

Microreview

Early steps of the hepatitis C virus life cycle

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Summary

To replicate its genome, a virus needs to cross the plasma membrane of a host cell and get access to cytosolic and/or nuclear components. For an enveloped virus, this involves binding to the plasma membrane, followed by migration of the virion to a microdomain or an endosomal vesicle where fusion between the virion envelope and a host cell membrane occurs. Increasing evidences indicate that virus entry is a tightly regulated process. Although we are still far from understanding the details of hepatitis C virus (HCV) entry, recent data show that this virus enters into target cells in a slow and complex multi-step process involving the presence of several entry factors. Initial attachment of the virion may involve glycosaminoglycans and the low-density lipoprotein receptor, and it is followed by the sequential interaction with the scavenger receptor class B type I, the tetraspanin CD81 and tight junction protein Claudin-1, -6 or -9. Furthermore, the identification of EWI-2wint as a new partner of CD81 which blocks E2–CD81 interaction provides additional evidence of the complexity of the HCV entry process. The current knowledge accumulated on HCV entry is summarized in this review.

Introduction

To infect a target cell, a viral particle needs to proceed through a multistep entry process, during which each step is tightly regulated in time and space. Virus entry is initiated by the binding of a protein present at the surface of the virion to a cell surface molecule. This process generally involves more than one type of entry factor. The surface structures that viruses bind to are of two general

types depending on the functional consequences of the interaction. Attachment factors serve to bind the particles and thus help to concentrate viruses on the cell surface whereas virus receptors actively promote entry. Indeed, after the initial attachment, a virus binds to high affinity receptor(s) or specific entry factor(s), which are responsible for initiating endocytosis, for transducing specific signalling events in the cytoplasm of the cell, or for inducing conformational changes in the structure of the envelope glycoproteins of the virus that lead to the release of the viral genome inside the cytosol.

Hepatitis C virus (HCV) is a small enveloped positive stranded RNA virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family (Lindenbach *et al.*, 2007). The HCV particle consists of a nucleocapsid surrounded by a lipid bilayer in which the two envelope glycoproteins, E1 and E2, are anchored as a heterodimer which plays a major role in HCV entry (Lavie *et al.*, 2007 for review). Infection by HCV often leads to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, which is due to the fact that viral replication occurs primarily, if not exclusively, within hepatocytes. Therefore, to initiate its life cycle, HCV has to cross the plasma membrane of hepatocytes and gain access to the cytosol of these cells.

For a long time, it has been difficult to study HCV entry because of the difficulties in propagating this virus in cell culture. However, the use of surrogate models and the recent development of a cell culture system for this virus are beginning to shed some light on the early steps of the HCV life cycle. In this review, we present the data that have recently been accumulated on the molecules involved in HCV entry as well as the sequence of events involved in the early steps of HCV entry.

Biochemical tools and cell culture systems to study HCV entry

Although the cloning of the HCV genome allowed a rapid analysis of the genomic organization and a biochemical characterization of its proteins, the lack of a cell culture system allowing efficient amplification of this virus has long been an obstacle for the study of its life cycle. Plasma-derived HCV particles have been used by some groups to study HCV entry. However, due to poor replication in cultured cells and heterogeneity of the material, this

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tool did not allow for exploration of the full process of the HCV life cycle. For this reason, several *in vitro* tools that give more robust read-outs have been developed. One that has been very useful is a truncated soluble form of E2 envelope protein (sE2) which aided identifying cell surface proteins potentially involved in HCV entry (reviewed in Cocquerel *et al.*, 2006). However, this tool as well as recombinant virus-like particles developed by others did not enable functional studies of E1E2-mediated cell entry up to the point of membrane fusion (Cocquerel *et al.*, 2006).

HCV pseudoparticles (HCVpp) were the first robust system used to study the full process of HCV entry into host cells. These particles consist of retroviral particles that have HCV E1E2 heterodimers instead of retroviral glycoproteins anchored in their lipid envelope (Bartosch *et al.*, 2003a; Drummer *et al.*, 2003; Hsu *et al.*, 2003). HCVpp are produced in 293T cells by expressing the HCV glycoproteins, retroviral core proteins and a packaging-competent retrovirus-derived genome containing a reporter gene. During budding, HCV envelope glycoproteins are incorporated into the pseudoparticle envelope. As no retroviral glycoproteins are present in this system, the cell entry properties of HCVpp are determined by HCV glycoproteins. HCVpp entry results in the delivery of the retroviral nucleocapsid into the cytosol followed by reverse transcription and integration of the retroviral-derived genome into the cellular genome. The reporter gene is then expressed from the integrated provirus making detection of successful entry into the target cell simple and reproducible.

Recently, the development of a cell culture system that enables a relatively efficient amplification of HCV (HCVcc) has finally been reported (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). This system is based on the transfection of the human hepatoma cell line Huh-7 with genomic HCV RNA derived from a cloned viral genome of a HCV isolate from a Japanese patient with fulminant hepatitis. HCVcc has been found to be infectious in chimpanzees and mice transplanted with human hepatocytes (Lindenbach *et al.*, 2005). Moreover, in contrast to HCV derived from the plasma of HCV-infected patients, virus recovered from HCVcc-infected animals are capable of replicating robustly in cell culture (Lindenbach *et al.*, 2005). With the development of HCVcc, the entire life cycle including entry, replication, assembly and release can now be studied in some hepatoma cell lines (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005) as well as in primary hepatocytes (Molina *et al.*, 2008).

HCV particle and association with lipoproteins

The HCV particle is thought to have a diameter of about 50 nm (Wakita *et al.*, 2005). It is composed of a nucleo-

capsid surrounded by a host cell-derived membrane envelope that contains the viral glycoproteins E1 and E2 (Lavie *et al.*, 2007 for review). HCV glycoproteins are type I membrane proteins with a C-terminal transmembrane domain anchored in the lipid envelope. In their functional form, E1 and E2 are thought to form a non-covalent heterodimer, and the transmembrane domains of HCV envelope glycoproteins are essential for E1E2 heterodimerization (Lavie *et al.*, 2007 for review). The ectodomains of HCV envelope glycoproteins are highly glycosylated, and these glycans have been shown to play a major role in protein folding, in virus entry and in protection against neutralizing antibodies (Goffard *et al.*, 2005; Falkowska *et al.*, 2007; Helle *et al.*, 2007).

Plasma-derived HCV have been reported to be in complex with low-density and very-low-density lipoproteins (LDL and VLDL) (Andre *et al.*, 2005 for review). Furthermore, it has recently been shown that HCV production in hepatoma cells is dependent on assembly and secretion of VLDL lipoproteins (Huang *et al.*, 2007). Apolipoprotein E is also required for infectivity and production of HCV in cell culture (Chang *et al.*, 2007). Thus, it appears that HCV particles interact with VLDL during the assembly of the lipoprotein particles and are secreted together with VLDL. However, the nature of the association between HCV and VLDL remains unclear. Whatever the nature of this interaction, it gives the virus the opportunity of using lipoprotein receptors to bind to target cells.

Attachment of HCV particle to the cell surface

Virus entry is initiated by the binding of the particle to an attachment factor, which helps to concentrate viruses on the cell surface. Interactions with attachment factors can be relatively non-specific and often involve binding to glycosaminoglycans. The role of glycosaminoglycans in HCV entry has been investigated and it has been shown that heparin, a heparan sulfate homologue, and heparinase, an enzyme able to degrade heparan sulfates at the cell surface, inhibit HCV glycoprotein E2 as well as HCVcc attachment to target cells (Barth *et al.*, 2003; Koutsoudakis *et al.*, 2006), suggesting that glycosaminoglycans may serve as an initial docking site for HCV attachment. However, there remains some controversy on the direct involvement of HCV envelope glycoproteins in this interaction (Helle and Dubuisson, 2007). Indeed, one cannot exclude a role of the lipoproteins associated with HCV particle in this initial binding to glycosaminoglycans.

As a result of the association between HCV and lipoproteins, the LDL receptor (LDL-R) has been proposed as another potential attachment factor for HCV (Agnello *et al.*, 1999). Cell surface adsorption of HCV particles isolated from patients and accumulation of viral RNA in cells can be inhibited by antibodies directed against the

LDL-R as well as purified LDL and VLDL. Furthermore, a correlation has been shown between the accumulation of HCV RNA into primary hepatocytes, expression of LDL-R mRNA and LDL entry (Molina *et al.*, 2007). Finally, the inhibition of HCVcc entry by anti-apolipoprotein E antibodies is another argument in favour of a role for the LDL-R in HCV entry (Chang *et al.*, 2007).

Specific entry factors

After the initial attachment to the host cell, a virus binds to high affinity receptor(s) or specific entry factor(s), which are responsible for initiating a series of events eventually leading to the release of the viral genome into the cytosol. In many cases, receptor interactions occur in an apparently programmed series of events using multiple receptors. With the development of tools to study HCV entry, several cell surface molecules have been described as specific entry factors for this virus. The first identified and best characterized entry factor is the tetraspanin CD81, which was initially shown to interact with HCV glycoprotein E2 (Pileri *et al.*, 1998). HCV entry is strongly reduced in the presence of anti-CD81 antibodies or in CD81 knock-down hepatoma cells (reviewed in Cocquerel *et al.*, 2006). Interestingly, cell lines like HepG2 or HH29 hepatoma cells that do not express CD81 are very useful tools for reverse genetic studies to identify CD81 residues involved in HCV entry (Zhang *et al.*, 2004; Bertaux and Dragic, 2006). Like all members of the tetraspanin family, CD81 is composed of four transmembrane spanning sequences, a small extracellular loop and a large extracellular loop (LEL) which contains an absolutely conserved CCG motif involved in the formation of disulfide bridges. The LEL domain is the critical region for the interaction with the E2 envelope glycoprotein and for virus entry. It is worth noting that many of the LEL mutations that abrogate binding to sE2 support entry of HCVpp and HCVcc, suggesting that the affinity of E1E2 binding to LEL may be higher on virion than in sE2 (Flint *et al.*, 2006). Furthermore, the access to the CD81 binding site on E2 is reduced by the presence of specific glycans (Falkowska *et al.*, 2007; Helle *et al.*, 2007), suggesting that these glycans surround the CD81 binding site on E2. One remarkable feature of tetraspanins is their ability to act as molecular organizers of specific proteo-lipidic membrane microdomains called tetraspanin-enriched microdomains (TEMs). Although cholesterol contributes to the organization of the TEMs as shown for CD81, these domains display a number of critical differences that distinguish them from conventional lipid rafts. It is worth noting that modifying the plasma membrane sphingomyelin/ceramide ratio of the target cell or depleting the cholesterol of these cells reduces the cell surface expression of CD81 as well as HCV entry (Kapadia *et al.*,

2007; Voisset *et al.*, 2007), suggesting that CD81 may need to be located in TEMs to function as an entry factor for HCV.

After the identification and characterization of CD81 as a molecule involved in HCV entry, the human scavenger receptor class B type I (SR-BI, also called CLA-1) has been proposed as another potential entry factor for this virus (Scarselli *et al.*, 2002). Indeed, HCV glycoprotein E2 has been shown to interact with SR-BI (Scarselli *et al.*, 2002) and HCV entry is strongly reduced in the presence of anti-SR-BI antibodies or in SR-BI knock-down hepatoma cells (reviewed in Helle and Dubuisson, 2007). SR-BI contains two short cytoplasmic domains, two transmembrane spanning sequences and a large and highly glycosylated extracellular loop. SR-BI was initially identified as the major physiological receptor for high-density lipoproteins (HDL) in the liver and is involved in selective lipid uptake (Connelly and Williams, 2004). Interestingly, the SR-BI gene allows expression of different isoforms by alternative splicing, and the SR-BII isoform which differs from SR-BI by its C-terminal extremity can also play a role in HCV entry (Grove *et al.*, 2007). SR-BI is a multi-ligand receptor (Connelly and Williams, 2004), and several SR-BI ligands have been found to affect HCV infectivity. Oxidized LDL and serum amyloid A have been shown to inhibit HCV entry; however, these molecules do not seem to compete with HCV for binding to SR-BI (Lavie *et al.*, 2006; von Hahn *et al.*, 2006; Cai *et al.*, 2007). In contrast, HDL enhances HCV entry, a process which depends on the lipid transfer function of SR-BI and the presence of apolipoprotein CI (Dreux *et al.*, 2007; Bartosch *et al.*, 2005; Meunier *et al.*, 2005; Voisset *et al.*, 2005), suggesting that HCV exploits the physiological functions of SR-BI during the entry process. Kinetics of inhibition with anti-SR-BI antibodies suggest that SR-BI might act concomitantly with CD81 (Zeisel *et al.*, 2007). However, it is likely that HCV particle encounters SR-BI before CD81. Indeed, HCVcc can bind to CHO cells expressing SR-BI but not to CHO cells expressing CD81, suggesting that a first contact with SR-BI might be necessary before the particle interacts with CD81 (Evans *et al.*, 2007). Although a direct interaction between E2 and SR-BI has been demonstrated (Scarselli *et al.*, 2002), the lipoproteins associated with the viral particle have also been proposed to interact with this entry factor (Maillard *et al.*, 2006). Indeed, SR-BI is also a receptor for LDL, a lipoprotein associated with HCV particle (Connelly and Williams, 2004). Whether this indirect interaction plays a major role in HCV entry remains, however, to be determined.

The observation that expression of CD81 and SR-BI was not sufficient to support HCV entry led to the hypothesis that at least one additional factor might be required (Bartosch *et al.*, 2003b; Hsu *et al.*, 2003). By screening a complementary DNA library derived from HCV permissive

cells for genes that render cells susceptible to HCVpp infection, the tight junction protein Claudin-1 (CLDN1) has recently been identified as a new entry factor for HCV (Evans *et al.*, 2007). Furthermore, other members of the CLDN family, CLDN6 and 9, are also able to mediate HCV entry (Zheng *et al.*, 2007). However, not all the members of the CLDN family can play a role in HCV entry. Indeed, CLDN2, 3, 4, 7, 11, 12, 15, 17 and 23 are unable to mediate HCV entry (Zheng *et al.*, 2007). Like CD81, these small proteins contain two extracellular loops, three intracellular domains and four transmembrane spanning sequences (Furuse and Tsukita, 2006). However, there is no sequence homology between CLDNs and tetraspanins. Residues involved in HCV entry have been identified in the first extracellular loop of claudin molecules (Evans *et al.*, 2007; Zheng *et al.*, 2007). No direct interaction between CLDNs and HCV envelope glycoproteins has been demonstrated, but one cannot exclude that such an interaction requires a conformational change in the envelope glycoproteins triggered by a first interaction with another entry factor. Kinetics of inhibition with antibodies suggest that CLDNs might play a role in a late step of the entry process, probably after virus binding and interaction with CD81 (Evans *et al.*, 2007). Experiments in polarized and unpolarized Caco-2 cells suggest that CLDN receptor activity may be independent of its tight junction function (Mee *et al.*, 2008). The precise role of CLDN proteins in HCV entry remains, however, to be determined.

The observation that expression of CD81, SR-BI and CLDN1 is not sufficient to support HCV entry into some cell lines suggests that other cellular molecules are involved in entry. Interestingly, a new partner of CD81 has recently been identified as a molecule which inhibits HCV entry into Huh-7 cells by blocking E2-CD81 interactions (V. Rocha-Perugini *et al.*, unpubl. data). This CD81 partner, called EWI-2wint, is a cleavage product of EWI-2, a major partner of CD81, which belongs to a family of immunoglobulin-like proteins. EWI-2wint, though expressed in different cell lines, is absent from hepatocytes suggesting that, in addition to the presence of specific entry factors in the hepatocytes, the absence of a specific inhibitor may contribute to the hepatotropism of HCV.

Internalization of HCV particle and fusion with an endosomal membrane

The binding of enveloped viruses to cell surface molecules is followed by fusion of the lipid envelope with a cellular membrane. This process is tightly co-ordinated in time and space and requires drastic conformational changes in the fusion proteins, which are triggered by cellular factors. Some viruses enter by fusing their envelope

directly with the plasma membrane, whereas others enter target cells by endocytosis. For HCV, it has been shown that the particle enters target cells by clathrin-mediated endocytosis (Blanchard *et al.*, 2006) and fusion has been proposed to occur in the early endosomes (Meertens *et al.*, 2006). Furthermore, the acidic pH of endosomes triggers the fusion process probably by inducing conformational changes in the envelope proteins (Bartosch *et al.*, 2003b; Hsu *et al.*, 2003; Blanchard *et al.*, 2006; Koutsoudakis *et al.*, 2006; Meertens *et al.*, 2006; Tscherne *et al.*, 2006). After fusion between the viral envelope and an endosomal membrane, the viral genome is released into the cytosol. It is worth noting that exposure of cell surface-bound virions to acid pH followed by a return to neutral pH does not affect HCV infectivity (Meertens *et al.*, 2006; Tscherne *et al.*, 2006), suggesting that HCV envelope proteins need an additional trigger, such as receptor interaction, to become sensitive to low pH. Based on its classification in the *Flaviviridae* family, it is currently thought that HCV envelope proteins have a folding pattern similar to class II fusion proteins (Lindenbach *et al.*, 2007). The proteins of this class are predominantly non-helical, having instead a beta-sheet type structure. They are not cleaved during their biosynthesis, and they possess an internal fusion peptide with a loop conformation. Furthermore, class II fusion proteins are synthesized as a complex with a companion membrane glycoprotein, which acts as a chaperone. Finally, cleavage of the companion protein activates the fusogenic potential of the fusion protein. The precise role of E1 and E2 in HCV entry has not been determined yet. In contrast to other class II viruses, both envelope glycoproteins seem to have a chaperone activity for their partner (Lavie *et al.*, 2007) and they both seem to play a role in the fusion process (Lavillette *et al.*, 2007). Indeed, it has recently been proposed that distinct regions in both HCV E1 and E2 may cooperate to drive the fusion process to completion (Lavillette *et al.*, 2007). In addition, mutations in the transmembrane domains of E1 and E2 affect the fusion properties of HCV envelope glycoproteins, possibly by affecting the oligomeric reorganization of the fusion protein (Ciczora *et al.*, 2007). However, a high-resolution structure of HCV envelope proteins will be necessary to understand how these envelope proteins function in the entry process.

Conclusion

The recent accumulation of data on the early steps of the HCV life cycle enables us to draw a model in which HCV entry appears to be a slow and complex multistep process (Fig. 1). Viral particles associated with lipoproteins, which circulate in the blood stream, use glycosaminoglycans and/or the LDL receptor (LDL-R) as attachment factors for

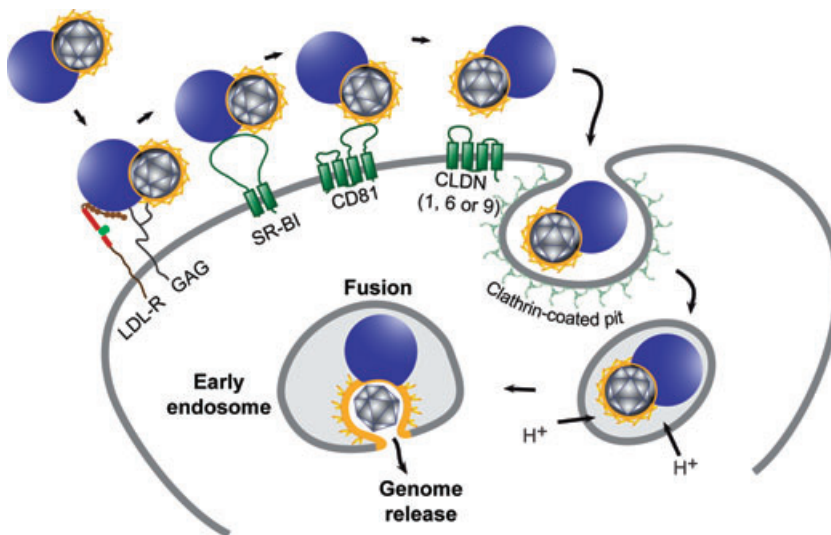


Fig. 1. Model of HCV entry into the hepatocyte. See text for details.

their initial binding to the host cell. After binding, the particle likely interacts with SR-BI and CD81. The interaction with SR-BI can potentially be direct or indirect through HCV-associated lipoproteins. CLDN1, 6 or 9 acts at a late stage of the entry process, after interaction with SR-BI and CD81. Then, HCV is internalized by clathrin-mediated endocytosis, and fusion probably occurs in early endosomes. Importantly, HCV entry process may even be more complex than already elucidated. Indeed, some human cell lines expressing CD81, SR-BI and CLDN1 remain resistant to HCV entry (Evans *et al.*, 2007), suggesting that one or more human-specific HCV entry factor(s) remain to be discovered. In line with this observation, the identification of EWI-2wint as a new partner of CD81 which blocks E2–CD81 interactions provides additional evidence of the complexity of the HCV entry process (V. Rocha-Perugini *et al.*, unpubl. data). The recent discovery of CLDN as an entry factor also suggests that cell polarization might play a role in HCV entry. Indeed, it has recently been shown that HCV particle preferentially interacts with the apical surface in polarized Caco-2 cells (Mee *et al.*, 2008). Furthermore, the tight junctions seem to provide a physical barrier for viral access to entry factors expressed on lateral and basolateral cellular domains (Mee *et al.*, 2008). Finally, the possibility of HCV transmission by cell–cell contact has also been raised (Timpe *et al.*, 2007), suggesting an alternative route for virus spreading. However, the mechanism of cell–cell transmission has not been characterized and the importance of this mode of transmission within the liver has not been determined yet. In conclusion, although rapid progress has recently been made on the identification of cellular factors involved in HCV entry, the precise role of each of these molecules in the early steps of the HCV life cycle remains to be determined.

Acknowledgements

We apologize to all authors whose work could not be cited due to space restrictions. We thank Sophana Ung for preparing the illustration and Johnathan Canton for critical reading of the manuscript. Our research was supported by the 'Agence Nationale de Recherche sur le Sida et les hépatites virales' (ANRS). J.D. is an international scholar of the Howard Hughes Medical Institute (HHMI).

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