Entry of hepatitis C virus pseudotypes into primary human hepatocytes by clathrin-dependent endocytosis

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Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. Studies of the early steps of HCV infection have been hampered by the lack of convenient in vitro or in vivo models. Although several cell-surface molecules that mediate the binding of HCV envelope proteins to target cells have been identified, mechanisms of viral entry into human hepatocytes are still poorly understood. Vesicular stomatitis virus/HCV pseudotyped viruses expressing the HCV envelope glycoproteins on the viral envelope were generated and it was found that their entry into human hepatocytes required co-expression of E1 and E2 on the pseudotype surface. Neutralization of pseudotype infection by anti-HCV antibodies suggested that cellular entry was mediated by HCV envelope glycoproteins and by previously characterized cell-surface molecules, including CD81. An entry assay based on the release of a fluorochrome from labelled HCV pseudotypes provided evidence for a pH-dependent fusion of the pseudotype envelope with a cellular compartment. By using a panel of endocytosis inhibitors, it is postulated that penetration of HCV into primary cultures of hepatocytes takes place by clathrin-mediated endocytosis.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of cirrhosis (Alter et al., 1989) resulting in hepatocellular carcinoma. Therapeutic options are of limited efficacy and high cost and a preventive vaccine is not yet available. HCV is a flavivirus with a precursor polyprotein processed by proteases to yield structural (Core, E1, E2 and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins. The viral envelope is formed by E1 and E2 glycoprotein heterodimers that localize in the endoplasmic reticulum (ER) (Deleersnyder et al., 1997; Dubuisson et al., 1994), where HCV budding occurs, and which are essential for virus entry into cells (Flint & McKeating, 2000).

Different host-cell receptors or ligands have been reported to interact with HCV, but the evidence appears to be contradictory. Thus, it has been shown that, in vitro, E2 can specifically bind human CD81, a member of the tetraspanin family (Pileri et al., 1998), although this interaction may not result in virion internalization (Petracca et al., 2000). However, CD81 expression in non-permissive hepatoma cells did restore the cells' susceptibility to HCV pseudotypes (Bartosch et al., 2003b), and CD81 silencing in Huh-7.5 cells inhibited human immunodeficiency virus (HIV)/HCV infection (Zhang et al., 2004). In contrast, expression of human CD81 by transgenic mice did not confer susceptibility to HCV infection (Masciopinto et al., 2002).

Another receptor candidate is the low-density lipoprotein receptor (LDLr) (Thomssen et al., 1992; Wünschmann et al., 2000). Accordingly, binding of HCV to LDLr has been reported to promote internalization of the virus (Agnello et al., 1999).

Cell-surface glycosaminoglycans may act as low-affinity receptors for HCV (Germi et al., 2002), allowing the interaction of HCV with specific receptors with a higher
affinity. Thus, HCV E2 was able to bind heparan sulfate proteoglycans with a high affinity (Barth et al., 2003).

Other molecules proposed as HCV receptors are the human scavenger receptor class B type I (SR-BI) (Bartosch et al., 2003b; Scarselli et al., 2002), the lectins L-SIGN and DC-SIGN (Gardner et al., 2003; Lozach et al., 2003; Pohlmann et al., 2003) or the asialoglycoprotein receptor (ASGP-R; Saunier et al., 2003).

After attachment, enveloped viruses enter cells by one of two major pathways: direct fusion at the plasma membrane, which is pH-independent, or endocytosis followed by fusion with the vesicle membranes, which is pH-dependent.

Flaviviruses enter cells via endocytosis and it was suggested early on that HCV uses this pathway. HCV E1 and E2 were expressed at the plasma membrane and fusion was measured by activation of a reporter gene in the target cell (Takikawa et al., 2000). In this model, it was shown that E1 and E2 fusion was enhanced at low pH, suggesting that HCV enters cells via the endosomal pathway and that E1 and/or E2 undergo conformational modifications that allow fusion of viral and cellular membranes. These observations have been confirmed by using HIV/HCV pseudotypes (Hsu et al., 2003).

Clathrin, a molecule implicated in endocytosis (Brodsky et al., 2001), forms invaginations named clathrin-coated pits. Caveolae, spherical invaginations of the plasma membrane, are also associated with vesicles, cholesterol and sphingolipid-rich structures (Stan, 2002). Internalization by caveolae seems to be slower than clathrin-dependent endocytosis and would only take place in activated cells (Thomsen et al., 2002). Macropinocytosis has been described as a non-specific internalization pathway because fixation of the virus on its receptor is not required.

To characterize the molecular mechanism of viral entry into human primary cultures of hepatocytes, we generated vesicular stomatitis virus (VSV)/HCV pseudotypes expressing the HCV envelope glycoproteins on the viral envelope. Here, by using an entry assay based on the release of a fluorochrome from labelled HCV pseudotypes, we provide evidence for the pH-dependent fusion of the pseudotype envelope with a cellular compartment and demonstrate that entry of HCV pseudotypes into primary cultures of human hepatocytes requires passage through the clathrin-dependent endosomal pathway.

**METHODS**

**Cells.** Baby hamster kidney cells (BHK-21, CCL-10), HepG2 human hepatoma cells (HB-8065) and Hekt 293 human embryonic kidney cells (CRL-1573) were obtained from the ATCC. The human hepatoma cell line Huh-7 was provided by R. Bartenschlager (University of Heidelberg, Germany). BHK-21 cells were maintained in Glasgow medium (GMEM; Invitrogen) with 5% fetal calf serum (FCS; Invitrogen), 10% tryptose phosphate broth and gentamicin (50 μg ml⁻¹). Huh-7 cells were maintained in DMEM with 10% FCS, 1 mM sodium pyruvate (Sigma) and gentamicin (50 μg ml⁻¹). Huh-7 cells were maintained in DMEM with 10% FCS, 1× glutamax, 1 mM sodium pyruvate, 1× non-essential amino acids (Invitrogen) and gentamicin (50 μg ml⁻¹). Primary human hepatocytes (David et al., 1998) were maintained in William’s E medium (Sigma Aldrich) with antibiotics, 1× glutamax, 4 μg porcine insulin ml⁻¹ (Sigma), 10% FCS, 2% DMSO (Merck) and 10⁻² M dexamethasone (Sigma).

**Construction of recombinant adenoviruses.** Plasmids pVSV-GLi (J. K. Rose, Yale University, New Haven, CO, USA) and p901-HCV-FL-long pU (strain H77) (C. M. Rice, Washington University, St Louis, MO, USA) (Kolykhhalov et al., 1997) were used to subclone the transmembrane and cytoplasmic domains of VSV G (Tmg) and the HCV E1 and E2 ectodomains, respectively, pEGFP-C1 (Clontech) was used as a template to amplify by PCR the enhanced green fluorescent protein (EGFP) coding sequence.

The chimeric gene encoding, from 5’ to 3’, E1 signal sequence, EGFP, E1 ectodomain and VSV G transmembrane and cytoplasmic regions was obtained by overlapping PCR (Fig. 1). The resulting PCR fragment was digested by NheI and MluI and cloned into pGTG13387 (Transgene SA) digested with the same two enzymes, downstream of a cytomegalovirus promoter sequence. This plasmid was called pVL520. The expression block containing the promoter and the transgene was then transferred into pTG6624, containing the entire adenovirus 5 genome (Transgene SA), by homologous recombination. The resulting plasmid is pVL521. A similar strategy was used to obtain pVL504 (E2 truncated at aa 661 fused with Tmg). The resulting fragment was cloned into pTG13387 and transferred into the adenovirus genome, generating plasmid pVL514 (E2–Tmg). Final plasmids pVL521 and pVL514 were then transfected into 293 cells by using the calcium phosphate precipitate method to obtain recombinant adenoviruses AdVL521-E1 and AdVL514-E2, respectively (Fig. 1).

**Viruses.** Isolate VSV νO45 (F. Lafay, Laboratoire de génétique des virus, Gif-sur-Yvette, France) is a normally enveloped VSV showing no virus particles budding at 40–5°C, but behaving as wild-type VSV when cultivated at 33–37°C, when virus progeny is found in supernatants. It was grown in BHK-21 cells with GMEM with 2% FCS. Infection was performed with an m.o.i. of 0.1 to 24 h at 33°C. Progeny VSV νO45 was titrated in cell supernatants by plaque assay with BHK-21 cells infected for 48 h at 33°C. VSV νO45 was also used as a control in cell fusion and infection assays performed at respectively 37 and 33°C. Recombinant vaccinia virus (VVIV205-E1E2) expressing HCV E1 and E2 was provided by F. Kien (Kien et al., 2003).

VSV/HCV pseudotypes were obtained as follows: BHK-21 cells were infected with AdVL521-E1 and AdVL514-E2 for 4 h at 37°C. Cells were then superinfected with VSV νO45 for 24 h at the non-permissive temperature of 40–5°C. Supernatants were ultracentrifuged at 110,000 g for 2 h at 4°C on a 25% (v/v) glycerol cushion. The pellets containing the pseudotypes (VSV/HCV-E1E2) were suspended in PBS and aliquots were frozen at −80°C. Pseudotypes bearing chimeric E1 (VSV/HCV-E1) or chimeric E2 (VSV/HCV-E2) were obtained by omitting one of the recombinant adenoviruses during the infection.

**Antibodies.** We used a conformation-sensitive mouse monoclonal antibody (mAb), H53 (Delaersnyder et al., 1997), against HCV E2 (J. Dubuisson, CNRS-FRE 2369, France), mAb A4 against HCV E1 (H. B. Greenberg, Stanford University School of Medicine, CA, USA), antibodies 18BS and 4G10 specific for VSV-G (A. Lamarrre, University of Zurich, Switzerland) and mAbs against CD81 and LDLr, EGFP and gp120 of Feline immunodeficiency virus (Immunotech, Tebu Santa Cruz, Clontech and Merial, respectively). Human sera came from patients initially positive for HCV RNA in whom...
viraemia became undetectable by RT-nested PCR. These samples were positive for anti-HCV antibodies as assessed by commercial diagnostic assays.

Radio-immunoprecipitation assay. Huh-7 cells (2 × 10⁶ cells) were infected with AdIV521-E1 or AdIV514-E2 (m.o.i. of 100) for 48 h at 37°C. After 1 h starvation with medium deficient in methionine and cysteine, cells were incubated with 10 μCi (370 kBq) 35S-labelled amino acids (NEN Life Science Products) for 5 h. Cells were then washed twice with PBS before lysis with N buffer [0-2% NP-40, 100 mM Tris/HCl (pH 8-8), 150 mM NaCl, 2 mM EDTA, 1μM PMSF, 1-5 μg aprotinin ml⁻¹]. Cell lysates were sonicated and cellular fragments were pelleted by centrifugation for 5 min at 13 000 g. Supernatants were incubated with mAb anti-EGFP, anti-E1 A4 or anti-E2 H53 (dilution 1/500) for 4 h at 4°C. Protein A/G Sepharose (Tebu Santa Cruz) was then added and samples were incubated overnight at 4°C. After centrifugation at 13 000 g for 5 min, pellets were washed three times with N washing buffer [0-2% NP-40, 100 mM Tris/HCl (pH 8-8), 500 mM NaCl, 1 mM Tris/HCl, pH 8-8]. Pellets were resuspended in SDS-PAGE sample buffer [360 mM Tris/HCl (pH 6-8), 1% SDS, 36% glycerol, 6% β-mercaptoethanol] and proteins were submitted to electrophoresis on a 7-5% SDS-polyacrylamide gel and autoradiographed.
**R18 labelling of viruses.** VSV/HCV pseudotypes and VSV\(\times\)O45 were diluted in PBS (1/10) and the amount of protein was determined (Bradford method). The lipophilic probe R18 (octadecylrhodamine B chloride) (Molecular Probes) was then added (1 mg ml\(^{-1}\)) and mixed with the viruses. Incubation was performed in the dark at room temperature for 20 min with frequent agitation. The non-adsorbed probe was eliminated by dialysis with a threshold of 10 kDa against 1 L PBS for 4 h, repeated three times. Labelled viruses were titrated and aliquots were stored at \(-80^\circ\text{C}\). Incorporation of R18 was estimated by fluorescence emitted at 560 nm.

**Flow cytometry.** Huh-7 cells (7\(\times\)10\(^5\)) infected with AdIV521-E1 or AdIV514-E2 (m.o.i. of 100) or with VVIV205-E1E2 (m.o.i. of 0-1) were removed at 48 h post-infection with PBS depleted in Ca\(^{2+}\) (Invitrogen) and pelleted at 400 g for 5 min. Cells were washed three times with PBS before incubation at 33 \(^\circ\text{C}\). Incubation was performed in the presence or absence of 0-3\% saponin with anti-E2 mAb H53 (1/100) or anti-E1 mAb A4 (1/100) for 30 min. Cells were then washed three times with PBS or saponin (0-1%) and incubated with phycoerythrin-conjugated goat anti-mouse IgG (Immunootech, 1/200 dilution) for 30 min. Cells were washed and fixed in 1-5\% paraformaldehyde and flow cytometry was performed (Becton Dickinson FACscan).

**Neutralization of VSV/HCV-E1E2 and VSV\(\times\)O45 cell fusion and infection.** Neutralization assays were performed on primary human hepatocytes, BHK-21 and Huh-7 cells, VSV\(\times\)O45 and VSV/HCV R18-labelled virus (50 and 0-001 p.f.u., respectively, for fusion and infection assays) were incubated with mAbs to VSV-G 18B5 (1/7500) or 4G10 (1/7500), mAb to CD81 (1/25), mAb to LDLr (1/25) or mAb to IV gp120 (1/200) and serum from HCV-seropositive, but HCV RNA-negative patients (H3439) (1/10) for 30 min at room temperature. Cells were then infected with the neutralized virus. If R18-labelled membranes from the pseudotypes fuse with other membranes, R18 can split up into target membranes and fluorescence is emitted. In this assay, incubation lasted for 20 min in the dark at 4 \(^\circ\text{C}\). Cells were then washed with PBS and incubated with the culture medium. After 30 min at 37 \(^\circ\text{C}\), cells were washed and fixed in 1-5\% paraformaldehyde. Fluorescence was measured by flow cytometry on a FACscan apparatus. For virus infection assays, adsorption was performed at room temperature for 45 min. Cells were washed three times with PBS before incubation at 33 \(^\circ\text{C}\). Supernatants were recovered at 48 h p.i. and production of progeny virus was titrated by plaque assay.

**Viral entry and infection.** BHK-21, HepG2 or Huh-7 cells (2\(\times\)10\(^6\)) or primary human hepatocytes (8\(\times\)10\(^3\)) were incubated with PBS adjusted to different pH values for 20 min at 4 \(^\circ\text{C}\). Labelled viruses were incubated with cells at 4 \(^\circ\text{C}\) for the dark. After 30 min, Cells were then washed and incubated in PBS adjusted to different pH values for 30 min at 37 \(^\circ\text{C}\) and fixed in 1-5\% paraformaldehyde. Fluorescence was determined by reading the emitted fluorescence on a Becton Dickinson FACscan. For virus infection assays, cells were incubated at a given pH for 30 min at 33 \(^\circ\text{C}\) and then with culture medium for 48 h at 33 \(^\circ\text{C}\). Production of progeny virus was measured by titration by plaque assay of the supernatants.

**Inhibition of VSV/HCV-E1E2 endocytosis.** Inhibition of VSV/HCV-E1E2 endocytosis was assessed by cell treatment with lysosomotropic drugs. BHK-21, HepG2 or Huh-7 cells (2\(\times\)10\(^3\)) or primary human hepatocytes (8\(\times\)10\(^3\)) were incubated with 10 \(\mu\)M monensin, 100 \(\mu\)M chloroquine or 300 \(\mu\)M NH\(_4\)Cl (Sigma Aldrich) for 30 min at 37 \(^\circ\text{C}\) and then infected with VSV\(\times\)O45 or VSV/HCV pseudotypes (m.o.i. of 50). After 45 min, cells were washed three times and culture medium was added. Incubation was performed at 33 \(^\circ\text{C}\) for 6 h, then supernatants were recovered and titrated for progeny VSV\(\times\)O45. The toxicity of the drugs tested was assessed by evaluating the cell viability by trypan blue exclusion.

In order to characterize the endocytic pathway used by VSV/HCV-E1E2 to enter host cells, we measured viral fusion to cells treated with different endocytosis inhibitors. Cells were incubated with 100 \(\mu\)M chlorpromazine, 10 \(\mu\)M cytochalasin D, 10 \(\mu\)M brefeldin A (BFA) or with 0-05 % filipin (Sigma Aldrich). After 20 min incubation at room temperature, medium was replaced with labelled viruses and cells were incubated for another 30 min at 4 \(^\circ\text{C}\) in the dark. Cells were then washed and incubated at 37 \(^\circ\text{C}\) for 30 min with culture medium containing the inhibitor tested. After washing, cells were fixed in 1-5\% paraformaldehyde and analysed by flow cytometry.

Assays of pH and lysosomotropic-drug sensitivity were carried out with cells exposed to different pH values for 30 min or to different lysosomotropic drugs for 75 min, the viability always being followed and showing high viable-cell percentages (>95\%). Under these conditions, previous experiments had shown that cell populations remained highly viable up to 6 h treatment (data not shown). In addition, data of VSV\(\times\)O45 titre (Fig. 2b) and endocytosis inhibition in BHK cells (Fig. 3), indicating, respectively, high virus titres and high percentage of fluorescent cells, ensured the good metabolic state of cells under, respectively, pH or drug treatment.

**RESULTS**

**Generation and characterization of HCV pseudotypes.** To produce VSV/HCV pseudotypes, truncated forms of the E1 and E2 glycoproteins were fused to the transmembrane domain and cytoplasmic region of VSV-G. The coding sequence for EGFP was fused to the 3’ extremity of the chimeric E1–TmG gene.

Chimeric E1 was detected by mAb A4 and modified E2 by conformation-sensitive mAb H53, suggesting that the transmembrane and cytoplasmic domains of VSV-G do not modify the correct E2 folding. Cells infected with AdIV521-E1 or AdIV514-E2 without saponin permeabilization exhibited strong staining by anti-E1 and anti-E2 antibodies. Native E1 and E2 expressed by VVIV205-E1E2 were only detected when cells were previously permeabilized by saponin. These results confirm the intracellular localization of native E1 and E2 expressed by VVIV205-E1E2 and indicate that both modified proteins are expressed at the cell surface by recombinant adenoviruses (Fig. 1). Similar results were obtained when modified E1 and E2 were expressed at 40-5 \(^\circ\text{C}\) (data not shown).

To generate HCV pseudotypes, BHK-21 cells were infected at 37 \(^\circ\text{C}\) with recombinant adenoviruses to express modified E1 and E2 and then superinfected by VSV\(\times\)O45 at the non-permissive temperature of 40-5 \(^\circ\text{C}\). At this temperature, VSV nucleocapsids can assemble normally, but VSV-G is retained in the ER. Budding VSV nucleocapsids will be enveloped by HCV E1 and E2 that are localized at the plasma membrane, leading to production of HCV pseudotypes.

VSV\(\times\)O45 is characterized by a single amino acid substitution in G, leading to its misfolding and retention in the ER at 40-5 \(^\circ\text{C}\) (Whitt et al., 1989). This results in the production
and budding of non-enveloped and, thus, non-infectious VSV virions (Schnitzer et al., 1979).

To test for VSV<sub>ts</sub> leakiness, we infected BHK-21 cells at 40-5 °C for 24 h and the supernatant was used to infect BHK-21 cells at 33 °C, a temperature that does not allow the production of VSV<sub>ts</sub>O45. Moreover, we were not able to detect any G at the surface of BHK-21 cells infected at 40-5 °C (data not shown). These results demonstrate the efficient retention of VSV-G in the ER at 33 °C.

**Entry of HCV pseudotypes into primary human hepatocytes and hepatoma cell lines is HCV envelope-dependent and requires CD81**

We performed experiments of virus fusion and infection, as well as neutralization by antibodies directed to viral or cellular structures by using the lipidic R18 probe.

Cells were exposed to VSV/HCV pseudotypes bearing either chimeric E1 (VSV/HCV-E1) or chimeric E2 (VSV/HCV-E2) or both E1 and E2 (VSV/HCV-E1E2). We observed VSV/HCV pseudotype entry only when both modified E1 and E2 were present at the virion surface (VSV/HCV-E1E2), indicating that co-expression of E1 and E2 on the HCV pseudotype surface is required for virus entry into cells. Results obtained with primary hepatocytes, HepG2 and BHK-21 cells (data not shown) were very similar to those shown for Huh-7 cells (Fig. 2).

Serum-derived anti-HCV antibodies efficiently neutralized entry of HCV pseudotypes into primary human hepatocytes or Huh-7 hepatoma cells (Fig. 2). Entry of VSV<sub>ts</sub>O45 was only neutralized by anti-VSV antibodies. Antibodies directed against the FIV gp120 did not affect the infection of either HCV E1/E2 pseudotypes or VSV<sub>ts</sub>O45. These results confirm the absence of residual VSV-G at the surface of the

**Fig. 2.** Fusion (a) and infection (b) of VSV/HCV pseudotypes and VSV<sub>ts</sub>O45 with primary hepatocytes, Huh-7 and BHK-21 cells and their neutralization by specific antibodies. Cells were treated with the indicated viruses and antibodies and maintained at 37 °C for fusion assays or 33 °C for infection assays. Fusion was determined by measuring the fluorescence emitted by the R18 probe when it was diluted into unlabelled membranes. Infection is measured by titrating the progeny virus at 48 h p.i. Data are the results of three independent experiments with SD. *Not determined.
virions and demonstrate that HCV pseudotype infection is mediated by HCV envelope E1 and E2. Inhibition of HCV pseudotype entry by anti-HCV antibodies was reproducible for a large series of sera from HCV-seropositive patients (data not shown).

Primary human hepatocytes and Huh-7 cells were pre-incubated with a mAb shown to block HCV E2–CD81 interaction (Pileri et al., 1998) or the uptake of serum-derived HCV virions into target cells (Agnello et al., 1999). HCV E1/E2 pseudotype entry into primary hepatocytes and Huh-7 cells was reduced markedly by these antibodies (Fig. 2). In contrast, VSV<sub>tsO45</sub> infection of primary hepatocytes, Huh-7 or BHK-21 cells was not altered by pre-incubation of cells with anti-CD81 or anti-LDLr. These data indicate that VSV/HCV entry is mediated, at least in part, by CD81 and LDLr.

**HCV pseudotype entry into human hepatocytes is pH-dependent**

Optimal pH required for VSV<sub>tsO45</sub> entry was 5.6 (Marsh & Helenius, 1980) (Fig. 3a). Viral entry was observed for all cell lines tested. VSV<sub>tsO45</sub> was able to fuse at a high extent to BHK-21 and to a lower extent to HepG2 membranes although, in both cases, a comparable degree of infection was observed. In contrast, VSV<sub>tsO45</sub> fusion to Huh-7 cells or to primary human hepatocytes was very low and gave rise to lower titres (Fig. 3).

The optimal pH for entry and infection of HCV pseudotypes was 6.0. It was similar for all cells, although the efficiency varied markedly between cell lines. VSV/HCV pseudotype entry was more efficient in human hepatocytes and in Huh-7 cells than in BHK-21 and HepG2 cells (Fig. 3). For
non-hepatic cells, such as BHK-21 cells, the VSV titres were higher, but for typically hepatic cells, such as primary hepatocytes, VSV/HCV infection led to higher titres (Fig. 3b).

The fact that entry and infection of VSV/HCV pseudotypes were more efficient at acidic pH indicates that they enter host cells via a pH-dependent pathway. Moreover, the observation that infection efficiency was dependent on the cell type may reflect the interaction of different cell-surface molecules required for entry of VSV<sub>ts</sub>O45 or VSV/HCV pseudotypes.

**HCV pseudotype entry into human hepatocytes is mediated by clathrin-dependent endocytosis**

Following cell treatment with lysosomotropic drugs, neither VSV<sub>ts</sub>O45 nor VSV/HCV pseudotype infection led to progeny virion synthesis (Table 1), indicating that virus cell entry occurs via endocytosis.

<table>
<thead>
<tr>
<th>Drug</th>
<th>BHK-21 (VSV&lt;sub&gt;ts&lt;/sub&gt;O45)</th>
<th>VSV/HCV</th>
<th>VSV&lt;sub&gt;ts&lt;/sub&gt;O45</th>
<th>VSV/HCV</th>
<th>VSV&lt;sub&gt;ts&lt;/sub&gt;O45</th>
<th>VSV/HCV</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1·90 ± 0·10</td>
<td>0·45 ± 0·4</td>
<td>1·25 ± 0·54</td>
<td>0·48 ± 0·42</td>
<td>0·7 ± 0·19</td>
<td>1·1 ± 0·64</td>
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<tr>
<td>Monensin</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;Cl</td>
<td>0·02 ± 0·02</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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We observed a strong reduction of VSV/HCV entry into Huh-7 cells following treatment with BFA and chlorpromazine, but not with cytochalasin D or filipin (Fig. 4). Similar results were obtained with primary human hepatocytes, suggesting that clathrin-mediated endocytosis is the major entry pathway used by pseudotypes. In human hepatocytes, filipin also induced a significant reduction of viral entry, indicating that VSV/HCV pseudotypes may also use a caveolae-dependent pathway to enter hepatocytes.

HCV-E1E2 pseudotype entry into HepG2 cells occurred at a much lower efficiency (Fig. 3a). In contrast to findings observed for Huh-7 cells and human hepatocytes, pseudotype entry into HepG2 cells was inhibited mainly by cytochalasin D, indicating that macropinocytosis could be an alternative entry pathway into HepG2 cells. Experiments with VSV<sub>ts</sub>O45 and BHK-21 cells showed that virus fusion was inhibited upon cell treatment with BFA or chlorpromazine, but not after cytochalasin D or filipin treatment.

**Table 1. Virus progeny after cell treatment with lysosomotropic drugs**

Cell cultures were treated with 10 μM monensin, 100 μM chloroquine or 300 μM NH<sub>4</sub>Cl before infection with VSV<sub>ts</sub>O45 or VSV/HCV pseudotypes. Supernatants were recovered after 6 h infection and titrated for progeny virus by plaque assay. Results are expressed as 10<sup>3</sup> p.f.u. ml<sup>−1</sup> and are means ± SD.

Fig. 4. Inhibition of VSV/HCV-E1E2 or VSV<sub>ts</sub>O45 endocytosis. Cells were treated for 30 min with PBS, 10 μM BFA, 100 μM chlorpromazine, 10 μM cytochalasin D or 0·05% filipin and incubated with R18-labelled VSV/HCV-E1E2 or VSV<sub>ts</sub>O45. Cells were then washed, fixed with 1·5% paraformaldehyde and virus fusion was measured. Results are expressed as a percentage of fluorescent cells (*P<0·05; $P<0·01$, significantly different from control).
confirming that VSVtsO45 enters host cells via clathrin-dependent endocytosis (Superti et al., 1987).

**DISCUSSION**

We report here the construction of VSV/HCV pseudotyped particles and experiments performed to understand HCV entry into primary human hepatocyte cells.

Previous studies have shown that E1 truncated at aa 311 and E2 truncated at aa 661 were secreted efficiently by cells (Michalak et al., 1997). In order to modify the normal subcellular localization of HCV E1 and E2, these secreted forms of glycoproteins were fused to the transmembrane domain and the cytoplasmic tail of VSV-G glycoprotein, to provide a membrane-anchor signal and to ensure HCV E1 and E2 glycoprotein attachment to the surface of VSV budding particles (Owens & Rose, 1993; Robison & Whitt, 2000). We show here that E1 or E2 ectodomain fusion to the heterologous transmembrane domain and cytoplasmic tail of VSV glycoprotein drove their localization to the cell surface. Although further studies on E1 and E2 fusogenic regions can be envisaged, the truncated E1 and E2, as employed in this study, led to the establishment of functional VSV/HCV pseudotypes, as measured by fusion and infection assays. Moreover, the recognition of E2 by a conformation-sensitive antibody indicates that the glycoprotein is folded correctly.

Different methods have been used to generate VSV/HCV pseudotypes (Lagging et al., 1998; Michalak et al., 1997; Seong et al., 1998), but the use of VSVtsO45 to generate HCV pseudotypes has recently been questioned, in view of the possible presence of VSV-G glycoprotein on the pseudotypes produced (Buonocore et al., 2002). In order to test for this possibility, we analysed BHK-21 cells infected with VSVtsO45 at 40–5 °C by flow cytometry; no residual VSV-G glycoprotein was detected in these experiments. Moreover, VSVtsO45 infections performed under the conditions chosen to produce the pseudotypes never led to infectious VSVtsO45 progeny. Lastly, our experiments on virus fusion, infection and neutralization confirmed the differential features of VSV/HCV pseudotypes when compared with the parental VSVtsO45 and excluded the possibility of the eventual presence of VSV-G glycoprotein on the pseudotype surface.

Our results on VSV/HCV-E1E2 interaction with hepatocytes and its neutralization by specific antibodies against viral or receptor molecules indicate that VSV/HCV fuses with and infect primary human hepatocytes, Huh-7 cells and, to a lower extent, HepG2 cells. VSV/HCV-E1 or VSV/HCV-E2 were unable to fuse and infect the studied cells, indicating that both E1 and E2 are required for HCV productive infection, as described in other studies (Bartosch et al., 2003a; Hsu et al., 2003; Matsuura et al., 2001). To generate chimeric proteins, we have conserved the 50 aa of the C-terminal extremity of VSV-G. Indeed, as the VSV-G fusion peptide was localized in the N-terminal region (Fredericksen & Whitt, 1995; Whitt et al., 1990), VSV/HCV-E1E2 fusion could only occur through HCV E1 and/or E2 and not by the transmembrane and cytoplasmic domains of VSV-G.

Our experiments show that antibodies directed against VSV-G only neutralize VSVtsO45 fusion and infection and that HCV patient sera containing antibodies against HCV only neutralize VSV/HCV-E1E2 fusion and infection, indicating that infection by VSV/HCV-E1E2 occurs following binding of E1 and/or E2 to the surface of target cells, further confirming our previous observations that they do not bear VSV-G glycoprotein.

The fact that VSV/HCV-E1E2 can fuse easily to primary hepatocytes and Huh-7 cells suggests that high-affinity receptor molecule(s) are present on the two cell types, whereas these molecules may not be present in sufficient numbers on the surface of BHK-21 and HepG2 cells, which are poor hosts for VSV/HCV-E1E2.

The tetraspanin CD81 was the first molecule proposed to be an HCV receptor (Pileri et al., 1998). However, its ubiquitous expression does not explain the restrained tropism of HCV and it seems that interaction of HCV with CD81 does not lead to internalization of the virus (Petracca et al., 2000; Sasaki et al., 2003). Our results show that VSV/HCV-E1E2 fusion and infection are more efficient on primary hepatocytes and on Huh-7 cells than on HepG2 cells and could be neutralized only partially by primary hepatocyte or Huh-7 cell treatment with antibodies directed to CD81. The fact that CD81 is not expressed on HepG2 cells (data not shown) may explain the differential magnitude of virus fusion and infection in these cell lines. One could also hypothesize that, in CD81-bearing cells, VSV/HCV-E1E2 binding to CD81 would allow the particle to move closer to another receptor responsible for internalization, thus enabling an efficient virus infection.

In agreement with our results, it has been shown that LDLr is involved in HCV adsorption (Germi et al., 2002; Triyatni et al., 2002; Wünschmann et al., 2000), suggesting that LDLr–HCV interaction may play a role in internalization, as HepG2 cell treatment with anti-LDLr was shown to inhibit HCV endocytosis (Agnello et al., 1999).

Our findings indicate that HCV may enter hepatic cells following binding to CD81 and/or LDLr, although the relevance of each ligand–receptor interaction is still unknown. No inhibition of VSVtsO45 fusion to or infection of BHK-21, primary hepatocytes or Huh-7 cells was detected upon treatment with antibodies directed to CD81 and LDLr. Altogether, our data on virus interaction with target cells showed clear-cut differential features between our VSV/HCV-E1E2 pseudotypes and their parent, VSVtsO45.

In kinetic assays, we found that pH 6.0 was optimal for VSV/HCV-E1E2 fusion and infection, thus confirming previous reports suggesting that HCV enters host cells via
the endosome (Takikawa et al., 2000; Meyer et al., 2000). In contrast to VSVsO45, VSV/HCV-E1E2 fusion and infection were more efficient in primary hepatocytes and Huh-7 cells than in BHK-21 and HepG2 cells.

Confirming that VSV/HCV-E1E2 entry required passage through the endosome, we have shown that treatment of cells with lysosomotropic amines, such as NH₄Cl or chloroquine, or with a carboxyl ionophore (monensin), currently used to modify the endosomal pH, inhibited viral production completely.

To inhibit clathrin-mediated endocytosis, we used BFA, known to inhibit GTPases implicated in the secretory pathway (Brodsky et al., 2001), and chlorpromazine, a calmodulin antagonist (Salisbury et al., 1980). Our results show that VSV/HCV-E1E2 fusion to primary human hepatocytes and Huh-7 cells was reduced strongly after treatment with BFA or chlorpromazine, indicating that HCV entry into host cells may occur preferentially via clathrin-dependent endocytosis.

Nevertheless, when the assay was carried out with HepG2 cells, a different pattern of endocytosis inhibition was observed. With these cells, the VSV/HCV-E1E2 fusion was reduced strongly by cytochalasin D, suggesting that, in this particular cell type, macropinocytosis may be the main pathway of virus entry. Therefore, primary human hepatocytes and Huh-7 cells are most appropriate to study HCV infection.

As lipid rafts, constituted primarily of cholesterol, are associated with caveolae formation, we tested the effect of filipin (Orlandi & Fishman, 1998), a sterol-binding drug, on VSV/HCV-E1E2 entry. In the presence of filipin, viral entry into Huh-7 cells and primary hepatocytes was partially inhibited, indicating that caveola-dependent endocytosis could be an alternative entry pathway used by HCV to enter host cells.

Hsu et al. (2003) and Lavillette et al. (2005) recently described a similar model by using pseudotyped retroviral particles. Consistent with our results, they show that both E1 and E2 glycoproteins are required to infect cells. The HIV/HCV pseudotyped particles infect Huh-7 cells and the fusion requires low pH exposure. On the other hand, the HIV/HCV pseudotypes do not infect HepG2 cells, whereas VSV/HCV pseudotypes can infect these cells at a low level. This discrepancy might be due to the fact that glycoproteins incorporated into HIV/HCV pseudotypes escape the ER and are transported to the cell surface, resulting in glycosylation modifications not representative of the native infection. In contrast to the study performed by Hsu et al. (2003), we have constructed our pseudotypes by using the VSV heterologous transmembrane domain, which was shown not to affect correct E2 folding. Nevertheless, our results are very similar and suggest that both the homologous and the heterologous transmembrane domains may lead to E1–E2 heterodimerization, allowing these proteins to exert their biological functions involved in cell fusion and infection.

In conclusion, the production of reliable VSV/HCV pseudotypes allowed us to bring new insights into early stages of HCV infection in primary human hepatocytes. We have shown that VSV/HCV pseudotype fusion and infection required both E1 and E2 HCV glycoproteins. Similarly to other flaviviruses (Andoh et al., 1998; Helenius et al., 1982), HCV entry seems to require an acidic environment occurring in the endosome–lysosome system. A clathrin-mediated endocytic mechanism is suggested to be the preferential pathway for HCV entry. However, a caveolae-dependent mechanism may also be involved in other cell types, depending on the specific receptors that recognize particular pseudotypes. As the natural host of HCV is the human hepatocyte, we believe that HCV infection mainly occurs by a clathrin-dependent endocytosis.

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