Ultrastructural analysis of hepatitis C virus particles

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Hepatitis C virus (HCV) is a major cause of chronic liver disease, with an estimated 170 million people infected worldwide. Low yields, poor stability, and inefficient binding to conventional EM grids have posed significant challenges to the purification and structural analysis of HCV. In this report, we generated an infectious HCV genome with an affinity tag fused to the E2 envelope glycoprotein. Using affinity grids, previously described to isolate proteins and macromolecular complexes for single-particle EM, we were able to purify enveloped particles directly from cell culture media. This approach allowed for rapid in situ purification of virions and increased particle density that were instrumental for cryo-EM and cryoelectron tomography (cryo-ET). Moreover, it enabled ultrastructural analysis of virions produced by primary human hepatocytes. HCV appears to be the most structurally irregular member of the Flaviviridae family. Particles are spherical, with spike-like projections, and heterogeneous in size ranging from 40 to 100 nm in diameter. Exosomes, although isolated from unfractoned culture media, were absent in highly infected, purified virus preparations. Cryo-ET studies provided low-resolution 3D structural information of highly infectious virions. In addition to apolipoprotein (apo)E, HCV particles also incorporate apoB and apoA-I. In general, host apolipoproteins were more readily accessible to antibody labeling than HCV glycoproteins, suggesting either lower abundance or masking by host proteins.

enveloped virus | hepacivirus | lipoviral particle | virus structure | virus assembly

Hepatitis C virus (HCV) is an important human pathogen that infects the liver and establishes chronic infection in the majority of cases, leading to cirrhosis and hepatocellular carcinoma (HCC) over the course of many years. More than 170 million people, ~3% of the world’s population, have been infected with HCV. Each year, 4–5% of patients with HCV-induced cirrhosis develop HCC, making HCV infection the leading indicator for liver transplantation in many areas of the world (1). Surgery, however, does not provide a cure because the donor organ universally becomes reininfected. A prophylactic vaccine is not available and despite the recent addition of HCV-specific protease inhibitors to the pegylated (peg)-IFN and ribavirin regimen, which has increased the cure rate, better therapies are still needed to solve the emergence of resistant variants, severe side effects and suboptimal response rates in cirrhotic patients (2).

HCV is a single-stranded, positive-sense RNA virus in the family Flaviviridae. The HCV genome is ~9.6 kb in length and encodes a long polypeptide of more than 3000 amino acids that is proteolytically processed to generate 10 mature viral proteins. Viral structural proteins are encoded by the first third of the polypeptide and include core or capsid protein (C) and the envelope glycoproteins E1 and E2. p7 (a viroporin) and non-structural proteins, encoded by the C-terminal two-thirds of the polypeptide, play various roles in virus assembly and RNA replication (3). The putative HCV particle consists of a nucleocapsid containing the viral genome, enveloped by an endoplasmic reticulum (ER)-derived lipid bilayer where E1 and E2 are assembled as heterodimers. The structure of the infectious unit of HCV, however, is likely more complex because several lines of evidence suggest that HCV circulates in the bloodstream as a hybrid lipoviral particle (LVP) (4). The majority of viral RNA in human-infected plasma coelutes with very low-density lipoproteins (VLDLs), whose size ranges from 30 to 80 nm. Moreover, the association of HCV to host lipoproteins may explain the atypically low buoyant density (<1.10 g/mL) for the most highly infectious material. HCV particles display a broad density profile, ranging from 1.03 to 1.25 g/mL. Higher density fractions are less infectious and can often be precipitated by anti-human IgG, suggesting the presence of immune complexes. In contrast, HCV RNA in low-density fractions can be captured by antibodies against the protein components of VLDLs and LDLs, and often exhibits higher specific infectivity for chimpanzee and cell culture infection (5). In this vein, HCV entry is strikingly linked to lipoproteins and their receptors (6). HDL enhances HCV infectivity, possibly by exploiting the lipid transfer function of scavenger receptor class B type I (SR-BI), a receptor for both HDL and HCV. Moreover, the LDL receptor was shown to play a role in the internalization of HCV. Given the link between lipoproteins and HCV infectivity and the potential implications for vaccine design or other therapeutic options, determining the structure and composition of the infectious HCV particle remains a priority.

Despite substantial progress in producing viral particles in cell culture and several biochemical and morphological studies, the structure of the HCV virion remains elusive. To date, no crystal structure of the HCV glycoproteins or 3D reconstruction of the virion exists. Classical approaches that proved successful for other members of the Flaviviridae (e.g., dengue virus, West Nile virus) have thus far failed to yield sufficient quantities of well-preserved, structurally intact HCV particles (7, 8). Here, we developed alternative strategies for purifying enveloped HCV virions produced in cell culture and by primary human hepatocytes, obtaining low-resolution 3D details of their ultrastructure. These results have implications for understanding HCV assembly, its interactions with the host cell, and the possible basis for escape from neutralization.

Results

Capture of HCV via Antibodies Targeting Envelope Glycoproteins. To capture and characterize extracellular virions, we used protein A–coated EM grids and α-HCV (AR4A) or α-HIV (B6) envelope antibodies as a negative control (Fig. L4). Particles were affinity-purified on EM grids exclusively by AR4A, which recognizes an a hybrid lipoviral particle (LVP) (4). The majority of viral RNA in human-infected plasma coelutes with very low-density lipoproteins (VLDLs), whose size ranges from 30 to 80 nm. Moreover, the association of HCV to host lipoproteins may explain the atypically low buoyant density (<1.10 g/mL) for the most highly infectious material. HCV particles display a broad density profile, ranging from 1.03 to 1.25 g/mL. Higher density fractions are less infectious and can often be precipitated by anti-human IgG, suggesting the presence of immune complexes. In contrast, HCV RNA in low-density fractions can be captured by antibodies against the protein components of VLDLs and LDLs, and often exhibits higher specific infectivity for chimpanzee and cell culture infection (5). In this vein, HCV entry is strikingly linked to lipoproteins and their receptors (6). HDL enhances HCV infectivity, possibly by exploiting the lipid transfer function of scavenger receptor class B type I (SR-BI), a receptor for both HDL and HCV. Moreover, the LDL receptor was shown to play a role in the internalization of HCV. Given the link between lipoproteins and HCV infectivity and the potential implications for vaccine design or other therapeutic options, determining the structure and composition of the infectious HCV particle remains a priority.

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A1/E2 conformational epitope, but not by the B6 monoclonal antibody. This indicates that both HCV glycoproteins are displayed on the surface of released HCV particles. Particles were roughly spherical and well preserved, but with heterogeneous and not always symmetrical structures (Fig. 1B) and diameters ranging from 40 to 100 nm (Fig. 1C). Their distribution across the grid was not homogeneous and overall particle density was low. This observation was confirmed in a quantitative virus capture assay in which protein G–coated magnetic beads were conjugated to AR4A, B6, or an apolipoprotein (apo)E-reactive antibody and the amount of precipitated HCV RNA measured by RT-quantitative PCR. ApoE was chosen for HCV RNA capture because it is both required for the production of cell–derived HCV (HCVcc) and is a component of HCVcc particles (9, 10). Although AR4A only captured about 2% of the input viral genomes, 10-fold more viral RNA was precipitated using an apoE-targeting antibody (Fig. 1D). Similarly, a small fraction of the beads conjugated to AR4A, but not B6, displayed bound particles by transmission EM (TEM), indicating that, albeit with low efficiency, intact virions were specifically captured using envelope-reactive antibodies (Fig. 1E).

Generation of E2-Tagged HCV Particles. The number of virions captured using envelope-specific antibodies was suboptimal for further ultrastructural analyses. We hypothesized that the low yield might be due to poor accessibility of the viral glycoproteins. To increase particle density on the EM grid, we generated a J6/JFH-1 derivative with a 39-aa-long, tandem affinity tag fused to the N terminus of E2 (tag-HCV) immediately upstream of the hypervariable region I. This region was shown to target insertions, is a target of neutralizing antibodies and therefore exposed on infectious virus (6). The first two residues of E2 were duplicated to ensure proper cleavage at the E1/tagE2 junction followed by six histidine residues (6xHis) and two copies of a streptavidin tag II (StrepII) tag (Fig. S1A). Immunoblot analysis of lysates prepared from cells electroporated with tag-HCV demonstrated correct processing, with both tags detectable (Fig. S1B). Importantly, the insertion did not markedly impair infectious virus production (Fig. S1C). Incubation of virus-containing media with beads recognizing either tag captured both E2 and capsid protein, as expected for isolation of assembled particles (Fig. S1D).

Ultrastructural Characterization of tag-HCV with Affinity grids. To purify tag-HCV, we developed custom-made EM affinity grids coated with a monolayer of lipids functionalized with a nickel-nitritotriacetic acid group (Ni-NTA) to interact with the 6xHis-tag (Fig. 2A). High specificity of capture was achieved during the binding step by using low concentrations of imidazole (20 mM), as demonstrated by the lack of WT-HCV particles on the grids (Fig. 2B). The captured tag-HCV particles had a broad size range (n = 317; mean = 64 nm, SD = 11 nm) similar to that observed with glycoprotein-specific monoclonal antibodies and WT-HCV (Fig. 2C) but particle density on the grid improved significantly (compare Fig. 2B with Fig. 1B). Similarly, beads coupled to α-His antibody precipitated about eightfold more HCV RNA than AR4A (Fig. 2D vs. Fig. 1D) and bead-bound virions were easily visualized by TEM (Fig. S2).

![Fig. 1. Characterization of HCV virions captured via glycoproteins-specific antibody. (A) Schematic of protein (prot) A EM grids. (B) Representative images of negatively stained HCV virions captured using protein A EM grids coated with α-HCV or α-HIV antibodies. (Scale bar: Upper, 100 nm; Lower, 20 nm.) (C) Size histogram of HCV particles (n = 111; mean = 62 nm, SD = 11 nm). (D) HCV RNA capture assay using protein G beads coupled to α-HCV, α-HIV, or α-apoE antibody and incubated with equal amounts of virus-containing media (2 × 10^5 viral genome copies). RNA was extracted from each pull-down and HCV genome copy numbers were determined by RT-quantitative PCR. Means and SD from three independent experiments are shown. The percent of input HCV RNA captured by each antibody is indicated. (E) Images of negatively stained HCV virions adsorbed to protein G beads coupled to α-HCV or α-HIV antibody (Scale bars: Upper, 100 nm; Lower, 20 nm.) A bead-bound HCV particle is indicated (arrow).](image-url)

![Fig. 2. Ultrastructural analysis of tag-HCV virions with affinity grids. (A) Schematic of the affinity grid, an EM grid coated with a Ni-NTA lipid monolayer. (B) Representative images of negatively stained HCV virions captured using 2% (vol/vol) Ni-NTA affinity grids. (Scale bars: Upper, 100 nm; Lower, 20 nm.) (C) Comparative analysis of HCV size distribution with different capture methods. The diameter of WT-HCV particles captured on protein A grids via α-E1/E2 antibody (black bars) and tag-HCV bound to affinity grids through the His-Ni interaction (gray bars) was measured and the number of virions in each size group is expressed as percent of total captured particles. (D) The copy number of tag-HCV genomes precipitated by His-, HIV-, or apo-specific antibodies coupled to protein G beads was measured by RT-quantitative PCR. Means and SDs from three independent experiments are shown and the percent of input HCV RNA captured by each antibody is indicated.](image-url)
HCV Virions Are Assembled as Lipoviral Particles. ApoE-specific antibodies were superior at capturing viral RNA than α-E1/E2 or His- and Strep-tag antibodies (Figs. 1D and 2D). Immunolabeling paralleled these results with particles staining more efficiently for apoE than E2 (Fig. 3A). Immunogold labeling revealed additional apolipoproteins associated with cell culture–produced HCVcc: apoA-I and apoB. Double-immunolabeling experiments for apoE and E2 showed that more than 95% of the stained particles were apoE+, whereas a low percentage (~3%) stained only for E2 (Fig. 3A). Single- and double-stained virus particles had similar size ranges (Fig. 3B). However, significantly more apoE- than E2-reacting gold particles was observed per virion in the apoE+ /E2+ population (Fig. 3C), suggesting that this viral envelope epitope may be less accessible and possibly masked. Taken together, our results are consistent with the LVP model for extracellular HCV.

HCV Produced by Primary Human Hepatocytes. Given that hepatoma cells are unable to produce authentic VLDLs (4), we were interested in characterizing HCV particles grown in more physiologic cultures, human fetal liver cells (HFLCs), which are polarized and may better recapitulate in vivo lipidoprotein and virus assembly. These cells are permissive for HCV but infection is short-lived with virus yield (11). To reduce possible background resulting from input cell culture virus, an additional capture step using a TBK1 (TBK1) inhibitor, BX795 (Fig. S3D) was employed. WT- and tag-HCV titers in HFLC approached those of HCVcc in Huh-7.5.1 cells by the third week posttransfection (Fig. S3). The visual amount of cells successfully infected over time (12). At peak virus titers (day 26), the majority of HFLC transfected with WT- and tag-RNA was HCV+ (Fig. S3C). Despite limited starting material, well-preserved, HFLC-produced virions (HFLC-HCV) could be captured using both protein A-coated EM grids with AR4A (Fig. S3D) or affinity grids (Fig. 4A), with the latter method being more efficient, as previously observed for HCVcc. When the same supernatant was applied to regular EM grids, no particles were observed, suggesting that the affinity purification in situ was crucial. As for HCVcc, no particles were detected with α-HIV antibody or when WT-HCV was applied to affinity grids. The size range of apoE+ particles captured from Huh-7.5.1 and HFLC culture media was similar (60.17 ± 21.03 nm and 65.13 ± 16.56 nm, respectively; P = 0.193) (Fig. 4B). Particles immunoreactive for apoA-I and apoB were detected, albeit to a lesser extent than apoE (Fig. 4C). These results suggest that virions derived from HFLC, like HCVcc, are assembled as LVPs incorporating host-derived apolipoproteins.

Cryo-EM of HCV. To attempt 3D reconstruction of the HCV virion, structurally preserved enveloped HCVcc particles were purified directly from cell culture media using affinity grids with 20% (vol/vol) Ni-NTA and frozen in their native state (Fig. 5A–C and Fig. S4). A total of 318 particle images were isolated and processed with RobEM software. Particle sizes ranged from 45 to 86 nm in diameter, with a mean diameter of 68 nm (Fig. 5C). A total of 150 particles were randomly selected to generate a random model using Auto3DEM. Random model generation yielded a model of 58 Å resolution as determined by 0.5 Fourier shell correlation (FSC). The resulting model appeared as a smooth sphere, as expected for a heterogeneous set of particles, where any identifying features would be averaged out. This model was used for iterative orientation search on the entire dataset using Auto3DEM. No result better than 58 Å was achieved. Because of the disparate particle size range, the dataset was sorted into three size classes (50–60, 61–70, and >71 nm) and the procedure was repeated on each size class with similar results. Efforts to use reconstructions of the alphavirus Ross River virus (22 Å) or the flavivirus dengue virus (30 Å) as initial models also yielded featureless spheres. Finally, the two smaller size classes were put through a common lines reconstruction method using the EMICOFV program. Orientation for particles was hand selected and the resulting reconstruction was yet another smooth sphere. Single-particle reconstruction methods failed because of extreme pleomorphism of the particles. Exosome-like structures 50–100 nm in diameter were occasionally visible, some of which were multivesicular with two or more distinguishable bilayers (Fig. 5B and Movie S1). Exosome-like particles were observed on affinity grids only when tag-HCVcc samples were applied, suggesting that they contain accessible HCV E2.

Ultrastructural Characterization of Purified HCV. With the goal of gaining insights into the ultrastructure of the infectious HCV particle, we generated highly purified HCVcc preparations. Infectious particles were concentrated by binding and elution from a heparin column followed by buoyant density fractionation on a 10–40% (wt/vol) iodixanol gradient. Particles with a density of ~1.13 g/mL contained more than 40% total infectivity, 20% of the HCV RNA, and thus a higher specific infectivity compared with the input (range 1:10–1:50 vs. 1:1,000–1:5,000; Fig. 6A). TEM analysis of peak infectious fractions captured on affinity grids revealed well-preserved, spherical particles with a broad size range (Fig. 6B and C). The average diameter and overall appearance of purified particles was similar to unpurified HCVcc (67 ± 12 nm vs. 64 ± 11 nm). However, virions 81–85 nm in size were enriched in purified samples (12.1% vs. 2.8% of total bound particles; Fig. 6B) and different structures were occasionally observed (Fig. 6C, Right). Given the 2D nature of TEM images, it was difficult to determine whether these structures represented...
To gain insights into the ultrastructure of fully assembled virions, we first used EM grids coated with AR4A, but only a very small percentage of HCV RNA was precipitated and very few particles were visualized by TEM. AR4A potently neutralizes the virus used in this study and displays one of the highest inhibitory effects among a panel of α-HCV antibodies screened (16, 17), arguing against insufficient binding properties (Fig. 1).

To increase the efficiency of particle capture, as an alternative approach we constructed a genome with a long, hydrophilic tandem affinity tag on E2 that was not impaired in infectious virus production (Fig. S1) and purified it directly on custom-made EM affinity grids interacting with the tags. The affinity grid, first described by Kelly et al. (18), enabled fast isolation, preservation, and enrichment of HCV virions that were instrumental to ultrastructural studies without the need for prior biochemical purification. Indeed, no particles were visualized if the same cell culture–derived media were applied to regular EM grids, indicating that the affinity purification in situ was a key step. Provided that tags can be inserted on accessible regions of the viral surface, the method described here can be applied to ultrastructural studies of other virions that are unstable, produced in low amounts, or not easily bound to conventional grids. The 6xHis tag allowed for better purification of HCV particles than the StrepII tag, possibly because of its higher accessibility (Fig. S1D); nevertheless, the presence of discrete particles with internal features or separate particles that overlapped. Interestingly, despite being present in cell culture supernatant, exosomes were not found in purified samples of high specific infectivity. Immunoblot analyses of purified virions (tag-HCV) captured with His-tag Dynabeads revealed apoE, apoA-I, and apoB100, confirming the immunolabeling results (Fig. 6D).

**Cryo-ET of Purified HCV.** In further attempts to obtain additional 3D structural information of purified HCV, we collected tomographic tilt series of vitrified, unfixed particles with high specific infectivity purified from iodixanol gradients. The quality of the tomograms was affected by the limited tilt range we were able to collect (–60° to +30°). The missing wedge made the tomograms streaky and limited the z-resolution of the reconstructions. Unfortunately, attempts at generating affinity grids using wider mesh grids resulted in insufficient binding of virus particles to enable cryoelectron tomography (cryo-ET) studies. Two reconstructed tomograms illustrating the general morphology of HCV particles are available in Movies S2 and S3. The majority of virions were round but not symmetrical with the outer rim heavily covered by globular proteins distributed unevenly around the viral envelope. Examples of particles with apparent patches of double-layer membrane, spikes, and internal structures are shown in Fig. 7A–D. Striations in the viral envelope, indicated by transversal densities are visible in Fig. 7E and might represent transmembrane proteins (Movie S4).

**Discussion**

TEM has been instrumental to the discovery and characterization of many clinically relevant viruses, some from infected cell cultures, as in the case of aden-, enter-, and reo-viruses, and others from unpurified clinical samples (i.e., hepatitis A and B) (13). HCV represents an unusual case in that it was first identified by molecular cloning and has proven very difficult to visualize from infected sera and tissues (14). Despite the development of systems to propagate infectious virus in cell culture (15), low titers and poor stability remained important challenges to the structural analysis of HCV. Attempts, by us and others, to scale up the number of purified particles applied to EM grids by concentration or precipitation were not successful because of aggregation and serum contaminants, whereas virus stocks produced in serum-free medium yielded fewer infectious particles.
the StrepII tag was confirmed by immunolabeling with peptide-specific antibody (Fig. 3A, f).

We were interested in characterizing HCV particles grown in primary hepatocytes (HFLCs) that represent more physiologic cultures. We previously showed that paramyxovirus V protein expression promotes productive HCV infection in HFLCs by antagonizing IFN induction and signaling following infection with HCV (11). Here, we report a further improvement of virus production in HFLCs using HCV RNA transfection rather than HCVcc infection. Productive infection was facilitated by a chemical inhibitor of TBK-1, a downstream signaling molecule of several pathogen-associated pattern recognition receptors including retinoic acid-inducible gene-I (RIG-I), a primary sensor for HCV. Increasing HCV titers were observed over time, indicative of virus spread, and high numbers of infectious particles [up to 10^7 50% tissue culture infectious dose per milliliter (TCID_{50/mL}) of uncentrated supernatant] were obtained without the potential for contamination by hepatoma-derived HCVcc inocula. The combination of optimized titers and affinity grid enrichment enabled the characterization of primary human hepatocyte-derived HCV virions (HFLC-HCV; Fig. 4).

Both HCVcc and HFLC-HCV purified with affinity grids appeared well preserved (as suggested by the lack of positive staining), spherical, and very heterogeneous in size (40–100 nm in diameter), with spike projections but no distinguishable arrays. Thus, HCV does not seem to have structural similarities with other members of the Flaviviridae, such as dengue or West Nile virus, which display a relatively smooth surface in their mature stage, an icosahedral symmetry and a diameter of 50 nm (7, 8).

Not only the size, but also the density of HCV particles was reported to be very broad both in vivo and in cell culture systems and to vary depending on the host cell in which the virus is assembled. This suggests that the host contributes to define the makeup of the virus in ways that might affect its structure and composition (5). Initial studies with human and chimpanzee plasma favored the hypothesis of association of HCV virions with the VLDL fractions (19, 20). Since then, a growing body of literature has emphasized how the maturation and release of HCV particles are tightly linked to the VLDL biosynthetic pathway, with the strongest evidence being that apoE is essential for virus assembly and found to be a component of HCVcc particles (9, 10).

In this study, we confirmed that apoE is incorporated in the majority of captured HCVcc and demonstrated that this is true for primary-derived HCV virions as well. In agreement with viral RNA immunocapture data (Fig. 1), double-immunolabeling experiments showed that significantly more copies of apoE than E2 were observed per particle (Fig. 3). These data indicate that a host-derived protein is better exposed on the outer rim of the viral envelope than the viral glycoproteins, which would be consistent with apoE playing a role in the initial attachment of HCV to the host cell. Interestingly, larger particles did not display stronger staining for apoE or E2. This may suggest that the sites accessible to antibody binding on the virion were saturated because of steric hindrance. Alternatively, a larger size may reflect higher lipid content or the presence of additional host proteins.

Other apolipoproteins were suggested to associate with serum or cell culture-derived HCV (4). In this study, we show incorporation of apoA-I and apoB-100 into both HCVcc- and primary hepatocyte-derived virions. ApoA-I, the major structural protein of HDL, interacts with SR-BI, which is the endogenous receptor for HDLs in the liver. Interestingly, SR-BI is also an HCV receptor, and HDLs were shown to facilitate HCV entry via SR-BI and to attenuate virus neutralization, suggesting that HCV has developed an advantageous strategy to hijack the physiological interaction of HDL with SR-BI (6). Our finding that apoA-I is exposed on the surface of virions may provide a link to explaining the complex interplay between HCV, HDL, and SR-BI. ApoB-100 is the longer isoform of the apoB protein.
specifically produced in the liver, required for VLDL biogenesis and found on VLDL, intermediate density lipoprotein (IDL), and LDL. Its role in the assembly and infectivity of HCV produced in cell culture systems is still being debated (4). Nevertheless, HCV RNA-containing particles purified from low-density fractions (i.e., VLDLs, LDLs) of chronically infected patients were found to be positive for apoB, supporting the incorporation of apoB into LVVs (4). Huh-7.5 cells were thought to be impaired in the formation of apoB-containing VLDLs and therefore unable to produce apoB-associated HCV virions (4). Notably, here we show that extracellular virions purified from Huh-7.5 cultures expose apoB on their envelope. ApoB is considered a nonexchangeable apolipoprotein; therefore, we can infer that viral particles acquire it in the liver cell and not from serum. Interestingly, most HCVcc- and Mary human hepatocytes, unlike HCVcc, are secreted in serum-free media and yet contains apoB. Interestingly, most HCVcc- and HFLC-derived virions reacted with only one apoB immunogold particle, which might indicate a similar stoichiometry to VLDL, which assembles around a single molecule of apoB.

In comparing the ultrastructure of HCV from cell culture media with that of purified samples with narrower density and higher specific infectivity, two differences were noticed. First, exosome-like structures were observed in HCV-containing supernatant but not in preparations enriched in infectious particles. These vesicles were captured via tag-specific methods, indicating that they exposed at least the E2 envelope glycoprotein on their surface. They were characterized by a relatively smooth surface compared with the particles found in highly infectious samples that were heavily covered by electron-dense globular material, suggesting a different composition and likely separate biogenesis. Second, when we looked at highly infectious HCV, we observed an enrichment in particles with a diameter of 81–85 nm displaying internal structures (Fig. 6). However, it was not possible to determine whether these particles accounted for the increase specific infectivity.

Interestingly, as one would expect for an enveloped virus, the cryo-ET images did not show evidence of a continuous bilayer. This could be due to the low resolution of the images or may indicate that the particle is embedded in a nonunique amount of lipoprotein, therefore delimited it by a lipid monolayer. In this context, the HCV envelope glycoprotein E1/E2 is located in a lipid bilayer. This transmembrane domain is parallel to the lipid monolayer, as suggested by Bartenschlager et al. (4), unless the capsid is in close proximity of the lipoprotein-detecting membrane. In that case, a partial bilayer might form and E1/E2 would be able to acquire a transmembrane topology, thus anchoring the lipoprotein to the capsid. This hypothesis would be consistent with some of our images (Figs. 6C and 7).

In summary, our results reveal the hybrid nature of HCV, assembled as a mixed particle with a thick shell of host-derived apolipoproteins coating the viral envelope that presumably aid both release and entry of the virus as well as escape from circulating neutralizing antibodies, ultimately allowing this veiled pathogen to fly under the radar.

Materials and Methods

**Virus Purification.** Virus-containing media was harvested every 4 h for 4 d after switching electroporated cells to low-serum media [1.5% (vol/vol) FBS]. High-titer HCVcc stocks were obtained by concentration of the infectious supernatant in a stirred ultracentrifugation cell (Model 8400 with 100-kDa MWCO membranes; Millipore). Concentrated samples were purified over heparin column (GE Hitrap Heparin) according to the manufacturer’s instructions. Heparin-eluted virus was fractionated over a 10–40% (wt/vol) iodixanol buoyant density gradient (Optiprep; Sigma) to isolate fractions with the highest infectivity (range 1.12–1.16 g/mL).

**Cryo-EM and Cryo-ET: Sample Preparation and Data Collection.** Holley carbon grids (400 mesh, Ted Pella) were coated with 20% (vol/vol) Ni-NTA lipid mesh to generate affin-ity grids suitable for cryo-EM. Ten-nanometer gold particles (Aurion Gold Sol, EMS) were added to the virus suspension to serve as fiducial markers for tomography. Grids were floated carbon side down on a 50-μL drop of virus solution containing 20 mM imidazole for 30 min, blotted for 2.0 s in a Gatan Cryoplunge C3p with 70–80% chamber humidity, and plunged into liquid ethane. Cryo-EM images were collected using a Titan Krios electron microscope (FEI) at 300 keV under low-dose conditions, using an Ultracat 950 4k CCD (Gatan). For cryo-ET, imaging was done on a JEOL 3200F electron microscope (JEOL USA) operating at 300 keV under control of SerialEM software using low-dose conditions. Images were collected on a Gatan Ultracat 4k camera at 50,000× nominal magnification and 2× binning, with a final pixel size of 4.40 Å and dose per frame of 2.4 e−/Å². An energy filter was inserted for all recorded images with slit width set to 20 eV. Tilt series were collected in 2° increments at the maximum range allowed by the grid: from −62° to +30° in 1024 frames, −64° to +64° in 256 frames, and −62° to +64° in 256 frames. The tilt range was limited by the mesh size of the grids. Tilt series were aligned and reconstructed using Pro- tomo software. Back-projected reconstructions were viewed using IMOD.

Detailed methods and the associated references can be found in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Cell Cultures and Virus Stocks. The human hepatoma cell lines Huh-7.5 and Huh-7.5.1 were cultured as described (1). Deidentified human fetal livers (16–24 wks gestation) were procured through Advanced Bioscience Resources or the Human Fetal Tissue Repository of The Albert Einstein College of Medicine. Human fetal liver cells (HFLCs) were isolated and cultured in hepatocyte-defined medium (HDM; BD Biosciences) as described (2). All protocols were reviewed and approved by the Rockefeller University Institutional Review Board. Cell culture–derived hepatitis C virus (HCVvec) stocks were produced by electroporation of hepatoma cells and titrated by limiting dilution as described (1). To generate primary cell-derived HCV stocks, HFLCs were plated at 10^5/cm^2 in 24-well plates and transduced with lentiviral pseudovirions generated from the pNL4-3NLac/WT vector. This is a live-cell reporter system capable of monitoring HCV infection in real time. It is based on the knowledge that the HCV protease NS3-4A cleaves the host protein IPS. The tagRFP-nls-IPS construct encodes a red fluorescent protein (RFP) fused to IPS such that upon HCV infection, cleavage of IPS results in the exposure of a nuclear localization sequence (nls) and re-localization of the RFP from the mitochondria to the nucleus. After 5–6 d of culture, cells were pretreated overnight with 1 μM of the tank binding kinase-1 (TBBK-1) inhibitor, BX795 (InvivoGen), then transfected with HCV genomic RNA by incubation for 2 h with 300 μL Opti-MEM (Invitrogen) containing 20 μL/mL DMRIE-C transfection reagent (Invitrogen) and 1 μg/mL HCV RNA. The cells were washed once and maintained in HDM containing 1 μM BX795. Supernatants were harvested and pooled every 2–3 d, concentrated ∼10-fold over Amicon-Ultra centrifugal units (100-kDa MWCO; Millipore) and stored at −80 °C.

Generation of Tagged HCV. The HCV genome Clone 2 has been previously described and is referred to in this study as WT HCV (4). A tagged Clone 2 derivative encoding a duplication of amino acids 384 and 385 of the HCV polyprotein, a 6-histidine repeat (6xHis), and a One-STrep-tag (OST), consisting of two copies of the streptavidin tag II (Strep II) [AG

Virion Pull-Down and Immunoblot Analysis. Fractionated WT- and tag-HCV samples were dialyzed to PBS and incubated with Dynabeads His-tag (Invitrogen) or MagStrep type 2HC beads (IBA) for 1 h at room temperature in the presence of protease inhibitors. For His-tag pull-downs, 20 mM imidazole was added during the binding step. Beads were washed six times with PBS and heated at 70 °C for 10 min in 1× SDS loading buffer or bound virions were eluted with 0.9 M imidazole. To prepare cell lysates, 72 h post-electroporation (post-ep), Huh-7.5.1 cells were lysed [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (vol/vol) Triton X-100, and Mini EDTA-free Protease Inhibitor Mixture (Roche)] for 30 min at 4 °C and lysates were clarified by centrifugation at 16,000 × g for 10 min. A total of 30 μg of protein lysate or bead-eluted virions was separated on 4–12% Bis/Tris NuPage polyacrylamide gels (Invitrogen) and transferred to PVDF membranes (Millipore). Blots were probed with primary antibodies against HCV E2 and core (1:500; Austral Biologicals), 6xHis (1:500; Qiagen), Strept-tag (1:500; IBA), apoE and apoA-I (1:1,000; Academic Biomedical), apoB100 (1:1,000; Calbiochem), or β-actin (1:10,000; Sigma) followed by HRP-conjugated secondary antibodies (1:10,000; Jackson Immuno Research) and developed using Super Signal West Pico substrate (Thermo Scientific).

Negative Staining and Immunogold Labeling. To capture WT-HCV particles, Protein A BioGirds (copper, 400 mesh; Dune Sciences, Inc.) were preincubated with an HCV-specific human IgG (AR4A; 1 μg) according to the manufacturer’s instructions. An HIV-specific human IgG (B6) (5) was used as a negative control. A total of 50 μL of 4% (vol/vol) paraformaldehyde (PFA)-fixed HCV-containing media [in 1.5% (vol/vol) FBS; titers ranging from 0.8 to 6 × 10^5 50% tissue culture infectious dose (TCID50)/mL] were incubated on grids for 30 min. To capture tag-HCV particles, affinity grids were prepared as previously described, using grids with a continuous carbon film (copper, 400 mesh; Electron Microscopy Sciences), chloroform reconstituted 1,2-Dilauryl-sn-glycero-3-phosphatidylcholine, and 1,2-dioleoyl-sn-glycero-3-[N(5-amino-1-carboxypentyl) iminodiacetic acid] succinyl-nickel salt (Ni-NTA lipid; Avanti Polar lipids) (6). Two percent (vol/vol) Ni-NTA affinity grids were used for transmission EM (TEM) studies and bound onto 25–50 μL of PFA-fixed tag-HCV containing 20 mM imidazole for 15–30 min at room temperature. For immunogold labeling, grids were blocked for 10 min in 3% (wt/vol) BSA-PBS, pH 7.4, incubated for 20 min with the following primary antibodies diluted in blocking buffer: α-E2 (1:15; 3/11), α-apoE (1:5; Academy Biomedical), α-apoA-I (1:45; Academy Biomedical), α-apoB (1:5; Calbiochem), α-α-His (1:20; Qiagen), and α-Strep-tag (1:20; IBA), then with secondary antibodies conjugated to colloidal gold (1:20; Jackson Immuno Research) for 20 min. Grids were negatively stained with 2% (vol/vol) uranyl acetate (EMS) and examined in a 100CX JEOL transmission electron microscope (JEOL TEM) with digital imaging system (XR41-C, Advantage Microscopy Technology Corp.) or in a TECNAI Bio-twin spirit G2 (FEI) with a built-in Gatan UltraScan 4000 camera (Gatan Inc.). Electron micrographs were analyzed with Digital Micrograph or Advantage Microscopy Technology software.


**Fig. S1.** Functional characterization of tag-HCV. (A) Schematic representation of the region of the HCV polyprotein containing the tag with the amino acid sequence of the insertion shown in black. At the N terminus of envelope glycoprotein E2, a duplication of residues 384–385 of E2, 6xHis, and an OST, which contains two copies of the streptavidin tag II (StrepII; peptide sequence: WSHPQFEK) separated by spacers and linker have been inserted. The protease cleavage site at the E1/E2 junction is indicated (arrow). (B) Immunoblot for E2, 6xHis, and StrepII tags and β-actin expression in Huh-7.5.1 cells electroporated with no RNA (mock), WT-HCV, or tag-HCV RNA. Cells were harvested 72 h post-ep and 30 μg total cell lysate was loaded per lane. (C) Kinetics of virus production in Huh-7.5.1 cells. The amount of infectious virus released in the supernatant (TCID_{50}) was measured at the indicated time points post-ep by limiting dilution assay. Means and SD from three independent experiments are plotted. (D) Immunoblot analysis for E2 and core of affinity-purified virions. WT- and tag-HCV extracellular virions were precipitated using magnetic beads directed against either 6xHis (Dynabeads) or StrepII (MagStrep) tags, and eluted proteins were loaded onto SDS/PAGE gel.

**Fig. S2.** Images of negatively stained HCV virions bound to Dynabeads. (Left) Bead-bound HCV particles are indicated by arrows and (Right) the framed boxes correspond to the enlarged images. (Scale bars: Left, 50 nm; Right, 20 nm.)
Fig. S3. Impact of TBK-1 inhibitor on HCV titers in HFLC. (A) Infectious virus production by HFLC transfected with WT-HCV RNA in the presence or absence of TBK-1 inhibitor (BX795). Cells were pretreated with 1 μM BX795, transfected as described in SI Materials and Methods and maintained in the continued presence of the drug. Culture medium was replaced every 2–3 d. Infectious virus in supernatants was assessed at the indicated time points (days) post-transfection by titration on Huh-7.5 cells. (B) Infectious virus production by HFLC transfected with WT- or tag-HCV RNA in the presence of BX795. (C) Representative images for HFLC transduced with lentiviral vector encoding tagRFP-nls-IPS at day 26 posttransfection with WT- or tag-HCV. RFP nuclear translocation is a signature of productive infection. (D) TEM images of WT-HCV virions from HFLC supernatant harvested at day 26 posttransfection, concentrated 10-fold, and captured using protein A EM grids coated with α-HCV antibody.

Fig. S4. Low magnification cryo-EM image of tag-HCV virions from Huh-7.5.1 cultures [in 1.5% (vol/vol) FBS] purified on 20% (vol/vol) Ni-NTA affinity grids. (Scale bar: 100 nm.)
Movie S1. Cryo-ET of HCV-containing supernatant showing HCV virions on the carbon film and exosome-like structures inside the holes.

Movie S1

Movie S2. Cryo-ET of purified, highly infectious HCV. Animations through sequential z-slices of a reconstructed tomogram show the general morphology of HCV virions.

Movie S2
Movie S3. Reconstructed tomogram of an additional field containing purified HCV particles.

Movie S4. Reconstructed tomograms of a particle with visible striations in the viral envelope.