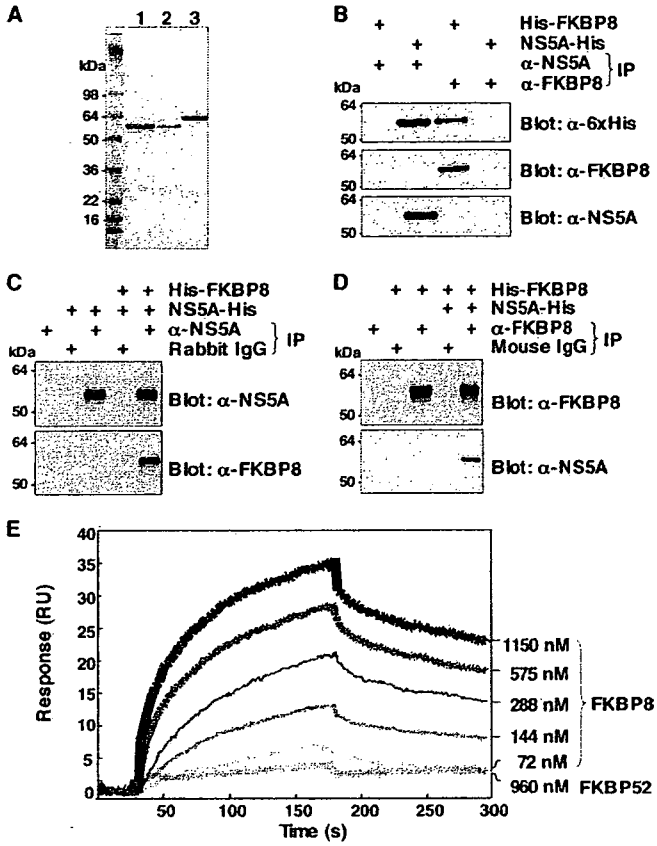

FIG. 1. Purification of recombinant NS5A, FKBP8, and FKBP52 and characteristics of their interaction. (A) Purified recombinant His-FKBP8 (lane 1), NS5A-His (lane 2), and His-FKBP52 (lane 3) were subjected to SDS-PAGE and stained with Coomassie brilliant blue G-250. The molecular size marker is shown 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

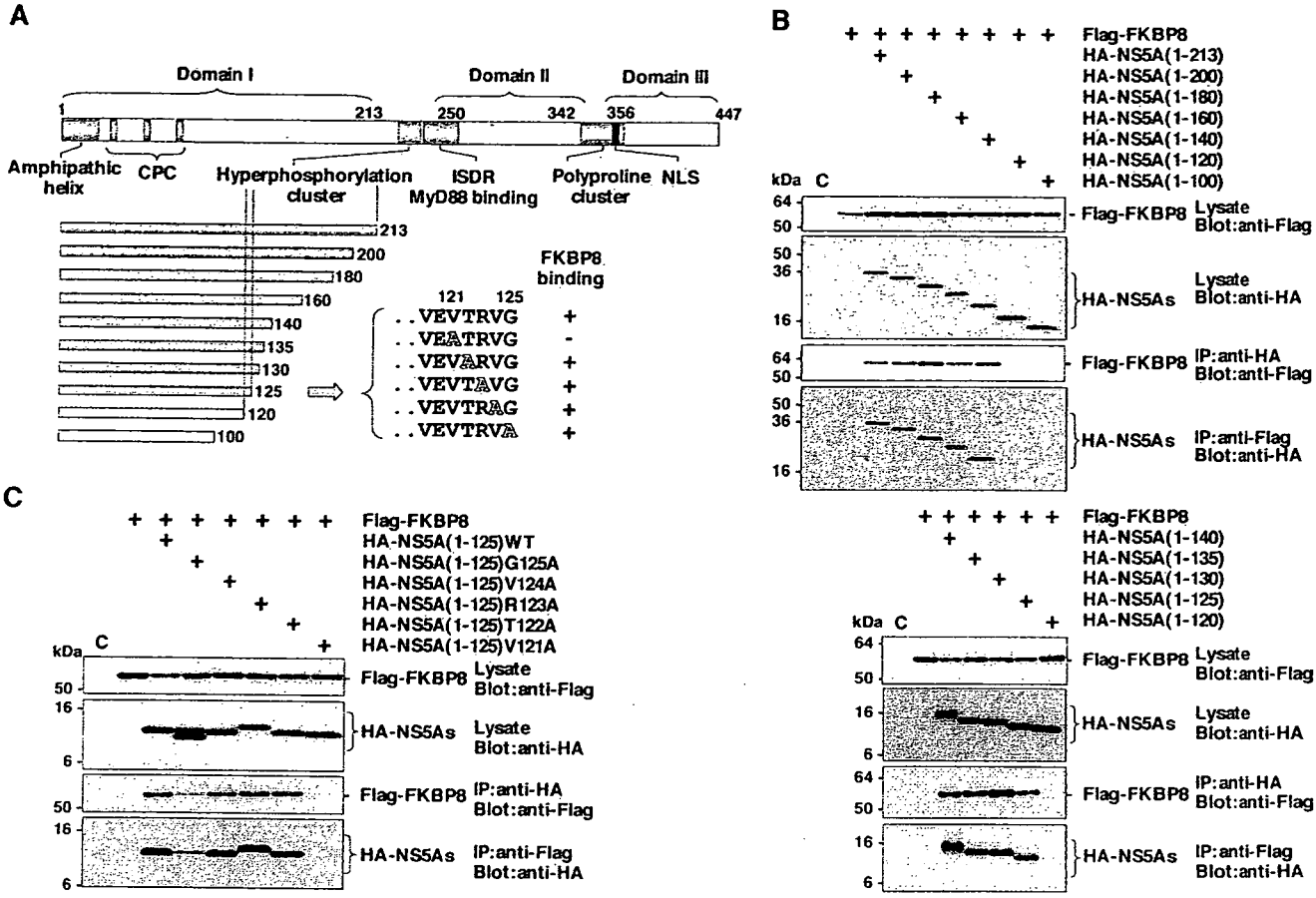

FIG. 2. Determination of an amino acid residue responsible for the interaction of FKBP8 and NS5A domain I. (A) Structure and functional domains of NS5A (top). The C-terminal deletion mutants of HA-tagged NS5A domain I used in the present study. Gray and white bars indicate ability and inability to bind to FKBP8, respectively. The site of Ala substitution of HA-tagged NS5A(1-125) and the results of binding to FKBP8 are summarized on the right. (B) The C-terminal deletion mutants of HA-tagged NS5A domain I were coexpressed with Flag-FKBP8 in 293T cells and immunoprecipitated with anti-HA or anti-Flag antibody. Immunoprecipitates were analyzed by immunoblotting. (C) Five substitution mutants of HA-NS5A(1-125) replacing each of the amino acid residues from 121 to 125 with Ala were coexpressed with Flag-FKBP8 in 293T cells, immunoprecipitated, and analyzed by immunoblotting. The data are representative of three independent experiments.

and NS5A. His-FKBP8 or His-FKBP52 was applied to a flow line at various concentrations on the sensor chip on which NS5A-His was immobilized. Each background signal was determined by flowing the FKBPs over a blank chip. The SPR signal of His-FKBP8 or His-FKBP52 was determined after subtraction by the background signals. The SPR was increased corresponding to the amount of His-FKBP8, but no response was observed with His-FKBP52 (Fig. 1D). The values of the dissociation constant, K_d (10^{-3} s^{-1}), and K_a (10^3 M^{-1} s^{-1}) were calculated to be 1.86 and 22.8, respectively. Therefore, the equilibrium dissociation constant (K_D) of the interaction between His-FKBP8 and NS5A-His was determined as 82 nM, suggesting a specific binding of the proteins. These results indicate that FKBP8 directly and specifically interacts with NS5A.

Val^{121} of NS5A is responsible for the specific interaction with FKBP8. The domain I of NS5A (amino acid residues 1 to 213) was shown to interact with FKBP8 (37). However, further analyses on the specific interaction of NS5A with FKBP8 have not yet been carried out. To determine the amino acid residues in NS5A responsible for specific interaction with FKBP8, Flag-FKBP8 was coexpressed with C-terminal deletion mutants of the hemagglutinin (HA)-tagged NS5A domain I in 293T cells and immunoprecipitated with appropriate antibodies (Fig. 2A). Although the C-terminal deletions up to the residue 141 in HA-NS5A exhibited no effect on the coimmunoprecipitation with Flag-FKBP8, further deletion beyond the amino acid residue 121 of HA-NS5A abrogated the coprecipitation with Flag-FKBP8 (Fig. 2B, upper panel), suggesting that residues from 121 to 140 in NS5A are responsible for the interaction with FKBP8. Further deletion mutants of HA-NS5A revealed that the amino acid residues from 121 to 125 are required for the interaction with Flag-FKBP8 (Fig. 2B, lower panel). To identify a specific amino acid residue critical for interaction with FKBP8, we generated substitution mutants of HA-NS5A(1-125) in which each of the amino acid residues from 121 to 125 were replaced with Ala. The mutant in which Val^{121} was replaced with Ala completely abrogated the interaction of HA-NS5A(1-125) with Flag-FKBP8, but the other substitution mutants did not (Fig. 2C). However, we could not obtain a clear reduction in the interaction of FKBP8 with a full-length of NS5A mutant substituted Val^{121} with Ala by immunoprecipi-

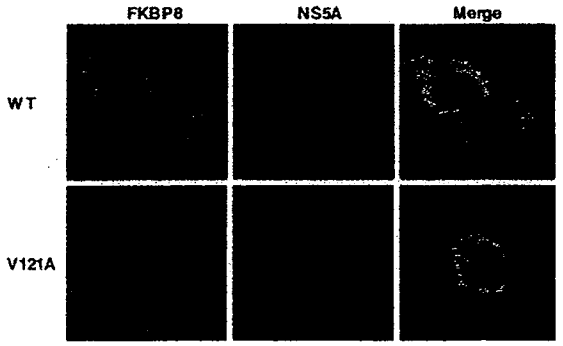

FIG. 3. Intracellular localization of wild-type and V121A mutant NS5A with FKBP8. Huh-7OK1 cells transfected with expression plasmids encoding HCV nonstructural proteins carrying a wild-type (WT) or mutant NS5A substituted Val^{121} with Ala (V121A) were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.25% saponin. Endogenous FKBP8 and NS5A were stained with anti-FKBP8 monoclonal antibody (KDM11) and rabbit anti-NS5A polyclonal antibody, followed by staining with AF488-conjugated anti-mouse IgG and AF594-conjugated anti-rabbit IgG antibodies, respectively.

tation analysis (data not shown). To examine the interaction of NS5A with FKBP8 in more functional setting, we examined the colocalization of the wild-type or mutant NS5A with an endogenous FKBP8 in Huh-7OK1 cells by transfection of the expression plasmids encoding HCV nonstructural proteins carrying a wild-type or mutant NS5A substituted Val^{121} with Ala. As shown in Fig. 3, colocalization of an endogenous FKBP8 with NS5A was reduced by the introduction of substitution of Val^{121} to Ala. These results suggest that Val^{121} of NS5A plays a critical role in the specific interaction with FKBP8.

Effect of the interaction of NS5A with FKBP8 on the replication of HCV. The amino acid alignment of NS5A derived from several genotypes on the basis of the European HCV database (http://euhcvdb.ibcp.fr/euHCVdb/jsp/index.jsp) revealed that the amino acid residue Val^{121} is well conserved among NS5A of various genotypes of HCV, with the exception of the genotype 1a strains, which have Ile in place of Val (Fig. 4A). We have previously shown that NS5A of genotype 1a (H77C strain), which has an amino acid residue Ile^{121}, was able to interact with FKBP8 (37). To determine the role of Ile^{121} on the binding of NS5A to FKBP8, HA-NS5A(1-125) of the genotype 1b Con1 strain in which Ile was substituted for Val^{121} was coexpressed with Flag-FKBP8 and immunoprecipitated with specific antibodies (Fig. 4B). The HA-NS5A mutant possessing the substitution of Val^{121} to Ile interacted with Flag-FKBP8 at the same level as the wild-type NS5A. Next, to determine the role of Val^{121} or Ile^{121} in the replication of HCV, we generated replicon RNAs in which Val^{121} of NS5A was replaced with either Ala or Ile. In vitro-transcribed RNAs from the pFK-I_{389} hRL/NS3-3′/5.1 carrying the mutation were introduced into the Huh-7 cell line by electroporation. The *Renilla* luciferase activity was measured at 4 and 48 h posttransfection and is represented as the ratio of luciferase activity measured at 48 h posttransfection to that measured at 4 h. The replacement of Val^{121} with Ala severely impaired the RNA replication, whereas substitution of Val^{121} to Ile, as seen in genotype 1a strains, had no apparent effect on the replication (Fig. 4C). These results suggest that Val^{121} and Ile^{121} of NS5A

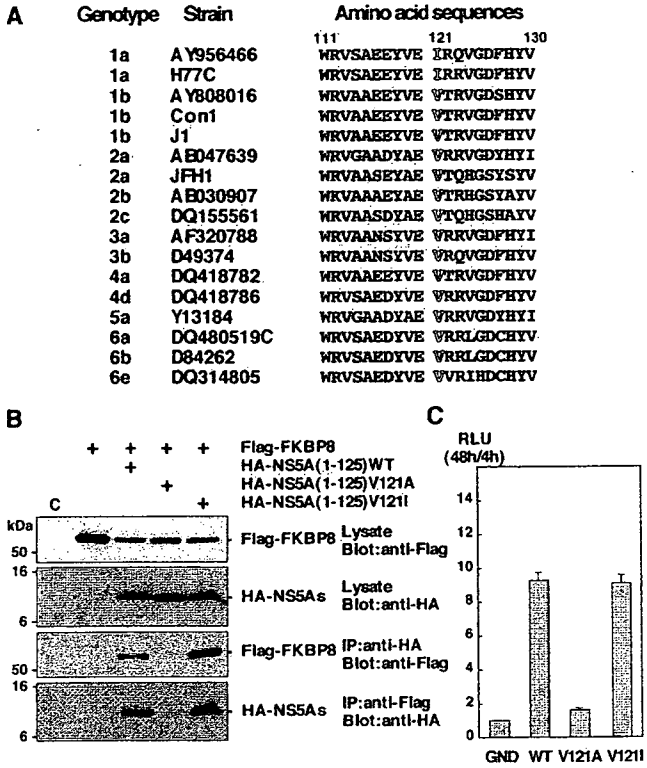

FIG. 4. Effect of the interaction of NS5A with FKBP8 on the transient replication of HCV. (A) Alignment of amino acid sequence of NS5A (111 to 130 amino acids) among different HCV genotypes. Outline letters indicate the amino acid residue at position 121. (B) The substitution mutants of HA-NS5A(1-125) replaced Val^{121} with Ala or Ile were coexpressed with Flag-FKBP8 in 293T cells and immunoprecipitated with anti-HA or anti-Flag antibody. Immunoprecipitates were analyzed by immunoblotting. (C) In vitro-transcribed RNAs from the pFK-I_{389} hRL/NS3-3′/5.1 (wild-type, WT) and those transcribed from the plasmids carrying the lethal mutation in NS5B (GND) or the substitution in Val^{121} to Ala (V121A) or to Ile (V121I) in NS5A were introduced into Huh-7 cells by electroporation. The relative luciferase value was calculated by determining the increase in *Renilla* luciferase activity at 48 h compared to that observed at 4 h after transfection. The relative activity is represented as the ratio of each value of replication efficiency to the corresponding value for GND mutant. The data are representative of three independent experiments.

play crucial roles in the interaction with FKBP8 and the transient replication of HCV replicons. We have previously reported that NS5A interacts with an endogenous FKBP8 in replicon cells harboring the subgenomic viral RNA (37). Therefore, we tried to demonstrate the lack of interaction of FKBP8 with the mutant NS5A substituted Val^{121} to Ala. However, we could not detect a sufficient amount of HCV proteins due to a low level of replication of the subgenomic replicon carrying the mutation in NS5A (Fig. 4C and 5A).

To further confirm the importance of Val^{121} and Ile^{121} in NS5A on the replication of HCV RNA, a colony formation assay was carried out. The replicon RNA carrying a neomycin resistance gene transcribed from pFKI_{389}/neo/NS3-3′/5.1 (23) was introduced into Huh-7 cells and cultivated under the pressure of G418. The number of remaining cell colonies was determined at 4 weeks posttransfection. There were more than

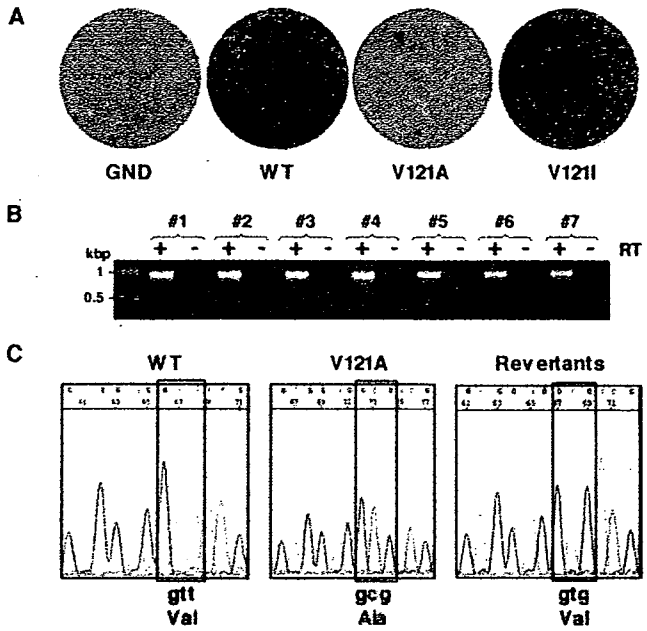

FIG. 5. Effect of the interaction of NS5A with FKBP8 on the colony formation by HCV replicon. (A) The replicon RNAs of the wild type (WT), a replication-deficient mutant (GND), and the substitution in Val^{121} to Ala (V121A) or to Ile (V121I) were transcribed from the plasmids based on $pFKI_{389}$ neo/NS3-3'/5.1, transfected into Huh-7 cells, and selected by G418 for 4 weeks. The remaining cells were fixed in 4% paraformaldehyde and stained with crystal violet. (B) Seven resistant colonies that appeared after transfection with the replicon RNA encoding substitution of Val^{121} to Ala (V121A) in NS5A were expanded, and the total RNAs were purified. The NS5A cDNAs were amplified by PCR with (+) or without (−) reverse transcription. (C) Sequence of NS5A genes derived from the wild type (WT), the V121A mutant, and seven resistant colonies (revertants).

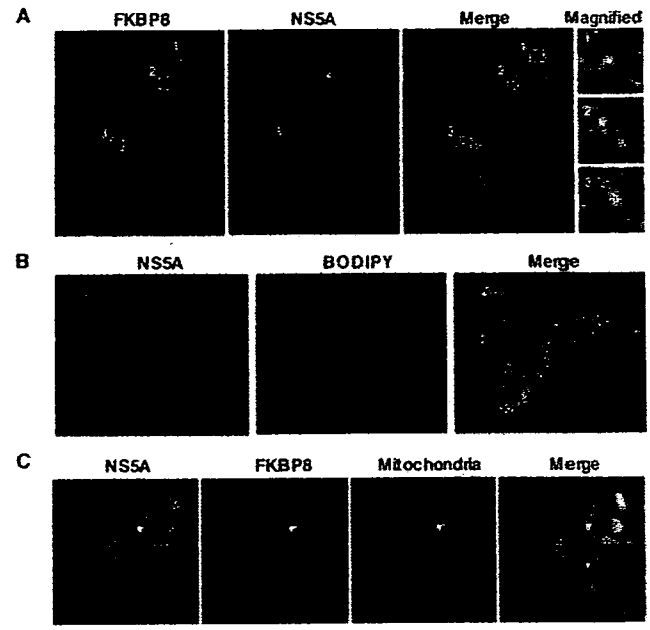

FIG. 6. Intracellular localization of FKBP8 and NS5A in the HCV replicon cells. (A) Huh-7 9-13 cells harboring an HCV subgenomic replicon were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.25% saponin. Endogenous FKBP8 and NS5A were stained with anti-FKBP8 monoclonal antibody (KDM11) and rabbit anti-NS5A polyclonal antibody, followed by staining with AF488-conjugated anti-mouse IgG and AF594-conjugated anti-rabbit IgG antibodies, respectively. Rectangles 1, 2, and 3 were magnified and are shown on the right. (B) NS5A was stained with the rabbit polyclonal antibody to NS5A and AF488 conjugated anti-rabbit IgG. Lipid droplets were specifically stained with Bodipy 558/568 C12. (C) Endogenous NS5A and FKBP8 were stained with stained rabbit anti-NS5A polyclonal antibody and anti-FKBP8 monoclonal antibody (KDM11), followed by staining with AF488-conjugated anti-rabbit IgG and AF546-conjugated anti-mouse IgG, respectively. Mitochondria were stained with Mitotracker Deep-Red. White rectangles indicate the magnified images of the small white inside boxes.

1,000 colonies on the plate of Huh-7 cells into which the parent replicon RNA or an RNA carrying the substitution of Val^{121} of NS5A to Ile was introduced, but only a few colonies appeared on the plate of cells into which RNA carrying the mutation of Val^{121} to Ala was introduced (Fig. 5A). To characterize the colonies emerging on the plate of Huh-7 cells into which the mutant Ala^{121} replicon RNA was introduced, the total RNAs were purified from the seven resistant colonies. The NS5A cDNAs were amplified after reverse transcription but not in the absence of reverse transcription (Fig. 5B), suggesting that the amplified cDNAs were derived from RNA but not from the remaining transfected plasmid DNA. The NS5A genes were subjected to direct sequencing and revealed that the transfected mutant replicon RNA had GCG corresponding to the Ala^{121}, in contrast to the parental replicon, which had GTT corresponding to the Val^{121}. On the other hand, all of the RNAs prepared from the individual resistant colonies had GTG encoding Val (Fig. 5C), indicating that the resistant colonies were not derived from the contamination of the wild-type replicon RNA but emerged by the mutation after replication. These results further support the notion that Val^{121} in NS5A is an indispensable amino acid and plays an important role in the replication of HCV though interaction with FKBP8.

Subcellular localization of FKBP8 and NS5A. Previous reports suggest that FKBP8 is mainly localized on mitochondria (7, 44), whereas NS5A is mainly localized on the endoplasmic reticulum (ER) and Golgi apparatus (2, 6, 16). HCV is reported to replicate in a raft-like intracellular compartment or the folded membranous compartment known as a membranous web in the replicon cells (8, 13, 15). In the present work, intracellular localization of FKBP8 was examined by immunofluorescence staining of the replicon cell line, Huh-7 9-13, which harbored an HCV subgenomic replicon, with the antibodies to NS5A and to FKBP8. Endogenous FKBP8 was mainly found in mitochondria and was partially colocalized with NS5A in a few compartments sharing a dot-like structure (Fig. 6A). Lipid droplets were required for production of infectious HCV (5) and were colocalized with NS5A and core protein (43), although NS5A formed as dot-like structures but was not found in lipid droplets stained with Bodipy 558/568 C12 in the replicon cell line (Fig. 6B). On the other hand, FKBP8 was mainly localized on mitochondria and partially together with NS5A on dot-like structures that were distinct from the mitochondria (Fig. 6C).

To further analyze the subcellular compartments where FKBP8 and NS5A were colocalized, the same fields of Huh-7 9-13 replicon cells were observed with FM and EM by using

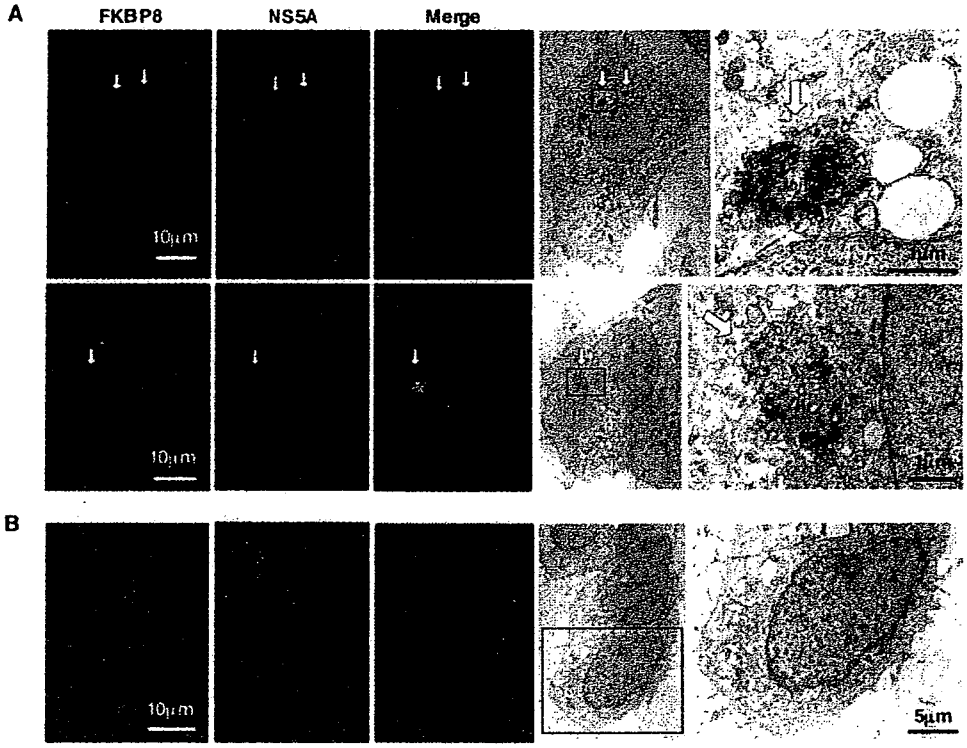

FIG. 7. FKBP8 interacts with NS5A in the membranous web. (A) The Huh-7 9-13 replicon cells were stained with specific antibodies to FKBP8 and NS5A as described in Fig. 6A. Identical fields were observed under EM by using the correlative FM-EM technique. Arrows indicate the areas NS5A and FKBP8 are colocalizing. Right panels indicate the magnified images of the small black boxes. Highly electron-dense and folded membranous structures were observed by a highly magnified EM. (B) Control cells in which the replicon cells were cured by IFN-α treatment were processed in the same procedures. No electron-dense structure was observed in the cytoplasm.

the correlative FM-EM technique described above. This method allowed us to examine the colocalization of the molecules by both FM and EM in the same samples, yielding two different but complementary data sets. The replicon cells were stained with antibodies to FKBP8 and NS5A and examined under FM (Fig. 7A, left panels), and the same fields were observed under EM (Fig. 7A, right panels). The compartments colocalizing FKBP8 and NS5A (arrows) exhibited a high electron density and a folded membranous structure that was similar to a membranous web (15, 32). In contrast, the replicon cells cured by IFN-α treatment did not have the electron-dense structure (Fig. 7B). These results suggest that FKBP8 interacts with NS5A on the membranous web in cells replicating HCV RNA.

DISCUSSION

HCV NS5A is a multifunctional protein involved in viral replication and pathogenesis (29). In a previous study, we have shown that NS5A specifically interacts with FKBP8 and recruits 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). Although we demonstrated that a TPR domain other than the carboxylate clump region of FKBP8 was responsible for the specific interaction with NS5A (37), the precise binding amino acid residue of the interaction was not determined. In the present study, FKBP8 exhibited a specific interaction with the immobilized NS5A in a dose-dependent manner with an equilibrium dissociation constant (K_d) of 82 nM as determined by the SPR, but no interaction with FKBP52 was detected. Furthermore, mutational analysis suggested that Val or Ile at the amino acid residue 121 of NS5A was responsible for the specific interaction with FKBP8. The subgenomic HCV replicon RNA harboring the mutation of Val^{121} to Ala within NS5A leads to severe impairment of RNA replication, and reversion from Ala^{121} to Val was detected, suggesting that interaction of FKBP8 with NS5A through the Val^{121} is crucial for HCV replication. The crystal structure of NS5A domain 1 revealed that Val^{121} is located on one of the β-sheet structures in the 1B subdomain and the side chain of the residue is located within the hydrophobic core (46); therefore, the Val^{121} may be involved in the maintenance of the β-sheet structure in the subdomain rather than the direct interaction with FKBP8. However, it remains feasible to speculate that unidentified host factors may be involved in the conformational change of region, including Val^{121} for direct interaction with FKBP8. Further studies, including a structural analysis of FKBP8, are needed to clarify the mechanisms by which HCV is replicated through the interaction of NS5A, FKBP8, and Hsp90.

The current combination therapy with pegylated IFN-α and ribavirin achieves a sustained virological response in half of the patients infected with a high viral load of HCV of genotype 1b (30). However, it is difficult to achieve the complete removal of viruses by antiviral drugs targeted to the viral enzymes, includ-

ing proteases and polymerases, from patients persistently infected with RNA viruses that exhibit a quasispecies nature, such as human immunodeficiency virus and HCV. Viral quasispecies are not a simple collection of diverse mutants but a group of interactive variants capable of adapting to new environments (48). Cyclosporine treatment has been shown to be effective for patients infected with HCV of genotype 1b (20) and suppresses HCV RNA replication in vitro (52). In addition, cyclosporine has been shown to disrupt the interaction between NS5B and cyclophilin B, which is required for an efficient RNA-binding of NS5B (53). Cyclophilins and FKBPs are classified as immunophilins capable of binding to the immunosuppressants cyclosporine and FK506, respectively (26). The family members do not share a homologous domain other than drug-binding and enzymatically active domains, based on their amino acid sequences, substrate specificities, and inhibitor sensitivities. However, cyclosporine-resistant RNA replicon was shown to exhibit mutations not only in NS5B but also in NS5A (12, 41), suggesting that cyclosporine might affect the viral replication through the nucleotide-binding ability of NS5B, as well as the function of NS5A. Recently, geldanamycin, an inhibitor of Hsp90, was shown to drastically impair the replication of poliovirus without any emergence of escape mutants (14). Therefore, the elucidation of host proteins, including immunophilins and chaperones, participating in the HCV replication complex may lead to the development of new therapeutics for chronic hepatitis C with a broad spectrum and a low possibility of emergence of revertant viruses. In particular, disruption of the specific interaction of Val^{121} of NS5A with the TPR domain of FKBP8 might be an ideal target for a novel therapeutic measure.

Egger et al. reported that NS4B alters the intracellular membrane to form a membranous web structure consisting of a membrane-associated multiprotein complex localized in the cytoplasmic compartments distinct from the mitochondria in vitro and in the liver of an HCV-infected chimpanzee, suggesting that the membranous web forms the viral replication complex (8). An N-terminal amphipathic helix of NS4B plays an important role in the viral replication, as well as in the correct localization of other NS proteins including NS5A (9). Furthermore, VAP-B was reported to interact with Nir2 protein through the FFAT (named for two phenylalanines [i.e., FF] in the acidic tract) motif and to remodel the ER structure to form a convoluted membrane structure resembling a membranous web (3). In addition, VAP-A and B interact with not only NS5A but also NS5B (13, 16, 47), suggesting that the complex of NS5A with FKBP8 might be recruited on the membranous web by NS4B and/or VAPs and participate in the HCV replication.

FKBP8 has been shown to be localized mainly on the mitochondria and to interact with Bcl-2 to sequester Bcl-2 on the mitochondria (7, 44). However, HCV RNA was suggested to be replicated in the membranous web structure in replicon cells (8, 13, 15), and NS5A was reported to localize on the ER, Golgi apparatus (2, 6, 16), and lipid droplets (43). Figures 6C and 7A clearly indicate that the intracellular compartment including NS5A and FKBP8 is distinct from mitochondria. The HCV core protein was shown to upregulate genes related to fatty acid biosynthesis through the interaction with proteasome activator PA28γ/REGγ in the nucleus (34) and to induce accumulation of cytoplasmic lipid droplets in the mouse liver (35). Recently, it was shown that the HCV core protein of the genotype 2a JFH1 strain recruits the replication complex to the lipid droplet-associated membranes, and HCV particles were detected in close proximity to the lipid droplets, suggesting that lipid droplets induced by core protein participate in the assembly of HCV particles (31). In addition, the lipid droplets including the core protein were surrounded by the nonstructural proteins was also detected in cells expressing the chimeric HCV genomes encoding core to a part of NS2 proteins of genotype 1b or 1a strain and the nonstructural proteins of JFH1 strain (31). In the present study, FKBP8 was shown to be colocalized with NS5A in a highly electron-dense intracellular compartment indistinguishable from the membranous web. Although the total amount of FKBP8 was not changed by the treatment of the replicon cells by IFN-α (data not shown), the membranous web structure where FKBP8 and NS5A had accumulated was removed by the treatment (Fig. 7B). These results suggest that the replication of the subgenomic HCV RNA induces the formation of a membranous web structure in which NS5A and FKBP8 are colocalized but has no effect on the expression level of FKBP8. Furthermore, we could not detect any colocalization of FKBP8 and NS5A with the lipid droplets in the replicon cells harboring a full-length genome of the genotype 1b Con1 strain (data not shown). Although the relationships between the membranous web and lipid droplets remain unknown, these discrepancies might be attributable to the difference in HCV genotypes of the nonstructural proteins that consist of the major components of the replication complex determining the efficiency of HCV replication.

In conclusion, our data indicate that NS5A directly binds to FKBP8 through the Val^{121} and colocalizes in the convoluted membrane structure known as the membranous web. Future studies on the role of FKBP8 in the replication of HCV might contribute to the development of a new type of anti-HCV drugs with a low frequency of emergence of drug-resistant breakthrough viruses.

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Human VAP-B Is Involved in Hepatitis C Virus Replication through Interaction with NS5A and NS5B

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The hepatitis C virus (HCV) nonstructural protein (NS) 5A is a phosphoprotein that associates with various cellular proteins and participates in the replication of the HCV genome. Human vesicle-associated membrane protein-associated protein (VAP) subtype A (VAP-A) is known to be a host factor essential for HCV replication by binding to both NS5A and NS5B. To obtain more information on the NS5A protein in HCV replication, we screened human brain and liver libraries by a yeast two-hybrid system using NS5A as bait and identified VAP-B as an NS5A-binding protein. Immunoprecipitation and mutation analyses revealed that VAP-B binds to both NS5A and NS5B in mammalian cells and forms homo- and heterodimers with VAP-A. VAP-A interacts with VAP-B through the transmembrane domain. NS5A interacts with the coiled-coil domain of VAP-B via 70 residues in the N-terminal and 341 to 344 amino acids in the C-terminal polyproline cluster region. NS5A was colocalized with VAP-B in the endoplasmic reticulum and Golgi apparatus. The specific antibody to VAP-B suppressed HCV RNA replication in a cell-free assay. Overexpression of VAP-B, but not of a mutant lacking its transmembrane domain, enhanced the expression of NS5A and NS5B and the replication of HCV RNA in Huh-7 cells harboring a subgenomic replicon. In the HCV replicon cells, the knockdown of endogenous VAP-B by small interfering RNA decreased expression of NS5B, but not of NS5A. These results suggest that VAP-B, in addition to VAP-A, plays an important role in the replication of the HCV genome.

Hepatitis C virus (HCV) infects 170 million people worldwide and frequently leads to cirrhosis or hepatocellular carcinoma (6, 29). HCV is classified in the family *Flaviviridae* and possesses a single-stranded positive-sense RNA with a length of 9.6 kb. The HCV genome encodes a single large precursor polyprotein composed of about 3,000 amino acids (aa) that is processed by cellular and viral proteases, resulting in at least 10 structural and nonstructural (NS) proteins (29). Details of HCV's replication cycle are unknown because of the low viral load in the sera of HCV-infected individuals and the lack of a reliable and robust cell culture system to support HCV infection and replication. The development of HCV RNA replicons in which a synthetic HCV genomic or subgenomic RNA replicates efficiently in the human hepatocarcinoma cell line Huh-7 has enabled the study of viral RNA replication in cell culture (4, 20, 24). The HCV RNA replication complex, composed of the viral NS proteins and host cellular proteins, replicates the viral RNA genome at the intracellular membrane. Thus far, the HCV replicon system has greatly contributed to the understanding of HCV replication and pathogenesis associated with the expression of viral NS proteins. Replication of positive-strand RNA viruses generally involves certain intracellular membrane structures, including the endoplasmic reticulum (ER), Golgi apparatus, endosome, and lysosome (39). Recently, several groups have succeeded in demonstrating cell-free replication activities of replication complexes in crude membrane fractions of HCV subgenomic replicon cells (2, 3, 14, 53). These cell-free systems provide semi-intact polymerase assays for biochemical dissection of HCV RNA replication and are a useful source for the isolation of HCV replication complexes. Replication complexes were detected in detergent-resistant membrane structures, most likely lipid raft structures (2, 14). Although HCV NS proteins presumably form a membrane-associated RNA replication complex with host proteins, the precise components and mechanisms for replication are poorly understood.

HCV NS5A is a phosphoprotein that appears to possess multiple and diverse functions in viral replication, interferon resistance, and pathogenesis (26, 35). Cell culture-adaptive mutations have been shown to cluster in the central portion of NS5A in subgenomic HCV replicons, indicating that NS5A is involved in the viral replication process either directly or by interacting with host cellular proteins (4, 55). This observation, together with the modulation of NS5A hyperphosphorylation by NS3, NS4A, and NS4B and physical interaction with other viral NS proteins, strongly supports the notion that NS5A is an essential component of the HCV replication complex (21, 30, 36). NS5A has been shown to be associated with a range of cellular proteins involved in cellular signaling pathways, such as interferon-induced kinase PKR (11), growth factor receptor-binding protein 2 (Grb2) (45), p53 (27, 37), phosphoinositide-3-kinase p85 subunit (15), and proteins in protein trafficking and membrane morphology, such as karyopherin β3 (8),

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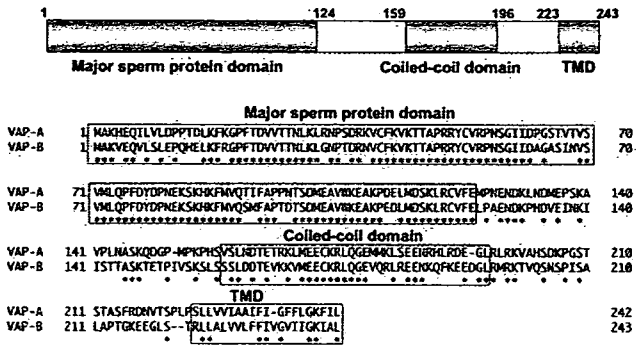

FIG. 1. Schematic representation of VAP-B and alignment of amino acid sequences of VAP-A and VAP-B. The major sperm protein domain, coiled-coil domain, and TMD are indicated. The asterisks indicate identical amino acid residues between VAP-A and VAP-B.

apolipoprotein A1 (40), amphiphysin II (56), and vesicle-associated membrane protein (VAMP)-associated protein (VAP) subtype A (VAP-A), also called VAP-33 (48). Host fatty acids and geranylgeranylation appear to modulate the host and viral proteins involved in HCV RNA replication (19, 49, 54). Gao et al. showed that small interfering RNA (siRNA) or the dominant-negative mutant of VAP-A resulted in relocation of NS5B from detergent-resistant to detergent-sensitive membranes and reduced HCV RNA replication (12). In addition, Evans et al. suggested that NS5A hyperphosphorylation disrupts interaction with VAP-A and negatively regulates HCV RNA replication (9). Like many of the fusion proteins, VAP is a tail-anchored protein with a globular amino-terminal domain followed by a stalk region containing a coiled coil (Fig. 1), and it is ubiquitously expressed in human tissues (7). In humans, there are two isoforms of VAP, VAP-A and VAP-B, encoded by separate genes, and VAP-C is a splicing variant of VAP-B missing the C-terminal two-thirds (23, 32). VAP-B shows 63% amino acid identity to VAP-A (32, 51). The first proposed function for VAP arose from its initial identification as an interactor with the membrane fusion protein synaptobrevin/VAMP in *Aplysia* (43). Since then, it has been shown to be involved in vesicle transport, including the regulation of COP-I vesicle transport in the ER/Golgi pathway (13, 44), VAMP/synaptobrevin-mediated neurotransmitter release (38), and VAMP-2-mediated Glut-4 trafficking at the plasma membrane (10); it is also involved in the interaction between the microtubule network and tight junctions (22). Recently, VAP has been linked to the function of mammalian neurons, where VAP is enriched on microtubules (42), because a mutation in human VAP-B causes familial amyotrophic lateral sclerosis type 8 (32).

To gain a better understanding of the interactions between NS5A and host proteins involved in HCV replication, we screened human libraries by a yeast two-hybrid system using NS5A as bait and identified VAP-B as an NS5A-binding protein. In this study, we examined the biological significance of the interaction between VAP-B and NS proteins in HCV replication and found that VAP-B binds to both NS5A and NS5B in mammalian cells and forms homo- and heterodimers with VAP-A. Immunodepletion of VAP-B suppressed the replication of HCV RNA in a cell-free replication assay, and the knockdown of endogenous VAP-B by siRNA decreased the expression of NS5B but not that of NS5A. These results suggest that VAP-B plays an important role in HCV replication through interaction with NS5A and NS5B.

MATERIALS AND METHODS

Cells. Human embryo kidney 293T, human cervical carcinoma HeLa, and human hepatoma Huh-7 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, Mo) containing 10% fetal calf serum (FCS), while the Huh-9-13 cell line, which possesses an HCV subgenomic replicon (4, 20, 23), was cultured in DMEM supplemented with 10% FCS and 1 mg/ml G418. All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Antibodies. Chicken anti-human VAP-B antibody was prepared by immunization using the synthetic peptides of residues from 188 to 203, KQFKEEDGL RMRKTVQ, of human VAP-B. A mouse monoclonal antibody to human VAP-A was purchased from BD Pharmingen (San Diego, CA). Mouse monoclonal antibodies to giantin, influenza virus hemagglutinin (HA), and GluGlu (EE) tag were from Covance (Richmond, CA). Mouse anti-FLAG antibody M2, horseradish peroxidase-conjugated antibody, and mouse monoclonal anti-beta-actin antibody were from Sigma. A mouse monoclonal antibody to protein disulfide isomerase (PDI) was from Affinity Bioreagents (Golden, CO). Rabbit polyclonal antibody to NS5A was prepared by immunization using peptides of residues from 409 to 422, DVESYSSMPPLEGE. Mouse monoclonal antibody to NS5B was described previously (41).

Plasmids. For expression in mammalian cells, a DNA fragment encoding NS5A was generated from HCV genotype 1b strain J1 (1) (GenBank database accession number D89815), and another was generated from genotype 1a strain H77 (52) (GenBank database accession number AF009606) by PCR using *Pfu* turbo DNA polymerase (Stratagene, La Jolla, CA). The fragments were then cloned into the appropriate sites in pEF-FLAG pGBK puro (18) and pEGFP-C3 (Clontech, Palo Alto, CA). The mutations of the NS5A gene were generated by a method known as "splicing by overlapping extension" (16, 17) and cloned into pEF-FLAG pGBK puro. The DNA fragment encoding NS5B of the J1 strain was generated by PCR and cloned into pCAGGs-PUR (33). The DNA fragment encoding human VAP-A was amplified by PCR from a human fetal-brain library (Clontech) and was introduced into pEF-FLAG pGBK puro, pEF-EE hygro (34), pCHA3 (34), and pcDNA3.1-N-HA, in which an HA tag is inserted in the N terminus of the cloning site of pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The cDNAs of human VAP-A and -B were amplified by PCR and cloned into pEF-FLAG pGBK puro, pEF-EE hygro, pcDNA3.1-N-HA, and pEGFP-C3. The genes encoding VAP lacking the transmembrane domain were amplified and cloned into pEF-FLAG pGBK puro. The DNA fragment encoding the human VAP-B protein lacking a coiled-coil region was introduced into pEF-EE hygro. All PCR products were confirmed by sequencing them with an ABI PRSM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Yeast two-hybrid assay and library screening. The NS5A-binding protein was identified by a yeast two-hybrid assay according to the user manual of MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech). The DNA fragment encoding amino acids 1973 to 2419 was amplified from HCV strain J1 by PCR and then was cloned into pGBKT7 (Clontech). The resulting plasmid was designated pGBK T7 HCV NS5A. A human brain library based on pACT2 was purchased from Clontech. The yeast *Saccharomyces cerevisiae* strain AH109, which secretes alpha-galactosidase under the control of MEL1 upstream activation sequence, was grown in yeast extract-peptone-dextrose medium and transformed with the bait and library plasmids. The transformed yeast cells were grown on 2.0% agar plates of dropout medium lacking tryptophan, leucine, histidine, and adenine. The resulting colonies were inoculated on the new dropout plate containing 20 μg/ml X-alpha-Gal (5-bromo-4-chloro-3-indolyl-alpha-*O*-galactopyranoside) and lacking leucine and tryptophan. The total DNA was prepared from all positive clones and then introduced into *Escherichia coli* strain JM109. The prey plasmids of isolated yeast cells were recovered from the clones grown on LB agar plates containing 10 μg/ml ampicillin and then purified. The insert DNA fragments of isolated clones were determined by sequencing. Finally, 48 alpha-galactosidase-positive clones were identified from 2 million clones screened in the fetal-brain library. One of the positive clones contained the complete cDNA of human VAP-B in frame.

Transfection, immunoblotting, and immunoprecipitation. Cells were seeded onto a six-well tissue culture plate 24 h before transfection. The plasmids were transfected into cells by liposome-mediated transfection using Lipofectamine 2000 (Invitrogen). Cells were harvested 36 h posttransfection, washed five times

with 1 ml of ice-cold phosphate-buffered saline (PBS), and suspended in 0.2 ml lysis buffer (20 mM Tris-HCl, pH 7.4, containing 135 mM NaCl and 1% Triton X-100) supplemented with 1 μg/ml leupeptin, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 5 mM $NaVO_4$. Cell lysates were sonicated at 4°C for 5 min, incubated for 30 min at 4°C, and centrifuged at 14,000 × *g* for 5 min at 4°C. The supernatant was immunoprecipitated with 1 μg of antibodies and 10 μl of Protein G-Sepharose 4B Fast Flow beads (Amersham Pharmacia Biotech, Franklin Lakes, NJ). The immunocomplex was precipitated with the beads by centrifugation at 14,000 × *g* for 30 s and then was washed five times with lysis buffer by centrifugation. The proteins binding to the beads were boiled in 30 μl of loading buffer and then subjected to sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and then reacted with primary antibody and secondary horseradish peroxidase-conjugated antibody. The immunocomplexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by using an LAS-3000 image analyzer (Fujifilm, Tokyo, Japan).

Immunofluorescence microscopy. Cells were seeded on an eight-well chamber slide at 2×10^4 per well 24 h before transfection. Transfected cells were washed twice with PBS, fixed with PBS containing 4% paraformaldehyde, and permeabilized with PBS containing 0.5% Triton X-100. The ER and Golgi apparatus of cells were stained with the mouse monoclonal antibody against luminal ER redox enzyme PDI and the rabbit polyclonal antibody against giantin, respectively, in PBS containing 5% bovine serum albumin. Bound primary antibody was revealed with Alexa Fluor 594-conjugated anti-mouse or anti-rabbit antibody. After additional washes with PBS, a coverslip was attached over PBS containing 50% glycerol and observed under an LSM 510 microscope (Carl Zeiss, Tokyo, Japan).

Gene silencing by siRNA. The siRNA target sequence against human VAP-B, 5′-GGUUAUGGAAGAAUGUAAGTT-3′, was synthesized and purified by Ambion (Austin, TX). Negative control siRNA, siCONTROL Non-Targeting siRNA-2, was purchased from Dharmacon (Lafayette, CO). The Huh-7 cells harboring a subgenomic HCV replicon on six-well plates were transfected with 80 nM or 160 nM of siRNA by using siFECTOR (B-Bridge International, Sunnyvale, CA) according to the manufacturer's protocol. Cells were incubated in DMEM supplemented with 10% FCS and harvested at 96 h posttransfection.

RNA replication assay. In vitro RNA replication was determined as previously described with some modification (3). Briefly, the Huh-7 cells harboring a subgenomic HCV replicon grown in a 100-mm dish were treated with lysolecithin (Wako, Osaka, Japan) (250 μg/ml in wash buffer; 150 mM sucrose, 30 mM HEPES [pH 7.4], 33 mM NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate), collected by scraping in 120 μl of incomplete replication buffer (100 mM HEPES [pH 7.4], 50 mM NH_4Cl, 7 mM KCl, and 1 mM spermidine), and centrifuged at 1,600 rpm for 5 min at 4°C. A total of 40 μl of cytoplasmic fraction (supernatant) was treated with 1% Nonidet P-40 (Boehringer Mannheim, Quebec, Canada) at 4°C for 1 h and incubated with antibody for 4 h at 4°C with rotation. Then, samples were incubated with 1 mM of ATP, GTP, and UTP; 10 μM CTP; [α-^{32}P]CTP (1 MBq; 15 TBq/mmol); 10 μg/ml actinomycin D; and 800 U/ml RNase inhibitor (Promega, Madison, WI) for 4 h at 30°C. RNA was extracted from the total mixture by TRI Reagent (Molecular Research Center Inc., Cincinnati, OH). The RNA was precipitated, eluted in 10 μl of RNase-free water, and analyzed by 1% formaldehyde agarose gel electrophoresis.

Real-time PCR. Total RNA was prepared from cell lines by using TRIzol LS (Invitrogen), and first-strand cDNA was synthesized by using a first-strand cDNA synthesis kit (Amersham) with random primers. Each cDNA was estimated by Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) according to the manufacturer's protocol. Fluorescent signals were analyzed with an ABI PRISM 7000 (Applied Biosystems). The HCV NS5A gene was amplified using the primer pairs 5′-AGTCAGTTGTCTGCGCTTTC-3′ and 5′-CGGGGAATTTCCTGGTCTTC-3′. The human beta-actin gene was amplified with the primer pairs 5′-TGGAGTCCTGTGGCATCCACGAAACTACCTTCAACTC-3′ and 5′-CGGACTCGTCATACTCCTGCTTGCTGATCCACATC-3′, which are located at different exons to prevent false-positive amplification from contaminated genomic DNA. The value of the HCV genome was normalized with that of actin mRNA. Each PCR product was found as a single band of the correct size on agarose gel electrophoresis (data not shown).

RESULTS

Isolation of VAP-B as a novel binding partner for HCV NS5A. To examine the protein(s) that interacts with NS5A in more detail, we screened a cDNA library of human fetal brain by a yeast two-hybrid system using a full-length NS5A of genotype 1b as bait. Among the 2 million transformants we screened, we obtained 48 positive clones containing cDNAs that encode proteins interactive with NS5A. A BLAST search against the GenBank database revealed each of two clones that have the cDNA encoding VAP-A and VAP-B in frame. Figure 1 shows the amino acid alignments of VAP-A and VAP-B and their predicted functional domains. VAP-A and VAP-B are composed of 242 and 243 amino acids, respectively. VAP-B shows 63% amino acid identity to VAP-A. VAP has three structural domains. The first 124 amino acids share high sequence similarity with the nematode major sperm protein and are conserved among all VAP family members (50). The central region on the protein contains an amphipathic helical structure and is predicted to form a coiled-coil protein-protein interaction motif (159 to 196 aa) and a hydrophobic carboxy-terminal transmembrane domain (TMD) (223 to 243 aa). The homology between their N-terminal regions is higher than that between their C-terminal regions (32, 48).

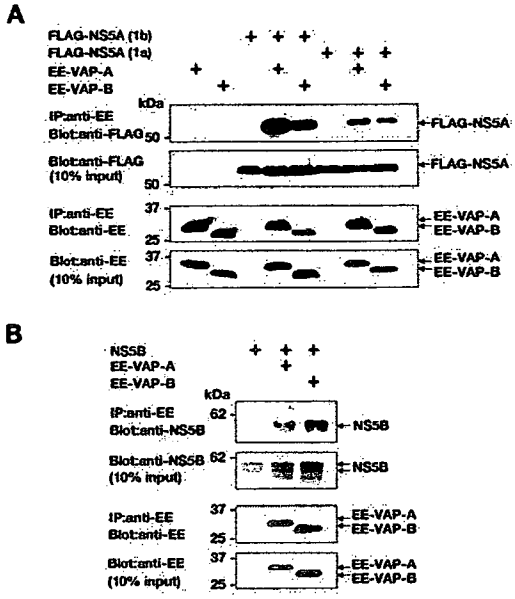

FIG. 2. VAP-A and VAP-B bind to both NS5A and NS5B in mammalian cells. N-terminally FLAG-tagged NS5A of genotype 1b, FLAG-NS5A (1b) of genotype 1a, FLAG-NS5A (1a), and N-terminally EE-tagged VAP (EE–VAP-A or EE–VAP-B) were coexpressed in HEK293T cells and immunoprecipitated with anti-EE antibody. The resulting precipitates were examined by immunoblotting using anti-FLAG antibody (A). NS5B was coexpressed with EE-tagged VAP-A or VAP-B and immunoprecipitated with anti-EE antibody, and NS5B in the precipitates was detected by anti-NS5B antibody (B). One-tenth of the lysates used in immunoprecipitation are shown as the 10% input. The data in each panel are representative of three independent experiments.

VAP-B interacts with NS5A and NS5B in mammalian cells. To confirm the specific interaction, FLAG-tagged NS5A was coexpressed with EE-tagged VAP-A or VAP-B in 293T cells, and cell lysates were immunoprecipitated by specific antibodies. NS5A was coprecipitated with VAP-A and VAP-B to similar extents (Fig. 2A). We also obtained the same results in the reverse experiments (data not shown). Recently, it was shown that hyperphosphorylation of NS5A disrupts interaction with VAP-A and negatively regulates HCV RNA replication, sug-

gesting that adaptive mutations detected in the HCV replicon prevent phosphorylation-dependent dissociation of the RNA replication complex (9). Amino acid residues at Tyr2185 and Lys2187 of NS5A genotype 1b were defined as key determinants for VAP-A binding, and the replacement of these residues with those of genotype 1a (Ala and Gly, respectively) reduced binding to VAP-A in yeast and enhanced hyperphosphorylation of NS5A (9). However, as shown in Fig. 2A, the NS5As of both the 1a and 1b genotypes were coimmunoprecipitated with VAP-A and -B in mammalian cells. Since a previous report indicated that VAP-A interacts with not only NS5A but also NS5B (12), we next examined the interaction of VAP-B with NS5B. EE-tagged VAP-A or VAP-B was coexpressed with NS5B in 293T cells and immunoprecipitated with anti-EE-tag antibody. NS5B was coprecipitated with VAP-B, as well as VAP-A (Fig. 2B). These results indicate that VAP-B participates in the complex of HCV NS proteins in a manner similar to that of VAP-A.

NS5A colocalizes with VAP-B in ER and Golgi compartments. To determine the subcellular localization of NS5A and VAP-B in mammalian cells, HeLa cells were cotransfected with plasmids encoding enhanced green fluorescent protein (EGFP)-tagged NS5A and FLAG-tagged VAP-B or FLAG-tagged VAP-A and examined by immunofluorescence analysis. EGFP-NS5A was colocalized exclusively with FLAG-VAP-B in the cytoplasm, as seen in FLAG-VAP-A (Fig. 3A). To further determine the precise subcellular localization of NS5A and VAP-B, the ER and Golgi apparatus were stained with specific antibodies against PDI and giantin, respectively. NS5A and VAP-B were colocalized with PDI and giantin in HeLa cells transfected with the plasmids (Fig. 3B), indicating that NS5A and VAP-B are colocalized in the membranes of the ER or ER-derived compartment. VAP-B was localized in a diffuse ER-like network, in small vesicles clustered around the nucleus, and predominantly in a perinuclear/Golgi region. Similar to the case with VAP-A, the colocalization of NS5A with VAP-B in the ER and Golgi apparatus suggests that NS5A specifically interacts with VAP-B under intracellular conditions.

Dimerization of VAP-A and VAP-B and interaction with NS5A. Immunoprecipitation analyses revealed that NS5A and NS5B interact with VAP-A and VAP-B. Therefore, it might be reasonable to speculate that VAP-A and VAP-B interact with each other and are involved in RNA replication through the formation of a replication complex. It has been demonstrated that VAP-A interacts with VAP-A or VAP-B through their TMDs and forms a homodimer and a heterodimer in vitro (32). We constructed expression plasmids encoding mutant VAP-A and VAP-B lacking their TMDs and examined their dimer formation with authentic VAPs in vivo. Although coprecipitation of authentic VAP (FLAG–VAP-B or FLAG–VAP-A) with VAP-B–HA was clearly detected, no interaction between TMD deletion mutants (FLAG–VAP-AΔTMD or FLAG–VAP-BΔTMD) and VAP-B–HA was observed (Fig. 4A and B). Furthermore, a TMD deletion mutant, HA–VAP-BΔTMD, which lost the ability to form a dimer with VAP-B and VAP-A, retained the ability to bind to FLAG-NS5A (Fig. 4C), although the efficiency of interaction with NS5A was reduced. These results indicate that TMDs of VAP-A and VAP-B are required for hetero- and homodimerization, but

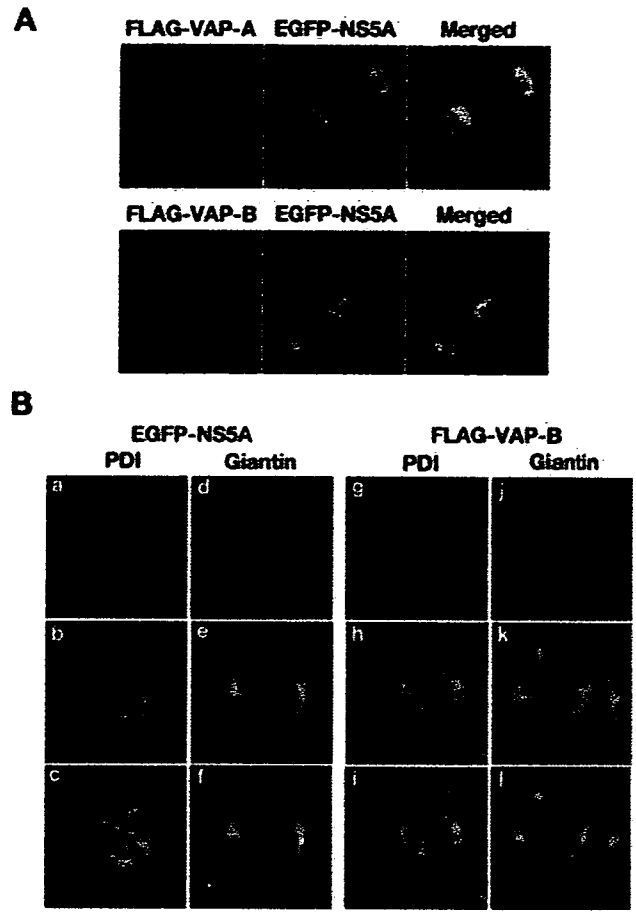

FIG. 3. Intracellular localization of VAPs and NS5A in mammalian cells. (A) N-terminally FLAG-tagged VAP (FLAG–VAP-A or FLAG–VAP-B) was coexpressed with N-terminally EGFP-fused NS5A of genotype 1b (EGFP-NS5A) in HeLa cells, fixed with 4% paraformaldehyde-PBS, permeabilized with 0.5% Triton X-100, and stained with anti-FLAG antibody and AlexaFluor 594-conjugated anti-mouse IgG antibody. (B) EGFP-NS5A of genotype 1b (b and e) or FLAG–VAP-B (h and k) was expressed and then stained with anti-PDI (a and g) or anti-giantin (d and j) antibodies and AlexaFluor 594-conjugated anti-mouse IgG antibody. FLAG–VAP-B was stained with biotinylated anti-FLAG antibody and fluorescein isothiocyanate-conjugated streptavidin. Overlapped images are shown in panels c, f, i, and l.

not for binding to NS5A. A region other than the TMD should be involved in the specific interaction between VAP-B and HCV NS5A. The coiled-coil domain of VAP-A was reported to be critical for binding to NS5A (48). Therefore, we examined whether the coiled-coil domain of VAP-B is also involved in interaction with NS5A. FLAG-NS5A was coimmunoprecipitated with EE–VAP-B but not with EE–VAP-BΔcoiled-coil, which lost the coiled-coil domain but retained the TMD (Fig. 4D), suggesting that the coiled-coil domain is also essential for interaction between NS5A and VAP-B.

Two separate domains in NS5A are critical for binding to VAP-B. Since NS5A specifically interacts with VAP-B, we tried to determine the region of NS5A responsible for interaction with VAP-B. Various deletion mutants of FLAG-tagged NS5A were prepared as shown in Fig. 5A. The mutants covering regions from amino acids 1 to 75, but not 1 to 50, and those

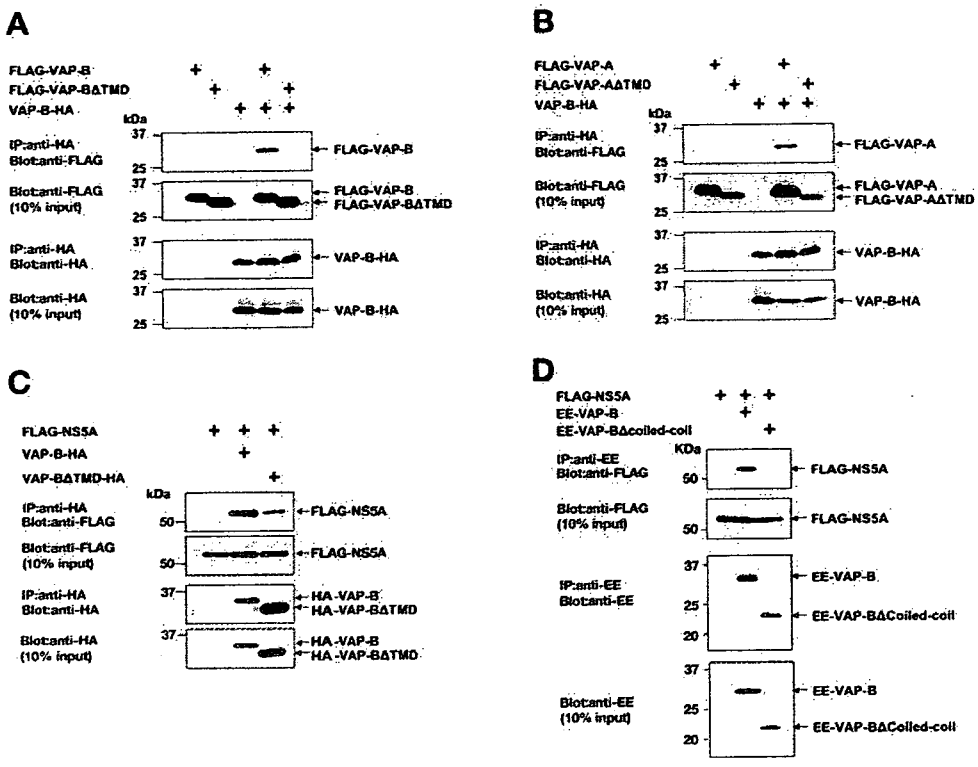

FIG. 4. VAP-B dimerizes with VAP-B and VAP-A through the TMD and interacts with NS5A via the coiled-coil domain. C-terminally HA-tagged VAP-B (VAP-B–HA) was coexpressed with FLAG–VAP-B or FLAG–VAP-B with TMD deleted (FLAG–VAP-BΔTMD). VAP-B–HA was immunoprecipitated with anti-HA antibody, and the immunoprecipitates were immunoblotted with anti-FLAG antibody (A). Interaction of VAP-B–HA with FLAG–VAP-A or FLAG–VAP-A with TMD deleted (FLAG–VAP-AΔTMD) was examined in a similar way (B). FLAG-NS5A was coexpressed with HA–VAP-B or HA–VAP-BΔTMD, and immunoprecipitates with anti-HA antibody and immunoprecipitates were immunoblotted with anti-FLAG antibody (C). FLAG-NS5A was coexpressed with EE–VAP-B or with EE–VAP-B in which the coiled-coil domain was deleted (EE–VAP-BΔcoiled-coil). EE-tagged VAP-B proteins were immunoprecipitated with anti-EE antibody, and immunoprecipitates were immunoblotted with anti-FLAG antibody (D). One-tenth of the lysates used in immunoprecipitation are shown as the 10% input. The data in each panel are representative of three independent experiments.

from amino acids 325 to 447, but not 350 to 447, exhibited binding to VAP-B, suggesting that two separate regions of NS5A (amino acids 51 to 75 and 325 to 349) are involved in physical association with VAP-B. Further mutational analyses of NS5A revealed that regions from amino acids 1 to 70, but not 1 to 65, and those from amino acids 340 to 447, but not 345 to 447, interact with VAP-B (Fig. 5B and C), suggesting that amino acids 66 to 70 and 340 to 344 are required for interaction with VAP-B. According to Tellinghuisen et al., NS5A consists of three domains, domain I (amino acids 1 to 213), domain II (amino acids 250 to 342), and domain III (amino acids 356 to 477) (46, 47). In our results, the region from amino acids 340 to 344, which is essential for the physical interaction with VAP-B, belongs to the connecting segment between domains II and III of NS5A. Ala substitution analyses revealed that an NS5A construct covering amino acids 260 to 447 that replaced the five amino acid residues between 340 and 344 with Ala abrogated interaction with VAP-B (Fig. 5D), whereas that covering 75 N-terminal amino acids carrying an Ala substitution of between 66 and 70 residues retained binding activity to VAP-B (data not shown). Therefore, we focused on the region between 340 and 344 to determine the amino acid residues in NS5A responsible for specific binding to VAP-B. A FLAG-tagged full-length NS5A carrying an Ala substitution between amino acid residues 340 and 344 (FLAG-NS5A/340-344A) exhibited a clear reduction of binding to EE–VAP-B compared with the authentic NS5A (Fig. 5E). To further determine the critical amino acids of NS5A responsible for specific binding to VAP-B, each amino acid between 340 and 344 of the NS5A construct covering amino acids from 260 to 447 was replaced with Ala, and the effect of each substitution on the interaction with VAP-B was examined by immunoprecipitation. As summarized in Fig. 5F, the four amino acid residues 341 to 344 in the polyproline cluster region of NS5A, which are highly conserved among HCV genotypes, are suggested to be involved in the interaction with VAP-B.

VAP-B plays an important role in HCV RNA synthesis. To determine whether VAP-B is involved in HCV replication, cell lysates isolated from Huh-7 cells harboring a subgenomic HCV replicon were used for an in vitro RNA synthesis assay. Chicken anti-human VAP-B antibody raised against synthesized peptides specifically detected endogenous and overexpressed VAP-B (Fig. 6A). Cytoplasmic fraction from the HCV replicon was added to an assay mixture containing [α-^{32}P]CTP and incubated at 30°C for 4 h in the presence or absence of antibodies. Labeled RNA was analyzed by 1% formaldehyde agarose gel electrophoresis as described previously (2). Replication of the subgenomic HCV RNA was inhibited by the

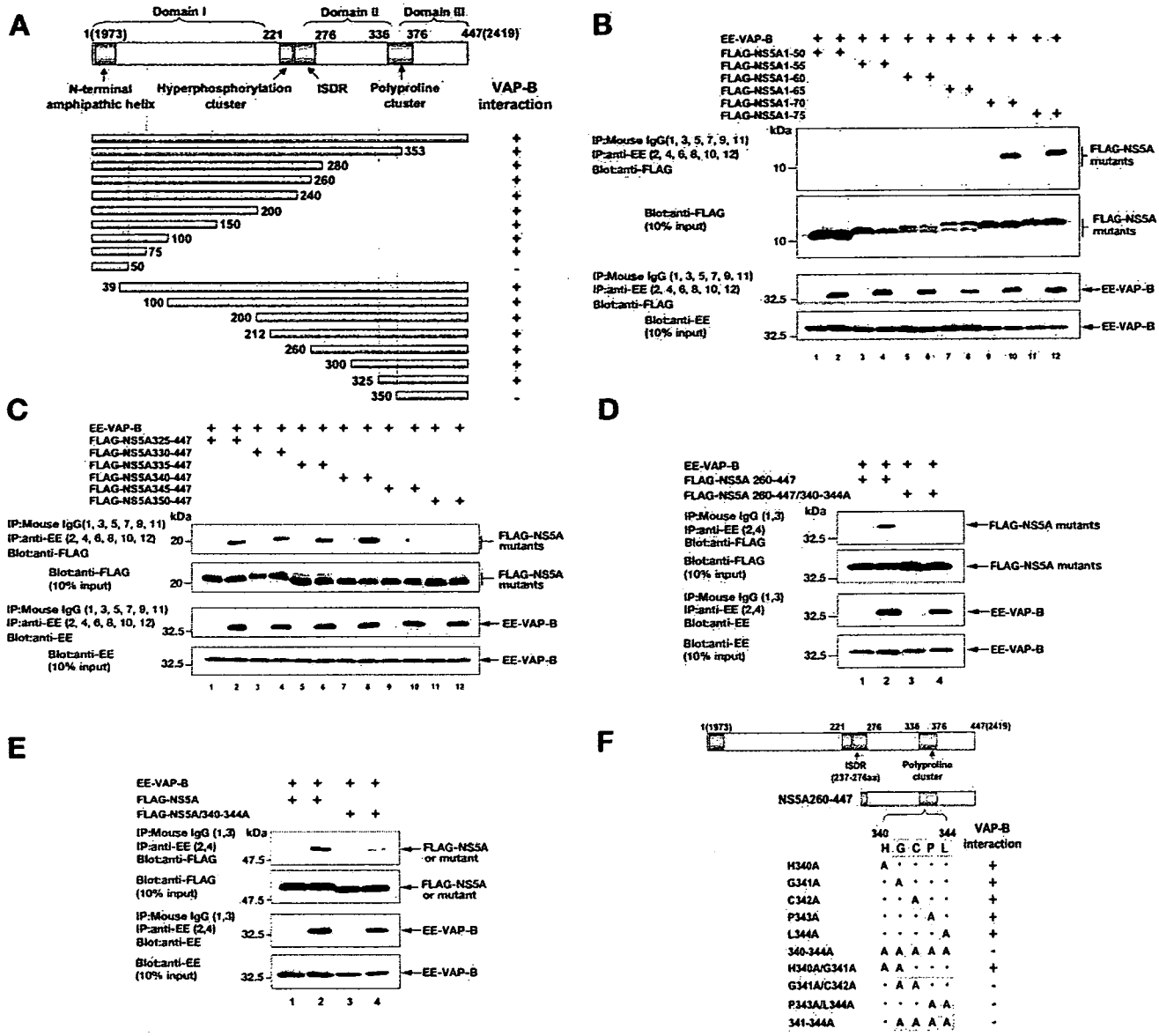

FIG. 5. Two regions of NS5A are required for VAP-B binding. N-terminal or C-terminal deletion mutants of NS5A were introduced into pEF-FLAG pGBK puro vector and coexpressed with EE–VAP-B. EE–VAP-B was immunoprecipitated with anti-EE antibody, and immunoprecipitates were immunoblotted by anti-FLAG antibody. The reverse combination of immunoprecipitation was also examined. The results are summarized in panel A. Four functional domains in the NS5A protein and three domains based on the locations of the blocks of low-complexity sequence (46) are indicated. The numbers in parentheses indicate amino acid residues in the HCV polyprotein. To further determine the critical amino acids of NS5A for specific binding to VAP-B, deletion mutants of the N-terminal region from residues 1 to 75 (B) or those of the C-terminal region from residues 325 to 447 (C) were immunoprecipitated with EE–VAP-B. Replacement of the five residues 340 to 344 with Ala was introduced into a truncated NS5A possessing residues 260 to 447, FLAG-NS5A 260-447/340-344A (D), or full-length NS5A, FLAG-NS5A/340-(E), to examine the interaction with VAP-B. Further precise mutations were introduced into NS5A possessing residues 260 to 447. The resulting mutants were coexpressed with EE–VAP-B and immunoprecipitated as described above. The results are summarized in panel F. Four amino acids (Gly, Cys, Pro, and Leu) responsible for interaction with VAP-B are indicated by dotted squares. Plus and minus indicate binding and nonbinding, respectively (A and F). One-tenth of the lysates used in immunoprecipitation are shown as the 10% input. The data in each panel are representative of three independent experiments.

antibody to VAP-B but not by a control chicken immunoglobulin G (IgG) (Fig. 6B), suggesting that VAP-B plays a critical role in HCV replication. Aizaki et al. suggested that VAP-A sequesters NS5A at an appropriate site, such as the raft-like domain on the intracellular compartment, and that the TMD of VAP-A plays an important role in subcellular localization and dimerization (2). We demonstrated that the TMD of VAP is required for hetero- and homodimerization of VAP-A and VAP-B but not for interaction with NS5A (Fig. 4). Gao et al. indicated that a truncated VAP-A mutant lacking the TMD inhibited the association of HCV NS proteins with insoluble membrane fractions and reduced both the expression level of

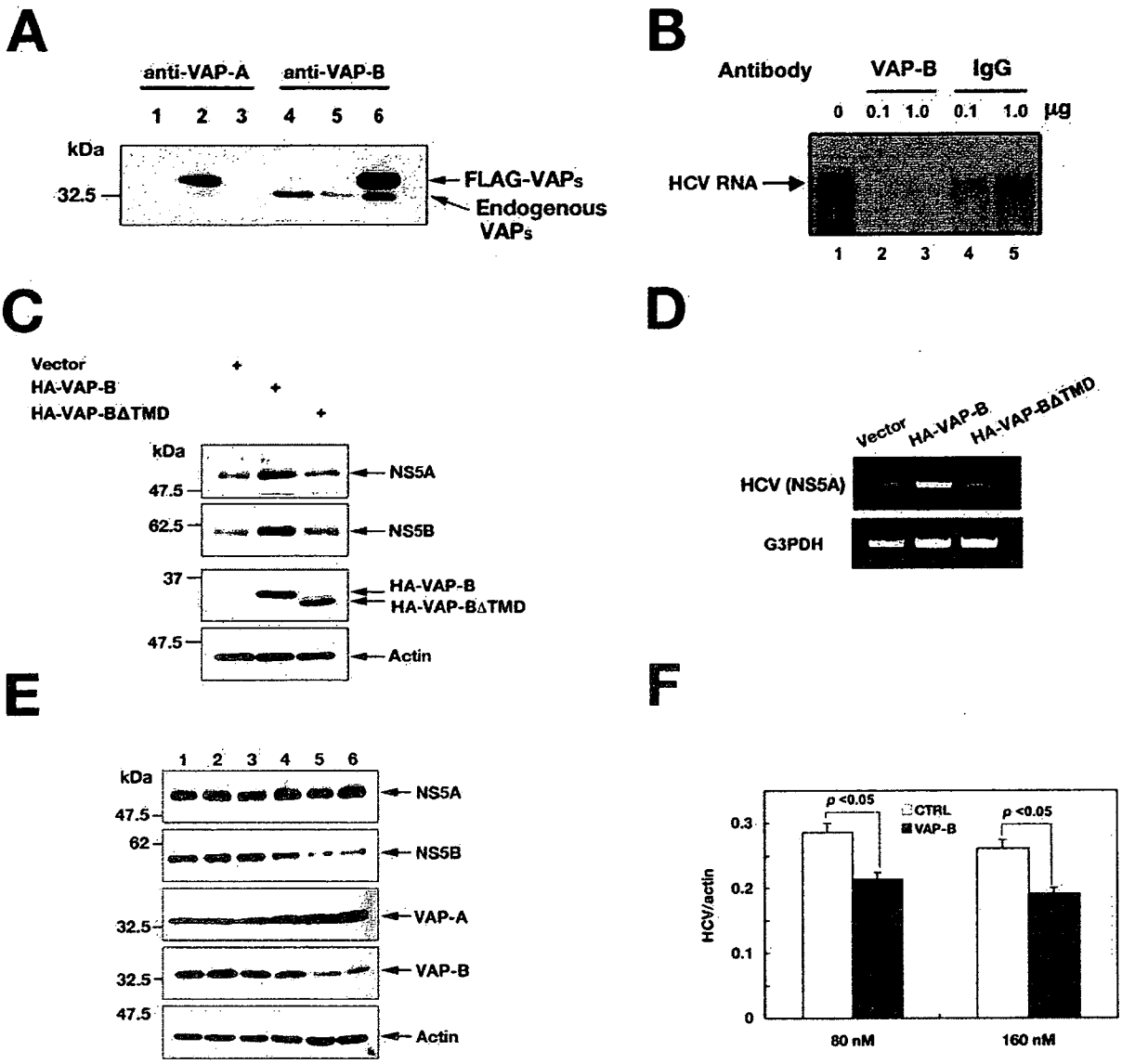

FIG. 6. VAP-B is involved in HCV replication. (A) FLAG–VAP-A (lanes 2 and 5) or FLAG–VAP-B (lanes 3 and 6) was expressed in HEK293T cells and examined by immunoblotting using anti-human VAP-A mouse monoclonal and anti-human VAP-B chicken polyclonal antibodies. (B) In vitro RNA synthesis was carried out in the presence of various concentrations of anti-human VAP-B chicken polyclonal antibody or control chicken IgG. RNA extracted from each fraction was analyzed by agarose gel electrophoresis and autoradiographed. (C) Empty plasmid, expression plasmid of N-terminally HA-tagged VAP-B (HA–VAP-B), or N-terminally HA-tagged VAP-BΔTMD (HA–VAP-BΔTMD) was transfected into HCV replicon cells. Expression of NS5A and NS5B was examined by immunoblotting. (D) HCV RNA was detected by reverse transcription-PCR using primer pairs against NS5A, and expression of G3PDH was used as a control. (E) siRNA against VAP-B or control was transfected into the HCV replicon cells. Lane 1, untreated; lane 2, treated with siFECTOR; lanes 3 and 4, control siRNA was transfected; lanes 5 and 6, VAP-B siRNA was transfected. Expression of NS5A, NS5B, VAP-A, VAP-B, and beta-actin was determined by immunoblotting at 96 h posttransfection. (F) siRNA against VAP-B or control was transfected into the HCV replicon cells. The results are expressed as standard deviations. The significance of the difference in means was determined by the Student *t* test. The data in each panel are representative of three independent experiments.

NS5A and HCV RNA replication in replicon cells (12). To determine the possible implication of VAP-B in HCV replication, VAP-B or VAP-BΔTMD was expressed in Huh-7 RNA replicon cells. In contrast with the previous data, overexpression of VAP-B increased NS5A and NS5B expression and enhanced the replication of HCV replicon cells, but no effect was observed in cells expressing VAP-BΔTMD (Fig. 6C and D). To confirm the role of VAP-B in HCV replication, we examined the effect of the knockdown of endogenous VAP-B from the HCV replicon cells by siRNA. At 96 h posttransfection, the expression of VAP-B in cells transfected with the siRNA targeted to VAP-B was reduced to half the levels of cells transfected with a control siRNA, whereas the expression of VAP-A was slightly increased. Although NS5B expression was reduced by the VAP-B knockdown, NS5A was not affected (Fig. 6E). HCV RNA

replication exhibited 25% and 27% reductions by the transfection of 80 and 160 nM siRNA, respectively, to VAP-B (Fig. 6F). Collectively, these results suggest that VAP-B plays an important role in the sequestration of NS5A and NS5B in the HCV RNA replication complex.

DISCUSSION

Although there are conflicting data in the literature, it is accepted that NS5A is a multifunctional protein with critical roles in HCV replication, as well as in the establishment and maintenance of persistent infection (26). Tu et al. were the first to successfully isolate VAP-33 (VAP-A) as a binding partner of NS5A by a yeast two-hybrid screening of the human liver library; they also indicated an association between VAP-A and not only NS5A, but also NS5B, in mammalian cells (48). Gao et al. (12) further demonstrated that NS5A interacts with NS4B, the only HCV NS protein possessing an intrinsic ability to associate with lipid rafts; and the interaction of NS5A, NS5B, NS4B, and other NS proteins with VAP-A on lipid rafts plays a crucial role in the formation of the HCV RNA replication complex (26). Evans et al. indicated that NS5A from the Con1 strain (genotype 1b) is strongly associated with VAP-A, whereas NS5A from the H77 strain (genotype 1a) was unable to bind VAP-A in yeast (9). The determinants of subtype-specific binding to VAP-A were mapped to amino acids 2185 and 2187, and the substitution of these amino acids of the Con1 strain into those of the H77 strain abrogated both the binding to VAP-A and the replication of the subgenomic replicon. However, these defects in binding to VAP-A and in the replication of the subgenomic replicon were suppressed in the highly adaptive S2204I mutation in NS5A. The S2204I adaptive mutation was shown to disrupt NS5A hyperphosphorylation (5), and the loss of NS5A hyperphosphorylation was shown to correlate with the adaptive mutation's ability to suppress the replication defect caused by the VAP-A-noninteracting mutations (9).

To gain more insight into interaction between NS5A and host proteins involved in HCV replication, we screened human libraries by the yeast two-hybrid system using NS5A as bait and identified VAP-B as a binding protein to NS5A. VAP-B is ubiquitously expressed as VAP-A in human tissues, including liver (32). NS5A can bind to both VAP-A and VAP-B and is colocalized in intracellular compartments, such as the ER and Golgi apparatus. The coiled-coil domain of VAP-B is responsible for their interaction with NS5A, as previously reported in VAP-A (48). In the present study, two regions in NS5A are suggested to be important for VAP-B binding. One region is the N-terminal 70 residues, especially from 66 to 70 (2037 to 2042 aa in the HCV polyprotein), although replacement of these 5 residues with Ala could not abrogate binding to VAP-B. The other is identified at the C-terminal polyproline cluster, and replacement of these four residues from 341 to 344 (2313 to 2316 aa in the HCV polyprotein) with Ala in a full-length NS5A reduced VAP-B binding. Two class II polyproline motifs (consensus PXXPXR) are identified in the polyproline cluster and can bind the SH3 domains of a number of cellular signaling proteins, including Grb2 (45), amphiphysin II (56), and Src family tyrosine kinases (25). Pro343 and Leu344 in the C-terminal VAP-B binding region are part of the first class II polyproline motif. The overlapping of VAP-B's binding region with other cellular signaling proteins may suggest interplay between cellular signaling and replication of HCV. A previous observation indicated that the interaction between NS5A and VAP-A was genotype specific, and amino acid residues critical for the interaction were mapped to amino acids 2185 and 2187 in yeast (9). However, the same authors indicated that NS5A derived from either the1a or 1b genotype expressed in Huh-7 cells interacted equally well with a glutathione *S*-transferase fusion VAP-A expressed in bacteria in vitro, and an attempt at selective interaction of hypophosphorylated NS5A from replicon cells with VAP-A was not successful (9). Furthermore, in our study, no clear difference was detected between native NS5A and the S2204I mutant in binding to VAP-A or VAP-B by immunoprecipitation analyses in mammalian cells (data not shown). In addition, the data in Fig. 2 clearly indicate that NS5A genotype 1a binds to both VAP-A and VAP-B, even though this genotype carries the VAP-A-noninteracting mutations (A2185 and G2187). This discrepancy might be explained by the differences between the experimental systems, including the condition of cell lines, the intracellular ratio of VAP-A and VAP-B, and the phosphorylation status of NS5A. Evans et al. proposed that hyperphosphorylated p58 NS5A represents a closed conformation that cannot interact with VAP-A, whereas hypophosphorylated p56 NS5A represents an open conformation capable of strong interaction with VAP-A (9). The phosphorylation of NS5A is a critical modification that controls not only its interaction with VAP-A, but also RNA replication in Huh7 replicon cells (9, 31). Further study will be needed to elucidate the relationship between the phosphorylation status of NS5A and the capability of binding to VAP-B.

The inhibition of HCV RNA replication by the specific antibody to VAP-B in vitro indicated that VAP-B is a component of the HCV RNA replication complex. Furthermore, the reduction of VAP-B expression by siRNA induced the suppression of NS5B expression but not of NS5A, as seen in the knockdown experiment with VAP-A (12). This suggested that VAP plays an important role in the participation of NS5B in the replication complex. VAP could form hetero- and homodimers through their TMDs and interact with NS5A through their coiled-coil domains (Fig. 4). VAP-C is a splicing variant of VAP-B missing 60% of the C terminus. Therefore, VAP-C cannot interact with VAP-A, VAP-B, or NS5A. Although it is difficult to determine precisely the participation of the monomer and dimer of VAP-A and VAP-B in the HCV replication complex, it might be plausible to speculate that VAP-A is expressed more abundantly than VAP-B and that the heterodimer of VAP-A and VAP-B is more active as an HCV replication complex than those of the monomeric or homodimeric forms. Therefore, overexpression of VAP-B, but not of VAP-A, enhanced HCV RNA replication by providing scaffolds in appropriate positions, like the raft-like domain in the ER/Golgi compartment, capable of changing the nonfunctional NS proteins into a replication-competent state, because only a small fraction of NS proteins are functional as replication complexes (20, 28). Furthermore, VAP-A might have a higher affinity to NS5B than VAP-B does, and overexpression of the TMD deletion mutant of VAP-A, but not that of VAP-B, exhibited a reduction of RNA replication (12). The

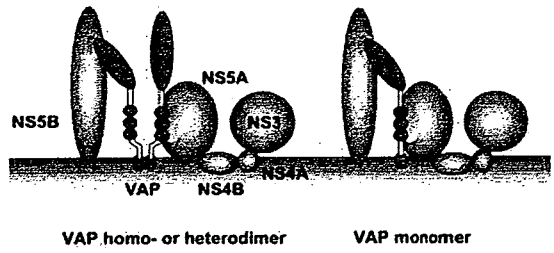

FIG. 7. Models of interaction between HCV NS proteins and VAP. Monomeric and hetero- or homodimeric forms of VAPs can interact with NS5A and NS5B through the coiled-coil domain and N-terminal region, respectively. NS4B can associate with lipid rafts and interact with NS5A (9). NS4A is a cofactor of NS3 and recruits NS3 to the HCV NS protein complex.

possible implication of monomeric and dimeric forms of VAPs in the replication complex of HCV is shown in Fig. 7.

In this study, we identified VAP-B as a novel binding protein to NS5A and NS5B and demonstrated its participation in HCV RNA replication. Elucidation of the precise roles of VAP-A and VAP-B in the phosphorylation of NS5A and in the formation of the replication complex through interaction with other HCV NS proteins and host proteins should provide clues to understanding the molecular mechanisms underlying the replication of HCV RNA and to developing novel therapeutics for chronic hepatitis C.

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Replication-Competent Recombinant Vesicular Stomatitis Virus Encoding Hepatitis C Virus Envelope Proteins▽

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Although in vitro replication of the hepatitis C virus (HCV) JFH1 clone of genotype 2a (HCVcc) has been developed, a robust cell culture system for the 1a and 1b genotypes, which are the most prevalent viruses in the world and resistant to interferon therapy, has not yet been established. As a surrogate virus system, pseudotype viruses transiently bearing HCV envelope proteins based on the vesicular stomatitis virus (VSV) and retrovirus have been developed. Here, we have developed a replication-competent recombinant VSV with a genome encoding unmodified HCV E1 and E2 proteins in place of the VSV envelope protein (HCVrv) in human cell lines. HCVrv and a pseudotype VSV bearing the unmodified HCV envelope proteins (HCVpv) generated in 293T or Huh7 cells exhibited high infectivity in Huh7 cells. Generation of infectious HCVrv was limited in some cell lines examined. Furthermore, HCVrv but not HCVpv was able to propagate and form foci in Huh7 cells. The infection of Huh7 cells with HCVpv and HCVrv was neutralized by anti-hCD81 and anti-E2 antibodies and by sera from chronic HCV patients. The infectivity of HCVrv was inhibited by an endoplasmic reticulum α-glucosidase inhibitor, *N*-(*n*-nonyl) deoxynojirimycin (Nn-DNJ), but not by a Golgi mannosidase inhibitor, deoxymannojirimycin. Focus formation of HCVrv in Huh7 cells was impaired by Nn-DNJ treatment. These results indicate that the HCVrv developed in this study can be used to study HCV envelope proteins with respect to not only the biological functions in the entry process but also their maturation step.

Hepatitis C virus (HCV) is the major causative agent of blood-borne chronic non-A, non-B hepatitis, infecting at least 3% of the world's population. The majority of HCV-infected individuals develop chronic hepatitis that eventually progresses to liver cirrhosis and hepatocellular carcinoma (36). HCV is an enveloped single-stranded plus-sense RNA virus belonging to the genus *Hepacivirus* in the *Flaviviridae* family, which also includes members of the genus *Flavivirus*, such as yellow fever virus, dengue virus, and West Nile virus, and of the genus *Pestivirus*, such as bovine viral diarrhea virus and classical swine fever virus. The genome of HCV encodes a polyprotein of approximately 3,000 amino acids, which is subsequently processed into at least 10 viral proteins. The HCV envelope glycoproteins E1 and E2 are cleaved from the polyprotein by host signal peptidases and play a crucial role in the initiation of infection through interaction with cell surface receptor(s) in the HCV life cycle (17, 38).

A number of cellular components have been shown to participate in HCV adsorption and/or internalization, including human CD81 (hCD81) (52), low-density lipoprotein receptor (LDLr) (1), human scavenger receptor class B type I (SR-BI) (57), dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), liver/lymph node-specific intercellular adhesion molecule-3-grabbing nonintegrin (L-SIGN or DC-SIGNR) (21, 34), glycosaminoglycans (2), and a tight junction component, claudin-1 (18). Recently, an in vitro cell culture system was developed for HCV of the genotype 2a JFH1 strain (HCVcc) isolated from a fulminant HCV patient (32, 63, 68). However, a robust cell culture system for HCV of the 1a and 1b genotypes, the most prevalent genotypes in the world, has not yet been successfully developed, except for the cell culture system of H77 or H77-S strain (1a genotype) (26, 65). Furthermore, it is currently not possible 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 cell culture. Thus, the relative contribution of these receptor candidates in HCV attachment and entry remains unclear (44).

As surrogate systems for the investigation of HCV infection mechanisms, HCV-like particles (HCV-LP) produced in insect or mammalian cells by recombinant baculovirus vectors have been developed (7, 37). Although the binding of HCV-LP to the target cells has been well characterized, HCV-LP are not suitable for the analysis of the HCV entry steps due to the absence of a clear distinction between binding and internalization. On the other hand, both murine leukemia virus (MLV)- and human immunodeficiency virus-based pseudotype retrovi-

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ral particles (HCVpp) bearing unmodified E1 and E2 proteins (5, 23) are capable of infecting human hepatoma cells, including Huh7 cells, and this infection can be inhibited by treatment with anti-hCD81 antibody and the soluble hCD81 protein or by a knockdown of hCD81 expression by small interfering RNAs (siRNAs) (67). Furthermore, the ectopic expression of hCD81 confers permissiveness to infection with HCVpp in normally nonpermissive HepG2 cells lacking expression of hCD81. These data suggest that expression of hCD81 is crucial for HCVpp infection (4). However, expression of this candidate receptor molecule is not sufficient to render nonhepatic cells permissive for HCVpp infection (6, 23). Indeed, it is also interesting to note that although neutralizing antibodies to HCVpp have been detected in the sera from persistently infected humans and chimpanzees (3, 23, 33, 66), these antibodies do not appear to play a significant role in the outcome of acute HCV infection (42). Therefore, further investigation is needed to assess the authenticity of the HCVpp as a surrogate system for HCV infection.

We and others have previously reported the generation of vesicular stomatitis virus (VSV)-based pseudotype viruses bearing chimeric or unmodified HCV E1 and E2 glycoproteins (HCVpv) in nonhepatic cell lines (27, 39, 60). Although HCVpv infected several cell lines, including human hepatoma cell lines (27, 39, 60), recombinant VSV bearing chimeric HCV E1 and E2 glycoproteins in place of VSV glycoprotein (G) was not infectious (9). This discrepancy in the cell tropism might be attributable to the differences in the constructs and strains of HCV envelope proteins or in the systems and cells in which the viruses were generated.

Human hepatocytes (Hc) are believed to be a main target for HCV replication, and it is reasonable to speculate that hepatocyte-specific host factors regulate the entry, replication, and assembly of HCV. Although HCVpp is an excellent system for examining the entry mechanisms of HCV, the system requires a high level of transfection of the expression plasmids, and thus production of HCVpp is limited to 293T cells due to their high transfectability. Furthermore, HCVpp are replication-defective and do not produce progeny virus in infected Hc, and thus reinfection with progeny viruses cannot be assessed. In this study, we generated replication-competent recombinant VSVs encoding the unmodified HCV E1 and E2 polyproteins of genotypes 1a and 1b in place of the G protein (HCVrv) in human cell lines. HCVrv was able to infect human hepatoma cell lines through an hCD81-dependent pathway and to form foci in Huh7 cells. Treatment with an ER α-glucosidase inhibitor was shown to inhibit not only infection but also focus formation of HCVrv, suggesting that modifications of envelope glycoproteins in the endoplasmic reticulum (ER) are required for infection with HCVrv.

MATERIALS AND METHODS

Plasmids and cells. The cDNAs encoding the C-terminal 60 amino acids of the core to the last residue of p7 protein (c60-p7; nucleotides 735 to 2746) of the H77 (provided by Bukh) and Con1 (provided by Bartenschlager) (residues atg + 521 to 2773 bp) strains were generated by PCR amplification. All PCR products were cloned into pCAGGS/MCS-PM, carrying the puromycin gene for the establishment of the cell lines derived from pCAGGS (45) and designated pCAGc60-p7. The plasmid used for construction of HCVrv was pVSVΔG-P/M2.6, which has additional transcription units with two multiple cloning sites (MCS) located between the P and M genes (MCS-2) and the M and L genes (MCS-1). The c60-p7 gene was subcloned into pBluescript SK(+) from pCAGc60-p7 by digestion with EcoRI and EcoRV and designated pBSc60-p7. To construct pVSVΔG-c60-p7, the c60-p7 gene was excised from pBSc60-p7 with KpnI and XbaI and ligated into the KpnI and NheI sites of MCS-2 of pVSVΔG-P/M2.6. The cDNA of hCD81 was amplified by PCR from Huh7 cells and cloned into the BamHI and XbaI sites of the pcDNA3.1 plasmid, resulting in phCD81. The hepatic (Huh7, HepG2, Hep3B, and PLC/PRF/5) and nonhepatic (293T, HeLa, Vero, BHK, and CHOK1) cell lines were obtained from the American Type Culture Collection (Rockville, MD). The FLC4 cell line was established as described previously (37). The Huh7.5.1 cell line was kindly provided by F. Chisari. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS). Human primary Hc were purchased from the Applied Cell Biology Research Institute (Kirkland, WA) and maintained using a CS-C serum-free medium kit (Applied Cell Biology Research Institute). To establish stable HepG2 or CHOK1 cell lines expressing hCD81, cells were transfected with phCD81 by the *Trans*IT-LT1 (Mirus, Madison, WI) reagent, selected with DMEM containing 10% FBS and 2 mg/ml (HepG2) or 3.5 mg/ml (CHOK1) of G418 (PAA Laboratories GmbH, Linz, Austria), and sorted twice by FACSCalibur (Becton Dickinson, San Jose, CA) after staining with anti-hCD81 monoclonal antibody (JS-81; BD Biosciences Pharmingen, Mountain View, CA) to obtain high-expressing clones. Anti-E1 (BDI198; Biodesign International, Saco, ME) and anti-E2 (AP33) (13, 49) monoclonal antibodies or anti-VSVG polyclonal antibody (ab34774; Abcam Inc., Cambridge, MA) was used for detection of E1 and E2 of the H77 strain or VSVG by immunoblotting, respectively.

Reverse genetics of VSV. Recombinant VSVs were generated as described previously (25, 30). Briefly, BHK cells were grown to 90% confluence on 35-mm tissue culture plates. The cells were infected with a recombinant vaccinia virus encoding T7 RNA polymerase (vTF7-3) (19) at a multiplicity of infection (MOI) of 5. After incubation at room temperature for 1 h, the cells were transfected with 3 μg of pBS-N, 5 μg of pBS-P, 1 μg of pBS-L, 8 μg of pBS-G, and 5 μg of pΔG-c60-p7 plasmids using a cationic liposome reagent (54). After 4 h, the supernatants were replaced with 10% FBS DMEM, and cells were incubated at 37°C for 48 h. The supernatants were then filtered through a 0.22-μm-pore-size filter (Millex-GS; Millipore) to remove vaccinia virus and were applied to BHK cells that had been transfected with pCAGVSVG (39) 24 h previously. Recovery of the virus was assessed by examining the cells for the cytopathic effects that are typical of a VSV infection after 24 to 36 h. Stocks of *G-complemented viruses, i.e., VSVΔG virus or recombinant viruses transiently bearing VSVG protein on the virion surface, were grown from single plaques on BHK cells transfected with pCAGVSVG and then stored at −80°C. The infectious titers of the recovered viruses were determined by a plaque assay.

Production and characterization of HCVpv, HCVrv, or HCVpp. The construction of HCVpv and HCVrv is summarized in Fig. 1. To generate HCVpv in 293T or Huh7 cells transiently expressing E1 and E2 proteins, cells were transfected with pCAGc60-p7 (H77 or Con1 strain) using *Trans*IT-LT1 (Mirus). After 24 h of incubation at 37°C, cells were infected at an MOI of 5 with the VSVΔG-GFP/G, in which the G envelope gene was replaced with the green fluorescent protein (GFP) gene and which was pseudotyped with the VSV G glycoprotein (39). The virus was adsorbed for 2 h at 37°C and then extensively washed four times with DMEM. After 24 h of incubation at 37°C, the culture supernatants were collected, centrifuged to remove cell debris, and stored at −80°C. HCVpp were produced as previously described from 293T cells cotransfected with an MLV Gag-Pol packaging construct, an MLV-based transfer vector encoding GFP, and the HCV envelope protein expression constructs (5). To generate HCVrv in various mammalian cell lines, cells were infected with the VSVG-complemented VSVΔG-c60-p7 at an MOI of 5 for 2 h at 37°C and then extensively washed four times with DMEM. After 48 h of incubation at 30°C, the culture supernatants were collected and stored at −80°C. The culture supernatants were pelleted through a 20% (wt/vol) sucrose cushion at 25,000 rpm for 2 h by using an SW28 rotor (Beckman Coulter, Tokyo, Japan). The pellets were resuspended in phosphate-buffered saline (PBS), mixed with 33% (wt/wt) cesium chloride, and centrifuged at 50,000 rpm for 48 h at 4°C by using an SW55Ti rotor (Beckman Coulter). After centrifugation, 12 fractions (0.5 ml each) were collected from the top and pelleted through a 20% (wt/vol) sucrose cushion by centrifugation at 50,000 rpm for 1 h at 4°C using an SW55Ti rotor. The pellets were resuspended in PBS and analyzed by immunoblotting to detect the incorporation of E1 or E2 proteins with anti-E1 (BDI198) or anti-E2 (AP33) monoclonal antibody, respectively. VSV N, P, and M were detected by anti-VSV polyclonal antibody, which was prepared by immunization of goats with purified VSVΔG. To determine the infectivities of HCVpv and HCVpp, infected cells were identified as GFP-positive cells under fluorescence microscopy or using FACSCalibur and expressed as infectious units (IU)/milliliter. The infectious