Mutagenesis of a conserved fusion peptide-like motif and membrane-proximal heptad-repeat region of hepatitis C virus glycoprotein E1

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The E1E2 glycoprotein heterodimer of Hepatitis C virus mediates viral entry. E2 attaches the virus to cellular receptors; however, the function of E1 is unknown. We tested the hypothesis that E1 is a truncated class II fusion protein. Mutated amino acids within a predicted fusion peptide (residues 276–286) and a truncated C-terminal stem-like motif, containing a membrane-proximal heptad-repeat sequence (residues 330–347). The fusion peptide mutation F285A abolished viral entry, while mutation of other hydrophobic residues had no effect. Alanine replacement of heptad-repeat residues blocked entry in three of five cases, whereas substitution with the helix breaker, Pro, led to loss of entry function in all cases. The mutations did not affect glycoprotein expression, heterodimerization with E2 or global folding, in contrast to the effects of mutations in the fusion motifs of prototypical class II fusion proteins. Our data suggest that E1 is unlikely to function in an analogous manner to other class II fusion glycoproteins.

Virus replication is initiated when viral attachment proteins bind to cellular receptors. In the case of enveloped viruses, water molecules bound to the polar headgroups of viral and cellular membranes exert strong repulsive forces when brought into close proximity. Viral fusion protein(s) overcome this hydration barrier by catalysing membrane fusion. Fusion proteins characteristically comprise two hydrophobic membrane-interactive regions: a C-terminal transmembrane domain (TMD) and a fusion peptide that is located either at the N terminus or internally (Kielian & Rey, 2006).

X-ray crystallography has thus far revealed two structural classes of fusion glycoprotein (Kielian, 2006; Kielian & Rey, 2006; Skehel & Wiley, 2000; Stiasny & Heinz, 2006). Class I fusion proteins [e.g. human immunodeficiency virus 1 (HIV-1) gp41, influenza virus HA2] are helical, trimeric rods that project as spikes from the viral envelope. In the fusion-activated state, their N (fusion peptide-proximal) and C (TMD-proximal) termini become juxtaposed at one end of a helical hairpin core domain. Class II fusion glycoproteins (e.g. flavivirus E, alphavirus E1) comprise three domains rich in β-strands that lie roughly parallel to the viral membrane. At neutral pH, the metastable state of E, which has dual receptor-binding and fusion functions, is maintained in a homodimer by monomer–monomer interactions that sequester the fusion loop. In the case of alphaviruses, glycoprotein E2 mediates receptor binding, whereas the associated E1 trimer mediates fusion. E1 metastability is maintained through E1–E2 interactions. At low fusion pH, E and E1 have almost identical trimeric structures where membrane-inserted fusion loops are atop three upilted protomers. Trimerization creates three surface-exposed hydrophobic grooves along the trimers axis for the antiparallel packing of the TMD-proximal amphipathic α-helical stem to form a hairpin. Thus, hairpin formation is employed by both classes of fusion glycoprotein to appose membrane-associated fusion peptides and TMDs, which leads to membrane fusion.

Hepatitis C virus (HCV), a member of the family Flaviviridae, is distantly related to flaviviruses and encodes two type 1 transmembrane glycoproteins, E1 (polyprotein residues 191–383) and E2 (384–746), which are cleaved from the viral polyprotein precursor by signal peptidases in the endoplasmic reticulum (ER). Entry of HCV occurs via clathrin-dependent receptor-mediated endocytosis, with the low pH environment of the endosome triggering membrane fusion (Blanchard et al., 2006; Codran et al., 2006; Lavillette et al., 2006; Tscherne et al., 2006). E1 and E2 mediate viral entry as a non-covalently associated heterodimer (Drummer et al., 2003); however, the mechanism whereby these glycoproteins mediate membrane fusion is not understood.

Several lines of investigation suggest that E2 functions as a class II fusion glycoprotein. For example, as for flaviviral E, E2 comprises a large ectodomain (residues 384–661) that can be divided further into three subdomains based on cross-competition studies with conformation-dependent mAbs (Keck et al., 2004, 2005). By using molecular
modelling approaches, Yagnik et al. (2000) proposed that E2 is a homologue of flaviviral glycoprotein E with receptor-binding, dimerization and fusion domains. Furthermore, we have shown that a membrane-proximal heptad repeat (residues 673–699) within the approximately 55-residue sequence linking the E2 ectodomain to the TMD is important for E1E2 heterodimerization and viral entry (Drummer & Poumbourios, 2004). This observation led us to propose that the membrane-proximal heptad repeat of E2 is analogous to the stem of flavivirus glycoprotein E (Drummer & Poumbourios, 2004). If E2 functions as a class II fusion protein, then it should also possess an internal fusion peptide. Recently, we examined a conserved class II fusion peptide-like sequence, C429NESLNTGWLAGLFYQHKFNSSGC452, located within the N-terminal portion of E2. Interestingly, even though this sequence has all the hallmarks of a class II fusion peptide (Delos et al., 2000), our analysis indicated that it functions as a CD81-binding site (Drummer et al., 2006). Thus, the role of E2 in membrane fusion remains unknown.

An alternative hypothesis is that E1 represents a truncated class II fusion glycoprotein that has lost receptor-binding function during its evolution (Garry & Dash, 2003). Based on this model, it was suggested that the conserved hydrophobic E1 sequence C272SALYVGDLC281 is a truncated class II fusion peptide. However, the region does not have strong homology to the class II fusion peptides of flavivirus glycoprotein E, e.g. Tick-borne encephalitis virus (TBEV) (CRKDQSDRGWGNHCGLFGKGSIVAC), or alphavirus E1, e.g. Semliki Forest virus (CKVYTVYFPFMWGGAYCF-CDSENTQ), underlined sequences representing the exposed fusion loop. However, C-terminal extension of the predicted HCV E1 fusion peptide, C272SALYVGLC-DG-SVFLVQALFSPRRHWTQDC, incorporates class II fusion peptide elements such as the VFLVG motif (underlined) and a total of three conserved cysteine residues (bold). In addition, the region contains a proline residue believed to be a feature of internal fusion peptides (Delos et al., 2000). Further analysis of E1 ectodomain sequences revealed a second truncated class II fusion glycoprotein motif: a conserved three hydrophobic-heptad repeat, A30ALVVAQLLRIPQAIMDM347, preceding the TMD (Fig. 1).

In this study, we examined the functional roles of these regions by site-directed mutagenesis. Overlap extension PCR was used to replace conserved hydrophobic residues within the original fusion peptide motif proposed by Garry & Dash (2003), at positions Tyr276 and Val277 as well as the downstream VFLVG sequence targeting the conserved hydrophobics Phe285 and Leu286 in the genotype 1a E1E2 expression vector pH77E1E2 (Drummer et al., 2003). Proline (helix-breaking) and alanine (helix-forming) mutations were introduced at the a and d positions of the heptad-repeat region to disrupt coiled-coil formation or hydrophobicity, respectively.

Previous studies with the prototypical class II fusion protein, glycoprotein E of TBEV, revealed that mutations in the fusion peptide disrupted native glycoprotein structure due to the ablation of monomer–monomer contacts, while mutations in the stem region affected heterodimerization between the viral glycoproteins prM and E, and the conversion of E dimers to trimers at the low pH of fusion (Allison et al., 1999, 2001; Rey et al., 1995). We therefore examined whether the E1 mutations altered the ability of E1 and E2 to be processed from the polyprotein, to form non-covalent heterodimers and to become incorporated into retroviral pseudotyped particles. The pseudotyping of HIV-1 with mature forms of E1E2 (E1E2-pp) confers infectivity for primary human hepatocytes and various human liver cell lines including HuH7 (Bartosch et al., 2003; Drummer et al., 2003; Hsu et al., 2003). These E1E2-pps have been validated as a surrogate model to study HCV entry (Bartosch & Cosset, 2006; Lindenbach et al., 2005). E1E2-pps were produced by co-transfecting 293T cells with pH77E1E2 and pNL4–3.LUC.R’E* (from N. Landau, NIH AIDS Reagent Bank, Germantown, MD, USA; He & Landau, 1995) as described previously (Drummer & Poumbourios, 2004). The E1E2-pps were labelled with 75 μCi Trans 35S-label for 18 h, and then pelleted from filtered (0.45 μm) culture supernatants at 23 000 g for 2 h. Immunoprecipitation of viral lysates with the conformation-dependent anti-E2 mAb, H53, and non-reducing SDS-PAGE indicated that similar amounts of non-covalently associated E1 and E2 were incorporated into
E1E2-pps for the wild-type and mutated glycoproteins (Fig. 2a). Notably, substitution of the a and d position heptad-repeat residues with proline, which can induce kinks of up to 30° in coiled coils (Chang et al., 1999), did not alter the overall level of glycoprotein expression and/or heterodimerization between E1 and E2. Immunoprecipitation with polyclonal IgG from an HIV-infected individual (IgG14) was used to confirm that similar amounts of HIV-1 structural protein were present in the E1E2-pps (Fig. 2b). The results indicate that the mutants were efficiently processed from the polyprotein, forming non-covalently associated E1E2 heterodimers that could be incorporated into E1E2-pps.

We next subjected E1E2-pps carrying mutations in E1 to a CD81-binding assay. The CD81-binding site comprises three discontinuous elements, W420, G436WLAGLFY and Y527SWGANDTD, that are brought together by the E2 fold (Drummer et al., 2006; Owsianka et al., 2006). The formation of this CD81-binding site is therefore critically dependent on correct glycoprotein folding and can be used to sense changes in E2 structure. We have previously shown that a recombinant form of the large extracellular loop fused to maltose-binding protein (MBP/LEL113–201) forms dimers that bind E2 with nanomolar affinity (Drummer et al., 2002). Recombinant MBP/LEL113–201 was used to detect binding of mature forms of E2 within E1E2-pp lysates to CD81 in a solid-phase enzyme immunoassay (Drummer et al., 2006; Drummer & Poumbourios, 2004). Fig. 2(c, d) shows that wild-type and mutated glycoproteins exhibited almost identical abilities to bind to CD81. Mutations in the E2 glycoprotein previously shown to reduce CD81 binding (W437A) or retain wild-type levels of CD81 binding (G436P) were included as negative and positive controls, respectively (data not shown). These data indicate that the mutations in E1 do not alter the conformation of the receptor-binding domain of E2, consistent with a wild-type-like E2 fold.

We next examined the effect of these mutations on viral entry. E1E2-pps produced in 293T cells were used to infect Huh7 cells for 4 h. After removal of the inoculum, the cells were cultured for 3 days prior to assay with the Promega luciferase substrate system in a Fluostar microplate reader (BMG LabTechnologies) (Drummer & Poumbourios, 2004). The V277A and L286A fusion peptide motif mutants exhibited wild-type levels of entry, while the entry competence of Y276A and Y276R mutations was not significantly different from V277A and L286A entry levels. However, the F285A mutation was not tolerated, suggesting that this amino acid plays a critical role in the entry process (Fig. 3a). Mutagenesis of the a and d positions of the membrane-proximal heptad repeat to proline
completely blocked viral entry while mutation to the helix-forming residue, alanine, resulted in site-specific entry defects (Fig. 3b). Significant reductions in entry were observed for V333A, L337A and M347A (*P*, 0.05), while I340A and I344A did not significantly alter viral entry competence (*P* > 0.05) (Fig. 3b). None of these defects in viral entry were associated with a loss of CD81 receptor-binding ability (Fig. 2c, d), suggesting that these mutations in E1 affect a non-CD81-dependent stage of viral entry.

Two recent studies have described multiple membrane-interactive regions in E1 and E2 based on the ability of synthetic peptides to effect fusion of model liposomes (Pacheco et al., 2006; Perez-Berna et al., 2006). One region spanning residues 268—298, encompassing the putative E1 fusion peptide, was able to mediate high levels of both hemifusion and complete fusion (Perez-Berna et al., 2006). Interestingly, a second synthetic peptide overlapping the heptad-repeat region analysed here, 317—339, was also shown to mediate liposome fusion (Perez-Berna et al., 2006). We observed that mutation of the conserved amino acids Y276 and V277 within the C272 SALYGDLC281 motif did not affect glycoprotein expression, global folding, heterodimerization or viral entry, as has been observed for other class II fusion proteins such as flaviviral glycoprotein E. However, mutagenesis of F285 within the downstream motif (V284 FLVG) resulted in a complete loss of viral entry, while mutagenesis of the adjacent residue L286 did not have a detectable effect on glycoprotein function. The fusion loops of class II fusion proteins form a hydrophobic ring that penetrates the hydrocarbon layer by approximately 6 Å (0.6 nm) (Modis et al., 2004). In dengue virus glycoprotein E, the exposed residues Trp101 and Phe108 form a membrane-penetrating hydrophobic bowl, with the hydrophobic side chains of Leu107 and Phe108 lining the bowl (Modis et al., 2004). It therefore remains possible that the E1 region 276—286 is a fusion loop sequence, with F285 mediating critical interactions with the target membrane and hydrophobic residues other than L286 contributing to a hydrophobic ring.

The stem region of flavivirus glycoprotein E is required for heterodimerization with prM in the metastable prefusion complex, the conversion of E dimers into homotrimers at low pH and the stability of the trimer of hairpins (Bressanelli et al., 2004; Allison et al., 1999; Modis et al., 2004). Proline mutagenesis of the a and d residues of the short membrane-proximal heptad repeat of E1 (A330 ALV VAQLLRIPQAIMDM) had no effect on heterodimerization, while Ala substitutions resulted in site-specific defects in viral entry (V333A, L337A and M347A), again with no effect on heterodimerization. By contrast, an earlier study found that Ala- and Pro-substitution mutagenesis of the membrane-proximal heptad repeat of E2 (675—699) resulted in loss of heterodimerization and loss of viral entry function. Thus, the E2 heptad-repeat region has features consistent with its function as a class II fusion protein stem region (Drummer & Poumbourios, 2004). A fusion peptide has thus far not been defined for E2. A synthetic peptide (residues 430—449) encompassing a candidate fusion peptide motif, G436 WLAGLFY, was shown to mediate liposome fusion (Pacheco et al., 2006). However, mutagenic analysis of the motif in the context of E1E2 revealed that it was essential for CD81 binding (Drummer et al., 2006). Receptor disengagement following internalization and trafficking to the late endosome would be required for this sequence to function as a fusion peptide. An alternative mechanism of E1E2-mediated fusion could involve E1 and E2 acting cooperatively through the contribution of the fusion loop by E1 and the stem by E2, potentially representing a new paradigm for viral fusion.

**Fig. 3.** Viral entry abilities of E1E2-pp containing mutations in the predicted fusion peptide sequence and heptad-repeat region. Viral entry of E1E2-pp containing mutations in the predicted fusion peptide motif (a) or hydrophobic heptad repeat (b). Insets show the amino acids targeted for mutagenesis in bold and the location of a and d amino acids in the heptad-repeat region. Data shown are the mean ± SEM of three independent experiments (b) or as indicated (a). *P* values were derived using the two-tailed Student’s *t*-test assuming unequal variances. *, *P* < 0.01 unless otherwise indicated.
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References


