The protonation of histidine in acidic environments underpins its role in regulating the function of pH-sensitive proteins. For pH-sensitive viral fusion proteins, histidine protonation in the endosome leads to the activation of their membrane fusion function. The HCV (hepatitis C virus) glycoprotein E1–E2 heterodimer mediates membrane fusion within the endosome, but the roles of conserved histidine residues in the formation of a functional heterodimer and in sensing pH changes is unknown. We examined the functional roles of conserved histidine residues located within E1 and E2. The E1 mutations, H222A/R, H298R and H352A, disrupted E1–E2 heterodimerization and reduced virus entry. A total of five out of six histidine residues located within the E2 RBD (receptor-binding domain) were important for the E2 fold, and their substitution with arginine or alanine caused aberrant heterodimerization and/or CD81 binding. Distinct roles in E1–E2 heterodimerization and in virus entry were identified for His<sup>691</sup> and His<sup>693</sup> respectively within the membrane-proximal stem region. Viral entry and cell–cell fusion at neutral and low pH values were enhanced with H445R, indicating that the protonation state of His<sup>445</sup> is a key regulator of HCV fusion. However, H445R did not overcome the block to virus entry induced by bafilomycin A1, indicating a requirement for an endosomal activation trigger in addition to acidic pH.

Key words: CD81, glycoprotein, hepatitis C virus (HCV), heterodimerization, protonation, virus entry.

INTRODUCTION

Membrane fusion is an obligate step leading to cellular entry by enveloped viruses and is mediated by a viral fusion protein. Viral fusion proteins have been divided into three main classes (I, II and III) on the basis of their structural features. Prior to membrane fusion, viral fusion proteins adopt a prefusion metastable structure on the surface of the virion such that the hydrophobic fusion peptide is sequestered from the aqueous solvent. Maintenance of the prefusion metastable structure is mediated through labile inter- and/or intra-molecular contacts that are broken in response to a specific biochemical activation trigger. Activation can occur at neutral pH at the plasma membrane or within the acidic environment of an endosomal compartment. For many viruses, including those containing class I and class II fusion proteins (e.g. influenza virus and the flaviviruses respectively), endosomal pH activates membrane fusion.

The HCV (hepatitis C virus) glycoproteins E1 (residues 171–383) and E2 (residues 384–746) are type I membrane proteins that form covalent and non-covalent heterodimers and mediate low-pH-dependent entry [1–3]. The lack of high-resolution structural information for E1 and E2 has hampered progress in understanding how these viral glycoproteins mediate membrane fusion. Glycoprotein E1 has been proposed to contain multiple hydrophobic regions important for viral fusion and a C-terminal membrane proximal heptad repeat essential for viral entry [4–7]. Glycoprotein E2 binds the cellular receptor CD81, leading to receptor-mediated endocytosis of virions [2,3,8]. Mutational studies suggest that glycoprotein E2 shares features in common with other class II viral fusion proteins. A conserved membrane-proximal hydrophobic heptad repeat in E2 is essential for heterodimerization with E1 and virus infectivity, indicating that this sequence is a functional homologue of the flaviviral glycoprotein E stem [9]. The disulfide bonding arrangement of monomeric soluble E2 (residues 384–716) has been determined, and, together with information obtained from functional and immunochemical studies, was used to thread the amino acid sequence of E2 on to a class II fusion protein structure [10]. In this model, E2 has a typical three-domain organization (domains I, II and III) and a putative fusion loop was predicted within domain II [10].

As the pK<sub>a</sub> of histidine approximates the pH of the endosome, it has been proposed that protonation of histidine residues at or below its pK<sub>a</sub> (pH 6.8) triggers the destabilization of the prefusion conformation of low-pH-dependent fusion proteins [11]. At neutral pH, histidine residues are usually uncharged, but may participate in hydrogen bonds. As the pH within the endosome decreases, histidine residues become protonated, perturbing hydrogen bonds while simultaneously facilitating the formation of new salt bridges with negatively charged amino acids. The net effect is disruption of the metastable prefusion conformation and the transition into a low-energy fusogenic structure that may be stabilized through the formation of new salt bridges involving protonated histidine(s). Mutational and structural studies of the fusion glycoproteins of influenza virus [12], the flavivirus TBEV (tick-borne encephalitis virus) [13], and the alphaviruses semliki forest virus [14] and chikungunya
virus [15] reveal a critical role for histidine residues in the low pH fusion activation mechanism. In contrast, studies on West Nile virus indicate that none of the individual histidine residues were important for maintenance of the prefusion structure or in virus entry and replication [16]. Structural information is not available to guide an examination of which HCV E1 and E2 histidine residues are important for maintenance of the prefusion structure and low-pH-dependent entry.

To address the question of whether histidine residues of HCV E1 and E2 contribute to the stability and function of the heterodimer, each histidine residue was mutated to either arginine, in order to artificially elevate the theoretical pK\textsubscript{a} of the amino acid side chain such that it is protonated at neutral pH while retaining side chain bulk, or to alanine. The results of the present study indicate that multiple histidine residues within E1 and E2 contribute to the formation of functional heterodimers and that the protonation state of amino acid 445 of E2 is a key regulator of the low-pH-dependent fusion mechanism employed by HCV.

**MATERIALS AND METHODS**

**Cell lines and antibodies**

HEK (human embryonic kidney)-293T, Huh-7 and Huh-7-Tat cells were maintained in DMF10 (Dulbecco’s minimal essential medium containing 10% fetal calf serum and 2 mM L-glutamine). Huh 7.5 cells were maintained in DMF10NEA (DMF10 supplemented with 0.1 mM non-essential amino acids). mAbs (monoclonal antibodies) A4, H53 and H52 [17,18] were produced in HEK-293T cells. At 72 h post-transfection, culture supernatants were filtered through a 0.45 μm pore filter size) and applied to Huh-7 cell monolayers. At 24 h post-infection, the medium was removed and replaced with DMF10. At 3 days later, virus in the tissue culture supernatant was filtered through a 0.45 μm syringe filter and luminescence was quantified using a FLUOstar Optima plate reader. Virus stocks were stored at −80 °C until further use.

**Vectors**

Construction of the pCDNA4HisMax (Invitrogen)-based expression vector pE1E2H77c has been described previously [19]. The HIV-1 luciferase reporter vector pNL4.3.LUC-R- E− was obtained from Dr N. Landau through the NIH AIDS Research and Reference Reagent Program [20]. The vector pHC-FLAG2(p7-NS-GLUC2A) was a gift from Professor Charles Rice (Rockefeller Institute, New York, U.S.A.). In vitro mutagenesis of the pE1E2H77c vector was carried out by standard overlap extension PCR techniques. Site-directed mutagenesis of pHC-FLAG2(p7-NS-GLUC2A) was performed using the QuikChange™ II Site-Directed Mutagenesis Kit (Stratagene). The sequence of the entire E1/E2 region was confirmed using Big Dye terminator chemistry.

**RIP (radioimmunoprecipitation) and Western blotting**

RIPs of metabolically labelled HCVpp (HCV pseudoparticles) were performed as described previously [19]. Briefly, HCVpp produced in HEK-293T cells were metabolically labelled with 75 μCi of tran–[35S]cysteine/methionine label in methionine- and cysteine-deficient DMF10 for 16 h. The tissue culture fluid was clarified by centrifugation at 14 000 g for 10 min, and radiolabelled viruses were pelleted by centrifugation at 14 000 g for 2 h at 4 °C. HCVpp were lysed in RIP lysis buffer (0.6 M KCl, 0.05 M Tris/HCl, pH 7.4, 1 mM EDTA, 0.02% sodium azide and 1% Triton X-100) and immunoprecipitated with mAb H53 or IgG14 and Protein G–Sepharose, prior to SDS/PAGE and phosphorimaging analysis. Western blotting of transfected cell lysates was performed as described previously [19].

**E1/E2-pseudotyped HIV-1 particle entry assay**

Pseudotyped particle entry assays were performed as described previously [19]. Briefly, HCVpp were produced in HEK-293T cells. At 72 h post-transfection, culture supernatants were filtered (0.45 μm pore filter size) and applied to Huh-7 cell monolayers. Luciferase activity was measured 72 h later with a FLUOstar plate reader (BMG Lab Technologies) fitted with luminescence optics using the Promega luciferase reagent system. Virus entry assays in the presence of bafilomycin A1 (Sigma) were performed using Huh-7.5 cells seeded at 50 000 cells/well in 48-well tissue culture plates (Nunc). At 24 h later, cells were pretreated with 12 nM bafilomycin A1 for 30 min at 37 °C prior to the addition of HCVpp. After 4 h, infected cells were washed with PBS and cultured in DMF10 containing 6 nM bafilomycin A1. Luciferase was measured 24 h later as described above.

**Solid phase E1/E2–CD81 binding assay**

The expression and purification of a chimaera composed of maltose-binding protein linked to CD81 large extracellular loop residues 113–201 (MBP–LEL113–201) has been described previously [21]. Radiolabelled HCVpp were produced as described above and lysed in RIP lysis buffer. A fraction of each lysate was subjected to H53 immunoprecipitation, non-reducing SDS/PAGE and phosphorimaging, and the amount of E2 present was determined by densitometry. The ability of the E2 present in HCVpp, normalized for E2 content, to bind MBP–LEL113–201 was determined as described previously [22].

**RNA transcription and transfection**

Wild-type and mutated HCV RNA was transcribed in vitro from XbaI-linearized pJC1FLAG2(p7-NS-GLUC2A) DNA using an Ampliscribe™ T7 high-yield transcription kit (Epigentre Biotechnologies). Huh-7.5 cells were seeded at 400 000 cells/well in six-well plates (Nunc) and transfected 24 h later with 6 μg of RNA using 10 μl of DMRIE (Invitrogen) and 1 μl of Opti-MEM® (Invitrogen). At 4 h post-transfection, the medium was removed and replaced with DMF10NEA. At 3 days later, virus in the tissue culture supernatant was filtered through a 0.45 μm syringe filter and luminescence was quantified using a FLUOstar Optima plate reader. Virus stocks were stored at −80 °C until further use.

**HCVcc (cell culture–derived HCV) infection**

Huh 7.5 cells were seeded on the day prior to infection at 30 000 cells/well in 48-well plates. Virus stocks of wild-type pJC1FLAG2(p7-NS-GLUC2A) and the pJC1FLAG2(p7-NS-GLUC2A)-based mutants, H445E and H445R, were adjusted to 2500 TCID\textsubscript{50} (50% tissue-culture infectious dose)/ml and incubated with Huh 7.5 cell monolayers for 4 h at 37 °C. The cells were then washed once with PBS and 500 μl of DMF10NEA was added. At 24, 48 and 72 h post-infection, luciferase activity associated with virus released in the tissue culture supernatant and infected cells was assayed using the Renilla luciferase kit (Promega) and read using a FLUOstar Optima plate reader.

For HCVcc infectivity experiments with bafilomycin A1-treated Huh 7.5 cells, cells were seeded at 80 000 cells/well in 24-well tissue-culture plates (Nunc) on the day prior to pretreatment with 25 nM bafilomycin A1 (1 h at 37 °C). Normalized amounts of wild-type or mutant HCVcc (2500 TCID\textsubscript{50}/ml) were then incubated with the treated cells for 2 h at 4 °C. The cells were then washed with citric acid buffer (15 mM citric acid and 150 mM NaCl) at either pH 7 or pH 5 and then incubated in DMF10NEA containing 12.5 nM bafilomycin A1 at 37 °C for
24 h. Luciferase activity in the tissue culture medium and cell lysates was determined using the Renilla luciferase kit using a FLUOstar Optima plate reader.

**Immunofluorescence assay**

Huh-7.5 cells were seeded on coverslips (12 mm diameter) in 24-well tissue-culture plates at 80 000 cells/well 24 h prior to infection. The medium was replaced with DMEM containing 10% FCS. After 48 h the cells were infected with a vaccinia virus expressing HCV p7. After 24 h, the cells were fixed with 100% cold methanol for 30 min at −20°C, incubated in blocking buffer (1% BSA and 0.2% non-fat dried milk powder in PBS), and incubated with anti-FLAG M2 antibody diluted in PBS. Primary antibody binding was detected with Alexa Fluor488 goat anti-mouse IgG (Molecular Probes). Nuclei were counterstained with propidium iodide (Sigma). Mounted samples were analysed by confocal microscopy (Bio-Rad MRC1024). The numbers of foci and nuclei per focus were counted manually from an entire coverslip.

**Cell–cell fusion assay**

HEK-293T ‘donor’ cells (2.5 × 10^6 cells/well seeded in six-well tissue culture dishes 24 h before transfection) were co-transfected using calcium phosphate reagent with 2 μg of pE1E2H77c and 20 ng of an HIV-1 LTR reporter plasmid. A negative control, cells were co-transfected with 2 μg of a plasmid encoding a defective form of E1/E2 and 20 ng of the HIV-1 LTR-luciferase reporter plasmid. The virus was removed 24 h later, the cells were washed with PBS and then incubated in DMEM for 3 days at 37°C. The cells were then fixed with 100% cold methanol for 30 min at −20°C, incubated in blocking buffer (1% BSA and 0.2% non-fat dried skimmed milk powder in PBS), and incubated with anti-FLAG M2 antibody diluted in PBS. Primary antibody binding was detected with Alexa Fluor488 goat anti-mouse IgG (Molecular Probes). Nuclei were counterstained with propidium iodide (Sigma). Mounted samples were analysed by confocal microscopy (Bio-Rad MRC1024). The numbers of foci and nuclei per focus were counted manually from an entire coverslip.

**RESULTS**

The alignment of E1 and E2 sequences representative of each of the six major genotypes (Los Alamos National Laboratory database) revealed the presence of five highly conserved histidine residues in E1 and eight conserved histidine residues in E2 are shown with amino acid numbering (vertical grey lines). Variable regions (black), CD81-binding segments (light grey), the stem regions (light grey cylinder) and the transmembrane domain (hatched) are indicated. The RBD spanning residues 384–661 is indicated (arrowed line).

**Mutagenesis of conserved histidine residues in glycoprotein E1**

We first investigated the effects of mutating the five conserved histidine residues in E1. Alanine or arginine mutations at positions 222, 298, and 352 all resulted in significant decreases in HCVpp entry (P < 0.05), whereas mutations at 312 and 316 to alanine or arginine retained wild-type entry levels (P > 0.05) (Figure 2A and Table 1). Although the mean entry abilities of H312R and H316R were ~50% with respect to wild-type, these differences were not significant (12 independent assays).

We next assessed whether the histidine to alanine or arginine mutations in E1 affected glycoprotein incorporation in metabolically labelled HCVpp. The ability of HCV glycoprotein mutants to form non-covalently associated E1–E2 heterodimers was determined by immunoprecipitation with the E2 conformation-dependent mAb H53 and native PAGE under non-reducing conditions. Viral incorporation of E1/E2 heterodimers was observed in all cases; however, reductions in E2-associated E1 were observed for H222A, H222R, H298R and H352A (Figure 2B and Table 1). These reductions were consistent with reduced levels of steady-state expression of E1 within transfected cells as determined by Western blot analysis (Figure 2C and Table 1) and significantly reduced viral entry (Figure 2A). Despite these variations, E2 incorporation into HCVpp occurred at wild-type levels and retained wild-type levels of CD81 binding for all mutants (Figure 2B and Table 1). Mutants H298A and H352R were entry-defective, despite the presence of near wild-type levels of E1–E2 heterodimers within pseudovirions and retention of CD81-binding function. The defects in virus entry observed for these latter mutants may therefore occur at a CD81-independent stage of virus entry. The functional effects of the mutations were not dependent on the pKa or shape of the substituting side-chain, suggesting that the E1 histidine residues are essential for the overall structure and function of the E1–E2 heterodimer.

**Mutagenesis of conserved histidine residues in glycoprotein E2**

Within E2, six histidine residues are located in the RBD (receptor-binding domain; residues 384–661) with His621 located directly adjacent to Trp620, a known component of the CD81-binding cluster [28], His659 and His660 are located C-terminal to the RBD within the membrane proximal stem-like region of E2 (residues 675–699), which plays a role in E1–E2 heterodimerization and virus entry [9] (Figure 1).

Mutation of any of the eight conserved histidine residues in E2 resulted in a significant reduction in virus entry (P < 0.05),
with the exception of H445A/R and H589R mutants. The H445A and H589R mutations reduced HCVpp entry by 50%, but this reduction was not significantly different to wild-type (P > 0.05). In contrast, H445R significantly enhanced entry almost 4-fold (P < 0.05) (Figures 3A and 3B and Table 2).

We next investigated the E1/E2 expression, heterodimerization and CD81-binding competence of the E2 mutants. The incorporation of [35S]methionine/cysteine-labelled non-covalently associated E1/E2 glycoproteins into HCVpp was examined by mAb H53-immunoprecipitation and non-reducing SDS/PAGE (Figures 3C and 3D and Table 2). With the exception of H617A/R, similar amounts of E2 were detected in HCVpp for wild-type and alanine mutants, whereas E1 incorporation was decreased for H589A/R, H638A/R and H691A/R, and was not detected for H488A/R and H617A/R. The defective incorporation of H617A/R-mutated E1/E2 into pseudovirions was also verified using polyclonal HCV-positive patient serum (results not shown). These results indicate that His421 is critical for the assembly of the E1–E2 heterodimer and incorporation into virions. In the case of His617, differences in E2 glycosylation are apparent, with a faster-migrating E2 species incorporated into HCVpp. In all cases, the overall expression of E1 and E2 in transfected cell lysates was similar to wild-type as detected by Western blotting using the non-conformation-dependent mAbs A4 (anti-E1) and H52 (anti-E2) (Figures 3E and 3F).

Figure 2. Biosynthesis, heterodimerization and virus entry function of histidine-to-alanine and histidine-to-arginine E1 mutants

(A) Single cycle entry of wild-type (WT) HCVpp and HCVpp containing histidine-to-alanine or histidine-to-arginine mutations in glycoprotein E1. Background entry with retroviral particles lacking envelope (empty) is shown. The results are the mean ± S.E.M. of 12 assays performed in quadruplicate. *P < 0.05, mutants with significantly different functional activity relative to wild-type (Student’s t test), RLU, relative light unit. (B) HCVpp were metabolically labelled with [35S]methionine/cysteine and lysates were immunoprecipitated with the conformation-sensitive E2-specific mAb H53. Proteins were separated by non-reducing SDS/PAGE on 10–15% gradient gels and phosphorimaged. The positions of molecular mass markers are shown on the left-hand side in kDa. (C) Transfected HeK-293T cell lysates separated by SDS/PAGE (10–15% gels) under reducing conditions were transferred to nitrocellulose, subjected to Western blotting with non-conformation-dependent mAbs A4 (anti-E1) and H52 (anti-E2) and visualized with Alexa Fluor® 680 goat anti-mouse antibody and an Odyssey infrared scanner. The positions of molecular mass markers are shown on the left-hand side in kDa.

Histidine residues at positions 488, 589, 617, 638 and 691 are essential for the biosynthesis of a functional virion-incorporated E1–E2 heterodimer.

The CD81-binding abilities of the HCVpp-incorporated glycoproteins were determined in an ELISA-based assay employing dimeric MBP–LEL113–201 [22]. A number of mutations within the RBD of E2 caused reductions in CD81-binding capacity (Figures 3C and 3D and Table 2). The most severe defect in CD81 binding was observed for H421A/R, which displayed a >80% reduction (P < 0.02, relative to H693R), despite wild-type levels of E1/E2 being incorporated into HCVpp. This suggests that the failure of H421A/R to mediate virus entry is due to its inability to interact with cellular CD81. This is further supported by the finding that replacement of His445 with arginine or alanine, in the context of the soluble RBD, severely reduces CD81 LEL-binding capacity (results not shown). The H488R, H589R and H638A/R mutants displayed both reduced E1 incorporation into HCVpp relative to co-precipitating E2 (Figure 3D, bottom panel) and ~50% decreases in CD81 binding (P < 0.02), consistent with defective folding. In contrast with their arginine-substituted counterparts, CD81 binding was not significantly affected by H488A and H589A, indicating that the CD81-binding defect is specifically due to the introduction of arginine at these positions. Finally, the H619A/R and H693R/R mutants within the E2 stem-like region did not significantly affect E2–CD81 interactions (P > 0.05). Taken together, these results demonstrate that His445 is directly involved in a CD81 interaction consistent with its location adjacent to a previously characterized CD81-binding residue, Trp280 [28], whereas substitution of other histidine residues within the RBD (488, 589, 617 and 638) affects E1/E2 heterodimerization and CD81 binding, indicative of structural changes induced by these mutations.

Histidine residues 45 and 465 regulate virus entry in a pH-dependent manner

The histidine-to-arginine scan revealed that H445R caused an approximately 4-fold enhancement in HCVpp entry. His445 is located downstream of the G436WLAGLFY motif previously shown to participate in CD81 binding [22] and upstream of hypervariable region 2 (Figure 1). Although His445 is completely conserved within genotype 3, the percentage conservation of histidine at this position ranges from 64 to 72% in genotypes 1, 4, 5 and 6, and is only 27% in genotype 2 strains (Figure 4). To further examine the role of His445 in virus entry, we substituted this residue with amino acids whose side chains have a lower pK_a (glutamate, pK_a = 4.1) or a higher pK_a (lysine, pK_a = 10.53) with respect to histidine, or possess a bulkier, partially polar ring structure (tyrosine). The H445E, H445Y, H445K and H445R mutations did not affect the
Table 1 Phenotype of E1 histidine mutants

<table>
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<tr>
<th>Residue</th>
<th>Alanine†</th>
<th>Arginine†</th>
<th>E1–E2 HCVpp‡</th>
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<th>Entry∥</th>
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<td>+</td>
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</tbody>
</table>

*Amount of E1 detected within transfected cell lysates in Western blotting. + +, wild-type-like expression; +, reduced expression.
†Substituting side-chain.
‡++, wild-type-like heterodimerization; +, reduced heterodimerization; −, absent heterodimerization.
§Ability of HCVpp to interact with CD81. ++, 75–100%; +, 25–75%; −, <25 % binding relative to wild-type.
∥+ +, entry similar to wild-type (P > 0.05); −, entry significantly reduced compared with wild-type (P < 0.05).

Figure 3 Biosynthesis, heterodimerization and virus entry function of histidine-to-alanine and histidine-to-arginine E2 mutants

Single-cycle entry of wild-type (WT) HCVpp and HCVpp containing histidine-to-alanine (A) or histidine-to-arginine (B) mutations in glycoprotein E2. Background entry with retroviral particles lacking envelope (empty) is shown. The results are the means ± S.E.M. of up to seven assays performed in quadruplicate. *P < 0.05, significantly different entry activity to wild-type (Student’s t test). HCVpp with histidine-to-alanine (C) or histidine-to-arginine (D) mutations were metabolically labelled with [35S]methionine/cysteine. Lysates were immunoprecipitated with the conformation-sensitive E2-specific mAb H55. Proteins were separated by non-reducing SDS/PAGE on 10–15 % gradient gels and phosphorimaged. The positions of molecular mass markers are shown on the left-hand side in kDa. The HIV-1 capsid protein p24 was immunoprecipitated from lysed HCVpp using IgG14 and is shown below. The ability of E1/E2 derived from HCVpp to bind to CD81 relative to wild-type (below the gels) was determined in a direct-binding immunoassay using recombinant CD81 MBP–LEL113–201. The results shown are the means ± S.E.M. from three independent experiments. Transfected cell lysates of alanine (E) or arginine (F) mutants separated by SDS/PAGE (10–15 % reducing gels) were transferred to nitrocellulose, subjected to Western blotting with the non-conformation-dependent mAbs A4 (anti-E1) and H52 (anti-E2) and visualized with Alexa Flour® 680 goat anti-mouse antibody and an Odyssey infrared scanner. The positions of molecular mass markers are shown on the left-hand side in kDa.

incorporation of non-covalent E1–E2 heterodimers into HCVpp (Figure 5A) nor significantly alter the ability of HCVpp-derived E1/E2 to interact with CD81 (Figure 5B). However, the level of virus entry correlated with the pKa of the substituting side chain, H445E reducing virus entry by ∼70 %, whereas 2.4- and 3.9-fold increases were observed for H445K and H445R respectively (Figure 5C). These results indicate that protonation at residue 445 may destabilize the envelope glycoprotein complex, promoting fusion activation. Tyrosine has both hydrogen-bonding capacity and can form π-electron interactions, but, unlike histidine, cannot be protonated at acidic pH. Interestingly, substitution of His445 with tyrosine led to a 1.7-fold increase in entry. This may suggest that the introduction of the slightly larger aromatic ring and hydroxy group causes an unfavourable geometry that destabilizes the prefusion glycoprotein complex, similar to the effect observed with protonation.
**Table 2** Phenotype of E2 His mutants

<table>
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<td>693</td>
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</table>

* + + + , wild-type-like heterodimerization; + , reduced heterodimerization; − , absent heterodimerization; Defect, defective expression of E1–E2 structure; NT, not tested.
† Ability of HCVpp to interact with CD81. + + + , 75–100%; + + , 25–75%; + , <25% binding relative to wild-type.
‡ Significantly enhanced entry (P < 0.05); + + + , wild-type-like entry (P > 0.05); − − − , significantly reduced entry relative to wild-type HCVpp (P < 0.05).

**His445 regulates infection by genotype 2a HCVcc in a pKₐ-dependent manner**

We next examined whether substitution of His445 with glutamine or arginine altered virus replication in the HCVcc system. In this case, we introduced H445E and H445R into pJC1FLAG2(p7-NS-GLUC2A), which encodes a full-length chimeric HCV genome comprising the J6 (genotype 2a) structural region and the JFH1 (genotype 2a) non-structural region [29]. The infectivity of wild-type and mutated cell-free HCVcc produced by transfection of Huh 7.5 cells with RNA was first confirmed by standard immunofluorescence staining of infected cells with the anti-FLAG mAb, M2, directed to an epitope tag inserted at the N-terminus of HVR1 of E2 (Figure 6A). Strong immunofluorescent staining was observed for wild-type, H445E and H445R, whereas reactivity to the FLAG epitope was not observed for the replication-incompetent NS5B mutant virus, GND (Figure 6A).

To quantify differences in virus replication initiated by cell-free HCVcc with mutations at His445, luciferase activity was measured in both the cell lysates and the tissue culture fluid at various times post-infection. The results show that H445R increased the amount of virus detected in the lysates of infected cells approximately 3-fold at 24, 48 and 72 h post-infection (Figure 6B). The increase in intracellular luciferase activity for H445R was reflected by an increase in luciferase activity in cell supernatants (Figure 6C). By contrast, H445E showed similar kinetics of replication to wild-type HCVcc (Figure 6B). We found that the increase in luciferase activity observed for H445R was reflected in a significant increase in the size of infectious foci as the average number of nuclei per focus was 4.25 ± 3.98 (24 foci) for wild-type and 7.37 ± 4.28 (37 foci) for H445R (P = 0.0004, Mann–Whitney test). These data indicate that the H445R mutation enhances the infectivity of genotype 2a HCV and confirms a conserved role for His445 in HCV entry.

**Cell–cell fusion of H445R is significantly enhanced at pH 7 and 5**

A cell–cell fusion assay was employed to determine the effect of H445R on the fusogenicity of the HCV E1–E2 envelope complex. Effector cells expressing wild-type or mutant HCV E1/E2 glycoproteins and the luciferase gene under the control of the HIV-1 LTR were mixed with target cells expressing HIV-1 Tat. After 24 h co-culture, cell populations were either treated at pH 7 or pH 5 and luciferase activity was measured 24 h later. To rigorously control this experiment, two defective E1/E2 controls were included containing mutations in the putative stem region of E2. The L692P mutation results in the expression of an E2 protein that retains wild-type levels of CD81 binding, but lacks heterodimerization with E1, whereas L682A retains normal biosynthesis, CD81 binding and heterodimerization, but fails to mediate viral entry [9]. The results (Figure 7) show that mutation of His445 to arginine significantly enhances cell–cell fusion at pH 7 and pH 5 relative to the H445E mutant (P < 0.05, Student’s t test). Consistent with previous observations, wild-type E1/E2 glycoproteins mediate cell–cell fusion at pH 7 and show an increase in fusion activity at pH 5 (15,30), but see [31]). In contrast, the heterodimerization-defective mutant L692P and the entry-defective mutant L682A failed to mediate fusion at pH 5 or 7, confirming that fusion is dependent on a functional E1–E2 heterodimer. Furthermore, H445A significantly reduced fusion function at pH 5 relative to H445E (P < 0.05). These results indicate that His445 is a key regulator of HCV glycoprotein fusion function.

**The H445R mutation does not overcome entry blockade due to bafilomycin A-mediated inhibition of endosomal acidification**

To delineate further how the H445K and/or H445R mutations affect E1/E2-mediated entry, we examined the effects of endosomal acidification and maturation by bafilomycin A1 on infectivity. The results show that bafilomycin A1 blocked virus entry for the wild-type and for each of the His445 mutants (Figure 8A). We confirmed that the entry block is likely to involve modulation of endosomal pH by bafilomycin A1, because pseudoparticle entry mediated by low-pH-dependent VSVg (glycoprotein G of vesicular stomatitis virus) was reduced 300-fold, whereas entry mediated by the pH-independent envelope

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**Figure 4** Consensus sequence of the E2 region proximal to one of the proximal discontinuous CD81-binding sites in HCV glycoprotein E2

*Consensus sequences derived using Multalin (49) from confirmed sequences of genotype 1a (51 strains), genotype 1b (220 strains), genotype 2 (47 strains), genotype 3 (six strains), and 11 strains representing genotypes 4, 5 and 6. The histidine residue at position 445 is shown in bold. The proximal CD81-binding site shown in dark grey [22] and the partial HVR2 region is shown in light grey. 1, asparagine; 2, asparagine, aspartate, glutamine or glutamate; upper case, amino acid present in >90 % of sequences; lower case, amino acid present in 50–90 % of sequences; †, no consensus. ‡Percentage of strains with a histidine residue at position 445.
shown are the means 
+− S.E.M. of three independent experiments. The p K a of the amino acid side 
chain at position 445 is shown below the histogram.

MBP–LEL113–201. The results shown are the means 
to wild-type (WT) was determined in a direct binding immunoassay using solid-phase dimeric 
immunoprecipitation with IgG14 is shown below. (Figure 8C, +Baf/pH 7). Although the results 
indicate that His445 is a regulator of HCV entry in two different genotypes, the artificial protonation of the side chain at this 
position at neutral pH in H445R does not overcome the need for 
a functional endocytic pathway and additional activation triggers 
therein.

DISCUSSION

Histidine plays a variety of structural and functional roles in 
protein folding and stability through the ability of its side-chain 
heteroatom to mediate diverse interactions with other amino acids, 
including the formation of salt bridges with acidic amino 
acids [32], co-ordination of metal ions [33] and to function 
as a catalytic base and/or acid in proton-transfer events of 
enzymatic reactions [34]. In addition, histidine can make π- 
contacts with other aromatic residues, as well as acting as an 
H-donor in forming classical hydrogen bonds with tyrosine, 
tryptophan and histidine [35]. In the case of viral fusion proteins, 
histidine can mediate interactions that contribute to the stability 
of the metastable prefusion structure and, in response to low-pH- 
induced protonation, trigger large conformational changes that 
are associated with fusion function.

Role of histidine residues in E1

Of the five conserved histidine residues in E1, only His322, 
His298 and His352 appear to play essential roles in glycoprotein 
structure and viral entry. Although E1 mutations did not affect the 
interaction between CD81 and virion-incorporated E1–E2, 
the amount of E1 expressed within transfected cells and the 
amount of E1 co-precipitated with E2 from viral particles was 
reduced. Overall, the results suggest that His322, His298 and His352 
are essential for the structural integrity of the E1–E2 heterodimer 
and its associated functions.

Role of histidine residues in E2

Mutational analysis of histidine residues in E2 revealed roles in 
receptor binding, E1–E2 assembly and virus entry. For example, 
H421A/R markedly reduced the ability of E1–E2 derived from 
HCVpp to interact with the large extracellular loop of CD81, and 
both mutations significantly reduced virus entry. This observation 
is consistent with the fact that His421 is adjacent to Trp420, 
which, together with the G436WLAGLFY, Y527SWGANDTD 
and Y613RLWHY sequences, is a critical component of the 
discontinuous CD81-binding site of E2 (Figure 1A) [22,28,36]. A 
previous report indicated that alanine substitution of this residue 
blocked CD81 binding by ∼ 70 % [28]. That removal of the 
imidazole ring in H421A or its replacement with the larger basic 
side-chain of arginine in H421R is poorly tolerated indicates 
that His421 makes an important contribution to the CD81-binding 
surface of E2. An examination of the amino acids contributing to 
the CD81-binding site of E2 reveals the presence of seven 
aromatic residues. Aromatic amino acids, including histidine, play 
important roles in stabilizing proteins through the formation of 
aromatic pairs that contribute between 0.6 and 1.3 kcal/mol 
(1 cal ≈ 4.184 J) to stability [37,38]. Aromatic pairs involving 
histidine commonly form a bridge between a β-strand and another 
secondary structural element [34]. The E2–CD81 interaction may 
be stabilized through aromatic pairing involving His421, Trp420, 
Trp437, Phe422, Tyr521, Trp529, Tyr547, Trp566 and Tyr548 of E2, and 
Phe186 present on the D-helix of the large extracellular loop of 
CD81 [21].

Proteins of MLV (murine leukaemia virus) was not affected by 
the treatment (Figure 8B).

We next confirmed this result using the replication-competent 
HCVcc. None of wild-type, H445E or H445Y HCVcc were able 
H421A/R markedly reduced the ability of E1–E2 derived from 
HCVpp to interact with the large extracellular loop of CD81, and 
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Trp437, Phe422, Tyr521, Trp529, Tyr547, Trp566 and Tyr548 of E2, and 
Phe186 present on the D-helix of the large extracellular loop of 
CD81 [21].
Distinct roles for two histidine residues in the stem region of E2

The putative stem region of E2 (residues 675–699) links the RBD to the transmembrane anchor and plays an essential role in E1–E2 heterodimerization and viral entry [9]. H691A/R and H693A/R, located within the E2 stem, did not affect the capacity of HCVpp to bind CD81, yet these mutants were unable to mediate HCVpp entry. The entry block observed for H691A/R may be attributed to the reduced amount of E1 in pseudovirions. These results are consistent with our previous proposal that the stem region of E2 is an essential domain for heterodimerization with E1 [9]. By contrast, wild-type levels of H693A/R E1–E2 heterodimer were incorporated into HCVpp, indicating a distinct function for His693. These distinct roles for His691 and His693 are reflected in the separable functions observed for the stem region of flaviviral glycoprotein E, where the N-terminal segment is important for heterodimerization with PrM during virion biosynthesis, whereas the C-terminal segment stabilizes the low-pH-activated E trimer [39]. In the latter case, the stem has been postulated to pack in an antiparallel orientation into hydrophobic grooves along the surface of the trimer, forming the hairpin conformation that mediates membrane merger [40,41]. By analogy, His693 of HCV E2 may play a critical role in a post-CD81-binding stage of entry such as formation and/or stabilization of the post-fusion hairpin structure.

His445 is a pH sensor

The substitution of His445 with arginine or lysine, which are likely to be protonated at neutral pH, led to increased HCVpp and HCVcc infectivity without detectable effects on E2 folding, E1–E2 heterodimerization and CD81 binding. The enhanced infectivity of H445R correlated with an enhancement in cell–cell fusion function at neutral pH, suggesting that the artificial protonation of position 445 by arginine led to a less stable prefusion glycoprotein complex that can be triggered to mediate fusion at neutral pH. The results of a recent study have indicated that the E2–CD81 interaction primes the HCV glycoprotein complex to undergo low-pH-triggered fusion activation [42]. In the case of the H445R mutant, CD81 ligation may be sufficient to trigger fusion in a cell–cell fusion context. However, wild-type HCVpp, H445K or H445R mutants could not enter cells blocked in endosomal acidification by bafilomycin A1, even though the H445R mutant exhibited enhanced fusion activity at pH 7. These results suggest that an unknown event, which is additional to acidification and dependent on a functional endosomal pathway, is a critical determinant of viral entry. In the case of the phylogenetically related dengue flavivirus, anionic lipids together with low pH trigger viral fusion within the late
Clues about the role of histidine residues in membrane fusion and viral entry come from studies of the structurally homologous glycoproteins E and E1 of flaviviruses and alphaviruses respectively, for which three-dimensional structures of pre- and post-fusion forms are available [40,41,44–46]. Flaviviruses are phylogenetically related to HCV and there is evidence that post-fusion forms are available [40,41,44–46]. Flaviviruses are membrane-active regions of the hepatitis C virus E1 and E2 envelope glycoproteins. 4 Perez-Berna, A. J., Moreno, M. R., Guillen, J., Bernabeu, A. and Villalain, J. (2006) The functional roles of HCV glycoprotein histidine residues. Hepatitis C virus entry depends on clathrin-mediated endocytosis. J. Gen. Virol. 87, 2583–2593


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REFERENCES


Figure 8 Mutation of His445 to arginine does not enable infection of cells blocked in endosomal acidification and maturation by bafilomycin A1

HCVpp pseudotyped with wild-type (WT) or mutant E1/E2 glycoproteins (A), or envelope glycoproteins derived from MLV or VSag (B), were incubated with bafilomycin A1-treated (grey bars) or untreated (open bars) Huh 7.5 cells. Luciferase activity present in cell lysates was quantified at 24 h post-infection. Mutants L441V and L685A are previously described E2 and E1 mutants respectively that fail to mediate virus entry [9,22]. The results shown are the means ± S.D. of an experiment performed in quadruplicate and are representative of two independent experiments. The results shown are relative to the mean of untreated wild-type pseudotype particle entry. RLU, relative light unit. (C) Huh 7.5 cells were treated with 25 nM bafilomycin A1 for 1 h prior to incubation with 2500 TCID50/ml of wild-type and mutant HCVpp on ice for 2 h. After washing to remove unbound virus, the cells were incubated at 37°C for 1 h, washed with citric acid buffer at pH 7 or pH 5 to induce fusion, and then incubated in medium containing 12.5 nM bafilomycin A1 for a further 24 h. Luciferase activity associated with cell-free virus was determined using the Ampla luciferase kit. The results shown are the means ± S.D. of an experiment performed in triplicate and are representative of two independent experiments. GND (open bars), wild-type (closed bars), H445E (grey bars) and H445R (hatched bars).

endosome [43]. Overall, the results suggest that His445 plays a conserved role as a pH sensor regulating viral entry. Interestingly, the substitution of His445 with the larger slightly polar aromatic ring of tyrosine led to a ~2-fold increase in entry. Although the presence of a ring structure at residue 445 may enable important interactions to be involved in low-pH activation involves histidine residues in E2 that contact the fusion loop. E2 His445 packs against the side-chain of E1 Trp98 within the fusion loop, whereas E2 His92 and E2 His256 hydrogen bond the fusion loop main chain [15]. Interestingly, His445 is not strictly conserved in all HCV genotypes (Figure 4; [49]). In the case of genotypes 1 and 2, for which sufficient sequence data are available to enable frequency analysis, 18% of genotype 1 isolates (50/273) and 36% of genotype 2 isolates (17/47) contain a basic residue at position 445 (Figure 4). That H445K and H445R are naturally occurring polymorphisms suggests that some HCV strains may have different pH thresholds that enhance infectivity. It is notable that the H445K and H445R mutations did not inactivate the E1–E2 complex, as has been observed with mutations of pH-sensing residues of other viruses, such as influenza virus, which induces transition of the fusion glycoprotein to the post-fusion conformation at neutral pH [50,51]. It is therefore likely that His445 is part of a cluster of histidine residues that co-operatively contribute to the metastable E1–E2 structure. The results of the present study suggest that artificial protonation at His445 by introduction of arginine or lysine at position 445 would alter the threshold of fusion activation resulting in enhanced viral entry and infectivity. In conclusion, the present study reveals essential roles for the histidine residues of E1 and E2 of HCV in the formation of a functional heterodimer and in regulating the threshold of fusion activation.

AUTHOR CONTRIBUTION

Irene Boo, Kevin teWierik and Florian Douam performed experimental work and assisted with proofreading the paper prior to submission. Dimitri Lavillette, Pantelis Poubourios and Heidi Drummer obtained funding support, designed experiments, supervised the conduct of experiments and wrote the paper.