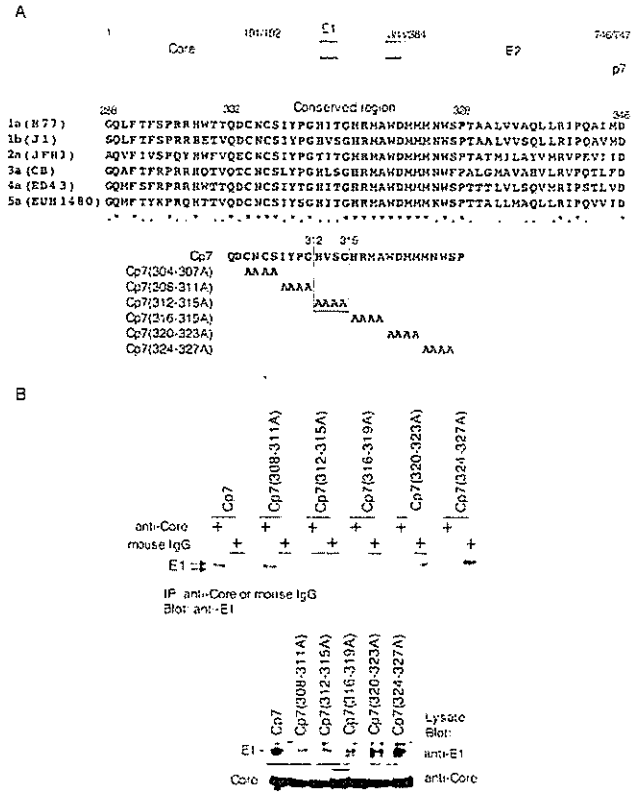

FIG. 6. Four amino acid residues, 312 to 315, in the cytoplasmic region of the E1 protein are important for interaction with the core protein. (A) Alignment of the amino acid sequence of the E1 cytoplasmic region among different HCV genotypes (1a, H77 [AF009606]; 1b, J1 [D89815]; 2a, JFH1 [AB047639]; 3a, CB [AF046866]; 4a, ED43 [Y11604]; 5a, EUH1480 [Y13184]). A conserved region from Gln^{302} to Pro^{328} 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^{312}, Val^{313}, Ser^{314}, and Gly^{315}, 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 $MgCl_2$ 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^{312}, Val^{313}, Ser^{314}, and Gly^{315}) 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^{312}, Val^{313}, Ser^{314}, and Gly^{315} 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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Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90

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

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Introduction

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

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

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

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

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

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

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

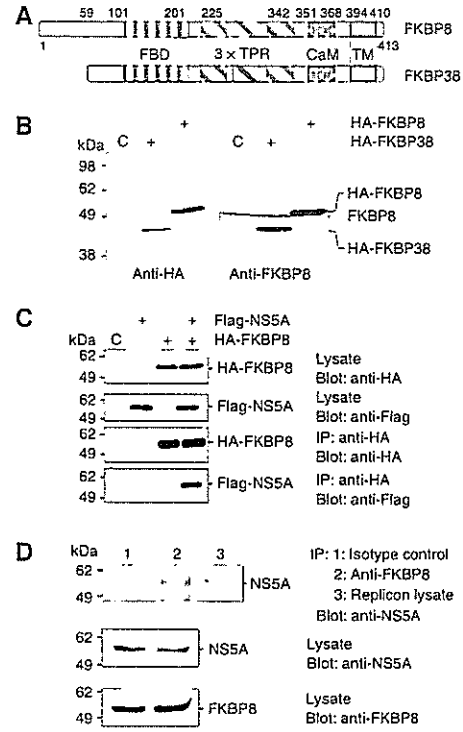

Figure 1 Expression of FKBP8 and FKBP38 in mammalian cells. (A) Schematic representation of FKBP8 and FKBP38. The FK506-binding domain (FBD), tetratricopeptide repeat (TPR), putative calmodulin binding motif (CaM), and transmembrane domain (TM) are shown. (B) N-terminally HA-tagged FKBP8 and FKBP38 were 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_6-tagged FKBP8 (His-FKBP8) and thioredoxin-fused domain 1 of NS5A (Trx-NS5A) prepared in *Escherichia coli* were examined by pull-down

analysis. Trx-NS5A was co-precipitated with His-FKBP8 by anti-FKBP8 antibody (Supplementary Figure 1), suggesting that FKBP8 can directly bind to NS5A domain I.

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

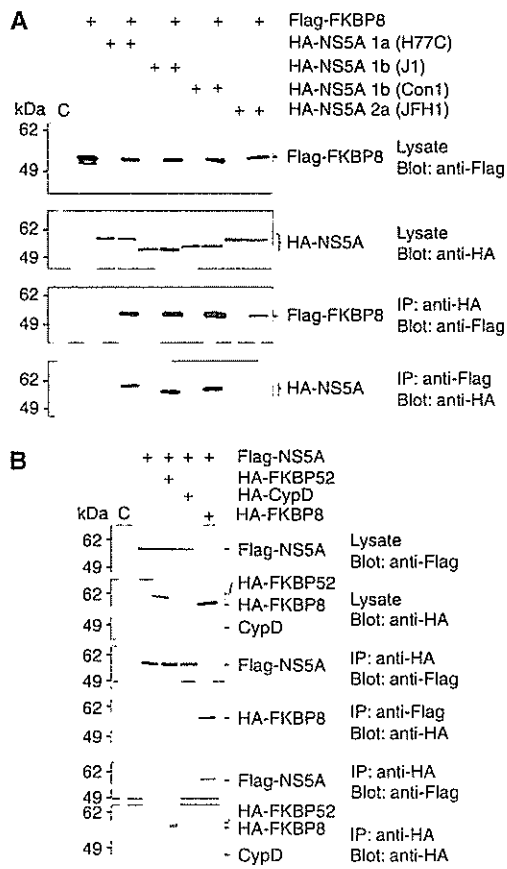

Figure 2 Specific interaction between FKBP8 and NS5A. (A) HA-NS5As were obtained from several genotypes of HCV and were expressed with Flag-FKBP8 in 293T cells. Proteins immunoprecipitated with anti-HA or Flag antibody were subjected to Western blotting. (B) Flag-NS5A was coexpressed with HA-FKBP8, -CypD,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

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

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

FKBP8 forms a homomultimer and a heteromultimer with NS5A

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

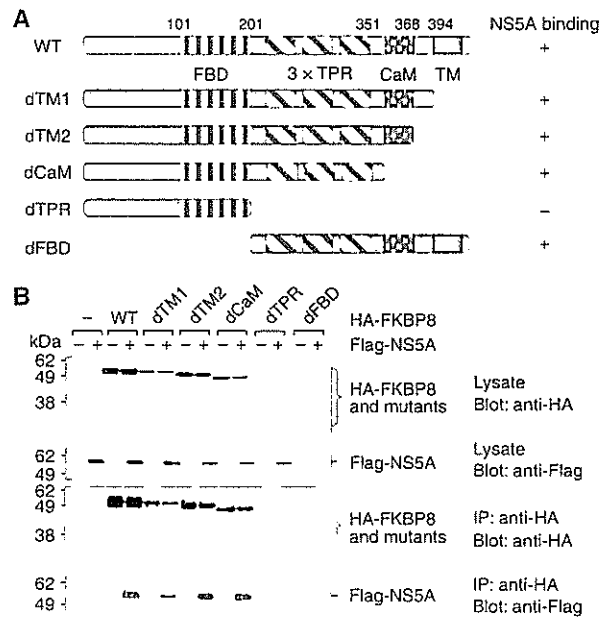

Figure 3 Determination of the NS5A-binding region in FKBP8. (A) Schematic representation of FKBP8 and deleted mutants. (B) Flag-NS5A was coexpressed with HA-FKBP8 and its mutants in 293T cells. Proteins immunoprecipitated with anti-HA antibody were subjected to Western blotting. The data shown in each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

Flag-FKBP8 was coexpressed with HA-FKBP52, HA-CypD, or HA-FKBP8 in 293T cells and it was immunoprecipitated with anti-Flag or anti-HA antibody. Flag-FKBP8 and HA-FKBP8 were co-immunoprecipitated with each antibody, but not with HA-FKBP52 or HA-CypD. It is known that Hsp90 forms a homodimer and also interacts with FKBP52 through TPR domain as FKBP8 (Chadli *et al*, 2000). If homodimer of FKBP8 is due to intermediating of Hsp90 as FKBP8-Hsp90-Hsp90-FKBP8 complex, FKBP52 would be co-precipitated with FKBP8 as FKBP8-Hsp90-Hsp90-FKBP52. However, we could not detect any association of FKBP8 and FKBP52 in the immunoprecipitation analysis (Figure 4A). These data suggest that FKBP8 can form a homomultimer without Hsp90 and associate with neither FKBP52 nor CypD through Hsp90. To examine the effects of the interaction with NS5A on the homomultimerization of FKBP8, HA-NS5A was 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

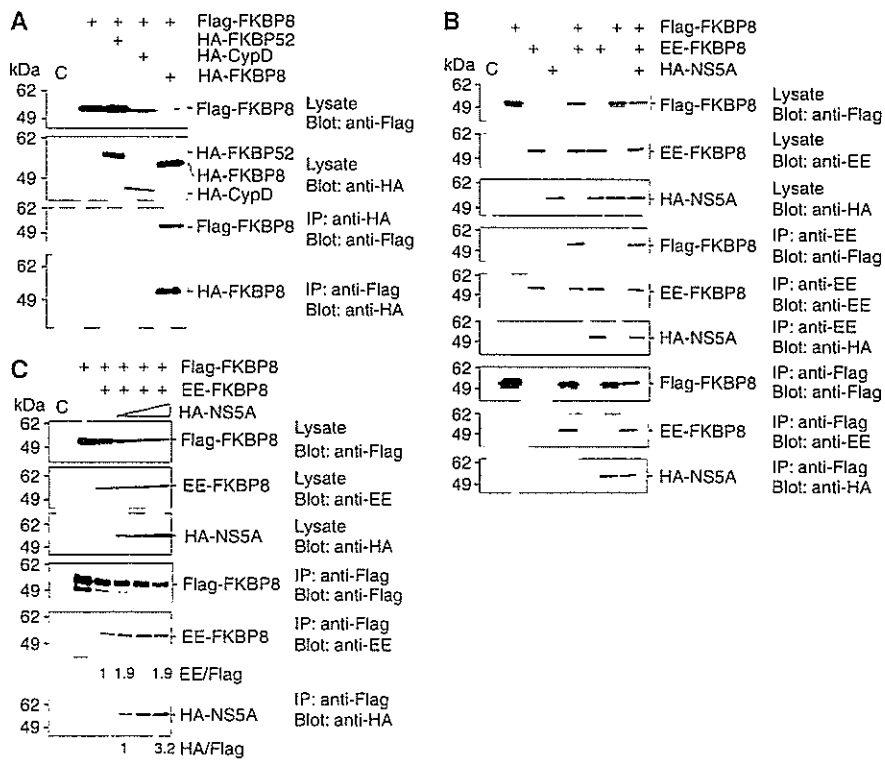

Figure 4 Homomultimerization of FKBP8. (A) Flag-FKBP8 was coexpressed with HA-FKBP52, -CypD, or -FKBP8 in 293T cells, and was immunoprecipitated with anti-HA or Flag antibody. Precipitates were analyzed by Western blotting. (B) Flag- or EE-tagged FKBP8 was coexpressed with HA-NS5A in 293T cells and was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

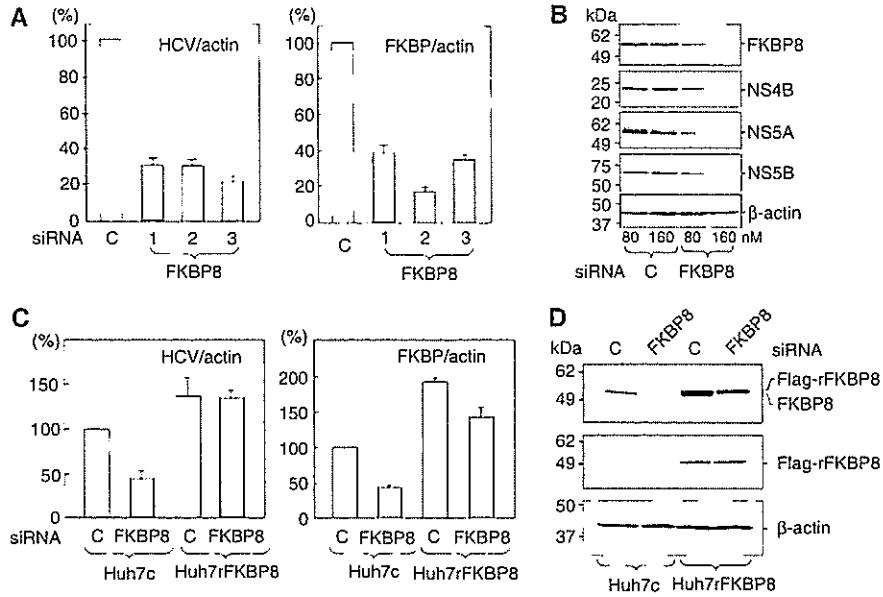

Figure 5 Decrease in HCV RNA by FKBP8-targeted siRNA. (**A**) HCV replicon cells (9–13 cells) were transfected with each of three kinds of siRNA targeted to FKBP8 or nontargeted siRNA at a final concentration of 80 nM. Transfected cells were collected at 72 h post-transfection, and FKBP8 mRNA and HCV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

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

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

FKBP8 forms a multicomplex with NS5A and Hsp90

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

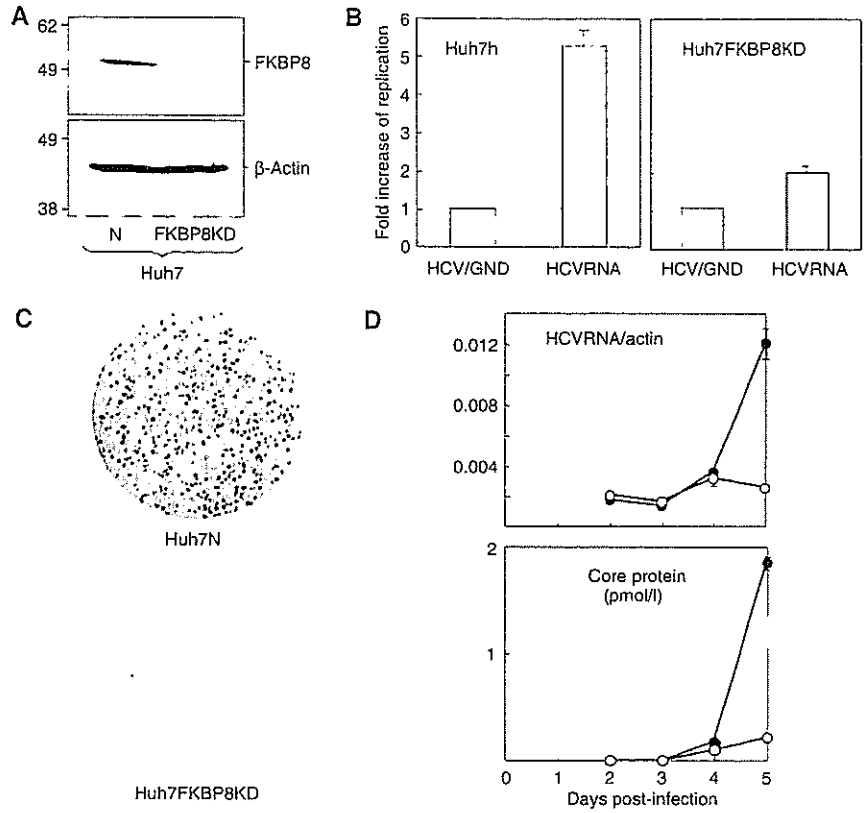

Figure 6 Effect of knockdown of FKBP8 on the transient replication, colony formation, and viral infection. (**A**) Levels of expression of FKBP8 and β-actin in Huh7N and Huh7 FKBP8KD cell lines bearing plasmids encoding shRNA for control mRNA (lane 1) and for FKBP8 mRNA (lane 2), respectively. (**B**)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

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

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

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

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

Hsp90 participates in the replication of HCV RNA

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

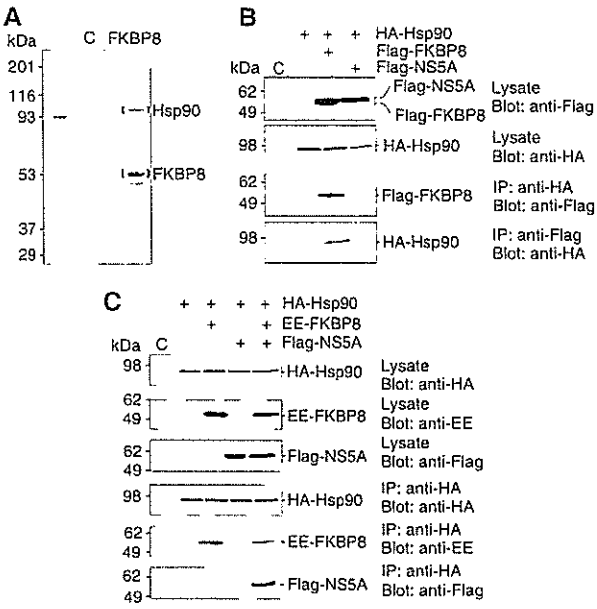

Figure 7 FKBP8 forms complex with NS5A and Hsp90. (A) An N-terminally myc-TEV-Flag-tagged FKBP8 was expressed in 293T cells and immunoprecipitated. The precipitated proteins were applied to SDS–PAGE and then stained with silver staining. Hsp90 and FKBP8 were identified by LC-MS/MS. (B) HA-Hsp90 was coexpressed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

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

Discussion

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

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

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

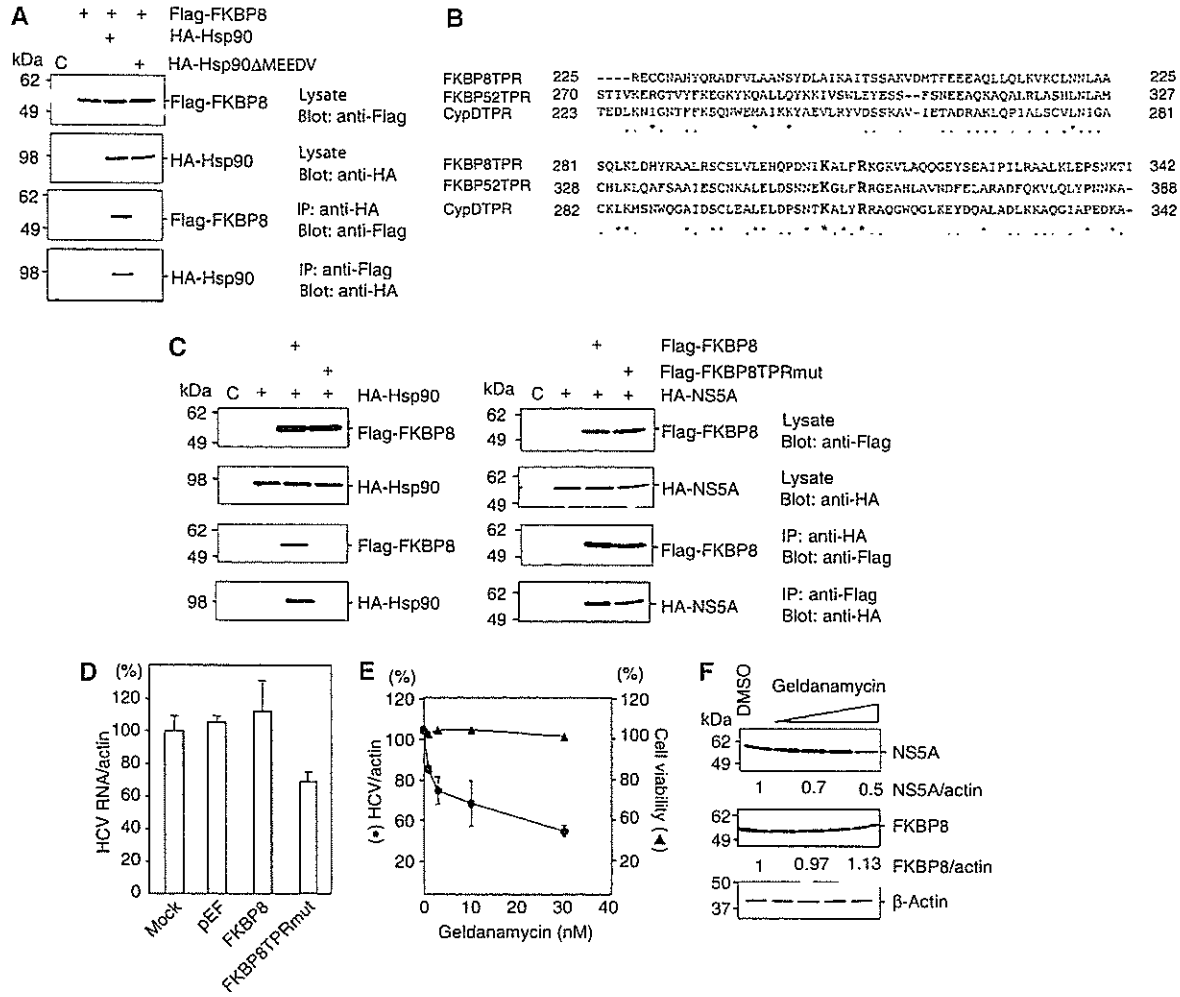

Figure 8 FKBP8 interacts with NS5A and Hsp90 via different sites in the TPR domain and participates in HCV replication. (**A**) Flag-FKBP8 was coexpressed with HA-Hsp90 or HA-Hsp90ΔMEEDV lacking the C-terminal MEEDV residues and was immunoprecipitated by anti-HA or anti-Flag antibody. Precipitates were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

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

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

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

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

Materials and methods

Yeast two-hybrid assays

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

Plasmids

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

Cell lines

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

Antibodies

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

Transfection, immunoblotting, and immunoprecipitation

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

Gene silencing by siRNA

The siRNA targeted to FKBP8, Target-1: 5′-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′-AGTCAGTTGTCTGCGCTTTC-3′ and 5′-CGGGGAATTTCCTGGTCTTC-3′,

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

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

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

Colony formation assay

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

Transient replication assay

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

Generation of infectious HCV particles

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

Determination of FKBP8-binding proteins

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

Supplementary data

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

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Overexpression of Hepatitis C Virus NS5A Protein Induces Chromosome Instability *via* Mitotic Cell Cycle Dysregulation

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Hepatocellular carcinoma (HCC) is a common primary cancer associated with high incidences of genetic variations including chromosome instability. Moreover, it has been demonstrated that hepatitis C virus (HCV) is one of the major causes of HCC. However, no previous work has assessed whether HCV proteins are associated with the induction of chromosome instability. Here, we found that liver cell lines constitutively expressing full-length or truncated versions of the HCV genome show a high incidence of chromosome instability. In particular, the overexpression of HCV NS5A protein in cultured liver cells was found to promote chromosome instability and aneuploidy. Further experiments showed that NS5A-induced chromosome instability is associated with aberrant mitotic regulations, such as, an unscheduled delay in mitotic exit and other mitotic impairments (e.g. multi-polar spindles). Thus, our results indicate that HCV NS5A protein may be directly involved in the induction of chromosome instability *via* mitotic cell cycle dysregulation, and provide novel insights into the molecular mechanisms of HCV-associated hepatocarcinogenesis.

Keywords: hepatitis C virus; NS5A; mitotic cell cycle; chromosome instability; hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is a common primary cancer that is often associated with hepatitis B (HBV) and hepatitis C (HCV) viral infections. In particular, more than 60% of HCV infections lead to chronic hepatitis, which can sequentially progress to chronic active hepatitis, liver cirrhosis, and HCC.[1–3] HCV is an envelope RNA virus and contains a single-stranded positive-sense RNA genome that encodes a precursor polypeptide of approximately 3000 amino acid residues. Host and viral proteases cleave this precursor polypeptide into at least ten individual proteins; namely, core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B proteins.[4,5] Moreover, recent studies indicate that HCV viral proteins can play direct roles in oncogenesis.[6–9] For example, transgenic mice harboring HCV core protein, structural proteins, or the full-length genome exhibit marked

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Abbreviations used: HCV, hepatitis C virus; NS, non-structural; HCC, hepatocellular carcinoma; APC/C, anaphase promoting complex/cyclosome; LOH, loss of heterozygosity; NEBD, nuclear envelope breakdown.

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liver steatosis and spontaneous HCC development after long periods of latency.[7–9] Interestingly, the development of HCC appears to depend on the host's genetic background, HCV genotype, and the expression levels of introduced HCV genes.[6,10–13] In general, it is believed that HCV proteins significantly cause and/or enhance the risk of liver cancer.

Previous studies suggest that in HCC, neoplastic cells originate from rapidly dividing hepatocytes or liver stem cells that have accumulated genetic alterations and thus demonstrate genomic instability.[14] Moreover, the majority of HCC cells display a high incidence of chromosome instability, including structural chromosomal alterations, and allelic losses and gains. In addition, chromosome instability is evident in cirrhotic liver tissues, and has been found to increase during the hepatocarcinogenesis process.[15,16] Although chromosome aneuploidization is found in many human cancers, it is unclear whether aneuploidy is a consequence of cell transformation, or whether it contributes to tumor progression by increasing tumor suppressor losses and protooncogene gains. Growing evidence indicates that aneuploidy occurs as a result of chromosome missegregation in response to a number of abnormalities, such as, double-strand DNA breaks and the loss of cell cycle checkpoint controls.[17,18] Faithful chromosome segregation is controlled by the mitotic machinery, which includes the kinetochore complex, spindle, centrosome and cohesion complex, and the inactivation or dysregulation of these components causes mitotic defects that can lead to chromosome missegregation and aneuploidy. Although studies have not yet established a direct molecular link between defects in the mitotic checkpoint and aneuploidy in cancer, a growing list of molecular components and processes known to cause chromosome missegregation *in vitro* and *in vivo* may be considered prime candidates.[19–22]

Normally, mitotic checkpoint controls trigger cell cycle arrest and/or the elimination of cells harboring cell cycle defects. However, some cells with constitutive checkpoint defects, such as those caused by DNA tumor virus infections, are able to escape apoptosis and adapt cell cycle progression, thus allowing the continuation of chromosome instability.[23,24] Interestingly, HTLV-1 TAX oncoprotein has been shown to target MAD1 mitotic checkpoint protein, and thus lead to impaired checkpoint function, multi-nucleation, and aneuploidy.[23] Other DNA tumor virus proteins, including E1A and E1B from adenovirus, E6 and E7 from papillomavirus, and large and small T antigens from simian virus 40 (SV40) have been shown to interact with major cellular regulators and induce mitotic abnormalities.[24] Furthermore, HBx protein, which is frequently integrated into the cellular genome, and which is expressed during the HCC development, has been reported to induce centrosome amplification, multi-polar spindles, and aneuploidy.[25] These previous results collectively suggest that mitotic checkpoint impairments may represent a common pathway for viral oncogenesis, and that viral proteins may affect chromosome segregation, leading to mitotic abnormalities and increased aneuploidy.

Previously, Smith *et al.*[14] performed microarray analyses on HCV-associated HCC specimens, and found an association between chromosomal segregation abnormalities and the improper expressions of potential HCC marker genes, such as, Aurora A, CDC2, mitotic cyclins, cyclin-dependent kinases, p53-related genes, and CENP-F. Furthermore, Kawai *et al.*[16] showed that HCC tissues associated with HCV infection show significant losses of heterozygosity (LOH), although the rate of LOH was higher in HBV-associated tissues. Consistent with this finding, LOH was detected more frequently in malignant tumors than in normal liver tissues[26,27] and was almost always accompanied by chromosome instability. These observations raise the possibility that HCV infection may be associated with chromosome instability, which is a known cause of neoplastic degeneration and hepatocarcinogenesis. However, due to the limitations of tissue culture and animal model systems, no previous work has directly examined whether HCV proteins are associated with the induction of chromosome instability.

Here, we examined whether one or more HCV proteins are directly involved in the acquisition of chromosome instability. Our analysis of HCV-associated HCC tumor tissues showed marked chromosomal aberrations, and liver cell lines constitutively expressing full-length or truncated versions of the HCV genome exhibited a high incidence of chromosome instability. In particular, the overexpression of HCV NS5A protein appeared to result in an unscheduled delay in mitotic exit and in mitotic abnormalities (e.g. multi-polar spindles). Moreover, these discrepancies were associated with chromosome instability and aneuploidy in NS5A-expressing cells. These results indicate that there is a direct link between HCV proteins and chromosome aneuploidy in hepatocytes.

Results

HCV non-structural proteins appear to act in an integrated manner during the induction of chromosome instability

Previously, we established stable human hepatoblastoma-derived HepG2 cell lines expressing the entire HCV ORF (Hep394), or ORF fragments including core to NS3 protein (Hep352) or NS2 (C-terminal 52 amino acid residues) to NS5B protein (Hep3294), and used immunochemical analyses to confirm the proper expressions and processing of these proteins in these cell lines.[28] Due to the lack of a cell culture system that supports the efficient propagation of HCV, these cell lines were developed with the intention of providing a suitable

in vitro model of HCV infection. To examine whether HCV proteins are involved in the induction of chromosome instability, we treated parental HepG2 cells or established Hep394, Hep352 or Hep3294 cell lines with colcemid. Giemsa staining was used to visualize chromosomes, which were counted to determine cellular chromosome number distributions (a marker of chromosome instability; Figure 1(a) and (b)). It was found that the Hep394 cell line (expressing the entire HCV ORF) showed a significant increase in cellular chromosome number distribution *versus* parental HepG2 cells. Moreover, Hep3294 cells (expressing NS2 to NS5B proteins) also showed a dramatic increase in cellular chromosome number distribution, whereas Hep352 cells (expressing core to NS3 protein) showed chromosome number distributions similar to those of control HepG2 cells. This observation

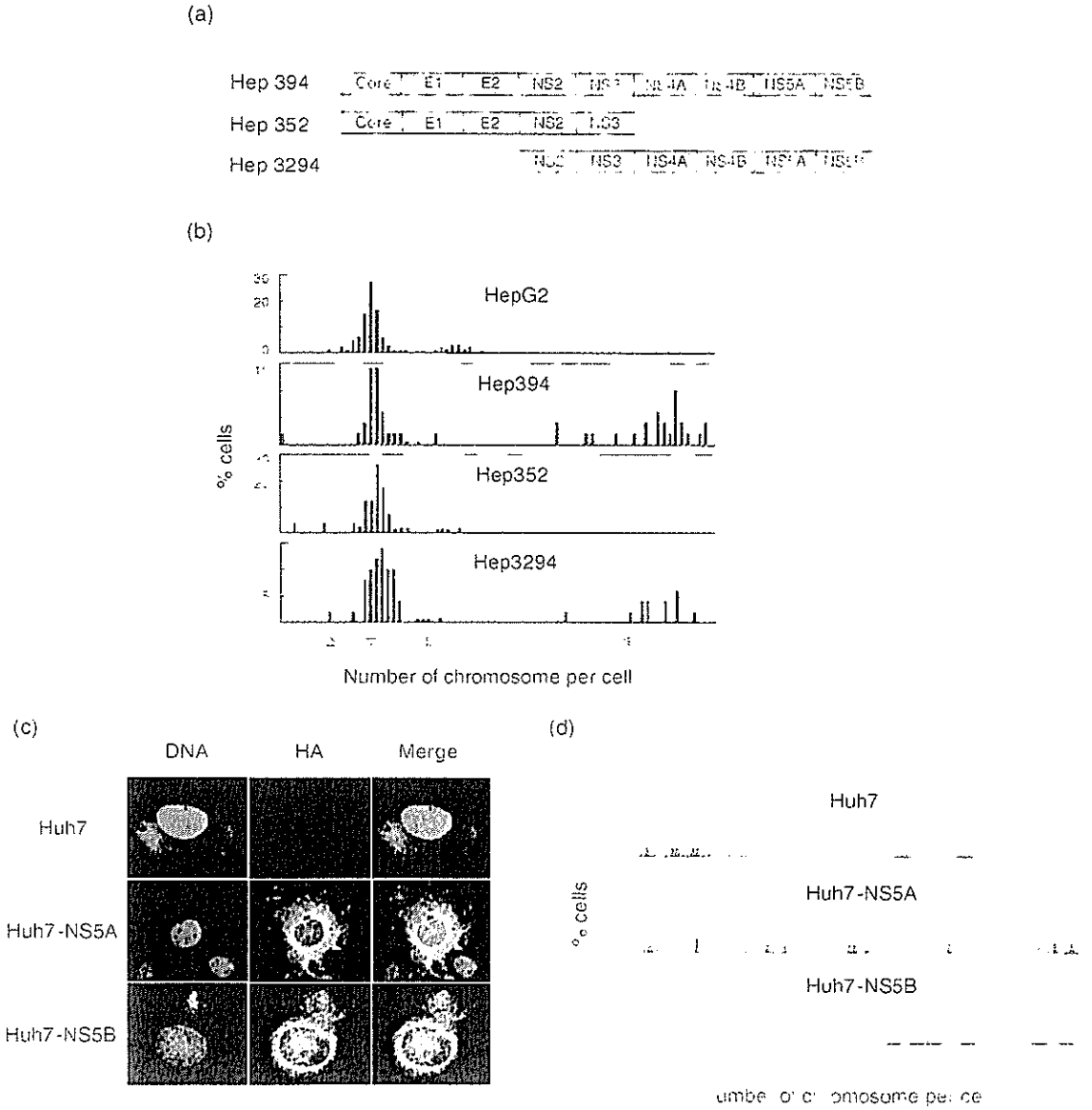


Figure 1. HCV non-structural proteins may be involved in the induction of chromosomal instability. (a) Schematic diagram showing the utilized independent HepG2-based cell lines constitutively expressing the full-length HCV ORF (Hep394), a truncated HCV including the core to NS3 sequences (Hep352), and a truncated HCV including the NS2 to NS5B sequences (Hep3294). The expression and processing of HCV proteins in these cell lines was previously confirmed by biochemical analyses.[28] (b) For metaphase chromosome analysis, parental HepG2 and established clones were cultured and treated with colcemid (0.04 mg/ml), and chromosomes were visualized by Giemsa staining. Chromosome number distributions were counted and analyzed in 200 cells per clone. (c) Expressions of HA-tagged NS5A or NS5B in selected Huh7 cell clones were assayed by immunofluorescence using an anti-HA antibody (red), and DNA was visualized by staining with Hoechst dye (blue). (d) Huh7 clones expressing HA-tagged NS5A (Huh7-NS5A) or NS5B (Huh7-NS5B) and control Huh7 cells were prepared (as described for (b)) for metaphase chromosome spreading analysis. Mitotic cells were randomly selected ($n = 200$ per clone) and chromosomes were counted.

provides first evidence that HCV proteins are directly involved in the induction of chromosome instability.

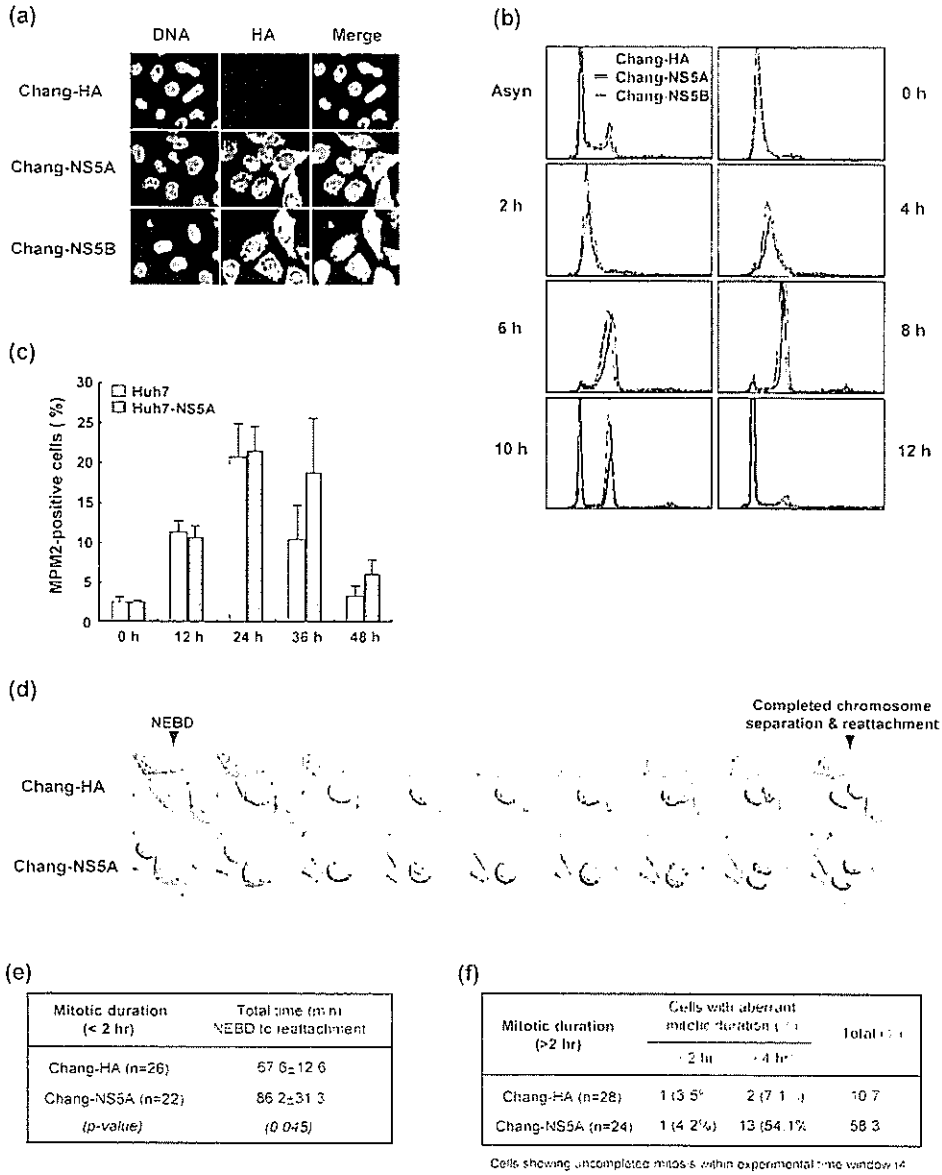
To identify the protein responsible for this effect, we compared the effects of expression plasmids encoding NS4A/4B or NS5A and NS5B on hepatocyte growth. Consistent with the previous findings,[29] flow cytometry revealed that cells co-transfected with NS5A and NS5B expression plasmids exhibited a shortened S phase and a significantly increased proportion of cells arrested in the G2 and M phases, as compared to cells transfected with NS4A/4B alone or with empty vector (data not shown), indicating that NS5A and/or NS5B may affect the cell cycle by regulating chromosome replication or chromosome segregation. To address whether HCV proteins, especially NS5A and NS5B, play roles in chromosomal instability and aneuploidy, we generated stable cell lines expressing HCV NS5A and NS5B in HBV genome-negative hepatocellular carcinoma-derived Huh7 cells (Figure 1(c) and (d)). Individual G418-resistant stable Huh7 cell clones were screened for NS5A and NS5B expression by immunofluorescence (Figure 1(c)) and immunoblotting (data not shown) using an anti-HA antibody. The data described below were obtained using single clones showing moderate levels of NS5A or NS5B expression, and all results were confirmed by examining two additional independent clones per cell line. NS5A-expressing and control Huh7 cells were cultured, treated with colcemid and subjected to Giemsa staining. As shown in Figure 1(d), Huh7-NS5A cells showed markedly increased chromosomal number distributions (143–155 chromosomes in ~60% of cells) as compared with control Huh7 cells grown under the same conditions (72–82 chromosomes in ~80% of cells). In contrast, Huh7-NS5B cells showed virtually the same chromosome number distribution profile as control Huh7 cells. To rule out the possibility that the induction of chromosome instability by NS5A protein may have been dependent on the cell line used, we performed parallel experiments in control Chang-HA (empty vector-transfected) and Chang-NS5A cells. As expected, chromosome spreading analysis revealed that Chang-NS5A cells showed a significantly higher incidence of chromosome instability than the control Chang-HA cells (data not shown). Collectively, these results demonstrate that HCV NS5A protein expression induces chromosomal instability.

HCV NS5A protein expression affects the normal timing of mitotic progression by delaying mitotic exit

Aberrant chromosome distributions were observed in cells expressing HCV NS5A, and thus we compared cell cycle progressions through S, G2, and mitosis (M) in the established Chang clones, i.e. vector-transfected control Chang-HA cells, Chang-NS5A cells, and Chang-NS5B cells (Figure 2(a)). Cells were synchronized at the G1/S boundary using a double thymidine block, and following release from G1/S arrest cells were harvested and analyzed for DNA content by flow cytometry (Figure 2(b)). Interestingly, at 10 h and 12 h after release, cells expressing NS5A showed a significant delay in mitotic exit, as evidenced by an accumulation of cells at G2/M and a decrease in the G1 population. In contrast to Chang-NS5A, Chang-NS5B cells and control Chang-HA cells progressed similarly. To determine whether NS5A expression is associated with the accumulation of cells in G2 or M, we treated cells with nocodazole and monitored the populations of MPM2-positive cells (an indicator of the mitotic phase of the cell cycle) (Figure 2(c)). In control Chang cells, mitotic indexes increased rapidly from 12 h to 24 h after nocodazole treatment and then decreased sharply at 36 h post-treatment. In contrast, cells expressing NS5A protein maintained a constant mitotic index for up to 36 h after nocodazole treatment, indicating that NS5A protein expression interferes with the normal timing of mitotic cell cycle progression by delaying mitotic exit. Next, we observed the effect of NS5A protein expression on the timing and morphology of NS5A-expressing mitotic cells using time-lapse photomicroscopy at 2 min intervals. As each cell entered mitosis, nuclear envelope breakdown (NEBD) was set as time zero, and the relative times for individual cell types to complete chromosome separation were then determined (Figure 2(d)). In control Chang-HA cells, the time to completion of chromosome separation was 67.6($\pm$12.6) min (Figure 2(e)). Interestingly, NS5A-expressing cells showed a significant retardation of mitotic cell cycle progress, with an 86.2($\pm$31.3) min interval between NEBD and complete chromosome separation (Figure 2(e)). In addition, a significant proportion of NS5A-expressing cells failed to complete chromosome separation over the duration of imaging (4 h); 57% of Chang-NS5A cells remained in metaphase at the end of observation period as compared with only 10% of Chang-HA cells (Figure 2(f)). These results collectively indicate that NS5A-expressing cells remain arrested in mitosis for an abnormally long time, thus delaying chromosome separation.

Delayed mitotic exit induced by NS5A expression may be associated with mitotic abnormalities

Normal cells display two centrosomes and two spindle poles during mitosis, ensuring bipolar microtubule attachment to sister chromatids, and abnormal centrosomes or mitotic spindles lead to unequal chromosome segregation and subsequent aneuploidy. Thus, since NS5A protein overexpression delays mitotic exit, it is possible that NS5A protein contributes to mitotic machinery defects associated with chromosome missegregation. Therefore, we examined whether aberrant mitotic arrest by NS5A overexpression is related to mitotic



(e)

Mitotic duration (< 2 hr)	Total time (min) NEBD to reattachment
Chang-HA (n=26)	57.6±12.6
Chang-NS5A (n=22)	86.2±31.3
(p-value)	*(0.045)*

(f)

Mitotic duration (>2 hr)	Cells with aberrant mitotic duration ( )		Total ( )
	> 2 hr	> 4 hr	
Chang-HA (n=28)	1 (3.5%)	2 (7.1)	10.7
Chang-NS5A (n=24)	1 (4.2%)	13 (54.1%)	58.3

Cells showing incomplete mitosis within experimental time window (4

Figure 2. Exogenous expression of HCV NS5A protein results in delayed mitotic exit. (a) Expression of HA-tagged NS5A (Chang-NS5A) or NS5B (Chang-NS5B) in selected Chang cell clones was assayed by immunofluorescence using an anti-HA antibody (green) and Hoechst dye (blue). (b) Chang liver cells expressing NS5A (Chang-NS5A, red) or NS5B (Chang-NS5B, green) and empty vector-transfected control cells (Chang-HA, gray) were synchronized at the G1/S boundary by a double thymidine block (see Materials and Methods). After release from G1/S arrest, cells were harvested and subjected to flow cytometry at the indicated time points. Asyn indicates asynchronized cells. (c) Control Chang-HA and Chang-NS5A cells were treated with nocodazole, harvested, stained with PI and an FITC-conjugated anti-MPM2 antibody, and examined by flow cytometry. Distributions of MPM2-positive cells after nocodazole treatment were graphed based on comparisons between Chang-HA and Chang-NS5A cells. The values shown are the means of three independent experiments, and bars indicate standard deviations. (d) Chang-HA and Chang-NS5A cells were cultured and imaged by time-lapse photomicroscopy during mitotic progression; nuclear envelope breakdown (NEBD) was designated time zero. Times from NEBD to complete chromosome separation were measured (an example is indicated by the arrowhead). Representative time-lapse images are shown. (e) Mitotic progression data from randomly selected Chang-HA and Chang-NS5A cells obtained from the image in (d). The percentages of cells showing aberrant mitotic durations of less than 2 h were compared. *P*-values were obtained using the Student's *t* test. (f) Percentages denote the population of cells showing incomplete mitosis at 4 h.

abnormalities, such as mitotic spindle defects, abnormal centrosome formation, or cytokinesis failure. Immunofluorescence analyses using α-tubulin and γ-tubulin were used to visualize mitotic spindles and centrosomes, respectively, in NS5A-expressing and control cells (Figure 3(a)). Interestingly, Huh7-NS5A cells exhibited microtubule disarray and increased numbers of cells

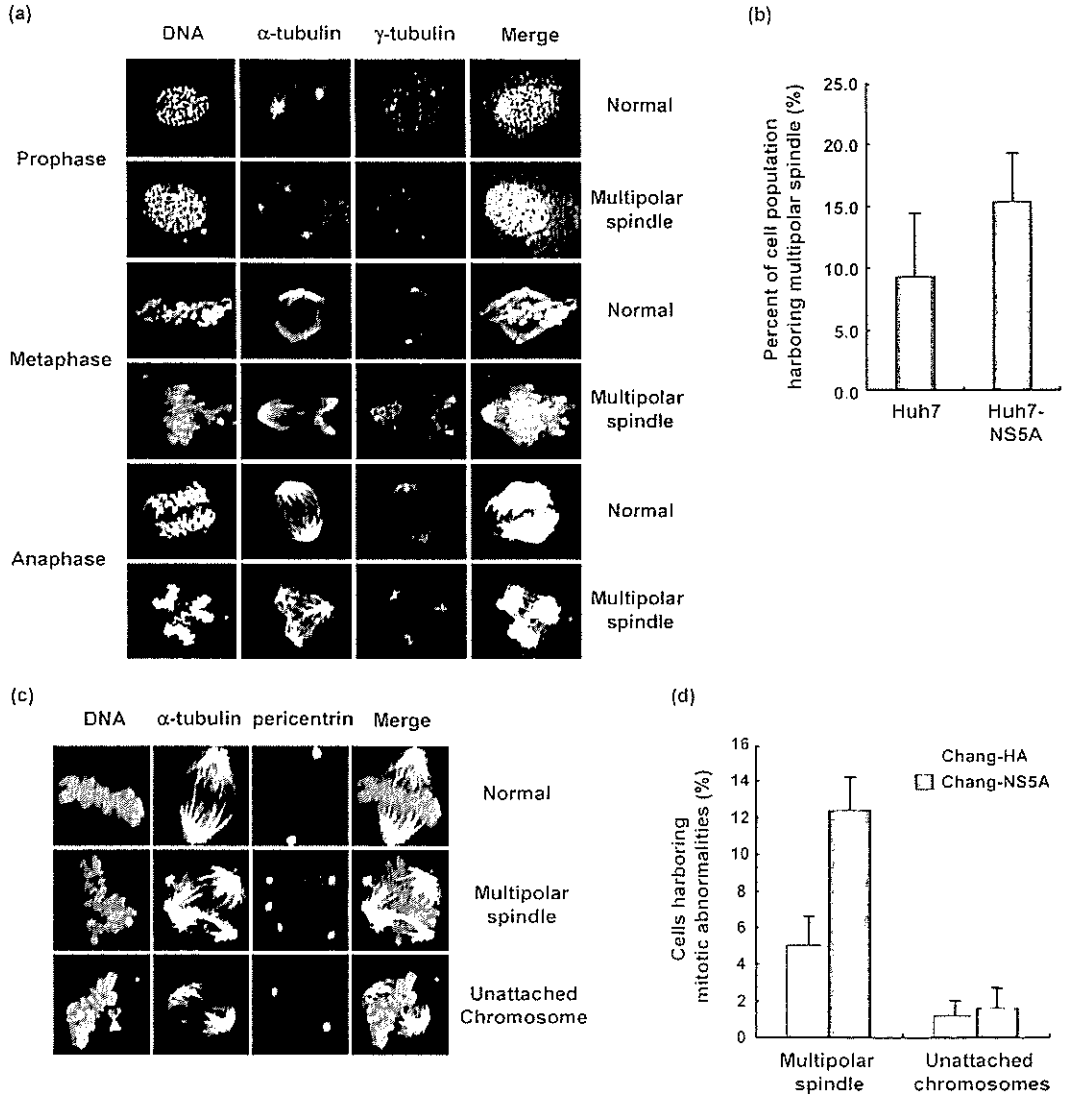


Figure 3. Cells expressing NS5A exhibited more mitotic spindle assembly defects. (a) To analyze mitotic spindles, control Huh7 and Huh7-NS5A cells were co-immunostained with anti-α-tubulin (green) and anti-γ-tubulin (red) antibodies. DNA was visualized with Hoechst dye (blue). Representative confocal microscopic images are shown. More than two centrosomes and spindles per cell were considered to indicate multi-polar (abnormal) spindles. (b) Graphical comparison of multi-polar spindle numbers in Huh7 and Huh7-NS5A cells. The values shown are the means of three independent experiments, in which more than 300 mitotic phase cells were counted, and bars indicate standard deviations. (c) Control Chang-HA and Chang-NS5A cells were co-immunostained with anti-α-tubulin (green) and anti-pericentrin (red) antibodies and DNA was visualized with Hoechst dye (blue). Populations of cells harboring mitotic abnormalities were determined by the presence of multi-polar spindles and/or unattached chromosomes. Representative confocal microscopic images are shown. The arrowhead designates unattached chromosomes. (d) Profiles of mitotic abnormalities in Chang-HA and Chang-NS5A cells. The frequencies of multi-polar spindles and unattached chromosomes were estimated from three independent experiments during which more than 331 mitotic phase cells were counted.

with more than two centrosomes and mitotic spindle poles (~15.5% of cells) as compared to vector-transfected control cells (~8.5%) (Figure 3(b)), suggesting that cells expressing NS5A protein show mitotic machinery abnormalities. Similarly, we immunoassayed control Chang-HA and Chang-NS5A cells using antibodies against α-tubulin and pericentrin (Figure 3(c)), and found that about 14% of NS5A-expressing cells displayed multi-polar spindles as compared with about 4% of control Chang cells (Figure 3(d)). The overexpression of NS5A also slightly increased the population of cells with unattached chromosomes (Figure 3(d)). In addition, recent reports show that cytokinesis failure and subsequent binucleation result in tetraploidization, which frequently is able to proceed to aneuploidy.[30,31] Therefore, we investigated whether the overexpression of NS5A contributes to cytokinesis failure and binucleation. However, cells expressing NS5A showed no