

remain noncovalently associated, interacting in part through their C-terminal transmembrane domains, which also mediate retention of the E1-E2 complex in the ER. Based on this model of membrane topology, the HCV envelope glycoproteins possess little or no cytoplasmic region. However, a physical association between E1 and the cytosolic core protein has been reported (25, 27), suggesting that the E1 glycoprotein is able to expose a cytoplasmic domain of sufficient length to interact with the core. In addition, the presence of the core protein has been shown to affect the folding of E1 (32).

We have previously suggested that the E1 glycoprotein may adopt a polytopic (double membrane-spanning) topology that coexists with the dominant type I form (35). In this study, we provide genetic evidence for a polytopic form of the E1 glycoprotein and for exposure of a centrally located cytoplasmic domain. Furthermore, we show that the cytoplasmic region of the polytopic form of E1 is required for interaction with amino acid residues 72 to 91 of the core protein.

#### MATERIALS AND METHODS

**Cell culture.** 293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 2 mM L-glutamine, penicillin, and streptomycin and supplemented with 10% fetal bovine serum.

**Plasmids.** A cDNA of E1 glycoprotein was amplified from HCV type 1b strain J1 (1) by PCR using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) and inserted between *Nhe*I and *Bam*III sites of pJW4303, which contains the signal sequence of tissue plasminogen activator and the bovine growth hormone polyadenylation signal (a kind gift from J. M. Mullins), to generate pJW383. For the deletion analysis, the plasmids pJW360 and pJW288 encoding residues 192 to 360 and 192 to 288, respectively, were amplified by PCR and cloned into pJW4303. The plasmids pJW383d1 and pJW383d2, containing deletions in residues 261 to 286 and 289 to 340, respectively, were generated from pJW383 by splicing of overlapping extensions (13, 15) as described previously (44). A cDNA fragment encoding core to E2 proteins of HCV strain J1 was amplified by PCR and cloned into pCAGGS-PUR (28), and glycosylation site mutations in the E1 protein were generated by the method of splicing by overlapping extension. For the yeast two-hybrid assay, pGBKT7HCVCore173 was used as bait, as described previously (38). The gene encoding amino acids 288 to 346 of HCV E1 protein was amplified from cDNA of strain J1 and introduced into *Nde*I and *Eco*RI sites of a pGADT7 vector (Clontech, Palo Alto, CA). In the same way, deletion mutants of core protein encoding residues 1 to 151, 1 to 25, 24 to 173, 38 to 173, 58 to 173, 72 to 173, and 92 to 173 were amplified by PCR and cloned into a pGBKT7 vector. The FLAG sequence was introduced between amino acids 195 and 196 of the cDNA encoding residues 1 to 383 of the HCV polyprotein and replaced Ala<sup>283</sup> with Arg to avoid processing by signal peptidase and spacer amino acids (Gly-Gly-Gly-Ser), and influenza virus hemagglutinin (HA) sequence was added at the C terminus. The resulting cDNA fragment encoding core protein, FLAG tag, E1, and HA tag was cloned into a pcDNA3.1(+) vector and designated Flag-core-E1-HA (see Fig. 2D, below) and used for in vitro transcription and translation. Similarly, the FLAG sequence was introduced into the cDNA encoding residues 151 to 383 of the HCV polyprotein, and the HA sequence was added at the C terminus. The resulting cDNA fragment encoding the C-terminal hydrophobic/transmembrane region of the core protein, FLAG tag, E1, and HA tag was designated Flag-E1-HA (see Fig. 3A, below). The DNA fragments encoding residues 1 to 191 with amino acids 72 to 91 deleted were generated by splicing via overlapping extension and cloned into pCAGGS (CoreΔ72-91) (see Fig. 4A, below) (42). The DNA fragment encoding the cytoplasmic domain of the E1 protein with a C-terminal HA tag was amplified by PCR and introduced at *Hind*III and *Sac*II sites of pEGFP-C3. pCAGGS plasmids encoding core to p7 replacing residues 304 to 307, 308 to 311, 312 to 315, 316 to 319, 320 to 323, 324 to 327, or 328 to 331 with Ala were generated by using splicing with overlapping extension (see Fig. 6A, below).

**Antibodies.** Mouse monoclonal antibody to HA tag (HA11) and anti-FLAG antibody (M2) were purchased from Covance (Richmond, CA) and Sigma, respectively. Mouse monoclonal antibodies to core protein (clones 11-7, 11-10, and 11-14) were gifts from S. Yagi (2). Anti-E1 mouse monoclonal antibody (clone 0726) was prepared by immunization using the membrane fraction of the

CHO L10 cell line, which constitutively expresses HCV envelope proteins (30). Anti-E2 monoclonal antibody (clone 187) was a generous gift from M. Kohara.

**Yeast two-hybrid assay.** A yeast two-hybrid assay was carried out by using Matchmaker system 3 (Clontech) according to the manufacturer's protocol. The bait vector pGBKT7HCVcore 173 (38) or empty plasmid was transfected into *Saccharomyces cerevisiae* strain AH109 together with the prey vectors, pGADT7-based constructs (see Table 1, below). The yeast cells possessing pGBKT7/p-53 and pGADT7/large T antigen were used as positive controls, while yeast cells possessing pGBKT7 and pGADT7 were the negative controls. These transfected yeast colonies were cultivated on dropout plates lacking Trp, Leu, His, and Ade (test plates) or plates lacking Trp and Leu (control plates) and then incubated at 30°C for 1 week.

**Transfection, immunoblotting, and immunoprecipitation.** Liposome-mediated DNA transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was described previously (38). Transfected cells were cultured at  $2 \times 10^5$  cells/well in a six-well plate, harvested 30 to 48 h posttransfection, washed twice with phosphate-buffered saline (PBS), and incubated at 4°C for 30 min in 0.25 ml of lysis buffer (20 mM Tris-HCl [pH 7.4], 135 mM NaCl, 1% Triton X-100, and 10% glycerol supplemented with 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 5 mM Na<sub>2</sub>VO<sub>4</sub>). After freezing and thawing, lysed cells were centrifuged at  $20,000 \times g$  for 5 min. The resulting cleared lysate was stored at -80°C prior to use for immunoprecipitation and blotting. Immunoprecipitation was carried out according to the method described previously (44). Briefly, lysates were preincubated at 4°C for 5 h in the lysis buffer with or without 1 mM MgCl<sub>2</sub> and 0.1 mg/ml of yeast tRNA (Sigma) prior to immunoprecipitation. The resulting lysates (0.2 ml) were gently rotated with 1.0 μg of anti-FLAG, anti-HA, or mixed mouse monoclonal anti-HCV core antibodies or mouse monoclonal antibody to the E1 protein at 4°C for 3 h with or without 1 mM MgCl<sub>2</sub> and 0.1 mg/ml of yeast tRNA. The immunocomplex was gently rotated at 4°C for 3 h with 10 μl of 50% (vol/vol) protein G-Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech, Franklin Lakes, NJ) with or without 1 mM MgCl<sub>2</sub> and 0.1 mg/ml of yeast tRNA and then centrifuged at  $20,000 \times g$  for 30 s. The precipitated beads were washed five times with 0.5 ml of lysis buffer containing or lacking 1 mM MgCl<sub>2</sub> and 0.1 mg/ml of yeast tRNA and then boiled in 50 μl of the loading buffer. The boiled samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins in gels were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA) and then blotted with primary antibody and secondary horseradish peroxidase-conjugated antibody. The immunocomplexes on membranes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by using an image analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

**Protease protection assay of HCV proteins synthesized by in vitro transcription/translation.** A plasmid encoding a FLAG-core-E1-HA protein was transcribed under the control of a T7 promoter by using the RiboMax large-scale RNA production system with Ribo m<sup>7</sup>G cap analog (Promega, Madison, WI). In vitro translation was carried out in the presence of [<sup>35</sup>S]methionine-cysteine (Amersham, Piscataway, NJ) by using rabbit reticulocyte lysate and canine pancreatic microsomal membrane (Promega). Translated sample was diluted sevenfold with PBS and then mixed with tosylsulfonil phenylalanyl chloromethyl ketone-treated trypsin (Sigma) at a final concentration of 2 μg/ml. The mixture was incubated at 30°C for 60 min with or without 0.5% Nonidet P-40, and then soybean trypsin inhibitor (Sigma) was added at a final concentration of 20 μg/ml. Digestion products were immunoprecipitated with anti-FLAG antibody.

**Indirect immunofluorescence analysis.** 293T cells were washed with PBS at 40 h after transfection and fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 for 3 min at room temperature and blocked with nonfat milk solution. Cells were incubated with the anti-E1 antibody for 60 min at 37°C and then with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG; TAGO, Burlingame, CA). HCV E1 protein was visualized by fluorescence microscopy (TE300; Nikon, Tokyo, Japan).

**Velocity sedimentation with sucrose gradients.** Transfected 293T cell were suspended in MNT buffer (20 mM 2-morpholinoethanesulfonic acid, 100 mM NaCl, 30 mM Tris-HCl [pH 8.6], and 0.1% Triton X-100) and then incubated at 4°C for 5 h with or without 0.1 mg/ml of yeast tRNA and 1 mM MgCl<sub>2</sub>. Each sample was layered on top of 12 ml of sucrose with a 20 to 60% gradient and then centrifuged in a Beckman SW 41Ti rotor (Beckman Coulter, Tokyo, Japan) at 30,000 rpm for 3 h at 4°C. Centrifuged lysates were collected from the bottoms of the tubes and then concentrated with trichloroacetic acid. After washing with ethanol, concentrated proteins were subjected to SDS-PAGE and immunoblotting.

## RESULTS

**Prediction of the topology of the E1 protein in the membrane.** Although a small fraction of the HCV envelope glycoproteins expressed in 293T cells is translocated onto the plasma membrane (3), the vast majority of E1 is retained in the ER membrane (6). Previously, we showed that both a central hydrophobic region of E1 (residues 260 to 288) and the C-terminal transmembrane domain (residues 360 to 383) are important for ER retention (29). As in the C-terminal hydrophobic region, the amino acid sequence of the central hydrophobic region is highly conserved among HCV isolates (4). To investigate the role of these two hydrophobic regions in the biogenesis of the E1 glycoprotein, we utilized the TMHMM algorithm (19), a computer program trained to identify potential transmembrane helical regions. The algorithm identified both hydrophobic regions as having a high probability of transmembrane helix (Fig. 1A). To examine the function of the hydrophobic regions as transmembrane domains, we constructed a series of deletion mutants in the E1 protein in which one or the other of the hydrophobic segments was absent (Fig. 1B). Mutant E1 glycoproteins were expressed in 293T cells, and the cellular localization of E1 proteins was determined by indirect immunofluorescence analysis (Fig. 1B). The full-length E1 (383) was detected only in permeabilized cells, consistent with its retention in the ER. The 383d2 mutant, which contains both hydrophobic regions but lacks the intervening hydrophilic region (residues 289 to 340), was also detected in the cytoplasm but not on the cell surface as the full-length E1. By contrast, deletion mutants lacking the central (383d1) or C-terminal (288 and 360) hydrophobic domains were detected on the cell surface in nonpermeabilized cells, suggesting that both the central and the C-terminal hydrophobic domains are required for retention of the E1 protein on the ER membrane. If the central hydrophobic domain traverses the ER membrane as predicted by the TMHMM program, the region between positions 288 and 360 would be expected to lie in the cytoplasmic space. Based on this model and on the results with E1 deletion mutants, we suggest that the E1 protein might be able to retain two membrane topologies: the conventional type I topology and a polytopic topology that spans the membrane twice with N and C termini in the ER lumen and an intervening cytoplasmic loop, as reported previously (35) (Fig. 1C). Recently, a similar polytopic form of the fusion glycoprotein of Newcastle disease virus was identified (31).

**Mutational analysis of putative N-glycosylation sites of the E1 glycoprotein.** To explore the membrane topologies of E1, we examined the utilization of potential glycosylation sites. The E1 protein of HCV strain J1 (1) contains seven N-glycosylation sequence motifs (Asn-X-Ser/Thr) at amino acid positions 196, 209, 233, 234, 250, 305, and 325 (Fig. 2A). The Asn residues at these possible N-glycosylation sites were individually replaced with Gln, and the mutant E1 glycoproteins were expressed as a core-, E1-, or E2-containing polyprotein in 293T cells. In all cases, the mutant polyproteins were expressed and properly processed by signal peptidase and signal peptide peptidase to generate the core, E1, and E2 proteins (Fig. 2B). The mutant E1 proteins displayed distinct glycoforms consistent with changes in glycosylation. The wild-type E1 glycoprotein exhibited a major band of 34 kDa and a minor band of 32 kDa.

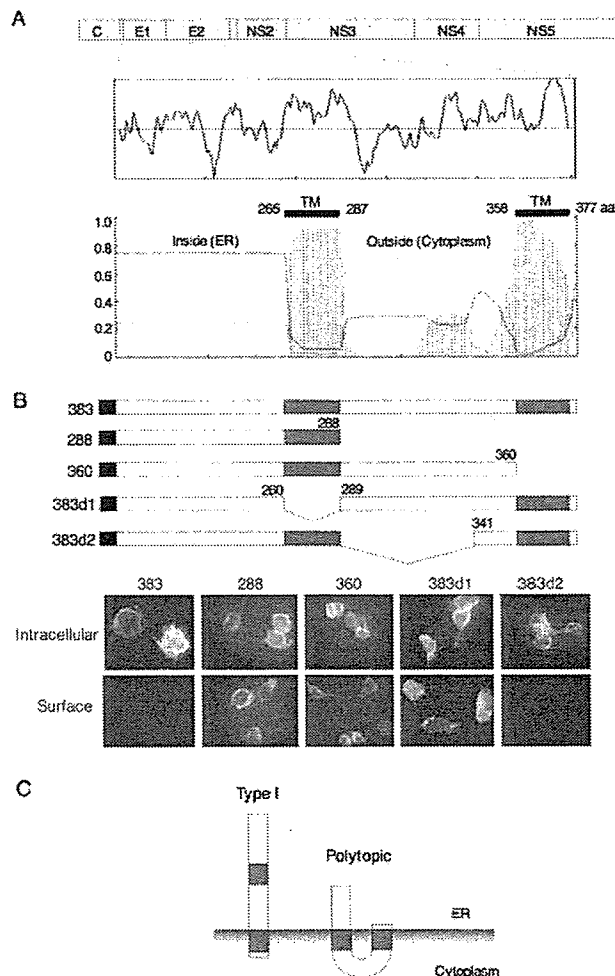


FIG. 1. Prediction of the membrane topology of the E1 protein. (A) Genome structure of HCV and a hydrophobic profile of the amino acid sequence of the E1 protein are shown at the top. The transmembrane helices in the E1 protein were predicted by the TMHMM program (19), and regions of high probability (amino acid residues 265 to 287 and 358 to 377) are indicated. (B) 293T cells transfected with the wild type (383) and deletion constructs were fixed with paraformaldehyde and permeabilized with Triton X-100 (intracellular) or not permeabilized (surface). E1 proteins were visualized with an anti-E1 monoclonal antibody and fluorescein isothiocyanate-conjugated anti-mouse IgG. (C) Possible topologies of the E1 protein on the ER. (Left) Type I topology model possessing a C-terminal transmembrane region; (right) a polytopic topology that spans the membrane twice, with both N and C termini in the ER lumen and with an intervening cytoplasmic loop.

The 325 mutant was unchanged from the wild-type E1, suggesting that the 325 position is not utilized, presumably due to an unfavorable NWSP motif in the genotype 1a protein (33). The 209, 233/234, and 250 mutants migrated faster than the authentic E1 protein and exhibited two bands of 32 and 30 kDa. The E1 of the 196 mutant was apparently not recognized by the monoclonal antibody directed to the N-terminal region of E1. In the 233 and 234 mutants, glycosylation occurred at the remaining Asn (234 or 233, respectively). These mutants comigrated with the wild-type E1 glycoforms, suggesting that

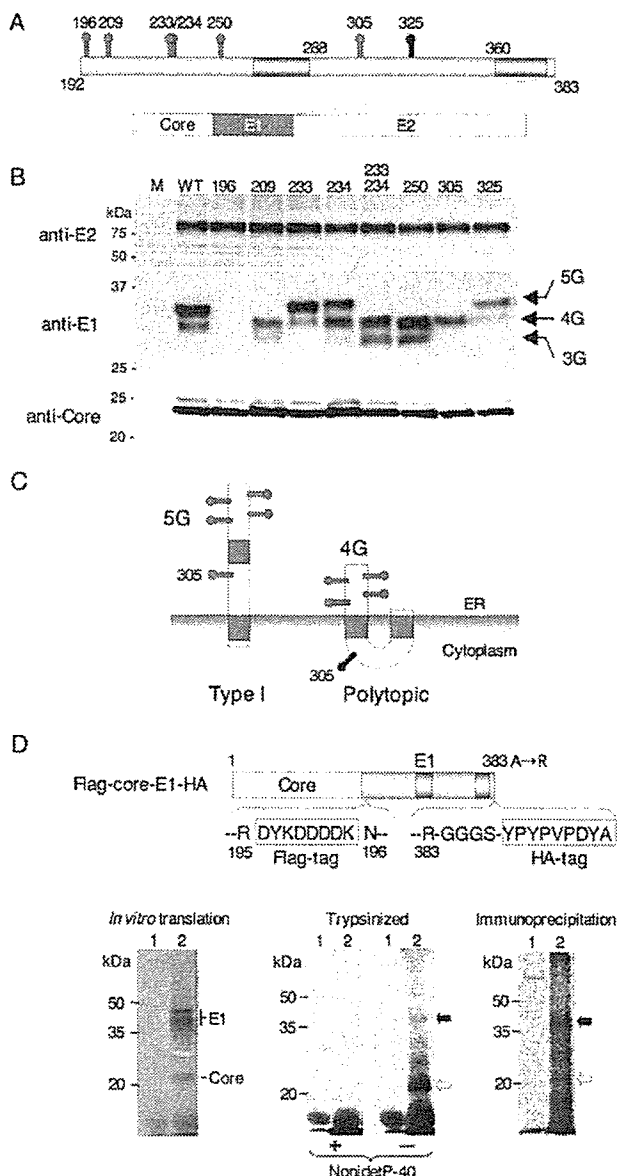


FIG. 2. Mutational analysis of N-glycosylation sites and protease protection assay of the E1 protein. (A) Positions of potential N-glycosylation sites (gray and black spikes) in the E1 protein are shown. (B) Asn residues in the possible N-glycosylation sites in the E1 protein were individually replaced with Gln. Mutant plasmids encoding the core, E1, and E2 polyproteins (A) were expressed in 293T cells, and processed core, E1, and E2 proteins were detected by immunoblotting. (C) Type I and polytopic topology models of E1 proteins bearing carbohydrates at positions of 196, 209, 234, 250, and 305 (5G) and 196, 209, 234, and 250 (4G), respectively. The 305 mutant would exhibit a single band of 4G irrespective of the topologic models. (D) Structure of the FLAG-core-E1-HA construct encoding the HCV core and E1 polyprotein carrying FLAG and HA tags in the N- and C-terminal regions of the E1 protein (top). (Bottom, left) In vitro translation of capped RNA transcribed from the FLAG-core-E1-HA (lane 2) and without RNA (lane 1) in the presence of [<sup>35</sup>S]methionine-cysteine using rabbit reticulocyte lysate and canine pancreatic microsomal membrane. (Bottom, middle) Translated products of FLAG-core-E1-HA (lane 2) and without RNA (lane 1) were digested with trypsin in the presence (+) or absence (-) of 0.5% Nonidet P-40. (Bottom, right) Digestion products were immunoprecipitated with control (lane 1) and anti-FLAG (lane 2) antibody. Black and white arrows indicate protected and digested E1 protein, respectively.

only one or the other of the overlapping motifs can be utilized in the wild-type molecule. Glycosylation in this region was absent in the double mutant (233/234). The existence of two glycoforms of E1 may reflect incomplete and stochastic use of the available glycosylation sites or, alternatively, the presence of two discrete topological forms of E1 protein. For instance, the major band of 34 kDa in the wild-type glycoprotein might correspond to the type I topology form, with glycosylation at 196, 209, 234, 250, and 305 (5G), whereas the minor band of 32 kDa might correspond to the polytopic form of E1, bearing glycans at positions 196, 209, 234, and 250 (4G). In this regard, it is noteworthy that the 305 mutant of E1 exhibited only a single band of 32 kDa. The absence of a second glycoform is consistent with the putative cytoplasmic localization of Asn305 in a polytopic form of E1 (Fig. 2C). Taken together, this mutational analysis provides support to the model in which the HCV E1 glycoprotein is able to exist in either the type I or polytopic form. In the latter form, an extended cytoplasmic domain in E1 would be available to interact with the core protein in the virion.

**Protease protection assay of the E1 protein.** To confirm the presence of the cytoplasmic domain in the E1 protein, in vitro translation products of the HCV core and E1 polyprotein carrying FLAG and HA tags in the N- and C-terminal regions of the E1 protein, respectively, were digested with trypsin, and the protected portion of the E1 glycoprotein was immunoprecipitated by anti-FLAG antibody. As shown below in Fig. 4D, treatment of the translation products with trypsin in the presence of Nonidet P-40 resulted in complete digestion, and a 22-kDa band (major) and several <35-kDa faint bands were detected in the absence of the detergent. When in vitro-translated HCV core protein was treated similarly, no band was detected, irrespective of the presence of detergent (data not shown); therefore, the protected bands from trypsin digestion were derived from the E1 protein. Although the 22- to 35-kDa bands were specifically immunoprecipitated with anti-FLAG antibody but not with control antibody, the 35-kDa protein corresponding to the type I topology of the E1 protein resistant to trypsin digestion was dominant. This might be due to the difference in the reactivity of the anti-FLAG antibody, which recognizes the intact E1 protein more efficiently than digested ones. These results further support the presence of the polytopic form of HCV E1 glycoprotein, which has a cytoplasmic region together with a type I topology in the ER.

**HCV core protein binds to the E1 protein in the presence of tRNA.** The HCV core protein undergoes extensive conformational changes upon binding to nucleic acid and self-assembling into nucleocapsid-like particles (20). To investigate the effects of nucleic acid on oligomerization of the core protein, lysates of 293T cells expressing HCV core protein were incubated in the presence or absence of yeast tRNA (20) and subjected to velocity sedimentation in a sucrose gradient. Oligomerized core protein was detected in fractions 1 to 4 in the presence of tRNA but not in those in the absence of tRNA (Fig. 3A). To specifically examine the interaction between HCV core and E1 proteins in the assembly of the nucleocapsid-like particles, we coexpressed the core protein with an E1 protein possessing a FLAG tag near its N terminus and an HA tag at the C terminus (Flag-E1-HA) (Fig. 3B, left). The transfected cells were lysed with Triton X-100, and the E1 protein

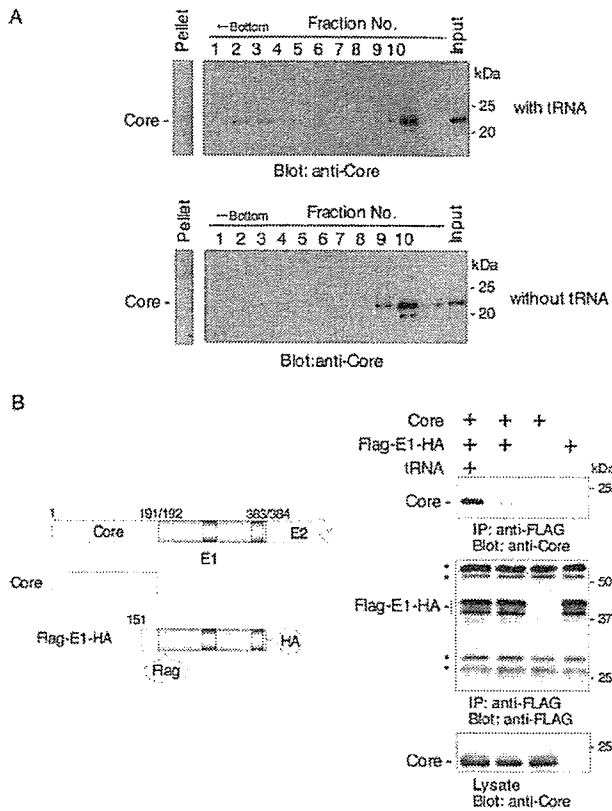


FIG. 3. HCV core protein binds to E1 protein in the presence of tRNA. (A) Cell lysates of 293T cells expressing HCV core protein were subjected to velocity sedimentation with a sucrose gradient in the presence or absence of tRNA. Oligomerized core protein was detected in fractions 1 to 4 in the presence of tRNA but not in those in the absence of tRNA. (B, left) cDNAs used for expression. FLAG-E1-HA encodes FLAG tag after the signal peptide and HA tag after the transmembrane region. (Right) Immunoprecipitation analyses. Cell lysates of 293T cells expressing core and FLAG-E1-HA proteins were immunoprecipitated by anti-FLAG antibody in the presence or absence of tRNA. The asterisks indicate nonspecific bands.

was immunoprecipitated by using an anti-FLAG antibody. Coprecipitation of core protein with E1 was assessed by Western blot analysis using a core-specific monoclonal antibody. Although HCV core protein was clearly coprecipitated with FLAG-E1-HA in the presence of tRNA, little association was seen in the absence of tRNA (Fig. 3B, right). Nonspecific precipitation of the core protein with tRNA was not observed (data not shown). Although a small amount of the intracellular core protein may already associate with viral RNA under the intracellular conditions, a large amount of RNA may be required for oligomerization that is detectable by the sedimentation assay. Together, our results suggest that tRNA facilitates oligomerization of the HCV core protein and potentiates the interaction between the core protein and E1.

**The region spanning amino acid residues 72 to 91 in the HCV core protein is crucial for binding to the E1 protein in yeast.** The interaction between the HCV core and E1 proteins likely occurs on the cytosolic side of the cell membrane and, thus, presumably involves the posited cytoplasmic loop region

in the polytopic form of the E1 glycoprotein. To investigate the possibility for this specific interaction in cells, core protein lacking the transmembrane region (Core1-173) was examined for interaction with the putative E1 cytoplasmic loop region in a yeast two-hybrid system (Table 1). When Core1-173 was expressed with the E1 cytoplasmic region (residues 288 to 346), the yeast was able to grow on the dropout plate lacking Trp, Leu, His, and Ade, suggesting that the core protein associates with the cytoplasmic loop of the E1 protein in yeast. To determine the region of the HCV core protein responsible for the interaction with the cytoplasmic domain of E1, deletion mutants of the core were tested. Association in the yeast two-hybrid system was seen with Core24-173, Core38-173, Core58-173, Core72-173, and Core1-151 mutants, but not with Core92-173 and Core1-25. Nonspecific interaction of the GAL4 activation domain with these core mutants was not observed. These results suggest that the region spanning from amino acid residues 72 to 91 in the HCV core protein is important for interaction with the cytoplasmic domain of the E1 protein in yeast.

**Amino acid residues 72 to 91 in the core protein are involved in oligomerization of the core protein and interaction with the E1 protein in mammalian cells.** To examine the involvement of amino acid residues 72 to 91 of the HCV core protein in the interaction with the E1 protein in mammalian cells, FLAG-E1-HA was coexpressed with either a wild-type core or a deletion mutant lacking amino acid residues 72 to 91 (CoreΔ72-91) in 293T cells (Fig. 4A). Cell lysates were incubated with yeast tRNA, and FLAG-E1-HA was immunoprecipitated with anti-FLAG antibody. As shown in Fig. 4B (left), only the wild-type core protein, but not CoreΔ72-91, coprecipitated with E1. Self-oligomerization was also prevented by the deletion in CoreΔ72-91 (Fig. 4B, right). These results suggest that amino acid residues 72 to 91 in the HCV core protein play a crucial role in the interaction with the E1 protein and oligomerization of the core protein.

**The E1 cytoplasmic domain interacts with the core protein in mammalian cells and inhibits the interaction with intact E1 protein in trans.** To assess the involvement of the E1 cytoplasmic region in the interaction with core protein in mammalian

TABLE 1. Interaction between the core and the E1 cytoplasmic region in yeast

Bait	Growth with prey <sup>a</sup>			
	E1 cytoplasmic loop		No insert	
	Dropout	Control	Dropout	Control
Core1-173	+	+	-	+
Core24-173	+	+	-	+
Core38-173	+	+	-	+
Core58-173	+	+	-	+
Core72-173	+	+	-	+
Core92-173	-	+	-	+
Core1-151	+	+	-	+
Core1-25	-	+	-	+
No insert	-	+	-	+

<sup>a</sup> HCV core mutants were expressed as fusion proteins with the DNA binding region by using a bait plasmid. The HCV E1 cytoplasmic region was expressed as a fusion protein with an activation domain by using a prey plasmid. Yeast growth was observed in dropout plates lacking Trp, Leu, Ade, and His (dropout) or plates lacking Trp and Leu (control). +, growth; -, no growth.

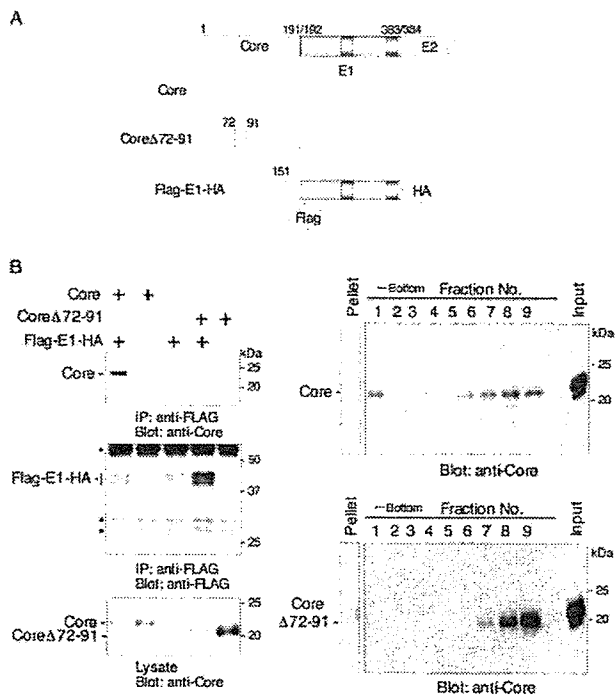


FIG. 4. Amino acid residues 72 to 91 in the core protein are involved in oligomerization of the core protein and interaction with the E1 protein. (A) cDNAs used for expression. Core $\Delta$ 72-91 is an HCV core protein carrying a deletion of amino acid residues 72 to 91. (B, left) FLAG-E1-HA was coexpressed in 293T cells with either a wild-type core or Core $\Delta$ 72-91, and the interaction was analyzed by immunoprecipitation in the presence of tRNA. The asterisks indicate non-specific bands. (Right) Oligomerization of a wild-type core or Core $\Delta$ 72-91 in the presence of tRNA. Wild-type core protein was self-oligomerized, but Core $\Delta$ 72-91 was not.

cells, we constructed an enhanced green fluorescent protein (EGFP) fusion protein carrying the E1 cytoplasmic domain followed by an HA tag (EGFP-cdE1-HA) (Fig. 5A). Upon coexpression of EGFP-cdE1-HA with the wild-type core protein in 293T cells, the two proteins could be coprecipitated using anti-HA antibody (Fig. 5B). The mutant Core $\Delta$ 72-91 protein was unable to associate with EGFP-cdE1-HA (Fig. 5B). Together, these studies demonstrate that the cytoplasmic loop region of E1 is able to interact with the core protein and that core residues 72 to 91 are required for this association.

To further confirm the specificity of the interaction of the E1 cytoplasmic region with the core protein, we examined the ability of the EGFP-cdE1-HA protein to inhibit the association of the intact E1 protein (in Flag-E1-HA) with the wild-type core protein (Fig. 5C). Expression of EGFP-cdE1-HA but not EGFP-HA competed strongly with the interaction between core and the FLAG-tagged Flag-E1-HA protein. These results suggest that the cytoplasmic loop in the intact E1 glycoprotein can directly bind to HCV core protein. Interestingly, the EGFP-cdE1-HA protein was unable to inhibit this interaction in the context of the intact core and E1 and E2 polyproteins (data not shown), suggesting that expression of the core and E1 proteins in *cis* may prevent subsequent interaction with E1 expressed in *trans*.

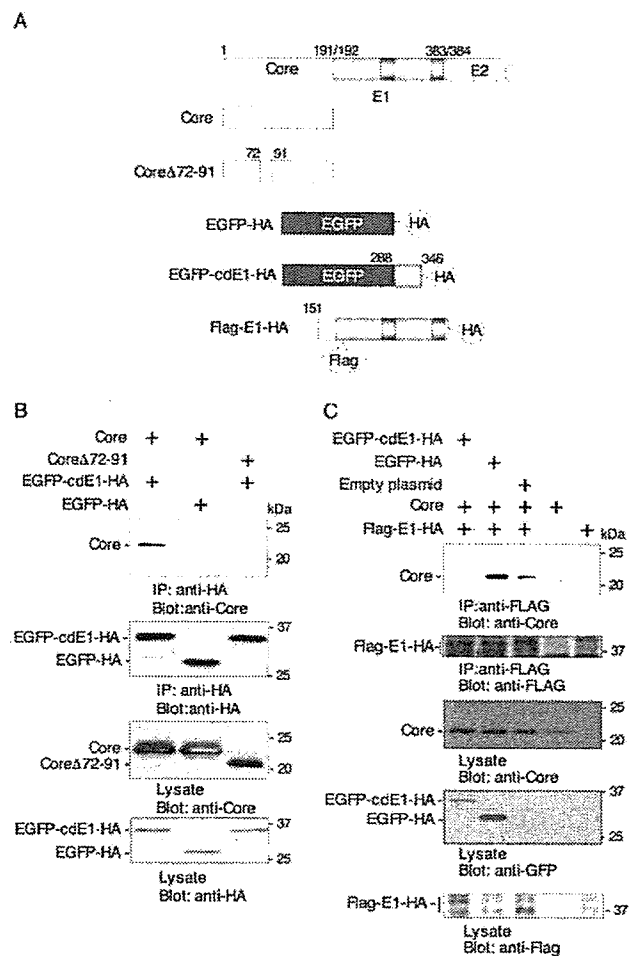


FIG. 5. Interaction of the E1 cytoplasmic loop with the core protein. (A) cDNAs used for expression. EGFP-cdE1-HA is an EGFP fusion protein carrying the E1 cytoplasmic region of amino acid residues 288 to 346 followed by an HA tag. (B) Wild-type core or Core $\Delta$ 72-91 was coexpressed with EGFP-cdE1-HA in 293T cells, and their interaction was analyzed by immunoprecipitation. EGFP-cdE1-HA coprecipitated with wild-type core protein, but not with Core $\Delta$ 72-91. (C) Inhibition of the interaction of the core protein with FLAG-E1-HA by expression of EGFP-cdE1-HA. Expression of EGFP-cdE1-HA but not of EGFP disrupted the interaction between core and E1 proteins.

**Four amino acid residues, 312 to 315, in the cytoplasmic region of the E1 protein are important for interaction with the core protein.** Alignment of the amino acid sequence of the E1 cytoplasmic region among different HCV genotypes revealed that the region from Gln<sup>302</sup> to Pro<sup>328</sup> is highly conserved (Fig. 6A). To determine residues in the E1 cytoplasmic region that are critical for interaction with the core protein, blocks of four residues each in the conserved region were replaced with Ala in the polyprotein (core, E1, E2, and p7) (Fig. 6A). These mutant polyproteins were expressed in 293T cells and immunoprecipitated with anti-core antibody; coprecipitated E1 protein was detected by immunoblotting using an anti-E1 monoclonal antibody (Fig. 6B). The replacement of four amino acid residues, 304 to 307, with Ala in the conserved region of the E1

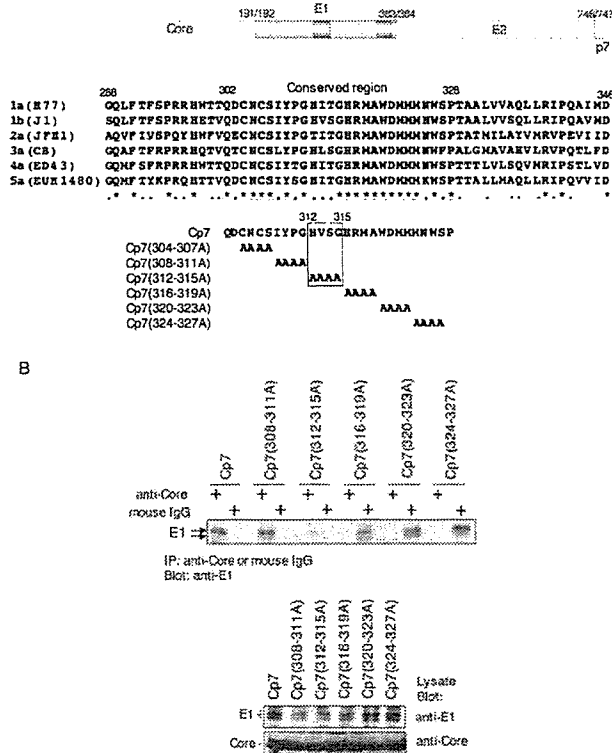


FIG. 6. Four amino acid residues, 312 to 315, in the cytoplasmic region of the E1 protein are important for interaction with the core protein. (A) Alignment of the amino acid sequence of the E1 cytoplasmic region among different HCV genotypes (1a, H77 [AF009606]; 1b, J1 [D89815]; 2a, JFH1 [AB047639]; 3a, CB [AF046866]; 4a, ED43 [Y11604]; 5a, EUH1480 [Y13184]). A conserved region from Gln<sup>302</sup> to Pro<sup>328</sup> is shown by gray shading. Mutant polyproteins consisting of the core, E1, E2, and p7 proteins with four residues each replaced by Ala in the conserved E1 region were constructed. Four amino acid residues, His<sup>312</sup>, Val<sup>313</sup>, Ser<sup>314</sup>, and Gly<sup>315</sup>, in the E1 cytoplasmic region of strain J1 and substitution of the amino acids with Ala in Cp7 (312-315A) are indicated by the box. (B) These mutant polyproteins were expressed in 293T cells and immunoprecipitated with anti-core antibody or nonspecific mouse IgG in the presence of MgCl<sub>2</sub> and tRNA. The E1 protein that coprecipitated with the core protein was detected by immunoblotting. The substitution of four amino acid residues, 304 to 307, with Ala in the conserved region of the E1 protein, Cp7 (304-307A), could not be examined due to the low level of expression.

protein could not be examined due to a low level of expression (data not shown). Among the mutant constructs examined, only the substitution at residues 312 to 315, Cp7 (312-315A), markedly diminished association with the core protein (Fig. 6B). These results suggest that this region in the E1 cytoplasmic domain of the J1 strain of HCV (His<sup>312</sup>, Val<sup>313</sup>, Ser<sup>314</sup>, and Gly<sup>315</sup>) is important for interaction with the core protein.

**DISCUSSION**

The biogenesis of the transmembrane glycoproteins involves a series of coordinated translation and membrane integration events that are directed by topogenic determinants within the nascent chains and that ultimately lead to the most favored topology for any given polypeptide (24). However, there is an

increasing number of examples of glycoproteins that can assume multiple topological orientations. The large envelope protein of the hepatitis B virus, for instance, has been suggested to adopt distinct topologies that enable the protein to serve in virus assembly as a matrix-like protein and in virus entry as a receptor binding protein (22). An unglycosylated form of the HCV E2 protein has been identified and shown to interact with protein kinase R in the cytosol (45). In Newcastle disease virus, type I and polytopic forms of the fusion protein are present in the same cell, and the polytopic form is suggested to be involved in the membrane fusion event (31).

HCV glycoproteins E1 and E2 were shown to possess transmembrane domains and associate to form noncovalent heterodimers that are statically retained in the ER membrane upon recombinant expression (10, 29, 46). Previously, the E1 protein of genotype 1a was suggested to possess a single C-terminal transmembrane domain, based in part on its utilization of potential glycosylation sites (33) and on a model of the transmembrane domains of the E1 and E2 proteins, in which the C terminus reorients, upon signal peptidase cleavage, from the ER lumen to protrude slightly into the cytoplasm (7). In our study, we have suggested that the E1 protein can also adopt a polytopic topology in which the protein spans the ER membrane twice and includes an intervening cytoplasmic region. In this model, the membrane orientation of the C-terminal transmembrane region is inverted and translocation of the signal peptidase-cleaved C terminus is not required.

Our analysis revealed that the 305 mutant of the 1b genotype expressed by transfection exhibited a single band of 32 kDa, whereas that of genotype 1a expressed by recombinant vaccinia viruses has been reported to contain two bands (33). Although we do not know the reason for this discrepancy, it may relate to differences in the expression systems. HCV proteins expressed by vaccinia virus and Sindbis virus vectors formed disulfide-linked aggregates (9, 11, 34), and coexpression of a large amount of vaccinia viral proteins also may alter the proper processing of the expressed proteins, as suggested by Merola et al. (32). However, further work will be necessary to clarify the reasons for the differences in glycosylation patterns of E1 mutants obtained in the different expression systems.

Mottola et al. analyzed the determinants for ER localization of the E1 protein and showed that the juxtamembrane region of E1, between amino acid residues 290 and 333, was required for ER retention (41). This region lies within the ectodomain of the E1 protein in the type I topology and in the cytoplasmic region of the protein in the proposed polytopic form. ER localization determinants of transmembrane proteins have in general been located either in the cytosolic or in the transmembrane domain, not in the luminal ectodomain, except for the yeast Sec20 protein (41). Therefore, assignment of the ER localization signal to the cytoplasmic region of the E1 protein might further support the possibility of the polytopic topology model. Affinity purification and membrane reconstitution of the E1 protein carrying an affinity tag (S-peptide) in the putative cytoplasmic region are also consistent with this model (35). Together, these findings provide indirect support that the E1 glycoprotein can adopt a polytopic form.

As previously reported (20), oligomerization of the HCV core protein to form nucleocapsid-like particles requires the presence of stem-loop RNA structures, such as those in tRNA.

Here, we have demonstrated that self-assembly of the core protein occurs without envelope protein in the presence of tRNA and that tRNA is required for the association of E1 glycoprotein with the core protein, suggesting that oligomerization of the core protein may be a prerequisite for this interaction during virus assembly. Based on hydrophobicity and the clustering of basic amino acids, the HCV core protein is proposed to possess three domains: the N-terminal basic and hydrophilic region (domain 1; residues 1 to 118), a central basic and hydrophobic domain (domain 2; residues 119 to 174), and the hydrophobic signal sequence for E1 (domain 3; residues 175 to 191) (14). Biophysical characterization of the core protein indicated that the C-terminal residues 125 to 179 were critical for the folding and oligomerization of the core protein (21). Although our mutant HCV polyprotein containing Ala substitutions at residues 312 to 315 in the cytoplasmic region of the E1 protein exhibited a clear reduction in its interaction with the core protein, a substantial amount of residual binding was retained. These results suggest that regions other than the residues from 312 to 315 in the E1 protein are also involved in the interaction with the core protein.

In Semliki Forest virus, the cytoplasmic domain of the E2 glycoprotein, which corresponds to the E1 protein in HCV, has been shown to interact with the capsid protein (26, 49). Assembly of alphaviruses has also been found to require the specific interaction between the C-terminal cytoplasmic domain of the E2 protein and the capsid protein (17). Although the functional significance of the two forms of the HCV E1 protein is still unclear, the E1 cytoplasmic region among different HCV genotypes is well conserved and four amino acid residues, His<sup>312</sup>, Val<sup>313</sup>, Ser<sup>314</sup>, and Gly<sup>315</sup> of strain J1, were shown to be important for interaction with the core protein. Although the four amino acid sequences identified in strain J1 of genotype 1b are not strictly conserved among the different HCV genotypes (Fig. 6A), a pattern of polar-hydrophobic-polar-glycine residues can be discerned in all of them. The interaction of the cytoplasmic E1 protein with the core protein may indicate that the polytopic form is a mature E1 protein that is incorporated into virions.

In conclusion, the polytopic topology model of the HCV E1 protein and the interaction of oligomerized core protein with the cytoplasmic region of the E1 protein may provide clues to aid in understanding the biosynthesis and assembly of the HCV structural proteins. HCV core protein is also involved in the development of liver steatosis, type II diabetes mellitus, and hepatocellular carcinoma in transgenic mice (39, 40, 48). A detailed knowledge of the assembly of HCV particles will provide the basis for the development of effective therapeutics for chronic hepatitis C.

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#### REFERENCES

- Aizaki, H., Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsuura, and T. Miyamura. 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. *Hepatology* 27: 621–627.
- Aoyagi, K., C. Ohue, K. Iida, T. Kimura, E. Tanaka, K. Kiyosawa, and S. Yagi. 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. *J. Clin. Microbiol.* 37:1802–1808.
- Bartosch, B., J. Dubuisson, and F. L. Cosset. 2003. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J. Exp. Med.* 197:633–642.
- Bukh, J., R. H. Purcell, and R. H. Miller. 1993. At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. *Proc. Natl. Acad. Sci. USA* 90:8234–8238.
- Cerny, A., and F. V. Chisari. 1999. Pathogenesis of chronic hepatitis C: immunological features of hepatic injury and viral persistence. *Hepatology* 30:595–601.
- Coquerel, L., S. Duvet, J. C. Meunier, A. Pillez, R. Cacan, C. Wychowski, and J. Dubuisson. 1999. The transmembrane domain of hepatitis C virus glycoprotein E1 is a signal for static retention in the endoplasmic reticulum. *J. Virol.* 73:2641–2649.
- Coquerel, L., A. Op de Beeck, M. Lambot, J. Roussel, D. Delgrange, A. Pillez, C. Wychowski, F. Penin, and J. Dubuisson. 2002. Topological changes in the transmembrane domains of hepatitis C virus envelope glycoproteins. *EMBO J.* 21:2893–2902.
- Dubuisson, J. 2000. Folding, assembly and subcellular localization of hepatitis C virus glycoproteins. *Curr. Top. Microbiol. Immunol.* 242:135–148.
- Dubuisson, J., H. H. Hsu, R. C. Cheung, H. B. Greenberg, D. G. Russell, and C. M. Rice. 1994. Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. *J. Virol.* 68:6147–6160.
- Dubuisson, J., and C. M. Rice. 1996. Hepatitis C virus glycoprotein folding: disulfide bond formation and association with calnexin. *J. Virol.* 70:778–786.
- Grakoui, A., C. Wychowski, C. Lin, S. M. Feinstone, and C. M. Rice. 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. *J. Virol.* 67:1385–1395.
- Hijikata, M., N. Kato, Y. Ootsuyama, M. Nakagawa, and K. Shimotohno. 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. *Proc. Natl. Acad. Sci. USA* 88:5547–5551.
- Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51–59.
- Hope, R. G., and J. McLauchlan. 2000. Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. *J. Gen. Virol.* 81:1913–1925.
- Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 77:61–68.
- Hussy, P., H. Langen, J. Mous, and H. Jacobsen. 1996. Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. *Virology* 224:93–104.
- Kail, M., M. Hollinshead, W. Ansoorge, R. Pepperkok, R. Frank, G. Griffiths, and D. Vaux. 1991. The cytoplasmic domain of alphavirus E2 glycoprotein contains a short linear recognition signal required for viral budding. *EMBO J.* 10:2343–2351.
- Koike, K., and K. Moriya. 2005. Metabolic aspects of hepatitis C viral infection: steatohepatitis resembling but distinct from NASH. *J. Gastroenterol.* 40:329–336.
- Krogh, A., B. Larsson, G. von Heijne, and E. L. Sonnhammer. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305:567–580.
- Kunkel, M., M. Lorinczi, R. Rijnbrand, S. M. Lemon, and S. J. Watowich. 2001. Self-assembly of nucleocapsid-like particles from recombinant hepatitis C virus core protein. *J. Virol.* 75:2119–2129.
- Kunkel, M., and S. J. Watowich. 2004. Biophysical characterization of hepatitis C virus core protein: implications for interactions within the virus and host. *FEBS Lett.* 557:174–180.
- Lambert, C., and R. Prange. 2001. Dual topology of the hepatitis B virus large envelope protein: determinants influencing post-translational pre-S translocation. *J. Biol. Chem.* 276:22265–22272.
- Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice. 2005. Complete replication of hepatitis C virus in cell culture. *Science* 309:623–626.
- Lipp, J., N. Flint, M. T. Haeuptle, and B. Dobberstein. 1989. Structural requirements for membrane assembly of proteins spanning the membrane several times. *J. Cell Biol.* 109:2013–2022.
- Lo, S. Y., M. J. Selby, and J. H. Ou. 1996. Interaction between hepatitis C virus core protein and E1 envelope protein. *J. Virol.* 70:5177–5182.
- Lopez, S., J. S. Yao, R. J. Kuhn, E. G. Strauss, and J. H. Strauss. 1994. Nucleocapsid-glycoprotein interactions required for assembly of alphaviruses. *J. Virol.* 68:1316–1323.
- Ma, H. C., C. H. Ke, T. Y. Hsieh, and S. Y. Lo. 2002. The first hydrophobic

- domain of the hepatitis C virus E1 protein is important for interaction with the capsid protein. *J. Gen. Virol.* 83:3085–3092.
28. Matsuo, E., H. Tani, C. K. Lim, Y. Komoda, T. Okamoto, H. Miyamoto, K. Moriishi, S. Yagi, A. H. Patel, T. Miyamura, and Y. Matsuura. 2006. Characterization of HCV-like particles produced in a human hepatoma cell line by a recombinant baculovirus. *Biochem. Biophys. Res. Commun.* 340:200–208.
  29. Matsuura, Y., T. Suzuki, R. Suzuki, M. Sato, H. Aizaki, I. Saito, and T. Miyamura. 1994. Processing of E1 and E2 glycoproteins of hepatitis C virus expressed in mammalian and insect cells. *Virology* 205:141–150.
  30. Matsuura, Y., H. Tani, K. Suzuki, T. Kimura-Someya, R. Suzuki, H. Aizaki, K. Ishii, K. Moriishi, C. S. Robison, M. A. Whitt, and T. Miyamura. 2001. Characterization of pseudotype VSV possessing HCV envelope proteins. *Virology* 286:263–275.
  31. McGinnes, L. W., J. N. Reitter, K. Gravel, and T. G. Morrison. 2003. Evidence for mixed membrane topology of the Newcastle disease virus fusion protein. *J. Virol.* 77:1951–1963.
  32. Merola, M., M. Brazzoli, F. Cocchiarella, J. M. Heile, A. Helenius, A. J. Weiner, M. Houghton, and S. Abrignani. 2001. Folding of hepatitis C virus E1 glycoprotein in a cell-free system. *J. Virol.* 75:11205–11217.
  33. Meunier, J. C., A. Fournillier, A. Choukhi, A. Cahour, L. Cocquerel, J. Dubuisson, and C. Wychowski. 1999. Analysis of the glycosylation sites of hepatitis C virus (HCV) glycoprotein E1 and the influence of E1 glycans on the formation of the HCV glycoprotein complex. *J. Gen. Virol.* 80:887–896.
  34. Michalak, J. P., C. Wychowski, A. Choukhi, J. C. Meunier, S. Ung, C. M. Rice, and J. Dubuisson. 1997. Characterization of truncated forms of hepatitis C virus glycoproteins. *J. Gen. Virol.* 78:2299–2306.
  35. Migliaccio, C. T., K. E. Follis, Y. Matsuura, and J. H. Nunberg. 2004. Evidence for a polytopic form of the E1 envelope glycoprotein of Hepatitis C virus. *Virus Res.* 105:47–57.
  36. Miller, R. H., and R. H. Purcell. 1990. Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. *Proc. Natl. Acad. Sci. USA* 87:2057–2061.
  37. Moriishi, K., and Y. Matsuura. 2003. Mechanisms of hepatitis C virus infection. *Antivir. Chem. Chemother.* 14:285–297.
  38. Moriishi, K., T. Okabayashi, K. Nakai, K. Moriya, K. Koike, S. Murata, T. Chiba, K. Tanaka, R. Suzuki, T. Suzuki, T. Miyamura, and Y. Matsuura. 2003. Proteasome activator PA28 $\gamma$ -dependent nuclear retention and degradation of hepatitis C virus core protein. *J. Virol.* 77:10237–10249.
  39. Moriya, K., H. Fujie, Y. Shintani, H. Yotsuyanagi, T. Tsutsumi, K. Ishibashi, Y. Matsuura, S. Kimura, T. Miyamura, and K. Koike. 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat. Med.* 4:1065–1067.
  40. Moriya, K., K. Nakagawa, T. Santa, Y. Shintani, H. Fujie, H. Miyoshi, T. Tsutsumi, T. Miyazawa, K. Ishibashi, T. Horie, K. Imai, T. Todoroki, S. Kimura, and K. Koike. 2001. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res.* 61:4365–4370.
  41. Mottola, G., N. Jourdan, G. Castaldo, N. Malagolini, A. Lahm, F. Serafini-Cessi, G. Migliaccio, and S. Bonatti. 2000. A new determinant of endoplasmic reticulum localization is contained in the juxtamembrane region of the ectodomain of hepatitis C virus glycoprotein E1. *J. Biol. Chem.* 275:24070–24079.
  42. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193–199.
  43. Ogino, T., H. Fukuda, S. Imajoh-Ohni, M. Kohara, and A. Nomoto. 2004. Membrane binding properties and terminal residues of the mature hepatitis C virus capsid protein in insect cells. *J. Virol.* 78:11766–11777.
  44. Okamoto, K., K. Moriishi, T. Miyamura, and Y. Matsuura. 2004. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. *J. Virol.* 78:6370–6380.
  45. Pavio, N., D. R. Taylor, and M. M. Lai. 2002. Detection of a novel unglycosylated form of hepatitis C virus E2 envelope protein that is located in the cytosol and interacts with PKR. *J. Virol.* 76:1265–1272.
  46. Ralston, R., K. Thudium, K. Berger, C. Kuo, B. Gervase, J. Hall, M. Selby, G. Kuo, M. Houghton, and Q. L. Choo. 1993. Characterization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia viruses. *J. Virol.* 67:6753–6761.
  47. Shimoike, T., S. Mimori, H. Tani, Y. Matsuura, and T. Miyamura. 1999. Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. *J. Virol.* 73:9718–9725.
  48. Shintani, Y., H. Fujie, H. Miyoshi, T. Tsutsumi, K. Tsukamoto, S. Kimura, K. Moriya, and K. Koike. 2004. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 126:840–848.
  49. Vaux, D. J., A. Helenius, and I. Mellman. 1988. Spike-nucleocapsid interaction in Semliki Forest virus reconstructed using network antibodies. *Nature* 336:36–42.
  50. Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11:791–796.
  51. Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari. 2005. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. USA* 102:9294–9299.



# 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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Keywords: FK506-binding protein; geldanamycin; hepatitis C virus; Hsp90; RNA replication

## 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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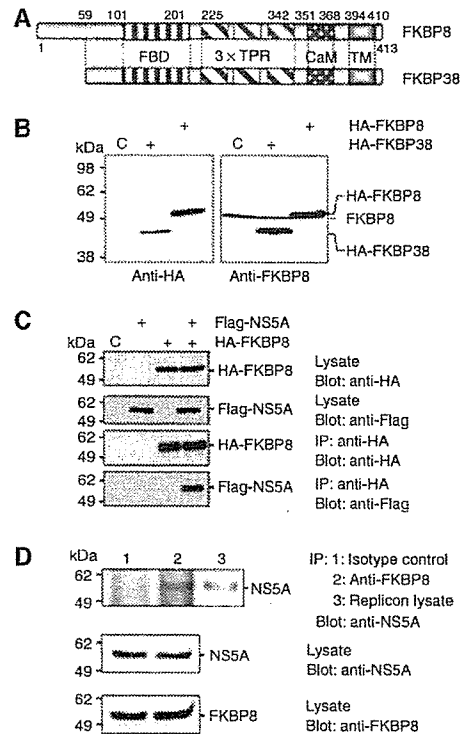
*et al*, 2001). RNA replication in HCV replicon cells has been shown to be inhibited by treatment with lovastatin, a drug that decreases the production of mevalonate by inhibiting 3-hydroxy-3-methylglutaryl CoA reductase; this inhibition of RNA replication was reversed by the addition of geranylgeraniol, 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.

## 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

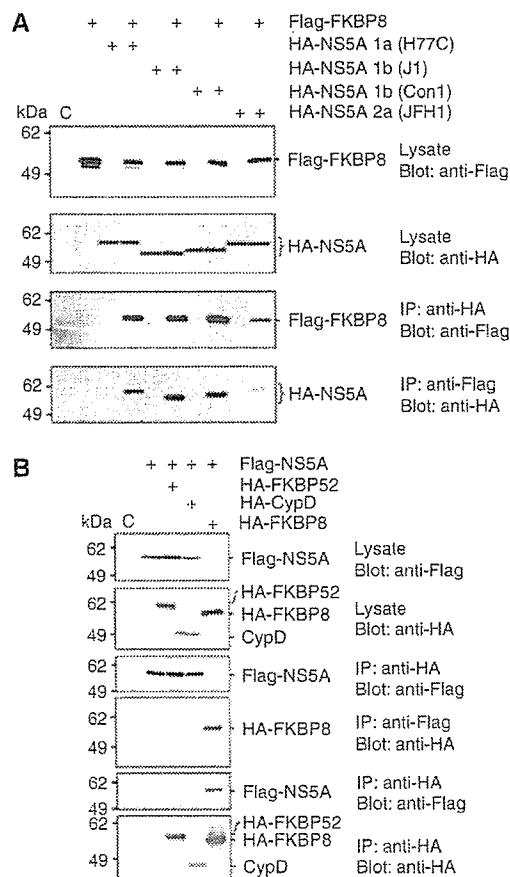


**Figure 1** Expression of FKBP8 and FKBP38 in mammalian cells. (A) Schematic representation of FKBP8 and FKBP38. The FK506-binding domain (FBD), tetratricopeptide repeat (TPR), putative calmodulin binding motif (CaM), and transmembrane domain (TM) are shown. (B) N-terminally HA-tagged FKBP8 and FKBP38 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.

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<sub>6</sub>-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.



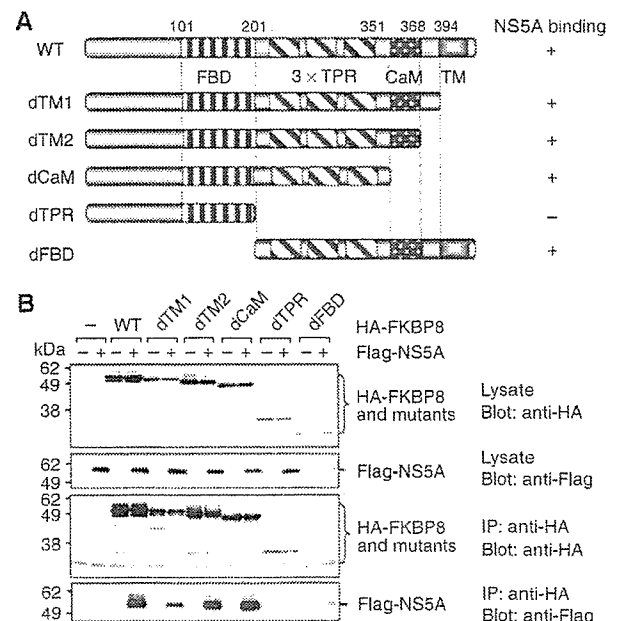
**Figure 2** Specific interaction between FKBP8 and NS5A. (A) HA-NS5As were obtained from several genotypes of HCV and were expressed with Flag-FKBP8 in 293T cells. Proteins immunoprecipitated with anti-HA or Flag antibody were subjected to Western blotting. (B) Flag-NS5A was coexpressed with HA-FKBP8, -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 NS5A 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,

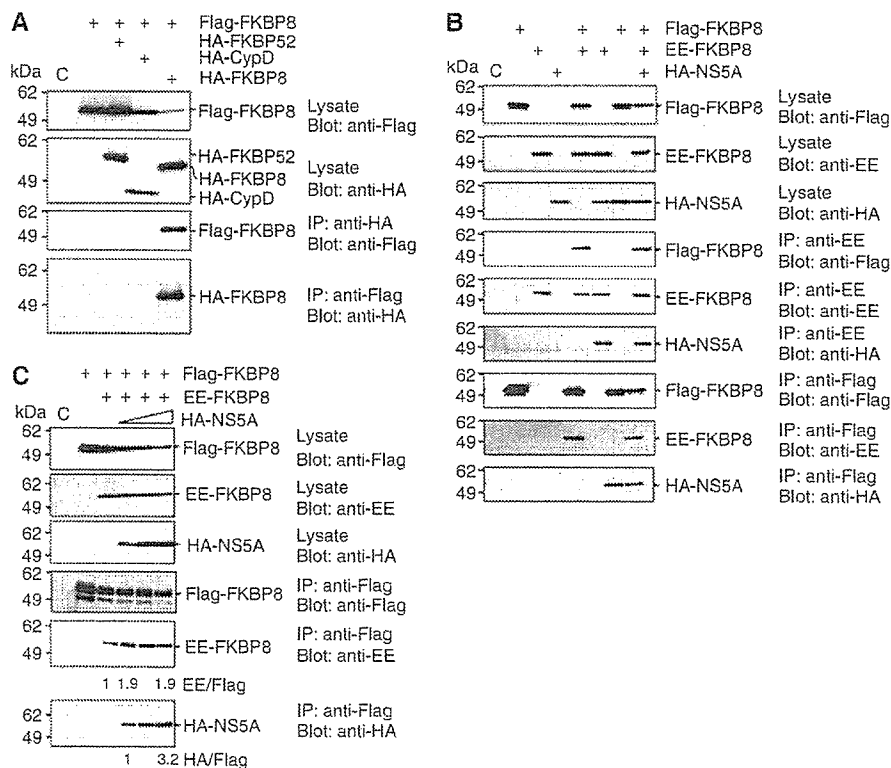


**Figure 3** Determination of the NS5A-binding region in FKBP8. (A) Schematic representation of FKBP8 and deleted mutants. (B) Flag-NS5A was coexpressed with HA-FKBP8 and its mutants in 293T cells. Proteins immunoprecipitated with anti-HA antibody were subjected to Western blotting. The data shown in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

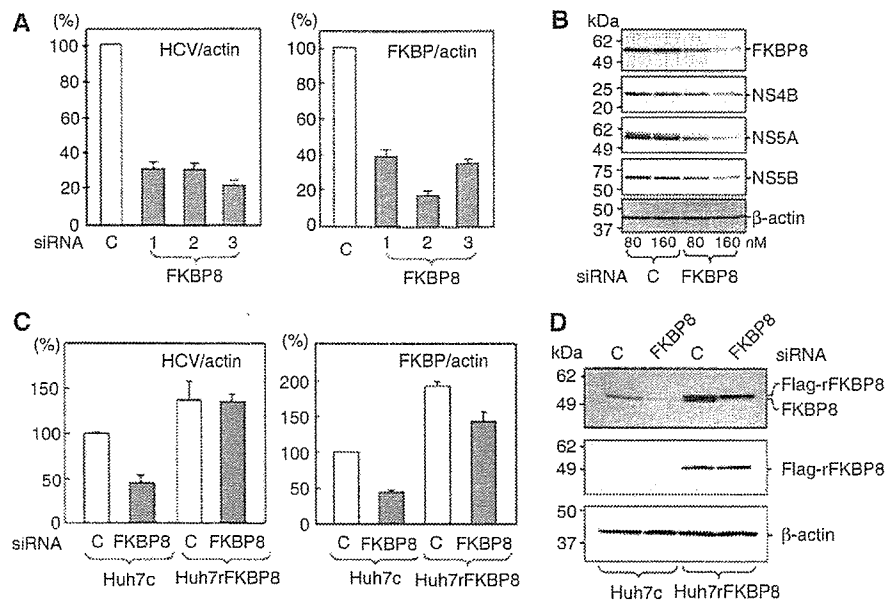
Flag-FKBP8 was coexpressed with HA-FKBP52, HA-CypD, or HA-FKBP8 in 293T cells and it was immunoprecipitated with anti-Flag or anti-HA antibody. Flag-FKBP8 and HA-FKBP8 were co-immunoprecipitated with each antibody, but not with HA-FKBP52 or HA-CypD. It is known that Hsp90 forms a homodimer and also interacts 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 co-expressed 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  $\mu$ g 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  $\beta$ -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  $\beta$ -actin mRNA. (D) Levels of expression of endogenous FKBP8, exogenous Flag-rFKBP8, and  $\beta$ -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.

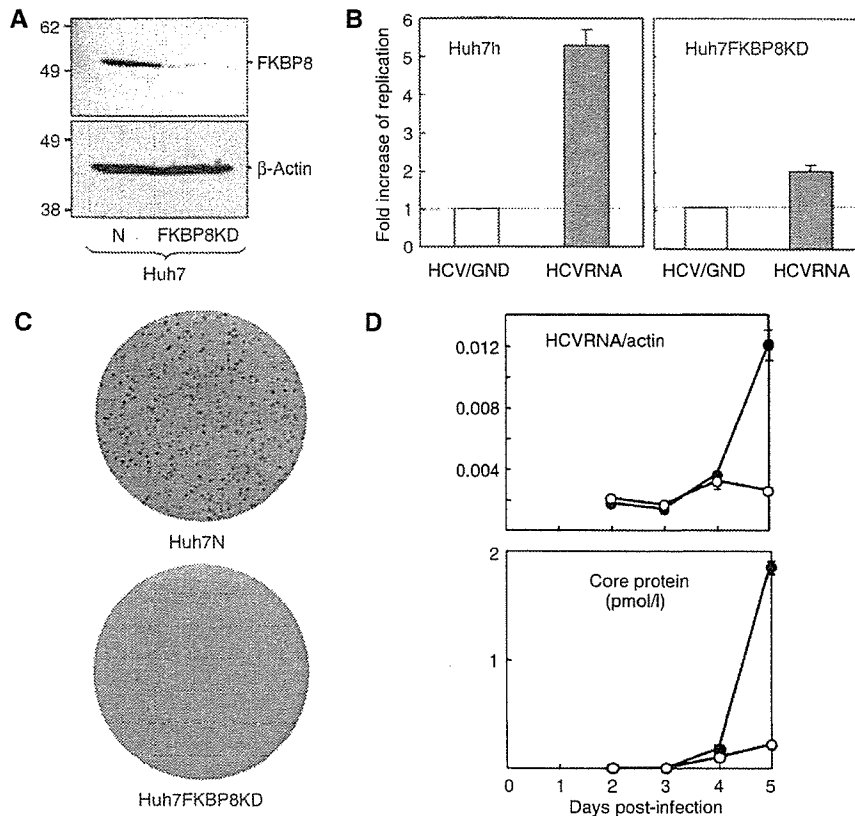
and empty plasmid, respectively, were pooled and then transfected with the FKBP8 siRNA (Target 1) or control siRNA. Although transfection of the FKBP8 siRNA led to a 60% reduction of HCV RNA and FKBP8 mRNA in Huh7c cells, in comparison with levels in cells transfected with the control siRNA, no reduction 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/ $\mu$ g 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  $\beta$ -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-1<sub>389</sub> hRL/NS3-3'/NK5.1 (HCVRNA), or a replication-negative mutant, pFK-1<sub>389</sub> 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-1<sub>389</sub> 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.

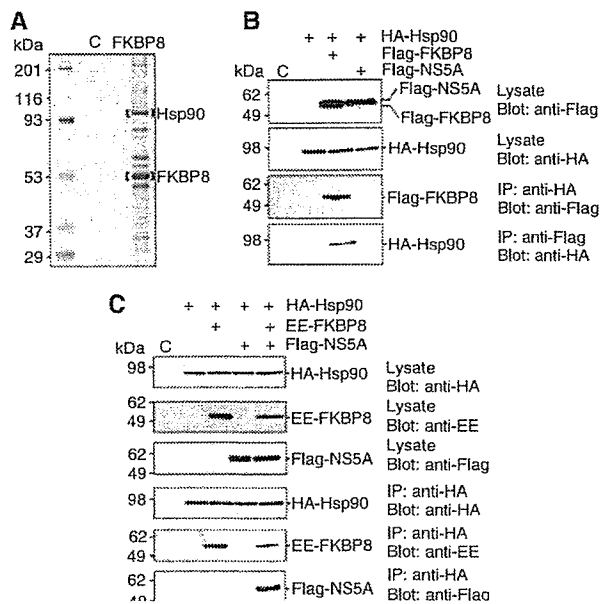
#### FKBP8 interacts with NS5A and Hsp90 via different sites in the TPR domain

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-Hsp90 $\Delta$ MEEVD) 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<sup>307</sup> and Arg<sup>311</sup> 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<sup>307</sup> and Arg<sup>311</sup> 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

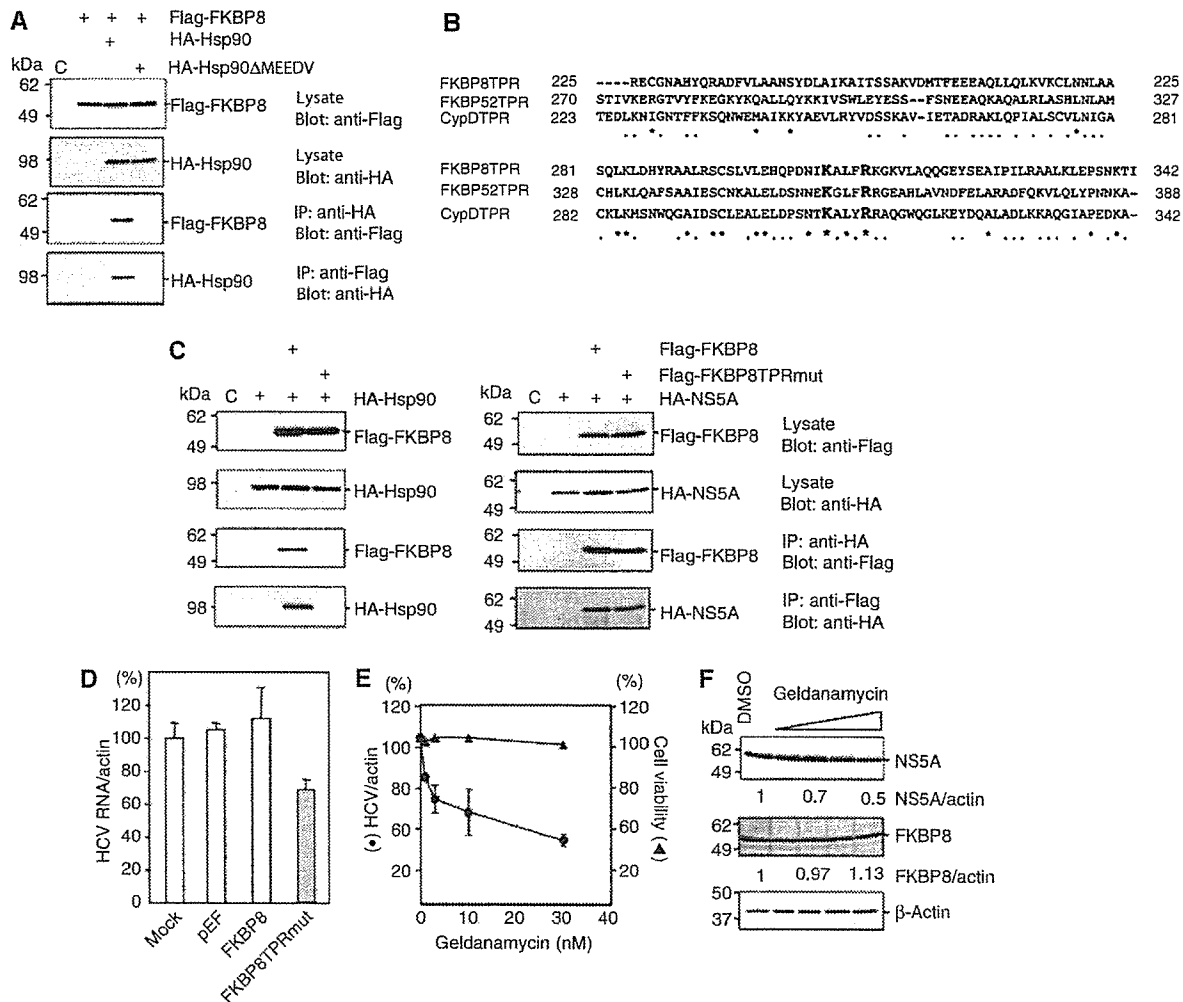
HCV replication by disruption of NS5A/FKBP8/Hsp90 complex. These results suggest that a protein complex composed of FKBP8, Hsp90, and NS5A is involved in HCV RNA replication.

## 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 Lys<sup>307</sup> and Arg<sup>311</sup> 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<sup>307</sup> and Arg<sup>311</sup> 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 PRISM 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<sub>2</sub>.

### 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'-GAGUGGCUGGACAUUC UGG-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'-AGTCAGTTGCTCGCCTTC-3' and 5'-CGGGGAATTTCTGCTTC-3',

5'-TGGAGTCTGTGGCATCCACGAACTACCTTCAACTC-3' and 5'-CGGACTCGTCATACCTCTGCTTGTCTGATCCACATC-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  $\beta$ -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  $\mu$ g/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'-GATCCGCTGGAACCTTCCAACAAGTTCAAGAGACTTGTGGGAAGTTCCAGCTTA-3', and 5'-AGCTTAAGCTGGAACCTTCCAACAAGTCTCTGAACTGTTGGAAGTTCCAGCG-3', were annealed and introduced between the *Bam*HI and *Hind*III sites of pSilencer<sup>TM</sup> 2.1-U6 hygro (Ambion, Austin, TX) according to the manufacturer's protocol. An HCV replicon cell line cured with IFN- $\alpha$  was transfected with 5  $\mu$ g of the plasmid by electroporation. The culture medium was replaced with DMEM supplemented with 10% FCS and 500  $\mu$ g/ml of Hygromycin B (Wako, Tokyo, Japan) at 24 h post-transfection. The remaining cells were re-seeded in 96-well plates and cloned for the colony formation and transient replication assays.

#### Colony formation assay

The plasmid pFK-I<sub>389</sub> 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  $\mu$ g) was introduced into Huh7 cells at 4 million cells/0.4 ml by electroporation at 270 V and 960  $\mu$ F using a Gene Pulser<sup>TM</sup> (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.

## References

- Aizaki H, Aoki Y, Harada T, Ishii K, Suzuki T, Nagamori S, Toda G, Matsuura Y, Miyamura T (1998) Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. *Hepatology* 27: 621–627
- Appel N, Pietschmann T, Bartenschlager R (2005) Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. *J Virol* 79: 3187–3194
- Boguski MS, Sikorski RS, Hieter P, Goebel M (1990) Expanding family. *Nature* 346: 114
- Brinker A, Scheufler C, Von Der Mulbe F, Fleckenstein B, Herrmann C, Jung G, Moarefi I, Hartl FU (2002) Ligand discrimination by TPR domains. Relevance and selectivity of EEVD-recognition in Hsp70  $\times$  Hop  $\times$  Hsp90 complexes. *J Biol Chem* 277: 19265–19275
- Chadli A, Bouhouche I, Sullivan W, Stensgard B, McMahon N, Catelli MG, Toft DO (2000) Dimerization and N-terminal domain proximity underlie the function of the molecular chaperone heat shock protein 90. *Proc Natl Acad Sci USA* 97: 12524–12529

#### Transient replication assay

The cDNA encoding *Renilla* luciferase was introduced between the *Asc*I and *Pme*I sites of the plasmid pFK-I<sub>389</sub> neo/NS3-3'/NK5.1, in place of the *neo* gene. The resulting plasmid, pFK-I<sub>389</sub> 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  $\mu$ g of *in vitro*-transcribed RNA at a 400- $\mu$ l volume; the cells were then electroporated at 270 V and 960  $\mu$ F by a Gene Pulser<sup>TM</sup> (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

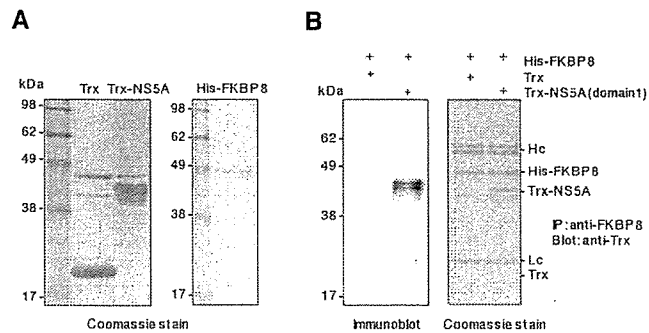
Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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- Cliff MJ, Harris R, Barford D, Ladbury JE, Williams MA (2006) Conformational diversity in the TPR domain-mediated interaction of protein phosphatase 5 with Hsp90. *Structure* 14: 415–426
- Edlich F, Weiwad M, Erdmann F, Fanghanel J, Jarczowski F, Rahfeld JU, Fischer G (2005) Bcl-2 regulator FKBP38 is activated by Ca(2+)/calmodulin. *EMBO J* 24: 2688–2699
- Evans MJ, Rice CM, Goff SP (2004) Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc Natl Acad Sci USA* 101: 13038–13043
- Fischer G, Aumuller T (2003) Regulation of peptide bond *cis/trans* isomerization by enzyme catalysis and its implication in physiological processes. *Rev Physiol Biochem Pharmacol* 148: 105–150
- Gao L, Aizaki H, He JW, Lai MM (2004) Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. *J Virol* 78: 3480–3488

- Hamamoto I, Nishimune Y, Okamoto T, Aizaki H, Lee K, Mori Y, Abe T, Lai MC, Miyamura T, Moriishi K, Matsuura Y (2005) Human VAP-B is involved in HCV replication through interaction with NS5A and NS5B. *J Virol* 79: 13473–13482
- Hirano T, Kinoshita N, Morikawa K, Yanagida M (1990) Snap helix with knob and hole: essential repeats in *S. pombe* nuclear protein nuc2+. *Cell* 60: 319–328
- Huang DC, Cory S, Strasser A (1997) Bcl-2, Bcl-XL and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. *Oncogene* 14: 405–414
- Ichimura T, Yamamura H, Sasamoto K, Tominaga Y, Taoka M, Kakiuchi K, Shinkawa T, Takahashi N, Shimada S, Isobe T (2005) 14-3-3 proteins modulate the expression of epithelial Na<sup>+</sup> channels by phosphorylation-dependent interaction with Nedd4-2 ubiquitin ligase. *J Biol Chem* 280: 13187–13194
- Inoue K, Sekiyama K, Yamada M, Watanabe T, Yasuda H, Yoshiba M (2003) Combined interferon alpha2b and cyclosporin A in the treatment of chronic hepatitis C: controlled trial. *J Gastroenterol* 38: 567–572
- Kapadia SB, Chisari FV (2005) Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc Natl Acad Sci USA* 102: 2561–2566
- Kashiwagi T, Hara K, Kohara M, Iwahashi J, Hamada N, Honda-Yoshino H, Toyoda T (2002) Promoter/origin structure of the complementary strand of hepatitis C virus genome. *J Biol Chem* 277: 28700–28705
- Koch JO, Bartenschlager R (1999) Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A, and NS4B. *J Virol* 73: 7138–7146
- Lackner T, Muller A, Konig M, Thiel HJ, Tautz N (2005) Persistence of bovine viral diarrhoea virus is determined by a cellular cofactor of a viral autoprotease. *J Virol* 79: 9746–9755
- Lam E, Martin M, Wiederrecht G (1995) Isolation of a cDNA encoding a novel human FK506-binding protein homolog containing leucine zipper and tetratricopeptide repeat motifs. *Gene* 160: 297–302
- Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM (2005) Complete replication of hepatitis C virus in cell culture. *Science* 309: 623–626
- Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285: 110–113
- Macdonald A, Harris M (2004) Hepatitis C virus NS5A: tales of a promiscuous protein. *J Gen Virol* 85: 2485–2502
- Maggioni C, Braakman I (2005) Synthesis and quality control of viral membrane proteins. *Curr Top Microbiol Immunol* 285: 175–198
- Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK (2001) Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 358: 958–965
- Mayer MP (2005) Recruitment of Hsp70 chaperones: a crucial part of viral survival strategies. *Rev Physiol Biochem Pharmacol* 153: 1–46
- Moriishi K, Inoue S, Koura M, Amano F (1999) Inhibition of listeriolysin O-induced hemolysis by bovine lactoferrin. *Biol Pharm Bull* 22: 1167–1172
- Moriishi K, Matsuura Y (2003) Mechanisms of hepatitis C virus infection. *Antivir Chem Chemother* 14: 285–297
- Nakagawa M, Sakamoto N, Tanabe Y, Koyama T, Itsui Y, Takeda Y, Chen CH, Kakinuma S, Oooka S, Maekawa S, Enomoto N, Watanabe M (2005) Suppression of hepatitis C virus replication by cyclosporin A is mediated by blockade of cyclophilins. *Gastroenterology* 129: 1031–1041
- Neckers L (2002) Hsp90 inhibitors as novel cancer chemotherapeutic agents. *Trends Mol Med* 8: S55–S61
- Neddermann P, Clementi A, De Francesco R (1999) Hyperphosphorylation of the hepatitis C virus NS5A protein requires an active NS3 protease, NS4A, NS4B, and NS5A encoded on the same polyprotein. *J Virol* 73: 9984–9991
- Nielsen JV, Mitchelmore C, Pedersen KM, Kjaerulff KM, Finsen B, Jensen NA (2004) Fkbp8: novel isoforms, genomic organization, and characterization of a forebrain promoter in transgenic mice. *Genomics* 83: 181–192
- Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108: 193–199
- Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, Strand D, Bartenschlager R (2002) Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J Virol* 76: 4008–4021
- Pietschmann T, Lohmann V, Rutter G, Kurpanek K, Bartenschlager R (2001) Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. *J Virol* 75: 1252–1264
- Pirkl F, Fischer E, Modrow S, Buchner J (2001) Localization of the chaperone domain of FKBP52. *J Biol Chem* 276: 37034–37041
- Qing K, Hansen J, Weigel-Kelley KA, Tan M, Zhou S, Srivastava A (2001) Adeno-associated virus type 2-mediated gene transfer: role of cellular FKBP52 protein in transgene expression. *J Virol* 75: 8968–8976
- Scheufler C, Brinker A, Bourenkov G, Pegoraro S, Moroder L, Bartunik H, Hartl FU, Moarefi I (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell* 101: 199–210
- Shirane M, Nakayama KI (2003) Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. *Nat Cell Biol* 5: 28–37
- Silverstein AM, Galigniana MD, Kanelakis KC, Radanyi C, Renoir JM, Pratt WB (1999) Different regions of the immunophilin FKBP52 determine its association with the glucocorticoid receptor, hsp90, and cytoplasmic dynein. *J Biol Chem* 274: 36980–36986
- Tellinghuisen TL, Marcotrigiano J, Gorbalenya AE, Rice CM (2004) The NS5A protein of hepatitis C virus is a zinc metalloprotein. *J Biol Chem* 279: 48576–48587
- Tellinghuisen TL, Marcotrigiano J, Rice CM (2005) Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature* 435: 374–379
- Tu H, Gao L, Shi ST, Taylor DR, Yang T, Mircheff AK, Wen Y, Gorbalenya AE, Hwang SB, Lai MM (1999) Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. *Virology* 263: 30–41
- Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 11: 791–796
- Wang C, Gale Jr M, Keller BC, Huang H, Brown MS, Goldstein JL, Ye J (2005) Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C Virus RNA replication. *Mol Cell* 18: 425–434
- Wasley A, Alter MJ (2000) Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin Liver Dis* 20: 1–16
- Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K (2003) Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* 38: 1282–1288
- Watashi K, Ishii N, Hijikata M, Inoue D, Murata T, Miyanari Y, Shimotohno K (2005) Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* 19: 111–122
- Waxman L, Whitney M, Pollok BA, Kuo LC, Darke PL (2001) Host cell factor requirement for hepatitis C virus enzyme maturation. *Proc Natl Acad Sci USA* 98: 13931–13935
- Wu B, Li P, Liu Y, Lou Z, Ding Y, Shu C, Ye S, Bartlam M, Shen B, Rao Z (2004) 3D structure of human FK506-binding protein 52: implications for the assembly of the glucocorticoid receptor/Hsp90/immunophilin heterocomplex. *Proc Natl Acad Sci USA* 101: 8348–8353
- Ye J, Wang C, Sumpter Jr R, Brown MS, Goldstein JL, Gale Jr M (2003) Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. *Proc Natl Acad Sci USA* 100: 15865–15870
- Yi M, Lemon SM (2004) Adaptive mutations producing efficient replication of genotype 1a hepatitis C virus RNA in normal Huh7 cells. *J Virol* 78: 7904–7915
- Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV (2005) Robust hepatitis C virus infection *in vitro*. *Proc Natl Acad Sci USA* 102: 9294–9299



**Supplementary Figure 1. NS5A directly binds to FKBP8**

Purified thioredoxin, Trx-NS5A (A) and His-FKBP8 (B) in gel were stained with Coomassie brilliant blue G-250. These proteins were mixed and subjected to immunoprecipitation with anti-FKBP8 antibody. Precipitates were immunoblotted with anti-thioredoxin antibody (C) and stained with Coomassie brilliant blue G-250 (D).