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REVIEW ARTICLE

Biogenesis of hepatitis C virus envelope glycoproteins

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Introduction

Enveloped viruses possess a membrane or 'envelope', which is a lipid bilayer containing viral proteins. The envelope proteins of a virus play a pivotal role in its lifecycle. They participate in the assembly of the infectious particle and also play a crucial role in virus entry by binding to a receptor present on the host cell and inducing fusion between the viral envelope and a membrane of the host cell. To fulfil these functions, viral envelope proteins have to adopt dramatically different conformations during the virus lifecycle. In addition, to ensure that these conformational changes occur at the appropriate time, folding and assembly of these proteins have to be tightly controlled. Studying the early steps of viral envelope protein biogenesis is therefore very useful to those who wish to understand how the different functions of these proteins can be controlled. Hepatitis C virus (HCV) encodes two viral envelope glycoproteins, E1 and E2. The lack of a cell culture system supporting efficient HCV replication and particle assembly has hampered the characterization of the envelope proteins present on the virion. However, indirect evidence, like the neutralization of virus infectivity by antibodies, supports the idea that HCV envelope glycoproteins are present on the surface of the virion (Flint & McKeating, 2000). Our current understanding of the biogenesis of HCV envelope glycoproteins is based on cell culture transient-expression assays with viral or non-viral expression vectors. Here, we present the knowledge that has recently been accumulated on folding, assembly and subcellular localization of HCV envelope glycoproteins.

Synthesis, folding and assembly of E1 and E2

As for the other members of the *Flaviviridae*, HCV, which belongs to the genus *Hepacivirus*, encodes a single polyprotein. For HCV, this polyprotein contains approximately 3000 amino acids and is cleaved by cellular (signal peptidase) and viral (NS2-3 and NS3/4A) proteases to generate at least 10 products: NH_2 C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B COOH (Fig. 1). The cleavages in the structural region of the polyprotein (C/E1, E1/E2, E2/p7 and p7/NS2) are catalysed by a host signal peptidase localized in the endoplasmic reticulum (ER) (reviewed in Reed & Rice, 2000). Although cleavages at the N termini of E1 and E2 proceed to completion rapidly after translation (Dubuisson *et al.*, 1994, 2000), cleavage is delayed at the p7/NS2 site and, at least for the H77 strain, is incomplete at the E2/p7 site, resulting in the production of fully processed E2 and uncleaved E2-p7 (reviewed in Reed & Rice, 2000). The significance of these two forms of E2 in the HCV lifecycle has yet to be determined. HCV glycoproteins are type I transmembrane (TM) proteins with an N-terminal ectodomain and a C-terminal hydrophobic anchor. During their synthesis, the ectodomains of HCV glycoproteins are targeted to the ER lumen where they are modified by *N*-linked glycosylation. E1 and E2 possess up to 6 and 11 potential glycosylation sites, respectively. It has been shown that, due to the presence of a proline immediately downstream of the consensus sequence for *N*-linked glycosylation, one of the potential *N*-glycosylation sites of E1 is not recognized by the oligosaccharyltransferase (Meunier *et al.*, 1999). Interestingly, the efficiency of glycosylation of E1 is dependent on the presence of a downstream sequence on the HCV polyprotein (Dubuisson *et al.*, 2000). For E2, it has not yet been shown whether all of the potential glycosylation sites are modified, but glycosidase treatment indicates that this protein is extensively glycosylated (Dubuisson *et al.*, 1994).

Studies using transient expression systems have shown that E2 interacts with E1 to form oligomers. In the presence of non-ionic detergents, two forms of E1E2 complexes are detected: a heterodimer of E1 and E2 stabilized by non-covalent interactions, and heterogeneous disulphide-linked aggregates (Dubuisson *et al.*, 1994) (Fig. 2). Extensive characterization of the non-covalent heterodimer supports the idea that this oligomer is most likely the prebudding form of the functional complex which will play subsequently an active role in the entry process into host cells (Deleersnyder *et al.*, 1997). Indeed, this non-covalent heterodimer is homogeneous and resistant to protease digestion. Its components have acquired intramolecular disulphide bonds and it no longer interacts with ER chaperones, indicating that it is extensively folded. The disulphide-linked aggregates are likely dead-end products (Dubuisson, 2000). Such aggregates were first observed when the proteins were expressed by virus vectors such as vaccinia virus and Sindbis virus, which can induce a high level of protein synthesis (Dubuisson *et al.*, 1994; Grakoui *et al.*, 1993).

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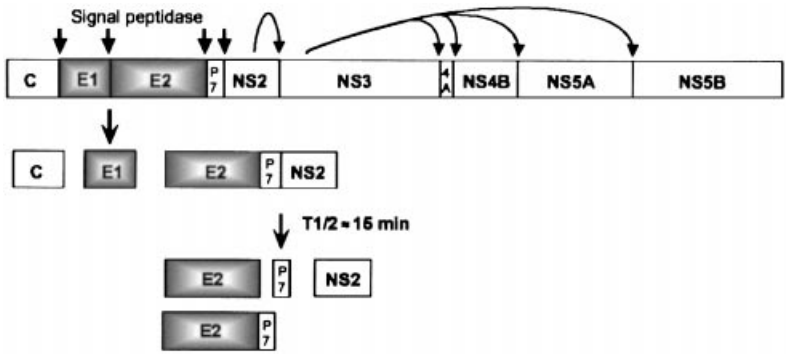

Fig. 1. Processing of the HCV polyprotein. Arrows above the polyprotein indicate the proteases involved in processing and their cleavage sites.

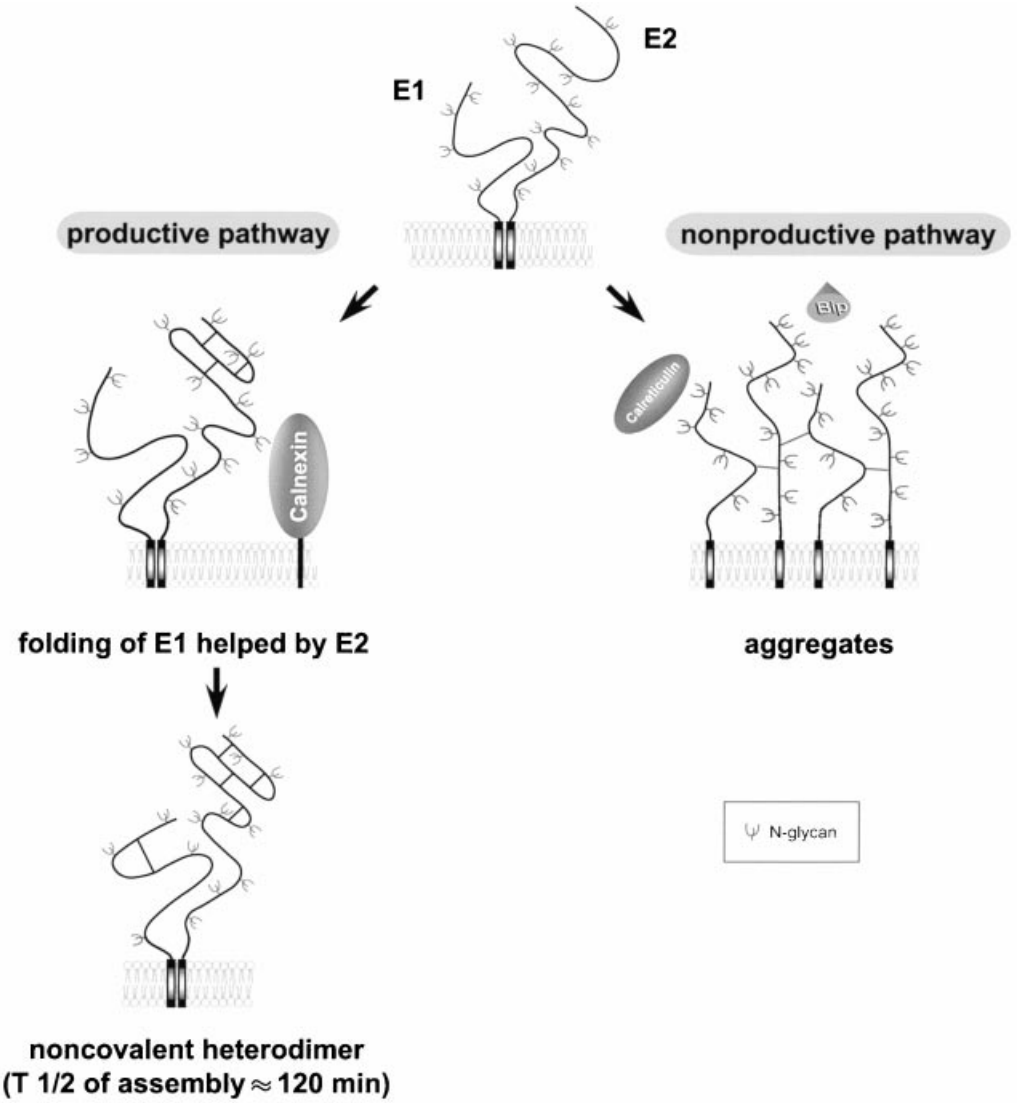

Fig. 2. Model summarizing the folding and assembly of HCV envelope glycoproteins.

However, aggregates have also been reported when HCV proteins are expressed in a non-virus vector which drives a lower level of protein synthesis (Choukhi *et al.*, 1999). This tendency to aggregate might therefore be an intrinsic property of HCV envelope glycoproteins. As recently proposed (Liberman *et al.*, 1999), the presence of such aggregates could play a role in the pathogenesis of HCV-induced diseases. However, because HCV does not replicate efficiently in cell

culture, it is hard to prove that, in the course of HCV infection, aggregates are also formed.

SDS–gel electrophoresis under non-reducing conditions has been used to monitor disulphide bond formation in HCV glycoproteins. When E1 was coexpressed with E2, an oxidized form of E1 was detected after 60 min of chase (Dubuisson & Rice, 1996). However, when E1 was expressed alone no oxidized form was observed (Michalak *et al.*, 1997). These observations indicate that the folding of E1 is slow and that coexpression of E2 is necessary for the proper folding of E1. In contrast to E1, disulphide bond formation in E2 appears to be complete by the time of E2-NS2 cleavage ($t_{1/2}$ 15 min). In addition, the kinetics of recognition of E2 by some conformation-sensitive human monoclonal antibodies (Habersetzer *et al.*, 1998) are similar to the kinetics of disulphide bond formation detected in E2. Together, these observations indicate that at least partial folding of E2 occurs rapidly. However, other conformation-sensitive mouse monoclonal antibodies showed slower kinetics of detection of E2, suggesting that their epitopes are located in a domain which folds slowly (Deleersnyder *et al.*, 1997; Duvet *et al.*, 1998; Michalak *et al.*, 1997). The epitopes recognized by these conformation-sensitive mouse monoclonal antibodies are dependent on the formation of intramolecular disulphide bonds (J. Dubuisson, unpublished data). Indeed, these antibodies recognize the non-reduced form of E2 but not its reduced form when analysed in Western blotting experiments.

Folding of proteins in the ER is assisted by chaperone molecules present in this compartment. ER chaperones calnexin, calreticulin and BiP have been shown to interact with HCV envelope glycoproteins (Choukhi *et al.*, 1998; Dubuisson & Rice, 1996). Calnexin and calreticulin are lectin-like chaperones which show an affinity for monoglucosylated *N*-linked oligosaccharides (Trombetta & Helenius, 1998). Binding of substrate glycoproteins to and release from calnexin and calreticulin depends on trimming and reglucosylation of the *N*-linked glycans. The absence of interaction between HCV glycoproteins and calreticulin or calnexin after tunicamycin treatment is consistent with the view that these chaperones act as lectins. In addition, the absence of glycans on E1 and E2 led to misfolding, demonstrating the essential role played by glycosylation for the folding of these proteins. Characterization of HCV envelope glycoproteins associated with ER chaperones indicates that calreticulin and BiP interact preferentially with aggregates of E1 and E2, and calnexin with non-covalently linked complexes (Choukhi *et al.*, 1998) (Fig. 2). These observations suggest that folding of the HCV envelope glycoproteins involved in the productive pathway of assembly is assisted by calnexin.

Regions involved in heterodimerization

The first attempts to identify amino acid residues involved in heterodimerization have led to the conclusion that the ectodomains of E1 and E2 play a major role in the contacts between these envelope glycoproteins. Indeed, coexpression of truncated forms of E1 and E2 led to secretion of E1E2 complexes (Lanford *et al.*, 1993; Matsuura *et al.*, 1994). Regions involved in these interactions have been mapped by far-Western blotting using bacterial recombinant proteins or by pull-down assays using proteins expressed in mammalian cells (Yi *et al.*, 1997). N-terminal sequences in E2 (amino acids 415 to 500 on the polyprotein) and also in E1 have been shown to be important for HCV glycoprotein interactions. However, deletion mutant analysis in the ectodomain of E2 has failed to identify any single region which is required for non-covalent interaction (Patel *et al.*, 1999). In addition, analysis of secreted complexes composed of C-terminally truncated forms of E1 and E2 has shown that in the absence of their TM domain, HCV envelope glycoproteins have a tendency to aggregate (Michalak *et al.*, 1997), indicating that one needs to be cautious in trying to identify amino acid residues involved in heterodimerization. Assembly of E1E2 oligomers has also been observed when the TM domains of HCV envelope glycoproteins were replaced by the TM domain of vesicular stomatitis virus G glycoprotein (Takikawa *et al.*, 2000), suggesting that the ectodomains of E1 and E2 contain the determinants for heterodimerization. In addition, these proteins were exported to the cell surface and were shown to be fusogenic, suggesting that they are functional for virus entry. However, in our hands, no detergent-resistant heterodimer could be observed with these chimeric proteins (C. Montpellier & J. Dubuisson, unpublished data). Further work will therefore be necessary to clarify the potential role of the ectodomains of E1 and E2 in heterodimerization.

Deletion of the TM domain of E2 or its replacement by the anchor signal of another protein has been shown to abolish the formation of E1E2 heterodimers (Cocquerel *et al.*, 1998; Michalak *et al.*, 1997; Patel *et al.*, 2001; Selby *et al.*, 1994), suggesting that the TM domains of HCV envelope glycoproteins are involved in heterodimerization. Recently, the role of these domains in the assembly of the E1E2 heterodimer has been analysed by alanine scanning insertion mutagenesis (Op De Beeck *et al.*, 2000). This technique has been shown to be a powerful method for detecting dimerization of TM α-helices (Braun *et al.*, 1997). Indeed, insertion of a single amino acid into a TM helix displaces the residues on the N-terminal side of the insertion by 110° relative to those on the C-terminal side of the insertion, disrupting a helix–helix packing interface involving residues on both sides of the insertion. Two distinct segments of the TM domain of E1 and one of the TM domain of E2 were very sensitive to alanine insertion (Fig. 3), demonstrating the essential role played by these domains in the assembly of HCV envelope glycoproteins. Interestingly, the amino-terminal segment of the TM domain of E1 contains a highly conserved GXXXG motif, which has been documented to ensure specific homodimerization of TM domains in TM proteins (Russ & Engelman, 2000).

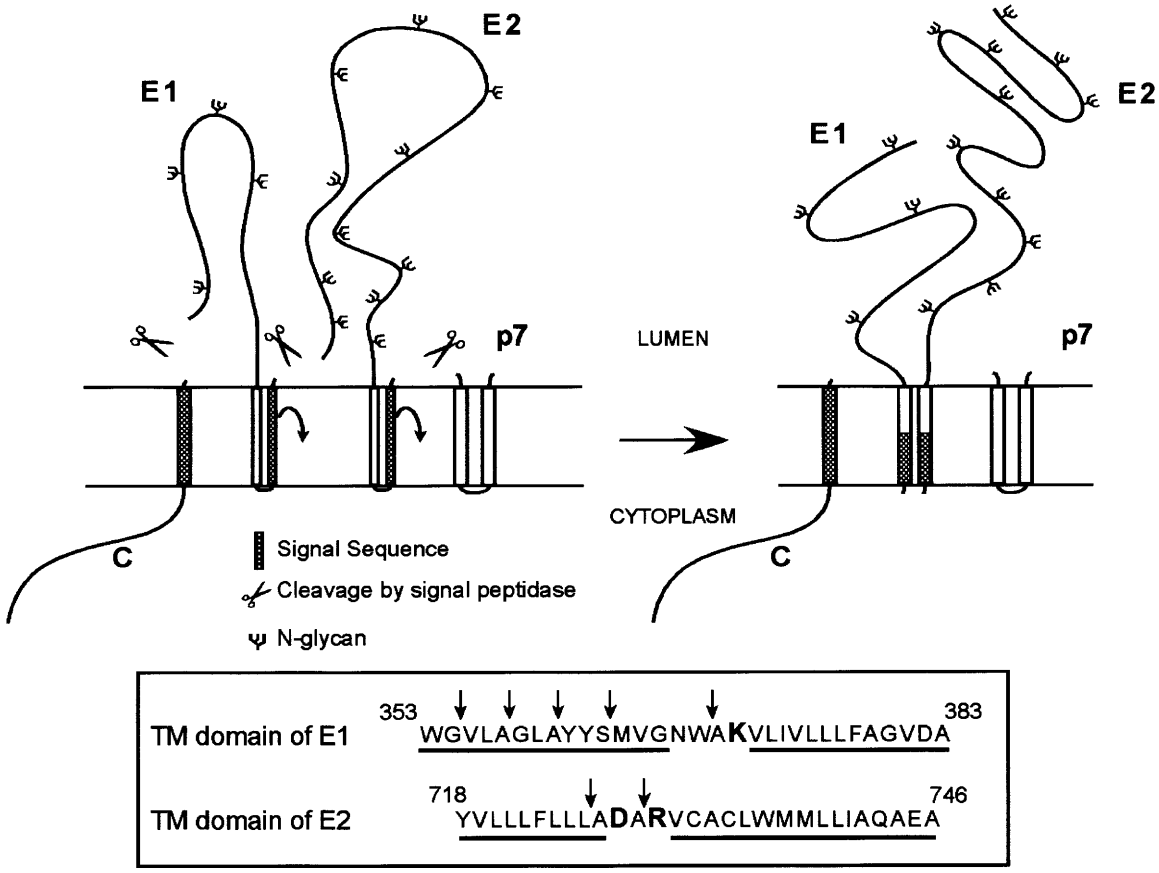

Fig. 3. Topology of the TM domains of HCV envelope proteins before and after signal sequence cleavage. The topology of p7 is putative. The sequences of the TM domains of HCV glycoproteins of the H strain are presented at the bottom of the figure. The numbers above the sequences of E1 and E2 indicate the positions of the N- and C-terminal limits of the TM domains within the HCV polyprotein. Charged residues present in the middle of the TM domains of E1 and E2 are in bold. Arrows indicate the positions of inserted alanine residues that disrupt E1E2 heterodimerization (Op De Beeck *et al.*, 2000). The two hydrophobic segments in the TM domains of E1 and E2 are underlined. The C-terminal hydrophobic segments of E1 and E2, including the positively charged residue immediately upstream, are signal sequences.

The TM domains of E1 and E2 are probably not the sole determinants of heterodimerization. Assisted folding of the ectodomain of E1 by E2 suggests that other region(s) than the TM domains might enter into contact. However, as discussed above, identifying these regions remains difficult. For many viral envelope proteins, the ectodomains have been shown to be involved in oligomerization (Doms *et al.*, 1993), which is important for the regulation of the fusogenic function of these proteins (Hernandez *et al.*, 1996). Why should there be different regions involved in heterodimerization of HCV envelope glycoproteins? Assembly and folding of HCV envelope glycoproteins seem to be interconnected events (Dubuisson, 2000). It is likely that an early contact between these proteins, probably initiated by their TM domains, is needed to bring their ectodomains into contact, which seems to be necessary for the formation of a native complex. This could explain why alanine insertion mutagenesis, which is disruptive for assembly, is also disruptive for the assisted folding of E1 by E2 (Op De Beeck *et al.*, 2000).

Subcellular localization of the E1E2 heterodimer

Enveloped viruses acquire their envelope by budding at cellular membranes. Virus budding can occur at the plasma membrane or at an intracellular membrane along the secretory pathway. In most cases, budding occurs at the cellular membrane to which the envelope proteins are directed. Immunolocalization studies and glycan analysis have shown that the non-covalent E1E2 heterodimer is located in an early compartment of the secretory pathway (Deleersnyder *et al.*, 1997). Immunolocalization of HCV glycoproteins has been analysed after treatment with nocodazole (Duvet *et al.*, 1998), a drug which disrupts microtubules, leading to a disintegration of the Golgi and interruption of traffic between the Golgi and the ER. Such a treatment did not lead to changes in the intracellular localization of HCV glycoproteins, suggesting that the heterodimer is retained in the ER. In addition, the glycans associated with these proteins are not modified by

Golgi enzymes, indicating that the ER localization of these proteins is not due to their retrieval from the *cis*-Golgi. These observations suggest that budding of HCV particles occurs in this compartment. However, due to the absence of a cell culture system supporting efficient HCV replication, it remains difficult to provide definitive proof that budding really occurs in the ER. Nevertheless, it has been shown for other viruses from the same family, i.e. the flaviviruses, that virions are detected in intracellular vesicles (probably modified ER) and are released from cells via the exocytic pathway (Pettersson, 1991). In addition, an electron microscopy study of a human T-cell line infected with HCV has also shown the accumulation of intracellular particles (Shimizu *et al*., 1996), reinforcing the idea that budding of HCV particles occurs in the ER or another early compartment.

ER retention of the E1E2 heterodimer suggests that specific signal(s) present in this complex are responsible for this subcellular localization. The presence of a retention signal was first demonstrated for E2 by Cocquerel *et al*. (1998). Indeed, E2 expressed in the absence of E1 can fold properly and is retained in the ER, as shown by the lack of complex glycans, its intracellular distribution and the absence of its expression at the cell surface. By using chimeric proteins constructed from E2 and proteins normally expressed at the cell surface, it has been shown that the TM domain of E2 is a signal for ER retention (Cocquerel *et al*., 1998; Flint *et al*., 1999; Forns *et al*., 2000; Patel *et al*., 2001; Takikawa *et al*., 2000). A similar approach has also been used to show that the TM domain of E1 is another signal for ER retention (Cocquerel *et al*., 1999; Flint & McKeating, 1999; Mottola *et al*., 2000). In addition to the TM domain, a second retention signal has also been identified in the ectodomain of E1 (Mottola *et al*., 2000). Indeed, a chimeric protein between CD8 and a 44 amino acid sequence of the ectodomain of E1 (located at position 290 to 333 of the polyprotein of the HCV BK strain) has been shown to be retained in the ER. However, data from other laboratories have shown that, when the TM domain of E1 is replaced by the TM domain of a protein normally expressed at the cell surface, E1 can be detected at the plasma membrane by immunofluorescence with anti-E1 antibodies (Forns *et al*., 2000; Takikawa *et al*., 2000), suggesting that this second retention signal is leaky. Alternatively, this retention signal might be buried in the structure of the ectodomain of E1. Immunolocalization of chimeric proteins containing the TM domain of E1 or E2 and analysis of their glycans have shown that these domains are signals for static retention in the ER (Cocquerel *et al*., 1999; Duvet *et al*., 1998; Patel *et al*., 2001). However, Martire *et al*. (2001) have observed that a small fraction of E2 migrates to the intermediate compartment and the *cis*-Golgi complex region, suggesting that the ER retention signal of E2 might be leaky. These data fit with the observation that a small fraction of E2 is not integrated in the ER membrane and follows the secretory pathway (Cocquerel *et al*., 2001).

The TM domains of E1 and E2 have been proposed to start at polyprotein positions 353 (Cocquerel *et al*., 1999) and 718 (Cocquerel *et al*., 2000), respectively (Fig. 3). These TM domains are composed of two hydrophobic stretches connected by a short hydrophilic segment containing one (Lys for E1) or two (Asp and Arg for E2) fully conserved charged residues (Cocquerel *et al*., 2000). In an attempt to identify residues in the TM domains of HCV envelope glycoproteins that are involved in ER retention, these charged residues have been mutated. Indeed, it has been shown for several non-viral proteins that a usual feature of membrane determinants for ER retention is the presence of one or several hydrophilic residues in the middle of the TM domain (Bonifacino *et al*., 1991). A mutagenesis study has confirmed that the presence of charged amino acid residues located in the middle of the TM domains of E1 and E2 plays a major role in ER retention of these proteins (Fig. 3) (Cocquerel *et al*., 2000). Indeed, replacement of the lysine residue in the TM domain of E1 by an alanine led to cell surface expression of a chimeric protein made of the ectodomain of CD4 in fusion with the TM domain of E1. Similarly, replacement of the aspartic acid and the arginine by alanine residues in the TM domain of E2 led to cell surface expression of E2. However, these mutations affected other functions of the TM domains of HCV envelope glycoproteins. They led to an alteration of the processing of the polyprotein and disrupted the heterodimerization of E1E2, indicating that these functions are tightly linked. In addition, these observations suggest that the charged residues located in the middle of the TM domains of HCV envelope glycoproteins play a crucial role in the structure of these domains.

Topology of the TM domains of E1 and E2

Because HCV synthesizes its polypeptides as a polyprotein that is cleaved co- or post-translationally by viral and host proteases, internal signal peptides must be used to target viral membrane proteins, e.g. the envelope glycoproteins, to the ER. As a consequence, after signal sequence cleavage, the signal peptides of these proteins remain bound to the C terminus of the protein located upstream on the polyprotein and contribute to the anchor function of these domains (Cocquerel *et al*., 2000). Indeed, deletion of the signal sequence present in the TM domain of E2 led to secretion of this protein. Similarly, deletion of the signal sequence present in the TM domain of E1 in a chimeric protein made of the ectodomain of CD4 in fusion with the TM domain of E1 led to secretion of this chimeric protein.

The topology adopted by the TM domains of HCV envelope glycoproteins is still unclear. The presence of a first hydrophobic stretch and a signal sequence function separated by charged residue(s) in the TM domains of E1 and E2 suggests that these domains are composed of two membrane-spanning segments with the charged residues facing the cytosol (Fig. 3). However, amino acid sequence analysis (Cocquerel *et al*., 2000) and recent data from our laboratory suggest that, after E1E2

heterodimerization, these TM domains likely exhibit a single membrane-spanning topology (Op De Beeck *et al*., 2000). How can we reconcile a single membrane-spanning topology for the TM domains of E1 and E2 with the signal sequence function present in the C-terminal half of these domains? The most likely explanation is that before signalase cleavage, the TM domains of E1 and E2 adopt a transient hairpin structure in the translocon with both their N- and C-termini facing the ER lumen. After cleavage, a reorientation of the second hydrophobic stretch would occur, leading to interaction of the TM domains of HCV envelope glycoproteins (Fig. 3). This model is supported by experimental data from our laboratory (L. Cocquerel, A. Op De Beeck, M. Lambot, J. Roussel, D. Delgrange, A. Pillez, C. Wychowski, F. Penin & J. Dubuisson, unpublished data). Indeed, we have shown that, in the absence of signal sequence cleavage, the TM domains of HCV envelope proteins form a hairpin structure. In contrast, when E1 or E2 was expressed alone and tagged at the C terminus with an epitope, the TM domain showed a single membrane-spanning topology. In addition, replacement of the charged residues present in the middle of the TM domains of E1 and E2 by alanine residues led to an alteration in the formation of the hairpin structures, indicating that these charged residues play a crucial role in the dynamic changes occurring in the TM domains of HCV envelope proteins. The dynamic behaviour of these TM domains is unique and it is linked to their multifunctionality. By reorienting their C terminus towards the cytosol and being part of a TM domain, the signal sequences at the C termini of E1 and E2 contribute to new functions: (1) membrane anchoring, (2) E1E2 heterodimerization, and (3) ER retention.

Concluding remarks

As for other envelope proteins that play a major role in virus assembly and entry, the biogenesis of HCV envelope glycoproteins is very complex. These proteins are targeted to the ER, where they fold and heterodimerize. In addition, they are retained in this compartment, where the virus particle is thought to acquire its envelope. The data that we have recently accumulated on the TM domains of E1 and E2 show that these domains are major players in these different events. Until recently, TM domains were mainly thought of as a stretch of hydrophobic residues whose only function was to anchor proteins in the lipid bilayer. The observation that short hydrophobic sequences like the TM domains of E1 and E2 can play multiple functions indicates that more attention should be given to TM domains in the functional studies of viral envelope proteins.

Because HCV does not replicate efficiently in cell culture, it is currently impossible to study later events in the maturation of HCV envelope glycoproteins that might occur during budding and maturation of the particle. However, the data that have been accumulated on closely related viruses, e.g. the flaviviruses and the pestiviruses (Rice, 1996), can provide information that might help us to understand the later steps of the HCV lifecycle.

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References

Bonifacino, J. S., Cosson, P., Shah, N. & Klausner, R. D. (1991). Role of potentially charged transmembrane residues in targeting proteins for retention and degradation within the endoplasmic reticulum. *EMBO Journal* **10**, 2783–2793.

Braun, P., Persson, B., Kaback, R. & von Heijne, G. (1997). Alanine insertion scanning mutagenesis of lactose permease transmembrane helices. *Journal of Biological Chemistry* **272**, 29566–29571.

Choukhi, A., Ung, S., Wychowski, C. & Dubuisson, J. (1998). Involvement of endoplasmic reticulum chaperones in folding of hepatitis C virus glycoproteins. *Journal of Virology* **72**, 3851–3858.

Choukhi, A., Pillez, A., Drobecq, H., Sergheraert, C., Wychowski, C. & Dubuisson, J. (1999). Characterization of aggregates of hepatitis C virus glycoproteins. *Journal of General Virology* **80**, 3099–3107.

Cocquerel, L., Meunier, J.-C., Pillez, A., Wychowski, C. & Dubuisson, J. (1998). A retention signal necessary and sufficient for endoplasmic reticulum localization maps to the transmembrane domain of hepatitis C virus glycoprotein E2. *Journal of Virology* **72**, 2183–2191.

Cocquerel, L., Duvet, S., Meunier, J.-C., Pillez, A., Cacan, R., Wychowski, C. & Dubuisson, J. (1999). The transmembrane domain of hepatitis C virus glycoprotein E1 is a signal for static retention in the endoplasmic reticulum. *Journal of Virology* **73**, 2641–2649.

Cocquerel, L., Wychowski, C., Minner, F., Penin, F. & Dubuisson, J. (2000). Charged residues in the transmembrane domains of hepatitis C virus glycoproteins play a key role in the processing, subcellular localization and assembly of these envelope proteins. *Journal of Virology* **74**, 3623–3633.

Cocquerel, L., Meunier, J.-C., Op de Beeck, A., Bonte, D., Wychowski, C. & Dubuisson, J. (2001). Coexpression of hepatitis C virus envelope proteins E1 and E2 in *cis* improves the stability of membrane insertion of E2. *Journal of General Virology* **82**, 1629–1635.

Deleersnyder, V., Pillez, A., Wychowski, C., Blight, K., Xu, J., Hahn, Y. S., Rice, C. M. & Dubuisson, J. (1997). Formation of native hepatitis C virus glycoprotein complexes. *Journal of Virology* **71**, 697–704.

Doms, R. W., Lamb, R. A., Rose, J. K. & Helenius, A. (1993). Folding and assembly of viral membrane proteins. *Virology* **193**, 545–562.

Dubuisson, J. (2000). Folding, assembly and subcellular localization of HCV glycoproteins. *Current Topics in Microbiology and Immunology* **242**, 135–148.

Dubuisson, J. & Rice, C. M. (1996). Hepatitis C virus glycoprotein folding: disulfide bond formation and association with calnexin. *Journal of Virology* **70**, 778–786.

Dubuisson, J., Hsu, H. H., Cheung, R. C., Greenberg, H. B., Russell, D. G. & Rice, C. M. (1994). Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. *Journal of Virology* **68**, 6147–6160.

Dubuisson, J., Duvet, S., Meunier, J. C., Op De Beeck, A., Cacan, R., Wychowski, C. & Cocquerel, L. (2000). Glycosylation of the hepatitis C virus envelope protein E1 is dependent on the presence of a downstream sequence on the viral polyprotein. *Journal of Biological Chemistry* **275**, 30605–30609.

Duvet, S., Cocquerel, L., Pillez, A., Cacan, R., Verbert, A., Moradpour, D., Wychowski, C. & Dubuisson, J. (1998). Hepatitis C virus glycoprotein complex localization in the endoplasmic reticulum involves a determinant for retention and not retrieval. *Journal of Biological Chemistry* **273**, 32088–32095.

Flint, M. & McKeating, J. A. (1999). The C-terminal region of the hepatitis C virus E1 glycoprotein confers localization within the endoplasmic reticulum. *Journal of General Virology* **80**, 1943–1947.

Flint, M. & McKeating, J. A. (2000). The role of hepatitis C virus glycoproteins in infection. *Reviews in Medical Virology* **10**, 101–117.

Flint, M., Thomas, J. M., Maidens, C. M., Shotton, C., Levy, S., Barclay, W. S. & McKeating, J. A. (1999). Functional analysis of cell surface-expressed hepatitis C virus E2 glycoprotein. *Journal of Virology* **73**, 6782–6790.

Forns, X., Allander, T., Rohwer-Nutter, P. & Bukh, J. (2000). Characterization of modified hepatitis C virus E2 proteins expressed on the cell surface. *Virology* **274**, 75–85.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M. & Rice, C. M. (1993). Expression and identification of hepatitis C virus polyprotein cleavage products. *Journal of Virology* **67**, 1385–1395.

Habersetzer, F., Fournillier, A., Dubuisson, J., Rosa, D., Abrigniani, S., Wychowski, C., Nakano, I., Trépo, C., Desgranges, C. & Inchauspé, G. (1998). Characterization of human monoclonal antibodies specific to the hepatitis C virus glycoprotein E2 with in vitro binding neutralization properties. *Virology* **249**, 32–41.

Hernandez, L. D., Hoffman, L. R., Wolfsberg, T. G. & White, J. M. (1996). Virus–cell and cell–cell fusion. *Annual Review of Cell and Developmental Biology* **12**, 627–661.

Lanford, R. E., Notvall, L., Chavez, D., White, R., Frenzel, G., Simonsen, C. & Kim, J. (1993). Analysis of hepatitis C virus capsid, E1, and E2/NS1 proteins expressed in insect cells. *Virology* **197**, 225–235.

Liberman, E., Fong, Y. L., Selby, M. J., Choo, Q. L., Cousens, L., Houghton, M. & Yen, T. S. (1999). Activation of the grp78 and grp94 promoters by hepatitis C virus E2 envelope protein. *Journal of Virology* **73**, 3718–3722.

Martire, G., Viola, A., Iodice, L., Lotti, L. V., Gradini, R. & Bonatti, S. (2001). Hepatitis C virus structural proteins reside in the endoplasmic reticulum as well as in the intermediate compartment/cis-Golgi complex region of stably transfected cells. *Virology* **280**, 176–182.

Matsuura, Y., Suzuki, T., Suzuki, R., Sato, M., Aizaki, H., Saito, I. & Miyamura, T. (1994). Processing of E1 and E2 glycoproteins of hepatitis C virus expressed in mammalian and insect cells. *Virology* **205**, 141–150.

Meunier, J.-C., Fournillier, A., Choukhi, A., Cahour, A., Cocquerel, L., Dubuisson, J. & Wychowski, C. (1999). Analysis of the glycosylation sites of hepatitis C virus (HCV) glycoprotein E1 and the influence of E1 glycans on the formation of the HCV glycoprotein complex. *Journal of General Virology* **80**, 887–896.

Michalak, J.-P., Wychowski, C., Choukhi, A., Meunier, J.-C., Ung, S., Rice, C. M. & Dubuisson, J. (1997). Characterization of truncated forms of hepatitis C virus glycoproteins. *Journal of General Virology* **78**, 2299–2306.

Mottola, G., Jourdan, N., Castaldo, G., Malagolini, N., Lahm, A., Serafini-Cessi, F., Migliaccio, G. & Bonatti, S. (2000). A new determinant of endoplasmic reticulum localization is contained in the juxtamembrane region of the ectodomain of hepatitis C virus glycoprotein E1. *Journal of Biological Chemistry* **275**, 24070–24079.

Op De Beeck, A., Montserret, R., Duvet, S., Cocquerel, L., Cacan, R., Barberot, B., Le Maire, M., Penin, F. & Dubuisson, J. (2000). Role of the transmembrane domains of hepatitis C virus envelope proteins E1 and E2 in the assembly of the noncovalent E1E2 heterodimer. *Journal of Biological Chemistry* **275**, 31428–31437.

Patel, J., Patel, A. H. & McLauchlan, J. (1999). Covalent interactions are not required to permit or stabilize the non-covalent association of hepatitis C virus glycoproteins E1 and E2. *Journal of General Virology* **80**, 1681–1690.

Patel, J., Patel, A. H. & McLauchlan, J. (2001). The transmembrane domain of the hepatitis C virus E2 glycoprotein is required for correct folding of the E1 glycoprotein and native complex formation. *Virology* **279**, 58–68.

Pettersson, R. F. (1991). Protein localization and virus assembly at intracellular membranes. *Current Topics in Microbiology and Immunology* **170**, 67–104.

Reed, K. E. & Rice, C. M. (2000). Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. *Current Topics in Microbiology and Immunology* **242**, 55–84.

Rice, C. M. (1996). *Flaviviridae*: the viruses and their replication. In *Fields Virology*, 3rd edn, pp. 931–959. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott–Raven.

Russ, W. P. & Engelman, D. M. (2000). The GxxxG motif: a framework for transmembrane helix–helix association. *Journal of Molecular Biology* **296**, 911–919.

Selby, M. J., Glazer, E., Masiarz, F. & Houghton, M. (1994). Complex processing and protein:protein interactions in the E2:NS2 region of HCV. *Virology* **204**, 114–122.

Shimizu, Y. K., Feinstone, S. M., Kohara, M., Purcell, R. H. & Yoshikura, H. (1996). Hepatitis C virus: detection of intracellular virus particles by electron microscopy. *Hepatology* **23**, 205–209.

Takikawa, S., Ishii, K., Aizaki, H., Suzuki, T., Asakura, H., Matsuura, Y. & Miyamura, T. (2000). Cell fusion activity of hepatitis C virus envelope proteins. *Journal of Virology* **74**, 5066–5074.

Trombetta, E. S. & Helenius, A. (1998). Lectins as chaperones in glycoprotein folding. *Current Opinion in Structural Biology* **8**, 587–592.

Yi, M., Nakamoto, Y., Kaneko, S., Yamashita, T. & Murakami, S. (1997). Delineation of regions important for heteromeric association of hepatitis C virus E1 and E2. *Virology* **231**, 119–129.

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