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Visualization of the structures of the hepatitis C virus replication complex

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ABSTRACT

Hepatitis C viral RNA synthesis has been demonstrated to occur on a lipid raft membrane structure. Lipid raft membrane fraction purified by membrane flotation analysis was observed using transmission electron microscopy and atomic force microscopy. Particles around 0.7 um in size were found in lipid raft membrane fraction purified from hepatitis C virus (HCV) replicon but not their parental HuH7 cells. HCV NS5A protein was associated with these specialized particles. After several cycles of freezing–thawing, these particles would fuse into larger sizes up to 10 um. Knockdown of seven proteins associated with lipid raft (VAPA, COPG, RAB18, COMT, CDC42, DPP4, and KDELR2) of HCV replicon cells reduced the observed number of these particles and suppressed the HCV replication. Results in this study indicated that HCV replication complexes with associated lipid raft membrane form distinct particle structures of around 0.7 um as observed from transmission electron microscopy and atomic force microscopy.

1. Introduction

Infection with hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1]. HCV belongs to the genus *Hepacivirus* in the family *Flaviviridae*. HCV genome is a single, positive-stranded RNA with a nucleotide length of about 9.6 kb, encoding a large polyprotein precursor of approximately 3000 amino acids. This polyprotein precursor is processed co- and post-translationally into at least 10 different viral proteins by host and viral proteases, which are arranged in the order of NH2-C(21Kd)-E1(31Kd)-E2(70Kd)-p7-NS2(23Kd)-NS3-(70Kd)-NS4A(8Kd)-NS4B(27Kd)-NS5A(58Kd)-NS5B(68Kd)-COOH [2,3]. C, E1, and E2 are structural proteins while NS2–NS5B are non-structural proteins. The mechanism and machinery of HCV RNA replication are not well characterized. HCV RNA replication occurs on the intracellular membrane, similar to the schemes utilized by other RNA viruses [4–8]. Most of the HCV NS proteins, with the probable exception of NS2, are involved in HCV RNA replication [9–11]. The replicating HCV RNA and the NS proteins colocalize on a cytoplasmic membrane structure, i.e., a lipid raft-associated membrane complex [12]. Lipid rafts are known to play an important role in virus entry and virus assembly of many viruses [13]. However, HCV is the first example of the association of lipid raft with viral RNA replication [12].

The relative insolubility of lipid rafts in cold nonionic detergents is the most widely used method to purify these fascinating membrane domains from intact cells or membranes [14]. Therefore, membrane flotation analysis is usually used to purify the lipid rafts with HCV replication complexes (RCs) derived from intracellular membranes [12,15,16]. To visualize the specialized structures of the HCV RCs with associated lipid rafts, transmission electron microscopy (TEM) and atomic force microscopy (AFM) were used in this study.

2. Materials and methods

2.1. Cell culture

HuH7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, USA). HCV subgenomic replicon cells were cultured in DMEM with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418 [17]. All cultured cells were maintained at 37 °C with 5% CO_2.

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2.2. Western blotting analysis

Our previous procedures were followed for Western blotting analysis [18–20]. The primary antibodies used for the analyses in this study were mouse anti-NS3 (Vector Laboratories, USA), anti-NS5A (Biodesign, USA), anti-GRP 78 (Santa Cruz Biotechnology, USA). A rabbit polyclonal antibody against ERK-2 was purchased from Santa Cruz Biotechnology (USA).

2.3. Membrane flotation analysis

The procedures of membrane flotation assay (discontinuous sucrose gradient centrifugation) from the previous reports were followed [12,15,16].

2.4. RNAi experiments

RNAi experiments were performed using the lentiviral expressing system (http://rnai.genmed.sinica.edu.tw), following the manufacturer's instructions. RNAi reagents were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica.

2.5. Real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR)

RNAi experiments using the lentiviral expressing system were performed in HCV replicon cells to knockdown some cellular genes. Twenty four hours after recombinant virus infection, 4 ug/ml puromycin was used to select the infected cells. After 72 h, cellular mRNAs were extracted and our previous procedures were followed for real-time RT-PCR [18]. Specific primers to detect individual cellular genes were listed in Supplementary Table 1.

2.6. Atomic Force Microscopy (AFM)

AFM images were generated by a commercial AFM (Nanowizard TM JPK, Germany) installed on an inverted optical microscope (Nikon, Japan). AFM probe was single-crystal silicon micro cantilever with a spring constant of 0.02 M/m (OMCL-TR400 PB-1, Olympus, Japan). Thirty microliters of diluted membrane fraction derived from the HCV replicon cells was dropped on freshly cleaved mica (Ted-Pella, CA), and placed at room temperature for 40 min for adsorption, the unadsorbed material was washed with PBS five times. Ten microliters of 0.01% BSA was added to prevent non-specific binding for 40 min, then washed with PBS five times. Primary anti-NS5A Ab was added to the mica for 3 h, then washed with PBS for five times. The gold particle-conjugated anti-mouse IgG Ab was added to incubate for 1 h, then washed with PBS for five times. The sample was then air dried at room temperature for 2–3 days. AFM images were collected by the AFM with scan rate of 1 Hz at 512 × 512 pixels. Images analysis was performed using the Image Processing software (JPK, Germany).

2.7. Transmission electron microscopy (TEM)

TEM images were generated by a commercial TEM (Hitachi H07500m, Japan). For sample preparation, 10 ul of samples were loaded onto 400 Mesh Formvar/Carbon Copper Grids (Ted Pella, Canada) and allowed to stay for 1 min. Excess fluid was wicked away with filter paper. 0.01%BSA was added for preblocking before Ab incubation. 10 ul 2% uranyl acetate was then added onto the sample containing copper grids for negative staining. After 30 s, the excess fluid was again wicked away with filter paper. The copper grids with samples were then air dried at room temperature for 2 days. The ready samples were imaged with the TEM at an operating voltage of 80 kV and direct magnifications of between 25,000× and 100,000×.

3. Results and discussions

3.1. Association of HCV NS proteins with detergent-resistant membrane fractions

To purify the HCV RCs with associated lipid raft membrane, the membrane flotation assay was performed using HCV replicon cells. HCV NS3 and NS5A proteins (components of HCV RCs) were detected mainly in the membrane fraction (fraction 2 in Fig. 1A) but not in the non-membrane fractions (fractions 6–10 in Fig. 1A). This assay was also performed in the parental HuH7 cells as the control (Fig. 1B). Interferon-treatment could remove HCV entirely (Fig. 1C). After the treatment of 1% Nonidet P-40 (NP-40) at 4 °C, unlike the endoplasmic reticulum-associated protein GRP78 shifted from membrane fractions to non-membrane fractions (Fig. 1D lower row), HCV RCs with associated lipid raft membrane retained in the membrane fraction (fraction 2 in Fig. 1D upper row). Thus, the purified HCV RCs were indeed associated with lipid raft in the detergent-resistant membrane fractions, the same as others' reports [12,15,16].

3.2. Distinct Particles (~0.7 um) were found in lipid raft membrane fraction from HCV replicon but not their parental HuH7 cells

Lipid raft membrane fractions from membrane flotation assay were observed using TEM. Particles around 0.7 um were found in fraction 2 purified from HCV replicon cells (Fig. 2A) but not from their parental HuH7 cells (Fig. 2B). These particles disappeared after HCV replicon cells were treated with interferon to remove HCV (data not shown). Therefore, these particles are probable lipid raft structures of HCV RCs. If these particles are indeed the structures for HCV RNA replication, then the HCV nonstructural proteins, e.g., NS5A should be in these particles. Indeed, HCV NS5A protein was associated with these specialized particles using ImmunoEM (Fig. 2C). Lipid raft membrane fraction 2 were also observed using AFM. Specialized particles were also found in samples purified from HCV replicon (Fig. 3A) but not from their parental HuH7 cells (data not shown, also see Fig. 3B). The sizes of the particles under AFM are less homogeneous than those in TEM, probably due to different procedures in sample preparations.

With two techniques (TEM and AFM), the size of these distinct particles (around 0.7 um) observed in the HCV RCs-containing lipid raft membrane fraction is similar to that of the speckle-like structures of HCV RCs in the cells [12].

3.3. After several cycles of freezing–thawing, the particles fused to larger ones up to 10 um

After the HCV RCs-containing membrane fraction (fraction 2 in Fig. 1A) went through several freezing–thawing cycles, the size of the particles increases from the initial around 0.7 um to 2–10 um as observed with AFM (Fig. 3B left column). This can be verified by the association of HCV NS5A protein in these particles (Fig. 3C). Also, treatment of interferon almost removed these structures completely (Fig. 3B right column). Therefore, these membrane structures derived from HCV replicon cells behave like liposomes, which will fuse to form larger vesicles after freezing–thawing cycles [21].

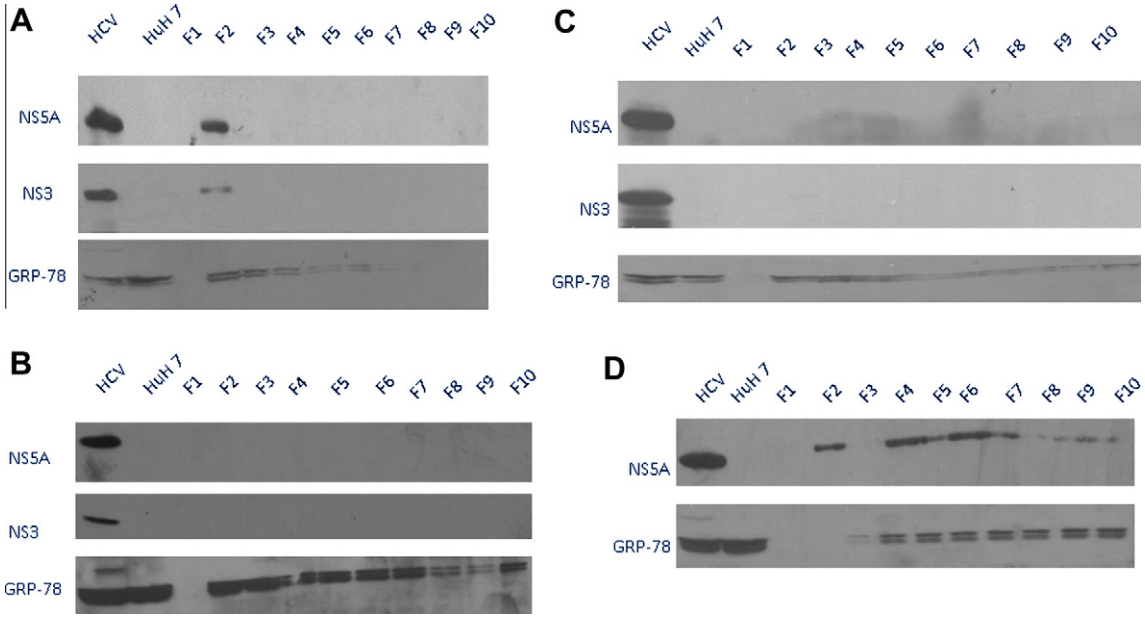

Fig. 1. Western blotting analysis of HCV NS5A, NS3 and E.R. -associated GRP78 proteins in cell lysates fractionated by membrane flotation assay of HCV replicon cells (A), HuH7 cells (B), HCV replicon cells treated with interferon (C), and HCV replicon cells treated with 1% NP-40 for 30 min on ice (D). Fractions are numbered as F1–F10 from top to bottom (light to heavy). HCV and HuH7 represent the total cell lysate derived from HCV replicon cells and its parental HuH7 cells.

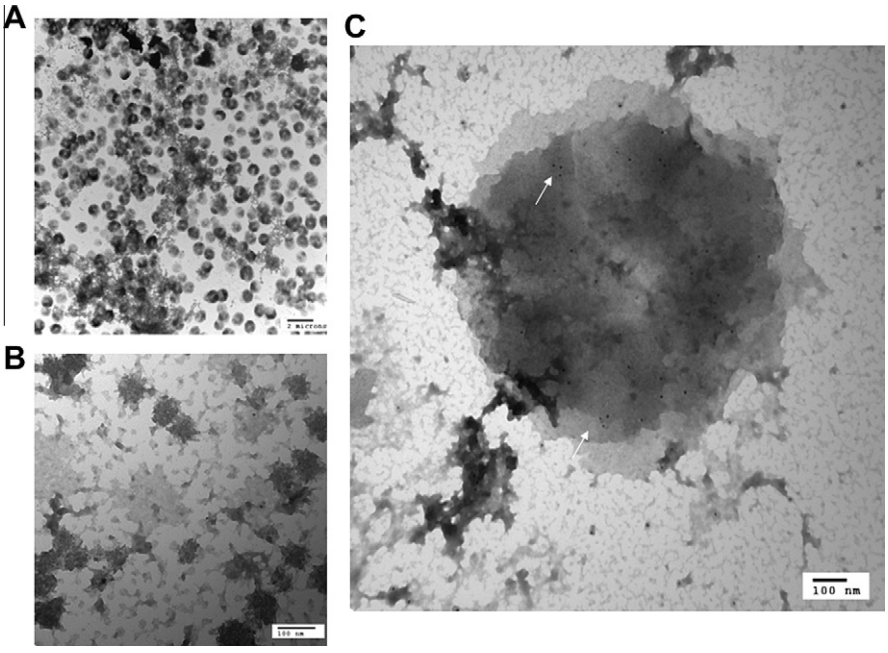

Fig. 2. Transmission electron micrograph of particles derived from fraction 2 of HCV replicon cell lysates (A) or that of HuH7 cell lysates (B) fractionated by membrane flotation assay. NS5A proteins were associated with the particles (the arrow marked the 10 nm gold particle-conjugated anti-NS5A Ab) (C).

3.4. Knockdown of proteins associated with lipid raft membrane in HCV replicon cells

VAPA (hVAP-33; the human homologue of the 33-kDa vesicle-associated membrane protein-associated protein), which binds to both NS5A and NS5B, has been demonstrated to play a critical role in the formation of HCV RCs [15]. Lots of other proteins up-regulated in lipid raft fraction of HCV replicon cells were also identified previously [22]. Seven randomly selected HCV RCs-associated cellular proteins (VAPA, COPG, RAB18, COMT, CDC42, DPP4, and KDELR2) were individually knocked-down using shRNAs to see their effects on HCV replication and the formation of these vesicles-like structures under AFM. It has been previously reported that miRNA122 could facilitate HCV replication [23]. Knockdown of Dicer would reduce the production of miRNA122 and, in turn, should repress the HCV replication [24]. Therefore, shRNA targeting Dicer was used as a positive control while shRNA targeting Luciferase gene as a negative control for the knockdown experiments. Reduced expression of each of these HCV RCs-associated cellular genes resulted from different shRNA clones was demonstrated using real-time RT-PCR (Supplementary Fig. 1). Knockdown of each of these selected lipid raft-associated proteins suppressed

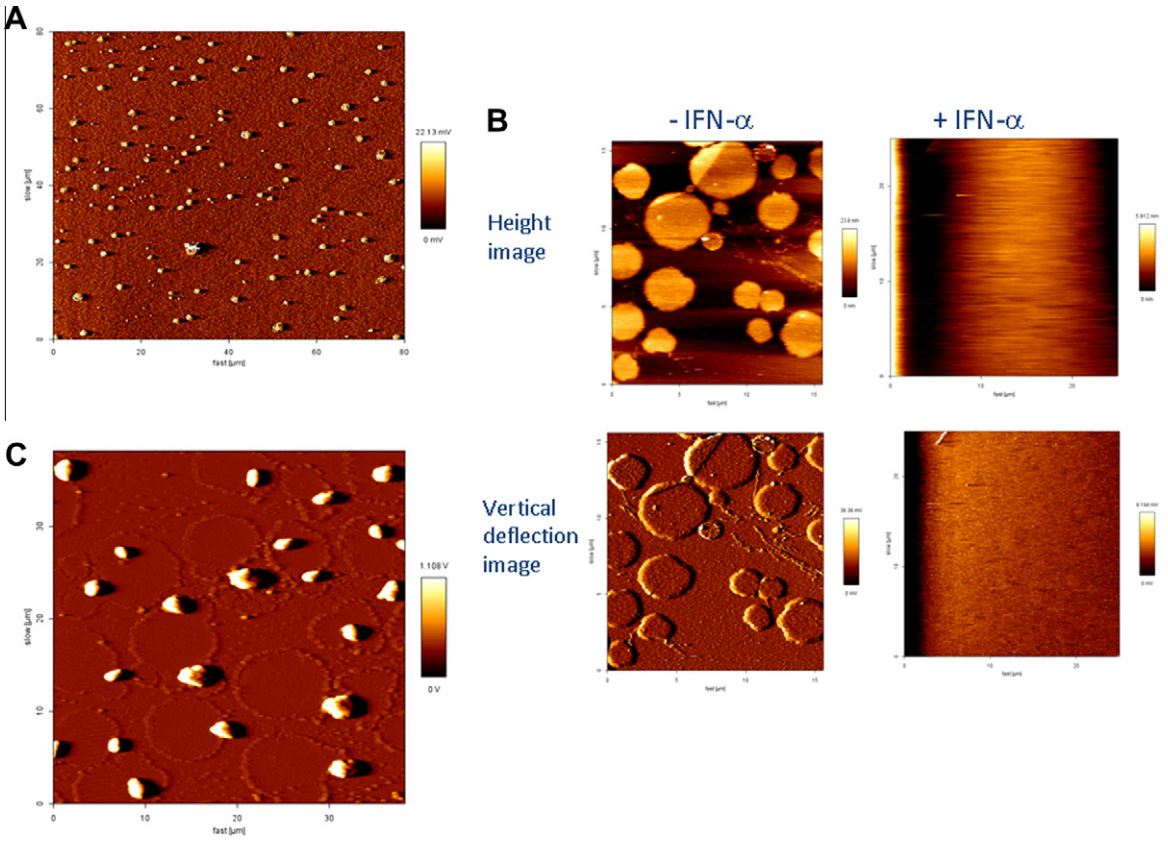

Fig. 3. Atomic force micrograph of particles derived from fraction 2 of HCV replicon cell lysates (A) fractionated by membrane flotation assay. After several cycles of freezing–thawing, particles with larger sizes were observed in fraction 2 of HCV replicon cell lysates before (left column of B) but not after the treatment of interferon (right column of B). NS5A proteins (the white spots represent the aggregated anti-NS5A Ab, the gold particles could not be resolved) were associated with the particles (C).

the HCV replication as demonstrated by the expression level of HCV NS5A proteins (Fig. 4), which correlated well with the knockdown efficiency of individual shRNA clones (Supplementary Fig. 1). The suppression of HCV replication was also coincided with the reduced number of observed particles from AFM (data not shown). However, the specialized particles were distorted and did not show the regular round shape after knockdown experiments were performed (data not shown). It is likely that infection with recombinant lentivirus would affect the structure of HCV RCs.

Caveolin-2 was also reported previously to associate with HCV RCs in lipid raft [12]. Interestingly, knockdown of Caveolin-2 (Supplementary Fig. 1) neither affected the HCV replication (Fig. 4) nor reduced the number of particles found in lipid raft membrane fraction (data not shown). This result suggests that either tiny amount

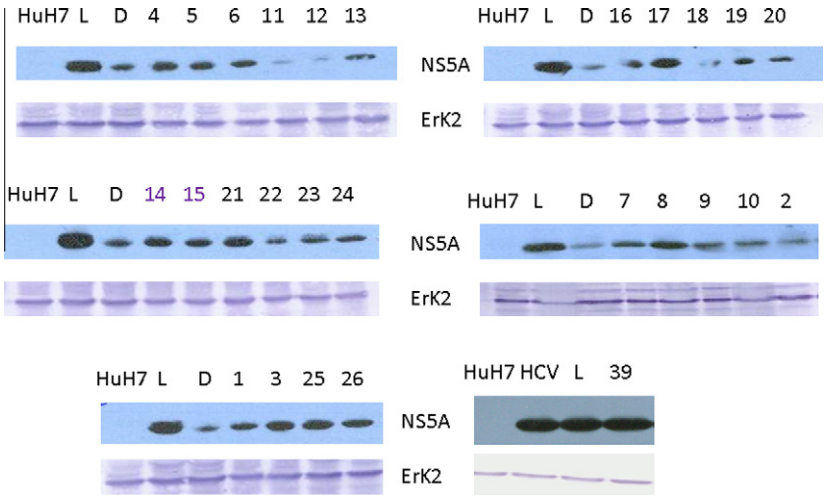

Fig. 4. Western blotting analysis of HCV NS5A protein in the cell lysates of HCV replicon cells from shRNA knockdown experiments. HCV and HuH7 represent the total cell lysate derived from HCV replicon cells and its parental HuH7 cells. L and D represent shRNAs targeting Luciferase gene and Dicer gene, respectively. shRNA clones 1, 2, 3 were designed for targeting COPG, clones 4, 5, 6 for RAB 18, clones 7, 8, 9, 10, 25, 26 for VAPA, clones 11, 12, 13 for COMT, clones 14, 15 for CDC42, clones 16, 17, 18, 19, 20 for DPP4, clones 21, 22, 23, 24 for KDELR2, clones 39, 40, 41, 42, 43 for Caveolin 2, respectively. ERK2 protein was used as a loading control.

of Caveolin-2 protein is enough for the support of HCV replication or caveolin isoforms (e.g., Caveolin-1) could compensate its function in supporting HCV replication.

In summary, results from this study indicated that purified HCV RCs with associated lipid raft membrane appeared as distinct particles of around 0.7 um under EM and AFM. Knockdown of proteins associated with lipid raft suppressed the HCV replication and reduced the number of these particles.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.037.

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