Only the non-glycosylated fraction of hepatitis E virus capsid (open reading frame 2) protein is stable in mammalian cells

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Hepatitis E virus (HEV) is a non-enveloped, positive-strand RNA virus, with the genome encoding three open reading frames (ORFs) of which ORF 2 directs translation of the capsid protein, PORF2. Following pulse-labelling and cell fractionation of PORF2 expressed in mammalian cells using the Semliki Forest virus replicon, the capsid protein was detected as three major species of 78 (PORF2), 82 and 86 kDa, with P82 and P86 being N-glycosylated (gPORF2 and ggPORF2, respectively). Although gPORF2 and ggPORF2 species represented 79% of total PORF2 after 20 min metabolic labelling and were largely membrane-associated, the glycosylated PORF2 species were much less stable than non-glycosylated PORF2, which was present in the cytosol and represented the major product accumulated in the cell. In the absence of detectable surface expression or export of PORF2, this suggests that glycosylated ORF 2 proteins may not be intermediates in HEV capsid assembly.

Hepatitis E virus (HEV) is endemic in many developing countries and has been associated with large epidemics of enterically transmitted hepatitis (Bradley et al., 1987; Khuroo, 1980; Wong et al., 1980). HEV is a non-enveloped virus which was originally classified in the family 
Caliciviridae

on the basis of overall genome organization and virion structure (Cubitt et al., 1995), but is now unclassified (Pringle, 1998) because of unique aspects of the detailed genome organization and sequence motifs therein, some of which are homologous to those found in alphaviruses (Purdy et al., 1993). HEV has a single-stranded, positive-sense genome encoding three open reading frames (ORFs), with ORF 3 overlapping both ORF 1 and ORF 2 (Bradley, 1992; Purdy et al., 1993; Tam et al., 1991). The non-structural proteins, including the putative methyltransferase, helicase and RNA polymerase are encoded by ORF 1 while the major structural protein (PORF2) is encoded by ORF 2 (Tam et al., 1991). A hydrophobic stretch of 22 amino acids is present at the amino terminus of PORF2 and it has been suggested that this functions as a signal peptide (Jameel et al., 1996; Tam et al., 1991).

HEV cannot be cultured routinely, although it has recently been propagated in primary macaque hepatocytes (Tam et al., 1996, 1997) and a virus resembling HEV has been cultured in A549 cells (Huang et al., 1995). As a result, studies of HEV protein synthesis, processing and assembly have been limited to heterologous expression systems.

Expression of PORF2 in Sf9 insect cells using the baculovirus system yields stable protein products with estimated molecular masses of 72 kDa and 59–62 kDa. The full-length products from insect cells are insoluble, whereas the truncated products, mapping to amino acids 112–600, assemble into virus-like particles, indicating that cleavage and assembly of the capsid protein occurs in this system (McAtee et al., 1996; Tsarev et al., 1993, 1996; Zhang et al., 1997). In addition, expression of the truncated 112–600 PORF2 in Tn5 insect cells leads to further processing at the carboxyl terminus and efficient secretion of a 50 kDa PORF2 species assembled into subviral particles (Li et al., 1997b).

Translation of HEV ORF 2 is expected to yield a protein with a molecular mass of approximately 72 kDa, including a putative signal sequence and potential sites for N-linked glycosylation (Tam et al., 1991). Multiple ORF 2-specific proteins have indeed been observed in mammalian cells in studies using both plasmid-based expression (Jameel et al., 1996) and the Semliki Forest virus (SFV) expression system (Torresi et al., 1997), with molecular masses for the three species estimated as 72–74, 79–84 and 84–88 kDa. Using tunicamycin and endoglycosidase treatments we have confirmed that the two larger ORF 2-specific proteins observed in cells infected with rSFV/HEV ORF 2 particles are modified by N-linked glycosylation (results not shown). In our current experiments, these species migrate with estimated molecular masses of 78, 82 and 86 kDa; for clarity, the smallest ORF2-specific protein will therefore be described as PORF2, and the larger proteins as gPORF2 and ggPORF2, respectively, to reflect their glycosylation states.

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As the capsid protein of natural HEV particles has not been characterized, it is unclear whether any of the processing events observed (proteolytic processing and assembly in insect cells, glycosylation in mammalian cells) are relevant to the replicative cycle of the virus in hepatocytes. To begin to address this question, we have further examined the processing of PORF2 in mammalian cells using the SFV system as previously described (Torresi et al., 1997).

We previously observed that a significant fraction of PORF2 remained unmodified and in the cytosol during 3 h labelling periods (Torresi et al., 1997) which suggested that not all PORF2 molecules are translocated and glycosylated. To determine the proportion of PORF2 which is glycosylated, and the fate of glycosylated and non-glycosylated PORF2 in the cell, HepG2 cells were infected with rSFV/HEV ORF 2 particles, and labelled with $^{35}$S-amino acids for 20 min at 15 h post-infection. Cells were then fractionated into cytosol and membrane fractions (Torresi et al., 1997), and analysed by (i) immunoprecipitation to detect newly synthesized PORF2 species, and (ii) Western blotting to detect accumulated PORF2. For these purposes, hyperimmune sera were raised in rats. Recombinant ORF2.1 protein, representing the HEV-specific sequences of GST–ORF2.1 (Li et al., 1994) with an amino-terminal hexa-histidine tag, was expressed in E. coli, purified by metal affinity chromatography and absorbed to alum (unpublished). Wistar rats were immunized with 50 µg of protein via the intramuscular route at 0 and 4 weeks. Blood was collected and serum pooled from three animals at 9–11 weeks after primary immunization. These sera were used for immunoprecipitation (Lin et al., 1994) and immunoblotting (Li et al., 1997a) as previously described. $^{35}$S-labelled proteins were quantified using a Fuji phosphorimager, while densitometry of scanned immunoblots was performed using NIH Image software.

Immunoprecipitation (Fig. 1a) revealed that gPORF2 and ggPORF2 together represent 79% of the newly synthesized
HEV ORF 2 protein processing

did not enter the glycosylation pathway is stable and accumulates in the cytosol, whereas the large proportion of PORF2 which is cotranslationally translocated and N-glycosylated is subsequently degraded within the cell. The detection of significant amounts of labelled gPORF2 and ggPORF2 in the cytosol fraction (Fig. 1a), whereas they do not accumulate in that fraction (Fig. 1b), suggests that HEV glycoprotein degradation may occur in the cytosol, perhaps via interaction with the mammalian homologue of yeast Sec61p (Pilon et al., 1997), which leads to the export of misfolded glycoproteins from the ER lumen to cytosolic proteasomes for degradation.

These studies raise the question of whether it is the glycosylated, membrane-bound PORF2 or rather the cytosolic, non-glycosylated PORF2 which is involved in HEV capsid assembly in virus-infected cells. Studies of SFV envelope protein expression have shown that the glycoproteins are unstable in the absence of core protein (Zhao & Garoff, 1992), which might also be true of HEV PORF2 expressed here in the absence of other viral proteins and RNA, and so the instability of the protein alone does not exclude it from a role in capsid assembly. However, three additional lines of evidence argue against a role for membrane-associated glycoproteins in HEV assembly. Firstly, HEV particles are only 27–34 nm in diameter and non-enveloped (Bradley, 1992), while the g- and ggPORF2 species are almost exclusively membrane-associated. Secondly, truncated HEV PORF2 species, lacking the first 111 amino acids and thus the signal peptide, can form virus-like particles in insect cells with high efficiency (Li et al., 1997 b). Finally, we have shown here that a significant fraction of PORF2 fails to be translocated, which seems incompatible with an essential role for the glycoprotein. Rather, we believe that translocation of the major fraction of PORF2 may be a result of heterologous expression in the absence of other viral products which might normally prevent association of the nascent protein with the translocation complex.

We conclude that the non-glycosylated, cytosolic form of PORF2 which accumulates in mammalian cells is more likely to be the authentic HEV capsid protein precursor, while high level production of membrane-associated, glycosylated forms of PORF2 (Jameel et al., 1996; Torresi et al., 1997) may be an artefact of heterologous expression. Further studies are required to resolve the relative roles of full-length and truncated forms of PORF2 in the replication and assembly of HEV, and the characterization of capsid proteins from native HEV particles should be a priority in this regard.

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