

## Assembly of HCV E1 and E2 glycoproteins into coronavirus VLPs

### Brief Report

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**Summary.** Hepatitis C virus (HCV) is believed to assemble by budding into membranes of the early secretory pathway, consistent with the membrane location where the viral envelope glycoproteins E1 and E2 accumulate when expressed. Coronavirus assembly also takes place at pre-Golgi membranes. Here, we generated coronavirus-like particles carrying in their envelope chimeric HCV glycoproteins composed of the ectodomains of E1 and E2, each fused to the transmembrane plus endodomain of the mouse hepatitis coronavirus spike glycoprotein. The chimeric particle system will enable structural and functional studies of the HCV glycoproteins.

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Hepatitis C virus (HCV) is the major etiological agent of human non-A, non-B hepatitis [4, 17] and has infected millions of people worldwide [20]. This flavivirus causes chronic liver disease for which treatment options are limited and no vaccine is yet available. The study of its biology has been severely hampered by the lack of viral culture systems, a situation that has recently changed by the generation of infectious HCV genomes that replicate efficiently in cell culture [21, 37, 38].

Little is still known about the biogenesis and structure of HCV particles. The virus is composed of a core (C) protein – that packages the positive-stranded RNA genome – and envelope proteins, E1 and E2, which are generated by cleavage

from the single precursor polyprotein, translated from the genomic RNA. E1 and particularly E2 are heavily N-glycosylated proteins anchored in membranes by their C-terminal hydrophobic domain. Expression studies have shown the glycoproteins to be retained in early compartments of the secretory pathway, consistent with the supposed localization of HCV assembly in the endoplasmic reticulum (ER) [7, 23, 26, 27].

Several workers have resorted to surrogate models to enable the analysis of interactions of E1 and E2 in particle assembly and their role in the initiation of viral infection. VSV pseudotype viruses were generated carrying HCV glycoprotein ectodomains joined to the VSV G protein's transmembrane and cytoplasmic domain [3, 19, 24, 25]. Unmodified HCV glycoproteins were assembled into retroviral and lentiviral particles [1, 6, 9, 14, 22, 36]. These pseudotype systems have one drawback in that particles are assembled at the plasma membrane rather than at the intracellular membranes or, as recently demonstrated, into multivesicular bodies [29], a late endosomal compartment that can fuse with the cell surface [12], whilst HCV assembly and budding most probably occur at the membrane of secretory pathway organelles. Under these different conditions, the glycosylation state of the assembling glycoproteins and the lipid composition of the resulting particles are clearly different, and the biological consequences of these differences are unknown. For this reason, the present study aimed at developing a coronavirus-based pseudotype system.

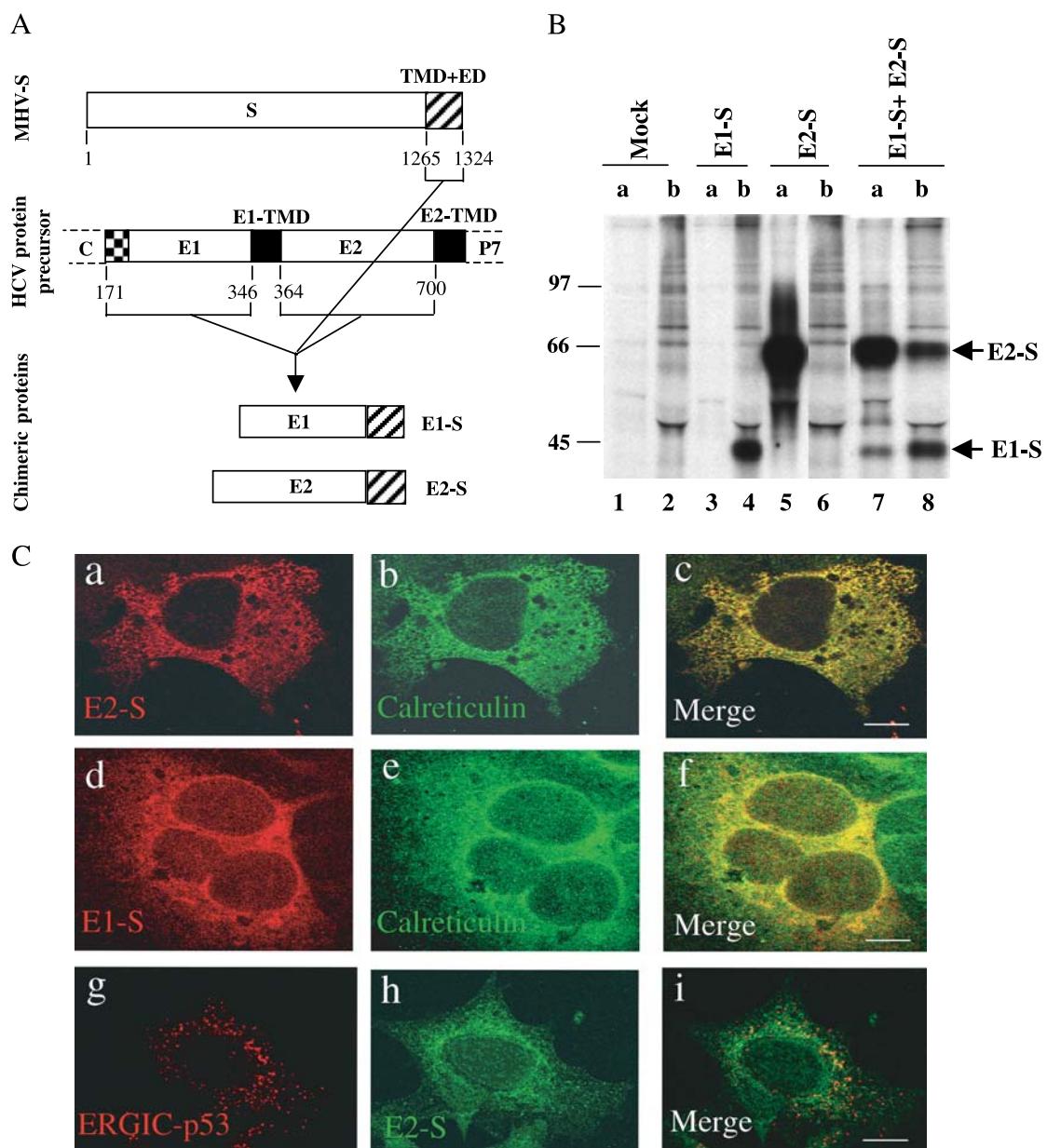
Coronaviruses are enveloped, plus-sense RNA viruses formed by budding into membranes of the intermediate compartment (IC) and ER [15, 16, 32]. They consist of a capsid, formed by the nucleocapsid (N) protein-packaged genomic RNA, surrounded by an envelope containing three proteins: M, E and S. The M and E protein, the major and minor membrane constituent, respectively, are able to

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**Fig. 1.** Construction and expression of chimeric E1-S and E2-S genes. **A** E1-S and E2-S proteins are composed of the signal sequence and ectodomain (aa 171–346 and 364–700) of the HCV precursor polypeptide, respectively, fused to the 60-residue transmembrane (*TMD*) plus endodomain (*ED*) from the MHV strain A59 S protein. **B** Cultures of vTF7-3 infected OST-7 cells, transfected or mock-transfected, were pulse labeled with  $^{35}\text{S}$ -labeled amino acids from 5 to 8 h post infection. Cell lysates were prepared and immunoprecipitations carried out with (a) the anti-E2 MAb 185 and (b) the anti-E1 MAb A4. The immunoprecipitates were subjected to SDS-PAGE. Numbers on the left indicate the positions in the same gel of marker proteins. **C** Similar expressions were performed in Huh-7 cells. At 5 h post infection the cells were fixed, permeabilized, double-stained by indirect immunofluorescence, and analysed by confocal microscopy. E1-S was immunostained with the anti-E1 MAb A4, E2-S with either the anti-E2 MAb 185 or the R21 polyclonal anti-E2 antibody (panels **a** and **h**, respectively), the ER marker calreticulin with a rabbit anti-calreticulin polyclonal antibodies (StressGen), and the intermediate compartment marker ERGIC-p53 with the MAb G1/93 directed against ERGIC-p53 [30]. Texas red- and fluorescein-conjugated secondary antibodies were from Jackson Laboratories and Bioatlantic. Fluorescence was observed with a Leica TCS Spectral (SP1) equipped with a DMR inverted microscope and 63 $\times$  objective with a numerical aperture of 1.4. For each line the same horizontal focus is shown. Colocalisation of Texas red and fluorescein was analysed in the merge image and quantified by using MetaMorph. Bars, 10  $\mu\text{m}$

self-assemble into VLPs that are secreted from cells [35]. The spike (S) protein is a type I membrane protein responsible for receptor binding and membrane fusion. It is not essential for particle assembly but is incorporated by interactions of its C-terminal domain with the M protein. Its ectodomain can be exchanged by that of other coronavirus S proteins or that of the VSV G protein, giving rise to chimeric viruses or VLPs [2, 11, 13, 18]. Here, we demonstrate that similar replacements allow the incorporation of the HCV glycoproteins into coronavirus VLPs.

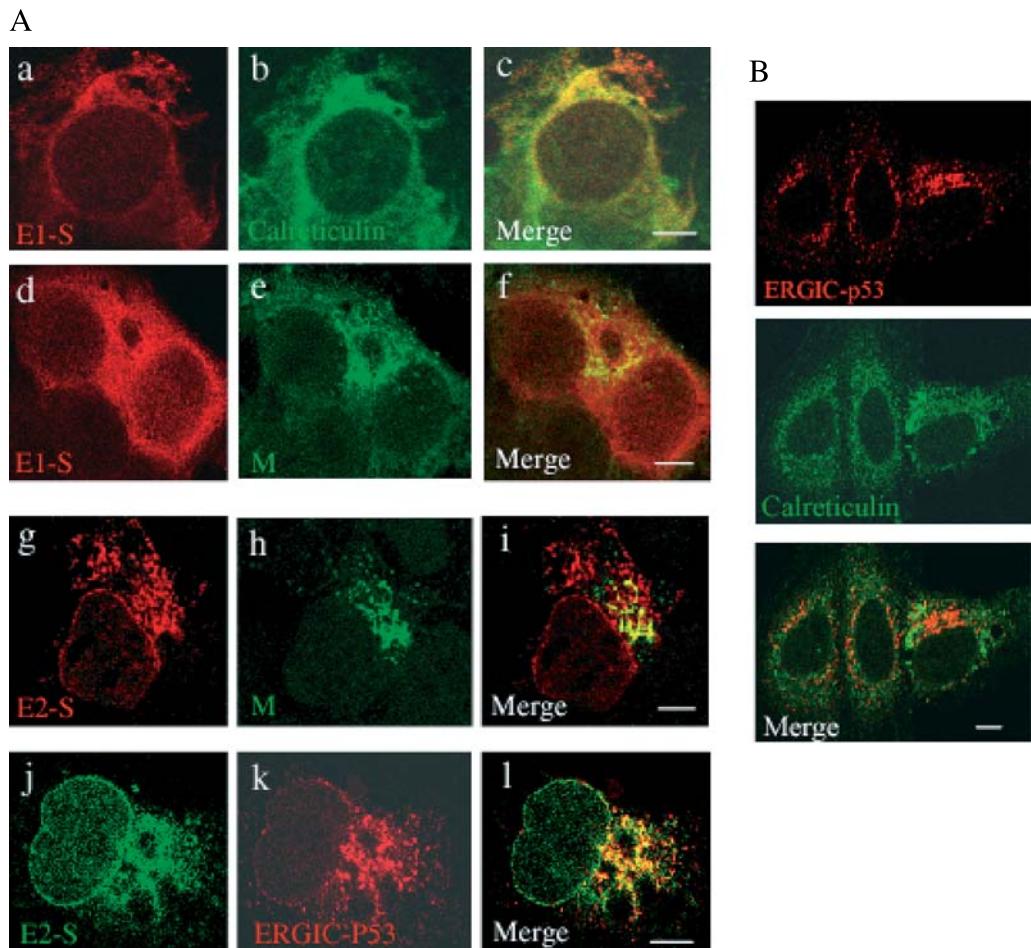
We generated chimeric HCV envelope constructs encoding the ectodomain of E1 or E2 protein fused to the transmembrane and endodomain of the mouse



hepatitis virus (MHV) S protein. The E1-S and E2-S chimeric genes were generated by a two-step PCR overlapping procedure using the S sequences from the plasmid pTUMS [33] and E1 and E2 sequences from the full-size cDNA of HCV strain BK [31]. The PCR products were cloned into pTUG3 [34], behind the bacteriophage T7 promoter for transcription. The resulting constructs are depicted in Fig. 1A. Expression of the chimeric proteins was studied in murine OST-7 cells [8] by first infecting with a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase (v-TF7-3) [10] and subsequently transfecting the cells with the plasmids, separately and together. The cells were labelled with  $^{35}$ S-amino acids from 5–8 h post-infection (p.i.), cell lysates were prepared, and immunoprecipitations carried out with anti-E1-specific (b) and anti-E2-specific (a) monoclonal antibodies (MAb), after which the resulting proteins were subjected to SDS-PAGE. As shown in Fig. 1B, both chimeric proteins were produced, E1-S and E2-S migrating with apparent molecular masses of approximately 44 and 64 kDa, respectively.

The subcellular distribution of E1-S and E2-S was analysed in Huh7 cells. Single- and double-transfected Huh7 cells were stained with specific antibodies and viewed by confocal immunofluorescence microscopy. In single-transfected cells (Fig. 1C) as well as in double-transfected cells (not shown), both E1-S and E2-S appeared in a perinuclear reticular pattern (panels a and d) fully superimposable by the staining pattern of calreticulin, an ER resident protein (panels c and f). This pattern was dramatically different from the punctuate profile exhibited by the IC marker ERGIC-p53, as illustrated for E2-S (panels g–i). It is clear that the chimeric proteins are retained in the ER, similarly to the native E1 and E2 glycoproteins.

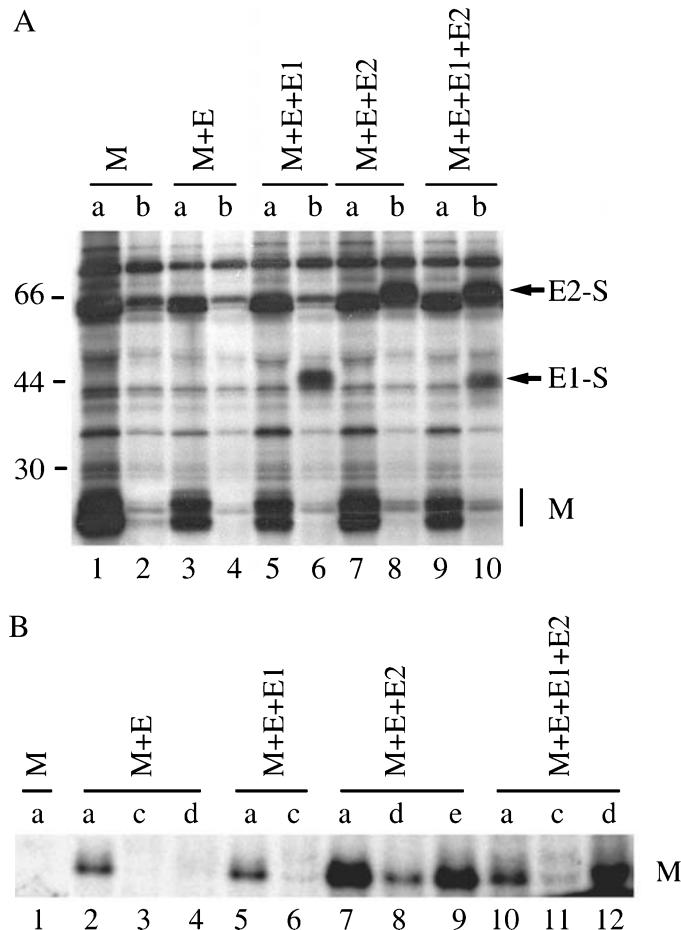
Next, we studied whether the E1-S and E2-S glycoproteins can be assembled into coronaviral VLPs. Initially, we analyzed whether the co-expression with the MHV strain A59 M and E proteins would change the E1-S and E2-S staining pattern (incorporation into VLPs should result in accumulation in the Golgi apparatus, where cargo destined for secretion concentrates). The E1-S protein co-expressed with M+E (Fig. 2A, panels a–f) or additionally with E2-S (not shown) was observed to display essentially the same reticular staining profile, overlapping with calreticulin (Fig. 2A, panel a–c), that we observed after single expression of the chimeric protein (Fig. 1C, panels d–f). However, a significant fraction, representing about 20% of the E1-S staining, as quantified using MetaMorph, was excluded from the ER staining as observed in the merge image. The same proportion of E1-S staining coincided with the coronavirus M protein (Fig. 2A, panels d–f), which is known to accumulate in the IC and Golgi complex [15, 16]. This shift in staining was even more significant when co-expressing the E2-S protein with M and E, both in the absence (Fig. 2A, g–l) and in the presence of E1-S (not shown). Now, about 60% of the E2-S staining signal colocalized with ERGIC-p53 and co-accumulated with the coronavirus M protein. Under the same experimental conditions, a dual labelling of both calreticulin and ERGIC-p53 (Fig. 2B) did not reveal any overlap of staining signal, indicating that the morphology and integrity of the organelles is well maintained. The appearance of E1-S and, particularly, E2-S in the IC and Golgi complex when co-expressed with



**Fig. 2.** Effect of co-expression of MHV M and E proteins on the subcellular localisation of E1-S and E2-S. Expressions were carried out in Huh-7 cells by vTF7-3 infection and transfections with plasmids encoding for M, E, E1-S, and E2-S proteins. At 5 h post infection, the cells were analysed by double-staining indirect immunofluorescence confocal microscopy. E1-S was immunostained with the anti-E1 MAb A4 and Texas Red® dye-conjugated goat anti-mouse IgG. The ER marker calreticulin was stained with rabbit anti-calreticulin polyclonal antibodies and fluorescein-conjugated sheep anti-rabbit IgG. The coronavirus M protein was stained with the rabbit polyclonal anti-MHV antibody k134 [28] and fluorescein-conjugated anti-rabbit IgG. E2-S was immunostained with either the anti-E2 MAb 185 (A, panel g) and Texas Red® dye-conjugated goat anti-mouse IgG or the R21 polyclonal anti-E2 antibody (A, panel j) and fluorescein-conjugated sheep anti-rabbit IgG. The intermediate compartment marker ERGIC-p53 was stained with the MAb G1/93 and Texas Red® dye-conjugated goat anti-mouse IgG. For each line, the same horizontal focus is shown and colocalisation of Texas red and fluorescein was analysed in the merge image and quantified by using MetaMorph. Bars, 10 μm

the M and E proteins, suggests that the proteins are indeed incorporated into VLPs, thereby enabling them to leave the ER and funnel into the post-ER compartments for secretion out of the cell.

Finally, incorporation of the chimeric E1-S and E2-S proteins into coronavirus VLPs was investigated by biochemical analysis as described before [11]. OST-7 cells were infected with vTF7-3 and transfected with combinations of plasmids



**Fig. 3.** Incorporation of chimeric E1-S and E2-S proteins into coronavirus VLPs. OST-7 cells were infected with vTF7-3 and transfected with different combinations of plasmids coding for the MHV M and E proteins and the chimeric E1-S and E2-S proteins. Cells were labeled with  $^{35}\text{S}$ -labeled amino acids from 5 to 8 h post infection, after which the cells and culture media were harvested separately. **A** Cells were lysed with detergent lysis buffer (10 mM Tris-HCl, (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X100, 20 mM N-ethylmaleimide, and 1 tablet of Protease Inhibitor Mixture (Roche) per 40 ml) and then processed for immunoprecipitation with the polyclonal anti-MHV rabbit serum k134 (lanes *a*) and with the peptide antibody W699 directed against the carboxy-terminal tail of the S protein (lanes *b*), and processed for SDS-PAGE. Molecular masses of marker proteins are indicated at the left, the positions in the gel of the chimeric proteins and of the M protein forms are indicated at the right. **B** VLPs isolated from the culture media were affinity purified with the polyclonal anti-MHV rabbit serum k134 (lanes *a*), the anti-E1 MAb A4 (lanes *c*), the conformation-independent anti-E2 MAb 185 (lanes *d*), or the conformation-dependent anti-E2 MAb 166 (lane *e*), and analysed by SDS-PAGE

encoding the coronaviral and chimeric proteins. They were labelled with  $^{35}\text{S}$ -amino acids for 3 h starting at 5 h p.i., after which (i) the cells were analysed to check the expression of the proteins, and (ii) the culture media were processed to study the presence and composition of VLPs. For the analysis of the expressed proteins, detergent lysates of the cells were prepared and subjected to immunoprecipitations. As shown in Fig. 3A, the synthesis of the M protein was obvious from the appearance of the characteristic bands representing different O-glycosylation forms, which were precipitated using a polyclonal anti-MHV serum (lanes a). The synthesis of the E1-S and E2-S proteins was analyzed using a peptide antiserum directed against the MHV S protein carboxy-terminal tail, which should be able to immunoprecipitate both chimeric proteins. The appropriate bands clearly appeared when the proteins were co-expressed with M and E either alone or together (lanes 6, 8 and 10) and were absent when E1-S and E2-S had not been expressed (lanes 2 and 4).

The VLPs released into the culture media were first purified by flotation through discontinuous sucrose density gradients as described earlier [11]. In this procedure, membranous particles and their associated proteins float to the top of the gradient during centrifugation, whereas proteins not associated with lipids remain in the more dense parts of the gradient. The presence, at the top of the appropriate gradients, of the typical spherical particles seen earlier for coronaviral VLPs [11] was confirmed by electron microscopy (data not shown). In order to establish whether the chimeric E1-S and/or E2-S proteins had been incorporated into these particles, we performed immunoprecipitations on samples of the top fraction of each gradient using antibodies directed against the E1 or E2 proteins, followed by PAGE. These analyses did not allow the direct demonstration of the chimeric proteins in any of the relevant samples, due probably to a too-low efficiency of incorporation. We therefore made use of an indirect assay used earlier for the sensitive detection of glycoproteins incorporated into coronaviral VLPs [2, 5, 11]. This assay involves the affinity-purification of intact VLPs using antibodies against the E1 or E2 protein and the subsequent analysis of the sample by PAGE, using the presence or absence of the M protein as the read-out parameter for VLP capture and, hence, for the presence or absence, respectively, of the chimeric protein in the particles. As a control to verify VLP release, a similar affinity-purification is done using the polyclonal anti-MHV serum, which recognizes epitopes in the M protein's ectodomain. The results of such analyses are shown in Fig. 3B, which first of all reveals that the negative control, single expression of the M protein, was indeed not productive (lane 1). The positive control, co-expression of M and E proteins, yielded VLPs that could be isolated with the MHV antiserum (lane 2), but these particles were not recognized by MAbs directed against E1 or E2 (lanes 3 and 4, respectively). In cells co-expressing M and E together with E1-S or E2-S or both, VLPs had been produced, as revealed by the anti-MHV serum (lanes 5, 7 and 10). These VLPs had incorporated E1-S and E2-S chimeric proteins, since they could be isolated by both anti-E1 (lanes 6 and 11) and anti-E2 MAb (lanes 8, 9 and 12). Though clearly positive as compared to the negative control (lane 3), the M protein band obtained after immuno-isolation with the anti-E1

MAb was always much weaker than with the antibodies directed against E2. This difference may relate to the different expression levels of the two proteins (see also Fig. 3A) or to the different qualities (affinities) of the antibodies, or it may indicate that E1-S is incorporated less efficiently than E2-S. The latter interpretation is consistent with the results of the immunofluorescence studies of Fig. 2, which showed that in the presence of the M and E proteins, a larger fraction of E2-S than of E1-S escapes from the ER.

In conclusion, the results obtained consistently indicate that E1-S and E2-S can be incorporated into coronavirus VLPs and subsequently secreted with these particles. This incorporation occurred in early membranes of the secretory pathway, as the chimeric proteins were able to move out from the ER only when co-expressed with the coronavirus envelope proteins that assemble at ER and IC membranes. Because these HCV chimeric particles have the lipid composition of early, i.e. pre-Golgi membranes, and because the E1 and E2 glycoproteins in these particles are processed and become mature in much the same way as during normal infection, they presumably mimic the native HCV particle very closely. They should therefore be suitable for studying early infection steps of HCV that require specific interactions with proteins and lipids of the target cell and might be tested for their vaccine potential. Finally it seems feasible now to integrate the chimeric E1-S and E2-S genes into the background of an attenuated MHV genome, replacing the S gene and generating chimeric viruses that allow safe studies of the early steps in the HCV life cycle.

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