terol (19) and SM (53). We further characterized the role of lipid on the plasma membrane in viral infectivity and found that cholesterol depletion by B-CD, as well as hydrolysis of SM by SMase, moderately inhibits HCV infectivity (Fig. 5). These results suggest that cholesterol and sphingolipid in the plasma membrane environment may assist HCV entry, while HCV virion-associated cholesterol and sphingolipid appear to play critical roles in viral infection.

We previously demonstrated that HCV RNA and nonstructural proteins are present in DRM structures, likely in the context of a lipid-raft structure, and that viral RNA is likely synthesized at a raft membrane structure in cells containing the genotype 1b HCV replicon (2, 10, 46). Here we observed that ISP-1 and HPA-12 suppress HCVcc production, but not viral RNA replication, by the JFH-1 replicon (Fig. 6). Impairment of particle assembly and maturation, rather than suppression of genome replication, by these drugs may account for the inhibition of HCV production in the JFH-1 system. Viral RNA replication of the HCV-N replicon, however, was efficiently inhibited by these compounds, as found in previous reports (43). The virus strain specificity of the anti-HCV activity of cyclosporine has recently been demonstrated: JFH-1 replication is less sensitive to cyclosporine than replication of genotype 1b strains. Furthermore, the requirement for interaction with a cellular replication cofactor, cyclophilin B, differs among HCV strains (18). It appears that ISP-1 and HPA-12 are further examples of diverse effects on HCV strain replication.

In summary, our data here demonstrate important roles of cholesterol and sphingolipid in HCV infection and virion maturation. Specifically, mature HCV particles are rich in cholesterol. Depletion from HCV or hydrolysis of virion-associated SM results in a loss of infectivity. Moreover, the addition of exogenous cholesterol restores infectivity. In addition, cholesterol and sphingolipid on the HCV membrane play key roles in virus internalization, and portions of structural proteins are localized at lipid-raft-like membrane structures within cells. Finally, inhibitors of the sphingolipid biosynthetic pathway efficiently block virion production. These observations suggest that agents capable of modifying virion-associated lipid content might function as antivirals by preventing and/or blocking HCV infection and production.

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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

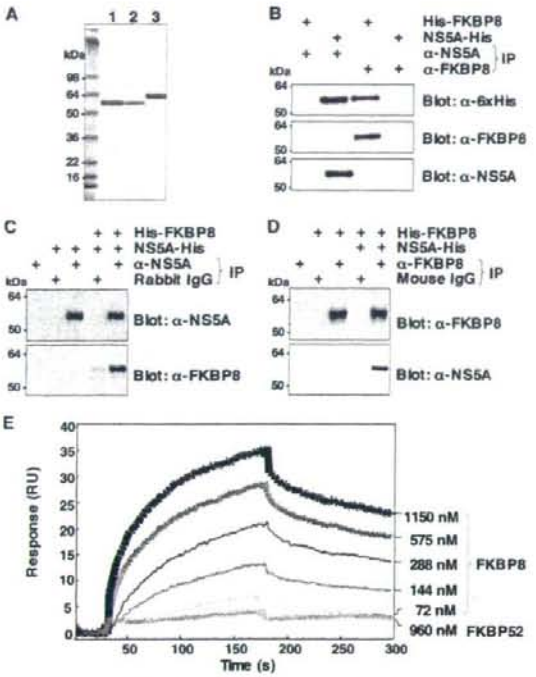

FIG. 1. Purification of recombinant NS5A, FKBP8, and FKBP52 and characteristics of their interaction. (A) Purified recombinant His-FKBP8 (lane 1), NS5A-His (lane 2), and His-FKBP52 (lane 3) were subjected to SDS-PAGE and stained with Coomassie brilliant blue G-250. The molecular size marker is shown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

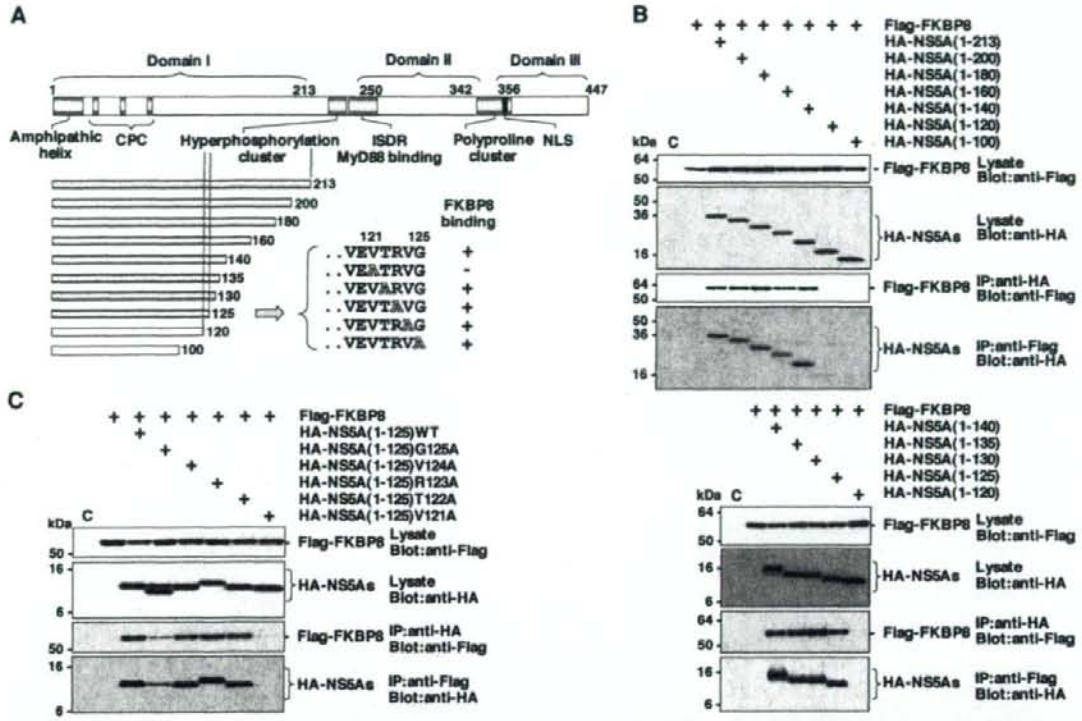

FIG. 2. Determination of an amino acid residue responsible for the interaction of FKBP8 and NS5A domain I. (A) Structure and functional domains of NS5A (top). The C-terminal deletion mutants of HA-tagged NS5A domain I used in the present study. Gray and white bars indicate ability and inability to bind 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-

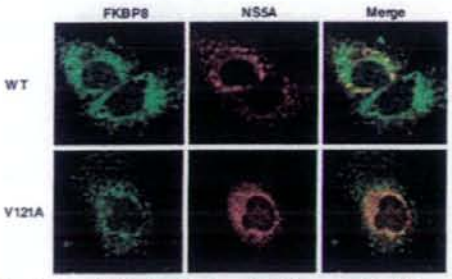

FIG. 3. Intracellular localization of wild-type and V121A mutant NS5A with FKBP8. Huh-7OK1 cells transfected with expression plasmids encoding HCV nonstructural proteins carrying a wild-type (WT) or mutant NS5A substituted Val^{121} with Ala (V121A) were fixed with 4% paraformaldehyde in PBS and permeabilized 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

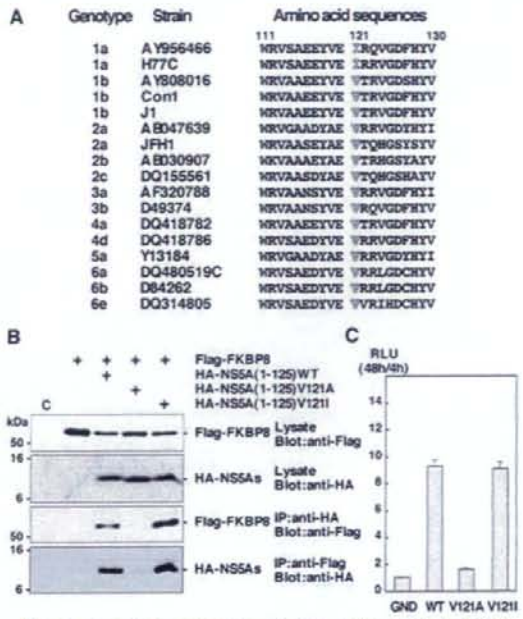

FIG. 4. Effect of the interaction of NS5A with FKBP8 on the transient replication of HCV. (A) Alignment of amino acid sequence of NS5A (111 to 130 amino acids) among different HCV genotypes. Outline letters indicate the amino acid residue at position 121. (B) The substitution mutants of HA-NS5A(1-125) 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

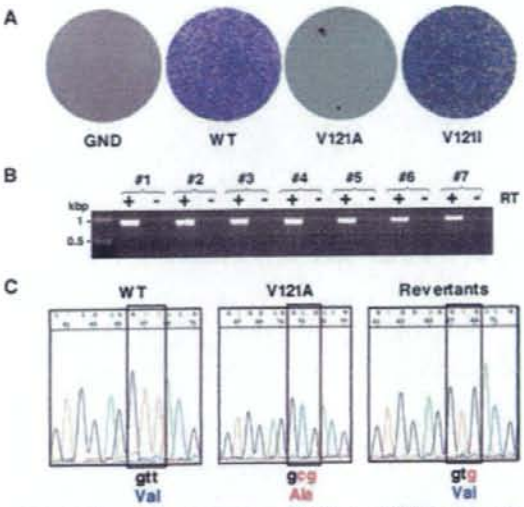

FIG. 5. Effect of the interaction of NS5A with FKBP8 on the colony formation by HCV replicon. (A) The replicon RNAs of the wild type (WT), a replication-deficient mutant (GND), and the substitution in Val^{121} to Ala (V121A) or to Ile (V121I) were transcribed from the plasmids based on $pFKI_{389}$ 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).

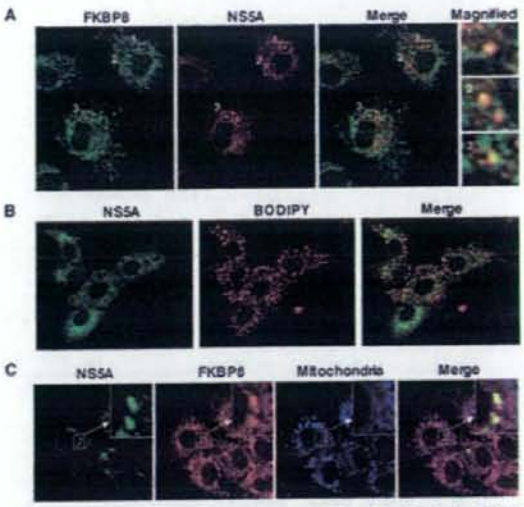

FIG. 6. Intracellular localization of FKBP8 and NS5A in the HCV replicon cells. (A) Huh-7 9-13 cells harboring an HCV subgenomic replicon were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.25% saponin. Endogenous FKBP8 and NS5A were stained with anti-FKBP8 monoclonal antibody (KDM11) 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

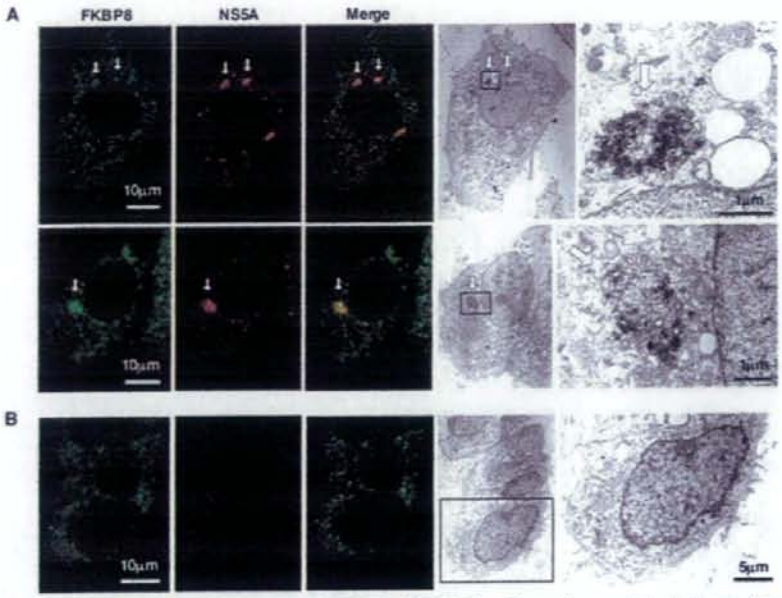

FIG. 7. FKBP8 interacts with NS5A in the membranous web. (A) The Huh-7 9-13 replicon cells were stained with specific antibodies to FKBP8 and NS5A as described in Fig. 6A. Identical fields were observed under EM by using the correlative FM-EM technique. Arrows indicate the areas NS5A and FKBP8 are colocalizing. 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 PA28-γ/REG-γ 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 Butyrate-Induced Transcript 1 Interacts with Hepatitis C Virus NS5A and Regulates Viral Replication▽

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Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) is required for the replication of the viral genome and is involved in several host signaling pathways. To gain further insight into the functional role of NS5A in HCV replication, we screened human cDNA libraries by a yeast two-hybrid system using NS5A as the bait and identified human butyrate-induced transcript 1 (hB-ind1) as a novel NS5A-binding protein. Endogenously and exogenously expressed hB-ind1 was coimmunoprecipitated with NS5A of various genotypes through the coiled-coil domain of hB-ind1. The small interfering RNA (siRNA)-mediated knockdown of hB-ind1 in human hepatoma cell lines suppressed the replication of HCV RNA replicons and the production of infectious particles of HCV genotype 2a strain JFH1. Furthermore, these reductions were canceled by the expression of an siRNA-resistant hB-ind1 mutant. Among the NS5A-binding host proteins involved in HCV replication, hB-ind1 exhibited binding with FKBP8, and hB-ind1 interacted with Hsp90 through the FxxW motif in its N-terminal p23 homology domain. The impairment of the replication of HCV RNA replicons and of the production of infectious particles of JFH1 virus in the hB-ind1 knockdown cell lines was not reversed by the expression of an siRNA-resistant hB-ind1 mutant in which the FxxW motif was replaced by AxxA. These results suggest that hB-ind1 plays a crucial role in HCV RNA replication and the propagation of JFH1 virus through interaction with viral and host proteins.

Hepatitis C virus (HCV) infects approximately 170 million people worldwide and induces serious chronic hepatitis that results in steatosis, cirrhosis, and ultimately hepatocellular carcinoma (7, 64). More than two-thirds of the HCV-positive population in Western countries and Japan face chronic infection by genotypes 1a and 1b. The current combination therapy using pegylated alpha interferon (IFN) plus ribavirin has achieved a sustained virological response in 50% of individuals infected with HCV genotypes 1a and 1b (37, 53).

HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae* and has a single-stranded, positive-sense RNA genome of approximately 9.6 kb, encoding a large polyprotein composed of approximately 3,000 amino acid residues. The polyprotein is cleaved by host and viral proteases, resulting in viral structural proteins (core, E1, and E2), a putative ion channel-forming protein (p7), and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (40, 55). Highly structured untranslated regions are flanked at both the 5′ and 3′ ends of the open reading frame. The initiation of translation of the viral RNA is dependent on an internal ribosome entry site (IRES) localized in the 5′ untranslated region (28, 58).

The HCV RNA is suggested to replicate in a replication complex composed of the viral nonstructural proteins and several host proteins. An HCV replicon system established as a representative functional system was composed of an antibiotic gene for selection and HCV genomic RNA for autonomous replication in the intracellular compartments of human hepatoma cell line Huh7 without production of infectious particles (34). Recently, cell culture systems for production of an infectious HCV have been established based on HCV genotype 2a (32, 62, 74). Furthermore, a mouse model consisting of an immunodeficient mouse xenotransplanted with human liver fragments has been established for the study of in vivo replication of HCV (38). These in vitro and in vivo systems have enabled us to investigate the life cycle of HCV and to develop antiviral drugs for chronic hepatitis C.

NS5A is a phosphoprotein that possesses multiple functions in viral replication, IFN resistance, and pathogenesis (35). Adaptive mutations to increase RNA replication are frequently mapped to the coding region of NS5A, indicating that NS5A is critical for HCV replication (1, 71). NS5A has been shown to be associated with a range of cellular proteins involved in cellular signaling pathways, such as IFN-induced kinase PKR (14), growth factor receptor-binding protein 2 (Grb2) (56), p53 (36, 48), and the phosphoinositide-3-kinase p85 subunit (18), and with proteins involved in protein trafficking and membrane morphology, such as karyopherin b3 (8), apolipoprotein A1 (52), amphiphysin II (73), F-box and leucine-rich repeat protein 2 (FBL2) (26, 63, 70), and vesicle-associated membrane protein-associated protein subtype A (VAP-A) (59). We have previously reported that the host proteins VAP-B and FKBP8, a member of the FK506-binding protein (FKBP) family, interact with NS5A and that these interactions are required for efficient replication of HCV (16, 45), further supporting the hypothesis that NS5A is a pivotal component of the HCV replication complex.

To gain a better understanding of the functional role of

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NS5A in HCV replication, we screened human libraries by employing a yeast two-hybrid system and using NS5A as the bait. We thus identified human butyrate-induced transcript 1 (hB-ind1) as an NS5A-binding protein. Murine B-ind1 has been identified as a transcript induced by treatment with sodium butyrate in BALB/c BP-A31 mouse fibroblasts (10). hB-ind1 is a multiple-membrane-spanning protein, consisting of 362 amino acids, that possesses significant homology with protein tyrosine phosphatase-like, member A (PTPLA), and co-chaperone p23 and is suggested to be involved in the Rac1 signaling pathway (10). In this study we examine the biological effects of the interaction of hB-ind1 with NS5A and other host proteins on the replication of HCV.

MATERIALS AND METHODS

Plasmids. The plasmids encoding NS5A, FKBP8, VAP-A, VAP-B, and heat shock protein 90 (Hsp90) have been described previously (45). The human FBL2 gene was amplified from the total cDNA of Huh7 by PCR. A cDNA clone containing hB-ind1 cDNA was isolated from a human fetal brain library (Clontech, Palo Alto, CA) by the advanced yeast two-hybrid system Matchmaker Two-Hybrid System 3 (Clontech) using an HCV NS5A protein as bait. Each cDNA of N-terminally FLAG-tagged hB-ind1 and its mutants was generated by cloning into pEF FlagGs pGKpuro (23). pSilencer-hB-ind1, carrying a short hairpin RNA (shRNA) targeted to hB-ind1 under the control of the U6 promoter, was constructed by cloning of the oligonucleotide pair 5′-GATCCGGAAAAGCGACCACTGTTTCTCAAGAGAAAACAGTGGTCGCTTTTCCTTTTTTGGAAA-3′–5′-AGCTTTTCCAAAAAAGGAAAAGCGACCACTGTTTTCTCTTGAGAAACAGTGGTCGCTTTTCCG-3′ between the BamHI and HindIII sites of pSilencer 2.1-U6 hygro (Ambion, Austin, TX). A plasmid encoding a mutant hB-ind1 resistant to shRNA was prepared by introduction of five silent mutations (nucleotides were changed from A to G, G to A, A to C, A to T, and C to T at positions 291, 294, 297, 300, and 301, respectively) into hB-ind1 cDNA by the method of splicing by overlap extension (19). The pSilencer negative-control plasmid (Ambion) has no homology to any human gene. The pFK-I_{389} neo/NS3-3′/NK5.1 plasmid (46) was kindly provided by R. Bartenschlager, and the neomycin-resistant gene was replaced with a firefly luciferase gene. The resulting plasmid was designated pFK-I_{389} FL/NS3-3′/NK5.1. The plasmids used in this study were confirmed by sequencing with ABI Prism genetic analyzer 3130 (Applied Biosystems, Tokyo, Japan).

Cells and virus infection. All cell lines were cultured at 37°C under a humidified atmosphere with 5% CO_2. Human embryo kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FCS). The human hepatoma cell line Huh7.5.1 was kindly provided by F. Chisari (74). The Huh7 and Huh7.5.1 cell lines were maintained in DMEM containing nonessential amino acids (NEAA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. The Huh9-13 cell line, an Huh7-derived cell line harboring a subgenomic HCV replicon (34), was maintained in DMEM containing 10% FCS, NEAA, and 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan). Huh7.5.1 cells were transfected with pSilencer-hB-ind1 or an empty plasmid, and drug-resistant clones were selected by treatment with hygromycin (Wako, Tokyo, Japan) at a final concentration of 10 μg/ml. Plasmids encoding a full-length or truncated (amino acid residues 101 to 277) version of hB-ind1 were transfected into Huh7.5.1 cells, and the cells surviving after selection with 0.1 μg/ml of puromycin for 1 week were used for virus infection. The viral RNA of JFH1 was introduced into Huh7.5.1 cells according to the method of Wakita et al. (62). The supernatant was collected at 7 days posttransfection and used as HCV particles that are infectious in cell culture (HCVcc).

Antibodies. A rabbit anti-hB-ind1 antibody was prepared by immunization with synthetic peptides corresponding to amino acid residues 106 to 117 of hB-ind1. A mouse monoclonal antibody to influenza virus hemagglutinin (HA) was purchased from Covance (Richmond, CA). The mouse anti-FLAG M2 antibody that was conjugated with a horseradish peroxidase and a mouse anti-β-actin monoclonal antibody were purchased from Sigma. The mouse monoclonal antibody to HCV NS5A was obtained from Austral Biologicals (San Ramon, CA).

Yeast two-hybrid assay and library screening. A human fetal brain library prepared with pAct2 was purchased from Clontech and was screened by the yeast two-hybrid system Matchmaker GAL4 Two-Hybrid System 3 (Clontech) according to the manufacturer's protocol. The NS5A cDNA fragment encoding amino acid residues 1973 to 2419 of HCV strain Con1 was amplified by PCR and cloned into pGBKT7 (Clontech); the resulting plasmid was designated pGBKT7 HCV NS5A. The yeast *Saccharomyces cerevisiae* strain AH109, which secretes α-galactosidase under the control of the MEL1 region, was transformed with pGBKT7 HCV NS5A and grown on a medium lacking tryptophan. The clone including the bait plasmid was transformed with the library plasmids. The transformed yeast cells were grown on 2% agar plates of a dropout medium lacking tryptophan, leucine, histidine, and adenine. The resulting colonies grown on the dropout plate were inoculated again on a new dropout plate containing 20 μg/ml X-α-Gal (5-bromo-4-chloro-3-indolyl-α-*O*-galactopyranoside) and incubated at 30°C for 7 days. The total DNA was prepared from all blue colonies and then introduced into *Escherichia coli* strain JM109. The prey plasmids were recovered from the clones grown on LB agar plates containing 10 μg/ml ampicillin. One positive clone was isolated from among 2 million colonies of the human fetal brain library, and the nucleotide sequence of this clone includes the complete cDNA of hB-ind1 in its frame.

Transfection, immunoblotting, and immunoprecipitation. Transfection and immunoprecipitation analyses were carried out as described previously (16, 45). Immunoprecipitates boiled in loading buffer were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and were reacted with the appropriate antibodies. The immune complexes were visualized with SuperSignal West Femto substrate (Pierce, Rockford, IL) and detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

Gene silencing by siRNA. The short interfering RNAs (siRNAs) Target-4 (5′-GCUGAGUGACGUACAGAAC-3′) and Target-6 (5′-GGAAAAGCGACCACUGUUU-3′) were obtained for knockdown of endogenous hB-ind1 (Ambion, Austin, TX). The negative control, siCONTROL Non-Targeting siRNA 2, which exhibits no downregulation of any human genes, was purchased from Dharmacon (Buckinghamshire, United Kingdom). Huh9-13 cells harboring a subgenomic HCV replicon grown on 6-well plates were transfected with 20 nM siRNA by using siFECTOR (B-Bridge International, Sunnyvale, CA) according to the manufacturer's protocol. The transfected cells were incubated in DMEM supplemented with 10% FCS and were then harvested at 96 h posttransfection.

Real-time PCR. The HCV RNA level was estimated by the method described previously (16, 45). Total RNA was prepared from cells by using the RNeasy minikit (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized using an RNA LA PCR kit (Takara Bio Inc., Shiga, Japan) and random primers. Each cDNA was estimated by Platinum Sybr green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA.) according to the manufacturer's protocol. Fluorescent signals were analyzed by an ABI Prism 7000 system (Applied Biosystems). The HCV IRES, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and hB-ind1 genes were amplified using primer pairs 5′-GAGTGTCGTGCAGCCTCCA-3′–5′-CACTCGCAAGCACCCTATCA-3′, 5′-GAAGGTGAAGGTCGGAGTC-3′–5′-GAAGGTGAAGGTCGGAGTC-3′, and 5′-CACCTGGAGTTCTTAGACCTTGTG-3′–5′-CAGTCGGAGTTTATTTAGGCGCTC-3′, respectively. The values for HCV genomic RNA and hB-ind1 mRNA were normalized to that for GAPDH mRNA. Each PCR product was detected as a single band of the correct size by agarose gel electrophoresis (data not shown).

In vitro transcription and RNA transfection. Plasmids pFK-I_{389} neo/NS3-3′/NK5.1 and pFK-I_{389} FL/NS3-3′/NK5.1 were linearized at the ScaI site and then transcribed in vitro using the MEGAscript T7 kit (Ambion) according to the manufacturer's protocol. To generate capped mRNA encoding *Renilla* luciferase, pRL-CMV was cleaved with BamHI and then transcribed using the mMESSAGE mMACHINE kit (Ambion) according to the manufacturer's protocol. These in vitro-transcribed RNAs were introduced into Huh7.5.1 cells at 4 million cells/0.4 ml by electroporation at 270 V and 960 μF using Gene Pulser (Bio-Rad, Hercules, CA).

Colony formation assay. The colony formation assay has been described previously (45). Briefly, in vitro-transcribed RNA was electroporated into Huh7 cells and plated in DMEM containing 10% FCS and NEAA. The medium was replaced with fresh DMEM containing 10% FCS, NEAA, and 1 mg/ml G418 at 24 h posttransfection. The remaining colonies were fixed with 4% paraformaldehyde and stained with crystal violet at 4 weeks after electroporation.

Luciferase assay. Transfected cells were seeded in a 12-well plate and then lysed in 200 μl of passive lysis buffer (Promega, Madison, WI) at 24 h posttransfection. Luciferase activity was measured in 20-μl aliquots of cell lysates using the Dual-Luciferase reporter assay system (Promega). Firefly luciferase activity was standardized to that of *Renilla* luciferase, and the results are expressed as the increases in relative luciferase units (RLU).

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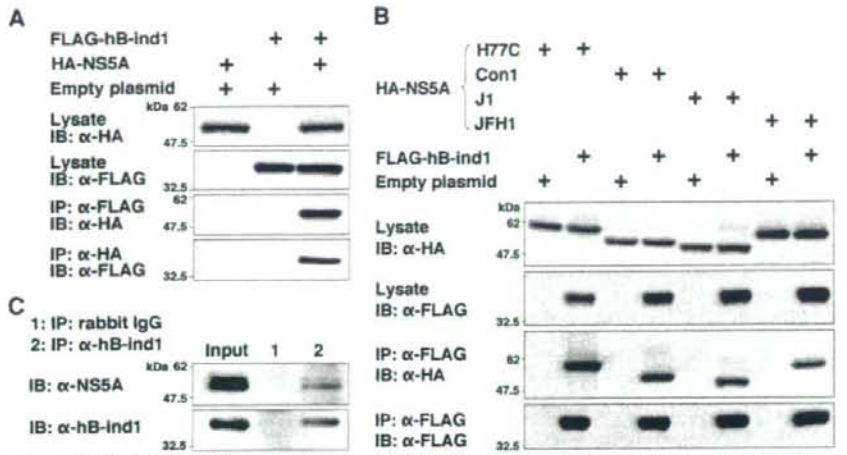

FIG. 1. Interaction of NS5A with hB-ind1 in mammalian cells. (A) HA-NS5A of strain Con1 and FLAG-tagged hB-ind1 were expressed in 293T cells and immunoprecipitated (IP) with an anti-HA or anti-FLAG antibody. Immunoprecipitates were subjected to Western blotting (IB) to detect coprecipitated counterparts. As a negative control, an empty plasmid was used instead of the plasmid encoding FLAG-hB-ind1 or HA-NS5A. Anti-FLAG and anti-HA antibodies did not recognize HA-tagged NS5A and FLAG-tagged hB-ind1, respectively. (B) HA-NS5A protein derived from genotype 1b strain Con1 or J1, genotype 1a strain H77C, or genotype 2a strain JFH1 was coexpressed with FLAG-hB-ind1 in 293T cells, immunoprecipitated with an isotype control or anti-FLAG antibody, and analyzed by Western blotting with an antibody to the FLAG or HA tag. An empty plasmid was used instead of the plasmid encoding FLAG-hB-ind1 as a negative control. (C) Endogenous hB-ind1 in Huh9-13 cells harboring subgenomic HCV replicon RNA was immunoprecipitated with normal rabbit immunoglobulin G (IgG) (lane 1) or anti-hB-ind1 rabbit IgG (lane 2), and immunoprecipitates were analyzed by Western blotting with specific antibodies.

Statistical analysis. Results are expressed as means ± standard deviations. The significance of differences between the means was determined by Student's *t* test.

RESULTS

hB-ind1 interacts with HCV NS5A of various genotypes. NS5A derived from the genotype 1b strain Con1 was used as bait to screen the human fetal brain cDNA library by a yeast two-hybrid system, and one clone including a gene encoding the open reading frame of the hB-ind1 gene was isolated. To examine whether hB-ind1 could interact with NS5A in mammalian cells, HA-tagged NS5A (HA-NS5A) was coexpressed with FLAG-tagged hB-ind1 (FLAG-hB-ind1) in 293T cells and immunoprecipitated with an antibody to the HA or the FLAG tag. FLAG-hB-ind1 and HA-NS5A were coimmunoprecipitated by either antibody (Fig. 1A). To determine the interaction of various genotypes of NS5A with hB-ind1, HA-NS5A of the genotype 1a strain H77C, the genotype 1b strain J1, or the genotype 2a strain JFH1 was coexpressed with FLAG-hB-ind1 and immunoprecipitated with the anti-FLAG antibody. An empty plasmid was used as a negative control. FLAG-hB-ind1 was immunoprecipitated with the anti-FLAG antibody at similar levels in cells coexpressing FLAG-hB-ind1 and HA-NS5A of all genotypes. HA-NS5A of various genotypes was coprecipitated with FLAG-hB-ind1 by the anti-FLAG antibody, whereas the anti-FLAG antibody did not precipitate any HA-NS5A of the various genotypes used in this study (Fig. 1B). To further confirm the interaction between hB-ind1 and HCV NS5A in the functional setting, lysates of Huh9-13 cells harboring subgenomic HCV replicon RNA were subjected to immunoprecipitation analysis with a rabbit polyclonal antibody raised against hB-ind1. NS5A was coimmunoprecipitated with endogenous hB-ind1 in the lysates of replicon cells (Fig. 1C). These results indicate that hB-ind1 interacts with NS5A of various HCV genotypes in mammalian cells.

hB-ind1 interacts with NS5A through the amino acid residues from 114 to 134 including the coiled-coil domain. hB-ind1 is composed of 362 amino acid residues and has domains homologous with p23 and PTPLA in the regions from Pro^{8} to Asp^{112} and from Gln^{196} to Leu^{346}, respectively (Fig. 2A). To determine the region responsible for the interaction with NS5A, various deletion mutants of FLAG-hB-ind1 were constructed (Fig. 2B). Each of the mutants was coexpressed with Con1 HA-NS5A in 293T cells and immunoprecipitated with an anti-HA antibody. An empty plasmid was used as a negative control in the immunoprecipitation analyses. HA-NS5A was coimmunoprecipitated with full-length hB-ind1 and with mutants possessing amino acid residues 114 to 134, corresponding to the coiled-coil domain, which generally participates in protein-protein interactions (Fig. 2B and C), whereas HA-NS5A was not coimmunoprecipitated with hB-ind1 mutants lacking the coiled-coil domain. The anti-HA antibody did not coprecipitate FLAG-hB-ind1 or its mutants. These results indicate that hB-ind1 interacts with HCV NS5A through the coiled-coil domain.

hB-ind1 participates in the replication of HCV RNA and the propagation of infectious HCV particles. To investigate the role(s) of endogenous hB-ind1 in the replication of HCV RNA, an siRNA targeted to hB-ind1 or a control siRNA was transfected into Huh9-13 cells harboring subgenomic HCV

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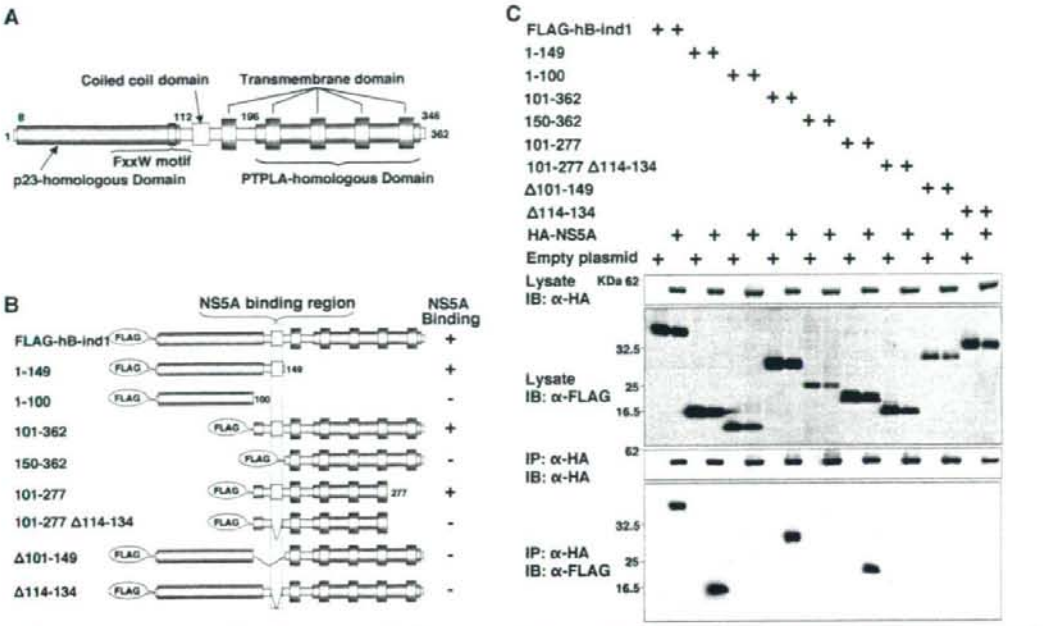

FIG. 2. Determination of the NS5A-binding region in hB-ind1. (A) Structure and functional domains of hB-ind1. (B) Deletion mutants of hB-ind1 used in this study and the results of binding to NS5A. N-terminally FLAG-tagged hB-ind1 mutants encoding the region from residue 1 to 149, 1 to 100, 101 to 362, 150 to 362, or 101 to 277 were designated 1-149, 1-100, 101-362, 150-362, or 101-277, respectively. An N-terminally FLAG-tagged hB-ind1 mutant spanning the region from residue 101 to residue 277 but lacking residues 114 to 134 was designated 101-277 Δ114-134. In addition, N-terminally FLAG-tagged hB-ind1 mutants lacking the region from 101 to 149 or from 114 to 134 were designated Δ101-149 or Δ114-134, respectively. The coiled-coil domain was located at residues 114 to 134. Each mutant gene was inserted into pEF FLAGGs pGKpuro. A summary of immunoprecipitation results is given on the right. (C) Each hB-ind1 mutant was coexpressed with Con1 HA-NS5A in 293T cells, immunoprecipitated with an anti-HA antibody, and analyzed by Western blotting with an anti-FLAG antibody. As a negative control, an empty plasmid was used instead of the plasmid encoding HA-NS5A. The anti-HA antibody did not recognize FLAG-tagged hB-ind1 or its mutants.

replicon RNA. Total RNA was extracted from the transfected cells, and levels of hB-ind1 mRNA and HCV RNA were determined by real-time PCR. At 72 h posttransfection, hB-ind1 mRNA and HCV subgenomic RNA levels in cells transfected with each of the hB-ind1 siRNAs were reduced more than 60% from the levels in cells treated with the control siRNA (Fig. 3A). The levels of expression of hB-ind1 and the HCV NS5A protein were decreased in HCV replicon cells transfected with the hB-ind1 siRNA but not in those transfected with the control siRNA (Fig. 3B).

To examine the effects of the knockdown of hB-ind1 on the replication of HCV RNA and the propagation of HCVcc, we established Huh7.5.1 cell lines stably expressing an shRNA targeted to hB-ind1. Dozens of colonies were obtained from cells transfected with a plasmid encoding the cDNA of the shRNA to hB-ind1 after selection with hygromycin. Although the levels of mRNA and expression of endogenous hB-ind1 were not changed in cells bearing a nonspecific shRNA, they were reduced in the clones bearing shRNAs targeted to hB-ind1, except for clone 1 (Fig. 3C and D). There was no significant difference in growth among the cell lines (Fig. 3E).

The replicon RNA transcribed from pFK-I_{389} neo/NS3-3'/NK5.1 was transfected into the hB-ind1 knockdown cell lines Huh-si2 and Huh-si5, which were cultured for 4 weeks in the presence of G418. The numbers of colonies in the knockdown cell lines were less than one-fourth of those in the control cell line (Huh-c) (Fig. 4A). A FLAG-tagged hB-ind1 wobble mutant (FLAG-rB-ind1), which is resistant to the shRNA targeted to hB-ind1 due to the introduction of silent mutations, was capable of expressing an siRNA-resistant hB-ind1 upon introduction into cells at a level similar to that of the endogenous hB-ind1 (eB-ind1) detected in the control cell line (Fig. 4B). The reduction of colony formation by the knockdown of eB-ind1 in the hB-ind1 knockdown cell lines Huh-si2 and Huh-si5 was canceled by the expression of FLAG-rB-ind1 (Fig. 4A). To further examine the involvement of hB-ind1 in the replication of HCV, a chimeric HCV RNA encoding a firefly luciferase gene under the control of HCV IRES (Fig. 4C) was transfected into the knockdown cell lines. Knockdown of hB-ind1 reduced the RLU in Huh-si2 and Huh-si5 cells by 40% and 70%, respectively, and this reduction was also canceled by the expression of FLAG-rB-ind1. To further examine the effect of hB-ind1 knockdown on the production of HCV infectious particles, HCVcc were inoculated into the hB-ind1 knockdown

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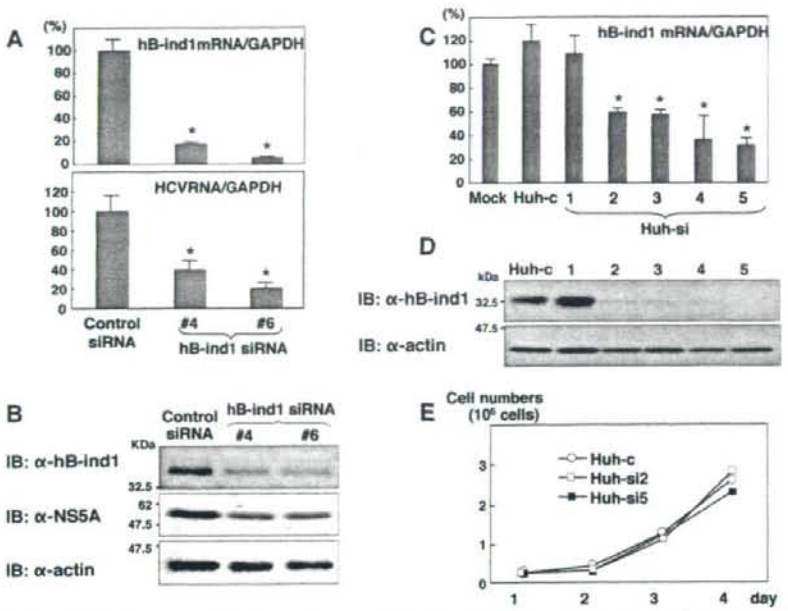

FIG. 3. Effects of hB-ind1 knockdown on HCV replication. (A) Huh9-13 cells were transfected with siRNA 4 or siRNA 6 (#4 or #6, respectively), targeted to the hB-ind1 gene, or with a nonspecific siRNA, at a final concentration of 20 nM, and were harvested at 72 h posttransfection. hB-ind1 mRNA and HCV RNA levels were determined by real-time PCR. The levels of hB-ind1 mRNA and HCV RNA were normalized to the amount of GAPDH mRNA and expressed as percentages of the control value. (B) Huh9-13 cells transfected with siRNAs were lysed at 72 h posttransfection and subjected to Western blotting (IB) with an antibody to hB-ind1, NS5A, or β-actin. (C) Establishment of hB-ind1 knockdown Huh7.5.1 cell lines. Plasmids encoding shRNAs targeted to hB-ind1 (siRNA 6) or nonspecific targets were transfected into Huh7.5.1 cells and cultivated in the presence of hygromycin. Independent clones were established by limiting dilution. The value for hB-ind1 mRNA was normalized to the amount of GAPDH mRNA and expressed as a percentage of the control value. Huh7.5.1 cell lines expressing siRNAs targeted to hB-ind1 (Huh-si1 to Huh-si5) and to a nonspecific target (Huh-c) were established. (D) Expression of hB-ind1 in knockdown cells. The knockdown cell lines were lysed and subjected to Western blotting with an antibody to hB-ind1 or β-actin. (E) Growth curves of the knockdown cell lines were determined by the method of trypan blue dye exclusion. Data in this figure are representative of three independent experiments. Error bars, standard deviations. Asterisks indicate significant differences ($P < 0.01$) from the control value.

cell lines. Both virus titers, determined by focus-forming units at 72 h postinfection in culture supernatants, and HCV RNA levels in Huh-si2 and Huh-si5 cells were significantly reduced, and these reductions were canceled by the expression of FLAG-rB-ind1 (Fig. 4D). These results suggest that hB-ind1 is involved in the replication of HCV RNA and the propagation of HCVcc.

An hB-ind1 mutant retaining the binding region to NS5A has a dominant-negative effect on the replication of HCV. To examine the involvement of hB-ind1 in the replication of HCV in greater detail, deletion mutants of hB-ind1 retaining or lacking the binding region to NS5A were expressed in Huh9-13 cells harboring subgenomic HCV replicon RNA (Fig. 5A). Although the hB-ind1 mutant possessing the NS5A binding region (101-277) and full-length hB-ind1 were detected at similar levels in replicon cells transfected with the expression plasmids (Fig. 5B), HCV RNA replication was reduced only in cells expressing the mutant retaining the binding region to NS5A, not in those expressing full-length hB-ind1 or the mutant lacking the binding region to NS5A (101-277 Δ114-134) (Fig. 5C). However, no significant difference in NS5A expression was observed in Huh9-13 cells transfected with the expression plasmids (Fig. 5B). Production of the infectious HCV particles was also reduced in the culture supernatants of Huh7.5.1 cells expressing the hB-ind1 mutant retaining the binding region to NS5A (101-277) but not in those expressing full-length hB-ind1 or the hB-ind1 101-277 Δ114-134 mutant (Fig. 5D). These dominant-negative effects of the hB-ind1 mutant retaining the binding region to NS5A on the replication of HCV RNA in Huh9-13 cells and on the production of infectious particles in Huh7.5.1 cells further support the notion that hB-ind1 regulates the replication of HCV RNA and the propagation of HCVcc.

hB-ind1 interacts with FKBP8 and Hsp90. Previous reports have suggested that HCV NS5A interacts with several host proteins such as FBL2 (63), VAP-A (59), VAP-B (16), and FKBP8 (45) and that these interactions participate in the replication of HCV. To determine the interplay of the NS5A-binding proteins, FLAG-tagged hB-ind1 was coexpressed with HA-tagged FBL2, VAP-A, VAP-B, or FKBP8 in 293T cells and immunoprecipitated with an anti-FLAG antibody, and FKBP8 was shown to specifically interact with hB-ind1 (Fig. 6A). We have

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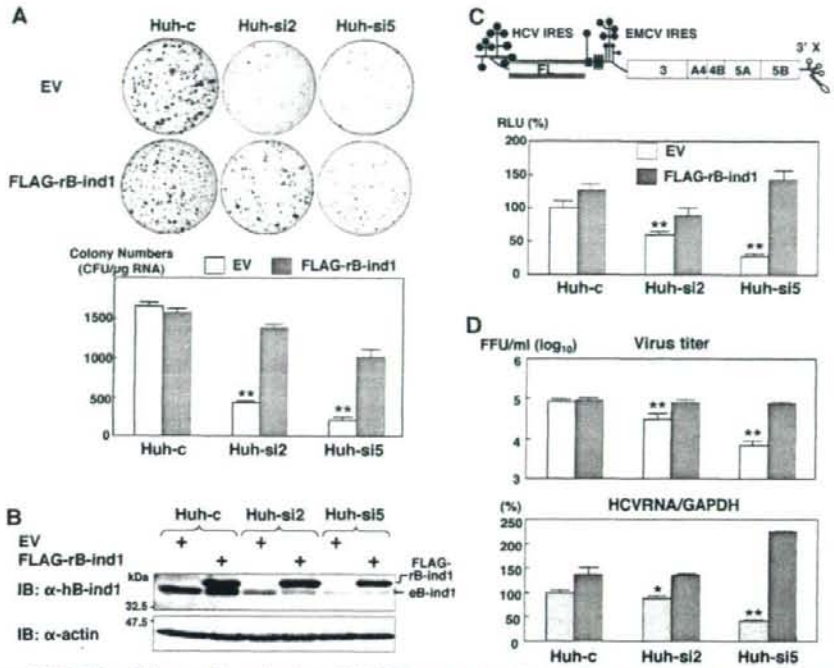

FIG. 4. Effects of hB-ind1 knockdown on the replication of HCV RNA and the production of infectious particles. (A) The hB-ind1 knockdown (Huh-si2 and Huh-si5) and control (Huh-c) cell lines were first transfected with either a plasmid encoding hB-ind1 resistant to siRNA by virtue of the introduction of silent mutations (FLAG-rB-ind1) or an empty vector (EV) and then further transfected with replicon RNA transcribed from pFK-I_{389} neo/NS3-3′/NK5.1. (Upper panel) The cell colonies remaining after cultivation for 4 weeks in the presence of G418 were fixed with 4% paraformaldehyde and stained with crystal violet. (Lower panel) The number of colonies was standardized to the amount of transfected RNA. (B) The expression of the siRNA-resistant hB-ind1 (FLAG-rB-ind1) and the endogenous hB-ind1 (eB-ind1) in Huh-c, Huh-si2, and Huh-si5 cells transfected with either a plasmid encoding FLAG-rB-ind1 or an empty vector was analyzed by Western blotting (IB) with an antibody to hB-ind1 or β-actin. (C) HCV subgenomic replicon RNA transcribed from pFK-I_{389} FL/NS3-3′/NK5.1 and capped *Renilla* luciferase RNA transcribed from pRL-CMV were cotransfected into Huh-c, Huh-si2, and Huh-si5 cells pretransfected with either a plasmid encoding FLAG-rB-ind1 or an empty vector. The firefly luciferase activity was normalized to that of *Renilla* luciferase. HCV IRES-dependent translational activity was expressed as a percentage of the RLU of Huh-c cells transfected with an empty plasmid. EMCV, encephalomyocarditis virus. (D) HCVcc were inoculated into Huh-c, Huh-si2, and Huh-si5 cells pretransfected with either a plasmid encoding FLAG-rB-ind1 or an empty vector. (Upper panel) The culture supernatants at 72 h postinoculation were subjected to a focus-forming assay, and virus titers are expressed as focus-forming units (FFU) per milliliter. (Lower panel) The amount of intracellular HCV RNA was measured by real-time PCR and normalized to the amount of GAPDH mRNA. The HCV RNA level is expressed as a percentage of that of Huh-c cells transfected with an empty plasmid. Data in this figure are representative of three independent experiments. Error bars, standard deviations. Asterisks indicate significant differences (**, $P < 0.01$; *, $P < 0.05$) from the control value.

previously shown that FKBP8 is capable of binding to both NS5A and Hsp90 through the tetratricopeptide repeat (TPR) domain and that the recruitment of Hsp90 to the replication complex plays a crucial role in the replication of HCV (45). Hsp90 is a molecular chaperone and requires various cochaperone proteins such as p23 for efficient chaperone activity. hB-ind1 shows homology to p23 (Fig. 2A), and the FxxW motif, essential for the binding to Hsp90, is conserved in residues Phe^{107}xxTrp^{110} of hB-ind1 (11, 27, 68). To determine whether hB-ind1 interacts with Hsp90 through the FxxW motif as reported for p23, FLAG-tagged hB-ind1 or an hB-ind1 mutant in which Phe^{107} and Trp^{110} had been replaced with Ala (FLAG-hB-ind1AxxA) was coexpressed with HA-tagged Hsp90 in 293T cells and immunoprecipitated with an anti-FLAG antibody. Hsp90 was coimmunoprecipitated with wild-type hB-ind1 but not with the mutant hB-ind1, indicating that hB-ind1 interacts with Hsp90 through the FxxW motif (Fig. 6B).

Previously, we showed that the amino acid residues of the carboxylate clump position in the TPR domain of FKBP8 attach to the C-terminal MEEVD motif of Hsp90 (45). To examine the interaction of hB-ind1 with Hsp90 in the absence of association with FKBP8, FLAG-tagged hB-ind1 was first coexpressed with HA-tagged Hsp90 or mutant Hsp90 lacking the MEEVD motif in 293T cells and then immunoprecipitated with an anti-FLAG antibody. Similar levels of hB-ind1 were coprecipitated with Hsp90 irrespective of the deletion of the MEEVD motif of Hsp90 (Fig. 6C), suggesting that hB-ind1 alone is capable of binding to Hsp90 through the FxxW motif irrespective of the association of FKBP8. To further clarify the interplay among hB-ind1, FKBP8, and Hsp90, FLAG-tagged

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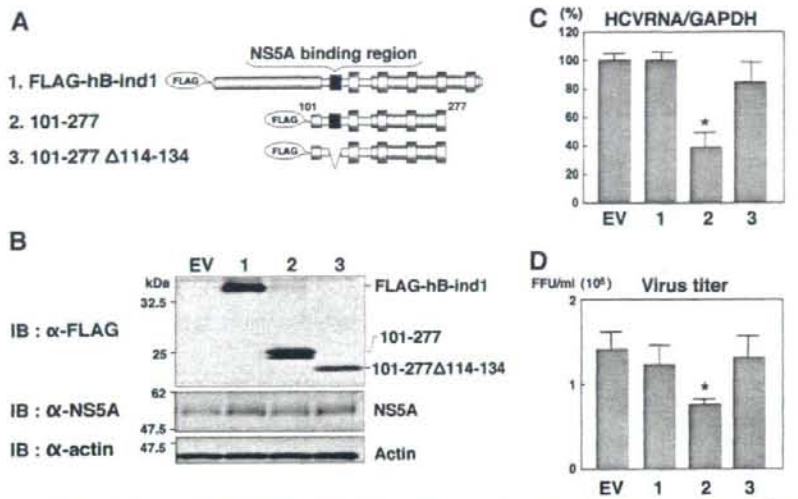

FIG. 5. Dominant-negative effect of an hB-ind1 mutant on the replication of HCV. (A) Plasmids encoding full-length hB-ind1 (construct 1) or deletion mutants of hB-ind1 retaining (construct 2) or lacking (construct 3) the NS5A binding region. (B) One of the three plasmids or an empty vector (EV) was transfected into Huh9-13 cells harboring a subgenomic HCV replicon RNA and was subjected to Western blotting (IB) with specific antibodies at 72 h posttransfection. (C) The amount of intracellular HCV RNA in the Huh9-13 cells was measured at 72 h posttransfection by real-time PCR, normalized to the amount of GAPDH mRNA, and expressed as the percentage of the value for control cells transfected with an empty plasmid. (D) One of the three plasmids or an empty vector was transfected into Huh7.5.1 cells, and then HCVcc were inoculated. Virus production in the culture supernatants at 72 h postinoculation was determined by a focus-forming assay. FFU, focus-forming units. Data in this figure are representative of three independent experiments. Error bars, standard deviations. Asterisks indicate significant differences ($P < 0.01$) from the control value.

hB-ind1 was coexpressed with HA-tagged Hsp90 and/or FKBP8 and then immunoprecipitated with an anti-FLAG antibody. Coprecipitation of Hsp90 with hB-ind1 was increased by additional expression of FKBP8 (Fig. 6D). These results suggest that hB-ind1 interacts with Hsp90 through the FxxW motif and that FKBP8 also participates in the complex formation to enhance the interaction.

hB-ind1 participates in HCV propagation through the interaction with Hsp90. Next, to examine the role of the interaction of hB-ind1 with Hsp90 in the replication of HCV RNA, the replicon RNA transcribed from pFK-I_{389} neo/NS3-3′/NK5.1 was transfected into hB-ind1 knockdown Huh-si5 cells expressing siRNA-resistant FLAG-rB-ind1 or FLAG-rB-ind1AxxA, in which the Hsp90 binding motif FxxW was changed to AxxA. The colony formation in Huh-si5 cells transfected with an empty plasmid was 10% of that in Huh-c cells. The expression of FLAG-rB-ind in Huh-si5 cells recovered the colony formation in Huh-si5 cells to 98% of that in Huh-c cells, although that of FLAG-rB-ind1 AxxA in Huh-si5 cells exhibited only 40% recovery (Fig. 7A). To further examine the role of the interaction between hB-ind1 and Hsp90 in the production of HCVcc, Huh-si5 cells expressing either FLAG-rB-ind1 or FLAG-rB-ind1AxxA were infected with HCVcc, and the virus titer in the culture supernatants and the intracellular HCV RNA level at 72 h postinfection were determined. Virus production was reduced in the culture supernatants, and viral RNA replication in the hB-ind1 knockdown cells was restored by the expression of FLAG-rB-ind1 but not by that of FLAG-rB-ind1AxxA, as seen in colony formation by the replicon RNA (Fig. 7B). Collectively, these results suggest that the interaction of hB-ind1 with Hsp90 through the FxxW motif is required for genomic RNA replication and particle production of HCV.

DISCUSSION

In this study we have shown that hB-ind1 participates in HCV RNA replication and particle production through interaction with NS5A, FKBP8, and Hsp90. hB-ind1 was initially identified as a downstream transducer of Rac1, a member of the small GTP-binding proteins, in mouse fibroblasts treated with sodium butyrate, a multifunctional agent known to inhibit cell proliferation and to induce differentiation by modulating transcription (6, 10). Rac1 possesses diverse biological functions, including cytoskeletal dynamics, membrane ruffling, cell cycle progression, gene transcription, and cell survival (4, 31, 49). Previous studies have suggested that hB-ind1 mediates Rac1 and Jun N-terminal protein kinase–NF-κB signaling and is involved in the regulation of gene expression (6, 10). Inhibition of Rac1 function leads to disruption of cytoskeleton dynamics, resulting in impairment of cell growth (17, 69).

Inhibition of cell growth downregulates HCV RNA replication in the replicon cell line (41, 51), and cell cycle regulation affects HCV IRES-mediated translation (20, 61). Furthermore, cytoskeletal regulation is required for HCV RNA synthesis (3). However, knockdown of hB-ind1 and expression of the deletion mutants exhibited neither morphological change nor suppression of cell growth, suggesting that the suppression

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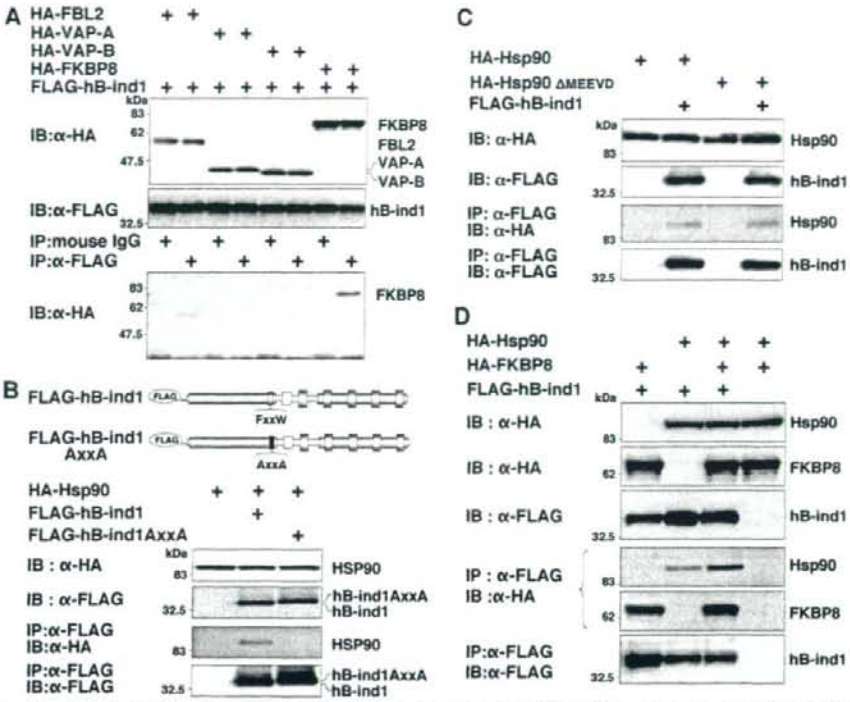

FIG. 6. Interaction of hB-ind1 with other NS5A-binding host proteins. (A) FLAG-hB-ind1 was first coexpressed with HA-tagged FBL2, VAP-A, VAP-B, or FKBP8 in 293T cells and then immunoprecipitated with an anti-FLAG or control antibody. The immunoprecipitates were detected by Western blotting (IB) with an anti-HA antibody. (B) FLAG-hB-ind1 or FLAG-hB-ind1AxxA, in which Phe^{107} and Trp^{110} had been replaced with Ala, was coexpressed with HA-Hsp90 in 293T cells and immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates were detected by Western blotting with an anti-HA or anti-FLAG antibody. (C) FLAG-hB-ind1 was coexpressed with HA-Hsp90 or mutant Hsp90 lacking the MEEVD motif (HA-Hsp90 ΔMEEVD) in 293T cells and was immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates were detected by Western blotting with an anti-HA or anti-FLAG antibody. (D) HA-Hsp90, HA-FKBP8, and FLAG-hB-ind1 were coexpressed in various combinations in 293T cells and immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates were detected by Western blotting with an anti-HA or anti-FLAG antibody. Data in this figure are representative of three independent experiments.

of HCV replication by dysfunction of hB-ind1 is not due to cell growth arrest or cytoskeletal disruption. Murine B-ind1 has been reported to be expressed in all mouse tissues examined, with abundant expression detected in the testis, kidney, brain, and liver (10). Significant levels of endogenous hB-ind1 expression have been detected in the human hepatic cell lines Huh7, HepG2, Hep3B, and FLC4 and in the nonhepatic human cell lines HeLa, 293T, and THP-1 (data not shown); therefore, the tissue specificity of HCV replication could not be explained by the expression of hB-ind1.

Combination therapy with IFN and cyclosporine A has been shown to be effective for patients infected with a high viral load of HCV genotype 1b (24), and cyclosporine A has been shown to suppress HCV RNA replication in vitro through deactivation of the interaction between NS5B and cyclophilin B (66). Cyclophilin and FKBP are classified as immunophilins capable of binding to immunosuppressants cyclosporine A and FK506, respectively (33). The immunophilins do not share a homologous domain with each other, based on their amino acid sequences, substrate specificities, and inhibitor sensitivities. We have recently reported that NS5A binds specifically to FKBP8 but not to other homologous immunophilins such as FKBP52 and cyclophilin D. FKBP8 forms both a homomultimer and a heteromultimer with the chaperone protein Hsp90. Mutation analyses of FKBP8 and Hsp90 suggest that FKBP8 acts as an intermediate between NS5A and Hsp90 via the different position of the TPR domain in FKBP8 and regulates HCV genome replication (45).

The molecular chaperone Hsp90 is one of the most abundant proteins in unstressed cells and generally requires various cochaperone proteins in multiple steps to promote the folding, functional maturation, and stability of its client proteins. Newly synthesized unfolded client proteins are delivered to the Hsp70 complex via Hsp40. In most cases, Hsp70 is able to process the client proteins on its own. Certain substrates require Hsp90 for proper folding or activation. In this case, the scaffold protein Hop connects elements of the Hsp70 and Hsp90 machineries to form an intermediate complex (2, 12, 13, 47). In the late stage, the Hsp70 component dissociates, and at the same time, p23 and immunophilins enter the complex (44, 54) and the

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