Hepatitis C virus envelope protein dynamics and the link to hypervariable region 1
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Conformational dynamics of viral envelope proteins seem to be involved in mediating evasion from neutralizing antibodies (NAb) by mechanisms that limit exposure of conserved protein motifs. For hepatitis C virus (HCV), molecular studies have only recently begun to unveil how such dynamics of the envelope protein heterodimer, E1/E2, are linked to viral entry and NAb evasion. Here, we review data suggesting that E1/E2 exists in an equilibrium between theoretical ‘open’ (NAb-sensitive) and ‘closed’ (NAb-resistant) conformational states. We describe how this equilibrium is influenced by viral sequence polymorphisms and that it is critically dependent on the N-terminal region of E2, termed hypervariable region 1 (HVR1). Finally, we discuss how it appears that the virus binding site for the HCV entry co-receptor CD81 is less available in ‘closed’ E1/E2 states and that NAb-resistant viruses require a more intricate entry pathway involving also the entry co-receptor, SR-BI.

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Current Opinion in Virology 2021, 50:69–75
This review comes from a themed issue on Engineering for viral resistance
Edited by Steven Foung, James Ou and Chris Walker

https://doi.org/10.1016/j.coviro.2021.07.006
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Introduction
More than 70 million people are chronically infected with hepatitis C virus (HCV) worldwide, resulting in at least 400,000 annual deaths from liver cirrhosis and cancer [1]. HCV is a positive-stranded RNA virus that belongs to the \textit{Hepacivirus} genus within the family \textit{Flaviviridae} [2]. For several reasons, such as frequent undiagnosed infection and high drug costs, treatment with direct acting antivirals of chronic HCV infection is only available to a small fraction of infected individuals, and there is a need for a prophylactic vaccine for global control of the disease, which is transmitted to over 1 million individuals annually [3]. A recent clinical phase I/II trial of a T-cell based vaccine, presenting the non-structural viral proteins, failed to reduce HCV chronicity rates despite eliciting a robust cellular immune response [4\textsuperscript{*}]. This suggests that the cellular arm of the immune system on its own is insufficient to consistently protect against persistence of the virus. However, humoral responses, either by themselves or combined with cellular immunity, may provide better protection from chronicity [3].

An early induction of HCV-specific neutralizing antibodies (NAb) correlates with spontaneous viral clearance [5,6,7\textsuperscript{,**}]. Such NAb target the HCV envelope glycoproteins 1 and 2 (E1 and E2), which assemble as heterodimers (E1/E2) in the lipid envelope on the surface of the virus particle. This E1/E2 complex likely depends on intra-complex interactions both within the transmembrane domains [9], as well as within the ectodomains [10]. The E2 glycoprotein is instrumental in the attachment of HCV particles to hepatocytes leading to direct interactions with several cell receptors (termed HCV entry co-receptors), such as tetraspanin CD81 [11] and scavenger receptor class B, type 1 (SR-BI) [12]. The E1 glycoprotein is not believed to engage HCV co-receptors directly, but contains a putative fusion peptide proposed to drive membrane fusion [13]. The mechanism of this process remains unresolved, and HCV E1/E2 driven fusion may function via a novel non-canonical mechanism [14]. Fusion proteins share an intrinsic pre-fusion state metastability, which is what enables the inherent structural rearrangement during the fusion process [14]. In line with this, emerging evidence suggests that the pre-fusion form of HCV E1/E2 is structurally dynamic [15,16**].

This review will discuss how these dynamics may also be linked to NAb evasion and entry co-receptor interactions, with a special focus on the N-terminal region of E2, termed hypervariable region 1 (HVR1; a.a. 384–410), which seems to play a critical role in the underlying mechanisms. Although E1/E2 structures are still missing, the development of infectious cell-culture systems representing the major genotypes of HCV, including variants lacking HVR1, has made it feasible to study these phenomena in great detail [18,19].
Conformational dynamics of viral proteins influence different processes of the infectious cycle

Viral membrane fusion, interaction with host receptors during the entry process and evasion from NABs are all processes that involve changes to virion structure and require structural rearrangements of the viral proteins. While fusion is not relevant in the context of non-enveloped viruses, conformational virion dynamics have been studied in detail for such non-enveloped viruses through cryo-EM and X-ray crystallography (reviewed in Refs. [20,21]). An example of this is the capsid rearrangement of Poliovirus, where a virion uncoating intermediate is stabilized upon binding to a cellular receptor. This structural rearrangement has also been observed for free viruses in solution, suggesting that the Polio virion naturally fluctuates between different conformational states [22]. Generating high-resolution structures for enveloped viruses is often more challenging due to extensive variation in size and composition of such particles. Exceptions to this are members of the Flaviviruses genus within the family Flaviviridae that exhibit a high degree of particle symmetry. Here, cryo-EM has been successfully applied on full virions, yielding high resolution structures of particles and of the envelope protein E (fusion class II) of West Nile virus (WNV), Zika virus, Yellow fever virus, dengue virus (DENV) and tick-borne encephalitis virus (reviewed in Ref. [22]). For DENV and WNV, compelling evidence supports that the E protein exists in a dynamic equilibrium on virus particles, termed virus ‘breathing’, in which the E protein fluctuates between ‘open’ (antibody accessible) and ‘closed’ (antibody inaccessible) states [23–25]. While this has been most extensively studied for DENV and WNV, these dynamics likely also play a role for other flaviviruses [22]. Antibody neutralization by binding to the E protein of DENV and WNV increases with temperature, and it has been proposed that the equilibrium is skewed toward ‘open’ conformational states under these conditions, where neutralization epitopes are exposed [24,26]. In line with this, NABs targeting DENV E epitopes that are not accessible in solved structures of complete virus particles (i.e. cryptic) were shown to bind particles at 37°C, but not at 4°C [22]. The structure of DENV E bound by a Fab derived from such a NAB (1A1D-2) depicted an arrangement and orientation on the virion that was widely different from previously solved DENV E structures [23]. The mentioned examples indicate how envelope protein dynamics may regulate epitope accessibility for these viruses, which in turn would impact NAB sensitivity [25,27].

Virions from many other enveloped viruses, like HIV and Influenza, lack the high degree of symmetry needed for direct structural work. However, heterologous expression of native-like envelope protein complexes of HIV and influenza (fusion class I) has enabled such studies at the individual protein complex level. Structural examination of HIV Env has revealed that this glycoprotein complex exists in an equilibrium between ‘open’ and ‘closed’ forms, in analogy to the ‘breathing’ described above for flaviviruses [28]. Similarly, single-molecule Förster resonance energy transfer imaging has uncovered reversible exchange between different pre-fusion intermediates of influenza hemagglutinin trimers [29]. Like HIV and influenza, HCV virions are too pleomorphic to generate high-resolution structures of whole virus particles [30,31]. In addition, while several studies have solved partial structures of E1 and E2, the structure of the functional E1/E2 complex has remained elusive [13,32–35,36]. Despite these shortcomings, much has been learned from various antibody studies using infectious HCV particles, as well as from studying solved structures of antibodies bound to partial HCV envelope proteins and their simulated dynamics [37].

Evidence of structural dynamics of the HCV envelope protein complex, E1/E2

As with DENV and WNV, antibody reactivity studies have been applied to investigate the dynamic features of HCV E1/E2 on virus particles. Initially, Sabo et al. showed that increased time and temperature of antibody binding also increased virus neutralization, hinting at the existence of such temperature-dependent structural dynamics [15]. We subsequently showed that temperature-dependent increases in neutralization were highly reliant on the presence of HVR1, and that this temperature effect was lower for a highly neutralization sensitive HCV recombinant, with several N-linked E2 glycans removed [17]. It was discovered that natural envelope polymorphisms give rise to broad differences in NAB sensitivity across HCV isolates [38–40]. We found large additive effects of such polymorphisms and showed that NAB sensitivity increased dramatically with temperature [16**]. Taken together, this suggests that E1/E2 undergoes structural rearrangement to modulate epitope exposure (Figure 1). In line with this hypothesis, recent studies suggest that HCV escape from NABs is not always mediated by direct alterations of specific neutralization epitopes, but also happens through an indirect mechanism that globally governs epitope accessibility on E1/E2 (for details on the mechanisms of HCV escape from NABs see our recent review [41]).

Structural studies of the ectodomain of E2 alone have suggested a high level of intrinsic disorder [42**], where several sites, like antigenic site 412 (AS412), antigenic site 434 and the CD81 binding loop, adopt several distinct conformations [36,43]. Additionally, it was recently shown that NABs can bind two distinct conformations of the E2 neutralizing face, which is a highly conserved and accessible surface on E2 [36*]. It has been proposed that these sites or regions of E2 are conformationally flexible to minimize the development of specific NABs in infected patients [37,44]. However, we recently
Proposed mechanism for how conformational dynamics of E1/E2 is linked to HCV NAb evasion and interaction with entry co-receptors. (a) The E1/E2 complex on circulating HCV particles may exist in a dynamic equilibrium between theoretical ‘open’ (NAb-sensitive) and ‘closed’ (NAb-resistant) conformational states. Envelope protein polymorphisms can modulate this equilibrium, providing a way in which HCV can regulate exposure of conserved epitopes. These dynamics only assert themselves in the context of native envelope protein complexes present on infectious HCV particles and are dependent on an unknown interplay between HVR1 and other parts of E1/E2. The β-hairpin conformation of AS412 is associated with ‘closed’ E1/E2 states. (b) HCV engages entry co-receptor CD81 through a conserved binding site on E2 that appears more exposed in ‘open’ states of the E1/E2 complex. HCV interactions with CD81 can occur via two routes, either directly or with prior engagement of SR-BI, which may be mediating ‘closed’ to ‘open’ E1/E2 transitions. SR-BI may also position E1/E2 close to CD81 on cells, increasing the likelihood of the critical interaction between E2 and CD81.

Hypervariable region 1 (HVR1) modulates NAb sensitivity by influencing E1/E2 conformational dynamics

The N-terminus of E2, HVR1, has proven to be an essential component of HCV NAb evasion, as we demonstrated that the β-hairpin conformation of AS412 is associated with broad NAb resistance [16**], suggesting that in this case, local conformations are linked to global E1/E2 states. This is supported by the finding that HCV escape from NAbs targeting the AS412 β-hairpin increase sensitivity to NAbs targeting other epitopes [45]. Similarly, viral escape from other antibodies with a high barrier to resistance was also found to modulate broad NAb sensitivity, suggesting that the escape from one antibody was altering resistance to other NAbs with non-overlapping target epitopes [46–48]. However, studying these phenomena at the structural level is made difficult by the fact that we and others have demonstrated that global epitope exposure is often not reflected in NAb binding to ectopically expressed E1/E2 or for soluble forms of the ectodomain of E2 by itself [16**,17,49,50**,51]. Thus, current knowledge indicates that HCV E1/E2 exhibits a high degree of intrinsic disorder and that structural dynamics may play a crucial role in NAb evasion and viral entry. Still, much remains unknown about the key players in these processes and how these phenomena are orchestrated.
recently described in a review [52]. Briefly, HVR1 is an important regulator of early HCV co-receptor interactions and HVR1-specific NAb s typically dominate the early phase of HCV infections [53]. Moreover, the virus accumulates non-synonymous mutations in this region in chronically infected patients, at a rate comparatively higher than in the remaining part of the envelope proteins [54,55]. Additionally, the accumulation of amino acid changes in HVR1 is greatly decreased in patients with various types of agammaglobulinemia (low concentrations of IgG) [56]. Thus, it has been proposed that HVR1 helps the virus establish persistent infection by acting as an immunological decoy that diverts the humoral response away from more conserved E1/E2 epitopes [57]. Indeed, it may even be the case that such HVR1-specific NAb s sterically hinder the binding of NAb s targeting other parts of the E1/E2 complex [58].

It has recently become clear that HVR1 also has a central role in governing E1/E2 dynamics. HVR1 is critical for broad NAb resistance, as its removal renders HCV highly sensitive to NAb s targeting a wide range of both conserved and non-conserved epitopes [18,59–61]. Our prior finding that NAb sensitivity of HVR1-deleted HCV is high and cannot be increased further by the removal of glycans, addition of NAb-sensitizing polymorphisms or increasing temperature [16**,17] implies that its removal completely destabilizes ‘closed’ neutralization-resistant conformational states (Figure 1a). Moreover, we and others have observed that HVR1-deleted HCV was more easily inactivated at higher temperatures [17,50**], a hallmark trait of the ‘open’ conformation of the E protein of DENV and WNV [27]. Little is known about the interactions between HVR1 and the remaining part of the E1/E2 complex. This is due to its inherent flexibility, which has so far made it intractable to include the complete HVR1 sequence in solved E2 structures [32–35], and perhaps the fact that HVR1 interactions may involve residues outside the E2 ectodomain. We recently adapted attenuated HVR1-swapped HCV recombinants in cell culture and identified envelope mutations that compensated for this attenuation, which suggested that residues at position 348 in the stem region of E1 and at position 385 in the N-terminus of HVR1 may be involved in HVR1-E1/E2 interactions [51]. In a separate approach, molecular dynamics experiments, based on solved E2 ectodomain structures, have suggested that HVR1 is an intrinsically disordered protein tail that influences distant E2 regions, possibly through allosteric linkages via disulfide bonds [42**]. Based on this, it was proposed that HVR1 serves as an entropic switch that regulates broad resistance by reducing the irreversible inactivation from NAb interactions [50**]. Nevertheless, a better understanding of the underlying mechanism awaits a complete structure of the native E1/E2 complex.

Finally, we and others have found that naturally occurring envelope protein polymorphisms in the C-terminal part (positions 400–405) of HVR1 are the main contributor to differences in HVR1-intrinsic effects on broad NAb protection [16**,39]. Thus, the well-documented antigenic drift in HVR1 [62,63] may stem either from direct evasion from HVR1-specific antibodies (i.e. HVR1 acting as an immunological decoy) or more indirectly from HVR1-dependent evasion from NAb s targeting other parts of the HCV E1/E2 complex.

**Conformational E1/E2 dynamics influence interaction of HCV particles with entry co-receptors SR-BI and CD81**

The tetratspan, CD81, has been shown to directly interact with the ectodomain of E2 [11], and antibodies against this receptor completely block infection in vitro [64] and in vivo [65], demonstrating that this receptor is important for viral entry. Although there is no high-resolution structure of this E2-CD81 complex, the binding site for CD81 (CD81bs) has been mapped by mutagenesis and antibody competition experiments to highly conserved E2 residues [32,66–69]. Most broadly reactive NAb s (antibodies that are able to cross-neutralize numerous HCV isolates from multiple virus genotypes) isolated from patients, target epitopes that overlap with this binding site [70]. Moreover, changes to the HCV envelope proteins that increase broad NAb sensitivity typically also increase virus sensitivity to neutralization by soluble large extracellular loop of CD81 (sCD81-LEL) [16**,59,71,72]. Thus, exposure of the CD81bs seems directly linked to the ‘open’ E1/E2 conformational state.

Following attachment of the virus to the susceptible cell, the interaction of HCV with CD81 is believed to occur either directly or with the prior engagement of another co-receptor, SR-BI [73], that is also bound directly by E2 [12]. The interaction between E2 and SR-BI likely involves HVR1, as soluble E2 without this region no longer binds CHO cells expressing SR-BI [67,74]. However, HVR1 removal does not always reduce the binding of virus particles to SR-BI-expressing CHO cells [74], which might indicate that other moieties of the HCV particle are important for viral interactions with SR-BI. Furthermore, removal of HVR1 drastically decreases entry dependency on this co-receptor for most HCV isolates [59,74,75]. However, our data support that HVR1-deletion compensatory adaptive substitutions were responsible for this effect, as their introduction into original viruses also decreased SR-BI dependency, whereas the removal of HVR1 itself had no effect on SR-BI dependency in the case of an HVR1-deleted HCV recombinant that did not require HVR1-deletion compensatory adaptive substitutions [75]. In general, there
seems to be a link between broad NAb resistance and SR-BI entry dependency, as broadly sensitizing cell-culture adaptive substitutions, polymorphisms and N-linked glycans have also been shown to decrease viral entry dependency on this receptor \cite{17,71,72}. Interestingly, we recently observed virtually perfect correlation between increased SR-BI dependency and decreased propensity of HCV to interact with sCD81-LEL \cite{16**}. This fits well with current hypotheses on the relationship between SR-BI and CD81 during virus entry and supports a function of SR-BI during viral entry in which it mediates conformational transitions of E1/E2 from ‘closed’ to ‘open’, thus priming HCV for CD81 engagement (Figure 1b). The more complex entry route involving SR-BI could have evolved as a way for the virus to conceal highly conserved residues involved in CD81 interactions from circulating NAbs. This might explain why, in the absence of immune pressure (e.g. in cell-culture), HCV acquires E1/E2 substitutions that increase exposure of the CD81bs while decreasing SR-BI entry dependency.

**Future perspectives**

Modifying various forms of the HCV envelope proteins based on findings from studies with infectious virus particles (such as removing HVR1 to expose underlying conserved epitopes) for vaccine development have either had detrimental effects or modest benefits to producing increased NAb titers in immunized animals (reviewed in Ref. \cite{76}). This might be related to the prerequisite requirement of higher-order structures for conformational E1/E2 dynamics, as we and others have observed that extracted E1/E2 do not represent virion-associated E1/E2 behavior \cite{17,49,51}. Thus, using the recently generated knowledge about NAb epitope exposure presented in this review in developing cross-genotype protective HCV B-cell vaccines may require more native-like immunogens. In contrast to recombinant envelope protein vaccines, whole virus vaccines present E1/E2 in their native form, and modifications that expose conserved neutralization epitopes may be more likely to improve the immunogenicity for these particles. Finally, as studies suggest that E1/E2 form noncovalently linked trimers on the virion surface, extraction and purification of such complexes may provide insights of key importance for future vaccine development and may prove to be superior vaccine antigens.

**Funding**

This work was supported by a Novo Nordisk Foundation Distinguished Investigator - Bioscience and Basic Biomedicine grant (Grant number: NNFI9OC0054518; JB), a Lundbeck Foundation Experiment grant (Grant number: R324-2019-1375; JP) and a Lundbeck Foundation Fellowship grant (Grant number: R335-2019-2052; JP).

**Conflict of interest statement**

Nothing declared.

**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

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The authors define a mechanism by which envelope protein polymorphisms identified in HCV infected patients modulate broad neutralizing sensitivity involving conformational E1/E2 protein dynamics. They show that this mechanism is linked to viral entry and define a novel association of the β-hairpin form of AS412 with 'closed', neutralization resistant conformational states of E1/E2.


This recent study found that some NABs with contact residues in the E2 back layer stabilizes a different conformation of the E2 neutralizing face, where the front-layer region is displaced and residues in the back-layer and the β-sandwich are exposed.


The authors perform detailed molecular dynamics simulation of sE2, showing among other things that motions of HVR1 may influence distant regions of E2.


Using molecular dynamics simulations of E2 as a tool to study differences in HVR1 structural flexibility, the authors propose that HVR1 is an intrinsically disordered protein that regulates NAb immunity by reducing irreversible inactivation mediated by NAB interactions.


