Hepatitis C virus entry and the tetraspanin CD81

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Abstract
CD81, a member of the tetraspanin integral membrane protein family, has been identified as an essential receptor for HCV (hepatitis C virus). The present review highlights recent published data on the role that CD81 plays in HCV entry, including the importance of actin-dependent lateral diffusion of CD81 within the cell membrane, CD81 endocytosis and the CD81-Claudin-1 receptor complex in HCV internalization. Additional functions for CD81 in the viral life cycle and the role of HCV–CD81 interactions in HCV-induced B-cell and CNS (central nervous system) abnormalities are discussed.

Introduction
HCV (hepatitis C virus) is an enveloped positive stranded RNA virus that infects an estimated 3% of the world’s population. HCV establishes a persistent infection in the majority of individuals that leads to progressive liver disease, including fibrosis, cirrhosis and hepatocellular carcinoma. Indeed, HCV is currently one of the leading indications for liver transplantation worldwide. Originally classified as non-A, non-B viral hepatitis, HCV was identified and sequenced in 1989 [1]. The 9.6 kb RNA genome encodes a polyprotein that is cleaved by viral and cellular proteases to generate the structural (core, envelope glycoproteins E1 and E2, p7) and non-structural proteins (NS2–NS5) located at the N- and C-termini respectively. The envelope glycoproteins E1 and E2 define particle interaction with target cells via interaction with cellular receptors that prime virus internalization and fusion with cellular membranes.

The first identified receptor for HCV was CD81, a member of the tetraspanin protein family [2]. A subsequent study reported that SR-BI (scavenger receptor BI) also bound HCV envelope glycoprotein E2 [3]. Successive reports highlighted that although both CD81 and SR-BI are essential molecules required for HCV entry, expression of both proteins was not sufficient to confer HCV entry. Recent reports demonstrate that the tight junction proteins Claudin-1 and Occludin [4–9] are also required along with CD81 and SR-BI to initiate virus infection, suggesting a complex multi-step entry process. HCV infects cells in a pH- and clathrin-dependent manner [10–12]. However, the specific roles of the individual receptors in the HCV entry process are not well understood. Over the last decade, considerable progress has been made and major technical advances to propagate HCV in vitro have contributed to our understanding of virus–host cell interactions. The development of HCVpps (HCV pseudoparticles) [13,14] and HCVcc (cell-culture-derived HCV) [15–17] have enabled studies on viral entry and the present paper focuses on recent studies addressing the role of CD81 in HCV entry.

The tetraspanin CD81
CD81, a 26 kDa integral membrane protein, consists of four hydrophobic transmembrane helices that separate two EC (extracellular) loops, defined as EC1 and EC2, and relatively short cytoplasmic N- and C-terminal tails. EC2 has four cysteine residues that form two disulfide bridges critical for correct folding and interaction with partner proteins. CD81 is ubiquitously expressed, although not on platelets, and can associate laterally to form homo- and hetero-interactions with each other and partner proteins in TEMs (tetraspanin-enriched microdomains; also known as the tetraspanin web). TEMs are reported to play an important role in many cellular processes, including adhesion, migration and cell signalling.

CD81–HCV-E2 interaction
The screening of a cDNA expression library from hepatoma cells that bound HCV-E2 in non-permissive T-cells identified CD81 as an E2-interacting molecule [2]. Functional studies confirmed the importance of CD81 in HCV infection using a variety of techniques, including anti-CD81 and sEC2 (soluble recombinant CD81-EC2) inhibition of HCVpp [13] and HCVcc infection [15–17]. Furthermore, silencing of CD81 in permissive cells rendered cells recalcitrant to infection [18] and ectopic expression of CD81 in HepG2 hepatoma cells, which do not endogenously express CD81, conferred permissivity to viral infection [13,15].

HCV-E2 interacts with the second EC loop of CD81, and high-affinity E2 binding is dependent on the integrity of the disulfide bonds formed by cysteine residues in CD81 [19]. The crystal structure of CD81-EC2 has been resolved and reveals five α-helices, A–E, with the HCV-E2-binding

Key words: CD81, central nervous system (CNS), Claudin-1, hepatitis C virus (HCV), hepatoma cell, partner protein, tetraspanin.
Abbreviations used: CNS, central nervous system; EC, extracellular; ERK, extracellular-signal-regulated kinase; FRET, Förster resonance energy transfer; HCV, hepatitis C virus; HCVcc, cell-culture-derived HCV; HCVpp, HCV pseudoparticle; HVR1, hypervariable region 1; MAPK, mitogen-activated protein kinase; NK, natural killer; PHH, primary human hepatocyte; sCD81, soluble recombinant CD81; sEC2, soluble recombinant CD81-EC2; sEC2, scavenger receptor BI; TEM, tetraspanin-enriched microdomains.

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region residing predominantly within the C and D helices [20]. Four amino acid residues, Leu162, Ile182, Asn184 and Phe186, within EC2 are essential for HCV-E2 binding [21]. In contrast the interactive region within the HCV E2 glycoprotein is less well defined and appears to be of a conformational nature, including amino acids 480–493 (region 1) and 544–551 (region 2) [22,23]. Recent results suggest that an additional E2 motif covering amino acids 612–619 also plays a role in binding CD81 [24]. The HCV envelope glycoproteins E1 and E2 form heterodimers that have been reported to interact with CD81 with a higher affinity compared with truncated E2, suggesting that E1 can modulate E2–CD81 binding [25].

The primary publication that identified CD81 as a receptor for HCV-E2 suggested that the interaction was specific to human CD81 [2]. However, more recent studies assessing the role of species CD81 in HCV infection demonstrate that CD81 proteins from mammalian and rodent species confer permissivity to HCVpp and HCVcc, although at significantly reduced levels [26,27]. A recent report demonstrated that several adaptive amino acid changes in the HCV envelope glycoprotein allowed HCV to more efficiently infect cells expressing murine CD81 [28].

The N-terminal 27 amino acids within the HCV E2 glycoprotein are defined as the HVR1 (hypervariable region 1) and are the target for strain-specific neutralizing antibodies. Deletion of the HVR1 reduces HCV entry and promotes CD81 binding [3,29]. In addition, viruses lacking a HVR1 are more susceptible to neutralization by anti-CD81 and sEC2 [30], suggesting that the HVR1 masks the viral CD81-binding site. The authors hypothesized that the CD81-binding site only becomes exposed after virus interaction with other host cell molecules. Masking of the CD81-binding site may protect important conserved epitopes necessary for CD81 binding from neutralization antibodies. An independent report demonstrated that mutation of specific N-linked glycans on HCV-E2 at positions 417, 532 and 635 increased E2–CD81 association, suggesting that a conformational change in E2 may be required to promote high-affinity CD81 association.

**CD81 and HCV infection of the liver**

CD81 has been shown to play an essential role in the infection of PHHs (primary human hepatocytes) with laboratory strains of HCV (HCVcc) and primary strains derived from the serum of infected individuals of PHH (primary human hepatocytes), where siRNA (small interfering RNA) silencing CD81 expression and treating cells with anti-CD81 antibodies inhibited infection [31]. However, sCD81-EC2, although able to neutralize HCVcc infection of PHHs, had minimal effect(s) on serum-derived virus infection. The authors hypothesized that serum-derived virus association with lipoproteins [32] masked the CD81-binding site, supporting earlier literature on the requirement of other host cell receptors such as SR-B1 or LDLR (low-density-lipoprotein receptor) in priming HCV CD81-dependent entry.

The species specificity of HCV infection has led to the development of the human liver uPA–SCID (urokinase-type plasminogen activator–severe combined immunodeficiency) mouse [33,34]. These immunodeficient mice with transgene-induced severe liver disease are transplanted with human hepatocytes which repopulate the diseased liver and the chimeric mouse–human liver can support the replication of hepatotropic viruses, including HCV and HBV (hepatitis B virus). Meuleman et al. [35] reported that anti-CD81 antibodies could protect mice from HCV challenge when administered before virus infection, demonstrating a role for CD81 in vivo.

**Interacting partners of CD81 important in HCV infection**

As a member of the tetraspanin protein family, CD81 interacts with a number of partner proteins, including tetraspanin CD9 and EWI-2. A cleavage product of EWI-2 was recently reported to inhibit HCV entry by reducing the association of E1–E2 with CD81 [36]. Interestingly, this cleavage product was not found in hepatocytes, leading the authors to suggest that expression in various non-permissive cell types may explain their inability to support HCV entry.

CD81 has been reported to form homodimers, which may promote higher-order oligomeric structures that are characteristic of the tetraspanin web [20,37]. In addition to CD81 homodimeric interactions, CD81 can associate with Claudin-1, and such complexes have been demonstrated in a variety of cell types that are permissive and non-permissive for HCV infection [37,38]. The homodimeric association(s) of CD81 appear to be highly conserved where a series of treatments, including cholesterol modulation and anti-CD81 antibody, have little effect on CD81–CD81 interaction(s) [39]. In contrast, depletion of cellular cholesterol with sequestering agents such as methyl-β-cyclodextrin inhibit CD81–Claudin-1 interaction, suggesting that the association is cholesterol-dependent. Further studies with fluorescently tagged CD81 and Claudin family members showed that CD81 not only interacts with Claudin-1 in a defined 1:1 stoichiometry, but with other HCV receptor active Claudin-6 and Claudin-9 molecules [39]. Mutation of amino acids 32 and 48 in Claudin-1 EC1 ablated its interaction with CD81 and HCV infection. Furthermore, introduction of the corresponding amino acids into the non-permissive Claudin-7 molecule promoted CD81 association and HCV entry. In summary, these data demonstrate that Claudin-1 EC1 residues 32 and 48 define CD81 interaction and CD81–Claudin-1 complexes are essential for HCV entry [39].

The primary target for HCV replication is hepatocytes within the liver. CD81, Claudin-1 and SR-B1 expression in normal and HCV-infected human livers is limited to the basolateral–sinusoidal hepatocellular surface [40]. Indirect FRET ( Förster resonance energy transfer) methodologies identified that CD81–Claudin-1 complexes are also present...
at the basolateral hepatocyte surface in normal and HCV-infected human livers [39]. Using an in vitro polarized hepatocyte model, Mee and co-workers demonstrated that CD81 is expressed predominantly at the basolateral surface and is excluded from tight junctions. Stoichiometric FRET analysis identified CD81–Claudin-1 complexes at the basolateral surface [39], concluding that HCV enters the liver via the sinusoidal blood, where it comes into direct contact with the basally expressed forms of the receptor complexes.

**Additional roles for CD81 in HCV infection**

Host cell signalling pathways are utilized by viruses to establish infection and HCV is no exception. Antibody or HCV-E2 engagement of CD81 has been reported to activate the Rho GTPase family members Rac, Rho and Cdc42 (cell division cycle 42) and MAPK (mitogen-activated protein kinase) signalling cascades, where inhibition of these pathways reduces HCV infection [41]. CD81-triggered Rho GTPase signalling has been suggested to induce actin remodelling, allowing lateral movement of CD81 necessary for HCV entry, whereas the Raf/MEK [MAPK/ERK (extracellular-signal-regulated kinase) kinase]/ERK MAPK pathway activated following CD81 engagement is believed to be important in as yet unidentified post-entry events. Conversely, our experiments to monitor the effects of antibody or HCV-E2 engagement of hepatoma expressed CD81 failed to activate the MAPK pathway. Our results have, however, revealed that, upon receptor engagement, CD81 is internalized in a dynamin- and clathrin-dependent manner to early endosomes (M.J. Farquhar, unpublished work), akin to the known movements of HCV [11], indicative of endocytosis of a virus–receptor complex.

SPT (single particle tracking) has proved useful to study the lateral movement and dynamics of tetraspanin CD9 at the surface of living cells [42]. We have recently performed similar experiments to study the movement of CD81 on the surface of HCV permissive cells and demonstrated that CD81 movement is under the distinct control of either protein–protein interactions within TEMs or the cytoskeleton. Cytoskeletal confinement of CD81 via its C-terminal cytoplasmic domain limits both its movement within the cell membrane and as a consequence reduces its capacity to promote HCV entry (H.J. Harris, unpublished work), suggesting that cytoskeletal control of CD81 is important for HCV entry supporting the requirement for actin remodelling.

More recently CD81 was implicated in HCV replication, where cells expressing low levels of CD81 exhibited poor replicative efficiency compared with highly expressing cells [43]. In addition, CD81 was implicated in the cellular trafficking of HCV glycoproteins during particle formation and release. In the absence of CD81, assembling virions were retained in the endoplasmic reticulum and in exosomes in the serum of HCV-infected patients [44].

Although the immune system in HCV-infected individuals mounts a significant response to the virus, its ability to clear the virus is infrequent. Recombinant HCV-E2 engagement of CD81 on NK (natural killer) cells has been reported to inhibit their antiviral response and reduce cytokine production and cytotoxic granule release [43]. However, subsequent studies have failed to observe any effects of HCVcc on NK function, suggesting that the earlier results may be an artefact of using recombinant HCV-E2 [45]. Extrahaepatic manifestations are associated with HCV such as B-cell and CNS (central nervous system) abnormalities. A minority of subjects chronically infected with HCV present with the B-cell proliferative disorder cryoglobulinaemia and an increased risk of non-Hodgkin’s lymphoma. This pathogenesis has been attributed to HCV engagement of CD81 on the surface of B-cells and the stimulation of the JNK (c-Jun N-terminal kinase) pathway to promote B-cell activation and proliferation [46]. However, further experiments to validate these observations with HCVcc are needed. HCV has been reported to bind to immune cells and, although the virus does not establish a productive infection of T- or B-cells, these cells may provide an important mechanism for virus delivery to hepatocytes in the liver [47,48]. Recent reports that cells derived from neuroepitheliomas are permissive to HCV infection in a CD81-dependent manner [49,50] suggest that the virus may infect the CNS in vivo.

**Conclusions**

Identification of CD81 as a receptor for HCV has allowed significant advancement in our understanding of HCV entry. Although CD81 plays a role in the attachment of HCV particles to the host cell, recent results highlight additional functions for CD81 in later steps in the entry process and possibly the life cycle of HCV itself. Consequently further work remains to fully understand the precise role that CD81 has to play in HCV infection.

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