15. Lindenbach BD, Evans MJ, Syder AJ, *et al.* Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**: 623–626.
16. Tomei L, Failla C, Santolini E, De Francesco R, La Monica N. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J Virol* 1993; **67**: 4017–4026.
17. Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J Virol* 1993; **67**: 2832–2843.
18. Hamill P, Jean F. Enzymatic characterization of membrane-associated hepatitis C virus NS3-4A heterocomplex serine protease activity expressed in human cells. *Biochemistry* 2005; **44**: 6586–6596.
19. Egger D, Wolk B, Gosert R, *et al.* Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* 2002; **76**: 5974–5984.
20. Evans MJ, Rice CM, Goff SP. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc Natl Acad Sci USA* 2004; **101**: 13038–13043.
21. Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. *Science* 2000; **290**: 1972–1974.
22. Behrens SE, Tomei L, De Francesco R. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J* 1996; **15**: 12–22.
23. Hamamoto I, Nishimura Y, Okamoto T, *et al.* Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. *J Virol* 2005; **79**: 13473–13482.
24. Tu H, Gao L, Shi ST, *et al.* Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. *Virology* 1999; **263**: 30–41.
25. Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T, Schmid FX. Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. *Nature* 1989; **337**: 476–478.
26. Siekierka JJ, Hung SH, Poe M, Lin CS, Sigal NH. A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* 1989; **341**: 755–757.
27. Siekierka JJ, Staruch MJ, Hung SH, Sigal NH. FK-506, a potent novel immunosuppressive agent, binds to a cytosolic protein which is distinct from the cyclosporin A-binding protein, cyclophilin. *J Immunol* 1989; **143**: 1580–1583.
28. Rahfeld JU, Schierhorn A, Mann K, Fischer G. A novel peptidyl-prolyl cis/trans isomerase from Escherichia coli. *FEBS Lett* 1994; **343**: 65–69.
29. Schiene-Fischer C, Habazettl J, Schmid FX, Fischer G. The hsp70 chaperone DnaK is a secondary amide peptide bond cis-trans isomerase. *Nat Struct Biol* 2002; **9**: 419–424.
30. Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 1991; **66**: 807–815.
31. Luban J, Bossolt KL, Franke EK, Kalpana GV, Goff SP. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* 1993; **73**: 1067–1078.
32. Wiegers K, Rutter G, Schubert U, Grattinger M, Krausslich HG. Cyclophilin A incorporation is not required for human immunodeficiency virus type 1 particle maturation and does not destabilize the mature capsid. *Virology* 1999; **257**: 261–274.
33. Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 2004; **427**: 848–853.
34. Sayah DM, Sokolskaja E, Berthoux L, Luban J. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature* 2004; **430**: 569–573.
35. Caroni P, Rothenfluh A, McGlynn E, Schneider C. S-cyclophilin. New member of the cyclophilin family associated with the secretory pathway. *J Biol Chem* 1991; **266**: 10739–10742.
36. Spik G, Haendler B, Delmas O, *et al.* A novel secreted cyclophilin-like protein (SCYLP). *J Biol Chem* 1991; **266**: 10735–10738.
37. Allain F, Denys A, Spik G. Characterization of surface binding sites for cyclophilin B on a human tumor T-cell line. *J Biol Chem* 1994; **269**: 16537–16540.
38. Rycyzyn MA, Reilly SC, O'Malley K, Clevenger CV. Role of cyclophilin B in prolactin signal transduction and nuclear retrotranslocation. *Mol Endocrinol* 2000; **14**: 1175–1186.
39. Cohen E, Taraboulos A. Scrapie-like prion protein accumulates in aggresomes of cyclosporin A-treated cells. *EMBO J* 2003; **22**: 404–417.
40. Ishii N, Watashi K, Hishiki T, *et al.* Diverse effects of cyclosporine on hepatitis C virus strain replication. *J Virol* 2006; **80**: 4510–4520.
41. Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* 2003; **38**: 1282–1288.
42. Inoue K, Sekiyama K, Yamada M, Watanabe T, Yasuda H, Yoshiba M. Combined interferon alpha2b and cyclosporin A in the treatment of chronic hepatitis C: controlled trial. *J Gastroenterol* 2003; **38**: 567–572.
43. Lam E, Martin M, Wiederrecht G. Isolation of a cDNA encoding a novel human FK506-binding

protein homolog containing leucine zipper and tetratricopeptide repeat motifs. *Gene* 1995; **160**: 297–302.
44. Edlich F, Weiwad M, Erdmann F, *et al.* Bcl-2 regulator FKBP38 is activated by Ca2+/calmodulin. *EMBO J* 2005; **24**: 2688–2699.
45. Edlich F, Weiwad M, Wildemann D, *et al.* The specific FKBP38 inhibitor N-(N′,N′-dimethylcarboxamidomethyl)cycloheximide has potent neuroprotective and neurotrophic properties in brain ischemia. *J Biol Chem* 2006; **281**: 14961–14970.
46. Nielsen JV, Mitchelmore C, Pedersen KM, Kjaerulff KM, Finsen B, Jensen NA. Fkbp8: novel isoforms, genomic organization, and characterization of a forebrain promoter in transgenic mice. *Genomics* 2004; **83**: 181–192.
47. Bulgakov OV, Eggenschwiler JT, Hong DH, Anderson KV, Li T. FKBP8 is a negative regulator of mouse sonic hedgehog signaling in neural tissues. *Development* 2004; **131**: 2149–2159.
48. Echelard Y, Epstein DJ, St-Jacques B, *et al.* Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 1993; **75**: 1417–1430.
49. Chang DT, Lopez A, von Kessler DP, *et al.* Products, genetic linkage and limb patterning activity of a murine hedgehog gene. *Development* 1994; **120**: 3339–3353.
50. Briscoe J, Ericson J. Specification of neuronal fates in the ventral neural tube. *Curr Opin Neurobiol* 2001; **11**: 43–49.
51. Chiang C, Litingtung Y, Lee E, *et al.* Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 1996; **383**: 407–413.
52. Chen Y, Struhl G. Dual roles for patched in sequestering and transducing Hedgehog. *Cell* 1996; **87**: 553–563.
53. Marigo V, Davey RA, Zuo Y, Cunningham JM, Tabin CJ. Biochemical evidence that patched is the Hedgehog receptor. *Nature* 1996; **384**: 176–179.
54. Price MA, Kalderon D. Proteolysis of cubitus interruptus in Drosophila requires phosphorylation by protein kinase A. *Development* 1999; **126**: 4331–4339.
55. Ohlmeyer JT, Kalderon D. Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature* 1998; **396**: 749–753.
56. Wijgerde M, McMahon JA, Rule M, McMahon AP. A direct requirement for Hedgehog signaling for normal specification of all ventral progenitor domains in the presumptive mammalian spinal cord. *Genes Dev* 2002; **16**: 2849–2864.
57. Goodrich LV, Milenkovic L, Higgins KM, Scott MP. Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* 1997; **277**: 1109–1113.
58. Shirane M, Nakayama KI. Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. *Nat Cell Biol* 2003; **5**: 28–37.
59. Waxman L, Whitney M, Pollok BA, Kuo LC, Darke PL. Host cell factor requirement for hepatitis C virus enzyme maturation. *Proc Natl Acad Sci USA* 2001; **98**: 13931–13935.
60. Naito T, Momose F, Kawaguchi A, Nagata K. Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. *J Virol* 2007; **81**: 1339–1349.
61. Momose F, Naito T, Yano K, Sugimoto S, Morikawa Y, Nagata K. Identification of Hsp90 as a stimulatory host factor involved in influenza virus RNA synthesis. *J Biol Chem* 2002; **277**: 45306–45314.
62. Burch AD, Weller SK. Herpes simplex virus type 1 DNA polymerase requires the mammalian chaperone hsp90 for proper localization to the nucleus. *J Virol* 2005; **79**: 10740–10749.
63. Kampmueller KM, Miller DJ. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells. *J Virol* 2005; **79**: 6827–6837.
64. Connor JH, McKenzie MO, Parks GD, Lyles DS. Antiviral activity and RNA polymerase degradation following Hsp90 inhibition in a range of negative strand viruses. *Virology* 2007; **362**: 109–117.
65. Hu J, Toft DO, Seeger C. Hepadnavirus assembly and reverse transcription require a multi-component chaperone complex which is incorporated into nucleocapsids. *EMBO J* 1997; **16**: 59–68.
66. Hu J, Seeger C. Hsp90 is required for the activity of a hepatitis B virus reverse transcriptase. *Proc Natl Acad Sci USA* 1996; **93**: 1060–1064.
67. Geller R, Vignuzzi M, Andino R, Frydman J. Evolutionary constraints on chaperone-mediated folding provide an antiviral approach refractory to development of drug resistance. *Genes Dev* 2007; **21**: 195–205.
68. Skehel PA, Martin KC, Kandel ER, Bartsch D. A VAMP-binding protein from Aplysia required for neurotransmitter release. *Science* 1995; **269**: 1580–1583.
69. Nishimura Y, Hayashi M, Inada H, Tanaka T. Molecular cloning and characterization of mammalian homologues of vesicle-associated membrane protein-associated (VAMP-associated) proteins. *Biochem Biophys Res Commun* 1999; **254**: 21–26.
70. Skehel PA, Fabian-Fine R, Kandel ER. Mouse VAP33 is associated with the endoplasmic reticulum and microtubules. *Proc Natl Acad Sci USA* 2000; **97**: 1101–1106.
71. Soussan L, Burakov D, Daniels MP, *et al.* ERG30, a VAP-33-related protein, functions in protein trans-

port mediated by COPI vesicles. *J Cell Biol* 1999; **146**: 301–311.
72. Kagiwada S, Hosaka K, Murata M, Nikawa J, Takatsuki A. The Saccharomyces cerevisiae SCS2 gene product, a homolog of a synaptobrevin-associated protein, is an integral membrane protein of the endoplasmic reticulum and is required for inositol metabolism. *J Bacteriol* 1998; **180**: 1700–1708.
73. Weir ML, Klip A, Trimble WS. Identification of a human homologue of the vesicle-associated membrane protein (VAMP)-associated protein of 33 kDa (VAP-33): a broadly expressed protein that binds to VAMP. *Biochem J* 1998; **333**(Pt 2): 247–251.
74. Nelson GA, Ward S. Amoeboid motility and actin in Ascaris lumbricoides sperm. *Exp Cell Res* 1981; **131**: 149–160.
75. Italiano JE Jr, Stewart M, Roberts TM. How the assembly dynamics of the nematode major sperm protein generate amoeboid cell motility. *Int Rev Cytol* 2001; **202**: 1–34.
76. Roberts TM, Stewart M. Acting like actin. The dynamics of the nematode major sperm protein (msp) cytoskeleton indicate a push-pull mechanism for amoeboid cell motility. *J Cell Biol* 2000; **149**: 7–12.
77. Tarr DE, Scott AL. MSP domain proteins. *Trends Parasitol* 2005; **21**: 224–231.
78. Loewen CJ, Roy A, Levine TP. A conserved ER targeting motif in three families of lipid binding proteins and in Opi1p binds VAP. *EMBO J* 2003; **22**: 2025–2035.
79. Pennetta G, Hiesinger PR, Fabian-Fine R, Meinertzhagen IA, Bellen HJ. Drosophila VAP-33A directs bouton formation at neuromuscular junctions in a dosage-dependent manner. *Neuron* 2002; **35**: 291–306.
80. Weir ML, Xie H, Klip A, Trimble WS. VAP-A binds promiscuously to both v- and tSNAREs. *Biochem Biophys Res Commun* 2001; **286**: 616–621.
81. Kaiser SE, Brickner JH, Reilein AR, Fenn TD, Walter P, Brunger AT. Structural basis of FFAT motif-mediated ER targeting. *Structure* 2005; **13**: 1035–1045.
82. Brickner JH, Walter P. Gene recruitment of the activated INO1 locus to the nuclear membrane. *PLoS Biol* 2004; **2**: e342.
83. Funakoshi T, Yasuda S, Fukasawa M, Nishijima M, Hanada K. Reconstitution of ATP- and cytosol-dependent transport of de novo synthesized ceramide to the site of sphingomyelin synthesis in semi-intact cells. *J Biol Chem* 2000; **275**: 29938–29945.
84. Fukasawa M, Nishijima M, Hanada K. Genetic evidence for ATP-dependent endoplasmic reticulum-to-Golgi apparatus trafficking of ceramide for sphingomyelin synthesis in Chinese hamster ovary cells. *J Cell Biol* 1999; **144**: 673–685.
85. Kawano M, Kumagai K, Nishijima M, Hanada K. Efficient trafficking of ceramide from the endoplasmic reticulum to the Golgi apparatus requires a VAMP-associated protein-interacting FFAT motif of CERT. *J Biol Chem* 2006; **281**: 30279–30288.
86. Quintavalle M, Sambucini S, Summa E, *et al.* Hepatitis C virus NS5A is a direct substrate of CKI-alpha, a cellular kinase identified by inhibitor affinity chromatography using specific NS5A hyperphosphorylation inhibitors. *J Biol Chem* 2007; **282**: 5536–5544.
87. Neddermann P, Quintavalle M, Di Pietro C, *et al.* Reduction of hepatitis C virus NS5A hyperphosphorylation by selective inhibition of cellular kinases activates viral RNA replication in cell culture. *J Virol* 2004; **78**: 13306–13314.
88. Burckstummer T, Kriegs M, Lupberger J, Pauli EK, Schmittel S, Hildt E. Raf-1 kinase associates with Hepatitis C virus NS5A and regulates viral replication. *FEBS Lett* 2006; **580**: 575–580.
89. Gao L, Aizaki H, He JW, Lai MM. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. *J Virol* 2004; **78**: 3480–3488.
90. Shi ST, Lee KJ, Aizaki H, Hwang SB, Lai MM. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. *J Virol* 2003; **77**: 4160–4168.
91. Zhang J, Yamada O, Sakamoto T, *et al.* Down-regulation of viral replication by adenoviral-mediated expression of siRNA against cellular cofactors for hepatitis C virus. *Virology* 2004; **320**: 135–143.
92. Amarilio R, Ramachandran S, Sabanay H, Lev S. Differential regulation of endoplasmic reticulum structure through VAP-Nir protein interaction. *J Biol Chem* 2005; **280**: 5934–5944.
93. Giese SI, Woerz I, Homann S, Tibroni N, Geyer M, Fackler OT. Specific and distinct determinants mediate membrane binding and lipid raft incorporation of HIV-1(SF2) Nef. *Virology* 2006; **355**: 175–191.
94. Brugger B, Glass B, Haberkant P, Leibrecht I, Wieland FT, Krausslich HG. The HIV lipidome: a raft with an unusual composition. *Proc Natl Acad Sci USA* 2006; **103**: 2641–2646.
95. Mannova P, Fang R, Wang H, *et al.* Modification of host lipid raft proteome upon hepatitis C virus replication. *Mol Cell Proteomics* 2006; **5**: 2319–2325.
96. Oomens AG, Bevis KP, Wertz GW. The cytoplasmic tail of the human respiratory syncytial virus F protein plays critical roles in cellular localization of the F protein and infectious progeny production. *J Virol* 2006; **80**: 10465–10477.
97. Chen BJ, Takeda M, Lamb RA. Influenza virus hemagglutinin (H3 subtype) requires palmitoylation of its cytoplasmic tail for assembly: M1 pro-

teins of two subtypes differ in their ability to support assembly. *J Virol* 2005; **79**: 13673–13684.
98. Kapadia SB, Chisari FV. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc Natl Acad Sci USA* 2005; **102**: 2561–2566.
99. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 2002; **109**: 1125–1131.
100. Barba G, Harper F, Harada T, *et al.* Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc Natl Acad Sci USA* 1997; **94**: 1200–1205.
101. Moriishi K, Mochizuki R, Moriya K, *et al.* Critical role of PA28gamma in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. *Proc Natl Acad Sci USA* 2007; **104**: 1661–1666.
102. Aizaki H, Lee KJ, Sung VM, Ishiko H, Lai MM. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* 2004; **324**: 450–461.
103. Ye J, Wang C, Sumpter R Jr, Brown MS, Goldstein JL, Gale M Jr. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. *Proc Natl Acad Sci USA* 2003; **100**: 15865–15870.
104. Wang C, Gale M Jr, Keller BC, *et al.* Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Mol Cell* 2005; **18**: 425–434.
105. Ilyin GP, Rialland M, Glaise D, Guguen-Guillouzo C. Identification of a novel Skp2-like mammalian protein containing F-box and leucine-rich repeats. *FEBS Lett* 1999; **459**: 75–79.
106. Shimakami T, Honda M, Kusakawa T, *et al.* Effect of hepatitis C virus (HCV) NS5B-nucleolin interaction on HCV replication with HCV subgenomic replicon. *J Virol* 2006; **80**: 3332–3340.
107. Goh PY, Tan YJ, Lim SP, *et al.* Cellular RNA helicase p68 relocalization and interaction with the hepatitis C virus (HCV) NS5B protein and the potential role of p68 in HCV RNA replication. *J Virol* 2004; **78**: 5288–5298.
108. Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM. Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. *J Virol* 2006; **80**: 7199–7207.
109. Kawamura H, Govindarajan S, Aswad F, *et al.* HCV core expression in hepatocytes protects against autoimmune liver injury and promotes liver regeneration in mice. *Hepatology* 2006; **44**: 936–944.
110. Yoshida T, Hanada T, Tokuhisa T, *et al.* Activation of STAT3 by the hepatitis C virus core protein leads to cellular transformation. *J Exp Med* 2002; **196**: 641–653.
111. Ogata H, Kobayashi T, Chinen T, *et al.* Deletion of the SOCS3 gene in liver parenchymal cells promotes hepatitis-induced hepatocarcinogenesis. *Gastroenterology* 2006; **131**: 179–193.
112. Moriya K, Fujie H, Shintani Y, *et al.* The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998; **4**: 1065–1067.
113. Lerat H, Honda M, Beard MR, *et al.* Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 2002; **122**: 352–365.
114. Koike K, Moriya K, Ishibashi K, *et al.* Sialadenitis histologically resembling Sjogren syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc Natl Acad Sci USA* 1997; **94**: 233–236.

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Intramembrane Processing by Signal Peptide Peptidase Regulates the Membrane Localization of Hepatitis C Virus Core Protein and Viral Propagation▽

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Hepatitis C virus (HCV) core protein has shown to be localized in the detergent-resistant membrane (DRM), which is distinct from the classical raft fraction including caveolin, although the biological significance of the DRM localization of the core protein has not been determined. The HCV core protein is cleaved off from a precursor polyprotein at the lumen side of Ala[191] by signal peptidase and is then further processed by signal peptide peptidase (SPP) within the transmembrane region. In this study, we examined the role of SPP in the localization of the HCV core protein in the DRM and in viral propagation. The C terminus of the HCV core protein cleaved by SPP in 293T cells was identified as Phe[177] by mass spectrometry. Mutations introduced into two residues (Ile[176] and Phe[177]) upstream of the cleavage site of the core protein abrogated processing by SPP and localization in the DRM fraction. Expression of a dominant-negative SPP or treatment with an SPP inhibitor, L685,458, resulted in reductions in the levels of processed core protein localized in the DRM fraction. The production of HCV RNA in cells persistently infected with strain JFH-1 was impaired by treatment with the SPP inhibitor. Furthermore, mutant JFH-1 viruses bearing SPP-resistant mutations in the core protein failed to propagate in a permissive cell line. These results suggest that intramembrane processing of HCV core protein by SPP is required for the localization of the HCV core protein in the DRM and for viral propagation.

The hepatitis C virus (HCV), which has infected an estimated 170 million people worldwide, leads to chronic hepatitis, which in turn causes severe liver diseases, including steatosis, cirrhosis, and eventually hepatocellular carcinoma (47). HCV possesses a positive-sense single-stranded RNA with a nucleotide length of 9.6 kb, which encodes a single large precursor polyprotein composed of about 3,000 amino acids. The viral polyprotein is processed by cellular and viral proteases into structural and nonstructural proteins (24). The development of efficient therapies for hepatitis C had been hampered by the lack of a reliable cell culture system, as well as by the absence of a small-animal model. Lohmann et al. established an HCV replicon, which consisted of an antibiotic selection marker and a genotype 1b HCV RNA, and showed that it replicated autonomously in the intracellular compartments of a human hepatoma cell line, Huh7 (16). The replicon system has been used as an important tool in the investigation of HCV replication, and it has served as a cell-based assay system for the evaluation of antiviral compounds. Recently, cell culture systems for in vitro replication and infectious-virus production were established based on the full-length HCV genome of a genotype 2a isolate, which was recovered from a fulminant hepatitis C patient (15, 45, 50). However, the molecular mechanism of the HCV life cycle in host cells has not been well characterized.

Several viruses have been reported to utilize a lipid raft composed of cholesterol and sphingolipids upon entry (34). The lipid raft is characterized by resistance to nonionic detergents at 4°C and includes caveolin, glycolipids, and other substances (40). Several nonenveloped viruses enter cells through a caveola/raft-mediated endosome, designated the caveosome, and then translocate to the endoplasmic reticulum (ER), endosome, or nucleus (34, 35), although enveloped viruses generally enter host cells through a clathrin-dependent pathway (18). HCV is enclosed by a host cell-derived membrane and belongs to the family *Flaviviridae*. Several reports suggest that HCV enters host cells through general endocytosis, such as by a clathrin-mediated pathway (5, 6, 22). However, HCV has been suggested to replicate on a detergent-resistant membrane (DRM), including some characteristic membrane structures such as lipid rafts and membranous webs (8, 9, 38). In a previous report, an HCV replication complex prepared from a cell fraction treated with a nonionic detergent was shown to be enzymatically active (2). HCV nonstructural proteins remodel the intracellular membrane to form a replication complex that includes several host proteins (8, 46). The HCV core protein has a C-terminal transmembrane region that is anchored on intracellular compartments such as the ER and mitochondria and on the surfaces of lipid droplets (10, 30, 42). Recent studies have indicated that assembly of HCV particles occurs around lipid droplets that are surrounded by the remodeled membranes (23). Although the HCV core protein functions as a capsid protein, it is found in the DRM fraction, which is

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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 polI 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 pcDNA3.1 (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 polI 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^{144} were replaced with Ala (JFH-1/VVL/3A); $Ile^{176}$and Phe^{177} were replaced with Ala and Leu, respectively (JFH-1/IF/AL); Ala^{180}, Ser^{183}, and Cys^{184} were replaced with Val, Leu, and Val, respectively (JFH-1/ASC/VLV); and Asp^{2736} 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

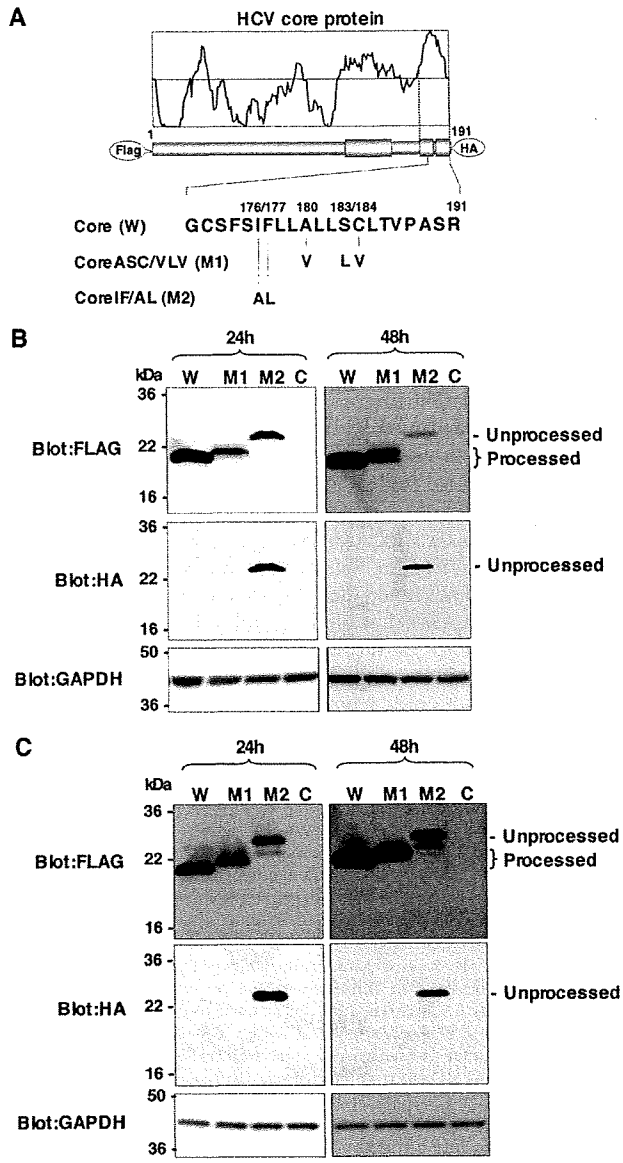
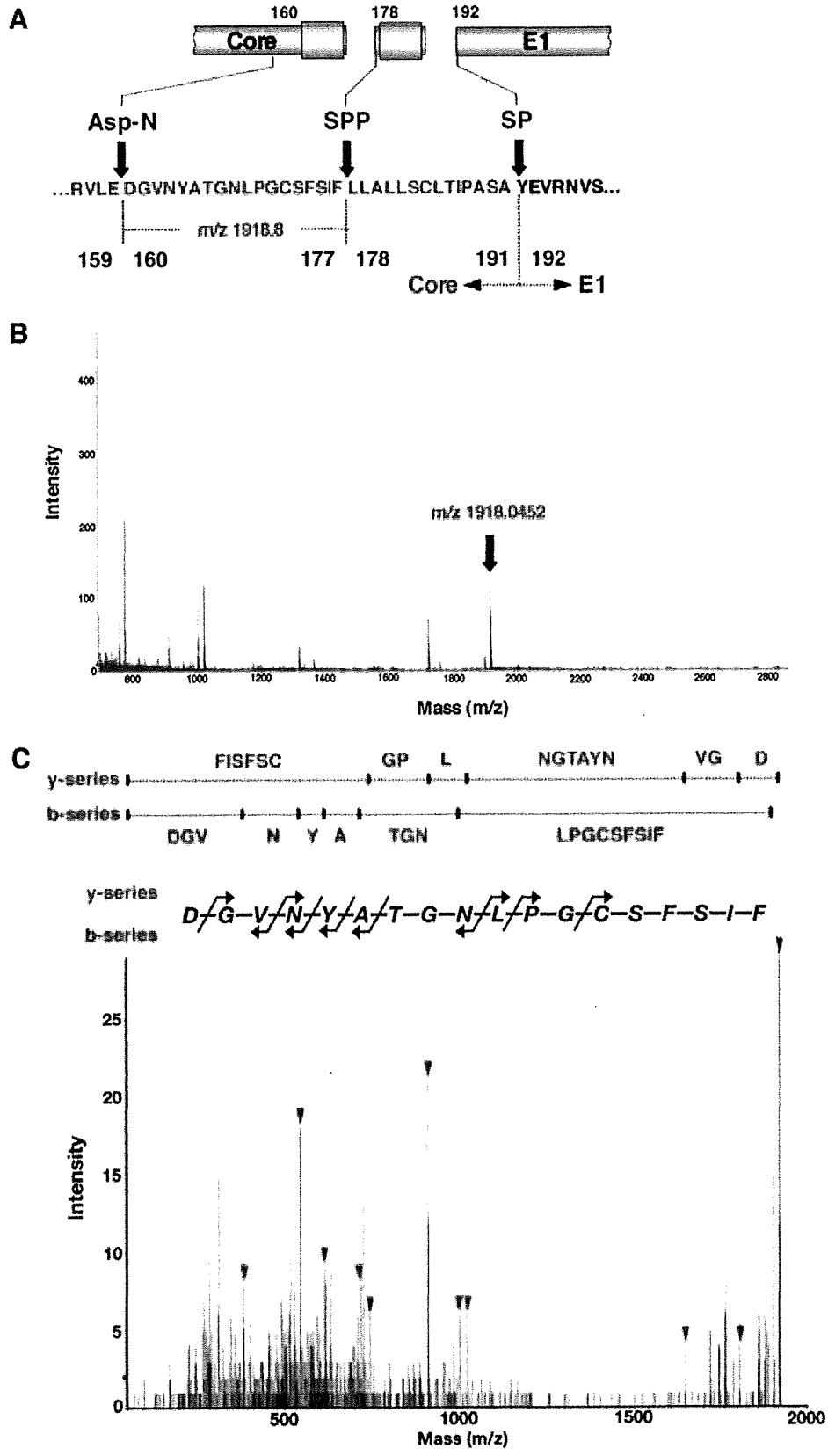


FIG. 1. Effects of mutations in the HCV core protein on cleavage by SPP. (A) cDNA constructs encoding the N-terminally FLAG- and C-terminally HA-tagged wild-type HCV core protein (W), Core ASC/VLV (M1), and Core IF/AL (M2). The Ala at amino acid residue 191 of all constructs was mutated to Arg in order 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

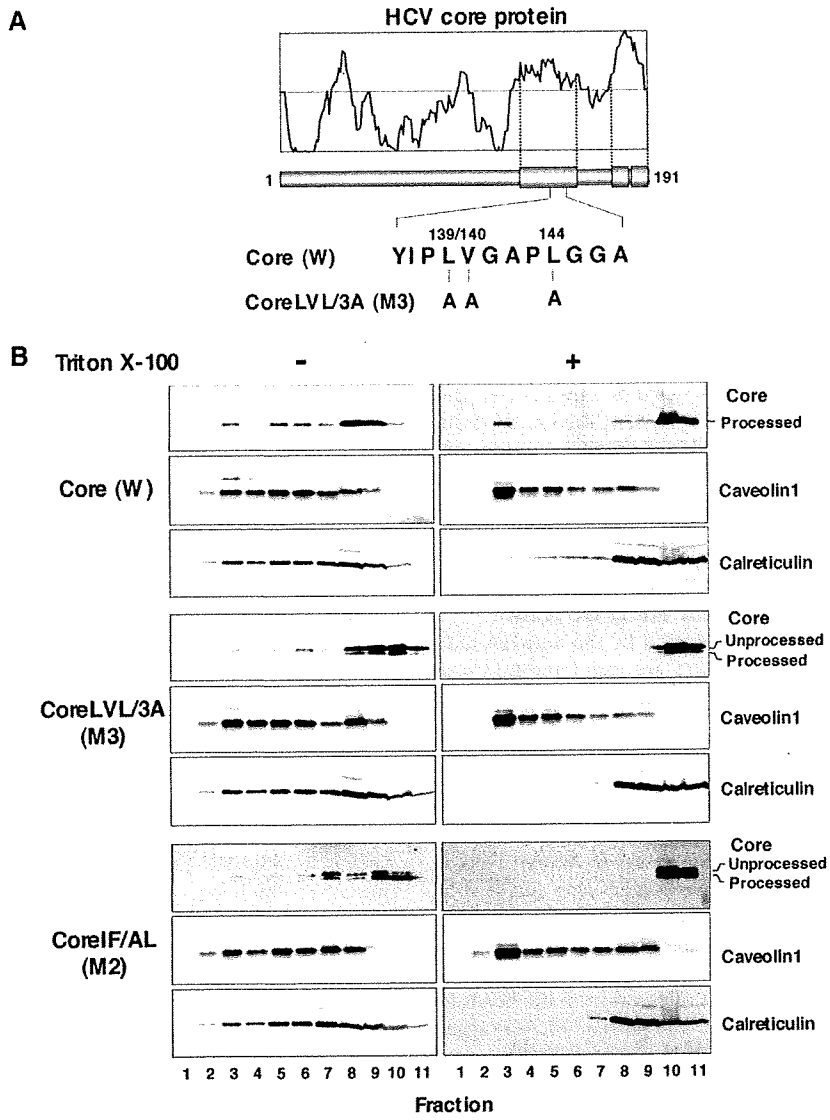


FIG. 3. HCV core protein partially migrates to the DRM after SPP processing. (A) cDNAs encoding authentic wild-type (W) and Core LVL/3A (M3) HCV core proteins. (B) Each plasmid was transfected into 293T cells, lysed with or without 1% Triton X-100, and then subjected to a flotation assay. Proteins in 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

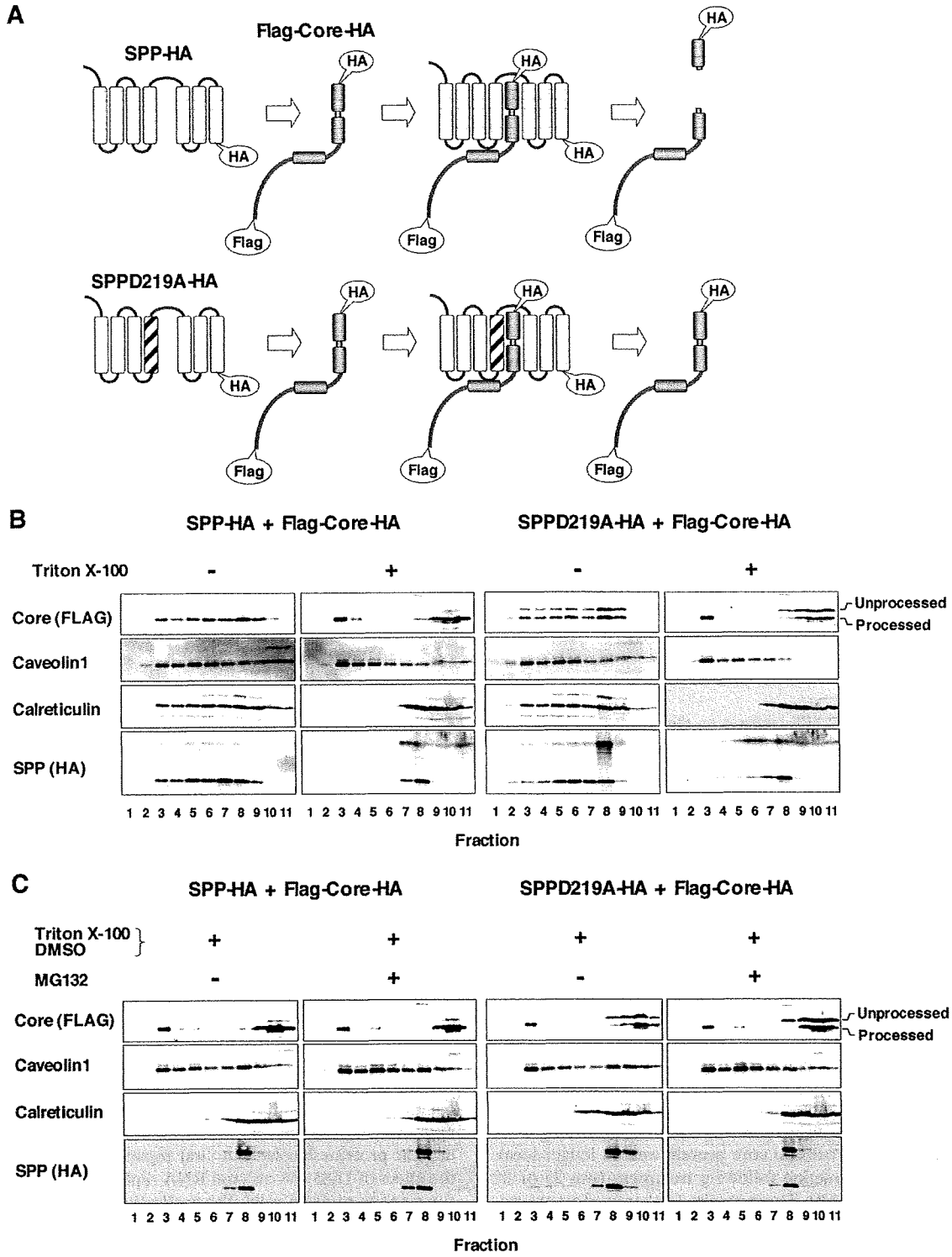


FIG. 4. The dominant-negative mutant of SPP prevents the cleavage of HCV core protein by SPP and its localization in the DRM. (A) Schematic representation of the processing of FLAG-core-HA by a wild-type SPP (SPP-HA) (top) and the dominant-negative effect of SPPD219A-HA (bottom). (B) FLAG-core-HA was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

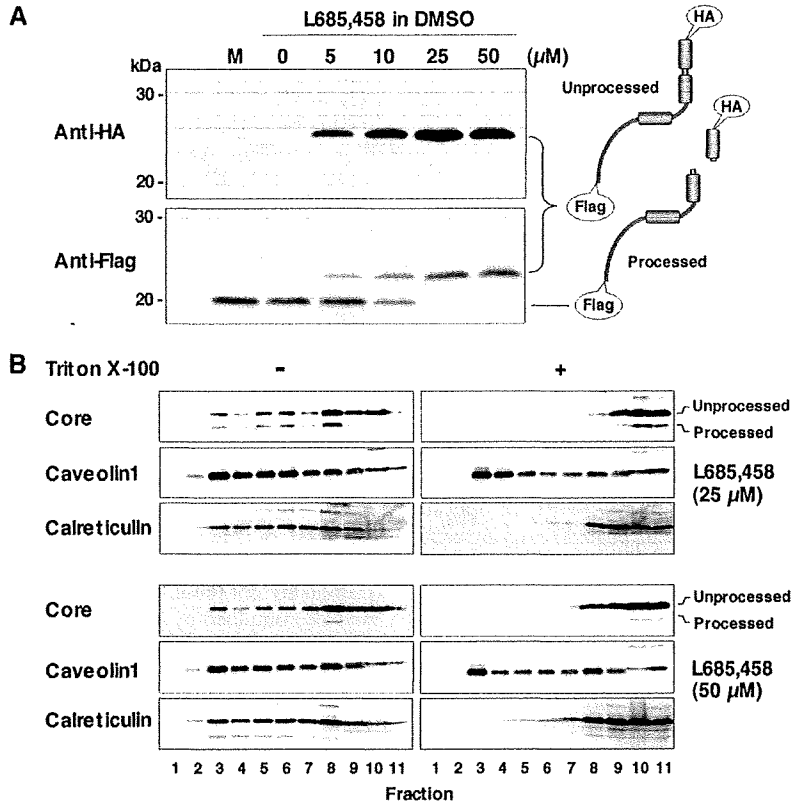


FIG. 5. Effect of an SPP inhibitor on the cleavage of HCV core protein by SPP. The HCV core protein was expressed in 293T cells, and L685,458 was added to the culture supernatant, at the indicated concentrations, at 5 h posttransfection. Cells harvested at 29 h posttransfection were lysed with 1% Triton 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

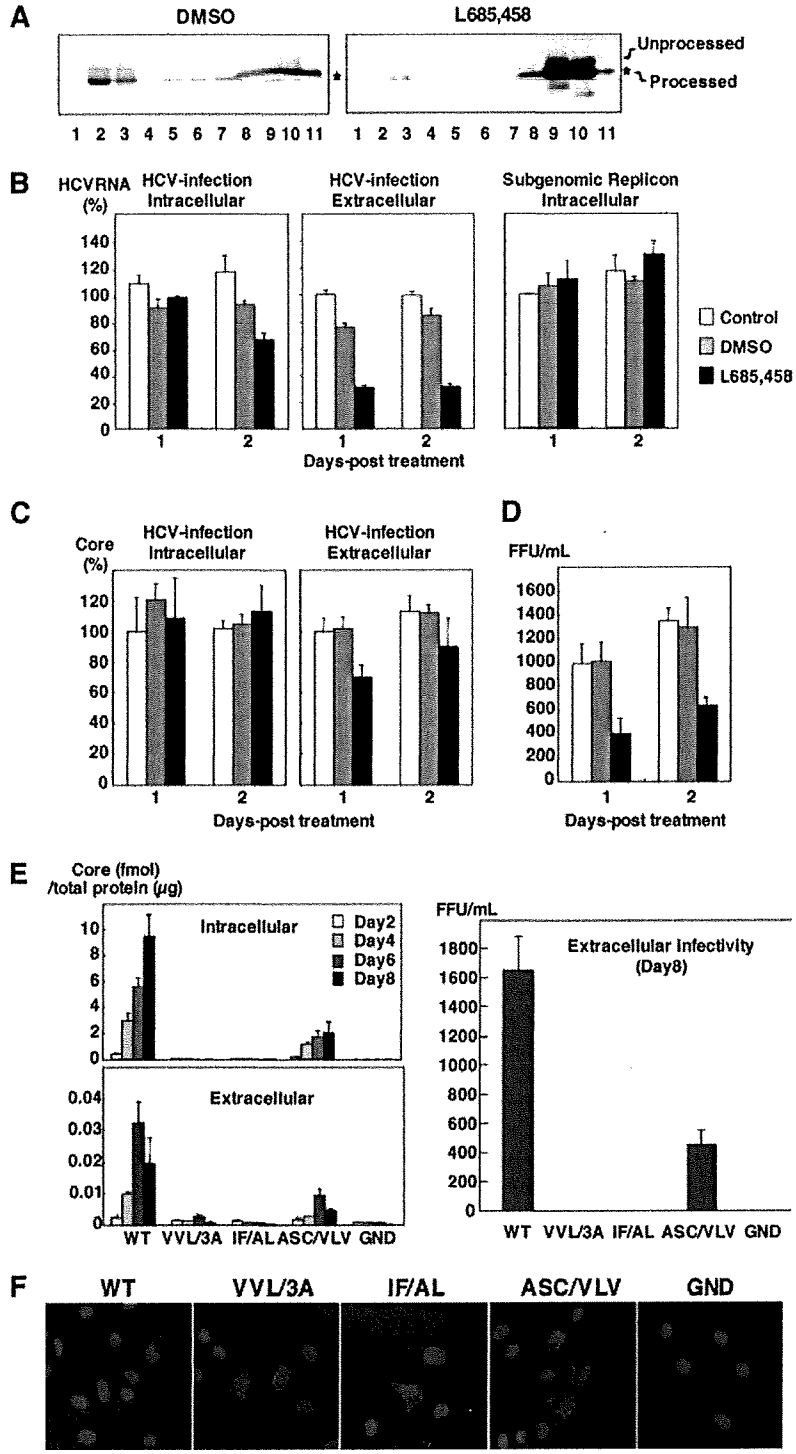


FIG. 6. Effect of the processing of HCV core protein by SPP on the propagation of JFH-1 virus. (A) L685,458 was added, at a concentration of 25 μM, to the culture supernatant of Huh7OK1 cells persistently infected with HCV strain JFH-1. Cells harvested at 24 h after treatment were lysed with 1% Triton 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^{178}. 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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REFERENCES

1. **Ait-Goughoulte, M., C. Hourioux, R. Patient, S. Trassard, D. Brand, and P. Roingeard.** 2006. Core protein cleavage by signal peptide peptidase is required for hepatitis C virus-like particle assembly. J. Gen. Virol. **87**:855–860.
2. **Aizaki, H., K. J. Lee, V. M. Sung, H. Ishiko, and M. M. Lai.** 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology **324**:450–461.
3. **Aizaki, H., K. Morikawa, M. Fukasawa, H. Hara, Y. Inoue, H. Tani, K. Saito, M. Nishijima, K. Hanada, Y. Matsuura, M. Lai, T. Miyamura, T. Wakita, and T. Suzuki.** 2008. A critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J. Virol. **82**:5715–5724.
4. **Barba, G., F. Harper, T. Harada, M. Kohara, S. Goulinet, Y. Matsuura, G. Eder, Z. Schaff, M. J. Chapman, T. Miyamura, and C. Brechot.** 1997. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc. Natl. Acad. Sci. USA **94**:1200–1205.
5. **Blanchard, E., S. Belouzard, L. Goueslain, T. Wakita, J. Dubuisson, C. Wychowski, and Y. Rouille.** 2006. Hepatitis C virus entry depends on clathrin-mediated endocytosis. J. Virol. **80**:6964–6972.
6. **Codran, A., C. Royer, D. Jaeck, M. Bastien-Valle, T. F. Baumert, M. P. Kieny, C. A. Pereira, and J. P. Martin.** 2006. Entry of hepatitis C virus pseudotypes into primary human hepatocytes by clathrin-dependent endocytosis. J. Gen. Virol. **87**:2583–2593.
7. **Dubuisson, J., S. Duvet, J. C. Meunier, A. Op De Beeck, R. Cacan, C. Wychowski, and L. Cocquerel.** 2000. Glycosylation of the hepatitis C virus envelope protein E1 is dependent on the presence of a downstream sequence on the viral polyprotein. J. Biol. Chem. **275**:30605–30609.
8. **Gao, L., H. Aizaki, J. W. He, and M. M. Lai.** 2004. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. J. Virol. **78**:3480–3488.
9. **Gosert, R., D. Egger, V. Lohmann, R. Bartenschlager, H. E. Blum, K. Bienz, and D. Moradpour.** 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J. Virol. **77**:5487–5492.
10. **Hope, R. G., M. J. McElwee, and J. McLauchlan.** 2006. Efficient cleavage by signal peptide peptidase requires residues within the signal peptide between the core and E1 proteins of hepatitis C virus strain J1. J. Gen. Virol. **87**:623–627.
11. **Horton, J. D., J. L. Goldstein, and M. S. Brown.** 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J. Clin. Investig. **109**:1125–1131.
12. **Hussy, P., H. Langen, J. Mous, and H. Jacobsen.** 1996. Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. Virology **224**:93–104.
13. **Kapadia, S. B., and F. V. Chisari.** 2005. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. Proc. Natl. Acad. Sci. USA **102**:2561–2566.
14. **Lecat, S., P. Verkade, C. Thiele, K. Fiedler, K. Simons, and F. Lafont.** 2000. Different properties of two isoforms of annexin XIII in MDCK cells. J. Cell Sci. **113**:2607–2618.
15. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309**:623–626.
16. **Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285**:110–113.
17. **Majeau, N., V. Gagne, M. Bolduc, and D. Leclerc.** 2005. Signal peptide peptidase promotes the formation of hepatitis C virus non-enveloped particles and is captured on the viral membrane during assembly. J. Gen. Virol. **86**:3055–3064.
18. **Marsh, M., and A. Helenius.** 2006. Virus entry: open sesame. Cell **124**:729–740.
19. **Masaki, T., R. Suzuki, M. Matsuda, T. Miyamura, T. Wakita, and T. Suzuki.** 2006. Production of infectious hepatitis C virus by using RNA polymerase I-mediated transcription, abstr. 209, p. 59. *In* Abstracts of the 13th International Meeting on Hepatitis C Virus and Related Viruses. Nola Miles-Clark, Cairns, Australia.
20. **Matto, M., C. M. Rice, B. Aroeti, and J. S. Glenn.** 2004. Hepatitis C virus core protein associates with detergent-resistant membranes distinct from classical plasma membrane rafts. J. Virol. **78**:12047–12053.
21. **McLauchlan, J., M. K. Lemberg, G. Hope, and B. Martoglio.** 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J. **21**:3980–3988.
22. **Meertens, L., C. Bertaux, and T. Dragic.** 2006. Hepatitis C virus entry requires a critical post-internalization step and delivery to early endosomes via clathrin coated vesicles. J. Virol. **80**:11571–11578.
23. **Miyanari, Y., K. Atsuzawa, N. Usuda, K. Watashi, T. Hishiki, M. Zayas, R. Bartenschlager, T. Wakita, M. Hijikata, and K. Shimotohno.** 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. **9**:1089–1097.
24. **Moriishi, K., and Y. Matsuura.** 2003. Mechanisms of hepatitis C virus infection. Antivir. Chem. Chemother. **14**:285–297.
25. **Moriishi, K., R. Mochizuki, K. Moriya, H. Miyamoto, Y. Mori, T. Abe, S. Murata, K. Tanaka, T. Miyamura, T. Suzuki, K. Koike, and Y. Matsuura.** 2007. Critical role of PA28γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. Proc. Natl. Acad. Sci. USA **104**:1661–1666.
26. **Moriishi, K., T. Okabayashi, K. Nakai, K. Moriya, K. Koike, S. Murata, T. Chiba, K. Tanaka, R. Suzuki, T. Suzuki, T. Miyamura, and Y. Matsuura.** 2003. Proteasome activator PA28γ-dependent nuclear retention and degradation of hepatitis C virus core protein. J. Virol. **77**:10237–10249.
27. **Murray, C. L., C. T. Jones, J. Tassello, and C. M. Rice.** 2007. Alanine scanning of the hepatitis C virus core protein reveals numerous residues essential for production of infectious virus. J. Virol. **81**:10220–10231.
28. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108**:193–199.
29. **Ogino, T., H. Fukuda, S. Imajoh-Ohmi, M. Kohara, and A. Nomoto.** 2004. Membrane binding properties and terminal residues of the mature hepatitis C virus capsid protein in insect cells. J. Virol. **78**:11766–11777.
30. **Okamoto, K., K. Moriishi, T. Miyamura, and Y. Matsuura.** 2004. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. J. Virol. **78**:6370–6380.
31. **Okamoto, T., Y. Nishimura, T. Ichimura, K. Suzuki, T. Miyamura, T. Suzuki, K. Moriishi, and Y. Matsuura.** 2006. Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. EMBO J. **25**:5015–5025.
32. **Okamoto, T., H. Omori, Y. Kaname, T. Abe, Y. Nishimura, T. Suzuki, T. Miyamura, T. Yoshimori, K. Moriishi, and Y. Matsuura.** 2008. A single amino acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. J. Virol. **82**:3480–3489.
33. **Okochi, M., S. Eimer, A. Bottcher, R. Baumeister, H. Romig, J. Walter, A. Capell, H. Steiner, and C. Haass.** 2000. A loss of function mutant of the presenilin homologue SEL-12 undergoes aberrant endoproteolysis in *Caenorhabditis elegans* and increases Aβ42 generation in human cells. J. Biol. Chem. **275**:40925–40932.
34. **Pelkmans, L.** 2005. Secrets of caveolae- and lipid raft-mediated endocytosis revealed by mammalian viruses. Biochim. Biophys. Acta **1746**:295–304.
35. **Pelkmans, L., D. Puntener, and A. Helenius.** 2002. Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. Science **296**:535–539.
36. **Randall, G., M. Panis, J. Cooper, T. Tellinghuisen, K. Sukhodolets, S. Pfeffer, M. Landthaler, P. Landgraf, S. Kan, B. Lindenbach, M. Chien, D. Weir, J. Russo, J. Ju, M. Brownstein, R. Sheridan, C. Sander, M. Zavolan, T. Tuschl, and C. Rice.** 2007. Cellular cofactors affecting hepatitis C virus infection and replication. Proc. Natl. Acad. Sci. USA **104**:12884–12889.
37. **Sastre, M., H. Steiner, K. Fuchs, A. Capell, G. Multhaup, M. M. Condron, D. B. Teplow, and C. Haass.** 2001. Presenilin-dependent γ-secretase processing of β-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. EMBO Rep. **2**:835–841.
38. **Shi, S. T., K. J. Lee, H. Aizaki, S. B. Hwang, and M. M. Lai.** 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J. Virol. **77**:4160–4168.
39. **Shirakura, M., K. Murakami, T. Ichimura, R. Suzuki, T. Shimoji, K. Fukuda, K. Abe, S. Sato, M. Fukasawa, Y. Yamakawa, M. Nishijima, K. Moriishi, Y. Matsuura, T. Wakita, T. Suzuki, P. M. Howley, T. Miyamura,**

and I. Shoji. 2007. E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. J. Virol. 81:1174–1185.
40. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. Nature 387:569–572.
41. Suzuki, R., Y. Matsuura, T. Suzuki, A. Ando, J. Chiba, S. Harada, I. Saito, and T. Miyamura. 1995. Nuclear localization of the truncated hepatitis C virus core protein with its hydrophobic C terminus deleted. J. Gen. Virol. 76:53–61.
42. Suzuki, R., S. Sakamoto, T. Tsutsumi, A. Rikimaru, K. Tanaka, T. Shimoike, K. Moriishi, T. Iwasaki, K. Mizumoto, Y. Matsuura, T. Miyamura, and T. Suzuki. 2005. Molecular determinants for subcellular localization of hepatitis C virus core protein. J. Virol. 79:1271–1281.
43. Suzuki, R., K. Tamura, J. Li, K. Ishii, Y. Matsuura, T. Miyamura, and T. Suzuki. 2001. Ubiquitin-mediated degradation of hepatitis C virus core protein is regulated by processing at its carboxyl terminus. Virology 280:301–309.
44. Vauloup-Fellous, C., V. Pene, J. Garaud-Aunis, F. Harper, S. Bardin, Y. Suire, E. Pichard, A. Schmitt, P. Sogni, G. Pierron, P. Briand, and A. R. Rosenberg. 2006. Signal peptide peptidase-catalyzed cleavage of hepatitis C virus core protein is dispensable for virus budding, but destabilizes the viral capsid. J. Biol. Chem. 281:27679–27692.
45. Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11:791–796.
46. Wang, C., M. Gale, Jr., B. C. Keller, H. Huang, M. S. Brown, J. L. Goldstein, and J. Ye. 2005. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. Mol. Cell 18:425–434.
47. Wasley, A., and M. J. Alter. 2000. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin. Liver Dis. 20:1–16.
48. Weihofen, A., K. Binns, M. K. Lemberg, K. Ashman, and B. Martoglio. 2002. Identification of signal peptide peptidase, a presenilin-type aspartic protease. Science 296:2215–2218.
49. Weihofen, A., M. K. Lemberg, E. Friedmann, H. Rueeger, A. Schmitz, P. Paganetti, G. Rovelli, and B. Martoglio. 2003. Targeting presenilin-type aspartic protease signal peptide peptidase with γ-secretase inhibitors. J. Biol. Chem. 278:16528–16533.
50. Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari. 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA 102:9294–9299.

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A Single-Amino-Acid Mutation in Hepatitis C Virus NS5A Disrupting FKBP8 Interaction Impairs Viral Replication▽

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Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) regulates viral replication through its interaction with host and other viral proteins. We have previously shown that FK506-binding protein 8 (FKBP8) binds to NS5A and recruits Hsp90 to form a complex that participates in the replication of HCV. In this study, we examined the biochemical characteristics of the interaction and the intracellular localization of NS5A and FKBP8. Surface plasmon resonance analysis revealed that the dissociation constant of the interaction between the purified FKBP8 and NS5A expressed in bacteria was 82 nM. Mutational analyses of NS5A revealed that a single amino acid residue of Val or Ile at position 121, which is well conserved among all genotypes of HCV, is critical for the specific interaction with FKBP8. Substitution of the Val^{121} to Ala drastically impaired the replication of HCV replicon cells, and the drug-resistant replicon cells emerging after drug selection were shown to have reverted to the original arrangement by replacing Ala^{121} with Val. Examination of individual fields of the replicon cells by both fluorescence microscopy and electron microscopy (the correlative fluorescence microscopy-electron microscopy technique) revealed that FKBP8 is partially colocalized with NS5A in the cytoplasmic structure known as the membranous web. These results suggest that specific interaction of NS5A with FKBP8 in the cytoplasmic compartment plays a crucial role in the replication of HCV.

Hepatitis C virus (HCV) infects more than 170 million people worldwide, a large percentage of whom suffer from persistent infection and severe chronic liver diseases, culminating in cirrhosis and hepatocellular carcinoma (51). Combination therapy with pegylated interferon (IFN) and ribavirin achieves a 40 to 50% sustained virological response in patients infected with genotype 1 HCV (30). Recently, therapeutics have been developed to target the protease and polymerase of HCV, as well as the host factors required for the viral replication (24, 42).

HCV belongs to the *Flaviviridae* family and has a single-stranded positive-sense RNA genome with a nucleotide length of 9.6 kb. The viral genome, translation of which depends on its own internal ribosomal entry site found within the 5′ nontranslated region, encodes a large precursor protein composed of about 3,000 amino acids. The polyprotein is cleaved by host and viral proteases, resulting in viral structural proteins (core, E1, and E2), a putative viropore protein (p7), and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (33). In the last decade, the mechanism by which HCV replicates in the hepatoma cell line Huh-7 has been partially revealed in studies using a cell culture system. The HCV replicon system, which encompasses the autonomously replicable genome of HCV in the Huh-7 cell line or other cell lines derived from it, has been established to accumulate information about the mechanism of HCV replication and to be utilized for screening antiviral drug candidates (27). In addition, the cell culture system for the propagation of infectious HCV particles was developed by using a full-length genome of HCV genotype 2a, JFH1 virus, which was isolated from a fulminant hepatitis C patient (25, 49, 57). However, a robust cell culture system for HCV of genotypes 1a and 1b, the most prevalent genotypes in the world, has not yet been successfully developed, with the exception of the cell culture systems for strains H77 and H77-S of the 1a genotype (21, 56). Furthermore, it is currently impossible to obtain a sufficient amount of HCV particles for biological and physiochemical studies due to the low viral load in the sera of hepatitis C patients and the low yield of HCV particles in the present cell culture system.

HCV NS5A is a membrane-anchored phosphoprotein that appears to possess multiple and diverse functions in viral replication, as well as in the establishment and maintenance of persistent infection (29, 38). Structural analyses suggest that NS5A forms a dimer and has a zinc-binding motif required for replication in the N-terminal domain (45, 46). NS5A has the IFN sensitivity-determining and MyD88-binding regions in the central domain (1, 10), and the SH3-binding region and nuclear localization signal in the C-terminal domain (28, 29). Adaptive mutations of NS5A have frequently been found in the replicon cells exhibiting efficient replication (4, 55). Several host proteins and lipids have been reported to interact with NS5A to upregulate the viral replication. For example, HCV replication was inhibited by treatment with lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, and this inhibition was restored by the addition of geranylgeraniol, suggesting that HCV replication requires geranylgera-

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nylated proteins (22, 54). In addition, the F-box and leucine-rich repeat protein 2 (FBL2) was identified as a binding partner of NS5A, and geranylgeranylation of FBL2 was shown to be required for replication of HCV RNA (50). Vesicle-associated membrane protein (VAMP)-associated protein (VAP) subtype A (VAP-A) and subtype B (VAP-B) were also shown to interact with NS5A and NS5B through the coiled-coil domain and the N-terminal major sperm protein domain, respectively (11, 16, 39).

Immunophilins are known to share the peptidyl prolyl *cis/trans* isomerase activity, thereby basically conserving the ability to interact with immunosuppressive drugs such as cyclosporine and tacrolimus (FK506). Cyclophilin B, one of the cyclosporine-binding immunophilins, can bind to NS5B and upregulate the replication of HCV (53). We have previously reported that NS5A specifically interacts with FK506-binding protein 8 (FKBP8) and recruits heat shock protein 90 (Hsp90) to the viral RNA replication complex through the interaction of the carboxylate clump structure of FKBP8 with the C-terminal MEEVD motif of Hsp90 (37). Knockdown of FKBP8 reduced the replication efficiency of the HCV genome in the replicon cells and the cells infected with JFH1 virus (37), suggesting that FKBP8 is required for the replication of HCV via formation of the replication complex. In the present study we identified an amino acid residue in NS5A responsible for specific interaction with FKBP8 and examined the biochemical interaction and intracellular localization of NS5A and FKBP8.

MATERIALS AND METHODS

Cells. Human embryo kidney 293T cells, and human hepatoma cell line Huh-7 and its derivatives were maintained in Dulbecco modified Eagle medium (DMEM; Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS) and nonessential amino acid (NEAA). The Huh-7 9-13 cell line, which harbors an HCV subgenomic replicon (4, 27), was cultured in DMEM supplemented with 10% FCS and 1 mg of G418 and NEAA/ml. The Huh-7 9-13 cell line was treated with IFN-α to deplete the HCV RNA replicon. A cell line exhibiting the highest efficiency of propagation of JFH1 virus was selected by limited dilution and designated Huh-7OK1. The Huh-7OK1 cell line retained the ability to produce type I IFNs 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. Detailed characteristics of this cell line are described elsewhere.

Antibodies. Rabbit antibody to NS5A was prepared by immunization with the NS5A peptide as described previously (16). Mouse monoclonal antibody to NS5A was purchased from Austral Biologicals (San Ramon, CA). Mouse monoclonal antibody to FKBP8 (KDM11) was described previously (37).

Plasmids. cDNA encoding NS5A was amplified from the HCV genotype 1b Con1 strain, kindly provided by R. Bartenschlager, by PCR using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA). The DNA fragment was cloned into pCAGGs-PUR/N-HA (36, 37). Human FKBP8 cDNA was amplified from the total cDNA of Huh-7 cells by PCR, and the fragment was introduced into pcDNA3.1 N-Flag, in which a Flag tag is introduced in the 5′ terminus of the cloning site of pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The point mutations of NS5A were generated by the method of splicing by overlap extension (17, 18) and introduced into pCAGGs-PUR/N-HA. The mutant NS5A cDNAs were amplified by PCR, digested with MluI and XhoI, and introduced into the replicon plasmid pFKI$_{389}$/neo/NS3-3′/5.1 (23), provided by R. Bartenschlager, or pFKI$_{389}$/hRL/NS3-3′/5.1 (37). The cDNA encoding NS3 to NS5A was excised from pFKI$_{389}$/neo/NS3-3′/5.1 and cloned into pCAGGs-PUR (36, 37). pET-UbCHis-del32-NS5A encoding an NS5A lacking the membrane-anchoring region (amino acid residues 1 to 32) and *Escherichia coli* strain BL21(DE3)/pCG1 was kindly provided by C. E. Cameron (19). The DNA fragment encoding the regions spanning from amino acid residues 2 to 389 of FKBP8 lacking the transmembrane region was amplified by PCR and replaced with the NS5A coding region of pET-UbNHis-del32-NS5A. The resulting plasmid encoding the amino acid residues from 2 to 389 of FKBP8 was designated pET-UbNHis-FKBP8(dTM) in this report. The DNA fragment encoding FKBP52 was amplified from the human fetal brain library (Clontech, Palo Alto, CA) by PCR and then was introduced into pET30a (Novagen, San Diego, CA) to be expressed in *E. coli*. The resulting plasmid was designated pET30a-FKBP52. The sequences of the plasmids were confirmed by using an ABI Prism 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Protein purification. The procedure used for protein purification was basically that of Huang et al. (19), with minor modifications that have been described previously (37). Briefly, overnight culture of *E. coli* strains transformed with pET-UbCHis-del32-NS5A, pET-UbNHis-FKBP8(dTM), or pET30a-FKBP52 were added at 1/100 volume into 250 ml of 2xYT medium and incubated at 37°C with shaking at 200 rpm. IPTG(isopropyl β-D-thiogalactoside) was added at a final concentration of 0.5 mM when the absorbance of the culture reached an optical density at 600 nm of 0.6 to 0.8, and then the culture solution was incubated at 20°C for 4 h with shaking at 200 rpm. After centrifugation of the culture at 3,000 × *g* for 5 min, the pellets were washed once with phosphate-buffered saline (PBS); suspended in 5 ml of 100 mM Tris-HCl (pH.8.0)–200 mM NaCl–10 mM 2-mercaptoethanol (lysis buffer) containing 0.5% Nonidet P-40, EDTA-free complete protease inhibitor (Roche, Indianapolis, IN), and 0.2 μg of lysozyme/ml; incubated at 4°C for 1 h; and subjected to freezing-thawing once. The resulting mixture was sonicated at 4°C for 5 min and was treated with 0.02 mg of DNase per ml at room temperature for 5 min. The suspension was centrifuged at 4°C at 30,000 rpm for 1 h in a Beckman SW50.1 (Beckman Coulter, Fullerton, CA), and the resulting supernatant was mixed with 0.5 ml of nickel agarose (Sigma) and gently rotated at 4°C for 60 min. The nickel resins were washed twice by spinning down with lysis buffer containing 10 mM imidazole. The recombinant protein was eluted from the nickel resin with lysis buffer containing 0.25 M imidazole and then dialyzed in 20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl. The dialyzed eluates were applied to a Resource Q Sepharose column (GE Healthcare, Tokyo, Japan), washed with a ten-column volume of 20 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl, and eluted under a linear gradient of 100 to 1,000 mM NaCl in 20 mM Tris-HCl buffer (pH 8.0). The peak fractions were pooled into a tube and concentrated by using Amicon Ultra-4 (Millipore, Bedford, MA). A half volume of the concentrated fraction was dialyzed against 10 mM HEPES (pH 7.4) containing 150 mM NaCl and 3 mM EDTA (HBS-E buffer) for analysis of the binding kinetics, while the remaining half was dialyzed in PBS for the immobilization on the sensor chip and pull-down assay. The protein concentration was measured using a Coomassie protein assay kit (Pierce, Rockford, IL).

Binding kinetics of NS5A and FKBP8. Surface plasmon resonance (SPR) measurements were made at 25°C by using a Bicore 2000 biosensor (GE Healthcare) in accordance with the manufacturer's instructions to determine the affinity between NS5A and FKBP8. Briefly, The NS5A-His was immobilized as ligand on a carboxymethyl-dextran (CM5) sensor chip with an amine coupling kit (Biacore). His-FKBP8 and His-FKBP52 were diluted with HBS-E buffer containing 0.0005% surfactant P20 (HBS-EP buffer) at the concentrations indicated in Fig. 1. The diluted sample was applied to the sensor chip at a flow rate of 20 μl/min in HBS-EP. The raw data were analyzed with a Biaevaluation software package (version 3.0; GE Healthcare).

Immunofluorescence microscopy. Huh-7 9-13 replicon cells cultured on glass slides overnight were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. After two washes with PBS, cells were permeabilized for 15 min at room temperature with PBS containing 0.25% saponin and blocked with PBS containing 1% bovine serum albumin (PBS-BSA) for 30 min at room temperature. The cells were then incubated with PBS-BSA containing mouse anti-FKBP8 antibody (KDM11) and/or rabbit anti-NS5A antibody at 37°C for 60 min, washed three times with PBS-BSA, and incubated with PBS-BSA containing Alexa Fluor 488 (AF488)-conjugated anti-mouse immunoglobulin G (IgG), AF488-conjugated anti-rabbit IgG, AF594-conjugated anti-rabbit IgG, and/or AF546-conjugated anti-mouse IgG antibody (Molecular Probes, Eugene, OR) at 37°C for 60 min. Finally, the cells were washed three times with PBS-BSA and observed on a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). For mitochondria and lipid droplet staining, cells were incubated with culture medium containing Mitotracker Deep-Red (200 nM; Molecular Probes) and Bodipy 558/568 C12 (20 μg/ml; Molecular Probes), respectively, for 20 min at 37°C. After staining, cells were washed once with fresh and prewarmed culture medium and incubated at 37°C for 20 min.

Correlative FM-EM. Correlative fluorescence microscopy-electron microscopy (FM-EM) allows individual cells to be examined both in an overview with FM and in a detailed subcellular structure view with EM (40). For the observation by FM-EM, the Huh-7 9-13 replicon or Huh-7OK1 cells were cultured on gridded, 35-mm glass-bottom dishes (Mat Tek, Ashland, MA) in 1 ml of DMEM containing 10% FCS at 37°C overnight. Cells on the grid were fixed and stained

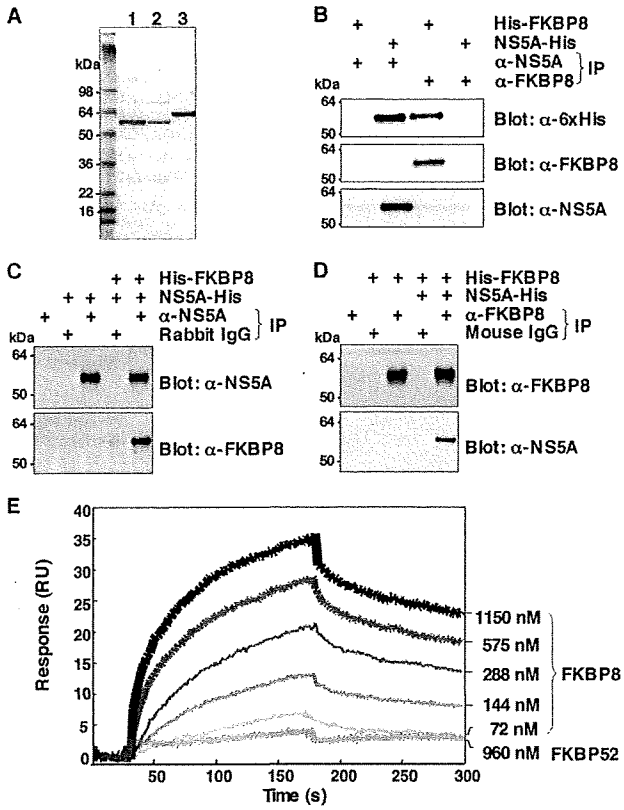

FIG. 1. Purification of recombinant NS5A, FKBP8, and FKBP52 and characteristics of their interaction. (A) Purified recombinant His-FKBP8 (lane 1), NS5A-His (lane 2), and His-FKBP52 (lane 3) were subjected to SDS-PAGE and stained with Coomassie brilliant blue G-250. The molecular size marker is shown on the left. (B) Antibodies to NS5A and FKBP8 specifically precipitated NS5A-His and His-FKBP8, respectively, and exhibit no cross-activity. The purified recombinant proteins (10 μg) were mixed, and immunoprecipitated with rabbit polyclonal IgG to NS5A or nonspecific rabbit IgG (C) or immunoprecipitated with mouse monoclonal antibody to FKBP8 (KDM11) or nonspecific mouse IgG (D). Immunoprecipitated proteins were subjected to immunoblotting with antibodies to NS5A and FKBP8. (E) The kinetics of interaction between His-FKBP8 and NS5A-His was estimated from SPR by using a Biacore 2000. The data are representative of three independent experiments.

with the specific antibodies as described above and then examined by using a confocal laser scanning microscope. The same specimens were then further incubated with 2.5% glutaraldehyde and 2% formaldehyde in PBS at 4°C overnight. After three washings with PBS, the samples were postfixed with 1% osmium tetroxide and 0.5% potassium ferrocyanide in PBS for 1 h, washed with distilled water three times, dehydrated in ethanol, and embedded in Epon812 (Structure Probe, West Chester, PA). Ultrathin sections of the cell (70-nm thick) were stained with saturated uranyl acetate and Reynolds lead citrate solution. The electron micrographs were taken with a JEOL JEM-1011 transmission electron microscope (JEOL, Ltd., Tokyo, Japan).

Transfection, immunoblotting, and immunoprecipitation. The transfection and immunoprecipitation tests were carried out as described previously (37). The immunoprecipitated samples were subjected to sodium dodecyl sulfate (SDS)–12.5 or 10% polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore) and reacted with the appropriate antibodies. The immune complexes were visualized with Super Signal West Femto substrate (Pierce) and were detected by using an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

Transient replication assay. The HCV replicon plasmid, pFK-I_{389} hRL/NS3-3′/5.1 (37), was cleaved with ScaI and transcribed in vitro by using a MEGAscript T7 kit (Ambion, Austin, TX). Then, 10 μg of the transcribed RNA was electroporated at 270 V and 960 μF by a Gene Pulser (Bio-Rad, Hercules, CA) into 10 million cells of Huh-7OK1 of cell line per ml, suspended in 25 ml of culture medium, and then seeded at 1 ml per well on 12-well culture plates. Luciferase activity was measured at 4 and 48 h posttransfection using a *Renilla* luciferase assay system (Promega, Madison, WI) according to the manufacturer's protocol. The relative luciferase activity was presented as the ratio of the luciferase activity measured at 48 h posttransfection to that at 4 h.

Colony formation. The plasmid pFK-I_{389} neo/NS3-3′/NK5.1 (23) was digested with ScaI, and 10 μg of the in vitro-transcribed RNA was electroporated onto 4 million Huh-7 cells per 0.4 ml and suspended in 10 ml of the culture medium as described above. A 3-ml aliquot of the resulting cell suspension was mixed with 7 ml of the culture medium and inoculated into a culture dish 10 cm in diameter. The culture medium was replaced with fresh DMEM containing 10% FCS and 1 mg of G418 (Nakarai Tesque, Tokyo, Japan)/ml at 24 h posttransfection. The medium was exchanged once a week with fresh DMEM containing 10% FCS and 1 mg of G418/ml, and the remaining colonies were fixed with 4% paraformaldehyde at 28 days posttransfection and stained with crystal violet.

Direct sequencing of the NS5A gene in a G418-resistant cell line. Total RNA was prepared from G418-resistant colonies by using an RNeasy minikit (Qiagen, Valencia, CA), and first-strand cDNA was synthesized with random primers by using a first-strand cDNA synthesis kit (GE Healthcare). The NS5A genes were amplified with the primer pair 5′-GACGGCATCATGCAAACCAC-3′ and 5′-CGTGGAGGTGGTATCGGAGG-3′. The PCR products were applied to agarose gel electrophoresis and purified by using a gel extraction kit (Qiagen). The purified PCR products were sequenced with the inside primer 5′-ATTAACGC GTACACCACGGG-3′ by using an ABI Prism 3130 genetic analyzer (Applied Biosystems).

RESULTS

Purification of recombinant NS5A, FKBP8, and FKBP52 and characteristics of their interaction. We have previously reported that the thioredoxin-tagged domain I of NS5A (Trx-NS5A) binds directly to His_6-tagged FKBP8 (37), although we could not obtain sufficient amounts of the recombinant FKBP8 for further biochemical analysis. Huang et al. reported that C-terminally His_6-tagged NS5A lacking the N-terminal 32 amino acid residues of the membrane anchoring region (NS5A-His) could be purified by using a pET-ubiquitin expression system, in which the NS5A-His fused with ubiquitin at the C terminus was cleaved off by a ubiquitin-specific protease, Ubp1, in *E. coli* and then purified by using nickel-charged resin (19). By using the pET-ubiquitin expression system, we could obtain 1 mg of the purified His-FKBP8 protein from 1 liter of a culture of *E. coli* harboring a pET-UbCHis-FKBP8-dTM encoding an N-terminally His_6-tagged FKBP8 lacking the transmembrane region (His-FKBP8), which is five times greater production than that achieved by the previous method we used (37). His-FKBP8, NS5A-His, and His-FKBP52 (10 μg) were purified with nickel-charged resin (Fig. 1A) and subjected to the pull-down assay. Immunoblotting, instead of protein staining, was used for detection of the precipitates due to the similar molecular sizes of NS5A-His and His-FKBP8 (Fig. 1A). To confirm the specificity of the antibodies to NS5A and FKBP8, NS5A-His and His-FKBP8 were immunoprecipitated and then subjected to immunoblotting by the antibodies. The antibodies to NS5A and FKBP8 specifically recognize NS5A and FKBP8, respectively (Fig. 1B). The antibody to NS5A, but not nonspecific rabbit IgG, precipitated NS5A together with FKBP8 (Fig. 1C). The reverse experiment was also successful in demonstrating that antibody to FKBP8, but not nonspecific mouse IgG, precipitated FKBP8 with NS5A (Fig. 1D). The binding kinetics was analyzed on the basis of the SPR technology to examine the specificity of interaction between FKBP8