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HEPATITIS C VIRUS ENTRY

Thomas von Hahn and Charles M. Rice
From Center for the Study of Hepatitis C
The Rockefeller University, 1230 York Avenue, New York, NY 10021
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Address correspondence to: Charles M. Rice, PhD, From Center for the Study of Hepatitis C, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA. Fax 212-3277048; Email ricec@rockefeller.edu.

Since its identification as the causative agent of non-A-non-B hepatitis in 1989 hepatitis C virus (HCV) has been recognized as a leading cause of chronic hepatitis, cirrhosis and liver cancer worldwide. Currently about 120 million individuals are chronically infected with a majority remaining undiagnosed or untreated (1). Current therapy with pegylated interferon and ribavirin is effective in just over half of cases and has significant side effects (2,3). Several novel drugs that specifically target viral enzymes are under development but have yet to reach the market (4).

THE HEPATITIS C VIRUS (HCV)

HCV is a small, enveloped virus with a single-stranded RNA genome of positive polarity. Together with GB virus B it forms the Hepacivirus genus within the Flaviviridae family (5). Within the infected host viral replication is thought to occur primarily if not exclusively within hepatocytes. Host cell infection is initiated by binding of the virion to cell-surface receptors followed by endocytosis and low pH-induced fusion of viral and cellular membranes that brings the nucleocapsid in contact with the cytosol (Fig. 1). The RNA genome of about 9.6 kb is decapsidated and directly translated by cellular ribosomes giving rise to a single polyprotein of around 3000 amino acids that is cleaved by host and viral proteases into the final gene products core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B and possibly an alternative reading frame protein termed AFP (5)(6). The structural proteins, core, E1 and E2, are components of the viral particle. The remaining non-structural (NS) gene products mediate genome replication inside the infected cell, a process that occurs exclusively in the cytoplasm in association with a virus-induced membrane structure known as the membranous web (6). Little is known about the mechanisms of viral morphogenesis, but it is thought that progeny virions bud at an intracellular membrane and are then exported through the cellular secretory pathway. This review describes the mechanisms of HCV cell entry, i.e. the earliest stages of the HCV lifecycle beginning with the infectious virion in the plasma and ending with the fusion of the viral envelope and the endosomal membrane.

TOOLS TO STUDY HCV ENTRY

Plasma derived HCV. For unknown reasons HCV derived from the plasma of infected individuals replicates poorly in cultured cells making the detection of productive infection cumbersome and unreliable. Detection of HCV RNA by standard reverse transcriptase polymerase chain reaction (RT-PCR) as a read-out for infection is problematic, as it does not discriminate between input viral RNA and RNA that is newly synthesized. To control for this, either an increase of RNA over time or a reduction by specific inhibitors of HCV replication (4) needs to be demonstrated. Alternatively, several authors have used RT-PCR-based detection of (-)strand RNA as this should not be found in the virion but only be generated during replication. However, to what extent this method can be trusted to be fully specific for (-)strand in everyday use is questionable (7,8).

Soluble E2. The above problems have limited the usefulness of plasma-derived HCV for the study of HCV entry. To circumvent these obstacles several in vitro tools that give more robust read-outs have been developed.

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One that has been very useful is soluble E2 (sE2), a form of the HCV glycoprotein (HCVgp) E2 from which the transmembrane domain has been deleted leading to its secretion (for more detail on HCV E2 see below). Truncations are usually made at amino acid 661, some 57 residues upstream of the beginning of the transmembrane domain, as this seems to be required for proper folding (9). sE2 can be generated in large quantities and has proven useful in many studies especially with regard to receptor discovery (see below and (10,11)). However, on the viral particle the HCV glycoproteins (HCVgps) E1 and E2 are thought to be present as a heterodimer, making it unlikely that sE2 recapitulates all characteristics and functions of the native HCVgps. To enable functional studies of E1E2 mediated cell entry up to the point of membrane fusion other tools were needed.

HCV pseudoparticles (HCVpp). HCV pseudoparticles (HCVpp), retroviral particles that have HCV E1E2 heterodimers instead of retroviral gps incorporated in their envelope, were the first robust in vitro assay of HCVgp-mediated entry (12-14). Pseudotyping takes advantage of the ability of retroviruses to incorporate heterologous glycoproteins into their envelope during budding. Specifically, HCVpp are generated by co-transfection of three plasmids into 293T producer cells: (1) the gag-pol genes of either human immunodeficiency virus (HIV) or murine leukemia virus (MLV), (2) a gutted provirus genome missing most genes but retaining the long terminal repeats and packaging signal and containing a reporter gene such as GFP or luciferase, and (3) the unmodified HCVgps. Gag-pol expression results in the formation of retroviral particles into which the provirus genome encoding the reporter is packaged. During budding an unknown number of HCV E1E2 heterodimers are incorporated into the pseudoviral envelope. As no retroviral gps are present in this system the cell entry properties of HCVpp are determined solely by the exogenous glycoproteins. Pseudoparticle entry results in the delivery of the retroviral nucleocapsid into the cytosol followed by reverse transcription and integration of the viral 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. Even after the development of an HCV strain that can readily be propagated in vitro (see below) HCVpp continue to offer certain advantages. Most notably, they do not depend on the ability of HCV to establish replication so that their use is not restricted to the few cell lines that allow high levels of HCV RNA replication.

Cell-culture grown HCV (HCVcc) and plasma-derived HCVcc. In what was a major breakthrough for HCV research, a genotype 2a genome isolated from a patient with an unusually severe course of acute hepatitis C has been found to replicate to high levels over prolonged periods of time in cell culture and to generate progeny virions in the process (15-17). This genome has been designated Japanese Fulminant Hepatitis 1 (JFH-1), and JFH-1-based HCV grown in cell culture is referred to as HCVcc. With the advent of HCVcc the entire HCV lifecycle including entry, assembly and release can now be recapitulated in vitro. HCVcc have been found to be infectious in chimpanzees and in mice with transplanted human hepatocytes (18). Moreover, in contrast to HCV derived from infected patient plasma (plasma-derived HCV), HCVcc recovered from infected animals (plasma-derived HCVcc) is capable of replicating robustly in cell culture (18). A remaining limitation is that robust viral growth is observed only in certain cell lines such as Huh-7.5 and Huh-7 Lunet cells. This is likely to be determined not at entry but at the level of viral replication since HCVpp entry occurs with comparable efficiency in a number of other cell types (19).

The infectious virion. Based on the limited imaging data available (16) and in analogy to the classical flaviviruses, the HCV particle is thought to have a diameter of about 50 nm. The nucleocapsid is built from copies of the core protein and contains the RNA genome. The nucleocapsid itself is surrounded by a host

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cell-derived membrane envelope that is studded with E1E2 heterodimers (5,16).

HCV GLYCOPROTEINS E1 AND E2

Both HCVgps are type I membrane glycoproteins with a C-terminal transmembrane domain anchored in the viral envelope (20). E1 and E2 make up residues 192 – 383 and 384 – 746, respectively, of the HCV polyprotein.

In their functional form E1 and E2 are thought to form a non-covalently linked heterodimer (21,22). While the molecular structure of the heterodimer is as yet unknown some features have been gleaned by indirect approaches: sE2 binds well to two of the viral (co-)receptors, scavenger receptor class B type I (SR-BI) (10) and CD81 (11). Thus E2 is thought to be primarily responsible for receptor binding. The SR-BI binding site seems to be located in the hypervariable region 1 (HVR-1), a 27 amino acid stretch that makes up the very N-terminus of E2 (10). Several non-contiguous sites further downstream in E2 have been implicated in CD81 binding, indicating that this binding site is conformational (23-25). Glycans have been found to be present in 4 and 11 sites in E1 and E2, respectively, and some of these are critical for HCVpp entry (26).The HCVgps are suspected to be class-II fusion proteins (for review (27)). Both E1 and E2 have been suggested to harbor the fusion peptide, a hydrophobic sequence that inserts into a cellular membrane to trigger fusion with the viral envelope.

ASSOCIATION BETWEEN HCV AND HOST SERUM FACTORS

Early studies of patient plasma-derived HCV reported that upon density gradient centrifugation HCV RNA migrated not as a discreet band but rather a broad smear with some of it having an unexpectedly low apparent density (28,29). A fairly small study observed that HCV RNA in plasma samples with high infectious titers tended to migrate at lower density, suggesting that low density may correspond to high infectivity (30). Interestingly, plasma derived HCVcc has been found to have a lower density and higher specific infectivty (infectious units per RNA copy) than the HCVcc used to inoculate the animal in the first place (18).

Several reports have found plasma-derived HCV to be bound to low and very low density lipoproteins (LDL and VLDL, respectively), an association that could account for the observed low density (see (31) for review). Some authors have also detected an association with high density lipoproteins (HDL) while others did not detect such an interaction. Recombinant E1 and E2 have been reported to be capable of binding all three species, VLDL, LDL and HDL, by interacting with a lipid moiety contained in the lipoproteins. The term "lipo-viroparticle" has been used to refer to complexes made up of viral and lipoprotein components and immunoglobulins that can be isolated from infected plasma; their precise structure and biological significance, however, remain unclear.

If host serum factors modulate HCV infectivity, it is unclear to what extent in vitro systems that may lack these can be trusted to recapitulate the in vivo situation. In this regard it is somewhat reassuring that HCVcc and plasma-derived HCVcc seem to have density characteristics similar to those of plasma-derived HCV (18).

INTERACTION WITH CELL-SURFACE FACTORS

Infection of the host cell is initiated through interactions between the HCVgps and several cell surface molecules. Currently, three host molecules, CD81, SR-BI and claudin-1 (CLDN1), are thought to be specific and required (co-)receptors for HCV entry. Moreover, glycosaminoglycans (GAGs) are likely to facilitate efficient entry. Other factors have been implicated although their role is less well established. Finally, since HCV-resistant cell lines expressing all of the above factors exist, at least one additional essential factor may still be missing.

CD81. Certainly the best studied of the HCV (co-)receptors, the tetraspanin CD81 is a 236

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amino acid protein with four transmembrane domains and intracellular N- and C-termini (Fig. 2B). The CD81 large extracellular loop (CD81LEL) is the critical region for the HCV co-receptor function. The crystal structure of CD81LEL shows 5 α-helices arranged into a head and a stalk subdomain with two intramolecular disulfide bonds stabilizing the head (32). A low-polarity patch in the head subdomain that involves residues previously reported to be important for the sE2 interaction (see below) has been suggested as a virus binding site.

A characteristic of tetraspanins is their ability to interact with each other and numerous other cell surface molecules to form molecular networks, referred to as "tetraspanin webs" (33). The physiological function of CD81 is only partly defined: it is part of the B-cell receptor complex but not essential for humoral immmune function (33). Additionally, it seems to be involved in but again not strictly required for sperm-egg fusion (34). Besides being an essential HCV co-receptor, CD81 has also been found to be required for invasion of hepatocytes by the sporozoites of Plasmodium falciparum, the etiologic agent of malaria tropica (35).

CD81 was initially suggested as an HCV receptor because of its sE2 binding properties (11) and its role was subsequently validated in functional assays. Antibodies against CD81 as well as soluble versions of its large extracellular loop (CD81LEL) are highly potent inhibitors of HCVpp of all HCV genotypes (13,14,36-38) as well as of HCVcc (15-17), plasma-derived HCVcc (39) and plasma-derived HCV (40). Moderate downregulation of CD81 by RNA interference abrogates HCV infectivity (19,38,41). In keeping with this, it has been determined that CD81 cell surface levels must be above a critical threshold for HCV entry to be efficient (41). Moreover, the CD81-negative human hepatoma cell line HepG2 is resistant to HCV entry but becomes fully susceptible when engineered to express CD81 (15,38,42). This cell line has been very useful in studies investigating the molecular determinants of CD81's function in HCV entry (38,43) and has allowed CD81LEL to be identified as the critical region for both sE2 binding and HCV infection (11,38,43). The closest relative of CD81, the tetraspanin CD9, does not support HCV entry, but a chimeric CD9 where CD9LEL has been replaced with CD81LEL supports entry as well as wild type CD81 (38). Several residues within CD81LEL have been have been found to be required for sE2 binding (44,45), however, mutation of these failed to show an effect on HCVpp entry, indicating that sE2 is an imperfect model of the interaction between the E1E2 heterodimer and cell-surface CD81 (43). Indeed, the CD81 sequence from African green monkey supports HCV entry about as well as human CD81 when expressed in human HepG2 cells despite being unable to bind sE2 (43).

It is widely believed that CD81 acts as an HCV co-receptor, i.e. interacts with the virion after binding to a primary cell-surface receptor has occurred. This notion is based on the observations that antibodies against CD81 are still able to inhibit HCV infection after initial attachment of the virus to the cell (19,46) and, conversely, anti-CD81 has little effect on binding of HCVpp and plasma-derived HCV to cells (47). The above-mentioned involvement of CD81 in sperm-egg fusion could be taken to hint at a role in the fusion between viral envelope and cellular membrane (34).

SR-BI and SR-BII. SR-BI is a 509 amino acid protein with two transmembrane domains and intracellular N- and C-termini ((48) and Fig. 2A). The product of an alternatively spliced mRNA, SR-BII differs from SR-BI in that the C-terminal 42 residues of SR-BI have been replaced by 40 residues encoded by a more downstream exon (48). The structure of the large extracellular domain that is identical for SR-BI and SR-BII has not been resolved, but it is known to be glycosylated at multiple sites. SR-BI was initially identified as the major physiological receptor for high-density lipoproteins (HDL) in the liver where, in a process termed selective lipid uptake, cholesteryl esters flow from the SR-BI-bound HDL-particle into the cell membrane. This process is thought to occur without endocytotic uptake of the HDL particle,

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although SR-BI-mediated endocytosis of HDL has also been observed. The physiological functions of SR-BI have been reviewed in detail in (48).

As with CD81, the implication of SR-BI as an HCV receptor came with the observation that it is the major binding site for sE2 on hepatoma cell lines (10). Numerous subsequent studies have established its functional role in HCV entry. Antibodies against SR-BI and siRNA-mediated down regulation of SR-BI expression result in a significant inhibition of HCVpp and HCVcc infectivity, although variability between HCV genotypes and incomplete inhibition are often observed (37,39,42,49). Plasma derived HCVcc has been found to be as sensitive to anti-SR-BI as cell culture grown virions (39) (50) . Unfortunately, the lack of an HCV resistant cell line where SR-BI expression confers susceptibility to HCV entry has significantly hampered the study of SR-BI's role in HCV entry.

SR-BI is a multiligand receptor with at least two distinct ligand binding sites (48). Several SR-BI ligands have been found to affect HCV infectivity. Oxidized LDL and serum amyloid alpha have been identified as inhibitors of HCV entry. Their mechanism of action is unclear, but direct competition for a binding site on SR-BI does not seem to be involved (51-53). In contrast, SR-BI's physiologically most important ligands, HDL, enhance cell entry of HCVpp and HCVcc when present during infection (54-56). HCVpp from which HVR1, the putative SR-BI binding site in E2, has been deleted are not responsive to HDL. Moreover, a moderate down-regulation of SR-BI on the cell is sufficient to abolish HDL enhancement and a drug termed blocker of lipid transfer (BLT-) 4, that allows HDL binding to SR-BI but inhibits selective lipid uptake, abolishes HDL enhancement without affecting baseline infectivity (53-55). This has led to the hypothesis that SR-BI might indirectly facilitate HCV entry by enriching the membrane in cholesterol. Indeed, HCVpp and HCVcc entry is mildly inhibited by cholesterol depletion, possibly because low membrane cholesterol causes a relocation of CD81 to intracellular sites (49). However, the ability of SR-BI to specifically bind sE2 suggests a direct receptor function. Both SR-BI and CD81 bind sE2 (10,11), however, when these molecules are over expressed in CHO cells, only SR-BI seems to be capable of binding HCVcc, leading to the hypothesis that it may act as a primary receptor for HCV (19).

CLDN1. Since the advent of HCVpp it had been noted that expression of the known HCV candidate receptors SR-BI and CD81 was in many cases insufficient to support HCV entry. This led to the hypothesis that at least one additional factor must be required (14,42). Using an expression cloning approach the tight junction component CLDN1 has recently been found to be required for HCV entry (19). Expression of CLDN1 confers susceptibility to HCVpp to the CD81-positive and SR-BI-positive, yet normally HCV resistant, cell lines 293T (human embryonic kidney) and SW13 (human adrenocortex carcinoma) (19). Conversely, in normally HCV susceptible cells down regulation of CLDN1 blocks entry.

Claudins constitute the backbone of the epithelial tight junctions that separate the apical from the basolateral membrane compartment (see (57) for review). A selection of the 24 claudin family members is expressed in all epithelial tissues, where claudins form strands in the plasma membrane that interact with partner strands on neighboring cells in a zipper-like manner to obliterate the intercellular space and form the epithelial barrier. While CLDN1 had not previously been implicated in host-pathogen interactions, CLDN3 and CLDN4 in the intestinal epithelium, more specifically their second extracellular loops, are known to be the molecular targets of Clostridium perfringens enterotoxin (57).

Like CD81, CLDN1 is a small (211 amino acid) cell-surface protein with four transmembrane domains and intracellular N- and C-termini (Fig. 2C). There is, however, no sequence homology between claudins and tetraspanins. Structural information on claudins has not been reported, but two amino acid residues in the first extracellular loop (EL1) of CLDN1 have been identified as critical for HCV entry (19). However, CLDN1

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does not interact with sE2 or HCVcc and, in fact, a direct interaction between CLDN1 and any viral component has not been demonstrated. No antibodies are available against extracellular CLDN1 epitopes, but by insertion of a flag epitope in the EL1 a CLDN1 mutant has been generated that supports HCV entry while allowing it to be blocked by anti-flag antibodies (19). These data indicate that HCV infection requires CLDN1 to interact with an extracellular partner that is likely to be the incoming virion. However, an interaction between CLDN1 and an unidentified intermediate factor rather than the virion itself is also possible.

Functional studies have suggested that CLDN1 acts after virus binding and likely functions downstream of CD81 in the HCV entry process (19). Moreover, cell surface expressed CLDN1 enhances syncytia formation with cells over expressing HCVgps. In the context of viral entry, however, it is unclear whether CLDN1 is directly involved in the fusion event or in some way primes the HCVgps for subsequent fusion.

Glycosaminoglycans (GAGs). GAGs are linear polysaccharides that are present on cell surface proteins throughout the human body. Like a number of other viruses, HCV has been found to interact with GAGs (58,59). The interaction is thought to be mediated by E2 and occur preferentially with highly sulfated GAGs such as eparin sulfate (HS) (58,60). Heparin (a short, very highly sulfated variant of HS that is produced by mast cells) and highly sulfated but not normally sulfated HS are weak inhibitors of HCVpp and HCVcc entry (46,60,61)and treatment of target cells with glycosidases reduces HCV infectivity (46), supporting that an interaction with GAGs does indeed promote viral entry. As GAGs are ubiquitous, it has been hypothesized that rather than being a high-affinity, high-specificity receptor, GAGs may temporarily retain the virion at the cell surface allowing for interactions with less abundant higher affinity receptors to occur.

Low density lipoprotein receptor (LDLr). In infected plasma HCV is associated with lipoprotein species of low density and numerous studies using plasma-derived HCV have suggested that LDLr, the major receptor for LDL and certain VLDL species on hepatocytes, is involved in HCV binding to and uptake into different cell types (59,62-65). This process may be mediated through an interaction between LDLr and the lipoprotein rather then the virion (66). However, it has not rigorously been tested whether the observed binding and/or uptake result in productive infection. This shortcoming is mostly due to difficulties detecting infection initiated by plasma-derived HCV in vitro. Thus LDLr could be involved in a non-productive pathway that leads to cellular uptake of HCV but not to HCV infection. For the more robust in vitro systems (HCVpp, HCVcc, and plasma-derived HCVcc) neither convincing evidence for LDLr usage nor data firmly ruling out its involvement have been published. An antibody against the apolipoprotein E (a component of VLDL) binding site on LDLr has been reported to moderately inhibit HCVpp entry (13) and while one study reported an inhibition of HCVpp by the LDLr ligands LDL and VLDL (67) most others did not observe such an effect (13,54,55). Clearly, more work will be required to define whether or not LDLr has a role in HCV entry.

Others. Two lectins, DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) and the related L-SIGN (liver-specific intercellular adhesion molecule 3-grabbing nonintegrin), have been demonstrated to bind sE2, plasma-derived HCV and also a number of unrelated viruses (68-70). Neither molecule is expressed on hepatocytes, thus they are unlikely to function as direct entry receptors. However, DC-SIGN- and L-SIGN-bound HCVpp can be transferred to susceptible cells in co-culture and it is therefore conceivable that these molecules aid HCV infection in vivo by capturing virions and passing them on to hepatocytes (71,72).

Asialoglycoprotein receptor has also been suggested to play a role in HCV-entry based on an observed interaction between this protein and baculovirus-expressed HCV

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structural proteins (73). The relevance of this observation in the context of functional HCV entry, however, has not been demonstrated.

Some human cell lines and all non-human cell examined so far remain resistant to HCV entry even when engineered to express human SR-BI, CD81 and CLDN1, indicating that these three do not constitute a complete set of HCV (co-)receptors. Thus at least one additional unknown factor may be required. Alternatively, there could be an unidentified cellular factor that restricts HCV entry in certain cell lines.

HOST RANGE AND TISSUE TROPISM OF HCV

HCV is characterized by a narrow host range and, probably, limited tissue tropism. Both these restrictions may be determined at the receptor level given that, when transfected into cells, RNA of the JFH1 isolate has been demonstrated to establish replication in non-hepatic (74) and even non-human (75) cell lines.

The natural host range of HCV is limited to humans and chimpanzees. CD81 sequences from tamarin and african green monkey, both of which are HCV resistant, have been found to efficiently support HCV entry indicating that CD81 is not a primary determinant of host range (43). Murine CD81, however, is 10-fold less effcient at supporting HCV entry compared to the human sequence (43). CLDN1 is even less likely to be a determinant of host range as mouse CLDN1 fully supports HCV entry when expressed in 293T cells (19). This leaves SR-BI as a potential human-specific factor. Indeed, sE2 binds to human but not mouse SR-BI (10). However, the caveat remains that, at least in the case of CD81, sE2 binding has been found to be poorly predictive of co-receptor function (43). Thus the known HCV (co-)receptors do not account for the observed host range restriction.

Within the human host, hepatocytes are thought to be the primary site of HCV replication. Transplantation of human hepatocytes into immunodeficient mice is sufficient to make these animals readily infectable with HCV, demonstrating that hepatocytes are indeed sufficient for HCV infection (76). However, there is an ongoing debate as to whether other tissues and cell types such as B-cells, T-cells, microglia and enterocytes harbor replicating HCV in vivo. Although it cannot be excluded that HCV might use distinct receptors and routes of entry in different cell types, what is currently known about its (co-)receptor requirements fits, at least partly, with hepatotropism. While CD81 is expressed in all nucleated cells and thus unlikely to define tissue tropism, both SR-BI and CLDN1 are most highly expressed in the liver. In fact, SR-BI serves as the hepatic receptor for circulating HDL making a convincing candidate primary receptor for circulating virions. However, SR-BI is also expressed at high levels in organs that synthesize steroid hormones and lower levels have been reported for other sites (48). Expression of CLDN1 is seen in a number of epithelial tissues but the levels are highest in the liver followed by the kidneys (77). As HCV entry seems to require high CLDN1 levels a threshold effect could conceivably dictate tissue tropism (19). DC-SIGN and L-SIGN although not expressed on hepatocytes have both been found on liver sinusoidal endothelial cells and thus might help to direct circulating HCV to its target (78).

POST CELL SURFACE EVENTS

Following virus-receptor interaction(s) at the cell surface both HCVpp and HCVcc are taken up by clathrin dependent endocytosis (79,80). Endocytotic uptake seems to proceed with very slow kinetics: only 50% of HCVpp were reported to have reached a proteinase K protected compartment (that may correspond to an endosome) 53 min after the entry process has begun and the half-time for membrane fusion was given as 73 min (79). Whether any cell-surface (co-)receptors accompany the virion in the endosome is currently unknown.

HCVpp entry is inhibited by dominant-negative Rab5 but not Rab7 indicating that delivery to early but not late endosomes is required (79). Within the endosome decreasing pH triggers fusion between the viral envelope and the endosomal membrane

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(14,42,46,80,81). Optimal fusion occurs at pH 5 – 5.5 with no fusion occurring above pH 6 (82,83). Although this is somewhat controversial (79,81), endocytotic uptake may not be absolutely required for fusion: in the presence of inhibitors of endosomal acidification HCVcc bound to the cell surface can to some extent be induced to fuse and initiate infection by a low pH wash (81). This low pH rescue, however, requires incubation of the bound virions for up to 1 h at 37°C suggesting that slow processes occurring at the cell surface may be required to render HCVcc pH sensitive. This hypothesis fits well with the observation that prior to cell binding HCVcc are not inactivated by low pH treatment (81). Interestingly, HCVgps incorporated into HCVpp have been shown to undergo low pH induced fusion with liposomes that are devoid of any HCV-specific (co-)receptors (83). However, it is unclear to what extent the presence of (co-)receptors would further enhance fusion efficiency in this system.

ONE VIRUS, MANY RECEPTORS

The development of HCVpp and HCVcc has made it possible to readily observe HCVgp mediated cell entry in vitro. Much information has been learned from these systems and many open questions remain or have newly arisen. Currently, it seems that HCVpp and HCVcc entry requires CD81, CLDN1 and likely SR-BI. Thus the group of HCV (co-)receptors is unexpectedly large, suggesting that HCV entry involves an intricate series of events. Although this is far from certain, SR-BI may act early in the entry process (19), possibly as a primary receptor, whereas CD81 and CLDN1 act at the post-binding stage (19,46,47). Moreover, the precise function(s) that each of these cellular factors fulfills in HCV entry and how these are orchestrated remains to be defined.

The involvement of CLDN1 suggests that the polarized nature of hepatocytes in vivo (Fig. 3) is an important factor to consider when modeling the interactions of HCV with its (co-)receptors. In hepatocytes and in polarized cells in culture CLDN1 is thought to localize strictly to the tight junction region (57,77). Neither SR-BI nor CD81 are known to be tight-junction associated but rather localize to the basolateral membrane compartment (84,85). If one assumes a direct interaction between CLDN1 and the entering virion, this would suggest that the virus needs to have a means to move about the cell surface in a directed manner to reach all of its (co-)receptors.

Tight-junction associated molecules have previously been implicated in the entry of other viruses including coxsackie-adenovirus-receptor (CAR) in the case of coxsackievirus and adenoviruses (86) and junction-adhesion molecule in the case of reoviruses (87). An elaborate route of entry into polarized cells has recently been demonstrated for coxsackievirus group B (CVB) entering the intestinal Caco-2 cell line (88). The primary CVB receptor, decay accelerating factor (DAF), is located on the luminal surface of intestinal epithelial cells. Upon virus binding a signaling cascade involving several cellular kinases triggers the actin-dependent relocation of the virion-DAF complex to the tight junction region, where CAR, the CVB co-receptor, is localized and endocytosis occurs. It will be important to understand whether HCV also manipulates cellular signaling networks in order to coordinate interactions with its multiple (co-)receptors.

Any insights learned from in vitro models of HCV entry will eventually have to be rigorously tested in vivo. Unfortunately, the only animal models of HCV infection are chimpanzees and immunodeficient mice with transplanted human hepatocytes (76). The usefulness of these animal models is limited for ethical, technical and economical reasons. The ideal system to study (co-)receptor contributions to the HCV entry process would be a small animal model that can be bred as well as genetically manipulated. Such as system, however, may not become available in the near future. An attractive and more readily attainable alternative may be to study plasma-derived HCVcc. As bona fide infectious HCV generated by human or chimpanzee hepatocytes in vivo it likely shares the properties of human plasma-derived HCV, and it can readily be studied due to its ability to establish robust infection in vitro. Initial

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experiments suggest that dependency of HCV entry on E2, CD81 and SR-BI, hold true in this system (39). However, much more work remains to be done to reach a better understanding of the mechanisms underlying HCV entry in the setting of human infection. Eventually, this will open up novel avenues for the rational design of anti-viral therapies targeting this critical first step of the HCV lifecycle.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Steps of HCV entry. (1) In the plasma the virion interacts with host lipoproteins. (2) Host cell entry is initiated through interactions with several (co-)receptors on the cell surface. (3) This leads to clathrin-mediated endocytosis followed by Rab5 dependent transport to (4) the early endosome compartment where acidification triggers (5) fusion between the viral envelope and the endosomal membrane resulting in release of the nucleocapsid and/or viral RNA genome into the cytosol. This is followed by the later steps of the HCV lifecycle: translation, genome replication, assembly of progeny virions and their release into the circulation.

Fig. 2. Membrane topology of HCV (co-)receptors. (A) SR-BI: asterisks indicate glycans; "P" indicates palmitoylations; the dashed line indicates the part that is different in the SR-BII splice variant. (B) CD81: the grey shaded area indicates the large extracellular loop (LEL), the only part of the molecule for which structiral information is available (32); residues that have been implicated in the interaction with E2 are indicated (see text); SEL – small extracellular loop. "P?" indicates potential palmitoylation sites. (C) CLDN1: EL1 - extracellular loop 1; residues that are critical for (co-)receptor function are indicated; FLAG denotes the site where a flag-epitope insertion allowed the generation of a CLDN1 mutant that supports HCV entry (19). In cells expressing CLDN1-flag HCV entry can be blocked using anti-flag antibodies. "P?" indicates potential palmitoylation sites. EL2 – extracellular loop 2.

Fig. 3. Location of HCV entry factors in the liver epithelium. As in all epithelial cells the plasmamembrane of hepatocytes is divided into a basolateral compartment that faces the "milieu interieur" or inside of the body and an apical compartment. The tight junction strand, where CLDN1 and other claudins are located, separates these compartments. In hepatocytes, the basolateral membrane compartment harbors CD81 and SR-BI and is in direct contact with the blood that fills the space of Disse and the sinusoidal lumen. The apical membrane compartments of neighboring hepatocytes form the walls of the bile canaliculi that drain the bile produced by the hepatocytes into larger bile ductules and, eventually, through the bile duct into the intestinal lumen.

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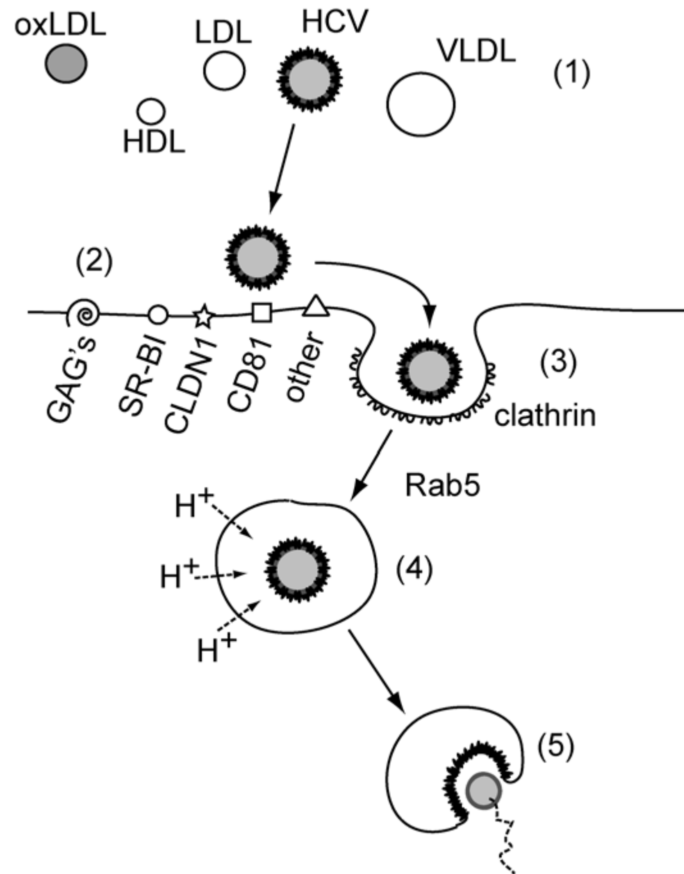

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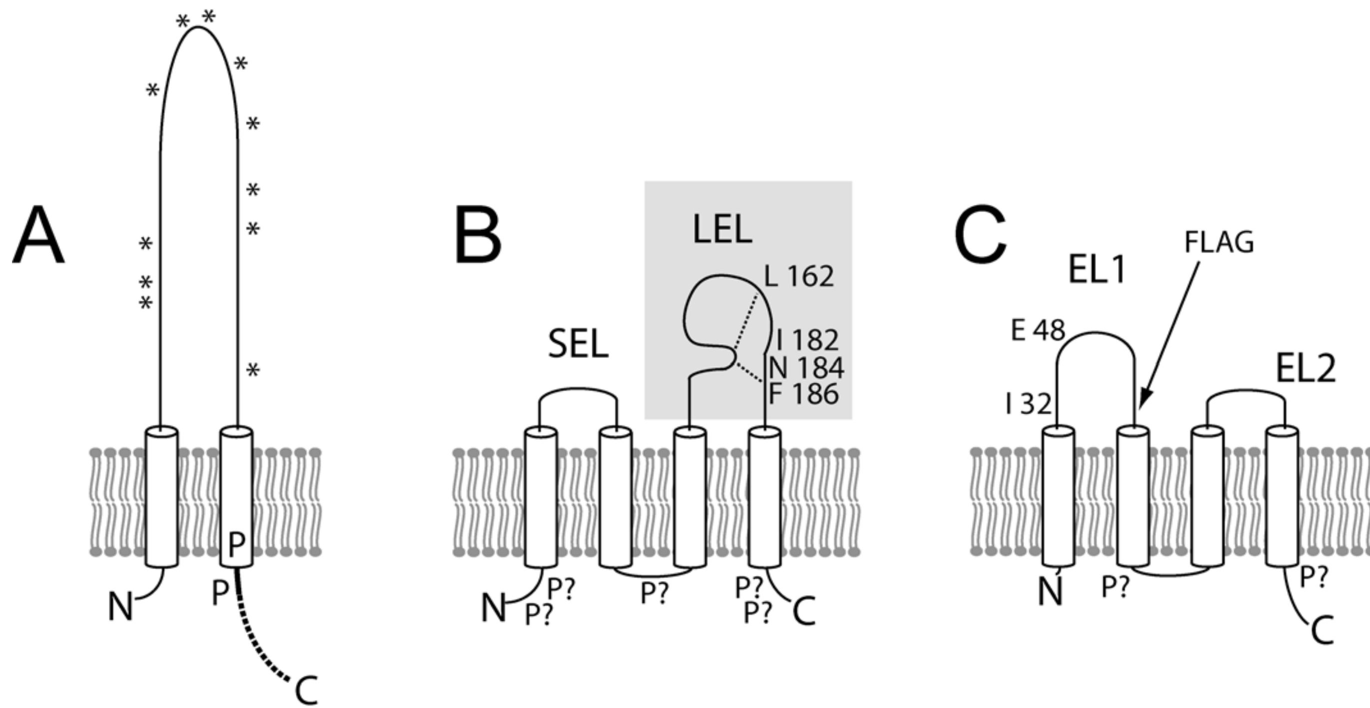

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