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Viral Hepatitis

Continuous *de novo* generation of spatially segregated hepatitis C virus replication organelles revealed by pulse-chase imaging

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Background & Aims: Like all positive-sense RNA viruses, hepatitis C virus (HCV) induces host membrane alterations for its replication. In chronically infected cells, it is not known whether these viral replication organelles are being continually resupplied by newly synthesized viral proteins *in situ*, or whether they are generated *de novo*. Here we aimed to study temporal events in replication organelles formation and maturation.

Methods: Here we use pulse-chase labeling in combination with confocal microscopy, correlative light electron microscopy and biochemical methods to identify temporally distinct populations of replication organelles in living cells and study the formation, morphogenesis as well as compositional and functional changes of replication organelles over time.

Results: We found that HCV replication organelles are continuously generated *de novo* at spatially distinct sites from preformed ones. This process is accompanied by accumulated intracellular membrane alteration, increased cholesterol delivery, NS5A phosphorylation, and positive-strand RNA content, and by eventual association with HCV core protein around lipid droplets. Generation of spatially segregated foci requires viral NS5A and the host factors phosphatidylinositol 4-kinase and oxysterol-binding protein, while association of foci with lipid droplets requires cholesterol.

Conclusions: Our results reveal that HCV replication organelles are not static structures, but instead are continuously generated and dynamically change in composition and possibly also in function.

Lay summary: Hepatitis C virus replication membrane structures are continuously generated at spatially distinct sites. New replication organelles are different in composition, and possibly also in function, compared to old replication organelles.

Keywords: Hepatitis C virus; Viral replication; Double membrane vesicles; SNAP; Cholesterol; Organelles; Pulse-chase imaging.

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Introduction

Hepatitis C virus (HCV) is a prevalent and globally distributed human pathogen. More than 170 million people are chronically infected, of whom many will develop cirrhosis and/or hepatocellular carcinoma. The positive-sense RNA genome encodes a single polyprotein that is processed by cellular and viral proteases to generate 10 mature proteins [1], of which the NS3-5B region is sufficient to sustain viral RNA replication [2].

Like all positive-sense RNA viruses, HCV induces cytoplasmic membrane alterations in infected cells [3], which have been termed 'replication organelles' and are believed to be sites of HCV RNA synthesis. The membranes that form the HCV replication organelle are likely derived from the cellular endoplasmic reticulum and are enriched in viral nonstructural proteins and HCV RNA [4,5]. At the ultrastructural level, HCV replication organelles are characterized mostly by double membrane vesicles (DMVs) and also by multi-membrane vesicles (MMVs) [6]. Recent studies have indicated that DMVs are the likely sites of HCV RNA replication and that NS5A is essential for their formation [6,7]. This function of NS5A may be partially attributable to its ability to interact with various host factors that are essential for viral RNA replication. Among them are the lipid kinase phosphatidylinositol 4-kinase IIIα (PI4KA) [8–10] and its downstream effector, oxysterol-binding protein (OSBP) [11,12]. These two proteins generate the unique lipid composition of the HCV replication organelle, which is enriched in phosphatidylinositol 4-phosphate (PI4P) [10,13] and cholesterol [7,14–16].

While progress has been made in defining early events of HCV replication organelle formation in acutely infected cells [6,17], there remain many questions regarding the temporal regulation of its function and composition, as well as the turnover of HCV replication organelles in chronically infected cells. For instance, are replication organelles relatively static structures that are continually resupplied by viral proteins, or are new ones continually generated *de novo*? Furthermore, while HCV RNA synthesis occurs at the replication organelle, virion assembly is believed to occur at or near lipid droplets (LDs) [18]; how are HCV genomes destined for encapsidation physically transferred between these sites?

In this study, we used a pulse-chase fluorescent labeling approach that allows us to discriminate 'old' from 'new' NS5A-positive membranous structures (NS5A foci). 'New' NS5A foci

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form at sites distinct from 'old' NS5A foci even at short chase intervals, supporting a model of continuous *de novo* replication organelle formation instead of resupply of previously formed ones. PI4KA, OSBP, and NS5A are required to initiate new NS5A foci. Cholesterol is preferentially trafficked to 'old' NS5A foci and is required for association of 'old' foci with HCV core protein and LDs. These findings collectively describe a process of continuous HCV replication organelle generation accompanied by alteration of lipid content and progressive association with sites of putative virion assembly that is dependent on cholesterol content.

Materials and methods

Viruses and viral replicons

A sequence encoding the SNAPf tag [19] was inserted into domain III of NS5A in the context of the full-length HCV genome and a subgenomic replicon based on the genotype 2a JFH1 strain, termed here as FL-JFH1(NS5A/SNAP) and SGR-JFH1 (NS5A/SNAP) respectively. Detailed information about these two constructs is described in the Supplementary material.

Labeling of SNAP-tagged NS5A with fluorescent SNAP-tag substrates

Labeling of SNAP-tagged NS5A with SNAP-tag substrates (SNAP-Cell 505, SNAP-Cell TMR-Star, and SNAP Cell-Block; New England Biolabs, Ipswich, MA), was performed according to the manufacturer's instructions. Briefly, cells were treated with complete medium containing 5 μM SNAP-Cell 505, 3 μM SNAP-Cell TMR-Star or 10 μM SNAP Cell-Block for 15 min before they were washed out and replaced with fresh medium.

Correlative light-electron microscopy

SGR-JFH1(NS5A/SNAP) replicon cells seeded onto glass bottom dishes with gridded coverslips (MatTek, Ashland, MA) were labeled with SNAP-Cell TMR-star (S_{TMR}) and SNAP-Cell 505 (S_{505}) and then evaluated by confocal microscopy to identify cells of interest; their positions were recorded and DIC/confocal fluorescent images were acquired. Cells were immediately fixed and processed for EM sectioning. The sections were viewed on a JEOL JEM-1400 Plus transmission electron microscope at 80 kV. Further details are provided in Supplementary material.

Quantitation of NS5A phosphorylation and negative: positive-strand RNA ratios

SGR replicon cells were first labeled with SNAP-Cell Block (S_{block}) and S_{TMR} to selectively label 'old' or 'new' NS5A before they were washed once with ice cold PBS, lysed with 100 μM digitonin in PBS containing protease inhibitors, phosphatase inhibitors and RNase inhibitors, and centrifuged for 5 min at 12,000 g. TMR-labeled NS5A-SNAP and associated RNA were then isolated by incubating the cell lysate with anti-TMR antibody for 1.5 h at 4 °C followed with Dynabeads Protein G (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Immunoisolated material was then subjected to immunoblotting with chemiluminescence quantitation on an Odyssey imager (Li-Cor, Lincoln, NE) or to strand-specific qRT-PCR as previously described [20], and is described further in the Supplementary material.

Statistics

The unpaired two-tailed Student's *t* test was used to compare the means of control and experimental groups.

Results

Characterization of a system for pulse-chase imaging of HCV NS5A as a marker of the HCV replication organelle

In order to study temporal aspects of HCV replication organelle formation and function, we genetically inserted a SNAP-tag into a known insertion-tolerant site within domain III of NS5A [21] as a marker of HCV replication organelles (Fig. 1A). NS5A and tagged NS5A proteins have been widely used as an HCV replication organelle marker [6,8–10,21–23]. The SNAP-tag specifically, rapidly, and irreversibly forms a covalent bond with benzylguanine derivatives [24]. A colony formation assay showed that insertion of the SNAP-tag into NS5A had minimal effects on replication compared to an untagged replicon (Fig. 1B), and this was further confirmed by measuring the luciferase activity of replicons encoding a *Renilla* luciferase reporter or intracellular HCV RNA content in cells stably expressing replicons (Supplementary Fig. 1A–C). Finally, NS5A-SNAP migrated as expected on SDS-PAGE (Fig. 1C).

Living NS5A-SNAP replicon cells were incubated with the green fluorescent cell-permeable SNAP-tag substrate S_{505} or the red fluorescent substrate S_{TMR}. Brightly labeled puncta were observed in cells stably expressing the NS5A-SNAP replicon but not in untagged replicon cells (Fig. 1D). No decrease in HCV RNA content (Fig. 1E) or reporter luciferase activity (Supplementary Fig. 1D) was seen in cells labeled with either S_{TMR} or S_{505} and incubated for up to an additional 72 h. In addition, labeling with S_{TMR} did not change the half-life of NS5A-SNAP protein (Supplementary Fig. 1E). There was near-complete overlap between NS5A immunostaining and NS5A-SNAP labeling by either S_{505} (Pearson's correlation coefficient 0.87) or S_{TMR} (Pearson's correlation coefficient 0.95) (Fig. 1F).

Pulse-chase imaging of HCV replication organelle formation

We then tested sequential labeling of NS5A-SNAP, first with S_{505} to label pre-existing ('old') NS5A, followed by S_{TMR} to label newly synthesized ('new') NS5A after a chase period. Newly synthesized protein could be detected with S_{TMR}, but not when protein synthesis had been halted by cycloheximide treatment during the chase period (Supplementary Fig. 1F), demonstrating specific labeling of newly synthesized protein. NS5A-SNAP labeling was efficiently blocked by the non-fluorescent compound S_{block} (Supplementary Fig. 1G), and S_{block} also did not inhibit HCV replication (Supplementary Fig. 1H).

Having confirmed that this system is suitable for pulse-chase imaging in live cells, we tested whether replication organelles are stable structures that are resupplied by newly synthesized NS5A, or whether newly synthesized NS5A is directed to new replication organelles formed *de novo*. This can be determined by quantitating the colocalization between 'old' and 'new' NS5A molecules at varying chase times, as depicted in Fig. 2A. Supplementary Fig. 2 depicts the two models and their predictions regarding 'old' and 'new' NS5A colocalization with increasing chase times. 'Old' and 'new' NS5A signals displayed significant overlap only when S_{block} was applied immediately after S_{TMR} labeling, while 'new' NS5A synthesized as little as 1 h after 'old' NS5A labeling was found at foci distinct from 'old' NS5A foci (Fig. 2B and C). The rapid loss of 'old' and 'new' NS5A colocalization was more consistent with newly synthesized NS5A being directed to *de novo* replication organelles formation rather than to resupplying previously formed ones. To exclude the possibility that the decrease in colocalization at later time points was due to variability in 'new' NS5A labeling duration, we fixed the 'new' NS5A labeling duration at 24 h and obtained similar results (Supplementary Fig. 3).