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Hepatitis C virus and host cell lipids
An intimate connection

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Hepatitis C virus (HCV) is a major human pathogen, persistently infecting more than 170 million individuals worldwide. The recent establishment of fully permissive culture systems allowed the unraveling of the close link between host cell lipids and HCV at each step of the viral replication cycle. HCV entry is triggered by the timely coordinated interaction of virus particles with cell surface receptors, including the low-density lipoprotein receptor. Viral RNA replication strictly depends on fatty acids and cholesterol biosynthesis. This process occurs on modified intracellular membranes, forming a membranous web. Their biogenesis is induced by the viral nonstructural proteins (NS) 4B and NS5A and requires the activity of cellular lipid kinases belonging to the phosphatidylinositol-4-kinase III family. A hallmark of HCV-induced membranes is thus the presence of phosphatidylinositol-4-phosphate (PI4P), which is synthesized by these kinases. Intriguingly, certain recently identified HCV dependency factors selectively bind to PI derivatives, suggesting a crucial role for PIPs in viral RNA replication and assembly. The latter occurs on the surface of lipid droplets and is tightly connected to the very low density lipoprotein pathway leading to the formation of unique lipoviro particles. Thus, HCV exploits lipid metabolism in many ways and may therefore serve as a model system to gain insights into membrane biogenesis, lipid droplet formation and lipid trafficking.

Introduction

About 170 million people worldwide are persistently infected with the hepatitis C virus (HCV), a major human pathogen responsible for a high proportion of acute and chronic liver diseases. Persistently infected individuals have a high chance to develop serious liver damage including steatosis, fibrosis, cirrhosis and eventually hepatocellular carcinoma.[1] No vaccine is available and current therapy for hepatitis C is based on a combination of polyethylene glycol-conjugated interferon-α and ribavirin. Unfortunately, this therapy has numerous side effects and in ~50% of cases fails to eliminate the virus. Thus, highly specific and efficient drugs to combat HCV infection are urgently needed.[2]

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HCV can be classified into 7 major genotypes, differing in their nucleotide sequence by 30–35%.[3] Most prevalent worldwide is genotype 1 that is also poorly responsive to interferon-α-based therapy.[2] Overall, the virus replicates inefficiently in cell culture and initially only subgenomic replicons could be designed that replicate to levels suitable for biochemical assays.[4] In such systems, the 5' portion of the HCV coding region—that is dispensable for RNA replication- was replaced by a selection marker, such as the neomycin phosphotransferase gene. This approach allowed for the selection of G418 resistant cell clones in which these HCV 'minigenomes' replicated persistently and to high levels.[4] However, even when using genomic replicons containing all viral sequences, virus production was not possible. This was due to the accumulation of replication-enhancing mutations (REMs) that are necessary to allow efficient HCV RNA replication. These REMs interfere with HCV assembly and thus prevent virus production.[5] Studies of the HCV replication cycle were therefore restricted to analyses of RNA replication and this limitation could only be overcome by the identification of an HCV isolate replicating to high levels without requiring REMs.[6] This molecular clone, belonging to genotype 2a and isolated from a Japanese patient with fulminant hepatitis (hence designated JFH-1), for the first time enabled production of infectious HCV particles.[7-9] More recently, a highly adapted genotype 1a clone has been developed also replicating to very high levels, but supporting virion production only poorly.[10] Thus, most studies are limited to the JFH-1 isolate or intergenotypic chimeras derived thereof.[11]

HCV belongs to the Hepacivirus genus within the Flaviviridae family, which also comprises flaviviruses, pestiviruses and the GB virus.[12] The HCV genome is a positive-sense single-strand uncapped RNA molecule of ~9.6 kb, containing a single open reading frame flanked at the 5' and 3' ends by non-translated regions (NTRs) (**Fig. 1**). Both NTRs form complex secondary structures and play multiple roles during the viral replication cycle.[13,14] The 5'NTR contains an internal ribosome entry site (IRES) essential for genome translation,[15] and possesses two binding sites for microRNA-122 (miR-122), required for RNA translation and genome replication.[16,17] The 3'NTR that lacks a 3'-terminal poly-A tract and contains instead an internal polyU/UC tract, is required for RNA replication and contributes to IRES-mediated RNA translation.[18,19]

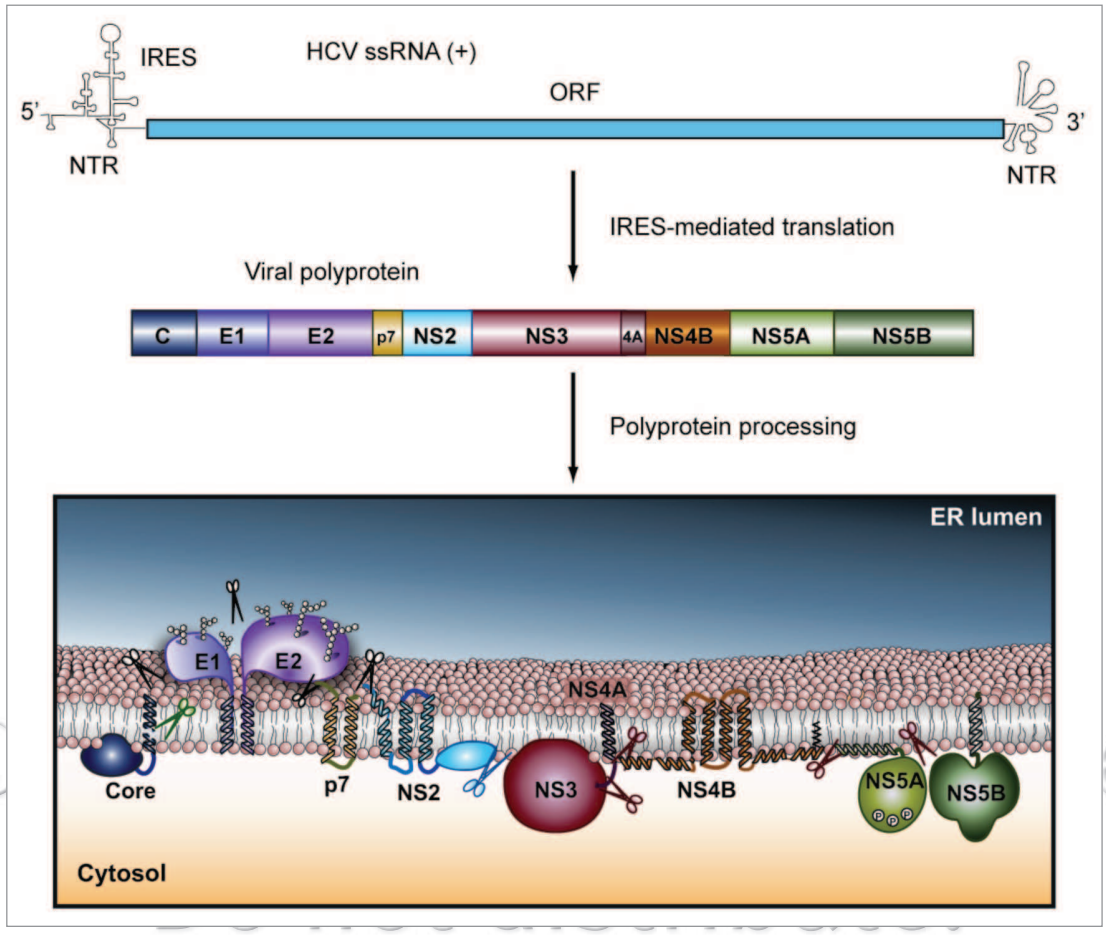

Figure 1. HCV genome organization, polyprotein processing and membrane topology of viral proteins. A schematic representation of the single strand (ss) HCV RNA genome of positive (+) polarity is given in the top panel. Simplified secondary structures in the non translated regions (NTR) at the 5' and 3' ends and the open reading frame are shown. Genome translation from the internal ribosome entry site (IRES) yields a polyprotein precursor (middle panel), which is co- and post-translationally processed by cellular and viral proteases into the structural and non-structural (NS) proteins at the rough endoplasmic reticulum (ER). In the bottom, the membrane topology of the viral proteins with their various transmembrane segments and amphipathic helices (NS5A) is depicted. Black and green scissors indicate cleavages by the signal peptidase and the signal peptide peptidase, respectively. The blue scissor indicates the autoproteolytic cleavage by the NS2-3 cysteine protease. Red scissors indicate NS3/4A-dependent cleavage sites. Glycosylation sites in E1 and E2 are depicted as sugar chain symbols. The palmitoylation at the C-terminus of NS4B is represented by a black zig-zag line. Encircled "P" symbols in NS5A denote its phosphorylation. For further details see main text.

Genome translation occurs at the rough endoplasmic reticulum (ER) and results in the synthesis of a ~3,000 amino acids (aa) long polyprotein precursor (**Fig. 1**).[20] It is co- and post-translationally processed by cellular and viral proteases (**Fig. 1**) into three structural proteins (core, envelope proteins E1 and E2), the small hydrophobic p7 protein and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B; reviewed in ref. 21). Core, E1 and E2 are constituents of the viral particle. P7 and NS2 are involved in virion assembly and release, whereas the NS3 to NS5B proteins are sufficient for RNA replication.[4]

HCV Proteins

The mature core protein (173–179 aa) mainly localizes on the monolayer membrane of lipid droplets (LDs), where HCV particle assembly is thought to take place.[22-24] Core can be divided into three functional domains: the N-terminally located, highly basic domain I (DI) that binds to the viral RNA via electrostatic interactions;[25] a hydrophobic central domain (DII), comprising two amphipathic helices (AHs) that are responsible for LD localization;[26] a C-terminal domain (DIII), corresponding to the signal peptide (SP) of the E1 protein. The SP targets the nascent polyprotein to translocation sites at the ER where cleavage by signal peptidase releases core from the N-terminus of E1.[27] Further cleavage of the signal peptide by signal peptide peptidase, located in the ER membrane (**Fig. 1**) is required for mobilization of mature core from the ER to the surface of LDs, which are the presumed sites where nucleocapsid assembly is triggered.[23,24] Beside its role in HCV assembly (see Section 7), core has been implicated in regulating host-cell lipid metabolism: overexpression of core is sufficient to induce formation of LDs in non hepatic cells[22] and steatosis in transgenic mice.[28]

The **envelope proteins E1 and E2** are highly glycosylated type I transmembrane (TM) proteins with an N-terminal ectodomain and a C-terminal hydrophobic membrane anchor.[29,30] By analogy to the related flaviviruses, it is assumed that E1 and E2 initially form heterodimers, but might rearrange into trimeric complexes required for entry.[31-34]

The **p7** protein, composed of two TM regions that are interconnected by a short cytoplasmic loop,[35] is a viroporin able to form hexa- and heptameric complexes serving as ion channels and required for virus assembly and release.[36,37]

NS2 is a dimeric integral membrane protein, composed of two functionally and topologically distinct domains: a highly hydrophobic, N-terminal, membrane anchor domain and a C-terminal cysteine protease domain, which liberates the C-terminus of NS2 from NS3.[38,39] NS2 is dispensable for genome replication,[4] but plays a central role in assembly, for which its protease activity is not required.[40-42]

NS3 is composed of an N-terminal serine-type protease domain and a C-terminally located domain endowed with RNA helicase and NTPase activities.[43,44] The NS3 protease domain forms a very stable heterodimer with **NS4A**, which acts as a protease cofactor and tethers NS3 to intracellular membranes (**Fig. 1**).[45] Protease activity is required for polyprotein cleavage at four sites: NS3-4A, NS4A-4B, NS4B-5A and NS5A-5B. In addition, this protease plays a pivotal role in HCV immune evasion by cleaving the Toll-like receptor-3 adaptor molecule 1 (TICAM-1/TRIF),[46] and the RIG-I adaptor MAVS,[47] two key adaptor proteins involved in innate immune response to viral infection. Recently, NS3 helicase has also been proposed to be involved in virion assembly.[48]

NS4B is the least characterized HCV protein, which is mainly due to its high hydrophobicity. It is believed to be an oligomeric ER membrane protein composed of an N-terminal region comprising two amphipathic helices (AHs), a central domain containing four TM segments and a C-terminal domain comprising two helices, H1 and H2, the latter of which has been shown to be amphipathic(**Fig. 1**).[49] Ectopic expression of NS4B in mammalian cells leads to membrane alterations resembling the so called "membranous web" (MW) that is induced upon HCV infection.[50] It has been reported that NS4B can directly bind to HCV RNA,[51] and possesses ATPase and GTPase activities for which an NTP-binding site, located in a cytosolic loop between TM helices 2 and 3 appears to be required.[52] However, the importance of these properties of NS4B for the viral replication cycle is still not clear.[53]

NS5A, a master regulator of HCV replication and assembly, is a dimeric zinc-binding metalloprotein.[54] It is composed of four domains:[55] an N-terminal AH essential for attachment to cellular membranes and contributing to LD targeting;[56,57] an RNA binding domain (D1); an intrinsically unfolded and poorly conserved D2 contributing to RNA replication;[58] and a C-terminally located D3, crucial for virus assembly (D3).[59,60] NS5A is the target of several cellular kinases, including casein kinase 1 (CK1), and CK2. Accordingly, it is detectable in infected cells as a basal and a hyperphosphorylated form with apparent molecular weights of 56 and 58 kDa, respectively.[63] NS5A binds to the viral RNA and to numerous host factors and colocalizes with core in close proximity of LDs.[59,60] It is assumed that newly synthesized viral RNA is transported to LDs by NS5A,[24] whose phosphorylation state possibly regulating the balance between viral RNA replication and particle assembly.[59,60,64-66]

NS5B, the RNA-dependent RNA polymerase, is composed of an N-terminal catalytic domain and a C-terminal TM segment anchoring it to intracellular membranes (**Fig. 1**).[67,68] Similar to many other polymerases,[69] NS5B is composed of finger, thumb and palm domains, but has a tightly encircled active site and an additional allosteric GTP binding site.[70] The finger and thumb subdomains form a tunnel through which the single-strand RNA is guided to the active site, while the NTPs enter the active site via a second positively charged tunnel. Importantly, RNA template binding and initiation of RNA synthesis are regulated by a highly flexible β-hairpin loop located in the thumb subdomain.[71] Accordingly, NS5B's catalytic activity is believed to be regulated by conformational changes and by its ability to form oligomers.[72] The latter hypothesis is, however, discussed controversially.[73] Enzymatic activity of genotype 1b-derived NS5B might be stimulated by conformational changes that are triggered by sphingomyelin binding,[74] which also appears to contribute to NS5B localization in lipid rafts.[75] Accordingly, inhibition of sphingomyelin biosynthesis has been shown to inhibit genotype 1b, but not 2a RNA replication.[74,75]

The HCV Replication Cycle and its Link to Lipids

HCV modulates lipid metabolism and alters the endomembrane system to create a lipid-rich environment favorable for viral replication. Indeed, each step of the HCV replication cycle appears to be connected to host cell lipids (**Fig. 2**). In this respect, HCV circulating in the blood of infected patients biochemically resembles a VLDL particle,[76] by being rich in cholesteryl esters and containing apolipoproteins such as ApoE and ApoB (**Fig. 2**). These apolipoproteins play an important role during virus entry since they are the ligands of the low-density lipoprotein (LDL) receptor, one of the entry molecules contributing to HCV internalization.[77] The scavenger receptor class B type I (SR-BI), another molecule involved in lipoprotein uptake, also participates in HCV entry by binding to the E2 glycoprotein.[78,79] In addition to the lipoprotein receptors, further cell-surface molecules including glycosaminoglycans, the tetraspanin CD81 and two tight junction proteins -claudin-1 and occludin- are essential for internalization of the virus (reviewed in ref. 80).

HCV RNA replication is strongly influenced by intracellular levels and composition of fatty acids including cholesterols (see section 4). RNA replication occurs in the MW, induced primarily by NS4B (see section 5).[50] The MW is characterized by the enrichment of specific phosphatidylinositol phosphate (PIP) species,[81] which is mediated by cellular kinases belonging to the PI4P-kinase family (see section 6).[81] RNA progeny is used for translation and thus production of new viral polyproteins, or packaged, together with cellular and viral proteins into new virions, a process that is thought to initiate on the surface of LDs (see section 7).[24,82] Finally, newly assembled viral particles are presumably released in a VLDL-dependent manner (see section 7).[83]

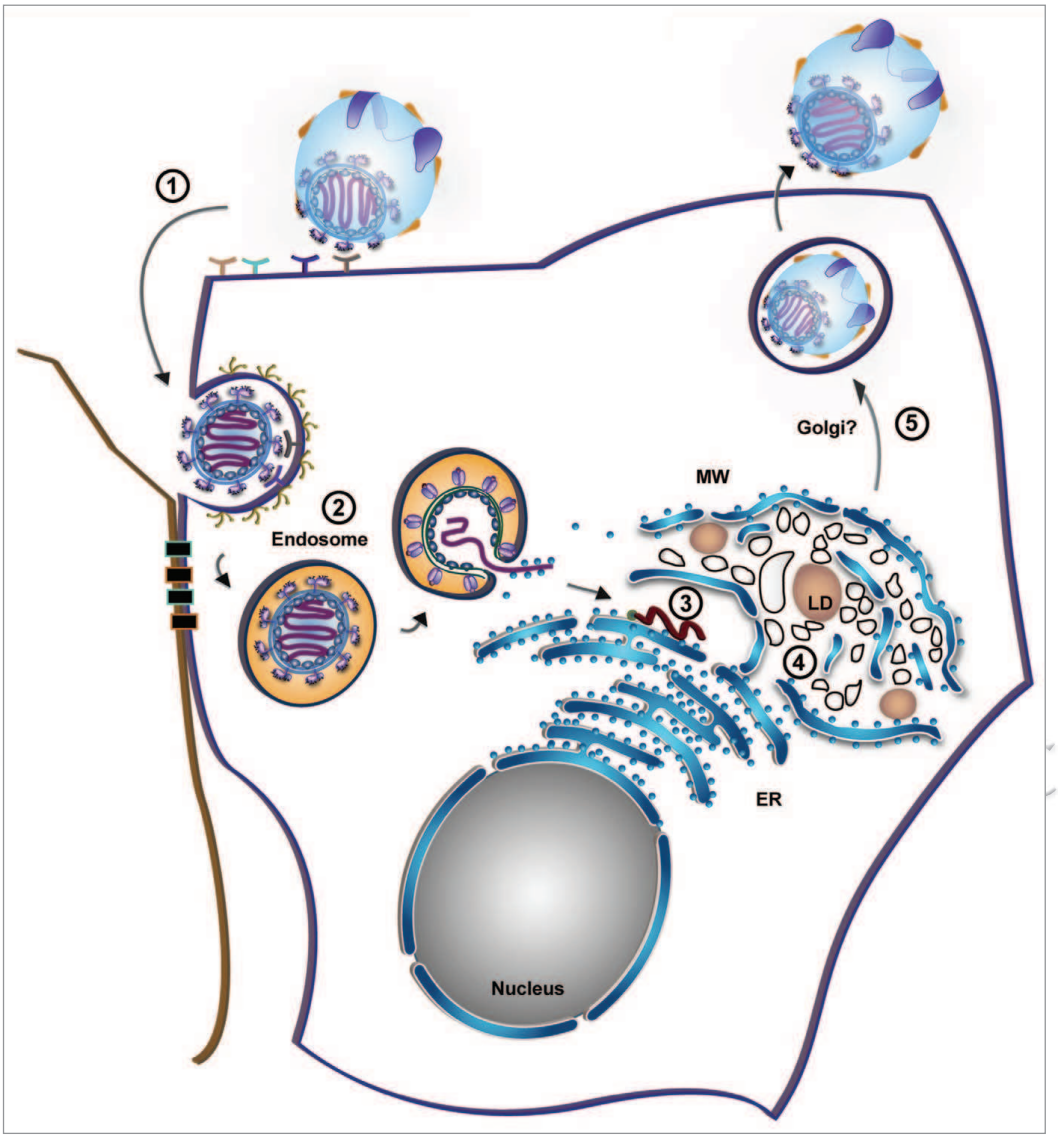

Figure 2. The HCV replication cycle. (1) Attachment of HCV to the cell surface. The HCV particle decorated with the glycoproteins E1 and E2 and cellular apolipoproteins, binds to a set of entry molecules at the surface of the hepatocyte. (2) Receptor-mediated endocytosis of HCV leads to internalization in a clathrin-dependent manner. The low pH (indicated in orange) in the endosome might trigger the fusion between the endosomal and the viral membrane, thus releasing the RNA genome (red waved line) into the cytoplasm. (3) Translation of the viral genome occurs at the rough ER giving rise to a single polyprotein precursor that is cleaved by cellular and viral proteases. (4) HCV RNA replication takes place at the membranous web (MW), consisting of clusters of virus-induced vesicles, ER membranes and lipid droplets (LDs), which are the presumed sites of HCV assembly. (5) Release of HCV is thought to be linked to the VLDL secretion pathway. For further details see main text.

Role of Lipid Metabolism in HCV RNA Replication

HCV infection is tightly associated with alterations in lipid metabolism and lipids have been shown to play important roles during the viral replication cycle,[84,85] (**Table 1**). Indeed, recent studies based on transcriptome and proteomic analyses have demonstrated that expression of host genes involved in the biosynthesis, degradation and transport of intracellular lipids is profoundly altered upon infection.[86-89] The expression of SREBPs, which control transcription of genes required for cholesterol biosynthesis,[90] is stimulated both by HCV infection and upon expression of individual viral proteins such as core, NS2 or NS4B,[91-94] and its inhibition by 25-hydroxycholesterol causes a decrease in fatty acid biosynthesis and a subsequent block of HCV replication.[95] In agreement with this, the expression of fatty acid synthase (FASN) and other genes related to the synthesis and transport of fatty acids is upregulated in infected cells.[87,88] Moreover, the inhibition of FASN activity blocks HCV RNA replication and production of infectious virus particles.[95,96] Finally, expression of genes regulating geranylgeranylation of cellular proteins important for HCV replication is also upregulated in HCV-containing cells.[87]

Table 1. HCV-induced alterations in lipid metabolism

Lipid metabolism alteration	HCV protein(s)/condition	Reference
Upregulation of SREBP and FAS expression	Infection; NS2; NS4B	92–94
Activation of SREBP1c in the presence of proteasome activator PA28γ (upregulation of lipid biogenesis)	Core protein	91
Activation and upregulation of PPARγ expression	NS5A	176
Increase in expression of genes involved in metabolism and transport of sphingolipids, phospholipids and fatty acids; geranylgeranylation of host proteins; decrease in expression of genes involved in cholesterol biosynthesis	Infection	87
Increase in cholesterol and free fatty acids	Infection	88
Downregulation of PPARα expression	Infection; core protein	87, 88, 98
Decrease in expression of genes involved in degradation and oxidation of fatty acids	Infection	89
Inhibition of AMP-activated protein kinase (decrease in fatty acid oxidation)	Infection; nonstructural proteins	89
Increase in fatty acid oxidation	Infection	86
Recruitment of PI4K-IIIα into the MW (alteration of PIP distribution and amounts)	Infection; NS5A	81, 131, 133
Intracellular accumulation of LDs	Infection; core protein	22, 28, 177
Rearrangement and morphological changes in LDs	Infection; core protein	152, 153, 178, 179
Inhibition of MTP activity (reduced assembly and secretion of VLDL)	Infection, core protein	180–182
Mitochondrial dysfunction and generation of reactive oxygen species (protein and lipid peroxidation)	Infection; core protein; NS5A	183, 184

Different results have been reported on how the genes controlling the oxidation of fatty acids are regulated. Several groups have shown that HCV infection leads to the reduction of β-oxidation by downregulating the expression of the transcription factor PPARα (peroxisome-proliferator-activated receptor α).[87,97,98] Moreover, the AMP-activated protein kinase (AMPK), a key regulator of lipid metabolism which augments fatty acid oxidation, appears to be inactive (dephosphorylated) in HCV-infected cells. Thus, inhibition of AMPK by HCV would favor a reduction in fatty acid oxidation and thus an increase in hepatic lipid accumulation.[89] In contrast, Diamond and co-workers reported a sustained increase in fatty acid oxidation upon HCV infection, but also a stimulation of lipid biosynthesis. Although the significance of this simultaneous induction in lipid anabolism and catabolism remains unclear, the authors argue that β-oxidation may help to maintain elevated ATP levels to support energy-dependent biosynthetic processes as well as to limit the accumulation and toxic effect of ceramide overload.[86]

In the last years, the role of cholesterol in HCV infection has gained much attention. In mammalian cells, cholesterol can be either synthesized via the mevalonate pathway,[99] or generated by internalization and degradation of LDL particles.[100] There are still controversial opinions about which step(s) of the HCV replication cycle requires cholesterol. The discrepancies are probably due to the use of different drugs and their mechanism of action. For instance, statins might inhibit HCV replication by preventing geranylgeranylation of the host factor FBL2 that is required for RNA replication.[101] In addition, methyl-β-cyclodextrin (MβCD), which depletes cholesterol without interfering with geranylgeranyl synthesis, might cause mislocalization and decreased surface expression of CD81 without affecting RNA replication or virus production.[102,103]

It was demonstrated that NS proteins associate with cholesterol-rich lipid rafts and that depletion of cholesterol with statins selectively reduces HCV RNA replication.[104] However, statins are inhibitors of the mevalonate pathway,[99] which produces not only cholesterol, but also non-sterol isoprenoids, including geranylgeranyl and farnesyl. Importantly, the restoration of replicon RNA levels upon addition of geranylgeraniol in cells treated with lovastatin, and the fact that inhibition of geranylgeranylation, but not farnesylation blocks viral replication, strongly support the involvement of geranylgeranylated protein(s) in HCV replication.[101] One candidate is FBL2, a ubiquitously expressed protein that belongs to the family of F-box proteins.[101] FBL2 interacts with NS5A in a geranylgeranylation dependent manner.[101] Geranylgeranylation is specific for proteins containing a C-terminal *CAAX* motif (i.e., Cysteine-A-A-X, where A is an aliphatic aa and X is leucine or isoleucine) and the modification frequently contributes to membrane association of the respective protein.[105] Accordingly, geranylgeranylation is assumed to target FBL2 to the membranes where NS5A resides, thus facilitating protein-protein interaction.[101] Although FBL2 is essential for HCV replication in cell culture, the mechanism by which it contributes to replication is still undefined. However, FBL2 contains a F-box motif promoting interaction with an ubiquitin ligase complex.[106] Thus, FBL2 might be involved in ubiquitin-mediated degradation of the NS5A-containing protein complex or its intracellular transport.

Palmitoylation is another post-translational lipid modification potentially involved in HCV replication. Indeed, it has been reported that individually overexpressed NS4B is palmitoylated at cysteine residues 257 and 261 located in its C-terminal part. Palmitoylation of Cys 261 was reported to

facilitate oligomerization of NS4B and its interaction with NS5A.[107] Since NS4B oligomerization is required for the formation of the MW and a functional replication complex,[53] NS4B palmitoylation could also be involved in HCV RNA replication.[107] However, further studies will be needed to confirm the role of NS4B palmitoylation in the context of an HCV infection.

Biogenesis of the Membranous Web: Remodeling of Intracellular Membranes by NS4B

Infection by many positive strand RNA viruses induces a dramatic remodeling of intracellular membranes generating specialized compartments where RNA replication takes place.[81,108] The origin of these membranes varies depending on the virus and they might be derived from e.g., the ER (Dengue virus),[109] the outer mitochondrial membrane (Flock house virus),[110] or the plasma membrane (alphaviruses).[111,112] It is assumed that the specific protein and lipid composition of these membranes play distinct roles in the replication cycle of a given virus. In case of HCV, a MW is induced containing, amongst others, double-membrane vesicles (DMVs) that are closely connected to ER membranes and that contain the NS proteins and the viral RNA.[50,113,114] Expression of NS4B triggers formation of membranous vesicles by an as yet unknown mechanism.[50] Nevertheless, based on NS4B structural data and similarities with other proteins inducing membrane alterations, several hypotheses can be put forward to explain the origin of the MW and how NS4B alters cellular membranes.

(1) NS4B may promote membrane curvature (a requisite for formation of membrane vesicles) by oligomerization.[115] In this case membrane bending might be induced by amphipathic α-helices located in the N-terminal region, which was found to translocate through the membrane post-translationally.[49] This asymmetric partitioning of amphipathic α-helices into the luminal leaflet of the membrane bilayer might induce membrane curvature triggered by the asymmetric distortion of the bilayer. Alternatively, by analogy to the E viroporin of the mouse hepatitis coronavirus, another oligomeric protein which possesses an amphipathic α-helix, NS4B might form membrane-integral pores inducing membrane curvature.[116,117]

(2) The recruitment of cell factors such as Arfs (ADP-ribosylation factors), which contain an N-terminal amphipathic helix or proteins containing BAR (Bin-Amphiphysin-Rvs) domains with a characteristic banana-like structure, might deform membranes.[118,119]

(3) The lipid composition of the membrane itself might directly trigger membrane curvature.[115] As described in detail below (see section 6), the membranous web is highly enriched in specific phosphatidylinositol (PI) species,[81] which can induce membrane curvature by different mechanisms.[115,120] Moreover, the fact that NS proteins are recruited to cholesterol-rich lipid rafts, likely by interacting with NS4B, and that replication complexes remain active after treatment with detergents, indicates enrichment of lipid rafts that might also contribute to vesicle formation.[104]

(4) DMVs might be induced, at least in part, by autophagy. Autophagosomes, which engulf long-lived proteins and damaged organelles, transport them to the lysosomes for degradation.[121] The origin of the autophagosomal membrane is still under debate and various sources including mitochondria, the Golgi apparatus and the ER have been proposed.[122] Autophagy can be activated in response to infection, starvation and different stress conditions.[123,124] Importantly, proteins of the autophagy pathway have recently been implicated in the HCV replication cycle. However, the lack of colocalization between HCV proteins and autophagosomal markers suggests that the viral replication complex does not assemble on autophagosomes.[125,126] Nevertheless, HCV might exploit certain components of the autophagy machinery to generate DMVs with a specific protein composition. In fact, it has been suggested that HCV prevents the degradation of long-lived proteins, by blocking the fusion between autophagosomes and lysosomes, thus leading to the accumulation of DMVs required for HCV RNA replication.[127] However, the literature about HCV and autophagy is very controversial and it is not clear to which step of HCV life cycle autophagy might contribute. For instance, it has been reported that autophagy contributes to RNA translation,[126] RNA replication,[126-128] or particle formation.[125] These discrepancies could be due, at least in part, to diversities of employed technical procedures such as cell lines, systems to introduce the viral RNA into cells (infection vs. transfection) or silencing conditions. Further studies are therefore required to define the contribution of autophagy to the HCV replication cycle.

In conclusion, MW formation might be induced by one of the mechanisms described above or a combination thereof, including a direct physical role of NS4B, but also recruitment of cellular factors altering membrane lipid composition or inducing membrane curvature directly.

Role of Phosphatidylinositol Phosphates and Phosphatidylinositol Phosphate-Binding Proteins in the HCV Replication Cycle

Apart from lipid metabolism, HCV infection alters the subcellular distribution of phosphatidylinositol phosphates (PIPs).[81,129] In eukaryotes, differential phosphorylation of PI on three of the five hydroxyl groups of the inositol ring (3', 4' and 5') results in the formation of seven different PIP species [PI(3)P, PI(4)P and PI(5)P; $PI(3,4)P_2$, $PI(3,5)P_2$ and $PI(4,5)P_2$ as well as $PI(3,4,5)P_3$]. The addition or the removal of phosphate groups at specific positions of the inositol ring is an extremely dynamic process catalyzed by specific PI kinases and phosphatases, respectively. Such enzymes are highly compartmentalized within the cell. Their activities and abundance differ locally, resulting in different concentrations of each of the seven PIPs in a specific compartment. Indeed each intracellular membrane can be defined by its prominent PI species, the so called "PI-signature". For instance, $PI(4,5)P_2$ is enriched at the plasma membrane; PI(4)P at the Golgi complex and PI(3)P at the early endosomes.[130] Similar to other positive strand RNA viruses,[81] HCV increases intracellular PI(4)P levels, presumably at the MW. It has therefore been suggested that viruses falsify the "PI-signature" of cellular

membranes.[81,131] Two cellular PI kinases are predominantly, if not exclusively, involved in this process: the ER-resident PI4K-IIIα and the Golgi-localized PI4K-IIIβ.[132] The importance of PI4K-IIIα for HCV replication is well established, because this kinase has been identified in several siRNA-based screens as one of the top hits, regardless of the viral genotype and the experimental system.[133-138] In contrast, the role of the Golgi-resident PIK4-IIIβ is still controversially debated; it appears to contribute to replication of genotype 1 replicons, but not to replication of the JFH-1 genotype 2a isolate.[131,135,136] Nevertheless, the two isoforms of the kinase are believed to perform complementary, non-redundant tasks, because silencing of each gene decreases the levels of PI(4)P in HCV replicon cells,[81] and because the inhibitory effect mediated by PI4K-IIIα knockdown on HCV replication cannot be rescued by overexpression of PIK4-IIIβ.[137]

A recently proposed model hypothesizes that during HCV infection, NS5A recruits PI4K-IIIα to the MW to increase local levels of PI(4)P necessary for MW integrity and hence viral replication.[129,131] This hypothesis is mainly based on three evidences. First, PI4K-IIIα colocalizes with both NS5A and double-strand (viral) RNA upon JFH-1 infection.[133] This recruitment is dependent on NS5A-D1, which is also sufficient to bind to PI4K-IIIα.[131] Second, NS5A can stimulate PI4K-IIIα activity in vitro.[131] Third, inhibition of PI4K-IIIα by RNAi alters both NS5A subcellular localization and MW architecture,[131,137] and eventually also its formation.[133]

What could be the role of elevated PI(4)P levels in the HCV replication compartment? In one scenario the specific PIP composition might directly influence membrane bending and thus contribute to membrane curvature necessary for MW formation.[120] In a second scenario PIPs might regulate intracellular processes including vesicle fusion, budding and sorting required for MW formation.[132] In fact, individual PIPs are recognized by distinct effector proteins via highly conserved, specialized domains, including the pleckstrin homology (PH), the PHOX homology as well as the FYVE, ENTH and ANTH domains.[130] Two proteins recently implicated in the HCV replication cycle can specifically recognize PIPs: first, Annexin AII, which is important for virus assembly and specifically binds $PI(4,5)P_2$;[139] second, oxysteroyl binding protein (OSBP), which appears to be essential for both HCV RNA replication and production of infectious virus, and which specifically recognizes PI(4)P via a PH domain.[140] OSBP and its related proteins have been implicated in regulating numerous cellular processes, which play a central role in HCV life cycle, such as sphingomyelin synthesis,[141] autophagy,[142] and LD biogenesis.[143]

In addition, OSBP contains a FFAT motif, conferring the ability to bind to the ER resident VAP (VAMP associated proteins) family of proteins.[140] VAPs represent another group of host cell factors whose involvement in the HCV replication cycle is well documented. This family comprises three type II integral ER membrane proteins: VAP-A, VAP-B and VAP-C.[144] They are capable of interacting with the SNAP receptor and FFAT-containing proteins such as OSBP or ceramide transfer protein, thus contributing to membrane fusion and trafficking as well as lipid metabolism.[145] VAPs possess an N-terminal immunoglobulin like β-sheet, which binds to NS5B.[146] VAP-A and VAP-B also possess a centrally located coiled coil domain, which binds to NS5A and a C-terminal TM domain, involved in homo- and heterodimerization.[146-148] It has been reported that these two VAPs are required for HCV replication complex formation on lipid rafts by promoting macromolecular complexes between NS5A, NS5B and NS4B.[146] In addition, VAP-A and VAP-B appear to be responsible for the recruitment of NS5B to detergent-resistant membranes.[146-148] This hypothesis is supported by the aberrant fractionation of NS5B in detergent soluble membranes and by the decreased HCV replication upon VAP-A and -B silencing or overexpression of dominant-negative VAPs.[146-150] Whether VAP-C also contributes to HCV replication remains to be confirmed,[149] as this protein is not expressed in liver tissue.

The VAP-A-NS5A interaction appears to depend on the phosphorylation status of the viral protein. Hypophosphorylation of NS5A correlates with its ability to bind to VAP-A and to promote efficient viral RNA replication, whereas hyperphosphorylation of NS5A leads to inefficient VAP-A-NS5A interaction and impairs RNA replication.[64] Mutations enhancing RNA replication decrease NS5A hyperphosphorylation and thus enable VAP-A binding.[64] In agreement with this assumption, pharmacological inhibition of NS5A hyperphosphorylation that is mediated by CK1, enhances RNA replication.[61,66,151] Point mutations preventing NS5A hyperphosphorylation enhance RNA replication, but strongly impair virus production, arguing that NS5A phosphorylation status regulates the switch between RNA replication and assembly.[60]

How could this regulation be achieved? HCV assembly is a process thought to occur on the surface of core-decorated LDs,[82] where NS5A is believed to 'deliver' newly synthesized viral genomes to trigger nucleocapsid formation.[24] It is therefore plausible that hyperphosphorylation might release NS5A from the replication complex because of dissociation from VAP-A. However, this hypothesis is challenged by the observation that mutations in NS5A selectively impairing its hyperphosphorylation and virus assembly do not prevent NS5A localization to LDs.[59,60] Alternatively, NS5A hyperphosphorylation might release VAPs that in turn could contribute, together with PI(4)P to recruitment of OSBP. Obviously, further studies will be required to define the exact role of VAPs and NS5A phosphorylation status for HCV replication and assembly.

HCV Assembly and Release

A prerequisite for HCV assembly is the proteolytic processing of core protein by signal peptide peptidase cleaving the C-terminal transmembrane segment that serves as signal sequence of E1. This cleavage triggers the translocation of core onto the surface of LDs,[23] and subsequent displacement of the LD-resident Adipose Differentiation Related Protein (ADRP).[152] This exclusion process leads to degradation of ADRP and microtubule-dependent transport of LDs towards the microtubule organizing center, which is in close proximity of HCV replication sites.[152-154] A cellular enzyme that is important for LD biogenesis has recently been implicated in the targeting of core to LDs. Both genetic

and pharmacological ablation of diacylglycerol acyltransferase-1 (DGAT-1) prevented HCV particle production without affecting viral replication arguing that assembly requires DGAT-1 mediated LD formation.[155]

NS5A is also targeted to the surface of LDs and presumably via a core—NS5A interaction the early step of assembly is triggered.[59] Indeed, point mutations interfering with core—NS5A co-localization at LDs strongly impair virus production.[24,59,156] Moreover, a comparative study of different HCV genomes revealed a correlation between LD localization of core and assembly efficiency. Mutations enhancing the production of infectious virus particles decreased the amount of core protein on LDs supporting the notion that LDs serve as platforms for HCV assembly.[82]

In the last few years, several host cell factors required for infectious HCV particle production have been described, including OSBP, Annexin AII and the heat shock cognate protein 70 (Hsc70).[157] The latter has been identified as a component of virus particles produced in cell culture. Surprisingly, Hsc70-specific antibodies can neutralize infectivity arguing that at least a fraction of this protein is accessible on the surface of virus particles.[157] In addition, Hsc70 appears to be required for LDs integrity because their volume was reduced upon silencing of Hsc70 expression.[157]

It is well established that apolipoproteins (Apo) play a key role in HCV assembly and release. RNAi-mediated knockdown of ApoB and ApoE as well as pharmacological inhibition of microsomal triglyceride transfer protein (MTP), an enzyme crucial for ApoB maturation and VLDL secretion, have been proven effective in inhibiting HCV production in Huh7 cells, suggesting that secretion of HCV relies on the VLDL pathway.[83,158-162] These results raise the question of how VLDL is formed and what the link is to HCV assembly. In hepatocytes, free fatty acids are converted to triacylglycerols (TGs) and incorporated into three different structures: the cytoplasmic lipid droplets (here simply referred to as lipid droplets; LDs); ApoB-containing precursors of VLDL (pre-VLDL particles or VLDL2); and luminal ApoB-free lipid droplets (luLDs). LDs and luLDs are important for the transfer of TGs to VLDL2.[163] They are composed of a core of neutral lipids and surrounded by a monolayer lipid membrane. Although LDs and luLDs share a very similar lipid composition, they are decorated with different sets of proteins, regulating their size, mobility and localization:[152] ADRP and the 47 kDa tail interacting protein (TIP47) are found on the surface of LDs, whereas MTP, ApoE and TG hydrolase cofractionate with luLDs, but not with LDs.[164] TGs stored in LDs can be hydrolyzed by cellular lipases and the lipids released can be either targeted to the mitochondria for β-oxidation or re-esterified in the ER lumen,[165] where MTP can either use them to produce a VLDL2 particle or luLDs. By fusion of VLDL2 with luLDs, a mature VLDL particle is generated when VLDL2 acquires the exchangeable apolipoprotein ApoE by fusion with luLDs.[166] Mature VLDL is transported to the Golgi and finally secreted.[163]

By using mass spectrometry analyses of affinity purified HCV particles produced in cell culture, it was found that the lipid composition of such particles is similar to that of VLDL.[167] This result is in keeping with the notion that assembly and release of HCV particles are tightly linked to host cell lipoproteins and lipids.[162,168-172] Moreover, the nature of the lipidic constituents of HCV particles appears to depend on the host cell in which the virus is produced.[173] This is best illustrated by the observation that cell culture grown HCV (HCVcc) produced in the human hepatoma cell line Huh7 differs from HCV circulating in patient serum as so-called lipoviro particles (LVPs). Both particle types have a heterogeneous density and in both cases density correlates inversely with infectivity. However, the average density of HCVcc is higher than that of LVPs (1.1 g/ml vs. ~1.05 g/ml, respectively) arguing for differences in lipid and lipoprotein contents. Importantly, infection of chimpanzees or mice containing human liver xenografts with (high density) HCVcc resulted in the production of low density particles. Importantly, HCV produced in primary human hepatocytes also has a lower density as compared to HCVcc, closely resembling that of LVPs.[173] Another difference between HCVcc and LVPs relates to their propensity to associate with ApoB. While HCV particles present in infected livers can be immunoprecipitated with ApoB-specific antibodies, this capture is inefficient in case of Huh7-derived HCVcc. However, both kinds of particles are tightly associated with other lipoproteins, most notably ApoE and ApoC1.[174,175] These discrepancies are probably due to a defect of Huh7 cells to secrete ApoB-containing VLDL particles,[173] supporting the notion of 'lipid imprinting' of HCV particles by the host cell.

Conclusions and Perspectives

The development of a fully permissive HCV cell culture system has provided new insights into the molecular mechanisms underlying the various steps of the viral replication cycle. However, many aspects are still unclear. For instance, what is the origin of the membranous web? Which cellular and viral factors contribute to its formation and regulate virus replication? What is the exact mechanism by which nucleocapsids assemble and how does the virus utilize the VLDL pathway? The growing knowledge about the link between HCV and host cell lipid metabolism led to the identification of a number of components contributing to viral replication, and at the same time helped to elucidate uncharacterized aspects of lipid biosynthetic pathways, such as VLDL assembly/release. Noteworthy, the important role of PIPs and PI4-kinases in HCV RNA replication will encourage new studies about the mechanisms underlying viral dependence on these phospholipids. Moreover, the design of new specific inhibitors targeting PI4-kinases could provide a new therapeutic alternative against HCV. In this respect, drugs targeting VLDL assembly and secretion, including MTP inhibitors, offer an interesting strategy for treatment of chronic hepatitis C and perhaps at the same time steatosis. Clearly, more studies are required to determine the potential of such compounds to arrest HCV infection without inducing toxic effects in patients.

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