cells where NS5A interacts with core protein (JFH-1 wild type) but not in cells where their interaction is impaired (CL3B/SA).

DISCUSSION

In the present study, we demonstrated the involvement of NS5A in the production of HCV particles via the interaction of NS5A with the core protein and identified its C-terminal serine cluster 3-B (aa 2428, 2430, and 2433), which is implicated in basal phosphorylation, as a key element for the interaction of NS5A with the core protein and for infectious virus production. Serine-to-alanine substitutions at the cluster, which have no impact on viral RNA replication, inhibit the interaction between NS5A and the core protein, thereby indicating that there is a connection between NS5A-core protein association and virus production. Finally, CL3B mutation leads to impairment of the association of the core protein with HCV RNA and, therefore, possibly RNA encapsidation.

Several reports have indicated that viral NS proteins are involved in the virion assembly of *Flaviviridae* viruses (25, 29, 30, 33). For instance, mutations in yellow fever virus NS2A block production of infectious virus, and this perturbation can be released by a suppressor mutation in NS3 (25), while the hydrophobic residues of Kunjin virus NS2A required for virus assembly have been mapped (26). Miyanari et al. have shown that HCV core protein recruits NS proteins to the LD-associated membranes and that the NS proteins around the LDs participate in the assembly of infectious viral particles (33). Furthermore, during preparation of the current article, two studies regarding participation of NS5A in the assembly of HCV particles were published. Appel et al. have demonstrated the essential role of domain III of NS5A in the formation of infectious particles, and deletions in this domain that disrupt colocalization of NS5A and the core protein abrogate virion production (2). Tellinghuisen et al. identified a serine residue in domain III as a key determinant for viral particle production