ourselves and others have identified a unique HCV genotype 2a isolate, JFH-1, that is able to replicate and produce high levels of infectious virus in culture (HCVcc)(54, 56), enabling us to investigate new aspects of the HCV life cycle.

In this study, we examine the importance of cholesterol and sphingolipid in association with the HCV membrane in virion maturation and virus infectivity. Mature HCV particles are rich in cholesterol. Cholesterol depletion or hydrolysis of sphingolipid from HCV particles results in a loss of infectivity. We further demonstrate a requirement for virion-associated cholesterol and sphingolipid for viral entry.

MATERIALS AND METHODS

Cell Culture. The human hepatoma cell line Huh-7, which is permissive to HCV infection, was obtained from Francis V. Chisari (The Scripps Research Institute). Human embryonic kidney 293T cells were cultured in DMEM-10% FBS. Huh-7 cell lines, which carry subgenomic replicon RNA of either the JFH-1 (20) or N strain (11, 17), were cultured as previously described (21, 46).

Reagents. The primary antibodies used in this study were mouse monoclonal antibodies against vesicular stomatitis virus-glycoprotein (VSV-G)(Sigma, St. Louis, MO), HCV E1 (54) and E2 (Biodesign International, Saco, ME), caveolin-2 (New England Biolads, Beverly, MA), CD81 (BD Pharmingen, Franklin Lake, NJ), as well as rabbit polyclonal antibodies against calnexin (Stressgen, Ann Arbor, MI) and HCV core (48). ISP-1/myriocin, cholesterol, and heparinase I were purchased from Sigma, and recombinant *Bacillus cereus* SMase was obtained from Higeta Shoyu (Tokyo, Japan). (1R, 3R)-N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl) dodecanamide (HPA-12), which was synthesized as described (24), is a gift from Dr. Shu Kobayashi (University of Tokyo).

Plasmids. pCAE1 and pCAE2 contain HCV cDNAs spanning E1 region (aa 192-383) with a FLAG tag at the N-terminus and E2 (aa 384-809) region with a Myc tag at the N-terminus of NIHJ1 strain (1), respectively, under the control of the CAG promoter (38). pCAV340V and pCAV711V consist of the ectodomains of E1 and E2, respectively, with the N-terminal signal sequences, transmembrane, and cytoplasmic domains derived from VSV-G, as described (50) (Fig. 4D).

Virus production. The plasmid pJFH1, containing full-length cDNA of the JFH-1 isolate, was used to generate HCVcc as described (23, 33, 34, 54). In vitro transcribed RNA from the linearized pJFH1 was delivered to Huh-7 cells by electroporation. Culture supernatants

were collected at 72 h post-transfection, clarified by low-speed centrifugation, passed through a 0.45-mm filter, and concentrated using Amicon Ultra-15 (Millipore, Bedford, MA) or by ultracentrifugation (23). Infectious titers, HCV RNA, and core protein concentrations of the viral stocks were ~ 5 x 10^3 focus forming units per ml, ~1 x 10^7 copies/ml, and ~1 x 10^4 fmol/L, respectively. HCVcc was isolated by a combination of ultrafiltration, ion exchange chromatography, heparin affinity chromatography, and sucrose density ultracentrifugation (33, K. Morikawa and T. Wakita, unpublished data). Pseudotyped VSV containing E1 and E2 proteins of HCV genotype 1a, H77c, isolate (HCVpv) was generated as previously described (51). Briefly, 293T cells transiently expressing E1 and E2 proteins (H77 strain) were infected with VSVdelG-GFP/G, in which the G envelope gene was replaced with a green fluorescent protein and pseudotyped with VSV-G.

Determination of cholesterol and phospholipid content of HCVcc and infected cells. Cellular and viral lipids were extracted from isolated HCVcc and from uninfected and infected Huh-7 cells. Cholesterol content was determined using the cholesterol oxidase method as previously described (15). Total phospholipid content was determined using the method of Rouser et al. (42).

Cholesterol depletion and replacement. To remove cholesterol from the HCV envelope, stock samples of HCVcc were treated with methyl-β-cyclodextrin (B-CD) in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and nonessential amino acids (Invitrogen, Carlsbad, CA) for 1 h at 37°C, followed by centrifugation at 100,000 g for 3 h to form a pellet, which was resuspended in 0.5 ml of the medium. In order to replenish cholesterol, the medium of HCVcc treated with 5 mg/ml B-CD was replaced with DMEM containing various concentrations of exogenous

cholesterol (Sigma) and incubated for 1 h, followed by centrifugation to form a pellet. In order to perform HCVcc infection assays, Huh-7 cells were infected with HCVcc with or without the above treatment for 1 h at 37°C and then washed as described above. Viral core protein in the cells and in the supernatant was quantified 72 h later using HCV core ELISA (Ortho-Clinical Diagnostics, Tokyo, Japan).

SMase treatment. HCVcc was treated with sphingomyelinase (SMase) at various concentrations in DMEM for 1 h at 37°C and then centrifuged at 100,000 g for 3 h to form a pellet, which was resuspended in 0.5 ml of medium for the infection assays.

HCVcc binding and internalization assays. To monitor binding, cells grown in a 6-well plate were preincubated for 1 h at 4°C, after which B-CD- or SMase-treated HCVcc were bound to the cells for 1 h at 4°C. As a measure of virus internalization, following the virus binding procedure, the cells were warmed to 37°C and maintained for 2 h, after which they were treated with 0.25% trypsin for 10 min at 37°C. Huh7-25, which is a CD81-negative Huh-7 subclone (3), was used to ensure removal of surface-bound virus by trypsin treatment. For both the binding and internalization assays, the resulting cells as described above were washed with ice-cold PBS, followed by lysis with TRIzol reagent (Invitrogen). Cell-associated virus was quantified by measuring the amount of HCV RNA in the cell lysate by the real-time RT-PCR method (2, 34). The heparinase treatment of cells was performed as previously described (33).

HCV replication assay in HCVcc infected- or replicon cells. HCV subgenomic replicon cells or cells infected with HCVcc were treated with various concentrations of inhibitors for 72 h. Total RNA was isolated from replicon cells using TRIzol Reagent (Invitrogen), followed by quantification of HCV RNA by real-time RT-PCR as previously described (2, 34). Levels of core protein in the culture supernatant of HCVcc-infected cells were tested

as described above.

Detection of cholesterol content of HCVcc. For [^{3}H]cholesterol labeling of viruses, HCVcc- or non-infected cells were incubated with 50 mCi of [1a, 2a-^{3}H] cholesterol in DMEM for 24 h. Culture supernatants of the cells were incubated in the presence or absence of B-CD at 5 mg/ml for 1 h at 37°C, followed by ultracentrifugation on a 60% sucrose cushion. The virus-containing fractions and corresponding ones from uninfected culture were lysed in the buffer containing 1% TX100 and radioactivity was quantified by scintillation counting. Radioactivities (cpm) of HCVcc samples were determined by subtracting that of uninfected cells from that of HCVcc-infected cells.

Metabolic labeling analysis of sphingolipid content. After 2 h incubation with [^{14}C] serine (0.5 mCi/ml) in Opti-MEM (Invitrogen), the cells were lysed with 0.1% SDS, after which total lipid was extracted with chloroform/methanol (1:2 v/v). The extracts were spotted onto Silica Gel 60 plates (Merck, Darmstadt, Germany) and chromatographed with methyl acetate/1-propanol/chloroform/methanol/0.25% KCl (25:25:25:10:9, v/v). Radioactive spots were quantitatively detected by BAS 2000 (Fuji Film, Japan).

Membrane flotation assay. The membrane flotation assay was performed as previously described (46).

RESULTS

Critical role of virion-associated cholesterol. A role of virion-associated cholesterol in infectivity has been demonstrated for several enveloped viruses (5). However, little is known about the role of lipids associated with the flavivirus virion including HCV and their contribution to the viral lifecycle. To determine lipid composition of mature HCV virions, we extracted total lipid from HCVcc (JFH-1 and chimeric J6/JFH-1) prepared from the culture supernatant of cells infected with HCV, as well as the total cellular membrane fractions of uninfected and infected Huh-7 cells. The cholesterol and phospholipid content was quantified, since these are the two major lipid constituents of biological membranes. The cholesterol to phospholipid molar ratio, which is known as a parameter of membrane viscosity (47), was significantly increased in virus samples (1.29 and 1.26, respectively), compared to cell membrane samples (0.40 and 0.42, respectively)(Table 1). The ratios of viral samples were similar or greater than those of mammalian plasma membranes where most cellular cholesterol is found. Minimal contamination of the viral samples with extracellular microvesicles likely occurred since only a small amount of lipid was detected in a sample prepared from culture medium of uninfected cells (data not shown). Thus, it is likely that HCV virions are enriched with cholesterol during assembly and maturation.

To investigate a potential role for the particular lipid composition of HCV particles, HCVcc was treated with increasing concentrations (0.1-5 mg/ml) of B-CD, which is known to extract cholesterol from membranes (40). The viral samples were then used to inoculate Huh-7 cells after removing B-CD by ultracentrifugation, after which infectivity was evaluated by quantifying the viral core protein in cells at 72 h post-infection (p.i.). Using an immunoassay that provide results indicative of HCV infectivity (25), we also

confirmed a good correlation between the core level and infectious titers (data not shown). As shown in Fig. 1A, core protein levels following B-CD treatment at 0.1, 1, and 5 mg/ml were reduced by 60, 83 and 98%, respectively, compared to the results obtained with untreated virus. The cholesterol level of HCVcc treated with 5 mg/ml B-CD was found to be ~50% relative to untreated virions (Table 2).

To demonstrate that the reduced infection efficiency of B-CD-treated virus was caused by the reduced cholesterol content in the viral envelope, we attempted to reverse the inhibitory effect by adding exogenous cholesterol. Following treatment of HCVcc with 5 mg/ml B-CD, the drug was washed out and increasing concentrations of cholesterol were supplemented in an attempt to reconstitute the normal virion cholesterol content. The addition of 1 mM of cholesterol completely reversed the total amounts of HCV core protein (Fig. 1B) and the viral RNA (data not shown) in infected cells.

To investigate the effect of cholesterol on the density of infectious HCV virions, B-CD-pretreated or untreated viral samples, as well as cholesterol-replenished treated viral samples, were subjected to sucrose density gradient centrifugation (Fig.1C). The peak density of core protein of HCVcc was ~1.17 g/ml. When virion-associated cholesterol was removed by B-CD, the peak density of HCVcc was shifted to 1.20 g/ml. Addition of exogenous cholesterol to this cholesterol-depleted sample restored lower density fraction (1.15 g/ml). Fig. 1D illustrates the infectivity of each gradient fraction. Untreated virus had maximum infectivity at ~1.13 g/ml (fraction 6), while, as expected, fractions from B-CD-treated viral samples exhibited minimal to no infectivity. Replenishment of depleted virus with cholesterol returned infectivity to untreated control levels, and cholesterol-replenished virus had a buoyant density of ~1.07 g/ml (fraction 4), suggesting that HCV-associated cholesterol is crucial for viral infectivity and that the effect of

cholesterol-depleting drug is reversible. We further observed that B-CD treatment of a pseudotyped VSV containing E1 and E2 proteins of HCV genotype 1a, H77c, isolate (HCVpv) resulted in a progressive loss of infectivity, while B-CD had significantly less impact on infectivity of the control virus VSVdelG-GFP/G (Fig. 1E).

The above results raise the possibility that the infectivity of HCV virions with relatively low levels of incorporated cholesterol might be enhanced by supplementation with exogenous cholesterol. Density gradient fractions of culture supernatant collected from HCV-infected cells were analyzed with regard to the presence of core protein and infectivity (Fig. 1F; left). As indicated above, maximum infectivity was obtained with fraction 6 (1.13 g/ml). In contrast, a major fraction of core protein banded at a higher density (1.17 g/ml) in fraction 8. We hypothesized that fraction 8 contains lipids at lower level compared to fraction 6. However, quantification of lipids including cholesterol in the fractions obtained failed presumably because of low sensitivity of detection. Thus, to extend our findings on involvement of cholesterol, we added exogenous cholesterol to fraction 8, followed by ultrafiltration to remove unincorporated cholesterol. A subsequent density gradient profile demonstrated a shift in the core protein peak to 1.13 g/ml (Fig. 1F; middle). A concomitant increase in infectivity of the fraction was observed, approaching that of untreated fraction 6 (Fig. 1F; right). In contrast, supplementing fraction 6 with exogenous cholesterol did not alter its infectivity (Fig. 1F; right) or change its density gradient (data not shown). These results suggest that exogenous cholesterol supplementation can reverse deficits in the infectivity of HCV virions due to low cholesterol content.

Sphingolipid dependence of HCV infectivity. In addition to cholesterol, sphingolipid is a major component of eukaryotic lipid membranes. We therefore investigated the functional

significance of sphingomyelin (SM), the most abundant sphingolipid, with regard to HCV infectivity. HCVcc was treated for 1 h with increasing concentrations (0.1-10 U/ml) of bacterial SMase, which is known to hydrolyze membrane-bound SM to ceramide. Following ultracentrifugation to remove the SMase, the Huh-7 cells were inoculated with the HCVcc. The amount of HCV core protein within the cells was quantified at 72 h p.i. Fig. 2A shows a reduction in HCV infectivity by 50 and 90% following incubation of the virion with 0.1 and 1 U/ml SMase, respectively. We further observed that SMase treatment of HCVpv resulted in a progressive loss of infectivity, while SMase had no effect on infectivity of the control virus (Fig. 2B). This demonstrates that sphingolipid, like cholesterol, play an essential role in HCV infectivity.

Requirement for virion-associated cholesterol and sphingolipid during HCV cell entry. These findings support the idea that virion-associated cholesterol and sphingolipid may influence viral entry into host cells by altering the interaction between viral particles and host cell factor(s). Viral entry is a multistep process including binding of the virion to the cell surface and internalization into the cytoplasm by endocytosis. To examine whether virion-associated cholesterol and SM might play a role in cell binding or post-binding events during viral entry, we used a binding assay in which Huh-7 cells pre-incubated for 1 h at 4°C were infected with B-CD- or SMase-treated HCVcc. Total RNA was extracted after a 1 h addition of the virions at 4°C, followed by quantification of HCV RNA. As shown in Fig. 3A, treatment of the virions with either B-CD or SMase had little influence on their ability to bind to cells.

It has been shown that CD81 plays an important role in HCV internalization but is not correlated with viral attachment (7, 33). An anti-CD81 antibody was used as a negative control in reducing the viral attachment. It is likely that heparan sulfate proteoglycan on

the target cell surface is needed for the initial attachment of HCV (33). Thus, heparinase I was used as a positive control for reduced HCV attachment to the cells. To examine the role of cholesterol and sphingolipid on the HCVcc membrane in viral internalization, a virus-cell mixture prepared at 4°C as described above was incubated for 2 h at 37°C, followed by trypsinization to remove virions that were surface-bound but not internalized (Fig. 3B). We verified that 94% of surface bound-viruses were removed by trypsinization using CD81-negative Huh-7 subclones. A marked reduction in viral RNA within cells was detected after pretreatment of the virus with either β-CD or SMase. These results strongly suggest that virion-associated cholesterol and sphingolipid function as key determinants of internalization but not cell attachment.

Association of HCV structural proteins with lipid rafts. Cholesterol and sphingolipid are major components of lipid rafts, which can be isolated as detergent-resistant membranes (DRMs) by cold Triton X-100 (TX100) treatment, followed by equilibrium flotation centrifugation. Matto et al. (30) reported that HCV core protein is associated with DRMs in cells carrying the full-length HCV replicon. To investigate whether HCV structural proteins are associated with DRMs in HCVcc-producing cells, lysate from cells infected with HCVcc was subjected to membrane flotation analysis. In the absence of detergent treatment, the majority of core (Fig. 4A) and E1 (Fig. 4B) proteins were detected in the membrane fractions. After treatment with cold TX100, significant amounts of both viral proteins were recovered from the DRM fraction. However, after treatment with TX100 at 37°C, the majority of E1 and core proteins had shifted to the detergent-soluble fractions. We also found that HCV genotype 1b E1 and E2 can be associated with the lipid raft in 293T cells transfected with E1 or E2 expression plasmid (Fig. 4C), and that cytoplasmic tails of envelope proteins are important for their interaction (Fig. 4D). These

data suggest that subpopulations of HCV structural proteins are associated with lipid rafts in cells generating the HCV particles.

Moderate inhibition of HCV infection by B-CD- or SMase treatment of host cells. It has recently been reported that cholesterol depletion or sphingomyelin hydrolysis from host cell membrane decreases HCV infection, in part by causing the decreased level of cell surface CD81 (18, 51). Involvement of lipid environment of host cell plasma membrane in the HCV infection was investigated in our HCVcc infection system. Prior to the infection, Huh-7 cells were treated with B-CD or SMase, followed by being washed with the medium five times. Cholesterol depletion by B-CD at 1 and 5 mg/ml from Huh-7 cells, respectively, inhibited HCV core levels by 20 and 75% when compared to untreated cells (Fig. 5A). We also found that hydrolysis of sphingomyelin by SMase at 1 and 10 U/ml on the cells, respectively, led to moderate reduction of the viral infection by 20 and 55% of untreated control (Fig. 5B). There was no influence on cell viability under the conditions with these treatments (data not shown). As compared with the results in Fig. 1A and 2A, it is likely that raft-like environment on the plasma membrane possibly serve as a portal for HCV entry, but HCV virion-associated cholesterol and sphingolipid more readily play a critical role in the viral infection.

Inhibitors of the sphingolipid biosynthetic pathway suppress the production of HCVcc, but not RNA replication, of a JFH-1-derived replicon. In the course of studying involvement of lipid metabolism in the HCV lifecycle, we observed that inhibitors of the sphingolipid biosynthetic pathway, including ISP-1 and HPA-12, which specifically inhibit serine palmitoyltransferase (31) and ceramide trafficking from the ER to the Golgi apparatus (55), influenced subgenomic replicons derived from the HCV-N isolate (genotype 1b), but not those derived from JFH-1. A dose-dependent decrease in

HCV RNA copy numbers among HCV-N replicon cells was observed upon exposure to ISP-1 or HPA-12, as previously reported (43, 52). In contrast, these compounds had little to no effect on viral RNA accumulation in JFH-1 replicon cells (Fig. 6A). Furthermore, these compounds did not affect luciferase activity within the lysate of Huh-7 cells transfected with an in vitro-transcribed JFH-1 replicon RNA containing a luciferase reporter gene (22) (data not shown). Fig. 6B shows the effects of ISP-1 and HPA-12 on de novo sphingolipid biosynthesis by replicon cells. No differences in the inhibitory effects of each compound were observed in replicon cells derived from HCV-N and JFH-1. When de novo synthesis of sphingolipids was examined by metabolic labeling with [^{14}C] serine, ISP-1 almost completely inhibited the production of both ceramide and SM, while HPA-12 greatly inhibited the synthesis of SM but not ceramide. Levels of phosphatidylethanolamine and phosphatidylserine, into which serine is incorporated by a pathway distinct from that of sphingolipid biosynthesis, were not influenced by these drugs. These results suggest that suppression of HCV RNA replication by inhibitors of sphingolipid biosynthesis might be viral genotype- or isolate-dependent.

This observation prompted us to investigate whether inhibitors of the sphingolipid biosynthetic pathway might have the ability to prevent HCV virion production. Interestingly, when Huh-7 cells producing JFH-1 HCVcc were treated with ISP-1 and HPA-12 under similar conditions as the replicon cells, viral core levels in the culture supernatants were greatly reduced in a dose-dependent manner. For example, exposure to 10 μM ISP-1 and 1 μM HPA-12 reduced viral core protein levels by more than 85% compared with control cells (Fig. 6C). The IC50 values for both drugs were less than 0.1 μM, 50-fold less than the values obtained for the RNA replication of HCV N-replicon. Together, these results suggest that the sphingolipid biosynthetic pathway plays an

1 important role in the production of HCV particles, but not genome replication, in JFH-1

2 based HCVcc.

DISCUSSION

In this study, we showed the role of HCV virion-associated cholesterol and sphingolipid in viral infectivity. Although a dependence on virion-associated cholesterol for virus entry has been shown for a number of viruses (5, 28, 49), this is the first study to demonstrate the importance of envelope cholesterol in a virus belonging to the flaviviridae family. Furthermore, to our knowledge, the functional role of virion membrane-associated SM has not been examined in viruses. Our previous studies using Chinese hamster ovary cell mutants deficient in SM synthesis have demonstrated that reduction of cellular SM enhances cellular cholesterol efflux to B-CD (9, 12). Thus, it may be possibly that SM plays a role in the retention of cholesterol on HCV particles due to interaction between cholesterol and SM. The finding that B-CD- or SMase-treatment of HCVcc markedly virus internalization but not cell attachment (Fig. 3) suggests that HCV membrane-associated cholesterol and sphingolipid are crucial for interaction of viral glycoproteins with the virus-receptor/co-receptor required for cell entry. Cholesterol depletion or sphingolipid hydrolysis might induce a conformational change in the viral envelope, resulting in instability of the virion structure. Since the cholesterol/phospholipid ratios of membranes affect bilayer fluidity, maturation of viral envelopes with high cholesterol/phospholipid ratios via association with rafts may be important for the stability of HCV particles. Replenishing the viral membrane with cholesterol following treatment with 5 mg/ml B-CD successfully restored viral infectivity to the same level as untreated virus (Fig. 1), suggesting that reversible B-CD-induced changes in HCV structure might critically influence viral infectivity. However, we were unable to restore viral infectivity by replenishing cholesterol after pretreatment of the virion with concentrations of B-CD exceeding 10 mg/ml (data not shown). Under these conditions, it is likely that large holes

in the viral membrane destroy the virus, which cannot be reversed by supplying exogenous cholesterol.

How are cholesterol and sphingolipid involved in the HCV virion during the process of the virus maturation? Like most positive-stranded RNA viruses, HCV is thought to assemble at the ER membrane. However, Miyanari et al. (32) reported that lipid droplets are important for HCVcc formation. The authors of this report have shown that the characteristics of lipid droplet-associated membranes in Huh-7 cells differ from those of ER membranes. In the case of flaviviruses, for which the mechanism of viral assembly and budding remains unclear (16), a few studies have demonstrated budding at the plasma membrane (13, 36, 37, 41) and it has been proposed that the site of budding may be virus- and cell type-dependent (27). We demonstrate here that subpopulations of HCV structural proteins partition into cellular detergent-resistant, lipid raft-like, membrane fractions in HCVcc-producing cells (Fig. 4), and that inhibitors of the sphingolipid biosynthetic pathway block HCV virion production (Fig. 6). Furthermore, a large proportion of HCV E2 protein incorporated into HCVcc is endoglycosidase H resistant (data not shown). Thus, membrane compartments containing cholesterol- and sphingolipid-rich microdomains may be involved in HCV virion maturation. Another explanation for recruitment of these lipids to the HCV membrane may be an association between the virus and very-low-density lipoprotein (VLDL)/LDL. Recently Huang et al. (14) demonstrated a close link between HCV production and VLDL assembly, suggesting that a HCV-VLDL complex is generated and secreted from cells.

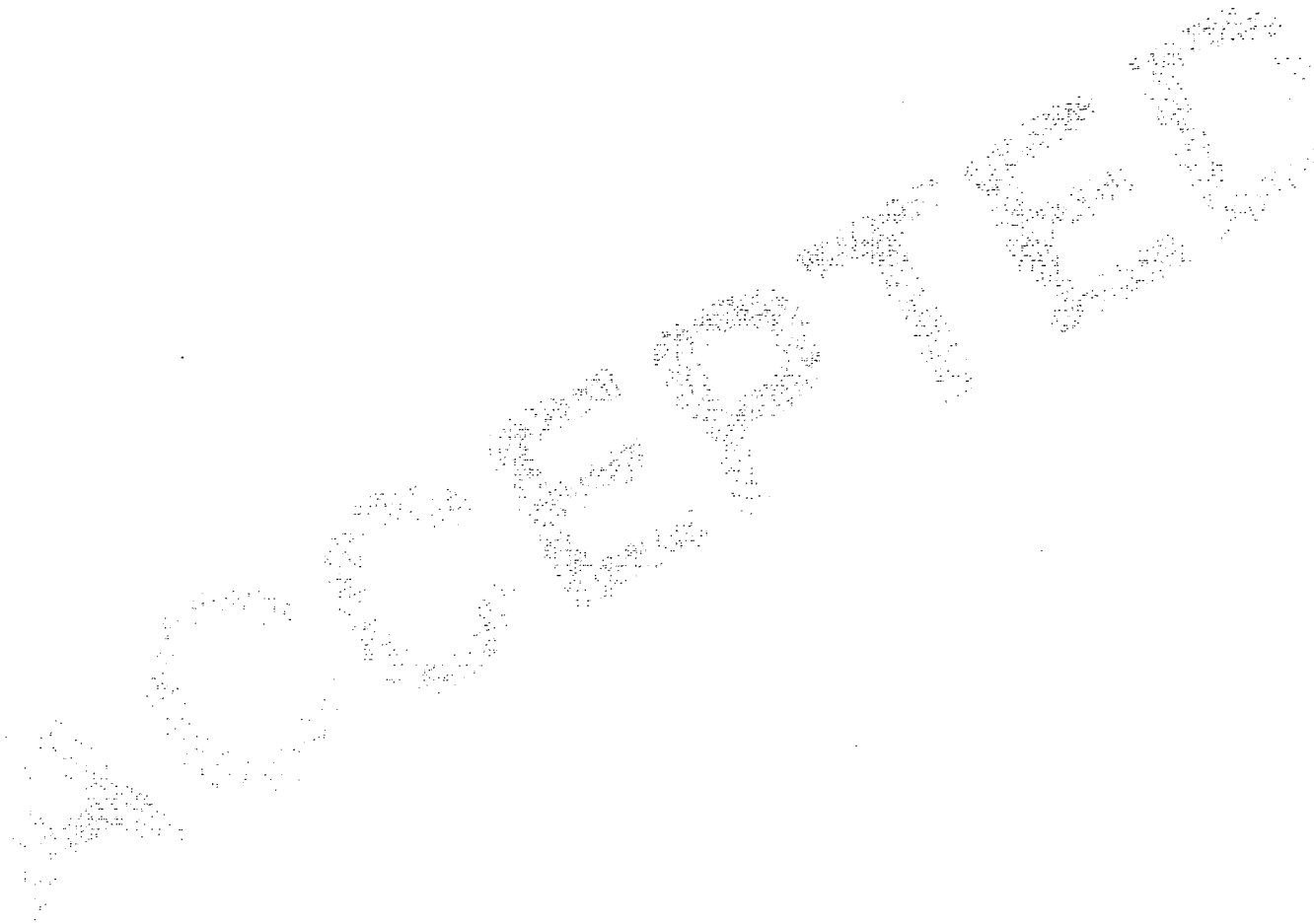
Recent reports have demonstrated that CD81-mediated HCV infection is partly dependent on cell membrane cholesterol (19) and SM (53). We further characterized the role of lipid on the plasma membrane in viral infectivity and found that cholesterol

depletion by B-CD, as well as hydrolysis of SM by SMase, moderately inhibits HCV infectivity (Fig. 5). These results suggest that cholesterol and sphingolipid on plasma membrane environment may assist HCV entry, while HCV virion-associated cholesterol and sphingolipid appear to play a critical role in viral infection.

We previously demonstrated that HCV RNA and nonstructural proteins are present in DRM structures, likely in the context of a lipid-raft structure, and that viral RNA is likely synthesized at a raft membrane structure in cells containing the genotype 1b HCV replicon (2, 10, 46). Here we observed that ISP-1 and HPA-12 suppress HCVcc production, but not viral RNA replication, of the JFH-1 replicon (Fig. 6). Impairment of particle assembly and maturation, rather than suppression of genome replication, by these drugs may account for inhibition of HCV production in the JFH-1 system. Viral RNA replication of the HCV-N replicon, however, was efficiently inhibited by these compounds as found in previous reports (43). Virus strain-specificity of the anti-HCV activity of cyclosporine has recently been demonstrated, with the JFH-1 replication being less sensitive to cyclosporine than genotype 1b strains. Furthermore, the requirement for interaction with a cellular replication cofactor, cyclophilin B, varies among HCV strains (18). It appears that ISP-1 and HPA-12 are further examples of diverse effects on HCV strain replication.

In summary, our data here demonstrate an important role of cholesterol and sphingolipid in HCV infection and virion maturation. Specifically, mature HCV particles are rich in cholesterol. Depletion from HCV or hydrolysis of virion-associated sphingomyelin results in a loss of infectivity. Moreover, the addition of exogenous cholesterol restores infectivity. In addition, cholesterol and sphingolipid on the HCV membrane play a key role in virus internalization and portions of structural proteins are localized at lipid raft-like membrane structures within cells. Finally, inhibitors of the

sphingolipid biosynthetic pathway efficiently block virion production. These observations suggest that agents capable of modifying virion-associated lipid content might function as antivirals by preventing and/or blocking HCV infection and production.



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REFERENCE

1. **Aizaki, H., Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsuura, and T. Miyamura.** 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. Hepatology **27:**621-7.

2. **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:**450-61.

3. **Akazawa, D., T. Date, K. Morikawa, A. Murayama, M. Miyamoto, M. Kaga, H. Barth, T. F. Baumert, J. Dubuisson, and T. Wakita.** 2007. CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. J Virol **81:**5036-45.

4. **Blanchard, E., D. Brand, S. Trassard, A. Goudeau, and P. Roingeard.** 2002. Hepatitis C virus-like particle morphogenesis. J Virol. **76:**4073-9.

5. **Bender, F. C., J. C. Whitbeck, H. Lou, G. H. Cohen, and R. J. Eisenberg.** 2005. Herpes simplex virus glycoprotein B binds to cell surfaces independently of heparan sulfate and blocks virus entry. J Virol **79:** 11588-97.

6. **Chazal, N., and D. Gerlier.** 2003. Virus entry, assembly, budding, and membrane rafts. Microbiol Mol Biol Rev **67:**226-37.

7. **Evans, M. J., T. von Hahn, D. M. Tscherne, A. J. Syder, M. Panis, B. Wolk, T. Hatziioannou, J. A. McKeating, P. D. Bieniasz, and C. M. Rice.** 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature **446:**801-5.

8. **Ezelle, H. J., D. Markovic, and G. N. Barber.** 2002. Generation of hepatitis C virus-like particles by use of a recombinant vesicular stomatitis virus vector. J Virol **76:**12325-34.

9. **Fukasawa, M., M. Nishijima, H. Itabe, T. Takano, K. and K. Hanada.** 2000. Reduction of sphingomyelin level without accumulation of ceramide in Chinese hamster ovary cells affects detergent-resistant membrane domains and enhances cellular cholesterol efflux to methyl-beta -cyclodextrin. J Biol Chem **275**: 34028-34.

10. **Gao, L., H. Aizaki, J. W. He, and M. M. Lai.** 2004. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. J Virol **78:**3480-8.

11. **Guo, J. T., V. V. Bichko, and C. Seeger.** 2001. Effect of alpha interferon on the hepatitis C virus replicon. J Virol **75:**8516-23.

12. **Hanada, K., T. Hara, M. Fukasawa, A. Yamaji, M. Umeda, and M. Nishijima.** 1998. Mammalian cell mutants resistant to a sphingomyelin-directed cytolysin.

Genetic and biochemical evidence for complex formation of the LCB1 protein with the LCB2 protein for serine palmitoyltransferase. J Biol Chem **273**: 33787-94.

13. **Hase, T., P. L. Summers, K. H. Eckels, and W. B. Baze.** 1987. An electron and immunoelectron microscopic study of dengue-2 virus infection of cultured mosquito cells: maturation events. Arch Virol **92:**273-91.

14. **Huang, H., F. Sun, D. M. Owen, W. Li, Y. Chen, M. Gale, 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**: 5848-53.

15. **Heider, J. G., and R. L. Boyett.** 1978. The picomole determination of free and total cholesterol in cells in culture. J Lipid Res **19:**514-8.

16. **Heinz, F. X., and S. L. Allison.** 2003. Flavivirus structure and membrane fusion. Adv Virus Res **59:**63-97.

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

18. **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:**4510-20.

19. **Kapadia, S. B., H. Barth, T. Baumert, J. A. McKeating, and F. V. Chisari.** 2007. Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. J Virol **81:**374-83.

20. **Kato, T., A. Furusaka, M. Miyamoto, T. Date, K. Yasui, J. Hiramoto, K. Nagayama, T. Tanaka, and T. Wakita.** 2001. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J Med Virol **64:**334-9.

21. **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:**1808-17.

22. **Kato, T., T. Date, M. Miyamoto, M. Sugiyama, Y. Tanaka, E. Orito, T. Ohno, K. Sugihara, I. Hasegawa, K. Fujiwara, K. Ito, A. Ozasa, M. Mizokami, and T. Wakita.** 2005. Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. J Clin Microbiol **43:**5679-84.

23. **Kato, T., T. Date, A. Murayama, K. Morikawa, D. Akazawa, and T. Wakita.** 2006. Cell culture and infection system for hepatitis C virus. Nat Protoc **1**: 2334-39.

24. **Kobayashi. S, K. Kakumoto, and M. Sugiura.** 2002. Transition metal