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The ins and outs of hepatitis C virus entry and assembly

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Preface

Hepatitis C virus, a major human pathogen, produces infectious virus particles with several unique features, such as an ability to interact with serum lipoproteins, a dizzyingly complicated process of virus entry, and a pathway of virus assembly and release that is closely linked to lipoprotein secretion. Here we review these unique features, with an emphasis on recent discoveries in virus particle structure, virus entry and virus particle assembly and release.

Introduction

Hepatitis C virus (HCV) is a major cause of acute and chronic liver disease, cirrhosis, hepatocellular carcinoma and liver failure, and it remains the leading indicator for liver transplantation[1]. A protective vaccine is not yet available, and the current therapies are suboptimal. HCV-specific, direct-acting antiviral compounds have recently made it into clinical practice. However, their use is limited to patients with a single subset of HCV genetic types, they are mainly used to augment the standard therapeutic regimen of pegylated interferon-alpha and ribavirin, and viral resistance to these compounds arises predictably and rapidly[2]. Therefore, a better understanding of the HCV life cycle is essential to develop additional strategies for antiviral intervention. The processes of virus entry and assembly are two important aspects of this life cycle that are being targeted for future antiviral development.

HCV is an enveloped, positive-strand RNA virus; it is the type member of the genus *Hepacivirus* within the family *Flaviviridae*[3]. Other genera in this family are: *Flavivirus*, which includes many arthropod-borne human pathogens, including dengue virus, West Nile virus, and yellow fever virus; *Pestivirus*, which includes several important pathogens of livestock; and *Pegivirus*, which includes blood-borne viruses that infect humans, non-human primates and other mammals with varying disease associations[4]. All viruses in the *Flaviviridae* share similarities in genome organization and overlapping aspects of viral replication.

The HCV RNA genome is 9.6 kb in length and encodes a single long open reading frame of 3,006 to 3,037 codons. The viral genome is directly translated to produce a polyprotein that is co- and post-translationally cleaved by viral and cellular proteases into ten gene products. The N-terminal region of the polyprotein encodes the structural proteins that are incorporated into the virus particle: the capsid-forming ‘core’ protein and the glycoproteins, E1 and E2. The C-terminal two thirds of the polyprotein encodes the non-structural (NS) proteins: p7, NS2, NS3, NS4A, NS5A and NS5B. By definition, the NS proteins are

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expressed in virus-infected cells but are not incorporated into virus particles; they serve to coordinate the intracellular aspects of HCV replication, including RNA synthesis, modulation of host defense mechanisms and virus assembly[3]. For instance, the heterodimeric complex of NS3 and NS4A proteins, referred to as NS3-4A, contains a serine protease domain and an RNA helicase domain. The serine protease activity is responsible for cleaving the viral polyprotein and cellular antiviral signaling proteins, thereby abrogating the induction of type I interferon in infected cells[5]. The RNA helicase domain is presumably responsible for unwinding double-stranded forms of the viral genome or clearing proteins from the genome during RNA replication[5]. As described below, NS3-4A also has an essential role in the assembly of infectious virus particles.

This Review presents an overview of properties currently known, and unknown, about infectious HCV particles, their entry and their assembly. Infectious forms of HCV have been historically difficult to study, so we briefly review the state of the art in experimental systems used to study HCV. HCV particles exhibit unusual and surprising properties; here we describe what is known about the structure of HCV particles and their entry, a multi-step process that involves a surprising number of receptors and remains to be fully worked out. We then review the other end of the virus life cycle, the assembly and release of HCV particle from infected cells. Emphasis is given to recent findings and current challenges to our understanding of HCV particle structure, entry and assembly. This Review does not describe in detail the intervening steps of RNA translation, modulation of host cell biology, RNA replication or subversion of innate cellular antiviral responses[6–8].

Evolving experimental systems

Since HCV was first proposed to be a distinct infectious virus in 1975[9] and formally identified in 1989[10], the rate-limiting step in understanding this virus has been limitations in suitable experimental systems. Thus, our knowledge has grown in a step-wise manner, with intermittent advances in technology rapidly progressing to new insights into HCV biology. The first functional cDNA clones of HCV were developed in 1997[11, 12] and allowed initial molecular characterization of the virus, but these systems only replicated in chimpanzees. Early HCV replication systems made use of drug-selectable ‘subgenomic’ replicons, which allowed the intracellular steps of HCV replication to be studied in cell culture, but infectious virus was not produced[13]. In the absence of cell culture-infectious virus, many groups began to characterize recombinant HCV-like particles produced in baculovirus[14] and to study the HCV entry process by using retroviruses pseudotyped with the HCV glycoproteins (HCVpp)[15–17].

Complete, reverse-genetic HCV cell culture (HCVcc) systems that produce infectious virus in cell culture have now been developed for a subset of viral genetic types[18–21]. It should be noted that most HCVcc studies are carried out in the Huh-7 hepatoma cell line, which efficiently supports HCV entry, replication and assembly. However, under current culture conditions this cell line lacks many features of bona fide hepatocytes, such as the ability to form polarized sheets or to produce normal serum lipoproteins[22]. For instance, HCVcc particles produced in Huh-7 cells have slightly different biophysical properties than HCV particles produced in vivo or in primary human hepatocytes[23, 24]. Ultimately, the gold standard for studying HCV biology remains the liver, and current efforts are underway to produce tractable and relevant cell culture and animal model systems[25, 26].

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The enigmatic HCV particle

Physical properties of HCV particles

HCV particles are membrane enveloped and display two surface glycoproteins, E1 and E2, which mediate binding and entry into host cells (Fig. 1a). As described below, HCV entry requires acidification of the endosomal compartment[27], presumably to induce rearrangement of the viral glycoproteins into a form that promotes fusion with the endosomal membrane. Beneath the envelope resides a nucleocapsid, which contains a single copy of the viral RNA genome in complex with multiple copies of the viral core protein.

Filtration and electron microscopy (EM) studies have indicated that HCV particles are 40–80 nm in diameter, pleiomorphic, lack obvious symmetry or surface features, and contain electron-dense cores[28–32](Fig. 1b). In the absence of a clear model of HCV particle structure, inferences have been drawn by comparison to the related flaviviruses dengue virus, tick-borne encephalitis virus, West Nile virus and yellow fever virus, which are much better characterized at the structural level. Enveloped flavivirus particles are 50 nm in diameter and display an icosahedral scaffold of 90 antiparallel E glycoprotein homodimers, which lie flat against the virus surface[33](Figs 1c, e). During flavivirus entry, virus particles are internalized and trafficked to the endosome, where the low pH causes E dimers to dissociate and reform into trimeric spikes, inserting an internal fusion peptide into target cell membranes[34]. Remarkably, the fusion glycoproteins of alphaviruses (family *Togaviridae*), rubella virus (family *Togaviridae*) and Rift Valley fever virus (family *Bunyaviridae*) have similar structural folds[35–37] and likely undergo similar acid-induced rearrangement into their fusogenic trimeric form, despite distant evolutionary relatedness and an absence of sequence homology. Such structural and functional similarities have led to the classification of the flavivirus, alphavirus, rubella virus, and Rift Valley fever virus glycoproteins as class II fusion proteins, distinct from class I and class III viral fusion proteins found in influenza virus, human immunodeficiency virus and herpes viruses, which exist as extendable, pre-formed trimeric complexes[38, 39].

Based on the relatedness of flaviviruses and HCV, it has been suggested that HCV may also utilize a class II fusion mechanism[40–42]. However, this assumption has been challenged by recent studies[43, 44] suggesting that HCV and pestiviruses share an uncharacterized mechanism of membrane fusion. As mentioned above, flavivirus particles encode a single class II fusion glycoprotein that is primed for low pH-activation during viral egress[45]. By contrast, HCV and pestiviruses encode small E1 glycoproteins and large, immunodominant E2 glycoproteins that interact with host cell receptors[46–48], and both HCV and pestiviruses require post-attachment priming steps before they can respond to low pH during viral entry[27, 49, 50]. The E2 glycoprotein of the pestivirus bovine viral diarrhea virus-1 (BVDV-1) was recently shown by x-ray crystallography to exhibit a linear domain topology and to form tail-to-tail disulfide-linked homodimers[43, 44] (Fig. 1d), making it structurally dissimilar to known fusion proteins, which indicates that it is unlikely to function as a class II fusion protein. One possibility is that the pestivirus E1 glycoprotein, rather than E2, is responsible for low-pH induced fusion, although E1 is much smaller than canonical class II fusion proteins, indicating that it may use a unique fusion mechanism. In this regard, it is notable that HCV E1 harbors a putative fusion peptide[43, 51, 52]. Clearly, structures of these viral glycoproteins in their pre- and post-fusion states are urgently needed to determine the mechanism by which HCV and pestiviruses mediate membrane fusion.

Virus + lipoproteins = lipoviroparticles?

The most striking feature of infectious HCV particles is their buoyant density, which is unusually low and heterogeneous for an enveloped RNA virus. Both serum-derived HCV

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and HCVcc particles with high specific infectivity (that is, low ratios of virus particles per infectious unit) have buoyant densities of 1.10 g/ml[18, 53], which is significantly lower than those of other enveloped RNA viruses (Table 1).

The low buoyant density of HCV particles is due to their interaction with serum lipoproteins[54, 55] (Box 1). Consistent with this interaction, serum-derived HCV particles have been found to associate with the lipoprotein components apoAI, apoB48, apoB100, apoC1 and apoE[54, 56–58]. Similarly, HCVcc particles interact with apoE and apoC1, whereas interaction with apoB has been only variably detected[31, 32, 59, 60]. Lipid profiling of highly purified HCVcc particles confirms that their lipid and cholesterol content is similar to that of serum lipoproteins, although similarity to a specific lipoprotein class could not be determined[31]. The interaction between virus particles and serum lipoproteins suggests that HCV may form hybrid ‘lipoviral’ particles (LVPs), which can facilitate virus entry into hepatocytes (see below) and may protect the virus from antibody neutralization. However, the overall architecture of HCV particles and LVPs remains obscure. Key unresolved issues include: how are the HCV glycoproteins arranged on infectious virus particles? What molecular interactions mediate HCV particle-lipoprotein association? Do LVPs represent virus particles transiently interacting with separable serum lipoprotein particles (Fig. 1f) or are they hybrid particles that share a common envelope (Fig. 1g)? In support of a two-particle model of transient, exchangeable interaction between HCV particles and serum lipoproteins, serum-derived HCV particles associate with apoB48-containing chylomicrons[58] and exhibit transient post-prandial shifts in buoyant density[57]. Still, the production of HCVcc particles depends on cellular components of lipoprotein assembly and release (described below), suggesting that the interaction of virus particles with serum lipoproteins begins at an early step.

BOX 1

Serum lipoprotein synthesis and function

Very low-density lipoprotein (VLDL; 30–80 nm in diameter) and low-density lipoprotein (LDL; 22–26 nm in diameter) particles are the major transporters of lipids in blood (see the figure, which shows models for the major classes of serum lipoproteins (VLDL, LDL and HDL) in approximate relative scales to an HCV particle). Both VLDL and LDL display one copy of the large, integral apolipoprotein B (apoB100) and variable numbers of the small exchangeable apolipoproteins C1 (apoC1) and E (apoE) on their surface, which consists of a phospholipid monolayer surrounding a hydrophobic core of triglycerides, cholesterol and cholesterol esters (VLDL and LDL particles contain only a single copy of the large apoB protein, which wraps around the particle but in the figure cross-section appears as three separate moieties).

Nascent VLDL particles are secreted by hepatocytes and are relatively short-lived in serum ($t_{1/2}$ = 3–4 hours); VLDL particles are converted to long-lived LDL particles ($t_{1/2}$ = 3–4 days) as they transit through the circulation and exchange lipids with tissues[157]. A related type of apoB-containing particle, the chylomicron, is secreted by the intestine and contains a truncated form of apoB (apoB48). By contrast, small (5–15 nm diameter) high-density lipoprotein (HDL) particles lack expression of apoB but display the exchangeable apolipoproteins apoAI, apoAII and/or apoE (see the figure). HDL is synthesized by the liver and intestine and plays a major part in trafficking cholesterol back to the liver[158].

VLDL particles are synthesized in hepatocytes by a multi-step process. In the first step, apoB is co-translationally translocated into the ER lumen, where it acquires lipids as they are transported into the ER lumen by microsomal triglyceride transfer protein (MTP); the timing of these events is crucial, as insufficient lipid transfer causes apoB misfolding and

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degradation[159]. Subsequently, the apoB-containing VLDL precursor undergoes further lipidation within the secretory pathway to form mature VLDL particles. The mechanisms of secondary lipidation are still under investigation, but may involve apoE- and apoC-containing microsome-associated LDs (maLDs), which can be produced by MTP or CideB[160]. It should be noted that there is compelling biochemical evidence for the existence of maLDs, although they have not been identified morphologically.

HCV entry

HCV circulates in the blood and thus has direct contact with the basolateral surface of hepatocytes, the cell type in which it replicates. This allows it to bind to receptors on the surface of these cells and trigger its entry. HCV is internalized via clathrin-mediated endocytosis. Clathrin is a cellular scaffold used for the budding of plasma membrane-bound vesicles, known as endosomes, into the cell cytoplasm. Once internalized, endosomes may be recycled back to the cell surface or progressively acidified and trafficked to lysosomes for ultimate degradation of their contents[61]. Many viruses use clathrin-mediated endocytosis for entry, and typically require interaction with a small number of receptors to initiate endocytosis[62]. However, HCV entry provides an exception to this simplistic model of virus entry.

Not the usual suspects: HCV entry requires several factors

Initial attachment of HCV to cells occurs via low affinity interaction with the LDL receptor (LDL-R) and with glycosaminoglycans (GAGs) present on heparan sulfate proteoglycans (HSPGs), both of which can interact with virion-associated apoE[63–65]. LDL-R is dispensable for HCV entry but may be needed for optimal replication of the virus within infected cells[66]. In addition to these attachment receptors, five cell surface molecules are essential for HCV particle entry: CD81, scavenger receptor class B type I (SR-BI, also known as SCARBI), claudin 1 (CLDN1), occludin (OCLN) and Niemann-Pick C1-like 1 cholesterol absorption receptor (NPC1L1). As described below, defined subsets of these molecules determine both the hepatocellular tropism and host species tropism of HCV entry.

CD81 was the first HCV co-receptor identified via expression cloning of a human cDNA that could confer binding of HCV E2 to a mouse fibroblast cell line[46]. CD81 is a ubiquitously expressed cell surface tetraspanin that directly binds HCV E2. Although the two proteins bind tightly[67], time course studies with CD81-specific antibodies, which block HCV infection, indicated that CD81 mediates a post-attachment event in HCV entry[68]. Consistent with this, the CD81-binding region of E2 is masked by its hypervariable region 1 (HVR1), suggesting that native E2 may need to undergo a conformational change before this co-receptor is engaged[69]. Treatment of virus particles with soluble CD81 renders them sensitive to acid, indicating that interaction with CD81 may help to prime the HCV glycoproteins for low pH activation during virus entry[70]. In addition, the function of CD81 in HCV entry requires interaction with CLDN1 (see below)[71]. Furthermore, CD81 helps to define the tropism of HCV for human cells[72, 73]. Specifically, HCV is capable of infecting mice that express human CD81 and OCLN in the liver [72, 73].

SR-BI was identified as a candidate co-receptor by its ability to bind recombinant HCV E2 on HepG2 cells, which lack CD81 expression[48]. This protein is highly expressed on hepatocytes, where it normally has important physiologic functions in binding HDL, VLDL and oxidized forms of LDL, and in promoting the uptake of cholesterol from these lipoproteins[74]. Because other HCV entry factors are expressed by multiple cell types, the high expression level of SR-BI in liver may help to define the hepatotropism of HCV entry. During viral entry, SR-BI seems to have multiple, step-wise roles. First, SR-BI contributes

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to virus attachment through interaction with virus-associated lipoproteins. Second, the lipid transfer activity of SR-BI mediates a post-binding event that is important for productive viral entry[75, 76]. Third, subsequent to lipid transfer, SR-BI binds to E2 by interacting with the E2 HVR1[48, 75], which exposes determinants in E2 that allow it to bind CD81[69].

CLDN1 was identified as an essential HCV entry factor through expression cloning of a cDNA that could confer HCVpp entry to the human kidney cell line, HEK293, which expresses CD81 and SR-BI[77]. CLDN1 is a multi-pass plasma membrane protein that localizes to tight junctions and in lower levels to the basolateral surface of hepatocytes and other polarized cells[78]. Although CLDN1 does not directly interact with the HCV glycoproteins, it contributes to the post-binding steps of HCV entry by interacting with CD81, which facilitates virus internalization[71, 77].

A similar cDNA expression cloning strategy was used to identify OCLN as a factor that could confer HCVpp entry to mouse cells engineered to express human CD81, SR-BI and CLDN1[79]. Together with CD81, OCLN defines the species tropism of HCV entry; mice engineered to express human CD81 and OCLN specifically in the liver are permissive for HCV infection[72, 73]. Similarly to CLDN1, OCLN is a tight junction protein that functions at a post-attachment step in HCV entry, although it is unclear whether OCLN directly interacts with HCV particles[80]. The precise role of OCLN in virus entry is still under investigation.

Based on the clinical observation that HCV patients often accumulate iron in the liver, one group found that the iron uptake receptor, transferrin receptor 1 (TfR1), acts as a cellular entry factor[81]. HCV infection is inhibited by TfR1 knockdown, TfR1-specific antibodies, or a small molecule inhibitor of TfR1 recycling; time of addition studies with these inhibitors showed that TfR1 is important at a post-CD81 binding event[81]. However, the mechanism by which TfR1 contributes to HCV entry remains to be determined.

Finally, NPC1L1 was identified as an HCV entry factor. Ezetimibe, a specific inhibitor of NPC1L1, blocks HCV entry in cell culture and in mice bearing human liver grafts, and knockdown of NPC1L1 inhibits HCV entry in cell culture[82]. NPC1L1 localizes to the apical, canalicular surface of polarized hepatocytes, where it functions in the re-uptake of cholesterol from bile[83], and can be internalized to endosomal compartments. The specific role of NPC1L1 in HCV entry is unknown, but likely involves cholesterol uptake. Given that HCV enters hepatocytes from the basolateral side, NPC1L1 may mediate these effects within endosomes.

Hail to the thief: signals announcing HCV entry

Recent studies show that the CD81-CLDN1 axis of HCV entry depends of the integrity of specific signal transduction pathways. Inhibitors of protein kinase A (PKA) have been shown to disrupt the interaction between CD81 and CLDN1, leading to CLDN1 internalization from the cell surface and inhibition of HCV entry[84], highlighting a key role for PKA in this process. Furthermore, HCV E2-CD81 engagement activates the Rho GTPase family members Rac1, RhoA and Cdc42, which modify cortical actin filaments and allow lateral mobility of HCV-CD81 complexes to sites of cell-cell contact[85]. Thus, Rho GTPases may be necessary for HCV-bound CD81 to interact with the tight junction protein CLDN1 prior to virus internalization. Consistent with this, an inhibitor of RhoA was found to inhibit the endocytosis of CD81-CLDN1 complexes[86].

In addition to the PKA and Rho signaling pathways, two receptor tyrosine kinases (RTKs) – epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) – were identified in a human kinome-specific siRNA library screen as host factors required for HCV entry[87]. Similar to PKA and Rho signaling, EGFR and EphA2 signaling promotes CD81-CLDN1

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interaction[87]. Moreover, HCV entry is blocked by the anti-cancer compounds erlotinib and dasatinib, which inhibit EGFR and EphA2, respectively, as well as by transient or stable knockdown of EGFR or EphA2 or by antibodies that block receptor-ligand interactions; in contrast, HCV entry is enhanced by the addition of EGFR and EphA2 ligands[87].

A follow-up study to clarify the role of EGFR in HCV entry found that binding of HCVcc particles to cells promotes the co-localization of CD81 with EGFR, which activates EGFR signaling, and that EGFR is required at a step between CD81-CLDN1 engagement and clathrin-mediated endocytosis[88]. Ras, a membrane-bound GTPase that regulates the timing and intensity of multiple signal transduction pathways, including EGFR signaling, acts as a major integrator of EGFR-mediated HCV entry[89]. Indeed, the anticancer compound tipifarnib, which prevents the membrane localization of Ras proteins, was found to potently inhibit HCV entry[89]. Moreover, Ras was found to associate with CD81 and promote its lateral diffusion into stable CD81-CLDN1 complexes[89]. Thus, it seems that EGFR and Ras contribute to the functional association of HCV co-receptors, thereby playing a key part in HCV uptake. In addition to its role in HCV entry, EGFR signaling may also render cells more permissive to viral infection by antagonizing the antiviral response of type I interferon[90].

An integrated model of HCV entry

Inherent in the above discussion is that HCV entry uses numerous cell entry factors in a temporally and spatially ordered manner (Fig. 2). Lipoprotein-associated HCV particles attach to the surface of hepatocytes by interacting with GAGs, LDL-R and SR-BI[63–66, 75]. The cholesterol-transfer activity of SR-BI may then serve to unmask virus particles from their associated lipoproteins, and interaction with SR-BI exposes the CD81-binding determinants on HCV E2[48, 69, 75, 76]. A key step in HCV entry is the lateral movement of CD81-bound HCV particles to tight junctions and their interaction with CLDN1. This cell surface trafficking is dependent on several signal transduction pathways, including EGFR and downstream Ras GTPase signaling and Rho GTPases, which remodel cortical actin[85–89]. The interaction of HCV-CD81 with CLDN1 then induces clathrin-dependent endocytosis[86]. Although the tight junction protein OCLN is also essential for HCV entry, its precise role in this process is currently unknown[79, 80].

Following clathrin-mediated endocytosis, HCV-coreceptor complexes are trafficked to Rab5-containing endosomal compartments[86, 91], where the lipid transfer activities of SR-BI[75] and NPC1L1[83] may further modify the virus and its associated lipoproteins. The interaction of E2 with CD81 may then prime the HCV glycoproteins to respond to the low pH within the endocytic compartment and induce fusion between the viral envelope and the bounding endosomal membrane[70]. Following fusion, the HCV genome is presumably released into the cytosol, where it is directly translated to produce viral proteins and initiate viral replication[7].

Given the role of tight junctions in HCV entry, it should be noted that the above model is largely based on experiments carried out in cultured Huh-7 cells, which do not faithfully mimic the architecture of polarized hepatocytes. Moreover, cell-to-cell transmission of HCV (as opposed to binding and entry of blood-borne, cell-free virus) may occur in infected liver tissue and confer resistance to antibody-mediated neutralization[92, 93]. Interestingly, cell-to-cell transmission of HCV may be less dependent on CD81, SR-BI and TfR1 expression, although this is still controversial[81, 94–97]. Ultimately, a comprehensive model of HCV entry will need to integrate information obtained from primary human hepatocytes in culture and in vivo.

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Virus assembly and release

In the later steps of the virus life cycle, following RNA replication, nascent HCV particles form by budding into the endoplasmic reticulum (ER), a process that must bring together viral genomes to be packaged, the viral core protein, and the E1-E2 glycoproteins in a temporally and spatially organized manner (Fig 3). Nascent virus particles transit through the secretory pathway, where they undergo maturation and acquire their low density before exocytosis at the cell surface[98, 99]. Several recent reports have revealed the role of host factors and the HCV NS proteins in coordinating these processes. Below, we briefly summarize the role of these viral and host factors, with emphasis on recent discoveries.

Trafficking of the viral core protein

The mature core protein contains a positively charged, N-terminal RNA-binding domain of unknown structure, and a C-terminal domain that facilitates peripheral membrane binding via two amphipathic helices and a palmitoylated cysteine residue[100, 101]. Following synthesis on the ER, the core protein homodimerizes and is trafficked to cytosolic lipid droplets (cLDs), which are cellular lipid storage organelles[102–104] (Fig. 3a). cLDs consist of a phospholipid monolayer, derived from the outer leaflet of the ER, surrounding a hydrophobic core of neutral lipids and cholesterol esters. The purpose of core cLD trafficking is unclear; perhaps it serves to sequester core protein until it is needed for virus assembly. Consistent with this model, mutations that prevent cLD trafficking of core protein strongly inhibit virus assembly[105–107].

Trafficking of core protein to cLDs requires the MAPK-regulated cytosolic phospholipase A2, PLA2G4A[108]. Moreover, a pharmacologic inhibitor of PLA2G4A potently blocks virus assembly, and this inhibition can be overcome by the addition of arachidonic acid, the product of PLA2G4A[108]. As arachidonic acid can act as a secondary messenger of membrane-bound signaling, one interesting hypothesis is that a signaling pathway downstream of PLA2G4A regulates core trafficking. The trafficking of the core protein to cLDs is also supported by diacylglycerol acetyltransferase 1 (DGAT1) (Fig. 3a), one of two DGAT enzymes required to synthesize the triglycerides that are stored within cLDs[109]. Indeed, inhibiting DGAT1 activity via genetic knockdown or a small molecule inhibitor caused partial decreases in core trafficking and virus production[109].

During virus particle assembly, the core protein must be retrieved from the surface of cLDs to the site of virus budding at the ER, and specific host factors have been shown to be involved in this retrieval process. For example, the core protein contains a conserved YXXϕ motif (in which X corresponds to any amino acid and ϕ corresponds to a hydrophobic amino acid) that interacts with clathrin adaptor protein complex 2 (AP2M1), which was shown to be essential for retrieval of core from cLDs and virus assembly[110]. Consistent with these observations, the anticancer compounds erlotinib and sunitinib, which can inhibit RTKs that regulate AP2M1 activation, were reported to be potent inhibitors of HCV assembly[110]. However, other groups found that erlotinib inhibits HCV entry but not assembly[87, 88]. Given that a major function of AP2M1 is to select cargo for clathrin-mediated endocytosis at the cell surface, its specific role in HCV assembly is not yet clear.

Recently, two groups have imaged live, virus-producing cells to obtain further insights into the trafficking of the viral core protein during virus assembly and release. One group developed an HCVcc variant encoding a core gene fused to a tetracysteine tag, allowing fluorescently labeling and imaging of functional core protein in live, virus-producing cells[111]. This study showed that the core protein is rapidly trafficked to cLDs and slowly recruited from cLDs into motile puncta that traffic on microtubules and likely represent virus particles within the secretory pathway; interaction between the viral NS2 and NS3-4A

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proteins was essential for this recruitment process[111]. By combining this imaging method with genetic and imaging analysis of host secretory components, another group provided further evidence that motile core puncta represent virus particles undergoing secretion[112].

Synthesis and trafficking of the E1-E2 glycoprotein complex

Following synthesis, the E1 and E2 glycoproteins form ER-retained, non-covalent heterodimers[113] (Fig. 3a). Folding of the HCV glycoproteins is interdependent, and formation of native heterodimers is a slow process[114, 115]. However, the E2 ectodomain can fold on its own into a native structure that binds cellular receptors and is recognized by conformation-specific antibodies[116–118]. Biochemical and biophysical characterization of the recombinant, folded E2 ectodomain has defined nine intramolecular disulfide bonds and shown that E2 contains three β-sheet-rich domains separated by regions of random coil and β-turns[41, 119]. Little is known about the structure of the E1 ectodomain, as it does not fold properly in the absence of E2. Further structural definition of the HCV E2 and E1-E2 ectodomains is eagerly awaited.

NS proteins as drivers of HCV assembly and egress

As mentioned above, NS proteins – p7, NS2, NS3, NS4A, NS5A and NS5B – have integral roles during virus assembly and egress, even though they are not themselves packaged into virus particles. One of the first key steps in HCV assembly is the interaction of NS5A with the cLD-bound core protein[106, 120, 121], which is enhanced by DGAT1[122]. NS5A is a homodimeric, RNA-binding, phosphoprotein that is important for RNA replication and virus assembly. It contains an N-terminal amphipathic helix, which anchors it to membranes, followed by a structured, Zn^{++}-binding domain that mediates homodimerization, and two long, flexible domains that are natively unfolded. NS5A has essential roles in virus particle assembly via determinants in its C-terminal unfolded domain[120, 123, 124]. Specifically, phosphorylation of a specific serine residue within this region by casein kinase IIα (CKIIα) is essential for virus assembly[124]. Interestingly, substitution of this serine residue with a phosphomimetic acidic residue renders HCV assembly insensitive to CKII inhibitors or genetic knockdown of CKIIα[124]. NS5A can also interact with apoE[125, 126], although the membrane topology of this interaction remains obscure, as well as with annexin A2, a membrane trafficking protein that enhances virus assembly[127].

Two additional NS proteins, p7 and NS2, have key roles in organizing the virus assembly complex[128–130]. NS2 is a polytopic membrane protein containing three N-terminal transmembrane (TM) domains and a C-terminal cysteine protease domain that causes NS2 to homodimerize[131]. Both the NS2 TM region and the protease domain are required for the production of virus particles; although the cysteine protease activity is required for viral gene expression and replication, it is dispensable for virus assembly when decoupled from viral gene expression[132, 133]. NS2 interacts with the small, membrane-bound NS protein p7, and this interaction is required to localize NS proteins and the core-containing cLDs to putative sites of virus assembly[128, 129].

NS2 (or the p7-NS2 complex) interacts with the NS3-4A enzyme complex, and this is a key step in the retrieval of the viral core protein from cLDs and into nascent virus particles. As mentioned above, NS3 contains an N-terminal serine protease domain that is required for viral gene expression and a C-terminal RNA helicase domain that is essential for viral genome replication[5]. NS3 interacts with NS4A, which anchors the complex onto membranes and functions as a cofactor for both NS3 serine protease and RNA helicase activities[5, 134, 135]. Indeed, mutations in the C terminus of NS4A, which modulates NS3 helicase activity, cause profound defects in virus assembly[134, 136]. Interestingly, mutations in the NS3 helicase domain that enhance RNA replication cause defects in virus assembly,

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implicating NS3 in virus assembly and suggesting that these two processes must be coordinated[137]. Furthermore, mutations in the NS3 helicase domain can act as genetic suppressors, overcoming virus assembly defects caused by genetic lesions in core, NS2, NS3 or NS4A[128, 138–140]. One intriguing possibility is that the NS3-4A RNA helicase activity may serve to package viral RNA during nucleocapsid formation.

Viral budding – a potential role for ESCRTs

The endosomal sorting complex required for transport (ESCRT) pathway is involved in budding and fission of membranous compartments that curve away from the cytosol, which many enveloped viruses use to bud into extracellular compartments[141]. Three groups found that HCVcc particle release is dependent on components of the ESCRT pathway[142–144], although, surprisingly, the assembly of intracellular infectious virus particles was independent of the late ESCRT pathway that mediates membrane fission. One possibility is that ESCRTs are involved in resolving the terminal membrane fission event during virus particle budding at the ER, but that incompletely budded virus particles were released during experimental preparation of cell extracts. Alternatively, the ESCRT pathway could be required for a post-assembly step during virus egress, such as trafficking into an intermediate secretory compartment. Consistent with this, HCV particles have been observed to traffic to recycling endosomes[112, 145].

HCV particle maturation

As the HCV particles transit through the secretory pathway, the E1-E2 glycoproteins are post-translationally modified. On purified HCVcc particles, E1-E2 contain both high mannose and complex N-linked glycans, indicating that virus particles transit through the Golgi[50], where glycan modification is known to occur. Furthermore, virion-associated E1-E2 are found in large, natively folded, disulfide-linked complexes[50], which may contribute to the acid resistance of HCV particles[27] and suggests that rearrangement of these disulfide bonds may be necessary to prime HCV particles for low pH-mediated fusion, similar to pestiviruses[49].

In addition to glycoprotein modifications, HCV particles interact with serum lipoproteins as they transit through the secretory pathway (Fig. 3c). As mentioned previously, Huh-7 cells produce VLDL-like particles that are underlipidated, which may explain why HCVcc particles have higher buoyant density and lower specific infectivity than HCV produced in bona fide hepatocytes[23, 24]. Despite this caveat, several lines of evidence support a link between HCV maturation and lipoprotein secretion. First, nascent HCVcc particles were found to have an intermediate buoyant density and to acquire their low buoyant density during egress, much like VLDL particles[98, 99]. Furthermore, HCV particle production is blocked by small molecule inhibitors of microsomal triglyceride transfer protein (MTP), which is involved in transporting lipids to the ER lumen (Box 1)[98, 146–148]. Although this was initially interpreted to mean that apoB expression is required for HCV assembly[98, 146, 148], careful titration of a MTP inhibitor showed that HCV assembly and release are dependent on the small exchangeable apoE protein, but independent of apoB[147]. Similarly, another study found that nascent virus particles traffic in the secretory pathway with apoE, but not with apoB[112]. These findings are intriguing, as apoB is essential for VLDL assembly, suggesting that HCV assembly may actually depend on apoE-containing microsome-associated LDs (maLDs) rather than on VLDL particle formation. Consistent with the essential role of apoE in HCV production, various cell lines were shown to produce infectious HCVcc particles in an apoE-dependent and apoB-independent manner[149, 150].

In addition to interacting with NS2 during virus assembly, p7 acts as a viroporin, oligomerizing to form hexameric and heptameric cation-specific ion channels that

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equilibrate pH gradients within the secretory pathway and thereby stabilize virus particles during maturation and egress[151–155]. Defects in p7 ion channel activity can be complemented by expressing the influenza virus M2 viroporin protein or by inhibiting the vacuolar-type proton-ATPase with Bafilomycin A1[154], arguing that p7 is a bona fide viroporin. NS2 also has an unknown role in viral egress, perhaps through its interaction with p7[156].

An integrated model of HCV particle assembly and release

HCV particles assemble by budding into the ER (Figure 3). An important early step in this process is the interaction between cLD-associated core and NS5A proteins, which may serve to shift RNA out of replication or translation and into virus assembly[106, 120, 121]. Similarly, p7-NS2 brings together the viral E1-E2 glycoprotein and NS3-4A enzyme complexes through the NS2 TM domains[55, 139]. The interaction between p7-NS2 and NS3-4A recruits the viral core protein from cLDs into sites of virus assembly[111], perhaps solely through protein-protein interaction, or intriguingly, by facilitating helicase-dependent RNA packaging and nucleocapsid assembly in concert with budding. Virus particles may complete the budding process independently of the ESCRT pathway, although this pathway does seem to be involved in virus release[142–144].

Virus particles are released from cells by transiting through the secretory pathway (Fig. 3c). During this process, HCV particles acquire their characteristic low buoyant density by interacting with apoE-containing VLDL or HDL particles[98, 99]. Furthermore, the viral surface glycoproteins are modified to contain complex glycans and their disulfide bonds are reorganized[50]. As they transit the secretory pathway, virus particles are protected from exposure to low pH by p7, which neutralizes intracellular compartments[154].

Perspectives

The past several years has seen enormous growth in our understanding of key steps in the HCV life cycle. We now know that HCV virus particles exhibit a defined composition despite their pleiomorphic nature. Several cell entry factors have been identified, including signal transduction pathways that are important for trafficking of virus to sites of endocytosis. Furthermore, we have learnt that virus particles assemble at the cLD-ER interface and require the concerted action of cellular enzymes and viral NS proteins, and then undergo maturation as they are trafficked through the secretory pathway.

Nevertheless, there are a number of key unanswered questions. We need to resolve the structure of the viral glycoproteins, understand how HCV particles are organized, and define the molecular basis for the interaction between virus particles and serum lipoproteins. Although numerous HCV co-receptors and entry factors have been defined, understanding how they are used temporally and spatially remains a priority, particularly in cells that model the architecture of hepatocytes.

On the virus assembly front, we need a clearer picture of how nucleocapsids are assembled, how NS proteins contribute to this process, and the relationship of virus particle assembly to lipoprotein secretion. Ultimately, answers to these questions must integrate our findings in the available cell culture systems with HCV biology in real hepatocytes and the liver.

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Suggested glossary terms

Buoyant density A physical characteristic of an object, describing its tendency to float above liquids that have greater densities than the object. Thus, at equilibrium, the buoyant density of an object is equal to the density of the surrounding liquid. Buoyant densities are determined by using isopycnic ultracentrifugation

Pleiomorphic heterogeneity in morphology, form, or shape

Secretory pathway a series of subcellular, membrane-bound compartments that traffic proteins and small molecules from the inside to the outside of cells

Subgenomic replicon A genetic unit capable of autonomous replication in a suitable host cell. An HCV subgenomic replicon is a viral RNA that is less than genome-length but still capable of autonomous replication

Tetraspanin a family of related cell-surface proteins that have four membrane-spanning domains. Tetraspanins display a small, N-terminal extracellular domain and a large, C-terminal extracellular domain that contains a conserved motif of disulfide-bonded cysteine residues

Tight junction (also known as the zonula occludens) A specialized plasma membrane compartment between two adjacent cells. Tight junctions are impermeable to small molecules and charged ions, thereby physically separating the apical and basoateral surfaces of cells

Type I interferon A class of cytokines induced by viral infection that interfere with viral replication. Type I interferons include multiple interferon-alphas, encoded by separate genes, as well as a single interferon-beta

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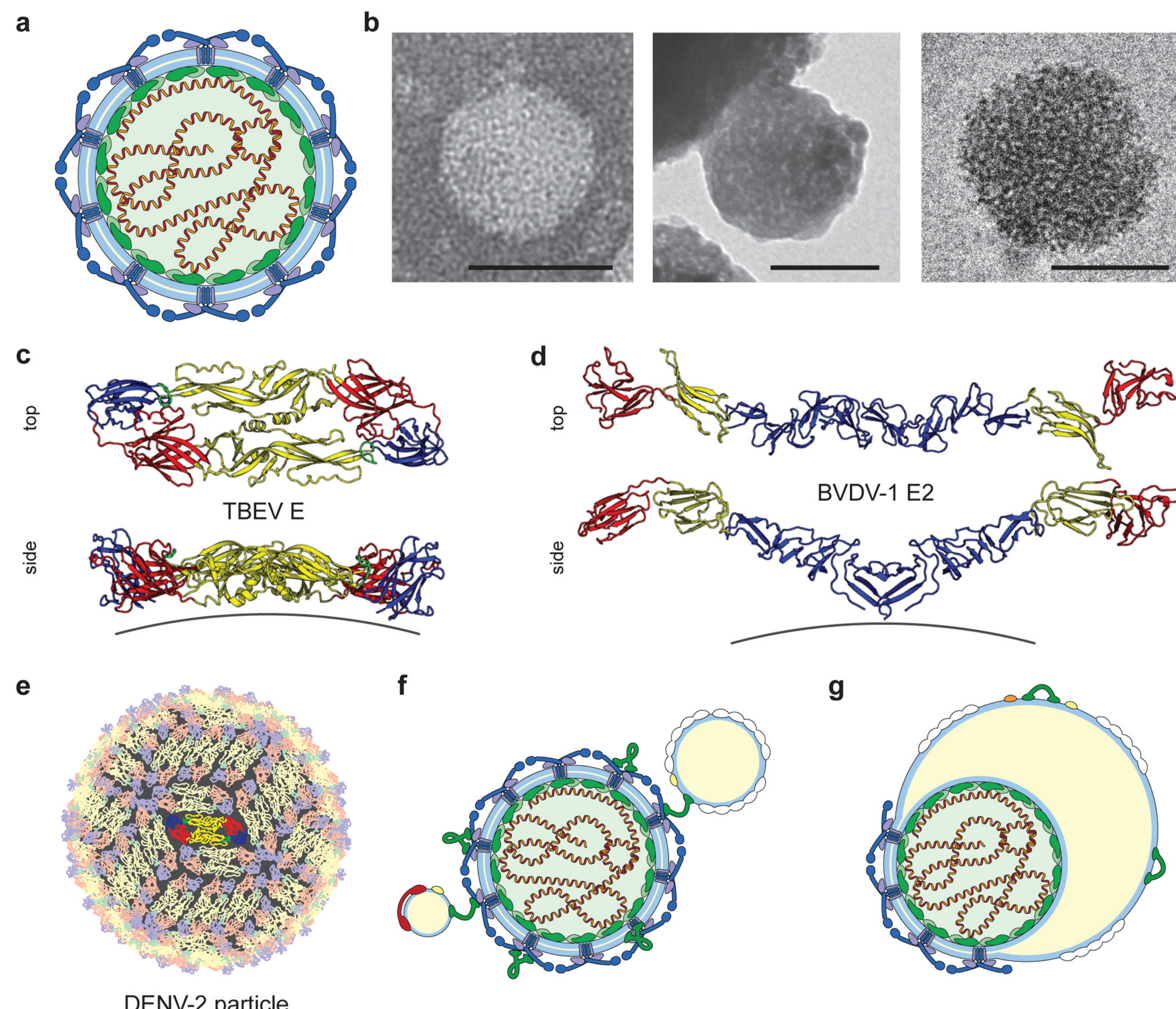

Figure 1.
Structural aspects of HCV and related viruses. a. Model of a HCV particle, showing the membrane bilayer envelope, E1-E2 surface glycoproteins, and nucleocapsid containing core protein and viral RNA. HCV-associated lipoproteins are not shown. b. Representative electron micrographs of negatively stained HCVcc particles captured on an affinity electron microscopy (EM) grid (left) or a magnetic bead recognizing the tagged E2 envelope glycoprotein (middle). Cryo-EM image of an HCV virion (right). Scale bars are 50 nm. These EM images, courtesy of M.T. Catanese, demonstrate the lack of regular surface features and pleiomorphic shape of HCV particles. c. Structure of the dengue-2 virus E glycoprotein homodimer as viewed from the top and side views. Domain I is colored red, domain II is yellow, the fusion loop is green and domain III is blue. Rendered from PDB 1OAN. d. Structure of the BVDV-1 E2 glycoprotein homodimer, rendered at the same scale and coloring as in c. Note that no fusion loop has been identified in BVDV-1 E2. Rendered from PDB 4ILD. e. Fitting of the E protein dimer structure into electron density map of purified DENV-2 particles. For clarity, one dimer is highlighted. **f**. Two-particle model for

LVP structure, which proposes that HCV particles and serum lipoproteins transiently interact. Particles are illustrated with approximate relative scales, and apoE is indicated in green. g. Single particle model for LVP structure, illustrating an HCV particle sharing an envelope with an LDL particle.

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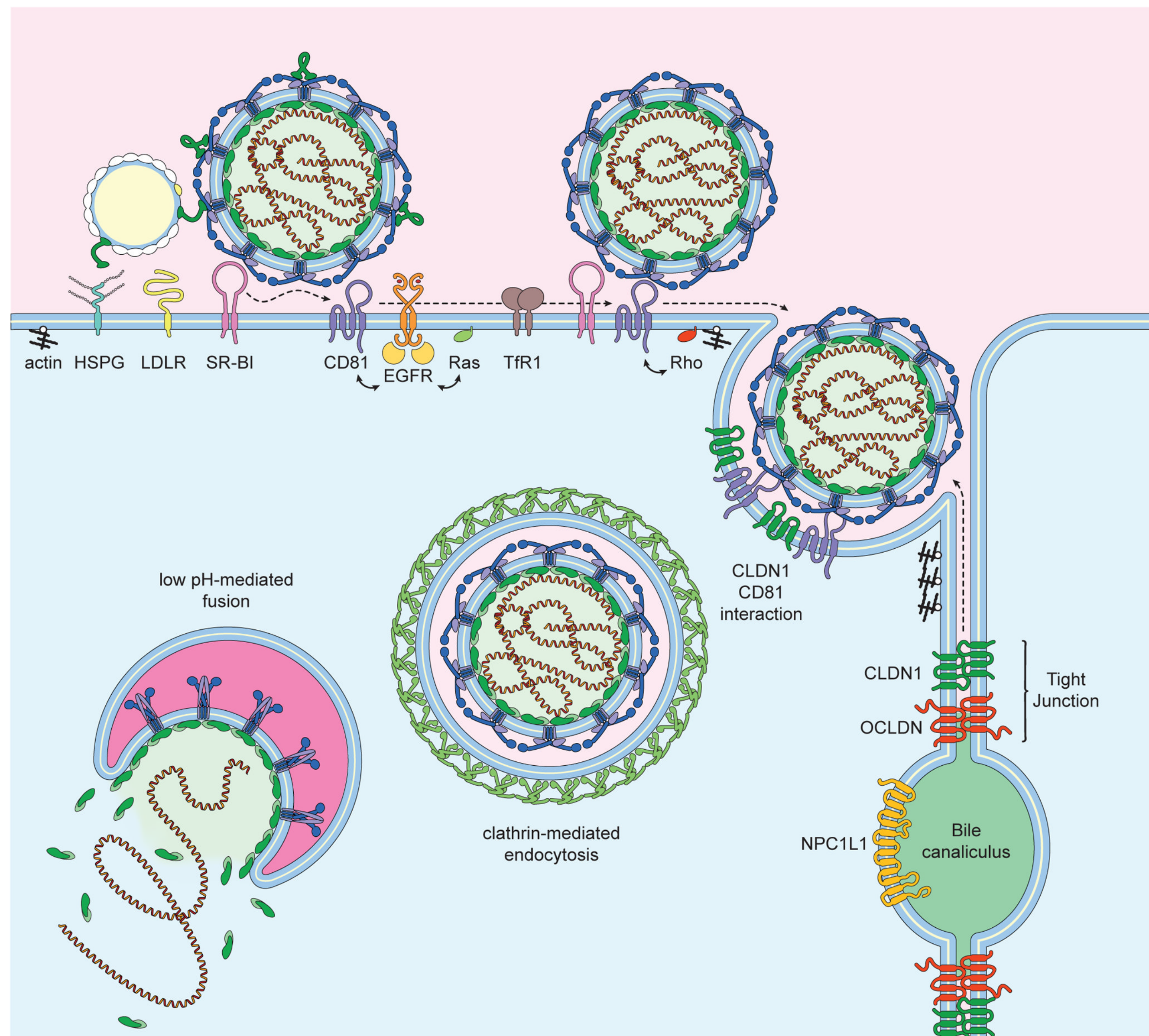

Figure 2.

HCV entry. HCV LVPs attach to the cell surface by interaction with HSPG, LDLR and SR-BI. SR-BI may delipidate HCV-associated lipoproteins and induces conformational changes in E2, exposing the CD81 binding site (step 1). TfR1 plays an unknown, post-CD81 role in HCV entry (not shown). Interaction of E2 with CD81 then activates signal transduction through EGFR and Ras as well as Rho GTPases (step 2). These signaling events promote lateral movement of CD81-HCV complexes to sites of cell-cell contact (step 3), interaction of CD81 with CLDN1, and HCV internalization via clathrin-mediated endocytosis (step 4). The low pH of the endosomal compartment induces HCV fusion (step 5).

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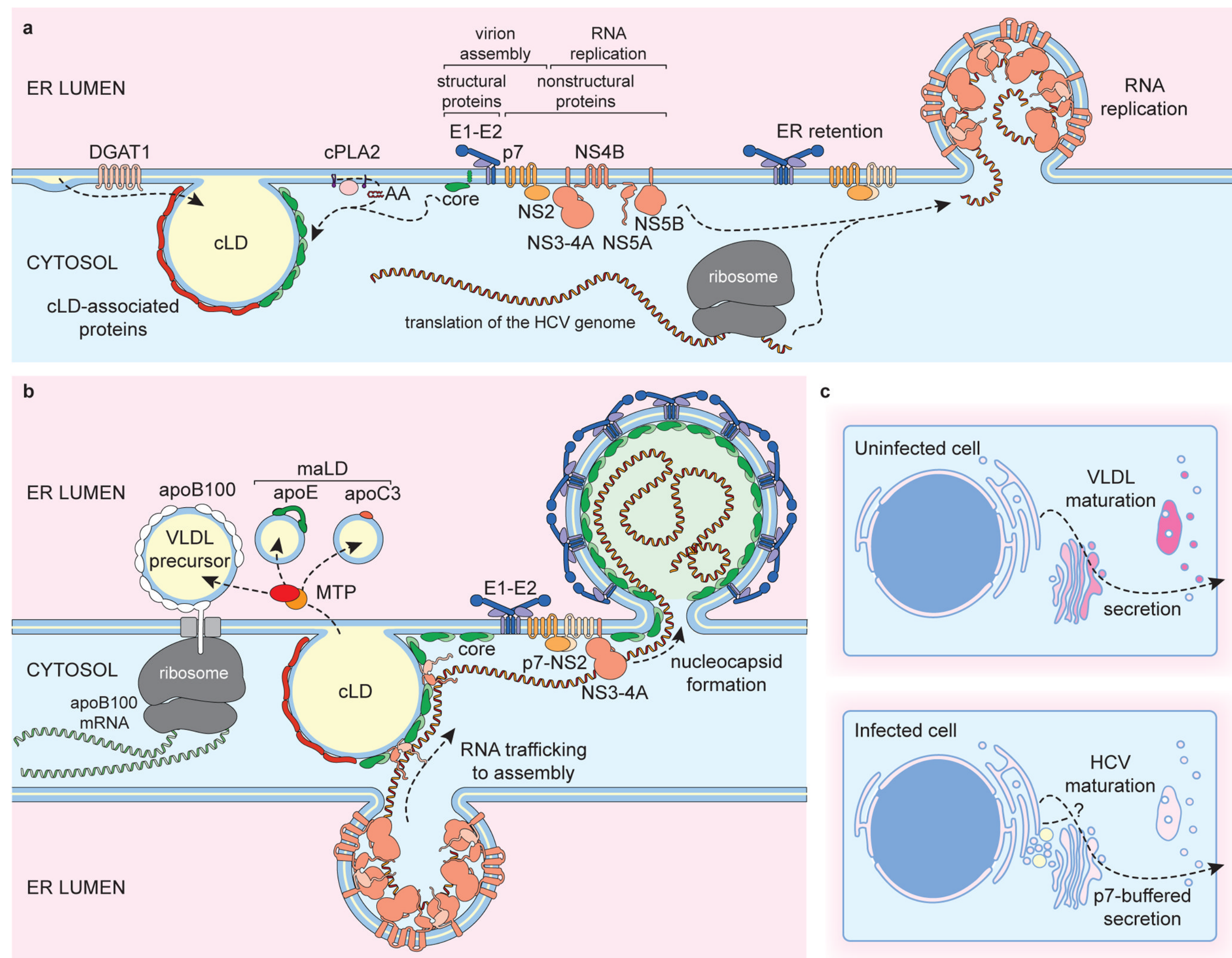

Figure 3.

HCV assembly and secretion. a. Early events in the HCV lifecycle, including gene viral expression, recruitment of the viral RNA into an RNA replication complex, ER-retention of the E1-E2 glycoprotein and p7-NS2 complexes, and cPLA2-mediated trafficking of core dimers to cLDs, which are formed by the action of DGAT1. b. Late events in the HCV lifecycle. Viral RNA is shifted out of replication and translation and towards virus assembly. The interaction of p7-NS2 with NS3-4A recruits the viral core protein to the site of virus assembly. Virus particles assemble by recruitment of the E1-E2 glycoproteins and budding into the ER. c. The pathways of VLDL and HCV particle secretion, including maturation into low-buoyant density particles, perhaps through specific lipidation steps and/or interaction with maLDs, and the secretion of particles through the p7-buffered secretory pathway in HCV-infected cells.

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Table 1

Buoyant density of enveloped RNA viruses and serum lipoproteins

	Buoyant density range (g/ml)*		References
Enveloped RNA Viruses			
HCV	1.03–1.10		3
Pestiviruses	1.12–1.15		3, 161, 162
Flaviviruses	1.20–1.23		3
Alphaviruses	1.19–1.22		162, 163
Rubiviruses	1.18–1.19		162, 164
Arteriviruses	1.13–1.17		165
Retroviruses	1.16–1.18		166
Bornaviruses	1.18–1.22		167
Rhabdoviruses	1.18–1.20		168, 169
Bunyaviruses	1.16–1.18		170
Serum Lipoproteins			
VLDL	0.950–1.006		171
LDL	1.019–1.063		171
HDL	1.061–1.210		171

*Sparklines represent the buoyant density ranges across a linear scale of 0.93 g/ml (left) to 1.27 g/ml (right).