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Mutational analysis of the hepatitis C virus E1 glycoprotein in retroviral pseudoparticles and cell-culture-derived H77/JFH1 chimeric infectious virus particles

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Summary

Cell entry by enveloped viruses is mediated by viral glycoproteins, and generally involves a short hydrophobic peptide (fusion peptide) that inserts into the cellular membrane. An internal hydrophobic domain within E1 (aa262–290) of hepatitis C virus (HCV) may function as a fusion peptide. Retrovirus-based HCV-pseudotyped viruses (HCVpp; genotype 1a) containing Ala or Pro substitutions at conserved amino acid positions within this putative fusion peptide were generated. Mutation of conserved residues significantly reduced efficiency of HCVpp entry into Huh-7 cells. The majority of amino acid substitutions appeared to disrupt necessary interactions between E1 and E2. For some mutants, reductions in HCVpp-associated E1 were associated with the incorporation of a high molecular weight, hyperglycosylated E2 that displayed decreased CD81-binding. Other entry-deficient mutants displayed normal E1E2 incorporation into pseudoparticles and normal CD81-binding, and therefore might affect viral fusion. One mutant (S283P) consistently displayed two- to threefold higher infectivity than did wild-type. Three mutations that decreased HCVpp infectivity also reduced levels of HCVcc infectious virus production. However, the S283P mutation had a different effect in the two systems as it did not increase production of infectious HCVcc. This comprehensive mutational analysis of the putative HCV fusion peptide provides insight into the role of E1 in its interaction with E2 and in HCV entry.

Keywords

glycoprotein; glycosylation; HCV entry; HCVcc; HCVpp; hepatitis C virus

Introduction

Hepatitis C virus (HCV) is a small, RNA virus in the genus *Hepacivirus* in the family *Flaviviridae* [1]. HCV is an enveloped virus that must undergo a membrane fusion reaction

between the viral and cellular membranes in order to gain entry. Fusion is via viral membrane glycoproteins and generally involves a short hydrophobic fusion peptide that inserts into the target membrane. HCV is assumed to contain a class II fusion protein, like those found in related alphaviruses and flaviviruses, whose envelope glycoproteins contain a hydrophobic fusion loop at the distal tip, which acts through a complex mechanism involving multiple conformational rearrangements resulting in the formation of a fusion-inducing homotrimeric molecule that inserts into the plasma membrane [2–4].

Hepatitis C virus (HCV) contains two envelope glycoproteins (E1 and E2) which form a non covalent heterodimer [5], which represents the functional glycoprotein structure essential for entry of HCV pseudoviruses (HCVpp) into Huh-7 cells [6–8]. However, a fusion peptide has not been conclusively demonstrated in either of these glycoproteins. E2 by itself binds CD81 [9], a putative HCV receptor, and is proposed to have mechanistic features in common with class II fusion proteins [10]. The function of E1 in viral entry is unknown, but its association with E2 is essential for this event [6–8]. This requirement for E1 suggests that HCV may resemble Semliki Forest Virus (SFV), in which the fusion peptide is contained within E1 [3, 4, 11].

The notion that HCV E1 contains a fusion peptide is supported by a number of reports. First, it contains an internal hydrophobic domain (aa262–290; Fig. S1A) that is conserved among HCV sequences, and alignment of this region with putative fusion peptides from flavivirus E proteins revealed a conservation of Cys and Gly residues [12]. Expression of aa259–298 of E1-induced membrane-permeabilization in an *Escherichia coli* system used to analyse membrane-active proteins. This ability to disturb the architecture of the lipid bilayer is intrinsic to viral fusion peptides [13]. More recently, studies involving induction of membrane leakage, hemifusion, and fusion by 18-mer peptide libraries derived from E1 and E2 have supported the theory that the HCV fusion peptide is located between aa265–296 of E1 [14]. And finally, liposome/HCVpp fusion studies identified three putative fusogenic regions; two in E2 and a third, encompassing the internal hydrophobic domain in E1 [15]. In the present study, we determined the effect of mutations in the putative fusion peptide on entry in the HCVpp system and production of cell-culture-derived infectious virus (HCVcc).

Materials and Methods

Cells

Transfections with DNA constructs to generate HCVpp stocks were performed in HEK-293T cells (ATCC), a human embryonic kidney cell line. Infections were performed in Huh-7 cells, a human hepatoma cell line [16], or in Huh-7 S24 cells, a subclone of Huh-7 cells (generated in-house by limiting dilution cloning) that were twofold more susceptible than Huh-7 cells to HCVpp infection. All cells were grown as monolayers in complete Dulbecco's modified Eagle's medium with L-glutamine (Invitrogen, Carlsbad, CA, USA) supplemented with penicillin/streptomycin (Sigma, St. Louis, MO, USA) and 9% foetal bovine serum (BioWhittaker, Walkersville, MD, USA) at 37 °C in the presence of 5% CO₂.

Plasmids and mutagenesis

The cytomegalovirus (CMV)-Gag-Pol murine leukaemia virus (MLV) packaging construct, the MLV-green fluorescent protein (GFP) plasmid, and the phCMV-7a expression vector encoding the C-terminal 60 amino acids of core and all of E1 and E2 (gifts from F.L. Cosset) were used to generate HCVpp. The HJ3/QL H77/JFH1 chimeric infectious clone [17,18] (gift from S. Lemon) was used to generate HCVcc. E1 mutations were introduced into the phCMV-7a and HJ3/QL plasmids by PCR-based mutagenesis and cloning strategies are

available on request. All HCV wild-type and mutant sequences were confirmed by double-stranded DNA sequencing.

Pseudotyped virus infectivity assays

HCVpp were produced by HEK-293T cells transfected with plasmids and Lipofectamine Plus reagents (Invitrogen) and infectivity assays were performed as described in the Supplementary Materials and Methods. As the absolute quantity of pseudoparticles can differ from transfection to transfection [19–21], each experiment was conducted with HCVpp produced concurrently.

Analysis of protein expression

Western blotting was performed on transfected cell lysates and pelleted HCVpp as described in the Supplementary Materials and Methods using monoclonal antibody A4 (gift from H. Greenberg) to detect E1 glycoprotein, hyperimmune rabbit serum LMF87 [22] to detect E2, and rabbit antiserum against MLV CAp30 (gift from A. Rein) to detect MLV Gag protein.

Endoglycosidase digestions

Pelleted HCVpp preparations were digested with endo- β -N-acetylglucosaminidase H (endo H; New England Biolabs, Ipswich, MA, USA) or Peptide: N-glycosidase F (NEB) as described in the Supplementary Materials and Methods and analysed by Western blotting.

CD81-binding assays

Selected HCVpp mutants were tested for binding to the large extracellular loop (aa116–202) of human CD81 [glutathione S-transferase (GST)-human CD81-LEL] or a CD81 double mutant F186L+E188K [23] (gifts from S. Levy). See Supporting Information for details.

HCVcc preparation and virus titration

Huh-7.5 cells (gift from C. Rice) were transfected with *in vitro*-transcribed ribonucleic acid (RNA) using DMRIE-C transfection reagent (Invitrogen) as recommended by the manufacturer. Virus stocks were harvested at 4 days post-transfection, passed through a 0.45- μ m filter, and virus titers were determined by endpoint dilution assays of focus-forming units as previously described [24,25]. See Supporting Information for details.

Virus sequencing

Viral RNA was extracted from 100 μ L of filtered transfection or infection culture media with TRIzol (Invitrogen) and long RT-PCR was performed as previously described [25–29] using a combination of H77- and JFH1-derived sequencing primers.

Results

Mutations within the internal hydrophobic domain of E1 affect efficiency of HCVpp entry

Glycine residues are important for the structure and function of fusion peptides of paramyxoviruses and retroviruses [30,31]; the putative HCV fusion peptide (aa262–290; Fig. S1A) contains two Gly residues with similar spacing as that of fusion peptides from paramyxoviruses, as well as a number of Cys residues that are conserved among putative fusion peptides from other flaviviruses [12]. A sequence alignment of 389 HCV E1 amino acid sequences contained in the Los Alamos HCV Sequence Database revealed that G278, C281, and G282 were the three most conserved Gly and Cys residues in this region (Fig. S1B). Substitution of Pro at each of these three positions practically abolished HCVpp infectivity of Huh-7 cells (entry <1% of wild-type). Replacement of the same three conserved residues with Ala also strongly reduced HCVpp entry to at the most 18% of wild-type (G282A) (Fig. 1a).

These results suggest that conserved Gly and Cys residues within the HCV E1 internal hydrophobic domain are important for E1 function.

Mutation of a single Leu residue in the tick-borne encephalitis (TBE) envelope protein abolishes fusion activity [32]. The HCV E2 glycoprotein contains a membrane-proximal Leu-rich heptad repeat (HR) sequence that is essential for E1E2 heterodimerization and viral entry [10]. The putative fusion peptide in E1 contains a discontinuous HR spanning aa262–289 (indicated by a/d in Fig. S1A) with a number of highly-conserved Leu residues (Fig. S1B). The high degree of hydrophobic amino acid conservation at these positions suggests that these residues might be involved in critical protein-protein or protein-lipid (i.e. membrane fusion) interactions during HCV entry. Substitution by Pro at L271, L280, or L286 almost completely abrogated infectivity (Fig. 1b). Substitution by Ala at L271, L280, or L286 reduced entry efficiency to 54%, 20%, and 36%, respectively (Fig. 1b). These results suggest that conserved hydrophobic amino acids within the internal hydrophobic domain are important for E1 function.

A number of genotype- and subtype-specific variations were noted within the E1 putative fusion peptide. For example, residue 271 is Leu in most genotype 1a sequences, whereas it is a Phe in all genotype 1b sequences. Similarly, at residue 280, whereas 81% of all HCV sequences listed in the database contained a Leu, most genotype 2b sequences had a Val. Substitution of the Phe from genotype 1b, or the Val from 2b, into the 1a sequence to create L271F and L280V, reduced HCVpp entry efficiencies to approximately 58% and 54%, respectively, as compared with wild-type (Fig. 1b). Therefore, subtype- and genotype-specific interactions may exist within the internal hydrophobic domain of E1.

Charged amino acids, such as Arg, located close to hydrophobic stretches affect membrane permeability and are proposed to affect pore formation and/or proper membrane-protein interaction [13]. The HCV putative fusion peptide is flanked by two short stretches of charged amino acids (259-RRH-261 and 296-RRH-298; Fig. S1A) that are fairly well-conserved. The most conserved Arg at either end of the putative fusion peptide (R259 and R296; Fig. S1B) was mutated to Ala, or the polarity at these positions was reversed by substituting the negatively charged Glu for Arg. Three of these mutants (R259A, R259E, and R296E) displayed infectivities <5% of wild-type. Only the R296A mutant showed significant efficiency of entry at 15% of wild-type (Fig. 1c). These reductions in HCVpp infectivity suggest that the positively charged amino acids flanking the internal hydrophobic domain are important for E1 function.

As Ser residues in E2 affect HCVpp entry [10], and because S268 and S283 are two of the lesser conserved amino acids in the internal hydrophobic region (Fig. S1B), the effects of mutating them were tested. As a high proportion of HCV sequences have Ala at both these positions, only Pro substitutions were tested. The S268P mutation completely abrogated HCVpp entry, while the S283P mutation actually increased entry two to three times over that of HCVpp containing wild-type E1 (Fig. 1d). Therefore, even less well-conserved Ser residues within the internal hydrophobic domain contribute to the function of E1.

Effects of E1 mutations on cellular and HCVpp-associated E1 and E2

These observed differences in HCVpp infectivity were not caused by variations in transfection efficiency or in the amount of pseudotyped virus particles produced as Western blotting analysis showed that transfected cell lysates and pelleted HCVpp contained similar levels of MLV Gag protein for all mutants tested (Fig. 2). Therefore, the levels of E1 and E2 in transfected cells and pelleted HCVpp were examined for altered glycoprotein expression (Fig. 2). For all mutants tested, no differences in the cellular levels of E2 were observed; thus, the differences among entry efficiencies were not caused by variations in E2 expression. However, when cellular E1 and HCVpp-associated E1 and E2 were examined, a number of patterns

became obvious. The majority of mutant E1 glycoproteins were expressed at levels similar to that of wild-type, but the levels of C281P, L271P, L286P, and R259E were lower (Fig. 2). Therefore, these mutations most likely affected the stability of the protein. These four mutants also contained little or no HCVpp-associated E1, consistent with defects in folding and/or stability of E1.

A second group of mutants, including G282P, L280A, L280P, and R296E, showed normal levels of cellular E1 expression, but low levels of E1 on pseudoparticles and reduced HCVpp entry efficiency. However, a higher level of E1 on the pseudoparticles does not guarantee infectivity, as other mutants, with normal levels of E1, also displayed reduced infectivity. As E1E2 heterodimers are required for infectivity [6–8], it was possible that incorporation of E2 glycoprotein was somehow affected by the mutations in E1. Western blotting analysis showed that E2 glycoprotein in the context of wild-type E1 migrated as a diffuse band of higher molecular weight than that of E2 found in the cell (Fig. 2). This is presumably attributable to different levels of glycosylation [33]. In contrast, HCVpp containing low levels of E1 displayed a narrower E2 band that migrated in the higher molecular weight range of E2 synthesized in the context of wild-type E1. These different migration patterns hereafter will be referred to as the high vs normal molecular weight forms of E2. When E2 was analysed, all mutants with low levels of HCVpp-associated E1 (C281P, L271P, L286P, R259E, G282P, L280A, L280P, and R296E), displayed low levels of HCVpp-associated E2 that was either high, or a mixture of high and normal, molecular weight E2. Despite normal E1 expression for the G282P, L280A, L280P and R296E mutants, HCVpp generated in the presence of these mutations incorporated lower levels of E1 and E2, suggesting that these mutations may have affected E1E2 assembly. In the absence of this interaction, a high molecular weight, presumably hyperglycosylated, form of E2 is incorporated onto the HCVpp.

The third group of E1 mutants, including G278A, G278P, C281A, L271A, L280V, R296A, and S268P, collectively displayed entry efficiencies ranging from 0.1–54.8%, showed normal cellular expression of E1 with slightly reduced but significant levels of HCVpp-associated E1, and reduced but significant levels of E2 that migrated either at the same molecular weight as E2 in the context of wild-type E1 or as a mixture of high and normal molecular weight E2 (Fig. 2). As these mutants displayed significant levels of HCVpp-associated E1 and E2, these mutations probably did not affect E1E2 heterodimerization, and the reduced entry efficiencies (Fig. 1) must have been caused by another aspect of E1E2 function.

The last group of mutants, composed of G282A, L271F, L286A, and R259A all showed reduced infectivities ranging from 0.4% to 58.0% (Fig. 1), but displayed normal levels of cellular and HCVpp-associated E1 and E2, with E2 migrating at the same molecular weight as that of wild-type (Fig. 2). For all intents and purposes, HCVpp containing these mutations resemble wild-type HCVpp, which suggests that the defects in entry efficiency observed in the context of these mutations must have been caused by a mechanism that was independent of glycoprotein assembly and incorporation into pseudoparticles.

The S283P mutant does not fit into any of the four groups described above because unlike all other mutants, this amino acid change actually increased HCVpp entry efficiency (Fig. 1). One possible explanation is that the S283P mutant incorporated E1E2 heterodimers more efficiently. However, a Western blot of S283P HCVpp revealed that mutant and wild-type pseudoparticles had similar amounts of E1 (Fig. 2). One noticeable difference, however, was that the band representing S283P-mutated E1 glycoprotein migrated slightly faster than that of wild-type and of the S268P-mutated E1. The reason for this is not clear, but it might be attributable to an altered glycosylation pattern. When the E2 glycoprotein found on HCVpp containing S283P-mutated E1 was analysed, the level of E2 appeared to be slightly lower than that of pseudoparticles generated with wild-type glycoproteins. Interestingly, the E2 band was

also less diffuse than that of wild-type. Therefore, the increased HCVpp efficiency of entry caused by the S283P mutation in E1 is apparently associated with distinct glycosylation patterns of both E1 and E2.

Mutations in E1 cause altered glycosylation patterns in E2

Endoglycosidase digestions of selected HCVpp mutants were performed in order to determine whether the high molecular weight E2 observed throughout this study represented hyperglycosylation. Western blotting analysis revealed that enzymatically treated E2 from mutant and wild-type HCVpp migrated at the same molecular weight (Fig 3). This result clearly demonstrates that the difference in molecular weight of E2 in the context of the various E1 mutations is attributable to differences in glycosylation. Moreover, these results confirm that the amino acid changes introduced into the internal hydrophobic domain in E1 indeed affected glycosylation of E2.

Effects of mutations in E1 on E2-CD81 binding

As HCVpp entry involves a number of steps, including virus attachment, endocytosis, and fusion, it was important to determine which of these was affected by the E1 mutations. The effect of mutations on the binding properties of HCV envelope glycoproteins is difficult to measure in the context of HCVpp because of non-specific interactions between the virus and the cell [34]. Therefore, an approach taken by others [21], a pull-down assay using GST-CD81-LEL [23] was chosen to analyse the mutant HCVpp. In a preliminary screen, mutant HCVpp containing severely reduced levels of E1 showed no E2-CD81 binding (data not shown). Therefore, CD81-binding was tested for only those mutants that incorporated at least 50% as much E1 as did wild-type. E2 glycoprotein from HCVpp made in the context of wild-type E1 bound well to the CD81 (Fig. 4). As controls, binding assays were performed with HCVpp lacking glycoprotein (Fig. 4; no E1E2), and with a GST-CD81-LEL double mutant (F186L + E188K) which does not bind E2 [23]. No wild-type E2 was pulled down by the CD81 double mutant (Fig. 4; Neg Ctrl), thereby demonstrating the specificity of the assay. For the majority of the mutants tested, the levels of E2-CD81 binding paralleled the levels HCVpp infectivity. For example, the G278A, G278P, C281A, and S268P mutants displayed low infectivities as compared with wild-type, and, the low level of E2 glycoprotein found on pseudoparticles containing these mutations bound inefficiently to CD81. This suggests that these pseudoparticles contain very little functional E1E2. Other mutants, such as L271A and L280V, showed intermediate levels of HCVpp infectivity along with reduced, but significant, levels of E2-CD81-binding. Based on these results, the decreased infectivities observed with these mutants might reflect a reduced ability to interact properly with cellular receptors. The S283P mutant displayed an E2 band that was less diffuse than that of even wild-type HCVpp and exhibited wild-type levels of CD81-binding. As this mutant was more infectious than wild-type, the faster migrating form of E2 observed on Western blots may represent the actual form that binds CD81. The G282A, L271F, L286A, and R259A mutants, on the other hand, displayed low infectivities despite normal or only slightly reduced levels of HCVpp-associated E1E2 and wild-type levels of E2-CD81-binding, indicating that these mutations in E1 might affect an entry step downstream of virus attachment, such as membrane fusion.

Mutations in E1 reduce the levels of infectious extracellular HCVcc

To confirm the relevance of these HCVpp mutagenesis studies in an infectious virus system, the H77/JFH1 chimeric virus containing a Q1251L adaptive mutation in NS3 (HJ3/QL) was obtained from S. Lemon [17,18] and further cultured in Huh-7.5 cells. Long-term culture of the HJ3/QL virus produced an additional I348S amino acid change in E1 (Kawaguchi *et al.*; unpublished results). The I348S and a previously reported Y361H mutation [18] were each combined with the Q1251L mutation to generate HJ3/QL/Y361H and HJ3/QL/I348S. When

RNA containing these viral sequences was transfected into Huh-7.5 cells and the infectivity of progeny virus was measured by immunofluorescence-based focus-forming assays [24] (Fig. 5), both mutations slightly increased the levels of extracellular infectious virus produced over that of the parental HJ3/QL (Fig. 5). As the I348S mutation was a novel adaptive mutation, it was included in the parent virus used in subsequent mutagenesis studies.

The G282A mutation in E1 is of specific interest because this mutation has been reported previously to affect membrane fusion in liposome/HCVpp fusion assays [15]. When the G282A mutation was substituted into the HJ3/QL/I348S infectious virus, the level of extracellular infectious virus produced was reduced by greater than 1 log (Fig. 5). The HJ3/QL/I348S/G282P mutant produced no detectable extracellular infectious virus. The L286A mutation, which significantly reduced HCVpp entry efficiency (Fig. 1b), also resulted in approximately 1 log lower levels of extracellular infectious virus (HJ3/QL/I348S/L286A) (Fig. 5). Taken together, these results are consistent with the reduced entry efficiencies observed for the G282A, G282P, and L286A HCVpp mutants (Fig. 1). The S283P mutation, which caused a two- to threefold increase in the efficiency of HCVpp entry (Fig. 1d), however, displayed an approximately 2 log reduction in the levels of extracellular infectious virus when incorporated into the HJ3/QL/I348S infectious virus (Fig. 5).

Discussion

The function of the HCV E1 glycoprotein remains unknown, but its associated with E2 is required for HCV pseudoparticle entry [6–8]. E1 has been proposed to function as a viral fusion protein based on the presence of an internal hydrophobic domain that resembles fusion peptides of other flaviviral E proteins, as well as paramyxovirus F proteins [12]. In this study, a panel of E1 mutants containing single amino acid substitutions at highly conserved residues within the putative HCV fusion peptide (aa262–290) was generated, and tested for the effects of these mutations on HCVpp entry into Huh-7 cells and on infectious virus production in the HCVcc system. This study clearly demonstrated that mutation of conserved residues within this region strongly impacted HCVpp assembly and/or entry, as well as production of extracellular infectious HCVcc.

Our results demonstrated that substitutions for a number of Gly, Cys, Leu, and Ser residues within the HCV putative fusion peptide, as well as of two Arg residues flanking this region, affected HCVpp entry to various extents. At this point, the underlying mechanisms responsible for the observed effects of these mutations are unclear, but some insight can be gained by grouping the mutants based on the observed results (Table 1). For example, group I, including C281P, L271P, L286P, and R259E, showed reduced levels of cellular E1 that resulted in little or no incorporation of E1 into HCVpp. Based on these effects on E1 itself, these mutations probably disrupted the glycosylation and folding of E1, causing premature degradation, and resulting in the production of HCVpp that lacked E1.

Another set of mutations (group II; Table 1), including G282P, L280A, L280P, and R296E, comprise a group that showed normal expression levels of E1 in transfected cells, but reduced levels of E1 (<50% of that of wild-type) on the pseudoparticles and at least 80% decreases in HCVpp efficiency of entry. HCVpp generated in the context of these mutations also contained only, or mostly, the high molecular weight form of E2. As dimerization with E2 is a prerequisite for efficient E1 incorporation into particles [35], and numerous studies have demonstrated that proper heterodimerization of E1 and E2 is absolutely required for HCVpp entry [6–8,10,19, 21,36–38], it seems logical to propose that the E1 mutations in this group may have affected E1E2 heterodimerization and E1 incorporation. However, it is unclear how these defects resulted in E2 hyperglycosylation.

The S268P, L271A, G278A, G278P, L280V, C281A, and R296A mutations (group III; Table 1) showed normal expression levels of E1 in transfected cells and HCVpp E1 levels that were at least 50% of that of pseudoparticles containing wild-type E1E2, with varied effects on E2 incorporation. However, these mutants also showed significant decreases in HCVpp efficiency of entry. The major question regarding these mutants was whether or not they contained functional E1E2 complexes and were capable of binding to the target cells at all. Previous studies have suggested that *in vitro* binding assays using GST-CD81 LEL may not be an accurate mimic of cell surface-expressed CD81, and that CD81 is an attachment receptor with poor capacity to mediate virus entry [39–41]. However, the role of CD81 as an entry receptor for HCV is now widely accepted, and CD81 LEL binding assays are considered to be a relevant way to study E2-CD81 interactions [9,12,23,38,42–48]. Therefore, these mutants were analysed in a GST-CD81 LEL pull-down assay that measured E2-CD81-binding [23]. For the most part, E2-CD81 binding paralleled infectivity in that mutants displaying low infectivities contained E2 glycoprotein that bound poorly to CD81, implying that these mutants were not able to infect Huh-7 cells because they contained very few functional E1E2 complexes and, therefore, did not bind to the target cells. Other mutants, such as L271A, L280V, and R296A showed intermediate levels of HCVpp infectivity along with reduced, but significant, levels of E2-CD81-binding. Infectivity in these may be compromised because of a reduced ability to properly interact with cellular receptors.

The fourth group (IV) of detrimental mutations, including R259A, L271F, L286A, and G282A, all showed close to wild-type levels of HCVpp-associated E1E2 and E2-CD81-binding, which suggests that these pseudoparticles contained functional E1E2 heterodimers. However, all of these mutants displayed significant reductions in HCVpp entry as compared with pseudoparticles generated in the presence of wild-type glycoproteins. Based on this pattern of results, we propose that these four amino acid substitutions affected a step downstream of virus attachment, such as membrane fusion, and we speculate that the Gly residue at position 282, the Leu residues at 271 and 286, and an Arg at amino acid 259 all play a critical role in the function of this internal hydrophobic domain. In a recent article, a Y276F mutation, and the same G282A mutation analysed herein, were both shown to reduce cell-cell fusion and impair lipid and content mixing in liposome-HCVpp fusion assays [15]. Based on these results the authors suggested that the region within E1 containing aa265–296 is a convincing candidate for virus-cell fusion. The Leu residues at positions 271 and 286 do not appear to align with an obvious heptad repeat, so it is unlikely that the mechanism by which they could affect fusion would involve such a structure. Instead, the hydrophobic nature of these amino acids might be important for protein–protein interactions such as those involved in structural rearrangements required for flavivirus fusion [3]. The mechanism by which R259 might affect fusion is also unclear, but charged amino acids flanking the fusion peptide may be important for membrane modification during the fusion event. Similarly, a positively charged amino acid (H230) is critical for membrane fusion of SFV [49].

The last mutation is the S283P substitution, which unexpectedly displayed higher HCVpp entry levels than pseudoparticles containing wild-type glycoproteins. The mechanism responsible for this phenomenon is unclear, but the observation of a slightly faster migrating E1 glycoprotein, and a less diffuse band for E2, suggests that their extent of glycosylation may be the key. In this regard, Western blotting analysis of E2 revealed the presence of a high molecular weight, overglycosylated form of E2 on other pseudoparticles (Fig. 2), which was associated with lower levels of HCVpp infectivity (Fig. 1). Interestingly, these higher molecular weight bands were not found on Western blots of CD81-bound E2 (Fig. 4). For example, HCVpp containing the G278A and G278P mutations in E1 predominantly incorporated the higher molecular weight form of E2 and showed the lowest levels of CD81-binding, but the lower molecular weight form was selectively bound to CD81. These results suggest that the highly glycosylated forms of E2 are not able to mediate HCVpp entry, and may explain the increased

infectivity observed with the S283P mutation. HCVpp containing this mutation in E1 consistently showed an E2 band that was less diffuse than that of even wild-type HCVpp, and showed wild-type levels of CD81-binding. Therefore, the faster migrating form of E2 apparently represents the true functional form. As this mutant displays higher infectivity but, does not show increased CD81-binding as compared with wild-type, we propose that this mutation induces a hyper-fusogenic E1E2 functional unit as has been proposed for other E2 mutants with infectivities higher than that of wild-type [21]. At this point, it is clear that the diminished infectivity associated with the appearance of a high molecular weight form of E2 is because of the fact that this highly glycosylated form does not bind well to CD81. However, the reason why E1 mutations cause this hyperglycosylation of E2 is currently not understood. It has been known for some time that E1 is more efficiently glycosylated when expressed along with E2 [50], and that E2 plays a role in the folding of E1 [51,52]. In agreement with the work of others [35], this study confirms that E1 can affect the glycosylation of E2. An interesting trend can be observed by examining Western blots representing HCVpp-associated E1 and E2. There appears to be an inverse relationship between the level of E1 found on HCVpp and the presence of the high molecular weight form of E2. As the endoglycosidase analyses of E2 showed that this difference in molecular weight is indeed attributable to the glycosylation state of E2, this trend suggests that E1 is somehow required for proper glycosylation of E2. However, at this point, it is not clear how the mutant forms of E1 are causing this modification of E2.

As HCVpp are chimeric viruses which contain non-HCV components and are released from cells by an unnatural route, it was of interest to test the effects a number of these E1 mutations in the context of a fully infectious HCV in cell culture [24,53–56]. The G282A, G282P, and L286A mutations tested all produced lower levels of extracellular infectious HCVcc than did the parent HJ3/QL/I348S virus (Fig. 5), which was consistent with the decreased infectivity displayed by corresponding mutants in the HCVpp system (Fig 1). However, the increased efficiency of entry induced by the S283P mutation in HCVpp was not observed in the context of HCVcc. One possible explanation for this discrepancy could be that in the HEK-293T cell-generated pseudoparticles, S283P affects the glycosylation of E1 and/or E2 in a manner that is not relevant for the HCVcc, which are generated in the Huh-7.5 hepatocyte cell line, and possibly exit the cell via a different pathway. It is also possible that there are inherent differences in the requirements for HCVpp vs HCVcc production that might explain the differing effects of individual mutations in these systems, such as the association of HCVcc with apolipoprotein C [57,58]. In any event, mutation of any conserved amino acid in this internal hydrophobic region is likely to decrease production of infectious virions, as we and others [38] have shown previously for production of infectious HCVpp. This region has been implicated as one of three affecting fusion of HCVpp with liposomes [15]. The sequence in this region is obviously critical for a function of E1, but whether this function involves fusion remains to be proved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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purification and binding assays. The HJ3/QL infectious clone was a gift from Stan Lemon (University of Texas Medical Branch, USA). Huh-7.5 cells were a gift from Charles Rice (Rockefeller University, USA).

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Abbreviations

HCV	hepatitis C virus
HR	heptad repeat
LEL	large extracellular loop
MLV	murine leukaemia virus
RNA	ribonucleic acid
SFV	semliki forest virus

Fig. 1.

Effects of E1 mutations on HCVpp entry. Plasmids encoding E1 mutated glycoproteins at (a) Gly or Cys, (b) Leu, (c) Arg, or (d) Ser residues, in the context of wild-type E2 were used to generate mutant HCVpp, which were then inoculated onto Huh-7 cells. Infected cells were detected by FACS analysis of GFP expression. HCVpp containing wild-type E1 and E2 typically infected 4–5% of the cultured Huh-7 cells. Infectivity of pseudoparticles generated in the presence of mutated E1 glycoproteins is expressed as a percentage of wild-type, which is arbitrarily set at 100%. Results shown represent three independent transfection/infection experiments and standard errors are indicated on the bars. Supernatants from cells transfected without E1E2-expressing plasmid (no E1E2) and untransfected cells (Untransf.) served as negative controls, which were never above 0.02% GFP-positive.

Fig. 2.

Western blot analyses of (a) Gly or Cys, (b) Leu, (c) Arg, or (d) Ser mutants were performed on transfected HEK-293T cell lysates (Cells) and HCVpp pelleted by ultracentrifugation (HCVpp) under reducing conditions. MLV Gag protein was detected with a rabbit antiserum against MLV CAP30. E1 and E2 glycoproteins were detected with Monoclonal antibody A4 and a hyperimmune rabbit serum, LMF87 respectively [22]. Western blot analyses of 'no E1E2' controls were always non-reactive with α -E1 and α -E2 Ab, and bands were not detected in lanes representing untransfected cells.

Fig. 3.

Analysis of glycans associated with HCV E2. Pelleted mutant and wild-type HCVpp were digested with endo H (H) or PGNase F (F). Undigested controls (U) were treated the same as endo H samples except enzyme was omitted. E2 glycoproteins were then analysed by Western blotting.

Fig. 4.

Effects of E1 mutations of E2-CD81 binding. Pelleted HCVpp were resuspended in 250 μ L of RSB and a 15 μ L aliquot was analysed by Western blotting for E2 (HCVpp-associated; upper panels) as described above. The remainder was subjected to a GST-CD81 pull-down assay and CD81-bound E2 was analysed by Western blotting (CD81-bound; lower panels). Wild-type and mutant HCVpp lysates were adsorbed with GST-human CD81-LEL. As negative controls, binding assays were performed with HCVpp lacking glycoprotein (no E1E2), as well as wild-type HCVpp in the presence of a GST-CD81-LEL double mutant (F186L+E188K) which does not bind E2 (Neg Ctrl) (23). Results shown are representative from two independent experiments. Mean HCVpp infectivity data for each mutant was transposed from Fig. 1 for comparison.

Fig. 5.

Effects of E1 mutations on production of extracellular infectious HCVcc. One million Huh-7.5 cells were transfected with in vitro-transcribed RNA representing HJ3/QL or each of the indicated HCVcc mutants. Extracellular infectious virus accumulated by day 4 was measured by focus-forming assays [24,25] in triplicate and means plus standard errors are plotted. 'Negative' represents titration of culture fluids from untransfected Huh-7.5 cells. The dotted line represents the cutoff of the assay, which was 10 ffu/mL. These data were consistent for two independent experiments and the results shown were generated from one transfection experiment performed in triplicate.

Table 1

Grouping of mutants based on their effects on E1 and E2

Group	Mutants	Infectivity (%) [*]	Cellular E1 levels	HCVpp E1 levels	HCVpp E2 levels	E2 band migration	CD81-binding [†]	Putative Mechanism
I	C281P	0.00	Reduced	None	Reduced	High MW	ND	E1 folding/stability
	L271P	0.00	Reduced	None	Reduced	High MW	ND	
	L286P	0.00	Reduced	None	Low	High MW	ND	
	R259E	4.80	Reduced	<50% of WT	Low	Diffuse	ND	
II	G282P	0.00	Normal	Low	Reduced	High MW	ND	E1E2 heterodimerization
	L280A	20.60	Normal	Low	Low	High MW	ND	
	L280P	6.40	Normal	None	Low	High MW	ND	
	R296E	2.90	Normal	<50% of WT	Reduced	Diffuse	ND	
III	G278A	1.70	Normal	Reduced	Reduced	Diffuse	Low	CD81-binding
	G278P	0.30	Normal	Reduced	Reduced	Diffuse	Low	
	C281A	4.10	Normal	Reduced	Reduced	Diffuse	Reduced	
	L271A	54.00	Normal	Reduced	Minor reduction	Normal	Low	
	L280V	54.80	Normal	Reduced	Reduced	Diffuse	Reduced	
	R296A	15.10	Normal	Normal	Low	Normal	Low	
IV	S268P	0.10	Normal	Reduced	Low	Diffuse	Reduced	Membrane fusion
	G282A	18.40	Normal	Normal	Normal	Normal	Normal	
	L271F	58.00	Normal	Minor reduction	Normal	Normal	Minor reduction	
	L286A	36.40	Normal	Normal	Normal	Normal	Normal	
V	R259A	0.40	Normal	Normal	Minor reduction	Normal	Normal	Membrane hyperfusion
	S283P	238.40	Normal	Normal	Normal	Normal	Normal	

^{*} Infectivity as a percentage of WT.[†] ND represents not determined.