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Review

Hepatitis C virus entry into host cells

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Abstract. The recent development of functional models to analyze the early steps of the hepatitis C virus (HCV) life cycle has highlighted that HCV entry is a slow and complex multistep process involving the presence of several entry factors. Initial host cell attachment may involve glycosaminoglycans and the low-density lipoprotein receptor, after which the particle appears to interact sequentially with three entry factors: the scavenger receptor class B type I, the tetraspanin CD81 and the tight-junction protein claudin-1. Several serum components may also modulate HCV entry, while the recently discovered CD81 partner EWI-2wint can block the interaction of the viral particle with CD81, potentially preventing infection in the cell types in which it is expressed. After binding to the host cell, the HCV particle is internalized by clathrin-mediated endocytosis, with fusion likely occuring in early endosomes. This review summarizes our current knowledge on HCV entry.

Keywords. Hepatitis C virus, virus entry, envelope glycoprotein, viral receptor, viral fusion, entry factor, virus attachment.

Introduction

The mission of a virion is to transport the viral genome from an infected cell to a naive host cell. To initiate an infectious cycle, the viral particle must cross the plasma membrane to gain access to the inner content of the target cell. Virus entry into host cells involves a complex series of events which are tightly coordinated in time and space. For an enveloped virus, this involves binding to cellular attachment factors and specific receptors as well as fusion of the lipid envelope with a cell membrane, which allow the release of the viral genome into the cytoplasm of the target cell. Since membrane fusion does not necessarily occur at the plasma membrane, virus entry can also involve endocytosis and vesicular trafficking. Regardless, once in the cytosol, the viral genome also needs to be transported to its final destination where replication can be initiated.

Hepatitis C virus (HCV) is a small, enveloped, positive-stranded RNA virus that belongs to the *Hepacivirus* genus in the Flaviviridae family [1]. Since the hepatocytes are the major target cells of HCV, infection by this virus often leads to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. To initiate its life cycle, HCV has to cross the plasma membrane of hepatocytes and gain access to the cytosol. The HCV particle consists of a nucleocapsid surrounded by a lipid bilayer where the two envelope glycoproteins, E1 and E2, are anchored. These two proteins form noncovalent heterodimers [2], which play a major role in HCV entry [3].

Due to the lack of a cell culture system supporting efficient production of infectious particles, studying HCV entry has been very difficult. For this reason,

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several surrogate models have been developed [reviewed in refs. 4–6]. Some of these models allowed the identification of cellular proteins potentially involved in HCV entry and helped to characterize the role of HCV envelope glycoproteins in virus entry. However, the recent development of a cell culture system for HCV now enables confirmation of the results obtained with surrogate models. Here, we summarize the knowledge that has recently been accumulated on the early steps of the HCV life cycle.

Recent model 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. Therefore, several laboratories used a truncated soluble form of E2 envelope protein (sE2) recombinant virus-like particles produced in insect cells or vesicular stomatitis virus pseudoparticles to identify cell surface proteins potentially involved in HCV entry [reviewed in refs. 4–6]. More recently, two major advances have enabled progress to be made in the study of HCV entry: the development of retroviral particles pseudotyped with HCV envelope glycoproteins (HCVpps) and native virus amplified in cell culture (HCVccs).

HCVpps consist of full-length HCV envelope glycoproteins assembled onto retroviral core particles containing a retrovirus-derived genome harboring a marker gene [7–9]. HCVpps are produced by transfecting 293T cells with three expression vectors encoding the E1E2 polyprotein, the retroviral core proteins and a packaging-competent, retrovirus-derived genome containing a marker gene. Retroviruses were chosen as platforms for HCV pseudotype particle assembly because their cores can incorporate a variety of cellular and viral glycoproteins [10, 11] and because they can easily package and integrate genetic markers into host cell DNA [12]. However, the assembly process of HCVpps is not well understood. Indeed, murine leukemia virus and HIV are supposed to assemble at the plasma membrane or in multivesicular bodies, whereas HCV glycoproteins are retained in the endoplasmic reticulum (ER) [2]. However, due to saturation of the ER retention machinery, the cells used to produce HCVpps have been shown to express a small fraction of HCV envelope glycoproteins at the plasma membrane and in multivesicular bodies [3, 7–9, 13]. This accumulation at the plasma membrane or in multivesicular bodies might therefore be sufficient to incorporate full-length HCV envelope glycoproteins into retroviral pseudotyped particles. Due to their preferential tropism for liver cells and to the specific neutralization of these particles by antibodies directed against E2 protein, HCVpps have been validated for the study of HCV entry [7, 14]. This model system is particularly useful for the functional characterization of large series of envelope protein mutants.

Recently, the development of a cell culture system that allows a relatively efficient amplification of HCV (HCVcc) has finally been reported [15–17]. 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 an HCV isolate from a Japanese patient with fulminant hepatitis. Interestingly, the viral particles produced in cell culture can infect chimpanzees and mice transplanted with human hepatocytes [15, 16]. HCVcc is now the most relevant system to study the HCV life cycle, but restriction to a single isolate remains a problem for some experiments. This can in part be overcome by making chimeric viruses containing structural proteins of different isolates or genotypes [16, 18–23].

HCV envelope glycoproteins

The genome of HCV encodes a single polyprotein of about 3000 amino acids, which is cleaved co- and post-translationnaly by cellular and viral proteases to yield at least ten mature products. Cleavage of the viral polyprotein by a cellular signal peptidase gives rise to the envelope glycoproteins, E1 and E2. These are type I membrane proteins containing a large N-terminal ectodomain and a C-terminal transmembrane domain. During their synthesis, E1 and E2 ectodomains are directed to the lumen of the ER and their transmembrane domains are inserted in the membrane of this compartment. Indeed, the C terminus of the immature form of the capsid protein is a signal sequence responsible for the translocation of the E1 ectodomain into the ER lumen [24]. Furthermore, the C-terminal sequences of E1 and E2 contain signals which lead to a reinitiation of translocation in the ER lumen [25].

E1 and E2 are anchored in the ER membrane by their transmembrane domains. These domains are multifunctional. They contain heterodimerization sequences and ER retention signals [26–29]. Thus, during their biogenesis, E1 and E2 assemble as noncovalent heterodimers which are retained in the ER [2]. Importantly, pseudotyped retroviral particles generated with E1 or E2 alone are noninfectious [7, 9], indicating that both proteins need to be coexpressed to be functional in virus entry. Therefore, the E1E2

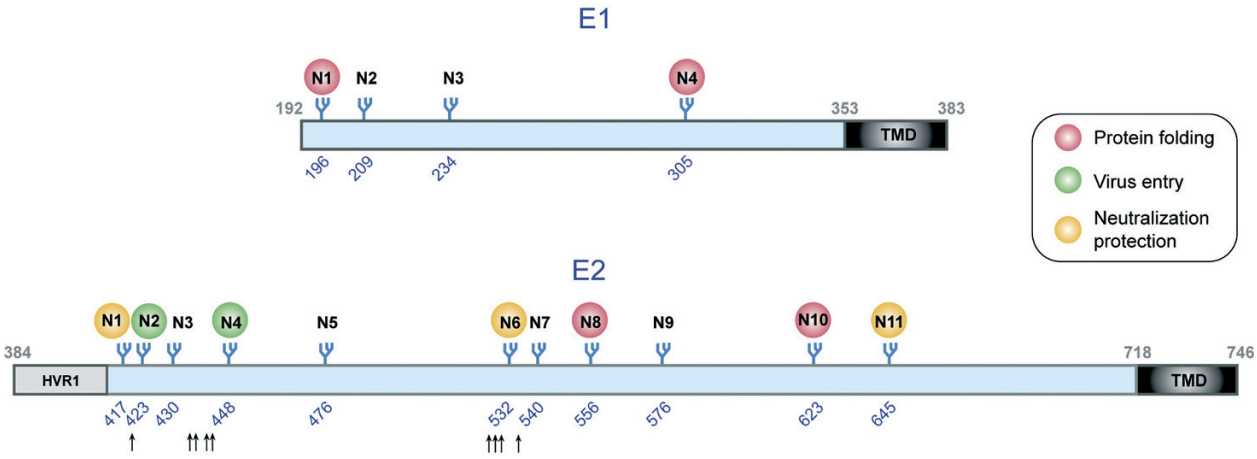

Figure 1. Schematic representation of HCV envelope glycoproteins E1 and E2. Glycosylation sites are indicated by N followed by the number of the site position in the sequence. The positions of the glycosylation sites in the polyprotein of reference strain H (GenBank accession no. AF009606) are shown. Glycans involved in protein folding, virus entry or protection against neutralization are indicated in pink, green and orange, respectively. Residues 420, 437, 438, 441, 442, 527, 530 and 535, involved in the E2-CD81 interaction [81, 82], are indicated by arrows. TMD, transmembrane domain; HVR1, hypervariable region 1.

heterodimer is probably the form of functional HCV glycoproteins present at the surface of viral particles and involved in the entry process [7, 9]. Interestingly, mutation of specific residues in the transmembrane domains of E1 and E2 alters the fusion property of these envelope glycoproteins, suggesting that these domains also play a major role in the fusion process [30].

The N terminus of E2 contains a hypervariable region called HVR1 (residues 384–410; Fig. 1) whose high variability may contribute to HCV escape from the immune response [31–35]. A virus with this region deleted remains infectious in chimpanzees, but has a lower infectivity [36]. Nevertheless, it seems that the properties of some residues are conserved across different genotypes [37]. Indeed, this region contains several basic amino acids which modulate HCVpp infectivity [38], suggesting that this region may be involved in HCV entry (see below). Other hypervariable regions in E2 glycoprotein that might also play a role in viral entry have been described: HVR2 (residues 474–482) and HVR3 (residues 431–466) [39–41].

E1 and E2 also contain 4 and 11 conserved glycosylation sites, respectively (Fig. 1) [42–44], all of which have been shown to be modified by N-glycosylation [45]. Despite variability in the sequences of HCV envelope glycoproteins, the glycosylation sites are highly conserved, suggesting that the glycans associated with these proteins play an essential role in the HCV life cycle. Studies of the functional role of these glycans have shown that they play a major role in protein folding, in HCV entry and in protection against neutralization (Fig. 1) [44–46]. Indeed, the lack of a glycan at position 196, 305, 556 or 623 (E1N1, E1N4, E2N8 or E2N10) strongly affects the incorporation of HCV glycoproteins into HCVpps, suggesting that these glycans are necessary for protein folding. Furthermore, mutation of the glycosylation sites at position 423 or 448 (E2N2 or E2N4) alters the infectivity of HCVpps despite normal incorporation into pseudoparticles, suggesting an essential role for the corresponding glycans in viral entry. Since E1E2 heterodimers produced in the absence of E2N2 or E2N4 glycans still interact with CD81, the best-characterized entry factor for HCV, these glycans might be important for E2 interaction with another entry factor or in the fusion process [45]. Finally, glycans at positions 417, 532 and 645 (E2N1, E2N6 and E2N11) were shown to reduce the sensitivity of HCVpps to antibody neutralization and to reduce the access of CD81 to its binding site on E2, suggesting that HCV glycans can contribute to the evasion of HCV from the humoral immune response by masking the CD81 binding site.

Cellular entry factors for HCV

Glycosaminoglycans

Glycosaminoglycans (GAGs) present at the surface of cells represent a first site of binding for several viruses including Flaviviridae [47–50]. There are several different types of GAG: chrondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, heparin and hyaluronan. By using different model systems (sE2, HCVpps, HCVccs, virus isolated from plasma), several authors have shown that heparin, a heparan

sulfate homolog, and heparinase, an enzyme able to degrade heparan sulfates at the cell surface, inhibit HCV attachment to target cells [22, 51–56]. However, other GAGs do not exhibit any inhibitory activity. The affinity of an intracellular form of sE2 for heparin is strong and HVR1 has been proposed as necessary for this interaction [52]. However, in the case of E1E2 heterodimers isolated from HCVpps, no interaction was observed between E2 and heparin [38], suggesting that the heparin-binding domain of E2 is not accessible on the functional heterodimer. To better understand the role of GAGs in HCV entry further experiments need to be done in the context of envelope glycoproteins isolated from HCVccs. It is worth noting that no heparin-binding motif has been identified in the E2 sequence [57]. However, it is possible that such a motif is formed at the surface of the folded E2 protein. Finally, one cannot exclude the possibility that HCV interacts indirectly with GAGs, e.g. through lipoproteins associated with HCV particles. Interestingly, a recent study suggests that the lipoprotein lipase plays an indirect role in the interactions between HCV and GAGs [58]. However, lipoprotein lipase-mediated binding of viral particles seems to promote nonproductive virus uptake.

Low-density lipoprotein receptor

The density of HCV in sera of infected patients is heterogeneous and surprisingly low. This has been attributed to the association of HCV with low-density and very low density lipoproteins (LDL and VLDL) even if the details of HCV-lipoprotein interaction remain unclear [59–62]. In favor of this association HCVcc production in Huh-7 hepatoma cells has been shown to depend on the assembly and secretion of VLDL [63]. As a result of the potential interaction between HCV and lipoproteins, the LDL receptor (LDL-R) has been proposed as a potential entry factor for HCV [64–66]. 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 by purified LDL and VLDL [51, 65, 67]. Furthermore, a correlation has been shown between the accumulation of HCV RNA into primary hepatocytes, expression of LDL-R mRNA and LDL entry [67]. Finally, the inhibition of HCVcc entry by anti-apolipoprotein B antibodies is another argument in favor of a role for the LDL-R in HCV entry [58]. In contrast, there is no evidence that HCVpps use the LDL-R to enter hepatocytes [7, 9]. However, unlike HCVccs [63], HCVpps are surmised to assemble independently of VLDL [13].

CD81

The cell surface protein CD81 has been identified as a potential entry factor for HCV using sE2 [68]. CD81 belongs to the tetraspanin family, whose members are involved in various cellular functions such as adhesion, morphology, proliferation or differentiation [69]. Like all members of the tetraspanin family, CD81 is composed of four transmembrane passages, a small extracellular loop (SEL) and a large extracellular loop (LEL) (Fig. 2) [70]. Since its identification as a molecule interacting with sE2, CD81 involvement in HCV entry has been confirmed in different models. Indeed, anti-CD81 monoclonal antibodies as well as a soluble form of CD81 LEL are able to inhibit HCVpp and HCVcc infectivity [7, 9, 15, 17, 71–74]. Furthermore, CD81 knock-down hepatoma cells are no longer permissive to HCVpp and HCVcc [72, 75]. Finally, HepG2 or HH29 hepatoma cells, which do not express CD81, become permissive to HCVpps and HCVccs after ectopic expression of CD81 [9, 16, 71–73, 76]. Other studies have also shown that susceptibility of cells to HCV infection is closely related to the CD81 expression level [75, 77]. Interestingly, enrichment of the plasma membrane in ceramide through sphingomyelin hydrolysis has a strong inhibitory effect on HCV entry [C. Voisset, M. Lavie, F. Helle, A. Op De Beeck, A. Bilheu, J. Bertrand-Michel, F. Tercé, L. Cocquerel, C. Wychowski, N. Vu-Dac and J. Dubuisson, unpublished data]. Likewise, ceramide enrichment of the plasma membrane lead to a decrease in the level of CD81 present at the cell surface, due to a massive ATP-independent endovesiculation of CD81, suggesting that ceramide enrichment of the plasma membrane can impair HCV entry by inducing CD81 internalization.

Residues of CD81 involved in the interaction with E2 have been mapped in the LEL [68, 72, 78–80]. On the E2 glycoprotein, amino acid residues 420, 437, 438, 441, 442, 527, 529, 530 and 535 have been shown to play a role in E2-CD81 interaction (Fig. 1) [81, 82]. Furthermore, it has been shown that E1E2 heterodimers have stronger CD81 interactions than sE2, suggesting that E1 can modulate the binding of E2 to CD81 [83]. As discussed above, access to the CD81-binding site on E2 is reduced by the presence of glycans at positions 417, 532 and 645 (E2N1, E2N6 and E2N11) [44, 46], suggesting that these glycans surround the CD81-binding site. Several studies suggest that CD81 acts at a post-binding step, and the half-maximal time of CD81-mediated HCVpp entry into target cells has been determined to be approximately 17 min [22, 71, 80, 84]. However, the exact role of CD81 in HCV entry remains to be elucidated.

The tropism of HCV is restricted to human liver cells expressing CD81 [7, 9, 71, 72], but ectopic expression

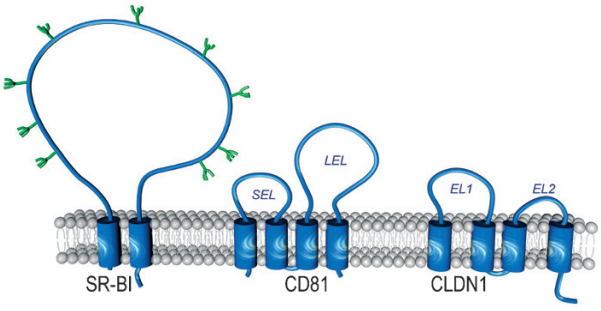

Figure 2. Schematic representation of SR-BI, CD81 and CLDN1. SR-BI is composed of two transmembrane passages, two intracellular domains [11 and 45 amino acids (aa)] and a large extracellular loop (411 aa). SR-BI contains nine potential glycosylation sites represented in green. CD81 is composed of four transmembrane passages, two intracellular domains (each of 12 aa), a small intracellular loop (5 aa) and two extracellular loops, SEL (30 aa) and LEL (89 aa). CLDN1 is composed of four transmembrane passages, a short intracellular N-terminal peptide (7 aa), an intracellular loop (13 aa), an intracellular C-terminal tail (27 aa) and two extracellular domains, EL1 (53 aa) and EL2 (27 aa).

of CD81 in nonhepatic cells does not render them permissive to infection [9, 76], indicating that other molecules are essential for HCV entry. Members of the tetraspanin family are able to interact with each other and with other protein partners to form multimolecular complexes called 'tetraspanin webs' [69, 85]. The two major partners involved in primary complexes with CD81 are EWI-F (also called CD9P-1, FPRP or CD315) and EWI-2 (also called PGRL, IgSF8 or CD316) which belong to a novel family of immunoglobulin proteins [86, 87]. Members of this family are type I transmembrane proteins, which share a conserved EWI motif and contain an ectodomain composed of four immunoglobulin domains. Very recently, a cleavage product of EWI-2 has been identified which is a partner of CD81 and inhibits HCVcc entry into Huh-7 cells [V. Rocha-Perugini, C. Montpellier, D. Delgrange, C. Wychowski, F. Helle, A. Pillez, H. Drobecq, F. Le Naour, S. Charrin, S. Levy, E. Rubinstein, J. Dubuisson and L. Cocquerel, unpublished data]. This protein, called EWI-2wint, corresponds to EWI-2 without its first immunoglobulin domain. Ectopic expression of EWI-2wint in permissive cells inhibits HCVpp and HCVcc infection by reducing E2-CD81 interactions. EWI-2wint though expressed in different cell lines is absent from hepatocytes, but it is not yet known whether this expression profile is due to a differential expression of the protease responsible for EWI-2wint production or if the accessibility of EWI-2 to protease is cell type specific. Nevertheless, these findings suggest 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.

SR-BI

The human scavenger receptor class B type I (SR-BI, also called CLA-1) is a cell surface protein which has been identified as another potential entry factor for HCV [88]. SR-BI is a 509-amino-acid protein containing two short cytoplasmic domains, two transmembrane passages and one large extracellular loop (Fig. 2) [89–91]. Although the interaction between sE2 and SR-BI seems specific [88, 92], no interaction with E1E2 heterodimers has yet been observed [6]. Nevertheless, the involvement of SR-BI in HCV entry has been confirmed with the HCVpp and HCVcc systems [74, 76, 93–96]. Indeed, it has been shown that pre-incubation of Huh-7 cells with anti-SR-BI antibodies significantly decreases HCV entry [74, 76, 96]. Furthermore, SR-BI knock-down hepatoma cells seem to be less permissive to HCVpps and HCVccs [93, 97]. SR-BI is expressed in the majority of mammalian cells, but its expression is particularly high in the liver [76, 91, 98–101]. Interestingly, the SR-BI gene allows expression of different isoforms by alternative splicing. In particular, the SR-BII isoform which differs from SR-BI at its C-terminal extremity can also play a role in HCV entry. Indeed, overexpression of SR-BII increases HCV infectivity, although to a lower extent than SR-BI [95].

SR-BI is a receptor for acetylated and oxidized LDL but also for high-density lipoproteins (HDLs) [90,98]. Although oxidized LDL inhibits HCVpp and HCVcc infectivity [102], HDL has been shown to facilitate HCV entry, a process which depends on the lipid transfer function of SR-BI [94, 103–105]. Several studies suggest that the presence of HVR1 on E2 is important for its interaction with SR-BI [7, 38, 88]. Along these lines, deletion of HVR1 abolishes the enhancing effect of HDL on HCVpp entry [94, 104]. Recently, SR-BI has also been shown to bind and internalize the serum amyloid A (SAA) apolipoprotein [106, 107]. SAA is a protein mainly produced by the liver immediately after infection, tissue damage or inflammation [108], suggesting a beneficial role for this protein in host defense. Interestingly, SAA inhibits HCV entry [109, 110]. However, its inhibitory effect is not due to competition between HCV and SAA for SR-BI binding, but rather to a direct interaction between SAA and the viral particle. HDL has also been shown to modulate the antiviral activity of SAA, suggesting a tight relationship between SAA and HDL in modulating HCV infectivity [109].

The exact role of SR-BI in HCV entry is not well understood. Recent data suggest a direct interaction between the viral particle and SR-BI [84, 96], but it has also been proposed that HCV may interact with SR-BI through its associated lipoproteins [111].

Kinetics of inhibition with anti-SR-BI antibodies suggest that SR-BI might act concomitantly with CD81 [97]. However, the binding of HCVccs to CHO cells expressing SR-BI together with the absence of binding of HCVccs to CHO cells expressing CD81 suggest that a first contact with SR-BI might be necessary before the particle interacts with CD81 [84]. Finally, SR-BI is able to modify the lipid composition of the plasma membrane [112–119], and it is possible that the enhancing activity on HCV entry is the consequence of such a modification, facilitating some early step in the HCV life cycle. In line with this hypothesis, it has been shown that HDL accelerates HCVpp endocytosis [103].

Claudin-1

By screening a complementary DNA library derived from HCV-permissive cells for genes that render cells susceptible to HCVpp infection, Evans et al. [84] have recently identified a new protein involved in HCV entry: claudin-1 (CLDN1). CLDN1, which is predominantly expressed in the liver [120], belongs to a family composed of 24 members responsible for the formation of tight junctions. These small proteins (between 20 and 27 kDa) contain two extracellular loops, three intracellular domains and four transmembrane passages (Fig. 2) and are characterized by the presence of a highly conserved motif W-GLWC-C in the EL1 [121]. Expression of CLDN1 in non-hepatic 293T cells renders them permissive to infection by HCVpps and HCVccs. Given that 293T cells naturally express CD81 and SR-BI [76], CLDN1 is thus the first protein described which confers susceptibility to HCV infection on nonhepatic cells. CLDN1 knock-down cells are less permissive to infection by HCVpps and HCVccs, although overexpression of CLDN1 in permissive cells does not increase infectivity. The region of CLDN1 involved in HCV entry corresponds to the first extracellular loop, particularly residues I32 and E48. It is worth noting that palmitoylation sites and the C-terminal intracellular domain, which allows interaction with other proteins involved in the tight junction, do not seem necessary for HCV entry. Lastly, an antibody directed against an epitope inserted in the first extracellular loop of CLDN1 inhibits HCV infectivity in a dose-dependent manner. Furthermore, in this approach, the half-maximal time of inhibition of HCVpp entry into target cells has been determined to be approximately 73 min [84], suggesting that CLDN1 plays a role in a late step of the entry process, probably after virus binding and interaction with CD81. Thus far, no direct interaction between CLDN1 and the HCV particle has been reported, but one cannot exclude the idea that such an interaction requires a conformational change in the envelope glycoproteins triggered by a first interaction between E2 and another entry factor, e.g. CD81 or SR-BI. The precise role of CLDN1 in HCV entry remains to be determined. However, since CLDN1 is strictly localized to the tight junctions in polarized hepatocytes, it is tempting to speculate that CLDN1 acts after lateral migration of a virus-receptor complex to the tight junctions.

Endocytosis of HCV particle

After binding to specific receptor(s) virus entry into host cells involves fusion of the lipid envelope with a cellular membrane. This process is tightly coordinated in time and space and requires drastic conformational changes in the fusion proteins, which are triggered by cellular factors. Enveloped viruses enter target cells in two different ways. Some of them (e.g. the majority of retroviruses) enter by fusing their envelope directly with the plasma membrane. This process does not depend on pH, and conformational changes in the envelope proteins are induced by interactions between the envelope proteins and a (co)receptor. Other enveloped viruses, e.g. influenza virus or vesicular stomatitis virus, enter target cells by endocytosis. In this case, the acidic pH of endosomes triggers conformational changes in the envelope proteins. The viral genome is released into the cytosol after fusion between the viral envelope and an endosomal membrane.

The use of endosome acidification inhibitors, such as bafilomycin A1, concanamycin A, ammonium chloride or chloroquine, has shown that HCV entry is pH dependent [9, 22, 23, 76, 122, 123]. Furthermore, the use of small interfering RNAs targeting clathrin indicates that HCV enters target cells by clathrin-mediated endocytosis [122, 123]. Finally, data obtained with dominant-negative mutants of proteins involved in endocytosis suggest that HCV fuses with early endosomes [122].

Fusion

Two major classes of viral fusion proteins have been described, class I and II [124]. Class I fusion proteins are synthesized as a precursor that is cleaved into two subunits by host cell proteases, with a membrane-anchored subunit containing an N-terminal (or N-proximal) fusion peptide. This proteolytic processing event creates a metastable state of the fusion protein. Class II fusion proteins have a completely different structure. They are predominantly nonhelical, having instead a β-sheet type structure. They are not cleaved

during their biosynthesis, and they possess an internal fusion peptide with a loop conformation [124]. Class II fusion proteins are synthesized as a complex with a companion membrane glycoprotein, which acts as a chaperone. Furthermore, cleavage of the companion protein activates the fusogenic potential of the fusion protein. Following a trigger by a cellular factor (low pH and/or receptor interaction) changes in the conformation of the fusion protein occur, leading to the formation of thermodynamically stable trimers. This oligomeric reorganization leads to the exposure of the fusion peptide and its insertion into a cellular membrane. Such a conformational change brings together the transmembrane domain and the fusion peptide, which is essential for the fusion process. It is worth noting that 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 [30]. As discussed above HCV entry is pH dependent, suggesting that the low pH of an endosomal compartment triggers the conformational changes in HCV envelope proteins which initiate virus-cell membrane fusion. Interestingly, an *in vitro* assay based on HCVpps and liposomes has recently been developed to better characterize the fusion process [125]. Fusion between HCVpps and liposomes is also pH dependent with a threshold pH of 6.3 and an optimum at about 5.5. The assay showed that fusion is dependent on temperature and facilitated by the presence of cholesterol but does not require the presence of any protein at the surface of liposomes [125]. For many enveloped viruses, an acidic pH induces an irreversible conformational change, which is necessary for fusion between viral and endosomal membranes. Such viruses are generally inactivated by acid pH treatment. Surprisingly, exposure of cell-surface-bound virions to acid pH followed by a return to neutral pH does not affect HCV infectivity [23, 122], 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 [126]. However, there remains some controversy regarding the identity of the HCV fusion protein. A potential structural homology with other class II fusion proteins suggests that E2 could be the fusion protein [127], making E1 the companion protein. On the other hand E1 has been proposed as a good candidate because sequence analyses suggest that it may contain a putative fusion peptide in its ectodomain [128, 129], raising the possibility that E1 is a truncated class II fusion protein [130]. It is worth noting that E1 and E2 chaperone each other to form the fully functional heterodimer [131, 132], suggesting that the interdependence of the two proteins is slightly different from other type II fusion proteins. By analyzing a peptide library of HCV envelope proteins for their activity on model membranes, Perez-Berna et al. [133] have identified peptides in both E1 and E2 which have potential fusion activity, suggesting that distinct regions in both HCV E1 and E2 may cooperate to drive the fusion process to completion. In keeping with this observation, a recent site-directed mutagenesis study in the HCVpp system has identified three regions important for fusion with liposomes [134]. The first one (residues 272–287) is located in E1, whereas the other two (residues 419–433 and 597–620) are in E2. Whether the involvement of these regions in fusion is direct or indirect remains to be determined. A high-resolution structure of HCV envelope proteins will be useful to solve this question.

Conclusion

The recent development of functional models to analyze the early steps of the HCV life cycle has led to the identification of several cell surface proteins involved in HCV entry. The data that have recently been accumulated suggest that HCV entry is a slow and complex multistep process. The exact role of each molecule involved in HCV entry remains to be determined, but our current knowledge allows us to draw a model (Fig. 3). GAGs and the LDL-R may facilitate initial attachment to the host cell. This interaction is probably mediated by the lipoproteins associated with HCV virions (represented by a beige sphere in Fig. 3). However, one cannot exclude direct contact between HCV envelope proteins and these cellular proteins. After the initial binding step, the particle likely interacts with SR-BI and CD81. Although the sequence of HCV interaction with these two entry factors has not been unequivocally determined, current understanding suggests that a first contact with SR-BI might be necessary before the particle interacts with CD81 (see above). The interaction with SR-BI can potentially be direct [88] or indirect, through HCV-associated lipoproteins [111]. Importantly, these early steps of HCV entry may be modulated by different components of the serum, which can enhance (HDL) or inhibit (oxidized LDLox, lipoprotein lipase and serum amyloid A) HCV infectivity. Furthermore, the presence of EWI-2wint in some cell types can block the interaction of the viral particles with CD81, thus potentially preventing these cells from being infected. As discussed above, CLDN1 acts at a late stage of the entry process,

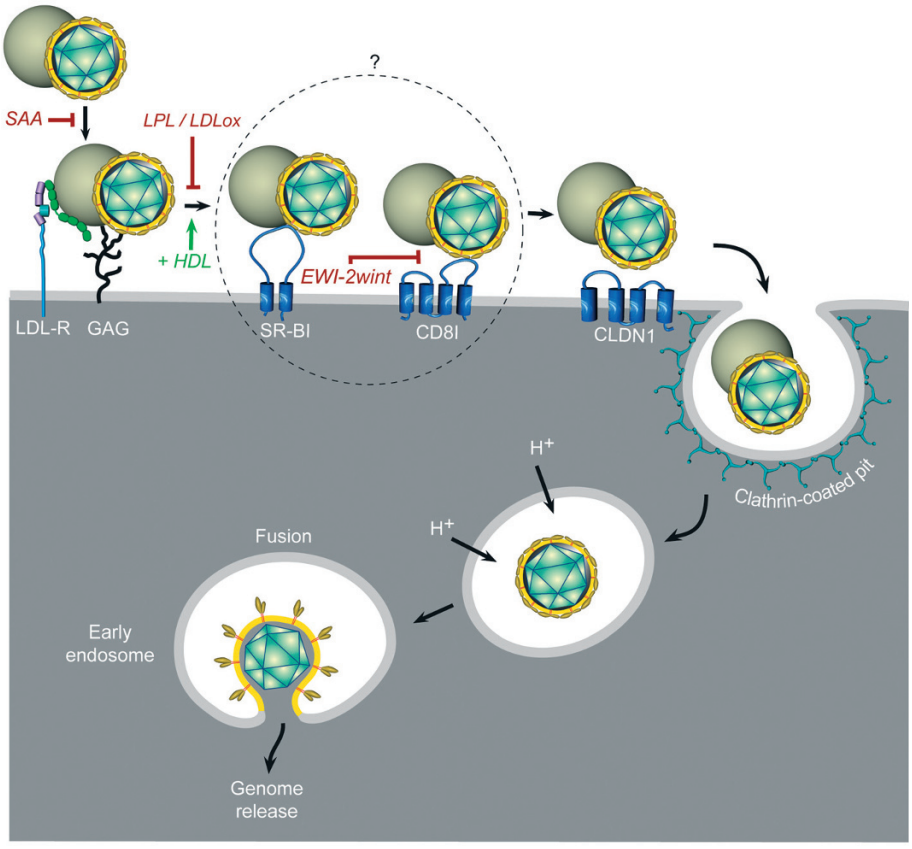

Figure 3. Model of HCV entry. See text for details.

after interactions with SR-BI and CD81 and probably after a lateral migration of the virus-receptor complex to the tight junctions. Then, HCV is internalized by clathrin-mediated endocytosis, and fusion probably occurs in early endosomes. HCV entry thus appears to be controlled by the presence of several entry factors and might also require the absence of a specific inhibitory factor.

The HCV entry process may be even more complex than already elucidated. Indeed, some human cell lines expressing CD81, SR-BI and CLDN1 remain resistant to HCV entry [84], suggesting that one or more human-specific HCV entry factor(s) remain to be discovered. Further studies will also be necessary to understand the precise role of each entry factor in the HCV life cycle. Indeed, it remains to be determined whether interactions with some entry factors lead to conformational changes in HCV envelope glycoproteins that are required for subsequent contacts with other molecules. Live cell imaging will also be necessary to analyze the dynamics of particle-receptor complex migration to microdomains and/or subcellular compartments of the host cell. Identifying the signaling events induced by early contacts between the HCV particle and the host cell will also contribute to the understanding of the entry process. Furthermore, high-resolution structures of the HCV particle and envelope proteins will be necessary to better understand the early events of the HCV life cycle, particularly the fusion process. These structures would also contribute to the molecular understanding of the interactions of the virion with lipoproteins and hence with some attachment factors. Finally, a detailed knowledge of HCV entry mechanisms will also be helpful for the characterization of new entry inhibitors, as already illustrated by the recent characterization of broad-spectrum antiviral compounds which have been shown to block HCV entry (e.g., cyanovirin-N, pradimicin-A and arbidol) [135–138].

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