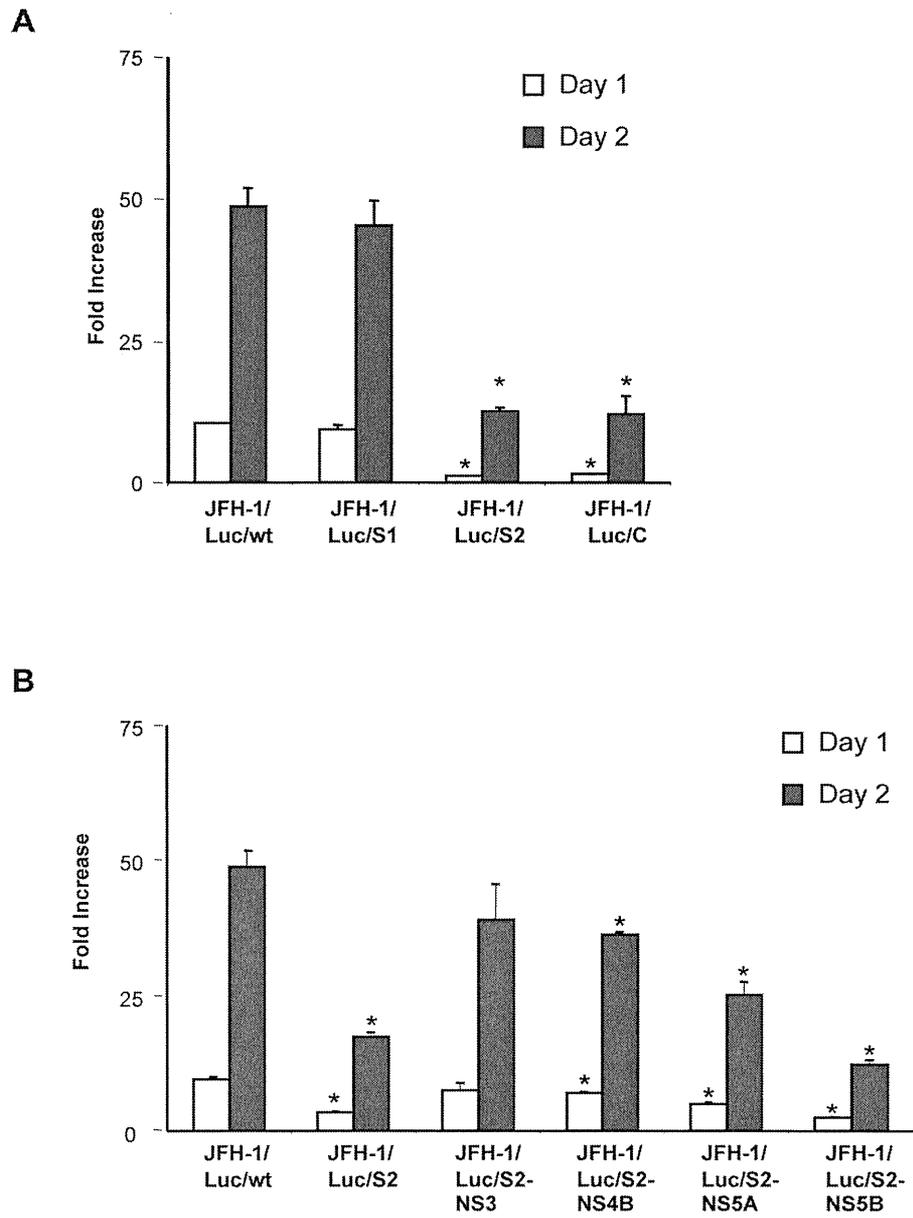Supporting Table 2.

Infectious virus production and release of JFH-1 variants with mutations detected in JFH-1/S2 strain
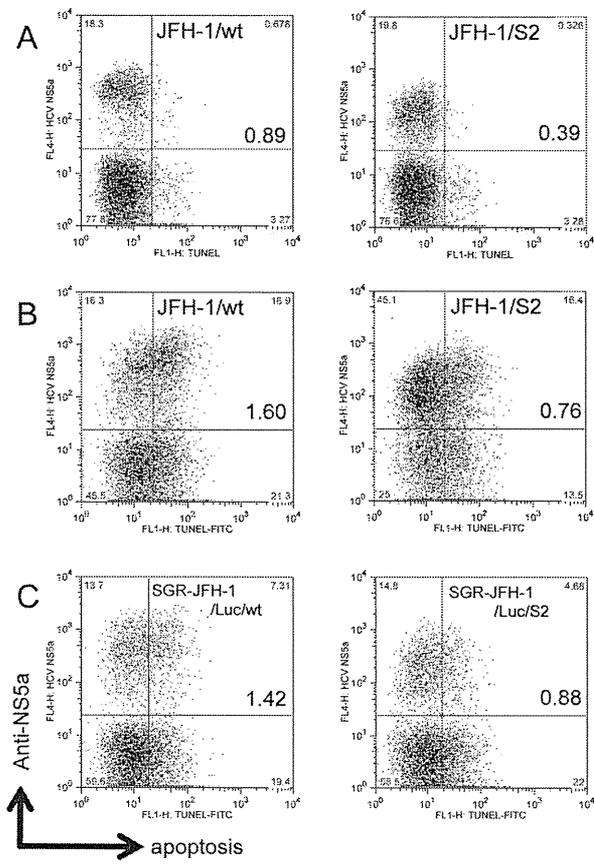
| Strain | Intracellular | | | Extracellular | Secretion Ratio (extra-/intra-) |
	HCV RNA (copies/ μg RNA)	Infectivity Titer (ffu/well)	Specific Infectivity (ffu/copies)	Infectivity Titer (ffu/well)	
JFH-1/wt	4.38×10^8 ± 1.43×10^8	1.60×10^2 ± 2.67×10^1	2.21×10^{-7} ± 9.39×10^{-8}	7.81×10^2 ± 5.74×10^1	4.9 ± 0.8
JFH-1/S2	2.91×10^8 ± 6.11×10^7	$2.72 \times 10^{3*}$ ± 4.25×10^1	$3.72 \times 10^{-6*}$ ± 4.97×10^{-7}	$1.60 \times 10^{4*}$ ± 3.31×10^3	5.9 ± 1.3
JFH-1/ S2-wt	4.47×10^8 ± 8.20×10^6	1.72×10^3 ± 6.54×10^2	$2.00 \times 10^{-6*}$ ± 8.53×10^{-7}	$9.50 \times 10^{3*}$ ± 1.02×10^3	6.0 ± 2.1
JFH-1/ N397S	5.32×10^8 ± 1.66×10^8	2.28×10^2 ± 4.25×10^1	2.12×10^{-7} ± 3.72×10^{-8}	$1.09 \times 10^{3*}$ ± 3.86×10^2	4.8 ± 1.5
JFH-1/ L752V	5.52×10^8 ± 1.60×10^8	$6.96 \times 10^{2*}$ ± 1.94×10^2	$8.90 \times 10^{-7*}$ ± 1.03×10^{-7}	$3.90 \times 10^{3*}$ ± 9.73×10^2	5.9 ± 2.2
JFH-1/ S2-NS2	5.14×10^8 ± 5.02×10^7	1.09×10^3 ± 5.54×10^2	1.20×10^{-6} ± 6.57×10^{-7}	$5.01 \times 10^{3*}$ ± 6.05×10^2	5.5 ± 2.8
JFH-1/ G838R	5.77×10^8 ± 1.24×10^8	$7.82 \times 10^{2*}$ ± 1.53×10^2	$6.80 \times 10^{-7*}$ ± 3.40×10^{-8}	$4.07 \times 10^{3*}$ ± 1.01×10^3	5.2 ± 1.0
JFH-1/ A878V	5.45×10^8 ± 6.35×10^7	1.86×10^2 ± 4.58×10^1	2.20×10^{-7} ± 6.09×10^{-8}	9.12×10^2 ± 1.16×10^2	5.0 ± 0.7

*$p < 0.05$ compared with JFH-1/wt

56

Supporting

Figure 1

57
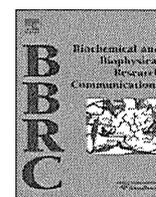
Development of recombinant hepatitis C virus with NS5A from strains of genotypes 1 and 2

Yuka Okamoto [a,b], Takahiro Masaki [a], Asako Murayama [a], Tsubasa Munakata [c], Akio Nomoto [d], Shingo Nakamoto [e], Osamu Yokosuka [e], Haruo Watanabe [b,f], Takaji Wakita [a], Takanobu Kato [a,*]

[a] Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan
[b] Department of Pathology, Immunology, and Microbiology, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan
[c] The Tokyo Metropolitan Institute of Medical Science, Setagaya-ku, Tokyo 156-8506, Japan
[d] Institute of Microbial Chemistry, Shinagawa-ku, Tokyo 141-0021, Japan
[e] Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba 260-0856, Japan
[f] National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan

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ABSTRACT

Nonstructural protein 5A (NS5A) of hepatitis C virus (HCV) plays multiple and diverse roles in the viral lifecycle, and is currently recognized as a novel target for anti-viral therapy. To establish an HCV cell culture system with NS5A of various strains, recombinant viruses were generated by replacing NS5A of strain JFH-1 with those of strains of genotypes 1 (H77; 1a and Con1; 1b) and 2 (J6CF; 2a and MA; 2b). All these recombinant viruses were capable of replication and infectious virus production. The replacement of JFH-1 NS5A with those of genotype 1 strains resulted in similar or slightly reduced virus production, whereas replacement with those of genotype 2 strains enhanced virus production as compared with JFH-1 wild-type. A single cycle virus production assay with a CD81-negative cell line revealed that the efficient virus production elicited by replacement with genotype 2 strains depended on enhanced viral assembly, and that substitutions in the C-terminus of NS5A were responsible for this phenotype. Pulse-chase assays revealed that these substitutions in the C-terminus of NS5A were possibly associated with accelerated cleavage kinetics at the NS5A–NS5B site. Using this cell culture system with NS5A-substituted recombinant viruses, the anti-viral effects of an NS5A inhibitor were then examined. A 300- to 1000-fold difference in susceptibility to the inhibitor was found between strains of genotypes 1 and 2. This system will facilitate not only a better understanding of strain-specific roles of NS5A in the HCV lifecycle, but also enable the evaluation of genotype and strain dependency of NS5A inhibitors.

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1. Introduction

Approximately 3% of the world's population is persistently infected with hepatitis C virus (HCV) and at increased risk of fatal chronic liver diseases such as decompensated liver cirrhosis and hepatocellular carcinoma. HCV have significant diversity in their genome and are grouped into six major genotypes. Among these genotypes, genotypes 1 and 2 are distributed worldwide and are predominant in Japan. The genotype is an important viral factor to predict the outcome of interferon (IFN)-based therapy. Because the efficacy of current therapy with pegylated IFN and ribavirin is insufficient, there is great interest in the development of novel HCV-specific inhibitors. The development of an HCV cell culture

system with strain JFH-1 has enabled the study of the viral lifecycle and research into anti-viral compounds [1]. However, the available strains used in the HCV cell culture system are still limited to JFH-1 (genotype 2a) and H77S (genotype 1a) [2]. Thus, JFH-1 based recombinant viruses harboring specific regions of other strains would be useful to assess the genotype or strain-specific sensitivity to novel anti-HCV compounds.

Although NS5A is an essential and involved in HCV RNA replication and virus assembly [3,4], it has been reported to be tolerable for trans-complementation in replication-defective mutants due to critical mutations in NS5A [5]. We hypothesized that the NS5A of strain JFH-1 could be replaced with those of other strains. In the present study, we developed a cell culture system with JFH-1 based intra- and inter-genotypic recombinant HCV harboring NS5A of strains H77 (genotype 1a) [6], Con1 (genotype 1b) [7], J6CF (genotype 2a) [8], and MA (genotype 2b) [9]. Through the use of these recombinant viruses, we evaluated the effects of NS5A replacement on the HCV lifecycle and susceptibility to the NS5A inhibitor BMS-790052.

* Corresponding author. Address: Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Fax: +81 3 5285 1161.
E-mail address: takato@nih.go.jp (T. Kato).

2. Materials and methods

2.1. Cell culture

The human hepatoma cell line, HuH-7, and derivative cell lines, Huh7.5.1 [10] and Huh7-25 [11], were cultured in complete growth medium as described previously [1,11].

2.2. Plasmid construction

Plasmids containing the full-genome of HCV strain JFH-1 (pJFH1) and of a replication defective mutant (pJFH1/GND) have been described previously [1]. The construction of the NS5A replaced recombinant viruses and subgenomic reporter replicons was described in Supplementary materials.

2.3. In vitro RNA synthesis and RNA transfection

In vitro synthesis of HCV RNA and RNA transfection were performed as described elsewhere [1].

2.4. Quantification of HCV core protein, luciferase activity, and extra- and intra-cellular infectivity

Quantification of these values was described in Supplementary materials.

2.5. Inhibition of HCV production by a specific NS5A inhibitor

Huh7.5.1 cells (3×10^6) were electroporated with 3 μg of synthetic HCV RNA, suspended in 15 mL complete growth medium, and seeded into 24-well plates. At 4 h after electroporation, the culture medium was replaced with medium containing 0.1% dimethyl sulfoxide (DMSO) with or without various concentrations of the specific NS5A inhibitor BMS-790052 (provided from Bristol-Myers Squibb Company, Plainsboro, NJ) [12]. After 44 h incubation, cells were harvested and HCV core protein was quantified.

2.6. Statistical analysis

Unpaired 2-tailed t-test was performed to evaluate the significance of results, and $p < 0.05$ was considered significant.

3. Results

3.1. Development of recombinant HCV with NS5A of genotypes 1 and 2

To establish an HCV cell culture system with NS5A of various strains, we generated recombinant viruses by replacing NS5A of strain JFH-1 with those of genotypes 1 and 2 strains. By transfection of in vitro transcribed RNA, efficient production of HCV core protein was detected in JFH-1 wild-type (JFH1/wt) and other recombinant viruses, but not in the replication defective mutant JFH1/GND (Fig. 1A). When compared between JFH1/wt and other recombinant viruses, intracellular core protein levels were comparable at days 2 and 3 after transfection, while extracellular core protein levels were very different. The extracellular core protein level of JFH1/wt-transfected cells increased exponentially up to 23,515 ± 1790 fmol/L at day 3. Similar kinetics was observed in JFH1/5A-H77-transfected cells. However, the extracellular core protein level of JFH1/5A-Con1-transfected cells was approximately 2.5-fold lower than that of JFH1/wt at days 2 and 3. Interestingly, the extracellular core protein levels of intra-genotypic recombinant viruses, JFH1/5A-J6CF and 5A-MA, were 2.5- to 3.5-fold higher than that of JFH1/wt at days 2 and 3. To evaluate the effect of these

NS5A replacements on HCV replication, we used recombinant subgenomic reporter replicons, SGR-JFH1/RLuc/wt, 5A-H77, 5A-Con1, 5A-J6CF, and 5A-MA. The Renilla luciferase activities of these recombinant subgenomic replicons were comparable to that of SGR-JFH1/Rluc/wt, suggesting similar levels of replication efficiency (Fig. 1B).

To further assess whether NS5A replacement affected other steps of the viral lifecycle, we used a single cycle virus production assay with Huh7-25 cells, a HuH-7-derived cell line lacking CD81 expression on the cell surface [11]. This cell line can support replication and infectious virus production upon transfection of HCV genomic RNA, but cannot be reinfected by produced HCV, therefore allowing the observation of a single cycle of infectious viral production without the confounding effects of reinfection [13]. As shown in Fig. 1C, JFH1/wt yielded an extracellular infectivity titer of 1585 ± 436 FFU/well at day 2 after transfection. JFH1/5A-H77 and 5A-Con1 showed significantly lower titers, while JFH1/5A-J6CF and 5A-MA showed significantly higher intracellular infectivity titers compared to JFH1/wt ($p < 0.05$). These data were consistent with the extracellular core protein levels of JFH1/wt and recombinant viruses (Fig. 1A). A similar tendency was observed in the intracellular infectivity titers of JFH1/wt and recombinant viruses (Fig. 1C). To estimate the efficiency of viral particle assembly, we determined the intracellular specific infectivity by calculating the ratio of the intracellular infectivity titer over the intracellular HCV core protein level. The intracellular specific infectivities of JFH1/5A-H77 and 5A-Con1 were 2.5- and 8-fold lower than that of JFH1/wt, respectively, while JFH1/5A-J6CF and 5A-MA showed 12- and 4-fold higher infectivities compared to JFH1/wt, respectively, suggesting a low assembly efficiency of JFH1/5A-H77 and 5A-Con1, and a high assembly efficiency of JFH1/5A-J6CF and 5A-MA (Fig. 1D). Taken together, all recombinant viruses could replicate and yielded infectious virus. Intra-genotypic recombinant viruses, JFH1/5A-J6CF and 5A-MA, had a higher ability to produce infectious virus than JFH1/wt in cultured cells.

3.2. The C-terminus of NS5A is responsible for enhanced viral assembly

The efficient infectious virus production of intra-genotypic recombinant viruses was unexpected. This prompted us to search for causes of the enhancement. To analyze the enhanced virus assembly of JFH1/5A-J6CF and 5A-MA, we focused on the C-terminus of NS5A of these strains, because this region influence the cleavage between NS5A and NS5B, and the cleavage is reported to be involved in virus assembly [14]. We generated recombinant JFH-1 viruses harboring 10 amino acids of the C-terminus of NS5A of J6CF and MA (JFH1/5AcJ6 and 5AcMA, respectively), and investigated replication and infectious virus production. In these 10 amino acids of the C-terminus of NS5A, JFH1/5AcJ6 and 5AcMA contain 2 and 6 substitutions, respectively, as compared with JFH1/wt, and 2 of them, T2438S and T2439V, are common (Fig. 2A). As shown in Fig. 2B, the extracellular core protein level of JFH1/5AcJ6-transfected cells was higher than those of JFH1/wt- and 5A-J6CF-transfected cells at the examined time points. A similar tendency was observed between JFH1/5AcMA and JFH1/wt or 5A-MA (Fig. 2C). In contrast to the extracellular core protein levels, the intracellular core protein levels were comparable for all NS5A recombinants at the examined time points.

We next assessed the replication of recombinant subgenomic luciferase reporter replicons on the basis of JFH1/5AcJ6 and 5AcMA (Fig. 2D). JFH1/5AcJ6 and 5AcMA showed similar levels of replication to JFH1/wt at day 2 after transfection. To investigate the effects of substitutions at the C-terminus of NS5A on infectious viral particle assembly, we determined the extra- and intracellular infectivity with the single cycle virus production assay with Huh7-25 cells. As shown in Fig. 2E, extra- and intracellular infectivities of
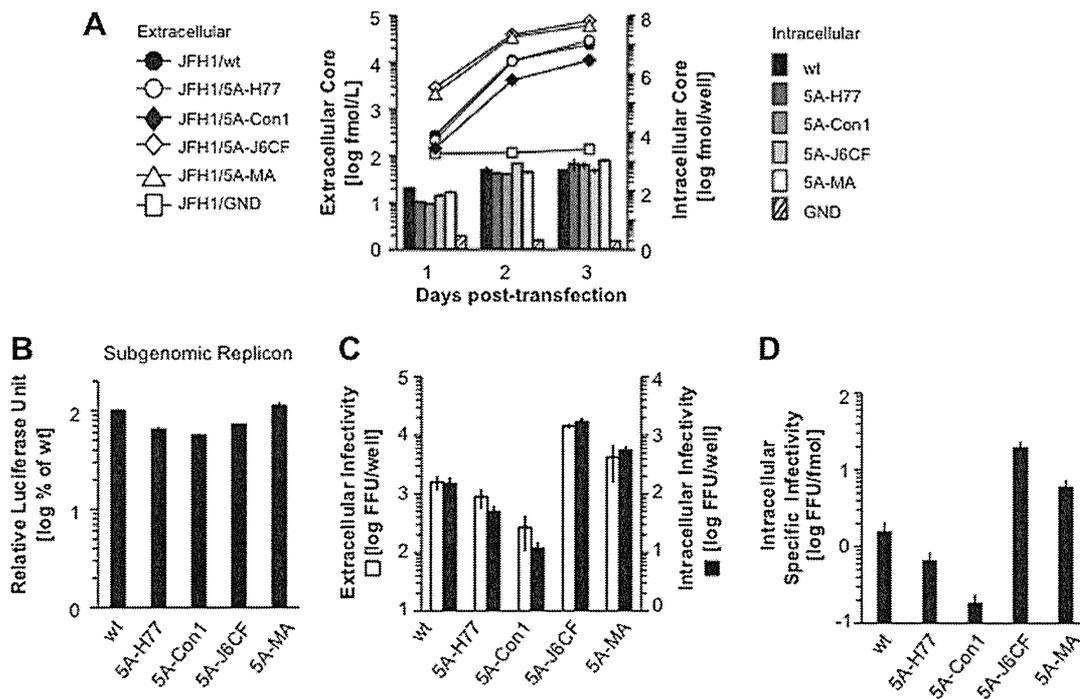
Fig. 1. Production and replication of recombinant viruses with NS5A of strains of genotypes 1 and 2. (A) Huh7.5.1 cells were transfected with in vitro synthesized RNA of JFH1/wt and indicated recombinants. The amount of extracellular (line graph) and intracellular (bar graph) HCV core protein was determined at the indicated time points. Assays were performed in triplicate, and means ± standard deviation are plotted. (B) Huh7.5.1 cells were transfected with subgenomic replicon RNA of JFH1/wt and indicated recombinants. Luciferase activity at day 2 was measured. Replication levels of JFH1/wt and indicated recombinants were calculated as fold increases at 4 h and are expressed as percentages of JFH1/wt. (C) Huh7-25 cells were transfected with RNA of JFH1/wt and recombinants. Forty-eight hours after transfection, extra- and intracellular infectivities were determined by inoculating into naïve Huh7.5.1 cells. (D) Intracellular specific infectivity of JFH1/wt and indicated recombinants.
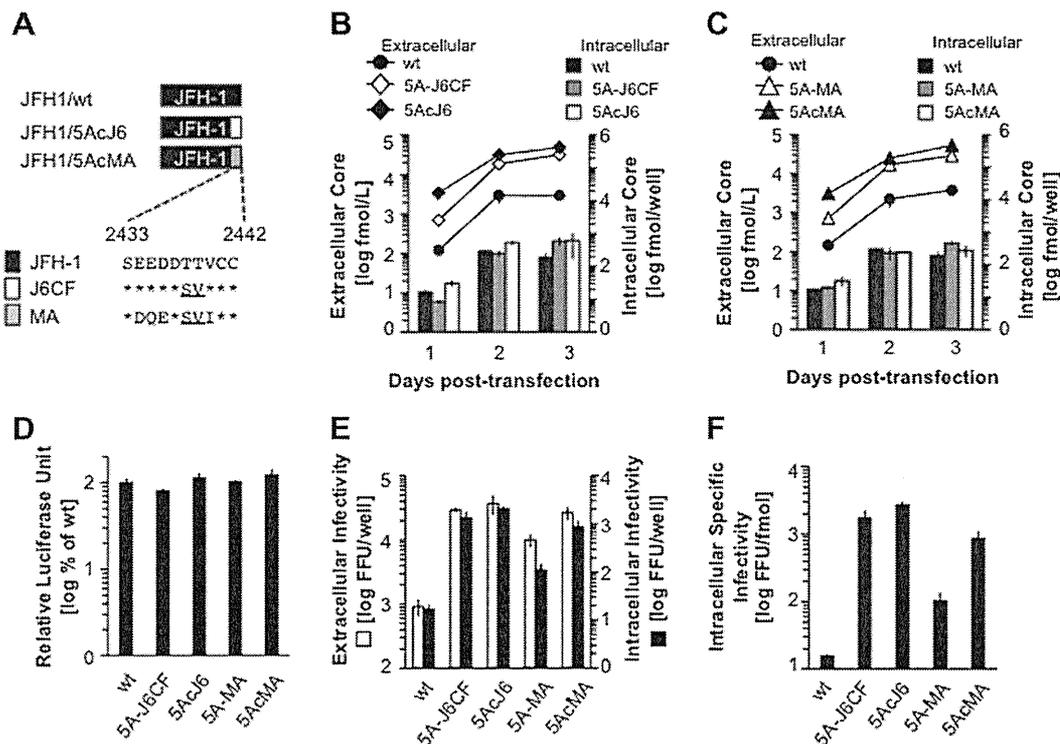
Fig. 2. C-terminal amino acids in NS5A were responsible for the enhanced virus production of recombinant viruses with NS5A of genotype 2 strains. (A) Alignment of C-terminal amino acids in NS5A of JFH-1, J6CF, and MA. Identical amino acids are indicated by asterisks. The indicated number represents the position of the amino acid in the entire polyprotein of JFH-1. (B) Huh7.5.1 cells were transfected with RNA of JFH1/wt, 5A-J6CF, and 5AcJ6. The amount of extracellular (line graph) and intracellular (bar graph) core proteins were quantified at the indicated time points. (C) Huh7.5.1 cells were transfected with RNA of JFH1/wt, 5A-MA, and 5AcMA. The amount of extracellular (line graph) and intracellular (bar graph) core proteins were quantified at the indicated time points. (D) Huh7.5.1 cells were transfected with subgenomic replicon RNA of JFH1/wt and indicated recombinants. Luciferase activity at day 2 was measured. Replication levels of JFH1/wt and indicated recombinants were calculated as the fold increase at 4 h and are expressed as percentages of JFH1/wt. (E) Huh7-25 cells were transfected with RNA of JFH1/wt and recombinant viruses. Forty-eight hours after transfection, extra- and intracellular infectivities were determined by inoculating into naïve Huh7.5.1 cells. (F) Intracellular specific infectivities of JFH1/wt and indicated recombinants.

JFH1/5AcJ6 and 5AcMA were approximately 20-fold higher than that of the JFH1/wt ($p < 0.05$), and were slightly higher than those of JFH1/5A-J6CF and 5A-MA. We also determined the specific intracellular infectivity of each recombinant virus to assess virus assembly (Fig. 2F). As with extra- and intracellular infectivities, the specific intracellular infectivities of JFH1/5AcJ6 and 5AcMA were more than 10-fold higher than that of the JFH1/wt ($p < 0.05$), and were slightly higher than those of JFH1/5A-J6CF and 5A-MA. These results suggest that these C-terminal amino acids of NS5A are responsible for enhanced assembly of intra-genotypic recombinant viruses JFH1/5A-J6CF and 5A-MA.

3.3. Amino acid substitutions at the C-terminus of NS5A accelerate cleavage kinetics between NS5A and NS5B

To investigate whether substitution of the C-terminus of NS5A affects the cleavage kinetics between NS5A and NS5B, we performed pulse-chase assays using a T7-based expression system. Immunoprecipitations were performed with an NS5B-specific antibody and immunocomplexes were analyzed on a 7.5% SDS–PAGE (Supplementary Fig. A). Fully processed NS5B and an uncleaved NS5A–NS5B precursor with a size of approximately 130 kDa could be detected for JFH1/wt and recombinant constructs JFH1/5AcJ6 and 5AcMA. In the case of JFH1/wt, the amount of uncleaved precursor was gradually decreased but still detectable at 4 h of the chase period. On the other hand, in the case of the recombinant constructs, JFH1/5AcJ6 and 5AcMA, the amounts of precursor were reduced more rapidly and were undetectable by 4 h of chase. To assess the kinetics of the cleavage, the percentages of uncleaved NS5A–NS5B precursor at the examined time points were plotted and analyzed using nonlinear regression (Supplementary Fig. B). Rapid cleavage kinetics was observed in JFH1/5AcJ6 and 5AcMA transfected cells as compared with JFH1/wt. These observations suggest that substitutions at the C-terminus of NS5A of these recombinant viruses are responsible for the accelerated cleavage kinetics between NS5A and NS5B, and might be associated with enhanced infectious viral particle assembly.

3.4. Susceptibility of recombinant HCV to the NS5A inhibitor BMS-790052

Using developed JFH-1 based inter- and intra-genotypic recombinant viruses, we assessed their susceptibility to the NS5A inhibitor BMS-790052 [12]. After transfection with synthesized HCV RNA, cells were treated with different concentrations of BMS-790052 for 2 days and intracellular HCV core protein levels were determined. No cytopathic effects were observed at the concentrations used (data not shown). As shown in Fig. 3, the intracellular core protein levels of JFH1/wt and recombinant viruses were inhibited to different extents. Recombinant viruses with NS5A of genotype 1, JFH1/5A-H77 and 5A-Con1, showed higher susceptibility to BMS-790052 as compared with JFH1/wt, while JFH1/5A-J6CF and 5A-MA showed much lower susceptibility. To compare the susceptibilities, the effective concentrations required to inhibit 50% of intracellular core protein level (EC_{50}) were determined, because the intracellular core protein levels of these recombinant viruses were at almost the same level at day 2 after transfection (Fig. 1A). The EC_{50} of JFH1/wt and recombinant viruses with NS5A of genotype 1, JFH1/5A-H77 and 5A-Con1, were 6.4, 3.1, and 1.4 pM, respectively, and do not conflict with results using replicon systems reported previously [12]. In contrast, recombinant viruses with NS5A of genotype 2, JFH1/5A-J6CF and 5A-MA, were more resistant to BMS-790052, and EC_{50} values were 1.5 and >5 nM, respectively. Collectively, the anti-HCV effect of the specific NS5A inhibitor BMS-790052 showed strain and genotype dependency. In particular, the NS5A of genotype 2 strains, J6CF and MA, excepting JFH-1, showed 300- to 1000-fold lower susceptibility to BMS-790052 compared with the NS5A of genotype 1 strains, H77 and Con1.

4. Discussion

HCV NS5A is essential for replication and infectious virus production, similar to other nonstructural proteins possessing enzymatic activities, including NS3 (a serine protease) and NS5B (an RNA-dependent RNA polymerase). Currently, these nonstructural proteins are being targeted to establish anti-viral compounds to improve the outcome of therapy for chronic HCV infection, and several inhibitors for these proteins are entering into clinical trials. A great deal of interest has also been shown in the development of NS5A inhibitors, and one potent inhibitor, BMS-790052, has recently been described [12]. In this study, to assess strain and genotype dependent susceptibility for this inhibitor, we generated recombinant HCV with NS5A from strains other than JFH-1, because a limited number of strains are available in the HCV cell culture system. We replaced NS5A of JFH-1 with those of genotype 1 and 2 strains, and observed efficient replication and infectious virus production in cell culture.

The replication efficiencies of these NS5A recombinant viruses were almost the same, whereas virus production levels into the culture medium were very different from JFH1/wt (Fig. 1A and
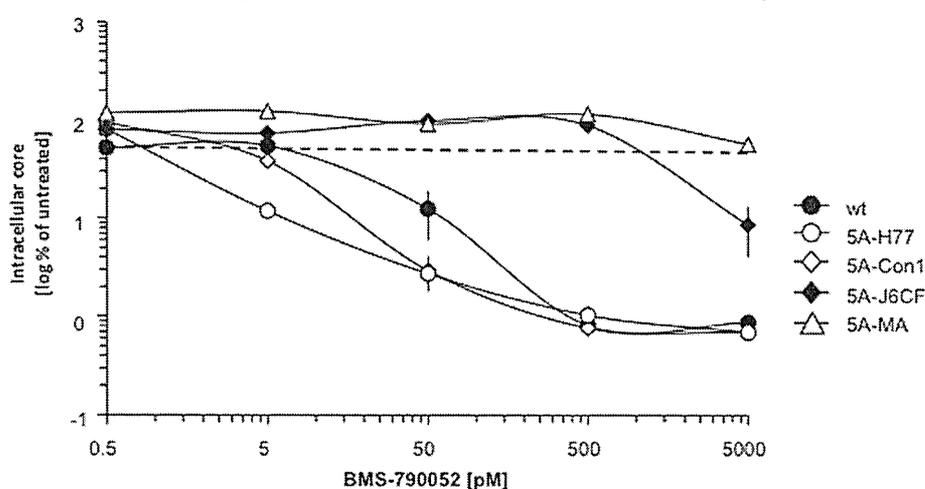

Fig. 3. Susceptibility of JFH1/wt and recombinant viruses to the NS5A inhibitor. Huh7.5.1 cells were transfected with RNA of JFH1/wt and recombinant viruses and treated with serially diluted BMS-790052 for 48 h. The amounts of intracellular HCV core protein were quantified and normalized against untreated control set to 100%.

Table 1
Amino acid substitutions in NS5A of strains used, and reported resistant mutations to BMS-790052.

AA[a]		Strains used in this study					Reported resistant mutations[b]			Ref.
Entire	NS5A	JFH1	H77	Con1	J6CF	MA	1a	1b	2a	
2004	28	F	M	L	F	L	T (683)	T (20)	–	[20]
2006	30	K	Q	R	K	K	E (24,933)	E (6)	–	[20]
							K (24,317)			
							H (1450)			
							R (1217)			
2007	31	L	L	L	M	M	M (350)	F (5)	M (170)	[12,20]
							V (3350)	M (3)		
								V (23)		
2008	32	P	P	P	P	P	L (233)	L (17)	–	[20]
2068	93	A	A	C	C	C	–	–	E (150)	[12]
2069	93	Y	Y	Y	Y	Y	C (1850)	H (19)	H (130–1400)	[12,20]
							H (5367)	N (28)		
							N (47,017)			

[a] AA, amino acid position which are according to entire polyprotein (Entire) and NS5A of JFH1.
[b] Fold resistance as compared with parental amino acid is indicated in parentheses.

B). Enhanced virus production was detected in recombinant viruses replaced with NS5A of genotype 2 strains, while reduced virus production was observed in recombinant viruses replaced with NS5A of genotype 1 strains (Fig. 1A). The single cycle virus production assay revealed that this enhanced virus production with NS5A of genotype 2 was due to efficient viral particle assembly (Fig. 1D). To analyze the mechanism of efficient virus assembly by NS5A of strains J6CF and MA, we focused on the cleavage between NS5A and NS5B. Several reports have shown that amino acids in the C-terminus of NS5A influence the cleavage [15,16]. Thus, we used recombinant JFH-1 viruses harboring amino acids of J6CF and MA in the C-terminus of JFH-1 NS5A, and assessed replication and infectious virus production. We found that both of these recombinant viruses, JFH1/5AcJ6 and 5AcMA, showed more enhanced virus assembly (Fig. 2F), and reasoned that the amino acid substitutions T2438S and T2439V were responsible for the enhanced infectious virus production. In pulse-chase assays for the cleavage of NS5A and NS5B, accelerated cleavage was observed in recombinant viruses JFH1/5AcJ6 and 5AcMA. Uncleaved NS5A–NS5B disappeared earlier in JFH1/5AcJ6- and 5AcMA-transfected cells than in JFH1/wt-transfected cells (Supplementary Fig. A). Taken together, the enhanced virus assembly observed with JFH1/5A-J6CF and 5A-MA depended on the C-terminal amino acid substitutions in NS5A, possibly through accelerated cleavage kinetics between NS5A and NS5B. The reason for the correlation between accelerated cleavage and enhanced virus assembly is still unknown. Accelerated cleavage may lead to an increased amount of mature NS5A used for virus assembly or affect the interaction with the core protein, which has been reported to be important for infectious virus assembly [17]. Another possibility is the interaction between the C-terminus of NS5A and some host factor(s) involved in virus particle assembly, such as apolipoprotein E [18]. Amino acid substitutions at this region may alter the potency of this interaction directly. A previous report has also shown that another mutation in this region, V2440L, is associated with delayed cleavage kinetics between NS5A and NS5B, but enhanced virus assembly [14]. Further investigation will be necessary to clarify this mechanism and to solve the discrepancy.

Using this cell culture system with NS5A recombinant viruses, we assessed strain and genotype dependent susceptibility to the novel NS5A inhibitor, BMS-790052. This potent inhibitor successfully inhibited replication of JFH1/wt and recombinant viruses with NS5A of genotype 1 strains (Fig. 3). However, it showed limited effectiveness on recombinant viruses with NS5A of genotype 2 strains. This high efficacy for genotype 1 strains makes sense because this inhibitor and its lead compound were identified using genotype 1a and 1b subgenomic replicons [19].

During preparation of this paper, another study was published describing an HCV cell culture system with NS5A-substituted recombinant viruses [20]. That study used a J6/JFH-1 chimeric virus that is known to have high virus production efficiency, but not natural viruses, and established nine recombinant viruses with NS5A from strains of eight different subtypes. They found that recombinant viruses with NS5A of strains of genotypes 1a, 1b, 4a, 5a, and 6a were sensitive, and strains of genotypes 2a and 3a were resistant to the NS5A inhibitor, data that are consistent with our own observations. In addition, we found that recombinant virus with NS5A of genotype 2b, which is the one of the predominant genotypes in Japan, was also resistant to the compound. Resistant mutations to BMS-790052 have been reported and are frequently observed in the N-terminus of NS5A, suggesting inhibition of membrane localization and dimerization of NS5A (Table 1) [12,20]. Among these reported mutations, one of the most potent, 2006E/K/H/R (amino acid position (AA) 30 in NS5A), is found in all strains but H77, and another potent resistant mutation, 2007F/M/V (AA 31 in NS5A), is also found in J6CF and MA. Thus, the lower susceptibilities of recombinant viruses JFH1/5A-J6CF and 5A-MA, as compared with JFH1/wt, might be due to the latter mutation. Based on an analysis of the database of submitted strains (Hepatitis Virus Database; http://s2as02.genes.nig.ac.jp/index.html), this resistant mutation, 2007M, is detected in 84.2% and 79.0% of genotype 2a and 2b strains, respectively, whereas it is observed in only 0.2% of genotype 1a and 3.8% of genotype 1b strains [20,21]. From these observations, most of genotype 2a and 2b strains may be resistant to BMS-790052, although these are known to be sensitive to interferon [22].

In conclusion, we established JFH-1 based recombinant viruses by replacement of NS5A with those from strains of genotypes 1 and 2. All the generated recombinant viruses could replicate and produce infectious viruses in cell culture, and were useful to assess the genotype and strain dependency to a novel NS5A inhibitor. The strategy of using recombinant virus will facilitate not only a better understanding of the strain-specific roles of NS5A in the HCV lifecycle, but also aid in developing and testing specific inhibitors against NS5A from different genotypes and strains.

Health, Labor and Welfare of Japan, from the Ministry of Education, Culture, Sports, Science and Technology, by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation, and by the National Institute of Biomedical Innovation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.144.

References

[1] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.G. Krausslich, M. Mizokami, R. Bartenschlager, T.J. Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.

[2] M. Yi, R.A. Villanueva, D.L. Thomas, T. Wakita, S.M. Lemon, Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells, Proc. Natl. Acad. Sci. USA 103 (2006) 2310–2315.

[3] M.J. Evans, C.M. Rice, S.P. Goff, Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication, Proc. Natl. Acad. Sci. USA 101 (2004) 13038–13043.

[4] N. Appel, M. Zayas, S. Miller, J. Krijnse-Locker, T. Schaller, P. Friebe, S. Kallis, U. Engel, R. Bartenschlager, Essential role of domain III of nonstructural protein 5A for hepatitis C virus infectious particle assembly, PLoS Pathog. 4 (2008) e1000035.

[5] N. Appel, U. Herian, R. Bartenschlager, Efficient rescue of hepatitis C virus RNA replication by trans-complementation with nonstructural protein 5A, J. Virol. 79 (2005) 896–909.

[6] M. Yanagi, R.H. Purcell, S.U. Emerson, J. Bukh, Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee, Proc. Natl. Acad. Sci. USA 94 (1997) 8738–8743.

[7] V. Lohmann, F. Korner, J. Koch, U. Herian, L. Theilmann, R. Bartenschlager, Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line, Science 285 (1999) 110–113.

[8] M. Yanagi, R.H. Purcell, S.U. Emerson, J. Bukh, Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras, Virology 262 (1999) 250–263.

[9] K. Murakami, M. Abe, T. Kageyama, N. Kamoshita, A. Nomoto, Down-regulation of translation driven by hepatitis C virus internal ribosomal entry site by the 3′ untranslated region of RNA, Arch. Virol. 146 (2001) 729–741.

[10] J. Zhong, P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D.R. Burton, S.F. Wieland, S.L. Uprichard, T. Wakita, F.V. Chisari, Robust hepatitis C virus infection in vitro, Proc. Natl. Acad. Sci. USA 102 (2005) 9294–9299.

[11] D. Akazawa, T. Date, K. Morikawa, A. Murayama, M. Miyamoto, M. Kaga, H. Barth, T.F. Baumert, J. Dubuisson, T. Wakita, CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection, J. Virol. 81 (2007) 5036–5045.

[12] M. Gao, R.E. Nettles, M. Belema, L.B. Snyder, V.N. Nguyen, R.A. Fridell, M.H. Serrano-Wu, D.R. Langley, J.H. Sun, D.R. O'Boyle 2nd, J.A. Lemm, C. Wang, J.O. Knipe, C. Chien, R.J. Colonno, D.M. Grasela, N.A. Meanwell, L.G. Hamann, Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect, Nature 465 (2010) 96–100.

[13] T. Kato, Y. Choi, G. Elmowalid, R.K. Sapp, H. Barth, A. Furusaka, S. Mishiro, T. Wakita, K. Krawczynski, T.J. Liang, Hepatitis C virus JFH-1 strain infection in chimpanzees is associated with low pathogenicity and emergence of an adaptive mutation, Hepatology 48 (2008) 732–740.

[14] A. Kaul, S. Stauffer, C. Berger, T. Pertel, J. Schmitt, S. Kallis, M. Zayas, V. Lohmann, J. Luban, R. Bartenschlager, Essential role of cyclophilin A for hepatitis C virus replication and virus production and possible link to polyprotein cleavage kinetics, PLoS Pathog. 5 (2009) e1000546.

[15] R. Bartenschlager, L. Ahlborn-Laake, K. Yasargil, J. Mous, H. Jacobsen, Substrate determinants for cleavage in cis and in trans by the hepatitis C virus NS3 proteinase, J. Virol. 69 (1995) 198–205.

[16] A. Urbani, E. Bianchi, F. Narjes, A. Tramontano, R. De Francesco, C. Steinkuhler, A. Pessi, Substrate specificity of the hepatitis C virus serine protease NS3, J. Biol. Chem. 272 (1997) 9204–9209.

[17] T. Masaki, R. Suzuki, K. Murakami, H. Aizaki, K. Ishii, A. Murayama, T. Date, Y. Matsuura, T. Miyamura, T. Wakita, T. Suzuki, Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles, J. Virol. 82 (2008) 7964–7976.

[18] W. Cun, J. Jiang, G. Luo, The C-terminal alpha-helix domain of apolipoprotein E is required for interaction with nonstructural protein 5A and assembly of hepatitis C virus, J. Virol. 84 (2010) 11532–11541.

[19] J.A. Lemm, D. O'Boyle 2nd, M. Liu, P.T. Nower, R. Colonno, M.S. Deshpande, L.B. Snyder, S.W. Martin, D.R. St Laurent, M.H. Serrano-Wu, J.L. Romine, N.A. Meanwell, M. Gao, Identification of hepatitis C virus NS5A inhibitors, J. Virol. 84 (2010) 482–491.

[20] T.K. Scheel, J.M. Gottwein, L.S. Mikkelsen, T.B. Jensen, J. Bukh, Recombinant HCV variants with NS5A from genotypes 1–7 have different sensitivities to an NS5A inhibitor but not interferon-alpha, Gastroenterology 140 (2011) 1032–1042.

[21] R.A. Fridell, D. Qiu, C. Wang, L. Valera, M. Gao, Resistance analysis of the hepatitis C virus NS5A inhibitor BMS-790052 in an in vitro replicon system, Antimicrob. Agents Chemother. 54 (2010) 3641–3650.

[22] K. Yoshioka, S. Kakumu, T. Wakita, T. Ishikawa, Y. Itoh, M. Takayanagi, Y. Higashi, M. Shibata, T. Morishima, Detection of hepatitis C virus by polymerase chain reaction and response to interferon-alpha therapy: relationship to genotypes of hepatitis C virus, Hepatology 16 (1992) 293–299.
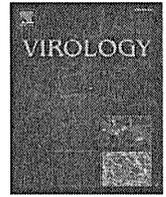
Chaperonin TRiC/CCT participates in replication of hepatitis C virus genome via interaction with the viral NS5B protein

Yasushi Inoue [a,b,c], Hideki Aizaki [a], Hiromichi Hara [a], Mami Matsuda [a], Tomomi Ando [a], Tetsu Shimoji [a], Kyoko Murakami [a], Takahiro Masaki [a], Ikuo Shoji [d], Sakae Homma [b], Yoshiharu Matsuura [e], Tatsuo Miyamura [a], Takaji Wakita [a], Tetsuro Suzuki [a,f,*]

[a] Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan
[b] Department of Respiratory Medicine, Toho University School of Medicine, Tokyo 143-8541, Japan
[c] International University of Health and Welfare, Mita Hospital, Tokyo 108-8329, Japan
[d] Division of Microbiology, Kobe University Graduate School of Medicine, Hyogo 650-0017, Japan
[e] Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan
[f] Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan

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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

* Corresponding author. Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan. Fax: +81 53 435 2337.
E-mail address: tesuzuki@hama-med.ac.jp (T. Suzuki).

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 electro-

phoresis (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
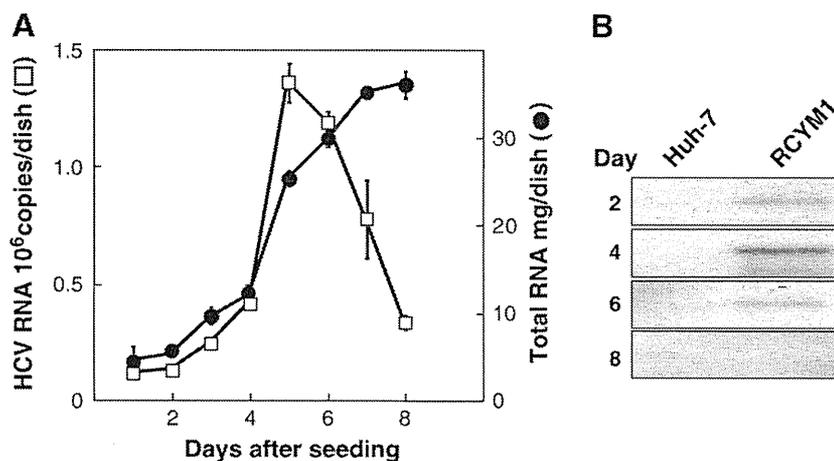
Fig. 1. Effect of cell growth on HCV RNA replication. (A) Measurement of HCV RNA (open squares) and total cellular RNA (closed circles) in RCYM1 cells at the time of harvest (days after seeding). (B) DRM fractions obtained from RCYM1 and parental Huh-7 cells harvested as indicated (day) were analyzed 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
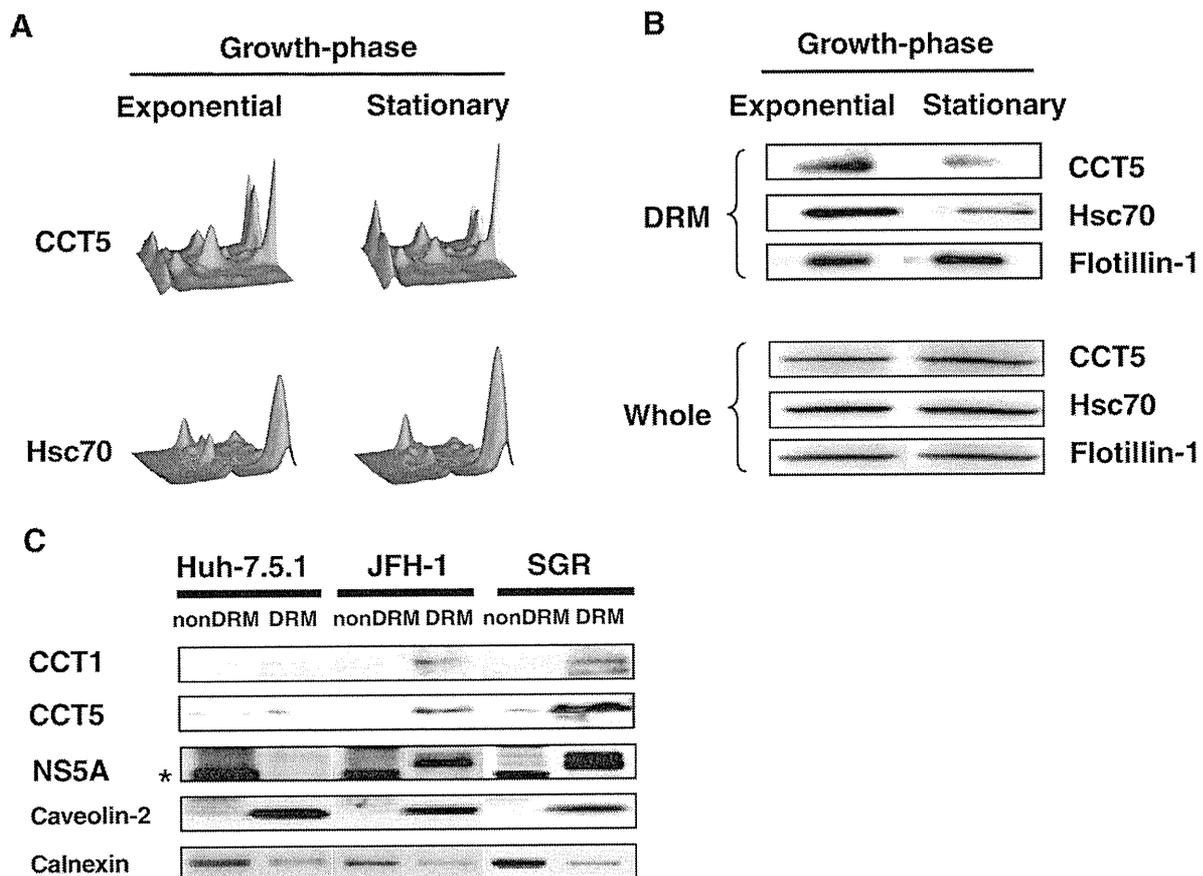
Fig. 2. Comparison of protein levels in DRM fractions prepared from RCYM1 cells at the exponential and stationary growth phases. (A) Three-dimensional images of CCT5 and Hsc70 analyzed by Ettan DIGE (GE Healthcare). Spots corresponding to CCT5/Hsc70 at exponential and stationary growth phases of the 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.
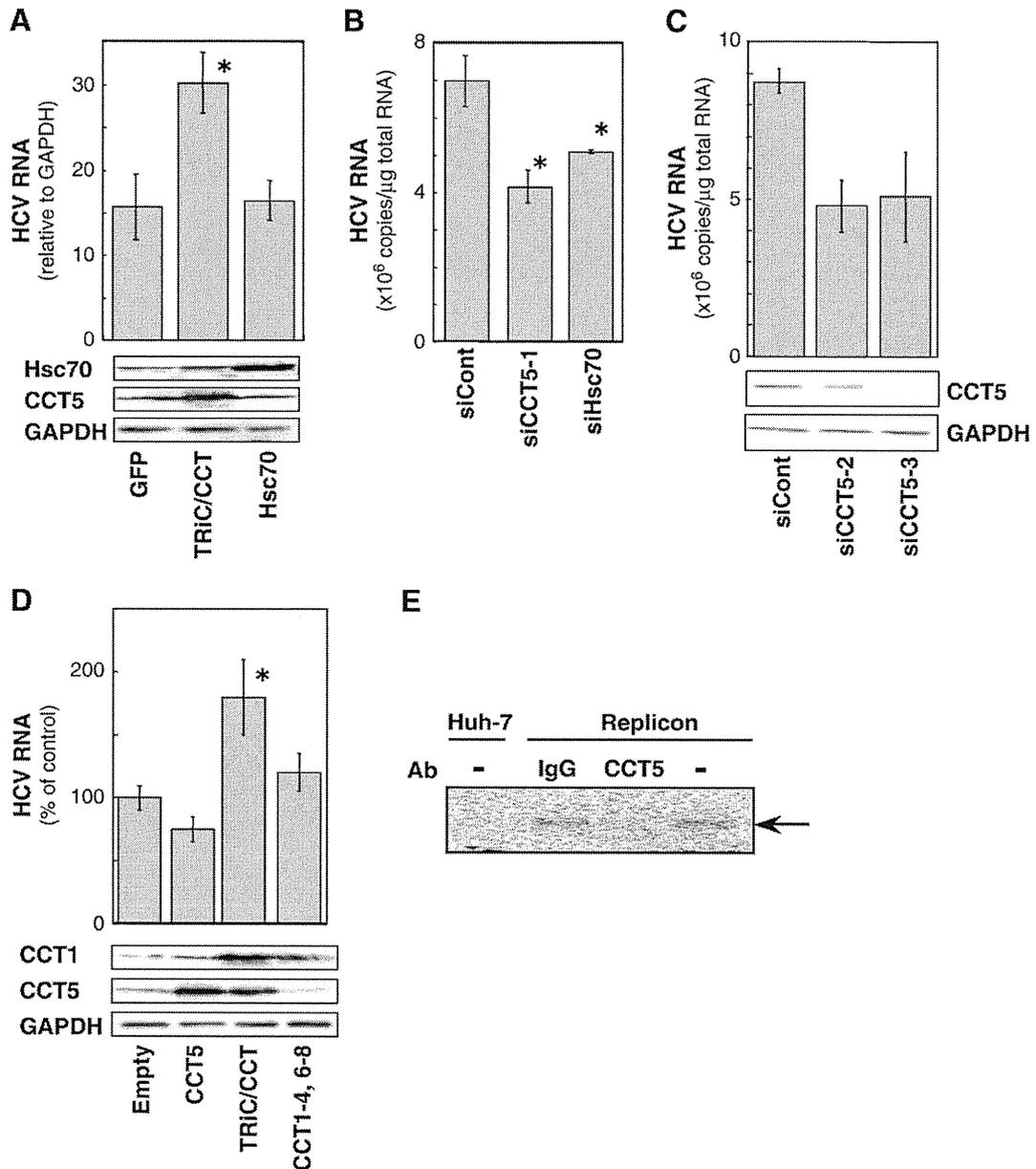
Fig. 3. Involvement of TRiC/CCT in HCV replication (A and D). Overexpression of all eight subunits of TRiC/CCT (TRiC/CCT); seven subunits, CCT1, 2, 3, 4, 6, 7, and 8 (CCT1–4, 6–8); subunit CCT5 only (CCT5); Hsc70; or control GFP in RCYM1 cells. HCV RNA levels were determined 48 h post-transfection (B 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
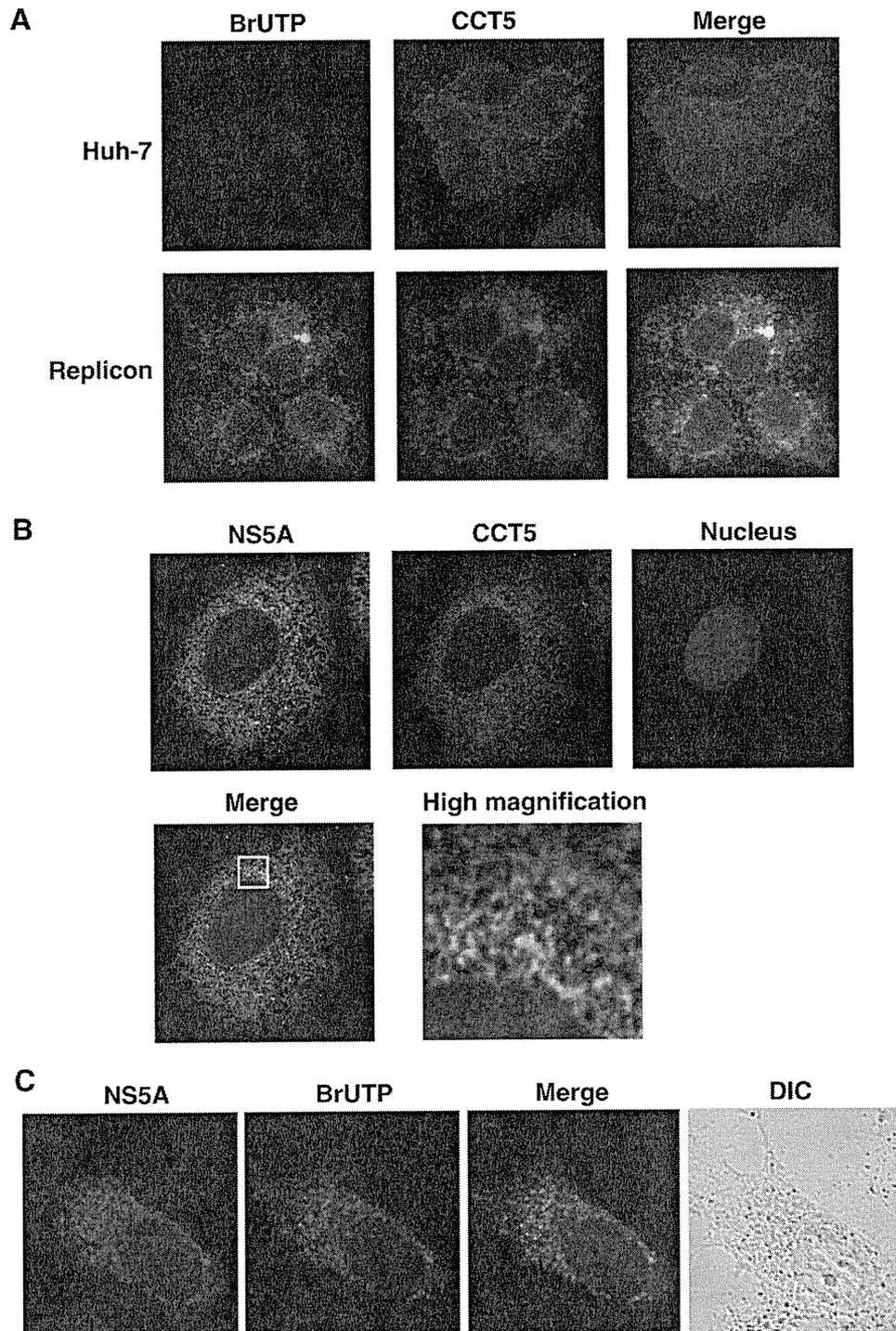
Fig. 4. Immunofluorescence analysis of CCT5 in SGR-N and Huh-7 cells (A) and HCVcc-infected cells (B). The primary Abs used were anti-CCT5 goat polyclonal Ab (red), anti-BrUTP monoclonal Ab (green), and anti-NS5A monoclonal Ab (green). Merged images of red and green signals (A) or of red, green and blue (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
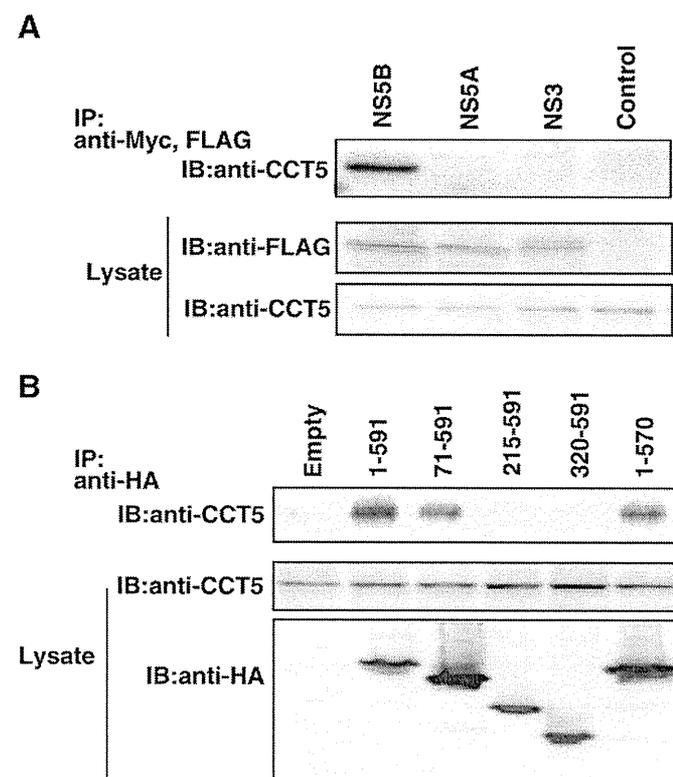
Fig. 5. CCT5 interacts with HCV NS5B. (A) CCT5 was co-expressed with MEF-tagged-NS5B, -NS5A, or -NS3 protein of strain NIHJ1 in cells, followed by two-step immunoprecipitation (IP) with anti-FLAG and anti-myc Abs. Immunoprecipitates were subjected to immunoblotting with anti-CCT5 Ab (IB). (B) Full-length 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.
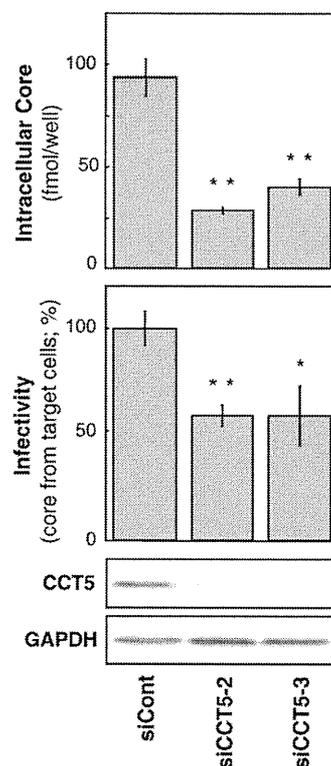
Fig. 6. Knockdown of endogenous CCT5 in HCVcc-infected cells. The cells were transfected with siRNAs against CCT5 (siCCT5-2, -3) or with control siRNAs (siCont). At 72 h post-transfection, the viral core protein levels in cells were determined (upper panel). Collected culture supernatants were inoculated 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 immuno-precipitation 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).

Acknowledgments

References

Aizaki, H., Aoki, Y., Harada, T., Ishii, K., Suzuki, T., Nagamori, S., Toda, G., Matsuura, Y., Miyamura, T., 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. Hepatology 27 (2), 621–627.

Aizaki, H., Lee, K.J., Sung, V.M., Ishiko, H., Lai, M.M., 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology 324 (2), 450–461.

Aizaki, H., Morikawa, K., Fukasawa, M., Hara, H., Inoue, Y., Tani, H., Saito, K., Nishijima, M., Hanada, K., Matsuura, Y., Lai, M.M., Miyamura, T., Wakita, T., Suzuki, T., 2008. Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J. Virol. 82 (12), 5715–5724.

Ali, N., Tardif, K.D., Siddiqui, A., 2002. Cell-free replication of the hepatitis C virus subgenomic replicon. J. Virol. 76 (23), 12001–12007.

Burch, A.D., Weller, S.K., 2005. Herpes simplex virus type 1 DNA polymerase requires the mammalian chaperone hsp90 for proper localization to the nucleus. J. Virol. 79 (16), 10740–10749.

Cuéllar, J., Martín-Benito, J., Scheres, S.H., Sousa, R., Moro, F., López-Viñas, E., Gómez-Puertas, P., Muga, A., Carrascosa, J.L., Valpuesta, J.M., 2008. The structure of CCT-Hsc70 NBD suggests a mechanism for Hsp70 delivery of substrates to the chaperonin. Nat. Struct. Mol. Biol. 15 (8), 858–864.

Daikoku, T., Kudoh, A., Sugaya, Y., Iwahori, S., Shirata, N., Isomura, H., Tsurumi, T., 2006. Postreplicative mismatch repair factors are recruited to Epstein–Barr virus replication compartments. J. Biol. Chem. 281 (16), 11422–11430.

De Francesco, R., Migliaccio, G., 2005. Challenges and successes in developing new therapies for hepatitis C. Nature 436 (7053), 953–960.

Dworniczak, B., Mirault, M.E., 1987. Structure and expression of a human gene coding for a 71 kd heat shock 'cognate' protein. Nucleic Acids Res. 15 (13), 5181–5197.

Farr, G.W., Scharl, E.C., Schumacher, R.J., Sondek, S., Horwich, A.L., 1997. Chaperonin-mediated folding in the eukaryotic cytosol proceeds through rounds of release of native and nonnative forms. Cell 89 (6), 927–937.

Feldman, D.E., Spiess, C., Howard, D.E., Frydman, J., 2003. Tumorigenic mutations in VHL disrupt folding in vivo by interfering with chaperonin binding. Mol. Cell 12 (5), 1213–1224.

Fislová, T., Thomas, B., Graef, K.M., Fodor, E., 2010. Association of the influenza virus RNA polymerase subunit PB2 with the host chaperonin CCT. J. Virol. 84 (17), 8691–8699.

Frydman, J., Hartl, F.U., 1996. Principles of chaperone-assisted protein folding: differences between in vitro and in vivo mechanisms. Science 272 (5267), 1497–1502.

Garcin, D., Rochat, S., Kolakofsky, D., 1993. The Tacaribe arenavirus small zinc finger protein is required for both mRNA synthesis and genome replication. J. Virol. 67 (2), 807–812.

Goh, P.Y., Tan, Y.J., Lim, S.P., Tan, Y.H., Lim, S.G., Fuller-Pace, F., Hong, W., 2004. Cellular RNA helicase p68 relocalization and interaction with the hepatitis C virus (HCV) NS5B protein and the potential role of p68 in HCV RNA replication. J. Virol. 78 (10), 5288–5298.

Guo, J.T., Bichko, V.V., Seeger, C., 2001. Effect of alpha interferon on the hepatitis C virus replicon. J. Virol. 75 (18), 8516–8523.

Hamamoto, I., Nishimura, Y., Okamoto, T., Aizaki, H., Liu, M., Mori, Y., Abe, T., Suzuki, T., Lai, M.M., Miyamura, T., Moriishi, K., Matsuura, Y., 2005. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. J. Virol. 79 (21), 13473–13482.

Hansen, J.L., Long, A.M., Schultz, S.C., 1997. Structure of the RNA-dependent RNA polymerase of poliovirus. Structure 5 (8), 1109–1122.

Hara, H., Aizaki, H., Matsuda, M., Shinkai-Ouchi, F., Inoue, Y., Murakami, K., Shoji, I., Kawakami, H., Matsuura, Y., Lai, M.M., Miyamura, T., Wakita, T., Suzuki, T., 2009. Involvement of creatine kinase B in hepatitis C virus genome replication through interaction with the viral NS4A protein. J. Virol. 83 (10), 5137–5147.

Hardy, R.W., Marcotrigiano, J., Blight, K.J., Majors, J.E., Rice, C.M., 2003. Hepatitis C virus RNA synthesis in a cell-free system isolated from replicon-containing hepatoma cells. J. Virol. 77 (3), 2029–2037.

Hirano, M., Kaneko, S., Yamashita, T., Luo, H., Qin, W., Shirota, Y., Nomura, T., Kobayashi, K., Murakami, S., 2003. Direct interaction between nucleolin and hepatitis C virus NS5B. J. Biol. Chem. 278 (7), 5109–5115.

Hoofnagle, J.H., 2002. Course and outcome of hepatitis C. Hepatology 36 (5 Suppl 1), S21–S29.

Hu, J., Flores, D., Toft, D., Wang, X., Nguyen, D., 2004. Requirement of heat shock protein 90 for human hepatitis B virus reverse transcriptase function. J. Virol. 78 (23), 13122–13131.

Huang, H., Chopra, R., Verdine, G.L., Harrison, S.C., 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science 282 (5394), 1669–1675.

Ichimura, T., Yamamura, H., Sasamoto, K., Tominaga, Y., Taoka, M., Kakiuchi, K., Shinkawa, T., Takahashi, N., Shimada, S., Isobe, T., 2005. 14-3-3 proteins modulate the expression of epithelial Na+ channels by phosphorylation-dependent interaction with Nedd4-2 ubiquitin ligase. J. Biol. Chem. 280 (13), 13187–13194.

Ikeda, M., Yi, M., Li, K., Lemon, S.M., 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J. Virol. 76 (6), 2997–3006.

Kampmueller, K.M., Miller, D.J., 2005. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells. J. Virol. 79 (11), 6827–6837.

Kim, S.J., Kim, J.H., Kim, Y.G., Lim, H.S., Oh, J.W., 2004. Protein kinase C-related kinase 2 regulates hepatitis C virus RNA polymerase function by phosphorylation. J. Biol. Chem. 279 (48), 50031–50041.

Kukihara, H., Moriishi, K., Taguwa, S., Tani, H., Abe, T., Mori, Y., Suzuki, T., Fukuhara, T., Taketomi, A., Maehara, Y., Matsuura, Y., 2009. Human VAP-C negatively regulates hepatitis C virus propagation. J. Virol. 83 (16), 7959–7969.

Kyono, K., Miyashiro, M., Taguchi, I., 2002. Human eukaryotic initiation factor 4AII associates with hepatitis C virus NS5B protein in vitro. Biochem. Biophys. Res. Commun. 292 (3), 659–666.

Laporte, M.G., Jackson, R.W., Draper, T.L., Gaboury, J.A., Galie, K., Herbertz, T., Hussey, A.R., Rippin, S.R., Benetatos, C.A., Chunduru, S.K., Christensen, J.S., Coburn, G.A., Rizzo, C.J., Rhodes, G., O'Connell, J., Howe, A.Y., Mansour, T.S., Collett, M.S., Pevear, D.C., Young, D.C., Gao, T., Tyrrell, D.L., Kneteman, N.M., Burns, C.J., Condon, S.M., 2008. The discovery of pyrano [3, 4-b] indole-based allosteric inhibitors of HCV NS5B polymerase with in vivo activity. ChemMedChem 3 (10), 1508–1515.

Liu, H.M., Aizaki, H., Choi, K.S., Machida, K., Ou, J.J., Lai, M.M., 2009. SYNCRIP (synaptotagmin-binding, cytoplasmic RNA-interacting protein) is a host factor involved in hepatitis C virus RNA replication. Virology 386 (2), 249–256.

Lohmann, V., Körner, F., Herian, U., Bartenschlager, R., 1997. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. J. Virol. 71 (11), 8416–8428.

Lohmann, V., Roos, A., Körner, F., Koch, J.O., Bartenschlager, R., 2000. Biochemical and structural analysis of the NS5B RNA-dependent RNA polymerase of the hepatitis C virus. J. Viral Hepat. 7 (3), 167–174.

Manns, M.P., Wedemeyer, H., Cornberg, M., 2006. Treating viral hepatitis C: efficacy, side effects, and complications. Gut 55 (9), 1350–1359.

Meyer, A.S., Gillespie, J.R., Walther, D., Millet, I.S., Doniach, S., Frydman, J., 2003. Closing the folding chamber of the eukaryotic chaperonin requires the transition state of ATP hydrolysis. Cell 113 (3), 369–381.

Momose, F., Naito, T., Yano, K., Sugimoto, S., Morikawa, Y., Nagata, K., 2002. Identification of Hsp90 as a stimulatory host factor involved in influenza virus RNA synthesis. J. Biol. Chem. 277 (47), 45306–45314.

Moradpour, D., Bieck, E., Hügle, T., Wels, W., Wu, J.Z., Hong, Z., Blum, H.E., Bartenschlager, R., 2002. Functional properties of a monoclonal antibody inhibiting the hepatitis C virus RNA-dependent RNA polymerase. J. Biol. Chem. 277 (1), 593–601.

Moriishi, K., Matsuura, Y., 2007. Host factors involved in the replication of hepatitis C virus. Rev. Med. Virol. 17 (5), 343–354.

Murakami, K., Ishii, K., Ishihara, Y., Yoshizaki, S., Tanaka, K., Gotoh, Y., Aizaki, H., Kohara, M., Yoshioka, H., Mori, Y., Manabe, N., Shoji, I., Sata, T., Bartenschlager, R., Matsuura, Y., Miyamura, T., Suzuki, T., 2006. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. Virology 351 (2), 381–392.

Naito, T., Momose, F., Kawaguchi, A., Nagata, K., 2007. Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. J. Virol. 81 (3), 1339–1349.

Nakagawa, S., Umehara, T., Matsuda, C., Kuge, S., Sudoh, M., Kohara, M., 2007. Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. Biochem. Biophys. Res. Commun. 353 (4), 882–888.

Nelson, H.B., Tang, H., 2006. Effect of cell growth on hepatitis C virus (HCV) replication and a mechanism of cell confluence-based inhibition of HCV RNA and protein expression. J. Virol. 80 (3), 1181–1190.

Niwa, H., Yamamura, K., Miyazaki, J., 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108 (2), 193–199.

Okamoto, T., Nishimura, Y., Ichimura, T., Suzuki, K., Miyamura, T., Suzuki, T., Moriishi, K., Matsuura, Y., 2006. Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. EMBO J. 25 (20), 5015–5025.

Okamoto, T., Omori, H., Kaname, Y., Abe, T., Nishimura, Y., Suzuki, T., Miyamura, T., Yoshimori, T., Moriishi, K., Matsuura, Y., 2008. A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. J. Virol. 82 (7), 3480–3489.

Parent, R., Qu, X., Petit, M.A., Beretta, L., 2009. The heat shock cognate protein 70 is associated with hepatitis C virus particles and modulates virus infectivity. Hepatology 49 (6), 1798–1809.

Pietschmann, T., Lohmann, V., Rutter, G., Kurpanek, K., Bartenschlager, R., 2001. Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J. Virol. 75 (3), 1252–1264.

Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., Strand, D., Bartenschlager, R., 2002. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J. Virol. 76 (8), 4008–4021.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q.L., Houghton, M., Kuo, G., 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl Acad. Sci. USA 87 (17), 6547–6549.

Seeff, L.B., Hoofnagle, J.H., 2003. Appendix: the National Institutes of Health Consensus Development Conference: management of hepatitis C 2002. Clin. Liver Dis. 7 (1), 261–287.

Shi, S.T., Lee, K.J., Aizaki, H., Hwang, S.B., Lai, M.M., 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J. Virol. 77 (7), 4160–4168.

Shimakami, T., Honda, M., Kusakawa, T., Murata, T., Shimotohno, K., Kaneko, S., Murakami, S., 2006. Effect of hepatitis C virus (HCV) NS5B-nucleolin interaction on HCV replication with HCV subgenomic replicon. J. Virol. 80 (7), 3332–3340.

Shirakura, M., Murakami, K., Ichimura, T., Suzuki, R., Shimoji, T., Fukuda, K., Abe, K., Sato, S., Fukasawa, M., Yamakawa, Y., Nishijima, M., Moriishi, K., Matsuura, Y., Wakita, T., Suzuki, T., Howley, P.M., Miyamura, T., Shoji, I., 2007. E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. J. Virol. 81 (3), 1174–1185.

Suzuki, T., Ishii, K., Aizaki, H., Wakita, T., 2007. Hepatitis C viral life cycle. Adv. Drug Deliv. Rev. 59 (12), 1200–1212.

Taguwa, S., Okamoto, T., Abe, T., Mori, Y., Suzuki, T., Moriishi, K., Matsuura, Y., 2008. Human butyrate-induced transcript 1 interacts with hepatitis C virus NS5A and regulates viral replication. J. Virol. 82 (6), 2631–2641.

Taguwa, S., Kambara, H., Omori, H., Tani, H., Abe, T., Mori, Y., Suzuki, T., Yoshimori, T., Moriishi, K., Matsuura, Y., 2009. Cochaperone activity of human butyrate-induced transcript 1 facilitates hepatitis C virus replication through an Hsp90-dependent pathway. J. Virol. 83 (20), 10427–10436.

Tian, G., Vainberg, I.E., Tap, W.D., Lewis, S.A., Cowan, N.J., 1995. Specificity in chaperonin-mediated protein folding. Nature 375 (6528), 250–253.

Tu, H., Gao, L., Shi, S.T., Taylor, D.R., Yang, T., Mircheff, A.K., Wen, Y., Gorbalenya, A.E., Hwang, S.B., Lai, M.M., 1999. Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. Virology 263 (1), 30–41.

Ujino, S., Yamaguchi, S., Shimotohno, K., Takaku, H., 2009. Heatshock protein 90 is essential for stabilization of the hepatitis C virus nonstructural protein NS3. J. Biol. Chem. 284 (11), 6841–6846.

Valpuesta, J.M., Martín-Benitoa, J., Gómez-Puertasa, P., Carrascosaa, J.L., Willison, K.R., 2002. Structure and function of a protein folding machine: the eukaryotic cytosolic chaperonin CCT. FEBS Lett. 529 (1), 11–16.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11 (7), 791–796.

Watashi, K., Ishii, N., Hijikata, M., Inoue, D., Murata, T., Miyanari, Y., Shimotohno, K., 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol. Cell 19 (1), 111–122.

Weng, L., Du, J., Zhou, J., Ding, J., Wakita, T., Kohara, M., Toyoda, T., 2009. Modification of hepatitis C virus 1b RNA polymerase to make a highly active JFH1-type polymerase by mutation of the thumb domain. Arch. Virol. 154 (5), 765–773.

Yaffe, M.B., Farr, G.W., Miklos, D., Horwich, A.L., Sternlicht, M.L., Sternlicht, H., 1992. TCP1 complex is a molecular chaperone in tubulin biogenesis. Nature 358 (6383), 245–248.

Yam, A.Y., Xia, Y., Lin, H.T., Burlingame, A., Gerstein, M., Frydman, J., 2008. Defining the TRiC/CCT interactome links chaperonin function to stabilization of newly made proteins with complex topologies. Nat. Struct. Mol. Biol. 15 (12), 1255–1262.

Yanagida, M., Miura, Y., Yagasaki, K., Taoka, M., Isobe, T., Takahashi, N., 2000. Matrix assisted laser desorption/ionization-time of flight-mass spectrometry analysis of proteins detected by anti-phosphotyrosine antibody on two-dimensional-gels of fibroblast cell lysates after tumor necrosis factor-alpha stimulation. Electorophoresis 21 (9), 1890–1898.

Yang, G., Pevear, D.C., Collett, M.S., Chunduru, S., Young, D.C., Benetatos, C., Jordan, R., 2004. Newly synthesized hepatitis C virus replicon RNA is protected from nuclease activity by a protease-sensitive factor(s). J. Virol. 78 (18), 10202–10205.