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Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts

Hideki Aizaki,[a,b] Ki-Jeong Lee,[a] Vicky M.-H. Sung,[a] Hiroaki Ishiko,[c] and Michael M.C. Lai[a,*]

[a] *Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA*
[b] *Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan*
[c] *Mitsubishi Kagaku Bio-clinical Laboratories, Inc., Tokyo 174-0056, Japan*

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Abstract

The mechanism and machinery of hepatitis C virus (HCV) RNA replication are still poorly characterized. Our previous study has shown that HCV RNA synthesis occurs on a lipid raft membrane structure [J. Virol. 77 (2003) 77 4160]. In this study, we further characterized these replication complexes (RCs) in Huh-7 cells that support active RNA replication of a subgenomic HCV replicon. Biochemical analysis showed that these membrane structures were resistant to Nonidet P-40 or Triton X-100 (TX-100) at 4 °C while solubilized by β-octylglucoside at 4 °C or Triton TX-100 at 37 °C, characteristic of lipid rafts. Cholesterol sequestration assay further demonstrated the association between HCV nonstructural (NS) proteins and cholesterol-rich lipid rafts. The RCs contained both minus- and plus-strand HCV RNA, with the plus-stranded RNA being approximately 10-fold more abundant than the minus-strand. Furthermore, the HCV RNA and NS proteins were resistant to RNase and protease digestion, respectively, but became sensitive after treatment with the raft-disrupting agents. These results suggested that the HCV RCs are protected within lipid rafts. Detergent-resistant membrane (DRM) fractions containing NS proteins and viral RNA were capable of HCV RNA synthesis using the endogenous HCV RNA template. NS proteins were distributed in both the ER and the Golgi, but the majority of the active RCs were detected in the Golgi-derived membrane. Depletion of cellular cholesterol selectively reduced HCV RNA replication. These findings provide further insights into the mechanism of HCV replication in vivo.

Keywords: HCV; Lipid raft; Replication complex; NS protein; Detergent-resistant membrane; ER; Golgi

Introduction

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Blood screening based on the cloned HCV genome and its gene products has reduced significantly the blood-borne HCV transmission (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990). However, detailed studies of HCV (e.g., virus replication, pathogenicity, and natural history of HCV) have been greatly hampered by the lack of efficient in vitro culture systems of HCV infection. Recently, the establishment of an HCV subgenomic replicon cell culture system (Lohmann et al., 1999) has enabled the studies of HCV RNA replication. This system has demonstrated that HCV RNA replication requires only the viral nonstructural NS proteins, NS3, NS4A, NS4B, NS5A and NS5B. But the detailed mechanism of HCV replication is yet to be characterized.

RNA replication of most RNA viruses involves certain intracellular membrane structure, including the ER (Restrepo-Hartwig and Ahlquist, 1996; Schaad et al., 1997; Van der Meer et al., 1998), Golgi (Shi et al., 1999), endosomes, and lysosomes (Froshauer et al., 1988). Recently, several groups have succeeded in showing the in vitro replication activities of the replication complexes (RCs) in crude membrane fractions of the HCV subgenomic replicon cells (Ali et al., 2002; Hardy et al., 2003; Lai et al., 2003). These crude cell-free systems provide a valuable complement to the in vitro polymerase

* Corresponding author. Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, 2011 Zonal Avenue, HMR-401, Los Angeles, CA 90033. Fax: +1-323-442-1721.
E-mail address: michlai@usc.edu (M.M.C. Lai).

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assays for biochemical dissection of HCV RNA replication and a useful source for isolation of viral RCs. However, the nature of these RCs has not been well characterized.

Recently, we have demonstrated that newly synthesized HCV RNA and the NS proteins colocalized with each other on distinct speckle-like structures in the cytoplasm of the replicon cell lines (Shi et al., 2003). Membrane flotation analysis further demonstrated that HCV RNA and NS proteins were present in detergent-resistant membrane (DRM) structures, most likely a lipid-raft structure. Lipid rafts are microdomains that are enriched in cholesterol and sphingolipid (Simons and Ikonen, 1997, 2000; Simons and Toomre, 2000). Lipid rafts are characterized biochemically by their insolubility in detergents, and are known to play a critical role in many biological processes such as regulators and organizing centers of signal transduction and membrane traffic pathways. Recent studies also demonstrated that many infectious agents, including viruses, bacteria, and parasites, utilize rafts to populate in the host cells (Fivaz et al., 1999).

In this study, we characterized the structure of the HCV RCs with an intact replication activity. We established that the active RCs, which consist of lipid rafts, existed in the Golgi-derived membrane. These studies provide important insights into the mechanism of RNA replication and pathogenesis of HCV and also suggest potential inhibitors targeting HCV RNA replication.

Results

Association of HCV NS proteins with DRM fractions

Previous studies on the HCV replicon cells have shown that the majority of HCV NS proteins were detected in membrane fractions (Bartenschlager and Lohmann, 2000). Shi et al. (2003) also suggested that the newly synthesized HCV RNA was present in the same membrane fractions. These findings provide strong evidence that HCV replication occurs through membrane-associated RCs, as is the case of several other positive-strand RNA viruses, including poliovirus, flavivirus, and flock house virus (Bolten et al., 1998; Westaway et al., 1997).

Furthermore, our data suggested that the HCV RCs were resistant to detergent [1% Nonidet P-40 (NP-40) at 4 °C] treatment. To further characterize the nature of the DRM fraction containing HCV RC, we performed additional membrane flotation analysis of the lysates of the HCV replicon cells under a different condition. In this experiment, each sucrose fraction was concentrated by passing through a Centriplus YM-100 filter unit, which allows free HCV NS proteins (< 100 000 Da) to go through. Thus, only those proteins existing in large complexes could be detected. As shown in Fig. 1, NS3, NS5A, and NS5B were found almost completely in the membrane fraction. When the cell lysates were treated with 1% NP-40, a nonionic detergent, on ice, a condition which released the ER transmembrane protein calnexin to the cytosol, most of the HCV proteins remained in the membrane fractions. Previously, Shi et al. (2003) has shown that by using a Centricon YM-10 filter unit, which detected all the individual HCV NS protein (>10 000 Da), only a small percentage of the NS5A protein and more than half of NS5B were detected in the cytosolic fractions after NP-40 treatment. These results combined indicated that most of the NS3, NS5A, and NS5B in the DRM were present as large complexes, which could be partially dissociated after treatment with the detergent.

To characterize the nature of these DRM fractions, we examined Caveolin-2 (Cav-2), which is localized in plasma membrane caveolae and associated with lipid rafts (Fujimoto et al., 2001; Ostermeyer et al., 2001; Pol et al., 2001). Cav-2 was present mostly in the DRM fractions, similar to the distribution of NS3, NS5A, and NS5B. These results indicate that the NS proteins are associated with the DRM structures

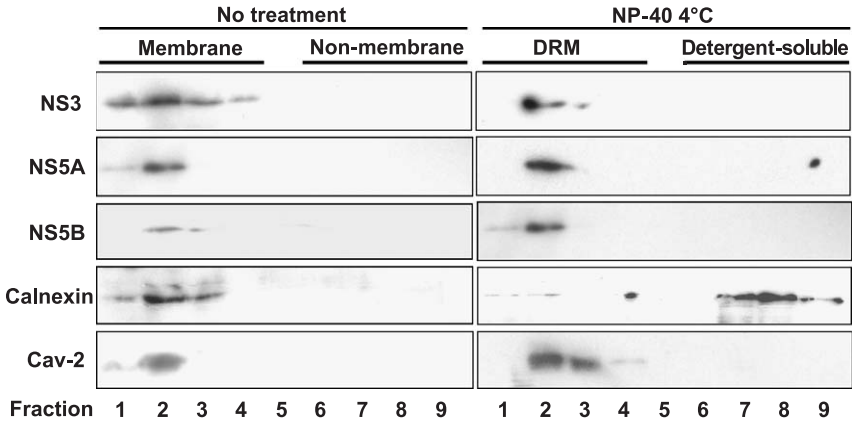

Fig. 1. Detection of HCV proteins in DRM fractions of HCV replicon cells. Cell lysates were either untreated or treated with 1% NP-40 for 30 min on ice and fractionated by discontinuous sucrose gradient centrifugation. Each fraction was concentrated in a Centricon YM-100 filter unit, except for Cav-2, for which YM-30 was used. Equal volumes of the recovered fractions were analyzed on a 12% SDS-PAGE, followed by immunoblotting with antibodies against NS3, NS5A, NS5B, Calnexin, or Cav-2. Fractions are numbered from 1 to 9 in order from top to bottom (light to heavy).

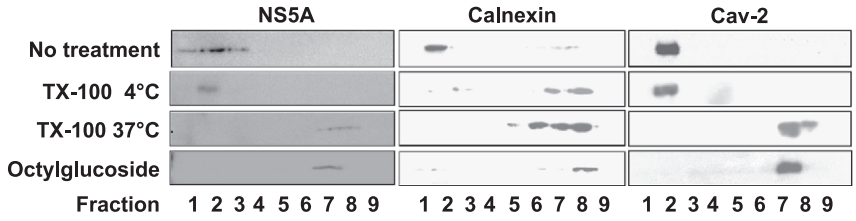

Fig. 2. Detergent solubilization of HCV proteins in HCV replicon cells. Cell lysates were untreated, treated with 1% TX-100 on ice, 1% TX-100 at 37 °C, or 60 mM octylglucoside on ice, and fractionated by discontinuous sucrose gradient centrifugation. Each fraction was concentrated in a Centricon YM-30 filter unit. Equal volumes of the recovered fractions were analyzed on a 12% SDS-PAGE, followed by immunoblotting with antibodies against NS5A, Calnexin, or Cav-2. Fractions are numbered from 1 to 9 in order from top to bottom (light to heavy).

that also contained Cav-2, strongly suggesting that these structures have properties of lipid rafts. Furthermore, these membrane structures are distinct from the ER membrane.

Detergent solubilization of HCV proteins in HCV replicon cells

To confirm that HCV NS proteins are associated with lipid rafts, cell lysates were treated with 1% Triton X-100 (TX-100) on ice before equilibrium flotation centrifugation; each fraction was concentrated by passing through a Centricon YM-30 filter unit. Immunoblotting analysis showed that both NS5A and Cav-2 were recovered exclusively in the membrane-containing fractions even after TX-100 treatment at 4 °C. In contrast, the ER protein Calnexin was solubilized by TX-100 (Fig. 2). However, after treatment with β-octylglucoside at 4 °C or TX-100 at 37 °C, the association of NS5A or Cav-2 with DRM structure was disrupted, as was observed for other raft-localized proteins (Brown and Rose, 1992). NS3 and NS5B were also shifted to the detergent-soluble fractions after treatment with β-octylglucoside at 4 °C or TX-100 at 37 °C (data not shown). Calnexin was solubilized by detergents under any one of these conditions. These results are consistent with the interpretation that the DRM structures containing the HCV NS proteins are lipid rafts.

Cholesterol sequestration assay

As a more stringent test for the association of HCV NS proteins with lipid rafts, we analyzed the effect of cholesterol disruption on the buoyant property of the NS proteins. It has been shown that the insolubility of lipid rafts in TX-100 on ice depends on cholesterol (Chamberlain et al., 2001), and that the removal of cholesterol from raft leads to dissociation of the raft proteins from the lipid (Simson et al., 1998). When replicon cell lysates were treated with a combination of saponin (pore-forming agent) and 0.5% TX-100 at 4 °C to disrupt cholesterol (Chamberlain et al., 2001), NS5A protein was effectively solubilized and no longer exhibited buoyancy in sucrose gradients (Fig. 3). As shown above (Fig. 2), in the presence of 0.5% TX-100 alone without saponin at 4 °C, most of the NS5A proteins remained in the membrane fraction. NS5B protein was also solubilized by saponin plus TX-100 (data not shown). The cholesterol-dependent insolubility of NS proteins further indicates that these proteins are associated with cholesterol-rich lipid rafts.

Both HCV positive- and negative-strand RNAs exist in DRM fraction in Huh-7/replicon cells

We next examined the association of HCV RNA with cellular membranes. We utilized tagged reverse trascriptase-polymerase chain reaction (RT-PCR) for detection of (−) and (+)-strand HCV RNA in the membrane flotation sucrose fractions. Strand specificity was examined using serially diluted synthetic (+) and (−)-strand HCV RNAs. Under these conditions, PCR detection was very strand-specific; even after 35 cycles of PCR amplification, no (+)-strand RNA could be detected using the (−)-strand-specific probe, although 10^6 copies of (−)-strand RNA could be detected (Fig. 4A). Conversely, (+)-strand-specific probe could distinguish (+) from (−)-strand RNA by at least 2 logs at 25 cycles of PCR amplification (Fig. 4B). Using these PCR conditions, (−)-strand RNA was detected in the membrane fractions 2–4, with the intensity comparable to that of 10^6 copies of (−)-strand RNA (Fig. 4A). (+)-strand RNA was detected after 25 cycles of PCR amplification in the membrane fractions 2 and 3, with an intensity compa-

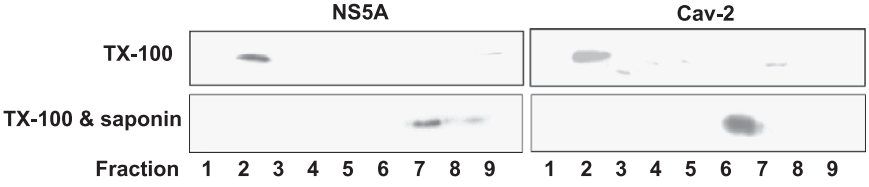

Fig. 3. Cholesterol dependence of raft association of HCV NS proteins. Cell lysates were treated with either 0.5% TX-100 (control) or 0.5% TX-100 + 0.5% saponin on ice and fractionated by discontinuous sucrose gradient centrifugation. NS5A and Cav-2 proteins were analyzed as described in the legend of Fig. 2.

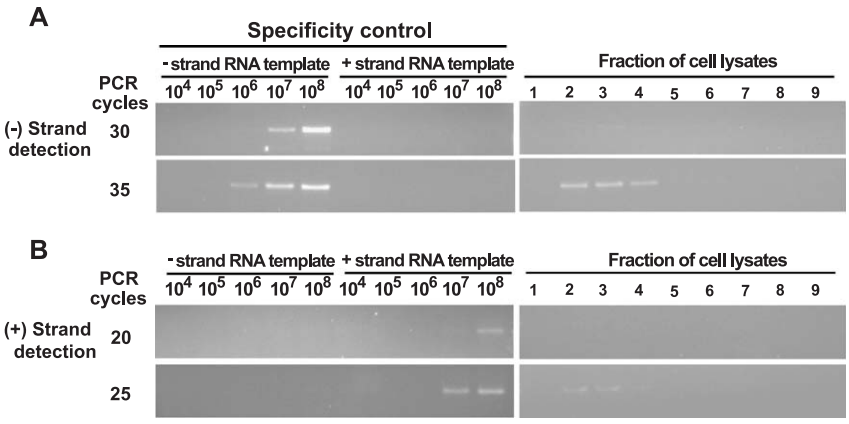

Fig. 4. Strand-specific RT-PCR assay to detect negative- and positive-strand RNAs in the RC. Negative (A)- and positive (B)-strand RNAs were analyzed by using the tagged RT-PCR method. PCR was conducted for one round of 30, 35 cycles or 20, 25 cycles for negative- and positive-strand RNA, respectively, followed by agarose gel electrophoresis. Synthetic positive- and negative-strand RNAs were prepared and diluted to 10^4, 10^5, 10^6, 10^7, 10^8 copies per reaction with total cellular RNA of Huh-7 cells as positive and negative controls. Cell lysates were fractionated by discontinuous sucrose gradient centrifugation. Total RNA was extracted from equal volumes of the recovered fractions. Fractions are numbered from 1 to 9 in order from top to bottom (light to heavy).

rable to that of 10^7 copies of (+)-strand RNA (Fig. 4B). Thus, (−)-strand RNA was present in approximately 10-fold lower molar amount than that of (+)-strand RNA, consistent with the previous report for the amounts of the HCV replicative intermediate in the replicon cells (Bartenschlager and Lohmann, 2000). Although this assay system is only semiquantitative within a narrow range, the result was highly reproducible. These results demonstrated that the membrane complex contained HCV RNA replication intermediates.

Characterization of the structure of the RCs

To characterize the structure of RCs, we first examined the RNase sensitivity of HCV RNA in the membrane fractions before and after treatment with detergents. The fractions separated by membrane flotation gradients were treated with 150 or 1000 ng/ml of RNase A under low-salt buffer conditions. The results showed that HCV RNA in fractions 1–5 was mostly resistant to RNase A treatment even after the membrane fraction had been treated with 1% NP-40 at 4 °C (Fig. 5). The distribution of HCV RNA treated with NP-40 was slightly narrower than that of the untreated sample (Fig. 5) and was consistent with the distribution of the NS proteins (Fig. 1). After treatment with either β-octylglucoside at 4 °C or TX-100 at 37 °C, the HCV RNA was completely digested by RNase (150 ng/ml). These data suggested that HCV RNAs were mostly enclosed within the DRM structures.

We next examined whether the HCV NS proteins were also enclosed within lipid rafts. We performed trypsin treatment of membrane fractions containing HCV NS proteins. As shown in Fig. 6, in the absence of detergent treatment, most of the NS5B protein was resistant to trypsin digestion even at a very high concentration of trypsin (100 μg/ml). After the membrane was treated with NP-40 at 4 °C, at least some NS5B was not digested. In contrast, after treatment with TX-100 at 37 °C, NS5B was completely digested by trypsin (100 μg/ml). This result suggested that at least part of NS5B was protected

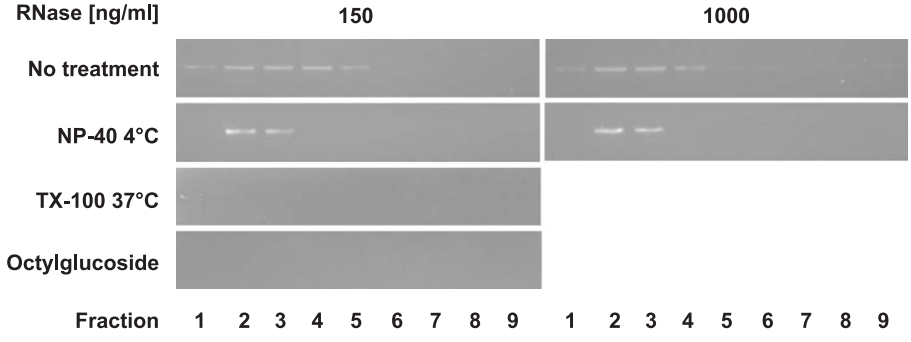

Fig. 5. RNase sensitivity of HCV RNA in the RC. Cell lysates were untreated, or treated with 1% NP-40 on ice, 60 mM octylglucoside on ice, or TX-100 at 37 °C for 30 min, and fractionated by discontinuous sucrose gradient centrifugation. Each fraction was incubated with 150 or 1000 ng/ml of RNase A for 5 min at room temperature. The digestion was terminated by phenol extraction. HCV RNA was detected by RT-PCR method and fractionated by 2% agarose gel electrophoresis. Fractions are numbered from 1 to 9 in order from top to bottom (light to heavy).

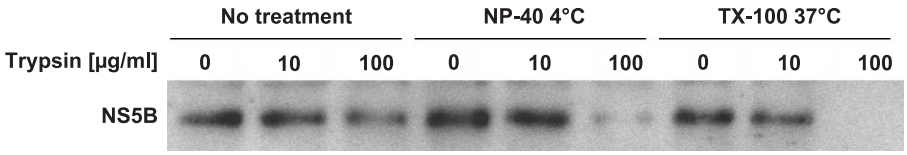

Fig. 6. Protease sensitivity of the HCV NS protein in the RC. Cell lysates were fractionated by flotation sucrose gradient centrifugation. Each fraction was concentrated in a Centriplus YM-100 filter unit. Fraction 2, which contains HCV proteins, was incubated without or with 1% NP-40 at 4 °C or 1% TX-100 at 37 °C for 1 h. Then samples were incubated without or with either 10 or 100 μg/ml of Trypsin for 5 min on ice. The digestion was terminated by adding soybean trypsin inhibitor and analyzed on a 12% SDS-PAGE with monoclonal antibody against NS5B.

by lipid raft. Alternatively, all of NS5B are in lipid rafts, but the protection may not be complete.

DRM fractions are active in HCV RNA synthesis

To determine whether the RCs contained all the components for RNA synthesis, each fraction from the membrane flotation gradient was used for in vitro RNA synthesis using the endogenous viral RNA template. As shown in Fig. 7A, HCV NS5B was predominantly present in fraction 2, with a lesser amount in 3, 5, and 6 (Fig. 7A). Each fraction was added to a replication assay mixture containing [α-^{32}P] CTP and incubated at 30 °C for 4 h. RNA was purified from the reaction mixture, denatured and analyzed by denaturing methylmercury agarose gel electrophoresis. A radiolabeled RNA species, which corresponded in size to that of the replicon RNA (8 kb), was detected in fraction 2 (Fig. 7B). A very weak signal was also detected in fraction 3. No RNA product was detected in fractions 5 and 6, although they contained some NS5B. These results indicate that fraction 2 contained the active RCs, whereas NS5B in fractions 5 and 6 were not in the active RCs. No radiolabeled RNA product corresponding to the template size was detected when fractions derived from mock Huh-7/Neo cells were used (Fig. 7C).

To determine whether these membranes were lipid rafts, the flotation gradients were prepared after the cellular lysates were treated with TX-100 4 °C for 1 h. Again, the radiolabeled RNA product was detected in DRM fraction 2 (Fig. 7D). These data suggested that the active HCV RCs are present in DRM fractions.

Subcellular locolization of RCs

To further analyze the nature of membrane locolization of RCs, lysates were examined by a subcellular membrane fractionation protocol (see Materials and methods) that can separate the ER from the Golgi. The trans-Golgi marker, Syntaxin 6, and the ER marker, calnexin, were partially separated under these conditions and were detected in fractions 2–4 and fractions 4–9, respectively (Fig. 8A). Significantly, most of NS5A and NS5B were detected in fractions 1–3, more closely resembling the distribution of syntexin 6. However, a significant portion of NS5A also overlapped with the distribution of calnexin. Each fraction was then subjected to in vitro RNA replication assay. Using this fractionation protocol, the in vitro RNA replication activity was relatively weak; nevertheless, the radiolabeled RNA product was detected only in fractions 1 and 2 (Fig. 8B). These data suggested that NS proteins were distributed

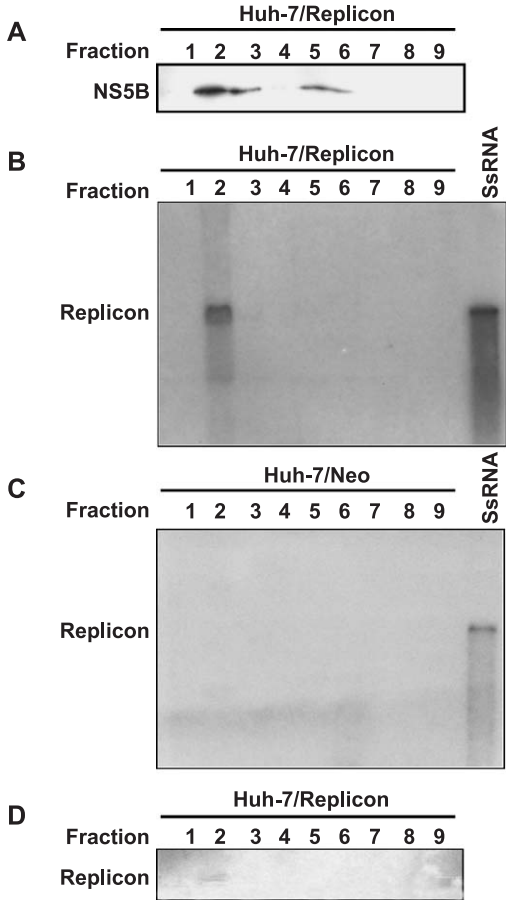

Fig. 7. Characterization of RNA synthesized from the endogenous templates in HCV RCs. Huh-7/replicon and Huh-7/Neo (mock) cells were fractionated by discontinuous sucrose gradient centrifugation as described in Materials and methods. Each fraction was concentrated in a Centricon YM-30 filter unit. Equal volumes of the recovered fractions were analyzed on a 12% SDS-PAGE, followed by immunoblotting with monoclonal antibody against NS5B (A). Each fraction isolated from Huh-7/replicon (B), Huh-7/Neo (control cell) (C), and Huh-7/replicon treated with TX-100 on ice for 1 h (D) was analyzed in a replication assay that included [α-^{32}P] CTP. RNA was extracted from each fraction and analyzed on a 1% methylmercury agarose gel and autoradiographed. An in vitro transcribed replicon RNA was used as an RNA marker (Replicon ss RNA). Fractions are numbered from 1 to 9 in order from top to bottom (light to heavy).

in both the ER and the Golgi fractions; however, the active RCs were found only in the Golgi-derived membranes, but not the ER fractions.

Effect of cholesterol depletion on replication of HCV replicon in Huh-7/replicon cells

Cholesterol sequestration assay (Fig. 3) suggests that the integrity of the lipid raft containing the NS protein depends on cholesterol. To examine the role of cholesterol-rich rafts in HCV replication, we treated replicon cells with 5 μM of lovastatin, which is an HMG-CoA reductase inhibitor, to decrease cellular cholesterol synthesis, and cultured the cells in the medium containing 10% delipidated calf serum (see Materials and methods). This treatment reduced cellular cholesterol levels by an average of 35% (Fig. 9A). Under the same condition, the growth and viability of cells were not altered when compared with the control cells for up to 18 h; however, longer treatment (24 h) caused slight reduction in cell growth rate (data not shown).

To determine the effect of cholesterol reduction on HCV RNA replication, HCV RNA titer in the replicon cells was measured in control and cholesterol-reduced replicon cells. The real-time PCR assay for HCV RNA used in this study showed linearity over a range of 10^6–10^1 copies per reaction and the correlation coefficient of the analysis was maintained above 0.96 for all experiments. Fig. 9B shows that HCV RNA titer was reduced by approximately 45% in the cholesterol-reduced cells, corresponding to the level of reduction of cholesterol under the same condition.

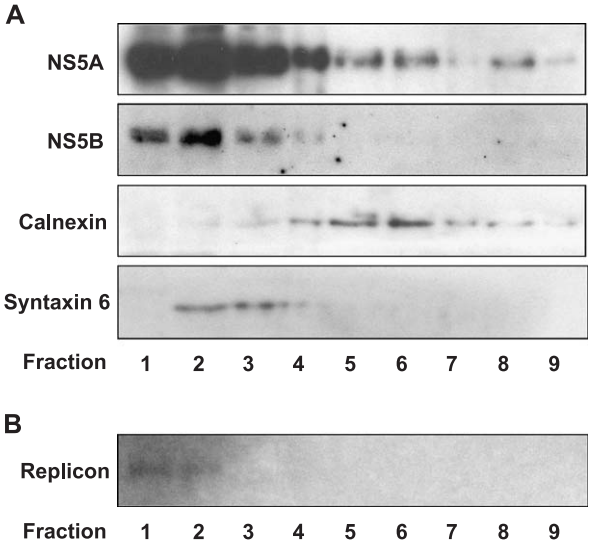

Fig. 8. Separation of membranes from the replicon cells. Cell lysates were fractionated to separate the ER and the Golgi by discontinuous sucrose gradient centrifugation as described in Materials and methods. (A) Each fraction was concentrated in a Centricon YM-10 filter unit and analyzed by immunoblotting with antibody against NS5A, NS5B, Calnexin, and Syntaxin 6. (B) In vitro replication assay of each fraction as in A. The RNA products were autoradiographed.

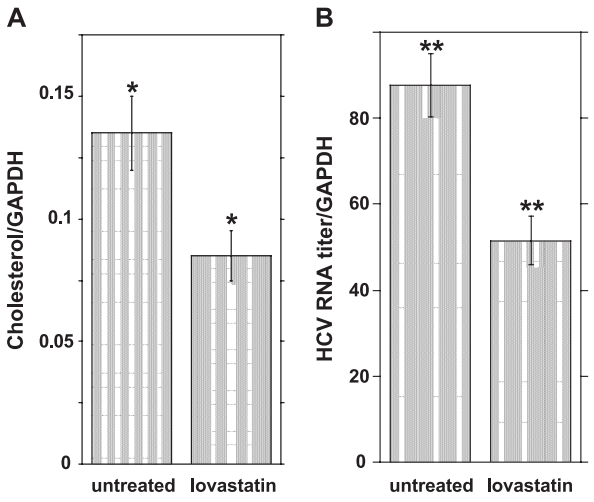

Fig. 9. Effects of cholesterol reduction on viral RNA replication. Cells were cultured in medium that contained 10% delipidated calf serum and 5 μM of lovastatin for 18 h. For control, cells were cultured in DMEM with 10% FBS. Total cellular cholesterol levels were measured (A). Quantification of HCV RNA in the cells was calculated by real-time RT-PCR method (B). GAPDH mRNA was used as the internal control. Error bars denote standard deviations with asterisks indicating statistical significance (*$P < 0.05$; **$P = 0.01$).

Discussion

HCV RNA replication occurs on the intracellular membrane (Bartenschlager and Lohmann, 2000), similar to the schemes utilized by other RNA viruses. Shi et al. (2003) reported that the newly synthesized, metabolically labeled HCV RNA in HCV subgenomic replicon cells was localized to distinct speckle-like structure, which may be equivalent to the membranous web, as reported by Gosert et al. (2003). In the present study, we characterized enzymatically active RCs by biophysical methods. The nature of the speckles was revealed to consist of lipid rafts and to be highly resistant to detergent.

Lipid rafts are known to play an important role in virus entry and virus assembly of many viruses, for example, influenza virus (Barman et al., 2001; Scheiffele et al., 1999; Zhang et al., 2000), human immunodeficiency virus type-1 (HIV-1) (Ding et al., 2003; Manes et al., 2000; Ono and Freed, 2001), Ebola, Marburg virus (Bavari et al., 2002), enterovirus (Stuart et al., 2002), avian sarcoma and leukosis virus (Narayan et al., 2003), Coxsackie B virus, adenovirus (Ashbourne Excoffon et al., 2003), measles virus (Manie et al., 2000), and respiratory syncytial virus (Brown et al., 2002). However, HCV is the first example of the association of lipid raft with viral RNA replication.

The results of our studies appear to be at odds with a reported study showing that NS proteins were associated with the ER in another replicon system (El-Hage and Luo,

2003; Gosert et al., 2003; Mottola et al., 2002; Pietschmann et al., 2001). The difference between the localization patterns of NS proteins in our studies and the previous reports may be attributed to the proportion of NS proteins existing in lipid rafts. When NS proteins are expressed alone, they are localized primarily in the ER. Most likely, they became relocalized to lipid raft membrane only when they are actively engaged in RNA replication. Thus, the proportion of NS proteins in lipid raft may correlate with the degree of RNA synthesis in the cells. We have established several replicon cell lines from different sources. Indeed, the proportion of NS5B protein associated with lipid rafts was variable in different replicon cell lines (data not shown). Furthermore, HCV RNA titers in different replicon cell lines appear to correlate with the amount of NS5B in lipid raft (data not shown). These results suggested that lipid raft might have an important role in HCV replication.

Expression of Cav-2, which is associated with lipid rafts, in Huh-7 cells was very low (Vainio et al., 2002); in contrast, the Cav-2 level in Huh-7/replicon was more than four times higher than that in Huh-7 cells (data not shown). These results suggested that our replicon cells might have been selected for the enrichment of lipid rafts to support HCV replication.

It is a widely held belief that the ER is the place where all HCV actions occur (Gosert et al., 2003; Hijikata et al., 1993; Mottola et al., 2002; Tu et al., 1999), but actual HCV replication site has never been precisely determined. We have previously shown by immunofluorescence studies that newly synthesized, metabolically labeled HCV RNA, together with all of NS proteins, were separated from the ER, although some HCV NS proteins did reside in the ER (Shi et al., 2003). In this report, we further showed by membrane separation that HCV NS proteins existed in both the ER and the Golgi, but HCV RNA replication activity was detected mainly in the Golgi. Recently, Choi et al. (2004) also found that the Golgi fraction, but not the ER, was where active RCs existed. How RC-raft participates in the life cycle of HCV is still not understood. HCV RC rafts may be formed and traffick in a similar way to other lipid rafts-binding proteins. Most likely, HCV NS proteins are synthesized in the ER (Fig. 10A) and then transported to the Golgi, where they distribute to lipid rafts. The cholesterol-sphingolipid rafts are believed to assemble in the Golgi, but not ER, because there is no sphingolipid in the ER (Simons and Ikonen, 1997, 2000; Simons and Toomre, 2000).

Our results of RNase and protease treatment suggested that HCV RNA and at least some of NS proteins are enclosed within the lipid rafts. We propose a model for the formation of HCV RCs in the lipid rafts. All of HCV NS proteins are synthesized in the ER (Fig. 10A). The NS proteins and host proteins then are transported to Golgi, where they associate with lipid rafts through protein–protein and protein–raft interactions (Fig. 10B). Many reports have demonstrated the interactions between HCV NS proteins (Bartenschlager and Lohmann, 2000; Bartenschlager et al., 1994; Flajolet et al., 2000). Dimitrova et al. (2003) suggested the existence of a complex network of interactions implicating all NS proteins. We have previously shown that NS5A bound to the C-terminus of a cellular vesicle-associated membrane protein (VAP-33), whereas NS5B protein bound to its N-terminus (Tu et al., 1999). We have also found that VAP-33 is localized in both the raft and non-raft membranes (Gao et al., 2004). Furthermore, NS4B was completely associated with lipid raft even when expressed alone, whereas NS3 or NS5B did not associate with lipid rafts when expressed individually (Gao et al., 2004) (Fig. 10B). NS5A was partially in the lipid raft. However, when these proteins were expressed together, they became colocalized in lipid raft. Therefore, NS4B, or its precursor peptide, may be the key protein that binds to lipid rafts first and then recruits other NS proteins sequentially to form RCs (Fig. 10B).

The rafts have a highly dynamic nature to coalesce to form the connected phase (Vaz and Almeida, 1993). NS4B also may bind each other by its own affinity because NS4B protein has an intrinsic ability to oligomerize (Dimitrova et al., 2003) (Fig. 10C). As a result, the connected phase of rafts may be stabilized. Indeed, clusters of the same protein have been observed within the same raft (Friedrichson and Kurzchalia, 1998; Varma and Mayor,

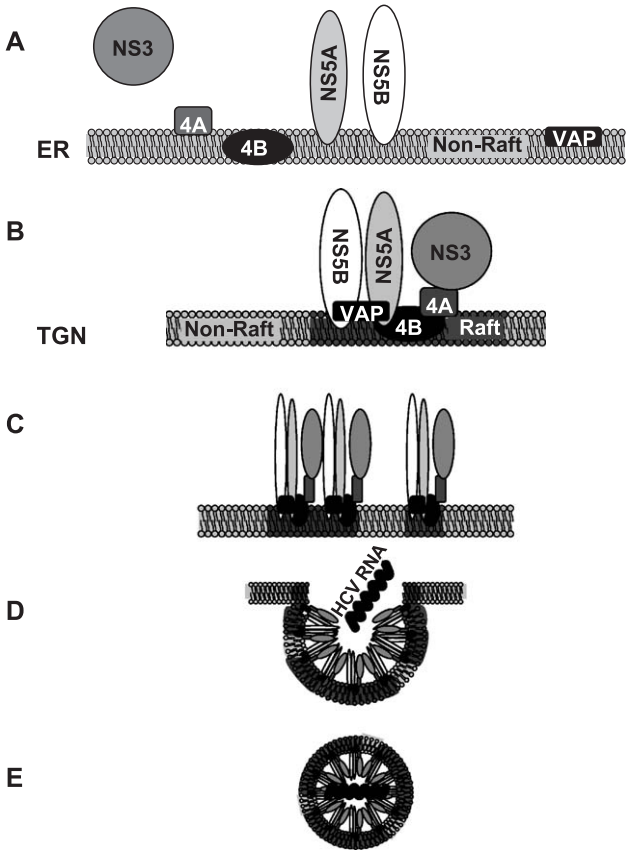

Fig. 10. A proposed model for the formation of HCV RC on lipid rafts.

1998). Generally, transmembrane proteins are thought to have a short residency time in rafts, spending most of the time outside rafts (Simons and Toomre, 2000). However, clusters of transmembrane proteins may display a stronger affinity for DRM because of an increased number of binding sites per complex. Several papers have reported that the protein affinity for rafts increased when the same protein was crosslinked or otherwise oligomerized (Harder et al., 1998; Janes et al., 1999; Ono and Freed, 2001; Schutz et al., 2000). In the case of HCV, rafts themselves may be stabilized and coalesced to form DRM domains by the oligomerization of NS4B. The connected rafts, in turn, facilitate recruitment and concentration of single RC to form polymerized RCs.

Generally, the formation of invaginated pits on the membrane and subsequent budding of vesicles is an energetically demanding process (Ford et al., 2002). The formation of a new vesicle is thought to be driven by both the polymerization of the coat protein into cage-like structure and selection/modification of membrane lipid (Brodsky et al., 2001; Ford et al., 2002; Schmidt et al., 1999). It was reported that NS4B protein alone induced membranous web in a membranous matrix (Egger et al., 2002) and that the newly synthesized HCV RNA existed in these membranous webs or speckles (Gosert et al., 2003; Shi et al., 2003). These findings suggested that NS4B might modify membrane curvature on binding lipid raft, in conjunction with oligomerization of NS4B (Fig. 10D). During this process, HCV RNA is enclosed within the membrane complex. NS4B may also modulate the late steps in the vesicle formation of RCs (Fig. 10E). Raft may modulate not only the formation of polymerization platforms for RCs but also vesicle formation of RCs. Polymerization platform model has been proposed for a raft-binding toxin (Marquardt et al., 1993) and for a raft-gag protein of HIV (Ono and Freed, 2001). With regard to the characteristic sizes of lipid raft microdomains, suggestions range from associations of a few molecules (Friedrichson and Kurzchalia, 1998) up to sizes of several hundred nanometers (Edidin, 1997; Simson et al., 1998; Yechiel and Edidin, 1987). Schutz et al. (2000) showed that lipid raft domain had a size of 0.7 μm (0.2–2.0 μm), which is compatible with the sizes of the speckles and membranous webs observed for HCV RC (Gosert et al., 2003; Shi et al., 2003).

Lai et al. (2003) demonstrated that HCV RC preparation could not use exogenous RNA as template and that the newly synthesized single-strand RNA served as the template for a further round of RNA synthesis. HCV proteins have been shown to be relatively stable (Pietschmann et al., 2001). Shi et al. (2003) suggested that HCV RNA replication did not rely on continuous protein synthesis. These data are consistent with our observation that RCs were covered with lipid rafts so that exogenous RNA could not be accessed by RCs. These NS proteins and HCV RNA in RCs are thus capable of multiple rounds of RNA synthesis.

Rafts may move out of the Golgi towards the plasma membrane, but may also move back to the ER (Brugger et al., 2000). Cholesterol is recycled to the ER by retrograde routes from the Golgi complex. The simian virus 40 uses caveolae and cytoskeletal elements to transport from the plasma membrane to the ER (Pelkmans et al., 2001). Microtubule network is the presumed transport route for vesicles trafficking. Bost et al. (2003) has suggested that cytoskeletal elements are important for HCV RNA synthesis in the HCV replicon cells. When HCV RC-raft comes to the ER, the rafts are decomposed because of the absence of sphingolipid in the ER. Conceivably, the newly synthesized RNA are then released and used for protein translation and for encapsidation with viral structural proteins.

Cholesterol can be released from the cell surface, at both non-raft and raft domains, the latter potentially involving caveolae (Simons and Ikonen, 1997, 2000; Simons and Toomre, 2000). Pietschmann et al. (2002) showed that HCV RNA in the replicon cells could be released into supernatants in lipid-containing structures. We have also found that some of RCs in our replicon cells are released into the supernatant (data not shown). It is possible that HCV uses the cholesterol–rafts circulation route to release its RCs into the supernatant.

Our results suggest that HCV replication is associated with lipid metabolism. Su et al. (2002) examined the host response to HCV in infected chimpanzees at multiple time points during the course of infection. Host genes involved in lipid metabolism were found to be differentially regulated during the early stages of infection. They also showed that drugs affecting lipid biosynthetic pathways could regulate HCV replication in HCV replicon system. HCV is known to cause steatosis (Lai, 2002; Lerat et al., 2002; Negro, 2002). The association of lipid raft with RNA replication thus may explain partially the abnormality of lipid metabolism associated with HCV infection.

In summary, we show here that HCV RNA and NS proteins are associated with lipid rafts. This association is likely to be a specific process that plays an important role in the formation of HCV RCs. These findings will facilitate further characterization of the components of HCV RC. In addition, analysis of HCV–raft interaction may provide new strategies for controlling HCV replication in vivo.

Materials and methods

Cells

Huh-7 cells were grown at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids. Huh-7/replicon harboring an HCV subgenomic replicon RNA derived from the HCV-N strain (Guo et al., 2001; Ikeda et

al., 2002) and Huh-7/Neo cells containing a neo gene were grown in the same medium containing 0.5 mg/ml of G418 (Shi et al., 2003).

Antibodies

The primary antibodies used for the analyses in this study were mouse anti-NS3 [Vector Laboratories (Burlingame, CA)], anti-NS5A [Biodesign (Saco, ME)], anti-Cav-2 [New England Biolads (Beverly, MA)], anti-Syntaxin 6 [Stressgen (Victoric, BC, Canada)] monoclonal antibodies and a rabbit anti-Calnexin polyclonal antibody (Stressgen). The mouse monoclonal antibody against NS5B was made by NS5B protein expressed from a recombinant baculovirus and was a generous gift of Dr. Soon Hwang (Hallym University, Korea) (Hwang et al., 1997; Shi et al., 2003).

Membrane flotation, detergent solubilization, and cholesterol sequestration assay

The membrane flotation assay was performed as previously described (Shi et al., 2003). Briefly, cells were first lysed in 1 ml of hypotonic buffer [10 mM Tris–HCV (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$] and passed through a 25-gauge needle 20 times. Nuclei and unbroken cells were removed by centrifugation at 1000 × *g* for 5 min in microcentrifuge at 4 °C. Cell lysates were then mixed with 3 ml of 72% sucrose in low-salt buffer [LSB, comprising 50 mM Tris–HCl (pH 7.5), 25 mM KCl, and 5 mM $MgCl_2$] and overlaid with 4 ml of 55% sucrose in LSB, followed by 1.5 ml of 10% sucrose in LSB. The sucrose gradient was centrifuged at 38 000 rpm in a Beckman SW41 Ti rotor for 14 h for 4 °C. After centrifugation, 1-ml fractions were taken from the top of the gradient, and each was added 1.7 ml of LSB to dilute sucrose and concentrated by being passed through a Centricon YM-30 or Centriplus YM-100 filter unit (Millipore, Bedford, MA). One half of each sucrose gradient fraction was separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. After blocking, the membrane was incubated with the primary antibody for 1 h at 37 °C, followed by the appropriate species-specific horseradish peroxidase conjugate, for an additional 1 h at 37 °C. Bound antibody was detected by the ECL-plus system (Amersham, Piscataway, NJ).

To separate the ER from the Golgi fractions, membrane flotation procedure was modified from the previous report (Xu et al., 2003). Briefly, the cell lysates were fractionated on a discontinuous sucrose gradient, which contained 1.5 ml of 70% sucrose, 3.5 ml of 45% sucrose, 3.5 ml of 35% sucrose, and 2.0 ml of 20% sucrose in LSB at 40 K rpm for 2 h. The fractions were diluted by LSB, concentrated by passing through a Centricon YM-10 filter unit, and analyzed by Western blotting and RNA replication assay.

To characterize lipid rafts, postnuclear supernatants of cell lysates were treated with 1% NP-40 (Boehringer Mannheim, Lavel, Quebec, Canada) at 4 °C, 1% TX-100 (Sigma-Aldrich, St. Louis, MO) at 4 °C, 1% TX-100 at 37 °C, or 60 mM β-octylglucoside (Calbiochem, La Jolla, CA) at 4 °C for 1 h and followed by flotation centrifugation.

To disrupt lipid rafts, cell lysate was treated with saponin (Sigma-Aldrich), which is a pore-forming agent. The cell lysates were solubilized in either 0.5% TX-100 (control) or 0.5% TX-100+ 0.5% saponin for 1 h at 4 °C and followed by flotation centrifugation.

Detection and quantitation of HCV RNA

To detect HCV RNA, RT-PCR was performed as previously described (Okamoto et al., 1990). HCV RNA was extracted from one tenth of each sucrose gradient fraction with the TRI Reagent (Molecular Research Center Inc., Cincinnati, OH). cDNA was synthesized by reverse transcriptase (Invitrogen, Carlsbad, CA) using antisense primer #36 as described. The PCR was performed with antisense primer #36 and sense primer #32 for 30 cycles. Amplified cDNA fragments were analyzed by electrophoresis on a 2% agarose gel and ethidium bromide staining.

Negative-strand-specific RT-PCR was performed as previously described (Lanford et al., 1994). cDNA was synthesized by RT using a forward primer TF, which contained a sequence unrelated to HCV at the 5′ end (the tag) and the 5′-untranslated sequences of HCV RNA at the 3′ end, and followed by heat inactivation of the RT for 1 h at 100 °C. The cDNA was amplified by 30 or 35 cycles of PCR using primer pairs of T (the tag sequence) and #36. PCR products were analyzed by electrophoresis on a 2% agarose gel and ethidium bromide staining.

Positive-strand-specific RT-PCR was modified from the negative-strand-specific PCR method. Briefly, cDNA synthesis was performed by RT using reverse primer #T36 (5′ TCA TGG TGG CGA ATA AAA CAC TAC TCG GCT AGC AGT 3′), which contained a sequence unrelated to HCV at the 5′ end (the tag) and the 5′-untranslated sequences of HCV at the 3′ end, and followed by heat inactivation of the RT for 1 h at 100 °C. The cDNA was amplified by 20 or 25 cycles of PCR using the primer pairs of T and #32. Strand-specific PCR assays were controlled by amplification of 10^4, 10^5, 10^6, 10^7, and 10^8 copies of the correct and the complementary strands of synthetic HCV RNA mixed with total cellular RNA of Huh-7 cells as positive and negative controls, respectively.

To determine the quantity of RNA by real-time PCR, we performed a single-tube reaction by using the TaqMan EZ RT-PCR Core Reagents (Applied Biosystems, Foster City, CA). Duplicate reactions for RNA standards and the samples were performed in 20-μl volume using 1.0 μl of HCV RNA, primers from HCV 5′ non-coding region (5′ GAG TGT CGT GCA GCC TCC A 3′ and 5′ CAC TCG CAA GCA CCC TAT CA 3′) of the HCV 1b sequence (Okamoto et al., 1992), and a fluorescent probe [5′ (FAM) CCC GCA AGA CTG CTA GCC GAG TAG TGT TGG (TAMRA) 3′] spanning these two regions. The RT step was performed at 60 °C for 50 min,

followed by 1 min at 50 °C. The amplification condition was as follows: 95 °C for 5 min and 50 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 10 s, and extension at 69 °C for 1 min.

Using the ABI Prism 7900 program, standard curves of the assays were obtained automatically by plotting the three hold values against each standard dilution of known concentration (10^1–10^6 copies per reaction) of HCV genotype 1b transcript. The same software was used to calculate the coefficients of regression. Using a standard curve, the Sequence Detector software calculated automatically the concentration of RNA copies in the experimental samples according to their three hold valves. Values were normalized to that of GAPDH (Applied Biosystems). Each test was done in triplicate and averages were obtained.

RNase treatment

One tenth of each sucrose gradient fraction was treated with 150 or 1000 ng/ml of RNase A (Ambion, Austin, TX) for 5 min at room temperature under the low-salt condition (30 mM NaCl, 3 mM sodium citrate). Following treatment, the RNA in the digestion mixture was purified with the TRI Reagent and precipitated. The samples were analyzed by RT-PCR.

Proteinase digestion assay

Proteinase digestion assay was performed as described previously (Polyak et al., 1998). After the membrane flotation assay, the membrane fraction 2 was treated with 1% NP-40 at 4 °C or 1% TX-100 at 37 °C for 1 h. Samples were incubated with or without trypsin (10, 100 μg/ml) at 4 °C for 5 min. The reactions were stopped by adding 100 μg/ml of soybean trypsin inhibitor. Then samples were analyzed by immunoblotting.

RNA replication assay

The RNA replication assay was similar to that described before (Bienz et al., 1992). Briefly, 20 μl of each sucrose gradient fraction was added to the following reagents in a final volume of 50 μl: 50 mM HEPES (pH 7.6), 10 mM dithiothreitol, 3 mM $MgCl_2$, 1.0 mM GTP, 1.0 mM ATP, 1.0 mM UTP, CTP to 0.04 mM with [α-^{32}P] CTP (Amersham) at 400 Ci/mmol and 0.1 mM unlabeled CTP, 10 μg of actinomycin D per milliliter, 25 μg of creatine phosphokinase per milliliter (Roche, Indianapolis, Ind.), 5 mM creatine phosphate (Roche), 800 U of RNase inhibitor per milliliter (Promega Corporation, Madison, WI), and 50 mM potassium acetate. The mixture was incubated at 30 °C for 4 h. Extraction of RNA from the total mixture was performed with the TRI Regent. The RNA was precipitated and eluted in 10 μl of RNase-free water. The replication products were analyzed by methylmercury agarose gel electrophoresis as described previously (Bailey and Davidson, 1976).

Cholesterol depletion assay

Cells were grown on six-well trays in DMEM supplemented with 10% delipidated calf serum and 5 μM of lovastatin (HMG-CoA reductase inhibitor) (Sigma) for 18 h. For control, cells were cultured in DMEM with 10% FBS. Total cellular cholesterol levels were measured by using Amplex Red Cholesterol Assay Kit (Sigma), which is based on an enzyme-coupled reaction that detected both free cholesterol and cholesteryl esters. Cell viability was assessed by Trypan Blue exclusion.

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