Hepatitis C Virus Entry: Protein Interactions and Fusion Determinants Governing Productive Hepatocyte Invasion

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Hepatitis C virus (HCV) entry is among the best-studied uptake processes for human pathogenic viruses. Uptake follows a spatially and temporally tightly controlled program. Numerous host factors including proteins, lipids, and glycans promote productive uptake of HCV particles into human liver cells. The virus initially attaches to surface proteoglycans, lipid receptors such as the scavenger receptor BI (SR-BI), and to the tetraspanin CD81. After lateral translocation of virions to tight junctions, claudin-1 (CLDN1) and occludin (OCLN) are essential for entry. Clathrin-mediated endocytosis engulfs HCV particles, which fuse with endosomal membranes after pH drop. Uncoating of the viral RNA genome in the cytoplasm completes the entry process. Here we systematically review and classify HCV entry factors by their mechanistic role, relevance, and level of evidence. Finally, we report on more recent knowledge on determinants of membrane fusion and close with an outlook on future implications of HCV entry research.

HCV ENTRY: A TEMPORALLY AND SPATIALLY CONTROLLED PROCESS

The entry of hepatitis C virus (HCV) into hepatocytes is among the best-studied virus entry processes to date. Starting in 1998 with the discovery of the tetraspanin CD81 as HCV receptor (Pileri et al. 1998), HCV entry research has identified a multitude of critical host factors and machineries involved in productive uptake into hepatocytes. On the one hand, HCV entry exemplifies widespread viral entry routes, such as

clathrin-mediated endocytosis (Blanchard et al. 2006; Codran et al. 2006; Meertens et al. 2006; Collier et al. 2009; Trotard et al. 2009). On the other hand, certain peculiarities of HCV entry such as the temporally and spatially controlled uptake pathway (Tscherne et al. 2006; Evans et al. 2007; Brazzoli et al. 2008; Ploss et al. 2009; Sourisseau et al. 2013; Zona et al. 2013; Baktash et al. 2018) and the lipid receptor interactions of the HCV lipo-viro-particle (Germi et al. 2002; Wünschmann et al. 2006; Molina et al. 2007; Owen et al. 2009; Albecka et al. 2012; Yamamoto

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et al. 2016) seem unique to HCV or are at least less common to other viruses.

Mechanistically, and based on current evidence, the HCV entry process can be classified into at least five distinct steps, which are studied in varying detail (Fig. 1). First, the HCV lipoviro-particles (André et al. 2002), that is, hybrid particles of apolipoproteins and HCV virions, attach to the basolateral side of hepatocytes. This is mediated by glycan and protein interactions, including binding to glycosaminoglycans (GAGs), low-density lipoprotein receptor (LDLR), scavenger receptor BI (SR-BI), and CD81 (Pileri et al. 1998; Agnello et al. 1999; Scarselli et al. 2002).

Second, the virions laterally translocate in complex with CD81 toward tight junctions, that is, the contact sites between two cells. This process is coordinated by epidermal growth factor receptor (EGFR) signaling (Brazzoli et al. 2008; Lupberger et al. 2011; Zona et al. 2013). At the tight junctions, the “late” entry factors CLDN1 and OCLN guarantee productive uptake of the virus into cells, presumably via transient interactions with the virions (Evans et al. 2007; Benedicto et al. 2009; Ploss et al. 2009; Krieger et al. 2010; Sourisseau et al. 2013; Douam et al. 2014).

In the third entry phase, clathrin-mediated endocytosis factors including the adaptor pro-

Figure 1. Model of time-dependent hepatitis C virus (HCV) cell-binding, uptake, and membrane fusion. Selected attachment factors, entry factors, and entry cofactors are shown. A comprehensive list of host factors implicated in HCV cell entry is presented in Table 1. GAG, Glycosaminoglycans; LDLR, low-density lipoprotein receptor; SR-BI, scavenger receptor BI; EGFR, epidermal growth factor receptor; CLDN1, claudin-1; OCLN, occludin.

tein complex 2 (AP2), Huntingtin-interacting protein 1-related protein (HIP1R) induce uptake of the virion into an endocytic vesicle. The virion containing vesicle translocates deeper into the cell via microtubular transport. Concomitantly, V-type ATPases acidify the endosomal lumen. This pH drop triggers the fourth entry step, which is the fusion of the viral envelope with the limiting endosomal membrane.

The fifth and final step in this classification of steps of entry, the disassembly of the viral protein capsid, has not been studied extensively yet, but is thought to rapidly occur after membrane fusion. This uncoating releases the positive strand viral RNA genome into the cytoplasm, where translation and replication occur.

The tropism of HCV to humans and specifically to the human liver is at least in part mediated by the usage of human liver expressed entry factors. While SR-BI and CLDN1 contribute to the liver tropism, CD81 and OCLN mediate species tropism, at least when comparing humans and mice (Ploss et al. 2009). Mouse orthologs of the entry factors CD81 and OCLN fail to support efficient HCV entry and consequently mice expressing human CD81 and OCLN become susceptible to HCV. Thus, the common notion that virus receptors and entry factors can confer species tropism also applies to the highly human adapted HCV.

In the HCV research field, the term “receptor,” referring to a host factor that binds the virion and is necessary for cell infection, is seldomly used. Nonetheless, at least three of the four essential entry factors largely fulfill this strict definition of a virus receptor. CD81, SR-BI, and CLDN1 bind the HCV glycoproteins and render cells susceptible when ectopically expressed in cells previously lacking either one of them (Pileri et al. 1998; Scarselli et al. 2002; Bartosch et al. 2003b). However, because at least four proteins are required in concert to permit HCV infection, the field typically uses the term “entry factor.” These four proteins are SR-BI, CD81, CLDN1, and OCLN. Recent work suggests that LDLR and very low-density lipoprotein receptor (VLDLR) can substitute SR-BI functionally during HCV entry, questioning whether SR-BI is an essential entry factor for

HCV (Yamamoto et al. 2016). We will provide a more detailed description of the terminology and suggest a definition of “entry factors” and “entry cofactors” below.

The vast majority of experimental evidence on HCV entry derives from cell culture studies in nonpolarized and polarized systems. More recently, 3D organoid culture systems confirmed the role and spatiotemporal requirements of the major entry factors, namely, SR-BI, CD81, CLDN1, and OCLN, in more physiological environments (Baktash et al. 2018). Finally, sophisticated mouse models using human xenotransplant mice, adenoviral overexpression of human factors, or genetic humanization of mice confirmed the role of these four HCV entry factors (Meuleman and Leroux-Roels 2008; Meuleman et al. 2008, 2012; Bissig et al. 2010; Dorner et al. 2011; Lacek et al. 2012; Ding et al. 2017). The lack of simple in vivo systems to study HCV infection and the sheer number of involved factors has provoked some skepticism as to which reported entry factors have physiological function. Importantly, congruent evidence from in vivo models, primary hepatocyte cultures, and stem cell-derived hepatocyte cultures clearly argues for the fact that HCV in vitro models can reliably mimic many parts of the HCV entry process into human hepatocytes (Fournier et al. 1998; Codran et al. 2006; Roelandt et al. 2012; Belouzard et al. 2017; Ding et al. 2017). We will specify in detail below which level of evidence exists for the most important entry factors and cofactors.

HOST FACTORS FOR HCV ENTRY: GLYCANS, LIPIDS, AND PROTEINS

The multitude of described HCV entry factors calls for a clear definition of their role in HCV infection and a classification of their physiological relevance. Here, we make an attempt to classify reported host proteins and proteoglycans depending on their functional and stage-dependent contribution in the HCV cell entry cascade. Clearly, lipids such as cholesterol are also contributing to productive HCV invasion, for example, by clustering proteinaceous entry

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factors in membrane microdomains (Kapadia et al. 2007) and promoting membrane fusion (Lavillette et al. 2006). For a comprehensive description of the role of cholesterol and other lipids in HCV entry, we refer the reader elsewhere (Felmlee et al. 2013).

For the proteinaceous host factors involved in HCV entry, we propose a nomenclature in which (1) attachment factors are classified as auxiliary factors, which help concentrate HCV particles at the cell surface, but which display redundancy and are thus neither necessary nor sufficient for infection. (2) Entry factors are defined as host factors, which are necessary for HCV infection as determined by genetic knock-out and complementation experiments. Because of the combined action of at least four entry factors in HCV infection, neither of them alone classifies as sufficient to confer susceptibility. In concert, however, HCV entry factors are sufficient to render nonsusceptible cells susceptible to HCV. (3) Entry cofactors aid in postattachment steps of HCV entry, but are not sufficient to confer susceptibility. Many of the entry cofactors are ubiquitously expressed and essential for cell proliferation or survival, for example, growth factor receptors and clathrin endocytosis factors. Thus, it is difficult to determine whether they are necessary for HCV entry. We will state experimental evidence for the necessary involvement of entry cofactors in detail below. Clearly, the entry cofactors, similar to the attachment factors, contribute to conferring full-fledged susceptibility to HCV. Table 1 and Figure 1 list HCV attachment factors, entry factors, and entry cofactors, state the level of evidence for the factors, and classify which entry-stage(s) they primarily participate in.

Attachment Factors

Initial attachment of HCV occurs via host lipid uptake receptors that can interact with the host lipoproteins incorporated in the HCV lipo-viro-particle (André et al. 2002). Apolipoprotein E (ApoE) mediates the cellular uptake of plasma lipids by binding to specific receptors, including LDLR and heparan sulfate proteoglycans (HSPGs) (Mahley 1988). ApoE is highly en-

riched in HCV lipo-viro-particles and ApoE-specific antibodies efficiently neutralize HCV infectivity, showing that HCV can use a virion incorporated host protein for binding to host cells (Chang et al. 2007). ApoE-specific and HCV glycoprotein E2-specific monoclonal antibodies directly bind HCV particles (Chang et al. 2007), and both proteins, ApoE and E2, are detected at the surface of the HCV envelope (Merz et al. 2011; Catanese et al. 2013; Lussignol et al. 2016). Thus, both lipo-viro-particle components can interact with host cell receptors.

Attachment factors for HCV, which are ApoE receptors at the same time, are LDLR and GAGs. LDLR, which classifies as an HCV entry factor, is discussed below. GAG chains critically support initial attachment of the HCV lipo-viro-particle as heparinase treatment reduces binding of HCV to the cell surface (Koutsoudakis et al. 2006). It was further shown that soluble purified HCV E2 ectodomains of various genotypes and virus-like particles can directly interact with highly sulfated heparin sulfate (HS) (Barth et al. 2003). Altering the receptor-binding site of ApoE leads to an inhibition of HCV attachment, suggesting that the lipo-viro-particle attaches to GAGs via its lipo-protein components. Besides GAGs and LDLR, the entry factor SR-BI also contributes to virus attachment, and ectopic expression of SR-BI in CHO cells enhances particle binding (Evans et al. 2007; Dreux et al. 2009; Bankwitz et al. 2014). Taken together, the first attachment of HCV lipo-viro-particles to the host membrane seems to be ApoE-mediated binding to cell surface HSs, in particular to syndecan 4 (Lefèvre et al. 2014; Xu et al. 2015). This binding concentrates the virus on the surface for further interactions with different host entry factors.

Viral attachment itself not always leads to successful entry. The C-type lectins liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN) and DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) can bind to E2 and to serum-derived HCV particles. L-SIGN is expressed on liver sinusoidal endothelial cells and DC-SIGN is present on DCs and liver-resident macrophages, which are refractory to

Table 1. Proteins and proteoglycans involved in HCV entry classified as attachment factors, entry factors, and entry cofactors

Cellular entry factor									
Protein name	Gene name	Entry step	Expression profile	Identification method	Experimental evidence	Binding/function	Species	Other ligands/entry factor for other viruses	References
Attachment factors									
Glycosamino-glycans (GAGs), syndecan, heparan sulfate		Initial attachment	Ubiquitous	ELISA-based binding of E2 to heparin	Hepatitis C virus lipoproteins (HCV-LPs) (insect derived), HCV _{cc} heparinase treatment	Direct binding of E2 to heparin in vitro		Broad range of viruses such as hepatitis B virus (HBV) (Leistner et al. 2008), herpes simplex virus (HSV) (Shieh et al. 1992), resveratrol (RSV) (Feldman et al. 2000), human cytomegalovirus (HCMV) (Compton et al. 1993), Dengue (Hilgard and Stockert 2000)	Barth et al. 2003; Koutsoudakis et al. 2006; Morikawa et al. 2007; Lefèvre et al. 2014; Xu et al. 2015
CD209 molecule	CD209 (DC-SIGN)	Capture, infection in “trans”	Dendritic cells (DCs)	Binding assay with sE2 (DC-SIGN/L-SIGN overexpressing HeLa cells incubated with labeled sE2)	sE2, HCVpp, HCV _{cc} , HCV ⁺ sera, mannan/blocking antibodies	Direct binding to E2		Mannan, human immunodeficiency virus (HIV) (Geijtenbeek et al. 2000), Ebola (Alvarez et al. 2002), CMV (Alvarez et al. 2002), Dengue (Tassaneetrithep et al. 2003)	Gardner et al. 2003; Lozach et al. 2003, 2004; Cormier et al. 2004

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Table 1. *Continued*

Cellular entry factor									
Protein name	Gene name	Entry step	Expression profile	Identification method	Experimental evidence	Binding/function	Species	Other ligands/entry factor for other viruses	References
C-type lectin domain family 4 member M	CLEC4M (L-SIGN)	Capture, infection in “trans”	Liver endothelial cells (LSECs)	Binding assay (DC-SIGN/L-SIGN overexpressing HeLa cells, labeled E2)	sE2 HCVpp HCVcc + sera, mannan/blocking antibodies	Direct binding to E2, binding to L-SIGN activates ERK pathway		Mannan, HIV (Geijtenbeek et al. 2000), Ebola (Alvarez et al. 2002), CMV (Alvarez et al. 2002), Dengue (Tassaneetrithep et al. 2003)	Gardner et al. 2003; Lozach et al. 2003, 2004; Cormier et al. 2004; Zhao et al. 2013
Entry factors									
Scavenger receptor class B member I	SCARB1	Multifunctional entry factor, initial recognition, lateral diffusion, endocytosis, postbinding	Highly expressed in liver hepatocytes and steroidogenic tissue	Co-IP, cross-linking of bound E2 to HepG2 cells	HCVpp, HCVcc, blocking antibodies (in vitro, in vivo), SR-BI siRNA	Direct binding to E2, E2-independent attachment, binding region of HCV and high-density lipoprotein (HDL) differ	Human (murine)	HDL, low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) (Van Eck et al. 2008)	Scarselli et al. 2002; Bartosch et al. 2003b; Catanese et al. 2007, 2010; Zeisel et al. 2007; Dao Thi et al. 2012; Lacek et al. 2012
CD81 molecule	CD81	Postbinding, endocytosis (clathrin mediated), signaling	Ubiquitous	cDNA library screen for E2 binding in mouse fibroblasts	sE2, HCVpp, HCVcc, HCV sera, blocking antibodies (in vitro, in vivo), soluble CD81-LEL (not in PHHs challenged with	Direct binding to E2, E2-binding site on CD81 LEL, CD81 colocalizes endosomal with CLDN1	Human, chimpanzees	Plasmodium falciparum (Silvie et al. 2003), influenza A virus (IAV) (He et al. 2013)	Pileri et al. 1998; Bartosch et al. 2003a; Zhang et al. 2004; Flint et al. 2006; Brazzoli et al. 2008; Meuleman et al. 2008; Molina et al.

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Table 1. *Continued*

Cellular entry factor		Protein name	Gene name	Entry step	Expression profile	Identification method	Experimental evidence	Binding/function	Species	Other ligands/entry factor for other viruses	References
Claudin 1	CLDN1	Late, postbinding, parallel to CD81/after CD81	Highly expressed in liver and skin	cDNA library screen for susceptibility of HCVpp in 293T	CD81/ SRBI	HCVpp, HCVcc, overexpression, anti-CLDN1 inhibit CD81-CLDN1 association	HCV sera), CD81 siRNA	No direct binding to E2, CD81/CLDN1 complexes during HCV infection	Human, murine		2008; Bankwitz et al. 2010; Farquhar et al. 2012
											Evans et al. 2007; Harris et al. 2008; Liu et al. 2009; Krieger et al. 2010
Claudin 6, claudin 9	CLDN6, CLDN9	Late, postbinding, parallel to CD81/after CD81	Expressed in liver, PBMCs	Overexpression of CLDN6 and CLDN9 in 293T		HCVpp, HCVcc		Complementation of CLDN1 in some cell types and for some genotypes			Zheng et al. 2007; Meertens et al. 2008; Haid et al. 2014
Occludin	OCLN	Postbinding, subsequently to CD81 and CLDN-1	Tissue-enhanced (thyroid gland)	cDNA library screen for HCVpp entry in mouse fibroblasts		HCVpp, HCVcc, OCLN siRNA, no AB available, only AB regulatable		Binding differs between isolates HCV entry dependent on second extracellular loop of OCLN	Human, primate, nonhuman primate		Ploss et al. 2009; Liu et al. 2010; Michta et al. 2010; Sourisseau et al. 2013
Low-density lipoprotein receptor (LDLR)	LDLR	Initial binding of virion-associated lipoproteins	Ubiquitous	Endocytosis of HCV correlated with LDL activity		HCVcc, HCV ⁺ sera, blocking antibodies		No direct interaction of E2 and LDLR, uptake of HCV-LDL or HCV-VLDL, ApoE-		LDL, BVDV, HRV2 (Hofer et al. 1994), VSV (Finkelshtein et al. 2013)	Agnello et al. 1999; Monazahian et al. 1999; Wünschmann et al. 2000;
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Table 1. *Continued*

Cellular entry factor		Protein name	Gene name	Entry step	Expression profile	Identification method	Experimental evidence	Binding/function	Species	Other ligands/entry factor for other viruses	References
Entry cofactors	Transferrin receptor	TFRC		Postbinding	Ubiquitous	Down-regulation of TfR during HCV infection	HCVpp, HCVcc, blocking antibodies, TFR siRNA	Binding to HCVcc, involved in HCVpp entry		NW arenaviruses (JUNV, Machupo) (Radoshitzky et al. 2007), canine parvovirus, feline panleukopenia (Parker et al. 2001)	Martin and Uprichard 2013
Epidermal growth factor receptor (EGFR), EPHA2 receptor A2 (EPHA2)		EGFR, EphA2		Postbinding	Tissue enhanced (placenta, esophagus) (RNA)	siRNA screen in Huh7	HCVpp, HCVcc, blocking antibodies, EGFR siRNA	No direct binding, co-internalization with CD81 after activation		EGF, transforming growth factor α (TGF- α) (Schneider and Wolf 2009), IAV (Eierhoff et al. 2010), HCMV (Chan et al. 2009)	Lupberger et al. 2011; Diaio et al. 2012
NPC1, like intracellular cholesterol transporter 1		NPC1L1		Postbinding, at or during fusion	Enriched in intestinal enterocytes, hepatocytes	Down-regulation during HCV infection	HCVcc, no effect on HCVpp, NPC1L1 siRNA, specific inhibitor ezetimibe	Cholesterol-associated binding, NPC1L1 LEL1 is involved in binding		Dengue (Osuna-Ramos et al. 2018)	Sainz et al. 2012
Cadherin 1		CDH1		Postbinding	Ubiquitous	siRNA screen, integrative	HCVpp, HCVcc, blocking antibodies	Important for cell surface localization of			Li et al. 2016

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Table 1. *Continued*

Cellular entry factor		Protein name	Gene name	Entry step	Expression profile	Identification method	Experimental evidence	Binding/function	Species	Other ligands/entry factor for other viruses	References
Serum response factor-binding protein 1	SRFBP1			Entry/early postentry	Ubiquitous	functional genomics	HCVcc, SRFBP1 siRNA, no effect on HCVpp entry	CLDN1 and OCLN			Gerold et al. 2015
						Quantitative proteomics of CD81 interacting proteins in HCVcc infected cells		No direct binding to E2 (HCVpp), colocalization with CD81			
Calpain 5	CAPN5			Postbinding	Ubiquitous	High-resolution quantitative proteomics, CD81 interactome	HCVcc, CAPN5 siRNA, CRISPR/Cas knockout cells				Bruening et al. 2018
Cbl proto-oncogene B	CBLB			Postbinding	Ubiquitous	High-resolution quantitative proteomics, CD81 interactome	HCVcc, CBLB siRNA, CRISPR/Cas knockout cells				Bruening et al. 2018
CD63 molecule	CD63			Early steps + endocytosis	Ubiquitous	Integrative-genomics approach	HCVpp, HCVcc, blocking antibodies, CD63 siRNA	Direct interaction with E2		Lujov virus (Raaben et al. 2017)	Park et al. 2013
HRas proto-oncogene, GTPase	Hras			Highly expressed in liver, GI tract and bone marrow	Highly expressed in liver, GI tract and bone marrow	Proteomic analysis of members of the EGFR signaling pathways in	HCVpp, HCVcc, HRas siRNA, specific inhibitor tipifarnib	Downstream from EGFR signaling, lateral membrane diffusion of CD81,		IAV (Karlas et al. 2010)	Zona et al. 2013

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Table 1. *Continued*

Cellular entry factor									
Protein name	Gene name	Entry step	Expression profile	Identification method	Experimental evidence	Binding/function	Species	Other ligands/entry factor for other viruses	References
Integrin subunit $\beta 1$	ITGB1c		Highly expressed in muscle tissue	association with CD81	HCVpp, HCVcc, ITGB1 siRNA, blocking antibodies	association with CD81-CLDN1 CD81-associated protein		Collagen	Zona et al. 2013
		Proteomic analysis of members of the EGFR signaling pathways in association with CD81							
Ras-related protein Rap2B	Rap2B		Ubiquitous (RNA)	Proteomic analysis of members of the EGFR signaling pathways in association with CD81	HCVpp, HCVcc, Rap2B siRNA	CD81-associated protein			Zona et al. 2013
Growth factor receptor-bound protein 2	Grb2		Ubiquitous	siRNA screen targeting EGFR adaptors and associated proteins	HCVpp, HCVcc, Grb2 siRNA	EGFR adaptors		EGFR	Zona et al. 2013
SHC adaptor protein 1	Shc1		Ubiquitous	siRNA screen targeting EGFR adaptors and associated proteins	HCVpp, HCVcc, Shc1 siRNA	EGFR adaptors			Zona et al. 2013
Clathrin heavy-chain like 1	CLTCL1	Endocytosis	Tissue enhanced (skeletal)	siRNA screen targeting proteins	HCVpp, HCVcc, CLTCL1 siRNA				Coller et al. 2009

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Table 1. *Continued*

Cellular entry factor							
Protein name	Gene name	Entry step	Expression profile (muscle, testis) (RNA)	Identification method	Experimental evidence	Binding/function	Species
Clathrin light-chain B	CLTB	Endocytosis	Ubiquitous	cellular membrane trafficking genes siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, CLTB siRNA		Other ligands/entry factor for other viruses
							Coller et al. 2009
Huntingtin-interacting protein 1 related	HIP1R	Endocytosis	Ubiquitous	siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, HIP1R siRNA		
							Coller et al. 2009
Huntingtin-interacting protein 1	HIP1	Endocytosis	Ubiquitous	siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, HIP1 siRNA		
							Coller et al. 2009
Epsin 1	EPN1	Endocytosis	Ubiquitous	siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, EPN1 siRNA		
							Coller et al. 2009
Epsin 3	EPN3	Endocytosis	Ubiquitous	siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, EPN3 siRNA		
							Coller et al. 2009

Continued

Table 1. *Continued*

Cellular entry factor							
Protein name	Gene name	Entry step	Expression profile	Identification method	Experimental evidence	Binding/function	Species
Cofilin 1	CFL1	Endocytosis	Ubiquitous	cellular membrane trafficking genes siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, CFL1 siRNA		Other ligands/ entry factor for other viruses
Cell division cycle 42	CDC42	Endocytosis	Ubiquitous	siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, CDC42 siRNA		
Rho-associated protein kinase 2	ROCK2	Endocytosis	Ubiquitous	siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, ROCK2 siRNA		
Synaptotagmin 1	SYT1	Endocytosis	Tissue enriched (cerebral cortex)	siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, SYT1 siRNA		
Cbl proto-oncogene	CBL	Endocytosis	Ubiquitous	siRNA screen targeting genes	HCVpp, HCVcc, CBL siRNA		

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Table 1. *Continued*

Cellular entry factor							
Protein name	Gene name	Entry step	Expression profile	Identification method	Experimental evidence	Binding/function	Species
Hepatocyte growth factor-regulated tyrosine kinase substrate	HGS	Endocytosis	Ubiquitous	cellular membrane trafficking genes siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, HGS siRNA		Coller et al. 2009
ATPase H ⁺ transporting V0 subunit a1	ATP6V0A1	Endocytosis	Ubiquitous	siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, ATP6V0A1 siRNA		Coller et al. 2009
Staufen double-stranded RNA-binding protein 1	STAU	Endocytosis	Ubiquitous	siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, STAU siRNA		Coller et al. 2009
RAB29, member RAS oncogene family	RAB29	Endocytosis	Ubiquitous	siRNA screen targeting cellular membrane trafficking genes	HCVpp, HCVcc, RAB29 siRNA		Coller et al. 2009

Tissue expression data from referenced publications or the Protein Atlas (Uhlén et al. 2015).

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HCV infection. Thus, the notion is that DC-SIGN and L-SIGN capture HCV from the serum and enable the infection of hepatocytes in *trans* (Gardner et al. 2003; Lozach et al. 2003, 2004).

Entry Factors

After attachment and concentration of HCV lipo-viro-particles on the cell surface of a susceptible hepatocyte, four entry factors are critically required for entry. These are CD81, SR-BI, CLDN1, and OCLN. SR-BI may be replaced functionally by LDLR and VLDLR (Yamamoto et al. 2016).

CD81 belongs to the large group of tetraspanins, characterized by four transmembrane domains. Tetraspanins lack signaling motifs, but interact with each other, cluster together in membrane microdomains, and take part in a numerous cellular functions such as cell adhesion, migration, and proliferation (Maecker et al. 1997; Hemler 2001, 2003; Yunta and Lazo 2003). The large extracellular loop (LEL) of CD81 directly binds the HCV glycoprotein E2 (Pileri et al. 1998). In cell culture–derived HCV particles, the CD81-binding site on E2 is partially masked by the hypervariable region 1 (HVR1) to shield it from neutralizing antibodies (Bankwitz et al. 2010; Prentoe et al. 2011). Interactions with other host factors, such as LDLR or SR-BI, are likely needed to induce a conformational change in E2 to expose the CD81-binding site. Soluble CD81 LEL blocks HCV pseudoparticle (HCVpp) and cell culture–derived HCV (HCVcc) infection strengthening the important role of this protein subdomain (Zhang et al. 2004). Furthermore, anti-CD81 antibodies protect against HCV infection in vitro and in vivo in a humanized mouse model (Meuleman et al. 2008; Molina et al. 2008). Finally, the crystal structure of CD81 revealed a cholesterol-binding site located within the fourth transmembrane domain (Zimmerman et al. 2016). A single amino acid exchange at the cholesterol-binding site of CD81, which reduces cholesterol binding of CD81, also reduces HCV susceptibility (Banse et al. 2018), suggesting that this direct CD81–lipid interaction may be important for HCV entry.

Interestingly, SR-BI, another described HCV entry factor, functions as a lipoprotein receptor and mediates bidirectional transport of cholesterol across the cell membrane (Gu et al. 1998). SR-BI directly binds to E2, as shown by binding assays with soluble recombinant E2 protein or E1/E2 dimers displayed on the surface of HCVpp and HCVcc (Scarselli et al. 2002; Bartosch et al. 2003b; Evans et al. 2007; Bankwitz et al. 2014). Some studies suggest that SR-BI can also mediate HCV entry in an E2-independent but lipoprotein-associated manner (Dao Thi et al. 2012). In contrast to CD81, where the HVR1 is shielding the CD81-E2-binding site, the HVR1 is important for the interaction with SR-BI (Scarselli et al. 2002; Bartosch et al. 2003b). However, as HCVcc particles lacking the HVR1 show SR-BI-dependent binding to CHO cells, the HVR1 seems not to be the sole viral determinant for binding to SR-BI (Bankwitz et al. 2014). Notably, HCV particles lacking HVR1 retain dependence on SR-BI for efficient infection supporting the notion that SR-BI plays a role in virion attachment and also later cell entry stages (Bankwitz et al. 2014). Despite the direct interaction of SR-BI with E2, infectivity of HCVpp and HCVcc mediated by SR-BI was enhanced by high-density lipoprotein (HDL) particles. The fact that natural ligand binding to SR-BI does not compete with binding to HCV suggests different binding sites for HDL and E2 on SR-BI (Bartosch et al. 2005; Voisset et al. 2005; Dreux et al. 2006; Zeisel et al. 2007). Blocking of the SR-BI E2-binding site by SR-BI specific antibodies prevents HCV infection in vitro and in vivo (Lacek et al. 2012; Vercauteren et al. 2014). In sum, SR-BI has a multifunctional role in HCV entry with, on the one hand, E2-independent attachment and postbinding functions and, on the other hand, E2-dependent enhancement of infectivity (Zahid et al. 2013). This is in line with the reported discrepancy that murine SR-BI cannot bind to sE2 but can mediate HCVcc uptake in the mouse model, supporting the multifaceted role of SR-BI (Barth et al. 2005; Ploss et al. 2009; Catanese et al. 2010; Dörner et al. 2011).

HCV not only hijacks the lipoprotein receptor SR-BI, but also the LDLR. The importance of

LDLR in HCV entry was first shown by inhibiting HCV endocytosis by anti-LDLR antibodies (Agnello et al. 1999). LDLR does not bind E2, but rather its natural ligand LDL in the HCV lipo-viro-particle (Wünschmann et al. 2000). It is controversial whether this LDLR uptake leads to a productive infection. Albecka and colleagues showed that LDLR-mediated uptake can be unproductive and that LDLR-mediated alterations in intracellular lipid content promote viral RNA genome replication (Albecka et al. 2012). Thus, blocking LDLR may rather inhibit viral replication instead of abolishing HCV entry (Seipp et al. 1997; Albecka et al. 2012).

Another essential entry factor for HCV is the tight junction protein CLDN1. Expression of CLDN1 rendered otherwise refractory nonhepatic HEK293 susceptible to HCVpp and HCVcc entry (Evans et al. 2007). More recent work additionally provides evidence for direct binding of the E1/E2 heterodimer to CLDN1 (Douam et al. 2014). Inhibition kinetics suggests that CLDN1 acts at a postattachment and post-CD81-binding step. Finally, CLDN1 can form a complex with CD81 and co-endocytoses with the HCV-CD81 complex (Harris et al. 2008; Krieger et al. 2010; Zona et al. 2013).

In vivo studies confirm the essential function of CLDN1 in HCV infection. As murine CLDN1 can replace human CLDN1 functionally in HCV entry, blocking CLDN1 in mouse models using antibodies abrogates HCV liver cell entry. Similarly, overexpression of human or mouse CLDN1 in the mouse liver by adenoviral delivery boosts HCV infection in the mouse model (Dorner et al. 2011).

Because CLDN1 facilitates HCV entry, other members of the CLDN family were analyzed for their role in HCV entry. Claudin-6 (CLDN6) and claudin-9 (CLDN9), albeit closely related to each other but not to CLDN1, could functionally substitute CLDN1 in 293Ts and to a moderate extent in hepatoma cell lines (Zheng et al. 2007; Meertens et al. 2008). However, the usage of CLDN6 and most presumably also CLDN9 appears to be HCV isolate specific (Haid et al. 2014). The physiological relevance of CLDN6 and CLDN9 in HCV entry is not yet defined. CLDN6 and CLDN9 mRNA expression was de-

tected not only in the liver but also in PBMCs, pinpointing to a possible role in extrahepatic HCV uptake (Meertens et al. 2008).

Finally, a second tight-junction molecule, OCLN, was discovered as a fourth and thus far final essential and nonredundant entry factor for HCV. Human OCLN, together with human CD81, confers HCV susceptibility to mouse hepatocytes. Similar to CLDN1, OCLN mediates the barrier function of the epithelium. Overexpression and silencing experiments clearly show the role of OCLN in HCV entry (Ploss et al. 2009). OCLN acts late in HCV entry, and although no direct interaction with E2 was shown, the isolate specific dependency on OCLN suggests a direct interaction with the virion (Sourisseau et al. 2013).

The strict liver tropism of HCV is in part mediated by entry factor expression. Whereas CD81 is ubiquitously expressed, SR-BI expression is highest in liver and steroid tissue (Cao et al. 1997). Of the two tight-junction proteins, OCLN is found in most polarized epithelial cells, while CLDN1 is highly expressed in liver (Furuse et al. 1993, 1998). The high expression levels of SR-BI and CLDN1 in the liver suggest that these two host factors are mainly responsible for the tissue tropism at the level of cell entry.

In addition to the explicit tissue tropism, HCV has a narrow host range and infects only humans and chimpanzees. In the mouse model, all four essential entry factors are necessary for a successful infection, but for CLDN1 and SR-BI the murine orthologs can substitute the human proteins and enable HCV entry (Ploss et al. 2009). In contrast, only human and primate OCLN can mediate HCV entry, but not murine OCLN. Specifically, a region in the second extracellular loop of OCLN was identified to mediate species tropism (Michta et al. 2010). For CD81, the LEL regions, which mediate HCV E2 binding, are only poorly conserved across species and only human and monkey LEL regions mediate binding to E2. Rat and mouse CD81 fail to bind soluble E2 (Meola et al. 2000; Flint et al. 2006).

Taken together, CD81, SR-BI, CLDN1, and OCLN represent the canonical entry factors for

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HCV and the wealth of data suggest that these four factors are sufficient to render cells susceptible to HCV. Whereas SR-BI and CDLN1 contribute to tissue tropism, CD81 and OCLN contribute to species tropism at least when comparing murine and human orthologs. Thus, the canonical set of entry factors not only reflects the highly organized entry process of HCV, but also the strong adaptation to the human host and its replication site in the liver.

Entry Cofactors

As previously described, HCV uses a broad range of usually ubiquitously expressed entry cofactors in addition to the essential entry factors. Two classes of cofactors exist: those cofactors that are not essentially required for HCV entry but can promote HCV uptake; and those that are essential to HCV entry but also to cell survival in general, that is, ubiquitous components of basic cellular machineries such as, for instance, clathrin. For some cofactors with poorly described cellular functions, a clear discrimination between both cofactor classes is impossible. In general, RNA interference screens, inhibitor studies, quantitative interaction proteomics, or phenotypical observations led to the discovery of these cofactors.

A functional siRNA screen identified EGFR and ephrin type-A receptor 2 (EphA2) as HCV entry cofactors (Lupberger et al. 2011). As receptor tyrosine kinases, both proteins regulate a broad range of cellular processes such as proliferation and differentiation (Schneider and Wolf 2009). EGFR does not directly interact with HCV; instead, binding of HCV to CD81 promotes EGFR activation. This EGFR-dependent signaling then leads to formation of CD81–CLDN1 complexes and the internalization of HCV–CD81 complexes. EGFR forms a complex with CD81 in hepatoma cells and primary human hepatocytes, suggesting that this cofactor is readily available after HCV–CD81 binding (Zona et al. 2013; Bruening et al. 2018). EGFR is required for internalization of HCV at the tight junction of polarized 3D organoid cultures, but not for HCV migration to the tight junction (Baktash et al. 2018). Erlotinib, a specific EGFR

inhibitor, blocks HCV entry in vivo, strengthening the important role of EGFR signaling (Lupberger et al. 2011; Diao et al. 2012).

The main receptor for cellular iron uptake, transferrin receptor 1 (TFRC), is down-regulated upon HCV infection. Knockdown and blocking of TFRC reduces HCV infectivity in cell culture. In addition, TFRC can bind HCV virions and is most likely involved in the endocytosis of the virus together with CD81 (Martin and Uprichard 2013). Interestingly, more recent proteomics studies reveal complex formation of TFRC with CD81 in primary hepatocytes and hepatoma cells (Bruening et al. 2018). This suggests that TFRC may be a secondary component of the entry receptor complex and HCV virion binding may be indirect.

Similar to the TFRC, Niemann–Pick C1-like 1 (NPC1L1) levels decrease in hepatoma cells during HCV infection (Sainz et al. 2012). NPC1L1 is a transmembrane cholesterol uptake receptor and highly abundant in intestinal enterocytes and human hepatocytes (Altmann et al. 2004). Because cholesterol is involved in several steps during HCV entry, a role of NPC1L1 in HCV infection seemed likely. Experiments including siRNA knockdown of NPC1L1, antibody-mediated blocking and usage of the NPC1L1-specific inhibitor ezetimibe confirmed the impact of NPC1L1 in a postbinding step in HCV entry and its relevance both in vitro and in vivo (Sainz et al. 2012). In vivo and in polarized cultures, the role of NPC1L1 remains enigmatic as it is expressed at the apical side of hepatocytes, which HCV infects from the basolateral side. This points toward an intracellular and/or indirect function of NPC1L1, possibly by providing the optimal lipid composition in endosomal fusion compartments.

Several CD81 interaction partners, in addition to EGFR and TFRC, are HCV entry cofactors. Serum response factor-binding protein 1 (SRFBP1) is recruited to the CD81 entry complex 15-min post-HCV infection, that is, during the uptake process. Silencing of SRFBP1 reduces HCVcc infectivity and SRFBP1 complementation restores HCV susceptibility, whereas entry of HCVpps does not depend on SRFBP1. The physiological role of SRFBP1 is linked to actin

and its function in HCV entry may be the retrograde transport of HCV particles (Gerold et al. 2015).

In the absence of HCV, CD81 coordinates the interaction of membrane proteins including integrins and EGFR, but also intracellular adaptors such as the calcium-dependent endopeptidase calpain-5 (CAPN5) and the E3 ubiquitin ligases casitas B-lineage lymphoma proto-oncogene (CBL) and CBL B (CBLB). RNA interference and CRISPR/Cas9-mediated knockout show a role for CAPN5 and CBLB as cofactors for HCV entry for all genotypes (Bruening et al. 2018).

Additional HCV entry cofactors, identified using proteomic analysis of CD81 complexes, include integrin $\beta 1$ (ITGB1), HRas, growth factor receptor bound protein 2 (Grb2), and Ras-related protein Rap-2b (Rap2b) (Zona et al. 2013). HRas is a GTPase downstream of EGFR and regulates CD81 lateral translocation and entry platform formation during HCV entry. ITGB1 and Rap2b are CD81-associated proteins and act as cofactors during HCV entry. Together with HRas, ITGB1 and Rap2b build a functional complex to regulate membrane organization and receptor mobility. Finally, a targeted RNA interference screen for endocytosis factors revealed that several components of the clathrin-mediated uptake pathway are important entry cofactors for HCV (Coller et al. 2009). Table 1 systematically lists the most prominent HCV attachment factors, entry factors, and entry cofactors with a confirmed role in HCV lipo-viro-particle entry.

PROTEIN-PROTEIN INTERACTIONS DURING HCV ENTRY: TOWARD A SYSTEMS BIOLOGY VIEW OF ENTRY

Virus entry, as other life cycle steps, is coordinated by protein-protein networks of the host cell, which may be hijacked and/or modified by viruses (Prusty et al. 2014; Gerold et al. 2015; Viswanathan et al. 2017; Bruening et al. 2018; Scaturro et al. 2018). Technological advances, in particular in interaction shotgun proteomics, allow the systematic analysis of protein interactions during virus entry and the construction of

a systems biology view of the entry process (Damm and Pelkmans 2006; Walther and Mann 2010; Cox and Mann 2011; Shah et al. 2015; Gerold et al. 2016, 2017; Lasswitz et al. 2018). Although alternative methods such as high throughput imaging and integrative analysis of RNA interference, haploid cell screens, and CRISPR knockout screens can provide indirect evidence on engaged protein networks (Damm and Pelkmans 2006; Park et al. 2013; Kilcher and Mercer 2014), we will in the following focus on evidence based on proteomic analyses of host networks engaged during HCV invasion.

The first protein interactions reported for HCV entry, were the interactions of the large HCV surface glycoprotein E2 with the cell surface entry factors CD81 and SR-BI (Pileri et al. 1998; Scarselli et al. 2002). Many HCV neutralizing antibodies recognize the conserved and structurally flexible CD81-binding site in E2, stressing the importance of this interaction for HCV entry but also vaccine design (Patel et al. 2000; Owsianka et al. 2001; Clayton et al. 2002; Law et al. 2008; Keck et al. 2009; Flyak et al. 2018; Kinchen et al. 2018). Subsequent studies revealed a close functional interplay between CD81 and SR-BI (Kapadia et al. 2007; Zeisel et al. 2007; Grove et al. 2008; Bankwitz et al. 2014). Later, proteomics and imaging studies showed complex formation of CD81 and SR-BI in polarized hepatoma cells, primary human hepatocytes, and 3D hepatocyte culture models (Zona et al. 2013; Baktash et al. 2018; Bruening et al. 2018), suggesting that early HCV entry factors form a preexisting complex on hepatocytes and can initiate the entry process immediately after virion attachment.

Interactions of the tight-junctional entry factors with HCV E2 proved more difficult to capture. Successful expression of E1/E2 ectodomain heterodimers in solution was the prerequisite to show a direct interaction with the large CLDN1 extracellular loop (Douam et al. 2014). On cells, interactions with CLDN1 and OCLN are likely transient and therefore attempts to show complex formation have thus far failed. Indirect genetic evidence, however, indicates that HCV also interacts with OCLN during the

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entry process (Sourisseau et al. 2013). Clearly, interactions with CLDN1 and other claudins as well as usage of OCLN is strain-dependent (Sourisseau et al. 2013; Haid et al. 2014).

Notably, the tetraspanin CD81 coordinates additional protein interactions critical for HCV entry apart from HCV E2 and SR-BI interactions. Historically, the first mapping of CD81 interactions stemmed from B cells (Levy et al. 1998; Cherukuri et al. 2004; Shoham et al. 2006). Later, Zona and colleagues confirmed CD81 complex formation with typical tetraspanin web proteins such as CD9, CD151, and the membrane protease ADAM10, as well as the HCV entry factors SR-BI and CLDN1 in polarized hepatoma cells (Zona et al. 2013). Additionally, integrins, the EGFR signaling molecule HRas, and Rap2B were found in a complex with CD81. ITGB1, HRas, and Rap2B contribute to full susceptibility to HCV (Zona et al. 2013). In a later study, Bruening and colleagues confirmed CD81 interactions with SR-BI, integrins, and CD151 in primary human hepatocytes from multiple donors, and additionally discovered EGFR and TFRC as CD81 complex constituents (Bruening et al. 2018). Several studies showed a critical role of EGFR signaling in HCV entry, in particular in triggering HCV entry factor complex formation (Zona et al. 2013), lateral translocation of the HCV-CD81 complex toward tight junctions (Brazzoli et al. 2008), and endocytosis of HCV (Diao et al. 2012). TFRC also contributes to full-fledged infection of hepatoma cells by HCV (Martin and Uprichard 2013). In addition to the proteomics studies, fluorescence resonance energy transfer (FRET) studies suggest an association between CD81 and CLDN1, which is important for HCV uptake (Harris et al. 2008; Krieger et al. 2010). Taken together, the preformed CD81 complex coordinates important entry factors (SR-BI, CLDN1) and cofactors (EGFR, TFRC, CBL, CBLB, CAPN5) on hepatocytes, which presumably enables productive HCV entry.

Clathrin-mediated endocytosis is the major route of HCV into the cell. CD81 interaction proteomics revealed the clathrin-coated pit component HIP1R and the E3-ubiquitin ligases CBL and CBLB as HCV entry cofactors for en-

docytic uptake (Gerold et al. 2015; Bruening et al. 2018). This confirmed previous RNA interference studies showing entry cofactor functions of CBL and HIP1R (Coller et al. 2009). Importantly, the association of HIP1R and CD81 occurs de novo 15-min post-HCV inoculation of cells (Gerold et al. 2015), suggesting that HCV actively translocates to clathrin coated pits or induces their formation.

Limited knowledge exists on postendocytosis protein network engagement. Although early interactions with CD81 prime the HCV E1/E2 glycoproteins for later low pH-dependent fusion, no essential additional protein interactions in the endosome have thus far been reported. Proteases, such as cathepsins, which other virus families strictly rely on, are dispensable for HCV entry (Tscherne et al. 2006). How the HCV capsid disassembles after membrane fusion is poorly understood. SRFBP1 is thus far the only protein reported to interact with CD81 at HCV-positive endosomes. Mechanistic studies further suggest that SRFBP1 is an entry cofactor for postfusion events in HCV entry (Gerold et al. 2015). The lack of uncoating assays for HCV, however, hampers pinpointing the exact role of such late entry cofactors to date. Figure 2 depicts protein complexes, and reported STRING interactions of HCV attachment factors, entry factors, and entry cofactors, as well as their functional clustering.

In addition to interactions with host dependency factors, protein interactions with restriction factors occur during entry and may determine tissue tropism. An example is the CD81 interaction partner EWI2, which in its cleaved form “EWI2-wnt,” blocks HCV entry. Notably, EWI2-wnt is not expressed in hepatocytes and hepatoma cells, which are the targets of HCV (Charrin et al. 2003; Montpellier et al. 2011; Potel et al. 2013). Moreover, cystatin A was identified as a protein, which dissociates from the HCV-CD81 complex during the entry process. Cystatin A, similar to EWI2-wnt, restricts HCV infection (Gerold et al. 2015). This suggests that the virus evolved to use host networks present in its primary target cells and additionally seems to induce dissociation of its CD81 entry factor from restricting factors.

Figure 2. Interaction network of hepatitis C virus (HCV) attachment factors, entry factors, and entry cofactors. Reported STRING interactions (all levels of evidence, confidence of 0.4) are depicted and the interaction strength is indicated (yellow, orange, and red edges). Additional reported interactions not yet annotated in STRING (Zona et al. 2013; Gerold et al. 2015; Bruening et al. 2018) are shown (black dotted edges). Proteins are assigned to their highest scoring DAVID cluster as indicated. Analysis performed using DAVID with high-classification stringency settings (Huang et al. 2009), STRING (Szklarczyk et al. 2015), a published Matlab algorithm (Mercer et al. 2012), and illustrated in Cytoscape (Smoot et al. 2011).

HCV MEMBRANE FUSION AND BEYOND

To deliver their viral genome into the cytosol of host cells, viruses have to pass cellular membranes. Although the sites where this occurs vary, penetration of at least one membrane barrier is crucial, as host-derived cofactors sustaining virus replication are only available after virus particles have been routed to the cytosol or even deeper into a cell. After membrane penetration, some viruses continue traveling on to reach different subcellular compartments for initiation of replication. These additional transport processes may be accompanied by viral uncoating events and they require complex networks of interactions between viral factors and cellular transport machineries (both actin and microtubule) so that the viral cargo is delivered to the final destination within a cell (Wang

et al. 2018; Walsh and Naghavi 2019). Many RNA viruses, however, including HCV, initiate their replication directly in the cytosol. Because HCV encodes a plus strand RNA genome that presumably comprises all information necessary for recruitment of cellular ribosomes, it is generally assumed that on membrane penetration, viral RNA is rapidly translated, protein synthesis occurs, and RNA replication is initiated. Although actin and microtubule-dependent processes are essential for HCV internalization and endocytosis (Brazzoli et al. 2008; Lupberger et al. 2011; Farquhar et al. 2012; Zona et al. 2013), it is not clear whether transport is required after membrane penetration and before protein translation and RNA replication. It is generally assumed that viral capsids at least partially uncoat before genome transcription/translation and for a few viruses this concept

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has been shown (Wang et al. 2018, and references therein). These requirements may also apply to HCV; however, the mechanism of HCV particle uncoating and involvement of host factors have not been reported. Some recent studies have shed light on essential steps that occur after RNA virus membrane penetration; for instance, a genetic screen revealed an important role of PLA2G16, a cellular phospholipase, that is required to prevent clearance of picornavirus particles directly after membrane penetration (Staring et al. 2017). This suggests that incoming virions may encounter cellular defense mechanisms. On the other hand, influenza A virus capsids incorporate unanchored ubiquitin chains to mimic misfolded protein aggregates, which allows recruitment of the cellular aggresome processing machinery to facilitate uncoating (Banerjee et al. 2014). Intriguingly, lymphocyte antigen 6 complex locus E (LY6E), an interferon-stimulated gene, was recently shown to enhance infection by several enveloped plus and minus-strand RNA viruses including dengue virus, chikungunya virus, and influenza A virus (Mar et al. 2018). For the latter, these investigators provided evidence that LY6E facilitated viral uncoating after endosomal escape. These studies suggest that yet-unknown processes may also occur after HCV has penetrated cellular membranes. They also underpin that virus particles carry all necessary information to recruit or connect to cellular machineries and pathways to ensure that their cargo, the viral genome, is delivered to exactly where replication can be initiated.

This also applies to virus membrane fusion, and virions are made exactly to ensure that membrane penetration is initiated precisely at the right point in time and cellular location so that all downstream replication steps can occur. To achieve this, some viruses encode envelope proteins that are synthesized and incorporated into nascent virus particles as immature protein precursors. These proteins can only induce membrane fusion on proteolytic cleavage ensuring that the frequently irreversible protein conformational changes needed for membrane fusion are only triggered at a specific location and point in time. In some cases, processing already

occurs in the virus-producing cell, whereas in other instances virus-incorporated envelope proteins are cleaved after attachment and entry into new host cells. In case of HCV, the complex network of virus entry factor interactions summarized above may be critical for delivering cues so that HCV particles elicit fusion only once inside of the susceptible cells.

Clearly, the HCV E1 and E2 glycoproteins are of pivotal importance as receivers of such cues and also as effectors that mediate the fusion between the viral lipid bilayer and a cellular membrane. However, also, host-derived lipoproteins that are incorporated during intracellular virus assembly (Fukuhara et al. 2015) and also after particle release (Bankwitz et al. 2017; Denolly et al. 2019) may influence virus membrane fusion. In the absence of comprehensive structural information about the complete E1/E2 heterodimeric glycoprotein complex, it is impossible to know how exactly membrane fusion occurs. Nevertheless, we have collected a range of information that allows drawing a model of membrane fusion. First, HCV membrane fusion depends on specific primers and triggers, which at least in part are provided by viral interactions on encounter with new host cells. Once the first functional HCV infection assays based on lentiviral or retroviral HCVpp were available, it was recognized that inhibition of cellular ATPases, enzymes that are required for acidification of cellular endosomes, precludes HCV infection (Hsu et al. 2003). This suggested that, as in many viruses, low pH triggers protein conformational changes that mediate virus membrane fusion. This notion was confirmed, and extended with arrival of HCVcc (Koutsoudakis et al. 2006; Tscherne et al. 2006). Tscherne and colleagues reported that low pH treatment of HCV virions did not inactivate particle infectivity, which sets HCV apart from other viruses, like for instance alphaviruses, which on exposure to low pH directly undergo irreversible conformational changes for membrane fusion. Congruently, when acidification of endosomes was blocked pharmacologically, cell surface-bound HCV particles could be activated for membrane fusion and cell entry by low pH treatment. However, membrane fusion and infection could only

be triggered on prolonged incubation at 37°C, suggesting that rate-limiting, postbinding events were required to render HCV particles competent for low-pH-triggered membrane penetration and infection (Tscherne et al. 2006).

Single-particle tracking of fluorescently labeled HCV virions into polarized hepatoma organoids recently revealed an ordered, time-structured, and sequential cell-entry process of HCV particles (Baktash et al. 2018). On cell binding to the basolateral pole of the organoids, virions are routed to the tight junction in an actin-dependent process and then internalized. In this system, accumulation of HCV particles at tight junctions increased to 90 min after inoculation, suggesting that virions may receive cues priming them for low pH-induced membrane fusion during their transport at the cell surface and before endocytosis (Baktash et al. 2018). The interaction between HCV glycoproteins and CD81, which occurs at the cell surface, has been implicated to serve as such a priming event. Sharma and colleagues observed that treatment of HCVpps with soluble CD81 induced conformational changes of the glycoproteins as evidenced by altered binding of E1/E2 to conformation-dependent antibodies (Sharma et al. 2011). On exposure to soluble CD81, acid treatment inactivated HCVpps, suggesting that the interaction with CD81 had rendered the glycoproteins receptive for pH-dependent conformational changes and fusion. Notably, recent structural study of the CD81 LEL points toward structural flexibility of the CD81 receptor itself, and suggests that pH and redox conditions control different CD81 conformations (Cunha et al. 2018). Thus, the CD81-binding platform itself may change conformation on arrival of HCV-CD81 complexes in acidified endosomes. This may provide a fusion-triggering cue to HCV or allow HCV dissociation, thus indirectly facilitating fusion. On the other hand, Esumi and colleagues reported that trypsin treatment enhanced HCVcc infection, whereas trypsin inhibitors decreased infectivity (Esumi et al. 2015). The investigators also noted that expression levels of transmembrane serine proteases, as for instance TMPRSS2, modulates HCV infectivity, suggesting that proteolytic cleavage

steps could be involved in HCV cell entry and possibly priming of HCV membrane fusion. However, it is currently not clear whether cleavage of viral proteins or of host factors by serine proteases is involved in HCV entry. In relation to host factors and HCV membrane fusion, it is worth noting that also lipoproteins and lipids themselves were implicated in modulating HCV fusion. On one hand, virus particle-resident ApoE enhances HCV particle attachment via GAG interactions (Wünschmann et al. 2006; Jiang et al. 2012, 2013; Lefèvre et al. 2014). Although this mechanism enhances HCV infection, a direct influence on fusion was so far not reported. On the other hand, apolipoprotein C1 (ApoC1), which like ApoE is also an exchangeable lipoprotein that naturally resides on HDL, seems to be directly involved in membrane fusion. Dreux et al. (2006) reported that ApoC1 enhances HCVpp and HCVcc infection. They observed that infection enhancement requires the HVR1 of HCV and that ApoC1 binds to HCVpp but not to pseudoparticles carrying alternative non-HCV envelope proteins. Using HCVpp and HCVcc fusion assays involving fluorescently labeled liposomes, the investigators provided evidence that addition of ApoC1 enhances the fusion rate. Again, the fusion rate of pseudoparticles with alternative envelope proteins was not affected. Under these conditions, HCVpp fusion remained low pH-dependent, and interestingly preincubation of liposomes did not enhance fusion. Thus, transfer of ApoC1 from HDL onto HCV particles seems to either modify membrane properties or envelope protein function, thereby stimulating membrane fusion. Similar biochemical assays were used to show the relevance of lipids in HCV membrane fusion. Both the lipid composition of target liposome membranes and also the buoyant density of HCV particles, and thus presumably the lipid content of HCV particles, affected HCV fusogenicity in these assays (Haid et al. 2009). It is generally assumed that abundance of cholesterol and lipid species modifies membrane fluidity and curvature, thereby influencing protein refolding and energy requirements for viral membrane fusion (Harrison 2015; Yang et al. 2016).

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So far, the absence of comprehensive structural information about HCV E1/E2 has prevented deeper insights into the molecular processes of HCV membrane fusion. Presently, we are not even sure which of the two proteins is the key driving force for membrane fusion. The recently published crystal structures of the E2 protein core domain revealed a surprisingly compact and globular fold with no obvious similarities to typical viral fusion proteins (Kong et al. 2013; Khan et al. 2014). Thus, many now believe that E1 may be a fusion protein. This notion is supported by early computational work that identified a putative fusion loop within a central domain of E1 by using sequence comparison between multiple viral fusion proteins (Flint et al. 1999). Interestingly, the idea that E1 is a fusion protein and that this particular domain is relevant for the fusion process received additional support by recent reports showing that viral resistance to membrane fusion inhibitors maps to this particular E1 protein domain (Perin et al. 2016; Banda et al. 2019). Moreover, strain-dependent natural resistance to fusion inhibitors maps to viral determinants within this region and correlates with differential requirements to low pH triggering of membrane fusion (Banda et al. 2019). These findings imply that the protein domain adjacent to this putative fusion loop influence the receptiveness of the E1/E2 protein complex to low pH-triggering of fusion and also the susceptibility to this class of HCV membrane fusion inhibitors. Clearly, in the absence of any structural information about this protein domain, it is impossible to conclude whether this region indeed carries a fusion loop and how the adjacent protein domain influences fusion. Nevertheless, these recent reports highlight the importance of this domain in regulating the membrane fusion process. More work will be needed to dissect in which way this protein segment influences the fusion process. Moreover, it is important to mention that careful reverse-genetics analyses have implicated additional regions within the E1/E2 proteins in the fusion mechanism (Lavillette et al. 2007). It will be exciting, once more structural information is available, to integrate these functional studies and results with a three-

dimensional structure of the glycoprotein complex. Ultimately, this should reveal the precise mechanism of HCV membrane fusion and possibly it will show another mechanism by which viruses penetrate across host cell membranes.

CONCLUDING REMARKS

Strong experimental evidence has accumulated supporting a functional role of numerous host factors in HCV cell entry. These studies depict a complex trajectory of HCV virions across the basolateral cell pole to the tight junction complex and via clathrin-mediated endocytosis into hepatocytes. Proteomic, cell signaling, and virus functional and imaging studies have implicated signaling cascades and described protein machineries involved in coordinating these steps. Clearly, lipid receptors like SR-BI, including its lipid transfer function and the CD81 protein that coordinates large protein networks, play a critical role in orchestrating these cell entry steps. The E2 protein is of critical importance for contacting these entry factors and likely for other protein interactions. The structure and function of E1 is less well understood although recent work suggests it is a central player in coordinating membrane fusion. Future structural studies of both E1 and E2 are likely to reveal details of how HCV contacts entry factors and possibly also how such interactions prepare HCV to fuse with host cells. What happens afterward and before RNA translation occurs remains essentially uncharted territory. Recent advances based on high-resolution proteomics and functional genomic screens have, at least for other viruses, showed unexpected mechanisms of disassembly and virus trafficking after fusion. Several proteomics and bioinformatics studies suggest that intricate protein networks operate during HCV entry, among them growth factor signaling networks, also usurped by other viruses from diverse families. More recent developments, such as proximity labeling, may enable a temporal and spatial subcellular resolution of virus entry processes by proteomics, similar to work on receptor recycling and dynamics (Lobingier et al. 2017). Combined with the novel imaging systems, based on liver organoids, this

provides unprecedented opportunities to drill deeper and later into the HCV cell entry stages. It is almost certain that this will reveal unexpected facets of virus host cell biology. Moreover, structure–function studies of the viral glycoprotein complex and its interplay with host factors and lipoproteins are likely to deliver important insights on conserved viral features crucial for cell entry. In the long run, this should help define vaccine antigens that induce robust and cross-protective antibodies thereby bringing us closer to solving one of the important translational research challenges in HCV.

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