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Reprint requests

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Acknowledgments

Participating investigators from the Hokuriku Liver Study Group are listed in Appendix A.

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Conflicts of interest

The authors disclose no conflicts.

Supplementary Materials and Methods

Plasma Amino Acid Analysis

Plasma sample amino acid concentrations were measured by high-performance liquid chromatography-electrospray ionization-mass spectrometry followed by derivatization.[1] An MSQ Plus LC/MS system (Thermo Fischer Scientific, Waltham, MA) equipped with an electrospray ionization source was used in positive ionization mode for selected ion monitoring. Xcalibur version 1.4 SR1 software (Thermo Fischer Scientific, Yokohama, Japan) was used for data collection and processing. The high-performance liquid chromatography separation system consisted of an L-2100 pump, L-2200 autosampler, and L-2300 column oven (Hitachi High-Technologies Corporation, Tokyo, Japan). A Wakosil-II 3C8-100HG column (100, 2.1, 3 mm; Wako Pure Chemical Industries, Osaka, Japan) was used for the separation, and the mobile phase consisted of eluent A (25-mmol/L ammonium formate in water, pH 6.0) and eluent B (water:acetonitrile = 40:60).

Western Blotting

The expression of HCV core protein, Socs3, Foxo3a, phospho-Foxo3a (Ser253) (pFoxo3a), STAT1, pSTAT1 (Tyr701), S6K, pS6K, p-mTOR (Ser2448), Raptor, and β-actin were evaluated with mouse anti-core (Affinity BioReagents, Golden, CO), mouse anti-Socs3 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Foxo3a, rabbit anti-β-actin (Sigma-Aldrich, St Louis, MO), rabbit anti-phospho-Foxo3a (Ser253), rabbit anti-STAT1, rabbit anti-p-STAT1 (Tyr701), rabbit anti-p70 S6K, rabbit anti-pS6K, rabbit anti-p-mTOR (Ser2448), and rabbit anti-Raptor (Cell Signaling Technology, Beverly, MA), respectively. Densitometric analysis was conducted directly on the blotted membrane using a charge coupled device camera system (LAS-3000 Mini; Fujifilm, Tokyo, Japan) and Scion Image software (Frederick, MD).

Primer Sequences for PCR and siRNA

Primer sequences for PCR and siRNA were as follows: 2′5′OAS: forward 5′- CTC AGA AAT ACC CCA GCC AAA TC-3′, reverse 5′-GTG GTG AGA GGA CTG AGG AA-3′; Socs3: forward 5′-TAC CAC CTG AGT CTC CAG CTT CTC-3′, reverse 5′-CCT GGC AGT TCT CAT TAG TTC AGC ATT C-3′; Foxo3a: forward 5′-TGC TGT ATG CAA GAA CTT TCC AGT AGC AG-3′, reverse 5′-ACT CTA GCC CCC ATG CTA CTA GTG-3′; glyceraldehyde-3-phosphate dehydrogenase: forward 5′-GAA GGT GAA GGT CGG AGT-3′, reverse 5′-GAA GAT GGT GAT GGG ATT TC-3′, siFoxo3a (SASI_Hs01_00119127; Sigma) sense: 5′-GAA UGA UGG GCU GAC UGA AdTdT-3′, antisense: 5′-UUC AGU CAG CCC AUC AUU CdTdT-3′. Small interfering Raptor was purchased as part of KIAA1303 siGENOME SMART pool siRNA reagents from Dharmacon, Inc (Lafayette, CO).

Construction of ISRE-Luc Reporter and FBEmut-luc Reporter Plasmids

Oligonucleotides containing the ISRE tandem repeat sequence (sense 5′-TCG AGA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA A-3′, antisense 5′-AGC TTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT C-3′, consensus 5′-GAA Ann GAA ACT-3′) were annealed, and integrated into Xho I and Hind III sites of the pGL4.23 luciferase vector (Promega). The human Socs3 promoter region (-109/+217) was amplified by genomic PCR using specific primers (forward, 5′-TGC TGC GAG TAG TGA CTA AAC ATT ACA AG-3′ and reverse, 5′-CCG TGA AGT CCA CAA AGG AGC CTT C-3′) and cloned into the EcoR V site of the pGL4.10-luc2 reporter vector (Promega). The Socs3 FBE mutant reporter vector was created by substituting 2 adenines in the putative FBE with guanines (wild-type sequence 5′-CTAAACA-3′, mutated sequence 5′-CTGAGCA-3′).

ChIP Assay

For the ChIP assay using the anti-ISGF3γ antibody, 1×10^6 Huh-7 cells were treated with IFN-alfa (0 or 100 U/mL) and BCAA (2 mmol/L) in low-amino-acid medium for 6 hours. For ChIP using the anti-Foxo3a antibody, 1×10^6 Huh-7 cells were cultured in low-amino-acid medium for 24 hours.

Cells were cross-linked with 1% formaldehyde in PBS for 10 minutes at 37°C, and the reaction was stopped with 250 mmol/L glycine for 10 minutes. Cells were suspended in sodium dodecyl sulfate-lysis buffer (1% sodium dodecyl sulfate, 10 mmol/L ethylenediaminetetraacetic acid [EDTA], 50 mmol/L Tris-HCl [pH 8.1]), complete protease inhibitor cocktail (Roche Applied Science), and incubated for 30 minutes at 10°C. Cell lysate was sonicated with Bioruptor (Cosmo Bio, Tokyo, Japan) to obtain chromatin fragments and diluted 10-fold in ChIP dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton-X 100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl [pH 8.1], 150 mmol/L NaCl, complete protease inhibitor cocktail). Chromatin fragments were incubated with 2 μg ISGF3γ antibody (Santa Cruz Biotechnology), 2 μg Foxo3a antibody (H-144; Santa Cruz Biotechnology), or normal rabbit immunoglobulin G for 18 hours at 4°C. Dynabeads (30 μL) protein G (Invitrogen) was added and incubated for 1 hour at 4°C. The beads were washed with low-salt-wash buffer (0.1% sodium dodecyl sulfate, 1% Triton-X 100, 2.0 mmol/L EDTA, 20 mmol/L Tris-HCl [pH 8.1], 150 mmol/L NaCl), high-salt-wash buffer (0.1% sodium dodecyl sulfate, 1% Triton-X 100, 2.0 mmol/L EDTA, 20 mmol/L Tris-HCl [pH 8.1], 500 mmol/L NaCl), LiCl wash buffer (250 mmol/L LiCl, 1% NP-40, 1% de-

oxycholate, 1.0 mmol/L EDTA, 1.0 mmol/L Tris-HCl [pH 8.1]) and Tris-EDTA buffer. Immunoprecipitated chromatin fragments were eluted with elution buffer (1% sodium dodecyl sulfate, 100 mmol/L NaHCO3, 10 mmol/L dithiothreitol), and reverse cross-linked by incubating for 6 hours at 65°C in elution buffer containing 200 mmol/L NaCl. DNA fragments were purified and quantified by real-time detection PCR with primers for putative ISRE in the 2′5′OAS promoter region (forward, 5′-AAA TGC ATT TCC AGA GCA GAG TTC AGA G-3′, reverse, 5′-GGG TAT TTC TGA GAT CCA TCA TTG ACA GG-3′) or putative FBE in the Socs3 promoter region (forward, 5′-TGC TGC GAG TAG TGA CTA AAC ATT ACA AG -3′, reverse, 5′-AGC GGA GCA GGG AGT CCA AGT C -3′). Values were normalized by the measurement of input DNA.

pH77S.3/GLuc2A

pH77S.2 is a modification of pH77S[2] containing an additional mutation within the E2 protein (N476D in the polyprotein) that promotes infectious virus yields from RNA-transfected cells (Yi et al, unpublished data). To monitor replication, the GLuc sequence, fused at its C terminus to the foot-and-mouth disease virus 2A autoprotease, was inserted between p7 and NS2 of pH77S.2 (Supplementary Figure 4). To insert the GLuc-coding sequence between p7 and NS2 in pH77S.2, followed by the foot-and-mouth disease virus 2A protein-coding sequence, Mlu I, EcoR V, and Spe I restriction sites were created between the p7 and NS2 coding sequences by site-directed mutagenesis. DNA coding for GLuc was subcloned into the Mlu I and EcoR V sites of the modified plasmid after PCR amplification using the primers: 5′- ATA ATA TTA CGC GTA *TGG GAG TCA AAG TTC TGT TTG CC*-3′ (sequence corresponding to the N-terminal GLuc is italicized and that corresponding to Mlu I is underlined) and 5′-ATA AAT AGAT ATC *GTC ACC ACC GGC CCC CTT GAT CTT*-3′ (C terminal GLuc is italicized and EcoR V is underlined). A DNA fragment encoding the 17 amino acids of the foot-and-mouth disease virus 2A protein was generated by annealing the following complementary oligonucleotides: 5′- ATA TGA TAT CAA CTT TGA CCT TCT CAA GTT GGC CGG CGA CGT CGA GTC CAA CCC AGG GCC CAC TAG CAT AT-3′ and 5′-ATA TGC TAG TGG GCC CTG GGT TGG ACT CGA CGT CGC CGG CCA ACT TGA GAA GGT CAA AGT TGA TAT CAT AT-3′ (underlined sequences indicate EcoR V and Spe I sites). The annealed oligonucleotides were digested by both restriction enzymes and the product inserted into the corresponding sites of pH77S.2 containing GLuc to generate pH77S.2/GLuc2A. Q41R is a cell-culture adaptive mutation within the NS3 protease domain of pH77S. Because it is not essential for production of infectious virus (Yi et al, unpublished data), pH77S.2 and pH77S.2/GLuc2A constructs underwent this mutation by site-directed mutagenesis of a PCR fragment spanning the Afe I and BsrG I sites to replace Gln_{41} with wild-type Arg. The resulting plasmids (pH77S.2/R41Q and pH77S.2/GLuc2A/R41Q) were redesignated pH77S.3 and pH77S.3/GLuc2A, respectively.[3,4] GLuc has several advantages over other luciferase reporter enzymes in that it is smaller and allows more sensitive detection than either firefly or Renilla luciferase.[3,4] In addition, a signal sequence directs its secretion into cell-culture media, allowing real-time dynamic measurements of GLuc expression without the need for cell lysis. H77S.3/GLuc2A RNA produces infectious virus, although with lower efficiency than H77S.3 RNA (10-fold less).

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Chaperonin TRiC/CCT participates in replication of hepatitis C virus genome via interaction with the viral NS5B protein

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ABSTRACT

To identify the host factors implicated in the regulation of hepatitis C virus (HCV) genome replication, we performed comparative proteome analyses of HCV replication complex (RC)-rich membrane fractions prepared from cells harboring genome-length bicistronic HCV RNA at the exponential and stationary growth phases. We found that the eukaryotic chaperonin T-complex polypeptide 1 (TCP1)-ring complex/chaperonin-containing TCP1 (TRiC/CCT) plays a role in the replication possibly through an interaction between subunit CCT5 and the viral RNA polymerase NS5B. siRNA-mediated knockdown of CCT5 suppressed RNA replication and production of the infectious virus. Gain-of-function activity was shown following co-transfection with whole eight TRiC/CCT subunits. HCV RNA synthesis was inhibited by an anti-CCT5 antibody in a cell-free assay. These suggest that recruitment of the chaperonin by the viral nonstructural proteins to the RC, which potentially facilitate folding of the RC component(s) into the mature active form, may be important for efficient replication of the HCV genome.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases, such as chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma (Hoofnagle, 2002; Manns et al., 2006; Saito et al., 1990; Seeff and Hoofnagle, 2003). HCV is an enveloped positive-strand RNA virus belonging to the *Hepacivirus* genus of the Flaviviridae family. Its genome of ~9.6 kb encodes a polyprotein precursor of ~3000 amino acids (aa) (Suzuki et al., 2007; Taguwa et al., 2008). The precursor polyprotein is post- or cotranslationally processed by both viral and host proteases into at least ten viral products. The nonstructural (NS) proteins NS3–NS5B are necessary and sufficient for autonomous HCV RNA replication. They form a membrane-associated replication complex (RC), in which NS5B is the RNA-dependent RNA polymerase (RdRp) that is responsible for copying the RNA genome of the virus during replication. The HCV RC has been detected in detergent-resistant membrane (DRM) structures, possibly in a lipid-raft structure (Aizaki et al., 2004; Shi et al., 2003). Cell-free RC replication activity has also been demonstrated in crude membrane fractions of HCV subgenomic replicon cells (Aizaki et al., 2004; Ali et al., 2002; Hara et al., 2009; Hardy et al., 2003; Yang et al., 2004); these cell-free systems provide semi-intact RdRp assays for biochemical dissection of viral replication.

In general, any process that occurs during viral replication is dependent on the host cell machinery and requires close interaction between viral and cellular proteins. Although evidence that host cell factors interact with HCV NS proteins and are involved in viral replication is accumulating (Moriishi and Matsuura, 2007), the cellular components of HCV RC and their functional roles in viral replication are not fully understood.

Recently, using comparative proteome analysis, we identified 27 cellular proteins that were highly enriched in the DRM fraction of HCV replicon cells relative to parental cells. Subsequent analyses demonstrated that one of the identified proteins, creatine kinase B, a key ATP-generating enzyme, is important for efficient replication of the HCV genome and for production of the infectious virus (Hara et al., 2009).

In this study, to extend our investigation and to increase our understanding of the precise components of HCV RC and the

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mechanisms of viral genome replication, we designed another comparative proteomic approach in which cells harboring genome-length bicistronic HCV RNA at the exponential growth phase (showing rapid replication of viral RNA) were compared with cells at the confluent-growth phase (showing poor replication of viral RNA). This strategy revealed that the chaperonin T-complex polypeptide (TCP1)-ring complex/chaperonin-containing TCP1 (TRiC/CCT) participates in HCV RNA replication and virion production possibly through an interaction between CCT5 (chaperonin-containing TCP1, subunit 5) and NS5B.

Results

CCT5 and Hsc70 are enriched in the DRM fraction containing the HCV RC

Recently, we analyzed the protein content of DRM fractions prepared from HCV subgenomic replicons and parental Huh-7 cells and identified 27 cellular proteins that were enriched in the DRM fraction prepared from the replicon cells (Hara et al., 2009). These were identified as factors that may be involved in the HCV RC and in viral replication. In fact, subsequent silencing of several genes coding for these proteins resulted in the inhibition of HCV RNA replication (Hara et al., 2009). However, it is likely that proteins unrelated to HCV replication are also included in the identified groups because long-term culture of the replicon cells under the selective pressure of G418 selects for a subpopulation of the parental cells and may induce changes in their protein expression profiles. Thus, to minimize interline differences in culture background, we further designed a comparative proteome analysis using a single cell line as follows.

HCV replication efficiency is dependent on the conditions of host cell growth. High cell density of the replicon culture has a reversible inhibitory effect on viral replication (Nelson and Tang, 2006; Pietschmann et al., 2001). Fig. 1A demonstrates that a high level of HCV RNA was detected in cells harboring the genome-length bicistronic HCV RNA, Con1 strain of genotype 1b (RCYM1) in the growth phase, whereas the RNA level declined sharply when the cells reached the stationary phase. We further compared the synthesis of HCV RNA in cell-free reaction mixtures containing the viral RC isolated from the RCYM1 cells at various cell densities (Fig. 1B). Replication activity was highest at the mid-log phase of cell growth (day 4 after seeding). By contrast, little or no RNA synthesis was observed under the confluent-growth cell culture (day 8), confirming the critical role of host cell growth conditions in the replication of the HCV genome.

Thus, to identify the host cell proteins required for HCV replication, we designed a two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)-based comparative proteomics analysis of RC-rich DRM fractions prepared from RCYM1 cells at the mid-log and confluent-growth phases. Protein spots that reproducibly showed a greater than 1.5-fold difference in the mid-log growth- and the confluent phases were excised and digested by trypsin or lysylendopeptidase. Matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry (MS), which allows identification of the corresponding proteins in 9 cases (Table 1). Two increased spots that showed an increase in levels (their stereoscopic images are shown in Fig. 2A) were identified as CCT5 and Hsc70. CCT5, an epsilon subunit of chaperonin TRiC/CCT, is a 900-kDa toroid-shaped complex consisting of eight different subunits (Valpuesta et al., 2002; Yaffe et al., 1992). Hsc70, a member of the HSP70 family, is a 71-kDa heat shock cognate protein (Dworniczak and Mirault, 1987). Independent of the proteome analyses, DRM fractions and whole cell lysates were prepared from RCYM1 cells at two different growth phases (as above) and were analyzed by immunoblotting (Fig. 2B). Steady-state levels of CCT5 and Hsc70 were obviously higher in the DRM fraction prepared from the cells that were at the mid-log growth phase compared with those at the confluent phase. However, in the whole cell analyses, they were shown to be present at comparable levels during the two different growth phases. These results suggest that expression of CCT5 and Hsc70 is not enhanced in proliferating cells and that the enrichment of these proteins in the DRM fraction is possibly due to their post-translational modification. It should be noted that in the previous proteome analysis, CCT5 and other TRiC/CCT subunits, such as CCT1 and CCT2, were identified as proteins that were enriched in the DRM fraction prepared from subgenomic replicon-containing cells compared with that prepared from parental cells (Hara et al., 2009). We showed that CCT5 and CCT1 were enriched in the DRM fractions of cells transfected with the HCV genomic RNA derived from JFH-1 isolate as well as of subgenomic replicon cells (Fig. 2C).

TRiC/CCT participates in replication of the HCV genome

We investigated gain- and loss-of-functions of TRiC/CCT and Hsc70 with respect to the replication of HCV RNA. Seventy-two hours after RCYM1 cells were transfected with eight plasmids corresponding to each of the TRiC/CCT subunits, the level of HCV RNA in the cells (determined by quantitative RT-PCR) significantly increased to 2-fold that observed in the control cells. However, exogenous expression of Hsc70 in the RCYM1 cells showed no effect on the viral RNA (Fig. 3A). siRNAs targeted to CCT5 or Hsc70 and consisting of pools of three target-specific siRNAs or control nonspecific siRNAs were transfected

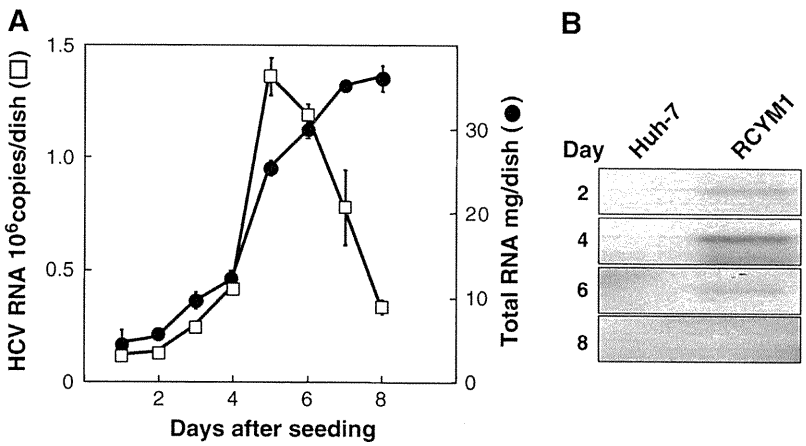

Fig. 1. Effect of cell growth on HCV RNA replication. (A) Measurement of HCV RNA (open squares) and total cellular RNA (closed circles) in RCYM1 cells at the time of harvest (days after seeding). (B) DRM fractions obtained from RCYM1 and parental Huh-7 cells harvested as indicated (day) were analyzed by cell-free RNA replication assay. RNA extracted from each sample was analyzed by agarose gel electrophoresis and autoradiograph.

Table 1
Selected cellular proteins that reproducibly increased and decreased in membrane fraction of RCYM1 cells at exponential growth phase.

Av. ratio	T-test	Coverage (%)	Protein name	Molecular function	GI
Increased proteins					
1.58	0.017	31	CCT5	Protein folding	33879913
1.54	0.005	35	HSPA8 (Hsc70)	Protein folding	24657660
Decreased proteins					
−1.95	0.028	44	Creatine kinase isozyme CK-B gene, exon 8	Energy pathway/metabolism	180568
−1.53	0.011	16	Chain C, Human Sirt2 Histone deacetylase	Cell cycle control	15826438
−2.14	0.001	33	Proteasome regulatory particle subunit p44S10	Metabolism	15341748
−1.71	0.004	21	Aldehyde dehydrogenase	Metabolism	178388
−1.85	0.004	40	Aminoacylase 1	Metabolism	12804328
−2.77	0.003	15	Eukaryotic translation initiation factor 3, subunit 3 gamma	Metabolism (translation regulator activity)	6685512
−2.43	0.014	20	Intraflagellar transport protein 74 homolog (Coiled-coil domain-containing protein 2)	Cell growth and/or maintenance	10439078

Three paired samples of RC-rich membrane fractions at the exponential- and confluent-growth phases of RCYM1 cultures were analyzed. The proteins representing a more than 1.5-fold increase or decrease (−) reproducibly and significantly are indicated.
Coverage (%): the ratio of the portion of protein sequence covered by matched peptides to the whole sequence.
GI: GenInfo Identifier number.

into RCYM1 cells. After 72 h, the HCV RNA level was reduced by 42% and 27% in the cells transfected with siRNAs against CCT5 and Hsc70, respectively, compared with controls (Fig. 3B). TRiC/CCT possibly interacts with Hsc70, and its complex formation contributes to increasing the efficiency of protein folding (Cuéllar et al., 2008). Our results suggest the involvement of TRiC/CCT and Hsc70 in the HCV life cycle. In particular, TRiC/CCT may play an important role in the replication of the viral genome.

To verify the specificity of the knockdown of CCT5 siRNA, we further synthesized two siRNAs targeted to different regions used in the above CCT5 siRNA and assessed their knockdown effect on HCV genome replication (Fig. 3C, upper panel). As expected, transfection of

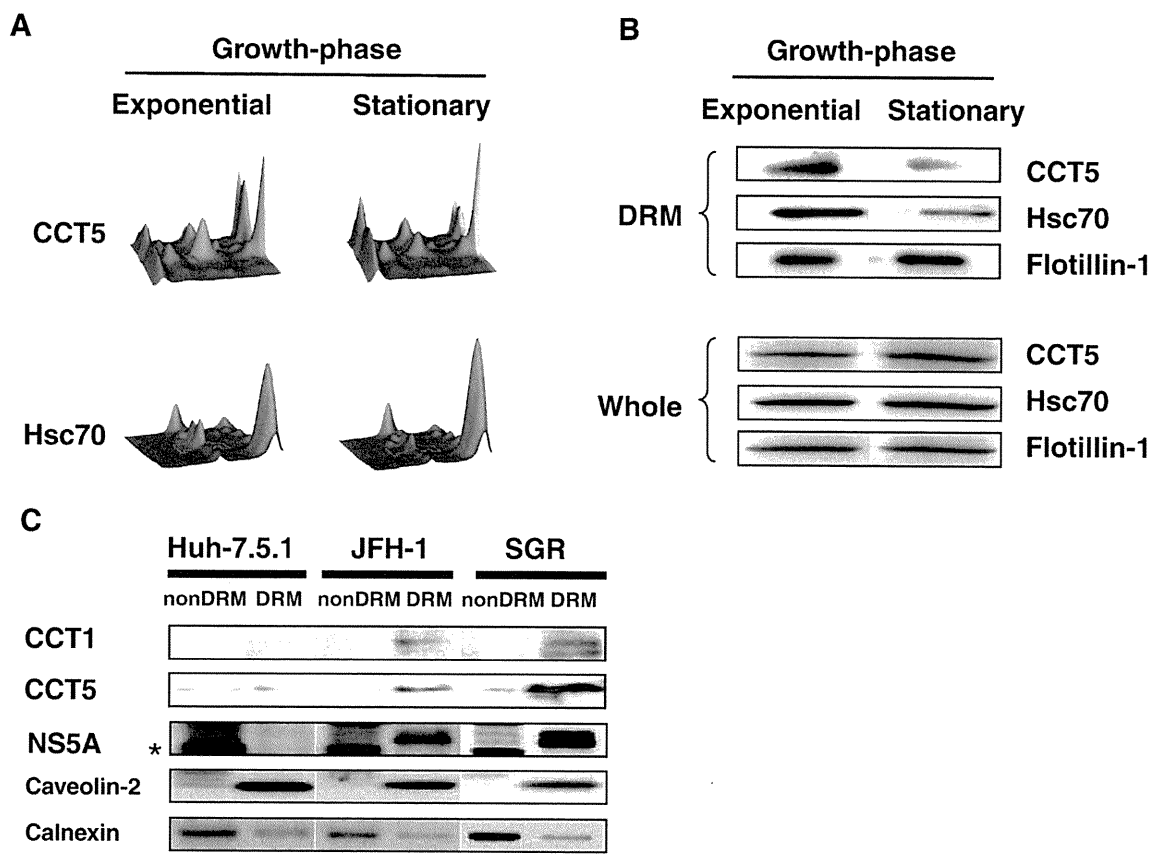

Fig. 2. Comparison of protein levels in DRM fractions prepared from RCYM1 cells at the exponential and stationary growth phases. (A) Three-dimensional images of CCT5 and Hsc70 analyzed by Ettan DIGE (GE Healthcare). Spots corresponding to CCT5/Hsc70 at exponential and stationary growth phases of the cells, respectively, are shown in green and red. (B) Equal amounts of protein in the DRM fractions prepared from RCYM1 cells at the exponential and stationary growth phases or corresponding whole cell lysates were analyzed by immunoblotting with Abs against CCT5, Hsc70 or flotillin-1. (C) Enrichment of CCT1 and CCT5 in the DRM fractions of HCV RNA replicating cells. Equal amounts of DRM or non-DRM fractions from full-length JFH-1 RNA transfected cells (JFH-1), subgenomic replicon cells (SGR) and parental Huh-7.5.1 cells were analyzed by immunoblotting with antibodies against CCT1, CCT5, NS5A, caveolin-2 or calnexin. *Non-specific bands.

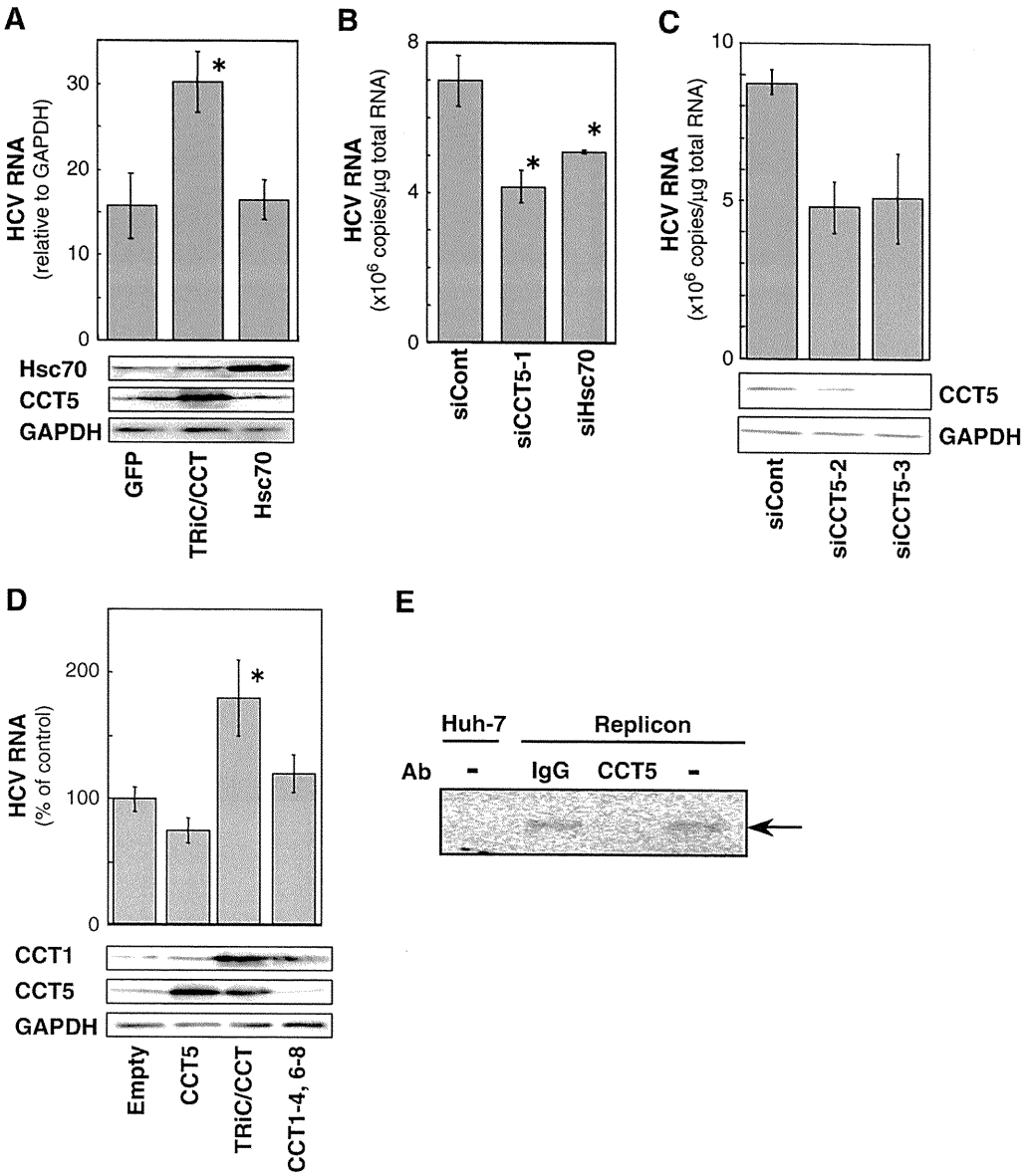

Fig. 3. Involvement of TRiC/CCT in HCV replication (A and D). Overexpression of all eight subunits of TRiC/CCT (TRiC/CCT); seven subunits, CCT1, 2, 3, 4, 6, 7, and 8 (CCT1–4, 6–8); subunit CCT5 only (CCT5); Hsc70; or control GFP in RCYM1 cells. HCV RNA levels were determined 48 h post-transfection (B and C). Knockdown of endogenous CCT5 or Hsc70 in RCYM1 cells, which were transfected with three types of siRNAs against CCT5 (siCCT5-1, -2, and -3), siRNA against Hsc70 (siHsc70), or control siRNA (siCont), and were harvested at 72 h post-transfection. siCCT5-1 and siHsc70 consisted of pools of three target-specific siRNAs. Immunoblotting for CCT1, CCT5, Hsc70 and GAPDH was performed (A, C and D; lower). (E) Cell-free de novo viral RNA synthesis assays were performed in the presence of anti-CCT5 Ab or control mouse IgG. Cytoplasmic fractions from SGR-N (replicon) and parental Huh-7 cells were used. An arrow indicates the synthesized HCV RNA. Error bars denote standard deviations with asterisks indicating statistical significance (*$P<0.01$).

RCYM1 cells with each CCT5 siRNA resulted in a reduction in viral RNA to a level of about 50% of that observed in cells treated with control siRNAs. Immunoblotting confirmed the efficient reduction in expression of endogenous CCT5 and the lack of cytotoxic effect exerted by the CCT5 siRNAs (Fig. 3C, middle and lower panels).

Having confirmed the upregulation of HCV RNA by ectopic expression of all the TRiC/CCT subunits, we further addressed the possibility that CCT5, independent of the complete TRiC/CCT complex, might have a role in promoting replication of HCV RNA. Transfection with either a CCT5 expression plasmid alone or with seven plasmids expressing all the TRiC/CCT subunits except CCT5 resulted in no or only a slight increase in the level of HCV RNA, indicating that all CCT subunits are required for HCV replication (Fig. 3D).

TRiC/CCT is generally known as a cytosolic chaperone (Valpuesta et al., 2002). However, it is enriched in the DRM fraction of HCV-replicating cells during the exponential growth phase (Fig. 2B). We used immunofluorescence staining to investigate whether TRiC/CCT is localized in the intracellular membrane compartments where replication of the viral genome occurs (Fig. 4). The de novo-synthesized RdRp was labeled by bromouridine triphosphate (BrUTP) incorporation in the presence of actinomycin D, and brominated nucleotides were detected with a specific antibody (Ab). Fluorescence staining in distinct speckles of various sizes was found in the cytoplasm of the HCV subgenomic replicon cells, whereas no signal was detected in the control cells, indicating that the observed BrUTP-incorporating RNA is mostly viral, newly synthesized viral RNA (Fig. 4A). Double immunofluorescence staining showed that a certain section of CCT5 co-distributed with the BrUTP-labeled RNA (Fig. 4A), which is known to co-exist with HCV NS proteins in viral replicating cells (Shi et al., 2003). We further observed that CCT5 was at least partially colocalized

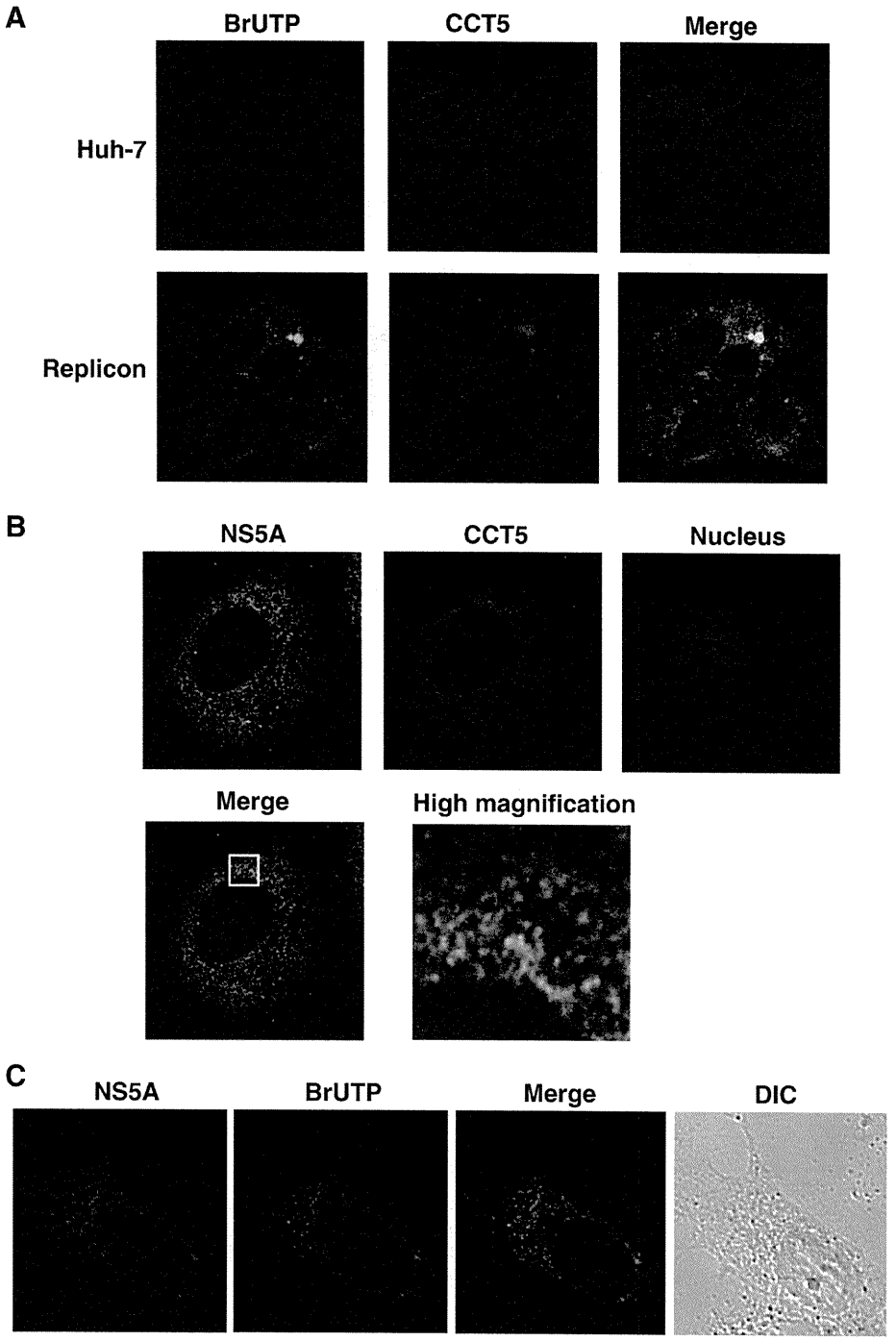

Fig. 4. Immunofluorescence analysis of CCT5 in SGR-N and Huh-7 cells (A) and HCVcc-infected cells (B). The primary Abs used were anti-CCT5 goat polyclonal Ab (red), anti-BrUTP monoclonal Ab (green), and anti-NS5A monoclonal Ab (green). Merged images of red and green signals (A) or of red, green and blue (nucleus) signals (B) are shown. The high magnification panel is an enlarged image of a white square of the merge panel. (C) Colocalization of NS5A protein with the viral RNA. The replicon cells were permeabilized with lysolecithin and labeled with BrUTP, followed by staining with anti-NS5A rabbit polyclonal Ab (red) and the anti-BrUTP monoclonal Ab (green). DIC, differential interference contrast.

with the viral NS protein in certain compartments sharing a dot-like structure in Huh-7 cells infected with HCV JFH-1 infectious HCV (HCVcc) derived from HCV genotype 2a (Fig. 4B) as well as in the replicon cells (data not shown). Fig. 4C indicated co-localization of BrUTP-labeled RNA with NS5A.

To further address the role of TRiC/CCT in HCV genome replication, we performed immunodepletion and in vitro replication analyses, which have been used for studying the genome replication of several viruses (Daikoku et al., 2006; Garcin et al., 1993; Liu et al., 2009). Cell extracts prepared from the HCV-replicating cells were reacted with either a mouse monoclonal Ab against CCT5 or mouse IgG derived from preimmune serum, followed by cell-free synthesis of HCV RNA. Fig. 3E shows that treatment with anti-CCT5 Ab inhibited viral RNA synthesis, whereas the control IgG did not affect the process, suggesting that TRiC/CCT participates directly in HCV RNA replication.

CCT5 interacts with HCV NS5B

The genome replication machinery of HCV is a membrane-associated complex composed of multiple factors including viral NS proteins. Given the involvement of TRiC/CCT in HCV RNA synthesis, we next examined its possible interaction with HCV NS proteins. A first attempt to immunoprecipitate the viral proteins with antibodies against TRiC/CCT subunits in the replicon cells was unsuccessful (data not shown), suggesting that endogenous levels of TRiC/CCT is not sufficient to pull out NS5B. Next, dual (myc/FLAG)-tagged NS3, NS5A, or NS5B proteins derived from the genotype 1b NIHJ1 strain were co-expressed with CCT5 in Huh-7 cells and then subjected to two-step immunoprecipitation with anti-myc and anti-FLAG Abs (Ichimura et al., 2005; Shirakura et al., 2007). An empty plasmid was used as a negative control in the analyses. As shown in Fig. 5A, CCT5 specifically interacted with NS5B. Little or no interaction was found between CCT5 and NS3 or NS5A. To determine the NS5B region required for the interaction with CCT5, various deletion mutants of HA-NS5B were constructed and their interactions with CCT5 were analyzed as described above. CCT5 was shown to be coimmunoprecipitated with either a full-length NS5B (aa 1–591), an N-terminal deletion (aa 71–591) or a C-terminal deletion (aa 1–570), but not with deletions aa 215–591 or aa 320–591 (Fig. 5B), suggesting that aa 71–214 of NS5B are important for its interaction with CCT5.

Knockdown of CCT5 results in the reduction of propagation of infectious HCV

We further examined whether the knockdown of CCT5 would abrogate the production of infectious HCV (HCVcc), derived from JFH-1 (Fig. 6). At 72 h post-transfection with each CCT5 siRNA, HCV RNA

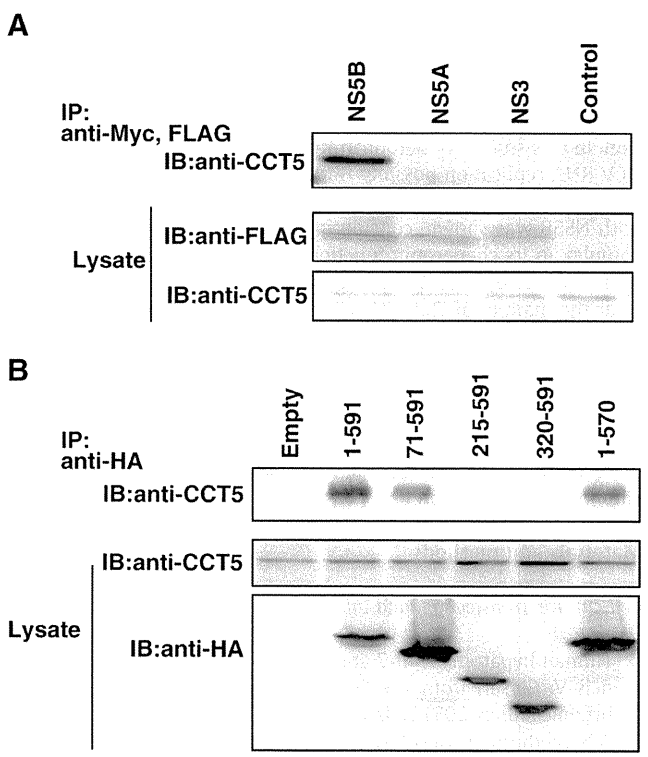

Fig. 5. CCT5 interacts with HCV NS5B. (A) CCT5 was co-expressed with MEF-tagged-NS5B, -NS5A, or -NS3 protein of strain NIHJ1 in cells, followed by two-step immunoprecipitation (IP) with anti-FLAG and anti-myc Abs. Immunoprecipitates were subjected to immunoblotting with anti-CCT5 Ab (IB). (B) Full-length NS5B (1–591) or its deletions (71–591, 215–591, 320–591, 1–570) along with a HA tag were co-expressed with CCT5. IP and IB were performed as described above.

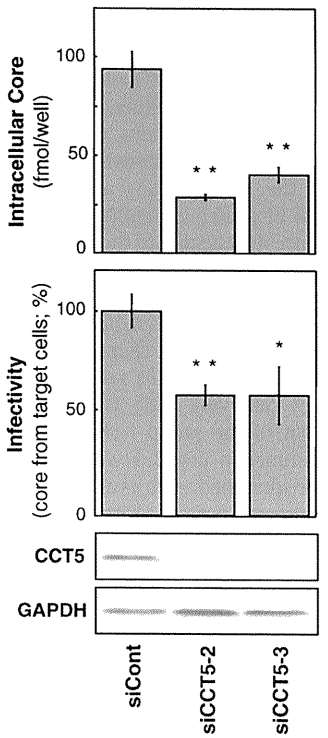

Fig. 6. Knockdown of endogenous CCT5 in HCVcc-infected cells. The cells were transfected with siRNAs against CCT5 (siCCT5-2, -3) or with control siRNAs (siCont). At 72 h post-transfection, the viral core protein levels in cells were determined (upper panel). Collected culture supernatants were inoculated into naïve Huh7.5.1 cells and intracellular core proteins were determined at 72 h post-infection (middle panel). Cells transfected with siRNAs were analyzed by immunoblotting with anti-CCT5 or anti-GAPDH Ab (lower panel). Error bars denote standard deviations with asterisks indicating statistical significance (*$P<0.05$; **$P<0.01$).

levels in Huh-7 cells infected with HCVcc were reduced by 25–35% compared with controls. Accordingly, virion production from CCT5 siRNA-transfected cultures was significantly decreased, as determined by intracellular HCV core protein levels at 72 h after the infection of naïve cells with culture supernatants taken from transfected cells. These results demonstrate that reduction of the HCV RNA replication by siRNA-mediated knockdown of CCT5 results in reduction of the propagation of the infectious virus.

Discussion

The chaperone-assisted protein-folding pathway is a process in living cells that results from coordinated interactions between multiple proteins that often form multi-component complexes. Several steps in the viral life cycle, such as protein processing, genome replication, and viral assembly, are regulated by cellular chaperones. Hsp90, one of the most abundant proteins in unstressed cells, has been implicated in HCV RNA replication (Nakagawa et al., 2007; Okamoto et al., 2006, 2008; Taguwa et al., 2008, 2009; Ujino et al., 2009). FKBP8, a member of the FKBP506-binding protein family, and hB-ind1, human butyrate-induced transcript 1, play key roles through their interaction with HCV NS5A and Hsp90 (Okamoto et al., 2006, 2008; Taguwa et al., 2008, 2009). Hsp90 has also been implicated in viral enzymatic activities including those of the influenza virus (Momose et al., 2002; Naito et al., 2007), herpes simplex virus (Burch and Weller, 2005), Flock house virus (Kampmueller and Miller, 2005), and hepatitis B virus (Hu et al., 2004).

In our former study, comparative proteome analyses of the viral RC-rich DRM fractions prepared from subgenomic replicon cells and Huh-7 cells were carried out to identify host factors involved

in HCV replication (Hara et al., 2009). We extended the proteomics by modifying our protocol of the analysis to reduce the interline differences in culture background and analyzed the DRM samples derived from the mid-log and confluent-growth phases of single cell line. Here, we identified two proteins, CCT5 and Hsc70, showing an increase in levels at the mid-log growth phase. Although CCT5 was also identified in the former study as expected, Hsc70 was not included in the list of proteins identified in the study (Hara et al., 2009). This difference may be due to the use of cells carrying the full-length replicon RNA in this study.

In this study, we demonstrated that TRiC/CCT participates in HCV RNA replication and virion production possibly through its interaction with NS5B. TRiC/CCT is a group II chaperonin that assists in protein folding in eukaryotic cells and forms a double-ring-like hexadecamer complex. Although relatively little is known about its function compared with that of the group I chaperonins such as bacterial GroEL, several mammalian proteins whose folding is mediated by TRiC/CCT have been identified, such as actin, tubulin, and von Hippel–Lindau tumor suppressor protein (Farr et al., 1997; Feldman et al., 2003; Frydman and Hartl, 1996; Meyer et al., 2003; Tian et al., 1995). With regard to viral proteins, the Epstein–Barr virus nuclear antigen, HBV capsid protein, and p4 of M-PMV have been identified as TRiC/CCT-interacting proteins (Yam et al., 2008). However, the functional significance of their interactions in the viral life cycles has yet to be determined. Here we demonstrated that the reduction in CCT5 expression in HCV replicon cells and in virus-infected cells inhibits HCV RNA replication (Figs. 3B and C) and virus production (Fig. 6) respectively. Gain-of-function was also shown by co-transfection of the replicon cells with eight constructs corresponding to all the TRiC/CCT subunits (Figs. 3A and D).

A recent study of the three-dimensional structure of the TRiC/CCT and Hsc70 complex has demonstrated that the apical domain of the CCT2 (CCT-beta) subunit is involved in the interaction with Hsc70 (Cuéllar et al., 2008). The complex formation created by the TRiC/CCT and Hsc70 interaction may promote higher efficiency in the folding of certain proteins (Cuéllar et al., 2008). In our comparative proteome analyses, both CCT subunits and Hsc70 were enriched in the HCV RC-rich membrane fraction of the replicon cells that showed high viral replication activity (Fig. 2B). Transfection of Hsc70 siRNA into the replicon cells moderately inhibited viral RNA replication (Fig. 3B). However, upregulation of HCV replication was not observed by ectopic expression of Hsc70 (Fig. 3A), and little or no interaction was observed between Hsc70 and HCV NS proteins in the co-immunoprecipitation analysis (data not shown). Thus, it is likely that TRiC/CCT acts as a regulator of HCV replication through participating in the de novo folding of NS5B RdRp, and Hsc70 might serve to assist in folding through its interaction with TRiC/CCT. It was recently reported that Hsc70 is associated with HCV particles and modulates the viral infectivity (Parent et al., 2009). Here we showed an additional role of Hsc70 in the HCV life cycle.

HCV genomic single-stranded RNA serves as a template for the synthesis of the full-length minus strand that is used for the overproduction of the virus-specific genomic RNA. NS5B RdRp is a single subunit catalytic component of the viral replication machinery responsible for both of these processes. It is known that the in vitro RdRp activity of recombinant NS5B expressed in and purified from insect cells and *Escherichia coli* is low in many cases. This could be due to the lack of a suitable cellular environment for favorable RdRp activity, although the particular conformational features dependent on the viral isolates may also be involved (Lohmann et al., 1997; Weng et al., 2009). In fact, besides interacting with HCV NS proteins, NS5B has been reported to interact with several host cell proteins. For example, human vesicle-associated membrane protein-associated protein subtype A (VAP-A) and subtype B (VAP-B), which are involved in the regulation of membrane trafficking, lipid transport and metabolism, and the unfolded protein response, interact with NS5B and NS5A and participate in HCV replication (Hamamoto et al., 2005). Recently, VAP-C, a splicing variant of VAP-B, was found to act as a negative regulator of viral replication through its interaction with NS5B but not with VAP-A (Kukihara et al., 2009). Cyclophilin A and B, peptidyl-prolyl isomerases that facilitate protein folding by catalyzing the *cis–trans* interconversion of peptide bonds at proline residues, play a role in stimulating HCV RNA synthesis through interaction with NS5B (Liu et al., 2009; Watashi et al., 2005). SNARE-like protein (Tu et al., 1999), eIF4AII (Kyono et al., 2002), protein kinase C-related kinase 2 (Kim et al., 2004), nucleolin (Kim et al., 2004; Hirano et al., 2003; Shimakami et al., 2006), and p68 (Goh et al., 2004) are also known to associate with NS5B and are possibly involved in HCV RNA replication.

We found that the aa 71–214 region in NS5B is important for interaction with TRiC/CCT. The catalytic domain of HCV RdRp has a "right-hand" configuration similar to other viral polymerases, such as HIV-1 reverse transcriptase (Huang et al., 1998) and poliovirus RdRp (Hansen et al., 1997), and is divided into the fingers, palm, and thumb functional subdomains (Lohmann et al., 2000). The region required for the interaction with TRiC/CCT has been mapped in a part of the fingers and palm domains of NS5B RdRp. To address how TRiC/CCT assists in the correct folding or disaggregation of NS5B through their interaction, leading to the formation of a functional RdRp, work based on an in vitro reconstitution system using purified proteins is under way. As all the TRiC/CCT subunits possess essentially identical ATPase domains, their protein-recognition regions are apparently divergent, allowing for substrate-binding specificity. It has recently been reported that TRiC/CCT interacts with the PB2 subunit of the influenza virus RNA polymerase complex and TRiC/CCT binding site is located in the central region of PB2, suggesting involvement of TRiC/CCT in the influenza virus life cycle (Fislová et al., 2010). Eukaryotic RNA polymerase subunit has also been identified as a binding partner of TRiC/CCT from interactome analysis (Yam et al., 2008). It would be interesting to examine how conserved the mechanisms of TRiC/CCT action that result in enhanced replication are among RNA polymerases.

The recruitment of a chaperonin by viral NS proteins may be important for understanding regulation of the viral genome replication. In this study, we demonstrated the involvement of TRiC/CCT in HCV RNA replication possibly through its interaction between TRiC/CCT and HCV NS5B. Although possible interaction of subunit CCT5 with NS5B was shown, considering involvement of whole TRiC/CCT complex in its chaperonin function, whether CCT5 directly interacts with NS5B is unclear. Further detailed studies are needed to make clear the manner of TRiC/CCT-NS5B interaction. NS5B RdRp is one of the main targets for HCV drug discovery. The search for NS5B inhibitors has resulted in the identification of several binding sites on NS5B, such as the domain adjacent to the active site and the allosteric GTP site (De Francesco and Migliaccio, 2005; Laporte et al., 2008). The findings obtained here suggest that disturbing the interaction between NS5B and TRiC/CCT may be a novel approach for an antiviral chemotherapeutic strategy.

Materials and methods

Cell culture, transfection, and infection

Human hepatoma Huh-7 and Huh-7.5.1 cells (kindly provided by Francis V. Chisari from The Scripps Research Institute) and human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Huh-7-derived SGR-N (Shi et al., 2003) and RCYM1 (Murakami et al., 2006) cells, which possess subgenomic replicon RNA from the HCV-N strain (Guo et al., 2001; Ikeda et al., 2002) and genome-length HCV RNA from the Con 1 strain (Pietschmann et al., 2002), were cultured in the above medium in the presence of 1 mg/ml G418. Cells were transfected with plasmid DNAs using FuGENE transfection reagents

(Roche Diagnostics, Tokyo, Japan). Culture media from Huh-7 cells transfected with in vitro-transcribed RNA corresponding to the full-length HCV RNA derived from the JFH-1 strain (Wakita et al., 2005) were collected, concentrated, and used for the infection assay (Aizaki et al., 2008).

Ab

Primary Abs used in this study were mouse monoclonal Abs against FLAG (Sigma-Aldrich, St. Louis, MO), c-myc (Sigma-Aldrich), CCT5 (Abnova Corporation, Taipei City, Taiwan), flotillin-1 (BD Biosciences, San Jose, CA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon, Temecula, CA), BrdU (Caltag, CA) and HCV NS5A (Austral Biologicals, San Ramon, CA), a rabbit polyclonal Ab against hemagglutinin (HA; Sigma-Aldrich), a sheep polyclonal Ab against bromodeoxyuridine (Biodesign International, Saco, ME), and goat polyclonal Abs against the individual subunits of CCT (Santa Cruz Biotechnology, Santa Cruz, CA) and Hsc70 (Santa Cruz Biotechnology). Anti Hsc70 and CCT5 monoclonal rat Abs were obtained from Abcam (Tokyo, Japan) and AbD serotec (Oxford, UK). Rabbit polyclonal antibody to NS5A was described previously (Hamamoto et al., 2005). Anti NS5B monoclonal Ab was kindly provided by D. Moradpour (Centre Hospitalier Universitaire Vaudois, University of Lausanne; Moradpour et al., 2002).

Plasmids

To generate expression plasmids for the NS proteins with dual epitope tags, DNA fragments encoding the NS3, NS5A, or NS5B proteins were amplified from HCV strain NIHJ1 (Aizaki et al., 1998) by PCR and cloned into the EcoRI–EcoRV sites of pcDNA3-MEF, which includes the MEF tag cassette containing the *myc* tag, TEV protease cleavage site, and FLAG tag sequences (Ichimura et al., 2005; Shirakura et al., 2007). To create a series of NS5B truncation mutants, each fragment was amplified by PCR and cloned into the EcoRI–XhoI site of pCMV-HA (Clontech, Mountain View, CA). To generate expression plasmids for the individual CCT subunits, cDNA fragments encoding human CCT1 through CCT8 were amplified from the total cellular RNA by RT-PCR and then cloned into the SmaI site of pCAGGS (Niwa et al., 1991). All PCR products were confirmed by nucleotide sequencing.

Proteome analysis

RC-rich membrane fractions from the cells were isolated as described previously (Aizaki et al., 2004). Briefly, cells were lysed in hypotonic buffer. After removing the nuclei, the supernatants were mixed with 70% sucrose, overlaid with 55% and 10% sucrose, and centrifuged at 38,000 rpm for 14 h. Proteins from the membrane fractions were then analyzed by 2D-DIGE as described previously (Hara et al., 2009). Briefly, protein samples were resolved in protein solubilization buffer (Bio-Rad Laboratories, Tokyo, Japan) and washed with pH adjustment buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris–HCl [pH 10.0]), before being labeled with fluorescent dyes; the dyes used were Cy3 for RCYM1 cells samples taken at the exponential growth phase, Cy5 for cells samples taken at the confluent phase, and Cy2 for a protein standard containing equal amounts of both cell samples. Aliquots of the labeled samples were pooled and applied to Immobiline DryStrip (GE Healthcare, Tokyo, Japan) for first-dimension separation and to 12.5% polyacrylamide gels for second-dimension separation. Images of the 2-D gels were captured on a Typhoon scanner (GE Healthcare), and analyzed quantitatively using DeCyder v5.0 software (GE Healthcare). Samples were analyzed in triplicate as independent cultures and the Student's *t*-test was applied using the DeCyder biological variation analysis module to validate the significance of the differences in spot intensity detected between the samples.

In vitro RNA replication assay

In vitro replication of HCV RNA was performed as described previously (Hamamoto et al., 2005). Briefly, cytoplasmic fractions of subgenomic replicon cells were treated with 1% NP-40 at 4 °C for 1 h, followed by being incubated with 1 mM of ATP, GTP, and UTP; 10 μM CTP; [^{32}P]CTP (1 MBq; 15 TBq/mmol); 10 μg/ml actinomycin D; and 800 U/ml RNase inhibitor (Promega, Madison, WI) for 4 h at 30 °C. RNA was extracted from the total mixture by using TRI Reagent (Molecular Research Center, Cincinnati, OH). The RNA was precipitated, eluted in 10 μl of RNase-free water, and analyzed by 1% formaldehyde-agarose gel electrophoresis. For the immunodepletion assay, the cytoplasmic fractions were incubated with anti-CCT5 Ab in the presence of NP-40 for 4 h before NTP incorporation.

MALDI-TOF MS analysis

Target spots were cut and collected from gels under UV luminescence and rechecked with Typhoon scanner. The spot gels of the target proteins were subjected to in-gel trypsin digestion and analyzed by MALDI-TOF MS meter (Voyager-DE STR, Applied Biosystems, Tokyo, Japan) as described previously (Yanagida et al., 2000). All proteins were identified by peptide mass fingerprinting.

Immunoblot analysis and immunoprecipitation

Immunoblot analysis was performed essentially as described previously (Aizaki et al., 2004). The membrane was visualized with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). For immunoprecipitation, cells transfected with plasmids expressing epitope-tagged HCV protein or CCT5 were lysed and then subjected to two-step precipitations with anti-myc and anti-FLAG Abs according to the procedures described previously (Ichimura et al., 2005). In some experiments, HA-tagged full-length NS5B (aa 1–591) or its deletion mutants (aa 71–591, 215–591, 320–591, 1–570) were co-expressed with CCT5 in cells, followed by single-step immunoprecipitation and immunoblotting.

Immunofluorescence staining

Cell permeabilization with lysolecithin and detection of de novo-synthesized viral RNA was performed as described previously (Shi et al., 2003). Briefly, Huh-7 cells were plated on 8-well chamber slides at a density of 5×10^4 cells per well. Cells were incubated with actinomycin D (5 μg/μl) for 1 h and were washed twice with serum-free medium, before being incubated for 10 min on ice. The cells were then incubated in a transcription buffer containing 0.5 mM BrUTP for 30 min. The cells were fixed in 4% formaldehyde for 20 min and then incubated for 15 min in 0.1% Triton X-100 in phosphate-buffered saline (PBS). Primary Abs were diluted in 5% bovine serum albumin in PBS and were incubated with the cells for 1 h. After washing with PBS, fluorescein-conjugated secondary Abs (Jackson Immunoresearch Laboratories, West Grove, PA) were added to the cells at a 1:200 dilution for 1 h. The slides were then washed with PBS and mounted in ProLong Antifade (Molecular Probes, Eugene, OR). Confocal microscopy was performed on a Zeiss Confocal Laser Scanning Microscope LSM 510 (Carl Zeiss MicroImaging, Thornwood, NY).

RNA interference

Small interfering RNAs (siRNAs) targeted to CCT5 or Hsc70 and scrambled negative control siRNAs were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Cells were plated on a 24-well plate with

antibiotic-free DMEM overnight, and each plate was transfected with 10 nM siRNAs by X-tremeGENE (Roche Diagnostics) according to the manufacturer's protocol. Forty-eight hours post-transfection, the total RNA and protein extracts were prepared and subjected to real-time RT-PCR and immunoblot analyses, respectively.

Quantitation of HCV RNA and core protein

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time RT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, CA) as described previously (Aizaki et al., 2004; Murakami et al., 2006). HCV core protein levels in the cells and in the supernatant were quantified using an HCV core enzyme-linked immunosorbent assay (Ortho-Clinical Diagnostics, Tokyo, Japan).

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Cross-species transmission of gibbon and orangutan hepatitis B virus to uPA/SCID mice with human hepatocytes

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ABSTRACT

To investigate the potential of cross-species transmission of non-human primate HBV to humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, in which the mouse liver has been engrafted with human hepatocytes, were inoculated with non-human primate HBV. HBV-DNA positive serum samples from a gibbon or orangutan were inoculated into 6 chimeric mice. HBV-DNA, hepatitis B surface antigen (HBsAg), and HB core-related antigen in sera and HBV cccDNA in liver were detectable in 2 of 3 mice each from the gibbon and orangutan. Likewise, applying immunofluorescence HBV core protein was only found in human hepatocytes expressing human albumin. The HBV sequences from mouse sera were identical to those from orangutan and gibbon sera determined prior to inoculation. In conclusion, human hepatocytes have been infected with gibbon/orangutan HBV.

1. Introduction

Hepatitis B is caused by hepatitis B virus (HBV), a hepatotropic virus of the family *Hepadnaviridae*. This family comprises two genera, Avihepadnavirus and Orthohepadnavirus which can infect birds and mammals, respectively (Mason et al., 2005). As for humans, approximately 350 million chronic carriers have been infected by HBV worldwide (Lavanchy, 2004) and 15–40 percent have developed liver cirrhosis and hepatocellular carcinoma (Lee, 1997; McQuillan et al., 1989; Sharma et al., 2005). In addition to humans, HBV also infects higher non-human primates (apes) such as orangutans (*Pongo pygmaeus*), gibbons (*Hylobates* sp. and *Nomascus* sp.), gorillas (*Gorilla gorilla*), and chimpanzees (*Pan troglodytes*) (Grethe et al., 2000; MacDonald et al., 2000; Makuwa et al., 2003; Noppornpanth et al., 2003; Sall et al., 2005; Sa-nguanmoo et al., 2008; Starkman et al., 2003; Warren et al., 1998). In comparison with human HBV, non-human primate HBVs contain a 33 nucleotide deletion in the *PreS1* gene and all non-human primate HBVs cluster within their respective group separate from each human HBV genotype (Grethe et al., 2000; Kramvis et al., 2005; Robertson, 2001; Takahashi et al., 2000).

Several experiments have been conducted to study cross-species transmission of human HBV to non-human primates. Human HBsAg positive sera were intravenously inoculated into chimpanzees. In all experiments, inoculated chimpanzees displayed HBsAg in their sera (Kim et al., 2008; Tabor et al., 1980). In 1977, Bancroft et al. inoculated pooled saliva collected from 5 human carriers into gibbons. Gibbons which received subcutaneous injections of the pooled saliva developed serological markers of HBV infection. In contrast, gibbons infected via either the nasal or oral route did not show evidence of HBV infection (Bancroft et al., 1977). However, the negative results in this study are probably attributable to the lack of a sufficiently sensitive test available at that time. Alter et al. transmitted semen and saliva of carrier patients to chimpanzees. Chimpanzees developed HBsAg and elevated ALT after inoculation (Alter et al., 1977). In 1980, Scott et al. inoculated semen donated by HBsAg and HBeAg positive patients

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into gibbons via the subcutaneous and vaginal route. Moreover, saliva of carrier patients was pooled and inoculated into gibbons via the subcutaneous and oral route. The results showed that semen and saliva from carrier patients cause asymptomatic disease in gibbons when transmitted via the subcutaneous or vaginal route, yet not via the oral route (Scott et al., 1980).

In addition to these experiments, Mimms et al. performed studies by infecting a chimpanzee with gibbon HBV. The HBV-DNA sequence from this chimpanzee was similar to that of gibbon HBV (Mimms et al., 1993). In conclusion, human HBV can be transmitted to non-human primates and cross-species transmission of non-human primate HBV can occur among various non-human primate species. However, cross-species transmission of non-human primate HBV to humans has not yet been supported by scientific evidence. To avoid performing experiments in humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, with the liver replaced with human hepatocytes (chimeric mice) serve as a suitable model for studies on human liver-specific pathogens such as HCV and HBV, human hepatic metabolism of pharmaceutical agents, and human hepatic toxicity of candidate anti-proliferative agents (Kneteman and Mercer, 2005). The mice present evidence that more fully characterizes the repopulation of the mouse liver with human hepatocytes (Meuleman et al., 2005). Histological studies have revealed that chimeric mice show evidence of human hepatocyte replace integration with infiltration into mouse liver. Moreover, human albumin and 21 other human specific proteins can be detected in mouse sera (Dandri et al., 2001; Mercer et al., 2001). Subsequently, these mice were used to support woodchuck and human hepatocyte culture and were supported infection with woodchuck hepatitis virus (WHV) and HBV (Meuleman et al., 2005; Petersen et al., 1998; Tabuchi et al., 2008).

The aim of this study has been to demonstrate that non-human primate HBV can be replicated in human hepatocytes in order to consider preventive measures in case of potential HBV transmission from non-human primates to humans.

2. Materials and methods

The study was approved by the Faculty of Veterinary Science, Animal Care and Use Committee, Mahidol University. All experiments were performed in a biosafety level 2 laboratory.

2.1. Gibbon and orangutan HBsAg-positive serum

To study cross-species transmission of non-human primate HBV to humans, the HBsAg and HBV-DNA positive sera of white-cheeked gibbon (*Nomascus leucogenys*) and orangutan (*P. pygmaeus*) were collected from Dusit zoo, Bangkok and Khao Pratub Chang Wildlife Breeding Center, Ratchaburi, Thailand, respectively. These sera constitute the stored surplus sera from a previous study (Sa-nguanmoo et al., 2008).

2.2. Chimeric mice inoculation

Twelve-week-old SCID mice transgenic for urokinase-type plasminogen activator with human hepatocytes (PhoenixBio Co, Ltd., Hiroshima, Japan) were used in this study (Tateno et al., 2004). Real-time PCR was employed to detect non-human primate HBV DNA concentration in gibbon and orangutan serum. This detection method has been shown elsewhere (Abe et al., 1999).

The minimum infectious dose of pre-acute and late acute HBV for HBV transmission to chimeric mice with human hepatocyte repopulation is approximately 10^0 and 10^2 copies (Tabuchi et al., 2008). In this study, 10^4 gibbon or orangutan HBV genome equivalents were intravenously inoculated into 3 chimeric mice of each group. However, none of the chimeric mice showed evidence of HBV markers until week 4 after inoculation. Then, all chimeric mice were re-inoculated with 10^5 genome equivalents.

2.3. Serum collection and HBV DNA extraction

Twenty microliter serum samples were collected once a week after inoculation. HBV DNA was extracted from 5 μl mouse sera by using the QIAamp® DNA Mini kit (QIAGEN, QIAGEN Sciences Inc., MD) following the manufacturer's recommendation.

2.4. HBV DNA quantitative method

HBV DNA quantity was determined by real-time PCR (ABI 7500 Fast Real-time PCR, Applied Biosystems, Foster City, CA). To that end, the small S region was amplified as previously described (Abe et al., 1999). Briefly, 5 μl of DNA were subjected to quantitative HBV DNA analysis by ABI 7500 Fast Real-time PCR (Applied Biosystems, Foster City, CA). The reaction mixture comprised 12.5 μl TaqMan® Universal PCR MasterMix (Applied Biosystems, Foster City, CA), 0.5 μl of 10 μM forward primer (HBSF2: 5′-CTTCATCCTGCTGCTATGCCT-3′), 0.5 μl of 10 μM reverse primer (HBSR2: 5′-AAAGCCCAGGATGATGGGAT-3′), 0.5 μl of 10 μM probe (HBSP2G: FAM-ATGTTGCC CGTTTGTCCTCTAATTCCAG-TAMRA) and 6 μl distilled water. The real-time PCR was performed under the following conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s, and 4 °C for the holding step. The HBV viral load in unknown samples was calculated by comparison with the standard curve. The detection limit in this study was 1000 copies/ml due to the small sample volume.

2.5. DNA extraction from mouse liver tissue and cccDNA detection in liver and sera of infected chimeric SCID mice

Mouse liver tissues from one HBV-DNA positive mouse each from the gibbon and orangutan HBV inoculation group were collected at week 15 after inoculation. To extract DNA from mouse liver tissue, 25 mg of liver tissue were extracted by using the DNeasy® Blood & Tissue kit (QIAGEN, QIAGEN Sciences Inc., MD) and eluted in 200 μl of elution buffer. HBV cccDNA was detected by conventional PCR (GeneAmp® PCR System 9700, Applied Biosystems, Foster City, CA). Primer sequences have been previously published (Suzuki et al., 2009). Partially double-stranded HBV DNA could not be amplified by these primers. The details have been previously described (Mason et al., 1998). Briefly, 5 μl of DNA were subjected to amplification by GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The reaction mixture comprised 1 U of Ampli Taq Gold® (Applied Biosystems, Foster City, CA), 2.5 μl of 10× PCR buffer containing 15 mM $MgCl_2$, 2 μl of GeneAmp® dNTP Mix (Applied Biosystems, Washington, UK), 1 μl of 10 μM forward primer (cccF2: 5′-CGTCTGTGCCTTCTCATCTGA-3′), 1 μl of 10 μM reverse primer (cccR4: 5′-GCACAGCTTGGAGGCTTGAA-3′), and 13.3 μl distilled water. The PCR was performed under the following conditions: 96 °C for 10 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, and 4 °C for the holding step.

2.6. Entire genome sequencing and phylogenetic analysis

Mouse serum samples positive for HBV DNA were subjected to further studies by sequencing the entire genome sequences. To amplify the entire genome, 1 μl of DNA re-suspended solution was used as template for round I PCR. The entire genome was distinguished into two segments (fragment A and fragment B). Fragment A was amplified by 10 μM forward primer (HBV17F-SARU: 5′-CAAACTCTGCAAGATCCCAGAG-3′) and 10 μM reverse

primer (HBV1799R-SARU 5′-GACCAATTTATGCCTACAGCCTC-3′). Fragment B was amplified by 10 μM forward primer (HBV1595F-SARU: 5′-CTTCACCTCTGCACGTTGCATGG-3′) and 10 μM reverse primer (HBV262R-SARU: 5′-CCACCACGAGTCTAGACTCTGTGG-3′). Both fragment A and fragment B used the same reaction mixture as follows: 5 μl of 2.5 mM dNTP, 2 μl of 10 μM forward primer, 2 μl of 10 μM forward primer, 0.33 μl of LA-Taq (TaKaRa BIO INC, Shiga, Japan), and 29.67 μl distilled water. The amplification method was performed on GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The thermal cycle was continued as follows: 95 °C for 2 min (pre-denaturation) and followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and 72 °C for 15 min (final extension).

For the second round PCR, 2 μl of round I PCR was used as template. Round I PCR product of fragment A was nested by HBV47F-SARU forward primer (5′-CTGTATTTTCCTGC-TGGTGGCTCCAG-3′) and HBV1760R-SARU reverse primer (5′-TAACCTCGTCTCCGCCCCAAACTC-3′). The first round I PCR product of fragment B was nested by HBV1608F-SARU forward primer (5′-GCATGGAGACCACCGTGAACG-3′) and HBV201R-SARU reverse primer (5′-TGTAACACGAGCAGGGGTCCTAGG-3′). Both fragment A and fragment B used reaction mixtures as round I PCR except increasing in the first round PCR template to 2 μl and adjusting distilled water to 28.67 μl. The amplification program was performed as follows: 95 °C for 2 min (pre-denaturation) and followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and 72 °C for 20 min (final extension).

The second round PCR products were segregated by electrophoresis on 1% agarose gel stained with ethidium bromide. The bands of PCR products were purified using the QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany). Purified products were further analyzed by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The genome was sequenced using the 8 primer sets previously published (Sugauchi et al., 2001). Cycle sequencing was performed using the BigDye Terminator 3.1V cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The conditions for sequencing were programmed into the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) as previously reported (Sugauchi et al., 2001). Nucleotide sequences were edited and assembled using SEQMAN 4.00 (LASERGENE program package, DNASTAR, DNASTAR Inc., Madison, WI). All complete HBV genomes isolated from mouse sera were compared to nucleotide sequences available at the GenBank database by using the Blast program (NCBI, Bethesda, MD). Moreover, the HBV sequences obtained from mouse sera were compared with gibbon and orangutan HBV strains determined prior to inoculation and also compared with other non-human primate HBVs and each human genotype from the GenBank database (NCBI, Bethesda, MD). Genetic comparison was performed by Clustal X program version 2.0.10 (European Bioinformatics Institute, Cambridge, UK). Subsequently, the phylogenetic tree was constructed using the Tamura – 3 parameter neighbor-joining method by Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ).

2.7. HBsAg, HBcrAg, and human albumin measurement in mouse sera

Mouse sera were diluted (1:10) and subjected to chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio Diagnostic, Inc., Tokyo, Japan) to detect HBV surface antigen (HBsAg) and HBV core – related antigen: – the antigen which includes both the HBV precore/core proteins (HBcrAg) (Kimura et al., 2005; Shinkai et al., 2006). HBcrAg measurement by this assay implies detection of precore/core proteins, including core protein and HBeAg (Kimura et al., 2002, 2005; Rokuhara et al., 2003; Wong et al., 2007). HBcrAg also showed a good correlation with HBV DNA levels in Asian patients (Kimura et al., 2002; Rokuhara et al., 2003, 2005) and intrahepatic parameters, including fibrosis scores, intrahepatic HBV, cccDNA and nuclear HBcAg (Wong et al., 2007). To expose the core protein and HBeAg, the diluted serum was fist incubated with the solution that contains sodium dodecylsulfate. Subsequently, the lysate was added to the plate coated with primary antibody to HBcAg and HBeAg. After incubation, the plate was washed to discard excess primary antibody and the second antibody labeled with alkaline phosphatase was added. Upon addition of substrate solution, the incubated reaction was measured by chemiluminescent enzyme immunoassay (CLEIA). Fully automated analysis was performed using the Lumipulse® System (Fujirebio Diagnostic, Inc., Tokyo, Japan). Human serum albumin (h-Alb) levels were determined applying a commercial enzyme linked immunosorbent (ELISA) test kit (Bethyl Laboratories Inc., Montgomery, TX).

2.8. Immunohistofluorescence assay

To detect HBcAg and human albumin, thick mouse liver tissue was prepared by cutting the frozen mouse liver with a Leica CM1900 Cryostat-microtome (Meyer Instruments, Inc., Houston, TX) and mounting the slices on glass slides. Histological analysis was performed by immunofluorescence assay as previously reported (Sugiyama et al., 2006). Briefly, mouse liver tissue was blocked by DakoCytomation antibody diluent (Dako North America, Inc., Carpinteria, CA) for 10 min at room temperature. After drying by air, the tissue was incubated in the dark with 50 μg/ml of polyclonal rabbit anti-hepatitis B virus core antigen (HBcAg) (Dako North America, Inc., Carpinteria, CA) for 1 h at 37 °C. After washing 5 times with 1× phosphate buffered saline (PBS) (GIBCO, Invitrogen Corporation, Carlsbad, CA) the tissue was incubated with 50 μg/ml of Cy3® goat anti-rabbit IgG (H + L) (Invitrogen Molecular Probes, Eugene, OR) or 5 μg/ml of goat anti-human albumin FITC (Bethyl Laboratories, Inc., Montgomery, TX) in the dark at 37 °C for 1 h. After washing 5 times with 1× PBS, the tissue was mounted by VECTASHIELD mounting medium with DAPI (Vector Laboratories, Inc., Birmingham, CA). The stained mouse tissue was examined under a Nikon Microscope ECLIPSE E800 (Nikon Instruments, Inc., Melville, NY).

3. Results

3.1. Serum HBV DNA, HBsAg, HBcrAg and human albumin level quantitation

Upon first inoculation with serum containing 10^4 copies of gibbon or orangutan HBV, none of the mice could be infected. Then, chimeric mice were re-inoculated with 10^5 copies. One mouse died before re-inoculation. After re-inoculation, mouse sera were collected once a week. Samples were subjected to quantitative HBV DNA analysis by real-time PCR while HBsAg and HBcrAg were quantitatively determined by CLIEA. Four of 5 mice could be infected with gibbon or orangutan HBV. Two mice each from the gibbon and orangutan groups showed levels of HBV DNA, HBsAg, and HBcrAg with the remaining mouse not displaying any of these markers. In detail, HBV DNA and HBcrAg could be detected in serum samples from two mice of the gibbon group (code 101 and 103) and two mice of the orangutan group (code 201 and 202) 4 weeks after inoculation. HBsAg was present in the orangutan group 4 weeks and in the gibbon groups 6 weeks after inoculation, respectively.

In this experiment, the expected HBV markers HBV DNA, HBsAg and HBcrAg could be detected in mouse serum around 4–5 weeks after inoculation. This finding matched previous studies that had

inoculated human HBV genotypes A2, C2, B1 and J into chimeric SCID mice (Sugiyama et al., 2009; Tatematsu et al., 2009). The time appearance and progression of non-human primate HBV markers presented as same as with human HBV markers (Ganem and Prince, 2004). Human albumin (h-Alb) was measured by ELISA as a quality control. Serum h-Alb levels prior to inoculation of all mice in this study exceeded 7 mg/ml indicating a human hepatocyte replacement index (RI) of over 70 percent (PhoenixBio Co, Ltd., Hiroshima, Japan) and were stable during the experiment (Fig. 1). Mean alanine aminotransferase (ALT) levels were around 200 IU/L in the uPA/SCID mouse sera. After non-human primate HBV inoculation, ALT levels slightly increased in this study (data not shown).

3.2. Intrahepatic cccDNA detection in liver tissue and mouse sera

Using the specific primers that amplify only cccDNA (Suzuki et al., 2009), HBV cccDNA was detected in mouse liver tissue from those mice that had been infected with gibbon and orangutan HBV (Fig. 2A). Moreover, cccDNA was found in the sera of mice infected with gibbon HBV (Fig. 2B).

3.3. Phylogenetic analysis of the entire HBV genome from mouse sera

HBV-DNA from all four mice was amplified and subjected to sequencing of the entire genome. The sequences from mouse sera were identical to HBV from gibbon or orangutan serum determined prior to inoculation (gibbon code GD14, GenBank ID: HQ603061; orangutan code OS25, GenBank ID: EU155824) (Fig. 3). Comparison between the complete HBV sequences from mouse sera and gibbon or orangutan sera prior to inoculation showed 99.9% and 100% similarity, respectively.

3.4. HBcAg and human albumin detection in mouse liver tissue

The mouse liver was also tested for HBcAg by staining with polyclonal rabbit anti-HBcAg and goat anti-rabbit IgG labeled with Cy3 (Fig. 4A). To locate the human hepatocyte area in chimeric mouse liver, the tissue was examined for human albumin. The same mouse liver tissue was stained with goat anti-human albumin conjugated with FITC (Fig. 4B). The study confirmed that HBcAg was found in the same area of human hepatocytes (Fig. 4C).

4. Discussion

In a previous study, Hu et al. (2000) constructed a phylogenetic tree and found that the *S* gene sequence from two chimpanzees clustered with human HBV genotypes A and C which could suggest possible virus transmission from human to chimpanzee. Currently, there is no evidence indicating natural infection of humans with non-human primate HBV (Noppornpanth et al., 2003). However, non-human primate HBV would be transmitted to humans because the respective HBV genomes are largely similar.

In this study, cross-species transmission was performed using chimeric mice containing human hepatocytes. The results showed that HBV-DNA, HBsAg and HBcrAg can be detected in sera of mice inoculated with HBV-DNA positive sera from orangutan or gibbon carriers. Detection of HBV cccDNA in liver as well as immune staining data have provided the evidence that gibbon and orangutan HBV can be replicated in human hepatocytes of the chimeric mice sero-positive for HBV DNA. HBsAg and HBV DNA concentrations could increase over time following inoculation. Interestingly, based on phylogenetic analysis, all strains of HBV sequences obtained from mouse sera inoculated with gibbon or orangutan HBV carrier sera grouped with HBV from gibbon and orangutan sera determined prior to inoculation. Nucleotide comparison between HBV in mouse sera and the HBV strain used for inoculation showed 100% identity.

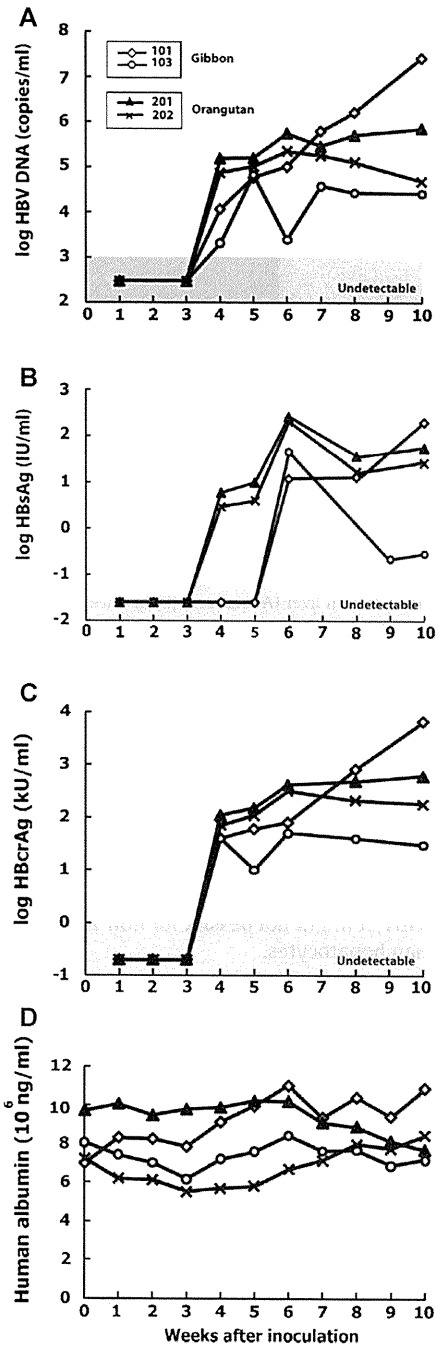

Fig. 1. HBV DNA, HBsAg, HBcrAg, and human albumin concentration in inoculated mouse sera on secondary inoculation. (A) Serum HBV DNA level. Gray zone indicates an area below the minimum sensitivity of real-time PCR (<10^3 copies/ml) (B) HBsAg concentration. The limitation of the test is 0.05 IU/ml. (C) HBcrAg level with the limited sensitivity at 1 kU/ml and (D) h-Alb concentration.

HBV infection depends on the infectious doses of HBV inoculums and host factors. In our experiment, one SCID mouse with human hapatocytes could not be infected with non-human primate HBV. This mouse lacks T- and B-lymphocytes as a protection from viral infection but still, it remains clear from viral infection. Some

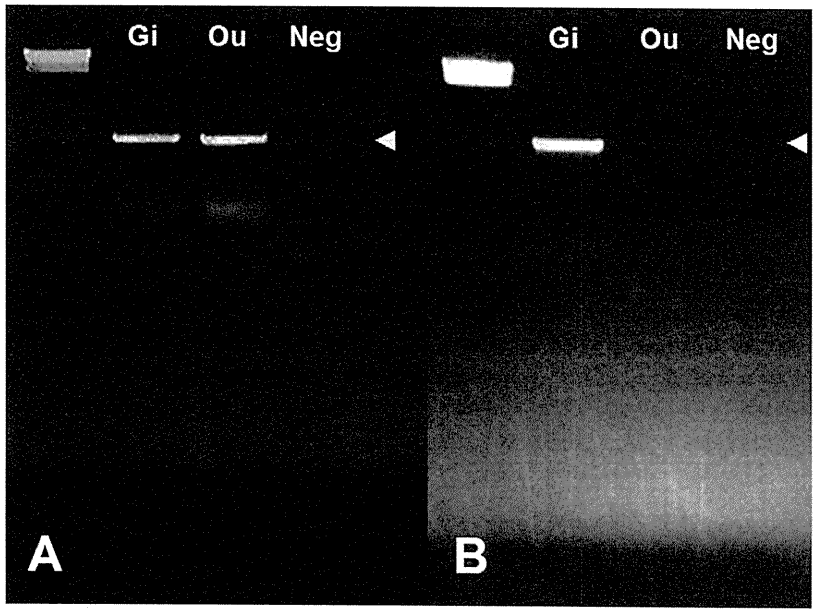

Fig. 2. CccDNA detection in liver (A) and sera (B) of mice that infected with gibbon HBV (Gi) or orangutan HBV (Ou). Neg represents negative PCR control (lacking DNA template). Arrow represents the target cccDNA PCR product.

researchers have attributed this to innate immunity of SCID mice (Lin et al., 1998). SCID mice have a normal innate immune system such as monocytes and macrophages (Ansell and Bancroft, 1989) which probably plays an important role in these mice. Moreover, infection of human hepatocytes with non-human primate HBV may be difficult due to the higher infectious dose required. Moreover, research on the early step of non-human primate HBV attachment to human hepatocytes has not been performed and the pathway of non-human primate HBV infection is still unclear. In comparison with human HBV, it might not be easy for non-human primate HBV to infect human hepatocytes.

Notably, a previous study has reported a new human HBV genotype (HBV-J) isolated from a Japanese patient with hepatocellular carcinoma (Tatematsu et al., 2009). The first HBV strain of interspecies HBV genotype J was closely related to gibbon and orangutan HBV strains and had a deletion of 33 nucleotides at the *preS1* region identical to non-human primate strains. Interestingly, this patient used to live in Borneo—a gibbon and orangutan habitat and hence, an endemic area (Tatematsu et al., 2009). He may have been infected with non-human primate HBV either by close contact or by eating raw meat of non-human primate HBV carriers (personal communication). However, infection of humans with non-human

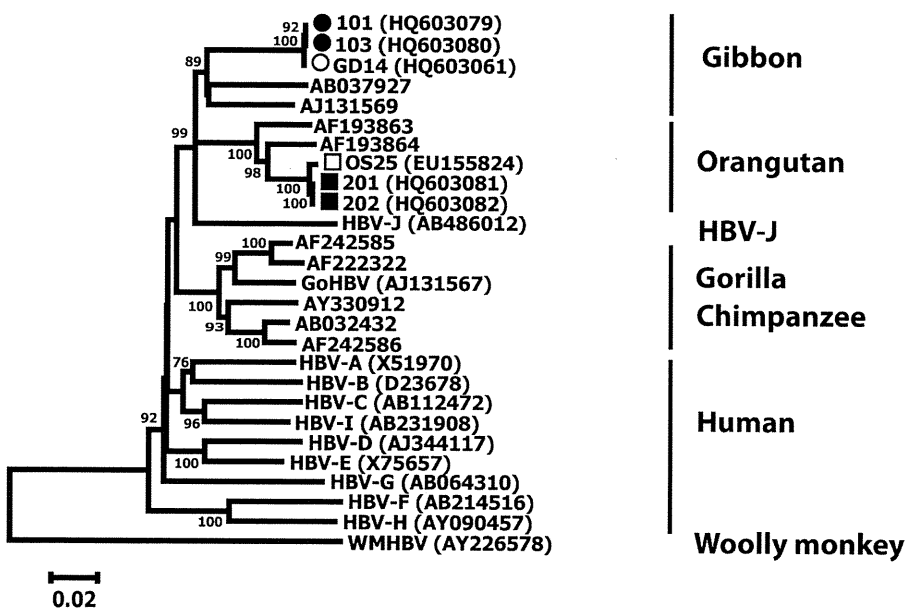

Fig. 3. Phylogenetic analysis of the entire HBV sequence obtained from mouse sera and available sequences of non-human primate HBV strains from GenBank database. Support of each branch as determined from 1000 bootstrap samples. Only 75% bootstrap values are indicated at each node. The scale bar at the bottom represents the genetic distance. Non-human primate HBV sequences obtained from our study are indicated by symbol (gibbon, ○; orangutan, □). HBV sequences obtained from mouse sera were ● and ■ for mice inoculated with gibbon and orangutan sera, respectively.

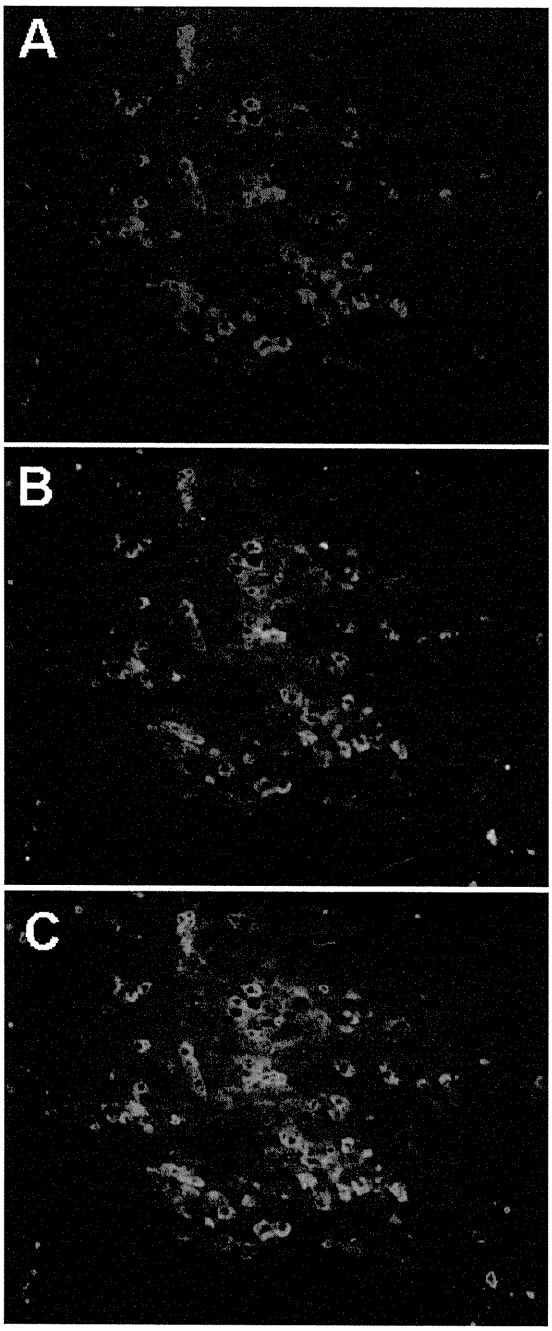

Fig. 4. Immunohistofluorescence of SCID mice infected with gibbon HBsAg-positive serum. Mouse liver tissue incubated for HBcAg (A), human albumin (B), and co-localization of HBcAg and human albumin (C).

primate HBV by eating raw meat or close contact with non-human primate HBV carriers would be hypothesis.

Yet, it has been reported that chimeric SCID mice with human hepatocytes can be infected by inoculation with HBV positive chimpanzee sera (Tabuchi et al., 2008) similar to what has been found in this study. In that previous study, human hepatocyte transplanted chimeric mice were used to study the HBV infectious titer in sera of pre-acute and late acute phase patients. These mice were inoculated with HBV infected chimpanzee sera. The chimeric mice also displayed HBV infection markers such as HBsAg, anti-HBc and anti-HBs as has been shown in this research. But the HBV in chimpanzee sera used to inoculate chimeric mice was human HBV, in contrast to the non-human primate HBV used in this study. Thus, this study is the first scientific evidence to prove and confirm that non-human primate such as gibbon and orangutan HBV can infect and replicate in human hepatocytes. Moreover, this finding can support the discovery of the HBV-J genotype which was found in the human and the assumption that humans can be infected with non-human primate HBV strains is still hypothesis.

Even though uPA-SCID mice with human hepatocytes constitute a useful animal model to study cross-species transmission, this model does not mirror the humoral and cellular immune response of the natural host. In real life, humans may be infected with non-human primate HBV and may clear this virus by their immune system. However, the results of this study indicated that human hepatocytes of chimeric mice have been infected with HBV from gibbon, orangutan and also with human HBV from infected chimpanzee sera as previously reported (Tabuchi et al., 2008). Previous studies have demonstrated cross-species transmission of human HBV to non-human primates, of non-human primate HBV to other species of non-human primates, and this study has demonstrated that non-human primate HBV can replicate in human hepatocytes. As non-human primates represent various virus reservoirs, not only of HBV but also lymphocryptovirus (LCV), Epstein-Barr virus (EBV), or simian foamy virus (SFV), people in close contact with animal HBV carriers should be aware and protect themselves from animal bites or exposure to infected blood or body fluids of non-human primates.

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