

1 as described above.

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

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

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

1 RESULTS

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

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

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

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

13 To investigate the effect of cholesterol on the density of infectious HCV virions,
14 B-CD-pretreated or untreated viral samples, as well as cholesterol-replenished treated viral
15 samples, were subjected to sucrose density gradient centrifugation (Fig. 1C). The peak
16 density of core protein of HCVcc was ~1.17 g/ml. When virion-associated cholesterol was
17 removed by B-CD, the peak density of HCVcc was shifted to 1.20 g/ml. Addition of
18 exogenous cholesterol to this cholesterol-depleted sample restored lower density fraction
19 (1.15 g/ml). Fig. 1D illustrates the infectivity of each gradient fraction. Untreated virus had
20 maximum infectivity at ~1.13 g/ml (fraction 6), while, as expected, fractions from
21 B-CD-treated viral samples exhibited minimal to no infectivity. Replenishment of depleted
22 virus with cholesterol returned infectivity to untreated control levels, and
23 cholesterol-replenished virus had a buoyant density of ~1.07 g/ml (fraction 4), suggesting
24 that HCV-associated cholesterol is crucial for viral infectivity and that the effect of

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

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

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

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

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

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

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

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

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

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

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

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

16 This observation prompted us to investigate whether inhibitors of the sphingolipid
17 biosynthetic pathway might have the ability to prevent HCV virion production.

18 Interestingly, when Huh-7 cells producing JFH-1 HCVcc were treated with ISP-1 and
19 HPA-12 under similar conditions as the replicon cells, viral core levels in the culture
20 supernatants were greatly reduced in a dose-dependent manner. For example, exposure to
21 10 μM ISP-1 and 1 μM HPA-12 reduced viral core protein levels by more than 85%
22 compared with control cells (Fig. 6C). The IC₅₀ values for both drugs were less than 0.1
23 μM, 50-fold less than the values obtained for the RNA replication of HCV N-replicon.
24 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.

ACCEPTED

1 DISCUSSION

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

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

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

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

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

5 We previously demonstrated that HCV RNA and nonstructural proteins are present in
6 DRM structures, likely in the context of a lipid-raft structure, and that viral RNA is likely
7 synthesized at a raft membrane structure in cells containing the genotype 1b HCV replicon
8 (2, 10, 46). Here we observed that ISP-1 and HPA-12 suppress HCVcc production, but not
9 viral RNA replication, of the JFH-1 replicon (Fig. 6). Impairment of particle assembly and
10 maturation, rather than suppression of genome replication, by these drugs may account for
11 inhibition of HCV production in the JFH-1 system. Viral RNA replication of the HCV-N
12 replicon, however, was efficiently inhibited by these compounds as found in previous
13 reports (43). Virus strain-specificity of the anti-HCV activity of cyclosporine has recently
14 been demonstrated, with the JFH-1 replication being less sensitive to cyclosporine than
15 genotype 1b strains. Furthermore, the requirement for interaction with a cellular
16 replication cofactor, cyclophilin B, varies among HCV strains (18). It appears that ISP-1
17 and HPA-12 are further examples of diverse effects on HCV strain replication.

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

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

ACCEPTED

1 **ACKNOWLEDGEMENTS**

2 We thank M. Matsuda, M. Sasaki, S. Yoshizaki, T. Shimoji, M. Kaga and T. Date for
3 their technical assistance and T. Mizoguchi for secretarial work. This work was partially
4 supported by a grant-in-aid for Scientific Research from the Japan Society for the
5 Promotion of Science, from the Ministry of Health, Labour and Welfare of Japan and from
6 the Ministry of Education, Culture, Sports, Science and Technology, as well as the
7 Research on Health Science Focusing on Drug Innovation from the Japan Health Sciences
8 Foundation.

1 REFERENCE

- 2 1. **Aizaki, H., Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y.**
3 **Matsuura, and T. Miyamura.** 1998. Full-length complementary DNA of hepatitis C
4 virus genome from an infectious blood sample. *Hepatology* **27**:621-7.
5
- 6 2. **Aizaki, H., K. J. Lee, V. M. Sung, H. Ishiko, and M. M. Lai.** 2004. Characterization
7 of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology*
8 **324**:450-61.
9
- 10 3. **Akazawa, D., T. Date, K. Morikawa, A. Murayama, M. Miyamoto, M. Kaga, H.**
11 **Barth, T. F. Baumert, J. Dubuisson, and T. Wakita.** 2007. CD81 expression is
12 important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C
13 virus infection. *J Virol* **81**:5036-45.
14
- 15 4. **Blanchard, E., D. Brand, S. Trassard, A. Goudeau, and P. Roingeard.** 2002.
16 Hepatitis C virus-like particle morphogenesis. *J Virol*. **76**:4073-9.
17
- 18 5. **Bender, F. C., J. C. Whitbeck, H. Lou, G. H. Cohen, and R. J. Eisenberg.** 2005.
19 Herpes simplex virus glycoprotein B binds to cell surfaces independently of heparan
20 sulfate and blocks virus entry. *J Virol* **79**: 11588-97.
21
- 22 6. **Chazal, N., and D. Gerlier.** 2003. Virus entry, assembly, budding, and membrane
23 rafts. *Microbiol Mol Biol Rev* **67**:226-37.
24
- 25 7. **Evans, M. J., T. von Hahn, D. M. Tscherne, A. J. Syder, M. Panis, B. Wolk, T.**
26 **Hatzioannou, J. A. McKeating, P. D. Bieniasz, and C. M. Rice.** 2007. Claudin-1 is
27 a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**:801-5.
28
- 29 8. **Ezelle, H. J., D. Markovic, and G. N. Barber.** 2002. Generation of hepatitis C
30 virus-like particles by use of a recombinant vesicular stomatitis virus vector. *J Virol*
31 **76**:12325-34.
32
- 33 9. **Fukasawa, M., M. Nishijima, H. Itabe, T. Takano, K. and K. Hanada.** 2000.
34 Reduction of sphingomyelin level without accumulation of ceramide in Chinese
35 hamster ovary cells affects detergent-resistant membrane domains and enhances
36 cellular cholesterol efflux to methyl-beta -cyclodextrin. *J Biol Chem* **275**: 34028-34.
37
- 38 10. **Gao, L., H. Aizaki, J. W. He, and M. M. Lai.** 2004. Interactions between viral
39 nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C
40 virus RNA replication complex on lipid raft. *J Virol* **78**:3480-8.
41
- 42 11. **Guo, J. T., V. V. Bichko, and C. Seeger.** 2001. Effect of alpha interferon on the
43 hepatitis C virus replicon. *J Virol* **75**:8516-23.
44
- 45 12. **Hanada, K., T. Hara, M. Fukasawa, A. Yamaji, M. Umeda, and M. Nishijima.**
46 1998. Mammalian cell mutants resistant to a sphingomyelin-directed cytolysin.

- 1 Genetic and biochemical evidence for complex formation of the LCB1 protein with
2 the LCB2 protein for serine palmitoyltransferase. *J Biol Chem* **273**: 33787-94.
3
- 4 13. **Hase, T., P. L. Summers, K. H. Eckels, and W. B. Baze.** 1987. An electron and
5 immunoelectron microscopic study of dengue-2 virus infection of cultured mosquito
6 cells: maturation events. *Arch Virol* **92**:273-91.
7
- 8 14. **Huang, H., F. Sun, D. M. Owen, W. Li, Y. Chen, M. Gale, and J. Ye.** 2007. Hepatitis
9 C virus production by human hepatocytes dependent on assembly and secretion of
10 very low-density lipoproteins. *Proc Natl Acad Sci U S A* **104**: 5848-53.
11
- 12 15. **Heider, J. G., and R. L. Boyett.** 1978. The picomole determination of free and total
13 cholesterol in cells in culture. *J Lipid Res* **19**:514-8.
14
- 15 16. **Heinz, F. X., and S. L. Allison.** 2003. Flavivirus structure and membrane fusion. *Adv*
16 *Virus Res* **59**:63-97.
17
- 18 17. **Ikeda, M., M. Yi, K. Li, and S. M. Lemon.** 2002. Selectable subgenomic and
19 genome-length dicistronic RNAs derived from an infectious molecular clone of the
20 HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. *J Virol*
21 **76**:2997-3006.
22
- 23 18. **Ishii, N., K. Watashi, T. Hishiki, K. Goto, D. Inoue, M. Hijikata, T. Wakita, N.**
24 **Kato, and K. Shimotohno.** 2006. Diverse effects of cyclosporine on hepatitis C virus
25 strain replication. *J Virol* **80**:4510-20.
26
- 27 19. **Kapadia, S. B., H. Barth, T. Baumert, J. A. McKeating, and F. V. Chisari.** 2007.
28 Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity
29 between CD81 and scavenger receptor B type I. *J Virol* **81**:374-83.
30
- 31 20. **Kato, T., A. Furusaka, M. Miyamoto, T. Date, K. Yasui, J. Hiramoto, K.**
32 **Nagayama, T. Tanaka, and T. Wakita.** 2001. Sequence analysis of hepatitis C virus
33 isolated from a fulminant hepatitis patient. *J Med Virol* **64**:334-9.
34
- 35 21. **Kato, T., T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, and T.**
36 **Wakita.** 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic
37 replicon. *Gastroenterology* **125**:1808-17.
38
- 39 22. **Kato, T., T. Date, M. Miyamoto, M. Sugiyama, Y. Tanaka, E. Orito, T. Ohno, K.**
40 **Sugihara, I. Hasegawa, K. Fujiwara, K. Ito, A. Ozasa, M. Mizokami, and T.**
41 **Wakita.** 2005. Detection of anti-hepatitis C virus effects of interferon and ribavirin by
42 a sensitive replicon system. *J Clin Microbiol* **43**:5679-84.
43
- 44 23. **Kato, T., T. Date, A. Murayama, K. Morikawa, D. Akazawa, and T. Wakita.** 2006.
45 Cell culture and infection system for hepatitis C virus. *Nat Protoc* **1**: 2334-39.
46
- 47 24. **Kobayashi, S, K. Kakumoto, and M. Sugiura.** 2002. Transition metal

- 1 salts-catalyzed aza-Michael reactions of enones with carbamates. *Org Lett* **18**:
2 1319-22.
- 3
- 4 25. **Koutsoudakis, G., E. Herrmann, S. Kallis, R. Bartenschlager, and T.**
5 **Pietschmann.** 2007. The level of CD81 cell surface expression is a key determinant
6 for productive entry of hepatitis C virus into host cells. *J Virol* **81**:588-98.
- 7
- 8 26. **Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R.**
9 **Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a
10 hepatoma cell line. *Science* **285**:110-3.
- 11
- 12 27. **Mackenzie, J. M., and E. G. Westaway.** 2001. Assembly and maturation of the
13 flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along
14 the secretory pathway, respectively. *J Virol* **75**:10787-99.
- 15
- 16 28. **Manes, S., G. del Real, R. A. Lacalle, P. Lucas, C. Gomez-Mouton, S.**
17 **Sanchez-Palomino, R. Delgado, J. Alcami, E. Mira, and A. C. Martinez.** 2000.
18 Membrane raft microdomains mediate lateral assemblies required for HIV-1 infection.
19 *EMBO J* **19**:190-6.
- 20
- 21 29. **Matsuo, E., H. Tani, C. Lim, Y. Komoda, T. Okamoto, H. Miyamoto, K. Moriishi, S.**
22 **Yagi, A.H. Patel, T. Miyamura, and Y. Matsuura.** 2006. Characterization of HCV-like
23 particles produced in a human hepatoma cell line by a recombinant baculovirus.
24 *Biochem Biophys Res Commun* **340**:200-8.
- 25
- 26 30. **Matto, M., C. M. Rice, B. Aroeti, and J. S. Glenn.** 2004. Hepatitis C virus core
27 protein associates with detergent-resistant membranes distinct from classical plasma
28 membrane rafts. *J Virol* **78**:12047-53.
- 29
- 30 31. **Miyake, Y., Y. Kozutsumi, S. Nakamura, T. Fujita, and T. Kawasaki.** 1995. Serine
31 palmitoyltransferase is the primary target of a sphingosine-like immunosuppressant,
32 ISP-1/myriocin. *Biochem Biophys Res Commun* **211**:396-403.
- 33
- 34 32. **Miyazawa, Y., K. Atsuzawa, N. Usuda, K. Watashi, T. Hishiki, M. Zayas, R.**
35 **Bartenschlager, T. Wakita, M. Hijikata, and K. Shimotohno,** 2007. The lipid
36 droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* **9**:
37 1089-97.
- 38
- 39 33. **Morikawa, K., Z. Zhao, T. Date, M. Miyamoto, A. Murayama, D. Akazawa, J.**
40 **Tanabe, S. Sone, and T. Wakita.** 2007. The roles of CD81 and glycosaminoglycans
41 in the adsorption and uptake of infectious HCV particles. *J Med Virol* **79**:714-23.
- 42
- 43 34. **Murakami, K., K. Ishii, Y. Ishihara, S. Yoshizaki, K. Tanaka, Y. Gotoh, H. Aizaki,**
44 **M. Kohara, H. Yoshioka, Y. Mori, N. Manabe, I. Shoji, T. Sata, R.**
45 **Bartenschlager, Y. Matsuura, T. Miyamura, and T. Suzuki.** 2006. Production of
46 infectious hepatitis C virus particles in three-dimensional cultures of the cell line
47 carrying the genome-length dicistronic viral RNA of genotype 1b. *Virology*

- 1 351:381-92.
2
- 3 35. Nakai, K., T. Okamoto, T. Kimura-Someya, K. Ishii, C. K. Lim, H. Tani, E.
4 Matsuo, T. Abe, Y. Mori, T. Suzuki, T. Miyamura, J. H. Nunberg, K. Moriishi,
5 and Y. Matsuura. 2006. Oligomerization of hepatitis C virus core protein is crucial
6 for interaction with the cytoplasmic domain of E1 envelope protein. *J Virol*
7 80:11265-73.
8
- 9 36. Ng, M. L., J. Howe, V. Sreenivasan, and J. J. Mulders. 1994. Flavivirus West Nile
10 (Sarafend) egress at the plasma membrane. *Arch Virol* 137:303-13.
11
- 12 37. Ng, M. L., S. H. Tan, and J. J. Chu. 2001. Transport and budding at two distinct sites
13 of visible nucleocapsids of West Nile (Sarafend) virus. *J Med Virol* 65:758-64.
14
- 15 38. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for
16 high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193-9.
17
- 18 39. Pessin, J. E., and M. Glaser. 1980. Budding of Rous sarcoma virus and vesicular
19 stomatitis virus from localized lipid regions in the plasma membrane of chicken
20 embryo fibroblasts. *J Biol Chem* 255:9044-50.
21
- 22 40. Pitha, J., T. Irie, P. B. Sklar, and J. S. Nye. 1988. Drug solubilizers to aid
23 pharmacologists: amorphous cyclodextrin derivatives. *Life Sci* 43:493-502.
24
- 25 41. Rahman, S., T. Matsumura, K. Masuda, K. Kanemura, and T. Fukunaga. 1998.
26 Maturation site of dengue type 2 virus in cultured mosquito C6/36 cells and Vero cells.
27 *Kobe J Med Sci* 44:65-79.
28
- 29 42. Rouser, G., G. Galli, and G. Kritchevsky. 1967. Lipid composition of the normal
30 human brain and its variations during various diseases. *Pathol Biol* 15:195-200.
31
- 32 43. Sakamoto, H., K. Okamoto, M. Aoki, H. Kato, A. Katsume, A. Ohta, T. Tsukuda,
33 N. Shimma, Y. Aoki, M. Arisawa, M. Kohara, and M. Sudoh. 2005. Host
34 sphingolipid biosynthesis as a target for hepatitis C virus therapy. *Nat Chem Biol*
35 1:333-7.
36
- 37 44. Sato, K., H. Okamoto, S. Aihara, Y. Hoshi, T. Tanaka, and S. Mishiro. 1993.
38 Demonstration of sugar moiety on the surface of hepatitis C virions recovered from
39 the circulation of infected humans. *Virology* 196:354-7.
40
- 41 45. Serafino, A., M. B. Valli, F. Andreola, A. Crema, G. Ravagnan, L. Bertolini, and
42 G. Carloni. 2003. Suggested role of the Golgi apparatus and endoplasmic reticulum
43 for crucial sites of hepatitis C virus replication in human lymphoblastoid cells infected
44 in vitro. *J Med Virol* 70:31-41.
45
- 46 46. Shi, S. T., K. J. Lee, H. Aizaki, S. B. Hwang, and M. M. Lai. 2003. Hepatitis C
47 virus RNA replication occurs on a detergent-resistant membrane that cofractionates

- 1 with caveolin-2. *J Virol* **77**:4160-8.
2
- 3 47. **Shinitzky, M., and M. Inbar.** 1976. Microviscosity parameters and protein mobility
4 in biological membranes. *Biochim Biophys Acta* **433**:133-49.
5
- 6 48. **Shirakura, M., K. Murakami, T. Ichimura, R. Suzuki, T. Shimoji, K. Fukuda, K.
7 Abe, S. Sato, M. Fukasawa, Y. Yamakawa, M. Nishijima, K. Moriishi, Y.
8 Matsuura, T. Wakita, T. Suzuki, P.M. Howley, T. Miyamura, and I. Shoji.** 2007.
9 E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus
10 core protein. *J Virol* **81**: 1174-85.
11
- 12 49. **Stuart, A. D., H. E. Eustace, T. A. McKee, and T. D. Brown.** 2002. A novel cell
13 entry pathway for a DAF-using human enterovirus is dependent on lipid rafts. *J Virol*
14 **76**: 9307-22.
15
- 16 50. **Takikawa, S., K. Ishii, H. Aizaki, T. Suzuki, H. Asakura, Y. Matsuura, and T.
17 Miyamura.** 2000. Cell fusion activity of hepatitis C virus envelope proteins. *J Virol*
18 **74**:5066-74.
19
- 20 51. **Tani, H., Y. Komoda, E. Matsuo, K. Suzuki, I. Hamamoto, T. Yamashita, K.
21 Moriishi, K. Fujiyama, T. Kanto, N. Hayashi, A. Owsianka, A. H. Patel, M. A.
22 Whitt, and Y. Matsuura.** 2007. Replication-competent recombinant vesicular
23 stomatitis virus encoding hepatitis C virus envelope proteins. *J Virol* **81**:8601-12.
24
- 25 52. **Umehara, T., M. Sudoh, F. Yasui, C. Matsuda, Y. Hayashi, K. Chayama, and M.
26 Kohara.** 2006. Serine palmitoyltransferase inhibitor suppresses HCV replication in a
27 mouse model. *Biochem Biophys Res Commun* **346**, 67-73.
28
- 29 53. **Voisset, C., M. Lavie, F. Helle, A. O. De Beeck, A. Bilheu, J. Bertrand-Michel, F.
30 Tercé, L. Cocquerel, C. Wychowski, N. Vu-Dac, and J. Dubuisson.** 2007.
31 Ceramide enrichment of the plasma membrane induces CD81 internalization and
32 inhibits hepatitis C virus entry. *Cell Microbiol* **10**:606-17.
33
- 34 54. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy,
35 A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J.
36 Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned
37 viral genome. *Nat Med* **11**:791-6.
38
- 39 55. **Yasuda, S., H. Kitagawa, M. Ueno, H. Ishitani, M. Fukasawa, M. Nishijima, S.
40 Kobayashi, and K. Hanada.** 2001. A novel inhibitor of ceramide trafficking from the
41 endoplasmic reticulum to the site of sphingomyelin synthesis. *J Biol Chem*
42 **276**:43994-4002.
43
- 44 56. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F.
45 Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C
46 virus infection in vitro. *Proc Natl Acad Sci U S A* **102**:9294-9

1 **FIGURE LEGENDS**

2 **FIG. 1.** Role of HCV-associated cholesterol in infection. (A) Effect of cholesterol
3 depletion on HCV infectivity. HCVcc particles (~2 fmol of the core protein) were treated
4 with B-CD at 0.1, 1, and 5 mg/ml for 1 h at 37°C. After removal of B-CD, Huh-7 cells were
5 infected with the treated virus particles, after which the core protein content of infected
6 cells at 72 h p.i. was determined as an indicator of infectivity, as previously established
7 (24). (B) Effect of cholesterol replenishment on infectivity. After treatment with 5 mg/ml
8 B-CD, virus was treated with medium alone or medium containing exogenous cholesterol
9 for 1 h at 37°C. (C) Effect of cholesterol depletion and replenishment on density gradient
10 profiles of the viral particles. The HCVcc treated with 5 mg/ml B-CD was replenished
11 with exogenous cholesterol (1 mM), followed by being separated by 10-60% sucrose
12 gradient ultracentrifugation. The core protein in each fraction was measured. Density of
13 each fraction was determined by refractive index measurement. (D) Effect of cholesterol
14 depletion and replenishment on viral infectivity. Each fraction (shown in panel C) was
15 subjected to infection, and then the core proteins in the cells were measured at 72 h p.i. (E)
16 Effect of cholesterol depletion on the infectivity of HCVpv (genotype 1a; gray bars) or
17 control VSVdelG-GFP/G (black bars). The viruses were preincubated with B-CD for 1 h at
18 37°C before infection. (F) Left; culture medium from HCVcc producing cells was
19 fractionated as above. For each fraction, the amounts of core and infectivity (intracellular
20 core) are plotted. Peaks of the core (arrow) and infectivity (arrowhead) were indicated.
21 Middle; an aliquot of fraction 8 (peak of the core; left panel) was treated with 1 mM
22 cholesterol for 1 h at 37°C. The resultant and non-treated aliquot of the fraction were
23 subjected to the sucrose gradient ultracentrifugation. The core in each fraction was plotted.
24 Right; infectivity of fraction 6 and 8, as depict in the left, with or without cholesterol