Critical Interaction Between E1 and E2 Glycoproteins Determines Binding and Fusion Properties of Hepatitis C Virus During Cell Entry

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Hepatitis C virus (HCV) envelope glycoproteins E1 and E2 are important mediators for productive cell entry. However, knowledge about their structure, intra- or intermolecular dialogs, and conformational changes is scarce, limiting the design of therapeutic strategies targeting E1E2. Here we sought to investigate how certain domains of E1 and E2 have coevolved to optimize their interactions to promote efficient HCV entry. For this purpose we generated chimeric E1E2 heterodimers derived from two HCV 1a strains to identify and characterize crosstalk between their domains. We found an E1E2 combination that drastically impaired the infectivity of cell culture-derived HCV particles, whereas the reciprocal E1E2 combination led to increased infectivity. Using HCV pseudoparticle assays, we confirmed the opposing entry phenotypes of these heterodimers. By mutagenesis analysis, we identified a particular crosstalk between three amino acids of E1 and the domain III of E2. Its modulation leads to either a full restoration of the functionality of the suboptimal heterodimer or a destabilization of the functional heterodimer. Interestingly, we found that this crosstalk modulates E1E2 binding to HCV entry receptors SR-BI and CD81. In addition, we found for the first time that E1E2 complexes can interact with the first extracellular loop of Claudin-1, whereas soluble E2 did not. These results highlight the critical role of E1 in the modulation of HCV binding to receptors. Finally, we demonstrated that this crosstalk is involved in membrane fusion. Conclusions: These results reveal a multifunctional and crucial interaction between E1 and E2 for HCV entry into cells. Our study highlights the role of E1 as a modulator of HCV binding to receptors and membrane fusion, underlining its potential as an antiviral target. (HEPATOLOGY 2014;59:776-788)
HCV entry and internalization but no direct interaction with E1E2 heterodimers has been demonstrated so far. During entry, the E1 and E2 glycoproteins are thought to undergo coordinated and fine-tuned conformational changes until membrane fusion, i.e., the merging of the viral and endosomal membranes, occurs. However, how E1 and E2 cooperate during HCV entry remains largely undefined. Studying the role of different domains of E1 and E2 and their temporal engagement during virus entry remains challenging and may reveal interesting information for the development of novel entry inhibitors.

The recent identification of E2 intramolecular disulfide bonds allowed the development of an E2 structural model that presents characteristics of a typical class II fusion protein like the E glycoprotein of flaviviruses, which possesses both binding and fusion properties. Accordingly, E2 functions include binding to CD81 and SR-BI, and some domains involved in membrane fusion have been proposed. Conversely, the role of E1 during HCV cell entry remains poorly understood. For Flaviviruses, the E companion protein, PrM, cleaved and incorporated onto viral particles as M protein, acts mainly as a chaperone protein with no reported active role during virus entry. However, no cleavage of E1 has been reported despite its size similar to PrM, which strongly suggests that E1 acts differently than PrM/M. Consistently, some segments of E1 located in both its ecto- and transmembrane domains were found to play a role during postbinding events. Moreover, both E1 and E2 have a chaperone activity essential for correct folding of the heterodimer complex. Altogether, the entry model of HCV seems quite original in its family.

In this study we aimed at identifying the nature of the interrelations between E1 and E2 domains during HCV entry. Although most functional studies addressing the role of E1E2 focused on conserved residues, recent findings demonstrated the importance of variable domains during entry. Our working hypothesis was that a conserved protein function that involves different segments requires the coevolution of variable domains in order to maintain the most optimal direct or indirect interactions (then qualified as “crosstalk” or “dialog”) between these domains. We first generated a nonoptimal chimeric E1E2 heterodimer in order to subsequently restore its full functionality, thereby identify residues of E1E2 involved in crosstalks critical for efficient cell entry. The functional characterization of this interaction indicated that a specific E1E2 dialog is involved in binding to HCV receptors and membrane fusion, highlighting a multifunctional role of E1 in entry.

Materials and Methods

Cell Lines and Antibodies. Human Huh-7.5 (kind gift of C. Rice, Rockefeller University, NY), BRL3A rat hepatoma (ATCC CRL-1442), and 293T kidney (ATCC CRL-1573) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen). The rat anti-E2 clone 3/11, the mouse anti-HCV E1 clone 7D3-B320 and the mouse anti-human SR-BI mAb A417 were used. HCVpp were produced and used to infect Huh-7.5 cells as described.

HCVpp Production, Infection, and Characterization. Chimeric E1E2 heterodimers were constructed by molecular cloning between the genotype 1a envelope H77 (AF009606) and a genotype 1a envelope A40 (Maurin et al. unpublished results). HCVpp were produced and used to infect Huh-7.5 cells as described.

Green fluorescent protein (GFP)-positive infected cells were quantified by FACs Canto II (BD Biosciences). Transfected 293T cells were lysed as described and pseudoparticles were purified by ultracentrifugation at 82,000g for 1 hour, 45 minutes
mixing was performed as described using R18-FluoroMax-4 Spectrofluorometer (Horiba Jobin Yvon). Dequenching of R18 due to lipid mixing phosphatidylcholine and cholesterol (Aventi). After pH labeled PC/Chol liposomes (R18 Molecular Probes; preincubated for 1 hour at room temperature with or for neutralization assay, HCVcc viral particles were using 3/11, A4, and anti-MLV-CA antibodies as described. Immunoprecipitation assays were performed as described using AR3A antibody.

**RESULTS**

**Identification of a Nonoptimal E1E2 Heterodimer for Cell Entry.** In order to identify nonconserved domains of E1 and E2 that may act together for efficient entry, we focused on E1E2 sequences from two genotype 1a strains, H77 and A40 (Fig. 1A) isolated from two different HCV-infected patients. We generated two chimeric heterodimers by swapping either E1 or E2 sequences between these two strains (E1H77/E2A40 and E1A40/E2H77; Fig. 1A) and introduced them in cell culture-derived HCV (HCVcc) particles. After infection with equal quantity of physical particles as measured by qRT-PCR, the titer of HCVcc harboring the E1H77/E2A40 heterodimer (then qualified as “over optimal”) was higher in comparison to the one of H77 HCVcc (4-fold), whereas the titer of HCVcc harboring the mirror heterodimer E1A40/E2H77 (“suboptimal”) was reduced (4-fold) (Fig. 1B).

Next, to assess whether these different E1E2 combinations specifically affect HCVcc cell entry properties, we derived HCV pseudoparticles (HCVpp) and infected Huh-7.5 cells (Fig. 1C). HCVpp harboring the E1A40/E2H77 heterodimer had a 20-fold reduction in entry efficiency compared to parental E1E2, whereas HCVpp harboring the inverse chimera, E1H77/E2A40, exhibited a 2-fold increased infectivity, correlating with the results obtained with the HCVcc chimeras (Fig. 1B). Western blot analysis and coimmunoprecipitation assays indicated that these differences of infectivity were not due to altered expression, heterodimerization, or incorporation of E1E2 complexes onto the viral particles (Fig. 1D). Altogether, these results indicated that the infectivity differences between the HCVcc particles harboring the E1H77/E2A40 and E1A40/E2H77 heterodimers were mainly due to altered or improved entry.

**Three Amino Acids in E1 Are Involved in a Cross-talk With E2.** Aiming to restore the functionality of the suboptimal heterodimer E1A40/E2H77, we first constructed five E1A40/E2H77 point mutant heterodimers in which each residue of E1A40 was substituted one-by-one by those that are different in E1H77 (Fig. 2A). Biochemical analysis indicated that all heterodimers were correctly expressed in cells and similarly...
incorporated into HCVpp (Supporting Fig. S1). We found that either of three mutations (M117I, T139A, or L154M) slightly improved the cell entry of the sub-optimal E1A40/E2H77 heterodimer (Fig. 2B) and that the 3M mutant, combining altogether these three mutations, significantly improved the functionality of the E1A40/E2H77 heterodimer (7-fold). This suggested that determinants encompassing these three amino acids in E1 are involved in a crosstalk with E2 important for heterodimer functionality.

**Domain III of E2 Is Involved in a Dialog With E1.** As E2H77 and E2A40 have many amino acid differences, we divided E2 in functional domains (Fig. 3A) according to the E2 structural model proposed by Krey et al. Thus, starting from the E1A40/E2H77 heterodimer, we constructed different chimeras by interchanging domains of E2H77 with domains of E2A40 (Fig. 3B). Biochemical analysis indicated that all chimeric heterodimers were correctly expressed and incorporated similarly into viral pseudoparticles (Fig. S2). Complementation of E1A40/E2H77 by the domain III of E2A40 (named DIII) restored HCVpp entry to the same level as A40 E1E2 (Fig. 3B). Other domain substitutions decreased HCVpp infectivity (HVR1, Domain I, Domain II, and IgVr) or did not have any significant effect (Stem Region) on heterodimer functionality. These results indicated that domain III of E2 plays an important role in the function of the E1E2 complex during cell entry.

**Identified Domains Are Important for E1E2 Function During HCVcc Entry.** To confirm our results using HCVcc, we generated recombinant HCV genomes harboring the E1E2 chimeric heterodimers described above (Figs. 2, 3). Equivalent amounts of viral RNAs were electroporated and subsequently quantified in cell supernatants at 48 and 72 hours postelectroporation. All viral genomes replicated in comparison to GND replication-defective HCV.

Fig. 1. Identification of E1E2 conformations modulating HCV entry. (A) Schematic representation of H77, A40, and of the E1H77/E2A40 and E1A40/E2H77 heterologous heterodimer. (B) HCVcc particles (JFH-1) harboring H77, A40, E1H77/E2A40, or E1A40/E2H77 envelope glycoproteins were quantified by qRT-PCR to determine the HCV RNA GE (genome equivalent) levels in the cell culture supernatants of electroporated Huh-7.5 cells. Equivalent amount (1 × 10^5 HCV GE) of HCVcc particles were used to infect naive Huh-7.5 cells. Infected cells were fixed and numbers of foci were counted 4 days postinfection. Results are expressed as foci-forming unit per mL (ffu/mL) (mean ± SD; n = 4). *P < 0.05. (C) HCV entry assays using HCVpp harboring H77, A40, E1H77/E2A40, or E1A40/E2H77 heterodimers E1E2. Huh-7.5 cells were inoculated with HCVpp harboring the indicated heterodimers E1E2 or no glycoprotein (no Env) and infectivity titers were determined 72 hours after inoculation. Results represent average infectious titers, expressed as infectious unit (IU) per mL (mean ± SD; n = 4). *P < 0.05 **P < 0.005. (D) Expression in transfected 293T cells (top) and incorporation on concentrated pseudoparticles (middle) of H77, A40, E1H77/E2A40, or E1A40/E2H77 heterodimers analyzed by western blot using AR3A to detect E2, A4 to detect E1, and p30 to detect MLV-CA. Folding and heterodimerization of E1 and E2 glycoproteins on HCVpp were analyzed by coimmunoprecipitation with the AR3A antibody (bottom), which recognizes a conformational epitope on E2, followed by western blot detection of pellets using anti-E1 antibody (A4).
genome (Fig. 4A). Viral particles harboring 3M and DIII heterodimers were correctly produced, showed no defect in viral release compared to control wild-type and chimeric heterodimers (Fig. 4A), and were all infectious compared to GND replication-defective and ΔE1E2 entry defective mutants (Fig. 4B). Viral particles harboring 3M and DIII heterodimers also presented an improved infectivity in comparison to viral particles harboring suboptimal E1A40/E2H77 heterodimers (Fig. 4B). Comparison of intracellular and extracellular infectivity of HCVcc indicated that E1E2 changes did not affect budding of viral particles (Fig. 4C). Based on the quantification of viral particle RNAs and on their infectivity, we then calculated the specific infectivity of viral particles harboring 3M and DIII heterodimer and confirmed that, similar to HCVpp infectivity, HCVcc harboring the 3M or DIII heterodimers presented a restored cell entry function (Fig. 4D). These observations were confirmed by HCVcc infection assays using equal amounts of viral particles (data not shown). Altogether, these data demonstrate that the identified amino acids in E1 and DIII in E2 are important for the functionality of E1E2 complex during HCVcc entry.

**Identified Amino Acids in E1 Are Involved in a Common Crosstalk With E2.** To address whether the three amino acids identified in E1 and DIII of E2 are partners of a crosstalk, we constructed H77 E1E2 mutants by substituting the three identified E1 amino acids (3M), the domain III of E2 (DIII), or both, by those of A40 E1E2 sequence. We first demonstrated that all heterodimers were expressed and incorporated onto viral particles in a similar manner (Fig. 4B). Results represent average infectious titers, expressed as infectious unit (IU) per mL (mean ± SD; n = 3). *P < 0.015; **P < 0.005.
HCVpp infectivity (Fig. 5). In contrast, substitution of DIII of H77 by that of A40 (#2) increased entry to a level similar to that of E1H77/E2A40 (Fig. 5). Substitution of 3M and DIII simultaneously in H77 heterodimer (#3) restored infectivity of HCVpp to a level similar to H77 HCVpp (Fig. 5), indicating that the crosstalk helps the regulation of E1E2 function. The same phenotype modulations were observed by mutating A40 heterodimer. Insertion of the three amino acids from E1 H77 overoptimized the A40 heterodimer for entry (#4). This overoptimization was conserved when E2A40 is exchanged with E2H77 harboring the DIII of A40 (#5). Altogether, these results underlined that the three identified amino acids in E1 and DIII of E2 are likely involved in a crosstalk essential for E1E2 conformation and/or virus cell entry.

**Identified Dialog Participates in the Modulation of Binding to CD81 and SR-BI.** We next analyzed at which step of the entry process the proposed E1E2 dialog could act. To address the possibility that it may modulate the interaction of E1E2 with HCV receptors, we performed HCVpp binding assays on BRL3A rat hepatocarcinoma cells expressing human CD81, SR-BI or Claudin-1 (Fig. S4A). The loss of binding of HCVpp harboring a H77 heterodimer mutated in the CD81 binding domain on BRL-CD81 cells (Fig. 6C; Fig. S4B) and the inhibition of HCVpp binding by anti-SR-BI antibody21 on BRL-hSR-BI cells (Fig. 6D) confirmed the specificity of these binding assays. Similar amounts of HCVpp were used in all assays as measured by the quantity of capsid and E2 by western blot (Fig. S4C). All binding assays were also confirmed with another anti-E2 antibody 18 (AR3A), suggesting that the differences in binding assays did not reflect affinity differences (data not shown). Interestingly, HCVpp harboring 3M and DIII mutations presented an improved binding to CD81 and SR-BI compared to HCVpp harboring suboptimal E1A40/ E2H77 heterodimer, which displayed a weaker binding.

**Fig. 3. Domain III of HCV E2 modulates viral entry.** (A) Amino acid alignment of E2H77 and E2A40. Proteins were divided in functional domains according to the E2 structure model proposed by Krey et al.7 Chimeric E1A40/E2H77 heterodimers were constructed by substituting domains of E2H77 by domains of E2A40. (B) Infectivity on Huh-7.5 cells of HCVpp harboring H77, A40, the heterologous heterodimers, E2 chimeric heterodimers, or no glycoproteins (no Env) was determined as described in Fig. 1B. Results represent average infectious titers, expressed as infectious unit (IU) per mL (mean ± SD; n = 3). ns = not statistically significant.
to these receptors (Fig. 6A,B; Fig. S5A,B). These results suggested that the 3M/DIII crosstalk modulates E1E2 binding to HCV receptors. Exposure of the viral CD81 binding site was also compared between chimeras by another complementary approach employing infection inhibition of HCVcc by soluble CD81 large extracellular loop (CD81-LEL). Overall, the CD81-LEL inhibition pattern of the HCVcc harboring the different chimera (Fig. 6E) was similar to the pattern of HCVpp binding on BRL-CD81 (Fig. 6A). Consistently, HCVcc harboring DIII mutation were less neutralized than HCVcc harboring suboptimal E1A40/E2H77. The differences with HCVcc harboring 3M mutations, however, were not significant ($P > 0.1$). These results suggest that the 3M/DIII crosstalk can participate in the uncovering of the viral CD81 binding site. However, there is no strict correlation between entry and binding phenotypes (Fig. 6A,B; Supporting Table 1), which indicates that other E1E2 interactions play a role within this dialog to modulate the binding to CD81 and/or SR-BI receptors.

**E1 Allows Binding of E1E2 Heterodimers to Claudin-1 at the Cellular Surface.** We then wondered whether Claudin-1 could also modulate binding of HCVpp. Interestingly, we found that HCVpp bound Claudin-1 at BRL cellular surface (Fig. 7A-C; Fig. S6A) and that this binding is specifically inhibited by a monoclonal antibody, known to inhibit HCV infection, targeting Claudin-1 (Fig. 7B). To better analyze the determinants of this binding, we generated chimeric Claudin-1 receptor harboring the first large extracellular loop (EL1) from Claudin-7 (nonfunctional for HCV entry) and an EL1-deleted Claudin-1 mutant (Fig. 7C). The Claudin-1 EL1 Claudin-7 chimeric reporter was more expressed at the BRL cell surface than the wild-type Claudin-1, which is similarly expressed to Claudin-1 EL1 deleted mutant (Fig. S6B). Interestingly, no binding were observed on either receptor mutants (Fig. 7C), suggesting that E1E2 probably bind to EL1 and that this binding plays a role during HCV entry. The binding analysis of the different chimeras (Fig. 7A) indicated that HCVpp harboring 3M and DIII mutations showed an improved binding to

Fig. 4. Identified E1 amino acids and DIII of E2 are important for HCVcc entry. HCVcc particles harboring the different E1E2 heterodimers or encoding for a replication (GND) defective genome were harvested at 48 and 72 hours after electroporation of Huh7.5 cells and analyzed from filtered cell culture supernatants for HCV RNA GE (genome equivalent) levels determined by qRT-PCR (A) and for HCV infectivity by titration (B) on naive Huh7.5 cells as foci-forming unit per mL (ffu/mL) (mean ± SD; n = 3). A genome deleted for E1 and E2 (DE1E2) was used as a negative control of infectivity (C). Intracellular and extracellular infectious titer of HCV viral particles harboring the different E1E2 heterodimers at 72 hours post electroporation. Viral particles were harvested either from cell supernatant (extracellular infectivity) or after disruption of electroporated cell membranes (intracellular infectivity) and titrated on naive Huh-7.5 cells. Results are expressed as foci-forming unit per mL (ffu/mL) (mean ± SD; n = 3). (D) Specific infectivity of HCVcc viral particles harboring the different E1E2 heterodimers was calculated as infectivity per HCV RNA GE at 72 hours post electroporation. For each envelope, the corresponding HCVpp infectivity is indicated below (mean ± SD; n = 4). *$P < 0.05$, ns = not statistically significant.
Claudin-1 (100% and 46%, respectively) as compared to HCVpp harboring suboptimal heterodimer (19%) (Fig. 7A; Fig. S6A, Table S1). These results demonstrated that the proposed 3M and DIII crosstalk modulates Claudin-1 binding.

To analyze the impact of E1 on E1E2 binding ability and regarding the technical limits to obtain a relevant folded soluble E1 protein,24 we compared binding of soluble E2A40 (sE2A40) and soluble E2H77 (sE2H77). As a control, we verified that a soluble E2H77 deleted for HVR1 (sE2H77 Δ HVR1) was able to bind CD81 but not SR-BI (Fig. S6C). When equivalent amounts of either sE2 was added to cells, no binding to Claudin-1 was observed (Fig. 7D). These observations highlighted the strict requirement of E1 to allow E1E2 binding to Claudin-1. In contrast, the binding of E1E2 to CD81 and SR-BI does not necessarily require E1. Our results indicated also that, consistent with the HCVpp binding phenotypes (Fig. 6A), sE2H77 bound CD81 with a higher ability than sE2A40 (ΔGEO mean = 6,000 versus 1,647, respectively) (Fig. 7D). On the contrary, sE2H77 and sE2A40 bound in a similar way SR-BI (ΔGEO mean = 3,940 versus 3,659, respectively) (Fig. 7D), which did not correlate with HCVpp binding results (Fig. 6B; Table S1).

In conclusion, E1 seems to determine the binding ability of heterodimers to CD81, SR-BI, and Claudin-1 differentially according to its sequence and its interactions with E2. E1 is mandatory for E1E2 binding to Claudin-1 and it influences binding to SR-BI and CD81. Altogether, these results suggest that E1 plays a global and active role in receptor binding modulation during entry.

Identified E1E2 Crosstalk Modulates Conformational Changes Necessary for Membrane Fusion. To assess the fusion abilities of our different heterodimers, we studied the ability of HCVpp to perform membrane fusion at low pH using a liposome-based fusion assay.10 Interestingly, we found that the fusion efficiency correlated with the infectivity of the pseudoparticles harboring the set of E1E2 heterodimers (Fig. 8; Table S1). Indeed, HCVpp harboring 3M and DIII heterodimers exhibited improved fusion rates (58% and 44%, respectively) than HCVpp harboring the E1A40/E2H77 envelope (20%). Moreover, E1H77/ E2A40 heterodimer induced-fusion was overoptimal (134%) in comparison to H77 and A40 fusion abilities (Fig. 8). Altogether, the identified crosstalk appeared to be crucial for viral fusion, highlighting that E1 and E2 may collaborate together during this process.

Discussion

Even if challenging without structure data, it remains important to dissect the role of viral glycoproteins at the molecular scale and potential conformational changes during the cell entry process. Several articles have already reported crosstalks15,16 and important domains3-11,13 for entry in HCV glycoproteins. However, these reports focused only on E1 or E2. Here, to our knowledge, we performed the first study focusing on both E1 and E2 functions and behaviors.
in the context of their interrelation. We identified a precise and multifunctional dialog, acting both during binding and fusion steps. These results contribute to a better understanding of how E1 and E2 may cooperate together during entry and suggest that E1 would not be a simple chaperone, but an active partner of E2 during entry, highlighting a very distinct entry mechanism compared to other members of the Flaviviridae family.\textsuperscript{12}

**Identified E1E2 Crosstalk Modulates the Binding to Cellular HCV Receptors.** Precise interactions between HCV E1 and E2 during receptor binding steps of the entry process are poorly characterized. We suspect that several conformational changes of the envelope glycoproteins occur and are triggered upon the interaction with the quite unique important number of identified HCV cellular receptors.

In this study, we showed that (1) a specific crosstalk between E1 and E2 participates to the modulation of binding site accessibility to several HCV receptors; (2) that the binding phenotype is dependent on the heterodimer conformation, governed by particular E1E2 interactions; and (3) that E1 plays an important role of modulation of binding, varying according to the receptor (Table S1).

The location of the CD81 and SR-BI binding domains in E2 are well characterized.\textsuperscript{2,3,7-10} Thus, it is likely that interactions with E1 do not create new heterodimeric conformational domains where both E1 and E2 would bind directly to receptors, but E1 probably modulates the accessibility of the existing different E2 binding domains by shaping the heterodimer conformation. These results are consistent with mutagenesis studies of conserved residues such as cysteins.\textsuperscript{25}
Fig. 7. Heterodimers are able to bind Claudin-1 at cell surface. (A) Binding ability of HCVpp harboring the different E1E2 heterodimers to Claudin-1-expressing BRL cells. Binding signals from native BRL were subtracted from the APC GEO mean signal observed with receptor expressing BRL (ΔGEO Mean) to determine heterodimer binding efficiencies as described in Fig. 6A (mean ± SD; n = 3). *P < 0.05. (B) Mean fluorescence intensities (MFI, x axis) of APC signal after binding of pseudoparticles harboring H77 heterodimer to native BRL (thin lines) or Claudin-1 expressing BRL cell lines (thick lines) previously incubated with a control rat IgG (30 μg/mL) or with the rat anti-human Claudin-1 mAb (30 μg/mL). Results are representative of 2 independent experiments. (C) Schematic representations of Claudin-1 and Claudin-1 chimeric constructions, and corresponding binding assays. Mean fluorescence intensities (MFI, x axis) of APC signal after binding of pseudoparticles harboring H77 heterodimers to BRL expressing either Claudin-1, Claudin-1 expressing the first large extracellular loop (EL1) of Claudin-7, or Claudin-1 deleted of EL1 (thick lines). Thin lines represent MFI signal after binding to native BRL. Results are representative of two independent experiments. (D) Mean fluorescence intensities (MFI, x axis) of APC signal after binding of soluble E2 H77 (sE2 H77) or E2 A40 (sE2 A40) to CD81, SR-BI or Claudin-1 expressing-BRL (thick lines). Thin lines represent MFI signal after binding to native BRL. Results are representative of three independent experiments.
which demonstrated that E1 structurally shapes E2 binding properties to CD81. However, our study used different genetically close E1 wild-type sequences and thus demonstrate that E1 functionally determines E2 binding to several receptors by way of their specific interactions between less conserved domains.

Due to technical limits, the identification of E1E2 conformations during entry steps remains difficult to assess. In previous studies, HCVcc binding could be detected only on SR-BI. Even if E1 or E2 domains in the context of HCVcc may mask binding domains to other receptors, it is possible that lipoprotein components also inhibit the direct interaction between the viral envelopes and the other receptors. Therefore, we used HCVpp, which are devoid of lipoprotein components as an alternative model in order to characterize E1E2 interactions with CD81 and/or Claudin-1. It is important to keep in mind that such systems still do not characterize E1E2 conformational changes after each successive binding to the different receptors, which remains an important technical issue to solve.

**E1 Is Critical for Binding of the E1E2 Heterodimer to Claudin-1.** We demonstrated for the first time that an E1E2 complex is able to interact with Claudin-1 at the cell surface. Whereas soluble E2 does not bind Claudin-1, our results suggest the crucial need of E1 interaction with E2 to allow effective binding to Claudin-1. This result supports a model where Claudin-1 is not only an entry factor needed for receptors clustering, but is an HCV entry factor that can interact directly with E1E2 complexes. Previous studies have suggested that viral envelope glycoprotein binds exclusively to SR-BI and CD81 with subsequent interaction of the virus with the CD81-Claudin-1 complex. Our results suggest a model where SR-BI and CD81 may transfer the HCV particle to entry competent Claudin-1 with subsequent formation of a virus-receptors complex crucial for virus internalization, as supported by the recent observation that CD81, SR-BI, and Claudin-1 are part of tetraspanin-enriched microdomains.

Further studies are needed to address three important questions. First, it would be relevant to determine whether E1E2 binding to Claudin-1 is critical for receptor clustering and internalization. Second, it would be of interest to map the Claudin-1 EL1 domains that are involved in E1E2 binding. Their identification would be eased by analyzing the amino acid differences between Claudin-1 and Claudin-7 EL1. In addition, Claudin-1-specific antibodies have been shown to inhibit CD81-Claudin-1 coreceptor associations and Claudin-1 residues involved in CD81 association have been identified. Further mapping studies are needed to determine whether these relevant residues have a role in E1E2-Claudin interaction. Third, the localization of Claudin-1 binding domain in E1E2 needs to be identified. E1E2-Claudin1 interaction could be mediated by E2 with E1 required for the proper conformation of E2 interacting with Claudin-1. However, as E1 appears to be indispensable for Claudin-1 binding, another possibility would be that the binding domain is constituted either by E1 alone or by a conformational domain formed by both E1 and E2.

**E1E2 Dialog Is Important for Conformational Changes Leading to Membrane Fusion.** In this study, we brought evidence that the identified crosstalk appears to be an actor of E1E2 conformational changes that are necessary for membrane fusion. E1 has been suggested in previous studies to be involved in viral fusion.

Through the identification of this specific interaction, we provide an explanation on how E1 may mediate fusion with E2. However, the precise effects of such dialogs on heterodimer conformations during membrane fusion...
remain enigmatic. First, domain III of class II fusion protein is known to fold back during fusion to approach the viral membrane of the endosomal membrane. We can hypothesize that E1, by interacting with DIII, may regulate this fold back. The modification or absence of this interaction would impair the ability of DIII to bring the two membranes closer. Second, E1 could also be essential to unmask a hypothetical E2 fusion peptide by way of induction of conformational changes. Finally, we cannot exclude the hypothesis that E1 represents the HCV fusion protein and harbors a fusion peptide, which requires unmasking induced by conformational changes in E2, as has been recently proposed for other Flaviviridae.

In conclusion, we characterized a specific and multifunctional dialog between E1 and E2 which modulates binding and which is crucial for fusion. These results strongly reinforce the knowledge of the roles of E1 during entry by characterizing its involvement in heterodimer functions and conformations. As entry represents a possible target for future therapy against HCV, an improved knowledge of E1E2 dialogs and conformations remains essential for the development of inhibitors targeting viral glycoproteins. It is likely that antibodies against domains involved in E1E2 crosstalks are generated in vivo. It will be important to isolate and characterize them, as the strong functional pressure on these domains probably limits their mutation in vivo, thereby providing relevant targets for B cell vaccines or immunotherapies with reduced risk of emergence of resistant viruses. Further work will be needed to validate the potential of the antiviral strategies targeting E1E2 crosstalk.

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