THE HEPATITIS C VIRUS E2p7 LOCALIZATION AND TOPOLOGY IN A RECOMBINANT SYSTEM

COSTIN I. POPESCU,[1, 2, 3*] ALINA S. GRIGORESCU,[4] RAYMOND A. DWEK,[5] NICOLE ZITZMANN[5]

[1]*Univ. Lille Nord de France, F-59000 Lille, France*
[2]*CNRS, Institut de Biologie de Lille (UMR8161), F-59021 Lille, France*
[3]*Institut Pasteur de Lille, F-59019 Lille, France*
[4]*University of Minnesota, Department of Civil Engineering, 500 Pillsbury DR-SE, Minneapolis, USA*
[5]*Oxford Glycobiology Institute, Department of Biochemistry, South Parks Road, Oxford, OX1 3QU, United Kingdom*

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The Hepatitis C virus (HCV) genome encodes a polyprotein that is processed co- and post-translationally. Incomplete processing at the E2/p7 junction generates the E2p7 product. Using a recombinant system, we analysed the processing, localization and topology of E2p7. By immunoprecipitation of proteins expressed by metabolically labelled cells, we confirm that E2p7 is a precursor of E2. E2p7 forms a native-like heterodimer with E1, and it is localized entirely to the endoplasmic reticulum, in contrast to fully processed E2 and p7 that leak to the plasma membrane. No change in the topology of p7 was observed upon processing of E2p7, indicating that incomplete cleavage at the E2/p7 site is not regulated by changes in p7 membrane topology.

Key words: hepatitis C virus, polyprotein processing, E2p7 glycoprotein, topology.

INTRODUCTION

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is a small, positive-stranded enveloped RNA virus. HCV chronically infects over 170 million people worldwide and is a major cause of liver cirrhosis and hepatocellular carcinoma. The HCV genome encodes a polyprotein precursor of approximately 3000 amino acids that is proteolytically processed co- and post-translationally by endogenous and virally encoded proteases to produce at least 10 mature viral proteins. The structural proteins, core (C), E1, and E2 are located towards the N-terminus of the polyprotein. These proteins constitute the viral particle, however, their role in the HCV morphogenesis process is not completely understood. While

* Corresponding author. E-mail: pop@biochim.ro

the core protein forms the viral nucleocapsid, the envelope glycoproteins E1 and E2 form a native heterodimer that mediates virus entry in the hepatocyte (1, 2). The non-structural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B are located towards the C-terminus of the polyprotein precursor and they are responsible for viral RNA replication. Located between the structural and non-structural proteins is the small (63 amino acids) hydrophobic, membrane-spanning protein p7. Cleavages in the structural region (C/E1, E1/E2, E2/p7 and p7/NS2) are catalysed by signal sequence peptidase(s) located in the endoplasmic reticulum (ER) of the host cell (3, 4). The non-structural proteins are cleaved from the polyprotein by HCV proteases.

Processing of a large polyprotein precursor to produce the mature protein products is a typical feature of positive strand RNA viruses. As such, both the kinetics and level of expression of the mature proteins are controlled post-translationally. The occurrence of precursors due to delayed and/or incomplete processing of the polypeptide may provide a means for the virus to control expression of the mature proteins and the virus life cycle, as reported for the post-translational processing of the flavivirus C-prM precursor (5, 6). In most HCV strains studied, the cleavage between E2 and p7 is incomplete, generating E2 and E2p7 (7, 8). It is not clear if E2p7 is a precursor of E2 and p7 or whether the two species appear co-translationally in a constant ratio. In previous studies of E2p7 it has been shown that, depending on the recombinant system used, E2p7 may be processed either co- (viral vectors) or post-translationally (plasmid vector) (7, 9). Other reports indicate that E2p7 topology, with its C-terminus facing the cytosol, may change upon processing, generating free p7 with the C-terminus facing the ER lumen (10).

Here, using a recombinant system expressing the HCV structural proteins including E2p7 in both its uncleaved and cleaved forms, we investigated the processing, localization, and topology of E2p7. We show that E2p7 is a precursor of E2, which associates with E1 in native heterodimers and localizes exclusively in the ER, adopting a similar topology to p7.

MATERIALS AND METHODS

REAGENTS, CELL LINES AND ANTIBODIES

HeLa, CHO and HEK293T cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). Cells were grown in RPMI 1640 medium (Invitrogen, Paisley, Scotland), containing 10% fetal calf serum (FCS, Sigma, Poole, Dorset, UK), 50 units/ml penicillin, and 50 mg/ml streptomycin (Invitrogen, Paisley, Scotland), and maintained at 37°C with 5% CO_2.

The mouse monoclonal anti-HCV E2 antibodies A11 and H53 were a kind gift from Dr. J. Dubuisson (CNRS-Institut Biologie de Lille, France) (1). The pTriEx-CE1E2p7 plasmid (HCV strain H77) and pTriEx-E2p7 were kindly supplied by Dr. David Durantel (INSERM, Lyon, France) (11). The related plasmids pTriEx-HCVE2 and pTriEx-E2mutp7, which contain a mutation (AEA to VEV) at the C-terminus of E2, were provided by Dr. Olivier Argaud (University of Oxford, UK). Mouse monoclonal anti-FLAG (M2) antiserum was from Stratagene (La Jolla, Ca., US). Mouse monoclonal anti-HA antiserum and Proteinase K were from Roche (Mannheim, Germany). [^{35}S] methionine/cysteine (Tran^{35}S-label, specific radioactivity 1100 Ci/mmol) was from MP Biomedicals (Asse-Relegem, Belgium). CHAPS (3-[3-cholamidopropyl]-dimethylammonino-1-propanesulfate) was from Pierce Chemicals Co (Rockford, IL). All other chemicals were from Sigma (Poole, Dorset, UK).

CONSTRUCTS

Using the pTriEx-CE1E2p7, pTriEx-HCVE2, pTriEx-E2p7 and pTriEx-E2mutp7 plasmids (Figure 1A) as templates, four new constructs – cMycCFLAGE1E2p7HA (H1);

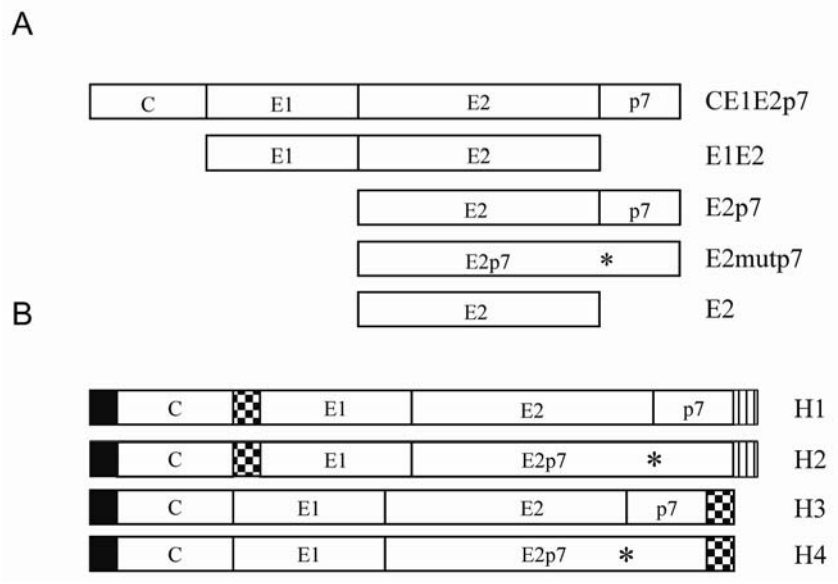

Fig. 1. – **Schematic representation of constructs used to express HCV proteins.**
(A) Constructs used as templates for cloning, as described in Materials and Methods. The E2mutp7 construct contains a mutation (AEA to VEV) at the C-terminus of the E2 protein that prevents cleavage between E2 and p7 (the asterisk denotes the site of mutation). (B) Constructs were created to incorporate cMyc (black rectangle), FLAG (checked rectangle) and HA (striped rectangle) tags. The H1, H2, H3, and H4 constructs were derived from CE1E2p7.

cMycCFLAGE1E2mutp7HA (H2); cMycCE1E2p7FLAG (H3); and cMycCE1E2mutp7FLAG (H4) – were created (Fig. 1B). Using basic molecular biology techniques, the sequences encoding the various HCV proteins (C, E1, E2, p7, and E2mutp7) were amplified from the appropriate template plasmid and cloned into the pTriEx-1.1 vector (Novagen EMD Biosciences, USA). The primers used incorporated sequences encoding for cMyc (EQKLISEEDL), FLAG (DYKDADDDK), and hemagglutinin (HA) (YPYDVPDYA) tags. The pTriex-E2mutp7, H2, and H4 constructs each contain a mutation (AEA to VEV) at the C-terminus of E2 that prevents cleavage between E2 and p7. The correct construction of the plasmids was verified by DNA sequencing (Department of Biochemistry, University of Oxford).

TRANSFECTION OF CELLS AND METABOLIC LABELLING

Constructs were transiently transfected in HEK293T, CHO and HeLa cells. Semi-confluent cells, grown for 1 day in 6-well dishes, were used to transiently express HCV cDNAs (1 μg DNA/well) using Lipofectamine Plus (Invitrogen, Paisley, UK). HEK293 cells were also transfected with polyethylimine (PEI)–DNA complexes: 3 μg DNA to 6 μl PEI (pH 9, 1 mg/ml). Cells were analyzed 24 hours after transfection. For metabolic labelling, transfected cells (10^7 cells/ml) were starved in cysteine- and methionine-free medium for 1 hour, pulse-labelled with 100-150 μCi [^{35}S] methionine/cysteine and chased for the times specified in the figure legends. Immediately after the chase, cells were harvested in cold PBS and lysed in CHAPS lysis buffer (50 mM HEPES buffer (pH 7.5), 2% CHAPS, 200 mM NaCl, and 0.5% protease inhibitor cocktail (Sigma), containing leupeptin, aprotinin, sodium EDTA, bestatin, AEBSF and E-64, for 1 hour, on ice.

IMMUNOPRECIPITATION AND SDS-PAGE

[^{35}S]-labelled cell lysates were clarified by centrifugation (30 min, 12000 g) and the supernatants incubated with H53 (1:100) or anti-FLAG (1:250) antibodies for 2 hours at 4°C, followed by the addition of 20 μl protein G Sepharose (GE Healthcare, Buckinghamshire, UK) and further incubation for 1 hour at 4°C. The slurry was washed 3 times with 0.5% CHAPS in 50 mM HEPES buffer. The antibody complexes were eluted by boiling the slurry for 5 min in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer with 5% (v/v) 2-mercaptoethanol and resolved by SDS-PAGE (12% gels). The gels were visualized by autoradiography.

PNGase F DIGESTIONS

Immunoprecipitated [^{35}S]-labelled samples, prepared as described, were eluted from protein G Sepharose in PNGase F (New England Biolabs, Hitchin,

UK) denaturing buffer (0.5% SDS, 1% 2-mercaptoethanol) by incubation for 5 min at 100°C. Samples were cooled and mixed with 1/10 (v/v) PNGase F reaction buffer (50 mM sodium phosphate, pH 7.5) and 1/10 (v/v) NP-40 (10%). To one half of each sample, 500 U PNGase F (1 µl) was added, while the other half contained PNGase F buffer alone. Digested samples and non-digested controls were incubated for 18 hours at 37°C. In Western blotting experiments, cell lysates containing 20 µg protein were denatured in PNGase F denaturing buffer (0.5% SDS, 1% 2-mercaptoethanol) for 5 min at 100°C, cooled and mixed with 1/10 (v/v) PNGase F reaction buffer.

WESTERN BLOTTING

Cells were harvested 24 hours after transfection and lysed in CHAPS lysis buffer. Proteins from transfected lysed cells were electrophoretically separated in 10% or 12% polyacrylamide gels, transferred to Immobilon membranes (Amersham, Buckinghamshire, UK) and detected with anti-HCV E2 antibody (A11; 1:250 dilution) in 5% milk, 0.1% PBS Tween-20. The membranes were incubated for 2 hours at room temperature (RT), followed by incubation with sheep anti-mouse IgG-HRP (1:8000 dilution). Proteins were detected using an enhanced chemiluminescent kit (ECL, Amersham Corp., UK), according to the manufacturer's instructions.

IMMUNOFLUORESCENCE

HeLa or HEK293T cells were plated on cover slips and transfected with the various constructs using the methods specified above. After 24 hours, the cells were rinsed with PBS and either fixed with paraformaldehyde (PFA) for 20 min at RT or fixed and permeabilized with methanol at -20°C for 5 min. For some samples, the PFA fixed cells were treated with Proteinase K (200 µg/ml) for 30 min at 4°C. After washing three times in PBS, cells were permeabilized with a) digitonin (5 µg/ml) for 15 min at RT, or b) Triton X-100 (0.1% in PBS) for 5 min at RT, or c) both digitonin and Triton X-100. After washing with PBS, the cells were incubated with primary antibodies, either anti-FLAG (1:100 dilution in PBS) or H53 (1:100 dilution in PBS), for 30 min at RT. Following three washes with PBS, the cells were further incubated with the appropriate Alexa 594-conjugated secondary antibodies (1:400 dilution; Molecular Probes, Invitrogen, Paisley, UK) in PBS, for 30 min at RT. Finally, cells washed three times with PBS were mounted in Vectashield mounting medium (Vector Laboratories, CA) and viewed with a Nikon Eclipse E 600 fluorescent microscope. Images were processed using Adobe Photoshop 5.0 software.

RESULTS

E2p7 IS A POST-TRANSLATIONALLY PROCESSED PRECURSOR OF THE E2 PROTEIN AND FORMS A NATIVE HETERODIMER WITH E1

In the absence of specific anti-p7 antibodies, to enable detection of the proteins of interest on Western blots, constructs were prepared to incorporate the well-characterised recombinant protein fusion tags cMyc, FLAG, and HA, as shown schematically in Fig. 1. To be able to distinguish between E2 and E2p7 products, the cleavage site at the C-terminus of E2 was mutated (AEA to VEV) and the constructs cMycCFLAGE1E2mutp7HA (denoted H2) and cMycCE1E2mutp7FLAG (denoted H4) prepared.

To investigate the kinetics of HCV polyprotein processing, HEK293 cells were transfected with the cMycCFLAGE1E2p7HA (H1) construct. Twenty hours post-infection, the cells were pulse-labelled for 10 minutes and chased for up to 4 hours. They were further lysed, digested or not with PNGase F, and immunoprecipitated with anti-E2 (H53) and anti-FLAG antibodies (Fig. 2A and B). Bound proteins were then analysed by SDS-PAGE under reducing conditions. As expected, glycoproteins treated with PNGase F had increased electrophoretic mobility due to the decrease in their molecular mass after glycan removal.

Processing of the E2p7 precursor to E2 was monitored using the anti-E2 (H53) antibody and is shown in Figure 2A. While at 0 min of chase the ratio of deglycosylated E2/E2p7 was approximately 0.1, at 2 hours of chase the ratio increased to 0.5 and remained unchanged, as measured by gel densitometry using in-house software. The processing of the E2p7 precursor to E2 in the plasmid based recombinant system is therefore post-translational and incomplete. This is in agreement with Brazzoli *et al*., who observed an increase in free E2 from 30%, at the zero chase point, to 61% at 2 h and up to 67%, at 6 h (9).

The formation of E1E2 heterodimers is thought to be important for mediating viral entry (1, 2). To understand the role of E2p7 in the HCV life cycle, processing of the HCV polypeptide was monitored using the H1 construct and an anti-FLAG antibody to detect E1 and associated products (Fig. 2B). Here processing of the polypeptide was monitored for 2 hours, as we had observed that the ratio of E2/E2p7 did not change after this period (Fig. 2A). Although detection was poor at the zero chase point, E1 and associated products were clearly detected at the 1 and 2 hour time points. A product migrating at approximately 30 kDa, the expected molecular mass for glycosylated E1, was immunoprecipitated confirming the correct processing of this protein (Fig. 2B). A sub-population (ΔE1) was also observed, probably due to partial E1 mRNA splicing, as previously reported (12).

The anti-E2 and anti-FLAG antibodies both pulled down a product of approximately 80 kDa with similar kinetics (Fig. 2A and B). An anti-HA immunoprecipitation confirmed that this product was E1E2p7, which is in agreement with other reports

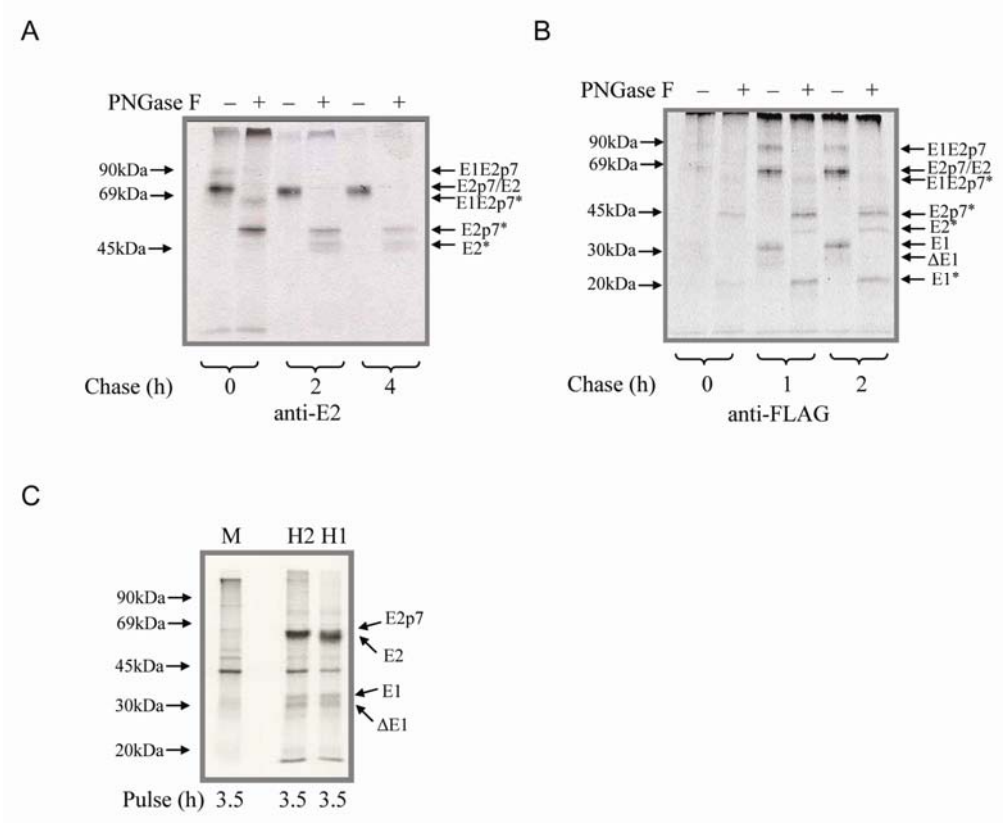

Fig. 2. – **E2p7 is a post-translationally processed precursor of E2 and forms native-like heterodimers with E1.**

HEK293 cells were transfected with the H1 construct (cMycCFLAGE1E2p7HA). Cells were pulse-chased (10 min pulse) 20 hours post-transfection. Samples were collected at the chase points indicated and lysed in HEPES/CHAPS lysis buffer as described in Materials and Methods. The samples were subjected to PNGase F digestion (+) or not (–) and immunoprecipitated with **(A)** anti-E2 (H53) or **(B)** anti-FLAG antibodies. The antibody complexes were separated electrophoretically by SDS-PAGE under reducing conditions and the gels visualized by autoradiography. Molecular weights and protein identities are shown on the left and right sides of the images, respectively. Asterisks denote deglycosylated products. **(C)** HEK293 cells were transfected with H2 (cMycCFLAGE1E2mutp7HA) and H1 (cMycCFLAGE1E2p7HA). Cells were pulsed for 3.5 hours, 20 hours post-transfection. Samples were collected and lysed in HEPES/CHAPS lysis buffer. The lysates were immunoprecipitated with the anti-E2 (H53) antibody, separated by reducing SDS-PAGE (12%) and autoradiographed. Molecular weights and protein identities are shown on the left and right sides of the images, respectively.

(10) (data not shown). A 60 kDa product corresponding to the molecular mass of glycosylated E2p7 and/or E2 was also immunoprecipitated by the anti-FLAG antibody (Fig. 2B). Digestions with PNGase F, to enable these glycosylated

products to be distinguished, showed that E1 interacted with both the E2 and E2p7 species throughout the chase period; bands corresponding to the molecular weights of each of these species are clearly detected. These data confirm the interaction of E2 with E1 (13) and show that E2p7 also interacts with E1. Between 1 and 2 hours of chase, when monitored using the anti-FLAG antibody, a decrease in the E2p7 species was observed accompanied by a corresponding increase in the E2 product. These data indicate that the E2p7 precursor interacts with E1 from the beginning of the chase period and that E2p7 is processed inefficiently whilst interacting with E1.

We next investigated whether E1 and E2p7 could form a native complex. The anti-E2 antibody (H53) is a conformation sensitive antibody, which recognises native E1E2 heterodimers that are non-covalently linked and do not recognize aggregated E2 molecules (1). Using this antibody, previous studies have shown that E2p7 and E2 interact with E1 (1, 10). However, these studies did not identify whether E2p7 was simply pulled down along with the E1E2 native heterodimers or if E1 and E2p7 form native-like heterodimers. To distinguish these possibilities, we analysed the processing of the HCV polypeptide in the presence and absence of the cleavage site between E2 and p7, to compare the interactions of E2 and E2p7 with E1.

Having confirmed the mutation of the cleavage site between E2 and p7 abrogated polyprotein processing in this region, resulting in the formation of E2p7 only and no free E2 (data not shown), the formation of E1E2 and E1E2p7 heterodimers was monitored. The cMycCFLAGE1E2p7HA (H1) and cMycCFLAGE1E2mutp7HA (H2) constructs were expressed in HEK293 cells. Twenty hours post-transfection, the cells were pulsed for 3.5 hours, lysed, and immunoprecipitated using the H53 antibody. The immunoprecipitated products were then electrophoretically separated by SDS-PAGE under reducing conditions. In addition to the expected bands observed for E2 and E2p7, in each case bands corresponding to the molecular weights of E1 and ΔE1 were also observed, indicating that both these species associate with E2 and E2p7 (Fig. 2C). These data indicate that the efficiency of E1E2 heterodimer formation is not affected by the removal of the E2p7 cleavage site and that E2p7 forms native-like heterodimers with E1.

E2p7 LOCALIZES EXCLUSIVELY TO THE ER MEMBRANE AND HAS A SIMILAR TOPOLOGY TO FREE p7

Although E1, E2 and p7 are mainly localized within the ER, there is some leakage to the plasma membrane (12, 14, 15, 16). Both p7 and E2p7 are thought to localize in the ER (14, 17) and ER membranes associated with mitochondria (18). Since ER-retention signals are reported for both E2 (19) and p7 (14), we hypothesised that E2p7 would be completely retained within the ER. To investigate the cellular localisation of E2p7, HeLa cells were plated on cover slips and transfected with constructs expressing E2p7 and E2mutp7. To allow visualisation of potential plasma membrane localization of the proteins, the transfected cells

were fixed with PFA without further permeabilization. These preparations were then compared with methanol fixed and permeabilized cells. The cells were immunofluorescently stained with the anti-E2 (H53) antibody and analysed by immunofluorescence (IF) microscopy. As shown in Figure 3A, while E2 leaked to the plasma membrane, E2p7 was not found at the cell surface. This is in agreement with other reports showing that a fraction of E2 (5%) and p7 (25%) leaks from the ER despite the retention signals present in both proteins (14, 19). However, unlike E2 and p7, E2p7 does not leak from the ER.

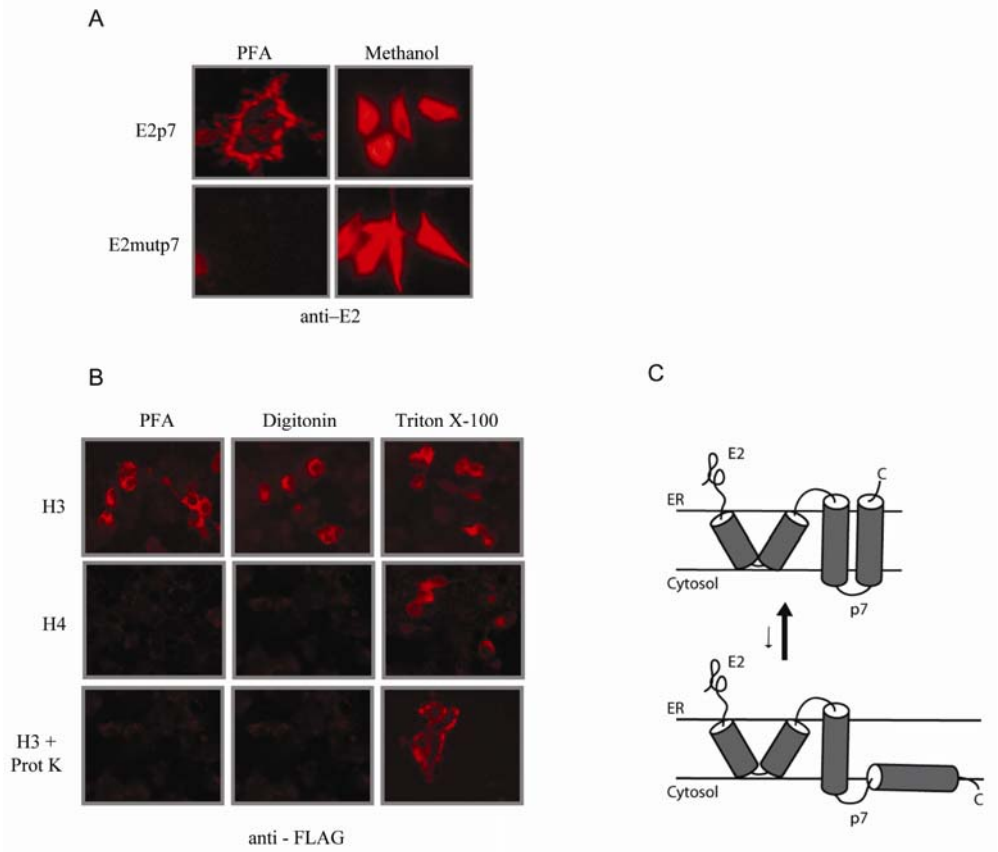

Fig. 3. – **E2p7 is completely retained in the ER and has a similar topology to free p7. (A)** HeLa cells transfected with plasmids expressing E2p7 and E2mutp7 were fixed either with PFA or methanol and stained with anti-E2 (H53) antibody. **(B)** HEK293 cells were transfected with H3 (cMycCFLAGE1E2p7FLAG) or H4 (cMycCFLAGE1E2mutp7HA) constructs as described in Materials and Methods. Transfected HEK293 cells were fixed with PFA (column 1), treated or not with Proteinase K and permeabilized either with digitonin (column 2) or Triton X-100 (column 3) and stained with an anti-FLAG antibody. **(C)** Proposed topological model of E2p7. The model shows both the C- and N- termini of p7 facing the ER lumen, however the possibility of a transient L-shaped form of p7 with the C-terminus facing the cytosol cannot be excluded. The torsion of the E2 transmembrane domains is hypothetical.

An earlier study of the topology of incompletely processed p7 showed that the C-terminus of p7, when expressed as E2p7, faces the cytosol (10). In contrast, p7 when expressed in mammalian cells has two membrane spanning domains and both the N- and C-termini are oriented towards the ER lumen (14). Here, to allow the topologies of E2p7 and p7 to be compared, two constructs, cMycCE1E2p7FLAG (H3) and cMycCE1E2mutp7FLAG (H4), were designed to enable E2p7 and p7 to be distinguished. These constructs were expressed in HEK293 cells and the localization of the processed proteins identified by IF microscopy using an anti-FLAG antibody for detection (Fig. 3C).

Staining of non-permeabilized cells transfected with the cMycCE1E2p7FLAG construct (H3) with the anti-FLAG antibody revealed the presence of either or both p7 and E2p7 at the plasma membrane (Fig. 3B, top left panel). To distinguish between these possibilities, cells were transfected with the cMycCE1E2mutp7FLAG construct, (H4), in which the cleavage site between E2 and p7 had been removed. In this case, no signal was detected. Together these data confirm that only p7 leaks to the membrane and E2p7 is localized to the ER, as observed previously (Fig. 3A).

When the cMycCE1E2p7FLAG (H3) transfected cells were treated with digitonin and stained in a similar manner, again the FLAG-tag was detected (Fig. 3B, top centre panel). Here, detection of the antibody is indicative of either leakage of p7 to the plasma membrane or the C-terminus of E2p7 and/or p7 being accessible to the antibody (10, 14). To eliminate the first possibility, that the signal arises due to leakage of p7 to the plasma membrane, proteinase K was used after cell fixation. Treatment with proteinase K would remove the C-terminus of p7 from the plasma membrane and subsequent digitonin permeabilization would allow the staining only of any cytosol accessible FLAG tags. As shown in Fig. 3B (bottom centre panel), treatment with proteinase K prior to digitonin permeabilization resulted in the disappearance of the IF signal. These data strongly suggest that the fluorescence signal observed when cells transfected with H3 are treated with digitonin stems exclusively from p7 molecules localized to the plasma membrane. As the C-terminal FLAG tag only becomes visible when all cellular membranes are permeabilized, these data indicate that the C-terminus of p7 faces the ER lumen. To confirm that the signal observed was due to p7 and not E2p7, HEK293 cells transfected with the cMycCE1E2mutp7FLAG (H4) construct were permeabilized with digitonin and stained with anti-FLAG antibodies. No fluorescence was detected, indicating that the C-terminus of E2p7 does not face the cytosol and that the C-termini of p7 and E2p7 share the same orientation, facing the ER lumen. In a further experiment, the cells were fixed and treated with Triton X-100, which permeabilizes both the plasma and ER membranes (Fig. 3B, right-hand panels). Here, for both the E2p7 and E2mutp7 expressing constructs, the Proteinase K-treated and untreated cells were fluorescent, showing the presence of the transfected proteins.

Taken together, these experiments indicate that the topology of p7 before and after cleavage of E2p7 remains unchanged. From these data we propose a topological model for E2p7 with the p7 C- and N-termini both facing the ER lumen (Fig. 3C). The relative orientation of the E2 TM domains to each other is not known and these domains are shown for illustrative purposes only. While our data suggest that the C-terminus of p7 always faces the ER lumen, others have hypothesized that p7 adopts an inactive L-shaped transport form, with the C-terminus facing the cytoplasm (18). Whilst we cannot at this stage exclude this possibility, our data suggest that this L-shaped form is either present transiently or at concentrations that cannot be detected using the methods described here.

DISCUSSION

E2p7 arises from the incomplete post-translational processing of the structural part of the HCV polyprotein precursor in the E2-NS2 region (7). Although the existence of the E2p7 product has been reported for some time, the role of this protein remained largely unknown. Here, using a recombinant system, we examined the processing, localisation, and function of E2p7. Until the recent development of a robust HCV cell culture system capable of sustaining the virus life cycle (20, 21, 22) studies of the HCV processing pathway and viral morphogenesis have been conducted solely in recombinant systems. However, data obtained using these systems have provided divergent observations. For example, incomplete time-dependent processing of E2p7 was observed in stable cell lines expressing HCV structural proteins and similarly for the E2p7 construct synthesized in an *in vitro* transcription/translation system (9, 23). Others, however, have reported a constant post-translational ratio between E2 and E2p7 using vaccinia based vectors (7, 24). It is possible that the different recombinant systems used in these studies account for the divergent observations. The effects of using different recombinant systems to analyze HCV viral proteins were described by Dumonceaux *et al.* (12), who observed differential localization and splicing of the HCV envelope proteins depending on the recombinant system used for delivery.

In keeping with earlier reports (7, 9), we observed that processing of E2p7 in our plasmid-based recombinant system was incomplete and gradual. In addition to previous studies (1, 10), it was shown that in a system where only the E2p7 species is present, a native E1E2p7 species could form. This interaction of E1 with E2p7 could potentially mean that E1E2p7, in addition to E1E2, may be a component of the viral envelope. However, data obtained with E2p7 from closely related pestiviruses do not favour this hypothesis. E2p7 from the pestivirus bovine viral diarrhoea virus (BVDV) was shown to be dispensable for the formation of infectious viruses (25). Lazar *et al*. have shown that only the E2 species, and not

E2p7, is present in the BVDV virion (26). E2p7 is also excluded from secreted virions of classical swine fever, another pestivirus (27). For the pestiviruses we therefore expect that E2p7 is not a structural element of the envelope. If the same were true for HCV, the presence of the E1E2p7 native-like heterodimer and the incomplete post-translational processing at the E2/p7 site may indicate a coordination mechanism regulating the timing between the generation of the structural proteins and viral morphogenesis.

Two possible causes for the incomplete cleavage of E2p7 have been proposed. A subset of E2p7 may co-translationally adopt a topology that makes the SSP cleavage site inaccessible (10), and/or there are structural constraints around the cleavage site that lead to the same result (28). To explore these possibilities, we compared the cellular localisation and topology of p7 alone and p7 within the context of E2p7. In agreement with other studies (14, 19), we showed that although E2 and p7 are mainly localized within the ER, both leak to the plasma membrane despite their ER retention signals. However, E2p7 is localized exclusively to the ER and does not leak to the plasma membrane, probably due to the presence of two rather than one ER retention signals. Further, we found that the topology of p7 does not change upon processing; the C-terminus of p7 when fully processed as well as in the context of E2p7 faces the ER lumen. Based on these data, we propose that structural constraints, as suggested by Carrere-Kremer *et al.* (28), are the cause of the partial cleavage of E2p7. These structural constraints may be another means of controlling the production of mature HCV proteins and consequently controlling the morphogenesis of the virus.

The morphogenesis of HCV has begun to be investigated recently when a cell culture system (HCVcc) which sustains the whole life cycle of the virus was reported (20, 21, 22). The functional significance of the E2p7NS2 processing is still unknown. In parallel with the recombinant systems, the existence of the delayed processing at the E2p7 junction was confirmed in the HCVcc system (29). The importance of the E2p7 product was also addressed. Jones *et al*. used a bicistronic HCV genome to prove that the E2p7 product is not required *per se* for virus infectivity of HCVcc (30). As postulated for bovine viral diarrhea virus (BVDV), the delayed processing of E2p7 may be advantageous for viral fitness *in vivo* (25). Although the timing of E2p7 processing seems not to be essential for the virus life cycle in cell culture, the actual cleavage event is most likely compulsory, as shown for the closely related BVDV (25). Moreover, it has been speculated that E2p7 is an inactive form of p7 (28). HCV p7 is crucial for the production of infectious virions in both the HCVcc system and *in vivo* (29, 31). HCVp7 oligomerises to form ion channels in artificial membranes (32–34). HCV p7 mutants which are devoid of ion channel activity in artificial electrophysiological systems, were shown to also impair the formation of infectious virions in HCVcc (29). As the E1E2 heterodimer is able to form in the context of non-processed

E2p7, we could imagine that p7 activation is preceded by E1E2 native heterodimer formation. Future experiments will aim to elucidate the exact role of E2p7 cleavage in the HCV morphogenesis process.

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