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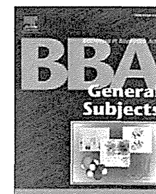

Different mechanisms of hepatitis C virus RNA polymerase activation by cyclophilin A and B in vitro

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ABSTRACT

Background: Cyclophilins (CyPs) are cellular proteins that are essential to hepatitis C virus (HCV) replication. Since cyclosporine A was discovered to inhibit HCV infection, the CyP pathway contributing to HCV replication is a potential attractive stratagem for controlling HCV infection. Among them, CyPA is accepted to interact with HCV nonstructural protein (NS) 5A, although interaction of CyPB and NS5B, an RNA-dependent RNA polymerase (RdRp), was proposed first.
Methods: CyPA, CyPB, and HCV RdRp were expressed in bacteria and purified using combination column chromatography. HCV RdRp activity was analyzed in vitro with purified CyPA and CyPB.
Results: CyPA at a high concentration (50× higher than that of RdRp) but not at low concentration activated HCV RdRp. CyPB had an allosteric effect on genotype 1b RdRp activation. CyPB showed genotype specificity and activated genotype 1b and J6CF (2a) RdRps but not genotype 1a or JFH1 (2a) RdRps. CyPA activated RdRps of genotypes 1a, 1b, and 2a. CyPB may also support HCV genotype 1b replication within the infected cells, although its knockdown effect on HCV 1b replicon activity was controversial in earlier reports.
Conclusions: CyPA activated HCV RdRp at the early stages of transcription, including template RNA binding. CyPB also activated genotype 1b RdRp. However, their activation mechanisms are different.
General significance: These data suggest that both CyPA and CyPB are excellent targets for the treatment of HCV 1b, which shows the greatest resistance to interferon and ribavirin combination therapy.
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1. Introduction

Hepatitis C virus (HCV[1]), which belongs to the *Flaviviridae* family, has a positive-strand RNA genome, and its replication is regulated by viral and cellular proteins [1]. The genome encodes a large precursor polyprotein that is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [2]. NS5B is an RNA-dependent RNA polymerase (RdRp) [3–5].

HCV frequently establishes a persistent infection that leads to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [6,7]. More than 170 million individuals worldwide are infected with HCV [8], and the challenge of developing HCV treatment continues. First, combination therapy with pegylated interferon α (Peg-IFNα) and ribavirin led to a sustained virological response (SVR) in approximately 55% of patients infected with any HCV genotype and 42–46% of patients with genotype 1 [9,10]. However, many patients could not tolerate the serious adverse effects. Triple therapy consisting of an NS3/NS4A protease inhibitor (boceprevir or telaprevir), Peg-IFN (α-2a or α-2b), and ribavirin was then introduced, and it has become the standard regimen for genotype 1 infection. SVR improved significantly (from 63% to 75%), and the treatment duration decreased from 12 to 6 months [11,12]. However, triple therapy is more toxic than combination therapy [13].

Nonimmunosuppressant cyclosporine A (CsA) analogues/CyP inhibitors such as DEBIO-025 (Alisporivir) [14], NIM811 [15], and SCY-635 [16] are also the most expected candidates for use as anti-HCV drugs because their resistance selection is rare compared with other direct-acting antiviral agents, and the HCV resistant to

Abbreviations: BSA, bovine serum albumin; CsA, cyclosporine A; CyP, cyclophilin; DTT, dithiothreitol; E, envelope; EDTA, ethylenediaminetetraacetic acid; GST, glutathione S-transferase; HCV, hepatitis C virus; NS, nonstructural protein; PPI, peptidyl prolyl *cis/trans*-isomerases; Peg-IFN, pegylated interferon-α; PMSF, phenylmethanesulfonylfluoride; RT-PCR, reverse transcription polymerase chain reaction; RdRp, RNA-dependent RNA polymerase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis; SVR, sustained virological response; ΔPPI, PPI knockout; wt, wild type

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CyP inhibitors acquired mutations that allowed for reduced dependence on CyPs [17,18].

CyP was originally discovered as a cellular factor with high affinity for CsA [19]. CyPs comprise a family of peptidyl prolyl *cis/trans*-isomerases (PPI) that catalyze the *cis–trans* interconversion of peptide bonds amino terminal to proline residues, facilitating protein conformation changes [20]. CyPs are potential antiviral targets because CyPA was found to play a critical role in human immunodeficiency virus-1 infection [21,22]. The role of human CyPs as cellular cofactors in HCV replication was first suggested upon discovery of the anti-HCV effect of CsA [23–26]. Although the completion of a binding assay and the mapping of resistance initially suggested that NS5B was a viral target for CsA [27–29], recent papers have pointed to CyPA and NS5A as the central virus–host interaction involved in HCV replication [30–36]. Despite this unfavorable evidence, we analyzed the effect of CyPA and CyPB on HCV RdRp of various genotypes in vitro and found differences in genotype specificity and the mechanism of HCV RdRp activation.

2. Materials and methods

2.1. Purification of HCV RdRp

HCV RNA RdRps with C-terminal 21 amino acid deletion of 1a (H77 and RMT), 1b (HCR6, NN, and Con1), and 2a (JFH1 and J6CF) were expressed in *E. coli* Rosetta/pLysS and purified as described previously [37–40]. The purified HCV RdRps (5 μM, >95% pure) were stocked in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 5% glycerol, and 1 mM phenylmethanesulfonylfluoride (PMSF) at −80 °C. The yield of HCV RdRps is approximately 1.7 mg from a 1-L bacterial culture. The purified HCV RdRps were as shown in Fig. S1 of Weng et al. [38]. The protein purities were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (SDS-PAGE), using ImageJ 1.46 (http://rsbweb.nih.gov/ij/).

2.2. Construction of CyP-expressing plasmids

Human CyPA and CyPB were cloned from total RNA extracted from 293T cells, using a reverse transcription-polymerase chain reaction (RT-PCR) kit (Takara, Dalian, China) as published previously [29]. After being digested with *Bam*HI and *Eco*RI, they were cloned into the same site of pGEX-6P-3 (GE Healthcare, Bucks, UK), resulting in pGEXCyPA and pGEXCyPB, respectively. CyPBΔPPI, the enzymatic inactive mutant of CyPB, was PCR cloned into pGEX-6P-3 from pCMV-CyPBΔPPIFL [29], resulting in pGEXCyPBΔPPI. CyPAΔPPI was produced by the introduction of the R55A and F60A mutations using a QuickChangeII Site-Directed Mutagenesis Kit (Stratagene, St. Clara, CA, USA) and primers (5′-GTTCCTGCTTTCACGCCATTATTCCAGGGG CCATGTGTCAGGGTG-3′ and 5′-CACCCTGACACATGGCCCCTGGAATAA TGGCGTGAAAGCAGGAAC-3′).

2.3. Purification of CyPs

E. coli Rosetta were transformed using pGEXCyPA, pGEXCyPAΔPPI, pGEXCyPB, and pGEXCyPBΔPPI. GST-tagged CyPA, CyPB, CyPAΔPPI, and CyPBΔPPI were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside at 18 °C for 4 h. The bacteria were harvested and stocked at −20 °C. After thawing on ice, the bacteria were lysed in 4 packed cell volumes of phosphate-buffered saline, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. After being clarified by centrifugation at 10,000×*g* for 30 min at 4 °C and filtered through a 0.45-μm nitrocellulose filter, the extract was incubated with Glutathione Sepharose 4B (GE Healthcare) for 30 min at 4 °C. After the resin was washed with 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF, the GST-CyP was eluted using 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM reduced glutathione, and 1 mM PMSF, followed by gel filtration through a Superdex 200 column (GE Healthcare) in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. The eluted GST-CyP were diluted to 50 mM NaCl and applied to a MonoQ (GE Healthcare) in 20 mM Tris–HCl (pH 9.0), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. GST-CyPB and GST-CyPBΔPPI were chromatographed using a continuous NaCl gradient of 50–1000 mM. The purified CyPs were stocked at −20 °C.

2.4. In vitro HCV transcription with CyPs

In vitro HCV transcription with CyPs was done as previously described [37–40]. Briefly, the indicated amounts of the CyPs were incubated in 50 mM Tris–HCl (pH 7.5), 200 mM monopotassium glutamate, 3.5 mM $MnCl_2$, 1 mM DTT, 0.5 mM GTP, 200 nM of a 184-nt in vitro transcribed model RNA template (SL12-1S), 100 U/mL of human placental RNase inhibitor, and 100 nM HCV RdRp at 29 °C for 30 min. After preincubation, RdRp was incubated for an additional 90 min with 50 μM ATP, 50 μM CTP, or 5 μM [α-^{32}P]UTP. The RNA products were analyzed using 6% PAGE containing 8 M urea after being purified by phenol/chloroform extraction and ethanol precipitation. The amount of RNA products was analyzed using Typhoon Trio (GE Healthcare).

2.5. RNA filter-binding assay with CyPA and CyPB

An RNA filter-binding assay with CyPA and CyPB was performed as previously described [37,38,40]. Briefly, [^{32}P]-SL12-1S was incubated in 25 μL of 50 mM Tris–HCl (pH 7.5), 200 mM monopotassium glutamate, 3.5 mM $MnCl_2$, 1 mM DTT, and 5 pmol of HCV RdRp with 375 pmol (75×) of CyPA and 25 pmol (5×) of CyPB at 29 °C for 30 min.

2.6. Chemicals and radioisotopes

[α-^{32}P]UTP (800 Ci/mmol, 40 mCi/mL) was purchased from PerkinElmer Life Sciences (Waltham, MA, USA). The nucleotides were purchased from GE Healthcare. The human placental RNase inhibitor T7 RNA polymerase and PrimeSTAR HS DNA polymerase were purchased from Takara. The bacteria were purchased from Novagen (Merck Chemicals, Darmstadt, Germany).

2.7. Statistical analysis

The statistical data were evaluated using Student's *t* test, with $p<0.05$ indicating statistical significance.

3. Results

3.1. Purification of CyPA and B

First, glutathione S-transferase (GST)-tagged CyPA, CyPB, the PPI inactive CyPA (CyPAΔPPI), and CyPB (CyPBΔPPI) were purified using Glutathione Sepharose 4B affinity chromatography. CyPA and CyPAΔPPI were further purified through a Superdex 200 column (Fig. S1). After the Superdex 200 gel filtration, to remove the contaminating nucleic acids, CyPB and CyPBΔPPI were further purified through MonoQ anion exchange chromatography by a continuous NaCl gradient of 50–1000 mM because CyPB has a strong affinity for nucleic acids. Each was eluted with 210–385 mM NaCl (Fig. S2). The purification scheme and purified CyPs are shown in Fig. 1. The yields of CyPA and CyPAΔPPI were approximately 3 mg from a 1-L bacterial culture. CyPA and CyPAΔPPI were >95% pure and stocked at 5 mg/mL in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. CyPB and CyPBΔPPI were stocked at 5 mg/mL in 20 mM Tris–HCl (pH 9.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT,

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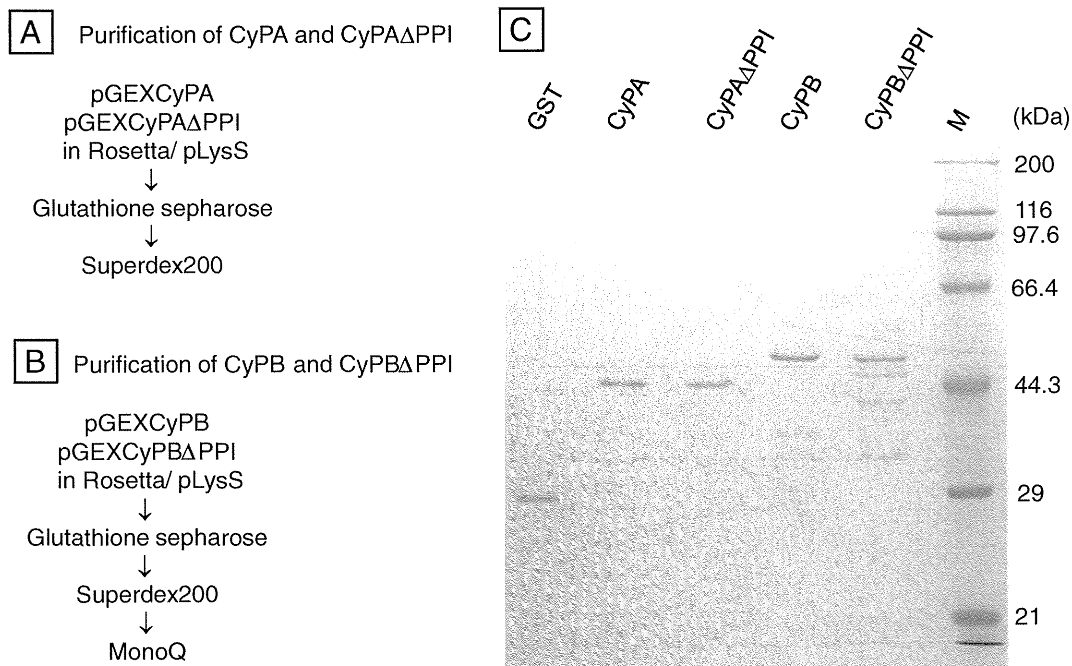

Fig. 1. Cyclophilin purification. The purification schemes of cyclophilin A (CyPA) and the peptidyl prolyl isomerase-inactive mutant protein of CyPA (CyPAΔPPI) (A), cyclophilin B (CyPB) and CyPBΔPPI (B), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (C) with 5 pmol each of purified glutathione S-transferase (GST; 28.3 kDa), GST-CyPA (44.9 kDa), GST-CyPAΔPPI (44.7 kDa), GST-CyPB (52.1 kDa), and GST-CyPBΔPPI (52 kDa) were separated through 10% SDS-PAGE and stained with Coomassie brilliant blue. The sizes of the molecular weight standards (M) are indicated on the right side of the gel. Their final elution profiles are shown in Figs. S1 and S2.

and 10% glycerol. The yields of CyPB and CyPBΔPPI were approximately 1 mg from a 1-L bacterial culture. The purities of CyPB and CyPBΔPPI were >95% and >90%, respectively.

3.2. HCV 1b and JFH1 (2a) transcription in vitro with CyPA and CyPB

The dose–response effects of CyPA and CyPB were examined using an in vitro transcription system of HCR6 (1b) and JFH1 (2a) RdRp wild type (wt). CyPA and CyPB were added to the optimal HCV in vitro transcription condition while the RNA synthesis was in the log phase [4,37]. RdRp (100 nM) was incubated with 0, 50 (ratio to RdRp: 0.5×), 100 (1×), 200 (2×), 500 (5×), and 1000 nM (10×) CyPA and CyPB, GST, or bovine serum albumin (BSA) in GTP (the initiating nucleotide) and an RNA template for 30 min, followed by elongation with ATP, CTP, and UTP for 90 min. CyPA enhancement was further tested using 2 (20×), 5 (50×), 7.5 (75×), and 10 (100×) μM because the enhancement effect of CyPA under 1 μM (10×) was unclear. Fig. S3 shows the autoradiography of HCV HCR6 (1b) and JFH1 (2a) RdRpwt with CyPA and CyPB, the graphs of which were drawn using the data from 3 independent experiments (Fig. 2).

The CyPA activation of both RdRps showed 2 reaction speeds. The first-order ratio of CyPA to HCR6 (1b) RdRpwt<50× is fitted as a linear regression curve, the equation for which is $y=0.07x$ (CyPA-to-RdRp ratio)$+0.7$. The linear regression curve fitting of the ratio >50× is $y=0.4x$ (CyPA-to-RdRp ratio)-17 when calculated from 3 points. That of CyPA to JFH1 (2a) RdRpwt is fitted to a similar linear regression, $y=0.09x$ (CyPA-to-RdRp ratio)$+0.9$ (the CyPA-to-RdRp ratio<50×). HCVR6 (1b) and JFH1 (2a) RdRps were activated by 100× CyPA to 25-±0.2- and 19-±1-fold, respectively.

The CyPB activation of HCR6 (1b) RdRpwt occurred in a dose-dependent manner and fitted a sigmoid curve, and the enhancement effect reached a plateau (9.4×) at the ratio of 5×. Neither GST nor BSA enhanced HCR6 (1b) RdRpwt. CyPB, GST, and BSA did not enhance JFH1 (2a) RdRpwt (<1.5×) at the concentrations described earlier.

3.3. Effect of the PPI inactive mutant proteins of CyPA and CyPB

CyP has PPI activity. To test the contribution of PPI activity to HCV HCR6 (1b) and JFH1 (2a) RdRpwt activation, the activation effect of the PPI inactive mutant proteins, CyPAΔPPI at 100× (10 μM) and CyPBΔPPI at 2× (200 nM), were tested together with 100× (10 μM) GST and BSA (Fig. 3). CyPA enhanced JFH1 (2a) RdRpwt 17.6×, whereas CyPAΔPPI enhanced it 16.2×. This difference is statistically significant (Student's t test, $p<0.05$). CyPA enhanced HCR6 (1b) RdRpwt activity 27.7×, whereas CyPAΔPPI enhanced it 16.0×. BSA slightly inhibited both RdRps at the same concentration in this experiment. As shown in Fig. 2C and D, it can be concluded that BSA has no effect on HCV transcription. GST enhanced JFH1 (2a) RdRpwt activity 5.0×, but it did not affect HCR6 (1b) RdRpwt activity. CyPB enhanced HCR6 (1b) RdRpwt activity 2.3×, whereas CyPBΔPPI enhanced it 1.7×. This difference is also statistically significant (Student's t test, $p<0.05$). JFH1 (2a) RdRpwt was not activated by CyPB or CyPBΔPPI.

3.4. CyP activation steps of HCV transcription

The HCV transcription steps of CyP enhancement were analyzed by the sequential addition of CyPs during in vitro transcription (Fig. 4). CyPA enhanced HCR6 (1b) and JFH1 (2a) RdRpwt, whereas CyPB enhanced HCR6 (1b) RdRpwt when HCV RdRps were incubated with them from the start of transcription (initiation). The CyP effect was then tested after their addition during the elongation period after HCV RdRps was initiated with GTP. CyPA (100×; 10 μM) and CyPB (5×; 500 nM) were added to HCV RdRps after the 30-min incubation with GTP, when 3 GTPs were incorporated at the 5′ end of the products. CyPB did not enhance HCR6 (1b) or JFH1 (2a) RdRp when added during the elongation period, although it enhanced HCV RdRp when added at the start of transcription. CyPA enhanced HCR6 (1b) and JFH1 (2a) RdRp activity only 1.6× (Student's t test, $p<0.05$) and 2.1× ($p<0.01$), respectively, when added during the elongation step. These results suggest that CyPA and CyPB activated only the transcription initiation step of HCV RdRps.

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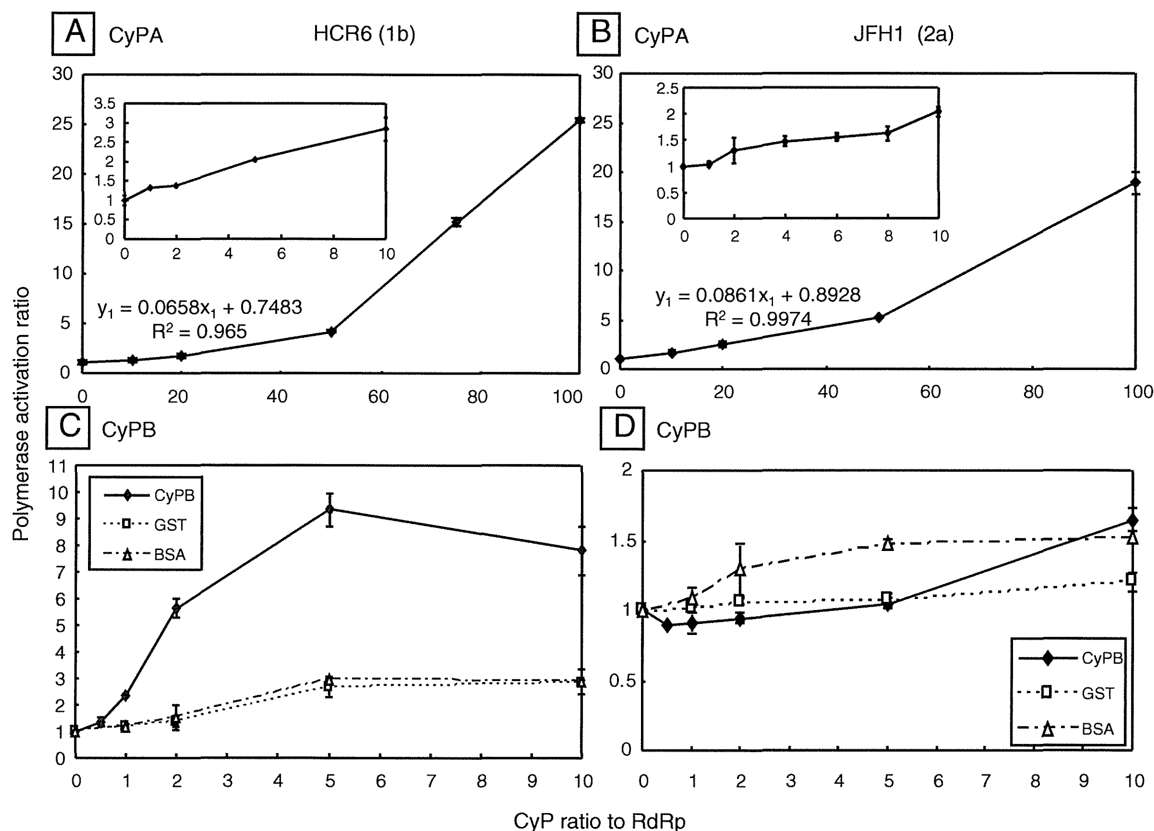

Fig. 2. Dose–response curve of cyclophilin A (CyPA) and cyclophilin B (CyPB) in hepatitis C virus (HCV) transcription in vitro. The dose–response curve of the HCV RdRp activation of CyPA in HCR6 (1b) RdRpwt (A) and JFH1 (2a) RdRpwt (B) CyPB in HCR6 (1b) RdRpwt (C) and JFH1 (2a) RdRpwt was drawn from the image analysis of Fig. S3. Insets A and B indicate that of 0, 0.5×, 1×, 2×, 5×, and 10× of CyPA to RdRp. The first-order ratio of the curves of A and B were fit by linear regression, and the calculated equations are indicated in the graph. The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements.

The effects of 75× CyPA and 5× CyPB on the RNA-binding activity of HCR6 (1b) and JFH1 (2a) RdRp were then tested (Fig. 4E). The effects of HCR6 (1b) and JFH1 (2a) RdRp with CyPA were 10.1-±0.56- and 6.6-±0.68-fold of that without CyPA, respectively. The effect of HCR6 (1b) RdRp with CyPB was 3.1-±0.3-fold of that without CyPB. The RNA-binding activity of HCV RdRps was thus enhanced by the addition of CyPA and CyPB.

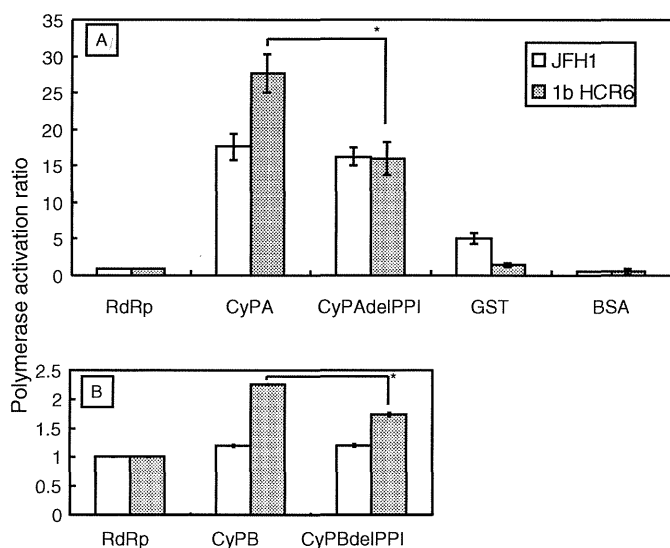

Fig. 3. Effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) with and without peptidyl prolyl isomerases activity on hepatitis C virus (HCV) JFH1 (2a) and HCR6 (1b) RdRp. HCV HCR6 (1b) and JFH1 (2a) RdRpwt (100 nM) were incubated with 100× (10 μM) of CyPA, CyPAΔPPI, glutathione S-transferase (GST), and bovine serum albumin (BSA) (A). HCV RdRps were incubated with 5× (500 nM) of CyPB, CyPBΔPPI, GST, and BSA (B). The mean relative polymerase activity and standard deviation (error bar) were calculated from 3 independent measurements. *p<0.01 (Student's *t* test).

3.5. *Effect of CyP activation on RdRp of various HCV genotypes*

The CsA sensitivity differed among the HCV genotypes [41]. Therefore, we tested the effects of CyPA and CyPB activation on NN (1b), H77 (1a), RMT (1a), and J6CF (2a) RdRp (Fig. 5). RdRp activity was compared with and without 50× (5 μM) CyPA and 5× (500 nM) CyPB. At their respective concentrations, CyPA activated all of the tested HCV RdRps by 3.9–5.3×, but CyPB activated only 1b RdRps (8–10×). CyPB slightly activated J6CF (2a) RdRp (approximately 4×), but it did not activate the 1a or JFH1 (2a) RdRps (1.4–1.8×).

4. Discussion

Since CsA was discovered to inhibit HCV infection [23–26], the CyP pathway contributing to HCV replication has been proposed as a potential stratagem for controlling HCV infection. Reports about the roles of CyPA in HCV replication via NS5A have been accumulating [33–35,42–44]. However, the effect of CyP inhibitors varied on the RNA-binding activity of NS5B [41,45], and DEBIO-025 decreased CyPB levels in patients [46]. Controversial results of CyPA and CyPB knockout experiments on HCV replicon activity were reported [29,30,47]. Therefore, the effects of CyPA and CyPB on HCV RdRp were carefully analyzed again in vitro.

In this study, we demonstrated that CyPA and CyPB activated HCV 1b RdRp in vitro by completely different kinetics using purified CyPs

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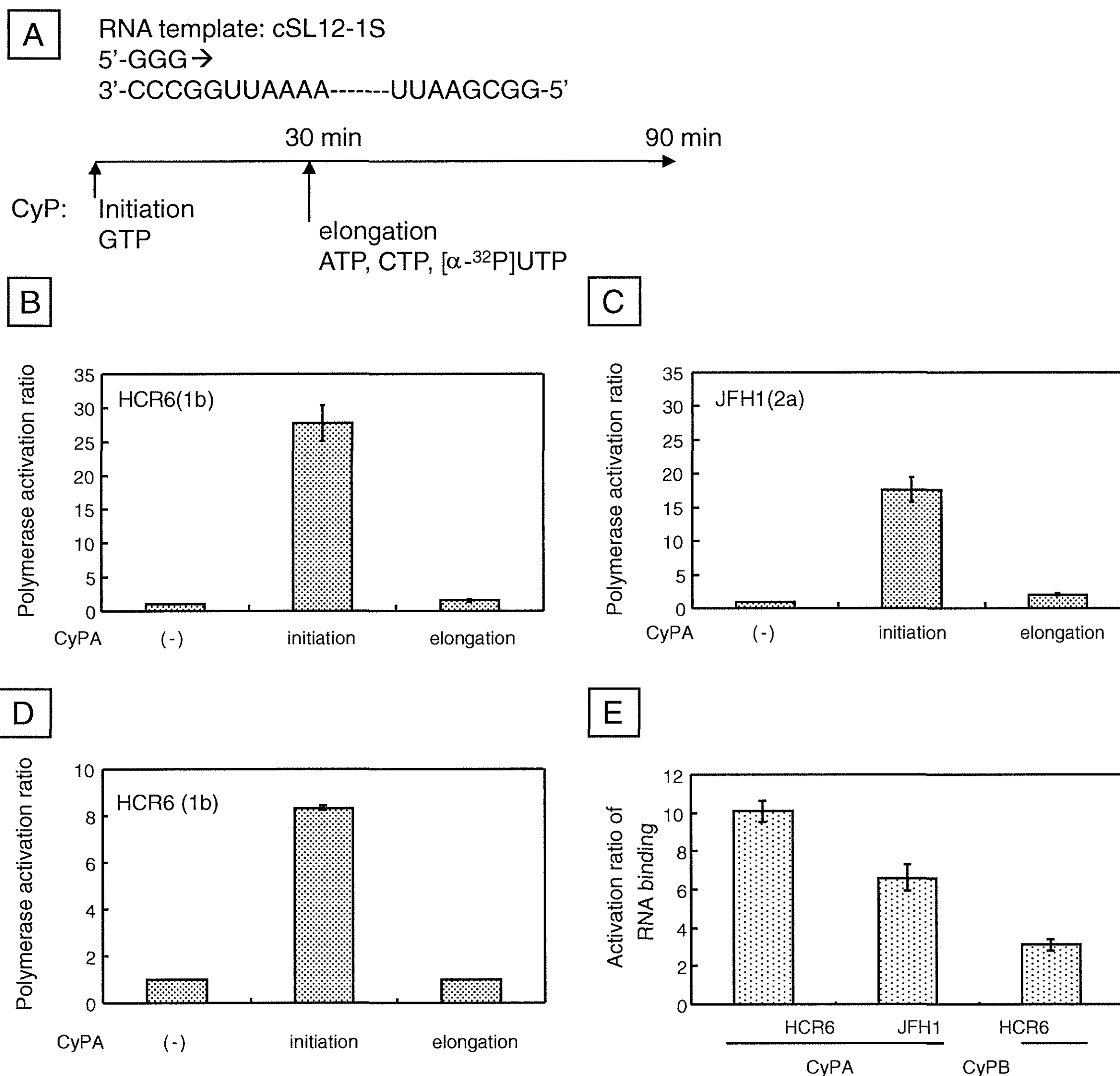

Fig. 4. Hepatitis C virus (HCV) RdRp activation effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) on transcription initiation and elongation. The polymerase activation effect of the timing of the CyPA or CyPB addition was examined. The sequence of the model RNA template (SL12-1S) and experimental design are shown in A. CyPA 100× (10 μM) was incubated with HCR6 (1b) RdRpwt (A) and JFH1 (2a) (B) RdRp during preincubation with 0.5 mM GTP (initiation) or after preincubation (elongation). CyPB 5× (500 nM) was incubated with HCVR6 (1b) RdRpwt during preincubation with 0.5 mM GTP (initiation) or after the preincubation (elongation) (C). The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements. The effect of the 100× CyPA and 5× CyPB on RNA template binding was examined (E).

and HCV RdRps (Fig. 2), which indicated that the mechanism of their HCV RdRp activation differed despite their similar structures [48–50]. Kinetic analysis of CyPA on HCR6 (1b) and JFH1 (2a) RdRp indicated that it had a similar activation mechanism on both HCV RdRps. CyPA did not activate HCV RdRp at low concentrations, but it did activate it at >50× molar excess to it. The unusual dose of CyPA activating HCV RdRp (Fig. 2) postulates that HCV RdRp may be surrounded by CyPA in vitro and factors involving CyPA and HCV RdRp interaction, such as NS5A, in the HCV replication complex of the infected cells [27,28,31,36,51–53] because the interaction of CyPA and HCV RdRp was weak (Fig. S4).

Although some controversial results were obtained from those of Heck et al. [54], the studies agree that CyPB also activated HCV 1b RdRp in vitro. The activation kinetics of CyPB on HCR6 (1b) RdRp showed a sigmoid-like curve (Fig. 2) that suggested an allosteric effect of CyPB on RdRp activity. CyPB may interact with HCV RdRp as a cofactor and directly activate HCR6 (1b) RdRp. The HCV RdRp–CyPB complex was likely to interact more with CyPB, and its activation plateaued at the CyPB/RdRp ratio of 5:1 (Fig. 2C). The CyPB activation curves of Heck et al. [54] also plateaued. These data from the 2 independent groups support the weak interaction between CyPB and HCV 1b RdRp (Fig. S4).

CyPA did not show genotype specificity in the current study (Fig. 5A), a finding that agrees with those of CyPA knockdown, DEBIO-025, and CsA experiments [30,43,55]. CyPB activation showed genotype specificity (Fig. 5B) [54]; CyPB activated 1b and J6CF (2a) RdRp but did not activate 1a or JFH1 (2a) RdRp. Both reports agreed with the finding that JFH1 (2a) subgenomic replicon was independent of CyPB [41]. Although mutations accumulated in the NS5A region of CsA- or DEBIO-025-resistant HCV replicons, some mutations were found in the NS5B region [18,27,28,33,45].

Another controversial result between that of Heck et al. [54] and ours is the Mg^{2+}-dependency of the CyPB activation. The Mg^{2+} concentration in cells is 14–20 mM, and Mg^{2+} ions are distributed almost equally throughout the nuclei, mitochondria, and cytosol/endoplasmic reticulum [56]. The Mn^{2+} concentration in cells varies from report to report [57,58]. The optimal Mn^{2+} and Mg^{2+} concentrations in the HCV in vitro transcription used in this study were

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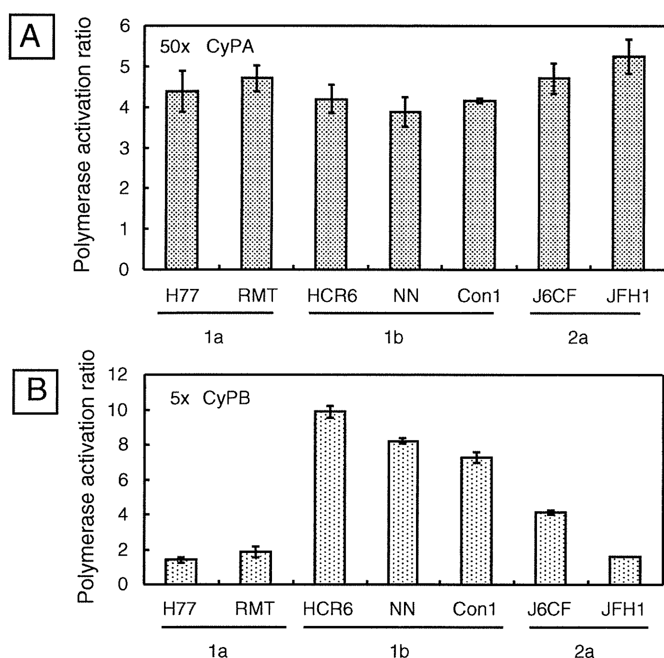

Fig. 5. Activation effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) on hepatitis C virus (HCV) RNA polymerase of genotypes 1a, 1b, and 2a. The polymerase activation effects of CyPA and CyPB on HCV 1a (H77 and RMT), 1b (HCR6, NN, and Con1), and 2a (J6CF and JFH1) were examined. HCV RdRp (100 nM) was incubated with 50× CyPA and 5× CyPB. The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements.

different from the physiological concentrations in cells [4,37]. However, under the optimal HCV transcription condition, HCV RdRp activation was observed by CyPA and CyPB (Fig. 1).

The amount of CyPA varies by cell type [59]. In some cells, CyPB may also contribute to HCV 1b replication because it localizes in the endoplasmic reticulum and plasma membranes [60,61], which form a membrane web in which an HCV replication complex exists [1].

PPI activity of CyPs is essential for HCV replicon activation [32,53]. CyP inhibitors (DEBIO-025, NIM811, and SCY-635) inhibit PPI activity. The PPI activity of CyPA contributed to HCV RdRp activation and CyP-NS5A binding [36]. The PPI activity of CyPA partly contributed to the activation of HCR6 (1b) RdRpwt in vitro (Fig. 3A, $p<0.01$). The PPI activity of CyPB may not be essential for RdRp activation because the activation ratio was not large between CyPB and CyPBΔPPI, although the experiment showed a statistically significant difference (Fig. 3B). There may be differences in the RdRp activation mechanisms of CyPA with and without PPI activity. This finding will help with the development of new CyPA inhibitors that target domains other than PPI.

The mechanism of HCV RdRp activation by CyPs is not clear. In the least, CyPA and CyPB enhanced the early stage of HCV transcription, including the template RNA binding of HCV RdRp (Fig. 4) [29,41,45]. The productive template-polymerase binding is the late-limiting step of transcription initiation by HCV RdRp in vitro, and a small fraction of HCV RdRp was active in vitro [62,63]. CyP may enhance this step on many HCV RdRp molecules to show apparent activation of RdRp in vitro.

Considering the controversial reports on CyP and HCV replication [29,33,35,41,43,44], it can be concluded that CyPA is the major factor of HCV genome replication and that the activation of HCV RdRp may require other factors such as NS5A to condense CyPA around the HCV RdRp. Although many HCV treatment approaches have been applied in addition to Peg-IFN, ribavirin, and NS3/NS4a protease inhibitor [64–67], more effort has to be made to ensure an HCV cure. This study and that of Heck et al. [54] demonstrated similar activation kinetics and genotype specificity of CyPB activation (Figs. 2 and 5). CyPB also has the potential to activate HCV 1b genome replication in a limited condition, and it should also be included as the target of inhibitor development because HCV 1b is the genotype that is most resistant to treatment [13].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2012.08.017.

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Self-Enhancement of Hepatitis C Virus Replication by Promotion of Specific Sphingolipid Biosynthesis

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Abstract

Lipids are key components in the viral life cycle that affect host-pathogen interactions. In this study, we investigated the effect of HCV infection on sphingolipid metabolism, especially on endogenous SM levels, and the relationship between HCV replication and endogenous SM molecular species. We demonstrated that HCV induces the expression of the genes (*SGMS1* and *2*) encoding human SM synthases 1 and 2. We observed associated increases of both total and individual sphingolipid molecular species, as assessed in human hepatocytes and in the detergent-resistant membrane (DRM) fraction in which HCV replicates. SGMS1 expression had a correlation with HCV replication. Inhibition of sphingolipid biosynthesis with a hepatotropic serine palmitoyltransferase (SPT) inhibitor, NA808, suppressed HCV-RNA production while also interfering with sphingolipid metabolism. Further, we identified the SM molecular species that comprise the DRM fraction and demonstrated that these endogenous SM species interacted with HCV nonstructural 5B polymerase to enhance viral replication. Our results reveal that HCV alters sphingolipid metabolism to promote viral replication, providing new insights into the formation of the HCV replication complex and the involvement of host lipids in the HCV life cycle.

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Introduction

Lipids have long been known to play dual roles in biological systems, functioning in structural (in biological membranes) and energy storage (in cellular lipid droplets and plasma lipoproteins) capacities. Research over the past few decades has identified additional functions of lipids related to cellular signaling, microdomain organization, and membrane traffic. There are also strong indications of the important role of lipids in various stages of host-pathogen interactions [1].

Sphingomyelin (SM) is a sphingolipid that interacts with cholesterol and glycosphingolipid during formation of the raft domain, which can be extracted for study as a detergent-resistant membrane (DRM) fraction [2]. Recently, raft domains have drawn attention as potential platforms for signal transduction and pathogen infection processes [3,4]. For instance, raft domains may serve as sites for hepatitis C virus (HCV) replication [5,6]. Additionally, *in vitro* analysis indicates that synthetic SM binds to the nonstructural 5B polymerase (RdRp) of HCV [7]. This association allows RdRp to localize to the DRM fraction (known to be the site of HCV replication) and activates RdRp, although the degree of binding and activation differs among HCV genotypes [7,8]. Indeed, suppression of SM biosynthesis with a serine palmitoyltransferase (SPT) inhibitor disrupts the association between RdRp and SM in the DRM fraction, resulting in the suppression of HCV replication [7,9].

Multiple reports have indicated that HCV modulates lipid metabolism (e.g., cholesterol and fatty acid biosynthesis) to promote viral replication [10–12]. However, the effect of HCV infection on sphingolipid metabolism, especially on endogenous SM levels, and the relationship between HCV replication and endogenous SM molecular species remain to be elucidated as there are technical challenges in measuring SM levels (for both total and individual molecular species) in hepatocytes.

To address these questions, we first utilized mass spectrometry (MS)-based techniques and analyzed uninfected and HCV-

Author Summary

One of the key components for hepatitis C virus (HCV) propagation is lipids, some of which comprise membranous replication complexes for HCV replication. Research on cofactors that are involved in the formation of the membranous replication complex has advanced steadily; on the other hand, the lipids constituting the membranous replication complex remain to be elucidated. Here, we report that HCV modulates sphingolipid metabolism by promoting sphingolipid biosynthesis, to enhance viral replication. Specifically a specific molecular species of sphingomyelin (SM), a type of sphingolipid interacts with HCV nonstructural 5B polymerase, enhancing HCV replication. This work highlights the relationship between specific molecular species of SMs and HCV replication, giving new insight into the formation of the HCV replication complex and the involvement of host lipids in the HCV life cycle.

infected chimeric mice harboring human hepatocytes. Second, we developed a hepatotropic SPT inhibitor, NA808, and used this tool to elucidate the effects of inhibition of sphingolipid biosynthesis on hepatocyte SM levels. Third, we tested the inhibitor's anti-HCV activity in humanized chimeric mice, and demonstrated the relationship between HCV and endogenous SM in human hepatocytes. Finally, we identified the endogenous SM molecular species carried by the DRM fraction, defining the association between these molecular species and HCV replication.

Results

HCV upregulates SM and ceramide levels in hepatocytes of humanized chimeric mice

First, we examined the effects of HCV infection on SM biosynthesis in hepatocytes using humanized chimeric mice. The study employed a previously described mouse model (SCID/uPA) into which human hepatocytes were transplanted (see Materials and Methods). The average substitution rate of the chimeric mouse livers used in this study was over 80% [13], and HCV selectively infected human hepatocytes. This model supports long-term HCV infections at clinically relevant titers [13,14]. Indeed, the HCV-RNA levels reached (at 4 weeks post-infection) 10^8–10^9 copies/mL in the genotype 1a group (**Figure 1A**) and 10^6–10^7 copies/mL in the genotype 2a group (**Figure 1B**).

Once serum HCV-RNA levels had plateaued, we observed elevated expression of the genes (*SGMS1* and *2*) encoding human SM synthases 1 and 2; this pattern was HCV-specific, as demonstrated by the fact that the increase was not seen in hepatitis B virus-infected mice (**Figure 1C and Figure S1**). SM synthases convert ceramide to SM, so we next examined SM and ceramide levels in hepatocytes of both HCV-infected and uninfected chimeric mice. SM and ceramide levels were assessed using MS spectrometry, which allows analysis of samples at the single lipid species level as well as at the whole lipidome level. MS analysis showed that the level of ceramide, the precursor to SM, was increased in hepatocytes obtained from chimeric mice infected with HCV of either genotype (**Figure 1D**). Further, MS analysis showed that infection of chimeric mice with HCG9 (genotype 1a) was associated with increased SM levels in hepatocytes (**Figure 1E**). Similarly, SM levels were elevated in the hepatocytes of HCR24 (genotype 2a)-infected chimeric mice. These results indicate that infection with HCV increases total SM and ceramide levels in human hepatocytes.

MS analysis was conducted to determine which of several molecular species of SM [15] are present in HCV-infected hepatocytes. SM molecular species were analyzed in extracts obtained from a human hepatocyte cell line (HuH-7 K4) and from hepatocytes derived from the humanized chimeric mice. We identified four major peaks as SM molecular species (*d*18:1-16:0, *d*18:1-22:0, *d*18:1-24:0, and *d*18:1-24:1), and other peaks as phosphatidylcholine (**Figure 1F**). Infection-associated increases were seen for all ceramide molecular species, with significant changes in three of four species (excepting *d*18:1-16:0; $p<0.05$) with genotype 1a, and in all four species with genotype 2a ($p<0.05$) (**Figure 1G**). Upon infection with HCV of either genotype, hepatocytes tended to show increased levels of all four identified SM molecular species, but the changes were significant only for one species (*d*18:1-24:1; $p<0.05$) in genotype 1a and for two species (*d*18:1-16:0 and *d*18:1-24:1; $p<0.01$) in genotype 2a (**Figure 1H**). In cell culture, negligible amount of SM was likely increased by HCV infection. With respect to each molecular species, d18:1-16:0 SM was likely increased by HCV infection (**Figure S2**). These results indicate that HCV infection increases the abundance of several SM and ceramide molecular species.

Relationship between the SGMS genes and HCV infection

To clarify the relationship between SGMS1/2 and HCV, we investigated the correlation between SGMS1/2 expression and liver HCV-RNA in humanized chimeric mice. We found that SGMS1, but not SGMS2, had a correlation with liver HCV-RNA in HCV-infected humanized chimeric mice (**Figures 2A and 2B**).

Next, to clarify whether HCV infection of human hepatocytes increases the expression of the genes (*SGMS1* and *SGMS2*), we examined the effect of silencing HCV genome RNA on the expression of these genes in HCV-infected cells (**Figures 2C and 2D**). We found that silencing the HCV genome RNA decreases the expression of *SGMS1* and *SGMS2*.

The above results motivated us to examine the relationship between SGMS1/2 and HCV replication. Therefore, we examined the effect of SGMS1/2 mRNA silencing on HCV replication using subgenomic replicon cells [7,16]. We observed that silencing SGMS1 mRNA suppressed HCV replication, whereas silencing SGMS2 mRNA had no such effect (**Figures 2E and 2F**). These results indicate that SGMS1 expression has a correlation with HCV replication.

Characterization of the hepatotropic SPT inhibitor NA808

Based on our data, we hypothesized that HCV might alter the metabolism of sphingolipids, providing a more conducive environment for progression of the viral life cycle. To explore the relationship between HCV and sphingolipids, we investigated the effect of sphingolipid biosynthesis inhibition on HCV and the lipid profiles of SM and ceramide using HCV-infected chimeric mice harboring human hepatocytes. To inhibit the biosynthesis of sphingolipids, we used NA808, a chemical derivative of NA255, which is an SPT inhibitor derived from natural compounds [7]. We found that NA808 (**Figure 3A**) suppressed both the activity of SPT (**Figure 3B**) and biosynthesis of sphingolipids (**Figure 3C**) in a dose-dependent manner.

The conventional SPT inhibitor myriocin is not clinically beneficial due to immunosuppression through restriction of T-cell proliferation [17,18]. However, NA808 showed little immunosuppressive effect at the concentration at which NA808 suppressed HCV replication (**Figures 3D and 3E**). Moreover, pharmacokinetic analysis using [^{14}C]-labeled NA808 in rat models showed

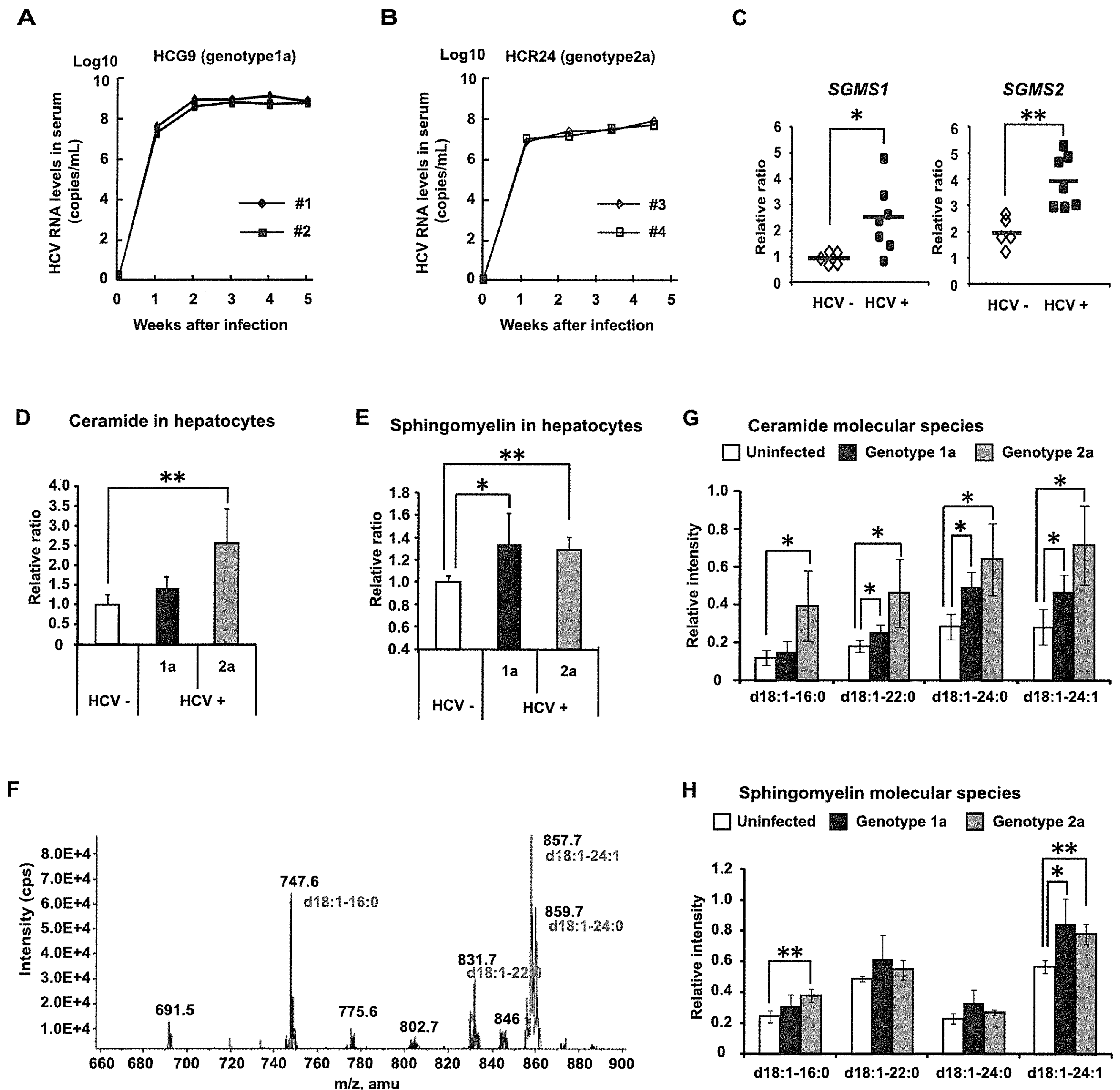

Figure 1. HCV alters sphingolipid metabolism. (**A, B**) Time-course studies of humanized chimeric mice inoculated with human serum samples positive for HCV genotype 1a (**A**) or 2a (**B**). (**C**) mRNA expression of *SGMS1* and *SGMS2* in uninfected (white, n = 5) and HCV genotype 1a-infected (black, n = 7) chimeric mice. (**D, E**) Effects of HCV infection on hepatocyte SM and ceramide levels in humanized chimeric mice. Relative intensity of total ceramide (**D**) and total shingomyelin (SM) (**E**) in uninfected mouse hepatocytes (white bar, n = 4), HCV genotype 1a-infected mouse hepatocytes (black bar, n = 5), and HCV genotype 2a-infected mouse hepatocytes (dark gray bar, n = 3). (**F**) Mass spectrum of SM in Bligh & Dyer extracts of a human hepatocyte cell line (HuH-7 K4). (**G, H**) Effects of HCV infection on hepatocyte SM and ceramide levels in humanized chimeric mice. Relative intensity of individual ceramide molecular species (**G**) and individual SM molecular species (**H**) in uninfected mouse hepatocytes (white bar, n = 3), HCV genotype 1a-infected mouse hepatocytes (black bar, n = 3), and HCV genotype 2a-infected mouse hepatocytes (dark gray bar, n = 3). In all cases, error bars indicate SDs. *$p<0.05$ and **$p<0.01$ compared with uninfected hepatocytes.
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that NA808 mainly accumulated in the liver and small intestine (**Table S1**). These results indicate that NA808 suppressed SPT activity, with hepatotropic and low immunosuppressive properties.

Based on these results, we then examined the effects of inhibition of sphingolipid biosynthesis with NA808 on HCV replication using subgenomic replicon cells [7,16]. The luciferase activity of FLR3-1 showed that replication was suppressed by NA808 in a dose-dependent manner with no effect on cell viability, as measured by the WST-8 assay (**Figure 3E**). Similarly, western blot and immunofluorescence analysis showed that NA808 effectively suppressed HCV replication (**Figures 3F and 3G**).

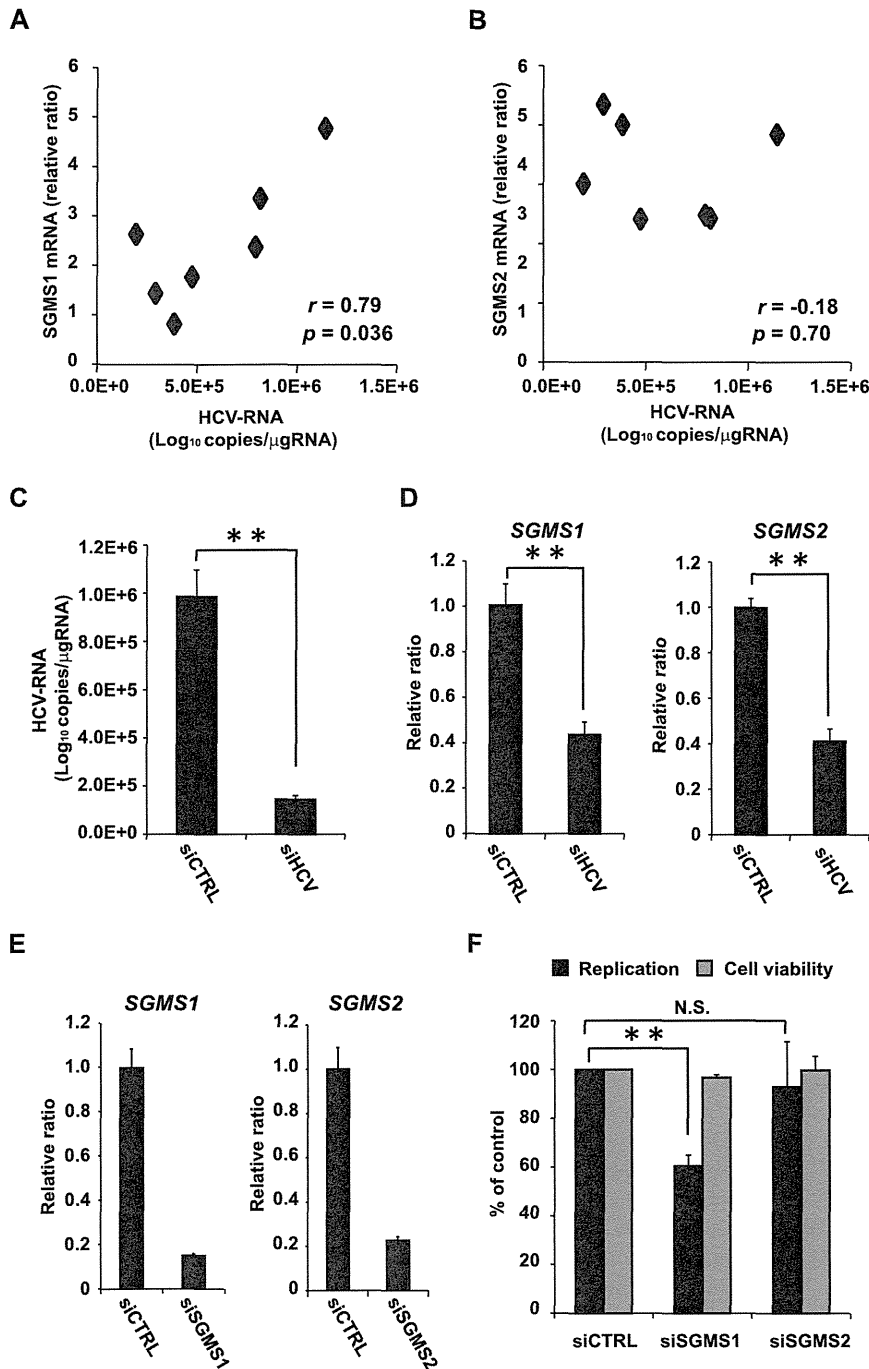

Figure 2. Relationship between the SGMS genes and HCV infection. (**A, B**) The correlation between SGMS1/2 and liver HCV-RNA of HCV infected humanized chimeric mice (n = 7). (**C**) The effect of silencing HCV genome RNA with siRNA (siE-R7: 1 nM) on HCV in HCV-infected cells. (**D**) The effect of silencing HCV genome RNA with siRNA (siE-R7: 1 nM) on the expression of SGMS1/2 mRNA measured by RTD-PCR. (**E**) The effect of silencing SGMS1/2 mRNA with siRNA (3 nM each) measured by RTD-PCR. (**F**) The effect of silencing SGMS1/2 mRNA with siRNA (3 nM) on HCV replication in FLR 3-1. In all cases, error bars indicate SDs. *$p<0.05$ and **$p<0.01$.
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Inhibition of sphingolipid biosynthesis impedes HCV infection of chimeric mice

To evaluate the effects of inhibition of sphingolipid biosynthesis in an animal model, we administered NA808 or pegylated interferon-α (PegIFN-α) via intravenous or subcutaneous injection to HCV-infected chimeric mice harboring human hepatocytes (**Table S2**). In chimeric mice infected with HCV genotype 1a, NA808 treatment led to a rapid decline in serum HCV-RNA (approximately 2–3 log units within 14 days). On the other hand, PegIFN-α produced less than a 1 log unit reduction, despite being delivered at 20 times the typical clinical dose (**Figure 4A**). Furthermore, results of 21-day NA808 treatment (5 mg/kg) in individual mice indicated that serum HCV RNA continued to decrease in all chimeric mice without viral breakthrough

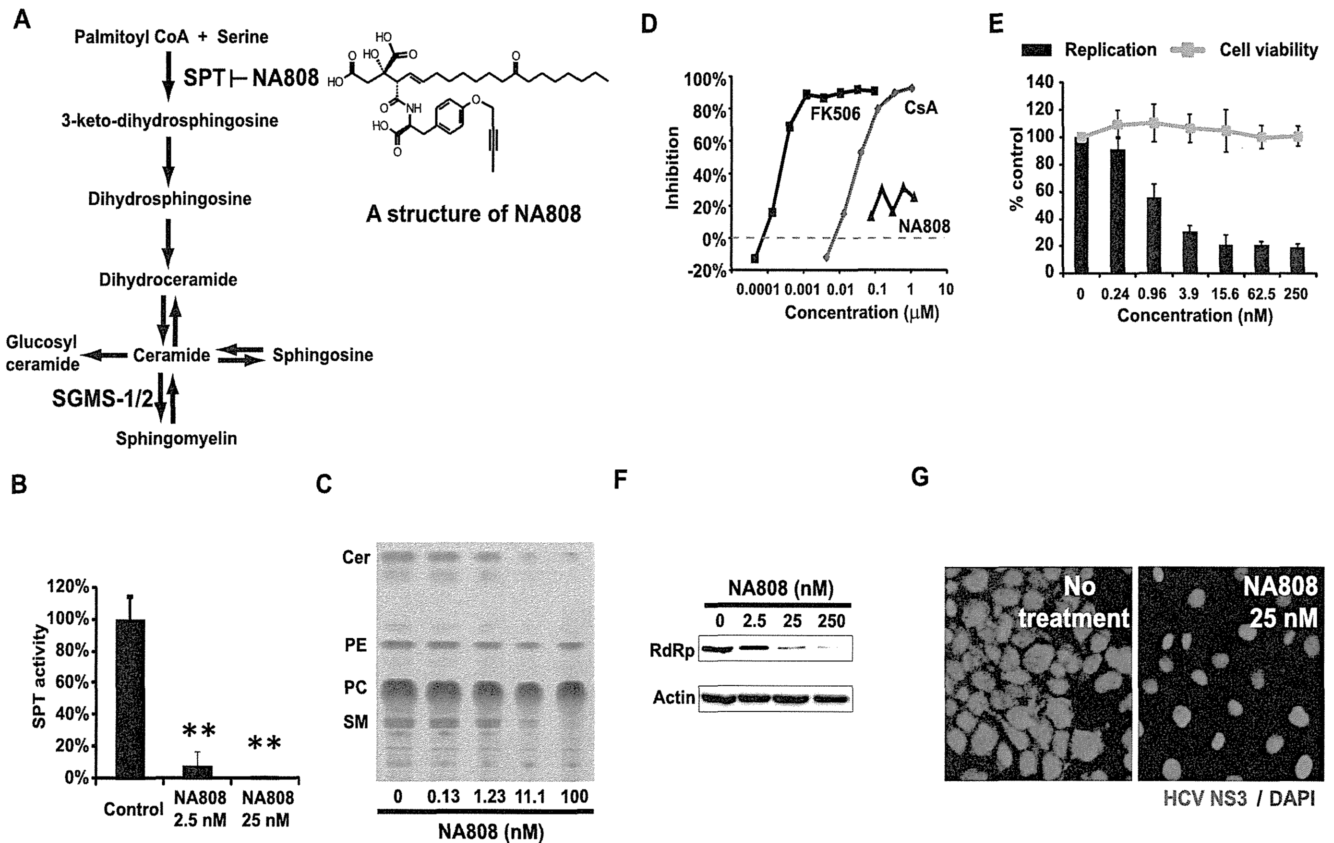

Figure 3. Characterization of the hepatotropic serine palmitoyltransferase inhibitor NA808. (**A**) Sphingolipid biosynthesis pathway and structure of NA808. (**B**) Activity of SPT in FLR3-1 cells after 72 h of NA808 treatment. ** $p<0.01$ compared with control. (**C**) Results of TLC showing *de novo* sphingolipid biosynthesis in the presence of NA808. Cer = ceramide, PE = phosphatidylethanolamine, PC = phosphatidylcholine, SM = sphingomyelin. (**D**) Immunosuppressive activity of NA808. Cyclosporin A (CsA) and tacrolimus (FK-506) were used as positive controls. (**E**) Effects of NA808 on HCV replication (black bars) and cell viability (gray symbols) in FLR 3-1 replicon-containing cells. Error bars indicate SDs. (**F**) Effects of NA808 on the level of the RdRp and β-actin, as assessed by Western blotting. (**G**) Effect of NA808 on the production of HCV NS3 protein (green) in FLR3-1 replicon-containing cells, as assessed by immunofluorescence analysis. Nuclear DNA was stained with DAPI (blue).
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(**Figure 4B**). Notably, in 2 of 5 chimeric mice, serum HCV-RNA was not detectable at the end of the 21-day regimen. Consistent with this observation, the levels of both hepatic HCV-RNA and HCV core protein decreased significantly ($p<0.01$ and $p<0.05$, respectively) following NA808 treatment, these effects being dose dependent (**Figure 4C**). Immunofluorescence analysis and immunohistochemistry confirmed the reduced abundance of HCV core protein after 14 days of treatment (**Figure 4D and Figure S3**).

In genotype 2a-infected chimeric mice, NA808 decreased serum HCV-RNA by approximately 3 log units within 14 days (**Figure 4E**). NA808-treated mice displayed a corresponding reduction in hepatic HCV-RNA (**Figure 4F**). NA808 did not affect body weight or human serum albumin levels (**Figures S4A and S4B**). Furthermore, hematoxylin and eosin (H&E) staining revealed little morphological change in response to treatment with NA808. Immunofluorescence analysis also indicated that NA808 did not affect the production of human albumin (**Figure S4C**). Thus, inhibition of sphingolipid biosynthesis by an SPT inhibitor impeded HCV replication in an animal infection model, regardless of HCV genotype.

Inhibition of SPT decreases ceramide and SM levels in hepatocytes of humanized chimeric mice

We next investigated the effects of sphingolipid biosynthesis inhibition on SM and ceramide levels in hepatocytes of humanized chimeric mice. Pharmacokinetic analysis in a rat model indicated that NA808 has hepatotropic properties (**Table S1**). Consistent with this analysis, our study in chimeric mice also indicated that the NA808 concentration was much higher in the liver than in serum (**Figure S5**). Furthermore, we observed that serum SM content was not decreased by NA808 treatment (**Figure S6**), in contrast to the effects previously observed for myriocin, another SPT inhibitor [19].

In HCV-infected chimeric mouse hepatocytes, MS analysis indicated that HCV infection resulted in increased ceramide and SM levels. However, treatment of infected animals with NA808 (5 mg/kg) attenuated this increase in ceramide and SM levels in hepatocytes, and the change in SM was significant ($p<0.05$) compared to the level observed in HCV-infected chimeric mice with no treatment. This effect of NA808 on ceramide and SM levels was dose-dependent (**Figures 5A and 5B**). We also found that SM levels and hepatic HCV-RNA were correlated (**Figure 5C**).

Interestingly, treatment with NA808 effectively decreased two specific SM and ceramide molecular species (*d*18:1-22:0 and *d*18:1-24:0), slightly decreased one other species (*d*18:1-24:1), and hardly decreased another (*d*18:1-16:0). Further, we found that among SM and ceramide molecular species, *d*18:1-16:0 did not change (**Figures 5D and 5E**). These results indicate that the

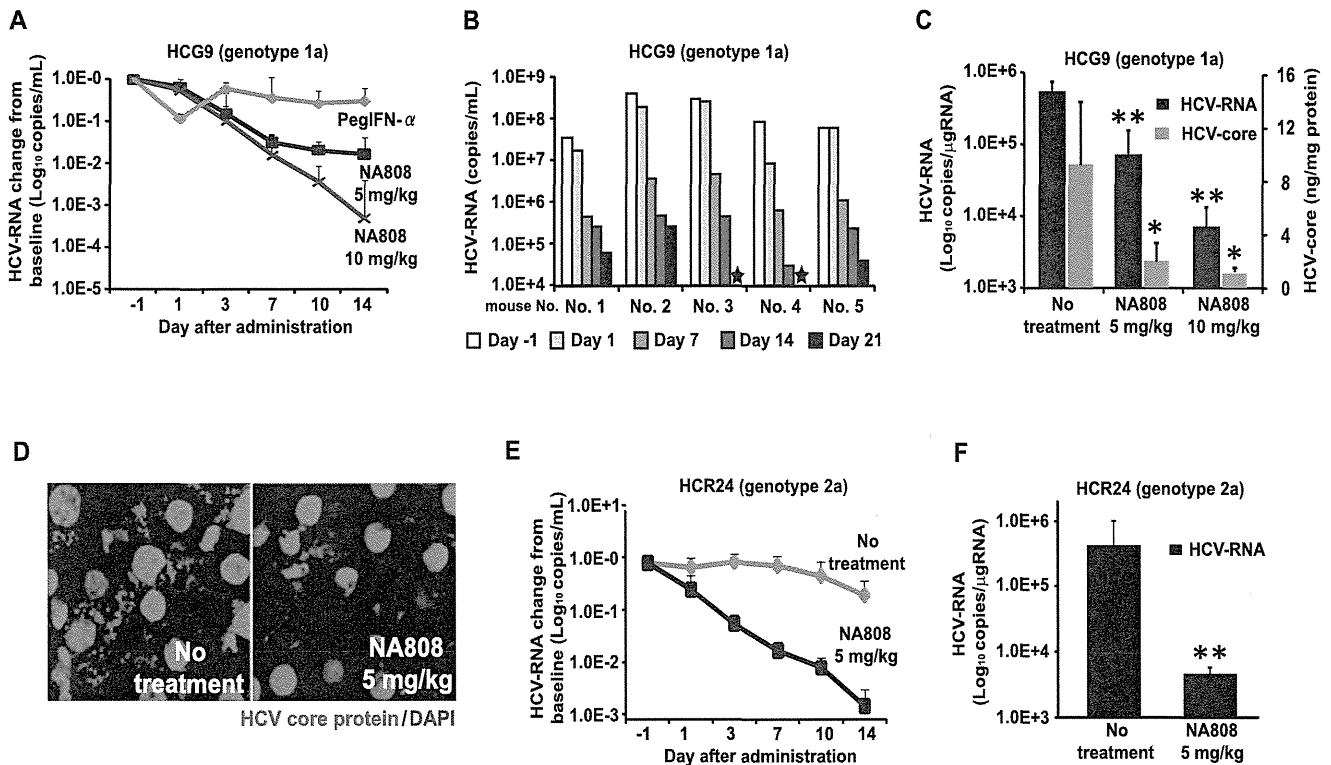

Figure 4. Inhibition of sphingolipid biosynthesis with hepatotropic serine palmitoyltransferase (SPT) inhibitor NA808 exerts anti-HCV effect. (**A**) Serum HCV-RNA levels in response to treatment with NA808 (blue, 5 mg/kg/day, purple, 10 mg/kg/day, n = 6 each), or PegIFN-α (pink, 30 μg/kg twice weekly, n = 4). (**B**) Effect of NA808 (5 mg/kg/day) on serum HCV-RNA levels. A star indicates that HCV-RNA was not detected. (**C**) Levels of liver HCV-RNA (black) and HCV core protein (gray) after the 14-day treatment. *$p<0.05$ and **$p<0.01$ compared with no treatment. (**D**) Histological analysis using immunofluorescent labeling of HCV core protein (green) and fluorescent staining of nuclei (blue). (**E**) Serum HCV-RNA levels in response to no treatment (pink, n = 3) or NA808 treatment (blue, 5 mg/kg/day, n = 4). (**F**) Liver HCV-RNA levels in genotype 2a-infected mice after the 14-day treatment. *$p<0.05$ and **$p<0.01$ compared with no treatment. In all cases, error bars indicate SDs.
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effects of sphingolipid biosynthesis inhibition varied among the molecular species.

Considering these results, we found a discrepancy in SM molecular species which were considered to be important for HCV replication. To elucidate the relationship between SM molecular species and HCV replication, we attempted to identify endogenous SM molecular species comprising the DRM fraction and to evaluate the effects of HCV infection and inhibition of sphingolipid biosynthesis on SM levels of the DRM.

Relationship between endogenous SM molecular species constituting the DRM and HCV replication

We previously reported that SM interacts with RdRp, allowing it to localize to the DRM fraction where HCV replicates and activates RdRp [7,8], and that suppression of SM biosynthesis disrupts the association between RdRp and SM in the DRM fraction, resulting in suppression of HCV replication [7,8]. In the present study, treatment with NA808 decreased SM levels in the DRM fraction; the decreased presence of SM correlated with decreased RdRp abundance, but the same effect was not seen for HCV nonstructural protein 3 (**Figures S7A–C**). Given these results, we investigated whether HCV replication was induced by elevated SM levels. Specifically, we compared SM levels in the DRM fraction between HCV-infected hepatocytes and uninfected hepatocytes. MS analysis showed that HCV increased SM levels in the DRM fraction more remarkably than in whole cells (**Figure 6A**). Next, we identified SM molecular species composing the DRM fraction and found that the composition ratio of SM molecular species was distinct between whole cells and DRM fractions in both HCV-infected and uninfected hepatocytes (**Figure 6B and Figure S8**). The DRM was composed primarily (69%) of *d*18:1-16:0, followed (in decreasing order) by *d*18:1-24:0, *d*18:1-22:0, and *d*18:1-24:1; the abundance of all SM molecular species increased upon HCV infection (**Figure 6C**). Further, NA808 treatment decreased all SM molecular species in the DRM fraction. Consistently, NS3 protease inhibitor decreased all SM molecular species in the DRM fraction of subgenomic replicon cells (**Figure S9**).

To address the association between RdRp and the endogenous SM molecular species composing the DRM, we used high-performance liquid chromatography (HPLC) to separate each SM molecular species from bulk SM derived from bovine milk and brain. We evaluated the relationship between RdRp and these endogenous SM molecular species using *in vitro* analysis. Enzyme-linked immunosorbent assay (ELISA) indicated that these endogenous SM molecular species bound to RdRp more readily than the bulk SM derived from milk as a positive control (**Figure 6D**). Further, *in vitro* HCV transcription analysis showed that three SM species (*d*18:1-16:0, *d*18:1-22:0, and *d*18:1-24:1) increased *in vitro* RdRp activation by approximately 5-fold, whereas the *d*18:1-24:0 species increased activation by 2-fold (**Figure 6E**). In a previous study, the soluble RdRp without its C-terminal hydrophobic 21-amino-acid sequence was used in *in vitro* analysis [8], and whether the relationship between RdRp and SM proved in this analysis

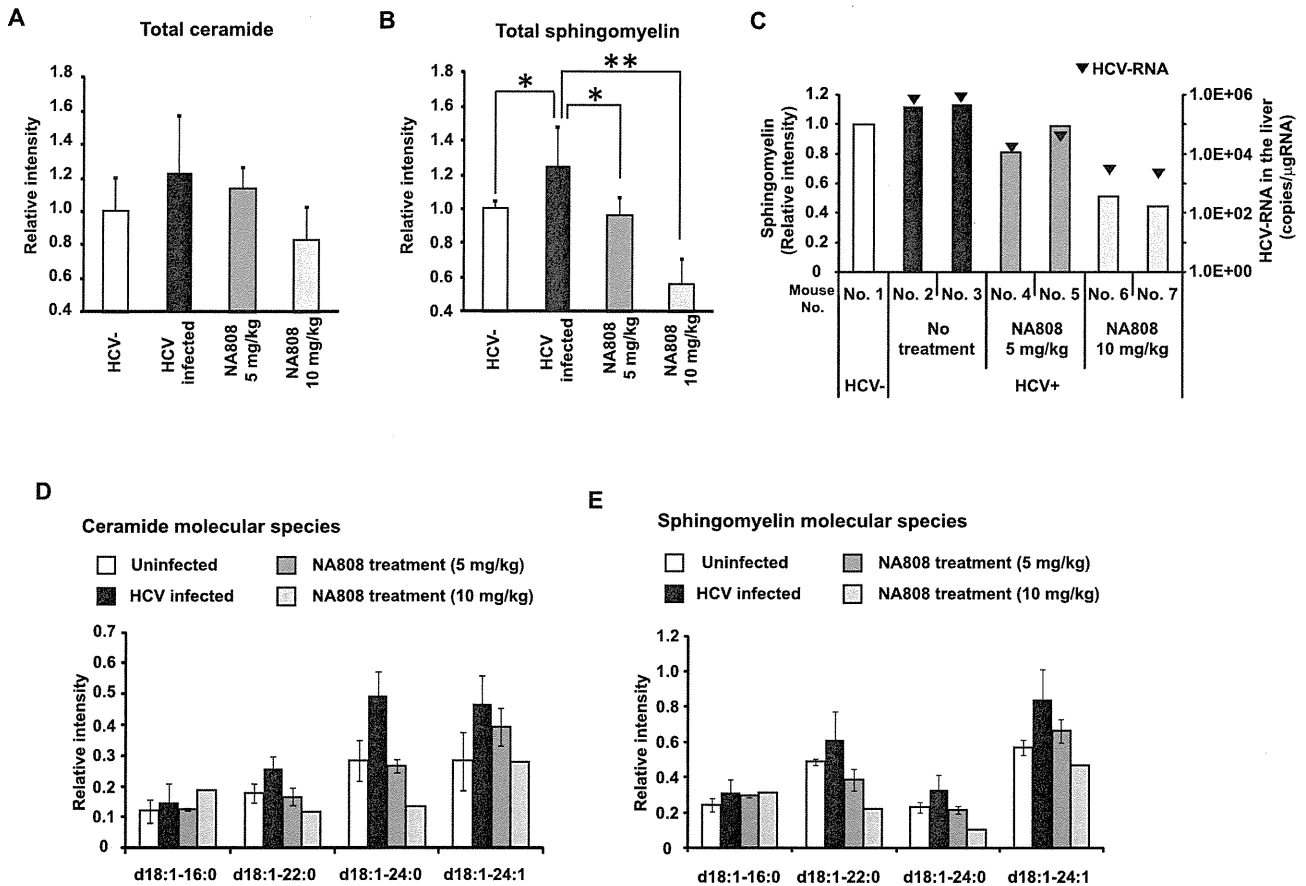

Figure 5. Effects of NA808 treatment on sphingomyelin (SM) and ceramide (total and individual molecular species). (**A, B**) Relative ratio of total ceramide (**A**) and SM (**B**) in uninfected mice (white, n = 4), HCV genotype 1a-infected mice (black, n = 5), and HCV-infected mice treated with NA808 for 14 days (dark gray, 5 mg/kg, n = 4; light gray, 10 mg/kg, n = 3). $^{*}p<0.05$ and $^{**}p<0.01$ compared with HCV-infected mice. (**C**) SM levels (bars) and HCV RNA levels (black arrowhead) in the livers of mice treated for 14 days with NA808 (5 or 10 mg/kg/day) and untreated chimeric mice. (**D, E**) Relative intensities of individual ceramide molecular species (**D**) and individual SM molecular species (**E**) in uninfected mice (white, n = 3), HCV-infected mice (black, n = 3), and HCV-infected mice treated with NA808 for 14 days (dark gray, 5 mg/kg, n = 2; light gray, 10 mg/kg, n = 1). In all cases, error bars indicate SDs.
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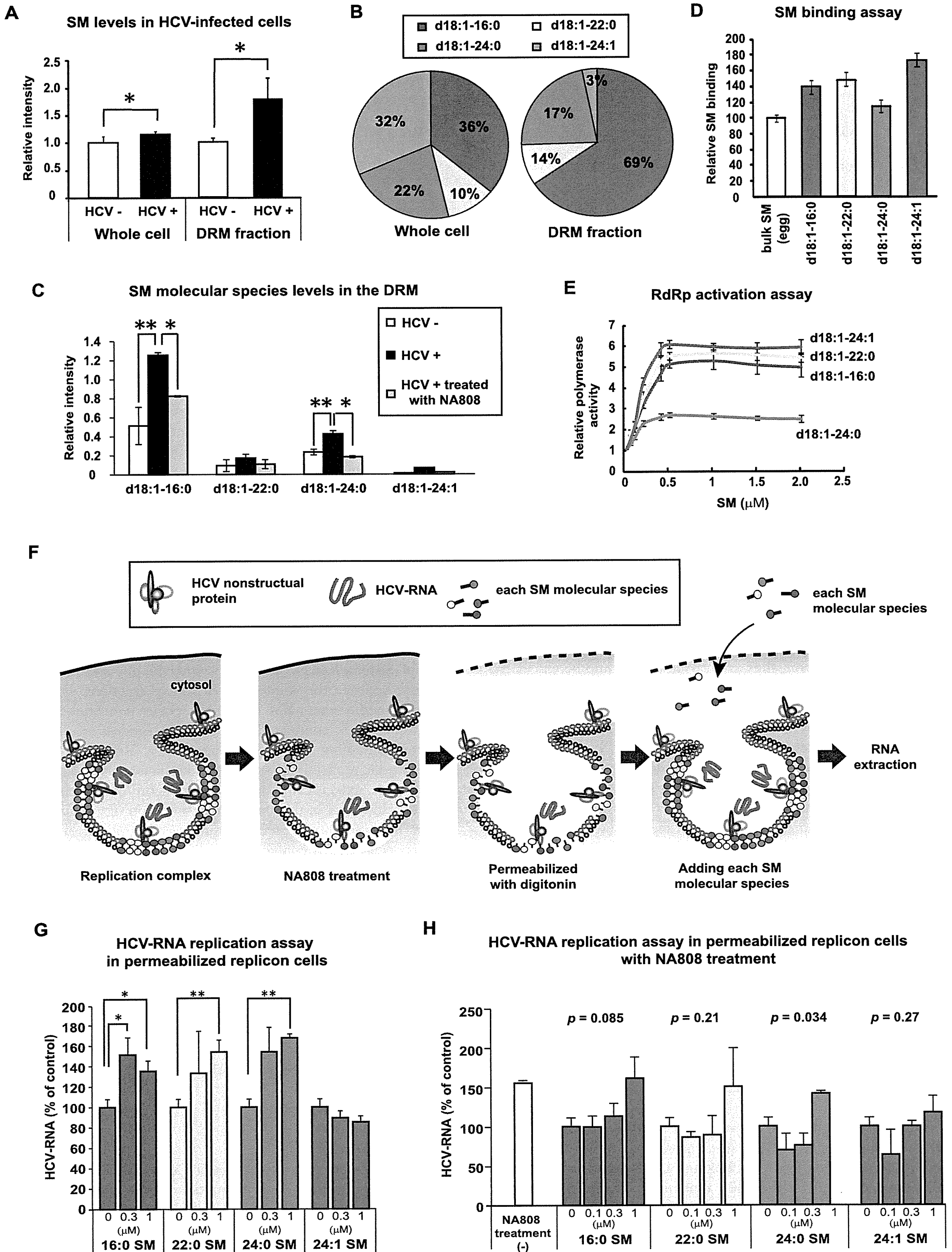
reflected the state in the membranous replication complex remains to be elucidated. Therefore, we attempted to examine the effect of endogenous SM molecular species on HCV replicase activity *in vivo* using digitonin-permeabilized semi-intact replicon cells, which permit monitoring of the function of the active HCV replication complex (**Figure 6F**) [20]. This *in vivo* analysis also enabled us to deliver the extrinsically added SM molecular species directly to the cytosol. This RNA replication assay indicated that the endogenous SM molecular species (*d*18:1-16:0 and *d*18:1-24:0) enhanced HCV-RNA replication, these species being consistent with the two SM molecular species that primarily constitute the DRM and are decreased significantly by NA808 treatment (**Figures 6G and 6H**). These results suggest that HCV infection modifies the levels of specific endogenous SM molecular species, which in turn enhance HCV-RNA replication by interacting with RdRp.

Discussion

In this study, we showed that HCV alters sphingolipid metabolism, resulting in a better environment for viral replication. Specifically, HCV increased SM content in the DRM fraction; this step is essential for viral replication since SM is a key component of the membranous replication complex and interacts with RdRp. Employing MS analysis, we identified endogenous SM molecular species (located in the DRM fraction) that increased upon HCV infection, and demonstrated that these endogenous SM molecular species interact directly with RdRp, enhancing HCV replication. Thus, we concluded that HCV modulates sphingolipid metabolism to promote viral replication.

We found that the expression levels of SGMS1/2 and the content of SM and ceramide in HCV-infected humanized chimeric mouse livers was increased (Figure 1). Our measurement revealed that chronic HCV infection promoted sphingolipid biosynthesis. HCV is known to induce cellular stress [21,22]. A variety of cell stressors increase intracellular ceramide content during the execution phase of apoptosis [23,24], indicating that ceramide is a proapoptotic lipid mediator. Furthermore, activation of ceramide-metabolizing enzymes such as glucosylceramide synthase and SM synthase can attenuate apoptosis by decreasing the intracellular ceramide content [25,26]. We found that HCV infection correlated with increased mRNA levels of the genes that encode human SM synthases (*SGMS1/2*) and glucosylceramide synthase (*UGCG*) (data not shown). Thus, the increase in ceramide levels observed in our study was likely to activate enzymes that transfer ceramide to other sphingolipids. On the other hand, Diamond et al. reported on lipidomic profiling performed over the


A
SM levels in HCV-infected cells
Relative intensity
HCV - HCV + HCV - HCV +
Whole cell DRM fraction
B
d18:1-16:0 d18:1-22:0
d18:1-24:0 d18:1-24:1
32% 36% 22% 10%
17% 3% 14% 69%
Whole cell DRM fraction
D
SM binding assay
Relative SM binding
bulk SM (egg) d18:1-16:0 d18:1-22:0 d18:1-24:0 d18:1-24:1
C
SM molecular species levels in the DRM
HCV -
HCV +
HCV + treated with NA808
Relative intensity
d18:1-16:0 d18:1-22:0 d18:1-24:0 d18:1-24:1
E
RdRp activation assay
Relative polymerase activity
d18:1-24:1
d18:1-22:0
d18:1-16:0
d18:1-24:0
SM (μM)
F
HCV nonstructual protein
HCV-RNA
each SM molecular species
each SM molecular species
cytosol
RNA extraction
Replication complex
NA808 treatment
Permeabilized with digitonin
Adding each SM molecular species
G
HCV-RNA replication assay in permeabilized replicon cells
HCV-RNA (% of control)
16:0 SM 22:0 SM 24:0 SM 24:1 SM
H
HCV-RNA replication assay in permeabilized replicon cells with NA808 treatment
p = 0.085 p = 0.21 p = 0.034 p = 0.27
HCV-RNA (% of control)
NA808 treatment (-)
16:0 SM 22:0 SM 24:0 SM 24:1 SM