

Fig. 1. Outline of the RFB system. RFB system consists of vessel, column and PC monitoring system. Culture condition was automatically controlled: oxygen concentration, temperature, pH, and oxygen level in the conditioning vessel are continuously monitored by PC and conditioned by mass flow controller.

retaining their differentiated hepatocyte function, as described above, enables evaluations of dynamic analysis of HCV replication and selection of viral variability and quasispecies. The potential of this culture system for screening HCV-positive sera was well suited for the viral infection.

Table 1 shows the serum samples (A–F) from six HCV carriers. The nucleotide complexity of HCV in serum samples was determined by sequencing the 1449–1598 nt region of the HCV genome, which includes HVR1 located at the N-terminal region of E2. Each serum was a mixture of a dominant HCV clone and related but distinct viral populations. The dominant species in

sera A, C, D, E, and F were found to be genotype 1, and that in serum B was genotype 2. Viral loads in A–F, respectively, were 2.4×10^6 , 8.6×10^6 , 5.9×10^6 , 2.5×10^6 , 1.0×10^7 and 1.4×10^7 copies/mL, which were determined by real-time RT-PCR, as previously described (Aizaki et al., 2003; Suzuki et al., 2005). HCV loads of 2×10^6 copies from each serum sample were mixed to prepare a pooled serum sample containing 1.2×10^7 copies of HCV RNA. After FLC4 cells were inoculated into the RFB and subjected to 2 weeks of pre-culture for the preparation of 3D culture, the cells were infected with the pooled serum. Cell number at infection was about 10^8 in the 30-

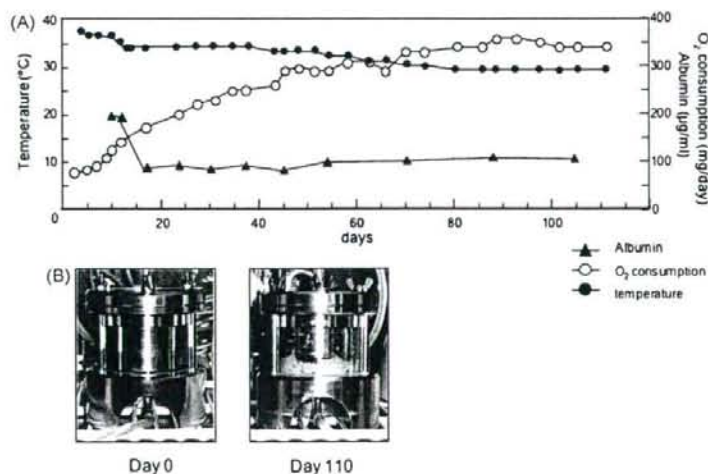


Fig. 2. Long-term culture of FLC4 cells in the RFB system. (A) Long-term culture of FLC4 cells in the RFB system. Temperature (closed circles) was gradually decreased from 37 to 30 °C. Oxygen consumption (open circles) was gradually increased from days 0 to 80 and reached the steady-state level. Albumin concentration (closed triangles) was constant from days 15 to 105. (B) The appearance of the RFB column at the beginning (day 0) and at the end (day 110) of culture.

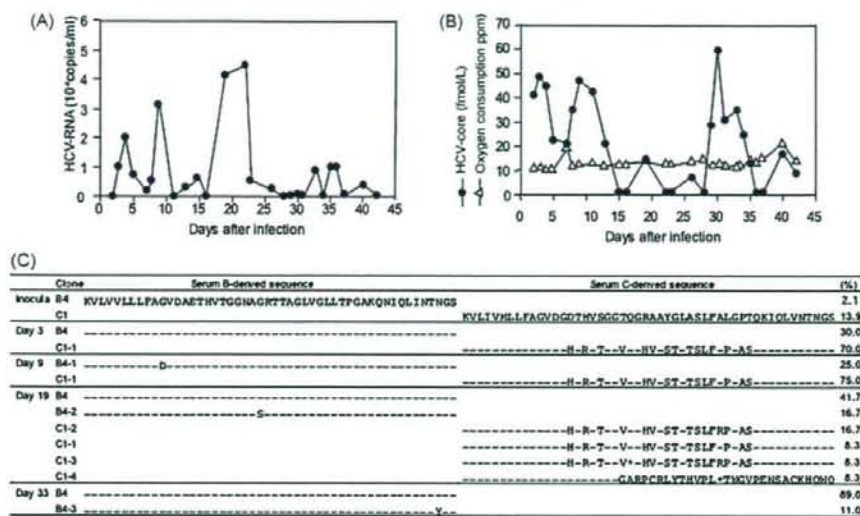


Fig. 3. HCV propagation in FLC4 cells cultured in the RFB system following inoculation with pooled sera obtained from HCV carriers. The 3D-cultured FLC4 cells were incubated with a pooled serum sample for 12 h, followed by changing the culture medium to fresh one. Culture medium was periodically collected for 42 days after inoculation, and HCV RNA and the viral core protein were quantified, respectively, by real-time RT-PCR and ELISA. (A) HCV RNA level in culture supernatant. (B) HCV-core protein (closed circles) and oxygen consumption (open triangles) levels in culture supernatant. (C) Changes in the viral quasispecies distribution after the inoculation. Percentages in the inoculum or in the culture medium at each time point (day 3, 9, 19, or 33 p.i.) are indicated at the right side. *, termination codon.

mL RFB column, as estimated from the glucose consumption (Kawada et al., 1998). Culture medium in the RFB was replaced with fresh medium 12 h post-infection (p.i.) and periodically sampled for 42 days.

Fig. 3A and B shows the levels of HCV RNA and viral core protein in the culture medium, respectively. HCV RNA was not observed on the first 2 days following infection, but was detectable from day 3 p.i. Viral RNA levels fluctuated, with peaks on days 3, 9, 19–21 and 33–36 p.i. At days 19–21 p.i., the average amount of HCV RNA detected in the culture supernatant was approximately 3×10^6 copies/day. Intermittent peaks were observed in HCV core protein levels in the culture supernatant, and the peak pattern of the core protein was largely consistent with that of viral RNA. During the infection experiment, the level of oxygen consumption was constant at approximately 12 ppm, thus suggesting that the desired conditions (constant or very gradually increasing cell number) were maintained.

3.3. Quasispecies analysis in RFB culture

The above results suggest that, although the environment was consistent in the pooled serum infection, there were periods in which the viruses actively replicated and released from the cells and periods in which they poorly replicated. The pooled serum used for the infection exhibited HCV populations had at least 26 distinct quasispecies (Table 1). To investigate whether the quasispecies distribution was altered due to infection, and whether HCV populations are selected during long-term culture in the RFB, total RNA was extracted from the culture supernatant samples collected on days 3, 9, 19 and 33 p.i., and the nucleotide sequence of the region containing HVR1 was deter-

mined, as described above. As shown in Fig. 3C, it is of interest that only two HCV species were detected in the sample at day 3 p.i.; the dominant clone C1-1, comprising approximately 70% of the viral population, and clone B4, comprising 30%. Although clone C1-1 was not detected in the sequence of the inoculum shown in Table 1, it was most similar to clone C1, a dominant clone in plasma C, among the HCV population observed in the inoculum; thus, it is possible that clone C1-1 is one of the minor species in serum C. Clone B4 was found to be derived from serum B. An almost identical HCV population was observed in the sample at day 9 p.i. In this sample, the dominant clone C1-1 and clone B4-1, which differs from clone B4 by only one amino acid, were detected. In contrast, more significant variation in quasispecies structure of the HCV species was observed in the sample at day 19 p.i. than that at day 9 p.i. With B4 as the dominant clone, the serum B-derived HCV species, clones B4 and B4-2, which differs from clone B4 by one amino acid, comprised 58% of the total population. Four types of HCV sequences derived from serum C were detected. Two of these (clones C1-3 and C1-4) contained lethal mutations. It was also found that the HCV species detected in the sample at day 33 p.i. included only two clones (clones B4 and B4-3), derived from serum B. The dominant clone, B4, was found to comprise 89% of the total population.

3.4. Potential use of the RFB system for evaluation of anti-HCV compounds

An experiment was carried out to determine whether this HCV infection experiment system was useful for the evaluation of anti-HCV drugs (Fig. 4). For this purpose, a small,

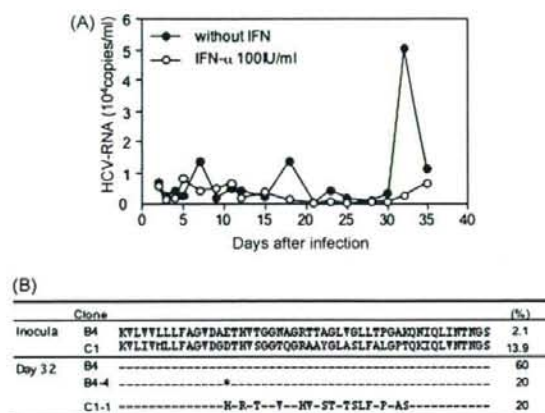


Fig. 4. A therapeutic effect of IFN in HCV infection model in the RFB cultures. HCV-infected FLC4 cells were treated with or without 100 IU/mL IFN- α . (A) Culture media were periodically collected, and HCV RNA levels were determined. Closed circles: without IFN treatment, open circles: treatment with IFN. (B) Changes in the viral quasispecies distribution in the cells without IFN treatment. Percentages in the inoculum or in the culture medium on day 32 p.i. are indicated at the right side. *, termination codon.

4-mL RFB column was adopted and a pair of RFB cultures infected with the HCV-positive pooled plasma (Table 1) was prepared. IFN- α was added to one culture at a final concentration of 100 IU/mL at 12 h p.i. No cytotoxicity was observed in FLC4 cells under these conditions (data not shown). Culture media from two cultures (12.5 mL each) were sampled periodically for 35 days and replaced by the same volume of fresh medium in the presence or absence of IFN- α . HCV RNA in the collected media was quantified by real-time RT-PCR, as described above. As shown in Fig. 4A, in the no-treatment culture, fluctuations in the viral RNA levels with the peaks on days 7, 18, and 32 p.i. ($1.5\text{--}5 \times 10^4$ copies/mL) were observed. However, while HCV RNA at $0.5\text{--}0.8 \times 10^4$ copies/mL was detected in the IFN-treated culture at days 5–11 p.i., no HCV RNA was detected at days 12–30 p.i. Serum levels of hepatic transaminases such as ALT and AST are known to be markers of liver damage. In the HCV-infection model with FLC4 cells cultured in RFB, the AST levels in the culture medium, which ranged from 5 to 10 IU/L without HCV infection, increased to 20–50 IU/L according to the viral infection (data not shown). Such increased AST levels were found to fall by the IFN treatment to lower than 10 IU/L at day 28 p.i. As reported previously, the ALT levels in the culture medium were constantly low; its levels were less than 10 IU/mL, with or without HCV infection (Aizaki et al., 2003). The viral nucleotide sequence in the no-treatment culture medium at day 32 p.i. was determined. It was found that serum B-derived clone B4 was dominant, and serum C-derived clone C1 was present as a minor clone (Fig. 4B); thus, the results corresponded well with those demonstrated in Fig. 3. An increase in viral RNA in the IFN-treated culture after day 32 p.i. was observed; although the degree of increase was only slight (Fig. 4A). It will be interesting to test whether HCV species grown in the IFN-treated culture is a variant resistant to IFN- α .

4. Discussion

At present an important limitation of the *in vitro* HCV infection system is that the only established culture system is based on genotype 2a, JFH-1 isolate, and Huh-7-derived cell lines. The development of alternate infection systems in which other HCV strains and host cells are available has been needed for the study of HCV dynamics and virus–host interactions, and for testing antivirals. This paper demonstrates that a long-term culture of the 3D RFB system is a useful tool for investigating HCV dynamics. The present results revealed that the viral quasispecies distribution altered in the HCV infection system in the RFB system. The change probably occurs in the following two stage process. The first change was observed on day 3 p.i.; thus, it is possible that the HCV species were selected according to infectivity in FLC4 cells. It has been reported that HCV particle populations in chronic hepatitis C patients consist of low-density virions and higher-density immune complex forms (Hijikata et al., 1993; Kanto et al., 1994). Inoculation of cultured cells with HCV has demonstrated that the immune complex forms were less infective than the antibody-unbound virions (Shimizu et al., 1994). Therefore, another hypothesis may be that a large number of HCV populations in sera A, D, E, and F are immune complex forms; thus, these sera are less susceptible to the cells than sera B and C. The second change was observed on days 19–33 p.i. While the serum C-derived clone was dominant in the early stages after infection, the serum B-derived HCV clone became dominant over time. In the absence of immunological selection pressure, viral nucleotide mutations at random positions are accumulated during viral replication, and the newly generated variant species are selected principally, if not solely, based on the intrinsic replicative advantages or disadvantages that these mutations confer. Thus, these results suggest that the use of pooled serum sample allowed for screening of infectious materials compatible for the RFB culture.

Evaluation methods for anti-HCV drugs using monolayer culture systems with various culture cells, such as the replicon system and the JFH-1 based virion production system, have been reported (Bartenschlager et al., 2003; Blight et al., 2000; Boriskin et al., 2006; Lanford et al., 2003; Lindenbach et al., 2005; Lohmann et al., 1999; Wakita et al., 2005; Zhong et al., 2005). These methods utilize viral markers, such as HCV RNA and antigens, as indicators of treatment efficacy. However, the utility of long-term cell culture systems for anti-HCV drug evaluation based on infection with human sera is still limited. The use of a chimpanzee model, the only non-human host for HCV infection, is restricted due to several reasons such as problematic availability and ethical consideration. Given intensive efforts to reduce and replace animal testing in the course of development of new therapies worldwide, the RFB-based HCV infection model is a potential alternative to animal models such chimpanzee for assessing anti-HCV compounds. According to the studies with regards to mathematical modeling of HCV kinetics (Dahari et al., 2005; Dixit et al., 2004; Layden et al., 2003; Layden-Almer et al., 2006; Perelson et al., 2005), IFN therapy against HCV infection generally generates a biphasic decline in viral load; there is a rapid decrease in the serum HCV RNA level over the

first 1 day of treatment, followed by the second phase, which is slower than the first-phase viral decline. To date, there were no such observable viral kinetics in the IFN treatment under such experimental settings. Further detailed kinetic analyses of the use of varying doses of IFN and of very early time points to evaluate the antiviral effect are in progress.

In summary, by investigating the dynamics of HCV populations in the RFB culture system, it was demonstrated that HCV was intermittently detected in the culture supernatants of long-term culture, and that changes in viral quasiespecies appear to be related to this fluctuation in the virus level. It was also shown that an HCV-infection model using the RFB system is useful for evaluating potential antivirals. Further investigation on the infection and growth of various HCV-positive sera is currently being conducted in order to obtain an adaptive clone with higher replication efficiency in this culture system.

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References

- Aizaki, H., Aoki, Y., Harada, T., Ishii, K., Suzuki, T., Nagamori, S., Toda, G., Matsuura, Y., Miyamura, T., 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. *Hepatology* 27, 621–627.
- Aizaki, H., Nagamori, S., Matsuda, M., Kawakami, H., Hashimoto, O., Ishiko, H., Kawada, M., Matsuura, T., Hasumura, S., Matsuura, Y., Suzuki, T., Miyamura, T., 2003. Production and release of infectious hepatitis C virus from human liver cell cultures in the three-dimensional radial-flow bioreactor. *Virology* 314, 16–25.
- Aoki, Y., Aizaki, H., Shimoike, T., Tani, H., Ishii, K., Saito, I., Matsuura, Y., Miyamura, T., 1998. A human liver cell line exhibits efficient translation of HCV RNAs produced by a recombinant adenovirus expressing T7 RNA polymerase. *Virology* 250, 140–150.
- Bartenschlager, R., Kaul, A., Sparacio, S., 2003. Replication of the hepatitis C virus in cell culture. *Antivir. Res.* 60, 91–102.
- Blight, K.J., Kolykhalov, A.A., Rice, C.M., 2000. Efficient initiation of HCV RNA replication in cell culture. *Science* 290, 1972–1974.
- Boriskin, Y.S., Pecheur, E.L., Polyak, S.J., 2006. Arbidol: a broad-spectrum antiviral that inhibits acute and chronic HCV infection. *Virology* 351, 56.
- Choo, Q.L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., Houghton, M., 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 244, 359–362.
- Choo, Q.L., Richman, K.H., Han, J.H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, R., Barr, P.J., et al., 1991. Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. U.S.A.* 88, 2451–2455.
- Dahari, H., Major, M., Zhang, X., Mihalik, K., Rice, C.M., Perelson, A.S., Feinstone, S.M., Neumann, A.U., 2005. Mathematical modeling of primary hepatitis C infection: noncytolytic clearance and early blockage of virion production. *Gastroenterology* 128, 1056–1066.
- Dixit, N.M., Layden-Almer, J.E., Layden, T.J., Perelson, A.S., 2004. Modelling how ribavirin improves interferon response rates in hepatitis C virus infection. *Nature* 432, 922–924.
- Grakoui, A., McCourt, D.W., Wychowski, C., Feinstone, S.M., Rice, C.M., 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polypeptide cleavage sites. *J. Virol.* 67, 2832–2843.
- Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., Shimotohno, K., 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. *Proc. Natl. Acad. Sci. U.S.A.* 88, 5547–5551.
- Hijikata, M., Shimizu, Y.K., Kato, H., Iwamoto, A., Shih, J.W., Alter, H.J., Purcell, R.H., Yoshikura, H., 1993. Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. *J. Virol.* 67, 1953–1958.
- Hongo, T., Kajikawa, M., Ishida, S., Ozawa, S., Ohno, Y., Sawada, J., Umezawa, A., Ishikawa, Y., Kobayashi, T., Honda, H., 2005. Three-dimensional high-density culture of HepG2 cells in a 5-ml radial-flow bioreactor for construction of artificial liver. *J. Biosci. Bioeng.* 99, 237–244.
- Ikeda, M., Sugiyama, K., Mizutani, T., Tanaka, T., Tanaka, K., Sekihara, H., Shimotohno, K., Kato, N., 1998. Human hepatocyte clonal cell lines that support persistent replication of hepatitis C virus. *Virus Res.* 56, 157–167.
- Iwahori, T., Matsuura, T., Maehashi, H., Sugo, K., Saito, M., Hosokawa, M., Chiba, K., Masaki, T., Aizaki, H., Ohkawa, K., Suzuki, T., 2003. CYP3A4 inducible model for in vitro analysis of human drug metabolism using a bioartificial liver. *Hepatology* 37, 665–673.
- Kanto, T., Hayashi, N., Takehara, T., Hagiwara, H., Mita, E., Naito, M., Kasahara, A., Fusamoto, H., Kamada, T., 1994. Buoyant density of hepatitis C virus recovered from infected hosts: two different features in sucrose equilibrium density-gradient centrifugation related to degree of liver inflammation. *Hepatology* 19, 296–302.
- Kawada, M., Nagamori, S., Aizaki, H., Fukaya, K., Niiya, M., Matsuura, T., Sujino, H., Hasumura, S., Yashida, H., Mizutani, S., Ikenaga, H., 1998. Massive culture of human liver cancer cells in a newly developed radial flow bioreactor system: ultrafine structure of functionally enhanced hepatocarcinoma cell lines. *In Vitro Cell Dev. Biol. Anim.* 34, 109–115.
- Kuo, G., Choo, Q.L., Alter, H.J., Gitnick, G.L., Redeker, A.G., Purcell, R.H., Miyamura, T., Dienstag, J.L., Alter, M.J., Stevens, C.E., et al., 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* 244, 362–364.
- Lanford, R.E., Guerra, B., Lee, H., Averett, D.R., Pfeiffer, B., Chavez, D., Notvall, L., Bigger, C., 2003. Antiviral effect and virus-host interactions in response to alpha interferon, gamma interferon, poly(i)-poly(c), tumor necrosis factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. *J. Virol.* 77, 1092–1104.
- Layden, T.J., Layden, J.E., Ribeiro, R.M., Perelson, A.S., 2003. Mathematical modeling of viral kinetics: a tool to understand and optimize therapy. *Clin. Liver Dis.* 7, 163–178.
- Layden-Almer, J.E., Cotler, S.J., Layden, T.J., 2006. Viral kinetics in the treatment of chronic hepatitis C. *J. Viral Hepat.* 13, 499–504.
- Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A., Rice, C.M., 2005. Complete replication of hepatitis C virus in cell culture. *Science* 309, 623–626.
- Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285, 110–113.
- Martell, M., Esteban, J.J., Quer, J., Genesca, J., Weiner, A., Esteban, R., Guardia, J., Gomez, J., 1992. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasiespecies nature of HCV genome distribution. *J. Virol.* 66, 3225–3229.
- Murakami, K., Ishii, K., Ishihara, Y., Yoshizaki, S., Tanaka, K., Gotoh, Y., Aizaki, H., Kohara, M., Yoshioka, H., Mori, Y., Manabe, N., Shoji, I., Sata, T., Bartenschlager, R., Matsuura, Y., Miyamura, T., Suzuki, T., 2006. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. *Virology* 351, 381–392.

- Pawlotsky, J.M., 2006. Hepatitis C virus population dynamics during infection. *Curr. Top. Microbiol. Immunol.* 299, 261–284.
- Perelson, A.S., Herrmann, E., Micol, F., Zeuzem, S., 2005. New kinetic models for the hepatitis C virus. *Hepatology* 42, 749–754.
- Poynard, T., Yuen, M.F., Ratzl, V., Lai, C.L., 2003. Viral hepatitis C. *Lancet* 362, 2095–2100.
- Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., 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.
- Shimizu, Y.K., Hijikata, M., Iwamoto, A., Alter, H.J., Purcell, R.H., Yoshikura, H., 1994. Neutralizing antibodies against hepatitis C virus and the emergence of neutralization escape mutant viruses. *J. Virol.* 68, 1494–1500.
- Suzuki, T., Omata, K., Satoh, T., Miyasaka, T., Arai, C., Maeda, M., Matsuno, T., Miyamura, T., 2005. Quantitative detection of hepatitis C virus (HCV) RNA in saliva and gingival crevicular fluid of HCV-infected patients. *J. Clin. Microbiol.* 43, 4413–4417.
- Tagawa, M., Kato, N., Yokosuka, O., Ishikawa, T., Ohto, M., Omata, M., 1995. Infection of human hepatocyte cell lines with hepatitis C virus in vitro. *J. Gastroenterol. Hepatol.* 10, 523–527.
- Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11, 791–796.
- Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T., Chisari, F.V., 2005. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9294–9299.

Critical Role of Virion-Associated Cholesterol and Sphingolipid in Hepatitis C Virus Infection[∇]

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In this study, we establish that cholesterol and sphingolipid associated with hepatitis C virus (HCV) particles are important for virion maturation and infectivity. In a recently developed culture system enabling study of the complete life cycle of HCV, mature virions were enriched with cholesterol as assessed by the molar ratio of cholesterol to phospholipid in virion and cell membranes. Depletion of cholesterol from the virus or hydrolysis of virion-associated sphingomyelin almost completely abolished HCV infectivity. Supplementation of cholesterol-depleted virus with exogenous cholesterol enhanced infectivity to a level equivalent to that of the untreated control. Cholesterol-depleted or sphingomyelin-hydrolyzed virus had markedly defective internalization, but no influence on cell attachment was observed. Significant portions of HCV structural proteins partitioned into cellular detergent-resistant, lipid-raft-like membranes. Combined with the observation that inhibitors of the sphingolipid biosynthetic pathway block virion production, but not RNA accumulation, in a JFH-1 isolate, our findings suggest that alteration of the lipid composition of HCV particles might be a useful approach in the design of anti-HCV therapy.

Hepatitis C virus (HCV) is recognized as a major cause of chronic liver disease, including chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma. It presently affects approximately 200 million people worldwide (26). HCV is an enveloped positive-strand RNA virus belonging to the *Hepacivirus* genus of the family *Flaviviridae*. Its genome of ~9.6 kb encodes a polyprotein precursor of ~3,000 residues, and the structural proteins (core, E1, and E2) reside in its N-terminal region.

Little is known about the assembly of HCV and its virion structure, because efficient production of authentic HCV particles has only recently been achieved. Nucleocapsid assembly generally involves oligomerization of the capsid protein and encapsidation of genomic RNA. This process is thought to occur upon interaction of the core protein with viral RNA, and this core-RNA interaction may induce a change from RNA replication to packaging. As with related viruses, the mature HCV virion likely consists of a nucleocapsid and an outer envelope composed of a lipid membrane and envelope proteins. Expression of the structural proteins in mammalian cells has been observed to generate virus-like particles with ultrastructural properties similar to those of HCV virions (5, 29). Packaging of these HCV-like particles into intracellular vesicles as a result of budding from the endoplasmic reticulum (ER) has also been observed (8, 34). However, HCV structural

proteins are observed both in the ER and in the Golgi apparatus (45). Moreover, complex N-linked glycans have been detected on the surfaces of HCV particles isolated from patient sera, suggesting that the glycans transit through the Golgi apparatus (44). Interactions between the core and E1/E2 proteins are thought to determine viral morphology and are mediated through a cytoplasmic loop present in the polytopic form of E1 (35). Recently, we 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 Dulbecco's modified Eagle medium (DMEM)-10% fetal bovine serum. Huh-7 cell lines, which carry subgenomic replicon RNA of either the JFH-1 (20) or the N (11, 17) strain, 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 Biolabs, Beverly, MA), and CD81 (BD Pharmingen, Franklin Lakes, NJ), as well as rabbit polyclonal antibodies against calnexin (Stressgen, Ann Arbor, MI) and HCV core (48). ISP-1 mycotoxin, cholesterol, and

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heparinase I were purchased from Sigma, and recombinant *Bacillus cereus* sphingomyelinase (SMase) was obtained from Higeta Shoyu (Tokyo, Japan). (1*R*,3*R*)-*N*-(3-Hydroxy-1-hydroxymethyl-3-phenylpropyl) dodecanamide (HPA-12), which was synthesized as described elsewhere (24), was a gift from Shu Kobayashi (University of Tokyo).

Plasmids. pCAE1 and pCAE2 contain HCV cDNAs spanning the E1 region (amino acids 192 to 383) with a FLAG tag at the N terminus and the E2 region (amino acids 384 to 809) with a Myc tag at the N terminus of strain NIHJ1 (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 domains, and cytoplasmic domains derived from VSV-G, as described elsewhere (50) (see Fig. 4D).

Virus production. Plasmid pJFH1, containing full-length cDNA of the JFH-1 isolate, was used to generate HCVcc as described elsewhere (23, 33, 34, 54). pJ6/JFH was obtained from JFH1 by replacement of the 5' untranslated region to the p7 region (EcoRI-BclI) of J6. In vitro-transcribed RNA from linearized pJFH1 or pJ6/JFH1 was delivered to Huh-7 cells by electroporation. Culture supernatants were collected at 72 h posttransfection, clarified by low-speed centrifugation, passed through a 0.45- μ m-pore-size filter, and concentrated using an Amicon Ultra-15 unit (Millipore, Bedford, MA) or by ultracentrifugation (23). Infectious titers, HCV RNA copies, and core protein concentrations of the viral stocks were $\sim 5 \times 10^7$ focus-forming units per ml, $\sim 1 \times 10^7$ copies/ml, and $\sim 1 \times 10^6$ fmol/liter, 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 the HCV genotype 1a isolate H77c (HCVpv) was generated as previously described (51). Briefly, 293T cells transiently expressing E1 and E2 proteins (strain H77) were infected with VSVdelG-GFP/G, in which the G envelope gene was replaced with green fluorescent protein (GFP) and pseudotyped with VSV-G.

Determination of cholesterol and phospholipid contents 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 (14). 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 DMEM (Sigma) supplemented with 10% fetal bovine serum (Sigma) and nonessential amino acids (Invitrogen, Carlsbad, CA) for 1 h at 37°C, followed by centrifugation at 100,000 \times 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 treatment described above, for 1 h at 37°C and then washed as described above. Viral core protein levels in the cells and in the supernatant were quantified 72 h later using an HCV core enzyme-linked immunosorbent assay (Ortho-Clinical Diagnostics, Tokyo, Japan).

SMase treatment. HCVcc was treated with SMase at various concentrations in DMEM for 1 h at 37°C and was then centrifuged at 100,000 \times 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 was 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. Huh-7-25, 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 phosphate-buffered saline, 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 reverse transcription-PCR method (2, 34). Cells were treated with heparinase 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 reverse transcription-PCR as previously described (2, 34). Levels of core protein in the culture supernatants of HCVcc-infected cells were tested as described above.

Detection of cholesterol content of HCVcc. For [3 H]cholesterol labeling of viruses, HCVcc-infected or uninfected cells were incubated with 50 mCi of

TABLE 1. Cholesterol and phospholipid contents of HCVcc and cells

Cell type or virus	Content (nmol/mg of protein) ^a		Chol/PL ratio
	Chol	PL	
Cells			
Uninfected	105.9 \pm 10.4	253.2 \pm 10.6	0.42
JFH-1 infected	116.5 \pm 10.0	292.0 \pm 18.4	0.40
Virus			
JFH-1	43.6 \pm 2.4	33.8 \pm 1.8	1.29
J6/JFH-1 ^b	28.7 \pm 4.8	22.7 \pm 2.9	1.26

^a Data are averages of three independent measurements \pm standard deviations. Chol, cholesterol; PL, phospholipids.

^b J6/JFH1 virus was produced from the pJ6/N2X-JFH1 construct and has structural proteins from the J6CF strain.

[1 α ,2 α - 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 fractions from an uninfected culture were lysed in the buffer containing 1% Triton X-100 (TX-100), and radioactivity was quantified by scintillation counting. Radioactivities (in counts per minute) of HCVcc samples were determined by subtracting the radioactivity of uninfected cells from that of HCVcc-infected cells.

Metabolic labeling analysis of sphingolipid content. After 2 h of incubation with [14 C]serine (0.5 mCi/ml) in Opti-MEM (Invitrogen), the cells were lysed with 0.1% sodium dodecyl sulfate, and total lipid was extracted with chloroform-methanol (1:2, vol/vol). 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, vol/vol). 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 (4). However, little is known about the role of lipids associated with the virions of flaviviruses, including HCV, and their contribution to the viral life cycle. To determine the lipid composition of mature HCV virions, we extracted total lipid from HCVcc (JFH-1 and chimeric J6/JFH-1) prepared from the culture supernatants of cells infected with HCV, as well as the total cellular membrane fractions of uninfected and infected Huh-7 cells. The cholesterol and phospholipid contents were quantified, because 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 higher in virus samples (1.29 and 1.26 for JFH-1 and J6/JFH-1, respectively) than in cell membrane samples (0.40 and 0.42 for JFH-1-infected and uninfected cells, respectively) (Table 1). The ratios in viral samples were similar to or greater than those in 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 the 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

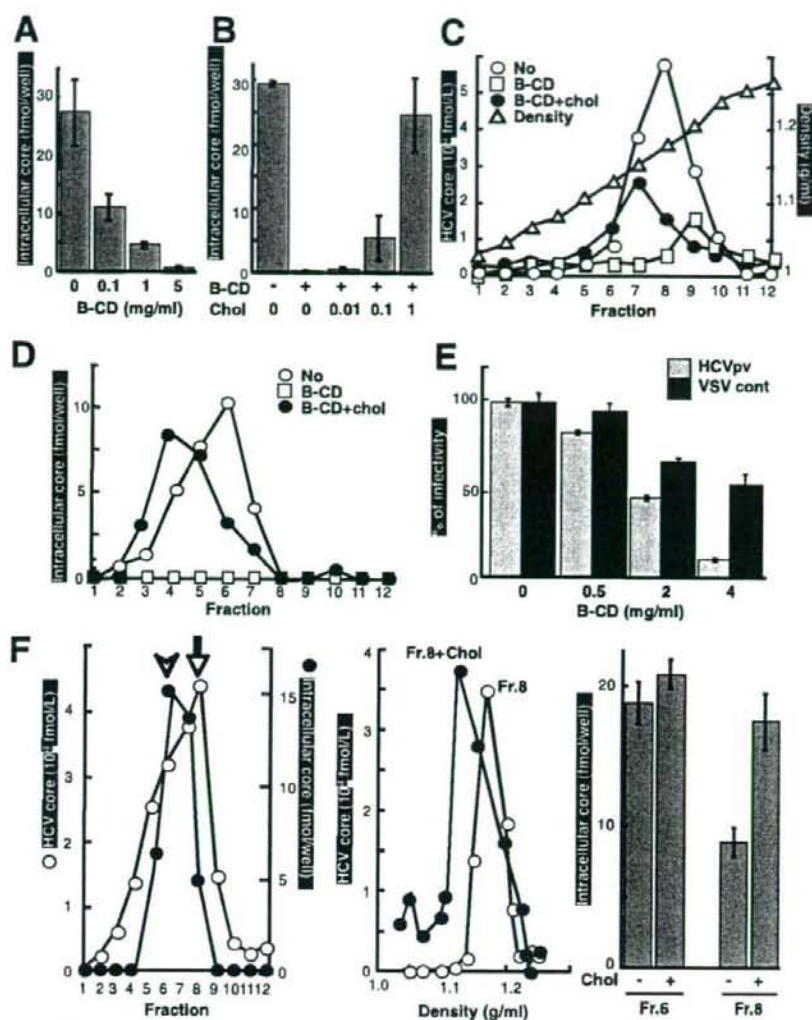


FIG. 1. Role of HCV-associated cholesterol in infection. (A) Effect of cholesterol depletion on HCV infectivity. HCVcc particles (≈ 2 fmol of the core protein) were treated with B-CD at 0.1, 1, and 5 mg/ml for 1 h at 37°C. After removal of B-CD, Huh-7 cells were infected with the treated virus particles, after which the core protein content of infected cells at 72 h p.i. was determined as an indicator of infectivity, as previously established (24). (B) Effect of cholesterol replenishment on infectivity. After treatment with 5 mg/ml B-CD, virus was treated either with medium alone or with medium containing exogenous cholesterol for 1 h at 37°C. (C) Effect of cholesterol depletion and replenishment on density gradient profiles of the viral particles. The HCVcc treated with 5 mg/ml B-CD was replenished with exogenous cholesterol (1 mM) and then separated by 10- to 60% sucrose gradient ultracentrifugation. The core protein in each fraction was measured. The density of each fraction was determined by refractive index measurement. (D) Effects of cholesterol depletion and replenishment on viral infectivity. Each fraction (see panel C) was infected, and then the core proteins in the cells were measured at 72 h p.i. (E) Effect of cholesterol depletion on the infectivity of HCVpv (genotype 1a) (shaded bars) or the control, VSV Δ delG-GFP/G (solid bars). The viruses were preincubated with B-CD for 1 h at 37°C before infection. (F) (Left) The culture medium from HCVcc-producing cells was fractionated as described above. For each fraction, the amounts of core and intracellular core (infectivity) are plotted. Peaks of the core (arrow) and infectivity (arrowhead) are indicated. (Center) An aliquot of fraction 8 (peak of the core) was treated with 1 mM cholesterol for 1 h at 37°C. The resultant aliquot and an untreated aliquot of the fraction were subjected to sucrose gradient ultracentrifugation. The core in each fraction was plotted. (Right) The infectivities of fractions (Fr.) 6 and 8 (see the left panel) with or without cholesterol treatment were determined as shown above. Data are means from four independent experiments. Error bars, standard deviations.

increasing concentrations (0.1 to 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 removal of B-CD by ultracentrifugation. Infectivity was

evaluated by quantifying the viral core protein in cells at 72 h postinfection (p.i.). Using an immunoassay that provides results indicative of HCV infectivity (25), we also confirmed a good correlation between the core level and

TABLE 2. Depletion of virion-associated cholesterol by B-CD

Treatment	Radioactivity (cpm) of HCVcc ^a		Avg (%) ^b
	Expt 1	Expt 2	
None	5,327	5,573	5,450 (100)
B-CD (5 mg/ml)	3,643	1,646	2,644 (48.5)

^a Determined by subtracting the radioactivity of uninfected cells from that of HCVcc-infected cells in two experiments.

^b Percentage of the radioactivity of the untreated sample.

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

To demonstrate that the reduced infection efficiency of B-CD-treated virus was caused by the reduced cholesterol content of 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 added in an attempt to reconstitute the normal virion cholesterol content. The addition of 1 mM cholesterol completely reversed the virus infectivity (Fig. 1B). After cholesterol was replenished, the viral RNA was restored to a level similar to that in the untreated control.

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 density of HCVcc core protein at its peak concentration in untreated virus samples was ~1.17 g/ml. When virion-associated cholesterol was removed by B-CD, the density of HCVcc core protein at its peak concentration was shifted to 1.20 g/ml. Addition of exogenous cholesterol to this cholesterol-depleted sample restored a lower-density fraction (1.15 g/ml). Figure 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 a cholesterol-depleting drug is reversible. We further observed that B-CD treatment of a pseudotyped VSV containing the E1 and E2 proteins of the HCV genotype 1a isolate H77c (HCVpv) resulted in a progressive loss of infectivity, while B-CD had significantly less impact on the infectivity of the control virus VSVdelG-G1P/G (Fig. 1E).

The results described above 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 supernatants collected from HCV-infected cells were analyzed with regard to the presence of core protein and infec-

tivity (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 levels than those in fraction 6. However, quantification of lipids, including cholesterol, in the fractions obtained failed, presumably due to a low sensitivity of detection. Thus, to extend our findings on the 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, center). A concomitant increase in the infectivity of the fraction, approaching that of untreated fraction 6, was observed (Fig. 1F, right). In contrast, supplementation of 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 to 10 U/ml) of bacterial SMase, which is known to hydrolyze membrane-bound SM to ceramide. Following ultracentrifugation to remove the SMase, Huh-7 cells were inoculated with the HCVcc. The amount of HCV core protein within the cells was quantified at 72 h p.i. Figure 2A shows 50 and 90% reductions in HCV infectivity after 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 the infectivity of the control virus (Fig. 2B). This demonstrates that sphingolipid, like cholesterol, plays 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 a 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 postbinding events during viral entry, we used a binding assay in which Huh-7 cells preincubated 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 for reduced 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 roles of cholesterol and sphingolipid on the HCVcc membrane in viral internalization, a virus-cell mixture

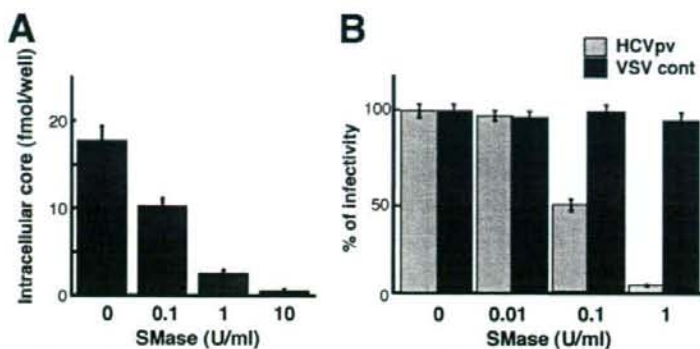


FIG. 2. Effect of SM hydrolysis on viral infectivity. (A) Effect on the infectivity of HCVcc. HCVcc was treated with 0.1, 1, or 10 U/ml SMase for 1 h at 37°C, after which SMase was removed by ultracentrifugation. Huh-7 cells were infected with the treated virus, and the core protein content of infected cells was determined at 72 h p.i. (B) Effect on the infectivity of HCVpv (genotype 1a) (shaded bars) or the control, VSVdelG-GFP/G (VSV cont) (solid bars). The viruses were preincubated with SMase for 1 h at 37°C before infection. Data are means from four independent experiments. Error bars, standard deviations.

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 levels within cells was detected after pretreatment of the virus with either B-CD or SMase. These results strongly suggest that virion-associated cholesterol and sphingolipid function as key determinants of internalization but not of 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 treatment with cold TX-100, followed by equilibrium flotation centrifugation. Matto et al. (30) re-

ported 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, lysates from cells infected with HCVcc were subjected to membrane flotation analysis. In the absence of detergent treatment, the majority of the core (Fig. 4A) and E1 (Fig. 4B) proteins were detected in the membrane fractions. After treatment with cold TX-100, significant amounts of both viral proteins were recovered from the DRM fraction. However, after treatment with TX-100 at 37°C, the majority of the 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 an E1 or E2 expression plasmid (Fig. 4C) and that the cytoplasmic tails of envelope

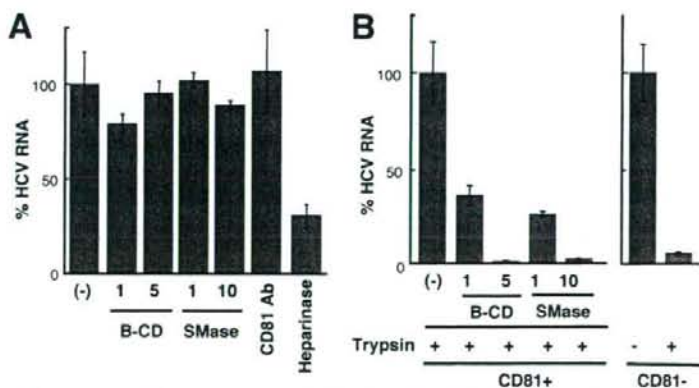


FIG. 3. Effects of B-CD or SMase on virus attachment and internalization. (A) Virus attachment to Huh-7 cells was determined at 4°C after treatment of HCVcc with B-CD (1 or 5 mg/ml) or SMase (1 or 10 U/ml). An antibody (Ab) against CD81 was used, in order to ensure that the antibody did not inhibit HCVcc binding (7, 33). Heparinase was used to reduce HCV attachment to the cell. Viral RNA copies were normalized to total cellular RNA, and the normalized RNA copies in the mock-treated sample (-) were arbitrarily set at 100%. (B) Virus internalization was measured in Huh7-25, a CD81-negative subclone (CD81⁻) (3), and Huh7-25-CD81, which stably expresses CD81 (CD81⁺), after treatment of the virions with B-CD or SMase. After internalization for 2 h at 37°C, cells were exposed to trypsin (trypsin +) or phosphate-buffered saline (trypsin -). Huh7-25 was used to ensure that surface-bound virus would be removed by trypsin treatment. The amounts of HCV RNA in Huh7-25 and Huh7-25-CD81 cells infected with untreated HCVcc were assigned the arbitrary value of 100%, respectively. Results are representative of four independent experiments.

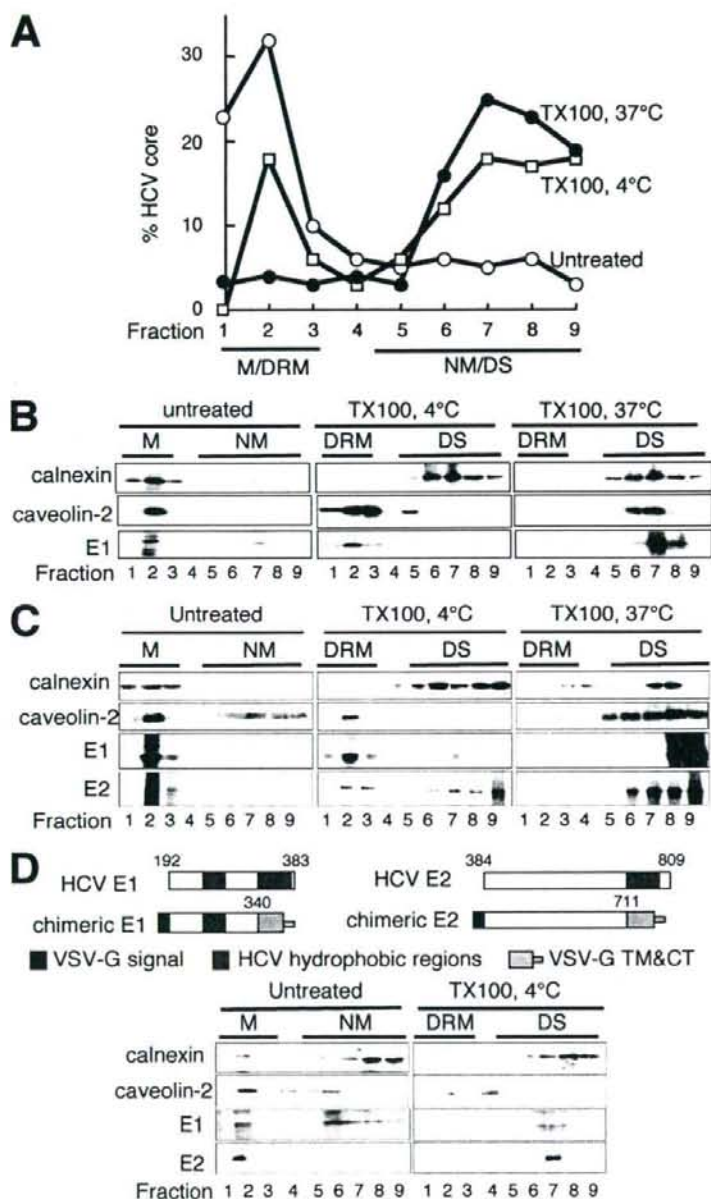


FIG. 4. Compartmentation of HCV structural proteins within DRM fractions. Lysates of HCVcc-infected cells were either treated with 1% TX-100, either on ice or at 37°C, or left untreated, followed by sucrose gradient centrifugation. (A and B) For each fraction, the amount of core protein was determined by an enzyme-linked immunosorbent assay (A), and E1, calnexin, and caveolin-2 were analyzed by Western blotting (B). The amount of core protein in each lysate (TX-100, 37°C; TX-100, 4°C; Untreated) was assigned the arbitrary value of 100%. M, membrane; NM, nonmembrane; DS, detergent soluble. (C) Lysates of 293T cells expressing HCV E1 or E2 protein were either treated with 1% TX-100, either on ice or at 37°C, or left untreated, followed by discontinuous sucrose gradient centrifugation. Each fraction was concentrated in a Centricon YM-30 filter unit and subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting with antibodies against calnexin, caveolin-2, Myc (E1), or FLAG (E2). (D) (Top) Structures of HCV envelope genes used. Amino acid positions of HCV are indicated. Signal sequence, transmembrane (TM), and cytoplasmic tail (CT) domains of VSV G protein are shown. (Bottom) Cell lysates expressing chimeric HCV E1 or E2 protein were treated with 1% TX-100 on ice or left untreated, followed by discontinuous sucrose gradient centrifugation. It has been reported that VSV-G is not associated with lipid (39). Calnexin, caveolin-2, and chimeric glycoproteins (chimeric E1 and chimeric E2) were analyzed by immunoblotting. Fractions are numbered from 1 to 9 in order from top to bottom (light to heavy).

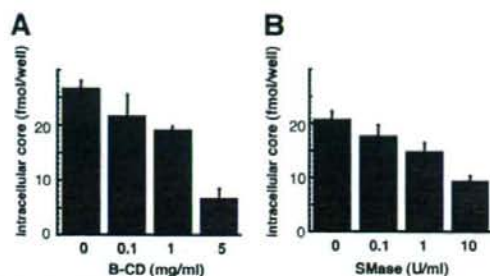


FIG. 5. Effects of B-CD or SMase treatment of cells on HCV infectivity. Huh-7 cells were either left untreated or treated with B-CD at 0.1, 1, or 5 mg/ml (A) or with SMase at 0.1, 1, or 10 U/ml (B) prior to HCVcc infection. Intracellular core levels were quantitated 72 h p.i. Data are means from four independent experiments. Error bars, standard deviations.

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 SM hydrolysis from the host cell membrane decreases HCV infection, in part by decreasing the level of CD81 on the cell surface (19, 53). The involvement of the lipid environment of the host cell plasma membrane in HCV infection was investigated in our HCVcc infection system. Prior to infection, Huh-7 cells were treated with B-CD or SMase and then washed with the medium five times. Cholesterol depletion from Huh-7 cells by B-CD at 1 or 5 mg/ml inhibited HCV core levels by 20 and 75%, respectively, compared to levels in untreated cells (Fig. 5A). We also found that hydrolysis of SM by SMase at 1 or 10 U/ml on the cells, respectively, led to moderate reduction of the viral infection, by 20 or 55% of the infection level of the untreated control (Fig. 5B). There was no influence on cell viability under the conditions of these treatments (data not shown). These findings, compared with the results in Fig. 1A and 2A, suggest that the raft-like environment on the plasma membrane likely serves as a portal for HCV entry, but HCV virion-associated cholesterol and sphingolipid more readily play more critical roles in viral infection.

Inhibitors of the sphingolipid biosynthetic pathway suppress the production of HCVcc, but not RNA replication, for a JFH-1-derived replicon. In the course of studying the involvement of lipid metabolism in the HCV life cycle, 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 or no effect on viral RNA accumulation in JFH-1 replicon cells (Fig. 6A). Furthermore, these compounds did not affect luciferase

activity in the lysates of Huh-7 cells transfected with an *in vitro*-transcribed JFH-1 replicon RNA containing a luciferase reporter gene (22) (data not shown). Figure 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 versus JFH-1. When *de novo* synthesis of sphingolipids was examined by metabolic labeling with [¹⁴C]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 dependent on the viral genotype or isolate.

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 or HPA-12 under conditions similar to those the replicon cells, viral core levels in the culture supernatants were greatly reduced in a dose-dependent manner. For example, exposure to 10 μ M ISP-1 or 1 μ M HPA-12 reduced viral core protein levels more than 85% from those for control cells (Fig. 6C). The 50% inhibitory concentrations of both drugs were less than 0.1 μ M, 50-fold less than those obtained for the RNA replication of the HCV-N-replicon. Together, these results suggest that the sphingolipid biosynthetic pathway plays an important role in the production of HCV particles, but not in genome replication, in JFH-1-based HCVcc.

DISCUSSION

In this study, we demonstrated the role of HCV virion-associated cholesterol and sphingolipid in viral infectivity. Although dependence on virion-associated cholesterol for virus entry has been shown for a number of viruses (4, 6, 28, 49), this is the first study to demonstrate the importance of envelope cholesterol in a virus belonging to the family *Flaviviridae*. 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 levels enhances cellular cholesterol efflux in the presence of B-CD (9, 12). Thus, it may be possible 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 inhibited virus internalization but not cell attachment (Fig. 3) suggests that HCV membrane-associated cholesterol and sphingolipid are crucial for the interaction of viral glycoproteins with the virus-receptor/coreceptor 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, the maturation of viral envelopes with high cholesterol/phospholipid ratios via association with rafts may be important for the stability of HCV

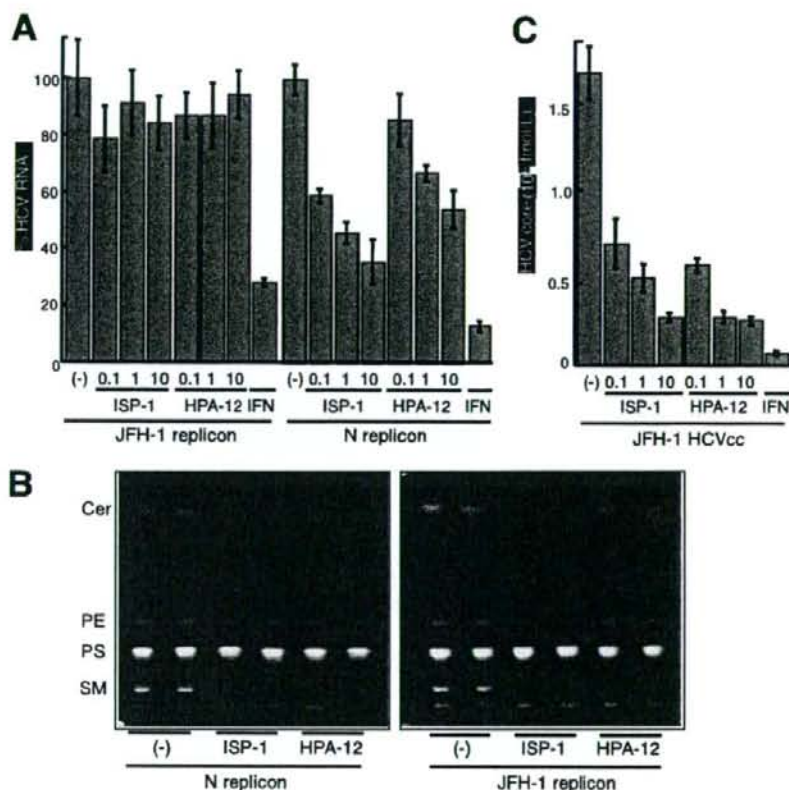


FIG. 6. Anti-HCV effects of inhibitors of the sphingolipid biosynthetic pathway. Subgenomic replicon cells derived from HCV isolate N or JFH-1, as well as HCVcc-producing cells, were treated with ISP-1 (0.1, 1, or 10 μ M), HPA-12 (0.1, 1, or 10 μ M) or alpha interferon (IFN) (100 U/ml) for 72 h. HCV RNA titers in the replicon cells (A) and the HCV core protein content of the culture medium of infected cells (C) were determined. Data are means from four independent experiments. Error bars, standard deviations. (B) De novo synthesis of sphingolipid in the absence or presence of ISP-1 (10 μ M) and HPA-12 (10 μ M) was monitored in duplicate by metabolic labeling with [14 C]serine for 2 h at 37°C. Cer, ceramide; PE, phosphatidylethanolamine; PS, phosphatidylserine.

particles. Replenishing the viral membrane with cholesterol following treatment with 5 mg/ml B-CD successfully restored viral infectivity to the same level as that of 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, a result that cannot be reversed by supplying exogenous cholesterol.

How are cholesterol and sphingolipid involved in the HCV virion during the process of virus maturation? Like most positive-stranded RNA viruses, HCV is thought to assemble at the ER membrane. However, Miyazaki et al. (32) reported that lipid droplets are important for HCVcc formation. These authors 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 (15), 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 II resistant (data not shown). Thus, membrane compartments containing cholesterol- and sphingolipid-rich microdomains may be involved in HCV virion maturation. Another explanation for the recruitment of these lipids to the HCV membrane may be an association between the virus and very-low-density lipoprotein (VLDL) or low-density lipoprotein. Recently, Huang et al. (16) demonstrated a close link between HCV production and VLDL assembly, suggesting that an HCV-VLDL complex is generated and secreted from cells.

Recent reports have demonstrated that CD81-mediated HCV infection is partly dependent on cell membrane choles-

terol (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 in the plasma membrane environment may assist HCV entry, while HCV virion-associated cholesterol and sphingolipid appear to play critical roles 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 HCV production, but not viral RNA replication, by the JFH-1 replicon (Fig. 6). Impairment of particle assembly and maturation, rather than suppression of genome replication, by these drugs may account for the 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). The virus strain specificity of the anti-HCV activity of cyclosporine has recently been demonstrated: JFH-1 replication is less sensitive to cyclosporine than replication of genotype 1b strains. Furthermore, the requirement for interaction with a cellular replication cofactor, cyclophilin B, differs 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 important roles 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 SM results in a loss of infectivity. Moreover, the addition of exogenous cholesterol restores infectivity. In addition, cholesterol and sphingolipid on the HCV membrane play key roles 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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REFERENCES

- Aizaki, H., Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsunaga, and T. Miyamura. 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. *Hepatology* 27: 621-627.
- Aizaki, H., K. J. Lee, V. M. Song, H. Ishiko, and M. M. Lal. 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* 324:450-461.
- 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 heterologous hepatitis C virus infection. *J. Virol.* 81:5036-5045.
- 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.* 79:11588-11597.
- Blanchard, E., D. Brand, S. Trassard, A. Goudeau, and P. Roingeard. 2002. Hepatitis C virus-like particle morphogenesis. *J. Virol.* 76:4073-4079.
- Chazal, N., and D. Gerlier. 2003. Virus entry, assembly, budding, and membrane rafts. *Microbiol. Mol. Biol. Rev.* 67:226-237.
- Evans, M. J., T. von Hahn, D. M. Tschernie, A. J. Syder, M. Panis, B. Wolk, T. Hatzioannou, 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* 446:801-805.
- 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.* 76:12325-12334.
- Fukasawa, M., M. Nishijima, H. Itabe, T. Takano, 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- β -cyclodextrin. *J. Biol. Chem.* 275:34028-34034.
- Gao, L., H. Aizaki, J. W. He, and M. M. Lal. 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.* 78:3480-3488.
- Guo, J. T., V. V. Bichko, and C. Seeger. 2001. Effect of alpha interferon on the hepatitis C virus replicon. *J. Virol.* 75:8516-8523.
- Hanada, K., T. Hara, M. Fukasawa, A. Yamaji, M. Umeda, and M. Nishijima. 1998. Mammalian cell mutants resistant to a sphingomyelin-directed cytotoxicity. Genetic and biochemical evidence for complex formation of the LCB1 protein with the LCB2 protein for serine palmitoyltransferase. *J. Biol. Chem.* 273:33787-33794.
- 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.* 92:273-291.
- Heldler, J. G., and R. L. Boyett. 1978. The picomole determination of free and total cholesterol in cells in culture. *J. Lipid Res.* 19:514-518.
- Helzlsouer, F. X., and S. L. Allison. 2003. Flavivirus structure and membrane fusion. *Adv. Virus Res.* 59:63-97.
- 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. USA* 104:5848-5853.
- 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.
- Ishii, N., K. Watanabe, 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.
- 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.* 81:374-383.
- Kato, T., A. Furnsaka, 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.* 64:334-339.
- Kato, T., T. Date, M. Miyamoto, A. Furnsaka, K. Tokushige, M. Mizokami, and T. Wakita. 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* 125:1808-1817.
- Kato, T., T. Date, M. Miyamoto, M. Sugiyama, Y. Tanaka, E. Orto, 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.* 43:5679-5684.
- 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.* 1:2334-2339.
- Kobayashi, S., K. Kakimoto, and M. Sugiyama. 2002. Transition metal salt-catalyzed aza-Michael reactions of enones with carbamates. *Org. Lett.* 18: 1319-1322.
- Kontsondakis, G., E. Herrmann, S. Kallis, R. Bartschlagler, and T. Pletschmann. 2007. The level of CD81 cell surface expression is a key determinant for productive entry of hepatitis C virus into host cells. *J. Virol.* 81:588-598.
- Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285:110-113.
- Mackenzie, J. M., and E. G. Westaway. 2001. Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. *J. Virol.* 75:10787-10799.
- Mancoske, S., G. del Real, R. A. Lacalle, P. Lucas, C. Gomez-Mouton, S. Sanchez-Palomino, R. Delgado, J. Alcami, E. Mira, and A. C. Martinez.

2000. Membrane raft microdomains mediate lateral assemblies required for HIV-1 infection. *EMBO J* 19:190-196.
29. Matsuo, E., H. Tani, C. Lim, Y. Komoda, T. Okamoto, H. Miyamoto, K. Morishiki, S. Yagi, A. H. Patel, T. Miyamura, and Y. Matsuura. 2006. Characterization of HCV-like particles produced in a human hepatoma cell line by a recombinant baculovirus. *Biochem. Biophys. Res. Commun.* 340:200-208.
30. Matto, M., C. M. Rice, B. Aroeti, and J. S. Glenn. 2004. Hepatitis C virus core protein associates with detergent-resistant membranes distinct from classical plasma membrane rafts. *J. Virol.* 78:12047-12053.
31. Myake, Y., Y. Kozutsumi, S. Nakamura, T. Fujita, and T. Kawasaki. 1995. Serine palmitoyltransferase is the primary target of a sphingosine-like immunosuppressant, ISP-1/myriocin. *Biochem. Biophys. Res. Commun.* 211:396-403.
32. Myanari, Y., K. Atsuzawa, N. Usuda, K. Watahi, 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.
33. Morikawa, K., Z. Zhao, T. Date, M. Miyamoto, A. Murayama, D. Akazawa, J. Tanabe, S. Sone, and T. Wakita. 2007. The roles of CD81 and glycosaminoglycans in the adsorption and uptake of infectious HCV particles. *J. Med. Virol.* 79:714-723.
34. Murakami, K., K. Ishii, Y. Ishihara, S. Yoshizaki, K. Tanaka, Y. Gotoh, H. Aizaki, M. Kohara, H. Yoshioka, Y. Mori, N. Manabe, I. Shoji, T. Sata, R. Bartenschlager, Y. Matsuura, T. Miyamura, and T. Suzuki. 2006. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. *Virology* 351:381-392.
35. Nakai, K., T. Okamoto, T. Kimura-Someya, K. Ishii, C. K. Lim, H. Tani, E. Matsuo, T. Abe, Y. Mori, T. Suzuki, T. Miyamura, J. H. Nunberg, K. Morishiki, and Y. Matsuura. 2006. Oligomerization of hepatitis C virus core protein is crucial for interaction with the cytoplasmic domain of E1 envelope protein. *J. Virol.* 80:11265-11273.
36. Ng, M. L., J. Howe, V. Sreenivasan, and J. J. Mulders. 1994. Flavivirus West Nile (Sarafend) egress at the plasma membrane. *Arch. Virol.* 137:303-313.
37. Ng, M. L., S. H. Tan, and J. J. Choo. 2001. Transport and budding at two distinct sites of visible nucleocapsids of West Nile (Sarafend) virus. *J. Med. Virol.* 65:758-764.
38. Nwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193-199.
39. Pessin, J. E., and M. Glaser. 1980. Budding of Rous sarcoma virus and vesicular stomatitis virus from localized lipid regions in the plasma membrane of chicken embryo fibroblasts. *J. Biol. Chem.* 255:9044-9050.
40. Pitth, J., T. Irie, P. B. Sklar, and J. S. Nye. 1988. Drug solubilizers to aid pharmacologists: amorphous cyclodextrin derivatives. *Life Sci.* 43:493-502.
41. Rahman, S., T. Matsuura, K. Masuda, K. Kanemura, and T. Fukunaga. 1998. Maturation site of dengue type 2 virus in cultured mosquito C6/36 cells and Vero cells. *Kobe J. Med. Sci.* 44:65-79.
42. Rouser, G., G. Galli, and G. Kritchevsky. 1967. Lipid composition of the normal human brain and its variations during various diseases. *Pathol. Biol.* 15:195-200.
43. Sakamoto, H., K. Okamoto, M. Aoki, H. Kato, A. Katsume, A. Ohta, T. Tsukada, N. Shimura, 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.
44. Sato, K., H. Okamoto, S. Athara, Y. Hoshi, T. Tanaka, and S. Mishiro. 1993. Demonstration of sugar moiety on the surface of hepatitis C virions recovered from the circulation of infected humans. *Virology* 196:354-357.
45. Serafino, A., M. B. Valli, F. Andreola, A. Crema, G. Ravagnan, L. Bertolini, and G. Carloni. 2003. Suggested role of the Golgi apparatus and endoplasmic reticulum for crucial sites of hepatitis C virus replication in human lymphoblastoid cells infected in vitro. *J. Med. Virol.* 76:31-41.
46. Shi, S. T., K. J. Lee, H. Alzaki, S. B. Hwang, and M. M. Lal. 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. *J. Virol.* 77:4160-4168.
47. Shintzky, M., and M. Inbar. 1976. Microviscosity parameters and protein mobility in biological membranes. *Biochim. Biophys. Acta* 433:133-149.
48. Shirakura, M., K. Murakami, T. Ichimura, R. Suzuki, T. Shimoji, K. Fukuda, K. Abe, S. Sato, M. Fukasawa, Y. Yamakawa, M. Nishijima, K. Morishiki, Y. Matsuura, T. Wakita, T. Suzuki, P. M. Howley, T. Miyamura, and I. Shoji. 2007. E6AP ubiquitin ligase mediates ubiquitination and degradation of hepatitis C virus core protein. *J. Virol.* 81:1174-1185.
49. Stuart, A. D., H. E. Eastace, T. A. McKee, and T. D. Brown. 2002. A novel cell entry pathway for a DAF-using human enterovirus is dependent on lipid rafts. *J. Virol.* 76:9307-9322.
50. Takikawa, S., K. Ishii, H. Aizaki, T. Suzuki, H. Asakura, Y. Matsuura, and T. Miyamura. 2000. Cell fusion activity of hepatitis C virus envelope proteins. *J. Virol.* 74:5066-5074.
51. Tani, H., Y. Komoda, E. Matsuo, K. Suzuki, I. Hamamoto, T. Yamashita, K. Morishiki, K. Fujiyama, T. Kanio, N. Hayashi, A. Owsianka, A. H. Patel, M. A. Whitt, and Y. Matsuura. 2007. Replication-competent recombinant vesicular stomatitis virus encoding hepatitis C virus envelope proteins. *J. Virol.* 81:8601-8612.
52. 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.
53. Volset, C., M. Lavie, F. Helle, A. Op De Boeck, A. Bilhen, J. Bertrand-Michel, F. Tercé, L. Cocquerel, C. Wychowski, N. Vu-Dac, and J. Dubuisson. 2008. Ceramide enrichment of the plasma membrane induces CD81 internalization and inhibits hepatitis C virus entry. *Cell. Microbiol.* 10:606-617.
54. Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11:791-796.
55. 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.
56. 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. USA* 102:9294-9299.

Interaction of Hepatitis C Virus Nonstructural Protein 5A with Core Protein Is Critical for the Production of Infectious Virus Particles[▽]

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Nonstructural protein 5A (NS5A) of the hepatitis C virus (HCV) possesses multiple and diverse functions in RNA replication, interferon resistance, and viral pathogenesis. Recent studies suggest that NS5A is involved in the assembly and maturation of infectious viral particles; however, precisely how NS5A participates in virus production has not been fully elucidated. In the present study, we demonstrate that NS5A is a prerequisite for HCV particle production as a result of its interaction with the viral capsid protein (core protein). The efficiency of virus production correlated well with the levels of interaction between NS5A and the core protein. Alanine substitutions for the C-terminal serine cluster in domain III of NS5A (amino acids 2428, 2430, and 2433) impaired NS5A basal phosphorylation, leading to a marked decrease in NS5A-core interaction, disturbance of the subcellular localization of NS5A, and disruption of virion production. Replacing the same serine cluster with glutamic acid, which mimics the presence of phosphoserines, partially preserved the NS5A-core interaction and virion production, suggesting that phosphorylation of these serine residues is important for virion production. In addition, we found that the alanine substitutions in the serine cluster suppressed the association of the core protein with viral genome RNA, possibly resulting in the inhibition of nucleocapsid assembly. These results suggest that NS5A plays a key role in regulating the early phase of HCV particle formation by interacting with core protein and that its C-terminal serine cluster is a determinant of the NS5A-core interaction.

Hepatitis C virus (HCV) infection is a major public health problem and is prevalent in about 200 million people worldwide (27, 40, 42). Current protocols for treating HCV infection fail to produce a sustained virological response in as many as half of treated individuals, and many cases progress to chronic liver disease, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (15, 31, 35, 43).

HCV is a positive-strand RNA virus classified in the *Hepacivirus* genus within the *Flaviviridae* family (55). Its approximately 9.6-kb genome is translated into a single polypeptide of about 3,000 amino acids (aa), in which the structural proteins core, E1, and E2 reside in the N-terminal region. A crucial function of core protein is assembly of the viral nucleocapsid. The amino acid sequence of this protein is well conserved among different HCV strains compared to other HCV proteins. The nonstructural (NS) proteins NS3-NS5B are considered to assemble into a membrane-associated HCV RNA replicase complex. NS3 possesses the enzymatic activities of serine protease and RNA helicase, and NS4A serves as a cofactor for NS3 protease. NS4B plays a role in the remodeling of host cell membranes, probably to generate the site for the replicase assembly. NS5B functions as the RNA-dependent RNA polymerase. NS5A is known to play an important but undefined role in viral RNA replication.

NS5A is a phosphoprotein that can be found in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms (49). Comparative sequence analyses and limited proteolysis of recombinant NS5A have demonstrated that NS5A is composed of three domains (52). Domain I is relatively conserved among HCV genotypes compared to domains II and III. Analysis of the crystal structure of the conserved domain I that immediately follows the membrane-anchoring α -helix localized at the N terminus revealed a dimeric structure (53). The interface between protein molecules is characterized by a large, basic groove, which has been proposed as a site of RNA binding. In fact, its RNA binding property has been demonstrated biochemically (17). Domains II and III of NS5A are far less understood. Domain II contains a region referred to as the interferon sensitivity determining region, and this region and its C-terminal 26 residues have been shown to be essential for interaction with the interferon-induced, double-stranded RNA-dependent protein kinase (6–10, 38, 39, 48). Domain III includes a number of potential phosphoacceptor sites and is most likely involved in basal phosphorylation. This domain tolerates insertion of large heterologous sequences such as green fluorescent protein (GFP) and is not required for function of NS5A in HCV RNA replication (1, 34). However, a study with the recently established productive HCV cell culture system using genotype 2a isolate JFH-1 (28, 56, 58) demonstrated that while insertion of GFP within the NS5A region does not affect RNA replication, it does produce marked decreases in the production of infectious virus particles (41). This suggests that the C-terminal region of NS5A may affect virus particle production independent of RNA replication. Re-

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cently, Miyazari et al. reported that the association of core protein with the NS proteins and replication complexes around lipid droplets (LDs) is critical for producing infectious viruses (33).

In the present study, we demonstrated that NS5A is a prerequisite for HCV particle production via its interaction with core protein, and we identified serine residues in the C-terminal region of NS5A that play an important role in virion production. Substitution of the serine residues with alanine residues inhibited not only the interaction of NS5A with core protein but also HCV RNA-core association and led to a decrease in HCV particle production with no effect on RNA replication.

MATERIALS AND METHODS

DNA construction. Plasmids pJFH1, which contains the full-length JFH1 cDNA downstream of the 7 RNA promoter sequence, and pSGR-JFH1/Luc, in which the neomycin resistance gene of pSGR-JFH1 has been replaced by the firefly luciferase reporter gene, have been previously described (24, 56). To generate the fluorochrome gene-tagged full-length JFH1 plasmid, pJFH1/NS5A-GFP, the region encompassing the RsrII site of NS5A and the BsrGI site of NS5B was amplified by PCR, the amplification product was cloned into pGEM-T Easy vector (Promega, Madison, WI), and the resultant plasmid was designated pGEM-JFH1/RsrII-BsrGI. A GFP reporter gene was amplified by PCR from pGreen Lantern-1 (Invitrogen, Carlsbad, CA) with primers containing the XhoI sequence and inserted, after restriction digestion with XhoI, into the XhoI site of pGEM-JFH1/RsrII-BsrGI. The resulting plasmid was digested by RsrII and BsrGI and ligated into pJFH1 similarly digested by RsrII and BsrGI to produce pJFH1/NS5A-GFP. For generation of the fluorochrome gene-tagged subgenomic reporter plasmid, pJFH1/NS5A-GFP was digested by RsrII and SnaBI and ligated into pSGR-JFH1/Luc similarly digested by RsrII and SnaBI. The mutations in the NS5A gene were generated by oligonucleotide-directed mutagenesis (57). To construct plasmids expressing N-terminally FLAG-tagged HCV core protein or hemagglutinin (HA)-tagged NS5A, DNA fragments encoding core protein or NS5A (wild type or mutants) were generated from the full-length JFH1 cDNA by PCR. The core protein coding sequence, together with a FLAG sequence linked to its N terminus, was cloned into the pCAGGS vector (57). The coding sequences of NS5A, together with an HA sequence linked to their N termini, were also cloned into pCAGGS vectors. All PCR products were confirmed by automated nucleotide sequencing with an ABI Prism 3130 Avant Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

Cells and viruses. The human hepatoma cell line, Huh-7, and JFH1-4-1 cells, which are Huh-7 cells carrying a subgenomic replicon of JFH1-1 (32), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with minimal essential medium nonessential amino acids (Invitrogen), 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ incubator. Huh-cp7 cells, which are Huh-7 cells stably expressing the proteins core to p7 derived from the JFH1-1 strain (18), were incubated in DMEM containing 300 µg/ml of zeocin (Invitrogen). HCV particles derived from JFH1-1 were produced by transient transfection of Huh-7 cells with *in vitro* transcribed RNA, as described previously (56, 58). Recombinant vaccinia virus strain DIs, which expresses the bacteriophage T7 RNA polymerase under the control of the vaccinia virus early-late promoter P7.5, was generated and propagated as previously described (19).

DNA transfection, immunoprecipitation (IP), and immunoblotting. For coexpression of FLAG-tagged core protein and HA-tagged NS5A, cells were seeded onto 35-mm wells of a six-well cell culture plate and cultured overnight. Plasmid DNAs (2 µg) were transfected into cells using TransIT-1 T1 transfection reagent (Mirus, Madison, WI). Cells were harvested at 48 h posttransfection, washed three times with 1 ml of ice-cold phosphate-buffered saline (PBS), and suspended in 0.25 ml lysis buffer (20 mM Tris-HCl [pH 7.4] containing 135 mM NaCl, 1% Triton X-100, 0.05% sodium dodecyl sulfate [SDS], and 10% glycerol) supplemented with 50 mM NaF, 5 mM Na₂VO₄, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were sonicated at 4°C for 5 min, incubated for 30 min at 4°C, and centrifuged at 14,000 × g for 5 min at 4°C. After preclearing, the supernatant was immunoprecipitated with 10 µl of anti-FLAG M2-agarose beads (Sigma, St. Louis, MO). For expression of the full-length HCV polyprotein, Huh-7 cells transfected with 10 µg of *in vitro* transcribed RNAs by electroporation were resuspended in 20 or 30 ml of culture

medium, and 10-ml aliquots were seeded into 100-mm culture dishes. At 72 h posttransfection, the cells were incubated in 0.5 ml of lysis buffer (20 mM Tris-HCl [pH 7.4] containing 135 mM NaCl, 1% Triton X-100, 0.05% sodium deoxycholate, and 10% glycerol) supplemented with 50 mM NaF, 5 mM Na₂VO₄, 1 µg/ml leupeptin, and 1 mM PMSF. After preclearing, the supernatant was immunoprecipitated with 5 µg of polyclonal anti-NS5A antibody (34a) or polyclonal anti-C/EBPβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and 20 µl of protein G-agarose beads (Invitrogen). The immunocomplex was precipitated with the beads by centrifugation at 800 × g for 30 s and then washed five times with lysis buffer by centrifugation. The proteins binding to the beads were boiled in 20 µl of SDS sample buffer and then subjected to SDS-12.5% polyacrylamide gel electrophoresis (PAGE). The proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA) and then reacted with a primary antibody and a secondary horseradish peroxidase-conjugated antibody. The immunocomplexes were visualized with an ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, United Kingdom) and detected using an LAS-3000 imaging analyzer (Fujifilm, Tokyo, Japan).

In vitro synthesis of HCV RNA and RNA transfection. Plasmid DNAs were digested with XbaI and treated with mung bean nuclease (New England Biolabs, Ipswich, MA) to remove the four terminal nucleotides, resulting in the correct 3' end of the HCV cDNA. Digested DNAs were purified and used as templates for RNA synthesis. HCV RNA was synthesized *in vitro* using a MEGAscript 17 kit (Ambion, Austin, TX). Synthesized RNA was treated with DNase I (Ambion), followed by acid guanidinium thiocyanate-phenol-chloroform extraction to remove any remaining template DNA. Synthesized HCV RNAs were used for electroporation. Trypsinized Huh-7 cells were washed with Opti-MEM 1 reduced-serum medium (Invitrogen) and resuspended at 3 × 10⁶ cells/ml with Cytomix buffer (54). RNA was mixed with 400 µl of cell suspension and transferred into an electroporation cuvette (Precision Universal Cuvettes; Thermo Hybaid, Middlesex, United Kingdom). Cells were then treated at 260 V and 950 µF using a Gene Pulser II unit (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred onto six-well culture plates or 100-mm culture dishes.

Luciferase assay. Cells were harvested at different time points posttransfection of subgenomic reporter replicons and lysed in passive lysis buffer (Promega). The luciferase activity in cells was determined using a luciferase assay system (Promega).

Quantification of HCV core protein. HCV core protein in transfected cells or cell culture supernatants was quantified using a highly sensitive enzyme immunoassay (Ortho HCV antigen ELISA Kit; Ortho Clinical Diagnostics, Tokyo, Japan). To determine intracellular core protein amounts, cell lysates were prepared as described previously (41). To determine the efficiency of core protein release, the ratio of extracellular core protein to total core protein (the sum of intra- and extracellular core protein amounts) was calculated.

Intra- and extracellular infectivity assay. Culture supernatants were harvested 72 h posttransfection, and virus titers were determined by a 50% tissue culture infectious dose (TCID₅₀) assay as described previously (25, 46). Virus titration was performed by seeding naïve Huh-7 cells in 96-well plates at a density of 1 × 10⁵ cells/well. Samples were serially diluted fivefold in complete growth medium and used to infect the seeded cells (six wells per dilution). At 72 h after infection, the inoculated cells were fixed and immunostained with a mouse monoclonal anti-core protein antibody (219) (56), followed by an Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (IgG) (Invitrogen). Wells that showed at least one core protein-expressing cell was counted as positive. Cell-associated infectivity was determined essentially as described previously (12, 47). Briefly, cells were extensively washed with PBS, scraped, and centrifuged for 3 min at 120 × g. Cell pellets were resuspended in 1 ml of DMEM containing 10% FBS and subjected to four cycles of freezing and thawing using dry ice and a 37°C water bath. Samples were then centrifuged at 2,400 × g for 10 min at 4°C to remove cell debris, and cell-associated infectivity was determined by TCID₅₀ assay.

Expression of HCV proteins using vaccinia viruses, metabolic labeling of cells, and radioimmunoprecipitation analysis. Metabolic labeling of cells and radioimmunoprecipitation analysis were performed as described by Huang et al. (17) with some modifications. A total of 4 × 10⁶ Huh-7 cells were seeded onto each well of six-well cell culture plates and cultured overnight. A 2-µg amount of subgenomic replicon DNAs carrying defined NS5A mutations was transfected into cells using TransIT-1 T1 transfection reagent, and at 12 h posttransfection the cells were then infected at a multiplicity of infection of 10 with recombinant vaccinia viruses expressing the 7 RNA polymerase. After 40 h of transfection, cells were incubated in methionine- and cysteine-deficient DMEM (Invitrogen) or phosphate-deficient DMEM (Invitrogen) for 2 h and labeled for 6 h with [³⁵S]methionine and [³⁵S]cysteine (200 µCi/well; GE Healthcare) or

[³²P]orthophosphate (250 μ Ci/well; GE Healthcare). The cells were then washed twice with cold PBS and lysed with SDS lysis buffer (50 mM Tris-HCl [pH 7.6], 0.5% SDS, 1 mM EDTA, 20 μ g/ml of PMSF). The cell lysates were passed through a 27-gauge needle several times to shear cellular DNA. After a 10-min incubation at 75°C, the lysates were clarified by centrifugation and diluted five-fold with HNAETS buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 0.67% bovine serum albumin, 1 mM EDTA, 0.33% Triton X-100). After preclearing by incubation with 20 μ l of protein G-agarose beads for 1 h at 4°C, the supernatant was incubated with 2 μ g of rabbit polyclonal anti-NS5A antibody overnight at 4°C. A 20- μ l aliquot of protein G-agarose beads was further added and incubated for 2 h at 4°C. The cell pellets were washed three times with 0.5 ml of HNAETS buffer (HNAET containing 0.5% SDS), followed by washing once with 0.5 ml of HNE buffer (50 mM HEPES [pH 7.5], 150 mM NaCl and 1 mM EDTA). After treatment with or without λ protein phosphatase (New England Biolabs), the cell pellets were suspended in 20 μ l of SDS sample buffer and boiled for 10 min. The proteins were resolved on 10% SDS-polyacrylamide gels and analyzed by autoradiography.

Subcellular fractionation analysis. All steps were carried out at 4°C in the presence of a protease inhibitor cocktail (Complete; Roche, Mannheim, Germany) as described previously (20), with some modifications. Cells were suspended in four cell volumes of homogenization buffer (50 mM NaCl, 10 mM triethylamine [pH 7.4], 1 mM EDTA), snap frozen in liquid nitrogen, stored at -80°C, and thawed in a water bath at room temperature. Supernatants (6.4 ml) were layered on linear 10-ml iodixanol gradients from 2.5 to 25% and centrifuged at 37,000 rpm for 3.5 h in an SW41 rotor (Beckman, Fullerton, CA), followed by collection of 0.8-ml fractions from the top. Each fraction was concentrated by Centricon YM30 (Millipore), separated by SDS-PAGE, and immunoblotted with a rabbit polyclonal anti-calnexin antibody (Stressgen Biotechnologies, Victoria, Canada), a mouse monoclonal anti-adipose differentiation-related protein (ADRP) antibody (Progen Biotechnik, Heidelberg, Germany), or a rabbit polyclonal anti-NS5A antibody. The core protein amount in each fraction was also determined by enzyme-linked immunosorbent assay (ELISA).

IP-RT-PCR. The process of cell lysis to RNA purification was carried out essentially as described by Johnson et al. (21) with some modifications. A total of 3×10^6 Huh-7 cells were transfected with 10 μ g of *in vitro* transcribed HCV RNAs and resuspended in 20 or 30 ml of culture medium, after which 10-ml aliquots were seeded into 100-mm culture dishes. At 72 h posttransfection, the cells were scraped and incubated in 500 μ l of hypotonic buffer (10 mM HEPES [pH 7.6], 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF) per dish. The cells were passed through a 20-gauge needle several times, lysed with Nondet P-40 at a final concentration of 1%, and incubated on ice for an additional 10 min. After centrifugation at 4,000 g at 4°C for 15 min, glycerol was added to the supernatants at a final concentration of 5%. The cell lysates were incubated with 20 μ l of protein G-agarose beads for 30 min at room temperature. After the cell lysates were removed from protein G-agarose beads, 5 μ g of mouse monoclonal anti-core protein antibody or normal mouse IgG (Sigma) as a negative control was added, and samples were incubated for an additional 1 h at room temperature. A 20- μ l aliquot of protein G-agarose beads per sample was added to the cell lysates and incubated for 1 h. After incubation, the beads were washed three times with wash buffer (10 mM Tris-HCl [pH 7.6], 100 mM KCl, 5 mM MgCl₂, and 1 mM dithiothreitol) and eluted in 100 μ l of elution buffer (50 mM Tris-HCl [pH 8.0], 1% SDS, and 10 mM EDTA) at 65°C for 10 min. After treatment with 100 μ g of proteinase K at 37°C for 30 min, the RNAs in immunocomplexes were isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. Reverse transcriptase PCR (RT-PCR) was carried out using random hexamer and Superscript II RT (Invitrogen), followed by nested PCR with LA *Taq* DNA polymerase (TaKaRa, Shiga, Japan) and primer sets amplifying the fragments of nucleotides (nt) 129 to 2367 and nt 7267 to 9463 of the JFH-1 genome. To amplify the fragment of nt 129 to 2367, the sense primer 5'-CTGTGAGGAACTACTGCTT-3' and the antisense primer 5'-TCAAGATGTCCTGGTGAAG-3' were used for first-round PCR; the sense primer 5'-CGGGAGAGCCATAGTGG-3' and the antisense primer 5'-CATTCGTTGGTAGAGTGC-3' were used for second-round PCR. To amplify the fragment of nt 7267 to 9463, the sense primer 5'-GTCCAGAGTGCCTTCTGGACT-3' and the antisense primer 5'-AGGGCTCACGGAGCTTTTCAT-3' were used for first-round PCR; the sense primer 5'-CAACGTTGGCTGGTGGTGGT-3' and the antisense primer 5'-GGTACCTAGTGGTGGTGGTGGT-3' were used for second-round PCR.

Indirect immunofluorescence analysis. Cells incubated for 3 days after transfection with JFH-1 RNAs were seeded in an eight-well chamber slide (BD Biosciences, San Jose, CA) and cultured overnight. The adherent cells were washed twice with PBS and fixed with 4% paraformaldehyde at room temperature. After a washing step with PBS, the cells were permeabilized with PBS containing 0.3% Triton X-100 and 2% FBS for 1 h at room temperature and

stained with a rabbit polyclonal anti-NS5A antibody and a mouse monoclonal anti-core protein antibody. The fluorescent secondary antibodies were Alexa Fluor 488- or Alexa Fluor 555-conjugated anti-rabbit or anti-mouse IgG antibodies (Invitrogen). Analyses of JFH-1 were performed on a Zeiss confocal laser scanning microscope LSM 510 (Carl Zeiss, Oberkochen, Germany).

RESULTS

Mutations of serine residues at the NS5A C terminus impair basal phosphorylation but have little effect on viral RNA replication. As demonstrated in a previous study, insertion of GFP into the NS5A C terminus does not significantly affect viral RNA replication but reduces the generation of infectious HCV particles (41). The C-terminal region of NS5A contains highly conserved serine residues that are involved in basal phosphorylation (1, 23, 49). To examine the involvement of the serine clusters (cluster 3-A [CL3A] and cluster 3-B [CL3B]) in the C-terminal region of NS5A in HCV particle production, we created mutated HCV genomes as well as subgenomic replicons carrying alanine substitutions for the conserved serine residues at aa 2384, 2388, 2390, and 2391 (residues are numbered according to the positions within the original JFH-1 polyprotein) (CL3A/SA); at aa 2428, 2430, and 2433 (CL3B/SA); or an in-frame deletion spanning aa 2384 to 2433 (Δ 2384-2433) (Fig. 1). A construct with an in-frame insertion of GFP (NS5A-GFP) was also generated as described previously for the Con1 isolate (34).

First, we analyzed the effects of the NS5A mutations on HCV RNA replication using a transient RNA replication assay using subgenomic luciferase reporter replicons (Fig. 2A) and found that the serine-to-alanine substitutions (CL3A/SA and CL3B/SA) did not affect viral RNA replication. NS5A-GFP and Δ 2384-2433 slightly reduced RNA replication, indicating that the mutations of the NS5A C terminus tested in this study do not critically affect RNA replication, which is consistent with previous reports (1, 34, 51).

Next, the phosphorylation status of the mutated NS5A was analyzed as described in Materials and Methods (Fig. 2B). NS5A was isolated from radiolabeled cells by IP and analyzed either directly by SDS-PAGE or after treatment with λ protein phosphatase. Analysis of ³²P-radiolabeled proteins revealed that the CL3A/SA, CL3B/SA, and Δ 2384-2433 mutations resulted in marked reduction of basal phosphorylation (Fig. 2B, compare lane 1 with lanes 3, 5, and 7 in the top panel). All ³²P-labeled NS5A proteins were sensitive to treatment with phosphatase (lanes 2, 4, 6, and 8). The possibility that loss of signal after dephosphorylation was due to contaminating proteases present in the phosphatase preparations can be ruled out because no degradation of the ³⁵S-labeled proteins was observed (Fig. 2B, bottom panel). These results suggest that mutations in the C-terminal serine cluster of NS5A impair basal phosphorylation but have no significant effect on viral RNA replication.

Effect of mutations introduced into the NS5A C terminus on the production of infectious HCV particles. To analyze HCV particle production from cells transfected with the *in vitro* transcribed viral genomic RNAs, we harvested supernatants and cells at 4, 24, 48, 72, and 96 h posttransfection and measured the amounts of core protein. As shown in Fig. 3A, comparable amounts of core proteins were detected in all transfected cells 4 h after transfection, reflecting unchanged

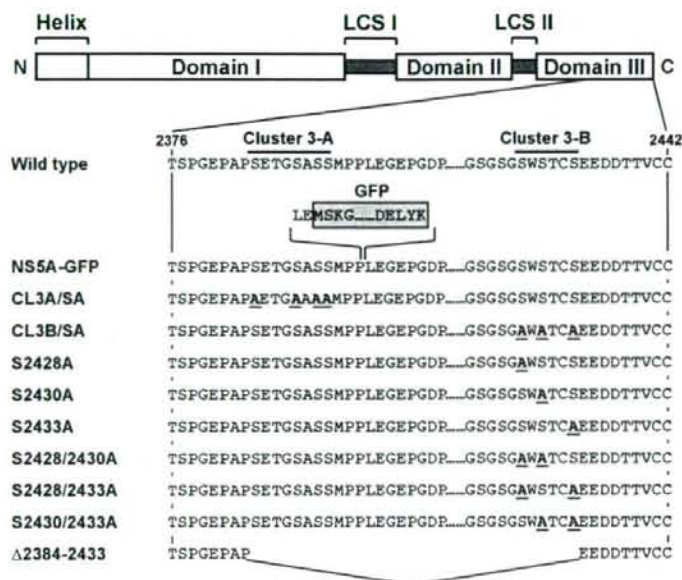


FIG. 1. Structures of HCV constructs used in this study. Schematic diagram of the NSSA structure according to Tellinghuisen et al. (52) is shown in the top panel. The three domains are indicated by white boxes and are separated by trypsin-sensitive regions with presumably low structural complexity (low-complexity sequence [LCS]). The numbers indicate amino acid residues within the original JFH-1 polyprotein. The names listed on the left represent full-length HCV constructs, subgenomic reporter replicons, or N-terminally HA-tagged NSSA constructs used in this study. NSSA-GFP carries a GFP insertion between aa 2394 and 2395 as indicated by a shaded box. CL3A/SA and CL3B/SA carry several serine-to-alanine substitutions in the NSSA C terminus constructed as described previously (1). HCV constructs from S2428A to S2430/2433A carry single or double serine-to-alanine substitutions generated by modification of the CL3B/SA construct. The Δ2384-2433 mutant possesses an in-frame deletion in the C-terminal region of NSSA. Amino acid substitutions are marked in bold and underlined. N and C represent N terminus and C terminus, respectively.

transfection efficiencies, and the kinetics of intracellular core protein levels was similar among transfectants. By contrast, core protein released from cells transfected either with the mutated genome of CL3B/SA, Δ2384-2433, or NS5A-GFP was more than 10-fold lower than that for the wild-type JFH-1 or CL3A/SA (Fig. 3B). Figure 3C shows the efficiency of core protein release from each transfectant, which is expressed as a percentage of the extracellular core protein level relative to the amount of total core protein (the sum of intra- and extracellular core protein). Core protein release efficiency with the wild type and CL3A/SA was 2 to 13% at 48 to 96 h after transfection, while only 1% or less of core protein was released in the cases of CL3B/SA, Δ2384-2433, and NS5A-GFP strains.

To further investigate production and release of infectious virus particles, naïve Huh-7 cells were infected with culture supernatants of cells harvested 72 h posttransfection, and infectious virus titers were determined by TCID₅₀ assay at 72 h after infection. Figure 3D shows that release of infectious virus particles from cells transfected with the genome of CL3B/SA or Δ2384-2433 mutants was markedly reduced (about 10,000-fold) compared to that from wild-type- or CL3A/SA-transfected cells (white bars). To examine whether such a decrease in infectious HCV in the culture supernatants was attributable to defective virion assembly or impaired release of virions, we determined cell-associated infectivity (Fig. 3D). Production of

intracellular infectious virions in CL3B/SA- and Δ2384-2433-transfected cells was strongly impaired in comparison with that in wild-type-transfected (~1,000-fold) and CL3A/SA-transfected (~100-fold) cells. Thus, the results suggest a potential role for the serine cluster at aa 2428, 2430, and 2433 of NSSA in assembly of infectious HCV particles. Among the NSSA mutations tested, CL3B/SA is of particular interest because this mutation leads to a marked reduction in HCV production with no impact on viral RNA replication.

Serine residues at aa 2428, 2430, and 2433 are important for the interaction between NSSA and core protein. Miyazari et al. reported that the association of core protein with NS proteins is critical for infectious HCV production and that mutations of the core protein and NSSA that cause these proteins to fail to associate with each other impair the production of infectious virus (33). Based on these observations and the findings noted above, we hypothesize that NSSA plays a key role in recruiting viral RNA, which is synthesized at the viral replication complex, to nucleocapsid formation via interaction between the NSSA C-terminal region and the core protein. To prove this, we analyzed the interaction of NSSA with the core protein by coimmunoprecipitation experiments. HA-tagged NSSA constructs carrying defined mutations were generated (Fig. 1) and coexpressed with the FLAG-tagged core protein in Huh-7 cells. As shown in Fig. 4A, coimmunoprecipitation of NSSA

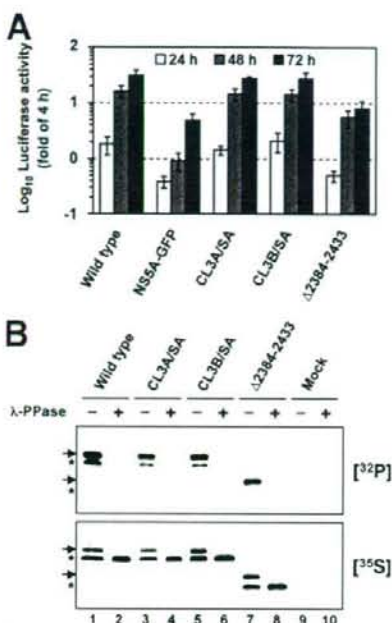


FIG. 2. Mutations at the C terminus of NS5A impair basal phosphorylation and have only a minor impact on RNA replication. (A) Replication of given mutants in transfected Huh-7 cells as determined by luciferase reporter assays performed at 24, 48, and 72 h posttransfection (white, gray, and black bars, respectively). Values given were normalized for transfection efficiency using the luciferase activity determined 4 h after transfection, which was set to 1. Mean values of quadruplicate measurements and the standard deviations are given. (B) Phosphorylation analysis of NS5A using the vaccinia virus T7 hybrid system. NS3-to-NS5B polyprotein fragments carrying the mutations specified above the lanes were transfected into Huh-7 cells, and proteins were radiolabeled with [³²P]orthophosphate or [³⁵S]methionine and [³⁵S]cysteine. NS5A proteins were isolated by IP and separated by SDS-PAGE (10% polyacrylamide). Mock-transfected cells served as a negative control (lanes 9 and 10). Half of the samples were treated with λ protein phosphatase (λ-PPase) (-) whereas the other half was mock treated (+) prior to SDS-PAGE. Arrows and asterisks indicate hyperphosphorylated and basally phosphorylated forms, respectively.

with the core protein was observed in cells expressing the wild-type NS5A and the CL3A/SA-mutated NS5A, but the amount of immunoprecipitated NS5A in the CL3A/SA-expressing cells was slightly lower than that in the wild-type-expressing cells. In contrast, the CL3B/SA- or the Δ2384-2433-mutated NS5A coimmunoprecipitated with the core protein only slightly or not at all.

We further examined the interaction of NS5A with core protein in cells expressing HCV genomes. At 72 h posttransfection with the wild type or CL3B/SA, cells were harvested and immunoprecipitated with an anti-NS5A antibody or an anti-C/EBPβ antibody as a negative control, followed by immunoblotting. Under these experimental conditions, the amount of extracellular core protein released from cells transfected with the CL3B/SA genome was about 10-fold lower than

that for the wild type, although comparable amounts of intracellular core protein were observed in both transfectants (Fig. 4B, left panels). As shown in the right panels of Fig. 4B, the core protein was specifically coimmunoprecipitated with NS5A in cells expressing the wild-type JFH-1 genome but not with the mutated NS5A in cells expressing the CL3B/SA genome. These results demonstrate that NS5A interacts with the core protein in cells producing infectious particles and that serine residues at aa 2428, 2430, and 2433 are important to the success of this interaction.

Two serine residues among aa 2428, 2430, and 2433 are responsible for regulating the interaction of NS5A with the core protein as well as HCV particle production. To further determine the critical residues in the C-terminal serine cluster of NS5A responsible for HCV particle production, we replaced one or two serine residues in the region with alanine (Fig. 1) and investigated which serine-to-alanine substitution influenced HCV particle production. Core protein levels in cells transfected with any construct were comparable over 4 days after transfection, indicating similar efficiencies of transfection and RNA replication from each construct (data not shown). As shown in Fig. 5A, we observed a slight delay in the kinetics of core protein release from cells transfected with the single-substitution genomes, S2428A, S2430A, and S2433A, up to 48 or 72 h posttransfection. However, core protein release from these cells reached comparable levels to that for the wild type at 96 h after transfection. In the cases of the double-substitution mutants (Fig. 5B), core protein release from cells transfected with the double-substitution genomes was markedly reduced, with 10- to 30-fold decreases compared to that for wild type observed. The kinetics of core protein release were similar to that for CL3B/SA.

Interaction of NS5A carrying single or double serine-to-alanine substitutions with the core protein was investigated by coimmunoprecipitation analysis using HA-tagged NS5A constructs. NS5A mutants carrying a single substitution were coimmunoprecipitated with the core protein (Fig. 5C), while none of the double-substitution NS5A mutants or the triple-substitution mutant, CL3B/SA, coimmunoprecipitated with the core protein (Fig. 5D). These results suggest that at least two serine residues in the C-terminal serine cluster of NS5A (aa 2428, 2430, and 2433) are necessary for the interaction between NS5A and the core protein as well as for regulation of HCV particle production and that there is positive correlation between their interaction and the amount of core protein released.

Glutamic acid partially substitutes for serine phosphorylation in the interaction of NS5A with the core protein and virus production. A consequence of phosphorylation is the addition of negative charge to a protein. In some cases, phosphoserine can be mimicked by glutamic or aspartic acid (14). To determine whether the introduction of negative charges into aa 2428, 2430, and 2433 instead of phosphoserines positively regulates the interaction of NS5A with the core protein and virus production, we replaced the serine residues with glutamic acid residues and constructed the CL3B/SA and S2428/2430E mutants (Fig. 6A). Cells transfected with the double-glutamic acid substitution, S2428/2430E, exhibited similar kinetics to the wild-type-transfected cells and released ~22-fold more core protein than S2428/2430A-transfected cells by 96 h posttransfection (Fig. 6B). In contrast,