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# Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90

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**Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) is a component of viral replicase and is well known to modulate the functions of several host proteins. Here, we show that NS5A specifically interacts with FKBP8, a member of the FK506-binding protein family, but not with other homologous immunophilins. Three sets of tetratricopeptide repeats in FKBP8 are responsible for interactions with NS5A. The siRNA-mediated knockdown of FKBP8 in a human hepatoma cell line harboring an HCV RNA replicon suppressed HCV RNA replication, and this reduction was reversed by the expression of an siRNA-resistant FKBP8 mutant. Furthermore, immunoprecipitation analyses revealed that FKBP8 forms a complex with Hsp90 and NS5A. Treatment of HCV replicon cells with geldanamycin, an inhibitor of Hsp90, suppressed RNA replication in a dose-dependent manner. These results suggest that the complex consisting of NS5A, FKBP8, and Hsp90 plays an important role in HCV RNA replication.**

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## Introduction

Hepatitis C virus (HCV) persistently infects approximately 170 million people worldwide, and it is responsible for most cases of severe chronic liver diseases, including cirrhosis and hepatocellular carcinoma (Wasley and Alter, 2000). Although treatment with interferon (IFN) alpha and ribavirin is available for about half of the population of HCV patients (Manns *et al*, 2001), therapeutic and preventative vaccines are still necessary for more effective treatment; however, such vaccines have not yet been developed. HCV belongs to the *Flaviviridae* family and possesses a positive-sense single-stranded RNA with a nucleotide length of 9.6 kb. The HCV genome encodes a single large precursor polyprotein composed of about 3000 amino acids, and the polyprotein is processed by cellular and viral proteases into at least 10 structural and nonstructural (NS) proteins (Moriishi and Matsuura, 2003).

The development of efficient therapies for hepatitis C has been hampered by the lack of a reliable cell-culture system, as well as by the absence of a non-primate animal model. The HCV replicon consists of an antibiotic selection marker and a genotype 1b HCV RNA, which replicates autonomously in the intracellular compartments in a human hepatoma cell line, Huh7 (Lohmann *et al*, 1999). This replicon system has functioned as an important tool in the investigation of HCV replication and it has served as a cell-based assay system for the evaluation of antiviral compounds. Recently, cell culture systems for *in vitro* replication and infectious viral production were established based on the full-length HCV genome of genotype 2a, which was isolated from an HCV-infected patient who developed fulminant hepatitis (Lindenbach *et al*, 2005; Wakita *et al*, 2005; Zhong *et al*, 2005). However, no robust *in vitro* culture systems for the 1a and 1b genotypes, which are the most prevalent HCV genotypes in the world, have been established to date.

Several viruses require viral and host molecular chaperones for entry, replication, and assembly, as well as for other steps in viral production (Maggioni and Braakman, 2005; Mayer, 2005). Cyclosporine A has been found to effectively inhibit viral replication in hepatitis C patients and in HCV replicon cells (Inoue *et al*, 2003; Watashi *et al*, 2003). Recently, it was shown that cyclophilin (Cyp) B specifically binds to NS5B and promotes association with the genomic RNA; furthermore, cyclosporine A was shown to disrupt interactions between NS5B and CypB (Watashi *et al*, 2005). CypB belongs to the immunophilin family, which shares peptidyl propyl *cis/trans* isomerase (PPIase) activity and an affinity for the immunosuppressive drug (Fischer and Aumuller, 2003). Furthermore, blockades of CypA, CypB, and CypC, as well as the induction of cellular stress responses, have been suggested to be involved in cyclosporine A-induced reduction of HCV RNA replication (Nakagawa *et al*, 2005). However, the involvement of other immunophilins in HCV RNA replication is not yet well understood.

HCV nonstructural protein 5A (NS5A) is a membrane-anchored phosphoprotein that possesses multiple functions in viral replication, IFN resistance, and pathogenesis (Macdonald and Harris, 2004). NS5A contains a zinc metal-binding motif within the N-terminal domain, and this zinc-binding ability is known to be essential for HCV replication (Tellinghuisen *et al*, 2004, 2005). Adaptive mutations frequently mapped in the coding region of NS5A have been shown to increase RNA replication (Yi and Lemon, 2004; Appel *et al*, 2005) and they are known to affect the hyperphosphorylation of NS5A by an unknown host kinase (Koch and Bartenschlager, 1999; Neddermann *et al*, 1999; Pietschmann

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*et al*, 2001). RNA replication in HCV replicon cells has been shown to be inhibited by treatment with lovastatin, a drug that decreases the production of mevalonate by inhibiting 3-hydroxy-3-methylglutaryl CoA reductase; this inhibition of RNA replication was reversed by the addition of geranylgeraniol, which suggests that HCV RNA replication requires geranylgeranylated proteins (Ye *et al*, 2003; Kapadia and Chisari, 2005). A NS5A-pull-down assay identified a geranylgeranylated protein, FBL2, as a NS5A-binding protein (Wang *et al*, 2005). Although several host proteins could potentially interact with NS5A, little is known about NS5A function.

To gain a better understanding of the functional role of NS5A in HCV replication, we screened human libraries by employing a yeast two-hybrid system and using NS5A as bait. We thereby successfully identified FKBP8 as an NS5A-binding protein. FKBP8 is classified as a member of the FK506-binding protein family, but it lacks several amino-acid residues thought to be important for PPIase activity and FK506 binding (Lam *et al*, 1995). We demonstrated here that FKBP8 forms a complex with Hsp90 and NS5A, and that this complex is critical for HCV replication, as based on the finding that treatment of the HCV replicon cells with geldanamycin, an inhibitor of Hsp90, suppressed RNA replication. These results therefore suggest that protein complex formation with NS5A, FKBP8, and Hsp90 plays a crucial role in HCV RNA replication.

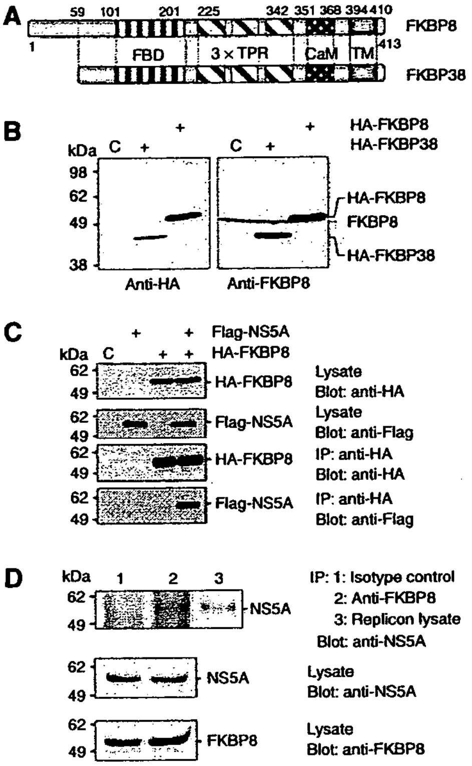

**Figure 1** Expression of FKBP8 and FKBP38 in mammalian cells. (**A**) Schematic representation of FKBP8 and FKBP38. The FK506-binding domain (FBD), tetratricopeptide repeat (TPR), putative calmodulin binding motif (CaM), and transmembrane domain (TM) are shown. (**B**) N-terminally HA-tagged FKBP8 and FKBP38 were expressed in 293T cells and visualized by immunoblotting using mouse monoclonal antibody to FKBP8 or the HA tag. (**C**) HA-FKBP8 was expressed together with Flag-NS5A of genotype 1b (J1) in 293T cells and was immunoprecipitated with anti-HA antibody. Immunoprecipitated proteins were subjected to immunoblot with anti-Flag or HA antibody. (**D**) Endogenous FKBP8 in HCV replicon (9-13) cells was immunoprecipitated with isotype control (lane 1) or anti-FKBP8 antibody, KDM-11 (lane 2). Endogenous FKBP8 was co-immunoprecipitated with HCV NS5A. The data shown in each panel are representative of three independent experiments.

## Results

### *Identification of human FKBP8 as an HCV NS5A-binding partner*

To identify host proteins that specifically interact with NS5A, we screened human brain and liver libraries using a yeast two-hybrid system that employs NS5A as bait. One positive clone was isolated from among 2 million colonies of the human fetal brain library, and the nucleotide sequence of this clone was determined. Several positive clones were isolated from the human liver library, but most of these clones included exon fragments of other than FKBP and/or noncoding regions. A BLAST search revealed that the positive clone encodes a full-length coding region of FKBP38, human FK506-binding protein 38 kDa. Although FKBP38 has been isolated from human and mouse mRNA (Lam *et al*, 1995), an additional sequence at the N-terminus of FKBP38 was revealed based on an analysis of the transcriptional start site in the genomic sequences of FKBP38 (Nielsen *et al*, 2004). The isoforms of FKBP38 were designated as FKBP8, which includes splicing variants of 44 and 46 kDa in mice, and 45 kDa in humans corresponds to the 44 kDa of the mouse FKBP8 (Nielsen *et al*, 2004). Human FKBP8 is identical to FKBP38 except for the extra 58 amino-acid residues at the N-terminus, and the FK506-binding domain in the N-terminal half, followed by three sets of tetratricopeptide repeats (TPRs), a calmodulin binding site, and a transmembrane domain (Figure 1A). Because the levels of expression of FKBP8 and FKBP38 have not been well characterized in human cell lines, we generated a mouse monoclonal antibody against human FKBP8, and we designated it as clone KDM19. This antibody recognizes a 50-kDa of endogenous FKBP8 in 293T cells, as well as exogenous HA-tagged FKBP8 (HA-FKBP8), which has slightly greater molecular weight (Figure 1B). Although the KDM19 antibody detected an exogenous HA-tagged FKBP38 (HA-FKBP38) in 293T cells, no protein band corresponding to endogenous FKBP38 was detected. Similar results were obtained in human liver tissue and in the hepatoma cell lines Huh7, HepG2, and FLC-4 (data not shown). These findings suggest that FKBP8, but not FKBP38, is a major product in human cells. In order to examine whether or not FKBP8 binds to NS5A protein in mammalian cells, Flag-tagged NS5A (Flag-NS5A) was expressed together with HA-FKBP8 in 293T cells. Cells transfected with the expression plasmids were harvested at 48-h post-transfection, lysed, and subjected to immunoprecipitation. Flag-NS5A was co-precipitated with HA-FKBP8 by anti-HA antibody (Figure 1C). Flag-NS5A was also immunoprecipitated together with HA-FKBP38, suggesting that the extra N-terminal sequence of FKBP8 is not critical for NS5A binding (data not shown). To further confirm the specific interaction of HCV NS5A with endogenous FKBP8, this interaction was examined in Huh7(9-13) cells harboring subgenomic HCV RNA replicon. Endogenous FKBP8 was co-precipitated with HCV NS5A by anti-FKBP8 antibody (Figure 1D). To determine the direct interaction between FKBP8 and NS5A, $His_6$-tagged FKBP8 (His-FKBP8) and thioredoxin-fused domain 1 of NS5A (Trx-NS5A) prepared in *Escherichia coli* were examined by pull-down analysis. Trx-NS5A was co-precipitated with His-FKBP8 by anti-FKBP8 antibody (Supplementary Figure 1), suggesting that FKBP8 can directly bind to NS5A domain I.

In order to investigate the interaction of FKBP8 with the NS5A of other HCV genotypes, HA-tagged NS5A (HA-NS5A) proteins of genotype 1a (H77C), 1b (Con1 and J1), or 2a (JFH1) were expressed together with Flag-tagged FKBP8 (Flag-FKBP8) in 293T cells (Figure 2A). Flag-FKBP8 was co-immunoprecipitated with the HA-NS5As of all of the genotypes examined here by anti-HA antibody, although it should be noted that the interaction between Flag-FKBP8 and the HA-NS5A of genotype 2a was weaker than that of the other genotypes tested. Furthermore, the HA-NS5As were co-precipitated with Flag-FKBP8 by anti-Flag antibody (Figure 2A, bottom panel). The TPR domain of FKBP8 is known to be responsible for protein–protein interactions. Among the immunophilins, FKBP8 shares high homology with CypD and FKBP52, both of which contain three tandem repeats of TPR, as does FKBP8 (Boguski *et al*, 1990; Hirano *et al*, 1990). However, co-immunoprecipitation of Flag-NS5A with HA-FKBP52 and HA-CypD by anti-Flag or anti-HA antibody was not successful (Figure 2B). These results indicate that FKBP8 specifically interacts with NS5A.

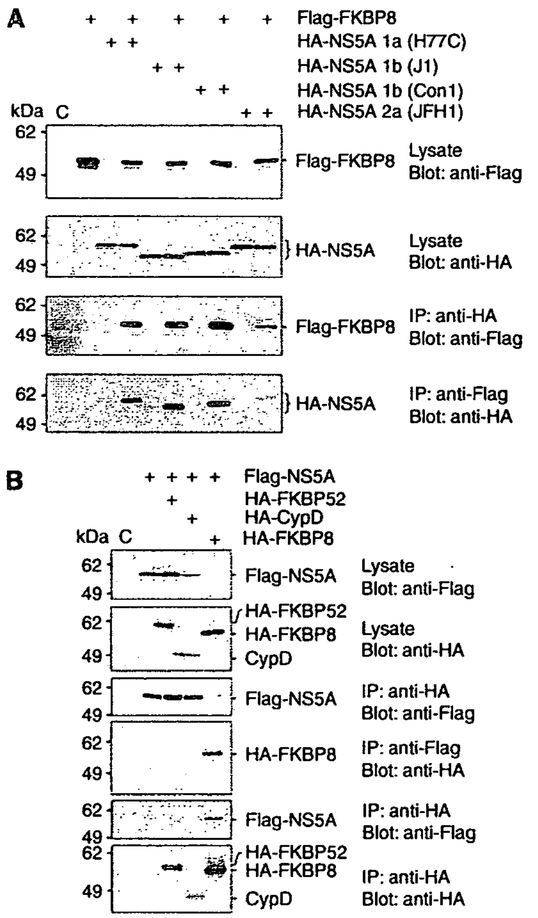

**Figure 2** Specific interaction between FKBP8 and NS5A. (**A**) HA-NS5As were obtained from several genotypes of HCV and were expressed with Flag-FKBP8 in 293T cells. Proteins immunoprecipitated with anti-HA or Flag antibody were subjected to Western blotting. (**B**) Flag-NS5A was coexpressed with HA-FKBP8, -CypD, or -FKBP52 in 293T cells. Proteins immunoprecipitated with anti-HA or -Flag tag antibody were subjected to Western blotting. The data shown in each panel are representative of three independent experiments.

### *The TPR domain is required for the interaction between NS5A and FKBP8*

FKBP8, CypD, and FKBP52 have high similarity and identity to each other within the TPR domain (Lam *et al*, 1995). Several FKBP8 mutants lacking the transmembrane region, the calmodulin-binding region, the TPR domains, and/or the FK506-binding domain were generated in order to identify the region responsible for the interaction with NS5A (Figure 3A). HA-tagged FKBP8 mutants were coexpressed with Flag-NS5A in 293T cells and were immunoprecipitated with anti-HA antibody. Flag-NS5A was co-immunoprecipitated with the FKBP8 mutants, except in the case of a dTPR mutant lacking the transmembrane, calmodulin binding, and TPR domains (Figure 3B). Although the level of expression of dFBD, an FKBP8 mutant with a deletion in the N-terminal region containing the FK506-binding domain, was lower than that of dTPR, co-immunoprecipitated NA5A was clearly detected. These findings suggested that the lack of an association of dTPR with NS5A was not due to the relatively low level of expression of dTPR, as compared to those of the other FKBP8 mutants. A specific interaction of NS5A with the TPR domain, but not with the transmembrane, calmodulin binding, or FK506-binding domains of FKBP8, was also observed using the yeast two-hybrid system (data not shown). These results indicated that FKBP8 interacts with HCV NS5A through the TPR domain.

### *FKBP8 forms a homomultimer and a heteromultimer with NS5A*

FKBP8 is similar to FKBP52 and CypD with respect to their amino-acid sequences and functional domains. In order to examine the interactions among FKBP8, FKBP52, and CypD,

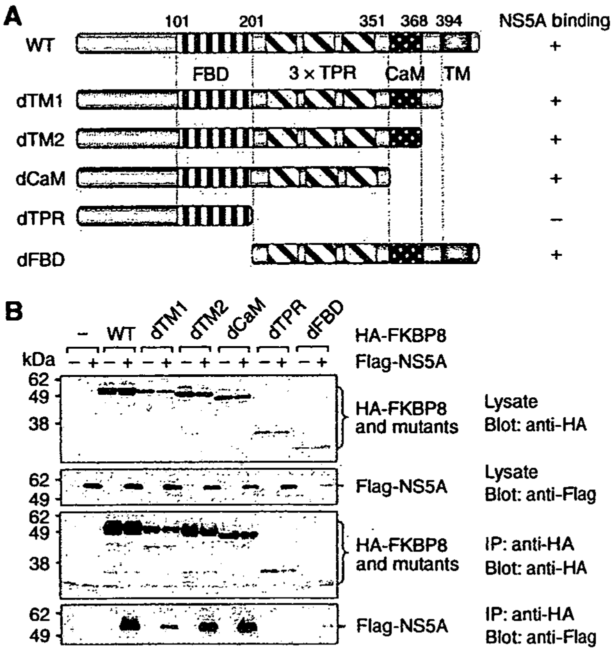

**Figure 3** Determination of the NS5A-binding region in FKBP8. (**A**) Schematic representation of FKBP8 and deleted mutants. (**B**) Flag-NS5A was coexpressed with HA-FKBP8 and its mutants in 293T cells. Proteins immunoprecipitated with anti-HA antibody were subjected to Western blotting. The data shown in each panel are representative of three independent experiments.

Flag-FKBP8 was coexpressed with HA-FKBP52, HA-CypD, or HA-FKBP8 in 293T cells and it was immunoprecipitated with anti-Flag or anti-HA antibody. Flag-FKBP8 and HA-FKBP8 were co-immunoprecipitated with each antibody, but not with HA-FKBP52 or HA-CypD. It is known that Hsp90 forms a homodimer and also interacts with FKBP52 through TPR domain as FKBP8 (Chadli *et al*, 2000). If homodimer of FKBP8 is due to intermediating of Hsp90 as FKBP8-Hsp90-Hsp90-FKBP8 complex, FKBP52 would be co-precipitated with FKBP8 as FKBP8-Hsp90-Hsp90-FKBP52. However, we could not detect any association of FKBP8 and FKBP52 in the immunoprecipitation analysis (Figure 4A). These data suggest that FKBP8 can form a homomultimer without Hsp90 and associate with neither FKBP52 nor CypD through Hsp90. To examine the effects of the interaction with NS5A on the homomultimerization of FKBP8, HA-NS5A was coexpressed with Flag-FKBP8 and Glu-Glu-tagged FKBP8 (EE-FKBP8) in 293T cells, and was then immunoprecipitated with anti-Flag or anti-EE antibody. HA-NS5A was co-immunoprecipitated with Flag-FKBP8 and EE-FKBP8 by anti-Flag or anti-EE antibody (Figure 4B). Although multimerization of EE-FKBP8 and Flag-FKBP8 was increased about 2 times in the presence of HA-NS5A, but no further increase of the multimerization of FKBP8 was observed by the increase of HA-NS5A expression (Figure 4C). These results further support the notion that NS5A binds to FKBP8 via the TPR domain and slightly influence homomultimerization exerted by the FK506-binding domain.

### *Knockdown of FKBP8 decreases RNA replication in HCV replicon cells*

In order to determine the role of endogenous FKBP8 on HCV RNA replication, 80 nM of small interfering RNA (siRNA) targeted to FKBP8 or control siRNA was transfected into Huh7 (9-13) cells harboring subgenomic HCV replicon RNA. To verify the specificity of the knockdown of FKBP mRNA, we synthesized three siRNAs targeted to different regions of FKBP8 (Targets 1-3). The total RNA was extracted from the transfected cells, and HCV RNA and FKBP8 mRNA levels were determined by real-time polymerase chain reaction (PCR). HCV subgenomic RNA and FKBP8 mRNA levels in the cells transfected with each of the FKBP8 siRNAs were reduced by more than 60%, as compared to the levels in cells treated with the control siRNA at 72 h post-transfection (Figure 5A). The levels of expression of FKBP8 and the HCV proteins (i.e., NS4B, NS5A, and NS5B) decreased in HCV replicon cells transfected with 80 or 160 nM of the FKBP8 siRNA (Target 1), but this was not observed in the cells with the control siRNA (Figure 5B). To further confirm the specificity of the reduction in HCV RNA replication in the replicon cells putatively achieved by the knockdown of FKBP8, a plasmid encoding Flag-FKBP8 containing either a silent mutation within the siRNA target sequence (Flag-rFKBP8) or empty plasmid was transfected into the HCV replicon cells and then selection was carried out with the appropriate antibiotics. The remaining cells, that is, Huh7rFKBP8 and Huh7c cells, harboring the Flag-rFKBP8

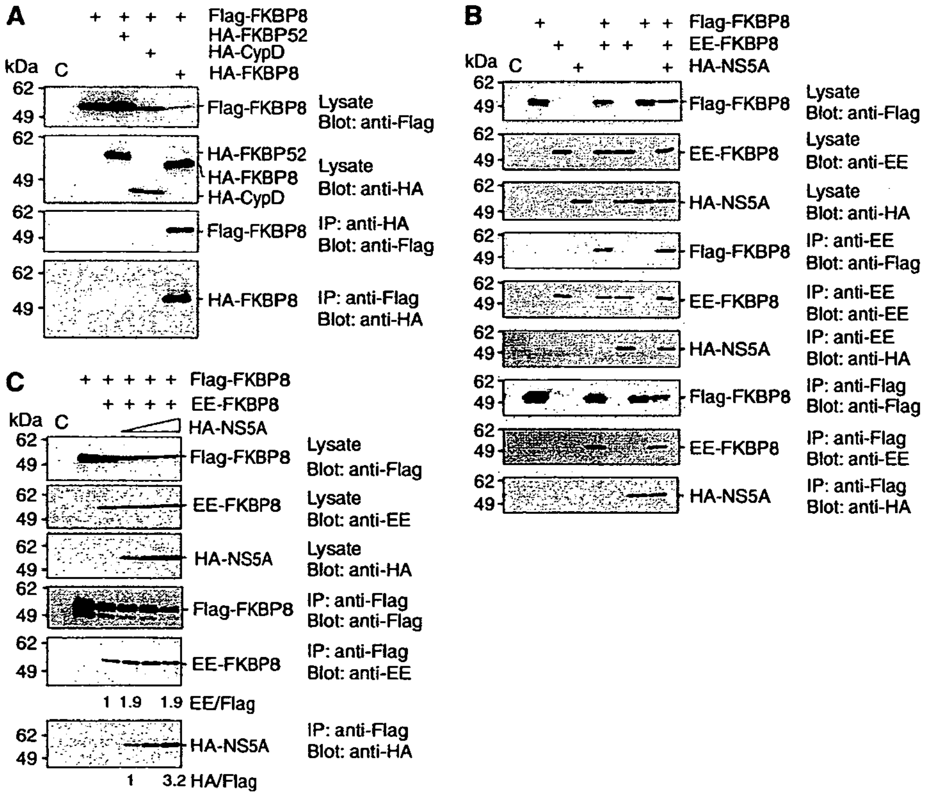

**Figure 4** Homomultimerization of FKBP8. (A) Flag-FKBP8 was coexpressed with HA-FKBP52, -CypD, or -FKBP8 in 293T cells, and was immunoprecipitated with anti-HA or Flag antibody. Precipitates were analyzed by Western blotting. (B) Flag- or EE-tagged FKBP8 was coexpressed with HA-NS5A in 293T cells and was immunoprecipitated with anti-EE or Flag antibody. Precipitates were analyzed by Western blotting. (C) Flag- and EE-tagged FKBP8 were coexpressed with increasing amounts of HA-NS5A (0.1, 0.2, and 0.4 µg of expression plasmid/well) in 293T cells. Immunoprecipitates with anti-Flag antibody were analyzed by Western blotting. The data shown in each panel are representative of three independent experiments.

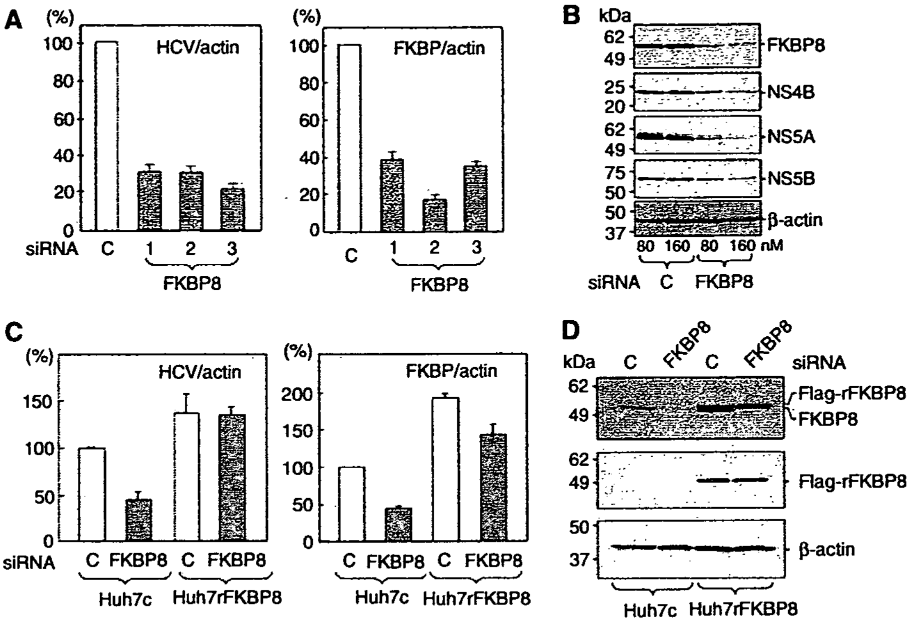

**Figure 5** Decrease in HCV RNA by FKBP8-targeted siRNA. (**A**) HCV replicon cells (9-13 cells) were transfected with each of three kinds of siRNA targeted to FKBP8 or nontargeted siRNA at a final concentration of 80 nM. Transfected cells were collected at 72 h post-transfection, and FKBP8 mRNA and HCV RNA levels were determined by real-time PCR after being normalized with β-actin mRNA. (**B**) HCV replicon cells transfected with 80 and 160 nM of Target 1 or nontargeted siRNA were harvested at 72 h post-transfection, and the samples were analyzed by immunoblotting. (**C**) HCV replicon cells expressing Flag-rFKBP8 mutant (Huh7rFKBP8) or control cells (Huh7c) were transfected with Target 1 (gray bars) or nontargeted (white bars) siRNA at a concentration of 80 nM. Transfected cells were harvested at 72 h post-transfection, and HCV RNA (left) and FKBP8 mRNA (right) were measured by real-time PCR and expressed as % increase after being normalized with the expression of β-actin mRNA. (**D**) Levels of expression of endogenous FKBP8, exogenous Flag-rFKBP8, and β-actin in the replicon cells after transfection of the siRNAs were determined by immunoblotting using specific antibodies. The data shown in each panel are representative of three independent experiments.

and empty plasmid, respectively, were pooled and then transfected with the FKBP8 siRNA (Target 1) or control siRNA. Although transfection of the FKBP8 siRNA led to a 60% reduction of HCV RNA and FKBP8 mRNA in Huh7c cells, in comparison with levels in cells transfected with the control siRNA, no reduction in HCV RNA, and only a slight reduction in FKBP8 mRNA levels were observed in Huh7rFKBP8 cells (Figure 5C). Flag-rFKBP8 expression was clearly detected in Huh7rFKBP8 cells after transfection with the FKBP8 siRNA or control siRNA, whereas the endogenous FKBP8 decreased in both Huh7rFKBP8 and Huh7c cells with the FKBP8 siRNA (Figure 5D). These findings suggest that the slight reduction of FKBP8 mRNA in the Huh7rFKBP8 cells was due to a loss of endogenous FKBP8. Knockdown of FKBP8 by siRNA induce no apoptosis in a hepatoma cell line (Supplementary Figure 2). These results therefore confirmed that the inhibition of HCVRNA replication by FKBP8 siRNA was due to a specific reduction in the mRNA of FKBP8, but was not due to a nonspecific reduction of any other host mRNA.

To further examine the involvement of FKBP8 on HCV replication, we established a line of Huh7 cells that stably expresses shRNA targeted to FKBP8. Huh7 was transfected with pSilencer 2.1 U6 hygro containing the cDNA of shRNA to FKBP8, and then selection was carried out with hygromycin. FKBP8 was detected in Huh7 cells harboring a control plasmid (Huh7N), whereas decreased expression of FKBP8 was clearly observed in cells expressing the shRNA to FKBP8 (Huh7FKBP8KD) (Figure 6A). In order to examine the effects of the knockdown of FKBP8 on HCV RNA replication, a chimeric HCV RNA containing the *Renilla* luciferase gene was transfected into these cell lines. Although the chimeric HCV RNA exhibited 5.5 times higher replication than a replication deficient GND mutant RNA in Huh7N, only a doubling of the levels of replication was observed in Huh7FKBP8KD (Figure 6B). Furthermore, HCV RNA containing a neomycin-resistant gene was transfected into the cell lines in order to examine the role played by FKBP8 in HCV RNA replication. The efficiency of colony formation in Huh7N and Huh7FKBP8KD cells with the HCV RNA were 1700 and 23 colonies/μg RNA, respectively (Figure 6C). We also examined the role of FKBP8 on the cell culture system for HCV infection. The siRNA-mediated knockdown of FKBP8 impaired both intracellular viral RNA replication and release of HCV core protein into the culture supernatants (Figure 6D). These results further confirmed that FKBP8 plays a crucial role in the efficient replication of HCV RNA.

### *FKBP8 forms a multicomplex with NS5A and Hsp90*

To identify the cellular proteins that associate with FKBP8, we employed a purification strategy using an MEF affinity tag composed of myc and FLAG tags fused in tandem and separated by a spacer sequence containing a TEV protease cleavage site (myc-TEV-FLAG) (Ichimura *et al*, 2005). The MEF expression cassette fused with FKBP8 was transfected into 293T cells and the cells were immunoprecipitated. The endogenous FKBP8-binding proteins bound to the Flag beads were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were then visualized by silver staining. The visible protein bands were excised and determined by a nanoflow LC-MS/MS system. Major protein bands with a molecular size of 94 and 53 kDa were identified as Hsp90 and FKBP8, respectively, although it should be

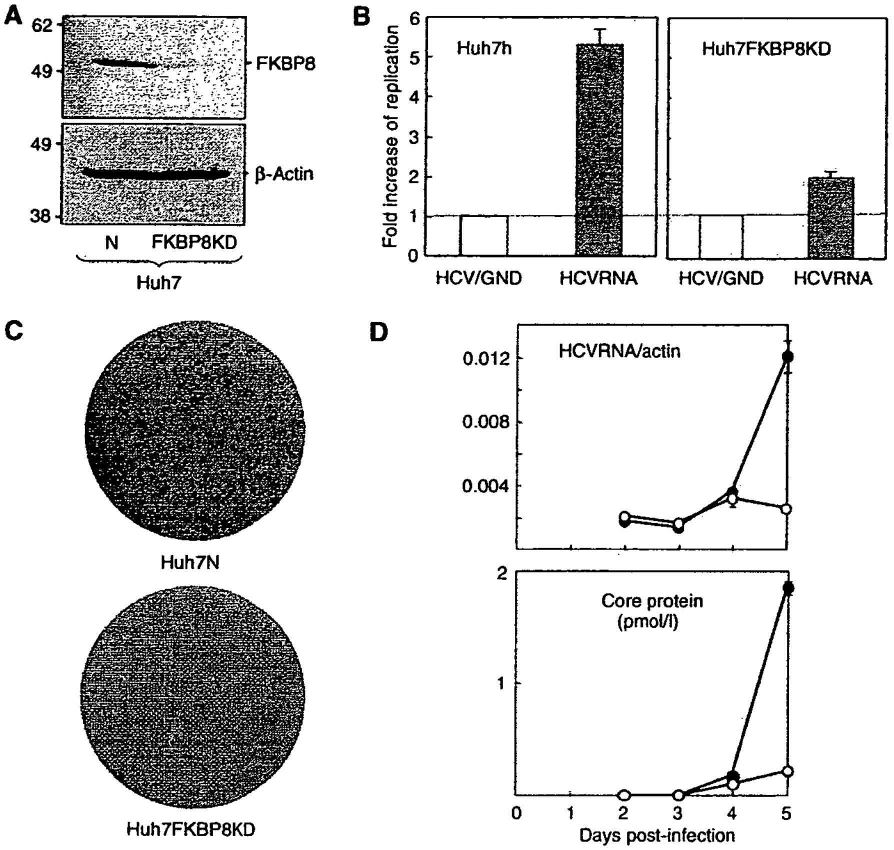

**Figure 6** Effect of knockdown of FKBP8 on the transient replication, colony formation, and viral infection. (**A**) Levels of expression of FKBP8 and β-actin in Huh7N and Huh7 FKBP8KD cell lines bearing plasmids encoding shRNA for control mRNA (lane 1) and for FKBP8 mRNA (lane 2), respectively. (**B**) Each cell line was transfected with *in vitro*-transcribed HCV replicon RNA, pFK-I$_{389}$ hRL/NS3-3′/NK5.1 (HCVRNA), or a replication-negative mutant, pFK-I$_{389}$ hRL/NS3-3′/NK5.1GND (HCV/GND). The fold increase in replication was determined by the increase in luciferase activity at 48 h compared with that observed 4 h after standardization, as based on the activity of the replication-deficient HCV/GND replicon. (**C**) Huh7N and Huh7 FKBP8KD cell lines were transfected with *in vitro*-transcribed replicon RNA (pFK-I$_{389}$ neo/NS3-3′/NK5.1) and the cells were incubated for 4 weeks. The remaining cells were fixed with 4% paraformaldehyde and then were stained. (**D**) Huh7.5.1 cells were transfected with either of siRNA targeted to FKBP8 (Target 1) or nontarget control at a concentration of 80 nM. The cells were inoculated with HCVcc at 24 h after transfection and cells and culture supernatants were harvested every day. Intracellular viral RNA (upper) and HCV core protein in the supernatant (lower) were determined. The data shown in each panel are representative of three independent experiments.

noted that the remaining bands detected in the samples could not be reliably identified (Figure 7A).

In order to elucidate the interaction of Hsp90 with FKBP8 in mammalian cells, Flag-FKBP8 was coexpressed with HA-Hsp90 and immunoprecipitated by anti-Flag or anti-HA antibody. HA-Hsp90 and Flag-FKBP8 were co-precipitated with each other by either of the antibodies but no interaction was observed between HA-Hsp90 and Flag-NS5A (Figure 7B). To examine the interplay among NS5A, FKBP8, and Hsp90, HA-Hsp90 was coexpressed with EE-FKBP8 and/or Flag-NS5A (Figure 7C). Co-immunoprecipitation of Hsp90 and NS5A was clearly detected in the presence but not in the absence of FKBP8. The increase in NS5A expression had no effect on the interaction between FKBP8 and Hsp90 (Supplementary Figure 3). These results suggest that Hsp90 does not directly bind to NS5A but forms complex with NS5A through the interaction with FKBP8.

### *FKBP8 interacts with NS5A and Hsp90 via different sites in the TPR domain*

Crystal structure of the TPR domain of Hop, an adaptor chaperone that binds both Hsp70 and Hsp90, revealed that C-terminal MEEVD motif of Hsp90 is held by amino-acid residues of the two-carboxylate clamp positions within the TPR domain (Scheufler *et al*, 2000; Brinker *et al*, 2002; Cliff *et al*, 2006). To examine the role of the C-terminal MEEVD motif of Hsp90 on the interaction with FKBP8, Hsp90 mutant lacking the MEEVD motif (HA-Hsp90ΔMEEVD) was coexpressed with Flag-FKBP8 (Figure 8A). Wild-type Hsp90 but not the mutant Hsp90 was co-precipitated with FKBP8, indicating that the FKBP8 interacts with Hsp90 via the C-terminal MEEVD motif. Lys$^{307}$ and Arg$^{311}$ residues in the two-carboxylate clamp positions of FKBP8 were conserved among the TPR domain of other immunophilins, such as FKBP52 and CypD (Figure 8B). To examine the role of the two-carboxylate clamp positions of FKBP8 for the interaction with Hsp90 and NS5A, FKBP8 mutant replaced Lys$^{307}$ and Arg$^{311}$ with Ala, designated as FKBP8TPRmut, was coexpressed with HA-Hsp90 or HA-NS5A (Figure 8C). FKBP8TPRmut exhibited no interaction with Hsp90, but still retained the capability of binding to NS5A, indicating that FKBP8 interacts with Hsp90 and NS5A through the conserved two-carboxylate clamp residues and other region in the TPR domain, respectively.

### *Hsp90 participates in the replication of HCV RNA*

To examine the role of Hsp90 in the replication of HCV RNA, FKBP8TPRmut lacking the ability to bind to Hsp90 was

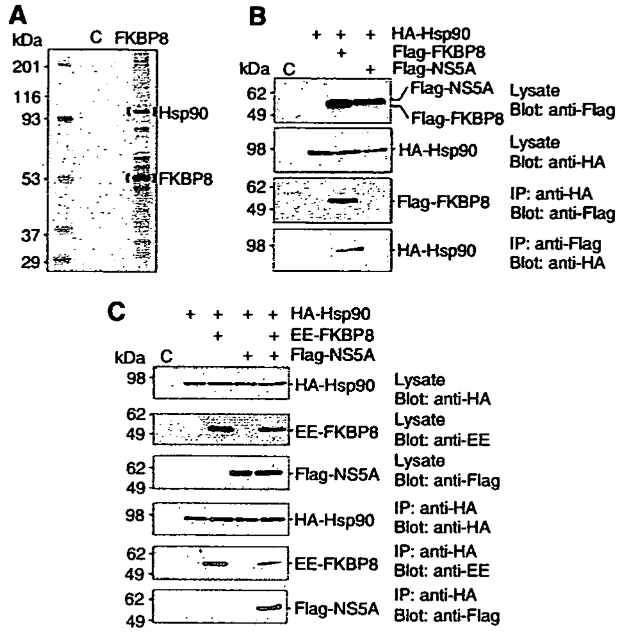

**Figure 7** FKBP8 forms complex with NS5A and Hsp90. (**A**) An N-terminally myc-TEV-Flag-tagged FKBP8 was expressed in 293T cells and immunoprecipitated. The precipitated proteins were applied to SDS–PAGE and then stained with silver staining. Hsp90 and FKBP8 were identified by LC-MS/MS. (**B**) HA-Hsp90 was coexpressed with Flag-FKBP8 or Flag-NS5A in 293T cells, and was immunoprecipitated by anti-HA or anti-Flag antibody. Precipitates were analyzed by Western blotting. (**C**) HA-Hsp90 was coexpressed with EE-FKBP8 and/or Flag-NS5A in 293T cells and was immunoprecipitated with anti-HA antibody. Precipitates were analyzed by Western blotting by anti-EE, -HA or -Flag antibody.

expressed in HCV replicon cells (Figure 8D). Expression of FKBP8TPRmut resulted in 30% reduction of HCV RNA replication, suggesting that FKBP8TPRmut works as a dominant negative. Geldanamycin is well known to bind to the ATP/ADP binding site of Hsp90 and specifically inhibits the enzymatic activity of Hsp90, resulting in the promotion of the degradation of client proteins for Hsp90 (Neckers, 2002). To determine the effects of Hsp90 inhibition induced by geldanamycin on the replication of HCV RNA, HCV replicon cells were treated with various concentrations of geldanamycin. Treatment with geldanamycin clearly reduced the levels of HCV RNA replication (Figure 8E); moreover, this treatment led to the slight suppression of NS5A without reducing the levels of FKBP8 expressed in the HCV replicon cells (Figure 8F). Although the inhibition of cleavage at the NS2/NS3 junction by geldanamycin has been demonstrated in both *in vitro* and *in vivo* assays (Waxman *et al*, 2001), the effects of geldanamycin on the replication of HCV RNA have not yet been examined in replicon cells. The HCV replicon cell line used in the present study does not contain an NS2-coding region, and NS2 has been shown to be unnecessary for the replication of HCV subgenomic replicon (Lohmann *et al*, 1999). Therefore, the observed reduction in RNA replication in the HCV replicon cells by treatment with geldanamycin was not due to an inhibition of HCV polyprotein processing. *In vitro* pull-down assays revealed that geldanamycin inhibited the binding of FKBP8 to Hsp90 and/or NS5A domain I (Supplementary Figure 4). Thus, geldanamycin may inhibit HCV replication by disruption of NS5A/FKBP8/Hsp90 complex. These results suggest that a protein complex composed of FKBP8, Hsp90, and NS5A is involved in HCV RNA replication.

## Discussion

HCV NS5A is a multifunctional protein involved in viral replication and pathogenesis (Macdonald and Harris, 2004). In this study, we demonstrated that NS5A specifically binds to FKBP8, but not to other homologous immunophilins such as FKBP52 and CypD, and that FKBP8 forms both a homomultimer and a heteromultimer with Hsp90. Mutation analyses of FKBP8 and Hsp90 suggest that FKBP8 intermediates between NS5A and Hsp90 via the different position in the TRP domain. FKBP8 has been shown to be expressed in several human tissues, including the liver (Lam *et al*, 1995); moreover, it has been demonstrated that FKBP8-knockout mice exhibit unusual morphological changes in brain development in the embryonic stage (Nielsen *et al*, 2004). However, the physiological function of FKBP8 has not been clarified to date.

Recently, the *in vitro* replication of the full-length HCV genome of genotype 2a (JFH1) isolated from an HCV-infected patient who developed fulminant hepatitis was reported (Lindenbach *et al*, 2005; Wakita *et al*, 2005; Zhong *et al*, 2005). Although binding of NS5A of the JFH1 clone to FKBP8 was weaker than that of genotypes 1a and 1b (Figure 2A), siRNA-mediated knockdown of FKBP8 impaired production of infectious HCV particles in JFH1 cell culture system (Figure 6D). In spite of a weaker interaction between FKBP8 and NS5A, these results suggest that FKBP8 is still required for HCV replication in the cell culture system of JFH1. The involvement of FKBP8 in mitochondria-mediated apoptosis remains controversial. Shirane and Nakayama (2003) reported that FKBP8 binds to Bcl-2 and that the Bcl-2/FKBP8 complex was sequestered in the mitochondria in order to suppress apoptosis. However, Edlich *et al* (2005) reported that FKBP8 binds to calmodulin via elevations in the calcium concentration, which in turn leads to the promotion of apoptosis in neuronal tissues. Knockdown of FKBP8 led to impaired HCV RNA replication, which was restored by the expression of an RNAi-resistant FKBP8 mutant. These results suggest that the impairment of HCV RNA replication induced by the knockdown of FKBP8 was not due to an induction of apoptosis, nor to any side effects of RNA transfection. The modulation of apoptosis by FKBP8 might be diverse in different tissue types and cell lines.

FKBP8 belongs to the FKBP family due to sequence similarity, but neither FK506 binding nor PPIase activity has been detected in the case of FKBP8 thus far (Lam *et al*, 1995). Apoptosis was induced in the SH-SY5Y neuroblastoma cell line by the treatment with mitochondria-mediated proapoptotic drugs, but was inhibited by the knockdown of FKBP8 and was enhanced by treatment with GPI1046, a nonimmunosuppressive FK506 derivative, whereas this result was not obtained with FK506 (Edlich *et al*, 2005). The inhibition constant of FKBP8 to FK506 was 50 times higher than that of FKBP12 to FK506 (Edlich *et al*, 2005), which suggests that the binding affinity of FKBP8 to FK506 is low. Furthermore, cyclosporin A, but not FK506, was shown to suppress HCV RNA replication via the interaction of NS5B with CypB

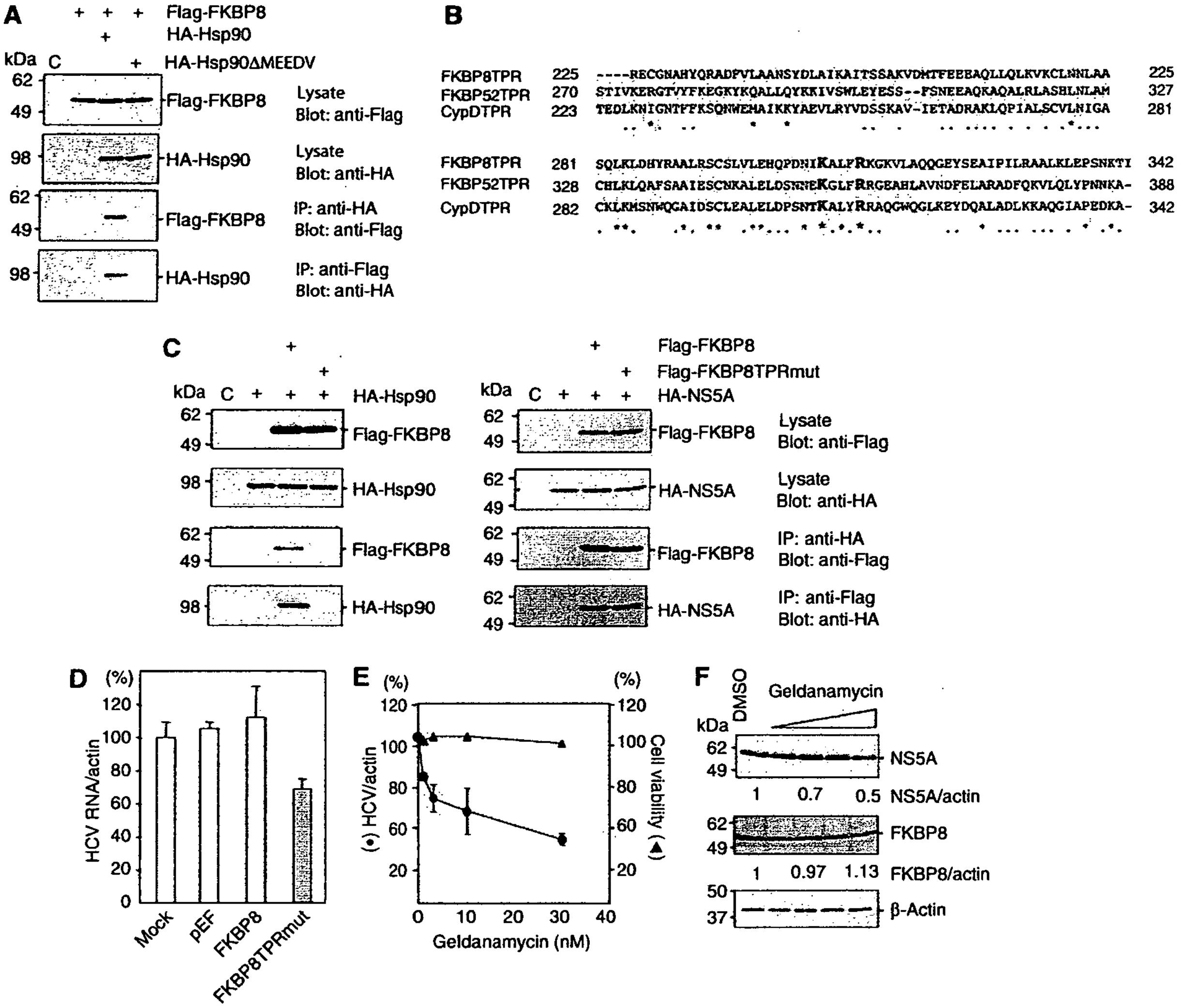

**Figure 8** FKBP8 interacts with NS5A and Hsp90 via different sites in the TPR domain and participates in HCV replication. (**A**) Flag-FKBP8 was coexpressed with HA-Hsp90 or HA-Hsp90ΔMEEDV lacking the C-terminal MEEDV residues and was immunoprecipitated by anti-HA or anti-Flag antibody. Precipitates were analyzed by Western blotting. (**B**) Sequence alignment of TPR domains of FKBP8, FKBP52, and CypD. The two bold characters (K and R) indicate amino-acid residues substituted to Ala in FKBP8TPRmut. (**C**) Flag-FKBP8 or Flag-FKBP8TPRmut substituted $\text{Lys}^{307}$ and $\text{Arg}^{311}$ to Ala was coexpressed with HA-Hsp90 (left) or HA-NS5A (right) in 293T cells, and was immunoprecipitated by anti-HA or anti-Flag antibody. Precipitates were analyzed by Western blotting. (**D**) Flag-FKBP8, Flag-FKBP8TPRmut, or empty plasmid was transfected into the replicon cells and HCV RNA was determined by real-time PCR after 48 h transfection. Relative replication was expressed as % increase after being normalized with the expression of β-actin mRNA. (**E**) The effect of geldanamycin on HCV RNA replication. HCV replicon cells (9–13 cells) were treated with 1, 3, 10, and 30 nM of geldanamycin and after 24 h treatment, HCV RNA replication was determined by real-time PCR. Relative replication was expressed as % replication after standardized by the expression of β-actin (closed circles). Cell viabilities were determined by trypan blue staining (closed triangles). (**F**) The effect of geldanamycin on the expression of NS5A and FKBP8. The replicon cells were examined by immunoblotting after treatment with various concentrations of geldanamycin. The data shown in each panel are representative of three independent experiments.

(Watashi *et al*, 2003, 2005). These results support the notion that FK506 preferentially binds to FKBP members other than FKBP8 *in vivo*, and that it does not participate in the inhibition of HCV replication.

Cellular and viral chaperones are implicated in the processing of viral proteins and viral assembly (Maggioni and Braakman, 2005; Mayer, 2005). The NS2 protein of bovine viral diarrhea virus (BVDV), a member of the *Flaviviridae* family as is HCV, exhibits autoprotease activity that leads to cleavage at the NS2 and NS3 junction (Lackner *et al*, 2005). A noncytopathogenic strain of BVDV is unable to cleave the NS2/3 junction in the absence of the interaction of a molecular chaperone, J-domain protein interacting with viral protein (Jiv); these previous findings suggest that Jiv is necessary for the replication of a noncytopathogenic strain of BVDV and is involved in the establishment of persistent infection (Lackner *et al*, 2005). Furthermore, FKBP52, which shares a high homology with FKBP8, was shown to regulate replication of adeno-associated virus type 2 by interacting with viral DNA (Qing *et al*, 2001). In this study, we demonstrated that HCV NS5A binds to FKBP8 and forms a complex with Hsp90. FKBP8 could directly bind to NS5A domain I *in vitro* (Supplementary Figure 1), suggesting that Hsp90 is not required for interaction between NS5A and FKBP8. FKBP52 forms a homodimer, binds to Hsp90 through TPR domain, and regulates chaperone activity of Hsp90 (Silverstein *et al*, 1999; Scheufler *et al*, 2000; Wu *et al*, 2004). FKBP8 may act as cochaperone of Hsp90 to regulate

HCV genome replication by interaction with NS5A. Hsp90 is a molecular chaperone that is highly expressed in most cell types in various organisms (Neckers, 2002). Here, Hsp90 was found to be able to bind to FKBP8 and form a complex with HCV NS5A. The suppression of NS5A, but not that of FKBP8, was observed in replicon cells treated with geldanamycin, thus suggesting that Hsp90 regulates the replication of HCV RNA via the interaction with FKBP8. It is well known that several host proteins such as VAPs and FBL2 interact with the HCV replication complex and regulate HCV RNA replication (Evans *et al*, 2004; Gao *et al*, 2004; Hamamoto *et al*, 2005; Wang *et al*, 2005). The TPR domain of FKBP8 is composed of 220 amino acids and is too long to determine the critical residues responsible for interaction with NS5A. Therefore, we tried to make a chimeric mutant carrying the TPR of FKBP52 to determine the critical amino-acid residues for binding to NS5A in FKBP8. However, expression of a chimeric FKBP8 possessing TPR of FKBP52 was much lower than the native form, suggesting that TPR domain is critical for stability and conformation of FKBP8. Amino-acid residues responsible for the binding to NS5A must be different from the two-carboxylate positions responsible for Hsp90 binding and locate within the TPR domain. The ternary complex consists of NS5A, FKBP8 and Hsp90 may be involved in the replication of HCV. FKBP52 possesses PPIase activity and chaperone activity in domain I (amino acids 1–148) and domain 3 (TPR domain, amino acids 264–400), respectively (Pirkl *et al*, 2001). Therefore, it is reasonable to speculate that the TPR domain is responsible for the chaperone activity of FKBP8, and that the FKBP8 and NS5A complex transports Hsp90 to the appropriate clients, including viral and host proteins, which in turn leads to the stabilization of the replication complex and the enhancement of HCV RNA replication.

In this study, we identified human FKBP8 as a binding partner of HCV NS5A. Our results suggest that the interaction between FKBP8 and HCV NS5A is essential for HCV replication. The NS5A protein forms a complex with FKBP8 and Hsp90, and an inhibitor of Hsp90 was shown to reduce the efficiency of HCV replication. The elucidation of the molecular mechanisms underlying the formation of the NS5A/FKBP8/Hsp90 complex may lead to the development of new therapeutics for chronic hepatitis C.

## Materials and methods

### *Yeast two-hybrid assays*

Screening for the gene-encoding host protein that interacts with HCV NS5A was performed with a yeast two-hybrid system, Matchmaker two-hybrid system 3 (Clontech, Palo Alto, CA), according to the manufacturer's protocol. Human fetal brain and liver libraries were purchased from Clontech. The cDNA of NS5A-encoding amino acids 1973–2419 of an HCV polyprotein of the J1 strain (genotype 1b) (Aizaki *et al*, 1998) was amplified by PCR and was cloned into the pGBKT7 vector (Clontech) (Tu *et al*, 1999; Hamamoto *et al*, 2005).

### *Plasmids*

DNA fragments encoding NS5A were amplified from HCV genotype 1b strains J1 and Con1 (provided by Dr Bartenschlager), genotype 1a strain H77C (provided by Dr Bukh), and genotype 2a strain JFH-1 (provided by Dr Wakita) by PCR using *Pfu* turbo DNA polymerase (Stratagene, La Jolla, CA). The fragments were cloned into pCAGGs-PUR/N-HA, in which the sequence encoding an HA tag is inserted at the 5′-terminus of the cloning site of pCAGGs-PUR (Niwa *et al*, 1991). The DNA fragment encoding human FKBP8 was amplified from the total cDNA of Huh7 cells by PCR, and this fragment was introduced into pEF-FLAG pGBK puro (Huang *et al*, 1997), pCAGGs-PUR/NHA, pcDNA3.1-N-HA (Tu *et al*, 1999; Hamamoto *et al*, 2005), and pcDNA3.1-N-EE, in which an Glu-Glu (EE) tag is inserted in the 5′-terminus of the cloning site of pcDNA3.1 (+) (Invitrogen, Carlsbad, CA). The DNA fragments encoding human Hsp90, FKBP52, and CypD were amplified from a human fetal brain library (Clontech) by PCR, and were introduced into pcDNA3.1-N-HA. The genes encoding the deletion mutants of human FKBP8 were amplified and cloned into pCAGGs-PUR/NHA. The gene encoding an FKBP8 mutant replaced Lys$^{307}$ and Arg$^{311}$ with Ala, designated as FKBP8TPRmut, was generated by the method of splicing by overlap extension and introduced into pEF-Flag pGBKpuro. The gene encoding an Hsp90 mutant lacking the C-terminal MEEVD motif of Hsp90, designated as Hsp90ΔMEEVD, was amplified and cloned into pcDNA3.1-N-HA. All PCR products were confirmed by sequencing by an ABI PRSM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan).

### *Cell lines*

Human embryonic kidney 293T cells and the human hepatoma cell lines Huh7 and FLC-4 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO) containing 10% fetal calf serum (FCS), whereas the Huh 9–13 cell line, which possesses an HCV subgenomic replicon (Lohmann *et al*, 1999), was cultured in DMEM supplemented with 10% FCS and 1 mg/ml G418. All cells were cultured at 37°C in a humidified atmosphere with 5% $CO_2$.

### *Antibodies*

Mouse monoclonal antibodies to the HA and EE tags were purchased from Covance (Richmond, CA). Anti-Flag mouse antibody M2, horseradish peroxidase-conjugated M2 antibody, and anti-β-actin mouse monoclonal antibody were purchased from Sigma. Mouse monoclonal antibody to NS5A was from Austral Biologicals (San Ramon, CA). Mouse monoclonal antibodies to NS4B and NS5B have been described previously (Kashiwagi *et al*, 2002). Rabbit polyclonal antibody to NS5A was prepared as described previously (Hamamoto *et al*, 2005). Rabbit polyclonal antibody to thioredoxin was described previously (Moriishi *et al*, 1999).

### *Transfection, immunoblotting, and immunoprecipitation*

The transfection and immunoprecipitation test were carried out by a previously described method (Hamamoto *et al*, 2005). The immunoprecipitates boiled in the loading buffer were subjected to 12.5% SDS–PAGE. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and were reacted with the appropriate antibodies. The immune complexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and they were detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan). The density of protein band was determined by using IMAGE-PRO PLUS 5.1 software (Media Cybernetics, Silver Springs, MD).

### *Gene silencing by siRNA*

The siRNA targeted to FKBP8, Target-1: 5′-GAGUGGCUGGACAUUCUGG-3′, and negative control siRNA, that is, siCONTROL Non-Targeting siRNA-2, were purchased from Dharmacon (Lafayette, CO). Target-2, 5′-UCCCAUGGAAGUGGCUGUU-3′, and Target-3, 5′-GACAACAUCAAGGCUCUCU-3′ were purchased from Qiagen (Tokyo, Japan). The Huh7 cells harboring a subgenomic HCV replicon grown on six-well plates were transfected with 80 or 160 nM of siRNA with siFECTOR (B-Bridge International, Sunnyvale, CA). The cells were grown in DMEM containing 10% FCS and were then harvested at 48 or 72 h post-transfection.

### *Real-time PCR*

Total RNA was prepared from cell lines by using RNeasy mini kit (Qiagen). First-strand cDNA was synthesized by using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Franklin Lakes, NJ) and random primers. Each cDNA was estimated by Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) according to the manufacturer's protocol. Fluorescent signals were analyzed by an ABI PRISM 7000 (Applied Biosystems). The HCV NS5A, human β-actin, and human FKBP8 genes were amplified using the primer pairs of 5′-AGTCAGTTGTCTGCGCTTTC-3′ and 5′-CGGGGAATTTCCTGGTCTTC-3′,

5′-TGGAGTCCTGTGGCATCCACGAAACTACCTTCAACTC-3′
and 5′-CGGACTCGTCATACTCCTGCTTGCTGATCCACATC-3′,
and 5′-GGCTGTTGAGGAAGAAGACG-3′
and 5′-CTTGGAGTCAGCAGTGACCA-3′, respectively. The FKBP8 primers are located at different exons in order to prevent the false-positive amplification of contaminated genomic DNA. The values of the HCV genome and FKBP8 mRNA were normalized with those of β-actin mRNA. Each PCR product was detected as a single band of the correct size upon agarose gel electrophoresis (data not shown).

### *Establishment of cell lines expressing an siRNA-resistant FKBP8 mutant and knockdown FKBP8 expression*

A, G, and T at nucleotides 273, 276, and 288 from the 5′ end of the open-reading frame of human FKBP8 were replaced with G, A, and C, respectively, according to a splicing method achieved by overlap extension; these silent mutations were then cloned into pEF-Flag pGBKpuro. The resulting plasmid encoding a mutant FKBP8 resistant to knockdown by siRNA was transfected into Huh7 cells harboring the HCV RNA replicon. The culture medium was replaced with DMEM supplemented with 10% FCS and 2 μg/ml of puromycin (Nakarai Tesque, Tokyo, Japan) at 24 h post-transfection, and the cells were cultured for 7 days. The surviving cells were used for the FKBP8 knockdown experiments. The shRNAs targeted to FKBP8, the target sequences of which were 5′-GATCCGCTGGAACCTTCCAACAAGTTCAAGAGACTTGTTGGAAGGTTCCAGCTTA-3′, and 5′-AGCTTAAGCTGGAACCTTCCAACAAGTCTCTTGAACTTGTTGGAAGGTTCCAGCG-3′, were annealed and introduced between the *Bam*HI and *Hin*dIII sites of pSilencer™ 2.1-U6 hygro (Ambion, Austin, TX) according to the manufacturer's protocol. An HCV replicon cell line cured with IFN-α was transfected with 5 μg of the plasmid by electroporation. The culture medium was replaced with DMEM supplemented with 10% FCS and 500 μg/ml of Hygromycin B (Wako, Tokyo, Japan) at 24 h post-transfection. The remaining cells were re-seeded in 98-well plates and cloned for the colony formation and transient replication assays.

### *Colony formation assay*

The plasmid pFK-$I_{389}$ neo/NS3-3′/NK5.1 (Pietschmann *et al*, 2002) was obtained from R Bartenschlager. The plasmid cleaved at the *Sca*I site was transcribed *in vitro* using the MEGAscript T7 kit (Ambion) according to the manufacturer's protocol. The linearized plasmid (10 μg) was introduced into Huh7 cells at 4 million cells/0.4 ml by electroporation at 270 V and 960 μF using a Gene Pulser™ (Bio-Rad, Hercules, CA). Electroporated cells were suspended at a final volume of 10 ml of culture medium. Three-milliliter aliquots of cell suspension were mixed with 7 ml of culture medium and then the cells were seeded on culture dishes (diameter: 10 cm). The culture medium was replaced with DMEM containing 10% FCS and 1 mg/ml of G418 (Nakarai Tesque) at 24 h post-transfection. The medium was exchanged weekly for fresh DMEM containing 10% FCS and 1 mg/ml G418. The remaining colonies were fixed with 4% paraformaldehyde at 4 weeks after electroporation, and the cells were stained with crystal violet.

### *Transient replication assay*

The cDNA encoding *Renilla* luciferase was introduced between the *Asc*I and *Pme*I sites of the plasmid pFK-$I_{389}$ neo/NS3-3′/NK5.1, in place of the *neo* gene. The resulting plasmid, pFK-$I_{389}$ hRL/NS3-3′/NK5.1, was cleaved with *Sca*I and was transcribed *in vitro* using a MEGAscript T7 kit (Ambion). Huh7 cells were suspended at 10 million cells/ml and the suspensions were mixed with 10 μg of *in vitro*-transcribed RNA at a 400-μl volume; the cells were then electroporated at 270 V and 960 μF by a Gene Pulser™ (Bio-Rad). The electroporated cells were suspended in 25 ml of culture medium and then were seeded at 1 ml/well on 12-well culture plates. Luciferase activity was measured at 4 and 48 h post-transfection using a *Renilla* Luciferase assay system (Promega, Madison, WI) according to the manufacturer's protocol. Luciferase activity at 4 h after electroporation was used to determine the transfection efficiency.

### *Generation of infectious HCV particles*

The viral RNA of JFH1 was introduced into Huh7.5.1 according to the method of Wakita *et al* (2005). The supernatant was collected at 7 days post-transfection and used as HCV particles that are infectious in cell culture (HCVcc). The naïve Huh7.5.1 cells were transfected with siRNA of nontarget control or FKBP8-Target 1 at a concentration of 80 nM. The siRNA-treated Huh7.5.1 cells were inoculated with HCVcc at 24 h post-transfection. Infected cells and culture supernatants were harvested every day until 5 days post-infection.

### *Determination of FKBP8-binding proteins*

MEF purification was carried out by a previously described method (Ichimura *et al*, 2005). The FKBP8 gene was amplified by PCR and introduced into pcDNA3.1 encoding the myc-TEV-Flag epitope tag (Ichimura *et al*, 2005). The resulting plasmid was transfected into 293T cells, which were then subjected to MEF purification. FKBP8-binding proteins were separated by SDS–PAGE and visualized by silver staining. The stained bands were excised, digested in gels with Lys-C, and analyzed by the direct nanoflow LC-MS/MS system (Ichimura *et al*, 2005).

### *Supplementary data*

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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# Critical role of PA28$\gamma$ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis

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**Hepatitis C virus (HCV) is a major cause of chronic liver disease that frequently leads to steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC). HCV core protein is not only a component of viral particles but also a multifunctional protein because liver steatosis and HCC are developed in HCV core gene-transgenic (CoreTg) mice. Proteasome activator PA28$\gamma$/REG$\gamma$ regulates host and viral proteins such as nuclear hormone receptors and HCV core protein. Here we show that a knockout of the PA28$\gamma$ gene induces the accumulation of HCV core protein in the nucleus of hepatocytes of CoreTg mice and disrupts development of both hepatic steatosis and HCC. Furthermore, the genes related to fatty acid biosynthesis and *srebp-1c* promoter activity were up-regulated by HCV core protein in the cell line and the mouse liver in a PA28$\gamma$-dependent manner. Heterodimer composed of liver X receptor $\alpha$ (LXR$\alpha$) and retinoid X receptor $\alpha$ (RXR$\alpha$) is known to up-regulate *srebp-1c* promoter activity. Our data also show that HCV core protein enhances the binding of LXR$\alpha$/RXR$\alpha$ to LXR-response element in the presence but not the absence of PA28$\gamma$. These findings suggest that PA28$\gamma$ plays a crucial role in the development of liver pathology induced by HCV infection.**

fatty acid | proteasome | sterol regulatory element-binding protein (SREBP) | RXR$\alpha$ | LXR$\alpha$

Hepatitis C virus (HCV) belongs to the Flaviviridae family, and it possesses a positive, single-stranded RNA genome that encodes a single polyprotein composed of $\approx$3,000 aa. The HCV polyprotein is processed by host and viral proteases, resulting in 10 viral proteins. Viral structural proteins, including the capsid (core) protein and two envelope proteins, are located in the N-terminal one-third of the polyprotein, followed by nonstructural proteins.

HCV infects $>$170 million individuals worldwide, and then it causes liver disease, including hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC) (1). The prevalence of fatty infiltration in the livers of chronic hepatitis C patients has been reported to average $\approx$50% (2, 3), which is higher than the percentage in patients infected with hepatitis B virus and other liver diseases. However, the precise functions of HCV proteins in the development of fatty liver remain unknown because of the lack of a system sufficient to investigate the pathogenesis of HCV. HCV core protein expression has been shown to induce lipid droplets in cell lines and hepatic steatosis and HCC in transgenic mice (4–6). These reports suggest that HCV core protein plays an important role in the development of various types of liver failure, including steatosis and HCC.

Recent reports suggest that lipid biosynthesis affects HCV replication (7–9). Involvement of a geranylgeranylated host protein, FBL2, in HCV replication through the interaction with NS5A suggests that the cholesterol biosynthesis pathway is also important for HCV replication (9). Increases in saturated and monounsaturated fatty acids enhance HCV RNA replication, whereas increases in polyunsaturated fatty acids suppress it (7). Lipid homeostasis is regulated by a family of steroid regulatory element-binding proteins (SREBPs), which activate the expression of $>$30 genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids. Biosynthesis of cholesterol is regulated by SREBP-2, whereas that of fatty acids, triglycerides, and phospholipids is regulated by SREBP-1c (10–14). In chimpanzees, host genes involved in SREBP signaling are induced during the early stages of HCV infection (8). SREBP-1c regulates the transcription of acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase, leading to the production of saturated and monounsaturated fatty acids and triglycerides (15). SREBP-1c is transcriptionally regulated by liver X receptor (LXR) $\alpha$ and retinoid X receptor (RXR) $\alpha$, which belong to a family of nuclear hormone receptors (15, 16). Accumulation of cellular fatty acids by HCV core protein is expected to be modulated by the SREBP-1c pathway because RXR$\alpha$ is activated by HCV core protein (17). However, it remains unknown whether HCV core protein regulates the *srebp-1c* promoter.

We previously reported (18) that HCV core protein specifically binds to the proteasome activator PA28$\gamma$/REG$\gamma$ in the nucleus and is degraded through a PA28$\gamma$-dependent pathway. PA28$\gamma$ is well conserved from invertebrates to vertebrates, and amino acid sequences of human and murine PA28$\gamma$s are identical (19). The homologous proteins, PA28$\alpha$ and PA28$\beta$, form a heteroheptamer in the cytoplasm, and they activate chymotrypsin-like peptidase activity of the 20S proteasome, whereas PA28$\gamma$ forms a homoheptamer in the nucleus, and it enhances trypsin-like peptidase activity of 20S proteasome (20). Recently, Li and colleagues (21) reported that PA28$\gamma$ binds to steroid receptor coactivator-3 (SRC-3) and enhances the degradation of SRC-3 in a ubiquitin- and ATP-independent manner. However, the precise physiological functions of PA28$\gamma$ are largely unknown *in vivo*. In this work, we examine whether PA28$\gamma$ is required for liver pathology induced by HCV core protein *in vivo*.

## Results

**PA28$\gamma$-Knockout HCV Core Gene Transgenic Mice.** To determine the role of PA28$\gamma$ in HCV core-induced steatosis and the development of HCC *in vivo*, we prepared PA28$\gamma$-knockout core gene transgenic mice. The PA28$\gamma$-deficient, PA28$\gamma^{-/-}$ mice were born without

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Author contributions: K. Moriishi, K.T., T.M., T.S., K.K., and Y. Matsuura designed research; K. Moriishi, R.M., K. Moriya, H.M., Y. Mori, and T.A. performed research; S.M. contributed new reagents/analytic tools; Y. Matsuura analyzed data; and K. Moriishi, K.K., and Y. Matsuura wrote the paper.

The authors declare no conflict of interest.

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Abbreviations: CoreTg, HCV core gene-transgenic; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LXR, liver X receptor; LXRE, liver X receptor-response element; MEF, mouse embryonic fibroblast; ROS, reactive oxygen species; RXR, retinoid X receptor; SRC-3, steroid receptor coactivator-3; SREBP, steroid regulatory element-binding protein.

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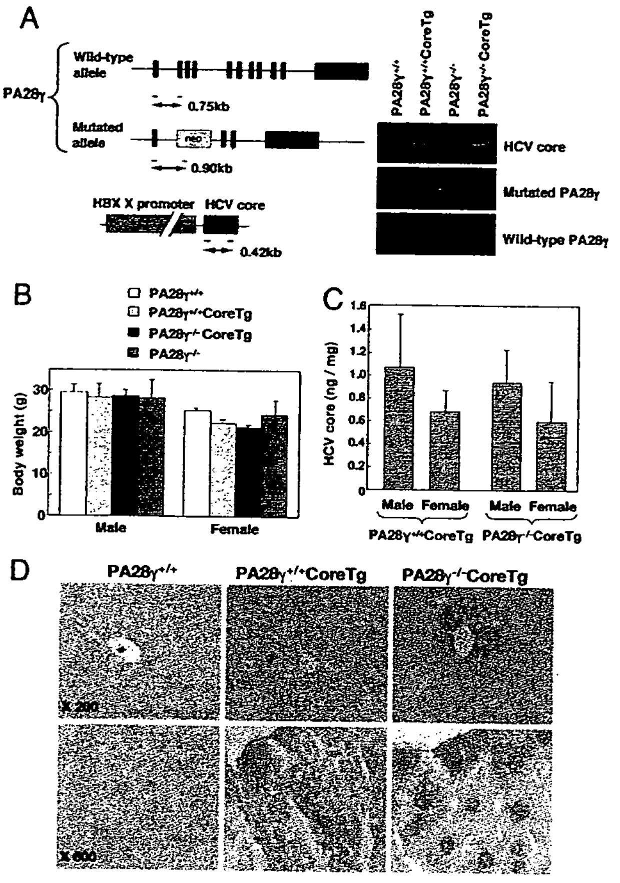

**Fig. 1.** Preparation and characterization of PA28γ-knockout HCV core-transgenic mice. (*A*) The structures of the wild-type and mutated PA28γ genes and the transgene encoding the HCV core protein under the control of the HBV X promoter were investigated. Positions corresponding to the screening primers and sizes of PCR products are shown. PCR products of the HCV core gene as well as wild-type and mutated PA28γ alleles were amplified from the genomic DNAs of PA28$\gamma^{+/+}$, PA28$\gamma^{+/+}$CoreTg, PA28$\gamma^{-/-}$, and PA28$\gamma^{-/-}$CoreTg mice. (*B*) Body weights of PA28$\gamma^{+/+}$, PA28$\gamma^{+/+}$CoreTg, PA28$\gamma^{-/-}$CoreTg, and PA28$\gamma^{-/-}$ mice at the age of 6 months. (*C*) HCV core protein levels in the livers of PA28$\gamma^{+/+}$CoreTg and PA28$\gamma^{-/-}$CoreTg mice were determined by ELISA (mean ± SD, *n* = 10). (*D*) Localization of HCV core protein in the liver. Liver sections of PA28$\gamma^{+/+}$, PA28$\gamma^{+/+}$CoreTg, and PA28$\gamma^{-/-}$CoreTg mice at the age of 2 months were stained with anti-HCV core antibody.

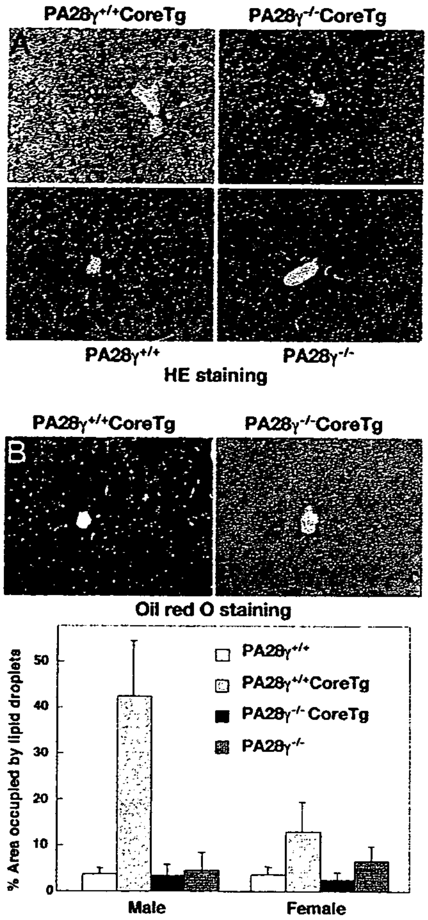

**Fig. 2.** Accumulation of lipid droplets by expression of HCV core protein. (*A*) Liver sections of the mice at the age of 6 months were stained with hematoxylin/eosin (HE). (*B*) (*Upper*) Liver sections of PA28$\gamma^{+/+}$CoreTg and PA28$\gamma^{-/-}$CoreTg mice at the age of 6 months were stained with oil red O. (*Lower*) The area occupied by lipid droplets of PA28$\gamma^{+/+}$ (white), PA28$\gamma^{+/+}$CoreTg (gray), PA28$\gamma^{-/-}$CoreTg (black), and PA28$\gamma^{-/-}$ (dark gray) mice was calculated by Image-Pro software (MediaCybernetics, Silver Spring, MD) (mean ± SD, *n* = 10).

appreciable abnormalities in all tissues examined, with the exception of a slight retardation of growth (22). HCV core gene-transgenic (PA28$\gamma^{+/+}$CoreTg) mice were bred with PA28$\gamma^{-/-}$ mice to create PA28$\gamma^{+/-}$CoreTg mice. The PA28$\gamma^{+/-}$CoreTg offspring were bred with each other, and PA28$\gamma^{-/-}$CoreTg mice were selected by PCR using primers specific to the target sequences (Fig. 1*A*). No significant differences in body weight were observed among the 6-month-old mice, although PA28$\gamma^{-/-}$ mice exhibited a slight retardation of growth (Fig. 1*B*). A similar level of PA28γ expression was detected in PA28$\gamma^{+/+}$CoreTg and PA28$\gamma^{+/+}$ mice (see Fig. 5*B*). The expression levels and molecular size of HCV core protein were similar in the livers of PA28$\gamma^{+/+}$CoreTg and PA28$\gamma^{-/-}$CoreTg mice (Fig. 1*C*; see also Fig. 5*B*).

**PA28γ Is Required for Degradation of HCV Core Protein in the Nucleus and Induction of Liver Steatosis.** HCV core protein has been detected at various sites, such as the endoplasmic reticulum, mitochondria, lipid droplets, and nucleus of cultured cell lines, as well as in hepatocytes of PA28$\gamma^{+/+}$CoreTg mice and hepatitis C patients (6, 23, 24). Although HCV core protein is predominantly detected in the cytoplasm of the liver cells of PA28$\gamma^{+/+}$CoreTg mice, as reported in ref. 6, in the present study a clear accumulation of HCV core protein was observed in the liver cell nuclei of PA28$\gamma^{-/-}$CoreTg mice (Fig. 1*D*). These findings clearly indicate that at least some fraction of the HCV core protein is translocated into the nucleus and is degraded through a PA28γ-dependent pathway. Mild vacuolation was observed in the cytoplasm of the liver cells of 4-month-old PA28$\gamma^{+/+}$CoreTg mice, and it became more severe at 6 months, as reported in ref. 25. Hematoxylin/eosin-stained liver sections of 6-month-old PA28$\gamma^{+/+}$CoreTg mice exhibited severe vacuolating lesions (Fig. 2*A*), which were clearly stained with oil red O (Fig. 2*B Upper*), whereas no such lesions were detected in the livers of PA28$\gamma^{-/-}$CoreTg, PA28$\gamma^{+/+}$, or PA28$\gamma^{-/-}$ mice at the same age. The areas occupied by the lipid droplets in the PA28$\gamma^{+/+}$CoreTg mouse livers were ≈10 and 2–4 times larger than those of male and female of PA28$\gamma^{+/+}$, PA28$\gamma^{-/-}$, and PA28$\gamma^{-/-}$CoreTg mice, respectively (Fig. 2*B Lower*). These results suggest that PA28γ is required for the induction of liver steatosis by HCV core protein in mice.

**PA28γ Is Required for the Up-Regulation of SREBP-1c Transcription by HCV Core Protein in the Mouse Liver.** To clarify the effects of a knockout of the PA28γ gene in PA28$\gamma^{+/+}$CoreTg mice on lipid

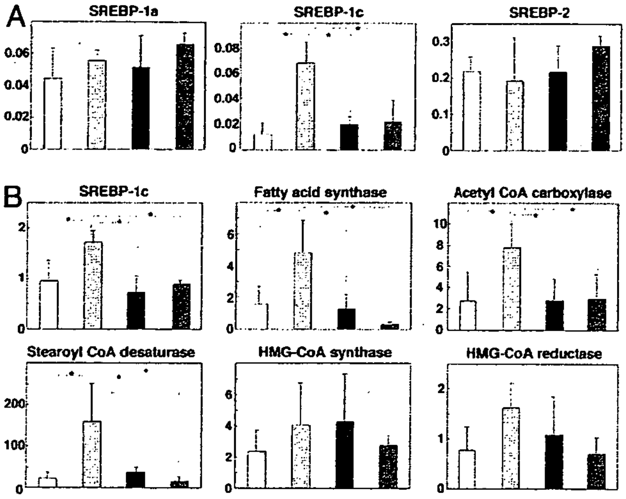

**Fig. 3.** Transcription of genes regulating lipid biosynthesis in the mouse liver. (*A*) Total RNA was prepared from the livers of 2-month-old mice; and the transcription of genes encoding SREBP-1a, SREBP-1c, and SREBP-2 was determined by real-time PCR. (*B*) The transcription of genes encoding SREBP-1c, fatty acid synthase, acetyl-CoA carboxylase, stearoyl-CoA desaturase, HMG-CoA synthase, and HMG-CoA reductase of 6-month-old mice was measured by real-time PCR. The transcription of the genes was normalized with that of hypoxanthine phosphoribosyltransferase, and the values are expressed as relative activity ($n = 5$; *, $P < 0.05$; **, $P < 0.01$). The transcription of each gene in PA28$\gamma^{+/+}$, PA28$\gamma^{+/+}$CoreTg, PA28$\gamma^{-/-}$CoreTg, and PA28$\gamma^{-/-}$ mice is indicated by white, gray, black, and dark gray bars, respectively.

metabolism, genes related to the lipid biosyntheses were examined by real-time quantitative PCR. Transcription of SREBP-1c was higher in the livers of PA28$\gamma^{+/+}$CoreTg mice than in those of PA28$\gamma^{+/+}$, PA28$\gamma^{-/-}$, and PA28$\gamma^{-/-}$CoreTg mice at 2 months of age, but no such increases in SREBP-2 and SREBP-1a were observed (Fig. 3*A*). Although transcription of SREBP-1c and its regulating enzymes, such as acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase, was also enhanced in the livers of 6-month-old PA28$\gamma^{+/+}$CoreTg mice compared with the levels in the livers of PA28$\gamma^{+/+}$, PA28$\gamma^{-/-}$, and PA28$\gamma^{-/-}$CoreTg mice, no statistically significant differences were observed with respect to the transcription levels of cholesterol biosynthesis-related genes that are regulated by SREBP-2 (e.g., HMG-CoA synthase and HMG-CoA reductase) (Fig. 3*B*). These results suggest the following: (*i*) the up-regulation of SREBP-1c transcription in the livers of mice requires both HCV core protein and PA28γ; and (*ii*) the nuclear accumulation of HCV core protein alone, which occurs because of the lack of degradation along a PA28γ-dependent proteasome pathway, does not activate the *srebp-1c* promoter.

**HCV Core Protein Indirectly Potentiates *srebp-1c* Promoter Activity in an LXRα/RXRα-Dependent Manner.** LXRα, which is primarily expressed in the liver, forms a complex with RXRα and synergistically potentiates *srebp-1c* promoter activity (16). Activation of RXRα by HCV core protein suggests that cellular fatty acid synthesis is modulated by the SREBP-1c pathway, although HCV core protein was not included in the transcription factor complex in the electrophoresis mobility shift assay (EMSA) (17). To analyze the effect of HCV core protein and PA28γ on the activation of the *srebp-1c* promoter, we first examined the effect of HCV core protein on the binding of the LXRα/RXRα complex to the LXR-response element (LXRE) located upstream of the SREBP-1c gene (Fig. 4*A*). Although a weak shift of the labeled LXRE probe was observed by incubation with nuclear extracts prepared from 293T cells expressing FLAG-tagged LXRα and HA-tagged RXRα, a clear shift was obtained by the treatment of cells with 9-*cis*-retinoic acid and 22(*R*)-hydroxylcholesterol, ligands for LXRα and RXRα, respectively. In contrast, coexpression of HCV core protein with LXRα and RXRα potentiated the shift of the probe irrespective of the treatment with the ligands. Addition of 500 times the amount of nonlabeled LXRE probe (competitor) diminished the shift of the labeled probe induced by the ligands and/or HCV core protein. Furthermore, coincubation of the nuclear fraction with antibody to FLAG or HA tag but not with antibody to either HCV core or PA28γ caused a supershift of the labeled probe. These results indicate that HCV core protein does not participate in the LXRα/RXRα–LXRE complex but indirectly enhances the binding of LXRα/RXRα to the LXRE.

The activity of the *srebp-1c* promoter was enhanced by the expression of HCV core protein in 293T cells, and it was further enhanced by coexpression of LXRα/RXRα (Fig. 4*B*). Enhancement of the *srebp-1c* promoter by coexpression of HCV core protein and LXRα/RXRα was further potentiated by treatment with the ligands for LXRα and RXRα. The cells treated with 9-*cis*-retinoic acid exhibited more potent enhancement of the *srebp-1c* promoter than those treated with 22(*R*)-hydroxylcholesterol. HCV core protein exhibited more potent enhancement of the *srebp-1c* promoter in cells treated with both ligands than in those treated with either ligand alone. These results suggest that HCV core protein poten-

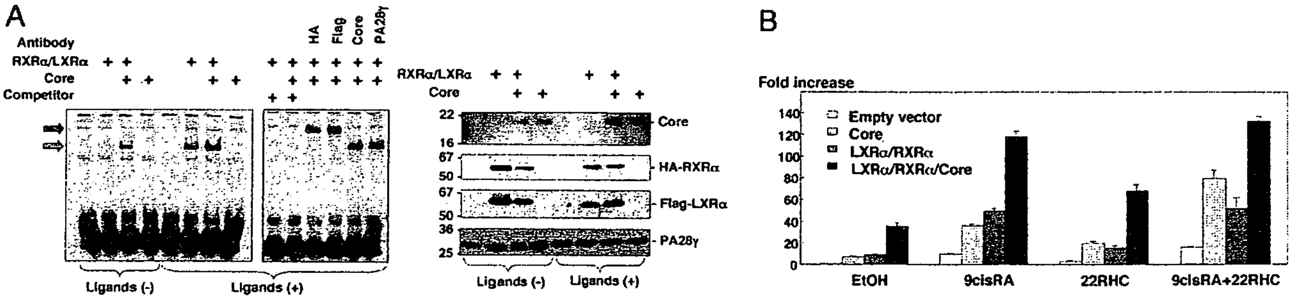

**Fig. 4.** Activation of the *srebp-1c* promoter by HCV core protein. (*A*) FLAG-LXRα and HA-RXRα were expressed in 293T cells together with or without HCV core protein. Ligands for LXRα and RXRα dissolved in ethanol [Ligands (+)] or ethanol alone [Ligands (−)] were added to the culture supernatant at 24 h posttransfection. Cells were harvested at 48 h posttransfection, and nuclear extracts were mixed with the reaction buffer for EMSA in the presence or absence of antibody (100 ng) against HA, FLAG, HCV core or PA28γ, or nonlabeled LXRE probe (Competitor). (*Left*) The resulting mixtures were subjected to PAGE and blotted with horseradish peroxidase/streptavidin. The mobility shift of the LXRE probe and its supershift are indicated by a gray and black arrow, respectively. (*Right*) Expression of HCV core, HA-RXRα, FLAG-LXRα, and PA28γ in cells was detected by immunoblotting. (*B*) Effects of ligands for RXRα, 9-*cis*-retinoic acid (9cisRA), and for LXRα, 22(*R*)-hydroxylcholesterol (22RHC), on the activation of the *srebp-1c* promoter in 293T cells expressing RXRα, LXRα, and/or HCV core protein. Ligands were added into the medium at 24 h posttransfection at a concentration of 5 μM, and the cells were harvested after 24 h of incubation.

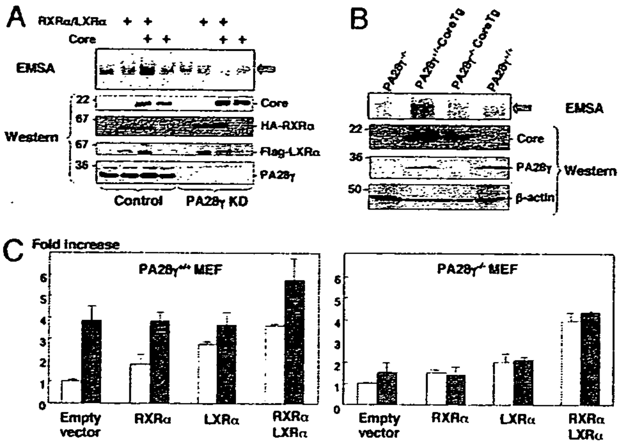

**Fig. 5.** PA28γ is required for HCV core-dependent activation of the *srebp-1c* promoter. (*A*) Effect of PA28γ knockdown on the LXRα/RXRα–DNA complex. FLAG-LXRα and HA-RXRα were expressed in FLC4 (control) or PA28γ-knockdown (PA28γ KD) cells together with or without HCV core protein. Cells were harvested at 48 h posttransfection, and nuclear extracts were mixed with the reaction buffer for EMSA. (*Upper*) The resulting mixtures were subjected to PAGE and blotted with horseradish peroxidase-streptavidin. The mobility shift of the LXRE probe is indicated by an arrow. (*Lower*) Expression of HCV core, HA-RXRα, FLAG-LXRα, and PA28γ in cells was detected by immunoblotting. (*B*) Effect of PA28γ knockout on the LXRα/RXRα–DNA complex in the mouse liver. (*Upper*) Nuclear extracts were prepared from the livers of 2-month-old PA28$\gamma^{-/-}$, PA28$\gamma^{+/+}$CoreTg, PA28$\gamma^{-/-}$CoreTg, and PA28$\gamma^{+/+}$ mice and subjected to EMSA. The mobility shift of the LXRE probe is indicated by an arrow. (*Lower*) The expression of HCV core, PA28γ, and β-actin in the livers of the mice was detected by immunoblotting. (*C*) Effect of HCV core protein on *srebp-1* promoter activity in PA28γ-knockout fibroblasts. A plasmid encoding firefly luciferase under the control of the *srebp-1c* promoter was transfected into MEFs prepared from PA28$\gamma^{+/+}$ (*Left*) or PA28$\gamma^{-/-}$ (*Right*) mice together with a plasmid encoding a *Renilla* luciferase. An empty plasmid or plasmids encoding mouse RXRα or LXRα were also cotransfected into the cells together with (gray bars) or without (white bars) a plasmid encoding HCV core protein. Luciferase activity under the control of the *srebp-1c* promoter was determined, and it is expressed as the fold increase in relative luciferase activity after standardization with the activity of *Renilla* luciferase.

tiates *srebp-1c* promoter activity in an LXRα/RXRα-dependent manner.

**HCV Core Protein Activates the *srepb-1c* Promoter in an LXRα/RXRα- and PA28γ-Dependent Manner.** To examine whether PA28γ is required for HCV core-induced enhancement of *srebp-1c* promoter activity in human liver cells, a PA28γ-knockdown human hepatoma cell line (FLC4 KD) was prepared. Enhancement of binding of the LXRE probe to LXRα/RXRα by coexpression of HCV core protein and LXRα/RXRα in FLC4 cells was diminished by knockdown of the PA28γ gene (Fig. 5*A*). Furthermore, formation of the LXRα/RXRα–LXRE complex was enhanced in the livers of PA28$\gamma^{+/+}$CoreTg mice but not in those of PA28$\gamma^{-/-}$, PA28$\gamma^{+/+}$, or PA28$\gamma^{-/-}$CoreTg mice (Fig. 5*B*). The expression of the HCV core protein in the mouse embryonic fibroblasts (MEFs) of PA28$\gamma^{+/+}$ mice induced the activation of the mouse *srebp-1c* promoter through the endogenous expression of LXRα and RXRα (Fig. 5*C Left*). Further enhancement of the activation of the *srebp-1c* promoter by HCV core protein in PA28$\gamma^{+/+}$ MEFs was achieved by the exogenous expression of both LXRα and RXRα. However, no enhancing effect of HCV core protein on *srebp-1c* promoter activity was observed in PA28$\gamma^{-/-}$ MEFs (Fig. 5*C Right*). These results support the notion that HCV core protein enhances the activity of the *srepb-1c* promoter in an LXRα/RXRα- and PA28γ-dependent manner.

**Table 1. HCC in mice at 16–18 months of age**

| Mouse and sex | Total no. of mice | No. of mice developing HCC | Incidence, % |
|---|---|---|---|
| PA28$\gamma^{+/+}$CoreTg | | | |
| Male | 17 | 5 | 29.4 |
| Female | 28 | 3 | 10.7 |
| PA28$\gamma^{+/-}$ | | | |
| Male | 16 | 0 | 0 |
| Female | 4 | 0 | 0 |
| PA28$\gamma^{-/-}$ | | | |
| Male | 23 | 0 | 0 |
| Female | 13 | 0 | 0 |
| PA28$\gamma^{-/-}$CoreTg | | | |
| Male | 15 | 0 | 0 |
| Female | 21 | 0 | 0 |

**PA28γ Plays a Crucial Role in the Development of HCC in PA28$\gamma^{+/+}$ CoreTg Mice.** The incidence of hepatic tumors in male PA28$\gamma^{+/+}$ CoreTg mice older than 16 months was significantly higher than that in age-matched female PA28$\gamma^{+/+}$CoreTg mice (6). We reconfirmed here that the incidence of HCC in male and female PA28$\gamma^{+/+}$CoreTg mice at 16–18 months of age was 29.4% (5 of 17 mice) and 10.7% (3 of 28 mice), respectively. To our surprise, however, no HCC developed in PA28$\gamma^{-/-}$CoreTg mice (males, 15; females, 21), although, as expected, no HCC was observed in PA28$\gamma^{+/-}$ (males, 16; females, 4) and PA28$\gamma^{-/-}$ mice (males, 23; females, 13) (Table 1). These results clearly indicate that PA28γ plays an indispensable role in the development of HCC induced by HCV core protein.

## Discussion

HCV core protein is detected in the cytoplasm and partially in the nucleus and mitochondria of culture cells and hepatocytes of transgenic mice and hepatitis C patients (6, 23, 24, 26). Degradation of HCV core protein was enhanced by deletion of the C-terminal transmembrane region through a ubiquitin/proteasome-dependent pathway (27). We previously reported (18) that PA28γ binds directly to HCV core protein and then enhances degradation of HCV core protein in the nucleus through a proteasome-dependent pathway because HCV core protein was accumulated in nucleus of human cell line by treatment with proteasome inhibitor MG132. In this work, accumulation of HCV core protein was observed in nucleus of hepatocytes of PA28$\gamma^{-/-}$CoreTg mice (Fig. 1*D*). This result directly demonstrates that HCV core protein migrates into the nucleus and is degraded through a PA28γ-dependent pathway. However, HCV core protein accumulated in the nucleus because knockout of PA28γ gene abrogated the ability to cause liver pathology, suggesting that interaction of HCV core protein with PA28γ in the nucleus is prerequisite for the liver pathology induced by HCV core protein. We have previously shown (18) that HCV core protein is degraded through a PA28γ-dependent pathway, and Minami *et al.* (28) reported that PA28γ has a cochaperone activity with Hsp90. Therefore, degradation products of HCV core protein by means of PA28γ-dependent processing or correct folding of HCV core protein through cochaperone activity of PA28γ might be involved in the development of liver pathology. We do not know the reason why knockout of the PA28γ gene does not affect the total amount of HCV core protein in the liver of the transgenic mice. PA28γ-dependent degradation of HCV core protein may be independent of ubiquitination, as shown in SRC-3 (21), whereas knockdown of PA28γ in a human hepatoma cell line enhanced the ubiquitination of HCV core protein [supporting information (SI) Fig. 6], suggesting that lack of PA28γ suppresses a ubiquitin-independent degradation but enhances a ubiquitin-dependent degradation of HCV core protein. Therefore, the total amount of HCV core protein in the liver of the mice may be unaffected by the knockout of the PA28γ gene.

Our results suggest that the interaction of HCV core protein with PA28γ leads to the activation of the *srebp-1c* promoter along an LXRα/RXRα-dependent pathway and the development of liver steatosis and HCC. HCV core protein was not included in the LXRα/RXRα–LXRE complex (Fig. 3*A*), suggesting that HCV core protein indirectly activates the *srebp-1c* promoter. Cytoplasmic HCV core protein was shown to interact with Sp110b, which is a transcriptional corepressor of RARα-dependent transcription, and this interaction leads to the sequestering of Sp110b in the cytoplasm, resulting in the activation of RARα-dependent transcription (29). The sequestration of an unidentified corepressor of the LXRα/RXRα heterodimer in the cytoplasm by HCV core protein may also contribute to the activation of the *srebp-1c* promoter. Although the precise physiological function of PA28γ-proteasome activity in the nucleus is not known, PA28γ has previously been shown (21) to regulate nuclear hormone receptors by means of the degradation of its coactivator SRC-3 and to participate in the fully Hsp90-dependent protein refolding (28). It appears reasonable to speculate that degradation or refolding of HCV core protein in a PA28γ-dependent pathway might be involved in the modulation of transcriptional regulators of various promoters, including the *srebp-1c* promoter. Saturated or monounsaturated fatty acids have been shown to enhance HCV RNA replication in Huh7 cells containing the full-length HCV replicon (7). The up-regulation of fatty acid biosynthesis by HCV core protein may also contribute to the efficient replication of HCV and to the progression of HCV pathogenesis.

Expression of HCV core protein was reported to enhance production of reactive oxygen species (ROS) (30), which leads to carbonylation of intracellular proteins (31). Enhancement of ROS production may trigger double-stranded DNA breaks and result in the development of HCC (30, 32, 33). HCV core protein could enhance the protein carbonylation in the liver of the transgenic mice in the presence but not in the absence of PA28γ (SI Fig. 7), suggesting that PA28γ is required for ROS production induced by HCV core protein. Development of HCC was observed in PA28γ$^{+/+}$CoreTg mice but not in PA28γ$^{-/-}$CoreTg mice (Table 1). Enhancement of ROS production by HCV core protein in the presence of PA28γ might be involved in the development of HCC in PA28γ$^{+/+}$CoreTg mice.

It is well known that resistant viruses readily emerge during the treatment with antiviral drugs targeting the viral protease or replicase, especially in the case of infection with RNA viruses. Therefore, antivirals targeting the host factors that are indispensable for the propagation of viruses might be an ideal target for the development of antiviral agents because of a lower rate of mutation than that of viral genome, if they have no side effects to patients. Importantly, the amino acid sequence of PA28γ of mice is identical to that of human, and mouse PA28γ is dispensable because PA28γ knockout mice exhibit no abnormal phenotype except for mild growth retardation. Therefore, PA28γ might be a promising target for an antiviral treatment of chronic hepatitis C with negligible side effects.

In summary, we observed that a knockout of the PA28γ gene from PA28γ$^{+/+}$CoreTg mice induced the accumulation of HCV core protein in the nucleus and disrupted the development of both steatosis and HCC. Activation of the *srebp-1c* promoter was up-regulated by HCV core protein both *in vitro* and *in vivo* through a PA28γ-dependent pathway, suggesting that PA28γ plays a crucial role in the development of liver pathology induced by HCV infection.

## Materials and Methods

Histology and immunohistochemistry, real-time PCR, and detection of proteins modified by ROS are discussed in *SI Materials and Methods*.

**Plasmids and Reagents.** Human PA28γ cDNA was isolated from a human fetal brain library (18). The gene encoding HCV core protein was amplified from HCV strain J1 (genotype 1b) (34) and cloned into pCAG-GS (35). Mouse cDNAs of RXRα and LXRα were amplified by PCR from the total cDNAs of the mouse liver. The RXRα and LXRα genes were introduced into pEF-FLAGGspGBK (36) and pcDNA3.1 (Invitrogen, Carlsbad, CA), respectively. The targeting fragment for human PA28γ knockdown (GGATCCGGTGGATCAGGAAGTGAAGTTCAAGAGACTTCACTTCCTGATCCACCTTTTTTGGAAAAGCTT) was introduced into the BamHI and HindIII sites of pSilencer 4.1 U6 hygro vector (Ambion, Austin, TX). Mouse anti-FLAG (M2) and mouse anti-β-actin antibodies were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antibody against synthetic peptides corresponding to amino acids 70–85 of PA28γ was obtained from AFFINITI (Exeter, U.K.). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgGs were purchased from ICN Pharmaceuticals (Aurora, OH). Rabbit anti-HCV core protein was prepared by immunization with recombinant HCV core protein (amino acids 1-71), as described in ref. 24. Mouse monoclonal antibody to HCV core protein was kindly provided by S. Yagi (37). The plasmid for expression of HA-tagged ubiquitin was described in ref. 27.

**Preparation of PA28γ-Knockout HCV CoreTg Mice.** The generation of C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b line C49 and that of PA28γ$^{-/-}$ mice have been reported previously (22, 25). Both strains were crossbred with each other to create PA28γ$^{-/-}$CoreTg mice. PA28γ$^{-/-}$CoreTg mice were identified by PCR targeted at the PA28γ or HCV core gene (22, 25). Using 1 μg of genomic DNA obtained from the mouse tail, the PA28γ gene was amplified by PCR with the following primers: sense, PA28-3 (AGGTGGATCAGGAAGTGAAGCTCAA); and antisense, PA28γ-5cr (CACCTCACTTGTGATCCGCTCTCTGAAAGAATCAACC). The targeted sequence for the PA28γ-knockout mouse was detected by PCR using the PA28-3 primer and the PAKO-4 primer (TGCAGTTCATTCAGGGCACCGGACAG). The transgene encoding HCV core protein was detected by PCR as described in ref. 25. The expression of PA28γ and HCV core protein in the livers of 6-month-old mice was confirmed by Western blotting with mouse monoclonal antibody to HCV core protein, clone 11-10, and rabbit antibody to PA28γ. Mice were cared for according to the institutional guidelines. The mice were given ordinary feed, CRF-1 (Charles River Laboratories, Yokohama, Japan), and they were maintained under specific pathogen-free conditions.

All animal experiments conformed to the Guidelines for the Care and Use of Laboratory Animals, and they were approved by the Institutional Committee of Laboratory Animal Experimentation (Research Institute for Microbial Diseases, Osaka University).

**Preparation of Mouse Embryonic Fibroblasts.** MEFs were prepared as described in ref. 22. MEFs were cultured at 37°C under an atmosphere of 5% $CO_2$ in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS, penicillin, streptomycin, sodium pyruvate, and nonessential amino acids.

**Transfection and Immunoblotting.** Plasmid vectors were transfected into the MEFs and 293T cells by liposome-mediated transfection by using Lipofectamine 2000 (Invitrogen). The amount of HCV core protein in the liver tissues was determined by an ELISA as described in ref. 37. The cell lysates were subjected to SDS/PAGE (12.5% gel), and they were then transferred onto PVDF membranes. Proteins on the membranes were treated with specific antibody and Super Signal Femto (Pierce, Rockford, IL). The results were then visualized by using an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan). The method of immunoprecipitation test is described in ref. 18.

**Reporter Assay for *srebp-1c* Promoter Activity.** The genomic DNA fragment encoding the *srebp-1c* promoter region (located from residues −410 to +24) was amplified from a mouse genome. The fragment was introduced into the KpnI and HindIII sites of pGL3-Basic (Promega, Madison, WI), and it was designated as pGL3-srebp-1cPro. The plasmids encoding RXR$\alpha$ and LXR$\alpha$ were transfected into MEFs together with pGL3-srebp-1cPro and a control plasmid encoding *Renilla* luciferase (Promega). The total DNA for transfection was normalized by the addition of empty plasmids. Cells were harvested at 24 h posttransfection. The ligand of RXR$\alpha$, 9-*cis*-retinoic acid (Sigma), and that of LXR$\alpha$, 22(*R*)-hydroxylcholesterol (Sigma) were added at a final concentration of 5 $\mu$M each to the culture medium of 293T cells transfected with pGL3-srebp-1cPro together with expression plasmids encoding RXR$\alpha$, LXR$\alpha$, and HCV core protein at 24 h posttransfection. Cells were harvested 24 h after treatment. Luciferase activity was measured by using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase, and the results are expressed as the fold increase in relative luciferase units.

**Electrophoresis Mobility Shift Assay (EMSA).** EMSA was carried out by using a LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturer's protocol. Nuclear extract of the cell lines and liver tissue was prepared with an NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce). Briefly, double-stranded oligonucleotides for EMSA were prepared by annealing both strands of each LXRE of the *srebp-1c* promoter (5′-GGACGCCCGCTAGTAACCCCGGC-3′) (16). Both strands were labeled at the 5′ ends with biotin. The annealed probe was incubated for 20 min on ice with nuclear extract (3 $\mu$g of protein) in a reaction buffer containing 10 mM Tris·HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 0.05 $\mu$g/$\mu$l poly(dI-dC), 2.5% glycerol, 0.05% Nonidet P-40, and 0.1 nM labeled probe, with or without 1 mM nonlabeled probe. The resulting mixture was subjected to PAGE (5% gel) at 120 V for 30 min in 0.5× TBE. The DNA–protein complex was transferred to a Hybond N+ membrane (Amersham, Piscataway, NJ), incubated with horseradish peroxidase-conjugated streptavidin, and visualized by using an LAS3000 imaging system.

**Statistical Analysis.** The results are expressed as the mean ± SD. The significance of differences in the means was determined by Student's $t$ test.

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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