Interaction of Stomatin with Hepatitis C Virus RNA Polymerase Stabilizes the Viral RNA Replicase Complexes on Detergent-Resistant Membranes

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Introduction

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease, leading to liver cirrhosis and hepatocellular carcinoma [16]. HCV is an enveloped virus with a positive-strand RNA genome of approximately 9.6 kilobases belonging to the Hepacivirus genus in the Flaviviridae family [2]. The HCV genome encodes a single polyprotein of approximately 3,010 amino acids, which is proteolytically processed by a combination of host and viral proteases into at least 10 distinct structural and nonstructural (NS) proteins. The structural proteins include C, E1, E2, and p7, and the NS proteins include NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Biochemical studies of each NS protein and reverse-genetic studies revealed that the NS3–NS5 proteins are essential for HCV replication [2]. Among the HCV NS proteins, NS5B with an RNA-dependent RNA polymerase (RdRp) activity is essential for viral RNA genome replication [25].

Viruses with positive-strand RNA genomes replicate on various intracellular membrane structures associated with the endoplasmic reticulum (ER) [26], Golgi apparatus [32], endosomes and lysosomes [19, 43], peroxisomes and chloroplasts [21], or mitochondria [13, 22]. Some RNA
viruses even induce the formation of distinct membrane structures derived from several membrane compartments to provide a structural scaffold for viral RNA replication [3, 9, 47]. HCV RNA replicate complexes (RCs), which contain the viral NS proteins NS3/4A, NS4B, NS5A, and NS5B and some cellular proteins, are also thought to be associated with membrane structures. It was proposed that HCV RCs may be formed on the ER [8, 23]. In addition, it was shown that newly synthesized HCV RNAs and NS proteins co-localize on distinct speckle-like structures, which are separate from the ER and distinct from lipid droplets [31]. These findings support the notion that HCV RNA RCs are separate from the ER and distinct from lipid droplets [31].

Here, we took a proteomic approach to identify cellular proteins that might participate in HCV RNA replication steps, including the formation of RCs at the intracellular viral replication sites. We have identified stomatin as one of the cellular proteins interacting with HCV NS5B. Stomatin has been known to participate in the formation of lipid rafts, which are membrane microdomains associated with protein complexes, cholesterolers, and sphingolipids [4, 5]. Our results suggest that stomatin functions in the assembly of HCV RCs on detergent-resistant membrane (DRM) structures.

Materials and Methods

Plasmids and Antibodies

An HCV subgenomic replicon, pZS2 [41], derived from the parental HCV Con-1 replicon I<sub>mp</sub>/NS3-3’ (AJ242652) was provided by Dr. Christoph Seeger. The plasmid pFHI1 [45], containing a full-length JFH1 cDNA under the control of the T7 RNA promoter, was provided by Dr. Takaji Wakita. The pEFStmyc5 vector [33], used to express myc-epitope-tagged stomatin, was provided by Dr. Rainer Prohaska (Medical University of Vienna, Austria). The anti-stomatin antibody GARP 50 was also kindly provided by Dr. Rainer Prohaska (Medical University of Vienna, Austria). Polyclonal rabbit antisera against HCV NS5B were raised by immunization with Ni-nitrilotriacetic acid (NTA)-Sepharose resin (Qiagen, Hilden, Germany), followed by chromatography using heparin-Sepharose and SP-Sepharose columns as described previously [10].

Expression and Purification of HCV NS5B

The (His)₆-tagged HCV NS5B lacking the 21 C-terminal amino acids (Δ21NS5B) was expressed in *Escherichia coli* at 25°C for 12 h by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. It was purified using Ni-nitrilotriacetic acid (NTA)-Sepharose resin (Qiagen, Hilden, Germany), followed by chromatography using heparin-Sepharose and SP-Sepharose columns as described previously [10].

Isolation of Cellular Proteins Interacting with HCV NS5B

The Huh7 cells were rinsed twice with phosphate-buffered saline (PBS) and incubated for 30 min at 4°C in lysis buffer A (100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 10 mM NaF, 1 mM Na<sub>2</sub>VO<sub>4</sub>, and 17.5 mM β-glycerophosphate) containing 10 mM imidazole and EDTA-free protease-inhibitor cocktail (Roche, Basel, Switzerland). Unbroken cells and nuclei were pelleted by centrifugation at 2,500 × g for 10 min. The supernatant was centrifuged at 30,000 × g for 30 min to pellet the detergent-insoluble membrane fraction. The pellet fraction was resuspended in buffer B (100 mM Tris-HCl (pH 8.0), 1 M NaCl, 1% Triton X-100, 1% NP-40, 10 mM NaF, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 17.5 mM β-glycerophosphate, 10 mM imidazole, and EDTA-free protease-inhibitor cocktail). The proteins in the detergent-insoluble membrane fraction were solubilized by stirring with a magnetic bar for 1 h. After centrifugation at 100,000 × g for 1 h, the supernatant was recovered, dialyzed against lysis buffer A, and pre-cleared by incubating three times with Ni-NTA resin that was pre-equilibrated with the same buffer. The pre-cleared cell lysate (2 mg) was incubated for 3 h at 4°C with either purified Δ21NS5B protein-bound Ni-NTA resin or empty Ni-NTA resin. The resins were washed five times with lysis buffer A. The proteins were eluted by boiling the samples in an SDS-sample buffer (60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromophenol blue) and detected by silver staining.
Identification of Proteins by Mass Spectrometry

The protein bands were excised from the SDS-PAGE gels and digested with trypsin. The digested samples were loaded onto a fused silica microcapillary C18 column (75 µm inner diameter × 10 cm; Magic C18 beads; Michrom BioResources, Auburn, CA, USA). An Agilent 1100 high-pressure liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) was used to deliver a gradient across a flow splitter to the column. Eluting peptides from the column were ionized by electrospray ionization (ESI) and subjected to mass spectrometry (MS) analysis using an LTQ ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). The acquired MS/MS spectra were searched against the non-redundant human protein database using the SEQUEST algorithm [39].

Immunoprecipitation and Western Blot Analysis

Huh7 cells grown in a 10 cm plate were harvested, washed with cold PBS, and suspended in 0.5 ml of lysis buffer A to prepare the solubilized proteins, as described above. The protein lysate was then mixed with 2 µg of purified Δ21NS5B and the anti-stomatin antibody GARPS0. After incubation for 1 h on a rotator at 4°C, the immune complexes were adsorbed to Protein A-Sepharose beads (Amersham Biosciences, Piscataway, NJ, USA). The myc-epitope-tagged stomatin was immunoprecipitated from the cell lysates using a monoclonal anti-myc-epitope antibody. Western blotting was performed as described previously [37] using the ECL chemiluminescence detection system (Amersham Biosciences).

Immunofluorescence Microscopy

R-1 cells were cultured in eight-well chamber slides (Nunc, Roskilde, Denmark) to 50% confluency. After incubation for 24 h, the cells were fixed with 1% formaldehyde in methanol for 12 h at −20°C, washed twice with cold PBS, and then permeabilized with PBS containing 0.2% Triton X-100 for 30 min at room temperature. After washing three times with PBS, the cells were treated with a blocking solution (PBS containing 1% BSA, 0.1% gelatin, and 5% goat serum) for 30 min at room temperature, and proteins were visualized by immunostaining. The nuclei were visualized by staining with 1 µM 4',6'-diamidino-2-phenylindole (DAPI) in PBS. Confocal images were obtained using an LSM 510 META confocal microscope (Carl Zeiss, Oberkochen, Germany).

Subcellular Fractionation

Subcellular organelles were fractionated as previously reported [28]. Briefly, cells were washed with buffer C (20 mM MOPS (pH 7.4), 100 mM sucrose, and 1 mM EGTA) and resuspended in buffer D (buffer C plus 5% Percoll (Sigma-Aldrich), 0.01% digitonin, 10 µM aprotinin, and 10 µM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and protease-cocktail inhibitor (Roche)). After incubation for 15 min on ice, unbroken cells and nuclei were pelleted by centrifugation at 2,500 ×g for 10 min. The nuclear pellet was resuspended in a buffer containing 10 mM Tris-HCl (pH 7.5), 2.5 mM KCl, and 2.5 mM MgCl₂. By centrifugation at 90,000 ×g for 30 min through 2.1 M sucrose in 50 mM Tris-HCl (pH 7.5) and 5 mM MgCl₂, a pure nuclear fraction was obtained. The nucleocleared supernatant obtained by centrifugation at 2,500 ×g was further centrifuged at 15,000 ×g for 15 min to obtain the P15 fraction enriched with mitochondria, which was resuspended in buffer E (20 mM MOPS (pH 7.4), 300 mM sucrose, 1 mM EGTA, and protease-inhibitor cocktail) for subsequent analysis. The supernatant recovered by centrifugation at 15,000 ×g was subjected to centrifugation at 100,000 ×g for 1 h. The resulting supernatant and the pellet were designated as the S100 fraction and the P100 fraction enriched with microsomes, respectively. The P100 fraction was resuspended in buffer E for subsequent analysis. Proteins (20 µg, unless otherwise specified) from each fraction were subjected to SDS-PAGE for western blot analysis. To further fractionate the P15 fraction, Percoll-density gradient centrifugation was performed as described previously [46].

RNA-Dependent RNA Polymerase Assay

The RdRp assay was performed using the P15 and P100 subcellular fractions. Briefly, 50 µg of each fraction was added to a 50 µl reaction mixture containing 50 mM HEPES (pH 7.6), 50 mM potassium acetate, 3 mM MgCl₂, 10 mM DTT, 20 µg/ml actinomycin D (Sigma-Aldrich), 40 U R N ase inhibitor (Promega, Madison, WI, USA), non-radioactive dNTP mixture (0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, and 50 µM UTP), and 10 µCl [α-32P] UTP (Amersham Biosciences). The reaction mixture was incubated at 28°C for 4 h, and then the RNA was extracted as described previously [25] and resolved on a formaldehyde denaturing 1% agarose gel. The gel was stained with ethidium bromide, photographed, and dried for autoradiography.

Membrane Flotation Assay

The P15 fraction, or the same fraction treated with 1% NP40 on ice for 30 min, was resuspended in 0.5 ml of hypotonic buffer (10 mM Tris-HCl (pH 7.5), 10 mM KCl, and 5 mM MgCl₂) prior to mixing with 4 ml of 72% sucrose in a low-salt buffer (50 mM Tris-HCl (pH 7.5), 25 mM KCl, and 5 mM MgCl₂). The resulting mixture was overlaid with 4.5 ml of 55% sucrose and 2 ml of 10% sucrose in the same buffer. The resulting sucrose-gradient preparation was centrifuged in a Beckman SW41 Ti rotor (Beckman Coulter, Brea, CA, USA) for 16 h at 4°C. Fractions (1 ml) were taken from the top of the gradient, and each fraction was concentrated using trichloroacetic acid (TCA) for subsequent immunoblotting experiments.

Cholesterol Analysis

The free cholesterol content of the fractions was estimated using the AmplexRed cholesterol assay kit (Molecular Probes, Eugene, OR, USA), as described previously [40].

RNA Interference

For the small interfering RNA (siRNA)-mediated silencing of stomatin, pSuper-ST plasmid was constructed by cloning the
annealed DNA oligonucleotides (5’-GATCCCC ATTGTTTTTTTC TCGCCCATCTCAAGGAGGGAAGGACGTTTT TTGCAAA-3’ and 5’-AGCTTTTCCAAAAATTTGTTTCTCCTTC TGGCCATCTCTGAAGGAGGGGAAAGACATTG-3’, siRNA targeting sequence (nucleotides 785–816) of stomatin (GenBank Accession No. bc010703) shown in bold) into the BglII and HindIII sites of the pSUPER vector (OligoEngine, Seattle, WA, USA). R-1 cells grown to 60% confluence were transfected with pSuper-ST or pSuper using FuGENE 6 reagent (Roche). Alternatively, a synthetic siRNA against the same target site of pSuper-ST was transfected into HCV-infected Huh7 cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

**TaqMan Real-Time Quantitative RT-PCR**

Total RNA was extracted with TRIzol reagent (Invitrogen) and purified according to the manufacturer’s recommendations. HCV RNA levels were quantified by real-time quantitative reverse-transcription (qRT)-PCR using a TaqMan probe, as described previously [17]. Cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA from the same RNA extract was used as an internal control.

**Northern Blot Analyses**

Northern blotting to detect HCV genomic RNA was performed as reported previously [10] with a 32P-labeled DNA probe (HCV NS3 (3,446 nt)-NS5B (9,265 nt)) generated with the Ready-To-Go DNA labeling kit (GE Healthcare Life Sciences, Piscataway, NJ, USA).

**HCV Infection**

Full-length HCV RNA was prepared by in vitro transcription using the JFH1 clone and electroporated into Huh7 cells as described previously [40]. At 72 h post-electroporation, the culture medium was collected, cleared by low-speed centrifugation, and passed through a filter with a 0.45 µm pore size. The filtered culture medium was used to infect Huh7 cells at a multiplicity of infection (MOI) of 0.25.

**Results**

**Identification of DRM-Associated Cellular Proteins Interacting with HCV RNA-Dependent RNA Polymerase**

It has been suggested that HCV RCs reside on DRMs [1]. In order to identify the cellular proteins interacting with HCV NS5B RdRp on DRMs, we extracted proteins from DRMs by solubilization with a combination of detergents (1% Triton X-100 and 1% NP40) and 1 M NaCl and applied the extracted proteins to an Ni-NTA resin carrying purified HCV ∆21NS5B (Fig. 1A). Upon silver staining of the proteins bound to the resin after SDS-PAGE, we detected six protein bands that were not present among the proteins retrieved from the control resin (Fig. 1B). The protein bands were in-gel digested with trypsin and analyzed by ESI-MS/MS. Among the six identified proteins, we chose stomatin for further characterization of its roles in HCV RC formation, because this integral membrane protein associated...
with DRMs is known to form high-order oligomers and could thereby have a scaffolding function [24, 34, 35]. An example of the ESI mass spectra of a peptide from stomatin is shown in Fig. 1C. We also identified α-actinin, belonging to the superfamily of actin crosslinking proteins, which was previously reported to interact with HCV NS5B [14]. The other proteins were not previously identified as interacting with NS5B and will be reported elsewhere.

Analysis of the Interaction Between Stomatin and HCV NS5B

To verify the interaction between NS5B and stomatin, we performed co-immunoprecipitation experiments. First, we immunoprecipitated the endogenous stomatin from the soluble protein mixture used for the aforementioned pull-down experiments to detect the purified Δ21NS5B spiked in the protein sample by western blot analysis. As shown in Fig. 2A, the NS5B was co-immunoprecipitated with the endogenous stomatin (lane 4). Additionally, when c-myc-tagged stomatin was immunoprecipitated from R-1 cells, which were transfected with an expression vector for the epitope-tagged stomatin, co-immunoprecipitated NS5B could also be detected (Fig. 2B, lane 4).

The cellular co-localization of these proteins in R-1 cells was confirmed by confocal microscopy. Stomatin exhibited a distinct cytoplasmic and perinuclear localization (Fig. 2C).

The HCV NS5B localized predominantly in perinuclear regions. There was extensive, but not complete, co-localization of stomatin with NS5B in the perinuclear regions within the R-1 cells.

Co-Fractionation of NS5B and Stomatin in the DRM Fraction

To examine the subcellular localization of HCV NS proteins and stomatin, we performed subcellular fractionation of R-1 cells harboring HCV subgenomic RNA replicons in which the coding region for the viral structural proteins was replaced by a drug-selection marker, enabling Huh7 hepatoma cells transfected with the subgenomic RNA transcript to grow in the presence of G418. The R-1 cells were fractionated into the nuclear, P15, P100, and S100 fractions by differential centrifugations. Equal amounts of protein from each fraction were analyzed by immunoblotting to detect the viral NS proteins NS3, NS5A, and NS5B, which are known to be essential for HCV RNA replication [18]. As shown in Fig. 3A, most of the HCV NS proteins were highly enriched in the P15 fraction (lane 3). This fraction was enriched with mitochondria, as evidenced by the presence of Hsp60, a mitochondrial matrix protein [28]. In this fraction, we also detected the ER marker Grp78, probably because of the physical linkages between some fractions of the ER and mitochondria. As expected, this

Fig. 2. Analysis of the interaction between HCV NS5B and stomatin.

(A) The soluble protein fraction (SP) prepared as described in the Materials and Methods was incubated with Δ21NS5B prior to immunoprecipitation (IP) of stomatin followed by western blot (WB) analysis for the indicated proteins. The input represents 1/10 of the proteins used for the co-immunoprecipitation experiments. (B) Huh7 and R-1 cells were transfected with pEFSTMyc5. The myc-epitope-tagged stomatin was immunoprecipitated from the transfected cells, and the immune complexes were analyzed for the indicated proteins as in (A). (C) The NS5B and stomatin in R-1 cells were immunostained. Nuclei were visualized by DAPI staining (blue). Merged images (yellow) are shown in the fourth panel.
marker was also present in the P100 fraction in which Hsp60 was not detected, indicating that the fraction was not contaminated with mitochondria. We also detected slightly lower levels of HCV NS proteins in the P100 fraction (lane 4; see also the Grp78), which is known to be enriched with microsomes [28]. Interestingly, we found that the subcellular localization of stomatin was altered in the R-1 cells; stomatin was detected in both the P15 and the

P100 fractions in the Huh7 cells, but it was primarily present only in the P15 fraction in the R-1 cells (Fig. 3B), demonstrating its subcellular redistribution in HCV-replicating cells.

Having detected HCV NS proteins in two different subcellular fractions, we investigated whether the HCV RCs in both fractions display RdRp activity. We performed an RdRp assay in a reaction mixture including $[\alpha^{32}P]$ UTP.

**Fig. 3.** Co-fractionation of stomatin with the HCV RNA replicase complex in a detergent-resistant domain of membranous structures.

(A) The nuclear (Nucl), P15, P100, and S100 subcellular fractions of R-1 cells (20 µg protein from each fraction) was resolved by SDS-PAGE and analyzed by immunoblotting for the indicated HCV NS proteins. Hsp60 and Grp78 were used as markers of the mitochondria and ER, respectively. (B) Subcellular localization of stomatin in R-1 and Huh7 cells was analyzed as in (A). (C) *In vitro* RdRp assays with the indicated fractions from R-1 and Huh7 cells. After the assays, the radiolabeled RdRp reaction products were resolved on a 1% denaturing agarose gel for autoradiography. An *in vitro* transcribed, internally labeled HCV subgenomic replicon RNA was used as an RNA marker and is indicated by the arrowhead. (D) The P15 fraction from the R-1 cells was further purified by Percoll-density gradient centrifugation. Equal volumes of each fraction were analyzed by immunoblotting as in (A). Fractions are numbered 1–4 in order from the top of the gradient to the bottom. (E) The P15 fraction, which was left untreated (top panel) or was treated with 1% NP40 for 30 min on ice (middle panel), was further fractionated by discontinuous sucrose-gradient centrifugation. Proteins in an equal volume of the recovered fractions were concentrated with trichloroacetic acid, resolved by SDS-PAGE, and analyzed by immunoblotting for the indicated proteins. The cholesterol concentration in each fraction from the NP40-treated P15 fraction is shown in the bottom panel. DRM, detergent-resistant membrane.
to label RNA synthesized by the HCV RCs using the endogenous HCV subgenomic RNA template. The radiolabeled RdRp products were analyzed by denaturing agarose gel electrophoresis followed by autoradiography. As shown in Fig. 3C, we could detect a labeled RNA product of ~8 kb in length, which was equivalent to the size of the subgenomic replicon RNA, from the reaction with the P15 fraction of the R-1 cells. In contrast, there was no detectable level of subgenomic replicon-RNA-size-labeled product from the reaction with the P100 fraction of the R-1 cells (compare lanes 3 and 4). None of the corresponding fractions from the Huh7 cells produced labeled products of HCV subgenomic replicon RNA size (lanes 1 and 2). The heterogeneous smear of labeled products shorter than the subgenomic RNA might be end-labeled products, possibly generated by host-cell-derived terminal transferase activity that co-fractionated particularly with the P100 fractions (lanes 2 and 4).

To investigate whether the HCV NS proteins, stomatin, and intracellular organelles or the membranous compartments derived from them were tightly associated with each other, we further fractionated the P15 fraction by Percoll-gradient centrifugation. Analysis of the resulting four fractions by immunoblotting revealed that HCV NS proteins had co-fractionated with stomatin, Hsp60, and calreticulin, an ER lumen marker, suggesting tight associations between the NS proteins and stomatin on a membranous structure and possible linkages among the organelles and/or membranous structures within the fraction.

To further define the subcellular compartment within which HCV NS5B and stomatin reside together, the P15 fraction from the R-1 cells was treated with the non-ionic...
detergent NP40 for 30 min on ice and subjected to the membrane flotation assay. In the membrane flotation assay, the DRM floats to the top of a sucrose gradient, while the non-membrane fractions remain among the bottom fractions. As shown in Fig. 3E, both NS5B and stomatin were found predominantly in the low-density membranous structure, which also contained Hsp60, suggesting that the membranous structure might be associated with yet-to-be-characterized mitochondria-derived compartments. When the P15 fraction was treated with 1% NP40, NS5B and stomatin remained in the same fraction, while Hsp60, which was released from mitochondria-derived compartments, was detected in the bottom fractions, suggesting that stomatin and NS5B were tightly associated with the intracellular DRM structures. Of note, the fraction enriched with HCV NS5B and stomatin contained a higher level of free cholesterol, suggesting a nature similar to that of DRMs.

Roles of Stomatin in HCV RNA RC Formation on DRM Structures

To investigate the function of stomatin in HCV replication, we silenced the expression of stomatin using a small hairpin RNA. The introduction of pSuper-ST designed to express a stomatin-specific shRNA in R-1 cells led to a significant decrease in the HCV subgenomic RNA copy number, as determined by qRT-PCR (up to 74 ± 22%, p < 0.05; Fig. 4A). The reduction of the HCV RNA abundance was further confirmed by northern blot analysis (Fig. 4B).

Next, we investigated whether the knockdown of stomatin expression alters the subcellular localization of NS5B to result in the inhibition of HCV RNA replication. We performed a membrane flotation assay with the P15 fraction from R-1 cells, which was pre-treated with 1% NP40 on ice for 30 min. In a parallel experiment, stomatin was silenced prior to the assay to investigate the impact of stomatin silencing on NS5B localization on DRMs. As shown in Fig. 4C, stomatin silencing induced the redistribution of NS5B in the P15 fraction. There was a remarkable reduction in the DRM-associated NS5B level, and the NS5B released from the DRM was detected in fractions 8-10. These results suggest that stomatin might help the formation of a stable RNA RC on the DRM through its interaction with HCV NS5B.

We further investigated whether stomatin silencing has an impact on HCV RNA replication in HCV-infected cells. Consistent with the result from the R-1 cells, transfection with a synthetic stomatin-specific siRNA resulted in the inhibition of HCV genomic RNA replication by 64 ± 2% (p < 0.05) in HCV-infected Huh7 cells (Fig. 4D), underscoring the important role of stomatin in HCV RNA replication.

Discussion

It was previously proposed that the HCV RNA RC, consisting of viral NS proteins and host proteins, replicates the HCV RNA genome on a DRM structure [7, 31]. Although several host factors interacting with HCV NS5B, the key enzyme in the RC, have been identified by various experimental approaches, cellular proteins playing a structural and/or scaffolding role in the viral RC formation on DRMs have not yet been identified and characterized. Furthermore, the mechanism by which the HCV RNA replicase forms a complex on an intracellular membranous structure has not been fully understood. In this study, we took a proteomic approach using solubilized proteins, which were extracted from DRMs by a combination of detergents and salts, in an attempt to uncover NS5B-interacting, DRM-associated host factors. We identified stomatin as one such protein that plays a role in the stabilization of the HCV RC on an intracellular DRM compartment.

The 31 kDa integral protein stomatin was originally identified as a major protein of the erythrocyte membrane, but it has been shown to be expressed in most tissues as well as in various cell lines [6, 11, 38, 42]. We showed that the stomatin in the P15 fraction of Huh7 and R-1 cells was associated with DRMs (Fig. 3E). This result is consistent with previous observations of stomatin’s association with DRMs in other cell types [27, 30]. Various proteins with a structural role in membrane compartments have the ability to oligomerize. Stomatin is one of the proteins belonging to the so-called SPFH (stomatin/prohibitin/flotillin/HfklK/C) protein family. The clustering of individual SPFH proteins can form a specific type of protein-defined microdomain. These “specialized rafts” are similar to caveolae and are suggested to provide platforms for the recruitment of multiprotein complexes [15]. In fact, stomatin has the ability to form oligomers [33, 48]. Additionally, it has some topological and biochemical properties that are shared with caveolin, which has a scaffolding function in caveolae membranes, forming homo-oligomers that further interact side-by-side to form more complex particles [29, 36]. Like caveolin, stomatin oligomers would also serve as a basis for building up a widespread microdomain structure on a membrane compartment to facilitate the formation of the multiprotein complex of the HCV RC. Supporting this possibility, we showed that HCV NS5B was released from...
the DRMs of R-1 cell-line-derived P15 fractions when the expression of stomatin was silenced. Stomatin silencing would hinder the RC formation by providing fewer than the number of platforms required for building HCV RC and by interfering with NS5B engagement to the DRM-associated RC, which is likely to be promoted through interaction with stomatin and other viral NS proteins. The stomatin silencing, as expected based on its possible role as a scaffolding protein for the building of HCV RCs, suppressed HCV replication. Interestingly, we also found that the subcellular distribution profile of stomatin was changed in R-1 cells; it was mainly enriched in the P15 fraction of the R-1 cells, whereas it was detected in both the P15 and P100 fractions of the HuH7 cells (Fig. 3B). This might be, in part, attributed to the generation of membrane web structures in the cytoplasm upon HCV infection, which may rearrange the cytoplasmic inter-organellar linkage patterns [20]. Additionally, molecular interactions between HCV NS proteins and stomatin on intracellular DRM compartments, which can in turn alter the interactions among organelles and/or the membranous compartment, might explain this subcellular-distribution reprogramming in HCV-replicating cells.

The results of subcellular fractionation experiments revealed that HCV RC could be associated with subcellular compartments that co-fractionated with the mitochondria and ER (Fig. 3). Further fractionation of the P15 fraction, which displayed the viral RdRp activity, showed that HCV NS proteins, stomatin, Hsp60, and calreticulin were all present together in the same fraction of the Percoll-density gradient centrifugation, further demonstrating that both the mitochondria and the ER appear to be associated with this fraction. It is important to note that the mitochondria marker Hsp60, which was detected in the membrane compartments of the P15 fraction, could be dissociated from the DRM by 1% NP40 treatment (Fig. 3E), suggesting reversible association, rather than membrane fusion, between the DRM compartments harboring the HCV NS proteins and the mitochondria. The membrane association of HCV RCs would provide a structural framework for replication; it fixes the RNA replication process to a spatially confined place, increasing the local concentration of the necessary components, and it offers protection for the alien RNA molecules against host defense mechanisms. Because positive-strand RNA viruses replicate within or on peculiar intracellular membranous structures or on the membranes of various cellular organelles, it would be of interest to investigate whether DRM-associated host factors interacting with virus-encoded replicate components fulfill a similar function in the formation of RCs.

Previous studies of the origins of the DRMs where HCV NS proteins reside together are controversial. As discussed above, our results suggest that the DRM in the P15 fraction might represent the mitochondria-associated membranes, which are known to be reversibly tethered to mitochondria and consist of a region of ER [44]. Contradictory to our observations, the ER was proposed to be the site of HCV replication, based on the subcellular localization of NS proteins on the ER [8, 23]. The ER is known to contain relatively low levels of cholesterol and sphingolipids compared with other organelles, but it still contains lipid raft-like domains (LRLDs) within its membranes [5]. Therefore, it is possible that a subpopulation of HCV NS proteins reside on the ER-associated LRLDs through a non-stomatin-mediated mechanism, considering that the P100 fraction of R-1 cells contains little stomatin but detectable levels of NS proteins. However, it is important to point out that we detected little RdRp activity associated with the P100 fraction of the R-1 cells, suggesting that HCV RCs, if there are any, present on the ER or some other membranous structures in this fraction are unlikely to be replication competent. This replication incompetence, in spite of the presence of NS5B RdRp, might be attributed to the differences in the architecture of the RC on the membranous structures. The three-party interactions; (viz., the molecular linkages among the viral NS proteins, the organelle membranes, and the cellular factors associated with the membranes) are likely to be different in the P15 and P100 subcellular fractions in terms of their ability to take up ribonucleotides and other host factors, including the small non-coding RNAs known to play a role in HCV genome replication. Additionally, we cannot rule out the possibility that the structural difference in the RC also affects the vulnerability of the viral RNA genome in the RC. The stomatin interacting with the NS5B in the DRM might be a crucial factor in determining these structural differences in the RC.

Taken together, our results suggest that the recruitment of stomatin to HCV RCs may be an important step in building the RC. Stomatin may help to build an active HCV RC on the DRM through its abilities to form oligomers and interact with NS5B. Stomatin, acting as a proviral host factor, could be useful for the development of specific antiviral therapies for the treatment of HCV infection.

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References


