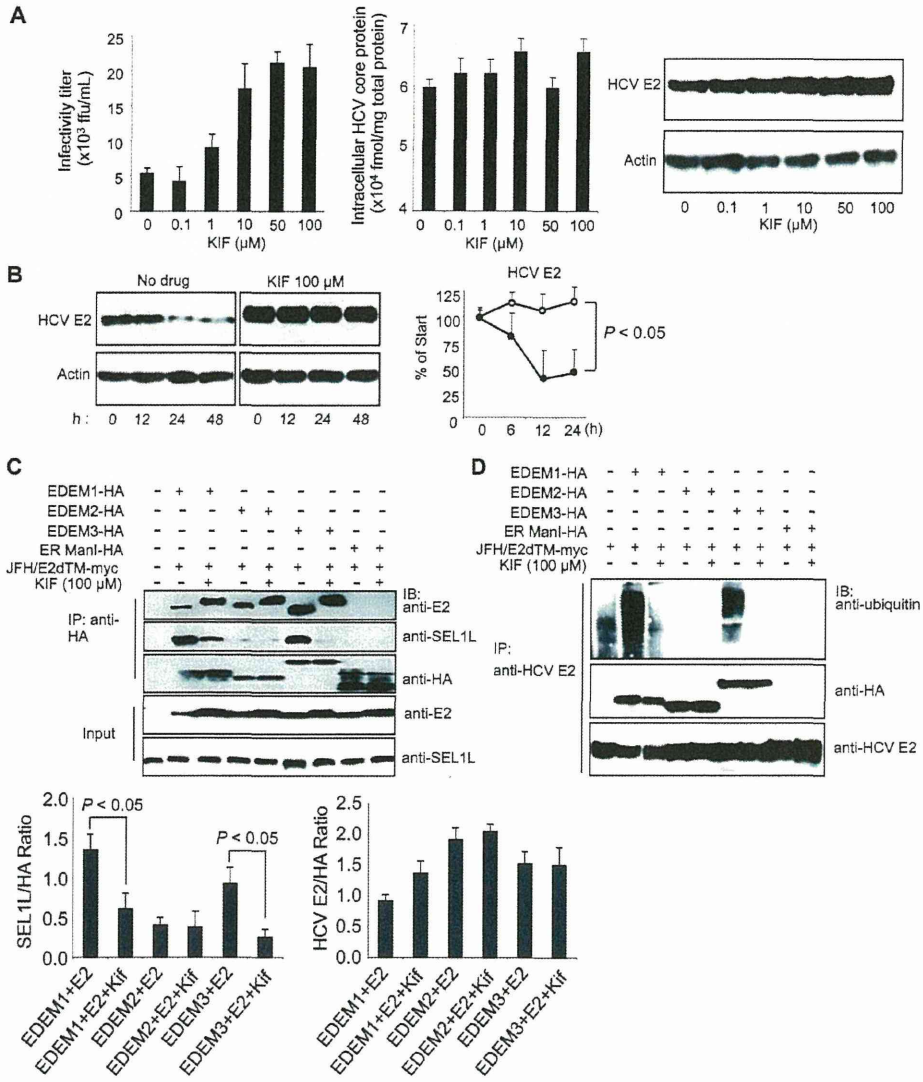

FIGURE 5. **Effect of KIF on HCV production and stability of E2.** *A*, extracellular HCV titer, intracellular HCV core protein expression, and steady-state level of HCV E2 in HuH-7 cells treated with different concentrations of KIF. *B*, CHX-based HCV protein stability assay of HCV E2 protein in KIF-treated cells as described in Fig. 3*E*. E2 protein levels normalized to actin levels are shown in the *graph* on the *right*. The *open* and *filled circles* indicate KIF-treated and nontreated cells, respectively. The mean ± S.D. (*error bars*) of two independent experiments are shown. *C*, binding of EDEMs and ER ManI with HCV E2 and SEL1L in 293T cells in the absence or presence of KIF. 293T cells were seeded in 6-well plates at a density of 3×10^5 cells/well. After overnight incubation, the cells were co-transfected with plasmids carrying HCV E2-myc (1 μg) and EDEM1-HA, EDEM2-HA, EDEM3-HA, or ER ManI-HA proteins (1 μg each). After 6 h, the culture medium was replaced with fresh or KIF-containing medium (100 μM). Forty-eight hours later, the cells were harvested and immunoprecipitated (*IP*) with anti-HA antibodies, after which Western blotting (*IB*) was performed with the indicated antibodies. Specific signals were quantified by densitometry, and the ratio between HCV E2 and HA (*right graph*) and between SEL1L and HA (*left graph*) in the same *lanes* is plotted on the graphs. The mean ± S.D. of three independent experiments are shown. *D*, EDEM protein-mediated ubiquitylation of HCV E2 protein in 293T cells in the absence or presence of KIF. The experimental procedure was the same as that described in Fig. 5*C*, except that immunoprecipitation was performed with anti-HCV E2 antibodies.

viruses is still unknown. To this end, we examined its role in the life cycle of JEV, another member of the Flaviviridae family. In contrast to HCV, KIF treatment had little effect on JEV production in infected cells (Fig. 6*A*) or the steady-state level of viral E glycoprotein (Fig. 6*B*). Interaction of EDEMs with JEV E was analyzed further. Neither EDEMs nor ER ManI was found to interact with JEV E in cells (Fig. 6*C*), indicating no significant role of the ERAD pathway in the JEV life cycle. Altogether, these results strongly suggest that the ERAD pathway is involved in the quality control of glycoproteins of specific viruses, possible through an interaction with EDEM(s), and subsequent regulation of virus production.

DISCUSSION

Accumulating evidence points to a role of the ERAD pathway in the pathogenesis of different genetic and degenerative diseases. However, the involvement of ERAD in the life cycle of viruses and infectious diseases remains poorly understood. Until recently, an experimental HCV cell culture infection system has been lacking such that studies evaluating the effect of HCV infection on the ERAD pathway were performed by either using HCV subgenomic replicons which lack structural proteins or by ectopic expression of one or multiple structural proteins (21, 22). However, this problem was solved by identifica-

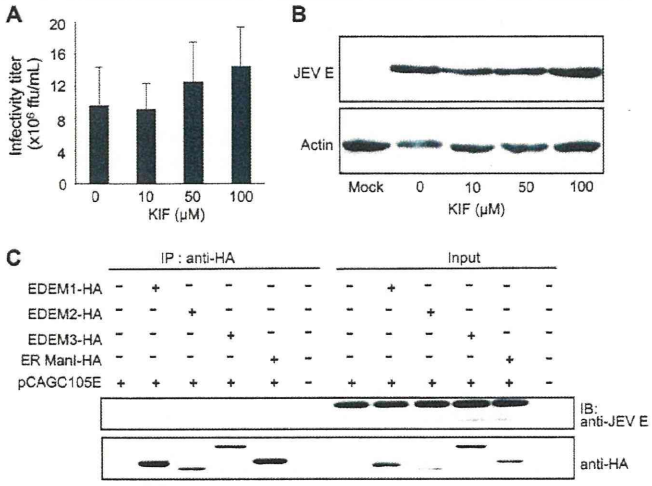

FIGURE 6. **Binding of JEV envelope glycoprotein with EDEMs and effect of KIF on JEV production.** *A*, JEV production in HuH-7 cells treated with KIF. The mean ± S.D. (*error bars*) of three independent experiments are shown. *B*, effect of KIF on the steady-state level of JEV envelope protein. *C*, binding of EDEMs with the JEV envelope.

tion of an HCV clone, JFH-1, capable of replicating and assembling infectious virus particles in cultured hepatocytes (15). In the present study, we used JFH-1 to examine the effect of HCV infection on activation of the ERAD pathway and its role in the virus life cycle. Our results show that the ERAD pathway is activated in HCV-infected cells, as evidenced by the maturation of XBP1 mRNA to its active form and up-regulation of EDEM1 (Fig. 1, *A–D*). Knocking down IRE1 reversed the induction of EDEM1, indicating that HCV infection-induced activation of the ERAD pathway is mediated through IRE1 (Fig. 1*F*). Loss- and gain-of-function analyses indicated that EDEM1 and EDEM3, particularly EDEM1, are involved in the post-translational control of HCV glycoproteins by which viral production is down-regulated (Figs. 3, *D* and *E*, and 4*A*). Our results suggest that EDEM1 and EDEM3 play a role in delivery of viral glycoproteins to the SEL1L-containing ubiquitin-ligase complex. It has recently been reported that coronavirus infection causes an accumulation of EDEM1 in membrane vesicles which are sites of viral replication, but that EDEM1 is not required for coronavirus replication (23). To our knowledge, the present study is the first to demonstrate regulation of the viral life cycle by ERAD machinery through interaction of EDEMs with viral glycoproteins.

We propose that the mechanisms described here are important during the early stages of establishing persistent HCV infection. ER stress caused by high levels of HCV infection during the acute phase presumably results in activation of the ERAD pathway. Induced EDEMs enhance the degradation of HCV envelope proteins, thereby reducing virus production. Maintenance of moderately low levels of HCV in the infected liver may contribute to the persistence of HCV infection, often associated with a lengthy asymptomatic phase that can last for decades. A range of viruses, including flaviviruses such as JEV, dengue virus, and West Nile virus, have been reported to induce XBP1 mRNA splicing triggered by ER stress (2, 3, 24). However, we demonstrate here that, in contrast to HCV, the envelope protein of JEV, which causes acute encephalitis, is not recognized by EDEMs, and the ERAD pathway does not control JEV production.

N-Linked glycoproteins displaying the glycan precursor Glc1Man9GlcNAc2 bind ER chaperones, such as calnexin or calreticulin, which facilitates protein folding. Removal of the terminal Glc from glycans disrupts this interaction with chaperones leading to Man trimming and delivery to ERAD machinery. A glucosyltransferase can transfer the terminal Man-linked Glc back to glycans, thereby allowing the "calnexin cycle" to continue until the glycoproteins are properly folded (for review, see Ref. 25). During this cycle, the decision of when to abandon additional folding attempts for immature polypeptides and to direct them instead toward the degradation pathway appears to be a crucial element of protein quality control. The basis by which this occurs, however, is not fully understood. Here, we demonstrate that stabilization of HCV envelope proteins and increased virus production occurs with KIF treatment (Fig. 5, *A* and *B*) and with gene silencing of either EDEM1 or EDEM3 (Figs. 3, *D* and *E*, and 4*A*). It is generally accepted that ERAD functions to eliminate proteins that are unable to adopt their native structure after translocation into the ER. From our results, however, one could argue that, during the HCV life cycle, at least a fraction of the competently folded viral glycoprotein intermediates may be released from the calnexin cycle before maturation and thereby be recognized as ERAD substrates. As suggested previously, the processes of protein folding and ERAD compete to some extent for newly synthesized polypeptides (26, 27). Under conditions in which high concentrations of ERAD-related factors are found in the ER due to induction of ER stress by viral infection, activated ERAD machinery may efficiently capture protein intermediates with folding/refolding capacity and cause premature termination of chaperone-assisted protein folding.

EDEM1 has recently been found to bind SEL1L, which is involved in the translocation of ERAD substrates from the ER to the cytoplasm (20). Our results demonstrate efficient binding of EDEM1 and EDEM3 to SEL1L, whereas EDEM2 exhibits only residual binding. In agreement with these results, increased ubiquitylation of HCV E2 protein was observed in cells overexpressing EDEM1 and EDEM3, but not in cells overexpressing the EDEM2 ortholog (Fig. 3*B*). Furthermore, KIF inhibited the binding of EDEM1 and EDEM3 with SEL1L, thus abrogating the ubiquitylation and enhancing the stability of HCV E2 protein (Fig. 5, *B* and *D*). It has been reported that KIF inhibits the interaction between EDEM1 and SEL1L, thus stabilizing ERAD substrates (4). Therefore, our results confirm previous findings and show that, along with EDEM1, KIF inhibits the binding of SEL1L to EDEM3. Furthermore, we have been the first to show that HCV E2 is a virus-derived ERAD substrate that can be used to analyze the mechanisms of this pathway. Taken together, our results indicate that EDEM1 and EDEM3, but not EDEM2, might be involved in targeting ERAD substrates to the translocation machinery, which may partly explain the different roles of the three EDEMs in HCV production. Although both EDEM1 and EDEM3 bind SEL1L and HCV envelope proteins, EDEM1 appears to have a larger role in regulation of HCV production than EDEM3. This is supported further by the finding that enhanced ubiquitylation of HCV E2 occurs in the presence

of EDEM1 overexpression (Figs. 3*B* and 5*D*). In EDEM3-knockdown cells, EDEM1 may take over the function of delivering ERAD substrates to the translocation machinery. We also speculate that EDEM1 may function as a helper for EDEM3. This is supported by the observation that EDEM1 and EDEM3 synergistically increase HCV production when knocked down together (data not shown). HCV glycoproteins are a suitable means by which to investigate differences and redundancies pertaining to the role of EDEMs in the ERAD pathway.

HCV-infected and TM-treated cells demonstrated the greatest activation of EDEM1 transcript production among EDEMs (Fig. 1, *C* and *D*, and supplemental Fig. S1). Although it is known that XBP1 binds to specific ER stress-responsive *cis*-acting elements to induce EDEMs (28, 29), the exact mechanism of transcriptional regulation is not fully understood. It will be interesting to examine regulatory mechanism(s) specific to individual EDEM homologs in an ER stress-dependent or -independent manner.

These findings highlight the crucial role of the ERAD pathway in the HCV life cycle. Further studies are needed to clarify the details of this complex pathway. The data generated in this work, however, further contribute to our understanding of the mechanisms that govern the maturation and fate of viral glycoproteins in the ER.

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

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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.

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.

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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 (3×10^5) 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

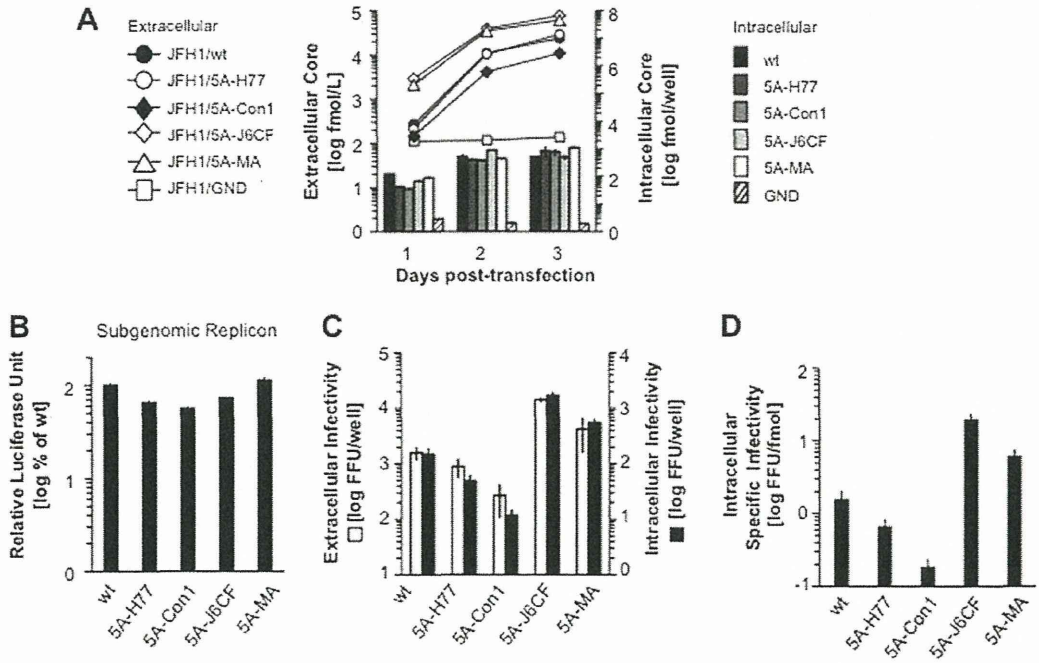

Fig. 1. Production and replication of recombinant viruses with NS5A of strains of genotypes 1 and 2. (A) Huh7.5.1 cells were transfected with *in vitro* synthesized RNA of JFH1/wt and indicated recombinants. The amount of extracellular (line graph) and intracellular (bar graph) HCV core protein was determined 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.

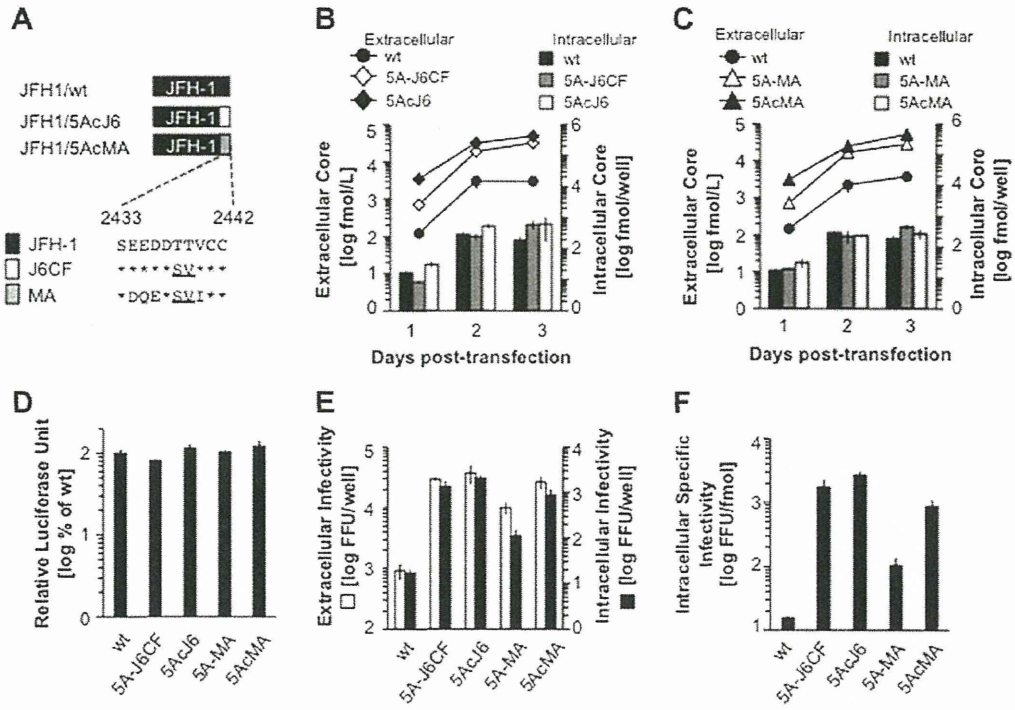

Fig. 2. C-terminal amino acids in NS5A were responsible for the enhanced virus production of recombinant viruses with NS5A of genotype 2 strains. (A) Alignment of C-terminal amino acids in NS5A of JFH-1, J6CF, and MA. Identical amino acids are indicated by asterisks. The indicated number represents the 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

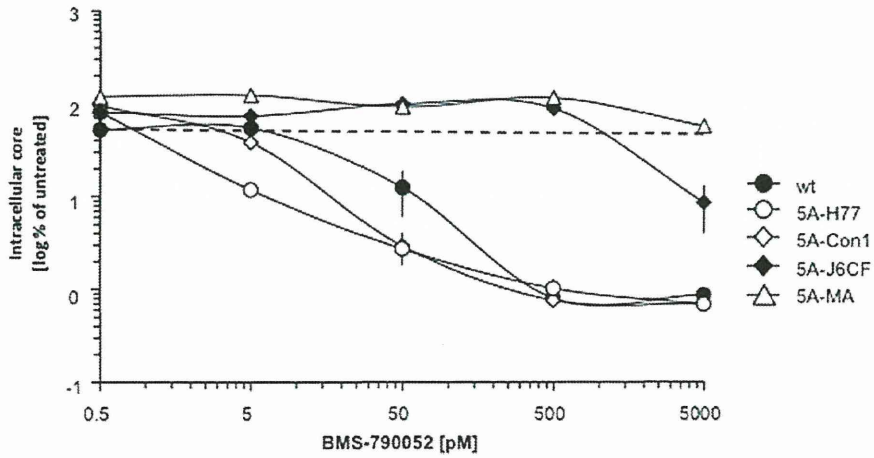

Fig. 3. Susceptibility of JFH1/wt and recombinant viruses to the NS5A inhibitor. Huh7.5.1 cells were transfected with RNA of JFH1/wt and recombinant viruses and treated with serially diluted BMS-790052 for 48 h. The amounts of intracellular HCV core protein were quantified and normalized against untreated 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) K (24,317) H (1450) R (1217)	E (6)	–	[20]
2007	31	L	L	L	M	M	M (350) V (3350)	F (5) M (3) V (23)	M (170)	[12,20]
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 (5367) N (47,017)	H (19) N (28)	H (130–1400)	[12,20]

[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.

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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.2011.05.144.

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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.

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.