Morphological and Biochemical Characterization of the Membranous Hepatitis C Virus Replication Compartment

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Like all other positive-strand RNA viruses, hepatitis C virus (HCV) induces rearrangements of intracellular membranes that are thought to serve as a scaffold for the assembly of the viral replicase machinery. The most prominent membranous structures present in HCV-infected cells are double-membrane vesicles (DMVs). However, their composition and role in the HCV replication cycle are poorly understood. To gain further insights into the biochemical properties of HCV-induced membrane alterations, we generated a functional replicon containing a hemagglutinin (HA) affinity tag in nonstructural protein 4B (NS4B), the supposed scaffold protein of the viral replicase complex. By using HA-specific affinity purification we isolated NS4B-containing membranes from stable replicon cells. Complementing biochemical and electron microscopy analyses of purified membranes revealed predominantly DMVs, which contained viral proteins NS3 and NS5A as well as enzymatically active viral replicase capable of de novo synthesis of HCV RNA. In addition to viral factors, co-opted cellular proteins, such as vesicle-associated membrane protein-associated protein A (VAP-A) and VAP-B, that are crucial for viral RNA replication, as well as cholesterol, a major structural lipid of detergent-resistant membranes, are highly enriched in DMVs. Here we describe the first isolation and biochemical characterization of HCV-induced DMVs. The results obtained underline their central role in the HCV replication cycle and suggest that DMVs are sites of viral RNA replication. The experimental approach described here is a powerful tool to more precisely define the molecular composition of membranous replication factories induced by other positive-strand RNA viruses, such as picorna-, arteri- and coronaviruses.

Hepatitis C virus (HCV) is a major human pathogen persistently infecting 130 to 170 million individuals worldwide, thereby increasing the risk for chronic liver diseases, including steatosis, fibrosis, liver cirrhosis, and hepatocellular carcinoma (1). Despite recent advances in the development of promising HCV-specific drugs (2), current therapies suffer from the occurrence of severe side effects and the risk of therapy resistance (3). Thus, more-efficient therapeutic treatments, for which a better understanding of the fundamental principles governing the viral replication cycle is necessary, are required.

HCV is the only member of the genus Hepacivirus within the family Flaviviridae (4). Owing to its high genetic variability, HCV is classified into 7 genotypes and more than 100 subtypes (5). An ~9.6-kb single-strand uncapped RNA molecule of positive polarity constitutes the HCV genome, which contains a single long open reading frame (ORF) that is flanked by 5’ and 3’ untrans- lated regions (UTRs). Both UTRs are highly structured and are implicated in viral RNA replication, while an internal ribosome entry site (IRES) contained in the 5’ UTR mediates translation of the positive-strand RNA viral genome (reviewed in reference 6). Upon translation of the ORF, at least 10 HCV proteins are generated from a polypeptide precursor that is co- and posttranslationally cleaved by cellular and viral proteases (6). The resulting cleavage products are three structural proteins (core, envelope protein 1 [E1], and E2), the viroporin p7, and six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). While p7 and NS2 are required for virus assembly and release (7, 8, 9), NS3 to -5B constitute the minimal viral replicase machinery (10, 11). Indeed, HCV “minigenomes” (termed subgenomic replicons) comprising both UTRs and encoding NS3 to -5B autonomously replicate in cell culture (10, 11). They have been used extensively to study basic principles of HCV replication and to develop directly acting antivirals (DAAs) (12).

Like for all other positive-strand RNA viruses, HCV RNA replication is thought to occur in tight association with remodeled cytoplasmic host cell membranes, which form distinct organelle-like structures designated the membranous web in the case of HCV (13, 14, 15) and “viral replication factories” for many other viruses (reviewed in references 16, 17, and 18). Recent electron tomography studies of infected cells revealed that HCV-induced membrane rearrangements are predominantly vesicular double-membrane protrusions of the endoplasmic reticulum (ER) (19). Such double-membrane vesicles (DMVs) have also been observed in cells containing subgenomic HCV replicon RNA (15, 19, 20). DMV formation is induced by a concerted action of several replicase proteins, with NS4B playing a key role (13, 14, 15, 19, 20). NS4B is a highly hydrophobic protein and is thought to remodel intracellular membranes by self-oligomerization (13, 14, 15; reviewed in reference 21). Notably, replication-impaired NS4B mutants exhibit an altered DMV morphology, suggesting the presence of this viral replicase factor in DMV membranes (15).

A major limitation in our understanding of HCV RNA replication is the lack of knowledge about the molecular composition of DMVs and the nature of the viral replication factor(s) that resides within them. Indeed, the identification of putative virally encoded scaffold proteins of HCV replication factories is hampered by the incomplete knowledge of the viral replicase machinery and the complex nature of DMVs. Here we present an experimental approach that provides insights into the biochemical properties of DMVs and the factors that associate with them. We describe the first isolation and biochemical characterization of HCV-induced DMVs, which may represent sites of viral RNA replication. This work defines the molecular composition of DMVs and offers a powerful tool to study the function of HCV replication factors and to better understand the process of viral RNA replication.
of the membranous replication compartment. In this study we developed an affinity purification method and present a detailed characterization of HCV replicase-containing membranes. We demonstrate that DMVs are associated with replicase activity and represent distinct virus-induced membranous compartments. Our method overcomes a major restriction and likely is applicable to the study of the membranous replication compartments of other positive-strand RNA viruses.

**MATERIALS AND METHODS**

**Antibodies.** Mouse monoclonal antibody against NS3 of the JFH1 isolate (2E3) was from BioFront Technologies (Tallahassee, FL). Mouse monoclonal antibody 9E10 recognizing NS5A domain III of the HCV isolates Con1 and JFH1 was a kind gift from Charles M. Rice. Rabbit polyclonal antibody raised against NS4B of Con1 and cross-reacting with NS4B of JFH1 was generated by the immunization of rabbits with recombinant hexahistidine-tagged NS4B produced in a baculovirus expression system in insect cells and affinity purified via Ni²⁺-nitrilotriacetic acid spin columns (Qiagen, Hilden, Germany) as described elsewhere (22). Rabbit polyclonal antibody against human calnexin (CANX; ADI-SPA-865) was obtained from Enzo Life Sciences (Memphis, TN). Rabbit polyclonal antibodies against human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), J2 mouse monoclonal antibody recognizing double-stranded RNA (dsRNA) was purchased from English & Scientific Consulting (Szirák, Hungary), and rat antibromodesoxyuridine (BrDU) antibody (M01269M), which cross-reacts with bromouridine (BrU), was obtained from Meridian Diagnostics, Uppsala, Sweden.

**Cell culture.** Huh7-Lunet cells (23) and Huh7.5 cells (24) were maintained in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, Carlsruhe, Germany) supplemented with 2 mM t-glutamine, nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (DMEM cplt). G418 (Geneticin; Invitrogen) was added at 400 μg/ml. For transduction experiments, Uppsala, Sweden.

**In vitro transcription and RNA transfection.** In vitro transcripts from given HCV plasmids were generated and purified and transfected into human hepatoma cells as described previously (15).

**Nuclease and protease protection assay.** In order to determine nuclelease protection of HCV RNA in purified membranous material, we subjected equal aliquots to nuclease (1 U/μl benzonase), protease (8 mg/ml proteinase K), and detergent (1% Triton X-100) treatment for 1 h at 25°C. HCV RNA was extracted and quantified as described below.

**Purification of HCV-resembled and control ER membranes.** Huh7-Lunet cells (7.5 × 10⁶) containing subgenomic replicon RNA encoding either wild-type (wt) or HA-tagged NS4B and control Huh7-Lunet cells...
stably overexpressing CANXHA were washed 3 times, scraped into PBS, and pelleted by centrifugation at 800 \times g for 10 min at RT. Cells were resuspended in 2 ml hypotonic buffer (20 mM Tris [pH 8], 1.5 mM MgCl₂, 10 mM Na-acetate) and incubated on ice for 30 min. Cells were lysed by 50 strokes with a dounce homogenizer, and nuclei and unbroken cells were removed by centrifugation at 800 \times g for 10 min at 4°C. Post-nuclear supernatants were layered on top of a discontinuous (70%) to (30%) sucrose gradient and centrifuged for 4 h at 130,000 \times g using a SW40 rotor (Beckman Coulter, Fullerton, CA). Thirteen fractions (1 ml each) were collected from the bottom and analyzed for density and for protein and HCV RNA content. For HA affinity capture, equal amounts (~1 mg protein) contained in pooled fractions 7 to 11 were equilibrated to 150 mM NaCl. Incubation with HA-agarose beads (Sigma-Aldrich, St. Louis, MO) was carried out for 4 h by continuously inverting tubes at 4°C. Beads were washed 3 times with 40 volumes of IP buffer (20 mM Tris [pH 8], 1.5 mM MgCl₂, 10 mM Na-acetate, 150 mM NaCl). Bound material was eluted by incubation with 100 μl IP buffer containing 250 μg/ml HA peptide (Sigma-Aldrich) 2 times, each for 15 min at RT. Elution fractions were further analyzed as described in the Results section.

Quantification of HCV RNA. Prior to quantification, RNA was extracted using a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. Five microliters of purified RNA was used for quantitative reverse transcription-PCR analysis using an ABI Prism 7000 sequence detector system (Applied Biosystems, Foster City, CA). HCV JFH1-specific reverse transcription-PCRs were conducted in triplicate using OneStep RT-PCR kits (Qiagen, Hilden, Germany), primers S-146 (5'-CTTCGCGAAACGGTGACT-3') and A-219 (5'-GGGGATCTGTTGGTATTCC-3'), and the probe 5'-6-carboxytetramethylrhodamine (TAMRA)-3' and 6FAM)-AAAGGACCCAGTCTTCCCGGCAATT-6'-carboxyfluorescein (FAM). Five microliters of total RNA served as the template for cDNA synthesis using the Expand long template PCR system (Roche, Mannheim, Germany). After digestion with NS1 and RsrII, amplicons were inserted into pFK_i389LucNS3-3'NsiI and RsrII, and cloned amplicons were analyzed by using the Expand long template PCR system (Roche, Mannheim, Germany) according to the instructions of the manufacturer. Five micrograms of total RNA served as the template for cDNA synthesis using oligonucleotide A/2A/9842 (5'-GGGACAGTTGATCATGGGATTCCC-3') and SuperScript III (Invitrogen) as specified by the manufacturer. A region spanning NS5 to NS5A was amplified with oligo (r_NS3_Nsil) (5'-TTACAAATGGGATCCCTACAG-3') and oligo (r_NS5A_Rsil) (5'-AGGGCGAACCTCTTCAGG-3') using the Expand long template PCR system (Roche, Mannheim, Germany). After digestion with NsiI and RsrII, amplicons were inserted into pFK_j389LucNS5_3'JFH bg and at least two DNA clones per cell clone were sequenced to confirm the presence of the inserted affinity tag and to identify conserved pseudoreversions.

Statistical analysis. Statistical analyses were performed as specified in the figure legends, and significance values were calculated by applying the two-tailed, unpaired Student’s t test available in the GraphPad Prism 5 software package (GraphPad Software, Inc., La Jolla, CA).

Transient replication and infectivity assays. Transient HCV RNA replication and the kinetics of viral progeny release were determined by measuring luciferase activity in cell lysates and by limiting dilution assay, respectively, as described recently (15).

RESULTS

Establishment of a functional HCV replicon encoding an affinity-tagged NS4B. Since NS4B is thought to be the scaffold protein of the HCV replicase complex and a key player in the induction of membrane rearrangements, we wanted to insert an affinity tag into this protein and express it in the context of a functional HCV replicon (Fig. 1A) to allow the isolation of membrane-associated viral replication factories. We inserted the HA or FLAG tag in frame after Ala-38, Gly-131, Leu-140, or Ile-258 of NS4B (Fig. 1B), which correspond to positions that previously have been shown to tolerate the insertion of a short heterologous sequence (29). As shown in Fig. 1C, all chosen insertions into NS4B severely diminished or completely abolished transient replication of a subgenomic JFH1 replicon (Fig. 1C). Only replicons containing the tag after Ala-38 still replicated to a detectable level, whereas the insertions at the other sites completely abrogated HCV RNA replication, as deduced from comparison with the nonrepetitive ΔGDD polymerase mutant. To improve the replication competence of these RNAs, we inserted tagged NS4B sequences into a selectable subgenomic replicon and passaged transfected cells under G418 selection for ~4 weeks (Table 1). In this way, we aimed to select for second-site compensatory mutations (pseudover-
sions) enhancing RNA replication. Indeed, we obtained single-cell clones for several NS4B tag insertion constructs (Table 1). By far the highest number of G418-resistant colonies was obtained with the HA tag insertion mutant after Ala-38, whereas no or only very few cell clones were obtained with all the other mutants. To confirm the presence of the inserted affinity tag and to identify pseudoreversions in selected replicons, for each given cell clone we determined the predominant nucleotide sequence of the NS3- to

FIG 1 HCV tolerates an HA tag insertion into the N-terminal region of NS4B. (A) Schematic representation of the bicistronic HCV subgenomic reporter replicon. Firefly luciferase (Fluc) or neomycin-phosphotransferase (neoR) is expressed as an N-terminal fusion with 16 amino acids of the N-terminal region of the core protein (black line) and is translated under the control of the HCV IRES contained in the 5′ UTR. The second cistron (NS3 to NS5B) is translated via the IRES of the encephalomyocarditis virus (EMCV-IRES). (B) Predicted NS4B membrane topology. N-terminal amphipathic α-helices AH1 and AH2, the four transmembrane segments (TM1-4), and the C-terminal α-helices H1 and H2 are schematically depicted. Numbers indicate amino acid positions of the JFH1 isolate. Affinity-tag insertion sites are highlighted by black arrows. (C) Huh7-Lunet cells were transfected with the in vitro-transcribed luciferase replicon RNAs specified at the bottom. Cells were lysed 4, 24, 48, and 72 h after transfection, and the luciferase activity in cell lysates was determined. Data were normalized to the 4-h value that reflects transfection efficiency. The background of the assay is determined by the NS5B active-site polymerase mutant (ΔGDD) (dashed line). Mean values of two independent experiments are shown. Error bars indicate standard deviations. (D) Huh7-Lunet cells were transfected with the in vitro-transcribed luciferase replicon RNAs specified at the bottom. Replication efficiency was determined as described for panel C. (E) Release kinetics of infectious HCV particles. Huh7-Lunet cells were transfected with the full-length HCV RNAs specified on the right. Culture supernatants were harvested at the given time points. Infectivity titers were determined by limiting-dilution assay and are expressed as 50% tissue culture infective doses (TCID₅₀)/ml. Mean values of two independent experiments are shown; error bars indicate standard deviations.
TABLE 1 Long-term selection assay of NS4B-tagged insertion constructs

<table>
<thead>
<tr>
<th>Affinity tag</th>
<th>Insertion site</th>
<th>No. of colonies</th>
<th>Pseudoreversions</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>38</td>
<td>~100</td>
<td>Q11R+D34A, Q31R, Q117R, Q117R</td>
</tr>
<tr>
<td>FLAG</td>
<td>38</td>
<td>1</td>
<td>KDDDD deletion in FLAG sequence</td>
</tr>
<tr>
<td>HA</td>
<td>131</td>
<td>3</td>
<td>PYDV deletion in HA sequence</td>
</tr>
<tr>
<td>FLAG</td>
<td>131</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>HA</td>
<td>140</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>FLAG</td>
<td>258</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>FLAG</td>
<td>258</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

* The affinity tag given in the first column was inserted in frame after the given NS4B amino acid residues.

** Pseudoreversions detected in replicons that were isolated from individual cell clones were identified by sequence analysis of amplicons spanning the NS3- to NS5A-coding region.

NS5A-coding region of the replicon contained therein. While the FLAG and HA epitopes inserted after Ala-38 and Gly-131, respectively, were deleted, the HA tag-encoding sequence inserted after Ala-38 was retained (Fig. 1C). In the case of the latter, we additionally identified four conserved mutations, all residing in the N-terminal half of NS4B (Table 1). Next we determined whether any of these second-site mutations enhanced the replication capacity of the primary sg4BHA mutant by inserting them individually or in combination into this mutant construct. Single mutations Q31R and Q117R, a combination thereof, and the double mutation Q11R plus D34A all rescued RNA replication of the NS4B alamine 38 HA tag insertion construct (Fig. 1D). Although delayed replication kinetics, which is most visible for the 24-h values, were observed, replication efficiencies at later time points were comparable to that of the wt replicon (Fig. 1D). Owing to its robust replication capacity and the requirement for only one adaptive mutation, the 4B-38HA mutant containing the Q31R pseudoreversion (construct sg4BHA31R) was used for all subsequent experiments. In order to confirm that this mutated NS4B supported the complete HCV replication cycle, we inserted it into the highly assembly-competent Jc1 full-length genome (27) and determined the release kinetics of infectious HCV progeny by limiting-dilution assay (Fig. 1E). The wt genome and a mutant that due to a deletion in the E1/E2-coding region was unable to produce infectious virus particles served as positive and negative controls, respectively. Considerably fewer infectious particles were released from cells transfected with the NS4B mutant at all time points (Fig. 1E), reflecting delayed RNA replication kinetics of Jc1-4BHA31R and, consistent with earlier reports, indicating a role of NS4B in virus production (15, 30). Nevertheless, a 30-fold increase in viral progeny release from transfected cells was detectable between 24 and 48 h after transfection, demonstrating that the HA-tagged version of NS4B supported the complete HCV replication cycle.

Characterization of HA-tagged NS4B and induced membranous morphotypes. Next we characterized the 4BHA31R mutant by using Western blotting, immunofluorescence, and electron microscopy (EM) analyses. In lysates of cells transfected with the subgenomic replicon sg4BHA31R and in cells infected with the Jc1-4BHA31R virus, NS4BHA was readily detectable via the HA tag (Fig. 2A and B, respectively). As expected, the HA tag slightly increased the apparent molecular weight of the NS4B protein. In replicon cells, the abundance of 4BHA31R was reduced concomitant with a reduction in NS5A steady-state levels, presumably as a result of delayed replication kinetics (Fig. 2A). Concurrently, in cells infected with Jc1, 4BHA31R and NS5A steady-state protein levels were reduced, reflecting delayed replication kinetics and reduced virus progeny production (Fig. 2B).

Immunofluorescence (IF) analyses of cells transfected with replicon RNA revealed NS4B-induced membrane-associated foci (MAF) (31, 32) that were detected with NS4B- and HA-specific antibodies (Fig. 2C). Their numbers did not differ considerably between wt and NS4BHA-replicating cells. This result confirmed HA tag accessibility and demonstrated that NS4B-induced MAF remained unaltered by insertion of the HA tag. Furthermore, the ability of the sg4BHA31R replicon to induce DMVs, the most prominent membrane alteration observed in cells containing replicating HCV RNA, was verified by TEM analysis (Fig. 2D).

For subsequent membrane purification assays, we additionally established a control cell line stably overexpressing the ER chaperone calnexin that was C-terminally fused to the HA-tag (CANXHA). Calnexin is a type I membrane protein with its ectodomain localizing to the ER lumen and a short cytoplasmic tail to which the HA tag was fused. We chose this protein because HCV-induced membrane alterations are ER derived, thus allowing the purification of control ER membranes. IF analysis confirmed recognition of CANXHA with the HA-specific antibody as well as unaltered ER morphology in cells overexpressing this protein (Fig. 2E).

Purification of NS4B-associated membranes from replicon-containing cells. Having established a cell line with the functional sg4BHA31R replicon and the CANXHA control cell line, we isolated intracellular membranes by using a combination of membrane enrichment via density gradient centrifugation and subsequent HA-affinity capture as schematically depicted in Fig. 3A. Cells containing an analogous subgenomic replicon without the HA tag served as an additional specificity control. First, we analyzed fractions after ultracentrifugation for the presence of HCV proteins and RNA. The majority of viral RNA (Fig. 3B) and proteins (Fig. 3C) was broadly distributed in fractions 7 to 11 (corresponding to densities 1.18 to 1.06 g/cm3), which also contained the ER marker calnexin. These fractions were well-separated from soluble cytosolic proteins, as exemplified with GAPDH. Moreover, gradient distribution of calnexin remained unaltered in the case of cells overexpressing CANXHA. Thus, we pooled fractions 7 to 11 of replicon or CANXHA control lysates and subjected them to HA-affinity capture. After elution under native conditions with the HA peptide, eluates were examined for HCV protein and RNA content. Western blot analysis revealed specific copurification of NS3 and NS5A along with HA-tagged NS4B (Fig. 3D). We also detected CANX in the NS4BHA-specific pull-down assay, corroborating the ER origin of HCV protein-containing membranes. Importantly, HCV RNA was significantly enriched in NS4BHA-captured material compared to that in the untagged negative control, even though slightly higher HCV RNA amounts, presumably reflecting a higher replication efficiency, were detected in the input for the untagged negative control (Fig. 3E).
FIG 2 Characterization of HA-tagged NS4B. (A) Huh7-Lunet cells were transfected with the \textit{in vitro} transcribed luciferase replicon RNAs specified at the top. Cells were harvested 72 h posttransfection and analyzed by immunoblotting, using the monospecific antibodies indicated on the right. The positions of molecular weight (MW; in thousands) markers are depicted on the left. (B) Huh7.5 cells were infected with the culture supernatants of cells transfected with the Jc1-derived full-length constructs specified at the top. After 72 h, cells were harvested and processed as described for panel A. (C) Huh7-Lunet cells were transfected with the \textit{in vitro} transcribed luciferase replicon RNAs specified at the top of each panel. After 48 h, cells were fixed, permeabilized with digitonin, and stained with NS4B- and HA-specific antibodies prior to confocal immunofluorescence microscopy. Only merged images are shown. Scale bars represent 5 μm. Numbers below each panel indicate the mean ± the standard deviation (SD) for NS4B-containing MAF per cell; for each condition, at least 10 different HCV-positive cells were analyzed. (D) Huh7-Lunet cells were transfected with \textit{in vitro} transcribed luciferase replicon RNAs as specified at the top of each panel. After 48 h, cells were fixed and flat embedded for TEM analysis. Scale bars represent 100 nm. (E) Naive Huh7-Lunet cells and those overexpressing CANXHA were fixed, permeabilized with Triton X-100, and stained with CANX- and HA-specific antibodies prior to confocal immunofluorescence microscopy. Only merged images are shown. Scale bars represent 5 μm.
nous structures, corresponding predominantly to spherical vesicles with a diameter of ~50 to 250 nm, were present in the NS4BHA-purified fraction (Fig. 4Ac and d, C, and 4Dd to f). Two mostly concentric membranes, reminiscent in size and morphology of DMVs detected in HCV-replicating cells (Fig. 2D), were clearly discernible for many of these structures. The numbers of membranous structures in NS4BHA preparations were lower than those in CANXHA control samples (Fig. 4B), but this was due to large amounts of overexpressed CANXHA (data not shown). Importantly, quantification of total membranous structures per area revealed significant enrichment in the NS4BHA-containing preparation compared to that in the untagged wt NS4B control (Fig. 4B). Moreover, classification of membrane structures according to their morphology and size revealed distinct differences for CANXHA- and NS4BHA-captured membranes (Fig. 4C), suggesting that for the latter mainly HCV-remodeled membranes had been purified.

In order to investigate the viral constituents of DMVs in more detail, we performed immunolabeling experiments for NS3 and NS5A as well as for dsRNA, which is a commonly accepted marker for viral RNA replication intermediates. Owing to the low number of membranous structures present after immunoprecipitation (IP) of the untagged wt NS4B control (Fig. 4B), we used membranes that were isolated by HA-specific affinity purification from HCV-naive CANXHA-expressing cells as the negative control for this and subsequent assays. A major portion of DMVs in the

FIG 3 Purification and biochemical characterization of NS4BHA-associated membranes. (A) Schematic overview of HCV membrane preparation. Cells containing a stably replicating wild-type replicon (HCV wt), the replicon sg4BHA31R (HCV 4BHA), and control cells stably overexpressing CANXHA were broken by hypotonic lysis. Postnuclear cytosolic supernatants were separated by discontinuous sucrose gradient ultracentrifugation (UC). Subsequently, membrane fractions were pooled and subjected to affinity capture using HA beads. After elution with the HA peptide, purified material was used for further analyses. (B) Distribution of HCV RNA (right y axis, solid lines) and corresponding density of each fraction (left y axis, dashed lines) along the gradient. Mean values and error bars indicating the standard deviations of three independent measurements are depicted. (C) Fractions analyzed for their protein content are shown for HCV NS4BHA. Proteins were separated by SDS-PAGE, and monospecific antibodies were used to detect calnexin (CANX), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), NS3, NS5A, and NS4B, as indicated on the right. The positions of molecular weight (MW) markers are depicted on the left. (D) Protein content after HA-specific affinity capture. Samples were analyzed as described for panel C. (E) HCV RNA content in purified material. Mean values with standard deviations, as indicated by the error bars, from at least three independent experiments, with three measurements each, are given. No HCV RNA was detected in CANXHA samples, as indicated by the inverted triangles (▼). *** P < 0.0001.

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NS4B<sup>HA</sup> elution fraction was found to be decorated with gold particles after immunolabeling (Fig. 4D to f), and 48%, 59%, and 63% of DMVs were positive for NS3, NS5A, and dsRNA, respectively. Only background labeling was detected on naive ER membranes (Fig. 4D to c). The specificity of immunolabeling was confirmed by quantification of gold particles on membranes, which revealed a considerably higher level of labeling for all tested antibodies on NS4B<sup>HA</sup>-containing membranes (Fig. 4E). Taken together, these results demonstrate that besides NS4B<sup>HA</sup>, NS3 and NS5A, as well dsRNA, are associated with HCV-induced DMVs, suggesting that DMVs might harbor viral replicase complexes.

HCV RNA in NS4B<sup>HA</sup> preparations resides in a nuclease-protected environment and is replication competent. With the aim to more precisely determine the localization of replicating HCV RNA, we exploited the possibility of isolating HCV-induced membranes by NS4B<sup>HA</sup>-specific affinity purification. Viral replicon RNA copurified with the membranes was resistant to nuclease and protease treatment but was destabilized upon the addition of detergent (Fig. 5). The combination of nuclease and detergent treatments led to complete degradation of HCV RNA, arguing for its localization in a membrane-protected environment. This observation is in good agreement with previous publications de-
of unspecifically captured HCV RNA present in the preparation translated NS4BHA-associated membranes contained functional HCV bated with rNTPs, arguing for \(-^32\text{P}]\text{CTP}\) into nascent viral RNA confirmed that iso-
to corroborate this assumption, we performed metabolic radio-
reactions using CANXHA membrane preparations that were pro-
replicase. This signal was specific, because it was not detected in
experiments are depicted. ns, error bars indicating the standard deviations for at least three independent
1 h at 25°C as indicated at the bottom. Subsequently, HCV RNA was extracted
(competitively), and DMVs were immunolabeled after
by using immunogold labeling of purified HCV-induced membranes and TEM. Membrane structures were double labeled for the viral replicase protein NS5A in order to unambiguously identify DMVs and additionally for VAP-A or VAP-B. While only a low level of the background label of NS5A (15-nm colloidal gold) was observed on ER control membranes (Fig. 7Aa and b), cellular proteins VAP-A and -B (10-nm colloidal gold) were readily detected. Importantly, HCV-induced DMVs were labeled intensively with NS5A and with VAP-A and VAP-B (Fig. 7Ac and d). Interestingly, 10- and 15-nm colloidal gold, corresponding to VAP-A or -B and NS5A, respectively, were frequently found next to each other (Fig. 7Ac and d), supporting earlier reports that these proteins interact with each other (37, 38, 39). Quantification of gold particles on membranes (Fig. 7B) confirmed the specificity of detection of NS5A and excluded cross-reactivity of used antibodies. Moreover, quantitative analysis of differently sized gold particles on membranes revealed that most NS5A-positive membranous structures from the NS4BHA capture also contained VAP-A or -B, while ER control membranes almost exclusively exhibited single labeling for VAP-A or -B (Fig. 7C). Finally, we calculated the number of 10-nm gold particles per micrometer of membrane of NS5A-positive structures in order to explore whether VAP-A and -B would be locally concentrated on HCV-induced DMVs. The results revealed an ~2-fold enrichment of both cellular proteins on HCV-induced DMVs compared to that on ER control membranes (Fig. 7D).

**Cholesterol is highly enriched in HCV-induced DMVs.** Apart from cellular proteins, specific host cell lipids likely contribute to HCV RNA replication and remodeling of intracellular membranes (reviewed in reference 40). For instance, HCV nonstructural proteins appear to reside in detergent-resistant membranes (DRMs) (41). Although by analogy to lipid rafts one might assume that HCV-induced DMVs are enriched for sphingolipids and cholesterol, their lipid composition is unknown. Hence we sought to determine if cholesterol is present in DMV membranes, resembling described DRM properties, by using NS4BHA-purified material. For this we employed biotinylated and noncytolytic P-O that has been developed to specifically detect cholesterol in cellular membranes by EM (28). Purified membranes were double la-

![FIG 5 HCV RNA associated with affinity-purified membranes is protected against nuclease. Equal aliquots of NS4BHA-purified and unspecifically bound (NS4B wt) material were subjected to treatment with nuclease (1 U/µl Benzonase), protease (8 mg/ml protease K), and detergent (1% Triton X-100) for 1 h at 25°C as indicated at the bottom. Subsequently, HCV RNA was extracted and quantified by quantitative reverse transcription-PCR. Mean values and error bars indicating the standard deviations for at least three independent experiments are depicted. ns, \(P > 0.4\); ***, \(P < 0.0003\).](http://jvi.asm.org/)

scribing protection of HCV RNA in enriched membrane fractions that were isolated from cells containing a stably replicating genotype 1 subgenomic RNA (33, 34, 35). Likewise, the small amounts of unspecifically captured HCV RNA present in the preparation with the nontagged NS4B were also nuclease resistant (Fig. 5).

Next we determined *in vitro* replicase activity in NS4BHA-purified membranes. Equal aliquots of elution fractions were incubated in the absence or presence of ribonucleotides (rNTPs), and HCV RNA was quantified by quantitative reverse transcription-PCR. A small but significant increase in the HCV RNA copy number was detected when NS4BHA-purified membranes were incubated with rNTPs, arguing for *in vitro* replicase activity (Fig. 6A).

To corroborate this assumption, we performed metabolic radio-labeling of the viral subgenomic RNA (Fig. 6B). The incorporation of \([\alpha-^32\text{P}]\text{CTP}\) into nascent viral RNA confirmed that isolated NS4BHA-associated membranes contained functional HCV replicase. This signal was specific, because it was not detected in reactions using CANXHA membrane preparations that were processed in parallel.

Taking advantage of purified HCV-induced membranes containing functional viral replicase, we next identified the sites of HCV RNA replication. NS4BHA-associated membranes were subjected to *in vitro* replicase assay using 5-bromouridine-5'-triphosphate (BrUTP), which was subsequently detected by immunogold labeling and TEM. Only background labeling of membranes was detected with CANXHA, which was subsequently detected by immunogold labeling and TEM. Membrane structures were double labeled against nuclease. Equal aliquots of NS4BHA-purified and unspecifically bound (NS4B wt) material were subjected to treatment with nuclease (1 U/µl Benzonase), protease (8 mg/ml protease K), and detergent (1% Triton X-100) for 1 h at 25°C as indicated at the bottom. Subsequently, HCV RNA was extracted and quantified by quantitative reverse transcription-PCR. Mean values and error bars indicating the standard deviations for at least three independent experiments are depicted. ns, \(P > 0.4\); ***, \(P < 0.0003\).
beled for cholesterol using P-O and for NS5A as a marker of DMVs, and membranous structures were examined by TEM. CANXHA-purified ER control membranes were only sporadically decorated with gold particles after P-O immunolabeling, indicating an overall low abundance of cholesterol in ER membranes (Fig. 8Aa). Interestingly, some mostly vesicular structures in the CANXHA preparation showed higher labeling densities, possibly representing cholesterol-rich domains (Fig. 8Ab). By contrast, NS5A-positive DMVs were heavily labeled for cholesterol, arguing for massive accumulation of this lipid in DMV membranes (Fig. 8Ac and d). Quantification of 10- and 15-nm colloidal gold particles present on membranes per area of the grid showed explicit tagging of cholesterol and NS5A and excluded cross-reactivity of used antibodies (Fig. 8B). Determination of cholesterol label density revealed a significant, ~9-fold enrichment of cholesterol in HCV-remodeled membranes compared to that in control ER membranes, with 3.5 ± 1.1 and 29.6 ± 2.6 (means ± standard error of the means [SEMs]) gold particles per micrometer of ER control and HCV DMV membranes, respectively (Fig. 8C). Labeling density was significantly reduced upon cholesterol depletion using methyl-β-cyclodextrin (MβCD) treatment, thus confirming the specificity of our labeling method (Fig. 8D and E).

FIG 6 HCV-induced double-membrane vesicles are sites of RNA replication. (A) Equal fractions of NS4BHA-purified material were incubated without (−) or with (+) exogenously added ribonucleotides in an in vitro replicase assay as specified at the bottom. Total HCV RNA was quantified before and after the in vitro replicase assay and is represented as the fold increase. Mean values and error bars representing standard deviations from six replicates in two independent experiments are shown. **, *P < 0.001. (B) Equal amounts of control CANXHA and NS4BHA-purified membranes were subjected to an in vitro replicase assay in the absence (−) or presence (+) of radioactively labeled [α-32P]CTP. After RNA purification, samples were analyzed by denaturing glyoxal agarose gel electrophoresis and autoradiography. (C) Membranes from the NS4BHA purification were used for in vitro replicase assay in the absence (−) or presence (+) of BrUTP. After immunolabeling with a BrdU–specific antibody and subsequent negative staining, samples were examined by TEM. White arrowheads show the locations of gold particles. The number below each panel indicates the percentage of non-specifically gold-labeled DMVs (a) or of DMVs with gold labeling on the exterior (b) or apparent in the interior (c) (n > 75). The scale bar in each panel corresponds to 100 nm. (D) Quantification of immunolabeling. At least 100 gold particles were counted, and the fraction of gold particles with respect to their location per area is depicted.
In order to investigate a functional role of cholesterol in HCV-remodeled membranes, we depleted it from DMV membranes by MβCD treatment and quantified HCV RNA copies as a measure of DMV integrity. The HCV RNA amount was reduced 2-fold (Fig. 8G), indicating a structural function of cholesterol for DMV and thus for HCV RNA stability. However, nuclease treatment of cholesterol-depleted membranes did not further decrease HCV RNA amounts, as seen in the case of detergent-only or detergent-plus-nuclease treatment (Fig. 8G), thus excluding a general membrane solubilization by MβCD. Indeed, when examined by TEM, MβCD-treated NS4BHA-captured membranes still exhibited a vesicular morphology (Fig. 8D). However, vesicle size was significantly decreased for cholesterol-depleted membranes, with a mean diameter of 121 ± 5.1 nm compared to 183 ± 5.5 nm (means ± SEMs) in the untreated control (Fig. 8F). These results emphasize the role of cholesterol as a structural component of DMV membranes and demonstrate a distinct lipid composition for HCV-induced DMVs.

DISCUSSION

An important role of HCV-induced DMVs in viral replication has been suggested by several studies (15, 19, 20, 42). However, viral and cellular constituents of DMVs, as well as their precise function in RNA replication, remain enigmatic. Here we report a functional HA-tagged version of NS4B that allows purification of HCV-remodeled membranes and their biochemical and morphological characterization. Our results provide evidence that HCV-induced DMVs harbor active viral replicate complexes and represent specialized membranous structures composed of distinct cellular protein and lipid constituents.

HCV replication is only moderately affected by the insertion of an HA affinity tag in NS4B. Epitope tagging is a commonly
FIG 8 DMV membranes contain large amounts of cholesterol. (A) Purified ER control (a and b) and HCV-remodeled membranes (c and d) were consecutively labeled for cholesterol using biotinylated perfringolysin O (10-nm gold) and for NS5A (15-nm gold). After negative staining, they were examined by TEM. Scale bars correspond to 100 nm. (B) Quantification of gold particles on membranes. At least 100 gold particles were counted, and the number of gold particles associated with membranes per area of the grid is depicted. (C) Amount of 10-nm colloidal gold particles per micrometer of membrane length of a given structure. Only membranes also positive for NS5A were considered for the analysis of NS4BHA-captured membranes. The analysis is based on at least 75 membranous structures with a total membrane length of more than 60 μm for each condition. Horizontal lines indicate mean values. ***, P < 0.0001. (D) Purified ER control (CANXHA) and HCV-remodeled membranes (NS4BHA) were treated with 10 mM MβCD for 1 h at 25°C as indicated, subsequently labeled for cholesterol using biotinylated perfringolysin O, and after negative staining examined by TEM. Scale bars correspond to 100 nm. (E) Amount of 10-nm colloidal gold particles per micrometer of membrane length of a given structure. The analysis is based on at least 50 membranous structures with a total membrane length of more than 50 μm for each condition. Horizontal lines indicate mean values. ***, P < 0.0001. (F) DMV diameters after MβCD treatment. Membranes from the NS4BHA capture were MβCD treated as described for panel D, and DMV diameters were measured. Horizontal lines indicate mean values. n > 100; ***, P < 0.0001. (G) Equal aliquots of NS4BHA-purified material were subjected to treatment with methyl-β-cyclodextrin (MβCD; 10 mM), nuclease (1 U/μl Benzonase), and/or detergent (1% Triton X-100) for 1 h at 25°C as indicated at the bottom. HCV RNA was extracted and quantified by reverse transcription-quantitative PCR. Mean values and error bars indicating the standard deviations for three independent experiments are depicted. #, P > 0.05; ***, P < 0.0001.
used method to allow purification of proteins with high efficiency. Although it has been used with success in the case of HCV (for examples, see references 43, 44, and 45), the main problem is to identify a site that tolerates the insertion of a heterologous sequence without affecting the functionality of the viral protein. In this respect, NS4B has proven to be very sensitive to sequence alterations, since the exchange of single amino acid residues often exhibits dramatic effects on viral RNA replication (14, 15, 30, 46). Thus, we based our initial analysis on sites within NS4B that previously were shown to tolerate the insertion of a short sequence (29) and inserted the FLAG or HA tag. While most of our insertion constructs were not viable, an NS4B containing an HA tag insertion after Ala-38 still supported HCV RNA replication when at least one additional mutation was present. Interestingly, these pseudoreversions eliminated a negative charge (D34A) or introduced an additional positive charge (Q11R, Q31R, Q117R) (Table 1), indicating compensation of the negative charge resulting from two aspartic acid residues within the HA-coding sequence. This might also explain why four of the five aspartic acid residues within the FLAG sequence were deleted after adaptation of the subgenomic replicon containing the FLAG-coding sequence after Ala-38 (Table 1). Hence, the introduction of negatively charged amino acids by an affinity tag into the NS4B N-terminal domain might interfere with its reported membrane association (47, 48) due to electrostatic repulsion with the negatively charged head groups of membrane phospholipids. Additionally, posttranslational membrane translocation of the NS4B N-terminal domain into the ER lumen (48, 49) could be inhibited. Nonetheless, NS4B^{HA}31R supported all NS4B-associated functions in the viral RNA we demonstrated that DMVs are sites of active HCV RNA replication when at least one additional mutation was present. Interestingly, these pseudoreversions eliminated a negative charge (D34A) or introduced an additional positive charge (Q11R, Q31R, Q117R) (Table 1), indicating compensation of the negative charge resulting from two aspartic acid residues within the HA-coding sequence. This might also explain why four of the five aspartic acid residues within the FLAG sequence were deleted after adaptation of the subgenomic replicon containing the FLAG-coding sequence after Ala-38 (Table 1). Hence, the introduction of negatively charged amino acids by an affinity tag into the NS4B N-terminal domain might interfere with its reported membrane association (47, 48) due to electrostatic repulsion with the negatively charged head groups of membrane phospholipids. Additionally, posttranslational membrane translocation of the NS4B N-terminal domain into the ER lumen (48, 49) could be inhibited. Nonetheless, NS4B^{HA}31R supported all NS4B-associated functions in the viral replication cycle, at least those required in cell culture (Fig. 1D and E and 2A to C). Thus, the advantages of the HA-tagged NS4B for specifically purifying HCV-remodeled membranous structures greatly outweigh the drawback of delayed replication kinetics for at least two reasons: first, because highly specific and efficient HA purification overcomes the inherent limitations of antibodies directly targeting viral proteins and, second, because the possibility of elution of immunocaptured samples with the HA peptide, i.e., under native conditions, is even more important and key to the success of the present study, which would not have been possible with an antibody directly targeting the NS4B protein.

Double-membrane vesicles contain active HCV replicase. Despite detailed knowledge about the three-dimensional architecture of DMVs in HCV-infected cells (19), determination of their viral and cellular constituents and their exact role in the viral replication cycle is lacking. This is due to difficulties in producing biochemical quantities of highly purified HCV-remodeled membranes. We have overcome this problem and provide conclusive evidence that DMVs contain the viral replicase machinery. First, we showed that replicase proteins NS3 and NS5A, as well as dsRNA, are associated with purified DMVs. Second, we showed that NS4B^{HA}83A8 categorically contains active viral replicase complexes. Third, by using metabolic labeling of nascent viral RNA we demonstrated that DMVs are sites of active HCV RNA replication. The latter result is of high importance, as detection of metabolically labeled viral RNA is the only reliable method of identifying viral replication sites. In situ hybridization or detection of viral RNA by dsRNA-specific antibodies does not discriminate between actively replicating RNA and RNA engaged in other reactions, such as translation or virion formation. In fact, the dsRNA-specific antibody can also bind to dsRNA structures present in the genomes of positive-strand RNA viruses, thus excluding specificity for detection of active viral RNA replication sites and explaining the disconnect between the signals of newly synthesized RNA and dsRNA described, e.g., for coronaviruses (50). BrUTP labeling in combination with TEM overcomes these limitations and has also been successfully applied to DMV replication factories of arteriviruses (51) and coronaviruses (52) and to spherule-type replication factories of rubella virus (53), alphaviruses (54), flock house virus (55), and brome mosaic virus (56).

Based on our findings, we propose a model of the membrane-associated HCV replicase complex (Fig. 9). DMVs are formed by an as-yet-unknown mechanism that requires the concerted action of several HCV replicase proteins (15, 19). Our data show that these DMVs contain viral replicase proteins NS3, -4B, and -5A, as well as viral RNA and replicase activity, thus representing a site of viral RNA storage and amplification (Fig. 9). It is not yet clear whether HCV RNA replication occurs primarily on the outer DMV membrane facing the cytoplasm as proposed for picornaviruses (57, 58) or whether it takes place mainly in the DMV lumen as suggested for coronaviruses (51, 52) or both (Fig. 9). Detection of incorporated BrU within the lumen of DMVs requires accessibility to relatively large antibodies and protein A-conjugated 10-nm colloidal gold particles, which might not be possible for the majority of DMVs that exhibit “sealed” inner and outer membranes (19). Alternatively, a subset of DMVs with apparent membrane discontinuity stained positive for BrU in their interiors, but we cannot determine whether those DMVs correspond to a subset of not yet fully closed structures as observed in HCV-infected cells (19) or whether membrane alterations have been introduced by purification or subsequent immunolabeling procedures. DMV lumenal replication sites would best fit theoretical assumptions that consider membranous replication factories to be shielded subcompartments protecting viral replication machinery from nucleases and intracellular RNA sensors (16, 18, 33, 35, 58, 59). This topology requires extensive transport of metabolites and viral RNA between the cytosol and the DMV lumen, which could occur directly for DMVs that are not yet closed and thus linked to the cytosol (19), or might be mediated via a previously unknown transport function of a viral and/or cellular protein (Fig. 9), as was also proposed for coronaviruses (60). In any case, we want to point out that our model does not exclude the possibility that viral RNA replication might also occur on other membranous structures, such as single membrane vesicles, that were either not enriched with our method or not detectable, e.g., because of low replicase activity. Further studies will be required to clarify this possibility.

Cellular components of HCV replication factories. Per their definition as obligate intracellular parasites, all viruses hijack infected host cells in order to replicate their genomes and generate viral progeny. Positive-strand RNA viruses, such as HCV, depend especially heavily on the exploitation of metabolic pathways. Thus, subversion of host cell factors by HCV and other positive-strand RNA viruses has been a major focus of research in recent years (36) and might ultimately lead to the development of host-targeting antiviral drugs (61). Despite detailed knowledge about virus dependency on certain cellular proteins and proven direct interactions between host and viral factors, little is known about their exact localization with respect to viral replication sites. As a proof of principle, we investigated the localization of two well-described HCV host dependency factors, VAP-A and VAP-B (37,
which in uninfected cells are implicated in ER homeostasis and vesicular trafficking (62, 63). We verified their presence in DMVs and demonstrated enrichment in the HCV-induced membranous compartment. Hence, we propose that other cellular replicase cofactors are recruited to and thus enriched in HCV-induced DMVs. Thus, determination of the host cell proteome associated with purified DMVs will be the next important task in identifying novel HCV–co-opted host factors.

Beside proteins, lipids constituting membranes of positive-strand RNA virus replication factories are of major interest. Although this group of viruses is known to alter cellular lipid homeostasis and depend on certain lipids for viral propagation (reviewed in reference 18), knowledge about the lipid composition of these specialized membrane compartments is scarce. A fundamental question is whether positive-strand RNA viruses simply reshape preexisting “donor” membranes (e.g., the ER in the case of HCV) without changing their properties or whether they create special membrane compartments with a distinct lipid composition. To address this point, we investigated the cholesterol content of HCV-induced DMVs in comparison to that of ER control membranes, since HCV nonstructural proteins are described to reside in detergent-resistant membranes (41). Strikingly, cholesterol was found to be highly concentrated in DMVs. The ~9-fold enrichment, as determined by quantitative immunolabeling, indicates a very high relative cholesterol concentration in DMV membranes. Such an enrichment of cholesterol in HCV-induced DMVs would result in local cholesterol concentrations that might be similar to that of endosomes or the plasma membrane (64). Since cholesterol enhances membrane rigidity and stability, it could serve as an important structural component of HCV-remodeled membranes (Fig. 9) and possibly for other DMV-inducing viruses, such as picornaviruses, coronaviruses, and arteriviruses, as well. The reduced DMV size and decreased HCV genome stability induced by cholesterol removal support this assumption.

How could HCV induce this high local concentration of membrane cholesterol? The striking difference between ER and DMV cholesterol contents argues against the simple usage of preexisting cholesterol in ER membranes and favors an active recruitment of cholesterol to DMVs. In spite of low ER cholesterol steady-state levels, this organelle is the site of cholesterol synthesis, implying a high turnover. Hence, ER-localized cholesterol could be recruited and thus concentrated during DMV formation by cholesterol-binding proteins associated with viral replicase proteins. Another appealing hypothesis is that cholesterol enrichment in HCV-remodeled membranes might be mediated via another lipid, phosphatidylinositol-4-phosphate (PI4P), and oxysterol-binding proteins (OSBPs). This protein family is implicated in nonvesicular sterol transport between different membrane compartments (65). Characteristic features are the N-terminal pleckstrin homology (PH) domain mediating PI4P binding and the C-terminal sterol-binding domain. OSBP's have been shown to...
exchange sterols for PI4P between different membranes (66) and thus, in a simplified view, transport cholesterol to PI4P-rich compartments, such as the Golgi compartment and the plasma membrane. However, HCV, as well as picornaviruses, prominently redistributes and induces intracellular PI4P pools by subversion of phosphatidylinositol-4-kinase class III alpha and beta (PI4KIII α/β), respectively (67, 68, 69). Furthermore, OSBPs interact via an FFAT motif with VAP-A and VAP-B (62, 70), which are also enriched in HCV-induced DMVs. Hence, virus-induced locally elevated PI4P as well as VAP concentrations might attract OSBPs, delivering cholesterol to viral replication organelles. Further studies are needed to address this unifying hypothesis.

In summary, the experimental approach reported here will help to unravel the biochemical properties of HCV replication factories. The methodology should also be applicable to other DMV-inducing viruses, such as picornaviruses, coronaviruses, and arteriviruses. High-throughput quantitative analyses will unravel the protein and lipid constituents of the membranous replication factories of viruses and thus provide fundamental insights into the cell biology of viral infection.

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