

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 Val¹²¹ 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 Val¹²¹ with Ala. As shown in Fig. 3, colocalization of an endogenous FKBP8 with NS5A was reduced by the introduction of substitution of Val¹²¹ to Ala. These results suggest that Val¹²¹ 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://eu.hcvdb.ibcp.fr/euHCVdb/jsp/index.jsp>) revealed that the amino acid residue Val¹²¹ 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 Ile¹²¹, was able to interact with FKBP8 (37). To determine the role of Ile¹²¹ on the binding of NS5A to FKBP8, HA-NS5A(1-125) of the genotype 1b Con1 strain in which Ile was substituted for Val¹²¹ was coexpressed with Flag-FKBP8 and immunoprecipitated with specific antibodies (Fig. 4B). The HA-NS5A mutant possessing the substitution of Val¹²¹ to Ile interacted with Flag-FKBP8 at the same level as the wild-type NS5A. Next, to determine the role of Val¹²¹ or Ile¹²¹ in the replication of HCV, we generated replicon RNAs in which Val¹²¹ of NS5A was replaced with either Ala or Ile. In vitro-transcribed RNAs from the pFKI-I₃₈₉ 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 Val¹²¹ with Ala severely impaired the RNA replication, whereas substitution of Val¹²¹ to Ile, as seen in genotype 1a strains, had no apparent effect on the replication (Fig. 4C). These results suggest that Val¹²¹ and Ile¹²¹ of NS5A

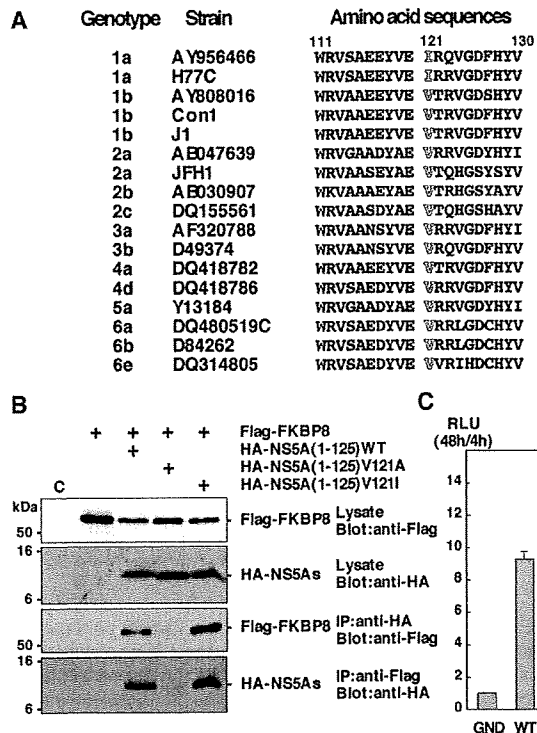


FIG. 4. Effect of the interaction of NS5A with FKBP8 on the transient replication of HCV. (A) Alignment of amino acid sequence of NS5A (111 to 130 amino acids) among different HCV genotypes. Outline letters indicate the amino acid residue at position 121. (B) The substitution mutants of HA-NS5A(1-125) replaced Val¹²¹ 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 pFKI-I₃₈₉ 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 Val¹²¹ 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 Val¹²¹ 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 Val¹²¹ and Ile¹²¹ 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 pFKI₃₈₉/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

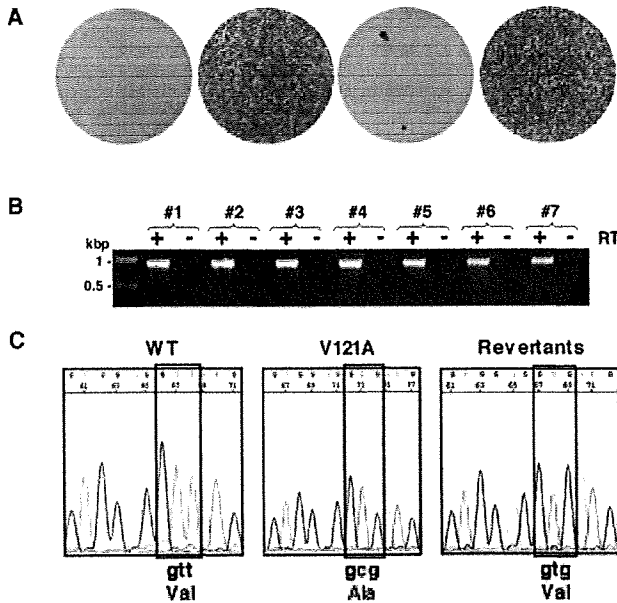


FIG. 5. Effect of the interaction of NSSA with FKBP8 on the colony formation by HCV replicon. (A) The replicon RNAs of the wild type (WT), a replication-deficient mutant (GND), and the substitution in Val¹²¹ to Ala (V121A) or to Ile (V121I) were transcribed from the plasmids based on pFKI₃₈₉ neo/NS3-3'/5.1, 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 Val¹²¹ to Ala (V121A) in NSSA 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).

1,000 colonies on the plate of Huh-7 cells into which the parent replicon RNA or an RNA carrying the substitution of Val¹²¹ of NS5A to Ile was introduced, but only a few colonies appeared on the plate of cells into which RNA carrying the mutation of Val¹²¹ to Ala was introduced (Fig. 5A). To characterize the colonies emerging on the plate of Huh-7 cells into which the mutant Ala¹²¹ 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 Ala¹²¹, in contrast to the parental replicon, which had GTT corresponding to the Val¹²¹. 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 Val¹²¹ 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

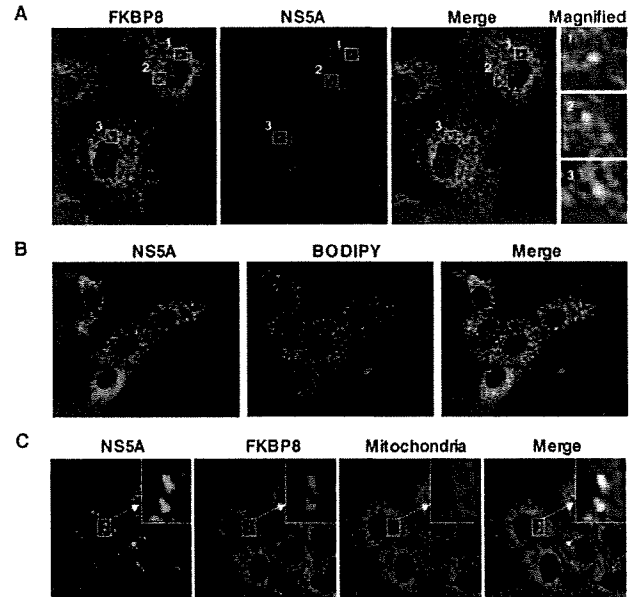


FIG. 6. Intracellular localization of FKBP8 and NS5A in the HCV replicon cells. (A) Huh-7 9-13 cells harboring an HCV subgenomic replicon were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.25% saponin. Endogenous FKBP8 and NS5A were stained with anti-FKBP8 monoclonal antibody (KDM11)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

(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

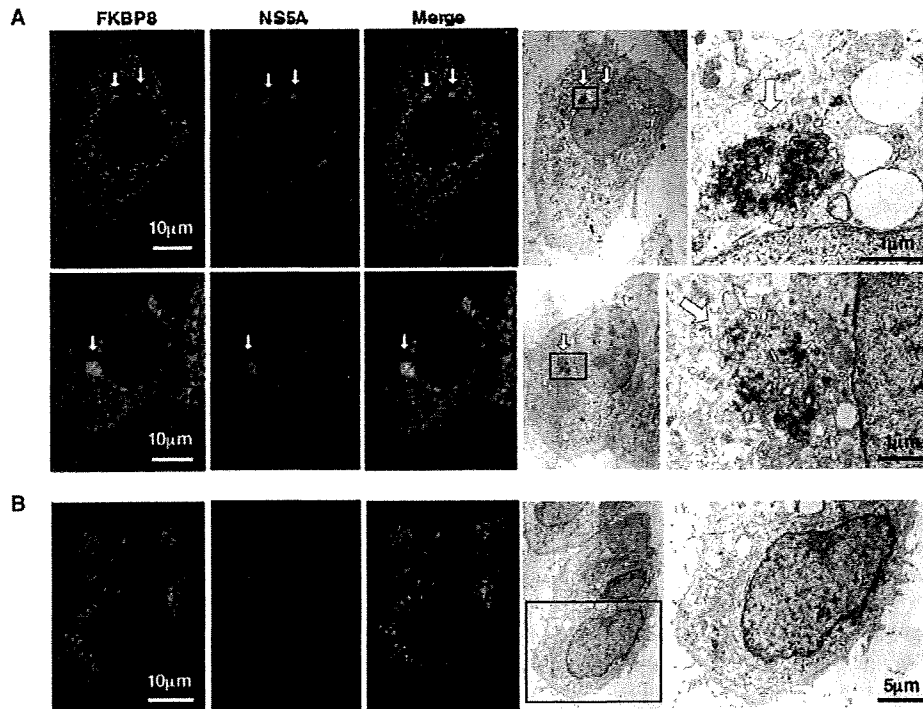


FIG. 7. FKBP8 interacts with NS5A in the membranous web. (A) The Huh-7 9-13 replicon cells were stained with specific antibodies to FKBP8 and NS5A as described in Fig. 6A. Identical fields were observed under EM by using the correlative FM-EM technique. Arrows indicate the areas NS5A and FKBP8 are colocalizing. 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 Val¹²¹ to Ala within NS5A leads to severe impairment of RNA replication, and reversion from Ala¹²¹ to Val was detected, suggesting that interaction of FKBP8 with NS5A through the Val¹²¹ is crucial for HCV replication. The crystal structure of NS5A domain 1 revealed that Val¹²¹ 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 Val¹²¹ 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 Val¹²¹ 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 FKBP8 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 Val¹²¹ 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 ac-

cumulation 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 Val¹²¹ 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'-GATCCGGA AAAGCGACCACTGTTTCTCAAGAGAAAACAGTGGTCGCTTTTCCTTT TTTGGAAA-3'-5'-AGCTTTTCCAAAAAGGAAAAGCGACCACTGTTT TCTCTTGAGAAACAGTGGTCGCTTTTCCG-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-I₃₈₉ 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-I₃₈₉ 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% CO₂. 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. Chusari (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 (Nacal 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) accord-

ing 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'-GGAAAAGCGAC CACUGUUU-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'-GAGTGTGCTGCAGCCTCCA-3'-5'-CACTCGCAAGCACCTATCA-3', 5'-GAAGGTGAAGGTCGGAGTC-3'-5'-GAAGGTGAAGGTCGGAGTC-3', and 5'-CACCTGGAGTCTTAGA CCTGTG-3'-5'-CAGTCCGAGTTTATTTAGGCGCTC-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-I₃₈₉ neo/NS3-3'/NK5.1 and pFK-I₃₈₉ 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 mMMESSAGE 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).

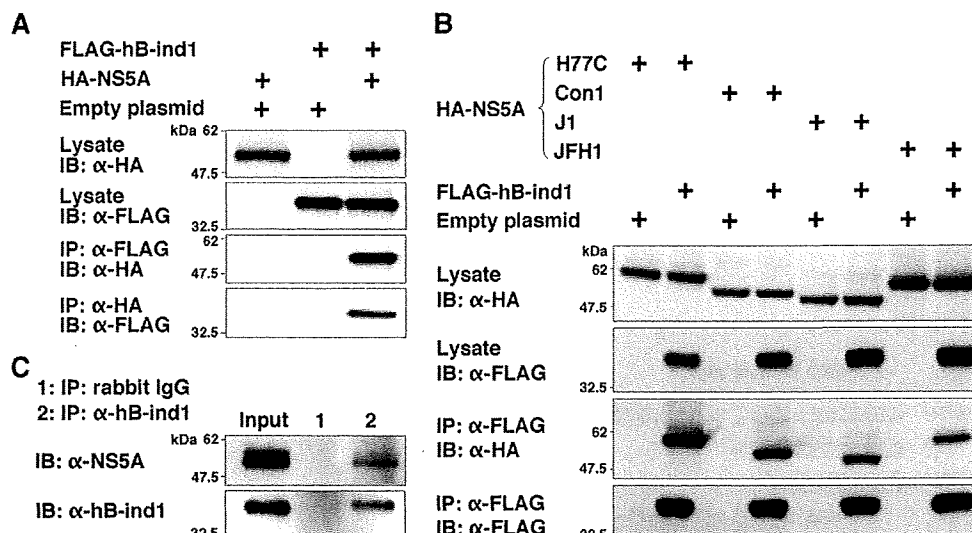


FIG. 1. Interaction of NS5A with hB-ind1 in mammalian cells. (A) HA-NS5A of strain Con1 and FLAG-tagged hB-ind1 were expressed in 293T cells and immunoprecipitated (IP) with an anti-HA or anti-FLAG antibody. Immunoprecipitates were subjected to Western blotting (IB) to detect coprecipitated counterparts. 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 \pm 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 im-

muno-precipitation 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 Pro⁸ to Asp¹¹² and from Gln¹⁹⁶ to Leu³⁴⁶, 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

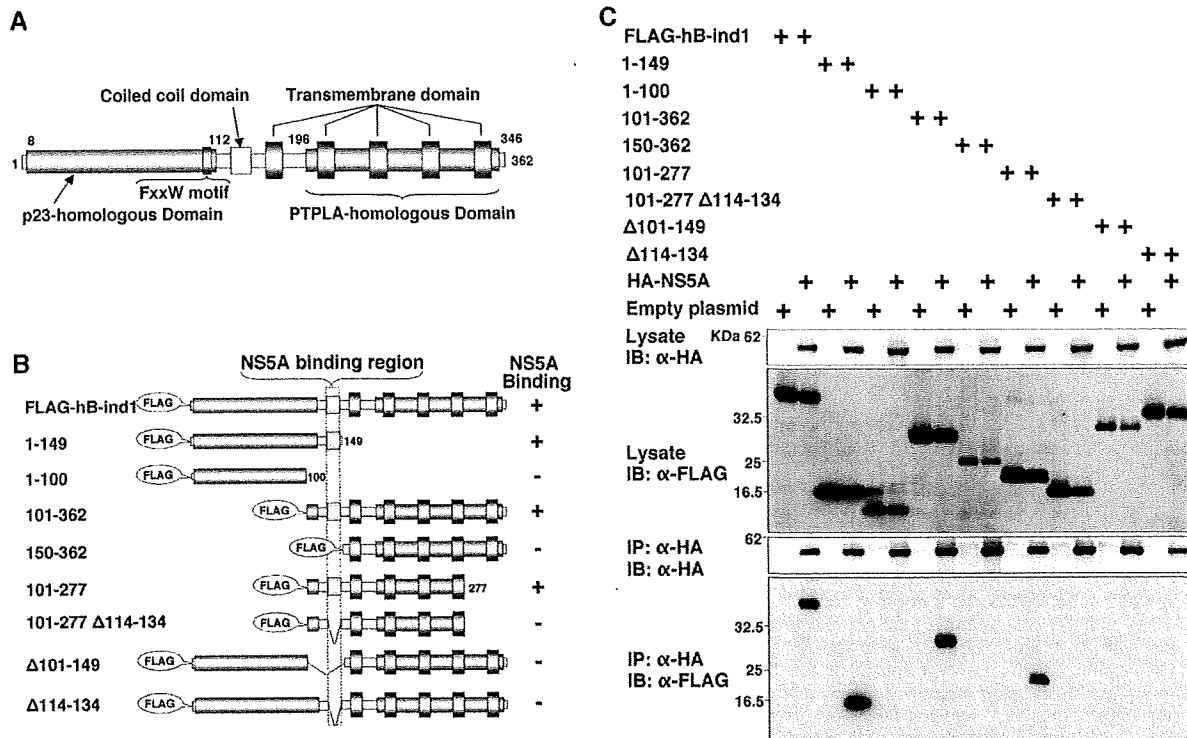


FIG. 2. Determination of the NS5A-binding region in hB-ind1. (A) Structure and functional domains of hB-ind1. (B) Deletion mutants of hB-ind1 used in this study and the results of binding to NS5A. N-terminally FLAG-tagged hB-ind1 mutants encoding the region from residue 1 to 149, 1 to 100, 101 to 362, 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-I₃₈₉ 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

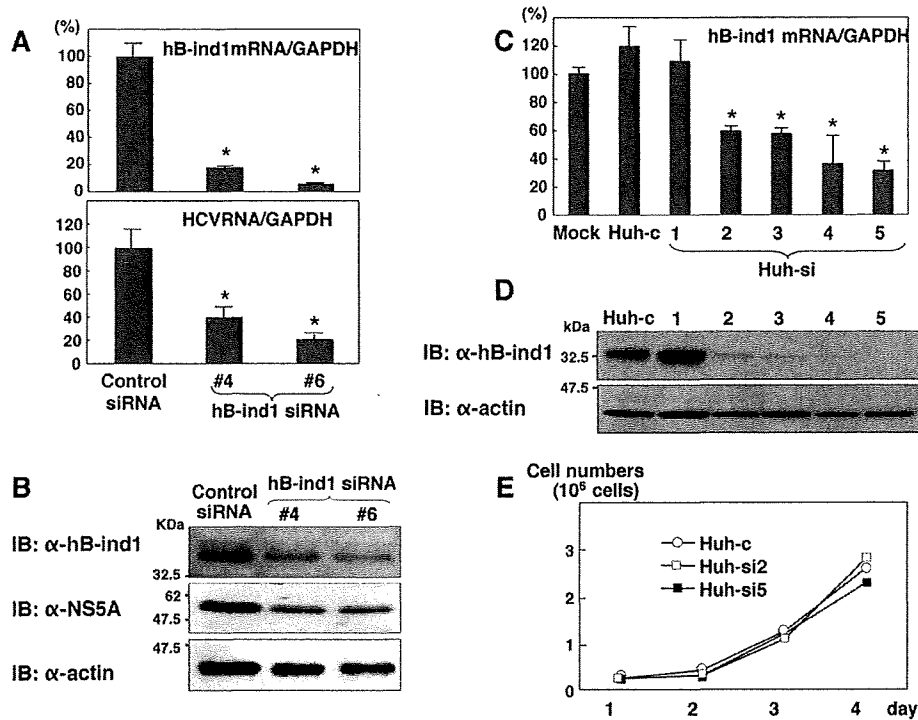


FIG. 3. Effects of hB-ind1 knockdown on HCV replication. (A) Huh9-13 cells were transfected with siRNA 4 or siRNA 6 (#4 or #6, respectively), targeted to the hB-ind1 gene, or with a nonspecific siRNA, at a final concentration of 20 nM, and were harvested at 72 h posttransfection. hB-ind1 mRNA and HCV 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 Δ 114-134) (Fig. 5C). However, no significant difference in NS5A expres-

sion 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 Δ 114-134 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

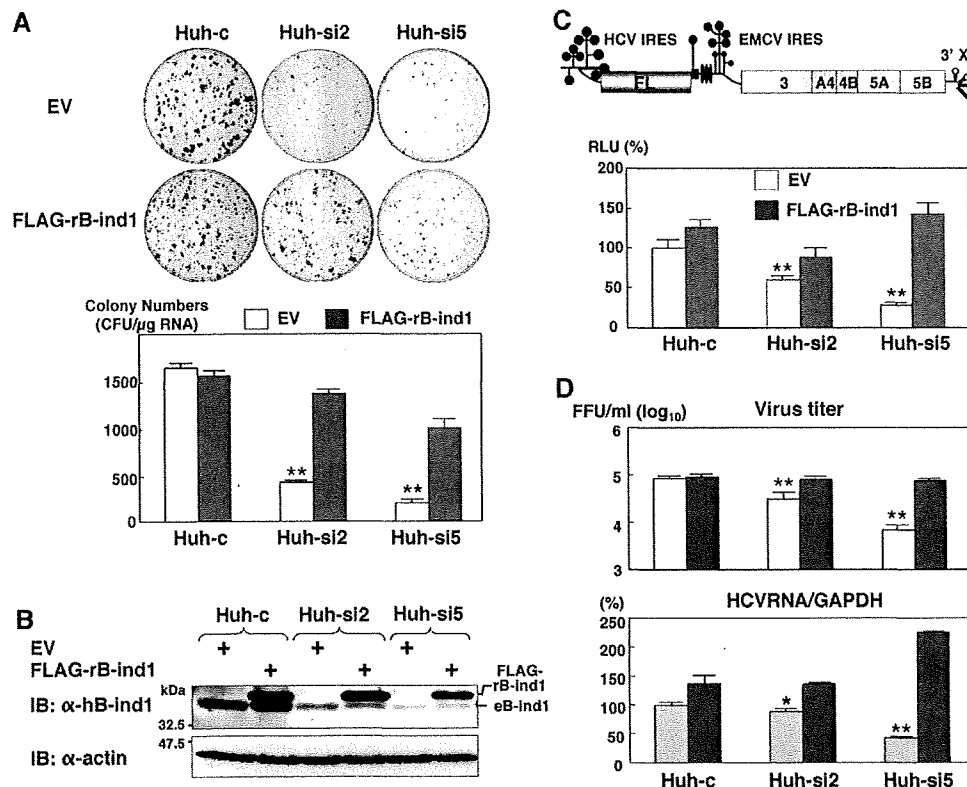


FIG. 4. Effects of hB-ind1 knockdown on the replication of HCV RNA and the production of infectious particles. (A) The hB-ind1 knockdown (Huh-si2 and Huh-si5) and control (Huh-c) cell lines were first transfected with either a plasmid encoding hB-ind1 resistant to siRNA by virtue of the introduction of silent mutations (FLAG-rB-ind1) or an empty vector (EV) and then further transfected with replicon RNA transcribed from pFK-I₃₈₉ 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-I₃₈₉ 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 NSSA 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 Phe¹⁰⁷xTrp¹¹⁰ 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 Phe¹⁰⁷ and Trp¹¹⁰ 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

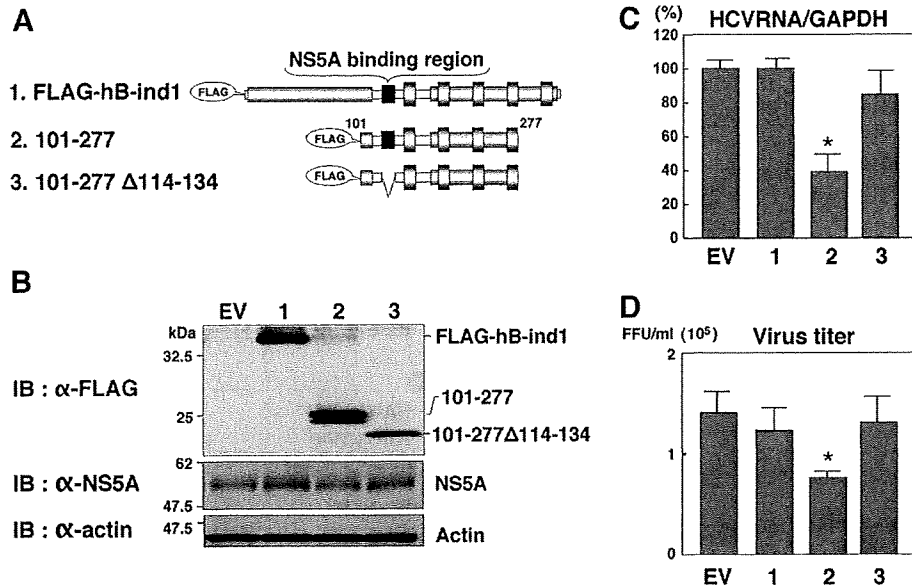


FIG. 5. Dominant-negative effect of an hB-ind1 mutant on the replication of HCV. (A) Plasmids encoding full-length hB-ind1 (construct 1) or deletion mutants of hB-ind1 retaining (construct 2) or lacking (construct 3) the NS5A binding region. (B) One of the three plasmids or an empty vector (EV) was transfected 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-I₃₈₉ 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- κ B 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

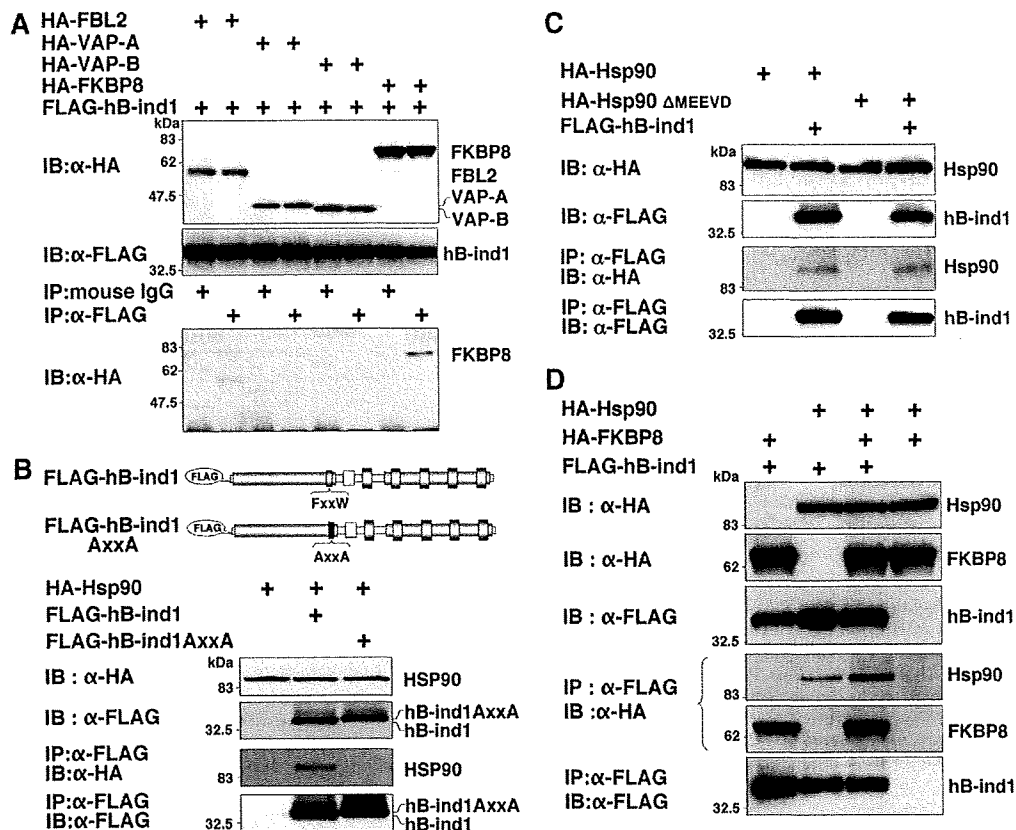


FIG. 6. Interaction of hB-ind1 with other NS5A-binding host proteins. (A) FLAG-hB-ind1 was first coexpressed with HA-tagged FBL2, VAP-A, VAP-B, or FKBP8 in 293T cells and then immunoprecipitated with an anti-FLAG or control antibody. The immunoprecipitates were detected by Western blotting (IB) with an anti-HA antibody. (B) FLAG-hB-ind1 or FLAG-hB-ind1AxxA, in which Phe¹⁰⁷ and Trp¹¹⁰ 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 Δ MEEVD) 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

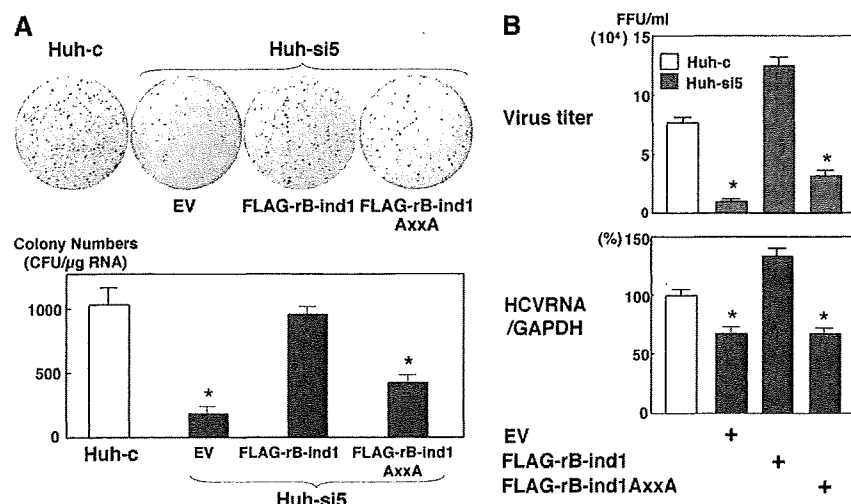


FIG. 7. Role of the interaction of hB-ind1 with Hsp90 in the replication of HCV. (A) hB-ind1 knockdown (Huh-si5) and control (Huh-c) cell lines were transfected either with a plasmid encoding the FLAG-tagged siRNA-resistant hB-ind1 (FLAG-rB-ind1) or FLAG-rB-ind1AxxA (with substitutions in the motif required for binding to Hsp90) or with an empty vector (EV) and were then further transfected with replicon RNA transcribed from pFK-I₃₈₉ 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) (Upper panel) Huh-si5 cells expressing either FLAG-rB-ind1 or FLAG-rB-ind1AxxA were infected with HCVcc, and virus production in the culture supernatants at 72 h postinoculation was determined by a focus-forming assay. (Lower panel) The amount of intracellular HCV RNA was measured at 72 h posttransfection by real-time PCR, normalized to the amount of GAPDH mRNA, and expressed as a percentage of the value for control cells transfected with an empty plasmid. Data in this figure are representative of three independent experiments. Error bars, standard deviations. Asterisks indicate significant differences ($P < 0.01$) from the control value.

client proteins are refolded by Hsp90 chaperone activity to achieve the mature form. After that, p23 enhances the dissociation of the mature client protein from the final complex, and the released Hsp90 enters in the next chaperone cycle (72). It has been reported that Hsp90 cochaperone frequencies differ among client proteins (50). FKBP8 interacts with the C-terminal MEEVD motif of Hsp90 through the carboxylate clump position in the TPR domain of FKBP8 (45).

The C-terminal region of hB-ind1 shares homology with PTPLA (60). Protein tyrosine phosphatases are generally involved in the signaling pathways regulating metabolism, cell growth, differentiation, and cytoskeletal dynamics through the conserved HC(x)₅R motif (57). NS5A also interacts with signal transducer and activator of transcription 1 (STAT1) and impairs IFN signaling through the suppression of STAT1 phosphorylation (30). In addition, intracellular uptake of apoptotic cells expressing NS5A by dendritic cells leads to an increase in the secretion of CXCL-8 and impairment of IFN-induced tyrosine phosphorylation of STAT1 and STAT2 (67). Although hB-ind1 lacks the conserved active motif, the interaction of NS5A with the coiled-coil domain in the central region of hB-ind1 may have an effect on the phosphorylation of host proteins involved in the replication of HCV.

Hsp90 has been shown to be involved in the enzymatic activity and intracellular localization of several viral polymerases, including those of influenza virus (39, 42), herpes simplex virus type 1 (5), and Flock house virus (25). Knockdown and treatment with an Hsp90 inhibitor have revealed that Hsp90 activity is important for the rapid growth of negative-strand RNA viruses (9). Furthermore, Hsp90 has been shown to be required for the activity of hepatitis B virus reverse

transcriptase (21, 22). Although the precise mechanisms by which Hsp90 and FKBP8 cooperate with NS5A to improve the in vivo replication of HCV have not been clarified yet, treatment with Hsp90 inhibitors in combination with IFN reduced HCV replication in mice xenotransplanted with human liver fragments (43).

In this study, hB-ind1 was shown to interact with Hsp90 through the FxxW motif in the N-terminal p23 homology domain, and the interaction of hB-ind1 with Hsp90 was shown to be further intensified by the expression of FKBP8, suggesting that FKBP8 and hB-ind1 cooperatively recruit Hsp90 to the HCV replication complex. Furthermore, hB-ind1 was shown to be involved in HCV genomic RNA replication and particle production through the interaction with NS5A and Hsp90. These results suggest that hB-ind1 may be involved in the Hsp90 chaperone pathway in a function similar to that of p23 in cooperation with immunophilins such as FKBP8 and that it plays a crucial role in HCV replication in terms of the correct folding of the replication complex required for efficient enzymatic activity. In addition, cyclophilin B may also participate in the translocation of NS5B, as seen in the polymerase subunits of influenza virus, to facilitate binding to the viral RNA. In contrast to cyclosporine A, FK506 per se exhibits no inhibition of RNA replication in HCV replicon cells (65). FKBP8 is a member of the FKBP family but lacks several amino acid residues required for peptidyl-prolyl *cis-trans* isomerase and FK506 binding activities (29). Therefore, nonimmunosuppressive FK506 derivatives that are capable of binding to FKBP8 may exhibit anti-HCV activity. Recently, geldanamycin, an inhibitor of Hsp90, was shown to drastically impair the replication of poliovirus without any escape mutant emerging (15).

Therefore, elucidation of host proteins, including immunophilins, cochaperones, 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 breakthrough viruses against antiviral drugs.

In conclusion, in this study we demonstrated that hB-ind1 is involved in HCV replication through interactions with NS5A, FKBP8, and Hsp90. Further clarification of the relationship between viral and host proteins is needed in order to understand the precise mechanism of HCV replication.

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Crystal structure of the catalytic domain of Japanese encephalitis virus NS3 helicase/nucleoside triphosphatase at a resolution of 1.8 Å

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Abstract

The NS3 protein of Japanese encephalitis virus (JEV) is a large multifunctional protein possessing protease, helicase, and nucleoside 5'-triphosphatase (NTPase) activities, and plays important roles in the processing of a viral polyprotein and replication. To clarify the enzymatic properties of NS3 protein from a structural point of view, an enzymatically active fragment of the JEV NTPase/helicase catalytic domain was expressed in bacteria and the crystal structure was determined at 1.8 Å resolution. JEV helicase is composed of three domains, displays an asymmetric distribution of charges on its surface, and contains a tunnel large enough to accommodate single-stranded RNA. Each of the motifs I (Walker A motif), II (Walker B motif) and VI was composed of an NTP-binding pocket. Mutation analyses revealed that all of the residues in the Walker A motif (Gly¹⁹⁹, Lys²⁰⁰ and Thr²⁰¹), in addition to the polar residues within the NTP-binding pocket (Gln⁴⁵⁷, Arg⁴⁶¹ and Arg⁴⁶⁴), and also Arg⁴⁵⁸ in the outside of the pocket in the motif IV were crucial for ATPase and helicase activities and virus replication. Lys²⁰⁰ was particularly indispensable, and could not be exchanged for other amino acid residues without sacrificing these activities. The structure of the NTP-binding pocket of JEV is well conserved in dengue virus and yellow fever virus, while different from that of hepatitis C virus. The detailed structural comparison among the viruses of the family *Flaviviridae* should help in clarifying the molecular mechanism of viral replication and in providing rationale for the development of appropriate therapeutics.

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Keywords: JEV; NTPase/helicase; Crystal structure; Replication

Introduction

The genus *Flavivirus* within the family *Flaviviridae* contains many arthropod-borne viruses, such as Japanese encephalitis virus (JEV), West Nile virus (WNV), dengue virus (DEN), yellow fever virus (YFV) and tick-borne encephalitis virus. JEV is still one of the most important flaviviruses in medical and veterinary fields despite the wide availability of inactive vac-

cines. JEV is distributed in the south and southeast regions of Asia and kept in a zoonotic transmission cycle between pigs or birds and mosquitoes (Solomon et al., 2003; Tsai, 2000). JEV spreads to dead-end hosts, including humans, through the bite of mosquitoes infected with JEV and causes infection of the central nervous system, with a high mortality rate. JEV has a plus-sense single-stranded RNA genome approximately 11 kb in length, which is capped at the 5' end but lacks modification of the 3' terminus by polyadenylation. The genome is translated into a single precursor polyprotein which is subsequently processed by host and viral proteases to produce three structural proteins, C, prM and E, and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Sumiyoshi et al.,

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1987). NS3 is a multifunctional protein of 619 amino acid residues. The N-terminal one-third of NS3 has a serine protease activity that participates in the processing together with a co-factor protein NS2B. The NS3 protein also has a catalytic domain for helicase, nucleoside 5'-triphosphatase (NTPase), as well as 5'-terminal RNA triphosphatase activities in the C-terminal two-thirds. Functionally, the enzymatic activities of helicase and ATPase have been confirmed in the NS3 proteins derived from several members of the family *Flaviviridae*, including viruses from the genus *Flavivirus*, such as JEV, DEN, YFV, and from the genus *Hepacivirus*, such as hepatitis C virus (HCV) (Cui et al., 1998; Kuo et al., 1996; Li et al., 1999b; Warrenner et al., 1993; Wengler and Wengler, 1991). Although the precise biological functions of the helicase/NTPase domain are unclear, one possible explanation is that the domain may resolve double-stranded RNA intermediates formed during transcription or replication of genomic RNA. The DEN mutants impaired in NTPase and helicase activities displayed little or no replication (Matusan et al., 2001), suggesting that flaviviral helicase/NTPase is essential for the viral life cycle and could be an ideal target for the development of antiviral drugs.

Helicases are motor enzymes that use energy derived from NTP hydrolysis to catalyze the unwinding and remodeling of double-stranded nucleic acids, and they are classified into three

superfamilies based on sequence comparison and conserved motifs (Luking et al., 1998). Helicase can be further classified into DEAD, DEXD, and DEXx subfamilies based on the sequence of motif II (Ahmadian et al., 1997; Luking et al., 1998; Schmid and Linder, 1992). All helicases bind NTP using two structural elements, the motif I/Walker A motif, which is a phosphate-binding P-loop, and the motif II/Walker B motif, which is a Mg^{2+} -binding aspartic acid loop (Koonin, 1991). NS3 helicases of flaviviruses and HCV are classified into superfamily 2 (Kim et al., 1997; Utama et al., 2000a,b). Superfamily 2 RNA helicases, also called DEA(D/H)-box helicases after the signature sequence in the Walker B motif, have seven conserved motifs, and these motifs are suggested to be associated with NTP hydrolysis and nucleic acid binding (Fig. 1A). Recently, a conserved Q motif upstream of the Walker A motif was reported to be essential for the ATPase activity of DEAD-box helicases (Tanner et al., 2003). Utama et al. have shown that the motif II (DEXH/D) of both JEV and HCV NS3 proteins is essential for RNA helicase and ATPase activities (Utama et al., 2000a,b). The crystal structures have been reported for several helicases, including the DNA helicase PcrA from *Bacillus stearothermophilus* (Subramanya et al., 1996) and Rep from *Escherichia coli* (*E.coli*) (Korolev et al., 1997) of the superfamily 1 and the NS3 helicases from DEN, YFV, and HCV (Cho et al., 1998; Kim et al., 1998; Yao et al., 1997; Yao et al.,

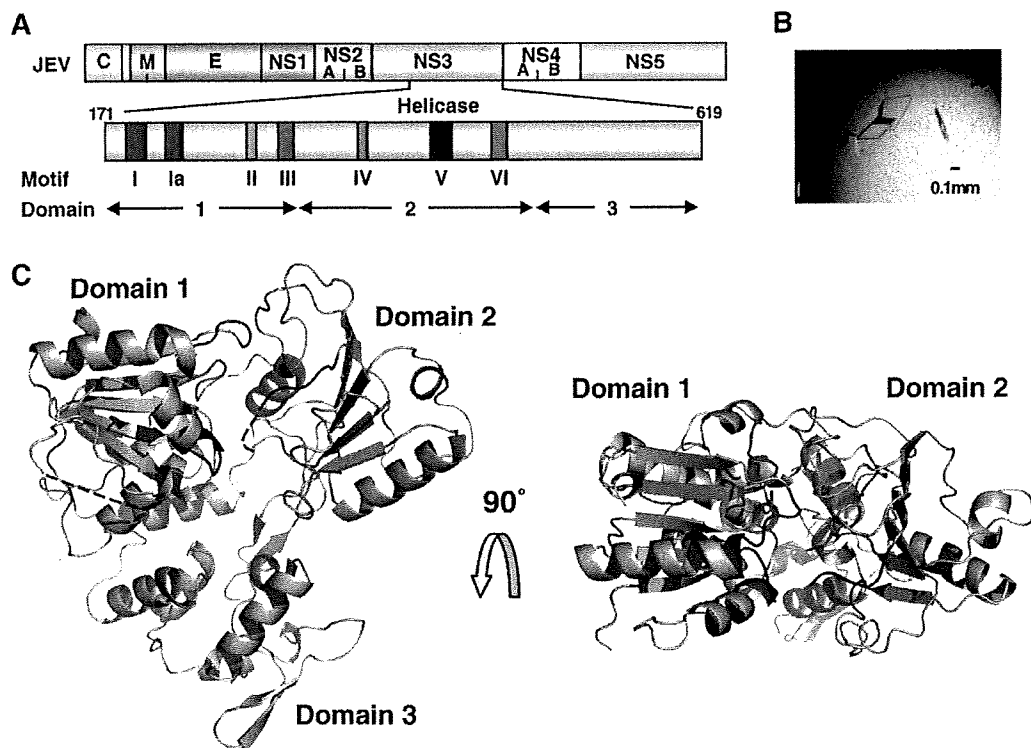


Fig. 1. Genome structure of JEV, crystals of JEV NS3 helicase/NTPase, and the three-dimensional structure. (A) Genome structure of JEV and the seven conserved motifs in JEV helicase domain. (B) Crystals of JEV NS3 helicase/NTPase domain obtained in the drop after 5 days of incubation. (C) The ribbon diagram of the JEV NS3 helicase/NTPase domain shows domain 1 and 2 at the top and domain 3 at the bottom (left). The seven helicase motifs are colored separately (motif I, purple; motif Ia, red; motif II, cyan; motif III, orange; motif IV, pink; motif V, blue; motif VI, green). Two regions, 245–252 and 413–415, are disordered and displayed as a broken line. The upper view of the JEV helicase/NTPase domain is shown at right.