

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

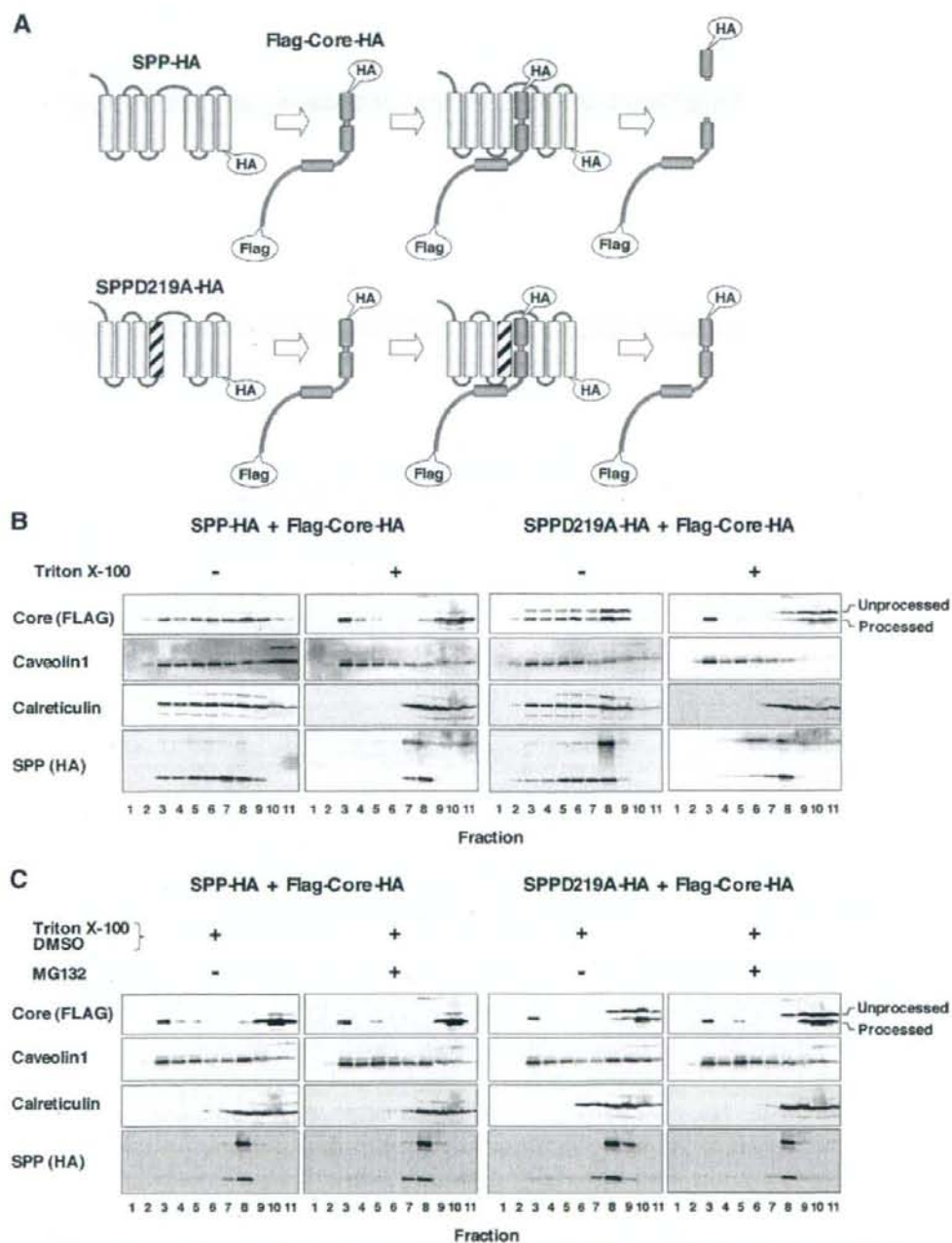


FIG. 4. The dominant-negative mutant of SPP prevents the cleavage of HCV core protein by SPP and its localization in the DRM. (A) Schematic representation of the processing of FLAG-core-HA by a wild-type SPP (SPP-HA) (top) and the dominant-negative effect of SPPD219A-HA (bottom). (B) FLAG-core-HA was 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.

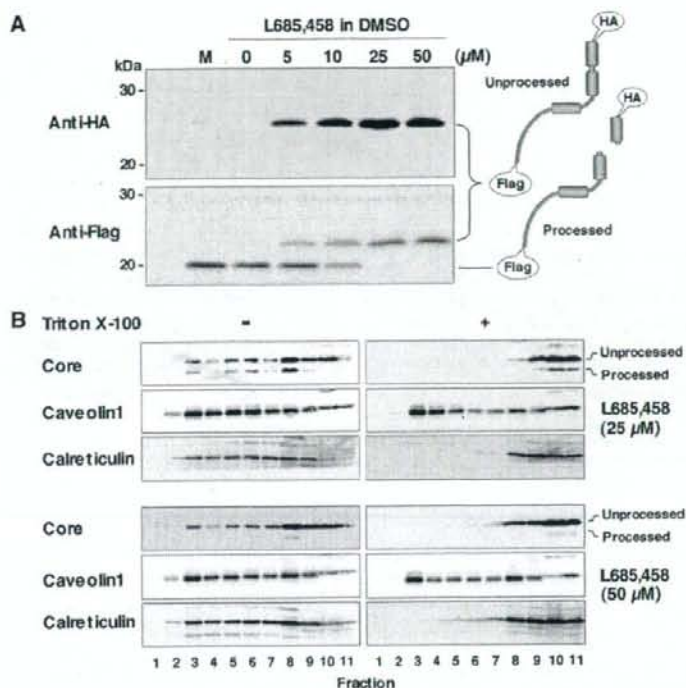


FIG. 5. Effect of an SPP inhibitor on the cleavage of HCV core protein by SPP. The HCV core protein was expressed in 293T cells, and L685,458 was added to the culture supernatant, at the indicated concentrations, at 5 h posttransfection. Cells harvested at 29 h posttransfection were lysed with 1% Triton 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-LL)₂ ketone and L685,458 on the processing of the HCV core protein. Although (Z-LL)₂ ketone was insoluble at a concentration of 10 μ M 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 μ M 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 μ M 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 L685,458 (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 Val¹⁴⁰, Leu¹⁴⁴, Ile¹⁷⁶, Phe¹⁷⁷, Ala¹⁸⁰, Ser¹⁸³, and Cys¹⁸⁴ 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 Val¹³⁹, Val¹⁴⁰, and Leu¹⁴⁴ to Ala (JFH-1/VVL/3A), of Ile¹⁷⁶ and Phe¹⁷⁷ to Ala and Leu (JFH-1/IF/AL), or of Ala¹⁸⁰, Ser¹⁸³, and Cys¹⁸⁴ 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 NSSB (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 Ala¹⁸⁰, Ser¹⁸³, and Cys¹⁸⁴, 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 Ala¹⁸⁰, Ser¹⁸³, and Cys¹⁸⁴ 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 Leu¹³⁹, Val¹⁴⁰, and Leu¹⁴⁴ in the hydrophobic region and Ile¹⁷⁶ and Phe¹⁷⁷ in

the region upstream of the cleavage site, but not Ala¹⁸⁰, Ser¹⁸³, or Cys¹⁸⁴, 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 Ile¹⁷⁶ and Phe¹⁷⁷ 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 Ala¹⁸⁰, Ser¹⁸³, and Cys¹⁸⁴ 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 Ala¹⁸⁰, Ser¹⁸³, and Cys¹⁸⁴ 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 Phe¹⁷⁷. 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

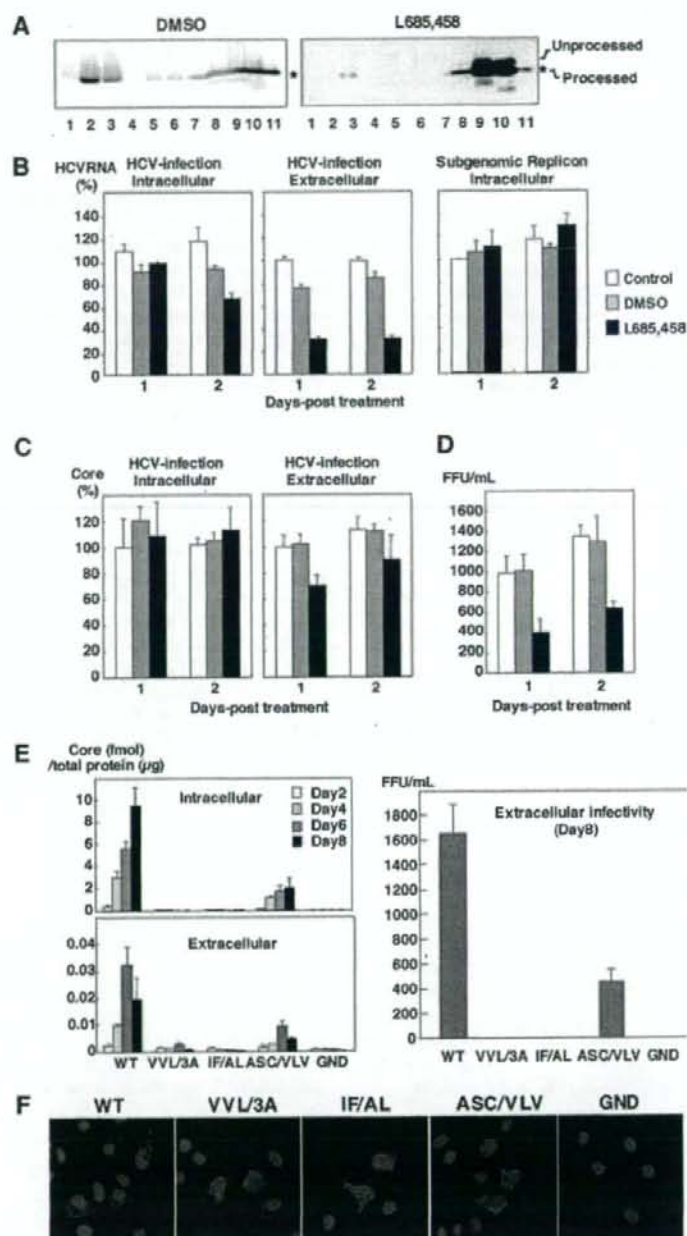


FIG. 6. Effect of the processing of HCV core protein by SPP on the propagation of JFH-1 virus. (A) L685,458 was added, at a concentration of 25 μ M, to the culture supernatant of Huh7OK1 cells persistently infected with HCV strain JFH-1. Cells harvested at 24 h after treatment were lysed with 1% Triton 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 Asp¹⁷⁸ and Ala¹⁹¹ rather than at that between Phe¹⁷⁷ and Leu¹⁷⁸. 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 VVL/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 Val¹³⁹, Val¹⁴⁰, and Leu¹⁴⁴ are all replaced with Ala, and by the JFH-1/IF/AL mutant, in which Ile¹⁷⁶ and Phe¹⁷⁷ 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 Ala¹⁸⁰, Ser¹⁸³, and Cys¹⁸⁴ 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 LXR α /RXR α -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 PA28 γ , 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[▽]

Takahiro Masaki,¹ Ryosuke Suzuki,¹ Kyoko Murakami,¹ Hideki Aizaki,¹ Koji Ishii,¹ Asako Murayama,¹ Tomoko Date,¹ Yoshiharu Matsuura,² Tatsuo Miyamura,¹ Takaji Wakita,¹ and Tetsuro Suzuki^{1*}

Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan,¹ and Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Suita-shi, Osaka 565-0871, Japan²

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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 *Hepadnavirus* 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-

* Corresponding author. Mailing address: Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81 3 5285 1111. Fax: 81 3 5285 1161. E-mail: tesuzuki@nih.go.jp.

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cently, Miyazari 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 NSSA is a prerequisite for HCV particle production via its interaction with core protein, and we identified serine residues in the C-terminal region of NSSA that play an important role in virion production. Substitution of the serine residues with alanine residues inhibited not only the interaction of NSSA 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 JFH1 cDNA downstream of the T7 RNA promoter sequence, and pSGR-JFH1/Lac, 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 JFH1 plasmid, pJFH1/NSSA-GFP, the region encompassing the RsrII site of NSSA and the BsrGI site of NSSB 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/NSSA-GFP. For generation of the fluorochrome gene-tagged subgenomic reporter plasmid, pJFH1/NSSA-GFP was digested by RsrII and SnaBI and ligated into pSGR-JFH1/Lac similarly digested by RsrII and SnaBI. The mutations in the NSSA gene were generated by oligonucleotide-directed mutagenesis (57). To construct plasmids expressing N-terminally FLAG-tagged HCV core protein or hemagglutinin (HA)-tagged NSSA, DNA fragments encoding core protein or NSSA (wild type or mutants) were generated from the full-length JFH1 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 NSSA, 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 JFH1 (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% CO₂ incubator. Huh-7 cells, which are Huh-7 cells stably expressing the proteins core to p7 derived from the JFH1 strain (18), were incubated in DMEM containing 300 µg/ml of zeocin (Invitrogen). HCV particles derived from JFH1 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 NSSA, 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-LTI 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, 0.5% sodium dodecyl sulfate [SDS], and 10% glycerol) supplemented with 50 mM NaF, 5 mM Na₂VO₄, 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 preclearing, 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 dodecyl sulfate, and 10% glycerol) supplemented with 50 mM NaF, 5 mM Na₂VO₄, 1 µg/ml leupeptin, and 1 mM PMSF. After preclearing, the supernatant was immunoprecipitated with 5 µg of polyclonal anti-NSSA 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 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 (Immobilion; 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 1 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 transfected into an electroporation cuvette (Precision Universal Cuvettes; Thermo Hybrid, 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 (TCID₅₀) assay as described previously (28, 46). Virus titration was performed by seeding naïve 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 (21P) (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 TCID₅₀ 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 4×10^6 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 NSSA mutations was transfected into cells using TransIT-LTI 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 [³⁵S]methionine and [³⁵S]cysteine (200 µCi/well; GE Healthcare) or

[³²P]orthophosphate (250 μ Ci/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 μ g/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 preclearing by incubation with 20 μ l of protein G-agarose beads for 1 h at 4°C, the supernatant was incubated with 2 μ g of rabbit polyclonal anti-NSSA antibody overnight at 4°C. A 20- μ l 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 HNAET 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 a protein phosphatase (New England Biolabs), the cell pellets were suspended in 20 μ l 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-NSSA 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 μ g 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 μ l of hypotonic buffer (10 mM HEPES [pH 7.6], 1.5 mM MgCl₂, 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 \times 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 μ l of protein G-agarose beads for 30 min at room temperature. After the cell lysates were removed from protein G-agarose beads, 5 μ g 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- μ l 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 MgCl₂, and 1 mM dithiothreitol) and eluted in 100 μ l 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 μ g 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'-CTGTGAGGAAC TACTGTCTT-3' and the antisense primer 5'-TCCACGATGTTCTGGTGAA G-3' were used for first-round PCR; the sense primer 5'-CGGGAGAGCCAT AGTGG-3' and the antisense primer 5'-CAITCCGTGGTAGAGTGCA-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'-CGGGCTCACGGACCTTTCAC-3' were used for first-round PCR; the sense primer 5'-CACGGTTGCTGGTTGTGCT-3' and the antisense primer 5'-GTGTACCTAGTGTGGCCGCTCA-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-NSSA 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 NSSA C terminus impair basal phosphorylation but have little effect on viral RNA replication. As demonstrated in a previous study, insertion of GFP into the NSSA C terminus does not significantly affect viral RNA replication but reduces the generation of infectious HCV particles (41). The C-terminal region of NSSA 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 NSSA 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 (Δ 2384-2433) (Fig. 1). A construct with an in-frame insertion of GFP (NSSA-GFP) was also generated as described previously for the Con1 isolate (34).

First, we analyzed the effects of the NSSA 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. NSSA-GFP and Δ 2384-2433 slightly reduced RNA replication, indicating that the mutations of the NSSA 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 NSSA was analyzed as described in Materials and Methods (Fig. 2B). NSSA was isolated from radiolabeled cells by IP and analyzed either directly by SDS-PAGE or after treatment with λ protein phosphatase. Analysis of ³²P-radiolabeled proteins revealed that the CL3A/SA, CL3B/SA, and Δ 2384-2433 mutations resulted in marked reduction of basal phosphorylation (Fig. 2B, compare lane 1 with lanes 3, 5, and 7 in the top panel). All ³²P-labeled NSSA 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 ³⁵S-labeled proteins was observed (Fig. 2B, bottom panel). These results suggest that mutations in the C-terminal serine cluster of NSSA impair basal phosphorylation but have no significant effect on viral RNA replication.

Effect of mutations introduced into the NSSA 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

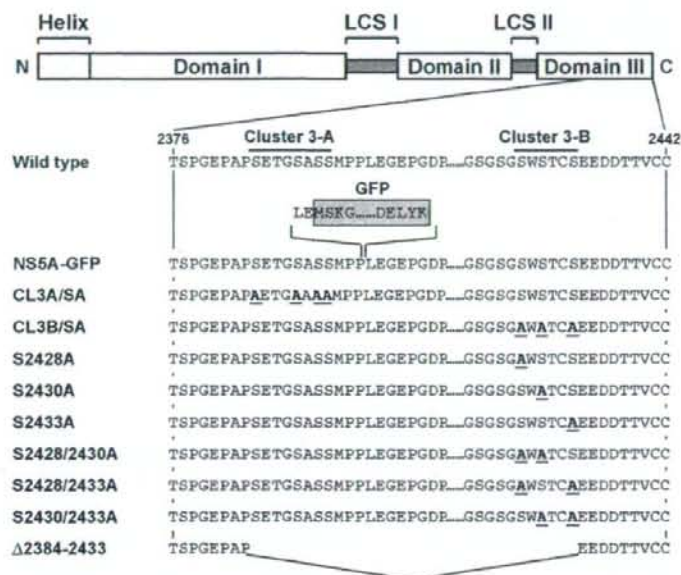


FIG. 1. Structures of HCV constructs used in this study. Schematic diagram of the NS5A structure according to Tellinghuisen et al. (52) is shown in the top panel. The three domains are indicated by white boxes and are separated by trypsin-sensitive regions with presumably low structural complexity (low-complexity 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 TCID₅₀ 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

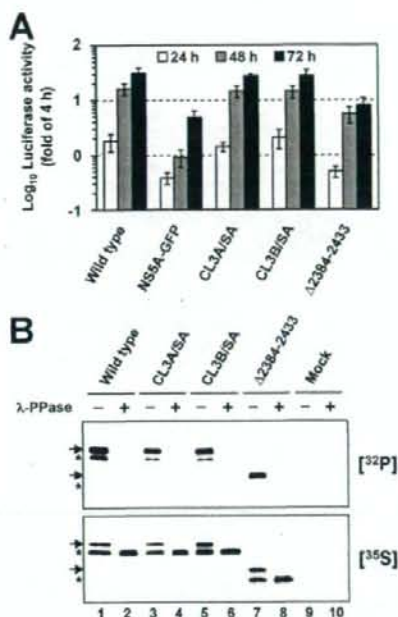


FIG. 2. Mutations at the C terminus of NS5A impair basal phosphorylation and have only a minor impact on RNA replication. (A) Replication of given mutants in transfected Huh-7 cells as determined by luciferase reporter assays performed at 24, 48, and 72 h posttransfection (white, gray, and black bars, 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 [³²P]orthophosphate or [³⁵S]methionine and [³⁵S]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,

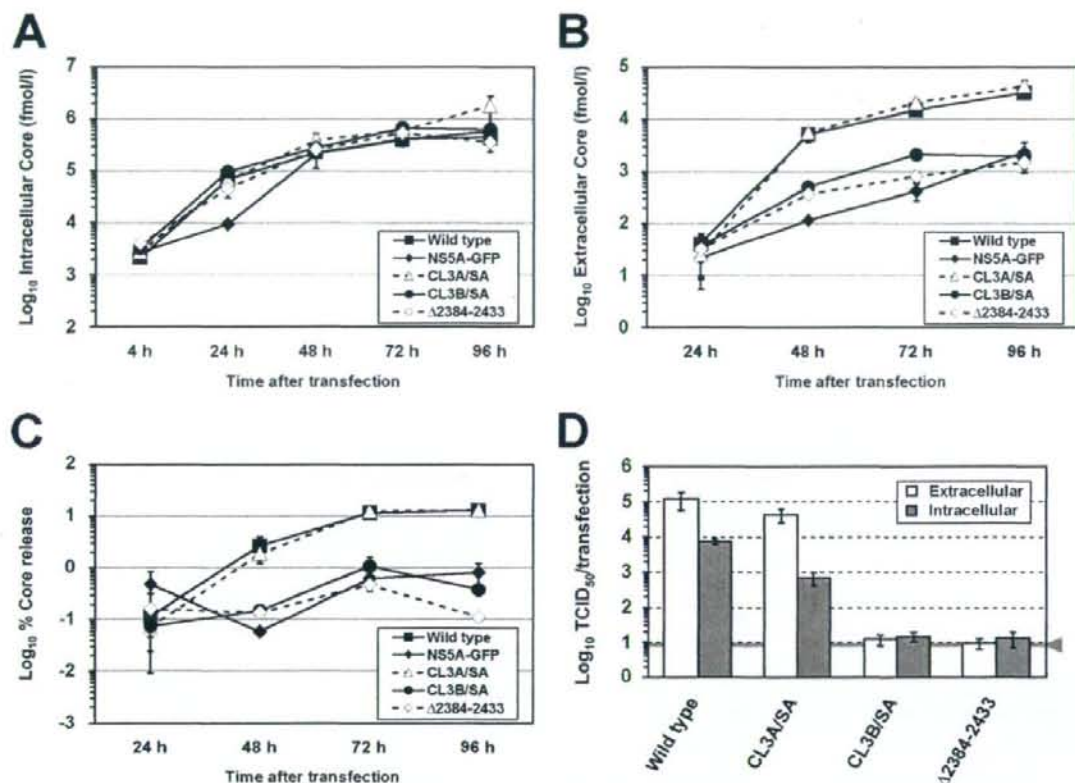


FIG. 3. Effect of mutations introduced into the NS5A C terminus on the production of infectious HCV particles. (A) Intracellular levels of core protein measured at various time points after transfection. A total of 3×10^6 Huh-7 cells were transfected with $10 \mu\text{g}$ of in vitro-transcribed HCV 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 $500 \mu\text{l}$ 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 TCID₅₀ 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

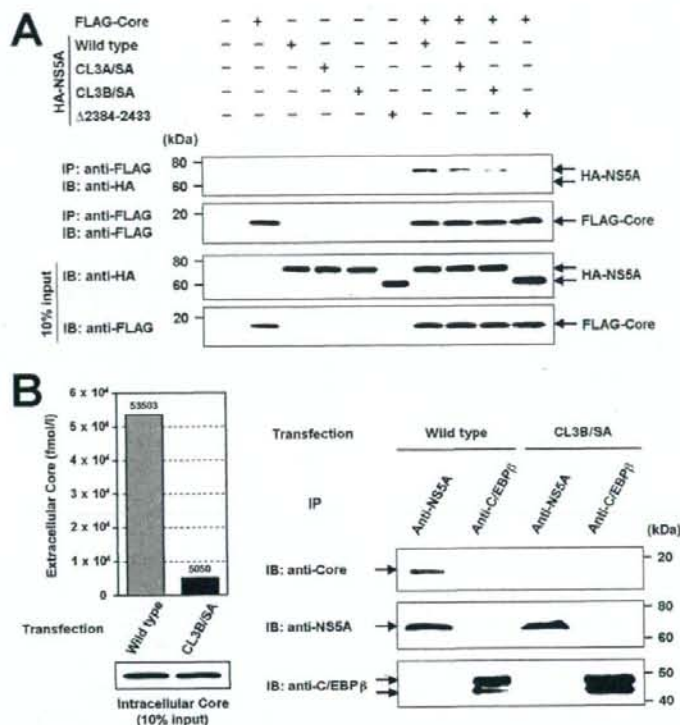


FIG. 4. aa 2428, 2430, and 2433 are essential for the interaction between NS5A and the core protein. (A) Effect of mutations at the NS5A C terminus on the interaction of NS5A with the core protein. N-terminally FLAG-tagged core protein and N-terminally HA-tagged NS5A carrying defined mutations were coexpressed 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/EBP β antibody as a negative control. The resulting precipitates were examined by immunoblotting using anti-core protein, NS5A, or C/EBP β 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-

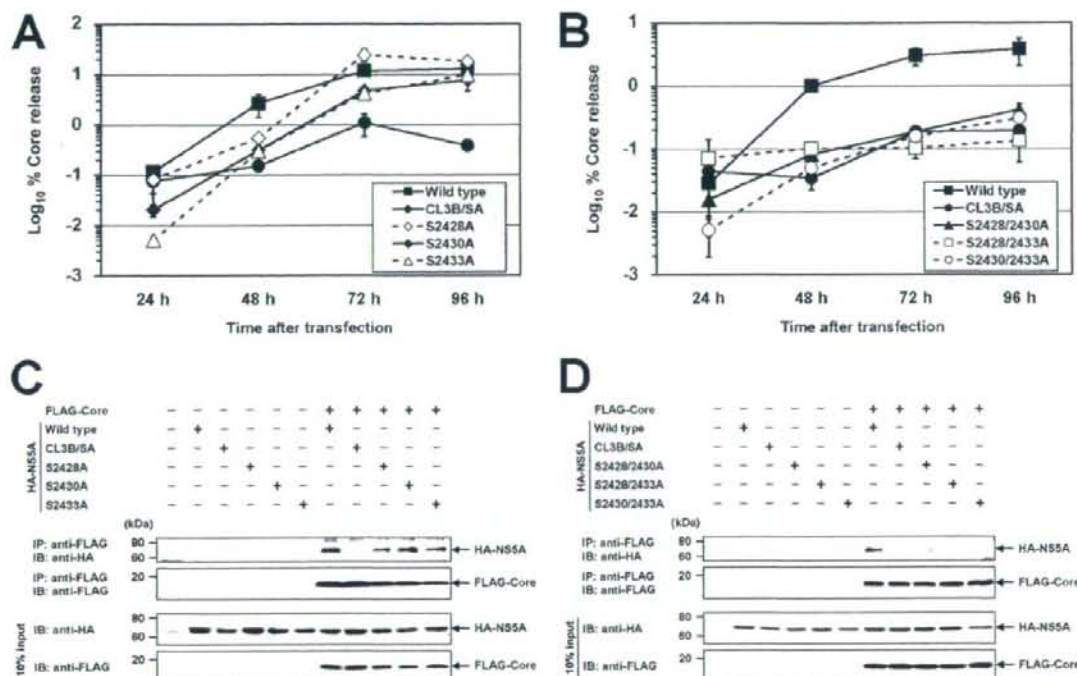


FIG. 5. Determination of critical amino acids responsible for virus production and the interaction of NSSA with the core protein. (A and B) Effect of single or double serine-to-alanine substitutions on virus production. After transfection of in vitro transcripts of the HCV genomes specified in the inset 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 NSSA and the core protein. N-terminally FLAG-tagged core protein and N-terminally HA-tagged NSSA carrying defined mutations were cocrossed 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.

precipitates 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 NSSA 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 NSSA in the production of HCV particles via the interaction of NSSA 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 NSSA 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 NSSA and the core protein, thereby indicating that there is a connection between NSSA-core protein association and virus production. Finally, CL3B mutation leads to impair-

ment 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 NSSA in the assembly of HCV particles were published. Appel et al. have demonstrated the essential role of domain III of NSSA in the formation of infectious particles, and deletions in this domain that disrupt colocalization of NSSA 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

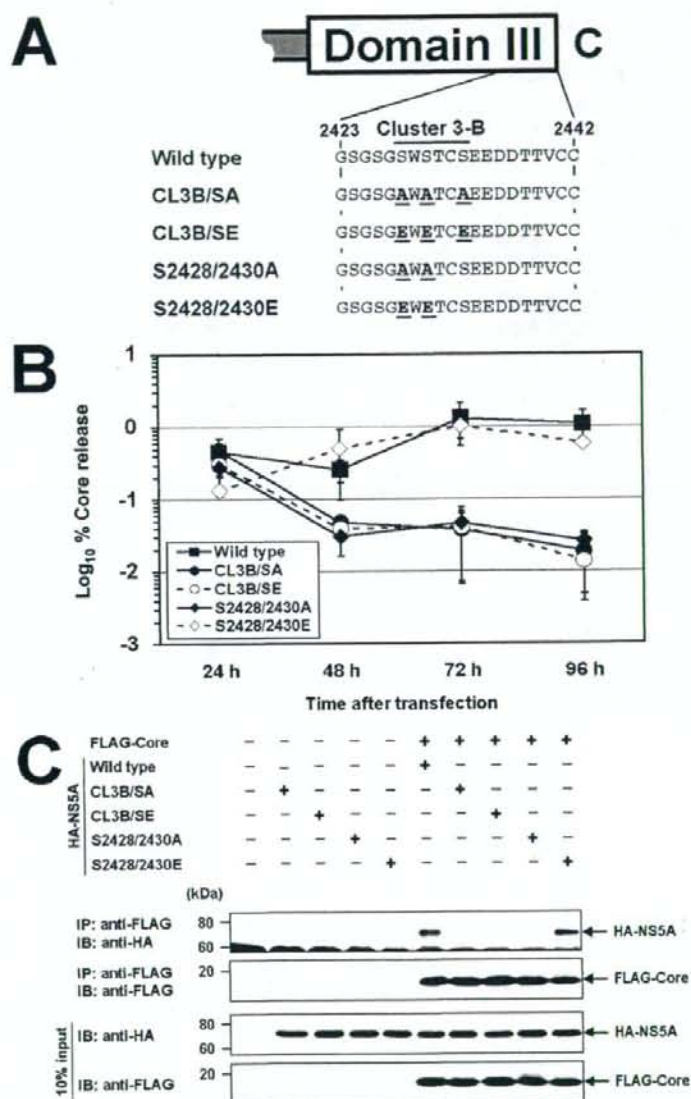


FIG. 6. Effect of glutamic acid substitutions for phosphoserines at aa 2428, 2430, and 2433 on virus production and the interaction of NS5A with the core protein. (A) Alanine or glutamic acid substitutions for serine residues at aa 2428, 2430, and 2433. The numbers indicate amino acid positions within the polyprotein of the JFH-1 isolate. The names shown on the left represent full-length HCV or N-terminally HA-tagged NS5A constructs used in this experiment. Amino acid substitutions are marked in bold and underlined. C represents the C terminus. (B) Effect of alanine or glutamic acid 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 the culture supernatants were harvested at the time points given, and the amounts of 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) Effect of alanine or glutamic acid 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 (IB) using anti-HA or FLAG antibody. One-tenth of the cell lysates used in IP is as shown as the 10% input.

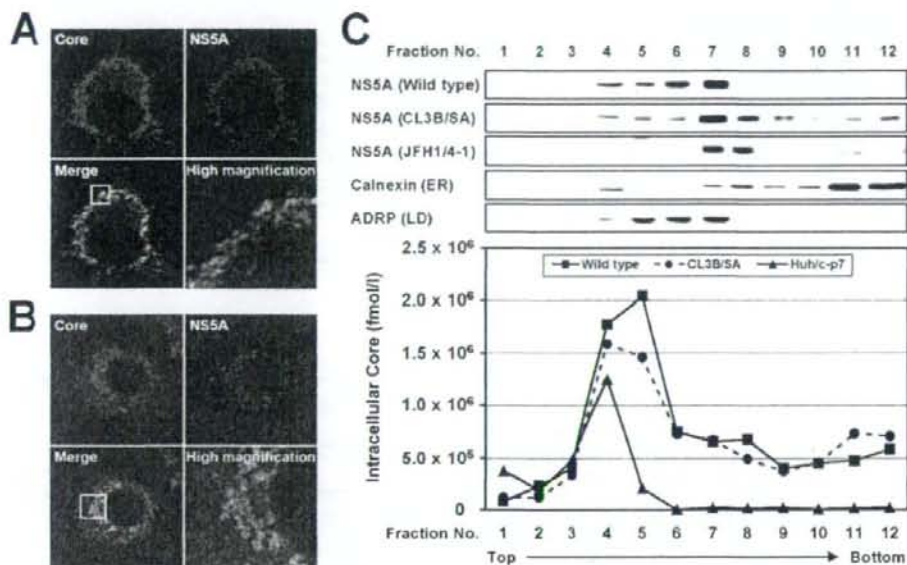


FIG. 7. Subcellular localization of NS5A and the core protein in HCV-replicating cells. Huh-7 cells were transfected with the *in vitro* transcript of the HCV genome, wild type (A) or CL3B/SA (B). Seventy-two hours after transfection, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and double stained with antibodies against the core protein (green) and NS5A (red), followed by staining with an Alexa Fluor 488- or Alexa Fluor 555-conjugated antibody. High-magnification panels are enlarged images of white squares in the merge panels. (C) HCV (wild type or CL3B/SA)-replicating cells, JFH1/4-1 cells harboring a subgenomic replicon of JFH-1, or Huh7c-p7 cells stably expressing JFH-1 structural proteins were lysed by freeze-thawing, and the cell lysates were fractionated on 5 to 25% iodixanol gradients. The distributions of NS5A, calnexin (ER marker), and ADRP (LD marker) were determined by immunoblotting, and those of the core protein were examined by core protein-specific ELISA.

(50). However, the mechanism by which NS proteins participate in virus assembly or the role of the interaction between structural and NS proteins in virus life cycles has not been fully elucidated. Here, we have clearly demonstrated that HCV NS5A interacts with the core protein in coimmunoprecipitation experiments not only with coexpression of each epitope-tagged protein but also with cells expressing the viral genome; and by using immunofluorescence and subcellular fractionation analysis, we have confirmed that mutations in CL3B abolish colocalization of NS5A and the core protein, presumably around LDs. In addition, the intracellular infectivity assay and IP-RT-PCR strongly suggest that impairment of the NS5A-core protein interaction results in disruption of virus production at an early stage of virion assembly. On the basis of the present results and findings in accompanying articles, one may infer the following events: newly synthesized HCV RNAs bound to NS5A are released from the replication complex-containing membrane compartment and can be captured by the core protein via interaction with domain III of NS5A at the surface of LDs or LD-associated membranes. Consequently, the viral RNAs are encapsidated, and virion assembly proceeds in the local environment. Recruitment of newly synthesized viral RNAs to the core protein could be important for efficient nucleocapsid formation in cells, where concentrations of the viral genome and the structural proteins are typically low, and may contribute to the selection of the viral genome to be

packaged. Interaction between NS5A and the core protein has been previously reported, and the NS5A region containing an interferon sensitivity determining region and the PKR-binding sequence (aa 2212 to 2330) has been mapped to that required for binding with core protein by yeast two-hybrid and *in vitro* pull-down assays (13). However, involvement of domain III in the NS5A-core protein interaction was not analyzed in detail, and a role for the NS5A-core protein interaction in the HCV life cycle was not examined in that study.

A growing body of evidence points to phosphorylation of NS5A as being important in controlling HCV RNA replication. Although the degree and the requirement for its hyperphosphorylation diverge between different HCV isolates, mutations that are associated with increased replicative fitness of HCV replicons frequently lead to a reduced level of NS5A hyperphosphorylation (1, 5, 36). Inhibitors of serine/threonine protein kinases that block NS5A hyperphosphorylation facilitate replication of a non-culture-adapted replicon (3, 36). One model that has been proposed suggests that NS5A hyperphosphorylation negatively regulates HCV RNA replication by disrupting the interaction between NS5A and the vesicle-associated membrane protein-associated protein subtype A, a cellular factor considered necessary for efficient RNA replication (5). However, the regulatory role of the basal phosphorylation of NS5A in the viral life cycle is poorly understood. It has been reported that the C-terminal region of NS5A (aa 2350 to 2419)

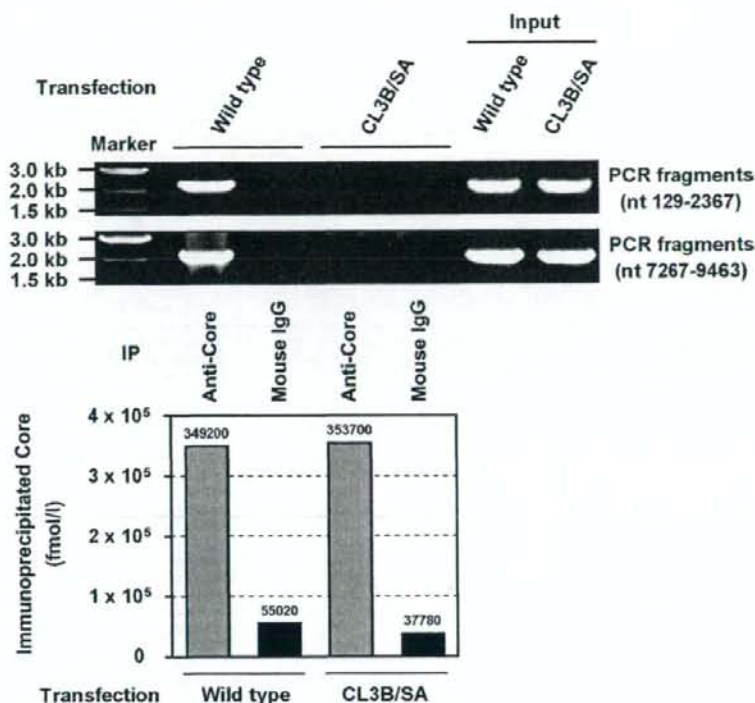


FIG. 8. IP-RT-PCR of HCV-replicating cells performed to examine the association between the core protein and the HCV genome RNA. Huh-7 cells were transfected with the *in vitro* transcript of the HCV genome (wild type or CL3B/SA) and lysed in 500 μ l of hypotonic buffer at 72 h posttransfection. After IP with an anti-core protein antibody or mouse IgG, immunoprecipitates were eluted in 100 μ l of elution buffer. RNAs in immunocomplexes were isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. PCR was carried out as described in Materials and Methods with primer sets amplifying the fragments of nt 129 to 2367 and nt 7267 to 9463 of the JFH-1 genome. One-tenth (10 μ l) of each eluted immunoprecipitate was used for assays of the core protein amounts to ensure IP efficiency (lower panel). RNA extracted from a small aliquot of each cell lysate used in IP-RT-PCR is shown as the input.

is involved in basal phosphorylation (23). There are highly conserved serine residues in this region, and alanine substitutions or in-frame deletion of the serine residues has been shown to impair basal phosphorylation but not to affect RNA replication in the genotype 1b isolate (1). Consistently, a metabolic ³²P labeling experiment in the present study demonstrated that NS5A mutants of the JFH-1 isolate in the region impair the basal phosphorylation. Nevertheless, Tellinghuisen et al. noted that the serine at aa 2433 of JFH-1 is involved in generating hyperphosphorylated NS5A, as shown by Western blotting (50). The basis for this difference is uncertain. To date, there is no clear evidence to determine which serine residues located in domain III are phosphoacceptor sites or whether these residues influence NS5A phosphorylation in an indirect fashion. Future study to map phosphoacceptor sites in the NS5A domain III by biochemical approaches is needed.

We found that two of the three serine residues at CL3B are responsible for regulating the interaction of NS5A with the core protein as well as for infectious virus production. To further evaluate the effect of constitutive serine phosphorylation at the cluster, we replaced the serine residues with glu-

tamic acid, which mimics the presence of phosphoserines. The S2428/2430E mutant led to restoration of the interaction of NS5A with the core protein and virus production up to levels similar to the wild type. Somewhat unexpectedly, the triple glutamic acid substitution (CL3B/SE) exhibited only a slight restoration effect or none at all. It is considered that the degree of negative charge on the glutamic acid residue is not completely equivalent to that of phosphoserine. It is likely that the range of acidity at the local environment of the NS5A domain III that will allow interaction with the core protein is rather narrow. Induction of a conformational change in NS5A by the incorporation of phosphate may also be important for its interaction with the core protein. Tellinghuisen et al. reported that a single serine-to-alanine substitution at aa 2433 blocks the production of infectious virus and that casein kinase II likely phosphorylates the residue (50). Although this seems inconsistent with our results, these investigators also showed that deletions producing a lack of all three serine residues in the cluster inhibited virus production more severely than a single mutation. We observed that a single substitution of S2428A, S2430A, or S2433A resulted in a moderate decrease

in the virus released from the transfected cells; however, more evident perturbation was obtained from double or triple substitutions (Fig. 5A and B). Tellinghuisen et al. determined the HCV production at 48 h after RNA transfection and found a marked inhibition by the single substitution S2433A. In our study, as indicated in Fig. 5A, the reduction caused by the S2433A mutant was approximately 90% at 48 h after transfection; however, the virus production from the mutant reached a similar level to that of the wild type at 96 h posttransfection.

Several previous studies have found that apolipoproteins B (apoB) and E (apoE), microsomal triglyceride transfer protein, and HCV p7 protein are key factors for production of the infectious HCV particles (4, 11, 16, 22, 47). Assembly and maturation of the viral particles appear to depend on the formation of very-low-density lipoprotein, a large particle containing apoB, apoE, and large amounts of neutral lipids in hepatic cells. p7 protein is primarily involved in a late step of virus particle production, and the findings support the idea that p7 acts as viroporin, which has the capacity to compromise cell membrane integrity and thus favors the release of viral progeny. How the early step in virion production regulated by the NSSA-core protein interaction links with the later step(s) involved in the very-low-density lipoprotein assembly or p7 function remains an interesting question to be addressed.

In summary, we demonstrated that the C-terminal serine cluster of NSSA (aa 2428, 2430, and 2433), which is involved in generating the basal phosphorylated form, is a determinant of NSSA interaction with the core protein and the subcellular localization of NSSA. Mutation of this cluster blocks the NSSA-core protein interaction, resulting in perturbation of association between the core protein and HCV RNA. It is thus tempting to consider that NSSA plays a key role in transporting the viral genome RNA synthesized by the replication complex to the surface of LDs or LD-associated membranes, where the core protein localizes, leading to facilitation of nucleocapsid formation. Structural analysis of the NSSA domain III-core protein complex should provide greater insight into the mode of interaction between these viral proteins. Identification of residues at the interface that are involved in important interactions will be of significant value in designing novel structure-based inhibitors to block the early step of HCV particle formation.

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