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Short Communication

Structural requirements of virion-associated cholesterol for infectivity, buoyant density and apolipoprotein association of hepatitis C virus

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Our earlier study has demonstrated that hepatitis C virus (HCV)-associated cholesterol plays a key role in virus infectivity. In this study, the structural requirement of sterols for infectivity, buoyant density and apolipoprotein association of HCV was investigated further. We removed cholesterol from virions with methyl β-cyclodextrin, followed by replenishment with 10 exogenous cholesterol analogues. Among the sterols tested, dihydrocholesterol and coprostanol maintained the buoyant density of HCV and its infectivity, and 7-dehydrocholesterol restored the physical appearance of HCV, but suppressed its infectivity. Other sterol variants with a 3β-hydroxyl group or with an aliphatic side chain did not restore density or infectivity. We also provide evidence that virion-associated cholesterol contributes to the interaction between HCV particles and apolipoprotein E. The molecular basis for the effects of different sterols on HCV infectivity is discussed.

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Hepatitis C virus (HCV) is a major cause of liver diseases, and is an enveloped, plus-strand RNA virus of the genus *Hepacivirus* of the family *Flaviviridae*. The mature HCV virion is considered to consist of a nucleocapsid, an outer envelope composed of the viral E1 and E2 proteins and a lipid membrane. Production and infection of several enveloped viruses, such as human immunodeficiency virus type 1 (HIV-1), hepatitis B virus and varicella-zoster virus (Bremer *et al.*, 2009; Campbell *et al.*, 2001; Graham *et al.*, 2003; Hambleton *et al.*, 2007), are dependent on cholesterol associated with virions. However, except for HIV-1 (Campbell *et al.*, 2002, 2004), there is limited information about the effects of replacing cholesterol with sterol analogues on the virus life cycle. We demonstrated the higher cholesterol content of HCV particles compared with host-cell membranes, and that HCV-associated cholesterol plays a key role in virion maturation and infectivity (Aizaki *et al.*, 2008). Recently, by using mass spectrometry, Merz *et al.* (2011) identified cholesteryl esters, cholesterol, phosphatidylcholine and sphingomyelin as major lipids of purified HCV particles.

To investigate further the effect of the structural requirement for cholesterol on the infectivity, buoyant density and apolipoprotein association of HCV, depletion of virion-associated cholesterol and substitution of endogenous cholesterol with structural analogues (Fig. 1a) was used in this study. HCVcc (HCV grown in cell culture) of the JFH-1 isolate (Wakita *et al.*, 2005), prepared as described previously (Aizaki *et al.*, 2008), was treated with 1 mM methyl β-cyclodextrin (B-CD), which extracts cholesterol from biological membranes, for 1 h at 37 °C. The cholesterol-depleted virus was then incubated with exogenous cholesterol or cholesterol analogues at various concentrations for 1 h. After removal of B-CD and free sterols by centrifugation at 38 000 r.p.m. (178 000 *g*) for 2.5 h, the treated particles were used to infect Huh7 cells, kindly provided by Dr Francis V. Chisari (The Scripps Research Institute, La Jolla, CA, USA), and their infectivity was determined by quantifying the viral core protein in cells using an enzyme immunoassay (Ortho-Clinical Diagnostics) at 3 days post-infection (p.i.). Virus infectivity, which fell to <20 % after B-CD treatment, was

A supplementary table and figure are available with the online version of this paper.

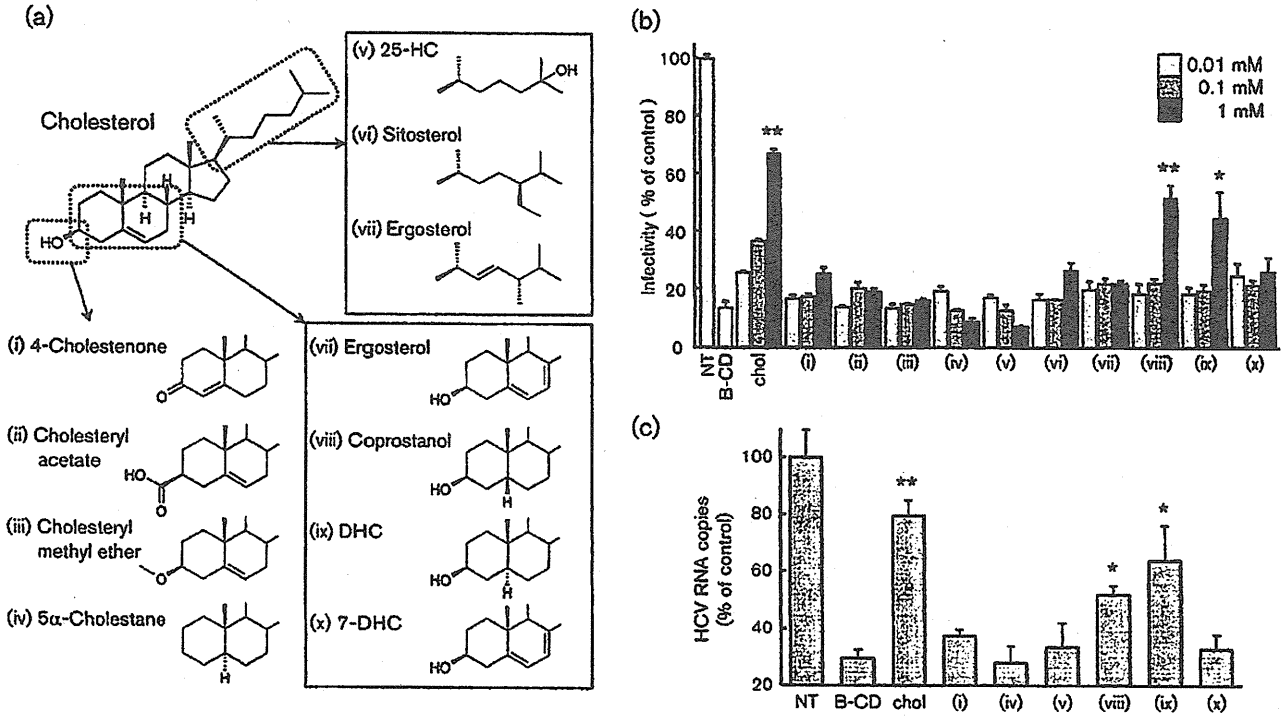

Fig. 1. Role of virion-associated cholesterol analogues in virus infection. (a) Structures of sterols used in this study. Variations in the 3β-hydroxyl group (lower left), aliphatic side chain (upper right) or ring structure (lower right) of cholesterol are shown. (i–x) Compounds studied in (b) and (c). (b) Effect of replenishment with sterols on HCV infectivity. Intracellular HCV core levels were determined at 72 h p.i. as the indicator of infectivity, which is represented as a percentage of the untreated HCVcc level (NT). (c) Effects of virion-associated sterols on virus internalization. HCV RNA copies in cells after virus internalization were quantified and are shown as percentages of the untreated HCVcc level (NT). (b, c) Means+SD of four samples are shown. $^*P<0.05$; $^{**}P<0.01$, compared with B-CD-treated virus (unpaired Student's *t*-test). Data are representative of at least two experiments.

recovered by addition of cholesterol at 0.01–1 mM in a dose-dependent manner (Fig. 1b). Among the cholesterol analogues tested, variants with a 3β-hydroxyl group (4-cholestenone, cholesteryl acetate, cholesteryl methyl ether and 5α-cholestane) or variants with an aliphatic side chain [25-hydroxycholesterol (25-HC), sitosterol and ergosterol] exhibited no or little effect on the recovery of infectivity of B-CD-treated HCV (Fig. 1b, lanes i–vii). In contrast, addition of variants in the structure of the sterol rings [coprostanol or dihydrocholesterol (DHC)] at 1 mM restored infectivity to around 50 % compared with non-treated virus control (Fig. 1b, lanes viii and ix). Other variants in the ring structure [7-dehydrocholesterol (7-DHC) and ergosterol, which is also a variant with an aliphatic side chain as indicated above] did not show any increase in the infectivity of B-CD-treated virus (Fig. 1b, lanes x and vii).

We demonstrated previously that HCV-associated cholesterol plays an important role in the internalization step of the virus, but not in cell attachment during virus entry (Aizaki *et al.*, 2008). The effect of virion-associated cholesterol analogues on virus attachment to cells and following internalization was determined. HCVcc, treated with B-CD with or without subsequent replenishment with sterols, was incubated with Huh7-25-CD81 cells, which stably express CD81 (Akazawa *et al.*, 2007), for 1 h at 4 °C. As an internalization assay, the incubation temperature was shifted to 37 °C post-binding procedure and maintained for 2 h. The cells were then treated with 0.25 % trypsin for 10 min at 37 °C, by which >90 % of HCV bound to the cell surface was removed (data not shown; Aizaki *et al.*, 2008). Internalized HCV was quantified by measuring the viral RNA in cell lysates by real-time RT-PCR (Takeuchi *et al.*, 1999). B-CD treatment or supplementation with sterols of B-CD-treated HCV had little or no effect on virus attachment to the cell surface (data not shown). Regarding virus internalization (Fig. 1c), treatment of HCVcc with 1 mM B-CD resulted in approximately 70 % reduction of viral RNA. The reduced level of the internalized HCV recovered markedly to approximately 80 % of the untreated HCVcc level by replenishment with 1 mM cholesterol. In agreement with the results shown in Fig. 1(b), addition of coprostanol or DHC to the B-CD-treated virus caused a significant recovery of virus internalization, suggesting that coprostanol and DHC associated with the

virion have the ability to play a role in HCV internalization into cells, in a manner comparable to cholesterol (Fig. 1c, lanes viii and ix). No or only a little recovery of virus internalization was observed by loading with other cholesterol analogues, such as 4-cholestenone, 5α-cholestane, 25-HC or 7-DHC (Fig. 1c, lanes i, iv, v and x).

To monitor the effect of cholesterol analogues on the physical characteristics of HCV, we next investigated buoyant-density profiles by using sucrose density-gradient centrifugation, in which untreated, B-CD-treated and sterol-replenished HCVcc were concentrated and layered onto continuous 10–60 % (w/v) sucrose density gradients, followed by centrifugation at 35 000 r.p.m. (151 000 *g*) for 14 h. Fractions were collected and analysed for the core protein. Fig. 2 shows that the virus density became higher after treatment with B-CD and that cholesterol-replenished virus shifted the density of B-CD-treated HCV to the non-treated level. Consistent with the result shown in Fig. 1(b), no effect on restoration of the buoyant densities of HCV was observed using variants with modifications in either the 3β-hydroxyl group (4-cholestenone, cholesteryl acetate and 5α-cholestane) or the aliphatic side chain (25-HC and sitosterol). In contrast, variants in the sterol ring structure (coprostanol, DHC and 7-DHC) had an ability to recover the density of B-CD-treated virus to that of non-treated virus.

Incorporation efficiency of the sterols into the cholesterol-depleted HCVcc was further determined by gas chromatography with flame ionization detection (see Supplementary Table S1, available in JGV Online). Under the experimental conditions used, exogenously supplied cholesterol after B-CD treatment was able to restore cholesterol content in HCVcc almost to initial levels. When 4-cholestenone, cholesteryl acetate, 25-HC, DHC or 7-DHC was added to B-CD-treated HCVcc, virion-associated sterol levels were 146, 157, 68, 96 or 73 %, respectively, of that of the non-treated control. The proportion of cholesterol analogues to the total sterols incorporated was ⩾30 % when 4-cholestenone, cholesteryl acetate, DHC or 7-DHC was used; however, the proportion in the case of 25-HC was only 3 %. It may be that the hydrophilic modification of the aliphatic side chain leads to poor association with HCVcc.

Collectively, exogenous variants with the 3β-hydroxyl group, such as 4-cholestenone and cholesteryl acetate, can be incorporated into B-CD-treated HCVcc, but resulted in no recovery of virus infectivity, indicating the importance of the 3β-hydroxyl group of cholesterol associated with the virus envelope in HCV infectivity. In contrast, two variants with modification in their sterol ring structures, coprostanol and DHC, have the ability to substitute for cholesterol. However, 7-DHC, another variant within the sterol ring, is incorporated readily into the depleted virion and restores the virus density, HCV replenished with 7-DHC is not infectious. These facts suggest that reduced forms of the sterol ring (coprostanol and DHC) in virion-associated cholesterol can be permitted for maintaining virus infectivity. However, a molecule with an additional double bond in the ring structure (7-DHC) seems to fail to exhibit infectivity, presumably because the change reduces structural flexibility in the

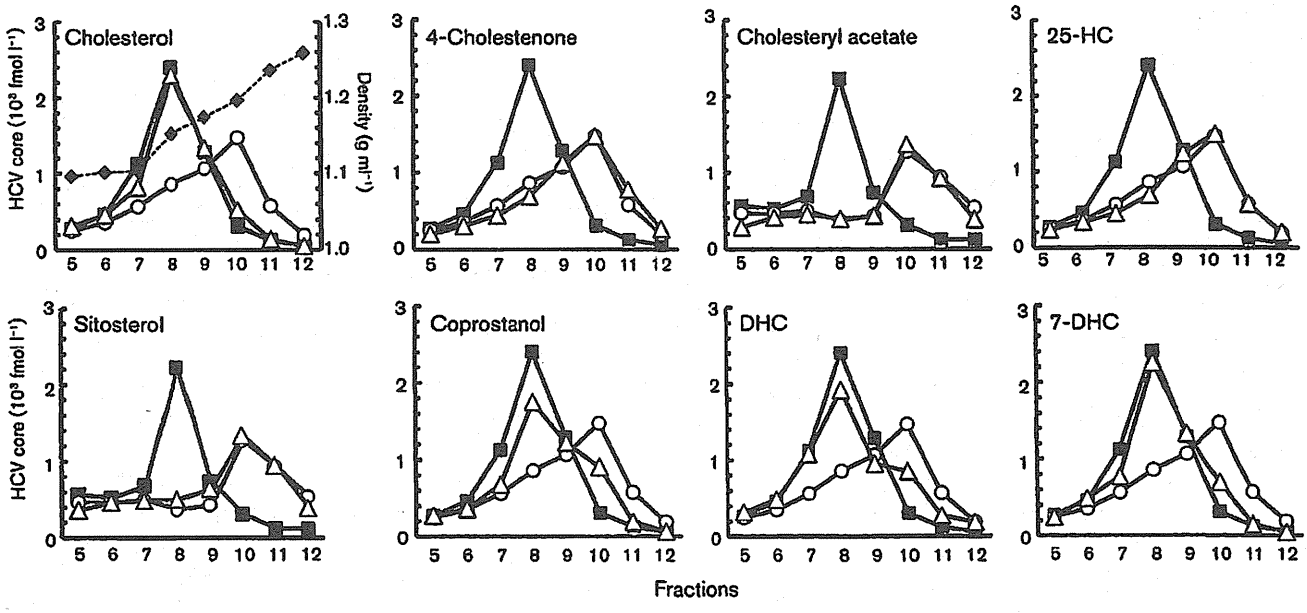

Fig. 2. Sucrose density-gradient profiles of lipid-modified HCV. Core protein concentration in each fraction of untreated HCVcc (■), B-CD-treated HCVcc (○) or HCVcc replenished with sterols (△) was determined. Corresponding densities of fractions are shown as a dashed line (◆).

sterol molecule and consequently in the virion structure. Coprostanol and DHC are *cis* and *trans* isomers, which are often known to have different physical properties. However, based on their molecular models, these two sterols, as well as cholesterol, possibly have similar spatial arrangements of the aliphatic side chain, the hydroxyl group and four-ring region because of their structural flexibility. In contrast, the spatial arrangement of 7-DHC does not seem comparable to that of cholesterol. Campbell *et al.* (2004) reported that replacement of HIV-1-associated cholesterol with raft-inhibiting sterols, including coprostanol, suppresses HIV-1 infectivity, whereas replacement with raft-promoting analogues such as DHC and 7-DHC (Megha *et al.*, 2006; Wang *et al.*, 2004; Xu & London, 2000; Xu *et al.*, 2001) maintains infectivity, demonstrating the importance of the raft-promoting properties of virion-associated cholesterol in HIV-1 infectivity (Campbell *et al.*, 2004). It is therefore likely that HCV-associated cholesterol is involved, at least in part, in virus infectivity via a molecular basis independent of lipid-raft formation.

The density of blood-circulating HCV is heterogeneous, ranging approximately from <1.06 to 1.25 g ml^{-1}, and it is proposed that low-density virus is associated with very-low-density lipoprotein (VLDL) and/or low-density lipoprotein (LDL) (André *et al.*, 2002; Thomssen *et al.*, 1993). It has recently been demonstrated that the pathway for VLDL assembly plays a role in assembly and maturation of infectious HCVcc (Icard *et al.*, 2009). HCVcc with low density, which is presumably associated with VLDL or VLDL-like lipoproteins, was found to possess higher infectivity than that with high density (Lindenbach *et al.*, 2006). This study, as well as our earlier work, indicated that removal of cholesterol from HCVcc by B-CD increased the buoyant density of the virus and reduced its infectivity. Thus, one may hypothesize that the virion-associated cholesterol plays a role in the formation of a complex with lipoproteins or apolipoproteins. To address this, the interaction between apolipoproteins and HCVcc with or without B-CD treatment was investigated by co-immunoprecipitation (Co-IP kit; Thermo Scientific). Virus samples were subjected separately to AminoLink Plus coupling resin, which was conjugated with a monoclonal antibody (mAb) against apolipoprotein E (ApoE) or apolipoprotein B (ApoB), and incubated at 4 °C for 4 h. After washing, total RNAs were extracted from the resulting resin beads by using TRIzol reagent (Invitrogen), followed by quantification of HCV RNA as described above (Takeuchi *et al.*, 1999). As indicated in Fig. 3(a), only a fraction of HCVcc was precipitated with an anti-ApoB mAb. In contrast, an anti-ApoE mAb was able to coprecipitate a considerable amount of the virus. It is of interest that B-CD-treated HCVcc hardly reacted with the mAb; however, the cholesterol-replenished virus was found to recover its reactivity, suggesting a role for virion-associated cholesterol in the formation of the HCV–lipoprotein/apolipoprotein complex. The results obtained are consistent with findings indicating that HCVcc can be captured with anti-ApoE antibodies, but capture with anti-ApoB antibodies is inefficient (Chang *et al.*, 2007; Hishiki *et al.*, 2010; Huang *et al.*, 2007; Jiang & Luo, 2009; Merz *et al.*, 2011; Nielsen *et al.*, 2006; Owen *et al.*, 2009), as well as with a recent model of structures of infectious HCV, in which HCVcc looks like ApoE-positive and primarily ApoB-negative lipoproteins (Bartenschlager *et al.*, 2011). We further tested the ApoE distribution in the density-gradient fractions of HCVcc samples (see Supplementary Fig. S1, available in JGV Online). With or without cholesterol depletion, ApoE was detected at a wide range of concentrations: 1.04 g ml^{-1} (fraction 1) to 1.17 g ml^{-1} (fraction 9). However, its level in the fractions at 1.10 g ml^{-1} (fraction 5) to approximately 1.17 g ml^{-1} was moderately decreased in the case of B-CD-treated virus.

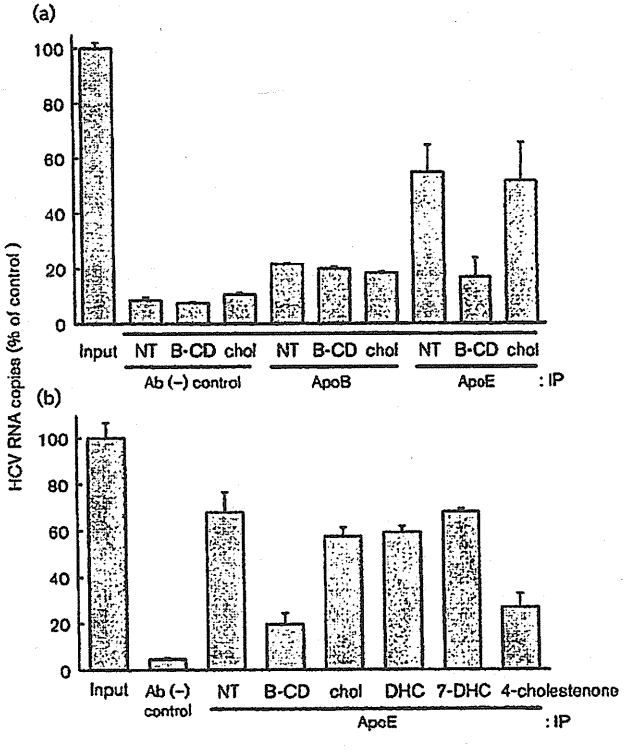

Fig. 3. Effect of virion-associated sterols on HCV–apolipoprotein interaction. (a) HCVcc samples with no treatment (NT), B-CD-treated (B-CD) or replenished with cholesterol (chol) were incubated with an amine-reactive resin coupling either an anti-ApoB mAb (ApoB) or an anti-ApoE mAb (ApoE). Control resin that is composed of the same material as above, but is not activated, was used as a negative control [Ab (–) control]. (b) B-CD-treated HCVcc was incubated with cholesterol (chol), DHC, 7-DHC or 4-cholestenone, followed by immunoprecipitation with the resin coupling with anti-ApoE mAb. (a, b) HCV RNAs in the immunoprecipitates were quantified and are indicated as percentages of the amount of input HCVcc RNA. Means+SD of three samples are shown. Data are representative of three experiments.

Whether cholesterol analogues could have a comparable role in HCV association with lipoprotein was examined further (Fig. 3b). Addition of DHC or 7-DHC, but not 4-cholestenone, to B-CD-treated HCVcc resulted in the recovery of coprecipitation of the virus with anti-ApoE. The results are correlated with the effect of sterols on the restoration of the buoyant densities of lipid-modified HCVcc (Fig. 2), suggesting that virion-associated cholesterol variants with modification in the sterol rings, but not in either the 3β-hydroxyl group or the aliphatic side chain, may tolerate the interaction between HCV and ApoE-positive lipoprotein.

Given that 7-DHC restored the association of HCV with ApoE and virion buoyant density, but did not restore infectivity, cholesterol and/or its analogues might affect the ability of virion membranes to fuse with the cell, independent of ApoE association. As cholesterol is an important mediator of membrane fluidity, one may hypothesize that HCV-associated cholesterol is involved in infectivity through modulation of the membrane fluidity. It has been reported that, in patients with Smith–Lemli–Opitz syndrome, a disorder of the cholesterol-synthesis pathway, cholesterol content decreases and 7-DHC increases in the cell membranes, leading to alteration of phospholipid packing in the membrane and abnormal membrane fluidity (Tulenko *et al.*, 2006).

It is now accepted that maturation and release of infectious HCV coincide with the pathway for producing VLDLs, which export cholesterol and triglyceride from hepatocytes. This study revealed roles for the structural basis of virion-associated cholesterol in the infectivity, buoyant density and apolipoprotein association of HCV. Although it was shown that HCV virions in infected patients, so-called lipo–viro particles, exhibited certain biochemical properties such as containing ApoB, ApoC and ApoE (Diaz *et al.*, 2006; Bartenschlager *et al.*, 2011), our studies provide useful information and the basis for future investigations toward a deeper understanding of the biogenesis pathway of infectious HCV particles.

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Hepatitis C virus RNA replication in human stellate cells regulates gene expression of extracellular matrix-related molecules

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ABSTRACT

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, including chronic hepatitis, fibrosis, and cirrhosis. Fibrosis often develops in HCV-infected livers and ultimately leads to cirrhosis and carcinoma. During fibrosis, hepatic stellate cells (HSC) play important roles in the control of extracellular matrix synthesis and degradation in fibrotic livers. In this study, we established a subgenomic replicon (SGR) cell line with human hepatic stellate cells to investigate the effect of HCV RNA replication on HSC. Isolated SGR clones contained HCV RNA copy numbers ranging from 10^4 to 10^7 per μg total RNA, and long-term culture of low-copy number SGR clones resulted in markedly increased HCV RNA copy numbers. Furthermore, HCV RNA replication affected gene expression of extracellular matrix-related molecules in both hepatic stellate cells and hepatic cells, suggesting that HCV RNA replication and/or HCV proteins directly contribute to liver fibrosis. The HCV RNA-replicating hepatic stellate cell line isolated in this study will be useful for investigating hepatic stellate cell functions and HCV replication machinery.

1. Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, including chronic hepatitis, fibrosis, and cirrhosis, with greater than 170 million individuals infected worldwide [1,2]. Presently, there is no preventive vaccine for HCV infection, and standard therapy involves the combination of pegylated interferon-alpha and ribavirin [3]. However, as the effects of this combination therapy approach are often insufficient to completely eliminate viruses from HCV carriers, novel antiviral therapies are desired to increase sustained virological response rates and reduce adverse effects.

Fibrosis is often observed in chronic HCV infections and is part of the dynamic process of extracellular matrix (ECM) remodeling that occurs continuously during chronic liver injury. Such remodeling results in excessive accumulation of ECM proteins, ultimately leading to cirrhosis and carcinoma [4,5]. Hepatic stellate cells are the main collagen- and ECM-producing cells and play a key role in liver fibrogenesis [6]. In liver tissue, the balance between ECM synthesis and degradation is regulated by gene expression of ECM-regulatory molecules, such as matrix metalloproteinases (MMP) and tissue inhibitors of matrix metalloproteinase (TIMP). On activation of hepatic stellate cells by inflammatory molecules, the balance of ECM regulation shifts towards progression of liver fibrosis. During fibrosis, however, little is known about the contribution of HCV to fibrogenesis.

HCV is a positive-strand RNA virus with a genome that encodes an approximately 3000-amino-acid (aa) polyprotein, which is co- and post-translationally processed by proteolysis into ten mature proteins, consisting of a capsid (core), two envelope (E1, E2), and seven non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [1,7]. Past investigations of HCV have been limited because no suitable HCV culture systems were available to observe all steps of the viral life cycle, including entry, replication, translation, assembly, and secretion. In 1999, however, Lohmann et al. [8] developed a subgenomic replicon (SGR) system using replicon RNA encoding NS proteins (NS3-NS5B) for the analysis of RNA replication and translation [8]. In addition, an HCV pseudo-particle system (HCVpp) based on HIV and MMLV was developed for viral entry analysis [9,10].

Recently, HCV strain JFH-1, which was isolated from a Japanese patient with fulminant hepatitis, has permitted all steps of the HCV life cycle to be examined in a cultured cell line [11]. Replication of the transfected JFH-1 genome in host cells was restricted to the human hepatoma cell line Huh7 or its derivatives. Hepatocytes

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are thought to be permissive for HCV infection because liver tissue is a major target organ for HCV infection. However, virus replication levels in HCV patients are typically too low to detect the distribution of viral proteins in liver biopsies by antibodies directed against HCV proteins, and no clear evidence indicates which cell groups are the major target of HCV infection. Thus, it is unclear whether hepatic cells are the only target of HCV infection in liver tissue, and if other cells, particularly HSCs, can serve as hosts for HCV replication.

Here, we established HCV-replicating hepatic stellate cells to address whether these cells can be a potential target of HCV infection. Furthermore, we analyzed gene expression profiles of ECM-related molecules in HCV-replicating stellate cell clones to investigate the effect of HCV RNA replication on hepatic stellate cell functions.

2. Materials and methods

2.1. Cells and reagents

Human hepatic stellate cells, TWNT-4 JP7 cells derived from the LI90 cell line [12,13], were maintained in D-MEM supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes (pH 7.4), 1 mM sodium pyruvate, and MEM Non-Essential Amino Acids. Electroporated TWNT-4 JP7 cells and isolated SGR cells were maintained in prepared D-MEM containing either 500 or 1000 μg/ml G418. SGR Huh7 cell lines (JFH-1/4-1, JFH-1/4-5, Con1 NK5.1/0-6, and Con1 NK5.1/0-11) were maintained in prepared D-MEM containing 500 μg/ml G418 [14]. Rabbit polyclonal antibodies against NS3 and NS5A proteins were raised by immunization with recombinant NS proteins (NS3, 1195–1661 aa; NS5A, 2001–2441 aa).

2.2. Transfection and isolation of SGR clones

JFH-1 SGR RNA was synthesized using a Megascript T7 Kit with linearized pSGR-JFH-1 and pSGR-JFH-1 GND plasmids as templates. Ten micrograms HCV RNA was electroporated into 2×10^6 TWNT-4 JP7 cells, as previously described [15], which were then cultured for 3 weeks under G418 selection (500 and 1000 μg/ml). Single colonies were isolated, and the selected SGR clones were expanded and stored at −80 °C until used for analysis.

2.3. Quantification of HCV RNA in SGR cells

Total RNA was purified from each SGR clone using ISOGEN (Nippon Gene), as directed by the manufacturer's protocol. The HCV RNA copy number of each SGR clone was analyzed by a real-time PCR method, as described previously [16].

2.4. Western blot analysis

Twenty micrograms of cell lysates were separated on 10% polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were first blocked with 2% skim milk in TBS-T (20 mM Tris–HCl [pH 7.2], 500 mM NaCl, and 0.01% Tween20) and then incubated with 2% skim milk in TBS-T containing primary antibody (α-NS3 or α-NS5A), followed by secondary antibody (α-rabbit IgG HRP-conjugated). After washing membranes, bands were detected by Enhanced Chemiluminescence (ECL) Plus reagent (GE Healthcare). Luminescence signals were analyzed using the LAS-3000 Mini image analysis system (Fujifilm).

2.5. Immuno-staining of non-structural HCV proteins

Isolated SGR cells were fixed with acetone-methanol. Fixed cells were dried and incubated with primary antibodies (rabbit polyclonal α-NS3 or α-NS5A), followed by secondary antibody (α-rabbit IgG-Alexa488). Both antibodies were diluted in Block Ace (DS Pharma Biomedical) prior to use. Fluorescence of NS proteins was visualized using a Biozero microscope (Keyence).

2.6. Gene expression analysis of ECM-related genes

Total RNA was purified from SGR clones in growth phase using ISOGEN-LS (Nippon Gene). cDNA was synthesized from total RNA using random primers and Superscript III (Invitrogen). TaqMan array plates (Custom Format) were purchased from Applied Biosystems. Information of ECM-related genes in the Custom Format plate is listed in Table 1S. cDNA (55 ng/reaction) was mixed with an equal volume of TaqMan Gene Expression Master Mix (Applied Biosystems) and real-time PCR was performed using a 7500 FAST Real-Time PCR System (Applied Biosystems).

3. Results

3.1. Isolation of SGR cells

To evaluate the potential of HCV to replicate in human hepatic stellate cells and examine the role of HCV replication and viral protein in liver fibrogenesis, we established HCV SGR cells from human hepatic stellate cells. We first examined the colony formation efficiency of HCV SGR transfected into hepatic stellate cells using JFH-1 SGR RNA (Fig. 1A), which was electroporated into the human hepatic stellate cell line, TWNT-4 JP7. The transfected cells were selected using G418 for 3 weeks, and the resulting colonies were stained by crystal violet. Interestingly, many colonies (~150) were formed on transfection of cells with 10 μg JFH-1 RNA; however, no colonies were detected among cells transfected with a HCV replication-defective mutant, JFH-1 GND (Fig. 1B). This result indicated that TWNT-4 JP7 cells can support HCV replication, although colony formation efficiency of TWNT-4 JP7 cells transfected with JFH-1 SGR was much lower than that in transfected Huh7 cells (data not shown).

Next, we selected colonies of JFH-1 SGR-transfected cells and established a number of SGR TWNT-4 JP7 clones; SGR clones #1–8 and clones #11–18 were isolated from 500 and 1000 μg/ml G418 selection, respectively. The HCV RNA copy number of each SGR clone was measured by real-time RT-PCR, which revealed that the clones contained RNA copy numbers ranging from 10^4 to 10^7 copies per μg total RNA (Table 1).

Among the isolated SGR clones, we focused on SGR #1 and #2 for further analyses, because these two clones allowed HCV RNA replication at a relatively low RNA copy number compared with the other examined SGR clones. SGR #1 and #2 were cultured for an additional 8 weeks in G418 selection medium, during which time the HCV RNA copy number in both clones increased from 10^4 to 10^7 copies, which represented increases of 3200- and 470-fold, respectively (Table 2). This observed increase suggested that the occurrence of viral adaptive mutations or modifications of cellular factors during cell passaging resulted in the increased efficiency of viral RNA replication in hepatic stellate cells. We thus determined the viral RNA sequences in clones SGR #1 and #2 and some other additional clones, and identified several synonymous and non-synonymous mutations in the HCV RNA sequences (data not shown). Although two common non-synonymous mutations were found in the NS4B and NS5A gene-encoding regions, these mutations did not increase HCV RNA replication in human

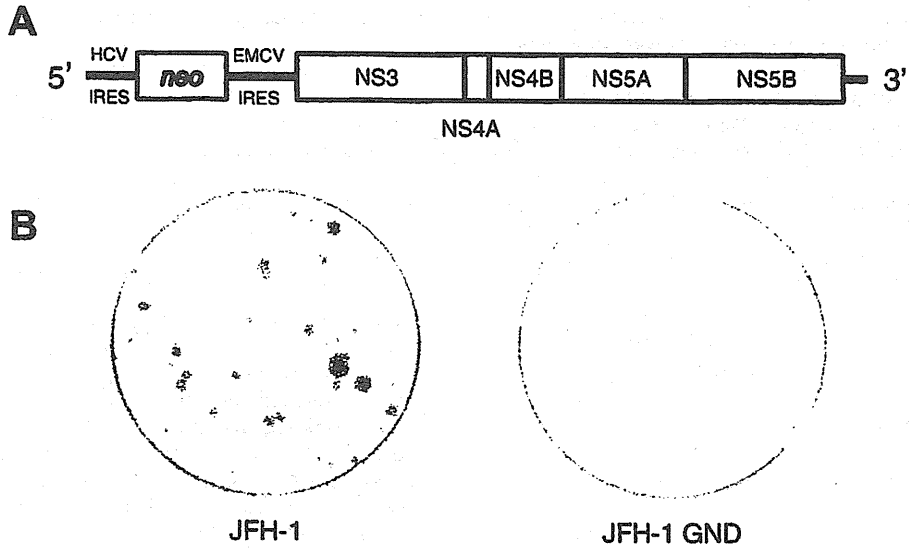

Fig. 1. Structure of the JFH-1 subgenomic replicon (SGR) and colony formation of SGR TWNT-4 JP7 cells. (A) Diagram of the JFH-1 SGR. (B) Colony formation of SGR TWNT-4 JP7 cells. TWNT-4 JP7 cells were transfected with either wild-type JFH-1 or replication-defective GND SGR RNA, and transfected cells were then cultured in G418 selection medium for 3 weeks. Cells were then fixed using formalin, and colonies were visualized by crystal violet staining. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
RNA copy number of isolated subgenomic replicon (SGR) clones.

SGR clone	HCV RNA copy number (copies/μg of total RNA)
#1	1.5×10^4
#2	5.3×10^4
#3	9.4×10^5
#4	2.3×10^5
#5	1.7×10^5
#6	1.5×10^4
#7	4.2×10^5
#8	1.3×10^6
Average	3.9×10^5
#11	3.8×10^5
#12	1.2×10^7
#13	4.8×10^5
#14	2.3×10^6
#15	4.7×10^5
#16	2.1×10^6
#17	5.1×10^5
#18	2.0×10^6
Average	2.5×10^6

Table 2
Change of RNA copy number in isolated subgenomic replicon (SGR) clones following long-term culture.

SGR clone	4 weeks*	12 weeks*
#1	1.5×10^4	4.9×10^7
#2	5.3×10^4	2.5×10^7

* Copies/μg of total RNA.

hepatic stellate cells when they were artificially introduced into the replicon genome (data not shown).

3.2. Expression of non-structural HCV proteins

The expression of several NS HCV proteins, from NS3 to NS5B, is required for HCV RNA replication in infected cells. It is reported that these NS proteins form complexes to replicate HCV RNA, with NS5A protein in particular localizing to specialized membranous web structures around lipid droplets, which are considered to serve as the scaffold for HCV RNA replication and assembly [17,18]. To confirm the expression of NS proteins in isolated SGR clones, we performed western blotting and immunostaining of HCV NS proteins (Fig. 2). As shown in Fig. 2A, NS3 and NS5A proteins of expected size were detected by western blotting in SGR #1 and #2 cells. The expression of both NS3 and NS5A in SGR #1 was higher than that in SGR #2, a result that is consistent with the determined viral RNA copy number of each clone (Table 2). Immunostaining of NS proteins clearly showed cytoplasmic distribution, similar to that observed in Huh7 cells (Fig. 2B). Specifically, the fluorescence of NS5A was distributed as a fine reticular pattern with occasional granular staining. These results demonstrated that HCV RNA replicated in hepatic stellate cells and that HCV NS proteins were expressed in hepatic stellate cells at identical levels as in Huh7 cells.

3.3. Gene expression profiles of ECM-related molecules in SGR cells

In liver tissue, once stellate cells are activated by inflammation mediators secreted from infected or injured cells, the activated cells differentiate into myoblast cells. In addition, ECM-related molecule gene expression is altered, which leads to the progression of liver fibrosis. Here, we examined if HCV RNA replication in stellate cells affects the gene expression of ECM-related molecules by measuring gene expression in both parental TWNT-4 JP7 and isolated SGR cells using a TaqMan array plate (Custom Format) consisting of 24 selected genes from the collagen (COL), MMP, and TIMP families (Table 1S) [19,20]. Among the 16 isolated SGR clones in this study, 10 in growth phase were examined for ECM-related molecule gene expression. All SGR clones exhibited a decrease of collagen gene expression compared with parental TWNT-4 JP7 cells. In particular, four collagen genes (COL1A1, COL4A2, COL5A1, and COL15A1) were expressed at levels approximately 0.5-fold lower than those of parental cells (Fig. 3A), whereas MMP1, MMP3, and MMP12 increased 4-, 5.6-, and 4.8-fold, respectively (Fig. 3B). These results suggest that HCV RNA replication or viral proteins in hepatic stellate cells down- and up-regulate COL and MMP gene expression, respectively.

To determine if gene expression changes of ECM-related molecules in isolated SGR clones were specific to hepatic stellate cells,

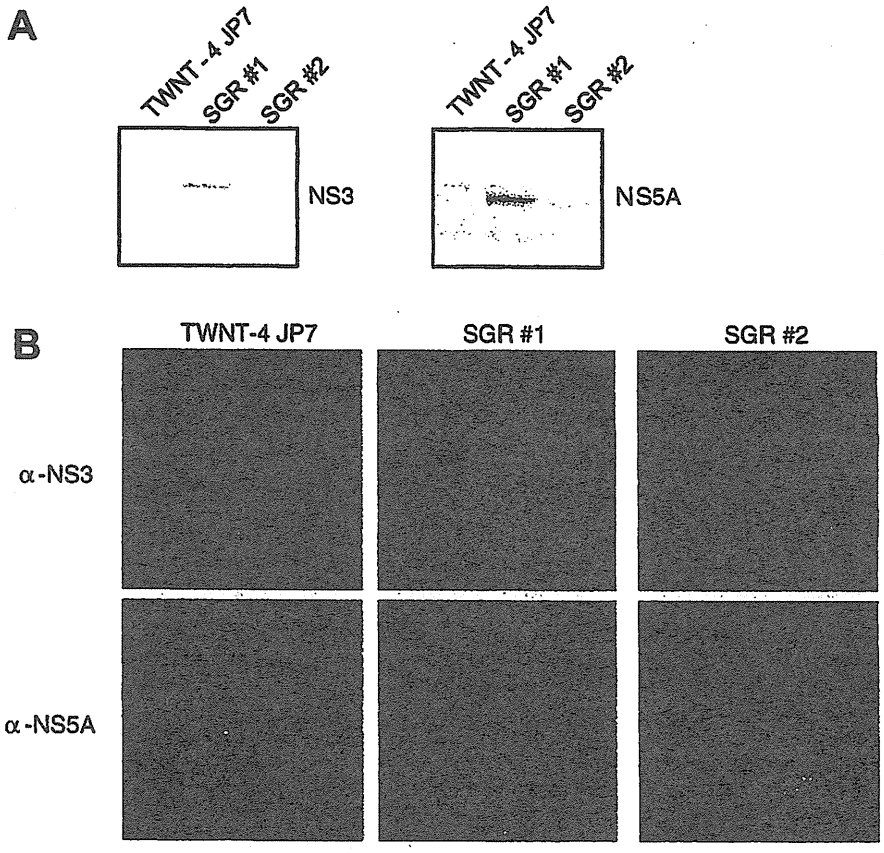

Fig. 2. Expression of non-structural (NS) NS3 and NS5A proteins in isolated SGR clones. (A) Total cell lysates of TWNT-4 JP7 and SGR clones were separated by SDS–PAGE and transferred to a nitrocellulose membrane. HCV NS proteins NS3 and NS5A were detected by western blot analysis with rabbit polyclonal α-NS3 and α-NS5A antibodies. (B) The expression of HCV NS proteins were also observed by immuno-staining with rabbit polyclonal α-NS3 and α-NS5A antibodies (blue, nuclear; green, NS proteins). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

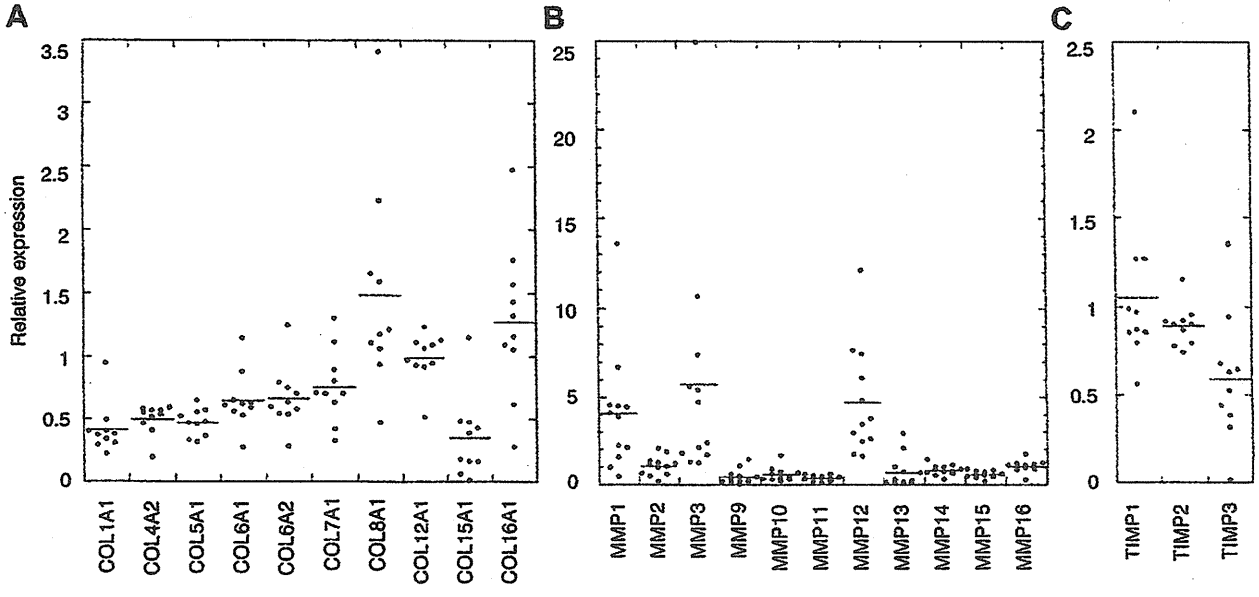

Fig. 3. Relative expression levels of extracellular matrix (ECM)-related genes in isolated TWNT-4 SGR clones. Gene expression levels of collagen (A), matrix metalloproteinase (B), and tissue inhibitor of metalloproteinase genes (C) were measured by TaqMan PCR. The expression levels of the indicated ECM-related genes were normalized to those of parental TWNT-4 JP7 cells. Values of gene expression level are summarized in Supplementary Table 2S. The bar in each column indicates the average.

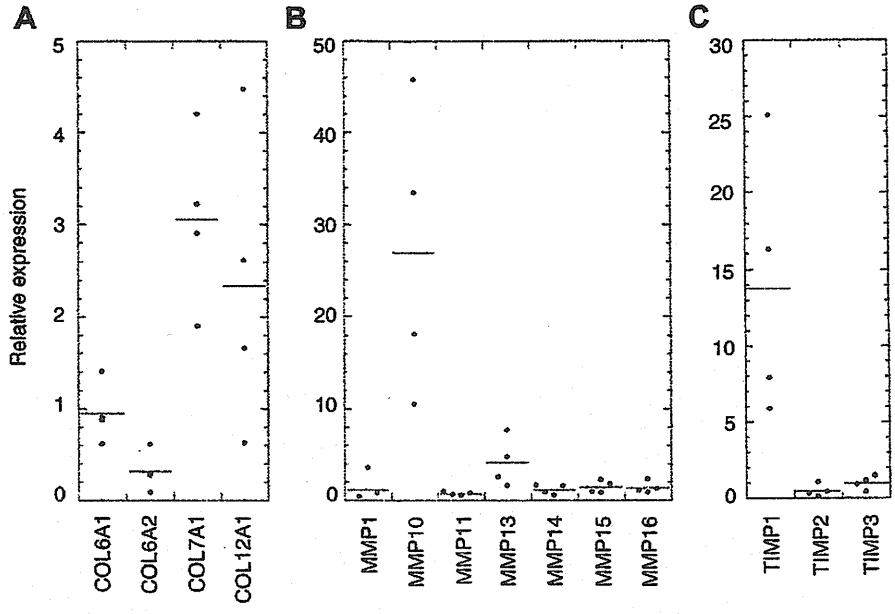

Fig. 4. Relative expression levels of extracellular matrix (ECM)-related genes in subgenomic replicon Huh7 clones. Gene expression levels of collagen (A), matrix metalloproteinase (B), and tissue inhibitor of metalloproteinase genes (C) were measured by TaqMan PCR. The expression levels of the indicated ECM-related genes were normalized to those of parental Huh7 cells. Values of gene expression levels are summarized in Table 3S. The bar in each column indicates the average.

we analyzed the expression of the identical 24 genes in SGR hepatic cells. The gene expression levels of four SGR hepatic cell clones derived from Huh7 cells (JFH-1/4-1, JFH-1/4-5, Con1 NK5.1/0-6, and Con1 NK5.1/0-11) were also measured by TaqMan array analysis (Fig. 4). Distinct differences in gene expression profiles were observed between hepatic and hepatic stellate cells, and the expression of several genes were not detected in Huh7 cells. In SGR Huh7 cells, COL7A1 and COL12A1 gene expression increased greater than 2-fold (Fig. 4A), while the expression of the COL1A1 gene increased from undetectable to detectable levels (data not shown). In addition, MMP10, MMP13, and TIMP1 gene expression was markedly increased in hepatic replicon cells compared with the levels in Huh7 cells (Fig. 4B and C).

4. Discussion

In previous studies, a few SGR cell lines were established from different HCV strains (JFH-1, H77, and Con-1) and several human cell lines (Huh7, HepG2, IMY-N9, HeLa, and 293 cells) [8,21,22]. The combination of JFH-1 SGR and Huh7 cells exhibited increased RNA replication without adaptive mutations compared with H77 and Con-1 strains. Furthermore, full genomic JFH-1 and its chimera virus have been shown to infect Huh7 and derivatives. Although we did not observe HCV infection using the combination of JFH-1 and the TWNT-4 JP7 cell line (data not shown), this is the first study to isolate SGR clones using hepatic stellate cells. Our findings indicate that hepatic stellate cells potentially support HCV replication in infected livers.

A total of 16 SGR clones were isolated from SGR RNA-transfected TWNT-4 JP7 cells in this study. The HCV RNA copy number in the selected clones clearly differed between the two concentrations of G418 used for selection, with 500 and 1000 μg/ml G418 yielding an average RNA copy numbers of 3.9×10^5 and 2.5×10^6, respectively (Table 1). Although RNA replication in SGR clones was lower than that observed in hepatic replicon cells, such as Huh7 and IMY-N9 cells, the RNA copy number of SGR clones selected using 1000 μg/ml G418 was comparable to that of non-hepatic replicon cells derived from HeLa and 293 cell lines [21,22]. Since hepatic stellate cells localize in liver tissue where HCV replicates, hepatic stellate cells are likely to be exposed to HCV from both the circulation system and neighboring hepatocytes. Cell-to-cell HCV transmission was also reported in recent studies [23,24], further supporting our results that hepatic stellate cells are a possible target of HCV infection.

It was reported that a number of adaptive mutations in the HCV genomic sequence increase viral production in infected cells and RNA replication in SGR cells [25–27]. Here, isolated replicon cells exhibited increased RNA replication during an additional 8 weeks of culture, and several mutations were identified in the sequenced HCV RNA of SGR cells. Clones SGR #1 and #2 contained four synonymous and three non-synonymous mutations, respectively. Although we performed a colony forming assay by transfection of SGR RNA containing these mutations into TWNT-4 JP7 cells, no differences in colony formation were observed between wild-type and mutant RNA-transfected cells (data not shown). This finding suggests that modification of certain cellular factors may have occurred in the SGR clones during cell passage, rather than the appearance of adaptive mutations in the replicon genome.

From the results of our TaqMan analysis, the expression of several collagen genes were significantly down-regulated and three MMP genes (MMP1, MMP3, and MMP12) were up-regulated in isolated SGR clones compared with parental TWNT-4 JP7 cells (Fig. 3). Type I and IV collagen are important for liver fibrogenesis, and MMP-1 degrades type I collagen to regulate ECM regeneration in liver tissue [19]. Furthermore, MMP-3 regulates MMP-1 activation [28,29], while overproduction of MMP-12 causes ECM protein breakdown and excessive remodeling, which has been implicated in a range of respiratory diseases [30–32]. Although no relationship between HCV RNA copy number and the expression of these genes in SGR cells was detected, a certain degree of HCV RNA replication may be sufficient to influence the regulation of ECM-related genes. Taken together, these findings suggest that HCV RNA-replicating hepatic stellate cells may suppress ECM production and negatively regulate hepatic fibrosis. In contrast, increased expression of type I collagen and TIMP-1 genes, an inhibitor of MMP-1, was observed in hepatic replicon cells, suggesting that hepatic cells up-regulate

ECM accumulation through collagen production and the inhibition of MMP activity. As hepatic cells are the major cell group in liver tissue, HCV-infected hepatic cells may be one of the factors promoting fibrosis by producing ECM proteins such as type I collagen and TIMP-1. Our results indicate that although HCV RNA replication and/or HCV NS proteins affect gene expression of EMC-related molecules in both hepatic stellate cells and hepatic cells, the gene expression profiles differ between these cell types. Further studies are necessary to analyze the mechanisms of ECM-related gene expression in HCV-infected livers.

Primary hepatic stellate cells isolated from liver tissue typically transform into myofibroblastic cells (active formation) during subsequent passages. As the TWNT-4 JP7 cells used in this study also exhibited an activated phenotype [13,33], the results of our gene expression analyses may reflect the phenotype of activated hepatic stellate cells. In fact, it is presently difficult to isolate and maintain quiescent hepatic stellate cell populations. Although *in vitro* culture systems involving primary cells and hepatic stellate cell lines are valuable tools for studying liver fibrosis, SGR cells generated from quiescent hepatic stellate cells are required for the detailed analysis of hepatic stellate cell functions in fibrosis.

In conclusion, our study has shown that HCV RNA replicates in human hepatic stellate cells and affects the gene expression of ECM-related molecules, suggesting the potential of HCV to infect and directly influence ECM-related gene regulation in hepatic stellate cells. The HCV RNA-replicating hepatic stellate cell line isolated in this study is useful not only for investigating hepatic stellate cell functions, but also for studying HCV replication machinery.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.125.

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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

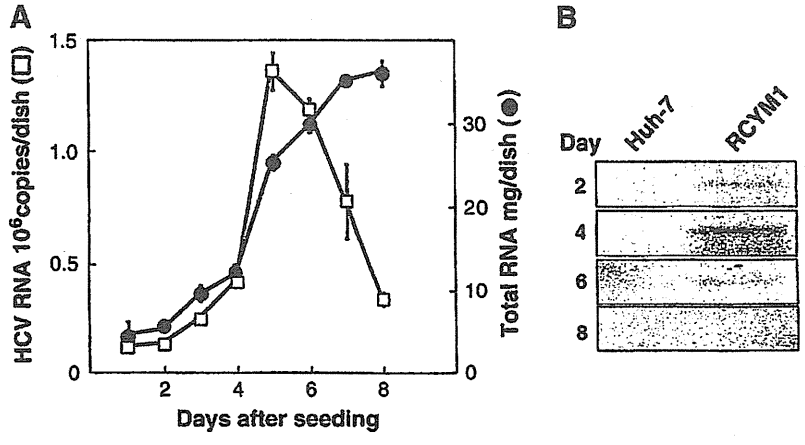

Fig. 1. Effect of cell growth on HCV RNA replication. (A) Measurement of HCV RNA (open squares) and total cellular RNA (closed circles) in RCYM1 cells at the time of harvest (days after seeding). (B) DRM fractions obtained from RCYM1 and parental Huh-7 cells harvested as indicated (day) were analyzed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

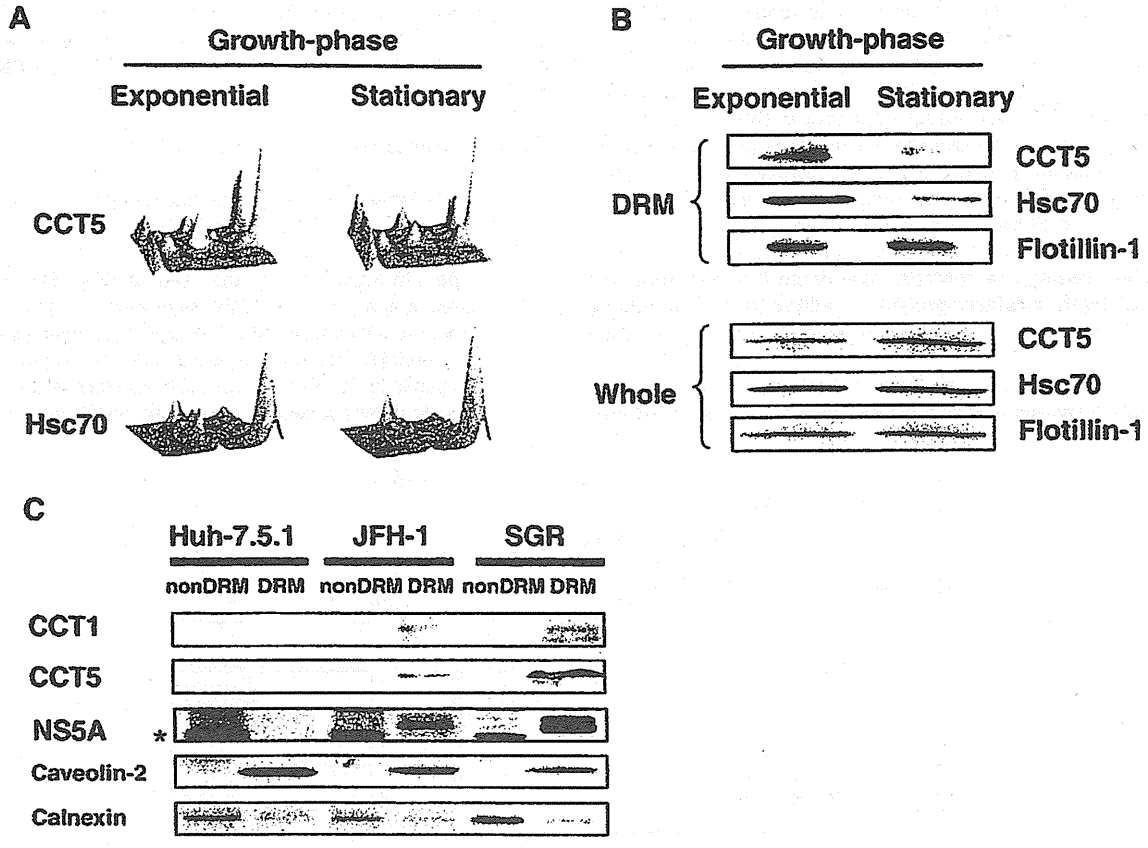

Fig. 2. Comparison of protein levels in DRM fractions prepared from RCYM1 cells at the exponential and stationary growth phases. (A) Three-dimensional images of CCT5 and Hsc70 analyzed by Ettan DIGE (GE Healthcare). Spots corresponding to CCT5/Hsc70 at exponential and stationary growth phases of the 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.

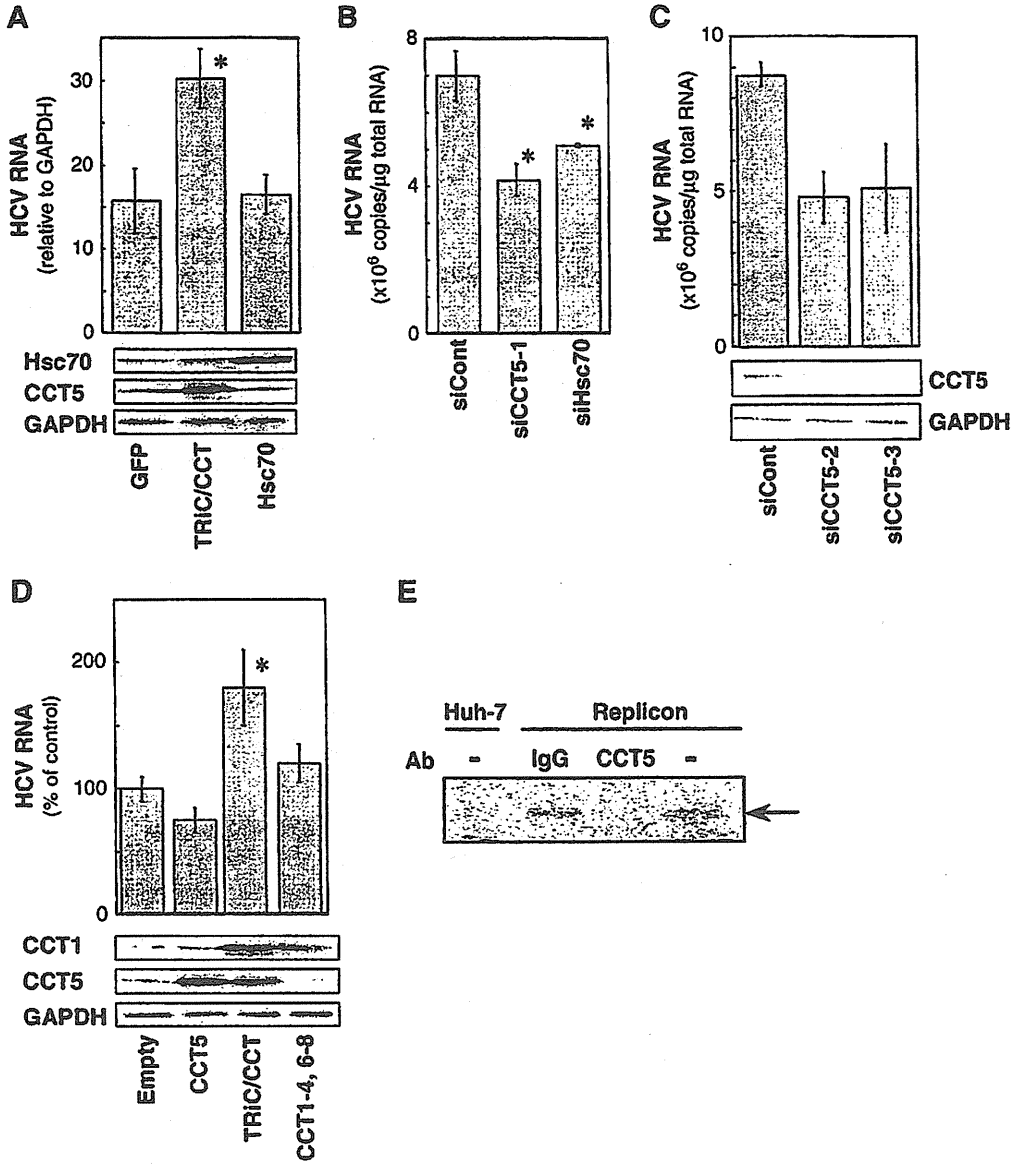

Fig. 3. Involvement of TRiC/CCT in HCV replication (A and D). Overexpression of all eight subunits of TRiC/CCT (TRiC/CCT); seven subunits, CCT1, 2, 3, 4, 6, 7, and 8 (CCT1–4, 6–8); subunit CCT5 only (CCT5); Hsc70; or control GFP in RCYM1 cells. HCV RNA levels were determined 48 h post-transfection (B 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

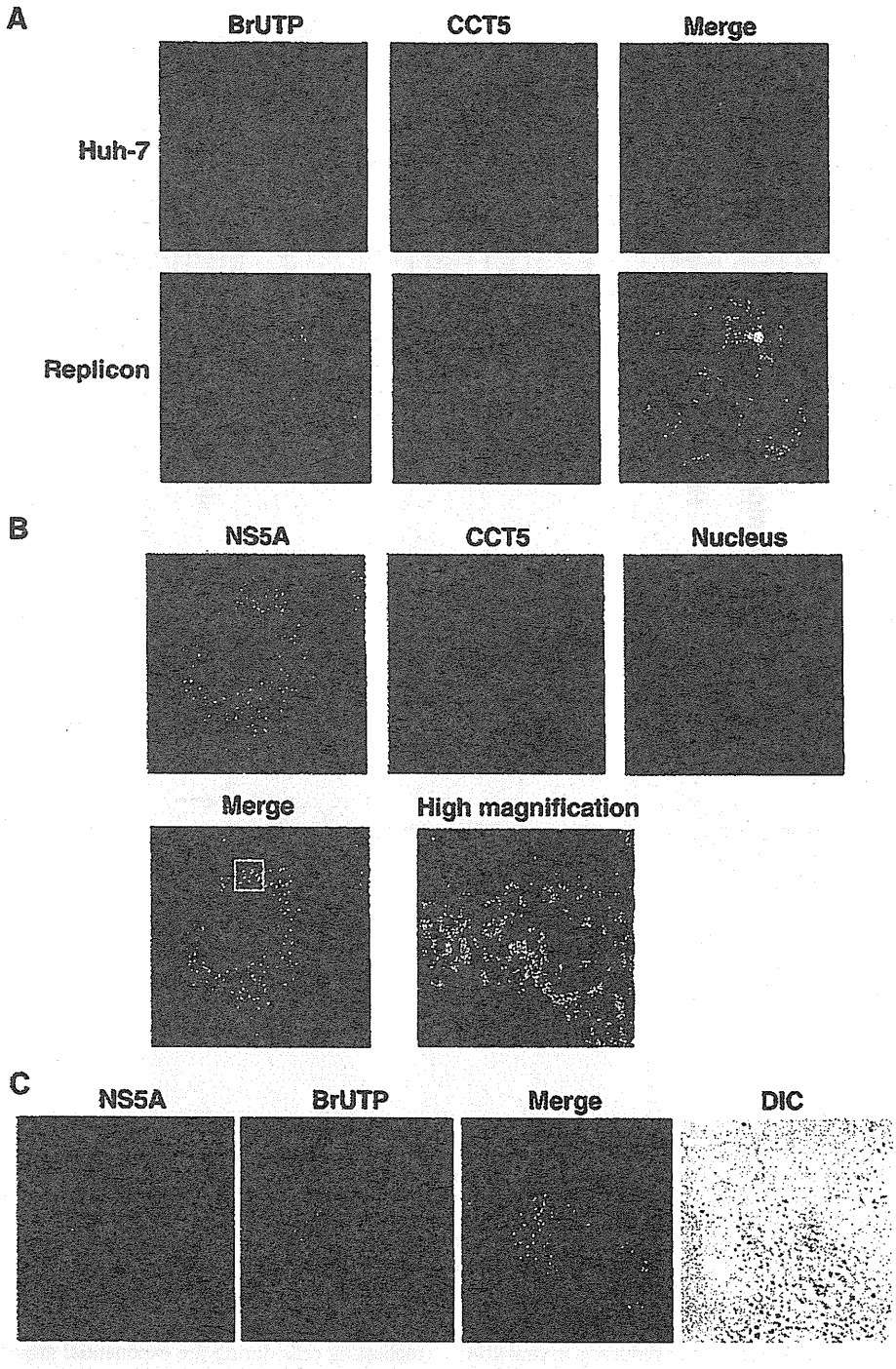

Fig. 4. Immunofluorescence analysis of CCT5 in SGR-N and Huh-7 cells (A) and HCVcc-infected cells (B). The primary Abs used were anti-CCT5 goat polyclonal Ab (red), anti-BrUTP monoclonal Ab (green), and anti-NS5A monoclonal Ab (green). Merged images of red and green signals (A) or of red, green and blue (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

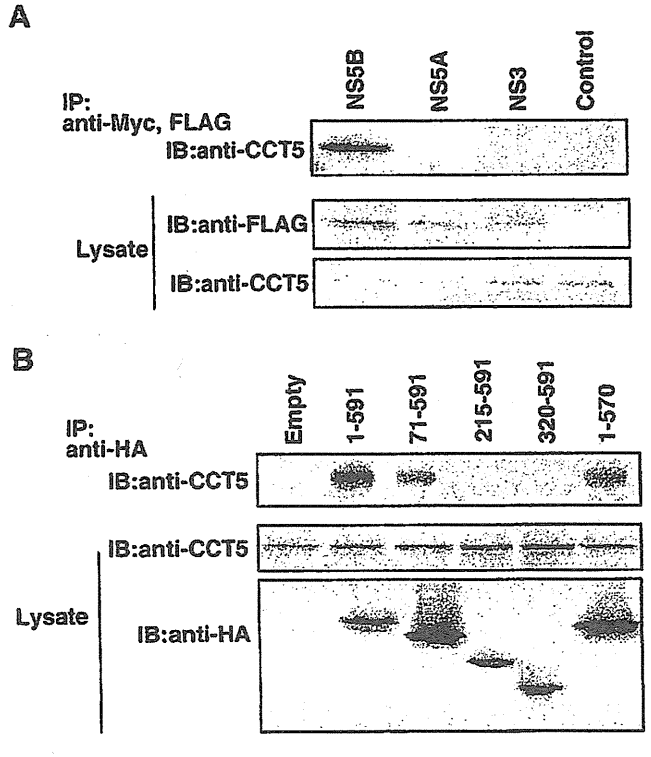

Fig. 5. CCT5 interacts with HCV NS5B. (A) CCT5 was co-expressed with MEF-tagged-NS5B, -NS5A, or -NS3 protein of strain NIHJ1 in cells, followed by two-step immunoprecipitation (IP) with anti-FLAG and anti-myc Abs. Immunoprecipitates were subjected to immunoblotting with anti-CCT5 Ab (IB). (B) Full-length 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.

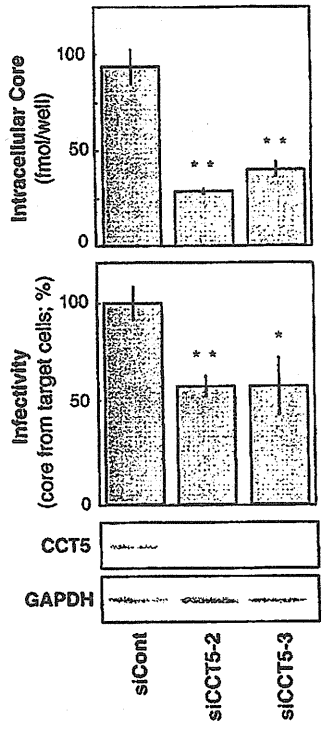

Fig. 6. Knockdown of endogenous CCT5 in HCVcc-infected cells. The cells were transfected with siRNAs against CCT5 (siCCT5-2, -3) or with control siRNAs (siCont). At 72 h post-transfection, the viral core protein levels in cells were determined (upper panel). Collected culture supernatants were inoculated 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