Hepatitis C Virus Entry*

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The Hepatitis C Virus

Hepatitis C virus (HCV)³ is a leading cause of chronic hepatitis, cirrhosis, and liver cancer. There are currently ~120 million individuals infected worldwide, with the majority remaining undiagnosed or untreated (1). The available therapy, a combination of pegylated interferon and ribavirin, has limited efficacy and significant side effects; drugs that specifically target viral enzymes have yet to reach the market (2).

HCV is a small, enveloped virus with a single-stranded RNA genome of positive polarity that replicates primarily, if not exclusively, within hepatocytes (3). Host cell infection is initiated by binding of the virion to cell-surface receptors, followed by endocytosis, low pH-induced fusion of viral and cell membranes, and release of nucleocapsid into the cytosol (Fig. 1, left). The 9.6-kb RNA genome is translated by cellular ribosomes to yield a single polyprotein of ~3000 amino acids, which 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 ARFP (3, 4). The structural proteins core, E1, and E2 are components of the viral particle, whereas the NS gene products mediate genome replication, a process that is thought to be primarily responsible for receptor binding. A 27-amino acid stretch at the very N terminus of E2, termed the hypervariable region 1, appears to contain the SR-BI-binding site (8). Several noncontiguous sites farther downstream in E2, including a site (8), has been identified by measuring reporter gene activity. Even after development of an HCV strain that can readily be propagated in vitro (see below), HCVpp continue to offer certain advantages. Most notably, because their use is independent of productive replication, they are not restricted to the few cell lines that support robust HCV RNA accumulation.

In what was a major breakthrough for HCV research, an HCV isolate termed Japanese fulminant hepatitis 1 was found to replicate to high levels over prolonged periods of time in cell culture and to generate infectious progeny (HCVcc) (15–17). With HCVcc, the entire HCV lifecycle, including entry, assembly, and release, can be recapitulated in vitro. Moreover, HCVcc is infectious in chimpanzees and in mice with transplanted human hepatocytes, and, in contrast to patient plasma-derived HCV, virus recovered from these animals (plasma-derived HCVcc) is capable of robust replication in cell culture (18). A remaining limitation is that efficient viral growth is observed only in certain cell lines such as Huh-7.5 and Huh-7 Lunet cells.

The Infectious Virion

Based on the limited imaging data available (16) and by analogy to related flaviviruses, the HCV particle is thought to have a diameter of ~50 nm. The nucleocapsid is built from copies of the core protein and the RNA genome and is surrounded by a host cell-derived membrane studded with E1/E2 heterodimers (3, 16). As sE2 binds well to two of the viral (co-)receptors, scavenger receptor class B, type I (SR-BI) (8), and CD81 (9), E2 is thought to be primarily responsible for receptor binding. A 27-amino acid stretch at the very N terminus of E2, termed hypervariable region 1, appears to contain the SR-BI-binding site (8). Several noncontiguous sites farther downstream in E2 have been implicated in CD81 association, suggesting a conformational binding site (19-21). Many studies have indicated that plasma-derived HCV is associated with LDL and VLDL, and there is some evidence that interactions with these lipoproteins enhance infectivity (reviewed in Ref. 22).
Interaction with Cell-surface Factors

Infection of a host cell is initiated through interactions between the HCV gp s and several cell-surface molecules. Currently, three host proteins, CD81, SR-BI, and claudin-1 (CLDN1), are thought to be essential (co-)receptors for HCV entry. In addition, glycosaminoglycans (GAGs) likely facilitate efficient virus uptake, and several other players have been implicated. Interestingly, cell lines exist that express all of the known entry factors but remain HCV-resistant, indicating that at least one additional essential molecule may still be undefined.

CD81—Certainly the best studied of the HCV (co-) receptors, the tetraspanin CD81 is a 236-amino acid protein with four transmembrane domains and intracellular N and C termini. The physiological function of CD81 is only partly defined: it is part of the B-cell receptor complex but not essential for humoral immune function (23). It also seems to be involved in, but not strictly required for, sperm-egg fusion (24). CD81 has also been found to interact with a primary receptor has already occurred. The involvement of CD81 in sperm-egg fusion might hint at a role for this molecule in fusing the viral envelope and cell membrane (24).

SR-BI and SR-BII—SR-BI is a 509-amino acid protein with two transmembrane domains and intracellular N and C termini (39). 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 downstream exon (39). SR-BI was initially identified as the major physiological receptor for HDL in the liver, where, in a process termed selective lipid uptake, cholesterol esters flow from the SR-BI-bound HDL particle into the cell membrane (reviewed in Ref. 39).

Like CD81, the implication of SR-BI as an HCV receptor came with the observation that it is the major binding partner for sE2 in hepatoma cell lines (8). Numerous subsequent studies have established its functional role in HCV entry. Antibodies against SR-BI, as well as small interfering RNA-mediated down-regulation of its expression, result in a significant inhibition of HCVpp and HCVcc infection, although variability between HCV genotypes and incomplete inhibition are often observed (27, 29, 33, 40). Plasma-derived HCVcc has been found to be as sensitive to anti-SR-BI antibodies as cell culture-grown virions (29, 41). Unfortunately, an HCV-resistant cell line rendered susceptible by SR-BI expression has not been identified, significantly hampering studies of the role of this molecule in viral entry.

SR-BI is a multiligand receptor with at least two distinct ligand-binding sites (39); several of these ligands have been found to impact HCV infectivity in different ways. Oxidized LDL and serum amyloid-α inhibit HCV entry through an unknown mechanism that does not seem to involve direct com-

MINIREVIEW: HCV Entry

interaction with cell-surface factors.
petition for a binding site on the receptor (42–44). In contrast, the physiologically most important SR-BI ligand, HDL, enhances entry of HCVpp and HCVcc when present during infection (45–47). Moderate down-regulation of SR-BI abolishes this HDL-mediated enhancement, as does deletion of hypervariable region 1, the putative SR-BI-binding site in E2, from the infecting HCVpp. A drug termed BLT-4 (blocker of lipid transfer), which allows HDL binding to SR-BI but inhibits selective lipid uptake, abolishes HDL enhancement without affecting base-line infectivity (44–46). This suggested 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 (40). However, the ability of SR-BI to specifically bind sE2 suggests a direct receptor function. Interestingly, although CD81 also interacts with sE2 (8, 9), only SR-BI is capable of binding HCVcc when these molecules are overexpressed in Chinese hamster ovary cells (32). This observation has led to the hypothesis that SR-BI may act as a primary receptor for HCV (32).

**CLDN1**—Since the advent of the HCVpp system, it had been noted that expression of the known candidate receptors SR-BI and CD81 was often insufficient to support HCV entry. This led to the hypothesis that at least one additional factor must be required (14, 33). The tight junction component CLDN1 has recently been found to be essential for HCV entry using an expression cloning approach (32). Subsequently, it was discovered that HCV can also utilize the related CLDN6 and CLDN9 (48). Expression of CLDN1 confers HCVpp susceptibility to the CD81- and SR-BI-positive yet normally HCV-resistant cell lines 293T (human embryonic kidney) and SW13 (human adenocarcinoma) (32). Conversely, in normally HCV-resistant cells, such as Hep3B and Huh-7.5, down-regulation of CLDN1 blocks viral entry.

Claudins constitute the backbone of the epithelial tight junctions, which separate the apical and basolateral membrane compartments (reviewed in Ref. 49). A selection of the 24 claudin family members is expressed in all epithelial tissues, forming strands in the plasma membrane that interact in a zipper-like manner with partner claudin strands on neighboring cells, obliterating the intercellular space and forming the epithelial barrier. Although CLDN1 had not previously been implicated in host-pathogen interactions, the second extracellular loops of CLDN3 and CLDN4 are known to be the molecular targets of Clostridium perfringens enterotoxin in the intestinal epithelium (49).

Like CD81, CLDN1 is a small (211 amino acid) cell-surface protein with four transmembrane domains and intracellular N and C termini. There is, however, no sequence homology between claudins and tetraspanins. The first, larger, extracellular loop of CLDN1 is critical for HCV entry (32), although it does not bind sE2 or HCVcc, and in fact, no direct interaction between CLDN1 and any viral component has been demonstrated. No antibodies are available against extracellular CLDN1 epitopes, but by insertion of a FLAG epitope in the first extracellular loop, a CLDN1 mutant has been generated that supports HCV entry while allowing it to be blocked by anti-FLAG antibodies (32). These data indicate that HCV infection requires CLDN1 to interact with an extracellular partner that is likely the incoming virion, but could also be an unidentified intermediate factor. CLDN1 seems to act at a late stage in the HCV entry process, downstream of CD81 (32). Interestingly, it has recently been reported that HCV can spread directly between adjacent cells in a manner that requires CLDN1 but not CD81 (50).

**GAGs**—GAGs are linear polysaccharides that are present on cell-surface proteins throughout the human body and have been shown to interact with a number of different viruses, including HCV (51, 52). This binding is thought to be mediated by E2 and to occur preferentially with highly sulfated GAGs such as heparan sulfate (HS) (51, 53). Heparin (a short, very highly sulfated variant of HS produced by mast cells) and highly but not normally sulfated HS are weak inhibitors of HCVpp and HCVcc entry (37, 53, 54). Treatment of target cells with glycosidases also reduces HCV infectivity (37), suggesting that an interaction with GAGs does indeed promote viral entry. As GAGs are ubiquitous, they are unlikely to be high affinity, high specificity receptors for HCV, but may instead temporarily retain the virion at the cell surface, facilitating interactions with less abundant high affinity receptors.

**LDL Receptor (**LDLR**)—In infected plasma, HCV is associated with LDL species, and numerous studies using plasma-derived HCV have suggested that LDLr, the major hepatocyte receptor for LDL and certain VLDL species, is involved in HCV binding and uptake (52, 55–58). This process may be mediated through interaction of LDLr with the lipoprotein rather than the virion (59). It has not been rigorously tested, however, whether this association results in productive infection. Indeed, for the more robust in vitro systems (HCVpp, HCVcc, and plasma-derived HCVcc), there is neither convincing evidence for LDLr usage nor data firmly ruling out its involvement. An antibody against the LDLr binding site for apoE (a component of VLDL) has been reported to moderately inhibit HCVpp entry (13), and one study reported an inhibition of HCVpp by the LDLr ligands LDL and VLDL (60), although most others did not observe such an effect (13, 45, 46). More work will be required to determine 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 (61–63). Because neither molecule is expressed on hepatocytes, they are unlikely to function as direct entry receptors. DC-SIGN- and L-SIGN-bound HCV pp can, however, be transferred to susceptible cells in co-culture, suggesting that these molecules may aid infection in vivo by capturing virions for transmission to hepatocytes (64, 65).

The 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 structural proteins (66). The relevance of this observation in the context of functional HCV entry has not been demonstrated.

Some human cell lines and all non-human cells 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. At least one additional unknown factor may therefore be required.
MINIREVIEW: HCV Entry

Post-cell-surface Events

Following virus-receptor interaction(s) at the cell surface, both HCVpp and HCVcc are taken up by clathrin-dependent endocytosis (67, 68). Endocytic uptake seems to proceed with very slow kinetics: only 50% of HCVpp were reported to have reached a protease K-protected compartment (possibly the endosome) 53 min after initiation of entry, whereas the half-time for membrane fusion was 73 min (67). Whether any cell-surface (co-)receptors accompany the virion into 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 (67). Within the endosome, decreasing pH triggers fusion between the viral envelope and the endosomal membrane (14, 33, 37, 68, 69). Prior to cell binding, HCVcc is not inactivated by low pH treatment, suggesting that the glycoproteins are primed to become pH-sensitive at some point between binding and the early endosome (69). Curiously, HCVgps incorporated into HCVpp have been shown to undergo low pH-induced fusion with liposomes that are devoid of any HCV-specific (co-)receptors (70). It is unclear, however, to what extent the presence of (co-)receptors would 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. Although much has been learned from these systems, many questions remain or have newly arisen. Currently, it appears that HCVpp and HCVcc entry requires CD81, CLDN1, and likely SR-BI; this unexpectedly large group of (co-)receptors suggests that HCV entry involves an intricate series of events. Although far from certain, SR-BI may act early in the entry process (32), possibly as a primary receptor, whereas CD81 and CLDN1 may function at a post-binding stage (32, 37, 38). The precise function(s) fulfilled by each of these cellular factors and how these events are orchestrated remain to be defined.

A recent study showed that disruption of tight junctions in confluent Caco-2 cells facilitates HCVpp and HCVcc entry (71), indicating that the nature of hepatocytes in vivo is an important consideration when modeling the interactions of HCV with its (co-)receptors (Fig. 1, right). In hepatocytes and in polarized cells in culture, CLDN1 is thought to localize strictly to tight junctions (49, 72). Neither SR-BI nor CD81 is believed to be tight junction-associated, but rather localizes to the basolateral membrane compartment (73, 74). If one assumes a direct interaction between CLDN1 and the entering virion, a means for the particle to move about the cell surface in a directed manner to reach all of its (co-)receptors must also be envisioned.

Tight junction-associated molecules have previously been implicated in the entry of other viruses, including the coxsackie-adenovirus receptor in the case of coxsackievirus and adenoviruses (75) and the junction adhesion molecule in the case of reoviruses (76). Coxsackievirus group B (CVB) has been shown to take a complex route into Caco-2 cells (77). The primary CVB receptor, decay-accelerating factor, 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-decay-accelerating factor complex to the tight junction region. The coxsackie-adenovirus receptor, the CVB co-receptor, is localized to these sites, where endocytosis can then occur. It will be important to understand whether HCV also manipulates cellular signaling networks 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 (78). The usefulness of these animal models is limited for ethical, technical, and economic reasons. It is encouraging, however, that dependence of HCV entry on E2, CD81, and SR-BI seems to hold true for plasma-derived HCVcc (29). Much work remains to be done in reaching 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 antiviral therapies targeting this critical first step of the HCV lifecycle.

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