Virion Assembly and Release

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Abstract Hepatitis C Virus (HCV) particles exhibit several unusual properties that are not found in other enveloped RNA viruses, most notably their low buoyant density and interaction with serum lipoproteins. With the advent of systems to grow HCV in cell culture, the molecular basis of HCV particle assembly and release can now be addressed. The process of virus assembly involves protein–protein interactions between viral structural and nonstructural proteins and the coordinated action of host factors. This chapter reviews our current understanding of these interactions and factors.

Contents

1 The Curious Nature of HCV Virus Particles

To understand the process of how hepatitis C virus (HCV) particles are assembled, it is important to first review the structure and physical properties of infectious virus particles. Serum-derived HCV particles are often complexed with antibodies and other serum components, making their characterization difficult; nevertheless, much has been learned through the study of chimpanzee serum samples with high specific

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infectivity. The advent of HCV cell culture (HCVcc) systems (reviewed in the chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann, this volume) now allows functional virus particles to be grown, purified, and characterized in much greater detail. However, it must be noted that the physical properties of particles produced in cultured hepatoma cell lines differ from those produced in vivo or in primary human hepatocytes (Lindenbach et al. 2006; Podevin et al. 2010). Thus, our understanding of HCV particles, and therefore virus assembly, remains incomplete.

HCV particles are enveloped and contain the viral core protein, which likely combines with the viral genome to form a nucleocapsid, and two surface glycoproteins, E1 and E2 (reviewed in the chapter "Hepatitis C Virus Proteins: From Structure to Function" by Moradpour and Peinin, this volume). A hypothetical model of an HCV particle is shown in Fig. 1a. Infectious, serum-derived particles have diameters between 30 and 80 nm (Bradley et al. 1985; He et al. 1987; Yuasa et al. 1991), while highly purified HCVcc particles have diameters between 60 and 75 nm (Gastaminza et al. 2010; Merz et al. 2011). By electron microscopy (EM), HCVcc particles are pleomorphic, contain electron-dense cores, and lack discernible surface features (Wakita et al. 2005; Gastaminza et al. 2010; Merz et al. 2011).

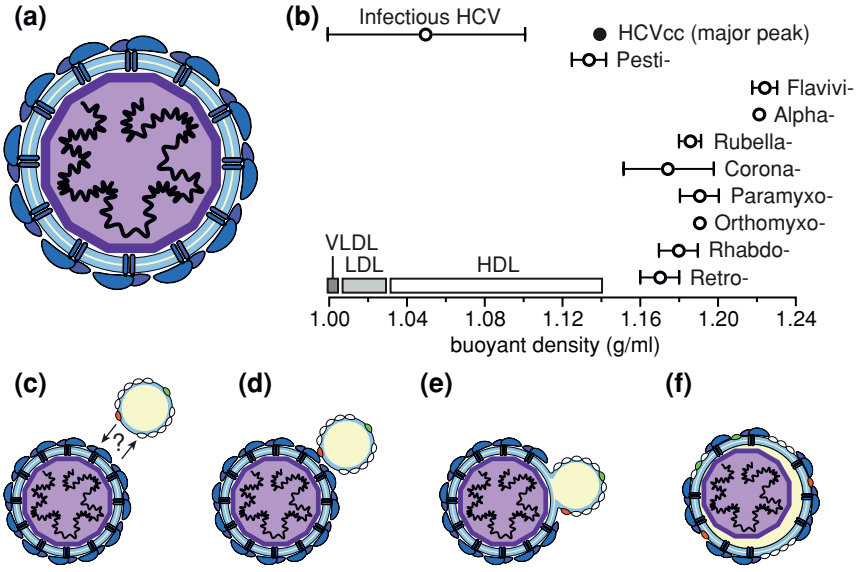

Fig. 1 HCV particles interact with low-density lipoproteins. **a** A model of an HCV particle, based on the structure of flaviviruses. The surface of the enveloped virus particle is decorated with the viral E1-E2 glycoproteins. Within the virus particle is a nucleocapsid formed by core protein and the viral RNA genome. **b** A comparison of enveloped RNA virus buoyant densities. Range bars indicate the buoyant density of viruses within each taxonomic group (data obtained from the International Committee on Taxonomy of Viruses website). The buoyant density of serum lipoproteins are indicated at the bottom for comparison. **c–f**. Illustrations show putative interactions between HCV particles and VLDL particles. See text for further description

The entry of HCV particles is dependent on the low pH of endosomal compartments (Tscherne et al. 2006), suggesting that the viral glycoproteins undergo acid-dependent conformational change, perhaps similar to the type II fusion mechanism of the flavivirus E protein (Bressanelli et al. 2004; Modis et al. 2004) (for further details see chapter "Hepatitis C Virus Entry" by Zeisel et al, this volume). However, HCVcc particles are remarkably resistant to low pH, indicating that virus particles may need to undergo a priming event before they become pH-responsive.

A key feature of infectious HCV particles is that they exhibit unusually low buoyant densities compared to other enveloped RNA viruses, while HCV particles with higher buoyant densities are less infectious (Fig. 1b). Highly infectious virus particles present in chimpanzee serum were found to have densities between 1.03 and 1.10 g/ml (Bradley et al. 1991; Hijikata et al. 1993b). Similarly, HCVcc particles with high specific infectivity have a peak buoyant density of approximately 1.10 g/ml (Cai et al. 2005; Lindenbach et al. 2005), although most cell culture-produced particles have low specific infectivity and buoyant densities near 1.15 g/ml (Cai et al. 2005; Lindenbach et al. 2005; Wakita et al. 2005; Zhong et al. 2005; Yi et al. 2006).

The low buoyant density of infectious HCV particles is thought to be due to their interaction with serum lipoproteins (Thomssen et al. 1992; Prince et al. 1996; André et al. 2002; Nielsen et al. 2006). Consistent with this, Apolipoprotein (Apo) AI, ApoB, ApoC1, and ApoE associate with serum-derived HCV particles (Thomssen et al. 1992; Kono et al. 2003; Nielsen et al. 2006). ApoE and ApoC1 have also been found in association with HCVcc (126, 568, 571), although reports of ApoB association have been variable (Chang et al. 2007; Meunier et al. 2008; Merz et al. 2011). Furthermore, lipid profiling revealed that highly purified HCVcc particles contain lipid and cholesterol contents similar to low-density lipoproteins (LDL) and very-low density lipoproteins (VLDL) (Merz et al. 2011).

The interaction of HCV particles with serum lipoproteins has led to the hypothesis that the virus exists as a hybrid "lipoviral" particle (LVP), which may protect virus particles from neutralizing antibodies (André et al. 2002). However, the precise nature of virus particle-lipoprotein association remains unclear and requires deeper understanding of three unanswered questions. First, what is the molecular basis for interaction of HCV particles with serum lipoproteins (Fig. 1c)? Second, what are the stoichiometric ratios of viral structural proteins and lipoprotein components within an infectious virus particle? Third, do virus particles and serum lipoproteins transiently or stably interact as separate particles, perhaps through specific protein–protein interactions (Fig. 1d), or are they in fact hybrid particles that share a single envelope (Fig. 1e and f)? While LVPs are frequently depicted in reviews and primary research articles as in Fig. 1f, it is unclear how neutral lipids and cholesterol esters would be enveloped by a charged phospholipid bilayer, suggesting that structures topologically similar to Fig. 1d or e are more likely.

One experiment that supports the two-particle model (Fig. 1d) is that HCVcc particles chemically stripped of cholesterol lose their infectivity, which can be restored by adding back exogenous cholesterol (Aizaki et al. 2008). Second, the buoyant density of HCV particles in serum rapidly shifts in relation to dietary triglycerides, suggesting that the interaction of HCV particles with serum

lipoproteins is transient and exchangeable (Felmlee et al. 2010). On the other hand, it is not clear from EM images whether purified HCVcc particles are decorated with VLDL/LDL particles (Gastaminza et al. 2010; Merz et al. 2011). Also, as described below, the production of HCVcc particles is dependent on many—but not all—components of the VLDL assembly pathway, suggesting that the interaction with lipoproteins begin at an early stage of virion assembly. Thus, while our knowledge of virus structure and virus assembly is still incomplete, advances in either area should inform our understanding of both areas.

2 Key Viral and Cellular Players in Assembly

2.1 Viral Structural Proteins

As mentioned, the HCV core, E1, and E2 proteins are structural components of virus particles. Interestingly, these proteins are targeted to distinct places within the cell, which suggests that virus particle assembly is regulated, at least in part, by the coordinated localization of viral structural proteins (see also chapter "Hepatitis C Virus Proteins: From Structure to Function" by Moradpour and Penin, this volume).

2.1.1 Core Protein

HCV core protein is generated from the viral polyprotein through C-terminal signal peptidase cleavage and subsequent C-terminal trimming by signal peptide peptidase (Santolini et al. 1994; Yasui et al. 1998; McLauchlan et al. 2002; Okamoto et al. 2004). Mature core protein consists of an N-terminal RNA-binding region (Domain I, ~120 amino acids) and a C-terminal membrane-binding region (Domain II, ~50 amino acids). Core protein forms homodimers (Boulant et al. 2005), which may be stabilized by an intermolecular disulfide bond (Kushima et al. 2010), as well as higher order multimers (Matsumoto et al. 1996; Kunkel et al. 2001; Klein et al. 2004). In vitro, core protein can interact with structured RNAs to form nucleocapsid-like structures (Kunkel et al. 2001; Klein et al. 2004), although preformed capsids have not been definitively identified in virus-producing cells. One possibility is that sequestration of core protein from viral RNA serves to prevent premature nucleocapsid formation, and that encapsidation occurs concurrent with budding.

Core protein interacts with cellular membranes through two amphipathic helices located within Domain II, as well as palmitoylation of a conserved cysteine residue (Boulant et al. 2005; Boulant et al. 2006; Majeau et al. 2009). This mode of peripheral membrane interaction allows mature core protein to migrate to the surface of lipid droplets (LDs) (Moradpour et al. 1996; Barba et al. 1997; McLauchlan et al. 2002; Boulant et al. 2006). LDs are cellular lipid storage organelles that contain a hydrophobic core of neutral lipids and cholesterol esters surrounded by a phospholipid monolayer that is derived from the outer leaflet of the

endoplasmic reticulum (ER). Targeting of core protein to LDs requires the MAPK-regulated cytosolic phospholipase A2, PLA2G4A (Menzel et al. 2012), and may be enhanced by the cellular enzyme diacylglycerol acetyltransferase 1, DGAT1 (Herker et al. 2010). Thus, core trafficking is functionally tied to specific lipid metabolism events. Furthermore, mutations in core protein that disrupt LD trafficking also abrogate virus production, indicating that LD localization is necessary for virus assembly (Boulant et al. 2007; Miyanari et al. 2007; Shavinskaya et al. 2007). Thus, targeting to LDs may serve to sequester core prior to virus assembly. Retrieval of core from LDs appears to involve recruitment of clathrin adapter protein complex 2 via a specific YXXø motif in core protein (Neveu et al. 2012), as well as specific interactions between viral NS proteins (described below).

To examine core trafficking during virus assembly, Counihan and colleagues developed methods to fluorescently label and image functional, tetracysteine-tagged core protein in live, virus producing cells (Counihan et al. 2011). These data, together with a subsequent paper by Coller and colleagues, show that core is recruited from LDs into virus particles that co-traffic within the secretory pathway in association with ApoE, and that the recruitment of core into this pathway is dependent on interactions between NS2 and NS3-4A (Counihan et al. 2011; Coller et al. 2012).

2.1.2 Envelope Glycoproteins

The HCV E1 and E2 glycoproteins are the major viral structural proteins expressed on the surface of virus particles. During their synthesis, E1 and E2 are translocated into the endoplasmic reticulum (ER), where they interact to form noncovalent heterodimers (Dubuisson et al. 1994; Duvet et al. 1998; Rouillé et al. 2006). E1-E2 dimerization is mediated via their C-terminal transmembrane (TM) domains (Op De Beeck et al. 2000; Patel et al. 2001; Ciczora et al. 2005; Ciczora et al. 2007) and regions within their ectodomains (Yi et al. 1997; Drummer and Poumbourios 2004; Albecka et al. 2011). Native heterodimer formation is a slow process, with the folding of each glycoprotein dependent on the other (Michalak et al. 1997; Patel et al. 2001; Brazzoli et al. 2005) as well as cellular chaperones (Dubuisson and Rice 1996). It has been predicted that E1-E2 heterodimer functions as a class II fusion protein complex (Yagnik et al. 2000); however, formal proof of this will require high-resolution structural information of the glycoprotein complex.

The ectodomain of E2 can independently fold into a near-native form that binds cellular receptors and is recognized by conformation-specific antibodies. Characterization of the recombinant E2 ectodomain revealed that it contains three β-sheet-rich domains separated by regions of random coil and β-turns (Whidby et al. 2009; Krey et al. 2010). Furthermore, the overall structure of E2 is the same at both acidic and neutral pH (Whidby et al. 2009; Krey et al. 2010), suggesting that E2 does not undergo pH-dependent conformational changes on its own. Because E1 does not fold properly in the absence of E2, little is known about the structure of the E1 ectodomain.

In HCVcc-producing Huh-7 cells (see chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann, this volume) and other cell lines, E1-E2 heterodimers are retained within the ER; the major determinants of ER-retention reside within the E1-E2 TM domains (Dubuisson et al. 1994; Cocquerel et al. 1998; Duvet et al. 1998; Cocquerel et al. 1999; Cocquerel et al. 2002; Ciczora et al. 2005; Rouillé et al. 2006). However, when expressed in polarized Caco-2 or HepG2 cells, a fraction of E1-E2 heterodimers is secreted in association with chylomicron-like and VLDL-like lipoproteins, respectively (Icard et al. 2009). These data suggest that the HCV glycoproteins contain undetermined signatures for lipoprotein association.

The development of HCVcc systems has allowed the functional, virion-associated forms of E1-E2 to be partially characterized. HCVcc-associated E1-E2 contain both high mannose and complex N-linked glycans, indicating that virus particles transit through the Golgi (Vieyres et al. 2010). Although intracellular E1-E2 forms non-covalent heterodimers, virion-associated E1-E2 are found in large, natively folded, disulfide-linked complexes (Vieyres et al. 2010). These covalent linkages may contribute to the acid-resistance of HCV particles (Tscherne et al. 2006) and suggest that disulfide rearrangement may be necessary to prime HCV particles for low pH-mediated fusion.

2.2 Viral Nonstructural Proteins

2.2.1 The p7 Ion Channel Protein

The small integral membrane p7 protein is considered to be a nonstructural (NS) protein, although definitive evidence is lacking whether or not p7 is virus-associated. This protein is required for the production of infectious virus particles and appears to play at least two essential roles in assembly and maturation (Sakai et al. 2003; Jones et al. 2007; Steinmann et al. 2007; Wozniak et al. 2010).

First, p7 is required for an early stage of virus assembly through interaction with NS2 (Jirasko et al. 2010; Boson et al. 2011; Ma et al. 2011; Popescu et al. 2011; Stapleford and Lindenbach 2011; Tedbury et al. 2011). The second major role of p7 involves its ability to oligomerize to form hexameric and heptameric cation-specific ion channels (Griffin et al. 2003; Pavlovic et al. 2003; Premkumar et al. 2004; Clarke et al. 2006; Luik et al. 2009; Montserret et al. 2010). Remarkably, p7 equilibrates pH gradients within the secretory and endolysosomal compartments of virus-producing cells (Wozniak et al. 2010). A p7 mutant lacking this activity was unable to produce infectious virus particles, but could be complemented by expressing the influenza M2 viroporin or by inhibiting the vacuolar-type H^+-ATPase with Bafilomycin A1 (Wozniak et al. 2010). These data strongly suggest that the ion channel activity of p7 acts as a viroporin to protect virus particles from premature exposure to low pH during virus maturation and egress.

2.2.2 NS2

NS2 is a polytopic membrane protein that contains three N-terminal TM domains and a C-terminal cysteine protease domain (Grakoui et al. 1993; Hijikata et al. 1993a; Lorenz et al. 2006; Jirasko et al. 2008; Schregel et al. 2009; Jirasko et al. 2010). Folding of the cysteine protease domain requires homodimerization to form a single enzyme with two composite active sites at the dimer interface (Lorenz et al. 2006). The only known substrate of this protease is the NS2/3 junction. While NS2 is dispensable for RNA replication of subgenomic replicons, NS2 protease activity is required for polyprotein processing and RNA replication of full-length HCV genomes (Kolykhalov et al. 2000; Welbourn et al. 2005). By using bicistronic constructs to overcome the requirement of NS2–3 cleavage for genome replication, it was shown that the NS2 protease domain, but not NS2 protease activity, is required for virus assembly (Jones et al. 2007; Jirasko et al. 2008).

NS2 plays an essential role at an early stage of virus assembly, bringing together E1-E2 glycoprotein complex, p7, and the NS3–4A enzyme complex (Phan et al. 2009; Jirasko et al. 2010; Boson et al. 2011; Ma et al. 2011; Popescu et al. 2011; Stapleford and Lindenbach 2011). Interestingly, many of these protein–protein interactions map to the N-terminal TM domains of NS2, suggesting that NS2 serves to bring together proteins within the plane of the ER membrane. By combining genetic analysis of NS2 with live cell imaging of functional core protein, Counihan and colleagues showed that the interaction between NS2 and NS3–4A is required to recruit core protein from LDs into sites of virus assembly (Counihan et al. 2011). Similarly, immunofluorescent studies on fixed samples showed that the interaction between NS2 and p7 is required to localize NS2 and core-containing LDs to putative sites of virus assembly (Jirasko et al. 2010; Boson et al. 2011; Popescu et al. 2011; Tedbury et al. 2011). Together, these data indicate that a p7-NS2 complex mediates the early stages of virus assembly through protein–protein interactions. Given that p7 and NS2 form hexamers and dimers, respectively, one intriguing possibility is that a higher order structure of p7-NS2 oligomers may template similar patterns of protein–protein interaction within the viral structural proteins during virion morphogenesis.

2.2.3 NS3–4A

The NS3-4A enzyme complex is essential for HCV polyprotein processing and genome replication. As reviewed in the chapter "Hepatitis C Virus Proteins: From Structure to Function" by Moradpour and Penin, this volume, NS3 contains an N-terminal serine protease domain and a C-terminal NTPase/RNA helicase domain (Morikawa et al. 2011). The small NS4A protein functions as a cofactor for both NS3 serine protease and RNA helicase activities (Failla et al. 1994; Bartenschlager et al. 1995; Lin et al. 1995; Kuang et al. 2004; Beran et al. 2009). NS4A contains an N-terminal TM domain, a central peptide that intercalates into the NS3 serine protease domain, and a C-terminal acidic peptide. Mutagenesis of

the C-terminal acidic domain showed that this region has dual roles in RNA replication and virus assembly, perhaps through its ability to modulate NS3 RNA helicase activity (Beran et al. 2009; Phan et al. 2011).

NS3–4A has been implicated in virus assembly through genetic and biochemical studies. Specifically, mutations in NS3 that enhance RNA replication cause defects in virus assembly (Pietschmann et al. 2009), suggesting that NS3 may contribute to the switch between RNA replication and virus assembly. Furthermore, mutations in the NS3 helicase domain were shown to suppress defects in virus assembly caused by mutations and/or genetic incompatibilities in NS2, NS3, or NS4A (Ma et al. 2008; Phan et al. 2009; Jirasko et al. 2010). As indicated above, the interaction between NS2 and NS3–4A is involved in recruiting LD-associated core protein into virus assembly (Counihan et al. 2011). Given that core protein is an RNA binding protein and that RNA helicases are processive RNA motors, one attractive hypothesis is that the NS3–4A RNA helicase packages HCV RNA during virus assembly. Consistent with this, genetic and biochemical evidence indicate that interaction between the NS3 helicase domain and core protein is essential for virus assembly (Jones et al. 2011; Mousseau et al. 2011). Nevertheless, definitive evidence is needed to show whether NS3–4A helicase activity is directly involved in nucleocapsid formation.

2.2.4 NS5A

NS5A is an RNA-binding phosphoprotein that plays multiple roles in the virus life cycle. It contains three domains: domain I, which folds into an unusual structure and mediates homodimerization; domain II, which is conserved but likely natively unfolded; domain III, which is less conserved and is tolerant of large insertions and deletions; and two low complexity regions that separate these domains.

NS5A plays essential roles in virus particle assembly, largely through determinants in domain III (Appel et al. 2008; Tellinghuisen et al. 2008; Kim et al. 2011). Specifically, virus assembly requires phosphorylation of a serine residue within domain III by casein kinase IIα (Tellinghuisen et al. 2008). Furthermore, genetic and biochemical data indicate that domain III encodes determinants for transient or weak association with the p7-NS2 complex (Jirasko et al. 2010; Ma et al. 2011; Popescu et al. 2011; Scheel et al. 2012). Similar to NS3, mutations in NS5A that enhance RNA replication cause decreases in virus assembly, suggesting that NS5A may contribute to the switch between replication and assembly (Pietschmann et al. 2009).

Virus particle assembly requires the recruitment of NS5A to LDs, where it interacts with core protein (Miyanari et al. 2007; Appel et al. 2008; Masaki et al. 2008). In addition to interacting with other viral proteins, NS5A interacts with ApoE, an apolipoprotein (Apo) that is required for virus assembly (Evans et al. 2004; Benga et al. 2010; Cun et al. 2010), and Annexin A2, a cellular membrane sorting protein that enhances virus assembly (Backes et al. 2010).

2.2.5 NS4B and NS5B

NS4B is a polytopic membrane protein that plays an essential role in the formation of HCV RNA replication complexes (Gouttenoire et al. 2010). In addition, genetic analysis of NS4B revealed a point mutation in the C-terminal region that increased HCVcc titers but had minimal effects on RNA replication (Jones et al. 2009). It is not yet clear whether NS4B plays a direct or indirect role in virus assembly.

NS5B is the RNA-dependent RNA polymerase that replicates the viral genome (chapter "Hepatitis C Virus RNA Replication" by Lohmann, this volume). It too has been implicated in virus assembly through genetic approaches. Specifically, a mutation at a surface loop residue in the "fingers" subdomain of NS5B was able to suppress virus assembly defects caused by genetic incompatibility of a heterologous p7 within a chimeric genome (Gouklani et al. 2012). Again, it remains to be determined whether this represents a direct or indirect role for NS5B in virus assembly.

2.3 Cellular Factors

2.3.1 VLDL Assembly

HCV particle assembly appears to share numerous features with the pathway of VLDL/LDL assembly. It is therefore worthwhile to briefly review the mechanisms of this pathway.

LDL particles are synthesized in hepatocytes as VLDL particles, which traffic lipids and cholesterol as they circulate through the blood. The surface of VLDL and LDL particles are coated with a single copy of ApoB, a large (≈4500 amino acids), essential, amphipathic glycoprotein; VLDL and LDL particles also transiently associate with the exchangeable apolipoproteins ApoA5, ApoC1, ApoC2, ApoC3, ApoE.

Biosynthesis of VLDL appears to occur in two steps. In the first step, newly synthesized ApoB acquires and encases lipids as it is translocated into the ER to form a VLDL precursor. Proper folding of ApoB requires the co-translational transport of lipids into the ER by microsomal triglyceride transfer protein (MTP) (Sakata et al. 1993; Yao et al. 1997). In the second step, the VLDL precursor undergoes further lipidation steps to form mature VLDL particles, which are secreted through the Golgi in a COPII-dependent manner (Gusarova et al. 2003; Siddiqi 2008). The mechanisms of secondary lipidation are still under investigation. Some models posit that VLDL precursors acquire lipids via fusion with (or catabolism of) ER-resident, ApoE- and ApoC-containing LDs, which may be produced by MTP and/or CideB (Rustaeus et al. 1998; Wang et al. 2007; Ye et al. 2009). However, direct evidence and mechanistic details of this fusion seems to be lacking. Alternatively, other evidence indicates that secondary lipidation occurs in a post-ER compartment, most likely the Golgi (Stillemark et al. 2000; Gusarova et al. 2003; Gusarova et al. 2007; Blasiole et al. 2008). Part of this debate stems from the fact that VLDL assembly has

been studied in different animal and cell culture systems, which may emphasize one part of a pathway over another. In this regard, it is notable that Huh-7 cells, which robustly support HCV RNA replication, do not produce authentic VLDL (Yamamoto et al. 1987; Meex et al. 2011); rather, they produce VLDL-like particles that are underlipidated, which may explain why HCVcc particles produced in Huh-7 cells have higher buoyant density and lower specific infectivity than HCV produced in bona fide hepatocytes (Lindenbach et al. 2006; Podevin et al. 2010).

Despite the above caveats of studying VLDL production in Huh-7 cells, several lines of evidence show that the assembly of HCVcc particles and VLDL are closely linked. First, small molecule inhibitors of MTP block virus particle production (Huang et al. 2007; Gastaminza et al. 2008; Nahmias et al. 2008; Jiang and Luo 2009). While some groups have reported that ApoB expression is required for HCV assembly (Huang et al. 2007; Gastaminza et al. 2008; Nahmias et al. 2008), Jiang and Luo found that HCV assembly was not dependent on ApoB expression, but was highly dependent on the small exchangeable protein ApoE (Jiang and Luo 2009). Similarly, Coller and colleagues showed that nascent virus particles traffic through the secretory pathway in association with ApoE, but not with ApoB (Coller et al. 2012). These are intriguing findings, since ApoB expression is essential for VLDL assembly, suggesting that HCV assembly may actually depend on ApoE-containing ER-luminal LDs rather than VLDL particle formation. Consistent with the essential role for ApoE in HCV production, mouse hepatoma cells were shown to produce infectious HCVcc particles in an ApoE-dependent manner (Long et al. 2011). In addition, intracellular HCVcc particles were shown to be immunoprecipitated and neutralized by antibodies against another small exchangeable apolipoprotein, ApoC1, indicating that they also associate at an early stage of virus production (Meunier et al. 2008).

2.3.2 ESCRT Pathway and Endosomal Release

The endosomal-sorting complex required for transport (ESCRT) pathway is a cellular machinery for the outward budding and fission of vesicles away from the cytoplasm, and is centrally involved in the formation of multivesicular bodies (Henne et al. 2011). In addition, many enveloped viruses utilize this pathway for budding into extracellular compartments (Welsch et al. 2007). Three groups found that the secretion of infectious HCVcc particles is dependent on components of the ESCRT pathway, although intracellular infectious virus particles were assembled when late steps of the ESCRT pathway were inhibited (Corless et al. 2010; Ariumi et al. 2011; Tamai et al. 2012). One interpretation of these data is that the ESCRT-III pathway is involved in resolving the terminal membrane fission event and that incompletely budded virus particles can be released during experimental preparation of cell extracts. An alternative interpretation is that the ESCRT pathway is required for a post-assembly step of virus release, perhaps through trafficking of virus particles into a late endosomal/multivesicular body compartment. In this regard, Tamai and coworkers propose that Hrs contributes to the release of virus particles through fusion of multivesicular bodies with the plasma membrane, similar to the release

of exosomes (Tamai et al. 2012). Consistent with an endosomal trafficking of virus particles, Lai and colleagues found evidence that HCVcc particles traffic through early and late endosomal compartments (Lai et al. 2010), although the timing of these experiments made it difficult to know whether the immunolabeled particles were on their way in or out of cells. More compelling evidence was provided by Coller and colleagues, who showed core protein trafficking with markers of recycling endosomal compartments, Rab11, transferrin, and dextran in live, virus-producing cells (Coller et al. 2012). These results are consistent with the flow of virus particles through the secretory pathway into a sorting endosomal compartment.

2.3.3 Other Host Factors

As mentioned above, trafficking of core protein to LDs requires PLA2G4A and is enhanced by DGAT-1 (Herker et al. 2010; Menzel et al. 2012). Interestingly, the block in virus assembly caused by a pharmacologic inhibitor of PLA2G4A was overcome by exogenous addition of arachidonic acid, the product of this enzyme. These data implicate specific lipid products in the pathway of virus assembly.

Another host factor involved in core protein trafficking is AP2M1, the μ1 subunit of clathrin adapter protein complex 2 (Neveu et al. 2012). This was a surprising result, as AP2M1 is involved in recruiting cargo into clathrin-mediated endocytosis at the plasma membrane. Further work will be needed to clarify the role of this pathway in virus assembly.

Backes and colleagues identified Annexin A2, a phospholipid-binding protein that anchors membranes to the actin cytoskeleton, within partially purified HCV RNA replication complexes (Backes et al. 2010). Surprisingly, knockdown of this gene had no effect on viral genome replication, but significantly inhibited the assembly of infectious virus particles within cells. Furthermore, Annexin A2 was found to bind to NS5A domain III that has been implicated in virus assembly (Backes et al. 2010).

The stress granule proteins G3BP1, TIA-1, and TIAR were recently implicated in virus particle production (Garaigorta et al. 2012). Knockdown of these genes moderately inhibited viral RNA replication but had stronger effects on virus assembly and release. Their mechanism of action is unknown, but may be independent of their roles in stress granule formation (Garaigorta et al. 2012).

3 Mechanism of Virus Particle Assembly

The details of HCV particle assembly are not yet fully clear, but must involve the coordinated action of the ER-resident E1-E2 glycoprotein complex, recruitment of LD-associated core protein to package viral RNA, and several viral and host factors described above. HCV particles form through budding into the ER, similar to other members of the *Flaviviridae*. Consistent with this, Gastaminza and colleagues showed that infectious HCVcc particles accumulate within cells when

treated with Brefeldin A, a potent inhibitor of ER-Golgi transport (Gastaminza et al. 2008). As described above, the interaction between LD-associated core and NS5A proteins is important at an early step of this process, perhaps by shifting RNA out of replication and into virus assembly (Miyanari et al. 2007; Appel et al. 2008; Masaki et al. 2008). Similarly, NS2 brings together the viral E1-E2 glycoprotein complex, p7, and the NS3-4A enzyme complex (Phan et al. 2009; Jirasko et al. 2010; Boson et al. 2011; Ma et al. 2011; Popescu et al. 2011; Stapleford and Lindenbach 2011), and the interaction between p7-NS2 and NS3–4A is necessary to recruit core protein from LDs into sites of virus assembly (Boson et al. 2011; Counihan et al. 2011). Given that preformed capsids have not been identified, nucleocapsid formation likely takes place in concert with budding. Furthermore, virus particle assembly may be intimately coordinated with RNA replication, as seen for other members of this virus family (Khromykh et al. 2001; Welsch et al. 2009). A model that brings together these considerations is presented in Fig. 2.

4 Maturation and Release of Virus Particles

As indicated above, E1-E2 present on extracellular virus particles contain some complex modifications (Vieyres et al. 2010), indicating that virus particles pass through the Golgi. Consistent with this, Coller and colleagues found that core protein trafficked to Golgi in a virus assembly-dependent manner prior to being released at the plasma membrane in a VAMP1-dependent manner (Coller et al. 2012). During egress, HCV particles depend on p7 to neutralize acidic compartments within the secretory pathway (Wozniak et al. 2010).

Gastaminza and colleagues showed that nascent, intracellular HCVcc particles have a higher buoyant density than extracellular particles and acquire their low

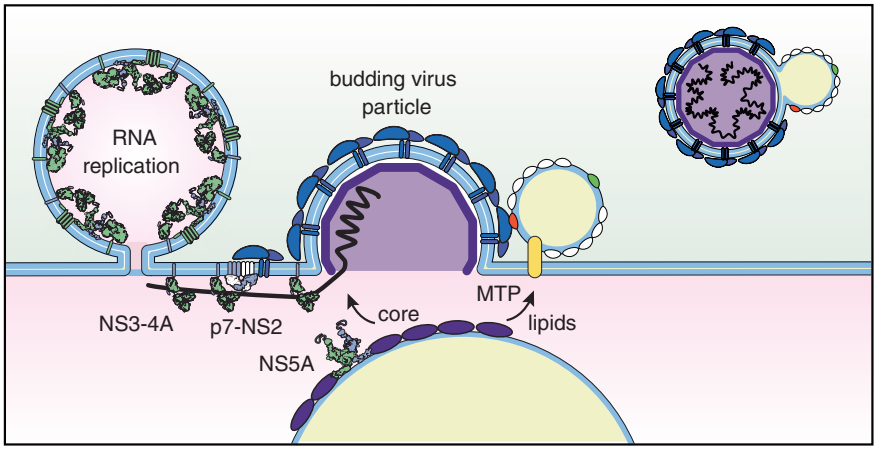

Fig. 2 Model of HCV particle assembly. See text for further description

buoyant density in a post-ER compartment (Gastaminza et al. 2006; Gastaminza et al. 2008). These data imply that virus particles undergo post-synthetic lipidation, similar to the VLDL assembly pathway.

In addition to producing extracellular virus particles, HCV has been reported to directly infect neighboring cells without releasing detectable virus particles or requiring the canonical HCV entry factors (Timpe et al. 2008; Witteveldt et al. 2009). The structural protein requirements and assembly and release pathways for cell–cell HCV transmission are not currently understood.

5 Conclusions

Much has been learned about the assembly and release of HCV particles in the past 7 years of research with HCVcc systems. Essential protein–protein interactions have been defined, and numerous host factors have been implicated. However, the structure of HCV particles and their interaction with lipoproteins remain to be addressed, and key questions need to be examined by using bona fide hepatocytes that make authentic lipoproteins. There is yet much work to be done!

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