

## Sphingomyelin Activates Hepatitis C Virus RNA Polymerase in a Genotype-Specific Manner<sup>∇†</sup>

Leiyun Weng,<sup>1</sup> Yuichi Hirata,<sup>2</sup> Masaaki Arai,<sup>3</sup> Michinori Kohara,<sup>2</sup> Takaji Wakita,<sup>4</sup> Koichi Watashi,<sup>4,5</sup> Kunitada Shimotohno,<sup>5,6</sup> Ying He,<sup>7</sup> Jin Zhong,<sup>7</sup> and Tetsuya Toyoda<sup>1\*</sup>

*Units of Viral Genome Regulation<sup>1</sup> and Viral Hepatitis,<sup>7</sup> Institut Pasteur of Shanghai, Key Laboratory of Molecular Virology and Immunology, Chinese Academy of Sciences, 411 Hefei Road, 200025 Shanghai, People's Republic of China; Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Biology, 3-18-22 Honkomagome, Bunkyo-Ku, Tokyo 113-8613, Japan<sup>2</sup>; Pharmacology Laboratory, Pharmacology Department V, Mitsubishi Tanabe Pharma Corporation, 1000 Kamoshida-cho, Aoba-ku, Yokohama 227-0033, Japan<sup>3</sup>; Department of Virology II, National Institute of Health, 1-23-1 Toyama, Shinjuku, Tokyo 132-8640, Japan<sup>4</sup>; Laboratory of Human Tumor Viruses, Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan<sup>5</sup>; and Chiba Institute of Technology, 2-17-1 Tsudamuna, Narashino, Chiba 275-0016, Japan<sup>6</sup>*

Received 25 March 2010/Accepted 27 August 2010

**Hepatitis C virus (HCV) replication and infection depend on the lipid components of the cell, and replication is inhibited by inhibitors of sphingomyelin biosynthesis. We found that sphingomyelin bound to and activated genotype 1b RNA-dependent RNA polymerase (RdRp) by enhancing its template binding activity. Sphingomyelin also bound to 1a and JFH1 (genotype 2a) RdRps but did not activate them. Sphingomyelin did not bind to or activate J6CF (2a) RdRp. The sphingomyelin binding domain (SBD) of HCV RdRp was mapped to the helix-turn-helix structure (residues 231 to 260), which was essential for sphingomyelin binding and activation. Helix structures (residues 231 to 241 and 247 to 260) are important for RdRp activation, and 238S and 248E are important for maintaining the helix structures for template binding and RdRp activation by sphingomyelin. 241Q in helix 1 and the negatively charged 244D at the apex of the turn are important for sphingomyelin binding. Both amino acids are on the surface of the RdRp molecule. The polarity of the phosphocholine of sphingomyelin is important for HCV RdRp activation. However, phosphocholine did not activate RdRp. Twenty sphingomyelin molecules activated one RdRp molecule. The biochemical effect of sphingomyelin on HCV RdRp activity was virologically confirmed by the HCV replicon system. We also found that the SBD was the lipid raft membrane localization domain of HCV NS5B because JFH1 (2a) replicon cells harboring NS5B with the mutation A242C/S244D moved to the lipid raft while the wild type did not localize there. This agreed with the myriocin sensitivity of the mutant replicon. This sphingomyelin interaction is a target for HCV infection because most HCV RdRps have 241Q.**

Hepatitis C virus (HCV) has a positive-stranded RNA genome and belongs to the family *Flaviviridae* (21). HCV chronically infects more than 130 million people worldwide (34), and HCV infection often induces liver cirrhosis and hepatocellular carcinoma (19, 28). To date, pegylated interferon (PEG-IFN) and ribavirin are the standard treatments for HCV infection. However, many patients cannot tolerate their serious side effects. Therefore, the development of new and safer therapeutic methods with better efficacy is urgently needed.

Lipids play important roles in HCV infection and replication. For example, the HCV core associates with lipid droplets and recruits nonstructural proteins and replication complexes to lipid droplet-associated membranes which are involved in the production of infectious virus particles (24). HCV RNA replication depends on viral protein association with raft membranes (2, 30). The association of cholesterol and sphingolipid with HCV particles is also important for virion maturation and infectivity (3). The inhibitors of the sphingolipid biosynthetic

pathway, ISP-1 and HPA-12, which specifically inhibit serine palmitoyltransferase (SPT) (23) and ceramide trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus (37), suppress HCV virus production in cell culture but not viral RNA replication by the JFH1 replicon (3). Other serine SPT inhibitors (myriocin and NA255) inhibit genotype 1b replication (4, 29, 33). Very-low-density lipoprotein (VLDL) also interacts with the HCV virion (15).

Sakamoto et al. reported that sphingomyelin bound to HCV RNA-dependent polymerase (RdRp) at the sphingomyelin binding domain (SBD; amino acids 230 to 263 of RdRp) to recruit HCV RdRp on the lipid rafts, where the HCV complex assembles, and that NA255 suppressed HCV replication by releasing HCV RdRp from the lipid rafts (29). In the present study, we analyzed the effect of sphingomyelin on HCV RdRp activity *in vitro* and found that sphingomyelin activated HCV RdRp activity in a genotype-specific manner. We also determined the sphingomyelin activation domain and the activation mechanism. Finally, we confirmed our biochemical data by a HCV replicon system.

### MATERIALS AND METHODS

**HCV RNA polymerase.** A C-terminal 21-amino-acid deletion was made to the HCV RdRps of strains HCR6 (genotype 1b) (36), NN (1b) (35), Con1 (1b) (5), JFH1 (2a) (36), J6CF (2a) (25), H77 (1a) (7), and RMT (1a), and the mutants

\* Corresponding author. Mailing address: Unit of Viral Genome Regulation, Institut Pasteur of Shanghai, Chinese Academy of Sciences, 411 Hefei Road, 200025 Shanghai, People's Republic of China. Phone and fax: 86 21 6385 1621. E-mail: ttoyoda@amber.plala.or.jp.

† Supplemental material for this article may be found at <http://jvi.asm.org>.

<sup>∇</sup> Published ahead of print on 15 September 2010.

were purified from bacteria as described previously (36). HCR6 (1b) RdRp with the mutation L245A [RdRp(L245A)] or I253A [RdRp(I253A)] or the double mutation L245A and I253A [RdRp(L245A/I253A)]; JFH1 (2a) RdRp with the mutation(s) A242C/S244D, A242, S244D, or T251Q; J6CF (2a) RdRp with the mutation(s) R241Q, S244D, or R241Q/S244D; and H77 (1a) RdRp(A238S/Q248E) were introduced using an *in vitro* mutagenesis kit (Stratagene) and the oligonucleotides listed in Table S1 in the supplemental material. HCR6 (1b) His<sub>6</sub>-tagged RdRp(L245A/I253A) was removed from pET21b/KM (36) and cloned into the BamHI/XhoI site of pGEX-6P-3 (GE), resulting in pGEXHCVHCR6RdRp(L245A/I253A).

**In vitro HCV transcription.** *In vitro* HCV transcription was performed as described previously (36). Briefly, following 30 min of preincubation without ATP, CTP, or UTP, 100 nM HCV RdRp was incubated in 50 mM Tris-HCl (pH 8.0), 200 mM potassium glutamate, 3.5 mM MnCl<sub>2</sub>, 1 mM dithiothreitol (DTT), 0.5 mM GTP, 50 μM ATP, 50 μM CTP, 5 μM [α-<sup>32</sup>P]UTP, 200 nM RNA template (SL12-1S), 100 U/ml human placental RNase inhibitor, and the lipid (amount indicated below) at 29°C for 90 min. <sup>32</sup>P-labeled RNA products were subjected to 6% polyacrylamide gel electrophoresis (PAGE) containing 8 M urea. The resulting autoradiograph was analyzed with a Typhoon Trio plus image analyzer (GE).

**RNA filter binding assay.** An RNA filter binding assay was performed as described previously (36). Briefly, 100 nM HCV RdRp and 100 nM <sup>32</sup>P-labeled RNA template (SL12-1S) were incubated with or without 0.01 mg/ml egg yolk sphingomyelin in 25 μl of 50 mM Tris-HCl (pH 7.5), 200 mM potassium glutamate, 3.5 mM MnCl<sub>2</sub>, and 1 mM DTT at 29°C for 30 min. After incubation, the solutions were diluted with 0.5 ml of TE (50 mM Tris-HCl [pH 7.5], 1 mM EDTA) buffer and filtered through nitrocellulose membranes (0.45-μm pore size; Millipore). The filter was washed five times with TE buffer, and the bound radioisotope was analyzed by Typhoon Trio plus after being dried.

**Enzyme-linked immunosorbent assay (ELISA).** Ninety-six-well microtiter plates (Corning) were coated with 250 ng of egg yolk sphingomyelin in ethanol by evaporation at room temperature. After the wells were blocked with phosphate-buffered saline (PBS) and 3% bovine serum albumin (BSA), they were incubated with 1 pmol of the HCV RdRp of HCR6 (1b) wild type (wt) or L245A, I253A, or L245A/I253A mutant; NN (1b); H77 (1a); RMT (1a); J6CF (2a); or JFH1 (2a) wt or A242C/S244D, A242, S244D, or T251Q mutant in Tris-buffered saline (50 mM Tris-HCl [pH 7.5] and 150 mM NaCl) for 1.5 h at room temperature. After being blocked with 3% BSA, the bound HCV RdRp was detected by adding rabbit anti-HCV RdRp serum (1:5,000) (see Fig. S1 in the supplemental material) (17) before incubation with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:5,000; Southern Biotech). The optical density at 450 nm (OD<sub>450</sub>) was measured with a Spectra Max 190 spectrophotometer (Molecular Devices) using a TMB (3,3',5,5'-tetramethylbenzidine) Liquid Substrate System (Sigma).

**HCV subgenomic replicon.** A D244S mutation was introduced into the HCV strain NN (1b) subgenomic replicon pLMH14 (35), resulting in pLMH(NN)5B(D244S) [where 5B(D244S) is the NS5B protein with the mutation D244S]. The A242C/S244D mutation was introduced into the HCV JFH1 (2a) replicon, pSGR-JFH1/luc (25), resulting in pSGR-JFH1/luc5B(A242C/S244D). The HpaI and XbaI fragment of pSGR-JFH1 (18) was replaced with that of pSGR-JFH1/luc5B(A242C/S244D), resulting in pSGR-JFH15B(A242C/S244D). The A238S/Q248E mutation was introduced into HCV H77 (1a) replicon pHCVrep13(S2204I)/Neo (7) after the neomycin gene was replaced by the firefly luciferase gene [pH77(I)/luc] by insertion of AflII and AscI sites (see Table S1 in the supplemental material), resulting in pH77(I)/luc5B(A238S/Q248E). Subgenomic replicon RNA was transcribed *in vitro* by T7 RNA polymerase using MegaScript (Ambion) after the replicon plasmids were linearized by XbaI (strain NN and JFH1 replicons) or HpaI (strain H77 replicon). Subgenomic replicon RNA was stored at -80°C after being purified by phenol-chloroform extraction and ethanol precipitation.

**Replicon assay with myriocin.** Huh7.5.1 cells were kindly provided by F. Chisari and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco). HCV replicon RNA (10 μg) was transfected into 4 × 10<sup>6</sup> Huh7.5.1 cells (1 × 10<sup>7</sup>/ml) in OptiMEM I (Gibco) by electroporation (GenePulser Xcell; Bio-Rad) at 270 V, 100 Ω, and 950 μF. After transfection, the cells were plated in 12-well plates incubated in DMEM-10% FBS. At 6 h after transfection, cells were treated with 0, 5, and 50 nM myriocin. At 4, 54, and 78 h after transfection (48 and 72 h after myriocin treatment), the cells were harvested, and luciferase activity was measured using a Dual-Glo luciferase assay kit and a GloMax 96 Microplate Luminometer (Promega). Luciferase activity was normalized against the activity at 4 h after transfection (26).

**HCV JFH1 wt and NS5B(A242C/S244D) replicon cells.** Huh7/scr cells were kindly provided by F. Chisari of the Scripps Research Institute and were maintained in Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum (Gibco). RNA (10 μg each) from SGR-JFH1 and SGR-JFH1 with the mutations A242C/S244D in NS5B [NS5B(A242C/S244D)] was transfected into 4 × 10<sup>6</sup> Huh7/scr cells (1 × 10<sup>7</sup>/ml) in OptiMEM I (GIBCO) by electroporation (GenePulser Xcell; Bio-Rad) at 270 V, 100 Ω, and 950 μF. After transfection, the cells were plated in 10-cm dishes and incubated in DMEM-10% FBS with 1.0 and 0.5 mg/ml G418 (Gibco). JFH1 wt and NS5B(A242C/S244D) replicon cells were maintained in DMEM-10% FBS and 0.5 mg/ml G418.

**Membrane floating assay.** JFH1 wt and NS5B(A242C/S244D) replicon cells were suspended in two packed cell volumes of hypotonic buffer (10 mM HEPES-NaOH [pH 7.6], 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 2 mM DTT, and 1 tablet/25 ml of EDTA-free protease inhibitor cocktail tablets [Roche]) and disrupted by 30 strokes of homogenization in a Dounce homogenizer using a tight-fitting pestle at 4°C. After nuclei were removed by centrifugation at 2,000 rpm for 10 min at 4°C, the supernatant (postnuclear supernatant [PNS]) was treated with 1% Triton X-100 in TNE buffer (25 mM Tris-HCl [pH 7.6] 150 mM NaCl, 1 mM EDTA) for 30 min on ice. The lysates were supplemented with 40% sucrose and centrifuged at 38,000 rpm in a Beckman SW41 Ti rotor (Beckman Coulter) overlaid with 30% and 10% sucrose in TNE buffer at 4°C for 14 h.

**Western blotting.** Western blotting using anti-HCV RdRp (17), rabbit anti-NS3 (32), anti-NS5A (16) and anti-caveolin-2 was performed as previously published (17).

**Reagent.** Egg yolk sphingomyelin, cholesterol phosphocholine, myriocin, and rabbit anti-caveolin-2 antibodies were purchased from Sigma. Hexanoyl sphingomyelin, C<sub>6</sub>-ceramide, C<sub>8</sub>-β-D-glucosyl ceramide, and C<sub>8</sub>-β-D-lactosyl ceramide were purchased from Avanti Polar Lipids. [α-<sup>32</sup>P]UTP was purchased from New England Nuclear.

**Statistical analysis.** Significant differences were evaluated using *P* values calculated from a Student's *t* test.

**Nucleotide sequence accession number.** The sequence of HCV RMT has been deposited in the GenBank under accession number AB520610.

## RESULTS

**Sphingomyelin activation of HCV RNA polymerases of various genotypes.** There are several sequence variations in the sphingomyelin binding domain (SBD; amino acids 231 to 260 of HCV RdRp) among HCV genotypes (see Fig. 7A). In order to compare the RdRps of different genotypes of HCV, we purified RdRp from genotypes 1b (strains HCR6, NN, and Con1), 1a (H77 and MRT), and 2a (JFH1 and J6CF) (see Fig. S2 in the supplemental material). First, the effect of ethanol on HCV HCR6 (1b) RdRp transcription was examined because lipids were suspended in ethanol before they were added to the HCV transcription reaction mixture. We found that 2% ethanol did not inhibit HCV transcription (see Fig. S3 in the supplemental material); therefore, all subsequent experiments were performed using less than 2% ethanol.

The kinetics of sphingomyelin activation were analyzed using egg yolk sphingomyelin for HCR6 (1b) RdRp wt (Fig. 1A) and subtype 2a (JFH1 and J6CF) RdRps (Fig. 1B), and *N*-hexanoyl-*D*-erythro-sphingosylphosphorylcholine (hexanoyl sphingomyelin) was used for HCR6 (1b) RdRp wt (Fig. 1C) and subtype 1a (H77 and MRT) RdRps (Fig. 1D). The egg yolk sphingomyelin activation curve of HCR6 (1b) RdRp wt at low concentrations (<0.01 mg/ml) was sigmoid. The transcription activity of HCR6 (1b) RdRp wt increased in a dose-dependent manner. It was activated 11-fold at 0.01 mg/ml and then plateaued (14-fold activation) at 0.1 mg/ml. However, JFH1 (2a) and J6CF (2a) RdRps were activated 2.5-fold and 2.2-fold, respectively, at 0.01 mg/ml sphingomyelin, at which point they plateaued.

Egg yolk sphingomyelin is a mixture. In order to obtain the optimal molar ratio for sphingomyelin activation of HCR6 (1b)

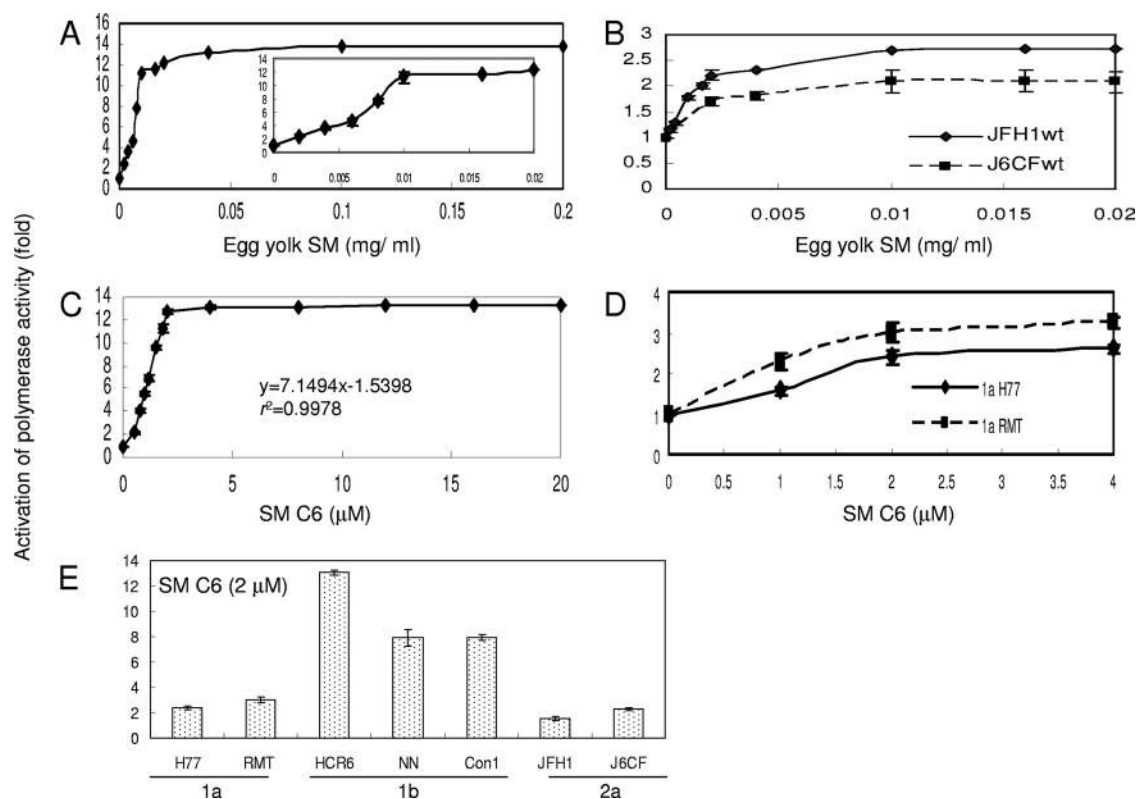


FIG. 1. Spingomyelin activation of HCV RNA polymerases. (A) Activation kinetics of HCV HCR6 (1b) RdRp wt by egg yolk spingomyelin (SM). The inset shows activation produced by 0 to 0.02 mg/ml egg yolk spingomyelin. Activation kinetics of HCV 2a (JFH1 and J6CF) RdRps by egg yolk spingomyelin (B) and of HCV HCR6 (1b) RdRp wt by hexanoyl spingomyelin (SM C6) (C). In panel C, the first order of the graph was fitted by linear regression; the calculated equation is indicated in the graph. (D) Activation kinetics of HCV 1a (H77 and RMT) RdRps by hexanoyl spingomyelin. (E) Activation effect of hexanoyl spingomyelin on HCV RdRp of various genotypes. HCV RdRp (100 nM) was incubated with or without 2  $\mu$ M SM C6. The names of the RdRps are indicated below the graph. Mean  $\pm$  standard deviation of the activation ratio was calculated from three independent experiments.

RdRp wt, its activation kinetics were calculated using hexanoyl spingomyelin (Fig. 1C, SM C6). The equation for the first-order ratio of hexanoyl spingomyelin activation according to linear regression fitting was as follows:  $y = 7.1494x - 1.5398$ , where  $y$  is the activation ratio and  $x$  is the spingomyelin concentration ( $r^2 = 0.9978$ ). RdRp activation had almost plateaued at 2  $\mu$ M hexanoyl spingomyelin. The activation kinetics of JFH1 (2a) and J6CF (2a) RdRps in egg yolk spingomyelin were biphasic and plateaued at 0.01 mg/ml. Those of RMT (1a) and H77 (1a) RdRps in hexanoyl spingomyelin were also biphasic and plateaued at 2  $\mu$ M. The curve of the first order was fitted by linear regression. The molar ratio of RdRp to hexanoyl spingomyelin at its plateau was calculated as 1:20.

Because RdRp activation had almost plateaued at 2  $\mu$ M hexanoyl spingomyelin, we compared the effect of spingomyelin on 100 M concentrations of RNA polymerases of the HCV 1a, 1b, and 2a genotypes using 2  $\mu$ M hexanoyl spingomyelin (Fig. 1E and Table 1).

**Helix-turn-helix structure for spingomyelin binding and activation.** Spingomyelin binds to the SBD peptide (see HCV SBD in Fig. 7) (29). Initially, we tested whether SBD was the spingomyelin binding site in HCV RdRp by ELISA (Fig. 2A and Table 1). When the L245 and I253 residues of the SBD

peptide were mutated to A, spingomyelin binding activity was lost (29). We introduced the same mutations in HCV HCR6 (1b) RdRp and purified HCR6 (1b) RdRp with mutations L245A, I253A, and L245A/I253A. Because the C-terminal His-tagged HCR6 RdRp(L245A/I253A) was not soluble, it was solubilized by tagging of glutathione *S*-transferase (GST) sequence at the N terminus but lost polymerase activity. As the L245A/I253A mutant had lost its polymerase activity, polymerase activation was tested only for L245A and I253A (Fig. 2B and Table 1). These results confirmed that SBD located in the finger domain (residues 230E to 263G) successfully achieved spingomyelin binding in HCV RdRp and that spingomyelin did not bind to the SBD when the helix-turn-helix structure had been destroyed by the L245A or I253A mutation (29).

The spingomyelin binding activities of genotype 1a and 2a RdRps were also tested (Fig. 2 and Table 1). Both JFH1 and J6CF were tested for genotype 2a because J6CF (2a) RdRp had an additional amino acid difference at position 241 in the SBD, and its spingomyelin binding activity was very low (Fig. 2A and 7A; Table 1). J6CF (2a) RdRp(R241Q) showed the same spingomyelin binding activity as HCR6 (1b) RdRp wt, indicating that 241Q was the critical amino acid for spingomyelin binding. J6CF (2a) RdRp(S244D) and RdRp(R241Q/S244D) also showed higher spingomyelin binding activity



TABLE 1. Summary of sphingomyelin activation of HCV RNA polymerase activities

Parameter	Value for the parameter by RdRp genotype, strain, and variant <sup>a</sup>																	
	1a							2a										
	HCR6		NN		Con1		RMT	H77		J6CF		JFH1						
	wt	L245A	I253A	L245A/I253A	D244S	D244S	wt	wt	A238S/Q248E	wt	R241Q	S244D	R241Q/S244D	wt	A242C	S244D	A242C/S244D	T251Q
SM binding (%) <sup>b</sup>	100	24.3	30.8	15.5	78.7	78.7	144	86.7	82.5	19.3	118	53.1	80.2	70.4	75.5	93.1	92.4	80.7
Activation of polymerase ( <i>n</i> -fold) <sup>c</sup>	13.0	(2.8) <sup>d</sup>	(2.5) <sup>d</sup>	ND	3.6	3.6	3.0	2.0	8.1	2.3	4.3	5.6	3.4	1.6	1.0	3.1	4.4	1.8
Activation of RNA binding ( <i>n</i> -fold) <sup>c</sup>	4.5	2.6	1.7	ND	1.9	1.9	ND	1.4	3.3	1.5	3.6	3.2	1.7	1.3	ND	ND	1.4	ND

<sup>a</sup> Numbers were averaged from three independent experiments. ND, not done.

<sup>b</sup> Egg yolk sphingomyelin (SM; 250 ng) was used.

<sup>c</sup> Hexanoyl sphingomyelin (2 μM) was used.

<sup>d</sup> Egg yolk sphingomyelin (0.01 mg/ml) was used.

than the wt ( $P < 0.001$ ) but lower binding than the R241Q mutant. However, S244D showed higher RdRp activation than R241Q ( $P < 0.005$ ), while the RdRp activation ratio of the double mutant (R241Q/S244D) was lower than that of S244D or R241Q, although all of them activated RdRp with sphingomyelin ( $P < 0.005$ ) (Fig. 2A and C and Table 1). For JFH1, when the JFH1 RdRp SBD was modified (A242C/S244D) to allow it to bind with more sphingomyelin than the wt ( $P < 0.005$ ), the mutant JFH1 RdRp(A242C/S244D) was activated more than the wt by sphingomyelin ( $P < 0.005$ ) (Fig. 2A and C; Table 1). The sphingomyelin binding activity of JFH1 RdRp(T251Q) was 80.7% of that of HCR6 (1b), and its activation ratio was 1.8-fold. These results agree that SBD is both the sphingomyelin activation and binding domain and that the domains for these two activities are somehow different.

We determined which amino acid, 242C or 244D, enhanced sphingomyelin binding by comparing HCR6 (1b) and JFH1 (2a) RdRps. Sphingomyelin binding of HCR6 (1b) RdRp(D244S) was 79% of that of the wt ( $P < 0.005$ ) (Fig. 2A and Table 1), and its activation by sphingomyelin was only 3.6-fold (Fig. 2C and Table 1). The sphingomyelin binding of JFH1 (2a) RdRp(A242C) and RdRp(S244D) increased to 75.5% and 93.1%, respectively, of HCR6 (1b) RdRp wt (Fig. 2A and Table 1). This was significantly higher than that of JFH1 (2a) RdRp wt ( $P < 0.005$ ), and the sphingomyelin activation of JFH1 (2a) RdRp(A242C) and RdRp(S244D) was increased 1.0-fold and 3.1-fold, respectively ( $P < 0.005$ ) (Fig. 2C and Table 1). From these mutation analyses of the J6CF and JFH1 RdRps, we concluded that 244D enhanced sphingomyelin binding and RdRp activation.

HCV 1a RdRps were not activated even though sphingomyelin bound to them (Fig. 1E and 2A and Table 1). We then tried to elucidate the domains responsible for sphingomyelin activation. There are 14 amino acids (residues 19, 25, 81, 111, 120, 131, 184, 270, 272, 329, 436, 464, 487, and 540) unique to genotype 1a RdRp in the region of residues 1 to 570 and two amino acid differences unique to 1a RdRp in SBD, i.e., 238A and 248Q (see Fig. 6A). Initially, we focused on the SBD and introduced the A238S and Q248E mutations into the H77 (2a) RdRp SBD (Fig. 2A and D and Table 1). The sphingomyelin binding activity of H77 (2a) RdRp(A238S/Q248E) was similar to that of H77 (2a) RdRp wt. The sphingomyelin activation ratio of H77 (2a) RdRp(A238S/Q248E) was increased 8.1-fold, leading us to conclude that these mutations are essential to sphingomyelin activation.

**Effect of lipids on HCV RNA polymerase activity.** In order to elucidate the structure of the lipids involved in activation of HCV RdRp, D-lactosyl-β-1,1'-N-octanoyl-D-erythro-sphingosine [C<sub>8</sub>-lactosyl(β) ceramide], D-glucosyl-β-1-17-N-octanoyl-D-erythro-sphingosine (C<sub>8</sub>-β-D-glucosyl ceramide), N-hexanol-D-erythro-sphingosine (C<sub>6</sub>-ceramide), and cholesterol were tested for their abilities to activate RdRp. The relative polymerase activities of 100 nM HCV HCR6 (1b) RdRp activated with 0.01 mg/ml egg yolk sphingomyelin, 2 μM hexanoyl sphingomyelin, 8 μM C<sub>8</sub>-lactosyl(β) ceramide, 12 μM C<sub>8</sub>-β-D-glucosyl ceramide, 12 μM C<sub>6</sub>-ceramide, and 0.02 mg/ml cholesterol were 11.2, 13.0, 5.66, 4.19, 1.12, and 2.25 of that without lipids, respectively (Fig. 3A). The amount of lipids that gave the maximum activation was calculated from the kinetics of the lipids bound to HCR6 (1b) and JFH1 (2a) RdRps (Fig. 3B and

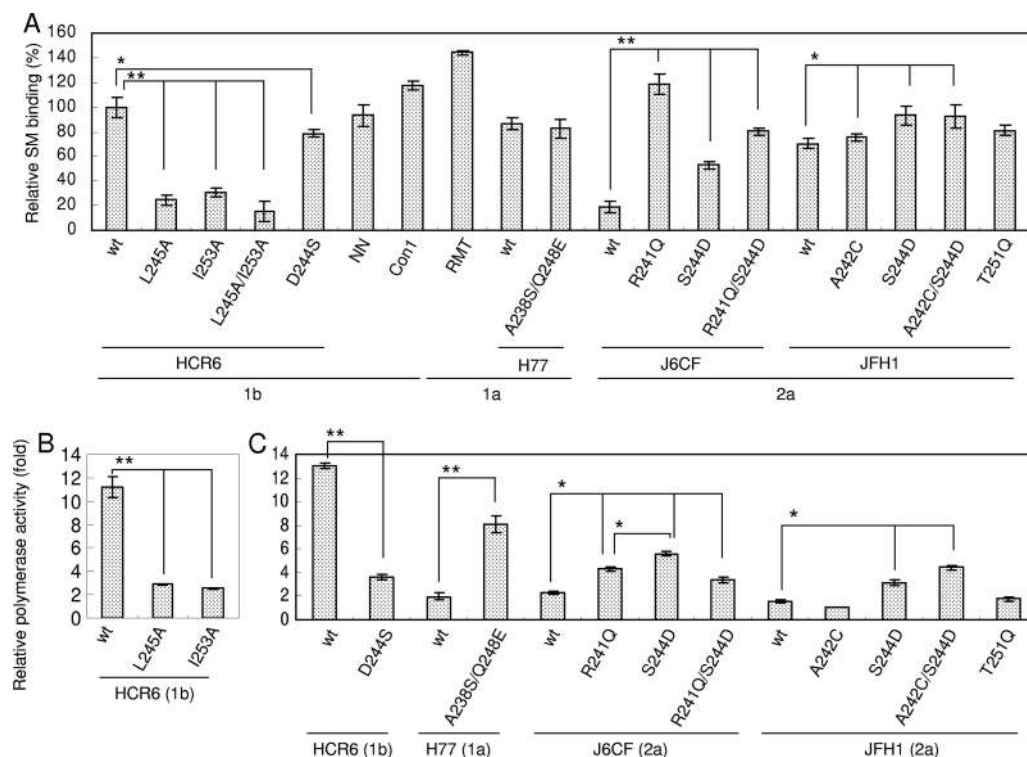


FIG. 2. SpHINGOMYELIN binding and activation of HCV RNA polymerase spHINGOMYELIN binding domain mutants. Names of RdRps are indicated below the graphs. (A) Egg yolk spHINGOMYELIN (SM) binding activity relative to that of HCR6 (1b) RdRp wt. Mean  $\pm$  standard deviation of the binding was calculated from three independent experiments. (B) Egg yolk spHINGOMYELIN activation of HCR6 (1b) RdRps. RdRps (100 nM) were incubated with or without 0.01 mg/ml egg yolk spHINGOMYELIN. (C) Hexanoyl spHINGOMYELIN activation of the RdRps (RdRp names are indicated below the graphs). HCV RdRps (100 nM) were incubated with or without 2  $\mu$ M hexanoyl spHINGOMYELIN. The mean  $\pm$  standard deviation of the activation ratio was calculated from three independent experiments. \*,  $P < 0.005$ ; \*\*,  $P < 0.001$ .

C).  $C_8$ -lactosyl( $\beta$ ) ceramide and  $C_8$ - $\beta$ -D-glucosyl ceramide activated HCR6 (1b) RdRp compared with the linear regression kinetics of the reaction with hexanoyl spHINGOMYELIN as it plateaued (Fig. 1C and 3B). Cholesterol activated HCR6 (1b) RdRp slightly but did not activate JFH1 (2a) RdRp (Fig. 3C). We therefore concluded that the phosphocholine of spHINGOMYELIN bound to the SBD of HCV RdRp because the order of HCV RdRp activation was hexanoyl spHINGOMYELIN >  $C_8$ -lactosyl( $\beta$ ) ceramide >  $C_8$ - $\beta$ -D-glucosyl ceramide, and  $C_6$ -ceramide did not activate HCV HCR6 (1b) RdRp. The polarity of the phosphocholine of spHINGOMYELIN is important for HCV RdRp activation (see Fig. S5 in the supplemental material).

In order to test whether phosphocholine activated HCV RdRp (Fig. 3D), HCR6 (1b) RdRp was incubated with 0.4, 2, 20, 100, and 400  $\mu$ g and 2, 4, 11, 54, and 100 mg of phosphocholine. Up to 400  $\mu$ g of phosphocholine did not affect RdRp activity, but more than 2 mg of phosphocholine inhibited RdRp activity.

**Effect of spHINGOMYELIN on the template RNA binding of HCV RNA polymerase.** The mechanism of HCV RdRp activation was analyzed. RNA polymerase changes its conformation throughout the different transcription steps, and template binding is the first step of transcription (9). Therefore, the effect of spHINGOMYELIN on template RNA binding activity was tested (Fig. 4A and Table 1). SpHINGOMYELIN enhanced the template RNA binding of HCR6 (1b) RdRp wt but not that of JFH1 (2a), H6CF (2a), or H77 (1a) wt RdRp. When the

A238S/Q248E mutation was introduced into H77 (1a) RdRp, the RNA binding was enhanced. J6CF (2a) RdRp R241Q and S244D mutants showed similar enhancement of RNA binding, but the R241Q/S244D double mutant did not. The activation effect of RNA binding of HCR6 (1b) RdRp wt and RdRp(L245A, I253A, and D244S) was lower than that of RdRp wt. JFH1 (2a) RdRp wt and RdRp(A242C/S244D) showed similar RNA binding activation levels. Based on a comparison of the spHINGOMYELIN activation of HCR6 (1b) RdRp wt and its mutants which lost spHINGOMYELIN binding with J6CF (2a) RdRp wt and the R241Q and S244D mutants and H77 (1a) RdRp wt and the A238S/Q248E mutant, we concluded that polymerase activation by spHINGOMYELIN was induced mainly via activation of the template RNA binding of RdRp. RNA binding activity of JFH1 (2a) RdRp wt and RdRp(A242C/S244D) was almost saturated because RNA binding of these RdRps was not activated by spHINGOMYELIN (see Fig. S4 in the supplemental material).

HCV RdRp has to be bound with spHINGOMYELIN before or at the same time as it binds to template RNA. After RdRp had bound to the template RNA, spHINGOMYELIN did not enhance template RNA binding strongly (Fig. 4B).

**Effect of the spHINGOMYELIN binding domain mutations for HCV replicon activity with myriocin.** In order to confirm spHINGOMYELIN activation of HCV polymerase activity in a viral replication system, HCV replicon activity of the loss-of-function mutant HCV NN (1b) NS5B(D244S) and the gain-of-

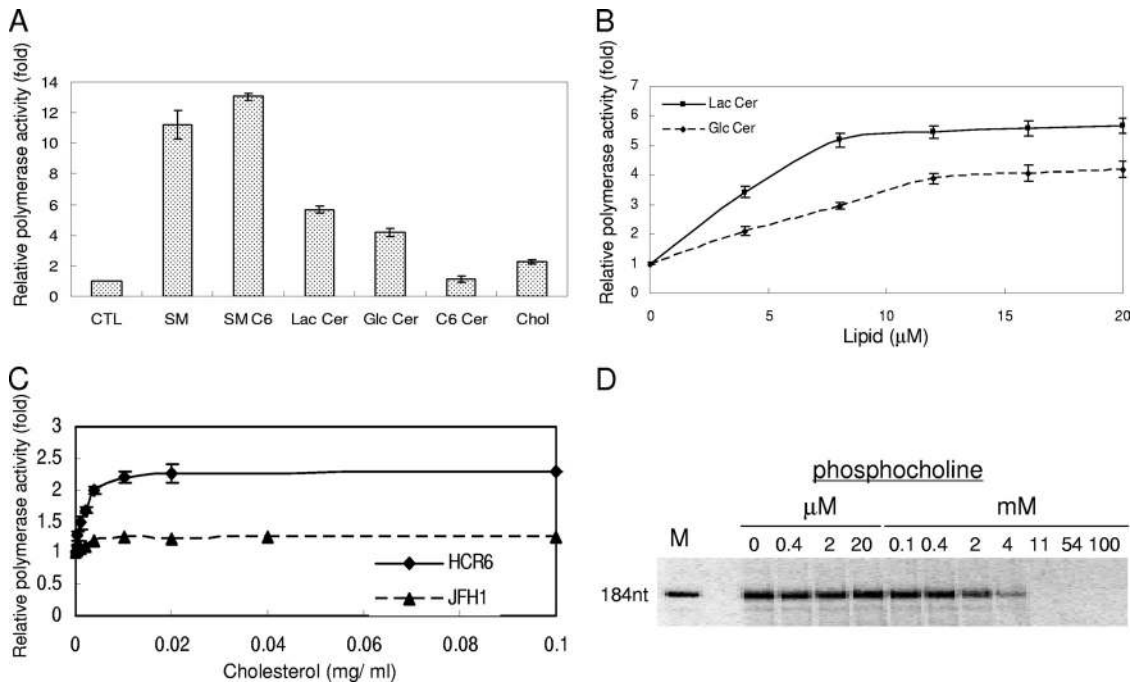


FIG. 3. HCV RNA polymerase activation effect of lipids. (A) Lipid activation of HCR6 (1b) RdRp wt. HCV HCR6 (1b) RdRp wt (100 nM) was incubated with or without (control [CTL]) 0.01 mg/ml egg yolk sphingomyelin (SM), 2 μM hexanoyl sphingomyelin (SM C6), 8 μM C<sub>8</sub>-lactosyl(β) ceramide (Lac Cer), 12 μM C<sub>8</sub>-β-D-glucosyl ceramide (Glc Cer), 12 μM C<sub>6</sub>-ceramide (C6 Cer), or 0.02 mg/ml cholesterol (chol). (B) Activation kinetics of C<sub>8</sub>-lactosyl(β) ceramide (Lac Cer) and C<sub>8</sub>-β-D-glucosyl ceramide (Glc Cer) on HCR6 (1) RdRp. (C) Activation kinetics of cholesterol on HCR6 (1b) and JFH1 (12a) RdRps. (D) The effect of phosphocholine on HCR6 (1b) RdRp. The mean ± standard deviation of the activation ratio was calculated from three independent experiments.

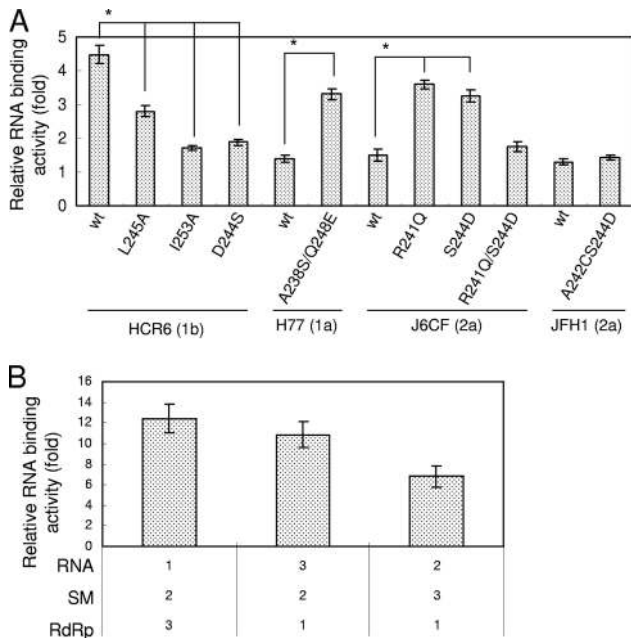


FIG. 4. Sphingomyelin activation of the RNA binding activity of HCV RNA polymerase. (A) Sphingomyelin activation of RNA filter binding of HCV RdRps (RdRp names are indicated below the graph). RdRps and <sup>32</sup>P-labeled RNA template (SL12-1S) were incubated with or without egg yolk sphingomyelin (SM), before filtration. (B) Effect of the order of sphingomyelin treatment. Numbers below the graph indicate the order in which the reagents were added. The graph represents the ratio to RNA binding without sphingomyelin. The mean ± standard deviation of the activation ratio was calculated from three independent experiments. \*, *P* < 0.01.

function mutants H77 (1a) NS5B(A238S/Q248E) and JFH1 (2a) NS5B(A242C/S244D) were compared with 5 and 50 nM myriocin treatment for 72 h (Fig. 5).

First, HCV replicon activity was compared as the relative luciferase activity (Fig. 5A). Both JFH1 (2a) wt and NS5B(A242C/S244D) replicons showed similar and strong replicon activity ( $133 \times 10^3 \pm 12 \times 10^3$  and  $138 \times 10^3 \pm 8.5 \times 10^3$ , respectively). JFH1 (2a) wt replicon was resistant to myriocin treatment, as reported by Aizaki et al. using other SPT inhibitors (3). The JFH1 (2a) NS5B(A242C/S244D) replicon became sensitive to myriocin but still showed higher replicon activity than NN (1b) or H77 (1a) replicons even at 50 nM myriocin.

To analyze the effect of mutations precisely, the replicon activity relative to each wt strain was compared (Fig. 5B). The JFH1 (2a) wt replicon with 50 nM myriocin showed the same luciferase activity as the wt without myriocin ( $102\% \pm 9.6\%$ ). JFH1 (2a) NS5B(A242C/S244D) replicon activity was the same as that of the wt without myriocin ( $103\% \pm 12\%$ ); with 5 nM myriocin it was  $84.1\% \pm 6.6\%$  of the wt level, but with 50 nM myriocin it was  $70.3\% \pm 5.3\%$  of the wt level, which was significantly lower (*P* < 0.01). NN (1b) wt replicon activity was  $45.3\% \pm 6.6\%$  with 5 nM myriocin and  $21.7\% \pm 2.9\%$  with 50 nM myriocin relative to the wt level without myriocin. NN (1b) NS5B(D244S) replicon activity was  $72.2\% \pm 12\%$  without myriocin (*P* < 0.05),  $44.0\% \pm 7.4\%$  with 5 nM myriocin, and  $38.1\% \pm 4.2\%$  with 50 nM myriocin relative to wt level without myriocin, which was significantly higher (*P* < 0.01). Thus, NN (1b) NS5B(D244S) showed lower replicon activity than the wt



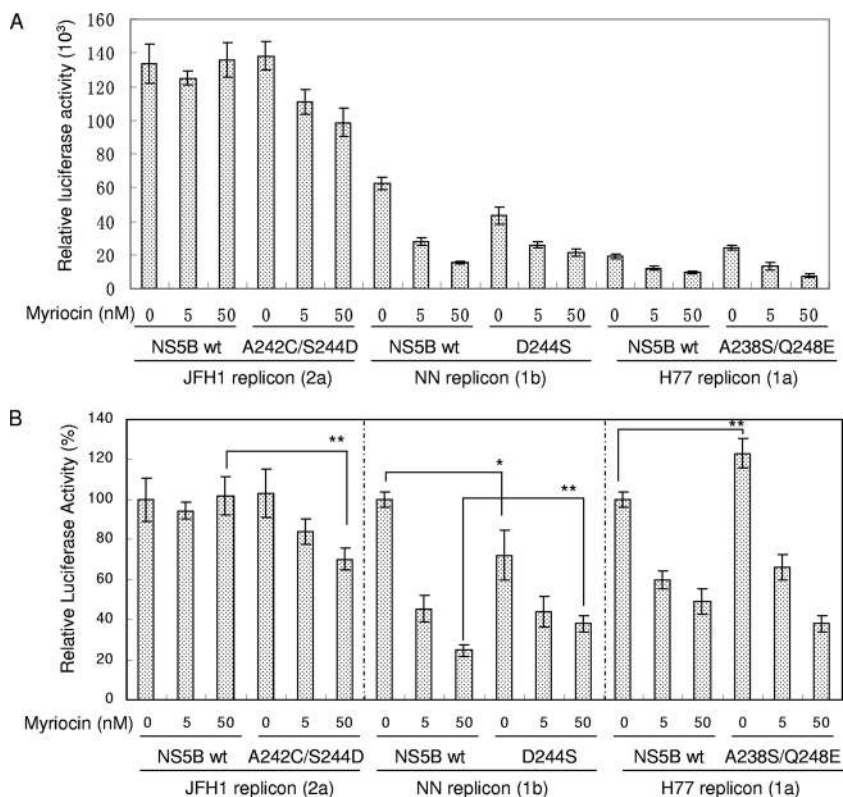


FIG. 5. Myriocin inhibition of HCV replicon activity. Huh7.5.1 cells were incubated with myriocin after transfection with the HCV replicons indicated below the graphs. Means  $\pm$  standard deviations of the relative luciferase activity at 72 h after myriocin treatment compared to activity at 4 h after transfection (A) and to that of each wt without myriocin (B) were calculated from three independent measurements. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

and was less sensitive to myriocin than the wt. H77 (1a) wt replicon activity was  $59.9\% \pm 4.2\%$  with 5 nM myriocin and  $49.2\% \pm 6.4\%$  with 50 nM myriocin relative to the wt level without myriocin. H77 (1a) NS5B(A238S/Q248E) replicon activity was  $123\% \pm 7.1\%$  without myriocin ( $P < 0.01$ ),  $66.1\% \pm 6.3\%$  with 5 nM myriocin, and  $38.0\% \pm 4.1\%$  with 50 nM myriocin relative to wt level without myriocin. Both H77 (1a) wt and NS5B(A238S/Q248E) replicons were sensitive to myriocin, and the replicon activity of NS5B(A238S/Q248E) was higher than that of the wt.

**JFH1 (2a) RdRp(A242C/S244D) localized in the DRM fractions.** Myriocin sensitivity of JFH1 (2a) NS5B(A242C/S244D) replicon indicates the importance of 244D in JFH1 NS5B for sphingomyelin binding. To further confirm the role of 244D for recruitment of HCV RdRp to the detergent-resistant membrane (DRM), where the HCV replication complex exists, we compared the distribution of NS5A and NS5B of JFH1 (2a) wt and NS5B(A242C/S244D) in their replicon cells by sucrose density gradient centrifugation of the DRM (Fig. 6). NS5A proteins of both JFH1 (2a) wt and NS5B(A242C/S244D) replicons localized in the DRM fraction where caveolin-2 was present (11, 27), but most of NS5B wt localized in the Triton-soluble fractions. NS5B of JFH1 (2a) NS5B(A242C/S244D) replicon was shifted to the DRM fraction from the soluble fraction. The shift of NS5B(A242C/S244D) localization into the DRM demonstrated that SBD was the DRM localization domain of NS5B and that residue 244D was important for this localization.

**DISCUSSION**

Hepatitis C virus is an envelope virus, and the lipid components of the virion play important roles in HCV infectivity and virion assembly (3, 15, 20, 24). HCV replication complexes localize in lipid raft structures/DRMs in the membrane frac-

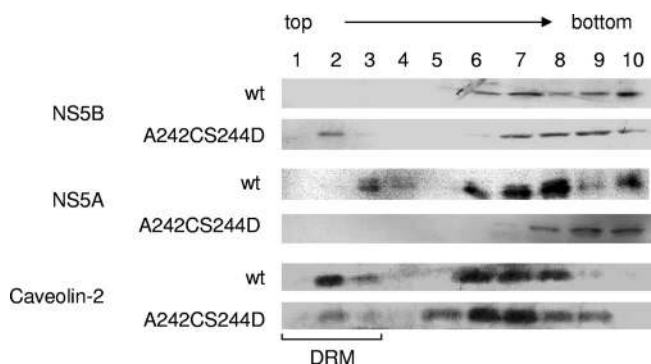


FIG. 6. Membrane floating assay of JFH1 wt and NS5B(A242C/S244D) replicon cells. The PNS fractions of HCV JFH1 (2a) wt and NS5B(A242C/S244D) replicon cells were treated with 1% Triton X-100 in TNE buffer for 30 min at 4°C and subjected to 10 to 40% sucrose gradient centrifugation in TNE buffer. Each fraction was subjected to 10% SDS-PAGE, followed by Western blotting with anti-NS5A, -NS5B, and -caveolin-2 antibodies. Fractions are numbered as indicated at the top of the panel. The DRM fractions (fractions 1 to 3) are indicated.

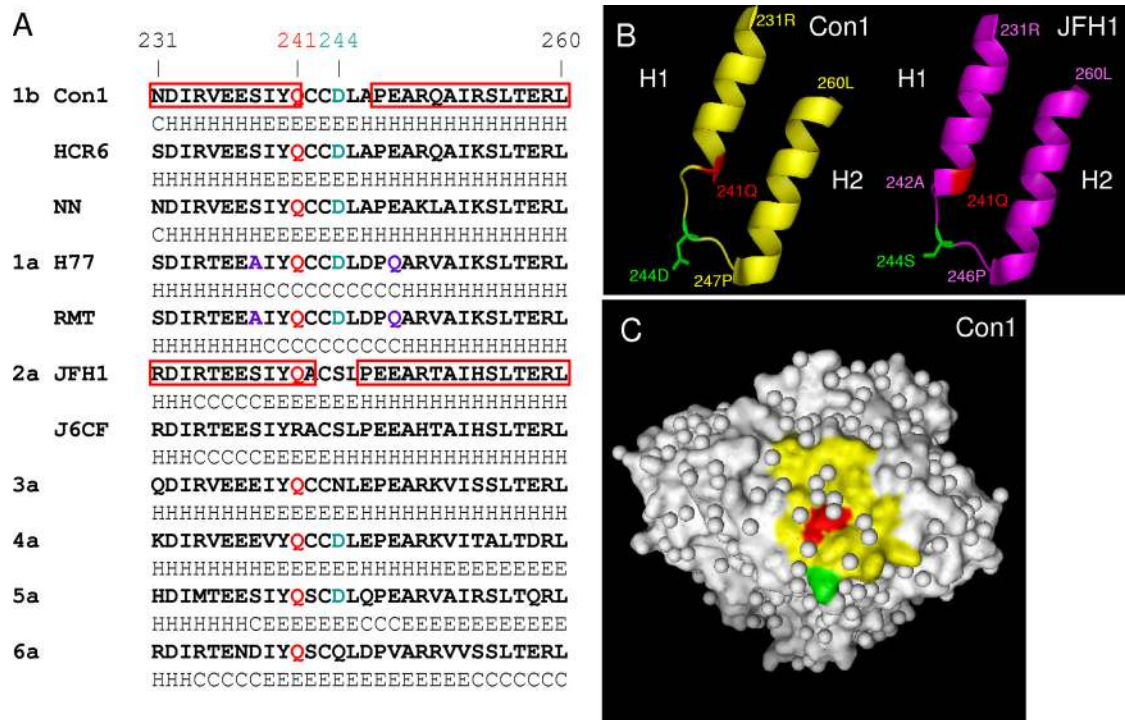


FIG. 7. Spingomyelin binding domain (SBD) of HCV RNA polymerase. (A) The SBDs (231N to 260L) of HCV RdRps are aligned together with their secondary structure predicted by the Chou-Fasman program (10). The predicted secondary structure is indicated below the sequence as follows: H,  $\alpha$ -helix; E,  $\beta$ -sheet; and C, coil. The  $\alpha$ -helix structures of HCV Con1 (1b) RdRp and JFH1 (2a) RdRp are boxed in red. Residues 241Q and 244D are indicated in red and green, respectively. The 238A and 248E of the H77 and RMT (1a) RdRps are indicated in purple. GenBank accession numbers of HCV genotypes 3a, 4a, 5a, and 6a are GU814263 (12), GU814265 (12), Y13184 (8), and Y12083 (1), respectively. (B) Comparison of the SBDs of HCV Con1 (1b) (yellow) and JFH1 (2a) RdRps (magenta). The starting and ending amino acids of H1 and H2 are indicated. The spingomyelin binding site, 241Q, is indicated in red, and 244D of Con1 (1b) and 244S of JFH1 (2a) RdRp are indicated in green. (C) Surface model of HCV Con1 (1b) RdRp. SBD is indicated in yellow, and 241Q and 244D are indicated in red and green, respectively. The structures of the Con1 and JFH1 RdRps were constructed by PyMOL, version 1.1.1 (<http://www.pymol.org/>). PDB numbers of Con1 (1b) RdRp and JFH1 (2a) RdRp are 3FQL (14) and 3I5K (31), respectively.

tions of subgenomic replicon cells (30). Lipid rafts are composed mainly of spingomyelin, cholesterol, and glycosphingolipids. Most reports regarding the relationship between lipids and HCV have examined virion assembly, infectivity, and the localization of HCV, but their biochemical interactions have not been reported. Our findings clearly demonstrate that spingomyelin plays an important role not only in HCV replication complex formation and its localization but also in HCV RdRp activity.

The helix-turn-helix structure of the SBD (residues 230 to 263), which is located between RNA polymerase motifs A and B, has been proposed as the spingomyelin binding domain of HCV RdRp (29). We compared the SBD of Con1 (1b) (Protein Data Bank [PDB] 3FQL) (14) and JFH1 (2a) (PDB 3I5K) (31) and the secondary structure of the amino acids (201 to 290) in the SBD predicted by the Chou-Fasman program (10) (Fig. 7; see also Fig. S5 in the supplemental material) because the helix structures of the SBD of Con1 (helix 1 [H1], 231N to 241Q; helix 2 [H2], 247A to 260L) and JFH1 (H1, 231R to 242A; H2, 246P to 260L) RdRp fit with those predicted by the Chou-Fasman program. The structures contributing to spingomyelin binding and activation are H1 and H2 and the junction (turn) between the two helix structures that are similar to the human immunodeficiency virus (HIV) gp120 V3 domain,

prion protein (PrP), and  $\beta$ -amyloid peptide (13, 22). Although Con1 (1b) RdRp has a shorter helix structure than JFH1 (2a) RdRp (Fig. 6B), the structures of their SBDs are very similar (Fig. 7; see also Fig. S5). When the helix-turn-helix structure of the SBD was destroyed (HCR6 genotype 1b RdRp mutants L245A and I253A), the RdRp lost spingomyelin binding activity and lost its activation (Fig. 2).

In order to study the structure-function relationship of the SBD and spingomyelin, we compared the SBD of genotype 1a, 1b and 2a RdRps and particularly focused on residue 244D in the turn and residues 241Q and 238S/248E in the helix domains. The polar amino acid 241Q and the negatively charged 244D of Con1 (1b) RdRp located on the surface of the RdRp molecule bind and interact with the positively charged choline residue of spingomyelin (Fig. 7C; see also Fig. S5 in the supplemental material). The positively charged 241R repels the choline residue of spingomyelin, and as a result, J6CF (a) RdRp wt did not bind to spingomyelin. J6CF (2a) RdRp(R241Q) showed almost the same spingomyelin binding activity as HCR6 (1b) RdRp wt. This ionic interaction between SBD and spingomyelin agrees with the activation of lipids with different sphingosine structures and fatty acid chains (Fig. 3A). JFH1 (2a) RdRp does not interact well with spingomyelin because it does not have the negatively charged



amino acids at the tip of its turn structure. Once its 244S was changed to D, more sphingomyelin bound to JFH1 (2a) RdRp and activated the RdRp (Fig. 2A and C). The reason for the low activation of J6CF (2a) RdRp(R241Q/S244D) is not clear. Sometimes mutations affect the entire conformation of the molecule. In conclusion, from the comparison of sphingomyelin binding and activation of HCR6 (1b), J6CF (2a), and JFH1 (2a) RdRp SBD mutants, 241Q is the essential amino acid for sphingomyelin binding in the SBD. Amino acid 244D enhanced both binding and RdRp activation.

The *in vitro* sphingomyelin binding and RdRp activation experiments indicate that sphingomyelin binding and its RdRp activation are different biochemical reactions because we found controversial activation rates for sphingomyelin binding and RdRp activation among J6CF (2a) RdRp mutants (Fig. 2). The relationship between sphingomyelin binding and the activation of polymerase activity was studied by comparing genotype 1b and 1a RdRps, both of which bind to sphingomyelin (Fig. 2). However, 1a RdRp is not activated by sphingomyelin because both of the helix structures of 1a RdRp are probably terminated at 238A and 248Q, making its helix structures shorter than those of 1b RdRp (Fig. 6A). The length of the helix structure may be essential for sphingomyelin activation because RdRp changes its structure to bind to template RNA when sphingomyelin binds to SBD (Fig. 4).

HCV RdRp changes its conformations at the early stages of transcription initiation, including the template RNA binding step (6, 9). Sphingomyelin binding is likely to change the conformation of 1b RdRp to recruit template RNA and initiate transcription efficiently. Comparison of the activation ratio of RNA binding and polymerase activity of 1b RdRp, J6CF (2a) RdRp wt and R241Q and S244D mutants, and JFH1 (2a) RdRp wt and mutant A242C/S244D suggests that steps other than RNA binding are also likely to be activated by sphingomyelin.

From a kinetic analysis of sphingomyelin activation (Fig. 1C and D), 20 sphingomyelin molecules are estimated to interact with the SBD of RdRp and activate it because sphingomyelin activation plateaued at 20 sphingomyelin molecules per HCV RdRp molecule. It is not clear whether 20 sphingomyelin molecules form a micelle or a layer structure. However, the structure of sphingomyelin is important for the activation of HCV RdRp because phosphocholine did not activate the RdRp (Fig. 3D).

To confirm these biochemical findings in HCV replication, we tested the effect of SBD mutations in HCV replicon systems with the SPT inhibitor myriocin (Fig. 5) (4, 33) because NA255 was not available. The loss-of-function mutant, HCV NN (1b) NS5B(D244S), showed lower replicon activity than NN (1b) wt and more resistance to 50 nM myriocin, which did not affect the viability of cells (4, 33), than the wt. The gain-of-function mutant, H77 (1a) NS5B(A238S/Q248E), showed higher replicon activity than H77 wt and retained myriocin sensitivity because it had the sphingomyelin binding sites 241Q and 244D. At 50 nM myriocin, another gain-of-function mutant, JFH1 (2a) NS5B(A242C/S244D), was inhibited although its activity was the same as that of JFH1 (2a) wt without myriocin because the JFH1 wt replicon had high replicon activity without myriocin (Fig. 5A). The JFH1 replicon activity may be maximal in the system; therefore, the JFH1 (2a) NS5B(A242C/S244D) replicon did not show higher activity than JFH1 (2a) wt with-

out myriocin while H77 (1a) NS5B(A238S/Q248E) showed higher replicon activity than H77 wt.

The binding and RdRp activation activity of the amino acid 244 mutants by sphingomyelin did not differ greatly from the wt *in vitro*. However, the myriocin sensitivity of JFH1 (2a) NS5B(S244D) was demonstrated clearly. That of H77 (1a) NS5B(A238S/Q248E) indicated that sphingomyelin binding was the target of myriocin inhibition, not the sphingomyelin activation of RdRp. These data confirm the importance of 241Q, 244D, and the helix structure in SBD for HCV replication in the cells.

Sphingomyelin is the major component of the lipid raft structure/DRM where the HCV genome replicates. To confirm that the SBD is the membrane binding site of HCV RdRp, we analyzed the localization of NS5B of JFH1 (2a) wt and NS5B(A242C/S244D) replicons by membrane floating assay (Fig. 6). JFH1 (2a) NS5B wt did not localize in the DRM. However, the localization of NS5B of the JFH1 (2a) NS5B(A242C/S244D) replicon shifted to the DRM from the soluble fractions. Previously, HCV NS5B was believed to localize in the DRM by its C-terminal hydrophobic sequences (21). However, our data demonstrate that the SBD is the membrane localization domain of HCV NS5B, which agrees with the myriocin sensitivity of JFH1 (2a) NS5B(A242C/S244D) replicons (Fig. 5) and the release of HCV 1b NS5B from the DRM by another SPT inhibitor, NA255 (29).

This is the first report of RNA polymerase activation by lipids. Twenty sphingomyelin molecules interact with SBD, particularly with residues 241Q and 244D of HCV (1b) RdRp, and change the conformation of the RdRp in order to recruit RNA templates. At the same time, HCV RdRp molecules may be aligned on the sphingomyelin layer formed via interactions between the hydrocarbon chains of sphingosine and fatty acids via placement of their SBD into the layer (Fig. 7C). Consistent with previous research (3, 23, 37), our findings explain why the inhibitors of the sphingolipid biosynthetic pathway influence subgenomic replicons derived from HCV genotypes 1a and 1b but not those derived from JFH1 (2a) (Fig. 5). Most HCV isolates have 241Q in NS5B, and some of them also have 244D (Fig. 7A). These sphingomyelin interactions are new targets for the treatment of HCV.

#### ACKNOWLEDGMENTS

We thank C. Rice and R. Bartenschlager for the HCV H77 and Con1 plasmids, respectively. We also thank F. Chisari for Huh7.5.1 and Huh7/src cells.

This work was supported by a grant-in-aid from the Chinese Academy of Sciences (O514P51131 and KSCX1-YW-10), the Chinese 973 project (2009CB522504), and the Chinese National Science and Technology Major Project (2008ZX10002-014).

#### REFERENCES

- Adams, N. J., R. W. Chamberlain, L. A. Taylor, F. Davidson, C. K. Lin, R. M. Elliott, and P. Simmonds. 1997. Complete coding sequence of hepatitis C virus genotype 6a. *Biochem. Biophys. Res. Commun.* **234**:393–396.
- Aizaki, H., K. J. Lee, V. M. Sung, H. Ishiko, and M. M. Lai. 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* **324**:450–461.
- Aizaki, H., K. Morikawa, M. Fukasawa, H. Hara, Y. Inoue, H. Tani, K. Saito, M. Nishijima, K. Hanada, Y. Matsuura, M. M. Lai, T. Miyamura, T. Wakita, and T. Suzuki. 2008. Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. *J. Virol.* **82**:5715–5724.
- Amemiya, F., S. Maekawa, Y. Itakura, A. Kanayama, A. Matsui, S. Takano, T. Yamaguchi, J. Itakura, T. Kitamura, T. Inoue, M. Sakamoto, K. Yamau-

- chi, S. Okada, A. Yamashita, N. Sakamoto, M. Itoh, and N. Enomoto. 2008. Targeting lipid metabolism in the treatment of hepatitis C virus infection. *J. Infect. Dis.* **197**:361–370.
5. Binder, M., D. Quinkert, O. Bochkarova, R. Klein, N. Kezmic, R. Bartenschlager, and V. Lohmann. 2007. Identification of determinants involved in initiation of hepatitis C virus RNA synthesis by using intergenotypic replicase chimeras. *J. Virol.* **81**:5270–5283.
  6. Biswal, B. K., M. M. Cherney, M. Wang, L. Chan, C. G. Yannopoulos, D. Bilimoria, O. Nicolas, J. Bedard, and M. N. James. 2005. Crystal structures of the RNA-dependent RNA polymerase genotype 2a of hepatitis C virus reveal two conformations and suggest mechanisms of inhibition by non-nucleoside inhibitors. *J. Biol. Chem.* **280**:18202–18210.
  7. Blight, K. J., J. A. McKeating, J. Marcotrigiano, and C. M. Rice. 2003. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. *J. Virol.* **77**:3181–3190.
  8. Chamberlain, R. W., N. J. Adams, L. A. Taylor, P. Simmonds, and R. M. Elliott. 1997. The complete coding sequence of hepatitis C virus genotype 5a, the predominant genotype in South Africa. *Biochem. Biophys. Res. Commun.* **236**:44–49.
  9. Chinnaswamy, S., I. Yarbrough, S. Palaninathan, C. T. Kumar, V. Vijayaraghavan, B. Demeler, S. M. Lemon, J. C. Sacchettini, and C. C. Kao. 2008. A locking mechanism regulates RNA synthesis and host protein interaction by the hepatitis C virus polymerase. *J. Biol. Chem.* **283**:20535–20546.
  10. Chou, P. Y., and G. D. Fasman. 1974. Prediction of protein conformation. *Biochemistry* **13**:222–245.
  11. Fujimoto, T., H. Kogo, K. Ishiguro, K. Tauchi, and R. Nomura. 2001. Caveolin-2 is targeted to lipid droplets, a new “membrane domain” in the cell. *J. Cell Biol.* **152**:1079–1085.
  12. Gottwein, J. M., T. K. Scheel, B. Callendret, Y. P. Li, H. B. Eccleston, R. E. Engle, S. Govindarajan, W. Satterfield, R. H. Purcell, C. M. Walker, and J. Bukh. Novel infectious cDNA clones of hepatitis C virus genotype 3a (strain S52) and 4a (strain ED43): genetic analyses and *in vivo* pathogenesis studies. *J. Virol.* **84**:5277–5293.
  13. Hammache, D., G. Pieroni, N. Yahi, O. Delezay, N. Koch, H. Lafont, C. Tamalet, and J. Fantini. 1998. Specific interaction of HIV-1 and HIV-2 surface envelope glycoproteins with monolayers of galactosylceramide and ganglioside GM3. *J. Biol. Chem.* **273**:7967–7971.
  14. Hang, J. Q., Y. Yang, S. F. Harris, V. Leveque, H. J. Whittington, S. Rajyaguru, G. Ao-Ieong, M. F. McCown, A. Wong, A. M. Giannetti, S. Le Pogam, F. Talamas, N. Cammack, I. Najera, and K. Klumpp. 2009. Slow binding inhibition and mechanism of resistance of non-nucleoside polymerase inhibitors of hepatitis C virus. *J. Biol. Chem.* **284**:15517–15529.
  15. Huang, H., F. Sun, D. M. Owen, W. Li, Y. Chen, M. Gale, Jr., and J. Ye. 2007. Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. *Proc. Natl. Acad. Sci. U. S. A.* **104**:5848–5853.
  16. Ishii, N., K. Watashi, T. Hishiki, K. Goto, D. Inoue, M. Hijikata, T. Wakita, N. Kato, and K. Shimotohno. 2006. Diverse effects of cyclosporine on hepatitis C virus strain replication. *J. Virol.* **80**:4510–4520.
  17. Kashiwagi, T., K. Hara, M. Kohara, K. Kohara, J. Iwahashi, N. Hamada, H. Yoshino, and T. Toyoda. 2002. Kinetic analysis of C-terminally truncated RNA-dependent RNA polymerase of hepatitis C virus. *Biochem. Biophys. Res. Commun.* **290**:1188–1194.
  18. Kato, T., T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, and T. Wakita. 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* **125**:1808–1817.
  19. Kiyosawa, K., T. Sodeyama, E. Tanaka, Y. Gibo, K. Yoshizawa, Y. Nakano, S. Furuta, Y. Akahane, K. Nishioka, R. H. Purcell, et al. 1990. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* **12**:671–675.
  20. Lambot, M., S. Fretier, A. Op De Beeck, B. Quatannens, S. Lestavel, V. Clavey, and J. Dubuisson. 2002. Reconstitution of hepatitis C virus envelope glycoproteins into liposomes as a surrogate model to study virus attachment. *J. Biol. Chem.* **277**:20625–20630.
  21. Lemon, S., C. Walker, M. Alter, and M. Yi. 2007. Hepatitis C virus, p. 1253–1304. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), *Fields virology*, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
  22. Mahfoud, R., N. Garmy, M. Maresca, N. Yahi, A. Puigserver, and J. Fantini. 2002. Identification of a common sphingolipid-binding domain in Alzheimer, prion, and HIV-1 proteins. *J. Biol. Chem.* **277**:11292–11296.
  23. Miyake, Y., Y. Kozutsumi, S. Nakamura, T. Fujita, and T. Kawasaki. 1995. Serine palmitoyltransferase is the primary target of a sphingosine-like immunosuppressant, ISP-1/myricin. *Biochem. Biophys. Res. Commun.* **211**:396–403.
  24. Miyanari, Y., K. Atsuzawa, N. Usuda, K. Watashi, T. Hishiki, M. Zayas, R. Bartenschlager, T. Wakita, M. Hijikata, and K. Shimotohno. 2007. The lipid droplet is an important organelle for hepatitis C virus production. *Nat. Cell Biol.* **9**:1089–1097.
  25. Murayama, A., T. Date, K. Morikawa, D. Akazawa, M. Miyamoto, M. Kaga, K. Ishii, T. Suzuki, T. Kato, M. Mizokami, and T. Wakita. 2007. The NS3 helicase and NS5B-to-3′X regions are important for efficient hepatitis C virus strain JFH-1 replication in Huh7 cells. *J. Virol.* **81**:8030–8040.
  26. Murayama, A., L. Weng, T. Date, D. Akazawa, X. Tian, T. Suzuki, T. Kato, Y. Tanaka, M. Mizokami, T. Wakita, and T. Toyoda. 2010. RNA polymerase activity and specific RNA structure are required for efficient HCV replication in cultured cells. *PLoS Pathog.* **6**:e1000885.
  27. Ostermeyer, A. G., J. M. Paci, Y. Zeng, D. M. Lublin, S. Munro, and D. A. Brown. 2001. Accumulation of caveolin in the endoplasmic reticulum redirects the protein to lipid storage droplets. *J. Cell Biol.* **152**:1071–1078.
  28. Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, et al. 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. U. S. A.* **87**:6547–6549.
  29. Sakamoto, H., K. Okamoto, M. Aoki, H. Kato, A. Katsume, A. Ohta, T. Tsukuda, N. Shimma, Y. Aoki, M. Arisawa, M. Kohara, and M. Sudoh. 2005. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. *Nat. Chem. Biol.* **1**:333–337.
  30. Shi, S. T., K. J. Lee, H. Aizaki, S. B. Hwang, and M. M. Lai. 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. *J. Virol.* **77**:4160–4168.
  31. Simister, P., M. Schmitt, M. Geitmann, O. Wicht, U. H. Danielson, R. Klein, S. Bressanelli, and V. Lohmann. 2009. Structural and functional analysis of hepatitis C virus strain JFH1 polymerase. *J. Virol.* **83**:11926–11939.
  32. Tsukiyama-Kohara, K., S. Tone, I. Maruyama, K. Inoue, A. Katsume, H. Nuriya, H. Ohmori, J. Ohkawa, K. Taira, Y. Hoshikawa, F. Shibasaki, M. Reth, Y. Minatogawa, and M. Kohara. 2004. Activation of the CKI-CDK-Rb-E2F pathway in full genome hepatitis C virus-expressing cells. *J. Biol. Chem.* **279**:14531–14541.
  33. Umehara, T., M. Sudoh, F. Yasui, C. Matsuda, Y. Hayashi, K. Chayama, and M. Kohara. 2006. Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model. *Biochem. Biophys. Res. Commun.* **346**:67–73.
  34. Wasley, A., and M. J. Alter. 2000. Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin. Liver Dis.* **20**:1–16.
  35. Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno. 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol. Cell* **19**:111–122.
  36. Weng, L., J. Du, J. Zhou, J. Ding, T. Wakita, M. Kohara, and T. Toyoda. 2009. Modification of hepatitis C virus 1b RNA polymerase to make a highly active JFH1-type polymerase by mutation of the thumb domain. *Arch. Virol.* **154**:765–773.
  37. Yasuda, S., H. Kitagawa, M. Ueno, H. Ishitani, M. Fukasawa, M. Nishijima, S. Kobayashi, and K. Hanada. 2001. A novel inhibitor of ceramide trafficking from the endoplasmic reticulum to the site of sphingomyelin synthesis. *J. Biol. Chem.* **276**:43994–44002.
  38. Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari. 2005. Robust hepatitis C virus infection *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **102**:9294–9299.