Assembly of functional hepatitis C virus glycoproteins on infectious pseudoparticles occurs intracellularly and requires concomitant incorporation of E1 and E2 glycoproteins

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Hepatitis C virus (HCV) E1 and E2 envelope glycoproteins (GPs) displayed on retroviral cores (HCVpp) are a powerful and highly versatile model system to investigate wild-type HCV entry. To further characterize this model system, the cellular site of HCVpp assembly and the respective roles of the HCV GPs in this process were investigated. By using a combination of biochemical methods with confocal and electron microscopic techniques, it was shown that, in cells producing HCVpp, both E1 and E2 colocalized with retroviral core proteins intracellularly, presumably in multivesicular bodies, but not at the cell surface. When E1 and E2 were expressed individually with retroviral core proteins, only E2 colocalized with and was incorporated on retroviral cores. Conversely, the colocalization of E1 with retroviral core proteins and its efficient incorporation occurred only upon co-expression of E2. Moreover, HCVpp infectivity correlated strictly with the presence of both E1 and E2 on retroviral cores. Altogether, these results confirm that the E1E2 heterodimer constitutes the prebudding form of functional HCV GPs and, more specifically, show that dimerization with E2 is a prerequisite for efficient E1 incorporation onto particles.

INTRODUCTION

Hepatitis C virus (HCV) has been classified within the genus *Hepacivirus*, family *Flaviviridae* (Robertson *et al.*, 1998), and is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Major *et al.*, 2001). The HCV genome encodes a single, approximately 3000 aa polyprotein precursor that is processed co- and post-translationally by cellular and viral proteases to yield at least 10 polypeptides, comprising structural components including the capsid protein and the envelope glycoproteins (GPs) E1 and E2, as well as non-structural proteins (Lindenbach & Rice, 2001; Penin *et al.*, 2004). HCV replicates its genome in a membrane-associated complex, which is derived from the endoplasmatic reticulum (ER) and has been designated ‘membraneous web’ (Gosert *et al.*, 2003). All HCV polypeptides have been found associated with these membraneous web structures in a direct or indirect fashion (Dubuisson *et al.*, 2002; Egger *et al.*, 2002), suggesting that genome replication occurs intracellularly within these structures (Egger *et al.*, 2002; Penin *et al.*, 2004). Morphogenesis of HCV is thought to occur within the secretory pathway, because particles containing HCV GPs have been detected in cytoplasmic vesicles of cells replicating the wild-type virus (Shimizu *et al.*, 1996). Furthermore, other viruses of the same family, including members of the genus *Flavivirus*, are thought to assemble intracellularly and bud into vesicles of the secretory pathway that are then released from the cell by exocytosis (Lindenbach & Rice, 2001; Mackenzie & Westaway, 2001).

The HCV envelope GPs expressed in heterologous systems were shown to be retained at the ER membrane by retention signals, including charged residues in the middle of their transmembrane domains (Cocquerel *et al.*, 1998, 1999; Flint & McKeating, 1999). Mutation of these charged residues has been shown to abolish ER retention, but also to interfere with heterodimerization of E1 and E2 (Cocquerel *et al.*, 2000; Michalak *et al.*, 1997; Op De Beeck *et al.*, 2000; Patel *et al.*, 2001). Indeed, the transmembrane domains of E1 and E2 play a major role in the assembly of E1 and E2 into

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non-covalently attached heterodimers, which are thought to be the prebudding form of the HCV GPs (Deleersnyder *et al.*, 1997; Dubuisson, 2000).

Since its discovery 16 years ago, HCV has been difficult to study because it does not replicate efficiently or form particles *in vitro*. To establish surrogate model systems for HCV particle production, several laboratories initially tried to develop virus-like particles or pseudotype viruses by, for example, incorporating the HCV GPs onto cores of heterologous viruses, including those of vesicular stomatitis virus (VSV) or influenza virus (Flint *et al.*, 1999; Lagging *et al.*, 1998; Matsuura *et al.*, 2001; Takikawa *et al.*, 2000). In such systems, assembly was thought to take place at the cell surface; therefore, the HCV GPs were retargeted away from the ER to the plasma membrane by mutation or replacement of their transmembrane domains to achieve relocalization and thus incorporation onto heterologous viral cores. Because assembly and functionality of HCV GPs are very sensitive to mutations and deletion within their transmembrane domains and because the HCV GPs have a tendency to misfold and aggregate (Cocquerel *et al.*, 2000; Dubuisson, 2000; Dubuisson *et al.*, 2000), these attempts were mainly unsuccessful (Buonocore *et al.*, 2002). Recently, production methods for replication-competent HCV particles *in vitro* (HCVcc) have been reported; however, they are restricted to safety-level 3 laboratories (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005).

We and others have recently described HCV pseudoparticles (HCVpp) that are assembled by incorporating unmodified, full-length HCV GPs onto oncoretroviral or lentiviral cores (Bartosch *et al.*, 2003b; Drummer *et al.*, 2003; Hsu *et al.*, 2003) that are highly infectious and that seem to mimic the viral entry and serological properties of wild-type HCV (Bartosch *et al.*, 2003a, c; Logvinoff *et al.*, 2004). HCVpp can be produced in large quantities at comparatively high titres and at a convenient safety level. Furthermore, they offer great flexibility in terms of incorporation of marker genes and allow investigation of viral entry independently of replication, as attachment and fusion are mediated by the HCV GPs and post-fusion steps are mediated by retro- or lentiviral core particles. Due to these features, HCVpp are likely to remain a valuable tool that will complement studies with the wild-type virus.

HCVpp are produced by expressing the E1E2 glycoproteins, the retroviral core proteins and a packaging-competent retroviral genome carrying a marker gene in human 293T cells (Bartosch *et al.*, 2003b). Viruses assembled by the 293T producer cells are collected from the supernatant and used to infect naive target cells. Within the 293T producer cells, E1 and E2 are expressed mainly at the ER, but a small fraction traffics to the cell surface (Bartosch *et al.*, 2003b; Drummer *et al.*, 2003; Hsu *et al.*, 2003). Recent insights into retroviral assembly show that assembly and incorporation of diverse viral GPs may not take place at the cell surface, but can occur intracellularly within the endocytic pathway (Nydegger *et al.*, 2003; Pelchen-Matthews *et al.*, 2003; Sherer *et al.*, 2003). In particular, budding of human immunodeficiency virus and *Murine leukemia virus* (MLV) has been shown to occur into multivesicular bodies (MVBs), a late endosomal compartment that can fuse with the cell surface (Gould *et al.*, 2003). We therefore investigated here the cellular site of assembly of MLV-based HCVpp. By using a combination of biochemical methods and confocal and electron microscopy (EM) to reveal the cellular localization of expressed E1 and E2 GPs, as well as MLV core proteins, we show that HCVpp bud intracellularly, presumably into MVBs. In addition, by studying the mutual requirement of E1 and E2 for the formation of infectious particles, we found that E2 can be incorporated efficiently onto retroviral core particles in the absence of E1, whilst the incorporation of E1 onto HCVpp is strongly dependent on the presence of E2.

METHODS

Cells and expression constructs. 293T human embryo kidney cells (ATCC CRL-1573), COS-7 African green monkey fibroblast kidney cells (ATCC CRL-1651) and Huh-7 human hepatoma carcinoma cells (Nakabayashi *et al.*, 1982) were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10 % fetal bovine serum and antibiotics. MLV-based based green fluorescent protein (GFP)-transfer vector CMV-GFP and GagPol expression vector CMV-GagPol have been described previously (Bartosch *et al.*, 2003b). phCMV-G, phCMVcE1E2, phCMVcE1, phCMVcE2 and phCMV-RD114 encode the VSV-G protein, the HCV GPs of genotype 1a as an E1E2 polyprotein or as E1 or E2 individual proteins and the GP of a feline endogenous retrovirus, respectively (Bartosch *et al.*, 2003b). An expression vector encoding the endosomal marker TI-VAMP-GFP (Alberts & Galli, 2003), was co-transfected with *env* and *gag* vectors.

Antibodies. The mAb p5D4 (Sigma-Aldrich), against VSV-G, was used diluted to 1 : 10 000 for Western blotting and to 1 : 2000 for immunofluorescence (IF) experiments. Anti-RD114 GP (ViroMed Biosafety Labs), a goat antiserum raised against the RD114 gp70 envelope surface protein (SU), was used at 1 : 3000 for IF experiments. The HCV-E2 GP was detected with mouse H52 at 1 : 1000 for Western blotting and with undiluted H53 hybridoma supernatant for IF studies. The HCV-E1 GP was detected with mouse A4 at 1 : 1000 for Western blotting and at 1 : 500 for IF studies. Anti-MLV capsid (MLV CA; ViroMed Biosafety Labs) is a goat antiserum raised against the Rauscher leukemia virus p30 capsid and was used at 1 : 10 000 dilution for Western blotting. A rabbit antiserum against MLV capsid p30 (a gift from A. Rein, National Cancer Institute, Frederick, MD, USA) was used at 1 : 10 000 dilution to identify MLV Gag in IF studies. The secondary Alexa antibodies used for IF were purchased from Molecular Probes.

Production of HCVpp and infection assays. Production of HCVpp and infection assays have been described previously (Bartosch *et al.*, 2003b). To analyse the incorporation of HCV envelope GPs into pseudoparticles, HCVpp were pelleted by centrifugation through 20 % sucrose cushions and analysed by Western blotting.

Biotinylation and Western blotting. Forty hours post-transfection, virion-producer cells were chilled on ice, washed twice with cold PBS (pH 8·0) supplemented with 0·7 mM $CaCl_2$ and 0·25 mM $MgSO_4$ (PBS^{++}) and incubated with 0·5 mg sulfo-NHS-LC-LC-biotin ml^{-1} (Pierce) for 30 min at 4 °C. Biotinylation was stopped by incubating the cells with 1 M glycine in PBS^{++} for 5 min at 4 °C. The cells were then washed with PBS/0·1 M glycine, lysed with

MacDougal buffer [20 mM Tris/HCl (pH 8·0), 120 mM NaCl, 200 μM EGTA, 0·2 μM NaF, 0·2 % sodium deoxycholate, 0·5 % Nonidet P-40] containing a protease-inhibitor cocktail (Complete Mini; Roche Diagnostics) and 0·1 M glycine, and centrifuged at 13 000 ***g*** for 30 min; 80 % of the cell lysates were incubated overnight at 4 °C with streptavidin–Sepharose beads (Pierce). The beads were then washed with MacDougal glycine buffer, resuspended in a denaturing buffer (1 % *β*-mercaptoethanol, 0·5 % SDS) and boiled for 5 min. Purified virus samples were obtained by ultracentrifugation of viral supernatants through a 1·5 ml 20 % sucrose cushion in a Beckman SW41 rotor (25 000 r.p.m., 2·5 h, 4 °C) and suspended in PBS. All samples were mixed 5 : 1 (v/v) with a loading buffer [375 mM Tris/HCl (pH 6·8) containing 6 % SDS, 30 % *β*-mercaptoethanol, 10 % glycerol and 0·06 % bromophenol blue], boiled for 5 min and then analysed by SDS-PAGE (12 % gel). Western blotting was performed by using standard procedures. SuperSignal West Pico chemiluminescent substrate (Pierce) was used to reveal proteins.

IF and confocal microscopy imaging. FuGENE 6 (Roche Diagnostics)-transfected virus-producer cells were grown on 35 mm diameter coverglass dishes coated with D-lysine (Mattek Corporation) or on uncoated 14 mm diameter glass coverslips. IF staining was performed at room temperature 40 h post-transfection. The cells were washed with PBS, fixed for 15 min in 3 % paraformaldehyde/PBS, quenched with 50 mM NH_4Cl and permeabilized in 0·2 % Triton X-100 for 8 min. Fixed cells were incubated for 1 h with primary antibody in 1 % BSA/PBS, washed and stained for 1 h with the corresponding fluorescent, Alexa-conjugated secondary antibody (at 0·5 μg ml^{-1}) in 1 % BSA/PBS. The cells were then washed several times with PBS and mounted on microscope slides with the antifading agent Prolong (Molecular Probes). Images were acquired with an LSM 510 confocal microscope equipped with an Axiovert 100 M microscope (Carl Zeiss) and a 63 × 1·3 numerical aperture Apocromat objective. Alexa 488 was excited with an argon laser line at 488 nm and emissions were collected with a band-pass filter (BP505–550). Alexa 546 or 555 was excited, independently of Alexa 488, with a HeNe laser line at 543 nm and emissions were collected with a long-pass filter (LP560).

EM. 293T virion-producer cells were harvested 48 h after transfection, pelleted, fixed with 2 % glutaraldehyde in 0·1 M sodium cacodylate buffer (pH 7·4) and post-fixed with osmium tetroxide [1 % in 0·1 M cacodylate buffer (pH 7·4)]. Cell specimens were dehydrated and embedded in Epon (Epon-812; Fulham). Sections were stained with 7 % uranyl acetate in methanol and post-stained with 2·6 % alkaline lead citrate in H_2O. Specimens were examined under a JEOL 1200-EX electron microscope and analysed with a MegaView II high-resolution TEM camera and the Soft Imaging system (Eloïse). For quantitive EM analysis, at least 50 different cell sections were examined and pseudoparticles were counted in the cytoplasm, in MVBs and at the plasma membrane. For determination of particle size and sphericity index (Gay *et al.*, 1998), a mean diameter for each individual particle was obtained by averaging at least three different diameters measured at 60° angles on the circle delineated by each sectioned particle. To establish the diameter of particles in a given cellular compartment, a minimum of 20 particles were measured.

RESULTS

HCV GPs colocalize with MLV core proteins intracellularly

In cells producing HCVpp, E1 and E2 species are retained mainly in the ER membrane, but, as we have shown previously by fluorescence-activated cell-sorting (FACS) analysis, a small fraction of these proteins reaches the cell surface (Bartosch *et al.*, 2003b). We asked whether HCV GP species are assembled onto MLV-derived core proteins to form HCVpp at the cell surface or intracellularly. For that purpose, we investigated the colocalization of MLV core proteins with HCV GPs by using IF confocal microscopic analysis. Cell-surface expression of HCV GPs could only be detected in non-permeabilized producer cells (Fig. 1a). In detergent-permeabilized cells, the large amount of intracellularly retained HCV GPs made surface detection impossible, confirming poor cell-surface expression of the HCV GPs (Bartosch *et al.*, 2003b). Interestingly, although intracellularly retained E1 and E2 proteins were concentrated mainly in the ER membranes, they were also observed in some intracellular vesicles (Fig. 1b, d–f and data not shown). This localization pattern of E1 and E2, observed here in cells expressing retroviral core proteins, was also observed in cells expressing the HCV GPs alone (data not shown). Co-staining with cellubrevin, a marker for recycling endosomes (Galli *et al.*, 1994), revealed no colocalization with the HCV GPs. In contrast, co-staining with the TI-VAMP marker (Alberts & Galli, 2003 and references therein) identified some of the HCV GP-containing vesicles as late endosomes and, more particularly, as MVBs (Fig. 1d–f and data not shown). MVBs constitute a cellular compartment that is involved in endocytosis and transport of cargo to lysosomes. However, MVBs also traffic to and fuse with the plasma membrane to release their contents into the extracellular medium (Gould *et al.*, 2003). In contrast to the HCV GPs, MLV core proteins, which form the cores of HCVpp, were not detected in non-permeabilized cells when expressed alone (data not shown). In detergent-permeabilized cells, the MLV core proteins appeared as a punctate and well-dispersed pattern throughout the cytoplasm, with some patches localized under the plasma membrane (Fig. 1c).

It has been shown previously that, depending on the cell type, different retroviral GPs are recruited onto retroviral cores in different cellular compartments (Nydegger *et al.*, 2003; Orenstein *et al.*, 1988; Pelchen-Matthews *et al.*, 2003; Raposo *et al.*, 2002; Sherer *et al.*, 2003). Consistently, as shown by confocal microscopy in Fig. 2, colocalization of GPs of the feline endogenous retrovirus RD114 with MLV core proteins was restricted to intracellular vesicles (Fig. 2a–c) (Sandrin *et al.*, 2004), whilst VSV-G GPs colocalized strongly with retroviral core proteins at the cell surface (Fig. 2d–f) (Guibinga *et al.*, 2004). In colocalization experiments using HCVpp-producing cells, we observed that both E2 and E1 GPs colocalized with MLV core proteins in intracellular vesicles (Fig. 2g–i and j–l), suggesting that assembly may occur intracellularly. The degree of colocalization between HCV GPs and MLV core proteins was less extensive than that observed between RD114 GPs and MLV core proteins. This finding is consistent with the lower infectious titres for HCVpp ($5 \times 10^5 \pm 1{\cdot}2 \times 10^5$ IU ml^{-1}) compared with RD114pp ($7 \times 10^6 \pm 2{\cdot}2 \times 10^6$ IU ml^{-1}) (Bartosch *et al.*, 2003b). The extent of colocalization of

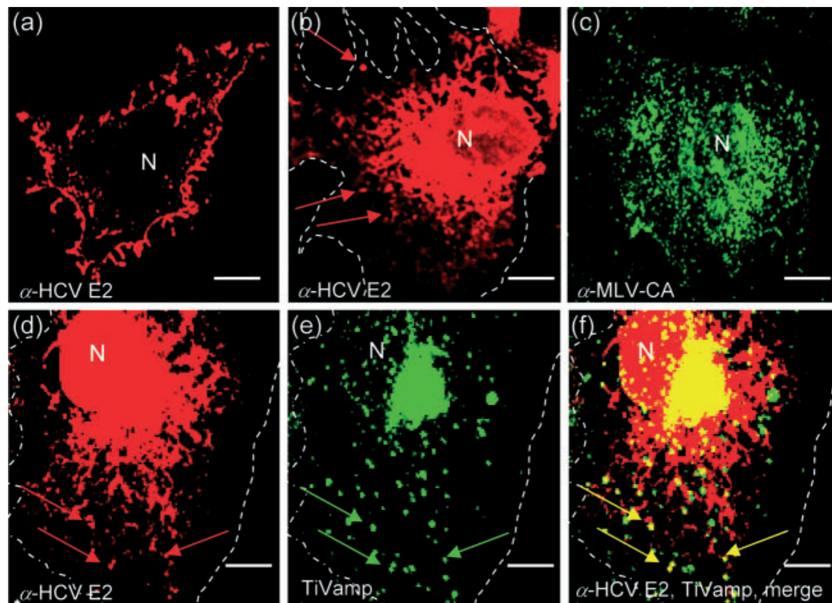

Fig. 1. Localization of viral envelope glycoproteins in HCVpp-producing COS-7 cells by IF. HCVpp-producing cells, expressing the HCV E1 and E2 GPs and MLV core proteins, were untreated (a) or permeabilized (b) and then stained with anti-E2 antibody. (c) Permeabilized cells expressing MLV core proteins stained with an anti-MLV CA antibody directed against the capsid component (CA) of MLV core particles. (d–f) Permeabilized HCVpp-producer cells, which had been co-transfected with the TI-VAMP marker, were stained with anti-E2 antibody (red signal) (d), the TI-VAMP marker (green signal) (e) or both (f). Arrows indicate vesicles where GPs and TI-VAMP colocalize. Outlines of the cell surface and nuclei (N) are indicated. Bars, 10 μm.

HCVpp with MLV core proteins was not dependent on the cell type used for production of HCVpp, as similar observations were made in 293T, COS-7, Huh-7 and HepG2 cells (data not shown).

HCV GPs assemble onto MLV core particles in MVBs

Further investigation of viral-producer cells by EM confirmed our observation that HCVpp formation may occur intracellularly (Fig. 3). By using cells expressing either the MLV core proteins alone or MLV core proteins and VSV-G or HCV GPs, we observed particles within the cytoplasm, in MVBs and at the cell surface (Table 1). Interestingly, the amount of viral particles present at any of these sites within a producer cell differed greatly, depending on which GPs were co-expressed with MLV core proteins. This finding was consistent with previous observations that showed that the viral GPs can influence the localization and assembly pattern of viral particles (Sandrin *et al.*, 2004). In VSV-Gpp-producing cells, particles with a diameter of $97{\cdot}31 \pm 19{\cdot}30$ nm were found exclusively at the cell surface; no particles were detected intracellularly (Table 1). This observation was consistent with our confocal microscopic studies, showing that VSV-G signal colocalized extensively with MLV core proteins at the cell surface (Fig. 2f). Similarly, in cells expressing the MLV core proteins alone, particles were detected mainly at the cell surface (Table 1). The diameter of these particles was $91{\cdot}94 \pm 7{\cdot}7$ nm (Table 1).

In HCVpp-producer cells, particles were present at the cell surface, but MVBs full of particles were found frequently (Fig. 3a, c). Furthermore, a thickening of the MVB membrane and inwards protrusions suggested that particle budding occurred in this cellular compartment (Fig. 3d) and that particles are secreted into the extracellular medium by fusion of these endocytic vesicles with the cell surface. When we investigated the size of viral particles released from cells expressing the MLV core proteins alone, the diameter was on average $91{\cdot}94 \pm 7{\cdot}7$ nm (Table 1). In contrast, in HCVpp-producing cells, we found that particles outside the cell, as well as in MVBs, had very similar diameters of $103{\cdot}76 \pm 9{\cdot}26$ and $103 \pm 10{\cdot}92$ nm, respectively. Thus, particles present at the cell surface, as well as in MVBs of HCVpp-producer cells, were about 10 % wider in diameter than MLV core particles devoid of viral glycoproteins. This suggests that the HCV GPs can be assembled onto retroviral core proteins within the cell.

Finally, we also observed particles within the cytoplasm of HCVpp-producing cells (Fig. 3b; Table 1). These intracellular particles, however, had a small diameter (approx. $82{\cdot}52 \pm 12{\cdot}74$ nm) when compared with particles produced from cells expressing the MLV core proteins alone ($91{\cdot}94 \pm 7{\cdot}7$ nm) (Table 1). They may represent non-enveloped, cytoplasmic core particles. Cells producing VSV-Gpp did not contain particles within MVBs or the cytoplasm, suggesting that these features are specific to HCVpp-producing cells (Table 1). Thus, to determine which HCV GP induced these phenomena, we investigated by EM the distribution of particles in cells expressing E1 or E2 individually with MLV core proteins. In cells co-expressing E2 and MLV core proteins, particles were observed at the cell surface and in MVBs, but not within the cytoplasm (Table 2). In contrast, in cells co-expressing E1 and MLV core proteins, the proportion of cytoplasmic particles had increased by approximately twofold (from 22 to 41 %; Table 2) compared with cells expressing both E1E2 GPs and MLV core proteins. Furthermore, by using confocal microscopy, whilst we detected some colocalization between MLV core proteins and E2 expressed in the absence of E1 (Fig. 4a–c), we did not detect any colocalization between MLV core proteins and E1 expressed in the absence of E2 (Fig. 4d–f).

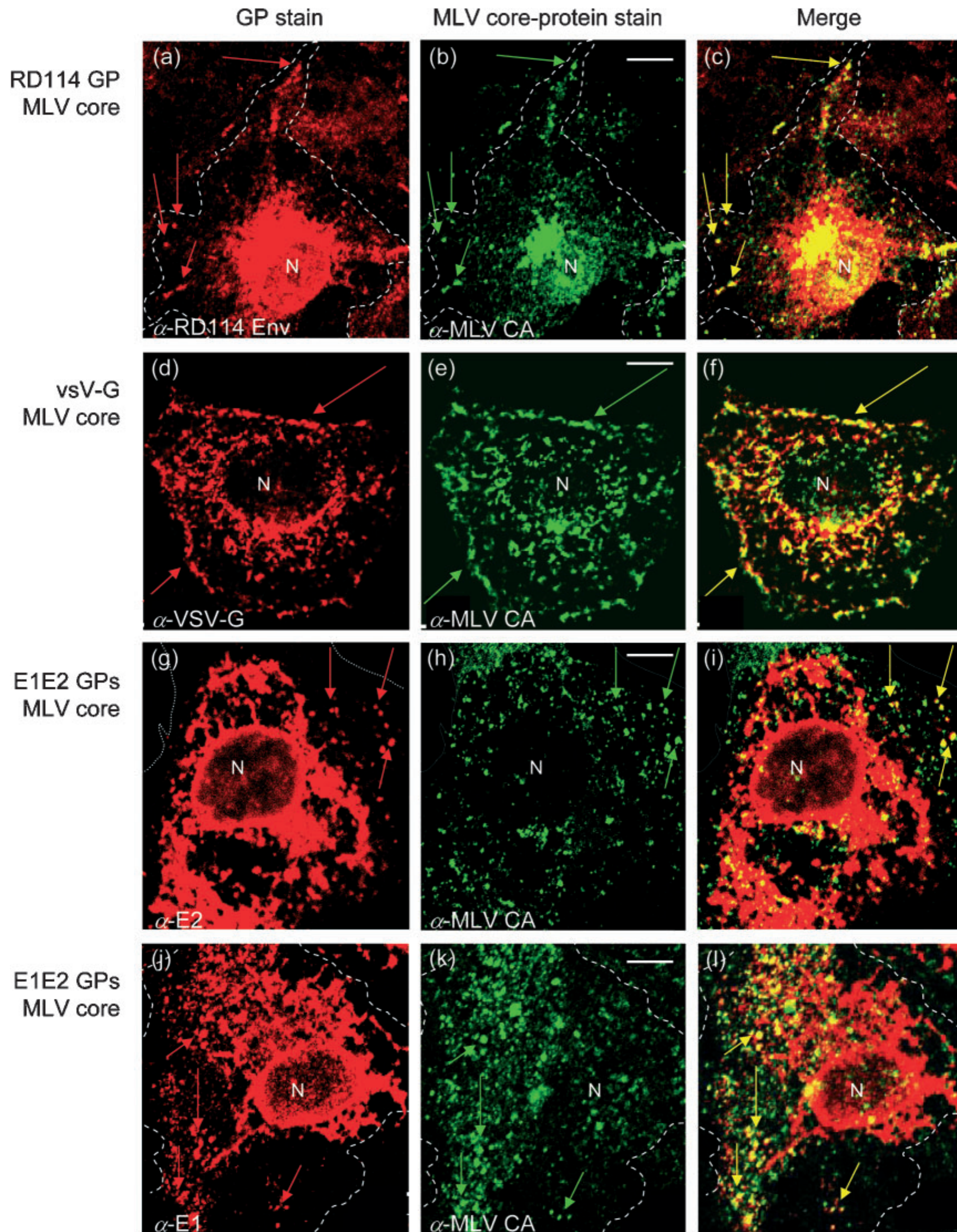

Fig. 2. Localization of viral envelope glycoproteins and MLV cores in COS-7 cells by IF. COS-7 cells producing RD114pp (a, b, c), VSV-Gpp (d, e, f) or HCVpp (g, h, i, j, k, l) were co-stained with the indicated anti-GP antibodies (a, d, g, j) as well as the anti-MLV CA antibody directed against the capsid component (CA) of MLV core particles (b, e, h, k). Merged images of GP and capsid stains are shown (c, f, i, l). Arrows indicate cell surface or intracellular vesicles where GPs and MLV core proteins colocalize. Comparable results were obtained by using 293T cells. Outlines of the cell surface and nuclei (N) are indicated. Bars, 10 μm.

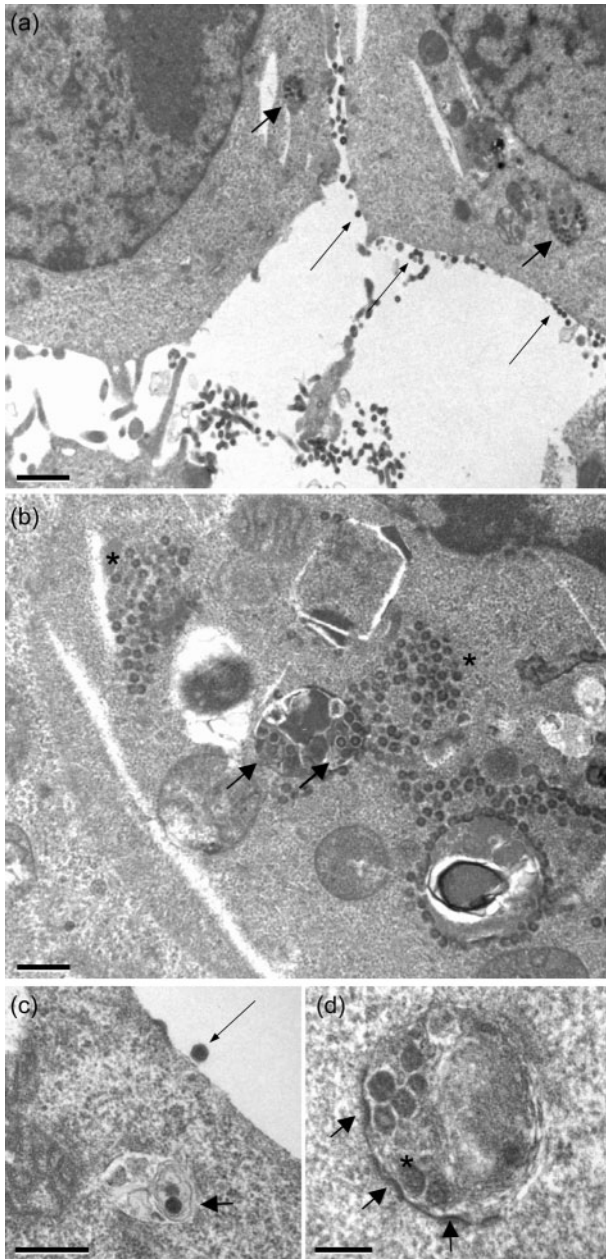

Fig. 3. EM studies of HCVpp-producing 293T cells. Transmission EM of 293T cells expressing the HCV E1 and E2 GPs and MLV core proteins. Several sites and patterns of viral assembly were observed: particles at the cell surface (long arrows), particles within MVBs (thick arrows) and cytoplasmic particles (asterisks). Note a thickening of the MVB membranes and inward protrusions (d), which suggest budding into MVBs. Bars, 1 μm (a); 500 nm (b, c); 250 nm (d).

Altogether, these data suggested that E2 colocalizes with MLV core proteins, is incorporated efficiently on particles and allows efficient particle egress. In contrast, E1 colocalizes and assembles efficiently onto MLV core proteins only in the presence of E2.

Intracellular forms of E2 are incorporated preferentially onto MLV core particles

To confirm and extend our microscopic observations on intracellular HCVpp assembly, we performed biotinylation studies of E1 and E2. Comparison of the amounts and electrophoretic mobilities of biotinylated E1 and E2 expressed at the cell surface to E1 and E2 present on viral particles or in total cell lysates by immunoblotting is shown in Fig. 5. Specificity of biotinylation for cell surface-expressed proteins was controlled by detection of the retroviral core proteins, which are protected from biotinylation by either cell or viral membranes (Fig. 5, bottom panels). Examination of the electrophoretic mobility of E2 revealed different isoforms on viral particles (Fig. 5b, right panel). Importantly, the E2 species found at the cell surface of HCVpp-producing cells migrated much faster in denaturing reducing SDS-PAGE than E2 species incorporated on virions. Because virion-associated E2 species are rather heterogeneous and sensitive to peptide : *N*-glycosidase F digestion (data not shown) (Op De Beeck *et al.*, 2004) whilst cell surface-expressed E2 species migrate with much higher mobility, E2 proteins must be incorporated into viral particles intracellularly at a stage before the trimming process is complete. After incorporation into viral particles, E2 must be protected from further trimming, whereas unincorporated, monomeric E2 protein is subject to further trimming before it finally reaches the cell surface. These biochemical data, suggesting intracellular recruitment of E2, are therefore fully consistent with our microscopic observations. In contrast, no clear variation of the electrophoretic mobility of E1 incorporated onto viral particles was observed when compared to E1 monomer in total cell lysate or on the cell surface of HCVpp-producing cells (Fig. 5b, left panel).

E1 requires the presence of E2 for efficient viral incorporation

The prebudding form of the HCV GPs has been described previously to be an E1E2 heterodimer (Deleersnyder *et al.*, 1997; Dubuisson, 2000; Op De Beeck *et al.*, 2001), supporting the notion that E1 should be recruited and assembled within the same intracellular compartment as E2. The fact that E1, expressed in the absence of E2, does not colocalize with MLV core proteins (Fig. 4d–f) indicates that E1 requires the presence of E2 for proper viral incorporation. To compare the incorporation of E1 in the presence or absence of E2, we co-transfected 293T producer cells with equal amounts of E1 and E2 expression constructs either separately or in combination, together with the MLV core and GFP-transfer vectors (Fig. 6). Consistent with previous data (Bartosch *et al.*, 2003b), E2 was incorporated efficiently onto particles both in the absence and the presence of E1 (Fig. 6a). However, in the absence of E1, E2 associated with pseudoparticles migrated with reduced mobility on SDS-PAGE, suggesting an altered glycosylation pattern of E2 in the absence of E1 (Fig. 6a). Pseudoparticle incorporation of E1 in the absence of E2 was detectable, but appeared to be

Table 1. Mean diameters of viral particles assembled at different cellular localizations in the presence of various viral envelope glycoproteins

Data are viral particle mean diameters ± SD (nm). Viral particles were counted in the cytoplasm, in MVBs and at the plasma membrane. Particles in the process of budding and egressing were included in the counting. Mean diameters of particles were determined as described in Methods. The number of particles analysed (*n*) is shown in parentheses. ND, Not detected in 50 different cell sections.

Envelope	Cellular localization		
	Plasma membrane	MVB	Cytoplasm
None	91·94 ± 7·70 (*n*=43)	ND	ND
VSV-G	97·31 ± 19·30 (*n*=50)	ND	ND
E1 + E2	103·76 ± 9·26 (*n*=23)	103·51 ± 10·92 (*n*=24)	82·52 ± 12·74 (*n*=28)

inefficient, whilst the co-expression of E2 strongly enhanced pseudoparticle incorporation of E1 (Fig. 6a, right panel). Co-expression of both GPs led to the assembly of HCVpp with a titre of about 10^5 IU ml^{-1} on Huh-7 cells, whilst pseudoparticles incorporating only E1 or only E2 had almost 1000-fold-reduced titres (Fig. 6d, left panel), confirming that both GPs are required to render pseudoparticles infectious (Bartosch *et al.*, 2003b).

To quantify the E2 dependence of E1 for its efficient incorporation and formation of infectious pseudoparticles, we transfected 293T cells with various ratios of E1- and E2-expression plasmids. By co-transfecting increasing amounts of phCMV-E1 expression plasmid together with steady amounts of phCMV-E2, we found the expression levels of E2 on virions to be the same in all conditions, confirming that E2 incorporation does not depend on E1 (Fig. 6b). However, when transfecting steady amounts of phCMV-E1 with increasing amounts of phCMV-E2, the incorporation levels of both GPs on pseudoparticles increased concomitantly (Fig. 6c). Thus, particle incorporation of E1 depends on the presence of E2 in a dose-dependent manner. In addition, whilst neither E1 nor E2 can form infectious pseudoparticles in the absence of their respective partners, particle infectivity increases concomitantly with increasing incorporation of both E1 and E2 (Fig. 6d, middle and right panels).

Table 2. Cellular distribution of viral particles assembled in the presence of the indicated HCV envelope glycoproteins

Data are percentages of VP in cellular compartments. Viral particles were counted in the cytoplasm, in MVBs and at the plasma membrane. Particles in the process of budding and egressing were included in the counting. The number of particles (*n*) analysed in each cellular compartment is shown in parentheses. ND, Not detected in 50 different cell sections.

Envelope	Cellular localization		
	Plasma membrane	MVB	Cytoplasm
E1 + E2	48 % (*n*=23)	29 % (*n*=24)	22 % (*n*=28)
E1	47 % (*n*=83)	11 % (*n*=20)	41 % (*n*=71)
E2	90 % (*n*=80)	10 % (*n*=22)	⩽1 % (ND)

DISCUSSION

The assembly of wild-type HCV is thought to occur intracellularly (Egger *et al.*, 2002; Greive *et al.*, 2002; Shimizu *et al.*, 1996), in analogy to other flaviviruses and because the structural HCV proteins have been described to be strictly retained in the ER or an ER-derived compartment (Dubuisson, 2000; Egger *et al.*, 2002). Indeed, the transmembrane domains of E1 and E2 contain strong ER-retention signals (Cocquerel *et al.*, 1998, 1999; Flint & McKeating, 1999) and the extremely short cytoplasmic tails of E1 and E2 display no canonical intracellular-trafficking signals. However, upon plasmid-based expression of E1 and E2 in HCVpp-producing cells, a fraction of the GPs trafficks from the ER to the cell surface (Bartosch *et al.*, 2003b; Drummer *et al.*, 2003; Dumonceaux *et al.*, 2003; Hsu *et al.*, 2003). This suggested that these surface-localized GPs were incorporated onto pseudoparticles (Flint *et al.*, 1999; Lagging *et al.*, 1998; Matsuura *et al.*, 2001; Takikawa *et al.*, 2000). However, in the present work, we showed that, in HCVpp-producing cells, besides localization at the cell surface, HCV GPs are also present in intracellular vesicles, including MVBs, a late endosomal compartment that can traffic and fuse with the cell surface. Moreover, colocalization of the HCV GPs with retroviral core proteins was restricted to intracellular vesicles, including MVBs. This finding is supported by our EM studies, which show that, in HCVpp-producing cells, MVBs contained many viral particles. Furthermore, inward protrusions and thickening of MVB membranes indicated particle formation and budding within this cellular compartment. In addition, the diameters of particles in MVBs of HCVpp-producing cells suggested that the particles contain viral GPs. Yet, the most conclusive evidence suggesting that assembly of HCVpp occurs mainly intracellularly is the results of our biotinylation experiments. In HCVpp-producing cells, surface-expressed forms of E2 do not seem to be incorporated on pseudoparticles because their glycosylation pattern is

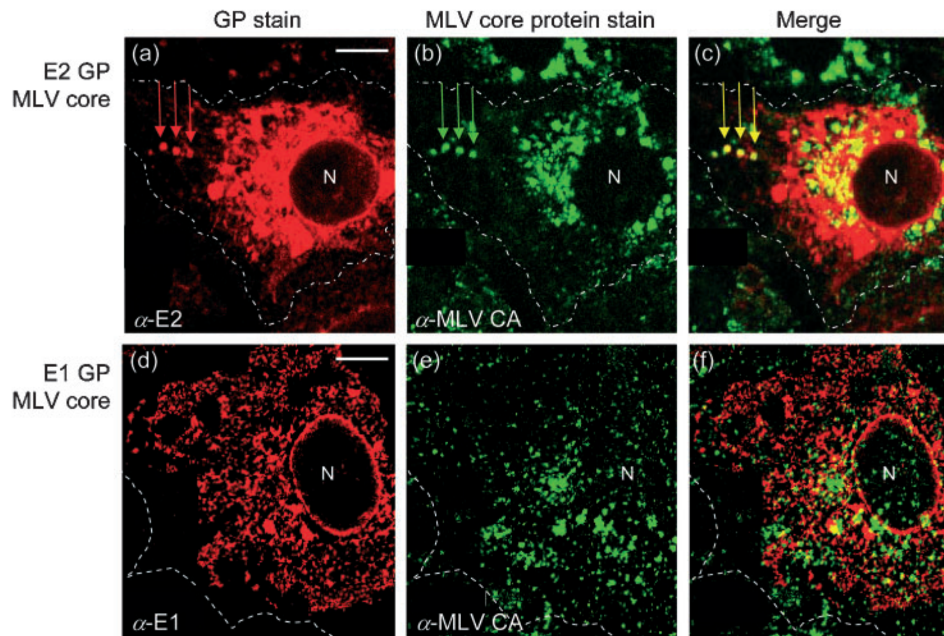

Fig. 4. Confocal microscopy of HCVpp-producing cells. COS-7 cells co-expressing E2 (in the absence of E1) with MLV core proteins (a, b, c) or co-expressing E1 (in the absence of E2) and MLV core proteins (d, e, f), were co-stained with the indicated anti-GP antibodies (a, d) as well as an anti-MLV CA antibody directed against the capsid component (CA) of MLV core particles (b, e). Merged images of anti-envelope and capsid stains are shown (c, f). Arrows indicate vesicles where envelopes and capsid proteins colocalize. Bars, 10 μm.

distinct from that of E2 species present on HCVpp, as revealed by their different electrophoretic mobilities. In summary, microscopic and biochemical data show that HCVpp assembly occurs in intracellular vesicles, including MVBs. This result is consistent with a growing body of evidence suggesting that the assembly of a large number of viruses, including retroviruses and filoviruses, takes place in MVBs (reviewed by Pornillos *et al.*, 2002).

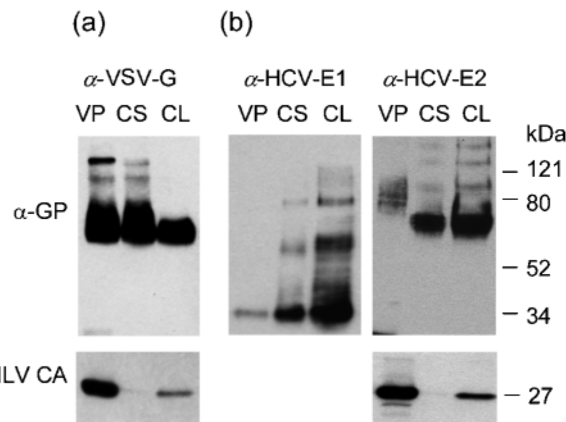

Fig. 5. Relative amounts of viral glycoproteins in producer cells and on virions. Viral GPs, VSV-G (a) or both E1E2 GPs (b) and MLV core proteins present in crude cell lysate (CL), at the cell surface (CS) and on sucrose cushion-purified viral particles (VP) were detected by immunoblotting with the indicated antibodies in VSV-Gpp (a)- or HCVpp (b)-producing cells. The cell surface-expressed proteins were biotinylated to allow their purification (see Methods for details).

Determination of structure and assembly of wild-type HCV *in vivo* remains a challenging issue. Reported data based on HCV viral-like particles and HCV replicons suggest that replication occurs in ER-derived compartments and HCV budding may be driven by the core protein (Baumert *et al.*, 1998; Blanchard *et al.*, 2002, 2003; Egger *et al.*, 2002). However, the cellular site of wild-type HCV assembly has remained elusive so far and the close association of HCV biology with lipoprotein metabolism further complicates current views on HCV morphogenesis (André *et al.*, 2005). Previous EM studies on HCV structure have shown that the virus measures 50–60 nm in diameter (Kaito *et al.*, 1994; Shimizu *et al.*, 1996). Wild-type HCV is thus considerably smaller than HCVpp (which has a diameter of 104 nm), a size difference that is probably due to the core proteins. MLV core particles devoid of viral GPs measure 92 nm, whilst the size of HCV nucleocapsid-like particles has been reported in the range 38–62 nm (André *et al.*, 2005). Regarding the assembly of wild-type HCV, Shimizu *et al.* (1996) have reported the detection of enveloped particles in cytoplasmic vesicles of HCV-replicating cells, which suggested that the morphogenesis of wild-type HCV may be vesicle-orientated. Interestingly, they detected HCV in cytoplasmic vesicles that potentially resemble MVBs. With

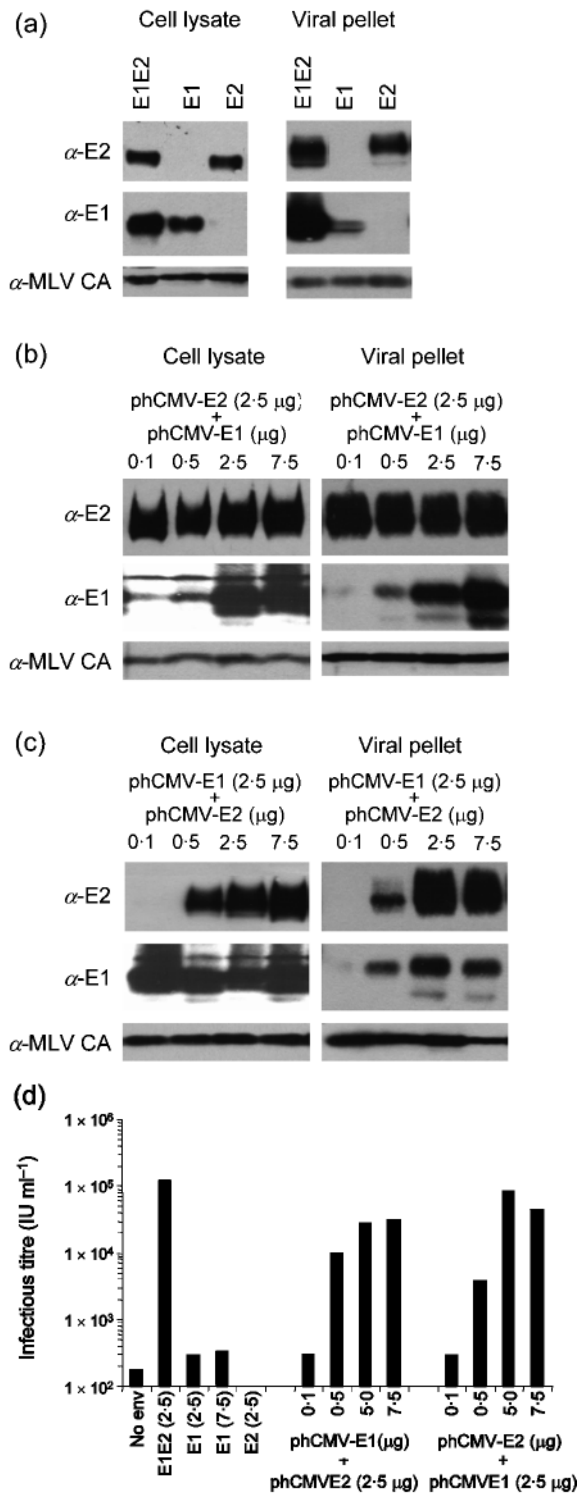

Fig. 6. Role of E2 in pseudoparticle incorporation of E1. (a–c) Immunoblots of HCVpp. Pseudotyped viruses were pelleted through 20 % sucrose cushions. Cell lysates and virus pellets were analysed by SDS-PAGE in reducing and denaturing conditions and revealed with mAbs against E1 and E2 or with an anti-MLV capsid (MLV CA) serum. (a) Immunoblots of HCV pseudotyped particles generated by transfection of $1{\cdot}8\times10^6$ cells with 2·5 μg of either E1 or E2 GP expression construct and 8 μg MLV core protein expression construct and 8 μg MLV GFP-transfer vector expression construct. phCMV-E1 and phCMV-E2 plasmids were co-expressed or expressed individually. Comparable results were obtained by using COS-7 cells for HCVpp production. (b) Immunoblots of HCV pseudotyped particles generated by co-expression of 2·5 μg phCMV-E2 with the indicated amounts of phCMV-E1 (μg). (c) Immunoblots of HCV pseudotyped particles generated by co-expression of 2·5 μg phCMV-E1 with the indicated amounts of phCMV-E2 (μg). (d) Infectivity of the generated HCVpp. Titres of corresponding viral supernatants were determined on Huh-7 target cells and are expressed as IU ml^{-1}.

the very recent development of systems that support wild-type HCV production *in vitro* (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005), it will be interesting to see whether an involvement of MVBs in wild-type HCV morphogenesis can be confirmed.

The respective roles of E1 and E2 in particle assembly were clarified in this study by investigating the expression patterns of the HCV GPs E1 and/or E2 expressed individually with MLV core proteins. In cells expressing E2 with MLV core proteins, most particles were observed at the cell surface, suggesting efficient particle assembly and egress. In contrast, in cells co-expressing E1 with MLV core proteins, an accumulation of non-enveloped particles in the cytoplasm was observed, suggesting that E1 may possibly inhibit particle assembly and egress by an unknown mechanism. Because the inhibition of E1 on particle assembly and/or egress can be overcome by co-expression of E2, and because E1 colocalizes efficiently with retroviral core only in the presence of E2, our findings suggest that E1 incorporation onto pseudoparticles occurs subsequent to E1E2 heterodimerization. This finding is consistent with previous studies, which showed that the prebudding form of E1E2 is a heterodimer (Cocquerel *et al.*, 2000; Michalak *et al.*, 1997; Op De Beeck *et al.*, 2000, 2004; Patel *et al.*, 2001). Furthermore, we found a direct correlation between the presence of both E1 and E2 and the infectivity of HCVpp, indicating that heterodimer formation and functionality of E1 and E2 are tightly linked processes.

In conclusion, the assembly of HCV GPs on pseudoparticles may be more similar to that of wild-type HCV than currently thought. The morphogenesis of HCVpp requires both HCV GPs and does not occur at the cell surface, but rather is 'vesicle-orientated' and leads to the formation of functional, fusogenic HCV GP complexes on the virion surface.

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