Hepatitis C Virus RNA Replication Occurs on a Detergent-Resistant Membrane That Cofractionates with Caveolin-2

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The mechanism and machinery of hepatitis C virus (HCV) RNA replication are still poorly understood. In this study, we labeled de novo-synthesized viral RNA in situ with bromouridine triphosphate (BrUTP) in Huh7 cells expressing an HCV subgenomic replicon. By immunofluorescence staining using an anti-BrUTP antibody and confocal microscopy, we showed that the newly synthesized HCV RNA was localized to distinct speckle-like structures, which also contain all of the HCV nonstructural (NS) proteins. These speckles are distinct from lipid droplets and are separated from the endoplasmic reticulum (ER), where some NS proteins also reside. Membrane flotation analysis demonstrated that almost all of the NS5A and part of the NS5B proteins and all of the viral RNA were present in membrane fractions which are resistant to treatment with 1% NP-40 at 4°C. They were cofractionated with caveolin-2, a lipid raft-associated intracellular membrane protein, in the presence or absence of the detergent. In contrast, the ER-resident proteins were detergent soluble. These properties suggest that the membranes on which HCV RNA replication occurs are lipid rafts recruited from the intracellular membranes. The protein synthesis inhibitors cycloheximide and puromycin did not inhibit viral RNA synthesis, indicating that HCV RNA replication does not require continuous protein synthesis. We suggest that HCV RNA synthesis occurs on a lipid raft membrane structure.

Hepatitis C virus (HCV) is an important human pathogen associated with non-A, non-B hepatitis and is the leading cause of chronic hepatitis and liver cirrhosis. As a member of the Flaviviridae family, HCV contains a positive-sense, single-stranded RNA genome of approximately 9.6 kb. The viral genome encodes a single polyprotein of about 3,010 amino acids, which is proteolytically processed by a combination of host- and virus-encoded proteases into 10 viral structural and nonstructural (NS) proteins arranged in the following order: (NH2-C)-C1-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-(COOH) (18, 28).

The establishment of the HCV subgenomic replicon and the subsequent analysis of the adaptive mutations revealed that most of the HCV NS proteins, with the probable exception of NS2, are involved in HCV RNA replication (6, 29, 37). NS3 is a helicase and a serine protease, whose function is dependent on NS4A. It is conceivable that the enzymatic activities of these proteins are key components of the HCV replication complex. The function of NS4B is thus far unknown, although it has been implicated in inducing transformation (34) and intracellular membrane alterations (13); the latter may play a significant role in the formation of the HCV RNA replication complex. NS3A is known to be a multifunctional protein implicated in the pathogenesis and interferon resistance of HCV infection. However, it has become evident that NS5A also plays an indispensable role in the replication of the HCV subgenomic replicon (6), but the underlying mechanism has yet to be identified. HCV NS5B is an RNA-dependent RNA polymerase (RdRp). All of these NS proteins, together with host proteins, are believed to form a membrane-associated RNA replication complex.

RNA replication of virtually all positive-strand RNA viruses involves certain intracellular membrane structures, including the endoplasmic reticulum (ER) (11, 40, 42, 54, 56), Golgi apparatus (47), endosomes, and lysosomes (14, 53). Most of these viruses induce distinct membrane structures derived from several membrane compartments to provide a structural scaffold for viral RNA replication (4, 5, 12, 17, 35, 54, 58). Previous studies have revealed an association of the HCV NS4B (23), NS5A (7, 39, 46, 52), and NS5B proteins (44) with the ER and/or the Golgi apparatus when they are expressed either alone or in the context of the entire HCV polyprotein. NS3 and NS5B also have been shown by biochemical studies to sediment with membrane fractions isolated from transfected cells (22, 24). In addition, NS5A has been shown by both cytological and biochemical approaches to be present on the surfaces of lipid droplets (46). NS4B, NS5A, and NS5B have all been found to be integral membrane proteins; the membrane association domains of NS5A and NS5B have also been defined (7, 44). A recent study further revealed that NS4B was able to induce a membranous web, with which all HCV proteins were found to be associated, forming a membrane-associated multiprotein complex (13). In HCV subgenomic replicon cells, all NS proteins, including NS3 and NS4A, were shown to be associated with the ER membranes by both fractionation experiments and immunomicroscopy (31). NS3 and NS4A are preferentially localized in the ER cisternae surrounding mitochondria, suggesting additional subcellular compartment-related functions for these viral proteins. Both studies (13, 31) revealed substantial alterations of the ER structure,

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resembling those observed in liver biopsy specimens of HCV-infected chimpanzees (36).

All of the evidence so far is consistent with the formation of a membrane-associated HCV RNA replication complex, which contains most of the HCV NS proteins. However, the considerable variation in the localization of the HCV proteins and the properties of the membrane among these reports left open the question of the nature of the truly functional HCV replication complex. So far, there is no evidence that these membrane structures are associated with active HCV RNA replication. Therefore, we set out to localize and characterize the HCV RNA replication machinery. In this study, we performed bromouridine triphosphate (BrUTP) labeling of de novo-synthesized HCV RNA in HCV subgenomic replicon cells. We found that the replicating HCV RNA and the NS proteins colocalize on a cytoplasmic membrane structure, which is distinct from the ER and the Golgi apparatus. Membrane flotation analysis further demonstrated that these membrane structures were resistant to detergent treatment and cofractionated with a lipid raft-associated protein, caveolin-2, suggesting that they are lipid rafts derived from intracellular membranes. These results suggest that the HCV replication complex may be recruited from the ER or Golgi apparatus to form a lipid raft-associated membrane complex.

MATERIALS AND METHODS

Establishment of HCV RNA replicon cells. Three different HCV subgenomic replicon constructs were used in this study. The original subgenomic replicon, HCV-1b NS3-3’ (29), containing an adaptive mutation, S1179I (6), was constructed by ligating the synthetic oligonucleotides according to the procedures of Blight et al. (6). Plasmids HCV1bneo and HCV1bneo/delS, which were the subgenomic replicons derived from the HCV-N strain (19, 25), were the kind gifts of C. Seeger (Fox Chase Cancer Center). In vitro-transcribed RNA was generated as previously described (19). Subconfluent Huh7 cells were trypsinized and washed once with Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.5 mM BrU. The suspension was diluted into DMEM supplemented with 10% fetal bovine serum and nonessential amino acids and once with serum-free DMEM. Cell pellets were resuspended in serum-free DMEM at a density of 10⁷ cells/ml. Forty micrograms of in vitro-transcribed RNA was added to 400 μl of the cell suspension in an electroporation cuvette (0.4-cm gap; Molecular Bio-rads, San Diego, Calif.). Cells were electroporated with a Gene Pulser II (Bio-Rad, Hercules, Calif.) set to 220 V and 975 μF. Subsequently, the cell suspension was diluted into DMEM supplemented with 10% fetal bovine serum, nonessential amino acids, and 1,25% dimethyl sulfoxide and seeded onto a 10-cm cell culture plate. After 24 h, the medium was replaced with the same medium containing G418 (Invitrogen, Carlsbad, Calif.) without dimethyl sulfoxide at 500 μg/ml (active dose), and the medium was changed twice per week. G418-resistant colonies were isolated after 2 to 3 weeks.

Antibodies. The monoclonal anti-bromodeoxyuridine (BrdU) antibody, which cross-reacts with bromouridine (BrU), was purchased from Roche Diagnostics Corporation (Indianapolis, Ind.). The mouse monoclonal antibody against NS3 was purchased from Vector Laboratories (Burlingame, Calif.). The mouse monoclonal antibody against NS3 was purchased from Vector Laboratories (Burlingame, Calif.). The mouse monoclonal antibody against NSA was purchased from Biodesign (Saco, Maine). The mouse monoclonal antibody against NSB was made by using NSB expressed from a recombinant baculovirus (24). The rabbit polyclonal antibody against calreticulin was obtained from Affini Bioreagents (Golden, Colo.). Mouse monoclonal antibodies against caveolin-1 and caveolin-2 were purchased from Novus Biologicals (Beverly, Mass.). M2 beads used for immunoprecipitation of Flag-tagged proteins were obtained from Sigma (St. Louis, Mo.). The rabbit polyclonal antibody against NSA has been described previously (52).

Cell permeabilization and labeling of de novo-synthesized viral RNA. Cell permeabilization with lysosolcin and detection of viral RNA synthesis were performed as described previously (47). Huh7 cells were plated on 8-well chamber slides at a density of 5 × 10⁵ cells per well. One day after seeding, cells were incubated with actinomycin D (5 μg/ml) for 1 h and washed twice with serum-free medium containing 50 μg/ml of bromouridine (BrU) added to the medium. Nuclei and unbroken cells were removed by centrifugation at 1,000 × g for 5 min in a microcentrifuge at 4°C. Cell lysates were then mixed with 3 ml of 70% sucrose in low-salt buffer (LSB, comprising 50 mM Tris-HCl [pH 7.5], 25 mM KCl, and 5 mM MgCl₂) and overlaid with 4 ml of 55% sucrose in LSB, followed by 1.5 ml of 10% sucrose in LSB. In some experiments, indicated, cell lysates were treated with 1% NP-40, 1 M NaCl, or 0.1 M NaClO₃ (pH 11.5) for 20 min before being loaded onto a sucrose gradient. The sucrose gradient was centrifuged at 38,000 rpm in a Beckman SW41 Ti rotor for 14 h at 4°C. One-milliliter fractions were taken from the top of the gradient, and each was concentrated by being passed through a Centricon YM-30 or YM-100 filter unit (Millipore, Bedford, Mass.). The pellet was resuspended in sodium dodecyl sulfate sample buffer and analyzed on a 12.5% polyacrylamide gel.

Detection of HCV RNA by reverse transcription-PCR (RT-PCR). HCV RNA was extracted from 50 μl of each sucrose gradient fraction with the TRI Reagent (Molecular Research Center Inc., Cincinnati, Ohio), according to the manufacturer’s protocol. cDNA was synthesized by reverse transcriptase (Invitrogen) using antisense primer #36 (32). The first-round PCR was performed with antisense primer #36 and sense primer #32 for 30 cycles. The second-round PCR was performed with nested primer pairs of #33 and #48 (32) for another 25 cycles. Amplified cDNA fragments were analyzed by electrophoresis on a 1% agarose gel and ethidium bromide staining.

RESULTS

HCV NS proteins are localized on both the ER and other membrane structures in replicon cells. To identify the nature and subcellular localization of HCV RNA replication, we first reexamined the localization of the NS proteins, which are likely involved in viral RNA replication, in Huh7 cells supporting a subgenomic RNA replicon. Three stable Huh7 cell lines containing different HCV-1b subgenomic replicon RNAs (see Materials and Methods) were used. Localization of NS proteins was determined by immunofluorescence staining with monoclonal antibodies against NS3, NS5A, and NS5B. Most of the NS3 and NS5A proteins showed diffuse perinuclear staining, which partially colocalized with calreticulin in 31 (Fig. 1), indicating that these proteins were localized partly in the ER. This distribution is similar to what was observed when these proteins were expressed in isolation or as the full-length HCV
polyprotein (39, 46, 59). However, in many of the replicon cells, besides the ER localization, these proteins were also detected on discrete speckles or patches in the cytoplasm (Fig. 1, right panels). These speckles did not colocalize with calreticulin. A similar pattern was also observed for NS5B (data not shown). In some cells, most of the NS proteins were present as prominent speckles, with fainter staining on the ER or the Golgi apparatus (Fig. 2). Similar results were obtained with three different replicon cells. The HCV1bneo/delS replicon was used in most of the subsequent experiments, since it appeared to yield the highest number of cells containing the speckle-like staining patterns. The relative distribution of NS proteins in the speckles and in the ER-Golgi apparatus varied from cell to cell. These speckles contained most of the NS proteins, as evidenced by the colocalization of NS3, NS5A, and NS5B on these structures (Fig. 2). It should be noted that NS3 had a wider distribution than NS5A, as some of the NS3 did not overlap with NS5A (Fig. 2). This finding is consistent with the potential role of NS3 in HCV protein processing, in addition to its possible role in RNA replication. However, the possibility that the NS3-specific antibody is more sensitive than the anti-NS5A antibody cannot be ruled out. Some of the speckle-like structures resembled oil droplets, which have previously been shown to colocalize with HCV NS5A (46); in

FIG. 1. The HCV NS3 and NS5A proteins partially colocalize with the ER marker calreticulin. One day after seeding, HCV1bneo/delS cells were double stained with the mouse monoclonal antibody against NS3 or NS5A and the rabbit polyclonal antibody against calreticulin. Arrows indicate localization of NS proteins on the cytoplasmic speckles that lack calreticulin staining. Bars, 5 μm.

FIG. 2. The NS proteins colocalize with each other in HCV replicon cells. One day after seeding, HCV1bneo/delS cells were double stained with the rabbit polyclonal antibody against NS5A and the mouse monoclonal antibody against NS3 or NS5B. Bars, 5 μm.
particular, some NS proteins appeared to be localized at the circumferences of the spherical particles (Fig. 1; see also Fig. 5 below). To determine whether these structures were lipid droplets, we performed Oil Red O staining of lipid droplets (46) in the replicon cells (Fig. 3). Clearly, the majority of NS5A did not colocalize with lipid droplets, in contrast to what was previously observed in cells expressing NS5A alone (46). Therefore, these speckle-like structures are not lipid droplets. None of the NS3, NS5A, or NS5B antibodies stained normal Huh7 cells or Huh7 cells expressing the neo gene and growing in the presence of G418 (data not shown).

Colocalization of de novo-synthesized HCV RNA and NS proteins in speckles. To test the possibility that these speckles may be associated with viral RNA synthesis, we adopted a system for labeling de novo-synthesized HCV RNA in lysolecithin-permeabilized cells (47). The HCV RNA was labeled by BrUTP incorporation in the presence of actinomycin D, followed by detection of brominated nucleotides with a monoclonal antibody. Under such conditions, fluorescent staining in distinct speckles of various sizes was found in the cytoplasm of Huh7 cells supporting an HCV replicon, whereas no signal was detected in Huh7 cells without a replicon (Fig. 4). These speckles most likely represent virus-specific RNA. Remarkably, this cytoplasmic staining appears very similar to the prominent speckles of the NS proteins (Fig. 1 and 2). Since BrUTP labeling was carried out for 15 to 30 min, the majority of the labeling observed most likely represents newly synthesized viral RNA, which is likely in the viral RNA replication machinery. We used three different replicon RNA constructs; similar patterns were observed (data not shown).

To establish that the NS proteins are associated with the replicon RNA, we performed dual labeling experiments using a polyclonal antibody against NS5A and a monoclonal antibody against the brominated RNA. The staining patterns of the NS proteins were similar in lysolecithin-permeabilized and untreated cells (compare Fig. 2 with Fig. 5), indicating that the lysolecithin treatment did not disrupt the normal cell morphology. Double immunofluorescence staining showed that the BrUTP-labeled RNA colocalized almost precisely with NS5A protein in the speckle-like structures. Since NS5A colocalized with NS5B, NS3 (Fig. 2), and other NS proteins, this result indicates that BrUTP-labeled speckles represent the RNA replication complex and that most of the HCV NS proteins are associated with the viral RNA replication machinery.

To examine the metabolic requirement of HCV RNA replication, we treated the cells with CHX for 3 h before permeabilization and BrUTP labeling; HCV RNA replication was not significantly affected (Fig. 6). Similar results were obtained for puromycin (data not shown). These results suggest that continuous protein synthesis is not necessary for HCV RNA replication. This is similar to findings for another flavivirus, Kunjin virus, which can carry out RNA replication in the absence of continued protein synthesis (57). The expression and localization patterns of the NS5A protein were not noticeably...
altered by the treatment, in agreement with the previous finding that HCV NS proteins are very stable (37).

**Association of HCV NS proteins with detergent-resistant membrane fractions.** To determine the properties of the structures that harbor the NS proteins and support HCV RNA synthesis, we performed membrane flotation analysis to separate membrane and cytosolic fractions (41, 46). The membrane-containing materials float to the top of the sucrose gradient, while the cytosolic fractions remain at the bottom. The presence of the NS proteins in each fraction was determined by immunoblotting. As shown in Fig. 7A, both NS5A and NS5B were found predominantly in the membrane fractions (fractions 2 and 3). Previously (46), an ER marker, GRP 78, was found to be distributed in both the membrane and cytosolic fractions. A similar distribution was observed for another ER marker, calreticulin (data not shown). The detection of some ER markers as cytosolic proteins by use of these procedures is common (33). When the cell lysates were treated on ice with 1% NP-40, a nonionic detergent—a condition which released all of the ER proteins to the cytosol (46)—only a small percentage of the NS5A protein was dissociated from the membrane and moved to the cytosolic fractions (Fig. 7B). This result indicates that NS5A is associated with a very special kind of membrane that is detergent insoluble. This type of membrane is referred to as a lipid raft (20, 48). However, more than half of the NS5B was detected in the cytosolic fractions after detergent treatment, indicating that not all of the NS proteins are in the detergent-insoluble complexes. Alternatively, NS5B may not be associated with the membrane as tightly as NS5A. Nevertheless, a substantial portion of NS5B was also in the membrane that is resistant to the detergent treatment.

To characterize the nature of these detergent-resistant membrane fractions, we examined several cellular proteins known to be associated with different lipid-containing membranes in the cells. Caveolin-1 (Cav-1), which is largely localized in plasma membrane caveolae and the Golgi apparatus (33), was detected mainly in the soluble fractions both before
and after NP-40 treatment (Fig. 7A and B). However, Cav-2, which is present on cytoplasmic lipid rafts as well as lipid droplets (15, 33, 38, 50), was mostly present in the detergent-resistant membrane fractions, similar to the distribution of NS5A and NS5B. It has been reported that only Cav-2, not Cav-1, was detected in the membrane fractions upon detergent treatment (15). Although Cav-1 and Cav-2 can interact with each other (43), it is possible that Cav-2 may be present in additional membrane structures resistant to nonionic detergents or that it may have a stronger association with the membranes than Cav-1. These results indicate that the NS proteins are associated with detergent-resistant membrane structures that cofractionate with Cav-2. The properties of these membrane structures resemble those of lipid rafts or lipid droplets. Since the NS protein did not colocalize with the vesicles stained with Oil Red O (Fig. 3), these membrane structures are most likely lipid rafts.

Finally, the distribution of these proteins was not affected by treatment with 1 M NaCl or 0.1 M NaCO₃ (Fig. 7C and D, respectively), indicating that they were integral membrane proteins, consistent with earlier findings for cells stably expressing HCV NS proteins in the absence of RNA replication (7, 44).

**Association of HCV replicon RNA with detergent-resistant membrane fractions.** We further examined the distribution of HCV RNA after membrane flotation analysis of HCV replicon cells. HCV RNA was detected by RT-PCR analysis. All of the HCV RNA was detected in the membrane fractions (fractions 1 to 4) (Fig. 8). The distribution of HCV RNA appeared to be slightly wider than that of the NS proteins. This difference could be due to the higher sensitivity of RT-PCR than of Western blotting. Significantly, none of the RNAs were solubilized by treatment with 1% NP-40. Although the amount of HCV RNA appeared to have decreased slightly after detergent treatment, no RNA was detected in the cytosolic fractions. These results combined suggest that both HCV RNA and NS proteins were present in the same membrane fractions and have similar detergent-resistant properties. They most likely reside in the same structures.

**DISCUSSION**

In the present study, we have characterized the localization of the HCV NS proteins and newly synthesized viral RNA in Huh7 cells that support active RNA replication of a subgenomic HCV replicon. We showed that most of the HCV NS proteins and RNA colocalized with each other on distinct...
speckle-like structures in the cytoplasm of the replicon cells, which may represent the sites of HCV RNA replication. This speckle-like appearance of the NS proteins is in contrast to their perinuclear ER and Golgi localization typically seen in cells that express only HCV NS proteins in the absence of RNA replication (26, 39, 45, 46, 52). These results suggest that, in subgenomic replicon cells, the HCV NS proteins may be recruited from a wider distribution in the cytoplasm, such as the ER and Golgi apparatus, to the more localized sites where active RNA replication occurs. Biochemical analysis further showed that the HCV NS proteins and RNA were present on detergent-insoluble membrane structures characteristic of lipid rafts or lipid droplets. Since there was no colocalization between the NS proteins and lipid droplets (Fig. 3) in the replicon cells, it is most likely that HCV RNA synthesis occurs on a lipid raft structure.

The use of BrUTP labeling has enabled us to characterize HCV RNA replication in situ for the first time. We clearly showed colocalization of the NS proteins with HCV RNA, strongly suggesting the involvement of the NS proteins in viral RNA synthesis. We found a strong correlation between the level of viral RNA replication and the number of cells, among the different HCV replicon cell clones, that exhibited the speckle-like distribution of NS proteins. The speckle-like structures were not seen in cells without HCV RNA synthesis. These results collectively support the notion that HCV RNA replication occurs on the speckle-like structures.

Continuous protein synthesis is required for RNA synthesis of some RNA viruses, such as coronaviruses (47). However, we found that HCV RNA synthesis was completely resistant to CHX or puromycin treatment for at least 3 h before BrUTP labeling, indicating that HCV RNA replication does not rely on continuous protein synthesis. HCV proteins have been shown to be relatively stable; even the most unstable protein (NS5A) was found to have a half-life of 7 h (37). These proteins are thus capable of multiple rounds of RNA synthesis in situ. Resistance of viral RNA synthesis to inhibitors of protein synthesis has also been demonstrated for another flavivirus, Kunjin virus (57).

The results of our present study appear to be at odds with a reported study showing that the NS proteins were associated with the ER in another replicon system (37). The difference between the localization patterns of NS proteins in these two reports may be attributed to the high level of HCV RNA replication in our replicon cells. We found that the more active the viral RNA synthesis was, the more cells exhibited such a speckle-like appearance of the NS proteins. Our finding that the de novo-synthesized HCV RNA was also localized to the speckles supports the notion that the HCV NS proteins are involved in HCV RNA synthesis and that the speckles represent the intracellular sites in which viral RNA is actively replicating in the replicon cells. The NS proteins that are localized on the ER may represent the proteins involved in the process of translation or transport but may not reflect the status of active viral RNA replication.

The nature of the speckles where HCV RNA and the NS proteins reside is unknown. All of the HCV structural and NS proteins were reportedly associated with the membranous webs (13). The nature of the speckles was suggested by the biochemical characterization, which revealed that they are highly resistant to detergent and high salt concentrations, properties characteristic of lipid rafts or lipid droplets. Lipid rafts are localized mainly at the level of plasma membrane but also can form within internal membrane compartments, such as the Golgi apparatus (16). The lipid droplets serve as energy storage sites (8, 55). They consist of a core of triacylglycerols and cholesterol esters, which is synthesized in the ER, surrounded by a phospholipid monolayer, which is also derived from the ER (8, 55). Lipid rafts differ from lipid droplets in their membrane compositions and contents, but both of them are rich in Cav-2 (15, 33, 38, 50). Our studies have shown that the HCV RNA replication complex was not localized to lipid rafts or lipid droplets.
droplets; thus, it is most likely associated with lipid rafts. Preliminary findings from our laboratory further showed that the membrane-associated HCV NS proteins and Cav-2 could be rendered soluble by treatment with β-ocytoglycocide, a non-ionic detergent known to disrupt the association of glyco-

sphingolipidinositol-linked proteins with lipid rafts (9) (data not shown), providing further evidence that the HCV NS proteins are indeed associated with lipid rafts. Furthermore, since the HCV RNA replication complex cofractionates with Cav-2 but not with Cav-1, the lipid raft where HCV RNA replicates is most likely derived from the internal cellular membranes. Several other viruses, including human immuno-
deficiency virus (10), murine leukemia virus (27), measles virus (30), Ebola virus, Marburg virus (3), influenza virus (2), and Epstein-Barr virus (21), have been reported to require lipid rafts in their life cycles. Although the internal lipid rafts are not well characterized, they have been implicated in various cellular functions, including vesicular trafficking and signal transduction (1, 50). HCV may induce the formation of these important intracellular structures to serve as the sites of viral RNA replication. Localization of the NS proteins on lipid rafts also may alter certain signal transduction pathways, as lipid rafts are strongly linked to signal transduction (49).

Taken together, the results of the present study demonstrated that the HCV NS proteins are associated with viral RNA replication machinery, which is localized on distinct speckle-like cytoplasmic membrane structures. These structures were detergent insoluble and corefractionated with Cav-2, suggesting that they may possess characteristics similar to those of lipid rafts. These findings will facilitate further characterization of the components of the HCV RNA replication complex.

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