Function of the HCV E1 envelope glycoprotein in viral entry and assembly

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HCV envelope glycoproteins, E1 and E2, are multifunctional proteins. Until recently, E2 glycoprotein was thought to be the fusion protein and was the focus of investigations. However, the recently obtained partial structures of E2 and E1 rather support a role for E1 alone or in association with E2 in HCV fusion. Moreover, they suggest that HCV harbors a new fusion mechanism, distinct from that of other members of the Flaviviridae family. In this context, E1 aroused a renewed interest. Recent functional characterizations of E1 revealed a more important role than previously thought in entry and assembly. Thus, E1 is involved in the viral genome encapsidation step and influences the association of the virus with lipoprotein components. Moreover, E1 modulates HCV–receptor interaction and participates in a late entry step potentially fusion. In this review, we outline our current knowledge on E1 functions in HCV assembly and entry.

First draft submitted: 25 October 2018; Accepted for publication: 11 January 2019; Published online: 4 March 2019

Keywords: envelope glycoproteins • glycoprotein structure • HCV • virus assembly • virus entry

HCV infects 70 million people worldwide, thus constituting a major health problem [1]. In most cases, HCV establishes chronic infection that can evolve into cirrhosis and hepatocellular carcinoma. The recent development of direct acting antivirals has been a breakthrough in the treatment of hepatitis C, showing potent efficacy against all HCV genotypes and being associated with elevated HCV clearance rates [2,3]. However, eliminating HCV by 2030, as proposed by the WHO, will be difficult to achieve without the use of a preventive vaccine.

HCV belongs to the hepacivirus genus of the Flaviviridae family. It is an enveloped virus that contains a positive stranded RNA genome [4]. Following the entry of the virus into host cell, the genome is translated in a single polyprotein that is processed into seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) and three structural proteins (the core and the two envelope glycoproteins E1 and E2) [5]. The viral particle is constituted of a nucleocapsid composed of the genomic RNA and the Core protein, which is surrounded by a lipid membrane in which the two envelope glycoproteins E1 and E2 are anchored [6]. E1 and E2 associate into heterodimers and play key roles in viral entry and assembly [7,8]. One of the specificities of HCV resides in its association with host lipoproteins to form LVPs. Thus, host lipoproteins participate in HCV particles composition, entry and assembly [9]. Studies have, for a long time, focused on E2 protein, which mediates the binding to the receptors and constitutes the main target of neutralizing antibodies. By analogy with the situation found in flaviviruses, E2 was initially thought to be responsible for the fusion step between the viral envelope and a host cell membrane. However the resolution of the structure of E2 core domain did not support this hypothesis, leading to the proposal that E1 alone or in combination with E2 was responsible for the fusion process [10,11]. As a consequence, there has been a surge of interest in studying E1 envelope glycoprotein in order to decipher its contribution to the different steps of the HCV life cycle. In this review, we summarize the recent advances made in the knowledge of the functions of HCV E1 glycoprotein during HCV entry and morphogenesis.

E1 synthesis & determinants for E1–E2 interactions

Similarly to E2, E1 is a Type 1 transmembrane protein but its N-terminal ectodomain corresponds to approximately half of E2 ectodomain length (160 and 330 residues respectively). E1 is addressed to the endoplasmic reticulum (ER), thanks to the signal sequences present in the C-terminal region of the Core protein that is encoded upstream of E1 on HCV polyprotein. Following polyprotein translation, E1 is cleaved from the polyprotein by a cellular signal peptidase. During the synthesis, E1 and E2 ectodomains are translocated in the lumen of the ER while their 30 residues transmembrane domains (TMDs) are anchored in the membrane. E1 and E2 have been shown to assemble as noncovalent heterodimers [12] and to cooperate for their folding [13]. Although E2 can be expressed alone with a functional folding recognized by conformational antibodies [1,14], the folding of E1 depends on the co-expression of E2 [2,3,15,16]. Nevertheless, several studies reported that E1 could also modulate the folding of E2 [4–6,17–20]. The TMDs of HCV envelope proteins are responsible for their ER retention as well as their heterodimerization [6–8,21]. In addition to the TMDs, several regions in E1 and E2 ectodomains have been shown to contribute to the interaction between the two proteins [9,20,22–25]. Indeed, several residues in E1 region aa278 to 309 have been shown to be involved in E1–E2 interaction [10,11,24,25]. Residues 308, 330 and 345 have also been shown to be important for the functional interaction between E1 and E2 [23]. In addition, several reports support a role for E1 N-terminal region in E1–E2 interaction [13,20,26]. Finally, the characterization of cell culture-derived HCV (HCVcc) virus harboring chimeric heterodimers revealed that certain genotype combinations are not functional for virus entry [22,23,27,28].

Altogether these data underline a functional complementarity between E1 and E2 during their synthesis and in several steps of the virus life cycle.

Folding, glycosylation & disulfide bonds formation

The maturation of E1 and E2 takes place in the ER and involves the formation of disulfide bonds with the help of the PDI as well as their glycosylation by the N-glycosylation machinery [29,30].

Glycosylation

HCV E1 and E2 are highly glycosylated with N-linked glycans contributing to a third of the mass of the heterodimer. Glycans are linked to asparagine (Asn) within the Asn–X–Thr/Ser motif where X corresponds to any residue except Proline. E1 harbors four N-linked glycans (reviewed in [31]) at amino acid positions 196, 209, 234 and 305 of genotype 1a (strain H77). A fifth glycosylation site can be found in genotypes 1b and 6 at position 250, or in genotype 2b at position 299 [8]. Although HCV genome sequence is highly heterogenic, most N-glycosylation sites are conserved among the various genotypes, indicating that occupation of these sites by glycans is crucial for the HCV life cycle. Interestingly, the complete E1 glycosylation requires the co-expression of E2 [30,32]. E1 N-glycans have been shown to influence its folding as well as its functions during the HCV life cycle. Indeed, N-linked glycans at positions 196 and 305 are required for E1–E2 proper folding and heterodimerization [33]. Moreover, N196 and N305 sites have been shown to be crucial for E1 folding and its incorporation in retroviral particles harboring HCV envelope glycoproteins (HCVpp) [34]. In the HCVcc system, N196 is the most critical glycan for infectivity and assembly [35]. Moreover, the glycosylation of E1 may modulate intramolecular disulfide bond formation. Thus, due to steric hindrance, N305 glycosylation site could hamper the formation of disulfide bonds involving C306 [33].

Importantly, envelope proteins associated glycans have been shown to modulate their immunogenicity by masking epitopes targeted by antibodies (reviewed in [31]). Thus, removal of E1 N305 glycosylation site has a positive effect on the anti-E1 humoral immune response [36,37].

Disulfide bonds

The ectodomains of HCV E1 and E2 contain several cysteine residues that form disulfide bridges in the oxidative environment of the ER. In heterologous expression systems, a large proportion of E1 and E2 proteins follows a nonproductive folding and forms misfolded aggregates stabilized by intermolecular disulfide bonds connecting E1 and E2 [38,39]. The process leading to the formation of a functional heterodimer is slow and assisted by the calnexin chaperone. In this context, E1 and E2 form noncovalent heterodimers and the cysteine residues are involved in intramolecular disulfide bridges within E1 and E2 [13]. Interestingly, HCVpp particles mainly harbor noncovalent heterodimers, whereas the envelope proteins associated with HCVcc particles form large covalent complexes stabilized by intermolecular disulfide bonds [40–42]. These covalent bonds are thought to contribute to the resistance of the viral particles to low pH. As a consequence, a rearrangement of the disulfide bonds might be

Figure 1. Schematic diagram of HCV E1. The transmembrane domain of E1 is represented by a black box containing the GxxxG motif [6,48]. Colored boxes indicate the different regions of E1 for which the secondary structure has been resolved (green boxes: alpha helices; blue boxes: beta strands) [45–47,49,50]. The putative fusion peptide region is indicated by a red box [51]. Residues that have been shown to be involved in the interplay of HCV with CLDN1, CD81 or SR-BI receptors are indicated [20,24,52] as well as residues that are important for E1–E2 cross-talk [20,23,24,53]. Positions identified with resistant mutations to entry inhibitors likely affecting fusion are shown in red [54–56]. Epitopes within E1 recognized by monoclonal antibodies are represented by black line segments [38,47,57–59].

required for low pH induced fusion during entry [43]. However, HCV entry weakly depends on its redox status [44]. Unexpectedly, individual mutation of E1 cysteines only attenuated virus infectivity while greatly increasing the sensitivity of the virus to freeze-thaw treatment [19]. This result suggests that disulfide bonds contribute to virion stability.

Finally, E1 and E2 envelope glycoproteins are subjected to important post-translational modifications that are of crucial importance for their contribution to viral entry and morphogenesis.

Global organization & structure of E1 glycoprotein

For decades, characterization of the structure E1 and E2 has been hampered by the difficulties encountered to express and purify the proteins in their native form. Despite these difficulties, the crystal structure of the N-terminal region of E1 protein (residues 192–270) could be solved in 2014 [45]. The overall fold of the N-terminal E1 monomer consists of a β -hairpin followed by a segment constituted of a 16 amino-acid long α -helix flanking a three-strand antiparallel β -sheet. In this β -sheet, the loop between β 4 and β 5 contains ten disordered residues. The crystal structure revealed complex network of intertwined E1 homodimers that associate through covalent bonds. Interestingly, the N-terminus of E1 presents some structural homology with a phosphatidylcholine transfer protein, which would support the ability of E1 N-terminus to interact with hydrophobic ligands [45]. Thus, this domain may mediate the association of HCV with lipoproteins. Nevertheless, since it has been previously shown that proper folding of E1 requires the co-expression of E2 [15,16], the relevance of this truncated E1 structure will have to be further validated experimentally.

In addition to the structural characterization of the N-terminal region of E1, NMR studies have also been performed on a peptide located in the C-terminal region of E1 ectodomain (aa 314–342). This study revealed the presence of two other α -helices (α 2 and α 3, Figure 1) at residues 319 to 323 and 329 to 338 [46]. In agreement with this finding, the co-crystallization of the Fab of the human monoclonal antibody IGH526 with a major component of its E1 epitope (aa314–324) confirmed that this peptide adopts a helical structure when stabilized with an antibody [47]. This peptide was also shown to interact with membranes suggesting that it can either interact with the envelope membrane during assembly or with the host membrane during fusion [46].

Figure 2. Theoretical arrangement of a trimer of E1–E2 heterodimers on the viral surface. E1 proteins form trimers by interacting through their transmembrane domains at the center of the structure. Each E1 subunit binds to an E2 protein, thereby forming a trimer of heterodimers. Ectodomains of E1 and E2 are represented as oval shapes in blue and yellow, respectively while transmembrane domains are depicted as perpendicular spirals to the membrane surface [48].

Finally, NMR studies of E1 TMD have shown that this domain adopts a helical conformation with helical stretches at residues 354–363 ($\alpha 4$) and 371–379 ($\alpha 5$) separated by a more flexible segment of residues 364–370 [49,50].

In the absence of further structural data, some *in silico* models have been developed. Indeed, starting from the partial structure of E1 and E2 and experimental data, Freedman *et al.* used computational methods to develop a model of the structure of the remaining parts of E1 and E2. In this model, residues 275–286 form an α -helix that adjoins E1 core at its C-terminus. Two more β -strands spanning residues 290–303 follow this helix [60]. Moreover, the stem region of E1 model harbors three α -helices, the first two overlapping with $\alpha 2$ and $\alpha 3$ at residues 315–324, 333–338 and the last one from 348 to 352.

Castelli *et al.* combined computational analysis of E1–E2 structure with functional characterization of a series of E1–E2 mutants to propose an *in silico* model for the ectodomain of E1–E2 [61]. In their model, E1 ectodomain is composed of three α -helices spanning residues 256–266, 269–291 and 317–324 surrounded by short β -strands of 3–5 residues.

Several studies demonstrated the functional importance of conserved residues of the structured regions identified in E1 at different stages of the virus life cycle [20,62]. Overall, the determination of the structure of E1–E2 heterodimer would be of great interest to further dissect E1 role and notably its involvement in HCV fusion.

Oligomerization

The oligomerization status of E1 and E2 might vary according to the viral life cycle steps. Thus, in HCV infected cells, E1 and E2 form noncovalent heterodimers, whereas they associate in large covalent complexes stabilized by disulfide bridges at the surface of viral particles [41]. Recently, the oligomeric state of HCV virion-associated envelope proteins was further investigated by SDS-PAGE in the absence of thermal denaturation [48]. This experimental setting allowed for the identification of SDS-resistant trimers of E1 on HCVcc as well as on HCVpp. The formation of E1 trimers required the co-expression of E2 and was mediated by the TMDs of the envelope proteins. The highly conserved N-terminal G354xxxG358 motif in the TMD of E1 was shown to be crucial for its trimerization as well as for the virus infectivity, indicating that the trimeric form of E1 is of great importance for the virus life cycle. The fact that no E2 homotrimers could be detected supports the hypothesis that the TMD of three E1 monomers contribute to the trimer formation while interacting in periphery with E2 to form a heterodimer (Figure 2) [48]. However, thermal unstable trimers could also be detected in the lysates of infected cells suggesting that trimers of E1–E2 heterodimers are already generated during the virus assembly intracellularly.

The ability of E1 to trimerize and the importance of this feature for HCV infection support a role for E1 in viral fusion.

Neutralizing epitopes of E1

The majority of identified HCV neutralizing antibodies target epitopes in the E2 glycoprotein. The difficulty to identify anti-E1 neutralizing antibodies could in part be due to the difficulty to express correctly folded E1 in

the absence of E2 [63]. Nevertheless, several studies have demonstrated the capacity of E1 to induce neutralizing antibodies. Thus, neutralizing E1-specific polyclonal antibodies could be raised in mice immunized with E1-HCVpp or recombinant E1 protein [64,65]. Moreover, synthetic peptides derived from the C-terminal region of E1 could be recognized by immunoglobulins present in the sera from infected patients [66,67]. Two main regions of E1 have been shown to be targeted by anti-E1 antibodies. The first region is the N-terminal part of the protein, which is targeted by the human monoclonal antibody H111 (aa192-207) [57] and the murine monoclonal antibody A4 (aa197-207) [38]. Although A4 is not neutralizing, H111 shows weak neutralizing activity. Recently, the A6 human monoclonal antibody was isolated from an HCV infected patient. This antibody recognizes an epitope located between residue 230 and 239 within the N-terminal region of E1. Although recognizing envelope proteins from a broad range of genotypes, this antibody could not neutralize infection [58]. The second immunogenic region recognized by the broadly neutralizing monoclonal antibodies IGH505 and IGH526 is located at the C-terminus of E1 ectodomain, from residues 313–327 [47,59]. Recently, the structure of the complex formed by IGH526 monoclonal antibody with a major component of its epitope (aa314-324) was reported [47]. This first antigenic epitope structure may be of great importance for future vaccine design.

Noteworthy, in addition to antibodies recognizing E1 alone, two human conformational neutralizing antibodies, AR4A and AR5A, recognize discontinuous epitopes on E1 and E2 [68] and are endowed with broad neutralization activity. Contrarily to most E2-specific antibodies, they are not targeting HCV-CD81 interplay but might inhibit conformational changes of E1–E2 heterodimer during virus entry.

Due to the relatively high level of conservation of E1 among genotypes, E1-specific antibodies might exert broad neutralization [19]. Moreover, several studies suggested that immune response to E1 was impaired in chronically infected patients and was crucial for HCV clearance [69]. In agreement with this hypothesis, immunization of chimpanzees with E1 protected from the evolution of infection to chronicity [70]. These findings led to perform a Phase I clinical study with a vaccine containing a recombinant truncated form of the E1 protein [71]. This vaccine reached the Phase III clinical trials. Vaccination induced humoral and cellular immune responses to E1 but had no effect on the histological progression of liver disease [72].

These studies demonstrated the potential immunogenicity of E1 and supported its importance for HCV infection. However, the use of a truncated form of E1 might limit immunogenicity to few epitopes and vaccine using both E1 and E2 might be more promising than vaccine using E1 alone. Supporting this hypothesis, vaccine strategy using recombinant HCV E1–E2 provided protective immunity against HCV challenge in chimpanzees [73,74] and induced neutralizing antibodies as well as proliferative CD4 T cells responses in human volunteers in a Phase I clinical trial [75,76]. Although this vaccine has been shown to be highly immunogenic in healthy volunteers and chimpanzees, its ability to protect from real-life exposures remains to be demonstrated.

Role of E1 in HCV entry

Viral envelope proteins are at the first line of the infection process by mediating virus entry into the host cell. HCV entry into target cells is a complex process that can be divided into several steps: attachment to the cell surface, interaction with specific receptors, internalization and fusion between viral and host cell membranes. Attachment of the virus to the hepatocyte is mediated by the negatively charged heparan sulfate proteoglycans that are plentiful on the liver surface and involves virion-associated ApoE [77–79]. The subsequent interaction of the particle with specific HCV receptors involves the envelope glycoproteins.

E1 & cellular receptors

A surprisingly large number of cell factors have been reported to participate in virus entry (reviewed in [80–83]). The contribution to HCV entry of four of them has been the most characterized. These are SR-BI, the tetraspanin CD81, and the tight-junction proteins CLDN1 and OCLN. Very recently, imaging of HCV entry in a 3D polarized hepatoma system revealed a sequential interplay of the virus with SR-BI and CD81 at the basolateral membrane followed by the migration and association of the virus with OCLN and CLDN1 at the tight junctions [84]. Subsequently, the virus has been shown to be internalized via clathrin mediated endocytosis (reviewed in [82,85]).

Among HCV envelope glycoproteins, E2 is considered as the receptor binding protein. However, a direct interaction could only be shown between E2 and CD81 and between E2 and SR-BI [86,87]. Several data suggest that E1 maintains E2 in a functional conformation, modulating the interaction of E2 with cellular receptors. Thus, the mutation of each of the eight conserved cysteines of E1 as well as some residues in the N-terminal part of E1 (JFH1 I212, T213, H222, W239) affects the interaction of E2 with CD81 [19,20]. Similarly, several mutations in

the residues of E1 $\alpha 2$ region affected the dependence of HCV on SR-BI [24]. Furthermore, the characterization of the capacity of E1–E2 chimera from different genotypes to interact with SR-BI and CD81 receptors revealed that the binding to these receptors requires a crosstalk between the two envelope proteins [23].

Interestingly, E1 seems to be involved in the interplay of HCV with CLDN1. Although there has been no evidence for direct interaction between E1 and CLDN1 until now, mutations in E1 can affect the binding of HCVpp to CLDN1-expressing cells [23]. Intriguingly, different residues of E1 have the opposite effect on the contribution of CLDN1 to HCV entry. Thus, T213A, I262A and H316N mutations in the N-terminal part of E1 and the $\alpha 2$ helix decrease the dependence of HCV on CLDN1 for entry while increasing its dependence on CLDN6 [20,52]. Conversely, replacement of residues L286, E303, M323 and P328 by alanine increases the sensitivity of the virus to neutralization by CLDN1-specific antibodies, suggesting that these mutants present a higher dependency on CLDN1 for cellular entry [24].

Supporting a role for E1 in HCV interplay with cellular receptors, E1 was recently shown to interact with the membrane protein related to lipid metabolism, CD36. Expression of this receptor was increased upon HCV infection. Moreover, CD36-specific antibodies inhibited virus entry and replication, suggesting that CD36 may constitute a new HCV co-receptor [88].

Thus, these data indicate that the interplay of HCV with cellular receptors is not only mediated by E2 but is also strongly modulated by E1.

E1 & membrane fusion

The fusion process is considered as the final step of HCV entry. Once the virus has entered the cells via clathrin-mediated endocytosis [89], fusion of the viral envelope with a host cell endosomal membrane occurs, leading to the release of the viral capsid into the cytosol. In endosomes, the fusion is induced by low pH, which causes conformational changes of the fusion protein. This has for consequence the exposure of the fusion peptide that can thus interact with cellular membranes [90]. Knowing that secreted HCV particles resist to acidic pH, it is believed that the interaction of CD81 with E2 is responsible for priming HCV glycoproteins to respond to low pH. This step would thus be required to induce the fusion between viral and endosomal membranes [91]. However, the precise molecular mechanism that drives HCV membrane fusion and the viral proteins involved remains unknown.

Fusion proteins are classified into three classes according to their structures and mechanism of fusion. In the Flaviviridae family, flaviviruses harbor class II fusion proteins [92]. Class II fusion proteins are also shared by viruses belonging to Togaviridae as well as Bunyaviridae families [90,93,94]. Class II fusion proteins are characterized by an elongated structure consisting predominantly of β -sheet. They are organized in three domains and form homo- or hetero-dimers at the surface of the particles. Domain II contains the fusion peptide, which is buried at the dimer interface in the prefusion conformation. Upon fusion induction at acidic pH, the fusion proteins rearrange into homotrimers that harbor a protruding trimeric spike that inserts into the endosomal membrane [95–98]. In flaviviruses, the fusion protein is also involved in the binding of the virus to the cellular receptors.

Due to the conservation of the genome organization in all members of the Flaviviridae family, it has been hypothesized that the viruses from the hepacivirus and pestivirus genera also encode class II fusion proteins [99]. Accordingly, HCV E2 was postulated to be the fusion protein [100]. However, the resolution of the pestivirus E2 glycoprotein structure that shows no structural homology with class II fusion proteins did not support this hypothesis [101,102]. Similarly, the crystal structure of the core domain of E2 does not present the characteristics shared by fusion proteins [10,11]. Instead, E2 presents a compact globular shape including several regions with no regular secondary structure. In addition, the potential fusion regions [100] are located in the hydrophobic core of the protein, which makes them unlikely to mediate fusion [10,11,103]. It was also reported that E2 does not undergo oligomeric or fold change at acidic pH. Altogether these findings suggest that E2 is not directly involved in the fusion process. This means that E1 alone or in combination with E2 mediates the fusion step. In agreement with this hypothesis, E1 presents the capacity to form trimers, which is a characteristic feature of viral fusion proteins [48]. Indeed, the post-fusion structures of class I, II and III viral envelope glycoproteins described so far are trimers [104]. Moreover, several regions of E1 present characteristics of fusion peptides. The first one corresponds to the highly conserved hydrophobic sequence from residues 272 to 291, which has been proposed to constitute a putative fusion peptide (pFP) [105,106]. This sequence is characterized by the presence of a highly conserved acidic residue (D279), which is present at a similar position in the fusion peptide of several flaviviruses. Moreover, this sequence contains two cysteines and two glycine residues that are essential for the fusion in paramyxoviruses [51]. The peptides corresponding to this region induce the fusion and disruption of liposomes and hinder HCVcc infectivity [51,107].

Interestingly, several mutations conferring resistance to novel inhibitors of a late step of HCV entry arose in the C-terminal part of this pFP [54,55]. This finding re-enforced the hypothesis that this peptide is of crucial importance during the fusion process. However, recent studies of the function of the conserved residues of the pFP in the HCV life cycle by mutagenesis approaches revealed an important role of this region in E1–E2 interaction as well as in virus assembly [24,108]. These findings are not incompatible with a contribution of this region to the fusion step. Indeed, as found for the Semliki Forest virus, mutations in the fusion peptide can have an impact on the envelope proteins interactions and affect the virion assembly [109,110]. However, since mutations in the pFP region affect different steps of the HCV life cycle, the question of the specific involvement of the pFP in entry and fusion is difficult to address. The second region that is potentially involved in the fusion step is located in the C-terminal part of E1 ectodomain encompassing residues 314–342 which comprises the $\alpha 2$ and $\alpha 3$ helices. This region contains highly conserved residues. Mutations conferring HCV resistance to inhibitors of late entry steps have also been shown to arise in this region [56]. In addition, this peptide has been reported to interact with membranes [46]. Recently, we showed that several point mutations in the $\alpha 2$ region abolished infectivity with no impact on E1–E2 folding nor on virus assembly [24]. Moreover, further characterization of some of these mutants in the HCVpp model revealed an effect of the mutations on viral entry. These findings support a direct or indirect contribution of the $\alpha 2$ helix in the fusion process, which would be in agreement with the high membrane affinity of this region [46]. Altogether, these findings suggest that several regions of E1 contribute to the fusion step. This is in agreement with the fact that fusion is a sophisticated process involving numerous membranotropic segments of envelope proteins. Indeed, while the fusion peptide triggers the initial step of fusion, further membranotropic segments have been shown to contribute to subsequent stages [90,111,112].

Although E1 presents some of the characteristics of fusion proteins, the crystal structure of the N-terminal part of E1 is not comparable to any known class of fusion proteins [45]. Since, a similar situation was found for the envelope protein E2 from the pestivirus bovine viral diarrhea virus, this suggests that viruses within the Flaviviridae family might employ quite different fusion mechanisms. Thus, HCV and pestiviruses fusion processes might differ from each other and from the better characterized mechanism employed by flaviviruses.

The strong cooperation between E1 and E2 during assembly and entry [15,16,113] suggests that the functional viral glycoprotein unit involved in fusion is the E1–E2 complex. In line with this hypothesis, a computational method of coevolution prediction suggested that E1 co-evolved with the E2 back layer domain, and that this genetic association was of great importance for membrane fusion. This prediction could be supported experimentally since a soluble back layer-derived polypeptide was shown to inhibit HCV entry by acting on viral particle [114]. Thus, the characterization of E1–E2 interplay together with the structure of the heterodimer might be crucial to further dissect HCV fusion mechanism.

E1 & HCV morphogenesis

As a component of the virion, HCV envelope glycoproteins play a crucial role in virus assembly. Thus, the formation of E1–E2 heterodimers seems to be a key step in HCV morphogenesis. Unfortunately, the precise characterization of HCV assembly is restricted by the weak yield of this step, which hampers the visualization of assembly events in live cells by high-resolution microscopy. The assembly of the particle requires the gathering of the three structural proteins Core, E1 and E2 and the viral RNA. As found for other members of the Flaviviridae family, this step involves nonstructural proteins, among which p7 and NS2 are the main coordinators. Once released from the virus polyprotein, the Core protein associates with lipid droplets (LD), whereas the viral genome replication takes place in the membranous web [115,116], derived from the ER. HCV envelope proteins reside mainly in the ER of infected cells. Subcellular fractionation studies have shown that RNA replication and virion assembly occur in distinct membranous compartments. Indeed, assembly components have been shown to concentrate in detergent resistant LD-associated membranes from the ER [117,118]. At later time of infection, NS5A is recruited from the ER to the LD where it interacts with Core. This step might constitute the transition between replication and assembly. NS5A supports the delivery of HCV genome to the Core protein. Through its interactions with E1, E2, and nonstructural proteins, NS2 has been proposed to play a critical role in the migration of E1–E2 and Core to the assembly site [119–123]. Recently, the characterization of a mutant in the highly conserved D263 residues in the N-terminal part of E1 supported an involvement of E1 in the viral RNA encapsidation step. Indeed while affecting E1–E2 interaction, D263A mutation led to the production of viral particles devoid of viral RNA and to a decrease in the co-localization of the viral RNA with E1 [20]. These findings support that through its interplay with Core, E1 participates to the genomic RNA encapsidation [124]. During or following viral RNA encapsidation, the

envelopment of the nucleocapsid takes place at ER membrane. This process requires the envelope glycoproteins [125] and their interplay with other viral proteins, since chimeric viruses with glycoproteins from a different genotype than the rest of the viral proteins are impaired for the capsid envelopment [126]. Interestingly, several E1 mutations have been shown to affect virus assembly without any effect on E1–E2 interaction, suggesting that E1 is endowed with specific assignments during that step [20]. In particular, two studies reported that mutations in several residues of the pFP region affect virus assembly, suggesting that this region is involved in HCV morphogenesis in addition to its potential role in entry [24,108].

HCV particles have a specific lipid composition that is similar to that of LDL and VLDL with an important proportion of cholesteryl ester [127]. Moreover, they have been shown to incorporate a certain number of apolipoproteins [59,127,128]. The association of HCV with lipoproteins led to define the HCV particle as the lipo-viro-particle (LVP) [129]. The interplay of HCV with the host lipoprotein pathway that leads to the formation of LVP is poorly understood. The incorporation of lipoprotein components is thought to occur during the budding in the ER or after the budding into the lumen of the secretory pathway. E1 and E2 glycoproteins determinant modulate virion–lipoprotein association [19,53,130]. Hence, cysteine mutations in E1 resulted in a change of the density of infectious viral particles [19]. Several proteins of the VLDL pathway have been shown to contribute to the production of infectious HCV particles (reviewed in [9]). Among them, ApoE, which is incorporated in HCV particles, is crucial for HCV morphogenesis [131–133]. Thus, interaction of ApoE with E1 and E2 is required at a HCV life cycle step between the nucleocapsid envelopment and the virions release from the cells [132,134]. Although a first study reported the interaction of E1 only with ApoE in enzyme-linked immunosorbent assay [135], further reports observed an intracellular interaction of ApoE with E1 and E2 glycoproteins [136,137]. Moreover, Lee *et al.* established that the TMD of E2 was necessary for ApoE–E2 interactions. The different experimental approaches used in these studies might be responsible for these discrepancies.

Finally, recent characterization of E1 functions revealed a more important role than previously thought for E1 in the virus morphogenesis.

Conclusion & future perspective

HCV entry into host cell and assembly are two sophisticated steps in the HCV life cycle, involving an important number of cellular factors. E1 and E2 envelope proteins that play central roles in these two steps are thus involved in complex interplays with lipoproteins components and an extensive list of cell surface receptors, which remain to be further characterized. In the recent years, great progresses have been made on the characterization of E2 and E1 structure. However, far from confirming the working hypothesis placing E2 at the center of the entry process, the results obtained raised new questions and revealed the underestimated diversity of viral fusion processes. Thus, HCV fusion might rely on a new type of membrane fusion machinery and E1 would play a central role during HCV fusion. Recent results obtained during the characterization studies of E1 support that it plays a more important role than previously thought in HCV entry and assembly. Moreover, an increasing number of evidence supports the functional interdependence of E1 and E2. Finally, additional structural studies aiming to resolve the full structure of the E1–E2 heterodimer in the pre- and post-fusion conformation will be necessary to fully characterize HCV specific fusion process. Furthermore, a better understanding of E1 and E2 crosstalks should greatly improve our understanding of HCV entry and assembly.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

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Executive summary

HCV envelope glycoprotein E1 is a potential fusion protein candidate

- Until recently, E2 was the most studied HCV envelope glycoprotein.
- E2 has been shown to interact with several HCV cellular receptors and is the main target of neutralizing antibodies.
- The crystal structure of E2 core does not support a role for this protein in fusion, which suggests that E1 or E1–E2 heterodimer is responsible for this step.
- E1 contains several regions with fusion peptide properties.
- Several mutations that confer resistance to inhibitors of late entry step are located in E1.
- In a similar fashion to fusion proteins, E1 can associate in trimers on the viral particle.

E1 contributes to the HCV life cycle

- E1 has been shown to modulate the interplay of the virus with several cell surface receptors.
- E1 modulates the association of the virus with lipoproteins.
- E1 participates in viral assembly, specifically in the encapsidation of the viral genome.

HCV E1–E2 heterodimers are potentially involved in a novel fusion mechanism

- The structure of the N-terminus part of E1 presents no homology with known fusion proteins.
- Similarly, no homology with known class of fusion proteins was found for E2 protein from the pestivirus bovine viral diarrhea virus, suggesting that Flaviviridae family gathers new classes of fusion proteins.
- E1 and E2 interplay seems to be important for the fusion process in HCV entry.

Future perspective

- The resolution of the complete structure of the entire E1–E2 heterodimer in pre- and postfusion conformation will allow for the characterization of the fusion process defined by HCV.

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