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Hepatitis C virus entry into the hepatocyte

Mini-Review

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Abstract: Hepatitis C virus (HCV) is a small enveloped virus with a positive stranded RNA genome belonging to the *Flaviviridae* family. The virion has the unique ability of forming a complex with lipoproteins, which is known as the lipoviroparticle. Lipoprotein components as well as the envelope proteins, E1 and E2, play a key role in virus entry into the hepatocyte. HCV entry is a complex multistep process involving sequential interactions with several cell surface proteins. The virus relies on glycosaminoglycans and possibly the low-density lipoprotein receptors to attach to cells. Furthermore, four specific entry factors are involved in the following steps which lead to virus internalization and fusion in early endosomes. These molecules are the scavenger receptor SRB1, tetraspanin CD81 and two tight junction proteins, Claudin-1 and Occludin. Although they are essential to HCV entry, the precise role of these molecules is not completely understood. Finally, hepatocytes are highly polarized cells and which likely affects the entry process. Our current knowledge on HCV entry is summarized in this review.

Keywords: *Hepatitis C virus • Virus entry • Viral receptor • Membrane proteins*

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1. Introduction

Hepatitis C is a global health problem. Hepatitis C virus (HCV) infects approximately 130 millions individuals worldwide with the majority remaining undiagnosed and untreated. In most infected individuals, the virus evades the immune system and establishes a chronic infection. As a consequence, hepatitis C is the leading cause of cirrhosis, end-stage liver disease, hepatocellular carcinoma and liver transplantation. To this day, there is no vaccine and the available treatment, a combination of pegylated α-interferon and ribavirin has a limited efficacy and significant side effects. However, specific direct-acting antivirals such as protease and polymerase inhibitors are under development and two NS3/4A protease inhibitors have already been approved for use in the USA.

HCV belongs to the genus Hepacivirus in the *Flaviviridae* family. It is a small enveloped virus with a positive sense single stranded RNA genome of 9.6 Kb. The genome is translated as a polyprotein of ~3000 amino acids, which is processed during translation by cellular and viral proteases to generate the structural (capsid, E1 and E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) [1]. The structural proteins are components of the viral particle which has the peculiar feature of being associated with low- or very low-density lipoproteins (LDL or VLDL) [2].

For many years, the study of the HCV life cycle has been impaired by the inability to efficiently grow HCV in cell culture. However, in recent years, several major advances have facilitated research on HCV. Among them, retroviral particles pseudotyped with HCV envelope glycoproteins, E1 and E2, (HCVpp) were the first robust *in vitro* assay for the functional study of HCV entry [3]. Following that, a major breakthrough in HCV research was the development of a cell culture system that allowed for the production of infectious particles (HCVcc) [4].

Hepatocytes are major target cells of HCV infection. The liver is a complex organ, containing at least a

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dozen different cell types. However, 70 to 80% of the liver cell population is composed of hepatocytes that are responsible for the major liver functions such as protein synthesis, lipid and carbohydrate metabolism, bile synthesis/secretion and detoxification. To function in this capacity, hepatocytes express specific genes and their functionality requires polarization with separated apical and basolateral membrane domains, which differ in their protein and lipidic composition. It is therefore very likely that the specific architecture of the liver plays a major role in HCV propagation within this organ.

The viral entry process relies on a fine interplay between the virion and the host cell. Infection is initiated by the interaction of the viral particle with specific proteins on the cell surface. These proteins belong to two general categories which are classified based on the functional consequences of their interaction: attachment factors and receptors. Attachment factors serve to bind and concentrate particles at the cell surface. Usually, these interactions have a rather low specificity. On the other hand, receptors actively promote virus entry by inducing conformational changes of the viral glycoproteins and/or by activating signaling pathways necessary for internalization of the virion. Currently, HCV entry is viewed as a complex multistep process as at least four specific cellular factors have been shown to be essential for HCV entry. These molecules are the scavenger receptor class B type I (SRB1), the tetraspanin CD81 and tight junction proteins, Claudin-1 (CLDN1) and Occludin (OCLN) (reviewed in [5]).

In this review, we will first address what we know of the composition of the virion that may be of significance for viral entry, then the host entry factors required for HCV entry and we will summarize current knowledge of the mechanisms of HCV entry.

2. Viral particle

The HCV RNA genome, core and envelope glycoproteins, E1 and E2, are the known viral components of the virion. The HCV genome interacts with the core to form the nucleocapsid that is surrounded by a host derived lipid membrane, called the viral envelope, in which are anchored the envelope glycoproteins. HCV envelope glycoproteins are key determinants of HCV entry with a role in receptor binding and in mediating the fusion process between the viral envelope and an endosomal host cell membrane. Their biogenesis has been extensively characterized in recombinant systems [6]. E1 and E2 are heavily N-glycosylated type I transmembrane proteins which form a noncovalent heterodimer. However, it has recently been shown that HCV envelope glycoproteins form large covalent complexes stabilized by disulfide bonds on secreted viral particles [7]. The presence of disulfide bridges between HCV envelope glycoproteins suggests that lateral protein-protein interactions assisted by disulfide-bond formation might play an active role during the budding process of HCV particles. Furthermore, functional non-infectious and capsidless structures, called subviral particles, can be produced when the HCV envelope glycoproteins are expressed alone in lipoprotein producing cell lines [8], supporting the idea that HCV envelope glycoproteins play an active role during the budding process.

The secondary and tertiary structures of envelope glycoproteins are proposed to be similar among the members of the *Flaviviridae* family, suggesting that HCV envelope glycoproteins belong to class II fusion proteins [9,10]. Recently, the identification of 9 intramolecular disulfide bonds in soluble E2 has shed light on its structure [11]. By compiling these results with various data relevant to E2 structure and functionality, Krey *et al*. proposed a model for the tertiary organization of E2, which is consistent with the structure of class II fusion protein (Figure 1). This model revealed the distribution of E2 amino acids among three different domains (DI, DII and DIII). The DI domain consists of eight β-strands and is extended on the N-terminus by hypervariable region 1 (HVR1). This domain, which contains determinants for CD81 interaction, has recently been functionally confirmed [10]. The DII domain includes hypervariable region 2 (HVR2) and its most conserved part is suggested to act as a fusion loop (a.a. 502-520) that inserts into the target membrane during the first step of membrane fusion. DI is connected to the DIII domain by a linker region called inter-genotypic variable region (IgVR). Finally, DIII is connected to the transmembrane domain by the flexible stem region. This latter region is potentially involved in envelope protein heterodimerization as well as in virus entry [10,12]. An amphipathic helix, which likely folds upon membrane binding has been identified in the C-terminal part of the stem region and it has been proposed that this region might be involved in the reorganization of glycoprotein complexes taking place during the fusion process [10].

Among the most variable regions, HVR1 has been shown to be essential for the interaction of HCV glycoprotein E2 with the known HCV receptor SRB1 [13]. Although they have an attenuated phenotype, most viruses lacking HVR1 remain infectious [14]. Furthermore, it has been proposed that this region obstructs the viral CD81 binding site as well as conserved neutralizing epitopes [14,15], potentially playing a role in some aspects of HCV immune evasion.

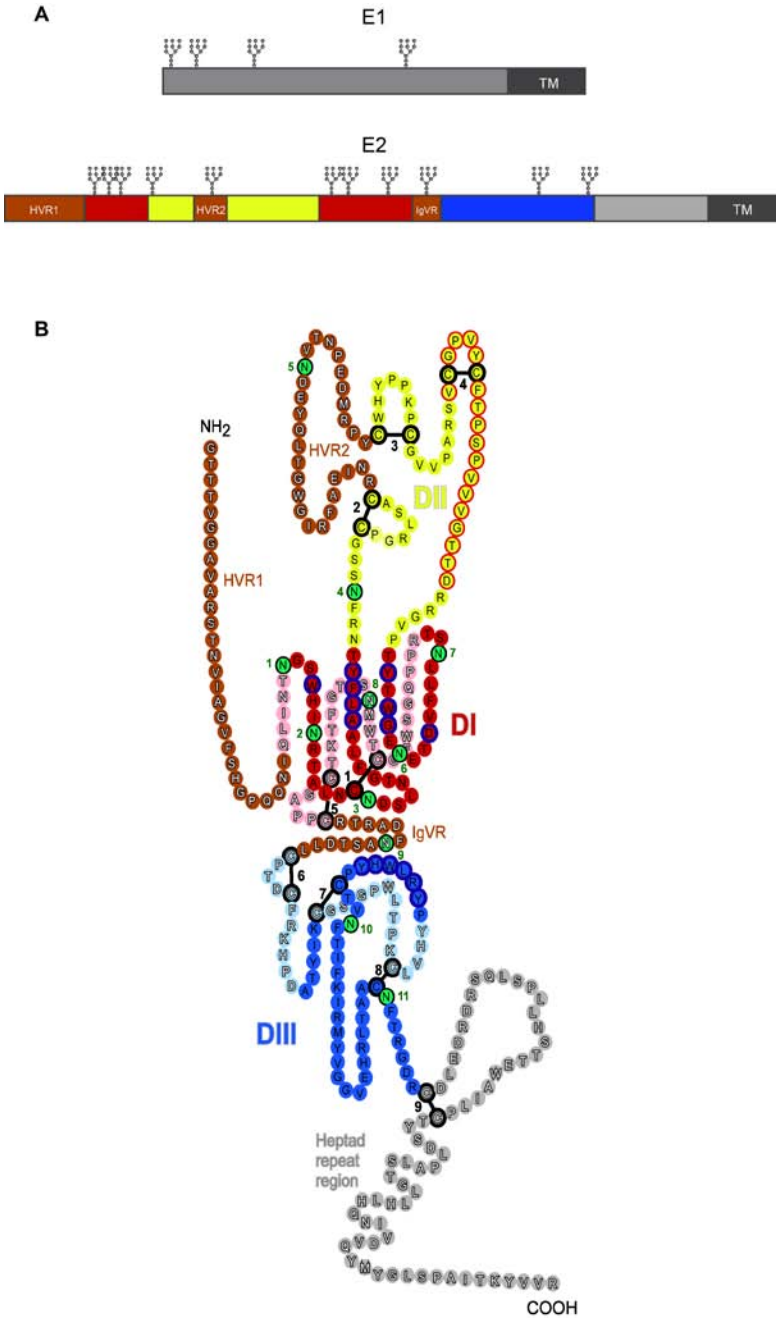

Figure 1. HCV envelope glycoproteins. (A) Schematic representation of HCV envelope glycoproteins E1 and E2. The transmembrane domains (TM) are indicated at the C-terminus of the proteins and the position of the glycosylation sites shown in the ectodomains. For E2 glycoprotein, hypervariable region 1 (HVR1) and 2 (HVR2) and intergenotypic variable region (IgVR) are indicated. Furthermore DI, DII and DIII domains and the stem regions are shown in red, yellow, blue and grey, respectively. (B) Model of E2 glycoprotein. The linear sequence of the E2 ectodomain (JFH1 strain; GenBank access number AB237837) is represented as a chain of beads (colored circles) labeled with the corresponding amino acid and threaded onto a class II fold, which is an adapted version of the model recently published by Krey *et al.* [11] (doi: 10.1371/journal.ppat.1000762.g006). The three putative domains are presented in red (DI), yellow (DII) and blue (DIII), and the variable regions (HVR1, HVR2 and IgVR) are indicated in brown, whereas the stem region is shown in grey. Circles in pale and bright colors represent residues in the background and foreground of the domains, respectively labeled in white and black fonts. Disulfide bonds are indicated by black bars. Glycosylation sites are shown by green circles numbered sequentially. DI domain residues that participate in CD81 binding are contoured in blue.

Surprisingly, other non-conserved regions like HVR2 and IgVR are essential for the assembly of functional E1E2 heterodimers [10,16,17].

HCV envelope glycoproteins, E1 and E2, contain up to 5 and 11 N-glycosylation sites, respectively. Most HCV glycans are highly conserved [16] and some have

been shown to play a role in virus assembly or entry [18]. Furthermore, they form a glycan shield which protects the virus from antibody neutralization [18].

HCV virions isolated from patients display a broad range of buoyant densities and are associated with host lipoproteins and antibodies. Virions and lipoproteins are believed to form complexes of very-low to low densities, called lipoviroparticles [19]. The exact nature of HCV association with lipoproteins remains elusive, yet it has been clearly demonstrated that infectivity is correlated with virus density with the highest infectivity being found in the lowest density fractions. A recent characterization of cell culture-produced particles indicates that their composition resembles the one of VLDL and LDL with cholesteryl esters accounting for almost half of the total HCV lipids. Thus, HCV particles possess a unique lipid composition that is very distinct from all other viruses analyzed so far [20]. Importantly, changes in the nature of HCV-associated lipoproteins by lipoprotein lipase affect HCV infectivity, suggesting that association of HCV with specific lipoproteins is important for infectivity [21,22]. It has also been shown that the HCV assembly pathway depends on components of the VLDL synthesis pathway. The contribution of different VLDL pathway components is still under debate. Some groups have found apoB and microsomal triglyceride transfer protein as major contributors while others indicate that apoE plays a more predominant role. Some explanations and models to accommodate the apparent conflicting data have recently been proposed [2]. ApoE is a ligand for two molecules that are involved in HCV infection: the low-density lipoprotein receptor (LDLR) and SRB1. Antibodies directed against apoE inhibit HCV infection [23,24], thus supporting a role for apoE in HCV entry. Furthermore, the apoE isoform associated with HCV particles has been shown to affect the level of virus infectivity [24]. The apoE gene is indeed polymorphic with different alleles leading to the production of 3 different isoforms, apoE2, apoE3 and apoE4. These isoforms show different affinities for LDLR, with apoE2 having a very low affinity for this receptor. Infectivity of virions produced in apoE knockdown cells could be properly restored by apoE3 and apoE4 expression but only very poorly by apoE2 [24]. Among other apolipoproteins, apoC1 is another factor that could also potentially play a role in HCV entry [25].

3. Receptors

3.1 SRB1

SRB1 is a major receptor for high-density lipoproteins (HDL). In addition, other ligands for SRB1 have been reported to include native and modified lipoproteins and modified serum proteins. This receptor plays a crucial role in selective lipid uptake and bidirectional transfer of free cholesterol. SRB1 contains two membrane spanning sequences flanking a large N-glycosylated extracellular loop and two short intracellular domains (Figure 2).

The role of SRB1 in HCV entry was first suggested by its ability to mediate soluble E2 binding [13] and it was later confirmed by the inhibition of HCV infection with anti-SRB1 antibodies and by silencing of the protein in hepatoma cells (reviewed in [5,26,27]). SRB1 residues potentially involved in E2 glycoprotein binding have recently been identified in its ectodomain [28]. Indeed, amino acids 70-87 and the residue E210 are required for E2 recognition. Alternative splicing of the transcript leads to the production of SRB2 which differs from SRB1 only in its C-terminal cytosolic domain. The SRB2 C-terminal domain is reported to mediate rapid internalization and intracellular localization of the protein. The C-terminus of SRB1 has recently been shown to play a role in HCV entry [29]. Deletion of this domain or introduction of the endocytosis motif of SRB2 into SRB1 impairs HCV entry. SRB1 is known to interact with the cytoplasmic adaptor molecule PDZK1 *via* an AKL motif in its C-terminal cytosolic tail. This interaction is involved in SRB1 stability in hepatocytes and it has recently been shown that PDZK1 plays an indirect role in HCV entry *via* its ability to interact with SRB1, which leads to an enhancement of its activity as an HCV entry factor [30].

Some SRB1 ligands are known to affect HCV infection (reviewed in [5]), notably HDL enhances infection by accelerating viral entry. This mechanism depends not only on the lipid transfer activity of SRB1, but also on the presence of CD81 [31], suggesting that an interplay between SRB1 and CD81 occurs during HCV entry. It is also possible that lipid transfer by SRB1 changes the membrane lipid composition, which in turn may affect HCV infection. In addition to the direct binding of soluble E2 to SRB1, it has also been suggested that the virus can bind SRB1 *via* its lipoprotein component [32]. Together with the observation that it interacts with HCV glycoprotein E2, the role of SRB1 in lipid metabolism has suggested that this entry factor may play a dual role during the early steps of the HCV life cycle [28,31]. More recently, the role of SRB1 in HCV uptake has been confirmed *in vivo* in a genetically humanized mouse model for HCV infection [33].

3.2 CD81

CD81 is a member of the tetraspanin family. Like the other members of this family, it contains 4 transmembrane

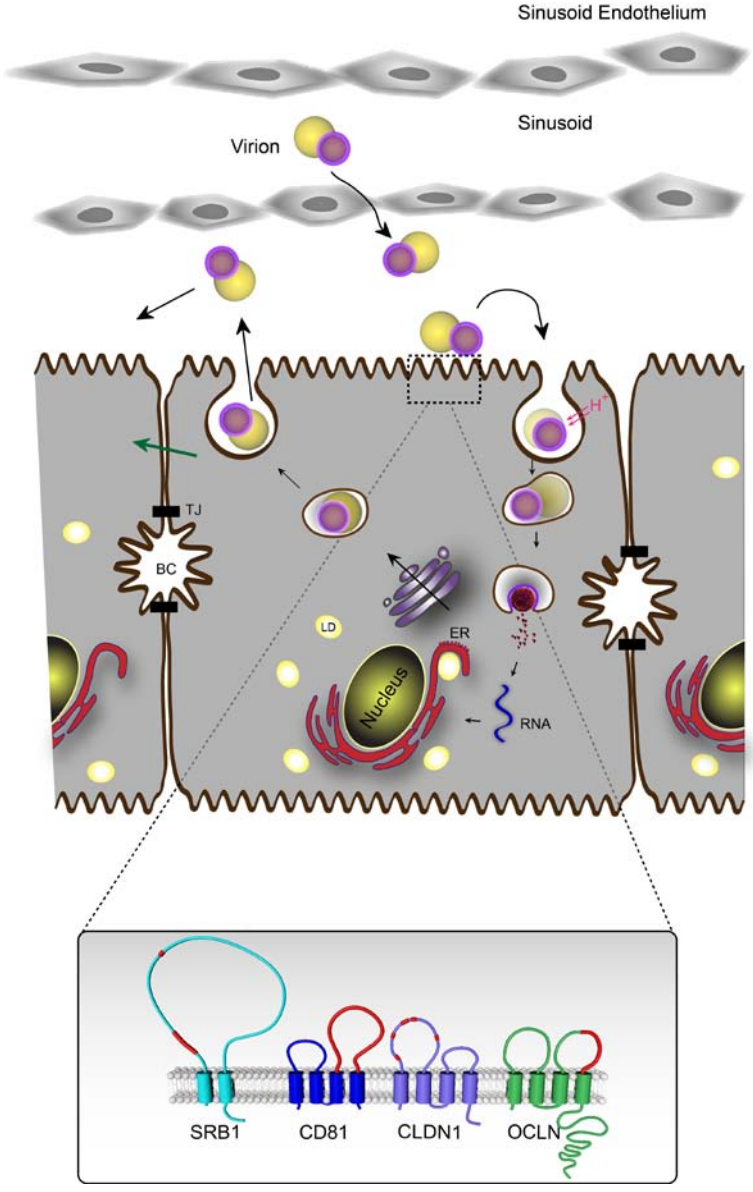

Figure 2. Schematic representation of HCV life cycle. HCV transported in the blood stream comes in contact with hepatocytes in the space of Disse after crossing the sinusoid endothelium. HCV attaches to cells and HCV entry is assisted by at least four cellular entry factors: the scavenger receptor SRB1 (light blue), the tetraspanin CD81 (dark blue) and the tight junctions (TJ) proteins: Claudin-1 (CLDN1; purple) and Occludin (OCLN; green). For each molecule, regions important for HCV entry are depicted in red (SRB1: amino acid 70-87 and E210, CD81 : the large extracellular loop, CLDN1: I32, D38, E48 and the conserved motif GLWxxC(8-10aa)C, OCLN: the second half of the second extracellular loop). The virus is internalized by clathrin-mediated endocytosis and fuses in early endosomes delivering its nucleocapsid into the cytosol. The genome of the virus is then released and translated to produce structural and non-structural proteins. The non-structural proteins are involved in viral genome replication. The mechanism of assembly is not completely understood but it is closely linked to endoplasmic reticulum (ER) and lipid droplets (LD). The lipoprotein-associated virus is then released in the surrounding medium to infect new cells. The virus can also be transferred directly to the neighboring cells by cell-to-cell transmission (green arrow). BC, bile canaliculi.

domains, a small (SEL) and a large extracellular loop (LEL) (Figure 2). Tetraspanins are involved in many cell functions such as adhesion, morphology, proliferation and differentiation. They are enriched in specific membrane microdomains called tetraspanin-enriched microdomains (TEMs), which differ from conventional raft domains, but the organization of which is dependent on cholesterol. Tetraspanins interact with partner proteins to form primary complexes which can interact together to form a network of interactions within TEMs.

The role of CD81 in HCV entry was first suggested after the demonstration that it interacts with a soluble form

of E2 [34]. Subsequently, it was extensively confirmed in HCVpp, HCVcc, HCV isolated from patient and in a humanized mouse model (reviewed in [5]). The CD81 binding site for E2 has been localized within the LEL [34] and characterization of chimeric proteins between CD81 and CD9, a related tetraspanin, confirmed that the LEL sequence is the region of CD81 defining HCV entry [35], with the binding site for E2 having been mapped to the variable double-helix subdomain [36]. Several studies have also shown that cell susceptibility to HCV infection is closely related to CD81 expression level [37-39]. Since tetraspanin molecules are known to interact with other membrane proteins, named partners, the involvement of CD81 partners in the modulation of HCV entry has been investigated. Indeed, the generation of a human cell line expressing murine CD81 (mCD81) permissive to HCV infection has allowed for the analysis of the role of TEM-associated CD81 in HCV infection [40]. The use of the MT81w antibody, which only recognizes TEM-associated mCD81 [41] and treatments with methyl-β-cyclo-dextrin or sphingomyelinase showed that TEM-associated CD81 is not preferentially used by HCV for its entry [40]. HCV entry is a multisequential process that is highly dynamic. CD81 association with TEM may lead to the sequestration of this tetraspanin which might impede its interaction with other coreceptors that are necessary for efficient HCV entry. Alternatively, tetraspanins may compete with coreceptors for their interaction with CD81. In addition, the lipid composition of TEMs might not be suitable for HCV entry.

A study of CD81-associated proteins led to the identification of EWI-2wint, a partner of CD81 that blocks E2 binding to CD81 [42]. EWI-2wint is a natural inhibitor that is produced following the cleavage of EWI-2, a major partner of CD81 that is expressed in most cells. EWI-2wint is notably absent in hepatocytes, enabling the binding of E2 to CD81 and allowing subsequent infection. In addition to the presence of specific entry factors, hepatotropism might be partly due to the absence of EWI-2wint. The regions involved in the interaction between EWI-2/EWI-2wint and CD81 have recently been identified, and it has been shown that EWI-2wint mutants, which no longer interact with CD81, are not able to exert their inhibitory effect on HCV infection [43].

3.3 Tight junction proteins CLDN1 and OCLN

Tight junctions (TJs) are membrane components specialized in sealing cells. They have the double function of controlling paracellular diffusion (barrier function) and restricting the diffusion of membrane proteins and lipids of the pole (fence function) [44]. In electron microscopy, TJs form a dense structure characterized by a close apposition of membranes of neighboring cells and a cytosolic plaque. Indeed, these structures are composed of transmembrane proteins and intracellular molecules. Transmembrane proteins play an important role in sealing the cells by interacting with similar molecules either in *cis* (on the membrane of the same cell) or in *trans* (on the membrane of facing cells). TJ functionality and architecture are regulated by signaling pathways that rely on the TJ cytosolic plaque. Transmembrane proteins interact with adapters of the cytosolic plaque that links the surface proteins to the actin cytoskeleton [44].

CLDN1 and OCLN are two transmembrane components of the TJs. CLDN1 is one of the twenty four members of the CLDN family [45] and evidence suggests that CLDNs constitute the backbone of tight junctions. These proteins consist of four transmembrane domains, two extracellular loops and two cytosolic domains. The C-terminus contains a PDZ-binding motif which binds to PDZ-containing plaque proteins such as the Zonula Occludens (ZO) proteins (Figure 2). The first extracellular loop (EC1) contains a conserved GLWxxC(8-10aa)C motif. OCLN was the first integral TJ protein to be identified and is a protein composed of four transmembrane domains, two extracellular loops, a short N-terminal cytoplasmic domain and a long C-terminal one (Figure 2). The first extracellular loop is rich (~60%) in glycine and tyrosine residues. The function of OCLN is not well understood but it may participate in the assembly and barrier function of TJs [45].

Recently, CLDN1 and OCLN have been identified as two essential entry factors for HCV [27,46,47]. CLDN1 was the first to be identified in a complementary DNA library screen for genes that confer HCVpp susceptibility to 293T cells [46]. Antibodies directed against CLDN1 were also shown to inhibit HCV entry [48]. Two other members of the CLDN family, CLDN6 and CLDN9, are also able to render 293T cells permissive for HCV entry [49,50]. The first extracellular loop of CLDN1 has been shown to be involved in HCV entry [46,51], and residues important for HCV infection have been identified in this domain (Figure 2). Indeed, mutations of the residues I32, D38 and E48 as well as in the GLWxxC(8-10aa)C conserved motif affect HCV entry [46,51]. Furthermore, it has been suggested that the second extracellular loop may also modulate CLDN1 function in HCV entry. The low efficacy of mouse CLDN1 in mediating HCV infection is linked to three amino acid residues in the second extracellular loop (L152, I155) and the fourth transmembrane helix (V180) [52]. In contrast, the C-terminal domain of CLDN1 is dispensable for HCV entry [46].

OCLN was the last entry factor to be identified, and is probably involved in a late post-binding step [47,53,54]. By using mouse and human OCLN chimeras, the species determinant for HCV entry was mapped to the second extracellular loop [55] (Figure 2). Furthermore, alanine-scanning mutagenesis of the two OCLN extracellular loops has revealed that the second part of the second loop is required for HCV entry. This region is flanked by two cysteines for which the mutation abolishes HCV infection, suggesting that this region might be folded by a disulfide bond. OCLN expression on hepatocytes as well as HCV entry is increased upon glucocorticoid treatment [56]. In contrast, OCLN expression is down-regulated upon HCV infection probably to prevent super-infection [54,57].

4. Entry mechanisms

In the liver, hepatocytes are organized in plates of single cell layers that are separated by sinusoid vessels. Endothelial cells and hepatocytes are separated by the space of Disse which drains lymph into the portal tract lymphatics (Figure 2). Basolateral domains of hepatocytes face the space of Disse whereas apical domains form tightly closed spaces called the canaliculi in which the bile is secreted. TJs act as a barrier between the two spaces and maintain the separation between both membrane domains maintaining their distinct molecular compositions. Cell polarity is achieved by a complex regulation of intracellular trafficking to deliver newly synthesized proteins to the suitable pole. It is believed that HCV enters the liver through blood flow and makes contact with hepatocytes at the basolateral membrane. In order to infect cells and propagate, the virus needs to overcome the complex organization of hepatocytes. The mechanisms leading to HCV entry into hepatocytes are still poorly understood. To date, accumulated data indicate that HCV entry is a complex multistep process involving numerous components as well as the constraints of hepatocyte polarity.

In vivo, HCV enters the liver through the sinusoidal blood. Before gaining access to the hepatocyte, this virus first needs to cross the sinusoidal endothelial cell barrier. C-type lectins have been proposed to play a role in assisting the virus cross the liver endothelium (reviewed in [5]), however, this remains to be demonstrated. HCV attachment to hepatocytes is likely first mediated by heparan sulfate proteoglycans (reviewed in [58]). Due to the association of the virion with lipoproteins, the LDLR has also been proposed to play a role in the early phase of HCV entry (reviewed in [5]). LDLR mediates internalization of low density lipoproteins that are released from the receptor upon pH decrease in endosomes. It has been shown that HCV infection is correlated with LDLR expression in hepatocytes and can be inhibited by LDLR ligands. It is therefore possible that the lipoviroparticles interact with LDLR through their apoB and/or apoE components, and that these interactions are likely modulated by the processing of LVP by LPL. Although recent data are in favor of the involvement of LDLR in the HCV life cycle [59,60], the role of this receptor in HCV entry remains unclear.

After binding to the cell surface with the help of heparan sulfate proteoglycans, the virus then interacts with specific entry factors. There is evidence that the E2 glycoprotein can interact with SRB1 and CD81. Recently, it has been proposed that HCV virions first interact with SRB1 [28]. This is in line with the observation that HCV virions can bind to CHO cells expressing SRB1, but not to cells expressing CD81 [46]. However, it remains to be determined whether SRB1 and CD81 form a co-receptor complex or whether the virus is transferred from SRB1 to CD81.

Currently, there is no evidence indicating that HCV particles directly interact with CLDN1 or OCLN. However, it has been shown that CLDN1 and CD81 interact in fluorescence resonance energy transfer (FRET) and fluorescence intensity ratio (FIR) assays [61,62], suggesting that CLDN1 may be a partner of CD81 in a HCV receptor complex. Concordant with this hypothesis, mutation of residues 32 and 48 in the first extracellular loop of CLDN1 that prevent HCV entry also abolish CLDN1-CD81 interaction [62]. Furthermore, antibodies directed against CLDN1 or CD81, inhibit HCV entry by perturbing CLDN1-CD81 interaction [62,63]. Recently, it has also been shown that epidermal growth factor receptor and ephrin receptor A2 act as host cofactors for HCV entry by regulating CLDN1-CD81 co-receptor association [64]. Analysis of CLDN1-CD81 interaction by surface plasmon resonance demonstrated a specific interaction between CLDN1 EC1 and CD81 LEL [62]. Finally, in polarized HepG2 cells, CD81 and CLDN1 association occurs at the basolateral domain of the cells in accordance with the virus coming in contact with the hepatocyte at the sinusoidal surface of the cells [62]. Although CLDN1 also interacts with OCLN, there is no clear relationship between CLDN1-OCLN association and HCV infectivity [62].

After initial binding to an attachment factor and prior uptake, viruses move laterally on the cell surface. These movements allow viruses to interact with secondary co-receptor(s) or with clathrin-coated pits. Single particle imaging during viral entry in Huh7.5 cells has revealed that HCV particles bind cells on filopodia and reach the

cell body by a mechanism that relies on retrograde actin transport [65]. On the cell surface, the virion interacts with a receptor complex and/or co-receptors and associates with clathrin-coated pits.

It has been proposed that CD81 may play a role in the transport of the virions from the basolateral face of the cells to the region of cell-cell contact and presumably the TJs where the virus could then interact with CLDN1 and OCLN [66]. This hypothesis is based on the observation that a soluble form of E2 glycoprotein is able to induce CD81 relocation in Huh-7 cells to the cell-cell contact areas where it colocalizes with CLDN1 and OCLN. This transport step involves actin cytoskeleton remodeling and Rho-GTPase activity. Therefore, not only does CD81 mediate virus binding, but it also triggers activation of signaling pathway(s) likely required during the virus life cycle.

It has been shown that HCV undergoes clathrin-mediated endocytosis and fuses in early endosomes [67,68] (Figure 2). HCV internalization into cells is a slow process. The half-maximal internalization of HCVpp is achieved in approximately one hour. A siRNA library screen led to the identification of host cofactors involved in HCV entry [65]. These proteins required for HCV infection are involved in clathrin-mediated endocytosis as well as in actin cytoskeleton dynamics or endosomal trafficking and acidification. In single particle tracking experiments, after surfing on the cell surface, virions colocalized with clathrin and the E3 ubiquitin ligase, c-Cbl [65]. The exact role of c-Cbl in HCV entry remains to be elucidated yet a role of this protein in clathrin-mediated endocytosis has already been shown for the bacteria *Listeria monocytogenes* [69]. After internalization, the virus has been shown to be transported to GFP-Rab5a positive early endosomes along actin stress fibers [65]. The role of microtubules in HCV infection has also been investigated [39] using pharmacological agents that are known to affect microtubules such as vinblastin, nocodazol or paclitaxel inhibit HCV infection. These molecules affect HCV entry as well as an early post-fusion step. Generally, virus endocytosis is mediated by receptor(s). However, to date, the exact role of HCV co-receptors in endocytosis of the virus remains undetermined.

Due to the lack of a robust polarized hepatocyte *in vitro* model that supports HCV replication, the role of TJ formation in HCV entry remains unclear. *In vivo,* OCLN and CLDN1 are concentrated in TJs, whereas CLDN is also detected at the basolateral face in polarized HepG2 cells or in liver tissue. Though the virus is probably internalized in the TJ area *in vivo*, formation of TJs is dispensable for HCV entry in cell culture. Some data suggest that the non-junctional pool of CLDN1 is involved in HCV entry [70,71], whereas others have shown that CLDN1 localization at cell-cell contacts is required for HCV infection. In addition, it has been shown that, in non-polarized Huh7.5 cells, cell density and formation of cell-cell contacts modulate receptor expression and HCV infection [72]. More precisely, SRB1 and CLDN1 expression is increased in confluent cells in which they are concentrated at cell-cell contacts whereas CD81 and OCLN expression levels remain unaffected by cell confluence. Interestingly, infection increased in confluent cells compared to subconfluent cells, and cell-cell contacts promoted virus internalization.

The relevance of cell polarity to HCV entry has only been addressed indirectly through the use of the HCVpp system. In HepG2-CD81 cells, the acquisition of polarity correlates with a reduction in HCVpp entry. HCV entry has also been studied in polarized Caco-2 cells [73]. These are colorectal adenocarcinoma cells that form polarized monolayers of cells displaying a columnar morphology with an apical domain and a basolateral domain. Caco-2 cells are poorly permissive to HCVpp and infection occurs at the apical surface of the cell, suggesting that TJs provide a barrier to virus diffusion. In these cells, receptor expression increased upon cell polarization and polarization induced relocalization of CLDN1 from the apical domain to the TJs and the basal domain. Unpolarized Caco-2 cells or polarized cells are infected at similar levels, suggesting that the CLDN1 receptor function is independent of TJ formation. These results suggest that establishment of cell-cell contacts and polarity may be of significance in HCV entry. As a consequence, a more suitable model of polarized hepatoma cell line is much needed to study HCV entry. Such a model will also be useful to understand virus spread in the liver.

Based on observations of HCV spread in cell culture in the presence of neutralizing antibodies, a mechanism of direct cell-to-cell HCV transfer has been proposed [74-77]. The role of the different HCV co-receptors in this transfer has been investigated. Both TJ proteins, CLDN1 and OCLN, are required. However, the role of CD81 in this mechanism is more controversial. It was first proposed that HCV cell-to-cell transmission occurs independently of CD81 [74-76]. Antibodies directed against CD81 are unable to inhibit HCV cell-to-cell transfer, and transfer to cells expressing almost undetectable levels of CD81 was observed. In addition, in a model of uPA(+/+)SCID mice with humanized liver [78], Meuleman *et al*. showed that prophylactic treatment with anti-CD81 antibodies prevented infection, but post-infection treatment had no effect. These results suggest that virus spread may be CD81-independent. However,

these data were challenged by other studies in which cell-to-cell transmission was abolished in cells lacking CD81 or treated with anti-CD81 antibodies [77,79]. Compared to cell-free infection, the role of SRB1 in cell-to-cell transmission is likely more important. Indeed, the neutralizing effect of anti-SRB1 antibodies and specific inhibitors was more pronounced on this infection route. In addition, the mutant JFH-1G451R, which displays less dependency on SRB1, demonstrated a reduced cell-to-cell transmission.

In the early endosome, fusion between the viral envelope and a host membrane occurs, thus allowing the nucleocapsid to be released into the cytosol. The mechanism of HCV fusion is poorly understood. Fusion of the viral envelope with a host cell membrane is driven by the viral fusion protein, which undergoes major conformational changes [9]. Fusion can be triggered by different mechanisms alone or in combinations of the following: receptor binding, low pH exposure and proteolytic processing. For HCV, it is believed that E2 is the protein mediating fusion. HCVpp entry is inhibited by neutralization of endosomal pH, suggesting that low pH exposure is required for HCV fusion [67,68,80]. By using an *in vitro* fusion assay between virion and fluorescently labeled liposomes, Haid *et al.* have reported that fusion is triggered by low pH and is dependent on virus buoyant density and the lipid composition of liposomes [80]. It has been shown that E1 and E2 on the viral surface are linked by intermolecular disulfide bridges [7]. These large oligomers may form a rigid protein lattice which may have consequences for the fusion process. Class II fusion proteins undergo an oligomeric rearrangement during the fusion process, which leads to the conversion into trimers. It is likely that disulfide rearrangements are required to prime HCV fusion process. Critical reshuffling of disulfide bonds has already been shown to occur for other viruses such as Sindbis virus and HIV [81,82]. For HIV, it has been proposed that protein disulfide isomerase (PDI) can act at a post-binding step to reduce two disulfide bonds in gp120 which consequently induces fusion competence. Further investigations on the potential role of PDI and the disulfide-bond rearrangement of HCV envelope proteins are needed to further understand the fusion process.

5. Concluding Remarks

With the development of a cell culture system to propagate the virus, considerable progress has been made in understanding the HCV life cycle. The data accumulated on this virus indicate that HCV enters into host cells in a tightly regulated and complex multistep process involving the presence of several entry factors. Another peculiarity of HCV is the hybrid nature of the lipoviroparticle. The recent progress on HCV entry has highlighted the contribution of both HCV envelope glycoproteins and lipoprotein moieties. Virus-associated lipoproteins are likely playing a role in the early phase of HCV entry, whereas envelope glycoproteins are believed to take the lead after this initial step. However, the details of successive events occuring during HCV entry remain to be revealed. The recent structural model of E2 glycoproteins provides a new framework for a better understanding of the structure-function relationship of HCV envelope glycoproteins, which will lead to a better mechanistic understanding of the entry process. In spite of the major progress in the field, it is still unclear how the virus overcomes the compact and highly organized environment of liver and the role of TJ formation in HCV infection remains to be elucidated.

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