Two conserved histidines (His490 and His621) on the E2 glycoprotein of hepatitis C virus are critical for CD81-mediated cell entry

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Hepatitis C virus (HCV) entry is a sequential and multi-step process that includes receptor interactions followed by pH-dependent membrane fusion. Specific and conserved histidine residues on the viral envelope proteins are involved in most pH-induced virus entries. In the case of HCV, some conserved histidines on the E1 and E2 proteins have been investigated in HCV pseudotype particle (HCVpp) systems. However, the roles of these histidines in cell-culture-derived HCV particle (HCVcc) systems remain unclear due to the different aspects of the viral life cycle emphasized by the two systems. In this study, the role of two conserved histidines (His490 and His621, located in domains II and III of E2, respectively) in HCV infection was evaluated in the context of JFH-1-based HCVcc using alanine substitutions. The infectivity of the H490A mutant decreased in spite of comparable initial RNA replication, protein expression and assembly efficiency as WT virus. The H621A mutant did not affect viral protein expression, but exhibited no obvious infectivity; there were fewer core proteins in the culture supernatant compared with WT virus, indicating the partially deficient virus assembly. The HCV receptor CD81-binding ability of the two mutant E2s was assessed further using enzyme immunoassays. The CD81-binding activity of H490A-E2 was reduced, and H621A-E2 was unable to bind to CD81. These data revealed the crucial role played by His490 and His621 in HCV infection, particularly during CD81 binding in cell entry. These results also contributed to the mechanical identification of the histidines involved in pH-dependent HCV entry.

INTRODUCTION

Hepatitis C virus (HCV) belongs to the genus Hepacivirus in the family Flaviviridae. It is a small enveloped virus with a positive-stranded RNA. The HCV genome encodes one polyprotein, which is subsequently processed into structural and non-structural proteins by host and viral signal peptidases (Carrère-Kremer et al., 2004; Lindenbach & Rice, 2005). The two envelope glycoproteins E1 (residues 192–383) and E2 (residues 384–746) are both transmembrane proteins that are retained mostly in the endoplasmic reticulum after their biosynthesis (Moradpour et al., 2007; Op De Beeck et al., 2004). HCV E1 and E2 assemble as non-covalent heterodimers in their intracellular forms, whereas they form large covalent complexes stabilized by disulfide bridges on the surface of mature HCV particles. These complexes also play a role in recognizing the large extracellular loop (LEL) of tetraspanin CD81, as well as conformation-sensitive antibodies (Gastaminza et al., 2008; Merz et al., 2011; Op De Beeck et al., 2000; Vieyres et al., 2010, 2014).

HCV envelope glycoproteins are responsible for the initial attachment as well as binding to receptors or entry factors on the surface of host cells. The HCV receptors include low-density lipoprotein receptor, SR-B1 (scavenger receptor class B type 1), CD81, claudin-1 and occludin (Agnello et al., 1999; Evans et al., 2007; Fénéant et al., 2014; Monazahian et al., 1999; Ploss et al., 2009; Scarselli et al., 2002). After the sequential interaction with receptors or entry factors, as well as possible conformational changes in HCV envelope proteins, the virion is then internalized via clathrin-dependent endocytosis (Blanchard et al., 2006). Upon exposure to low pH in the early endosome, the conformation of the virion

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rarranges, allowing it to fuse with the endosomal membrane. This leads to the release of the HCV genome into the cytoplasm and infection of the cell (Haid et al., 2009).

Previous studies assessing low-pH-dependent virus entry demonstrated that the protonation of one or more histidine residues is critical for the maturation of progeny virions as well as the conformational alteration of fusion proteins (Carneiro et al., 2003; Fritz et al., 2008; Mueller et al., 2008; Qin et al., 2009). A ‘histidine switch’ hypothesis was proposed recently, whereby some highly conserved histidines and their interaction partners play a central role in initiating the structural transition that leads to viral fusion (Kampmann et al., 2006). These specific histidines are usually located around positively charged residues in the pre-fusion form, whereas their translocation to nearby negatively charged residues leads to the formation of salt bridges in the post-fusion form. To date, this hypothesis has been verified in several pH-induced virus fusions, such as paramyxovirus, human metapneumovirus, dengue virus (DENV) and vesicular stomatitis virus (VSV) fusion; in contrast, West Nile virus uses a different fusion mechanism, where histidine protonation appears to be not required for its pH-dependent entry (Nelson et al., 2010; Prakash et al., 2009; Schowalter et al., 2009).

Recently, some conserved histidine residues in the HCV E1 and E2 proteins were investigated, and the data revealed that they were involved in E1/E2 folding, CD81 binding and viral entry based on an HCV pseudotype particle (HCVpp) system (Boo et al., 2012). Unlike HCVpp, which mimics only the early entry steps, cell-culture-derived HCV particle (HCVcc) systems can mimic the natural virion because they allow the complete replication and production of viral particles. Although most entry characteristics obtained from HCVpp systems, such as receptor usage and pH-dependent entry, are consistent with those from HCVcc systems, it is necessary to clarify the entire virus life cycle using HCVcc systems (Lindenbach et al., 2005; Wakita et al., 2005; Zhang et al., 2004; Zhong et al., 2005). To evaluate the role of the two conserved histidine residues (His490 and His621), located in domains II and III of E2, respectively, in HCV infection and the possible underlying mechanisms by which they affect virus infectivity, two point mutations were made by substituting the histidines for alanines. The mutants were then assessed using an infectious HCVcc system based on the JFH-1 strain. These studies identified the crucial role played by these two histidine residues in efficient HCV infection and also shed light on the influence of mutations on the ability of the E2 protein to bind to CD81 during HCV entry.

RESULTS

Sequence comparison and initial characterization of the two histidine residues (His490 and His621) in HCV E2

Sequence alignments of the HCV E2 protein from the six major HCV genotypes (genotypes 1–6) revealed that there were eight highly conserved histidine residues. Amongst these, His490 and His621 were conserved completely; therefore, they were analysed in this study (Fig. 1a). The two residues His490 and His621 are located in domains II and domain III of E2, respectively, as determined using a structural model of the HCV E2 ectodomain (E2e) from the H77 strain (Krey et al., 2010) (aa 490 and 621 of JFH-1 correspond to aa 488 and 617 of H77, respectively; Fig. 1b). Structural analysis of the HCV E2 protein indicated that its domain II contains a putative fusion peptide similar to that of class II fusion proteins and plays critical roles in the conformational changes in E2 during membrane fusion. Interestingly, His490 is located close to the fusion peptide, suggesting that this residue might be involved in pH-dependent HCV entry. Domain III, an immunoglobulin-like fold, is connected to domain I via a flexible linker region, which is believed to facilitate the translocation of domain III to the side of the trimer during the conformational changes that occur during fusion, as expected for a class II fusion protein. His621 lies at the contacting interface with domain I and its surrounding fragments also form an essential component that interacts with the receptor CD81. As the protonation of histidine is involved in virus fusion and maturation, and also because His490 and His621 are located at the key conformational transition of E2, we hypothesized that these two histidine residues might be involved in HCV infection, such as during the conformational transition or the interaction of E2 with receptors during HCV entry.

H490A and H621A mutants do not affect viral protein expression

To investigate the role of His490 and His621 in the infection of hepatocytes, we substituted histidine for alanine using site-directed mutagenesis in an HCVcc system. The full-length infectious HCV RNAs transcribed from the WT, GND or mutant pJFH-1 plasmids were electroporated into Huh7.5.1 cells. The viral proteins expressed in the electroporated cells were then examined using immunofluorescence assays. Data revealed that the H490A and H621A mutants were expressed in a similar number of cells as WT after staining with anti-core antibody, anti-E2 antibody or HCV-positive serum from patients 72 h after electroporation (Fig. 2a). The expression kinetics of WT and mutant HCV proteins was also compared at different time points (48, 72 and 96 h) after electroporation using Western blotting. Data revealed that expression levels of HCV core and NS3 proteins from the H490A and H621A mutants were similar to WT at the analysed time points. No expression of HCV proteins was observed in the mock or GND electroporated cells; gliceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was adopted to control for protein load between WT and mutant samples (Fig. 2b). The WT and mutant E2 proteins were also expressed in the electroporated cells (Fig. S1, available in the online Supplementary Material). These results suggested that the H490A mutant exhibited similar viral protein expression as WT HCV, whilst the H621A mutant exhibited relatively constant protein expression.
H490A and H621A mutants exhibit reduced infectivity of HCV particles

Next, we compared the infectivity of the WT and H490A and H621A mutant HCV particles. The titres of infectious HCV particles present in culture supernatants were determined using end-point dilution and immunofluorescence assays. As shown in Fig. 3(a), the infectious titres of the H490A mutant were ~18-fold lower than the parental WT HCV 72 h after electroporation, whereas no infection was observed for the H621A mutant and GND control.

The reduction in extracellular viral infectivity exhibited by the two histidine mutants might have resulted from defects in the particle assembly or entry processes for the progeny HCV particles. We first explored the intracellular viral infectivity in the infected cells, and evaluated whether the assembled virus particles were intact by analysing HCV RNA and the amount of core protein released into the supernatants 72 h post-electroporation. Intracellular titres were evaluated by infecting Huh7.5.1 cells with clarified lysates harvested from electroporated cells by triple freeze/thaw cycles. The results demonstrated that compared with the WT intracellular virus titres, the H490A mutant lysate titre was decreased ~7.1-fold, and no infection was observed for the H621A mutant and GND control (Fig. 3b). The results indicated that the H490A mutant was able to assemble infectious viral particles the same as WT despite of reduced intracellular infectivity.

HCV RNA quantification in the supernatants using quantitative real-time (qRT)-PCR showed that the H490A mutant RNA levels were identical to those of the H621A mutant, which was statistically around twofold higher than the parental WT HCV RNA (Fig. 3c). The relative change in the amount of core protein released into the supernatants was assessed using an adapted ELISA by measuring A450. No core protein was released by the GND control, but the amount of core protein released by the H490A mutant was slightly higher than that of the WT and less core protein was observed in supernatants from cells transfected with the H621A mutant compared with the WT (Fig. 3d), suggesting that the H621A mutant might not be intact and defective in...
the formation of infectious viral particles. Taken together, these data suggested that the particles assembled by the H490A mutant should be intact, whereas those assembled by the H621A mutant contain less core protein compared with WT HCV.

**HCV protein expression kinetics of the H490A and H621A mutants is reduced during single-cycle HCV infection**

As Huh7.5.1 cells support a second round of infection by the HCV virion, the results of infection titres from these cells reflect not only the ability of electroporated viral RNA to produce progeny virus, but also of the progeny to infect naive host cells. In contrast, CD81-deficient Huh7 cells do not support HCV reinfection due to the absence of the entry receptor CD81 on the cell surface; instead, they allow the replication and production of single-cycle viruses (Rocha-Perugini et al., 2009; Russell et al., 2008). To determine which function the mutation of histidine affected, we used CD81-deficient Huh7 cells to evaluate and compare the two mutants and WT during a single viral replication cycle. The electroporated CD81-deficient Huh7 cells were fixed and then stained with anti-core antibody or HCV-positive serum from patients; the electroporation efficiency was then assessed 72 h after electroporation. Immunofluorescence assays revealed that the H490A and H621A mutants yielded similar amounts of positive cells as the WT, whilst no positive cells were observed for the mock or GND control (Fig. 4a). We then compared the expression kinetics of the WT and mutant HCV proteins in a single cycle using Western blotting. The results revealed that expression levels of HCV core and NS3 protein of the H490A and H621A mutants were both lower than that of the WT at each time point. No expression of HCV protein was observed in the mock or GND electroporated cells; GAPDH expression was used for protein load control (Fig. 4b). These data demonstrated that the H490A and H621A mutants exhibited lower protein expression kinetics than WT HCV during a single-cycle infection.
H490A and H621A mutations reduce the specific infectivity of HCV particles

As HCV protein expression was affected by the H490A and H621A amino acid substitutions in electroporated CD81-deficient Huh7, we next analysed the specific infectivity of extracellular viruses in a single cycle. Culture supernatants from CD81-deficient Huh7 cells electroporated with WT, GND or mutant RNA were collected 72 h after electroporation, and were then titrated for infectious virus particles using end-point dilution and immunofluorescence assays. As shown in Fig. 5(a), the H490A mutant produced around fourfold fewer infectious particles than did WT HCV. No infectivity was detected in supernatants from cells transfected with the H621A mutant and GND control (Fig. 5a), which is consistent with the results of the extracellular virus infectivity experiments in Huh7.5.1 cells. To further evaluate whether the assembled HCV particles were intact in a single cycle, we assessed the extracellular-specific infectivity by analysing the presence of HCV RNA in the supernatant. As shown in Fig. 5(b), qRT-PCR revealed that the levels of extracellular RNA in the supernatants from the H490A or H621A mutants were comparable with that of the WT (Fig. 5b). Results of adapted ELISA showed that the amount of core protein released by the H490A mutant was also slightly higher than that of the WT and less core protein was observed for the H621A mutant (Fig. 5c). Extracellular-specific infectivity was then calculated as the ratio of infectious titres to the copies of HCV RNA. As extracellular virus containing the H621A mutant showed no infectivity at all, it was not necessary to calculate its specific infectivity because data suggested that the H621A mutant could not reinfect naive host cells. The specific infectivity of the H490A mutant was lower than that of WT viruses (Fig. 5d), suggesting that the H490A mutation decreased the entry efficiency of the progeny HCV particles.

H490A and H621A mutations reduce the CD81-binding activity of the HCV E2 protein

As the interaction of E2 with the receptors is the first and most important step during HCV entry, the entry deficiency of the two mutants might result from a defective interaction with the HCV receptors. Human CD81, a critical entry receptor, is vital for HCV entry, particularly for the initial attachment or binding of the E2 protein. To further clarify the molecular mechanism by which the H490A or H621A mutants decreased or diminished the ability of virus particles to enter host cells, we investigated the ability of the HCV E2 protein containing the histidine mutations to bind to CD81. Recombinant plasmids encoding the WT or mutant E2 proteins were constructed and expressed transiently in
human embryonic kidney (HEK) 293T cells. An haemagglutinin (HA)-tag on the recombinant E2 proteins was detected using Western blotting (Fig. 6a). The purified thioredoxin (TRX)–hCD81-LEL recombinant fusion proteins (see Methods) were then used to test the CD81-binding activity of the WT and mutant HCV E2 proteins using enzyme immunoassays (EIAs). As shown in Fig. 6(b), compared with the WT-E2 protein, the H490A-E2 protein exhibited lower binding activity to human CD81 LEL, whereas no CD81-binding activity was detected for the H621A-E2 protein. These results suggested that the reduction or loss of H490A or H621A mutant infectivity was partially due to deficient CD81 binding.

**DISCUSSION**

Our results demonstrated that the specific infectivity of the H490A mutant in a JFH-1 HCVcc system was decreased by around fourfold compared with the WT, which was partially due to reduced CD81-binding activity. In contrast, no infectivity was detected with the H621A mutant, despite similar protein expression levels as in WT HCV at early time points. These results suggest that His490 and His621 on the E2 protein play roles in HCV entry, particularly during binding to the CD81 receptor.

HCV entry involves four essential and sequential steps that are regulated both spatially and temporally (Hsu et al., 2003; Lavillette et al., 2006; Sharma et al., 2011). Some conserved histidine residues in the E1 and E2 proteins are involved in E1/E2 folding and viral entry, including His222 in E1, and His421 and His445 in E2 (Boo et al., 2012). In the current study, intracellular HCV protein expression, HCV RNA levels and core content in the culture supernatants were similar in the H490A mutant and WT viruses (Figs 2, 3c, d and 4). However, the infectivity of extracellular multi-cycled H490A mutant virus decreased by ~18-fold and its specific infectivity decreased by around fourfold (Figs 3a and 5d). This suggests that the H490A mutation exhibited less efficient entry, rather than defective virus assembly. These data are inconsistent with a study by Boo et al. (2012), who found that the corresponding His488 in the H77 strain was essential for the biosynthesis of a functional virion-incorporated E1/E2 heterodimer. The differences in HCV genotypes and the study systems might contribute to the discrepancy between the two studies. In the H621A mutant, intracellular HCV protein expression was comparable with that of the WT (Fig. 2b). The extracellular and intracellular virus particles were not infectious (Figs 3a and 5d), suggesting that the H621A mutant might exhibit deficient virus assembly as well as cell
entry. Extracellular HCV RNA of the H621A mutant was similar or even higher than that of the WT, whereas its core content decreased to ~50% of WT HCV (Figs 3c and 5b, c), indicating that the efficiency of the H621A virus assembly was lower than the WT. A previous study revealed that the corresponding His617 in the H77 strain was critical for the assembly of the E1/E2 heterodimer and its incorporation into virions (Boo et al., 2012). Although data on the heterodimerization of this mutant are absent in the current study, we could not exclude the possibility that the H621A mutant exhibits deficient heterodimer formation in the JFH-1-based HCVcc system. Nevertheless, further experimental data are required to support the hypothesis that His621 in the JFH-1 strain might also be involved in E1/E2 heterodimer formation and virion incorporation, as occurs in the H77 strain.

CD81 binds to a soluble form of the E2 protein and plays a post-cell-binding role during HCV entry (Heo et al., 2006; Krieger et al., 2010; Zhang et al., 2004). Although there is no direct evidence of CD81 binding to the HCV virion, their interaction could also prime the fusogenic activity of HCV glycoproteins into a ‘more open’ or ‘primed’ conformation required for efficient membrane fusion (Harris et al., 2008; Petracca et al., 2000; Sharma et al., 2011). The D-helix of CD81 LEL is mainly responsible for the stability of the E2–CD81 interaction via aromatic pairing between critical amino acids (Cao et al., 2007; Drummer et al., 2002; Zhu et al., 2012). The CD81-binding sites in E2 are composed of several discontinuous fragments including Trp^{420}G{136}WLALFY, Y{527}SWGANDTD and Y{613}RLWH{617}Y (based on the amino acid numbering in the H77 strain) (Drummer et al., 2006; Owsianka et al., 2006). In the current study, H621A-E2 was unable to bind to CD81 (Fig. 6b), suggesting that this histidine residue is critical for CD81 binding.

Although the expression of HCV E2 proteins was not affected dramatically by the histidine mutations, H490A-E2 exhibited reduced CD81-binding activity compared with WT-E2 (Figs 2a and 6). This could partially explain why the H490A mutant had decreased entry efficiency. However, Boo et al. (2012) used an H77 strain-based HCVpp system and demonstrated that H488A did not affect CD81 binding significantly. In contrast, an arginine-substituted counterpart H488R exhibited ~50% reduced binding to CD81, suggesting that the introduction of arginine specifically led to a CD81-binding defect. The discrepancy between these observations and the results of the current study might be partially due to differences amongst HCV genotypes (Jardim et al., 2009; Weiner et al., 1999). Nevertheless, it remains unclear whether these different genotypes or strains share a common entry mechanism, including receptor use and binding or membrane fusion.

Low-pH-induced HCV fusion is also an important step after being endocytosed into host cells. The protonation of key histidines is believed to be critical for the structural transition and subsequent pH-dependent fusion. For example, VSV fusion was driven by the protonation of two histidines (His148 and His149) on the G protein (Carneiro et al.,

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**Fig. 5.** Extracellular-specific infectivity of single-cycle WT, H490A and H621A mutant HCVs 72 h post-electroporation. (a) Extracellular virus titres were determined using immunofluorescence assays in Huh7.5.1 cells. (b) HCV RNA in the supernatant was quantified using qRT-PCR. (c) HCV core proteins in the supernatant were detected using an adapted ELISA. The dashed horizontal line indicates the cut-off value based on negative controls. (d) Extracellular-specific infectivity was determined in the supernatants. Data are presented as the mean ± SD of three independent experiments. *P<0.05; **P<0.01.
and CD81 binding contributed to the non-infectivity of the reduced CD81 binding. Deficiencies in virus assembly infectivity compared with the parental virus mainly due to although viral RNA replication or proteins expression was HCV fusion. His621 are involved directly in, and how they participate in, interaction required for membrane fusion. Nevertheless, additional studies are required to determine whether His490 and His621 are involved directly in, and how they participate in, HCV fusion.

Although viral RNA replication or proteins expression was not varied significantly, the H490A mutant decreased infectivity compared with the parental virus mainly due to the reduced CD81 binding. Deficiencies in virus assembly and CD81 binding contributed to the non-infectivity of the H621A mutant. Taken together, these results suggest that both His490 and His621 in the E2 protein in the HCVcc system are involved in the HCV life cycle, particularly during cell entry.

**METHODS**

**Cells.** HEK293T cells, human hepatoma Huh7.5.1 cells and CD81-deficient Huh7 cells (Zhong et al., 2006) (kindly provided by Dr ling Zhong, Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China), were grown at 37 °C in complete Dulbecco’s modified Eagle’s medium containing 10% (v/v) FBS (Gibco-BRL) supplemented with 100 nM non-essential amino acids (Invitrogen), 1 mM l-glutamine, 100 μg streptomycin ml⁻¹ and 100 U penicillin ml⁻¹.

**Plasmid construction.** The plasmids pJFH-1 encoding the full-length HCV JFH-1 genome (genotype 2a) and pJFH-1/GND (kindly provided by Dr Takaji Wakita, National Institute of Infectious Diseases, Tokyo and L. Janet Milton, Apath, Brooklyn, NY, USA) were used as the WT and negative control virus, respectively. The alanine-to-histidine mutations were introduced into the WT pJFH-1 as described previously (Qin et al., 2013b). Briefly, the H490A or H621A mutation was introduced first into the subcloned plasmid poBNI using a QuikChange Lightning Site-Directed Mutagenesis kit (Stratagene). The mutated fragments were then cloned into pJFH-1 using restriction enzyme digestion. The presence of the desired mutations was confirmed using nucleotide sequencing. (Invitrogen). Using the WT and histidine-mutated pJFH-1 plasmids as template, the DNA sequences encoding the WT and mutated C-terminal truncated E2 (residues 364–687 of the HCV polyprotein) of strain JFH-1 were amplified using PCR. The resulting fragment was HA-tagged, inserted into pCI-neo vector (Promega) after digestion with EcoRI and SalI restriction enzymes, and then sequenced for confirmation. The prokaryotic recombinant plasmid expressing human CD81 (hCD81) LEL was designated pET32a-hCD81-LEL and was described previously (Tong et al., 2011).

**In vitro transcription and RNA electroporation.** The WT, GND and mutated RNAs were transcribed and electroporated into Huh7.5.1 or CD81-deficient Huh7 cells as described previously (Kato et al., 2006; Qin et al., 2013b). Briefly, plasmids were linearized using XbaI and treated with mung bean nuclease (New England Biolabs). The infectious full-length RNA was transcribed in vitro at 37 °C for 3 h using a MEGAscript T7 kit (Ambion) according to the manufacturer’s instructions. The DNA template was degraded by DNase treatment for 15 min at 37 °C. The synthesized RNA was then precipitated by treatment with LiCl, purified, quantified by measuring A260 and aliquoted at 10 μg per tube. In total, 7.5 × 10⁶ cells (for Huh7.5.1) or 3.0 × 10⁶ cells (for CD81-deficient Huh7) were resuspended in Opti-MEM (Invitrogen), mixed with 10 μg viral RNA and transferred to an electroporation cuvette. The cells were electroporated using a Gene Pulser II apparatus (Bio-Rad) under the following conditions: 270 V, 950 μF and 100 Ω. Cells were then cultured at 37 °C for the indicated times to allow the production of viral progeny.

**Indirect immunofluorescence assays.** The electroporated or infected cells were incubated with fresh complete medium for 48 h. After washing, they were fixed with ice-cold methanol and incubated with anti-core mAb (1:500 dilution), anti-E2 mAb (AP33, kindly provided by Dr Arvind Patel, University of Glasgow, UK; 1:500 dilution) or HCV-positive serum from patients (1:100 dilution), followed by FITC-conjugated goat anti-mouse or human IgG antibodies. Images were captured or infected foci were counted under a fluorescence microscope (IX81; Olympus).

**Titrating infectious HCV using immunofluorescence.** The concentration of infectious virus particles in the supernatant or in
Critical histidine residues for hepatitis C virus entry

The electrotoporated cells was determined 72 h post-electroporation using end-point dilution and immunofluorescence assays as described previously (Qin et al., 2013b). Briefly, HCVcc-containing culture supernatants or cell lysates obtained by three freeze/thaw cycles were serially diluted at 10-fold increments in complete medium and 100 μl aliquots were used to infect ~1 × 10^4 HuH7.5.1 cells in 96-well plates. After co-culturing at 37 °C for 3 h, the infected cells were washed and incubated with fresh complete medium for an additional 48 h. Cells were then fixed and HCV infection was assayed using indirect immunofluorescence as described above. Infectious titres of extracellular and intracellular viruses were calculated and expressed as f.f.u. ml⁻¹.

Western blotting. Western blotting was performed as described previously with some modifications (Qin et al., 2004, 2007). Briefly, cells were lysed using SDS lysis buffer (20 mM Tris, pH 8.0, 250 mM NaCl, 3 mM EDTA, 10 %, v/v, glycerol, 1 %, w/v, SDS, 0.5 %, v/v, NP-40, 1 mM PMSF and protease inhibitor cocktail) on ice. After centrifugation, protein concentrations were determined using the Bradford or bicinchoninic acid method (Beyotime). The proteins were then separated using 12.5 % (w/v) SDS-PAGE and transferred onto PVDF membranes (Millipore) using a Trans-Blot apparatus. The proteins from supernatants or cell lysates obtained by three freeze/thaw cycles were blocked with milk buffer (5 %, v/v, non-fat milk and 0.05 %, v/v, Tween-20 in PBS) for 1 h, and incubated with serial dilutions of WT and mutated HCV E2 at 4 °C overnight. After extensive washing, the bound E2 was detected using mouse anti-HA mAb at a 1:1000 dilution in milk buffer for 1 h at room temperature, followed by HRP-conjugated anti-mouse IgG and tetramethylbenzidine substrate. A450 was measured using an ELISA reader.

Expression of recombinant E2. The WT and mutated E2 plasmids were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen). After 48 h, the transfected cells were detached from the tissue culture dishes by treatment with PBS/EDTA, resuspended in PBS supplemented with protease inhibitor cocktail (Roche) and lysed using ultrasonication. The clarified lysates were analysed using the EIAs described below after being assessed by Western blotting with anti-HA mAb.

EIAs for the binding of E2 to recombinant human CD81. EIAs were performed to evaluate the binding of E2 to recombinant hCD81 LEL as described previously (Flint et al., 1999; Tong et al., 2011). Briefly, ELISA plates (Nunc) were coated overnight with 1 μg purified TRX-hCD81-LEL recombinant protein per well. The plates were then blocked with milk buffer (5 %, v/v, non-fat milk and 0.05 %, v/v, Tween-20 in PBS) for 1 h, and incubated with serial dilutions of WT and mutated HCV E2 at 4 °C overnight. After extensive washing, the bound E2 was detected using mouse anti-HA mAb at a 1:1000 dilution in milk buffer for 1 h at room temperature, followed by HRP-conjugated anti-mouse IgG and tetramethylbenzidine substrate. A450 was measured using an ELISA reader.

References


