The C-terminal region of the hepatitis C virus E1 glycoprotein confers localization within the endoplasmic reticulum

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Expression of the hepatitis C virus glycoprotein E1 in cultured cells localizes it to the endoplasmic reticulum, suggesting that E1 contains a signal mediating retention. Fusion of the C-terminal region of E1 to the ectodomain of CD4 prevented it from being transported to the cell surface. Fusion of this region of E1 resulted in localization of CD4 and influenza virus haemagglutinin chimeric molecules to a pre-medial Golgi compartment. This signal was present within E1 residues 311–383. Retention was not due to misfolding since the chimeric molecules did not form disulphide-linked aggregates indicative of misfolded proteins, and could be recognized by MAbs specific for conformational epitopes.

Members of the Flaviviridae family, such as hepatitis C virus (HCV), are believed to bud into the endoplasmic reticulum, from where virions are carried through the secretory transport pathway of the host cell, before release from the cell. The HCV genome encodes two putative envelope glycoproteins, E1 (polyprotein residues 192–383) and E2 (residues 384–746). Both are heavily modified by C-terminal hydrophobic anchor domains. Expression of E1 and E2 is believed to be type I integral transmembrane proteins, with C-terminal linked glycosylation and are believed to be type I integral transmembrane proteins, with C-terminal hydrophobic anchor domains. Expression of E1 and E2 in mammalian cell lines demonstrates their endoplasmic reticulum (ER) retention, with no cell surface expression detectable (Dubuisson et al., 1994; Ralston et al., 1993; Selby et al., 1994; Spaete et al., 1992). Immuno-electron microscopic studies localized the glycoproteins to the ER (Dubuisson et al., 1994; Deleersnyder et al., 1997). These observations are consistent with a model of HCV particle morphogenesis by budding into the ER. Truncation of both E1 and E2 glycoproteins at their C termini leads to their secretion from expressing cells (Matsuura et al., 1994; Michalak et al., 1997; Selby et al., 1994; Spaete et al., 1992), suggesting that signals mediating localization reside within their C termini. Furthermore, chimeric molecules containing the E2 ectodomain fused to a heterologous transmembrane domain sequence are expressed at the cell surface (Cocquerel et al., 1998; Flint et al., 1999). It has recently been reported that fusion of the C-terminal E2 region (residues 662–746) to CD4 resulted in ER retention of the chimeric protein (Cocquerel et al., 1998; Duvet et al., 1998).

Truncation of E1 after residue 311 causes it to be secreted from expressing cells (Michalak et al., 1997), suggesting the ER-retention signal resides between E1 residues 311 and 383. To determine if this region of E1 could confer ER localization on heterologous proteins, chimeric glycoproteins consisting of the ectodomains of the cell surface-expressed glycoproteins influenza A virus haemagglutinin (HA) and CD4, fused to the HCV sequence, were expressed. To achieve this, cDNA cassettes were constructed by PCR mutagenesis, where unique restriction sites were introduced at the sequence encoding the N and C termini of CD4 and HA, and at the junction between the ectodomain and the transmembrane region (Fig. 1a). Introduction of an Apal site at the junction between the ectodomain-transmembrane domains caused the substitution of an Ala residue for an Ile in the HA cassette (HA/CAS) and a Met for Gly in the CD4 cassette (CD4/CAS). PCR was used to generate inserts, which were ligated with the cassette DNA to form cDNA encoding a chimeric glycoprotein (Fig. 1a). Sequencing confirmed the identity of the cDNA clones.

To ascertain that the addition of any sequence to the HA ectodomain did not cause non-specific retention, the transmembrane domain and cytoplasmic tail of CD4 were fused to the HA ectodomain (HA/CAS+CD4CYT). The reciprocal chimeric protein was also constructed (CD4/CAS+HA_CYT). As a positive control for ER retention the cytoplasmic tail of the E3/19K protein of adenovirus (IMWACKYKSRRSFID-EKKMP-COOH) was fused to the CD4 ecto-and transmembrane domains (CD4/CAS+E3_CYT). The adenovirus E3 protein contains the well-characterized KXXX ER-retention signal (Nilsson et al., 1989). To test for the presence of a retention signal in E1, residues 311–383 were fused to both the HA and CD4 ectodomains.

To determine if the chimeric glycoproteins were expressed at the cell surface, 100 mm dishes of subconfluent HEK (293)
cells were transfected with 10 µg plasmid DNA encoding the CD4 chimeras using the calcium phosphate method. Forty-eight hours post-transfection cells were harvested and resuspended in PBS containing 1% FCS and 0.05% sodium azide. 1 x 10^6 cells were incubated with the anti-CD4 MAb Q4120 (5 µg/ml). Cells were washed and incubated with anti-mouse FITC (1:250) and analysed by flow cytometry. Both the wild-type CD4 molecule and the CD4_CAS_HA_TMCT protein were detected at the surface of unfixed cells, whilst chimeric CD4/CAS+E1311–383 and CD4/CAS+E2661–746 glycoproteins were not (Fig. 1b). This demonstrated that introduction of the mutation during construction of the CD4 cassette did not prevent export to the cell surface. To confirm expression of the chimeric glycoproteins, cells were fixed and permeabilized (Leucoperm, Serotec) before incubation with antibody (Fig. 1b). When visualized using fluorescence microscopy, the permeabilized cells expressing CD4/CAS+E1311–383 and CD4/CAS+E2661–746 exhibited a perinuclear staining characteristic of ER localization (data not shown).

To monitor the intracellular transport of the chimeric glycoproteins, the sensitivity of their glycans to endoglycosidase treatment after pulse–chase labelling was assayed. Endo-β-N-acetylglucosaminidase H (endo-H) removes high mannose, but not complex forms, of N-linked glycans (Tarentino & Maley, 1974), whereas peptide N-glycosidase F (PNGase F) removes both high mannose and complex N-linked sugars. Resistance to endo-H, and sensitivity to PNGase F, indicates transport of a protein from the ER to at least the medial Golgi compartment.

Subconfluent monolayers of HEK cells grown in six-well plates were transfected with 5 µg plasmid DNA using lipofectamine (Life Technologies, GibcoBRL). Twelve hours after transfection, the cells were incubated with Cys/Met-free DMEM for 20 min at 37 °C. Pulse–chase labelling was carried out by incubating cells with Cys/Met-free DMEM supplemented with 50 µCi/ml [35S]-PROMIX (Amersham) for 30 min, followed by DMEM supplemented with the usual amount of Cys and Met for 3 h. Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) and clarified by centrifugation in a microfuge for 15 min at 4 °C. The chimeric glycoproteins were immunoprecipitated from the supernatants using antibodies specific for the ectodomains of HA (Fig. 2a) or CD4 (Fig. 2b) and protein G–agarose. Digestion with PNGase F and
endo-H (New England Biolabs) was carried out according to the manufacturer’s instructions. HA derived from both the wild-type and cassette vectors (HA/WT, HA/CAS; Fig. 2a) became resistant to endo-H within a 3 h chase period, indicative of their transport through the cis-Golgi. Wild-type HA attained resistance to endo-H slightly faster than the HA/CAS protein. The mutation introduced during the construction of HA/CAS may have altered the kinetics of folding or transport. Since HA/CAS became resistant to endo-H during the 3 h chase period this phenomenon was not investigated further. The negative control molecule with the HA ectodomain fused to the transmembrane and cytoplasmic domains of CD4 (HA/CAS › CD4™CT; Fig. 2a) also became resistant to endo-H during the chase period. Thus, addition of a heterologous sequence to the HA ectodomain could be tolerated without causing misfolding and inducing ER retention by a quality control mechanism. In contrast to the wild-type and cassette molecules, the HA fusion protein containing E1 residues 311–383 remained sensitive to endo-H after a 3 h chase period (HA/CAS + E1311–383; Fig. 2a). Sensitivity to endo-H was also observed for the HA fusion protein containing E2 residues 661–746 (HA/CAS + E2661–746). These results indicate that E1 residues 311–383 induce retention in a premedial Golgi compartment, probably the ER.

To provide further evidence for the existence of a retention signal within E1, a similar analysis was carried out for the CD4 chimeras. The CD4 protein contains two N-linked glycans, and only one becomes endo-H resistant (Shin et al., 1991), hence endo-H-resistant and -sensitive forms are less obvious than with HA. Similar results were observed as for the HA chimeras, with wild-type and cassette CD4 (CD4/WT and CD4/CAS; Fig. 2b) becoming resistant to endo-H during the chase, whilst chimeras containing both E1 and E2 sequences (CD4/CAS + E1311–383 and CD4/CAS + E2661–746) remained sensitive. The negative control molecule consisting of CD4 ectodomain fused to the HA transmembrane and cytoplasmic regions (CD4/CAS + HA™CT) became resistant to endo-H (Fig. 2b). As a positive control for ER retention the endo-H sensitivity of CD4 with the cytoplasmic tail of the adenovirus E3/19K protein (CD4/CAS + E3™CT) was tested. This molecule remained sensitive to endo-H after the chase period, consistent with its retention in the ER. Thus, data obtained for both the HA and CD4 chimeric molecules suggest that E1 contains a signal mediating retention in a pre-Golgi compartment.

Since quality control mechanisms ensure that misfolded glycoproteins are not exported from the ER, it was important to establish if the observed retention was simply due to misfolding. To determine if the HA chimeras were misfolded, the proteins were expressed in HEK cells, labelled and immunoprecipitated under non-denaturing conditions (150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 10 mM Tris pH 7.5) with a MAb against a conformational epitope (HC67; Daniels et al., 1983). All of the chimeras, including HA/CAS + E1311–383 and HA/CAS + E2661–746 could be immuno-
precipitated by HC67 (Fig. 3a), suggesting that the ectodomains of these glycoproteins had adopted a native conformation. In addition, we determined if the CD4/CAS+E1<sub>311–383</sub> chimeric glycoprotein could be recognized by MAbs directed against conformational epitopes within the CD4 ectodomain (Fig. 3b). Both CD4/CAS and the chimeric protein could be immunoprecipitated by all five MAbs tested; 63G4 interacts with a linear epitope, and the four remaining MAbs recognize conformational epitopes. These data suggest that the CD4 ectodomain adopts a native conformation within the context of the CD4/CAS+E1<sub>311–383</sub> chimeric protein.

Formation of disulphide-linked aggregates is a common fate of misfolded proteins which are retained in the ER (Machamer & Rose, 1988). To determine if such aggregates were formed during the folding of CD4/CAS+E1<sub>311–383</sub> which would suggest that retention occurred by virtue of misfolding, radiolabelled chimeric molecules were analysed by reducing and non-reducing SDS–PAGE (Fig. 3c). These proteins were immunoprecipitated under non-denaturing conditions in buffer supplemented with 20 mM iodoacetamide. Under these conditions aggregates of HCV E1 and E2 are observed (Dubuisson et al., 1994); however, no disulphide-linked aggregates were apparent for either CD4/CAS or CD4/CAS+E1<sub>311–383</sub>. Together, these results suggest that CD4/CAS+E1<sub>311–383</sub> is retained in the ER by a mechanism that does not involve the ER quality control system or aggregation due to misfolding.

Localization of HCV glycoproteins within the ER has been observed using a number of different expression methods, including recombinant vaccinia and Sindbis viruses (Dubuisson et al., 1994) and stable expressing cell lines (Duvet et al., 1998). Duvet and colleagues (1998) recently reported that the C-terminal region of E2 contains a signal mediating retention in the ER. We have demonstrated a similar property for the C-terminal region of E1. It is interesting that both the HCV glycoproteins contain sequences that mediate retention. Whilst the folding of E1 and E2 occurs slowly, these proteins associate whilst folding (Deleersnyder et al., 1997). Since unfolded proteins are not permitted to leave the ER, the reason for two independent signals is not obvious.

Multiples mechanisms for retention of proteins within the ER have been described, including retention mediated through KDEL, di-lysine and di-arginine motifs. These motifs induce retention by signalling for proteins containing them to be retrieved from post-ER compartments. However, many ER resident proteins do not bear these retrieval sequences, suggesting that additional mechanisms for ER targeting also exist. The regions of HCV E1 and E2 which mediate ER retention do not possess similarity with other known ER localization signals, or with each other, except for the fact that many of these sequences contain stretches of hydrophobic residues, possibly functioning as transmembrane domains. The C-terminal regions of E1 and E2 shown to confer ER retention must contain the membrane-spanning domains for these type I transmembrane proteins. Whilst targeting of proteins to the Golgi apparatus may be mediated through the length of the transmembrane domain of a protein (Bretscher & Munro, 1993; Munro, 1995), this property may also have a role in localizing proteins to the ER (Pedrazzini et al., 1996; Yang et al., 1997). This lipid-based sorting model suggests that, in the absence of dominant lumenal or cytosolic associations, proteins partition according to differences in membrane thickness and

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**Figure 3.** The chimeric molecules are not misfolded. (a) Recognition of HA chimeric proteins by MAb (HC67) specific for ‘native’ trimeric HA was assayed by immunoprecipitation. HEK cells were transfected with the indicated plasmid and metabolically labelled. Cells were lysed under non-denaturing conditions, and the chimeric proteins immunoprecipitated with a polyclonal rabbit serum (P), HC67 (C) or an irrelevant control MAb (E1<sub>311–383</sub> by MAbs specific for conformational epitopes was tested. Immunoprecipitation was carried out with anti-CD4 MAbs: lanes 1, 63G4; 2, 12.16.A2.F9; 3, JAH 7.3F11; 4, Q4084; 5, Q4120; 6, 38.96k (α-p24 irrelevant antibody control). (b) The recognition of CD4/CAS+E1<sub>311–383</sub> by MAbs specific for conformational epitopes was tested. Immunoprecipitation was carried out with anti-CD4 MAbs: lanes 1, 63G4; 2, 12.16.A2.F9; 3, JAH 7.3F11; 4, Q4084; 5, Q4120; 6, 38.96k (α-p24 irrelevant antibody control). (c) To determine if the CD4–E1 chimera formed disulphide-bridged aggregates indicative of misfolded proteins, CD4/CAS (lanes 1) and CD4/CAS+E1<sub>311–383</sub> (lanes 2) were immunoprecipitated with anti-CD4 MAb (Q4120) and analysed by reducing and non-reducing SDS–PAGE as indicated.
deformability between compartments of the secretory pathway. It may be that the HCV glycoproteins are ER-localized because of the properties of their transmembrane domains. This model predicts that localization is mediated in a sequence-independent manner, and consequently may be tested experimentally.

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References


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