The variable regions of hepatitis C virus glycoprotein E2 have an essential structural role in glycoprotein assembly and virion infectivity

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The three variable regions of hepatitis C virus (HCV) glycoprotein E2 can be removed simultaneously from the E2 ectodomain (residues 384–661) without affecting folding or CD81 binding. In this study, we show that deletion of hypervariable region (HVR) 2 or the intergenotypic variable region (igVR) in the context of the E1E2 polyprotein eliminates formation of heterodimers, reduces CD81 binding and abolishes virus entry. The replication competence of genomic RNA transcribed from the JFH1 infectious HCV clone was not affected by the HVR1, HVR2 or igVR deletions in transfected Huh7.5 cells. However, infectivity of the resultant cell-culture-derived HCV (HCVcc) was abolished by HVR2 or igVR deletions, while deletion of HVR1 led to a 5- to 10-fold reduction in infectivity. Serial passage of cells transfected with genomes lacking HVR1 generated reverted viruses with wild-type levels of infectivity. Sequencing of viral cDNA obtained after full reversion revealed mutations in E1 (I262L) and E2 (N415D) that were present in 35 and 27 % of clones, respectively. Insertion of N415D into HVR1-deleted HCV genomes conferred wild-type levels of infectivity, while I262L increased infectivity by 2.5-fold. These results suggest that HVR2 and the igVR, but not HVR1, are essential for structural integrity and function of the HCV glycoprotein heterodimer.

INTRODUCTION

The hepatitis C virus (HCV) glycoproteins E1 and E2 are present on the surface of virions as a heterodimer that mediates viral attachment to host-cell receptors and facilitates virus fusion and entry. The folding of E1 and E2 is dependent on their coexpression (Brazzoli et al., 2005; Cocquerel et al., 2003; Michalak et al., 1997; Patel et al., 2001). Glycoprotein E1 is encoded by residues 171–383 of the HCV polyprotein and is a typical type I glycoprotein. Its precise role in virus entry has not been determined, although a role in virus fusion has been suggested (Drummer et al., 2007; Lavillette et al., 2007; Li et al., 2009). Glycoprotein E2 is composed of a receptor-binding domain (RBD; 384–661) that is linked to the C-terminal transmembrane domain (715–746) via a stem region (662–714) containing a heptad repeat (675–699). HCV entry proceeds via initial attachment to glycosaminoglycans (Barth et al., 2003; Morikawa et al., 2007), followed by binding to scavenger receptor type B class I (SR-B1) and the tetraspanin CD81 (Bartosch et al., 2003; Pileri et al., 1998; Scarselli et al., 2002; Zeisel et al., 2007). The tight-junction molecules claudin 1 (Evans et al., 2007) and occludin (Pless et al., 2009) are also essential cofactors required for successful virus entry via clathrin-mediated endocytosis and low-pH-dependent fusion within an early endosome (Blanchard et al., 2006; Meertens et al., 2006).

The structural organization of E2 has been proposed based on disulfide mapping studies and modelling on the known flavivirus glycoprotein E structure (Krey et al., 2010). The E2 RBD is believed to comprise three subdomains (DI, DII and DIII). DI is predicted to contain eight antiparallel β-sheets (β0–I0) and contains the majority of known CD81-binding determinants (Fig. 1a). DII is connected to DI via strands D0 and E0, while DIII separates DI from the stem region and includes part of the CD81-binding domain. Located within the RBD are three highly variable sequences termed hypervariable region 1 (HVR1; 384–411), HVR2 (460–485) and the intergenotypic variable region (igVR; 570–580) (Fig. 1a) (Hijikata et al., 1991; McCaffrey et al., 2007). Whilst HVR1 is an immunodominant region that elicits type-specific neutralizing antibody, HVR2 and the igVR are not known targets of the humoral immune system.
response (Facex et al., 1996). The predicted structure of glycoprotein E2 suggests that HVR1 precedes DI and HVR2 forms a loop-like structure via flanking cysteine residues within DII, while the igVR links DI and DIII (Fig. 1a) (Krey et al., 2010).

Previously, we showed that replacement of the three variable regions, individually or in combination, with Gly–Ser–Ser–Gly (GSSG) in the context of a recombinant soluble form of E2 (residues 384–661; E2661) did not affect expression and secretion of E2, its recognition by a conformation-sensitive monoclonal antibody (mAb H53) or binding to a recombinant form of the CD81 large extracellular loop fused to the maltose-binding protein (MBP–LEL113–201). The replacement of all three variable regions with GSSG resulted in the expression of a minimal core domain of E2 (D123) (McCaffrey et al., 2007). Here, we examine the role of the three variable regions in the expression and function of the E1E2 heterodimer in virion-incorporated forms of the glycoproteins and show an essential role for HVR2 and the igVR in the structural integrity of virion-incorporated forms of E2.

**RESULTS AND DISCUSSION**

**HVR2 and the igVR, but not HVR1, are essential for E1E2 heterodimerization and the conformation of E2**

The three variable regions of E2 were replaced with GSSG linkers using overlap extension PCR in the context of a plasmid encoding genotype 1a glycoproteins, pH77c E1E2, as shown in Fig. 1(b) (Drummer et al., 2003). The first three residues of E2 (384-Glu–Thr–His) were maintained in ΔHVR1 constructs to enable polyprotein processing. Cysteine residues, present on either side of HVR2 and the igVR, were maintained to enable disulfide formation (Fig. 1b). Retroviral pseudovirions containing HCV glycoproteins (HCVpp) were produced by cotransfection of 293T cells with either pH77c E1E2 wild-type or pH77c E1E2 with variable region GSSG replacements and the HIV-1 luciferase reporter genome pNL4.3R2E2Luc (Fig. 1b). The transfected cells were metabolically labelled with Tran-35S-label as described previously (Drummer et al., 2003). The HCVpp were immunoprecipitated with conformation-dependent mAb H53 and examined under non-reducing SDS-PAGE (Fig. 2a). mAb H53 recognizes non-covalently associated E1E2 on HCVpp that is similar to that recognized by neutralizing antibodies that bind to HCV particles from human plasma, but has a higher affinity and improved sensitivity (Cocquerel et al., 1998; Deleersnyder et al., 1997; Drummer et al., 2003; Op De Beeck et al., 2004). The results show that deletion of HVR1 (ΔHVR1) alone did not affect E1E2 heterodimerization, as wild-type levels of E1 were co-precipitated in non-covalent association with E2. By contrast, deletion of HVR2 (ΔHVR2) or the igVR (ΔigVR) eliminated E1E2 heterodimerization, evident by the lack of E1 co-precipitated with E2 (Fig. 2a). Combining the deletions to produce double (ΔHVR1 + 2, ΔHVR1 + igVR, ΔHVR2 + igVR) or triple (ΔHVR1 + 2 + igVR) deletion mutants did not rescue E1E2 heterodimerization. In all cases, E2 expression and

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**Fig. 1.** (a) Schematic representation of the proposed three-dimensional structure of E2 predicted by disulfide mapping and molecular modelling (Krey et al., 2010). Variable regions are shown in red and the nine intramolecular disulfides are indicated by dashed lines. (b) Schematic representation of E2 constructs used in this study. The three variable regions (red) were deleted individually and in combination from the pH77c E1E2 expression vector, or individually from pJFH1 or JC1flag, and replaced with GSSG linkers as indicated. The first three amino acids of E2 were retained in the ΔHVR1 constructs to allow polyprotein processing. Glycoprotein E1 (light grey), E2 (dark grey) and the transmembrane domains (filled boxes) are shown.
recognition by mAb H53 was maintained. A reduction in the apparent molecular mass of E2 as a result of single and multiple variable region deletions, and the consequent deletion of one glycosylation site from each of HVR2 and the igVR, was evident in SDS-PAGE (Fig. 2a). The overall expression of E1 and E2 in cell lysates was examined in reducing SDS-PAGE followed by immunoblotting with mAbs to linear epitopes within E1 (A4) and E2 (H52) (bottom panel). Total expression of E1 and E2 in cell lysates was examined in reducing SDS-PAGE followed by immunoblotting with mAbs to linear epitopes within E1 (A4) and E2 (H52) (bottom panel).

Deletion of HVR1 alone increased binding by domain A- and C-reactive antibodies, by approximately 60 and 40%, respectively (Fig. 2b). In a previous study, deletion of HVR1 from genotype 2a glycoproteins in HCVcc increased neutralization sensitivity to CBH-7 (Bankwitz et al., 2010). Thus, deletion of HVR1 increases the exposure of antibody epitopes within glycoprotein E2, increasing neutralization sensitivity. By contrast, deletion of either HVR2 or the igVR abolished recognition by the domain A antibody CBH-4D and reduced recognition by domain C antibody CBH-7. This suggests that deletion of HVR2 or the igVR affects the folding of at least two of the three immunogenic domains of E2 when derived from the full-length E1E2 polyprotein.

Fig. 2. (a) Expression, folding and heterodimerization of E1E2 containing variable region deletions. 35S-Labelled HCVpp incorporating the H77c E1E2 glycoproteins or no glycoproteins (empty) were immunoprecipitated with the conformation-sensitive anti-E2 mAb H53 and examined under non-reducing SDS-PAGE conditions (top panel). The HIV-1 p24 capsid protein was immunoprecipitated from radiolabelled HCVpp using IgG purified from plasma derived from an HIV-1-infected patient (middle panel). Total expression of E1 and E2 in cell lysates was examined in reducing SDS-PAGE followed by immunoblotting with mAbs to linear epitopes within E1 (A4) and E2 (H52) (bottom panel). (b) 35S-Labelled HCVpp were immunoprecipitated with conformation-sensitive anti-E2 mAbs specific to domains A (CBH-4D) and C (CBH-7) and examined under non-reducing SDS-PAGE conditions. RO4 is an irrelevant human mAb. (c) CD81-binding by HCVpp-incorporated E1E2 containing variable region deletions. HCVpp viral lysates normalized for E2 expression were applied to immobilized MBP-LEL13–201. Bound E2 was detected by the conformation-sensitive antibody H53. Data shown are the mean ± SEM percentage binding relative to wild-type (WT) of three independent preparations of HCVpp. (d) Huh7 cells were infected with HCVpp incorporating the H77c E1E2 glycoproteins containing single or multiple deletions of the E2 variable regions. Luciferase activity in cell lysates was quantified 72 h post-infection. Data shown are means ± SD of one experiment and are representative of two independent experiments.
Deletion of HVR2 or the igVR reduces the ability of pseudovirion-incorporated forms of E2 to bind CD81

The CD81-binding site on E2 has been mapped to reside within immunogenic domains B and C, which are likely to be equivalent to DI and DIII, respectively, on the predicted structure of HCV E2 (Drummer et al., 2006; Krey et al., 2010; Owsianka et al., 2006; Roccasecca et al., 2003). We examined the ability of E2 with deletions of one or more variable regions to bind MBP–LEL113–201 in order to monitor CD81-binding capacity and the conformation of immunogenic domains B and C. This form of CD81 has been used extensively to characterize E2–CD81 interactions (Drummer & Poumbourios, 2004; Drummer et al., 2002, 2005, 2006; Grove et al., 2008; Harris et al., 2010; McCaffrey et al., 2007) and has been shown to be a mimic of native CD81, as it retains the ability to interact with the first extracellular loop of claudin 1 (Harris et al., 2010). In addition, virion-incorporated E2 can be examined directly for its ability to bind CD81 (Drummer et al., 2006, 2007). The ability of virion-incorporated E2 with variable region deletions to bind CD81 was compared with HCVpp containing wild-type glycoproteins and HCVpp containing the L441M mutation, which eliminates binding to CD81 (Drummer et al., 2006). Deletion of HVR1 alone did not alter the ability of genotype 1a E1E2 within HCVpp to bind MBP–LEL113–201 (Fig. 2c). This result contrasts with the increased binding observed previously for recombinant HVR1-deleted E2661 (strain H, genotype 1a, and strain N2, genotype 1b) to the large extracellular loop of CD81 fused to GST (GST–LEL) or CD81 present on the surface of Molt-4 cells (Roccasecca et al., 2003). In addition, Bankwitz et al. (2010) also demonstrated that deletion of HVR1 increases the sensitivities of genotype 2a-derived HCVcc neutralization by GST–LEL. Together, the data suggest that HVR1 modulates exposure of the CD81-binding site on virion-incorporated forms of E2 in a strain-dependent manner. By contrast, deletion of either HVR2 or the igVR resulted in a 50% reduction in the CD81-binding activity of HCVpp. Deletion of two (ΔHVR1 + 2, ΔHVR1 + igVR, ΔHVR2 + igVR) or all three (ΔHVR1 + 2 + igVR) variable regions from E2 did not further affect the ability of virion-incorporated E2 to bind CD81, maintaining maximal binding at 50% that of wild-type E2 (Fig. 2c). Taken together, these data indicate that deletion of HVR2 or the igVR compromises the structure of all three immunogenic domains of full-length HCV E2 when co-expressed with E1.

Deletion of HVR1, HVR2 or the igVR disrupts virus entry of HCVpp

The ability of HCVpp containing deletions of the variable regions to mediate entry into Huh7 cells was examined. The results show that, although deletion of HVR1 did not affect CD81 binding or E1E2 heterodimerization within HCVpp, no significant entry could be detected relative to retroviral pseudotypes lacking envelope glycoproteins (empty) (Fig. 2d). Deletion of HVR2 or the igVR and multiple deletions thereof rendered HCVpp non-infectious, consistent with a lack of E1E2 heterodimers within HCVpp and reduced levels of CD81 binding (Fig. 2d and data not shown).

HVR1 is not essential for replication of cell-culture-derived HCV

To examine the role of the variable regions in HCV replication, the HVR1 (384–410) sequence was deleted while HVR2 and the igVR were replaced with GSSG in the context of the replication-competent genome of genotype 2a pJFH1 (Wakita et al., 2005). RNA was transcribed in vitro and used to transfect Huh7.5 cells. Seventy-two hours post-transfection, cells were fixed and HCV-infected cells were visualized by immunofluorescence with anti-NS5A antibody. All constructs were able to replicate efficiently in RNA-transfected Huh7.5 cells (Fig. 3a, b; post-transfection). Similar amounts of core protein could be detected in the supernatant fluid of transfected cells for all constructs, indicating that the E2 deletions did not block assembly and release of viral particles (Fig. 3c).

The transfected cells were split every 3–4 days and HCV NS5A was detected using immunofluorescence as described above. Passaging of cells allows both cell-free and cell-associated infection to occur and is less stringent than passaging of cell-free virus alone. Furthermore, as cell–cell transmission of HCV is CD81 independent, this method does not rely on E2 retaining its CD81-binding properties (Witteveldt et al., 2009). The JFH1 virus with a deletion of HVR1 was capable of maintaining successive rounds of infection and replication (Fig. 3a). Quantification of the infectious foci observed after passaging of cell-free HVR1-deleted virus revealed approximately 5- and 3-fold lower levels of infection relative to wild-type JFH1 in P1 and P2, respectively (not shown). In a previous report, Bankwitz et al. (2010) attributed the decreased infectivity of HVR1-deleted HCVcc to a decrease in the infectivity of low-density particles (1.02–1.08 g ml⁻¹) and impaired fusion activity. We conclude that deletion of HVR1 does not affect glycoprotein structure significantly and that virions containing a deletion of HVR1 replicate efficiently but have 10-fold-reduced infectivity, this effect being more severe in HCVpp.

HVR2 and the igVR are essential for replication of HCVcc

Deletion of HVR2 or the igVR from pJFH1 severely reduced viral infectivity (Fig. 3b). Isolated foci of infection could be observed after cell passages 2 and 3 (Fig. 3b) but not in cell passages 4–10 (not shown).
Infectious foci were also not observed following infection of Huh7.5 cells using cell-free JFH1 virus with deletions of HVR2 or the igVR (not shown). HVR2 and the igVR are therefore dispensable for genome replication and translation, but are essential for virion infectivity.

**Fig. 3.** Replication of HCVcc containing deletions of variable regions. (a) Immunofluorescence of wild-type or HVR1-deleted pJFH1 RNA-transfected Huh7.5 cells. Viral replication was detected using immunofluorescence with anti-NS5A antibody and Alexa 488-conjugated anti-mouse immunoglobulin. (b) Immunofluorescence of wild-type or HVR2- or igVR-deleted pJFH1 RNA-transfected Huh7.5 cells. The replication-defective plasmid pJFH1/GND was used as a control (GND). (c) Quantification of core particles released from transfected Huh7.5 cells. Core protein present in supernatant fluid was quantified using the ORTHO HCV assay.
HVR1-deleted HCVcc acquire adaptive mutations in E1 and E2

We employed the vector pJC1FLAG2(p7-NS-GLUC2A) (JC1flag) to examine how viruses containing deletions of HVR1 can revert following passaging. The vector incorporates a *Guassia* luciferase gene that allows virus replication to be monitored by measurement of luciferase activity in the tissue-culture supernatant fluid and incorporates a FLAG epitope tag at the N terminus of E2, preceding HVR1 (Ploss et al., 2009). Luciferase activity in tissue-culture fluid of JC1flag-infected cells is directly proportional to the TCID$_{50}$ ml$^{-1}$ (not shown; $r^2=0.9988$). The entire HVR1 region and the N-terminal FLAG epitope tag were simultaneously deleted (JC1ΔHVR1). Deletion of the FLAG epitope tag alone from the parental JC1flag vector did not alter genome replication, secretion of viral particles or the infectivity of virions compared to wild-type JC1flag (not shown). The transfected Huh7.5 cells were initially split every 3–4 days for four passages. The supernatants from passage 5 were then used to infect naive Huh7.5 cells, that were then split every 3–4 days for a further five passages. The infectivity of cell-free virus obtained after each passage was monitored by infection of naive Huh7.5 cells and assayed for luciferase activity 48 h later. As was observed for JFH1 lacking HVR1 (Fig. 3a), cell-free virus

![Graph](attachedGraph.png)

**Fig. 4.** HCVcc viruses containing a deletion of HVR1 acquire mutations in E1 and E2. (a) Huh7.5 cells were transfected with either wild-type or HVR1-deleted JC1flag RNA and cells were passaged a total of 10 times. Cell-free virus obtained at 48 h from each cell passage of WT JC1flag (■), JC1ΔHVR1 (□) or replication-defective JC1flag GND (○) was used to infect naive Huh7.5 cells. Luciferase activity in the supernatant fluid was measured at 48 h. (b) Nucleotide sequence of viral cDNA clones obtained at passage 10 from JC1ΔHVR1 virus.
obtained from JC1ΔHVR1 cultures displayed approximately 10-fold lower levels of infectivity compared with wild-type JC1flag at passages 1–4 (Fig. 4a). At passage 5, cell-free virus was used to infect naive Huh7.5 cells and resulted in a 100-fold drop in infectivity for JC1ΔHVR1, suggesting that reversion had not yet occurred. Cell passaging was therefore continued until wild-type levels of infectivity were observed. Wild-type levels of replication were observed for JC1AHVR1 virus from passage 9 onwards (Fig. 4a). Sequencing of cloned cDNA prepared from cell-free JC1AHVR1 viral RNA obtained from passage 10 revealed the presence of two predominant second-site mutations within E1 (I262L) and E2 (N415D), occurring at a frequency of 35 and 27%, respectively (Fig. 4b).

A mutation in E1 partially restores infectivity, while a mutation in E2 fully restores infectivity to JC1ΔHVR1 virions

The I262L and N415D mutations were inserted individually into the JC1ΔHVR1 vector in order to determine whether they could restore viral infectivity. Huh7.5 cells transfected with JC1flag, JC1ΔHVR1, JC1ΔHVR1 (I262L) or JC1ΔHVR1 (N415D) RNA showed equivalent levels of RNA replication, indicating that the mutations did not alter genome replication significantly (Fig. 5a).

The infectivity of the viruses produced 48 h post-transfection was examined by incubation of naive Huh7.5 cells with an equivalent infectious dose of each virus and measurement of luciferase activity at 48 h post-infection. The insertion of I262L into the parental JC1ΔHVR1 genome resulted in a modest, 2.5-fold improvement of viral infectivity, whereas N415D restored cell-free virus infectivity to wild-type levels (Fig. 5b). The N415D mutation has been reported previously to arise after long-term propagation of HCV in the cell-culture system (Dhillon et al., 2010). However, sequencing of wild-type virus subjected to the same number of passages did not identify any second-site mutations in this study. The N415D mutation has been shown previously to increase the affinity of JFH-1 for CD81 (Dhillon et al., 2010), suggesting that a possible mechanism of reversion of the HVR1-deleted viruses carrying N415D in this study is through increased binding to CD81. In the study by Bankwitz et al. (2010), an alternative mechanism of reversion of virions lacking HVR1 was identified. The second-site mutation I347L partially restored fusion activity and partially increased the infectivity of low-density particles lacking HVR1 (Bankwitz et al., 2010). Our study indicates that mutations within E1 and/or E2 are sufficient to restore infectivity of viruses lacking HVR1. The HVR1 region is therefore dispensable for virus replication but improves virion infectivity.

The results of this study suggest that deletion of either the igVR or HVR2 disrupted folding of all three immunogenic domains in the E1E2 heterodimer, consistent with a global folding defect. These results contrast strongly with those observed for deletion of the variable regions in E2αα1, where all three regions could be deleted individually and in combination without affecting MBP–LEL113–201 binding or recognition by domain A-, B- and C-specific mAbs (McCaffrey et al., 2007, 2009). Our results suggest that, when full-length E2 is co-expressed with E1, the productive folding pathway of E2 is heavily influenced by the presence of E1 and the additional C-terminal E2 sequence (residues 662–746) and is dependent on the presence of both HVR2 and the igVR. These studies provide further insights into the role of HVR2 and the igVR in E1E2 folding and function.

**METHODS**

**Cell lines and antibodies.** HEK 293T and Huh7 cells were maintained in Dulbecco’s minimal essential medium containing 10% fetal calf serum (FCS) and 2 mM L-glutamine (DMF10). Huh7.5 cells were maintained in DMF10 supplemented with 0.1 mM non-essential amino acids (DMF10NEA). mAbs A4 (anti-E1), H53 (anti-E2) and H52 (anti-E2) (Deleersnyder et al., 1997; Dubuisson et al., 1994) were a kind gift from Dr Jean Dubuisson (Institut Pasteur de Lille, Lille, France), while E2-specific mAbs CBH-4D and CBH-71 (Foung (Stanford University, California, USA). Immunoglobulin G (IgG14; Amersham Pharmacia Biotech).

**Fig. 5.** Infectivity of ΔHVR1 JC1flag viruses is partially restored by I262L and restored completely by N415D. (a) Huh7.5 cells were transfected with equivalent amounts of in vitro-synthesized RNA transcripts of JC1flag, JC1AHVR1, JC1AHVR1 (I262L) or JC1AHVR1 (N415D) or JC1flag GND (GND). Luciferase activity in cell lysates was measured 48 h post-transfection. (b) Naive Huh7.5 cells were infected with cell-free virus obtained 48 h after transfection. Luciferase activity in tissue-culture fluid was measured 48 h later.
Vectors and mutagenesis. Construction of the pCDNA4HisMax (Invitrogen)-based expression vector pH77c E1E2 has been described previously (Drummer et al., 2003). The HIV-1 luciferase reporter vector pNL4-3.LUC.R.E was obtained from Dr Nathaniel R. Landau (New York University School of Medicine, New York, USA) through the NIH AIDS Research and Reference Reagent Program (He et al., 1995). The vector pJFH1 (Wakita et al., 2005) was a kind gift from Takaji Wakita (Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan). The vector pJC1FLAG2(p7-NS-GLUC2A) (JC1flag) was a kind gift from Charles Rice (Rockefeller Institute, New York, USA). pJC1FLAG2(p7-NS-GLUC2A) comprises the structural region (core–p7) of HCV-J6 (genotype 2a) and the non-structural region (NS2A–NS5B) of JFH1 (Ploss et al., 2009). A FLAG epitope tag (GSGADYKDDDDK) is located at the N terminus of HVR1, adjacent to the signal peptide cleavage site between E1 and E2. In vitro mutagenesis of the pH77c E1E2 vector was carried out by standard overlap extension PCR techniques. Deletion of the E2 variable regions or insertion of point mutations in pJC1FLAG2(p7-NS-GLUC2A) or pJFH1 was performed using overlap extension mutagenesis. Sequences of primers can be obtained on request. For pJFH1, the amplified fragment was digested with BstWI and KpnI restriction enzymes and cloned into similarly digested pJFH1. For JC1flag, the amplified fragment was digested with BstWI and NotI and cloned into similarly digested JC1flag. The sequence of the entire subcloned fragment was confirmed using Big Dye terminator chemistry.

E1E2-pseudotyped HIV-1 particle entry assay. Pseudotyped particle entry assays were performed as described by Drummer et al. (2003). Briefly, retroviral pseudotypes containing the HCV glycoproteins (HCVpp) were produced by cotransfecting HEK 293T cells with wild-type or variable-region-deleted forms of pH77c E1E2 or cDNA4 empty vector (empty) plus pNL4-3.LUC.R.E. At 72 h post-transfection, culture supernatants were fractionated (0.45 mm) and applied to Huh7 cell monolayers seeded the day before at 3 × 10^6 cells per well. Luciferase was measured 72 h later with a Fluostar (BMG LabTechnologies) fitted with luminescence optics using the Promega luciferase reagent system.

Western blotting and radioimmunoprecipitation. To examine overall E1 and E2 expression, 293T cells were transfected with 2 μg wild-type or variable-region-deleted forms of pH77c E1E2 and lysed 48 h later (PBS containing 1 % Triton X-100 and 1 mM EDTA). Lysates were examined in reducing SDS-PAGE following transfer to 0.2 μm nitrocellulose membranes. The E1 and E2 proteins were visualized with either 4 or 1 μg RNA using 10 or 2.5 μM DMRIE reagent (Invitrogen) and 1 or 0.25 ml Optimem (Invitrogen), respectively. At 6 h post-transfection, the medium was removed and replaced with DMF10NEA.

RNA transcription and transfection. Wild-type and mutated HCV RNA was transcribed in vitro from Xbal-linearized pJFH1 or JC1flag using Ampliscribe T7 High Yield Transcription kits (Epicient Biotechnologies). Huh7.5 cells were seeded at either 700 000 cells per well in 6-well culture dishes or 125 000 cells per well in 24-well culture dishes (Nunc) and transfected 24 h later with either 4 or 1 μg RNA using 10 or 2.5 μM DMRIE reagent (Invitrogen) and 1 or 0.25 ml Optimem (Invitrogen), respectively. For JC1flag, the sequence of the entire subcloned fragment was confirmed using Big Dye terminator chemistry.

Solid phase E1E2–CD81 binding assay. The expression and purification of a chimera composed of maltose-binding protein linked to CD81 large extracellular loop residues 113–201 (MBP–LE1.13–201) has been described previously (Drummer et al., 2002). Solid-phase CD81-binding assays using HCVpp were performed as described by Drummer et al. (2006). The amount of viral lysate applied was normalized according to E2 content as described by Drummer et al. (2006). The plates were read at 450 nm and the background absorbance at 650 nm was subtracted. Binding is represented as the percentage relative to wild-type HCVpp and assays were performed on three independent preparations of HCVpp.

Culture of JFH1 and JC1ΔHVR1 viruses. Huh7.5 cells were seeded at a density of 7 × 10^5 cells per well in 6-well culture dishes and transfected with 4 μg in vitro-synthesized RNA transcripts using DMRIE reagent (Invitrogen). After 3–4 days, cells were trypsinized and transferred into T25 flasks (Becton Dickenson) and trypsinized 3–4 days later and transferred into a T75 flask. Afterwards, cells were passaged at 3- to 4-day intervals by trypsinization followed by reseeding at a 1:3 to 1:4 split into fresh culture vessels. For pJFH1 cultures, a subset of cells at each split was seeded onto coverslips for immunofluorescence analysis. For JC1flag and JC1ΔHVR1 constructs, cells were split every 3–4 days post-transfection. Cell-free virus present in supernatants from passage 5 was used to infect naïve Huh7.5 cells and split into fresh culture vessels every 3–4 days. For JC1flag and JC1ΔHVR1 constructs, lucimunisence in tissue-culture supernatants was quantified using the Renilla luciferase system (Promega) in a Fluostar Optima fitted with luminescence optics (BMG Labtechnologies). Virus stocks were stored at −80 °C until further use. The TCID50 ml−1 was determined according to the method of Lindenbach et al. (2005) using the Reed–Muench calculator (Reed & Muench, 1938).

Immunofluorescence analysis. Cells obtained after transfection or at each cell passage were seeded onto glass coverslips in 24-well cell culture plates. Monolayers were washed three times with PBS and fixed with 4 % paraformaldehyde for 10 min at room temperature, permeabilized in 0.5 % Triton X-100 in PBS for 5 min and washed three times. Prior to addition of antibody, blocking was performed with PBS containing 5 % FCS. Cells were incubated with primary anti-NS5A antibody (9E10) (1: 5000) and bound antibody was detected with Alexa Fluor 488 goat anti-mouse antibody (Invitrogen) (1:1000) for 1 h in the dark. Cellular nuclei were counterstained with propidium iodide diluted 1 : 5000 for 10 min in the dark. Fluorescent slides were examined using the Bio-Rad MRC1024 confocal microscope at the AMREP imaging facility.

Identification of cell culture suppressor mutations. Total viral RNA was extracted from 140 μl supernatant fluid harvested after passage 10 for JC1ΔHVR1 using a QIAamp viral RNA kit (Qiagen) in accordance with the manufacturer’s specifications. cDNA of the respective viral RNA was synthesized using the SuperScript III One-Step RT-PCR system (Invitrogen), according to the manufacturer’s instructions, digested with EcoRI/XhoI and cloned into similarly digested pcDNA4 (Invitrogen). The DNA sequence of the entire E1 and E2 region of 25 independent clones was determined using Big Dye terminator chemistry.
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