Hepatitis C virus (HCV) E1 and E2 glycoproteins assemble intracellularly to form a non-covalently linked heterodimer, which is retained in the endoplasmic reticulum (ER). To study the subcellular localization of E2 in live cells, the enhanced green fluorescent protein (EGFP) was fused to the N terminus of E2. Using fluorescence and confocal microscopy, we have confirmed that E2 is located in the ER, where budding of HCV virions is thought to occur. Immunoprecipitation experiments using a conformation-sensitive antibody and a GST pull-down assay showed that fusion of EGFP to E2 interferes neither with its heterodimeric assembly with E1, nor with proper folding of the ectodomain, nor with the capacity of E2 to interact with human CD81, indicating that the EGFP–E2 fusion protein is functional. As a tool to study binding of E2 to target cells, we also described the expression of an EGFP–E2 fusion protein at the cell surface.

The specific receptor allowing penetration of HCV into target cells has not been unambiguously identified to date. Low-density lipoprotein receptor (Agnello et al., 1999), human CD81 (hCD81) tetraspanin (Pileri et al., 1998) and glycosaminoglycans (Chen et al., 1997) may all act as receptors for HCV, either sequentially or for different viral quasispecies. However, several reports suggest that additional, as yet unidentified cellular proteins are involved in virus binding and entry (Meola et al., 2000; Petracca et al., 2000). Recently, Scarselli et al. (2002) have proposed the human scavenger receptor class B type I as a novel candidate receptor for HCV.

Previous studies of the subcellular localization of HCV glycoproteins have used indirect immunofluorescence and immunoelectron microscopic techniques (Deleersnyder et al., 1997; Dubuisson et al., 1994). Utilization of the Aequorea victoria green fluorescent protein (GFP) for visualizing gene expression and protein localization has provided a powerful tool to investigate subcellular localization of various recombinant proteins in live cells (Chalfie et al., 1994). To examine the subcellular localization of HCV E2 glycoprotein in live mammalian cells, we have constructed a recombinant vaccinia virus (VV) expressing the enhanced green fluorescent protein (EGFP) fused to E2. Since the TM domain of E2 is known to be multifunctional, EGFP was fused to the N terminus rather than to the C terminus of E2 (Fig. 1a). The corresponding coding sequences were assembled using overlap extension PCR amplification on infectious p90/HCV-FL-long pU.
HCV clone cDNA (Kolykhalov et al., 1997) and pEGFP-C1 plasmid vector (Clontech). The resulting sequence was cloned within the thymidine kinase gene of plasmid pTG9148 (Transgene). The recombinant VV expressing the EGFP–E2 fusion protein (vvIV215) or the core, E1 and E2 proteins (vvIV205) were generated by homologous recombination (Kieny et al., 1984). A VV recombinant (vvIV218) expressing the EGFP protein was used as a control. Expression and size of the recombinant proteins in human hepatic cell lines were confirmed using Western blot analysis with a GFP-specific monoclonal antibody (mAb) (Clontech) and a conformation-insensitive E2-specific mAb (H47) (not shown).

In order to examine the proper folding and conformation of the protein, EGFP–E2 was expressed and labelled with 100 μCi ³⁵S-Protein Labelling Mix (NEN) ml⁻¹ in HepG2 cells, immunoprecipitated with a conformation-sensitive E2-specific mAb (H53) and analysed by SDS-PAGE (Dubuisson & Rice, 1996). The EGFP–E2 protein, like native E2 (Fig. 1b), was recognized by mAb H53, indicating that the conformation of the EGFP–E2 fusion protein was similar to that of native E2.

As an indicator of intracellular trafficking of the protein, we examined the sensitivity of EGFP–E2 to endoglycosidase treatment after pulse–chase labelling with 100 μCi ³⁵S-Protein Labelling Mix ml⁻¹ and immunoprecipitation with mAb H53. Immunelectrophoretic precipitates were digested with either endo−β-N-acetylglucosaminidase H (endo H; Roche Boehringer Mannheim) or peptide N-glycosidase F (PNGase F; New England Biolabs), or left untreated. Both native E2 and EGFP–E2 were shown to be sensitive to endo H and PNGase F endoglycosidases, indicative of their retention in the ER (Fig. 1b). This result indicates that fusion of EGFP to the N terminus of the E2 protein does not modify the localization of this glycoprotein.

Having demonstrated that addition of EGFP to its N terminus interferes neither with the correct folding nor with the sensitivity of E2 to endoglycosidase treatment, we investigated whether the fusion could affect known
functional properties of E2. Indeed, E2 has been reported to interact with E1 to form a stable, non-covalently linked heterodimer and E2 has been shown to interact with hCD81.

Association of the EGFP–E2 fusion protein with E1 was investigated by co-immunoprecipitation of E2 and E1 using mAb H53. The proteins were expressed in trans (Cocquerel et al., 2001) using vvIV215 and AdIV243 (a recombinant adenovirus expressing HCV E1 protein) in HepG2 cells, labelled and immunoprecipitated as described above. vvIV205 was used as a control. As shown in Fig. 2(a), E1 was co-immunoprecipitated with EGFP–E2, demonstrating that this protein interacts with E1. Similar results were obtained using a GFP-specific polyclonal antibody (data not shown). This result demonstrated that addition of EGFP to the N terminus of the E2 protein does not alter its association with E1.

We then investigated whether fusion of E2 with EGFP would affect its binding to hCD81. Indeed, among the possible disadvantages of GFP as a protein tag is its large size (29 kDa), which could mask regions of E2 involved in interactions with hCD81. Lysates of BHK-21 cells infected with vvIV205 or vvIV215 were incubated with a soluble recombinant glutathione S-transferase (GST) fusion protein containing the large extracellular loop of hCD81 (GST–CD81) (Higginbottom et al., 2000) or with GST protein for 2 h at 4 °C. CD81–E2 and CD81–EGFP–E2 complexes were recovered by incubation (pull-down) with glutathione–Sepharose 4B beads for 2 h at 4 °C. Interaction with CD81 was then analysed by Western blot detection of native E2 and EGFP–E2 using mAb H47. As shown in Fig. 2(b), both E2 and EGFP–E2 were detected when incubated with GST–CD81. No binding of E2 or EGFP–E2 to GST was observed. This result showed that the EGFP–E2 fusion protein conserves its capacity to bind hCD81.

Expression of EGFP–E2 in HepG2 cells was detected in live cells using conventional fluorescence microscopy (not shown). In addition, infected cells were examined by laser scanning confocal microscopy (LSCM) at 24 h post-infection (p.i.). As shown in Fig. 3(g), EGFP staining was localized uniformly throughout the cytoplasm and nucleus (Ogawa et al., 1995). In contrast, the EGFP–E2 fusion protein-specific staining was localized at restricted areas in the cell (Fig. 3c). Moreover, EGFP–E2 fluorescence showed a diffuse granular pattern indicative of a vesicular localization and was concentrated mainly in the perinuclear space, reminiscent of the subcellular localization of native E2 (Deleersnyder et al., 1997; Dubuisson et al., 1994; Duvet et al., 1998). Immunoelectron microscopy using mAb H53 confirmed this ER localization (data not shown). This observation of the retention and accumulation of EGFP–E2 in the ER of live cells supports the hypothesis that budding of HCV particles, like that of flaviviruses (Mackenzie & Westaway, 2001), occurs into this compartment.

In order to study the trafficking of the fluorescent protein in live cells, HepG2 cells were infected with vvIV215 and examined at various times after infection. Staining was first observed in the majority of cells at 4 h p.i. (not shown). At 8 h p.i. (Fig. 3a), EGFP–E2 displayed a vesicular pattern compatible with an ER-like distribution. At 18
and 24 h p.i. (Fig. 3b, c), EGFP–E2 accumulated in the perinuclear space and in the ER as expected. Fusion of EGFP thus provides a powerful tool for the analysis of the temporal and spatial trafficking of the HCV E2 envelope protein in live mammalian cells.

In the absence of an efficient tissue culture system to replicate HCV, studying HCV interactions with host cell-surface proteins can prove difficult. With the intention of developing a reagent to study HCV E2 interactions with host cell-surface components, we constructed a recombinant VV expressing an EGFP–E2 fusion protein transported to the cell surface. This fusion protein consisted of EGFP fused to the N terminus of a modified E2 protein resulting from the fusion of the ectodomain of E2 (truncated E2 protein ending at aa 661) to the TM and cytoplasmic domains of the rabies virus G glycoprotein, which is naturally exported to the cell surface (Fig. 1a) (Dietzschold et al., 1978). Recombinant vvIV279 expressing the EGFP–E2–TM<sub>rabies</sub> protein was generated and expression of the fusion protein was demonstrated by Western blot using GFP-specific and E2-specific (H47) mAbs (data not shown). The EGFP–E2–TM<sub>rabies</sub> fusion protein was further analysed with mAb H53 in pulse–chase experiments as described above. EGFP–E2–TM<sub>rabies</sub> was shown to be recognized by mAb H53 (Fig. 1b) and two bands were detected after immunoprecipitation: a fast-migrating form of the expected size, which was sensitive to both endo H and PNGase F, and a slow-migrating form, which corresponds to the EGFP–E2–TM<sub>rabies</sub> protein harbouring additional glycan modifications, acquired during translocation of the recombinant protein to the plasma membrane. This slow-migrating species was indeed found to be resistant to endo H and sensitive to PNGase F, suggesting that EGFP–E2–TM<sub>rabies</sub> reaches at least the medial- or trans-Golgi apparatus. Interaction with hCD81 was further analysed using a GST pull-down assay, as described above. Detection of the fast-migrating form of EGFP–E2–TM<sub>rabies</sub> by Western blot following incubation with GST–CD81 (Fig. 2b) demonstrated that this protein is expressed in a functional configuration and can interact with hCD81. It is of note that no interaction between the slow-migrating form of

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**Fig. 3.** Expression and subcellular localization of EGFP-tagged HCV proteins. HepG2 cells were infected with vvIV215 (EGFP–E2), vvIV279 (EGFP–E2–TM<sub>rabies</sub>), or vvIV218 (EGFP). EGFP or its fusion derivatives were observed at 8, 18 and 24 h p.i.
EGFP–E2–TMrabies and hCD81 was detected using this technique. This result is consistent with previous reports demonstrating a modulation of the E2–hCD81 interaction following translocation of this HCV glycoprotein to the plasma membrane (Flint et al., 2000; Heile et al., 2001). Indeed, E2 isoforms harbouring complex glycans have been shown to bind hCD81 with poorer affinity.

Cell-surface expression of EGFP–E2–TMrabies was analysed by fluorescence and LSM. At 8 h p.i. (Fig. 3d), the recombinant protein displayed a vesicular pattern of fluorescence compatible with an ER-like distribution. At 18 and 24 h p.i. (Fig. 3e, f), the EGFP–E2–TMrabies protein displayed some residual granular staining indicative of a vesicular localization, but was mainly localized on the plasma membrane. Immunochemistry with antibody to E2 demonstrated a modulation of the E2–hCD81 interaction followed by translocation of the fusion protein to the plasma membrane. The contribution of Anne Chaffie is gratefully acknowledged. The fluorescence compatible with an ER-like distribution. At 8 h p.i. (Fig. 3d), the recombinant protein displayed a vesicular pattern of fluorescence compatible with an ER-like distribution. Translocation of the fusion protein to the plasma membrane was followed by a modulation of the E2–hCD81 interaction.

REFERENCES


Scarselli E., Ansuini, H., Cerino, R. & 7 other authors (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J 21, 5017–5025.