

How does hepatitis C virus enter cells?

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CD81; envelope proteins; exosomes; hepatitis C virus (HCV); lipoproteins; low density lipoprotein receptor; scavenger receptor class B type 1 (SR-BI)

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Hepatitis C virus (HCV) exists in different forms in the circulation of infected people: lipoprotein bound and lipoprotein free, enveloped and non-enveloped. Viral particles with the highest infectivity are associated with lipoproteins, whereas lipoprotein-free virions are poorly infectious. The detection of HCV's envelope proteins E1 and E2 in lipoprotein-associated virions has been challenging. Because lipoproteins are readily endocytosed, some forms of HCV might utilize their association with lipoproteins rather than E1 and E2 for cell attachment and internalization. However, vaccination of chimpanzees with recombinant envelope proteins protected the animals from hepatitis C infection, suggesting an important role for E1 and E2 in cell entry. It seems possible that different forms of HCV use different receptors to attach to and enter cells. The putative receptors and the assays used for their validation are discussed in this review.

The World Health Organization estimates that ~170 million people, 3% of the world population, are infected with hepatitis C virus (HCV) [1]. The majority of those infected (55–85%) fail to clear the virus and become chronic carriers manifested by the persistent presence of detectable virus in the serum [2]. The clinical course of chronic hepatitis C is highly variable ranging from mild hepatitis (inflammation of the liver), fibrosis (scarring of the liver), cirrhosis (end-stage fibrosis) to hepatocellular carcinoma (liver cancer). Liver damage is not directly caused by the virus, but by the interplay between the virus and the immune system that results in the replacement of healthy liver tissue with fibrous scar tissue. About 20% of patients with chronic hepatitis C will develop liver cirrhosis within 20 years. Once cirrhosis is established, the rate of hepatocellular cancer development is 1–4% per year [3]. The standard treatment for chronic HCV infection is pegylated α -interferon in combination with the nucleo-

side analogue ribavirin. About 55% of patients respond to the therapy and show a sustained reduction in viral titer [4]. Few treatment options exist for non-responders. Ribavirin and α -interferon have general antiviral properties not specifically related to HCV. Drugs interfering specifically with HCV RNA replication or translation and processing of HCV proteins are not available yet, but a few promising candidates are in clinical testing [5,6].

Since the discovery of HCV in 1989, the major bottleneck in HCV research has been the lack of a robust and reliable cell culture system for the propagation of the virus, and the absence of a nonprimate animal model. While cultured liver cells can be infected with clinical HCV isolates, the process has been inefficient, transient and not always reproducible [7]. Our current knowledge about the mechanism of viral cell entry comes from several different approaches including vaccination of chimpanzees, structural studies of

Abbreviations

ASGPR, asialoglycoprotein receptor; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; HCV, hepatitis C virus; HCVpp, HCV pseudotyped particles; HCVcc, cell culture-derived HCV particles; HDL, high-density lipoprotein; HSV, herpes simplex virus; LDL, low-density lipoprotein; MLV, murine leukemia virus; SR-BI, scavenger receptor class B type 1; VLDL, very-low-density lipoprotein; VSV, vesicular stomatitis virus.

clinical isolates, binding studies with recombinant envelope proteins, and the use of clinical isolates or recombinant, pseudotyped viruses in infectivity assays. Results from these different approaches have not always been consistent and point towards a complex mechanism for HCV cell entry involving more than one host protein.

HCV genome and viral proteins

HCV is a single-stranded, positive-sense RNA virus belonging to the genus *Hepacivirus* in the Flaviviridae family. Its genome is ~9600 nucleotides in length and contains a single open reading frame encoding a polyprotein of ~3010 amino acids. Naturally occurring variants of HCV are classified into six major genotypes and multiple subtypes. The amino acid sequences of different genotypes vary by ~30%, whereas sequences of subtypes within a given genotype differ by 5–10%. Additional variants, known as quasispecies, are present in infected individuals and are a result of the high error-rate of the viral RNA polymerase during replication.

The HCV polyprotein is co- and post-translationally processed by host and viral proteases into at least 10 mature proteins: Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. A ribosomal frame shift during the translation of the viral polyprotein can result in the synthesis of an additional protein termed F or ARFP (for frame shift and alternative reading frame protein, respectively), but the functional relevance of this protein is not known. The structural proteins include the core, which forms the viral nucleocapsid, and the envelope proteins E1 and E2. They are cleaved from the polyprotein by the endoplasmic reticulum (ER)-resident host enzymes signal peptidase and signal peptide peptidase. The core protein is mainly found on the cytosolic side of the ER membrane and on the surface of lipid droplets that bud from the ER membrane [8]. E1 and E2 are type-I membrane proteins with extensively glycosylated ectodomains. Both proteins form a heterodimer and are retained in the ER [9]. The accumulation of the structural proteins on the ER membrane suggests that the viral capsid and envelope are formed in this compartment, although direct experimental evidence is not available. The nonstructural proteins are NS2, NS3, NS4A, NS4B, NS5A and NS5B. NS2-3 is an autoprotease, which cleaves the NS2-NS3 junction. Further proteolytic processing of the NS3-NS5 region is catalyzed by the NS3 protease and its cofactor NS4A. In addition to the N-terminal protease domain, the carboxy-terminal domain of NS3 consists of an RNA helicase and NTPase activity.

NS4A serves as a cofactor for NS3. The functions of NS4B and NS5A are largely unknown. NS5B is an RNA polymerase and catalyzes the synthesis of the viral RNA. Expression of the nonstructural proteins in the liver cell line Huh7 resulted in the formation of vesicular membrane structures similar to alterations of the ER membrane observed in hepatocytes from HCV-infected liver [10,11]. These structures are thought to be the viral replication complex.

Physicochemical properties of HCV

Little is known about the structure and morphogenesis of HCV. Electron microscopy studies of virions isolated from sera of infected patients yielded variable results with diameters for putative HCV particles ranging from 20 to 100 nm [12–14]. There is evidence that both enveloped and nonenveloped HCV virions exist in serum. Virus-like particles were detected by immunoelectron microscopy using antibodies against the viral core and envelope proteins [12,15–17]. It is not known whether all of the different HCV forms are infectious or if some of them are noninfectious, defective viral particles. Structural heterogeneity of HCV particles is also a result of their variable binding to serum components such as lipoproteins and immunoglobulins [18–21]. In many infected sera, HCV RNA could be quantitatively precipitated with lipoprotein-specific antibodies [19,22,23]. Removal of lipoproteins from infected sera by apheresis reduced HCV RNA levels by 77%, further suggesting that the majority of viral particles are associated with lipoproteins [24]. Upon separation of infected serum by density centrifugation, HCV RNA was detected in fractions containing very-low-density lipoprotein (VLDL, $d = 0.95\text{--}1.006\text{ g mL}^{-1}$), low-density lipoprotein (LDL, $d = 1.006\text{--}1.063\text{ g mL}^{-1}$), high-density lipoprotein (HDL, $d = 1.063\text{--}1.21\text{ g mL}^{-1}$) as well as in the lipoprotein-free fraction. The relative amounts of HCV RNA in these fractions vary greatly between infected people. Several factors cause this variability. HCV virions associated with VLDL are fragile and density centrifugation alters their structure and can partially destroy these particles [22,25]. The occurrence of HCV RNA-containing material in the LDL fraction and fractions of higher density might be, at least in part, an artifact of the purification procedure. Biological reasons such as the HCV genotype [23] and lipid metabolism might also influence the extent to which HCV virions interact with lipoproteins. The binding of immunoglobulins to lipoprotein–HCV complexes further affects the density of these particles [19,23,26]. For most HCV-positive sera, the majority of HCV RNA

banded at buoyant densities of about 1.03–1.08 g·mL⁻¹ and 1.17–1.25 g·mL⁻¹, which represent densities of VLDL/LDL and lipoprotein-free particles, respectively [12,18–22]. Occasionally, a third population of HCV RNA-containing material was observed at a medium density of about 1.13–1.16 g·mL⁻¹ [15,27]. Treatment of HCV RNA-containing material from low density fractions with strong detergents or chloroform which remove lipoproteins and the viral envelope shifted the density of HCV RNA-containing material to buoyant densities of 1.17–1.25 g·mL⁻¹ [20,21,28]. Low concentrations of mild detergents shifted the buoyant density of lipoprotein-associated HCV RNA-containing particles to 1.11 g·mL⁻¹. These particles lost apolipoprotein E and some of the associated lipids, but were still bound to apolipoprotein B and remained enveloped, as they reacted with antibodies directed against both envelope proteins [16,22].

HCV RNA was also found to be associated with exosomes in the serum of infected people [29]. Exosomes are 50–100 nm large vesicles and are formed by many cells (including hepatocytes) by inward budding of endosomal membranes. Upon fusion of endosomes with the plasma membrane, exosomes are released into the extracellular space. Putative functions of exosomes are in the elimination of obsolete proteins and in intercellular communication. The nature of the HCV RNA–exosome complex is not known. It might be derived from free virions that bind to exosomes in the circulation (association of two independent particles), or HCV particles might become integrated into the center of exosomes during their formation in infected hepatocytes (formation of a fused virus-exosome particle). The buoyant densities of exosomes and lipoproteins overlap, and it is possible that at least part of the lipoprotein-associated HCV RNA observed upon density centrifugation of infected sera is in fact exosome-associated HCV RNA.

Correlation of infectivity and lipoprotein association of HCV

Two studies analyzed the correlation between the buoyant density of HCV RNA-containing material and infectivity in chimpanzees [20,30]. Bradley *et al.* [30] separated infected human serum into five fractions by density centrifugation and determined the infectious titer of each fraction by injecting chimpanzees with 10-fold serial dilutions of the fractions. Almost all infectious particles were contained in the fraction with the lowest density (< 1.10 g·mL⁻¹). In the second study, human sera with known infectious titers were separated by density centrifugation and the distribution of

HCV RNA was determined by RT-PCR [20]. HCV RNA in highly infectious serum was predominantly found in fractions with low density (1.06 g·mL⁻¹), whereas HCV RNA in less infectious plasma was found at a higher density (1.17 g·mL⁻¹). Both studies suggest that HCV particles associated with lipoproteins represent the species with highest infectivity, whereas lipoprotein-free virions are poorly infectious.

Role of E1 and E2 in viral infection

What is the composition of the virus in lipoprotein-associated infectious particles? Viral components that were repeatedly detected in the VLDL/LDL fractions of infected sera are HCV RNA and the core protein suggesting that at least the viral capsid is present [12,14,17,26,31]. Surprisingly, the detection of the envelope proteins E1 and E2 within infectious viral particles has been challenging. Several studies showed an association between E2 and HCV RNA in infected sera using either E2-specific antibodies or the E2-binding protein CD81 as capturing reagent [32–35]. However, it was not investigated if the captured HCV RNA was bound to lipoproteins. Three reports provided evidence that E2 can be part of lipoprotein-associated HCV particles [16,22,36]. Nielsen *et al.* [22] used several different antibodies against E2 and lipoproteins to precipitate HCV RNA from the VLDL/LDL fractions of infected serum. Antibodies against lipoproteins captured >90% of HCV RNA in these fractions, whereas several anti-E2 antibodies precipitated = 25% of HCV RNA. The majority of lipoprotein-associated HCV RNA was not recognized by antibodies against E2. Others failed to detect E2 at all in HCV RNA-containing low-density particles [12,26,29]. It remains puzzling that it has been so difficult to detect envelope proteins in infectious viral particles. Several scenarios seem possible: (a) The methods used to detect E1 and E2 did not have sufficient sensitivity. (b) The epitopes recognized by the detection reagents were masked, e.g. by lipoproteins. However, this scenario cannot explain the failure to detect the envelope protein by western blotting [26]. (c) As noted above, the viral envelope in lipoprotein-associated particles might be labile and was lost during purification of these particles. However, Bradley *et al.* [30] demonstrated that viral particles isolated from low-density fractions of sucrose gradients remained infectious, arguing against major structural changes or loss of viral components required for infectivity during centrifugation. (d) Alternatively, some of the lipoprotein-associated viral particles might not be enveloped. Enzymatic digestion of lipoproteins in HCV-positive sera made HCV RNA vulnerable to

ribonucleases [37], whereas viral RNA in enveloped viruses is usually protected by the envelope and capsid from enzymatic degradation. This result suggests that lipoprotein-associated virions might have a different structural organization than classical enveloped viruses.

The absence of envelope proteins in lipoprotein-associated virions would certainly explain the difficulties to detect them. However, as there is no precedent for an enveloped virus that does not use its envelope proteins for cell entry, the hypothesis that these particles exist remains unpopular.

Despite the difficulties in visualizing the envelope proteins in clinical HCV isolates, functional data suggest that E1 and E2 can be present in infectious particles. Antibodies specific for E2 block the binding of HCV from infected serum to human cell lines [38,39]. Vaccination of chimpanzees with recombinant E1 and E2 either protected the animals from subsequent HCV infection or enabled them to resolve the infection [40]. Coinjection of HCV and an antiserum against E2 also protected chimpanzees from infection [41]. These examples show that antibodies against E1 and E2 can be generated that block the interaction between HCV and host cells.

Infectivity assays with HCV particles

In order to validate a cell surface protein as a viral cell entry receptor, an infectivity assay is required. It should be shown that (a) a nonpermissive cell line which does not express this protein is rendered permissive upon expression of the protein; and (b) an antibody against the protein, a recombinant form of the protein or other methods that down-regulate or inactivate the receptor candidate can block viral infection. Assays to measure HCV infection have used three different types of HCV particles: clinical HCV isolates, HCV pseudotyped particles (HCVpp), and cell culture-derived HCV particles (HCVcc). The following section describes the advantages and disadvantages of these particles for infectivity assays.

Clinical isolates

The use of clinical isolates in infectivity assays has the advantage that these particles should closely resemble the virus as it occurs in infected people, as little or no manipulation of the infected serum is required to isolate the particles. However, HCV from infected sera infects and replicates in cultured cells only with very low efficiency and makes the quantification of infection challenging [7,42]. It has been difficult to

unambiguously distinguish between virus bound to cell surface receptors and virus having gained access to the cytoplasm. PCR amplification and *in situ* hybridization were used to detect plus-strand HCV RNA associated with cells. The detection of plus-strand HCV RNA does not discriminate between bound and internalized HCV and necessary controls to eliminate cell surface-bound virus (e.g. low pH wash) were not always performed. Another assay to quantify virus internalization relies on the uptake of the protein biosynthesis inhibitor α -sarcin. α -Sarcin does not enter cells with intact cell membranes. However, co-entry occurs with internalization of several animal viruses [43–45]. The inhibition of protein synthesis therefore correlates with the infectivity of the viruses. Cells became sensitive to α -sarcin upon incubation with HCV-infected serum and it was concluded that this assay could be used to evaluate the effect of several compounds on HCV infectivity [46]. Critics may argue that there is no proof that sensitivity to α -sarcin directly correlates with HCV entry. Moreover, even if internalization of virions can be unambiguously demonstrated, the absence of a robust cell culture system makes it difficult to prove that the internalized viral genome is in a replication-competent form. In light of the technical difficulties, experiments measuring infection of cultured cells with clinical isolates should be interpreted with caution.

HCV pseudotyped particles (HCVpp)

HCVpp are recombinant viral particles. Their capsids are derived from a retrovirus that efficiently assembles in cell culture, such as HIV or murine leukemia virus (MLV). Instead of displaying HIV or MLV envelope proteins, they integrate native HCV glycoproteins E1 and E2 into their envelope and therefore should resemble native HCV virions in terms of cell entry pathways [47–49]. HCVpp do not have a higher infectivity than native HCV virions, but they are engineered to code for a reporter protein such as green fluorescence protein or luciferase. Despite the low infectivity of HCVpp, the number of infected cells can be determined by means of highly sensitive fluorescence assays. For HCVpp with HIV or MLV capsids, both HCV envelope proteins, E1 and E2, were required for infectivity [47,48]. They preferentially infected hepatocytes and thus reflect the tropism of HCV. Sera from patients chronically infected with HCV, but not sera from healthy donors, were able to neutralize the infectivity of HCVpp further, suggesting that the E1–E2 complex on HCVpp mimics the structure of the envelope proteins in native HCV [48,50,51]. However,

structural analysis of HCVpp showed that they were not bound to lipoproteins and therefore lack an important feature associated with infectivity of clinical HCV isolates [52]. HCVpp were produced in 293 cells, which do not synthesize lipoproteins, thus explaining the lack of lipoprotein association. The production of HCVpp in VLDL-synthesizing cells such as liver cells or intestinal cells might lead to the assembly of lipoprotein-associated HCVpp. However, the inefficient transduction of these cells and the resulting low expression levels of E1 and E2 have prevented such an approach so far. Another potential problem that might prevent the association of HCVpp with lipoproteins is that HCVpp assemble at the plasma membrane, whereas both HCV virions and lipoproteins in infected liver cells are thought to assemble at the ER membrane [7,10,14,53,54]. It is also possible that the HCV core protein, which is not present in HCVpp, is required for lipoprotein association.

Cell culture-derived HCV particles (HCVcc)

Very recently, three groups developed robust cell culture systems for the propagation of a HCV strain isolated from a patient with fulminant hepatitis [55–57]. Two groups used the wild-type genome, one group generated a chimeric clone replacing the core-NS2 gene region with the corresponding region from another clone of the same genotype. Hepatoma cells transfected with the full-length HCV genome produced HCV particles, which could infect naive hepatoma cells. The nonstructural protein NS5A was reliably detected in infected cells by western blotting and immunocytochemistry, thus allowing for the unambiguous identification of infected cells. The buoyant densities of the produced virions differed between the three systems, probably due to the use of different subclones of the hepatoma cell lines Huh7 as viral host. In one system, chimeric virions had a broad density distribution ranging from 1.01 to 1.18 g·mL⁻¹, suggesting an association with lipoproteins [56]. Virions with highest infectivity banded at 1.10 g·mL⁻¹. The majority of particles banded at densities of 1.14 g·mL⁻¹ and above, but were poorly infectious. Thus, the correlation observed in chimpanzees between the density of viral particles and their infectivity was also observed in this cell culture system. Viral particles produced in the other two systems were homogenous with densities of 1.10 g·mL⁻¹ and 1.16 g·mL⁻¹, respectively [55,57]. Virions with buoyant densities of 1.16 g·mL⁻¹ were used to infect a chimpanzee [55]. The buoyant density suggests that these virions were not associated with

lipoproteins. The virus was infectious in chimpanzees and viral RNA was detected in the serum up to 5 weeks postinfection. Thereafter, infection was cleared without signs of liver inflammation.

The described cell culture systems are an important breakthrough in HCV research and should enable the analysis of individual steps of cell entry such as cell attachment, internalization, and fusion. It is important to show how representative this HCV strain is and if the findings apply to other strains. The nucleotide sequences that set this viral strain apart from others and allow its propagation in cell culture need to be identified and will probably lead the way to a more general cell culture system.

HCV receptor candidates

Despite the difficulties in detecting the envelope proteins in infectious particles, the most common assumption has been that the envelope proteins E1 and E2 are responsible for viral attachment to cells and subsequent cell entry. Consequently, recombinant E1 and E2 were used to screen for cell-surface receptors with high affinity to these proteins. Five cell surface proteins were described as potential HCV receptors based on their affinity to recombinant HCV envelope proteins: CD81, the scavenger receptor class B type I (SR-BI), L-SIGN, DC-SIGN and the asialoglycoprotein receptor (ASGPR). Heparan sulfate, a glycosaminoglycan in the plasma membrane of many cells, also binds to recombinant E2 with high affinity [58] and blocks binding of HCV from infected sera to Vero cells [38], although no binding to E1–E2 heterodimers on HCVpp was observed [59]. Finally, the LDL receptor is another receptor candidate based on the finding that HCV particles in serum associate with lipoproteins and infectivity correlates with lipoprotein association. These potential receptors can be grouped into three categories according to the nature of their interaction with HCV: CD81 binds directly to amino acids of the envelope protein E2; L-SIGN, DC-SIGN and ASGPR bind to carbohydrate residues of E1 or E2; the LDL receptor probably does not interact directly with any viral components, but binding is mediated by lipoproteins. SR-BI might play a dual role in HCV binding, i.e. it can directly interact with E2 and it can bind HCV via lipoproteins.

CD81

CD81 belongs to the family of tetraspanins. It is expressed in most human tissues with the exception of red blood cells and platelets. Several functions have

been attributed to CD81 including cell adhesion, motility, metastasis and cell activation [60]. CD81 was identified as a potential HCV receptor by screening a cDNA expression library with recombinant E2 as a probe [33]. The interaction between both proteins has been extensively studied and the binding sites on both proteins were mapped [61–63]. CD81 has a small and a large extracellular loop. The large extracellular loop is sufficient to mediate binding to recombinant E2 [33,65] and is mainly responsible for HCVpp cell entry [64]. The dissociation constant K_D between the large extracellular loop of CD81 and the ectodomain of E2 is ~ 2 nM [65]. CD81 might also facilitate the release of HCV virions from infected cells by binding to E2 in the ER and recruiting viral particles into exosomes. When expressed in Chinese hamster ovary (CHO) cells, E1 and E2 were retained in the ER. Co-expression of human CD81 caused the release of both envelope proteins into exosomes, which are secreted from cells [29].

Results from infectivity assays with HCVpp, HCVcc and clinical isolates relating to CD81 are summarized in Table 1. CD81 is necessary but not sufficient for cell entry of HCVpp. The CD81-negative cell line HepG2 was resistant to infection, but became permissive upon transfection with a CD81 expression construct [64,66,67,72]. To date, no CD81-negative cell line has been identified that can be significantly infected with HCVpp. However, not all CD81-positive cell lines can be infected [47,64,66]. Antibodies to CD81 inhibited infection with HCVpp by at least 90% [47,48,68].

Recombinant CD81 caused at least 50% reduction of infection. CD81-specific siRNA that down-regulated cell surface expression of CD81 by $\sim 70\%$ completely inhibited infection [64].

Expression of CD81 in host cells is also required for infectivity of HCVcc. Recombinant CD81 and antibodies to CD81 neutralized infection [55–57]. CD81-negative HepG2 cells were resistant to infection, but infectivity was restored in HepG2 cells transfected with CD81 [56].

In contrast to promoting infectivity of HCVpp and HCVcc, the role of CD81 in binding and internalization of clinical HCV isolates is not as clear. Antibodies against CD81 or recombinant CD81 had no or only a marginal effect on the binding and internalization (as measured by the α -sarcin assay) of HCV from infected sera to Huh7 cells, HepG2/CD81 cells and Molt4 cells [38,46,68,69]. Overexpression of CD81 in Huh7 cells enabled binding of HCV particles from infected sera to these cells, but CD81 by itself was not capable of facilitating viral entry. However, if the endocytic activity of CD81 was increased by fusing the cytoplasmic domain of the transferrin receptor to CD81, HCV was internalized and replicated in these cells [36]. This suggests that CD81 requires an endocytotic cofactor in order to promote HCV cell entry.

SR-BI

SR-BI is primarily expressed in the liver and steroidogenic tissues. It is a multiligand receptor, binding a

Table 1. Inhibition of cell binding and infection by CD81 antagonists.

Source of virus	Reference	Inhibition of infection			Detection method
		Cell	Antagonist	% inhibition	
Clinical isolate	38	Huh7	Anti-CD81 (JS81)	0 ^a	RNA (+) strand by RT-PCR
		Huh7	Anti-CD81 (1.3.3.22)	30 ^a	RNA (+) strand by RT-PCR
	68	Huh7	Anti-CD81 (JS81)	20 ^a	RNA (+) strand by RT-PCR
		HepG2/CD81	Anti-CD81 (JS81)	0 ^a	RNA (+) strand by RT-PCR
	46	3T3/CD81	Anti-CD81 (JS81)	70 ^a	RNA (+) strand by RT-PCR
		Molt4	Recombinant CD81	0	α -Sarcin assay
HCVpp with HIV core	69	Huh7	Anti-CD81 (JS81)	0–20 ^a	RNA (+) strand by RT-PCR
		Huh7	siRNA	100	Fluorescence assay
	64	Huh7	Anti-CD81 (5A6)	>90	Fluorescence assay
		Huh7	Recombinant CD81	100	Fluorescence assay
	47	Huh7	Anti-CD81 (JS81)	100	Fluorescence assay
		Huh7	Anti-CD81 (JS81)	100	Fluorescence assay
HCVpp with MLV core	48	Huh7	Anti-CD81 (JS81)	90	Fluorescence assay
		Huh7	Recombinant CD81	50	Fluorescence assay
HCVcc	55	Huh7	Anti-CD81 (JS81)	>90	Fluorescence assay
		Huh7.5	Recombinant CD81	80	RNA (+) strand by RT-PCR
	57	Huh7.5.1	Anti-CD81 (5A6)	>95	RNA (+) strand by RT-PCR

^aOnly cell binding was analyzed.

variety of lipoproteins including HDL, LDL and VLDL, and proteins such as β -amyloid and maleylated BSA [70]. SR-BI facilitates the cellular uptake of lipids from both LDL and HDL, although the underlying mechanisms are different. Upon binding to SR-BI, LDL is internalized by receptor-mediated endocytosis and degraded in lysosomes. This process is similar to, although less efficient than the LDL-uptake by the LDL receptor. Binding of HDL to SR-BI does not lead to lysosomal degradation. Instead, SR-BI selectively extracts the lipids and subsequently releases lipid-depleted HDL into the extracellular space.

SR-BI was identified as potential HCV receptor by coprecipitation with recombinant E2 [71]. SR-BI probably interacts with the hypervariable region 1 (HVR1) of E2, as recombinant E2 lacking HVR1 did not bind to SR-BI and antibodies to HVR1 competed with SR-BI for E2 binding [66,71]. The involvement of SR-BI in cell entry of HCV particles is summarized in Table 2. Transfection of 293 cells with SR-BI increased their susceptibility to infection with HCVpp about 20-fold. However, the susceptibility of 293/SR-BI cells was still \sim 200- and 20-fold lower than the susceptibility of the hepatocellular carcinoma cells Huh7 and HepG2/CD81, respectively [66]. The hepatocarcinoma cell line SK-Hep1, which is CD81-positive and SR-BI-negative [74], is resistant to HCVpp infection [66]. It has not been investigated whether ectopic expression

of SR-BI in SK-Hep1 cells restores infectivity. A polyclonal antiserum against SR-BI inhibited infection of Huh7 cells with HCVpp by \sim 70% [66,72]. A 90% down-regulation of SR-BI expression in Huh7 cells by RNA interference caused a 30–90% inhibition of HCVpp infection, depending on the HCV genotype [72,74]. In another study, no siRNA-mediated inhibition of infection was observed, although SR-BI expression was down-regulated by 68% [73]. HDL, the natural ligand of SR-BI, enhanced infectivity of HCVcc and HCVpp about four-fold and up to nine-fold, respectively, although it did not act as a carrier for HCVpp because no association between both particles was found [73–75]. HDL specifically inhibited neutralizing antibodies that block the binding of E2 to CD81, whereas the activity of other neutralizing antibodies was not impaired [74,75]. The stimulating effect of HDL on infectivity and its inhibiting effect of neutralizing antibodies depended on functionally active SR-BI, since inhibitors of SR-BI-mediated lipid transfer abrogated the stimulation of infectivity and fully restored the potency of neutralizing antibodies.

Expression of SR-BI also facilitated binding of HCV clinical isolates to cells and their subsequent uptake into the endocytic compartment. SR-BI-transfected CHO cells bound twice as many virions as parental CHO cells, and the SR-BI-mediated increase in binding was completely inhibited by a SR-BI antiserum

Table 2. Inhibition of cell binding and infection by SR-BI antagonists. Additional references for the effect of LDL and VLDL are shown in Table 3.

Source of virus	Reference	Inhibition of infection			Detection method	
		Cell	Antagonist	% inhibition		
Clinical isolate	78	HepG2	HDL	0	RNA (+) strand by <i>in situ</i> hybridization	
		Vero	HDL	0 ^a	RNA (+) strand by RT-PCR	
		HepG2	HDL	10 ^a	RNA (+) strand by RT-PCR	
	76	HepG2	Anti-SRBI (polyclonal)	20 ^a	RNA (+) strand by RT-PCR	
		HepG2	Anti-HCV (polyclonal)	0 ^a	RNA (+) strand by RT-PCR	
HCVpp with HIV core	47	Huh7	Anti-SRBI (C25)	0	Fluorescence assay	
HCVpp with MLV core	66	Huh7	Anti-SRBI (polyclonal)	70	Fluorescence assay	
72	72	Huh7	siRNA	30–90 ^b	Fluorescence assay	
		Huh7	Anti-SRBI (polyclonal)	40–80 ^b	Fluorescence assay	
		Huh7	siRNA	0	Fluorescence assay	
	73	Huh7	HDL	4x increase in infectivity	Fluorescence assay	
		Huh7	LDL	0	Fluorescence assay	
		74	Huh7	siRNA	80	Fluorescence assay
			Huh7	HDL	9x increase in infectivity	Fluorescence assay
			Huh7	VLDL	0	Fluorescence assay
		Huh7	LDL	0	Fluorescence assay	
HCVcc	75	Huh7	HDL	4x increase in infectivity	Fluorescence assay	

^aOnly cell binding was analyzed. ^bDepending on E1/E2 genotype.

[76]. Surprisingly, a HCV antiserum, which contained E1- and E2-specific antibodies and was shown to neutralize infectivity of HCVpp, did not inhibit binding of clinical isolates to CHO/SR-BI cells, whereas VLDL and antibodies to beta-lipoproteins did. Similar results were obtained with HepG2 cells, although the role of SR-BI in HCV binding was less pronounced. A SR-BI antiserum inhibited HCV binding by 20%, whereas the HCV antiserum did not have any effect. These data suggest, that clinical isolates can interact with SR-BI through associated lipoproteins and not through E2.

LDL receptor

Most mammalian cells take up lipoprotein particles such as LDL from the extracellular space because they need phospholipids and cholesterol stored in LDL to build new membranes. LDL binds to the LDL receptor on the plasma membrane of cells and is internalized by receptor-mediated endocytosis. As HCV in infected sera is associated with LDL and VLDL, the virus might piggyback on lipoproteins and use their interaction with the LDL receptor to bind to and enter cells [18,46,77,78]. It was shown that the removal of free lipoproteins from serum and cell-bound lipoproteins from target cells is a crucial step for the efficient

binding of clinical HCV isolates to hepatoma cell lines and subsequent infection [26,79]. The viral component interacting with LDL or VLDL is not known. Attempts to detect a direct interaction between LDL/VLDL and recombinant core protein [80], recombinant E2 ectodomain [46] and noncovalently linked E1–E2 heterodimer (which is thought to be the native conformation) incorporated into liposomes [81] have failed. Recombinant E1–E2 heterodimers (including their transmembrane domains) interacted with lipoproteins in the absence of detergents, but this probably reflects a nonspecific, hydrophobic interaction in a hydrophilic solvent [81]. Both lipoproteins and HCV assemble in the ER of hepatocytes and intestinal cells. It seems possible that the interaction between both particles is established during their assembly [14], but that fully assembled E1–E2 dimers do not have an affinity for lipoproteins.

Table 3 summarizes the effect of reagents binding to the LDL receptor on HCV attachment and infectivity. An anti-LDL receptor antibody inhibited binding and/or internalization of HCV from infected sera by at least 60%, as measured by *in situ* hybridization or PCR detection of the HCV RNA plus strand [38,78]. An excess of LDL and VLDL, both natural ligands of the LDL receptor, inhibited binding and/or internal-

Table 3. Inhibition of cell binding and infection by LDL receptor antagonists.

Source of virus	Reference	Inhibition of infection			Detection method	
		Cell	Antagonist	% inhibition		
Clinical isolate	78	HepG2	Anti-LDL receptor (C7)	100	RNA (+) strand by <i>in situ</i> hybridization	
		HepG2	Antiapolipoprotein B/E	65	RNA (+) strand by <i>in situ</i> hybridization	
		HepG2	VLDL	100	RNA (+) strand by <i>in situ</i> hybridization	
		HepG2	LDL	100	RNA (+) strand by <i>in situ</i> hybridization	
		HepG2	HDL	0	RNA (+) strand by <i>in situ</i> hybridization	
	38	Vero	Anti-LDL receptor (C7)	60 ^a	RNA (+) strand by RT-PCR	
		Vero	VLDL	80 ^a	RNA (+) strand by RT-PCR	
		Vero	LDL	80 ^a	RNA (+) strand by RT-PCR	
		Vero	HDL	0 ^a	RNA (+) strand by RT-PCR	
	46	Molt4	LDL	28	α -Sarcin assay	
	26	PLC	VLDL	75	RNA (+) strand by RT-PCR	
		HepG2	Antiapolipoprotein B/E	85	RNA (+) strand by RT-PCR	
		76	HepG2	VLDL	50 ^a	RNA (+) strand by RT-PCR
			HepG2	LDL	20 ^a	RNA (+) strand by RT-PCR
			HepG2	HDL	10 ^a	RNA (+) strand by RT-PCR
HepG2			Anti β -lipoprotein	90 ^a	RNA (+) strand by RT-PCR	
HepG2		Anti-HCV	0 ^a	RNA (+) strand by RT-PCR		
HCVpp with HIV core		47	Huh7	Anti-LDL receptor	0	Fluorescence assay
HCVpp with MLV core	48	Huh7	VLDL	20	Fluorescence assay	
		Huh7	LDL	<10	Fluorescence assay	
		Huh7	Antiapolipoprotein E	50	Fluorescence assay	

^aOnly cell binding was analyzed.

ization to the same extent. HDL, which does not interact with the LDL receptor, had no effect. In agreement with these results, it was shown that only HCV RNA-containing particles with buoyant densities of $<1.06 \text{ g}\cdot\text{mL}^{-1}$, which corresponds to densities of VLDL and LDL, could infect cultured cells as measured by *in situ* hybridization and by co-entry of α -sarcin [26,46]. Particles with higher densities corresponding to HDL and lipoprotein-free fractions were not infectious. These results are in agreement with the aforementioned infectivity studies in chimpanzees. A role for the LDL receptor in HCV entry is further supported by findings that HCV binding to fibroblast and entry into Molt-4 cells (as measured by the α -sarcin assay) correlated with the expression level of the LDL receptor [46,77,78]. Cos7 cells, which do not bind HCV, gained this property after ectopic expression of the LDL receptor [77].

Conflicting results were obtained with HCVpp regarding the role of the LDL receptor. An antibody against the LDL receptor did not inhibit infectivity of HCVpp with HIV core [47]. In the MLV system, VLDL showed a 20% inhibition of infection. This effect was probably nonspecific, as pseudotyped particles displaying the envelope protein of vesicular stomatitis virus (VSV) were similarly affected by VLDL although VSV does not use the LDL receptor to enter cells [48]. An antibody against apolipoprotein E, which is part of VLDL, neutralized infection by $\sim 50\%$. This neutralization was specific for HCVpp, as the antibody did not neutralize infectivity of VSV-pseudotyped viruses. However, the sedimentation property in sucrose gradients suggests that pseudotyped viruses were not associated with lipoproteins and, therefore, antiapolipoprotein E antibodies should not affect infectivity [48].

L-SIGN, DC-SIGN and ASGPR

L-SIGN and DC-SIGN were shown to interact with recombinant E2, HCVpp and clinical HCV isolates [82–84]. ASGPR binds to recombinant E1 and E2 produced in insect cells [85]. L-SIGN, DC-SIGN and ASGPR are C-type (calcium-dependent) lectins and their binding to HCV is mainly mediated by carbohydrate residues of E1 and E2. In case of ASGPR, direct interactions with amino acids of E1 and E2 further increase the affinity. L-SIGN is largely expressed on endothelial cells in liver sinusoids, whereas DC-SIGN is expressed on dendritic cells. Both proteins are not expressed on hepatocytes, the main target of HCV. It is therefore unlikely that they function as direct entry receptors for HCV. However, liver endothelial cells and Kupffer cells (dendritic cells in the liver) are

localized adjacent to hepatocytes. A possible function of L-SIGN and DC-SIGN is the capture and transfer of HCV to hepatocytes, reminiscent of DC-SIGN's role in infections with HIV [86–88]. DC-SIGN enhances infection of T-cells by capturing HIV on dendritic cells and transferring the virus to T-cells.

ASGPR is most commonly found on liver cells. It facilitates the clearance of glycoproteins that lack terminal sialic acid residues from the circulation through receptor-mediated endocytosis [89]. Because insect cells do not attach sialic acid residues to glycoproteins, the binding of ASGPR to recombinant E1 and E2 produced in insect cells might be an artifact. It remains to be seen if ASGPR can bind to HCV envelope proteins produced in human cells.

A model for HCV cell entry

The cell entry of HCV has been analyzed using clinical isolates, HCVpp and HCVcc. The different model systems predict different requirements for HCV cell entry. Infectivity assays with HCVpp demonstrate the importance of CD81 and SR-BI, which both bind to envelope protein E2 [64,66,67,72,74]. CD81 is also required for cell entry of HCVcc [55–57], whereas the role of SR-BI has not been analyzed in this system. However, expression of both proteins is not sufficient for viral entry. There are several cell lines positive for CD81 and SR-BI that are nonpermissive for infection with HCVpp [64,66]. These cells lack at least one protein acting in the CD81 or SR-BI pathways. A putative entry pathway involving an interaction of HCV-associated lipoproteins with lipoprotein receptors cannot be analyzed with current HCVpp, because they do not contain lipoproteins.

Binding and infectivity assays with clinical HCV isolates point towards the LDL receptor, rather than towards CD81, as the main attachment receptor for HCV. If the α -sarcin assay is indeed an indicator for viral internalization, the LDL receptor might also mediate HCV cell entry. SR-BI can also mediate cell attachment of clinical isolates and their internalization into endosomes [76]. Rather than being mediated by E2 (as in the case of the interaction between SR-BI and HCVpp), this interaction depends on HCV-associated lipoproteins and is probably very similar to the interaction of clinical isolates with the LDL receptor. Other cellular proteins beside the LDL receptor or SR-BI might be required for the internalization of lipoprotein-associated virions, but their identification will be difficult without an infection assay for clinical isolates. Such an assay will also be required to demonstrate that the internalization of virions via lipoprotein receptors can lead to viral replication.

It seems difficult to merge the results from the different model systems into one mechanism for cell entry. Potential problems of the model systems, such as the lack of lipoprotein association of HCVpp and the difficulty to distinguish between cell attachment and internalization of clinical HCV isolates, have been mentioned and might explain the different predictions for attachment and entry receptors. On the other hand, HCV is a heterogeneous virus and more than one entry pathway might exist. It is not uncommon for viruses to use alternative receptors to enter cells. Examples are HIV and herpes simplex virus (HSV). HIV usually uses CD4 and either CXCR4 or CCR5 as receptors to infect cells. Recently, an isolate was identified that can infect CD8 T-cells which are CD4-negative [90]. HSV can use different entry receptors belonging to evolutionary unrelated classes of cell surface molecules such as glycosaminoglycans, the tumor necrosis alpha receptor family, and the immunoglobulin superfamily [91].

Figure 1 shows a model of HCV cell entry that takes into account the heterogeneity of the virus and the results obtained from the different infection assays. Several forms of HCV have been proposed to exist: lipoprotein-free enveloped virus, lipoprotein-free non-

enveloped virus, lipoprotein-associated enveloped virus and lipoprotein-associated nonenveloped virus. These different forms might use different pathways to infect cells. HCVpp most likely resembles lipoprotein-free enveloped viruses. Results from assays with HCVpp suggest that lipoprotein-free enveloped virions are infectious and require CD81, SR-BI and an as yet unidentified protein for infectivity. However, if the correlation between infectivity and lipoprotein association observed in chimpanzees can be generalized, this form of the virus only plays a minor role. Its infectivity *in vivo* is probably too low to cause a sustained infection.

Lipoprotein-associated, enveloped viral particles are probably resembled by HCVcc produced in a recently described cell culture model [56]. Their infectivity was dependent on CD81 expression on host cells and inversely correlated with their density, indicating that lipoproteins promote infectivity. Lipoprotein receptors might facilitate the efficient capture of these virions and transfer them to CD81 or SR-BI in order to initiate fusion of the viral and host cell membranes. At this point, the entry pathways of enveloped virions with and without associated lipoproteins would merge. Without lipoprotein association, the capture of virions

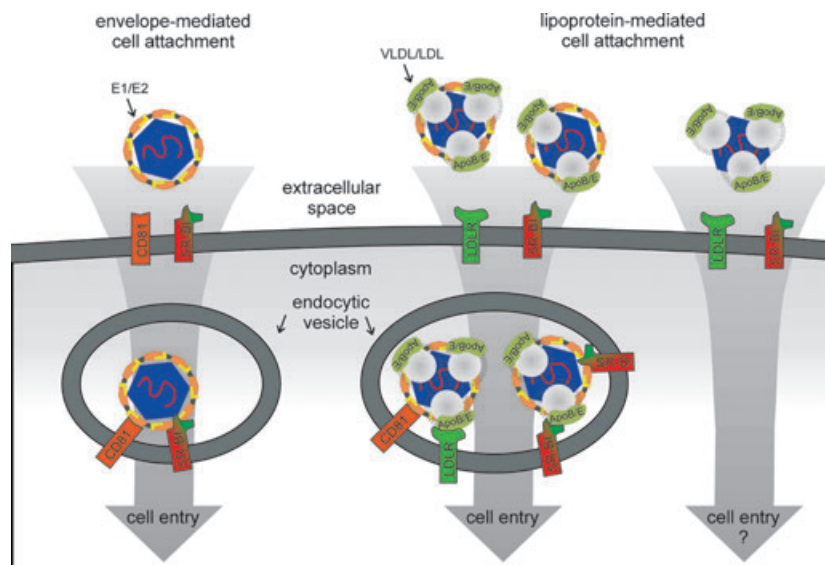


Fig. 1. Model of HCV cell attachment and entry. HCV particles in the circulation can be either enveloped or nonenveloped, and either bound to or free of lipoproteins. The different forms of HCV might use different receptors for cell attachment and entry. Enveloped virions might interact with CD81 via envelope proteins E2, whereas the interaction between lipoprotein-associated virions and the LDL receptor might be independent of the envelope proteins. SR-BI might have a dual role and facilitate binding of enveloped virions via E2, and of lipoprotein-associated virions via a lipoprotein-mediated mechanism. Upon endocytosis of lipoprotein-associated enveloped virions, E2 might interact with CD81 or SR-BI and the entry pathways for enveloped virions with and without associated lipoproteins merge. At least one additional host protein, which has not yet been identified, is required for cell entry of enveloped virions via the CD81/SR-BI pathways. The existence of nonenveloped, lipoprotein-associated virions and whether they can establish a productive infection is controversial. For simplicity, immunoglobulins, which can also bind to HCV particles, are not shown.

would be less efficient, explaining the requirement of lipoproteins for efficient infection.

Do all lipoprotein-associated virions require CD81 for cell entry? There are at least doubts. Hadlock *et al.* [34] cloned several antibodies from a patient's B-cells that prevented the binding of CD81 to recombinant E2 from genotypes 1a, 1b, 2a and 2b. If the patient had high titers of potentially broadly neutralizing antibodies, why did he continue to exhibit plasma viremia? The authors speculated that CD81 might not be the primary receptor for some HCV strains. Alternatively, the epitopes recognized by the neutralizing antibodies might not be accessible on HCV particles in the circulation (see below).

Do all HCV particles require an envelope for cell entry? Again, there are at least doubts. The detection of both envelope proteins in lipoprotein-associated virions has been challenging. It will be difficult to unambiguously demonstrate the existence of lipoprotein-associated, nonenveloped HCV particles, as the failure to detect the envelope can also be the result of technical problems of the detection methods. However, there are indications that these particles might exist [12,22]. Further analysis will be needed to decide whether nonenveloped, lipoprotein-associated virions exist and are infectious. How these particles would deliver their viral genome into the cytoplasm is not known. If such a cell entry mechanism exists, lipoprotein receptors will probably play an important role.

Electron microscopy studies and separation of viral particles on density gradients suggest the existence of lipoprotein-free, nonenveloped virions in infected serum, but there is no evidence that these particles are infectious.

The use of lipoproteins for internalization into endocytic vesicles might explain the inefficiency of the humoral immune response to clear an HCV infection. Viral epitopes required for the delivery of the viral genome into the cytoplasm might be covered by lipoproteins. If the interaction between lipoproteins and viral particles is already established during their assembly inside infected cells, then these epitopes will not be accessible in the circulation to neutralizing antibodies. Upon internalization of virions via lipoprotein receptors, the environment of endocytic vesicles might induce a conformational change of the virus-lipoprotein complex and expose these epitopes.

Association with exosomes has been suggested as another means for HCV to enter cells [29,92], but this hypothesis remains highly speculative. Exosomes contain many host proteins involved in cell adhesion and membrane fusion. Although experimental evidence is missing, it is widely believed that exosomes can fuse

with target cells and thus transport cytosolic and membrane components from one cell to another. If HCV particles are integrated into the center of the exosome and not just adsorbed to the outside of the membrane (which remains to be demonstrated), the virus might use the potentially fusogenic properties of exosomes for cell entry. This mechanism would be independent of HCV's envelope proteins. A similar mechanism has been proposed for HIV as a low-efficiency pathway for cell entry [93].

The hypothesis that different forms of HCV particles use different mechanisms for cell entry is further supported by sequence analysis of the genome of viral particles isolated from different tissues. Amino acid changes in the N-terminal domain of E2 occurred more frequently in virions isolated from whole plasma and liver than from lipoprotein-associated virions in plasma [94]. The N-terminus of E2 in the latter particles was not subject to any selection pressure from the immune system and therefore is probably not involved in receptor binding. In contrast, the majority of viral particles in plasma and in the liver appear to use that region of E2 for cell entry. This result further suggests that viral particles in serum cannot easily switch from the lipoprotein-associated state to the lipoprotein-free state and vice versa. It is likely that the interaction between lipoproteins and virions is established during viral assembly inside infected cells. It will be important to learn more about the different forms of HCV and their correlation with disease progression, to understand why some particles associate with lipoproteins and others do not, and to identify which cell types the different forms preferentially infect and replicate in.

Conclusions

Many pieces of the mechanism of HCV cell entry have been identified in recent years. However, it is unclear how these pieces fit together. The involvement of several proteins in HCV cell entry either points towards a complex entry pathway including many sequential steps, or the virus might enter cells through more than one pathway. Firstly, enveloped HCV might enter cells through an interaction between the viral envelope proteins and cellular receptors like CD81 and SR-BI. Second, HCV associated to lipoproteins attaches to lipoprotein receptors on the plasma membrane and might gain access to the cytoplasm without utilizing CD81 and potentially even without involvement of the viral envelope proteins. The extent to which these putative entry pathways are used and genetic or environmental factors that shift the virus from one pathway to the other remain difficult to analyze in the

absence of a general cell culture system for HCV. Such a system will also be required to analyze which of the different forms of the virus are able to establish a productive infection once they have entered cells. The developments of pseudotyped viral particles displaying native HCV envelope proteins and of a cell culture system for one viral strain were important steps for the validation of some receptor candidates. However, these model systems have several limitations. Pseudotyped particles produced by current methods do not bind lipoproteins and thus lack an important feature associated with HCV infectivity. The current cell culture model supports the propagation of only one HCV strain whose properties may or may not be representative of the majority of HCV strains. Therefore, infectivity assays using these systems might not measure all of HCV's properties. Until a general culture system for the propagation of the majority of clinical HCV isolates will be developed, the pathways for HCV cell entry remain speculative.

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