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The hepatitis B x antigen anti-apoptotic effector URG7 is localized to the endoplasmic reticulum membrane

A. Ostuni [a,1], P. Lara [b,1], M.F. Armentano [a], R. Miglionico [a], A.M. Salvia [a], M. Mönnich [b], M. Carmosino [a], F.M. Lasorsa [c], M. Monné [a], I. Nilsson [b], F. Bisaccia [a,*]

[a] Department of Sciences, University of Basilicata, 85100 Potenza, Italy
[b] Department of Biochemistry and Biophysics, Stockholm University, 106 91 Stockholm, Sweden
[c] CNR Institute of Biomembranes and Bioenergetics, 70125 Bari, Italy

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ABSTRACT

Hepatitis B x antigen up-regulates the liver expression of URG7 that contributes to sustain chronic virus infection and to increase the risk for hepatocellular carcinoma by its anti-apoptotic activity. We have investigated the subcellular localization of URG7 expressed in HepG2 cells and determined its membrane topology by glycosylation mapping *in vitro*. The results demonstrate that URG7 is N-glycosylated and located to the endoplasmic reticulum membrane with an N_{lumen}–$C_{cytosol}$ orientation. The results imply that the anti-apoptotic effect of URG7 could arise from the C-terminal cytosolic tail binding a pro-apoptotic signaling factor and retaining it to the endoplasmic reticulum membrane.

1. Introduction

Approximately two billion people worldwide have been infected with hepatitis B virus (HBV) and the consequences of acute and chronic infections can lead to chronic liver disease, cirrhosis and cancer, which cause 600 000 deaths every year [1]. An important factor encoded by the virus, HBV x antigen, acts by up- and down-regulating host gene expression and thereby it induces a number of cellular responses that are involved in the pathogenesis of chronic infection and the development of hepatocellular carcinoma (HCC). One of the genes whose expression is induced by HBV x antigen in hepatocytes infected by HBV is the up-regulated gene clone 7 (*URG7*) encoding the protein URG7 [2]. It has been reported that URG7 inhibits TNFα-mediated apoptosis through blocking one or more caspases, probably by activating anti-apoptotic signaling through phosphoinositol-3-kinase and β-catenin [3]. Antibodies against URG7 are detected in the serum of HBV carriers before the appearance of HCC. Therefore URG7 could be used as a preneoplastic marker to identify HBV carriers at risk of developing HCC [4] and could represent a molecular target for anti-tumor therapies.

Although URG7 has an important role in the HBV x antigen induced cellular response, very little is known about it. URG7 consists of 99 amino acids [2], of which the N-terminal 74 residues are identical to the N-terminal of the multidrug-resistance protein 6 (MRP6/ABCC6), while the remaining 25 C-terminal residues are unrelated. MRP6 is a member of the ATP binding cassette transporter family in which mutations cause pseudoxanthoma elasticum [5]. The similarity between URG7 and MRP6 can be explained by URG7 being encoded by the so-called pseudogene 2 of *ABCC6*. The peptide sequence in common between the two proteins contains one predicted transmembrane segment preceded by an N-linked glycosylation site that has been shown to be glycosylated in MRP6 expressed in MDCKII cells [6]. In these polarized cells, MRP6 is localized to the basolateral membrane exclusively but recent studies have suggested that it is localized to mitochondria-associated membranes [7]. However, the cellular localization of URG7 is still unknown.

We have investigated the subcellular localization and topology of URG7. Results obtained in HepG2 cells transfected with URG7 demonstrate that this protein is glycosylated and localized to the

* Corresponding author. Address: Department of Sciences, University of Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy. Fax: +39 0971 205678.
E-mail address: faustino.bisaccia@unibas.it (F. Bisaccia).
[1] These authors have contributed equally to this work.

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endoplasmic reticulum (ER). Studies with URG7 expressed in an *in vitro* system confirm its ER targeting and show that its only predicted transmembrane segment is integrated into the ER membrane with an N_{lumen}–$C_{cytosol}$ orientation. These findings suggest that URG7 might act by anchoring a pro-apoptotic agent or signal to the ER membrane and thereby have an anti-apoptotic effect.

2. Materials and methods

Detailed protocols for the methods can be found in Supplementary materials.

2.1. Construction of URG7 expression vector and transfection in HepG2 cells

Total RNA was isolated from human hepatoblastoma cell line HepG2 and was transcribed to cDNA corresponding to the encoding sequence for URG7 (GenBank AY078405) with a flag tag (DYKDDDDK) at the C-terminus. The amplified DNA was cloned into pcDNA3 vector and the inserts were confirmed by DNA sequencing. HepG2 cells were grown in Dulbecco's modified essential medium until ~70% confluent and then transfected with pcDNA3/URG7flag.

2.2. Immunoblotting, deglycosylation assay and tunicamycin treatment

Proteins from RIPA lysed HepG2 cells transfected with empty vector pcDNA3 and with pcDNA3/URG7flag were resolved by SDS–PAGE and transferred onto nitrocellulose membranes that were used for Western blots with monoclonal Anti-Flag M2 and HRP-coupled anti-mouse secondary antibodies. For the deglycosylation assay, URG7flag expressing cells were denaturated and treated with protease inhibitors and PNGase F. To inhibit the N-linked glycosylation of proteins, transfected HepG2 cells were grown in medium containing tunicamycin.

2.3. Immunofluorescence

Forty-eight hours after transfection, the cells were fixed, washed and incubated with monoclonal Anti-FlagM2 antibody, calnexin antibody or anti-TGN46 antibody. Bound antibodies were detected with Alexa Fluor-conjugated anti-mouse, anti-goat and anti-rabbit IgG antibody, respectively. To label mitochondria, cells were incubated with MitoTracker Red CMXRos. Confocal images were obtained with a laser scanning fluorescence microscope Leica TSC-SP2.

2.4. In vitro expression

The human *URG7* and *MRP6* (TM1–TM2) [8] genes were cloned in the pGEM1 plasmid by themselves or as fusion proteins with the P2 domain of Lep [9,10], similar to what has been described previously with other proteins [11] and with similar considerations [9,12]. N-glycosylation acceptor sites were designed as described [13,14]. To introduce additional glycosylation acceptor sites into the URG7 gene near the N-terminal of the protein, $P_7C_8A_9G_{10}$ was substituted to ANAT, and near the C-terminal, the glycosylation site sequence NST was introduced between the positions K_{72}–M_{73}. The P2 domain of Lep had an additional glycosylation acceptor site $N_{97}S_{98}T_{99}$ [10,15] in the wild type sequence of Lep. All inserted fragments and mutants were confirmed by sequencing of plasmid DNA.

Constructs cloned in pGEM1 were transcribed and translated in the TNT® SP6 Quick Coupled System as previously described [10,15] in the presence and absence of dog pancreas rough microsomes (CRMs) [16]. To demonstrate N-linked glycosylation by oligosaccharyl transferase, Endo H treatment was performed as described [10]. Translation products were analyzed by SDS–PAGE, visualized in a Fuji FLA-300 phosphoimager and quantified with the MultiGauge (Fujifilm) software.

3. Results

3.1. Cellular localization of URG7 expressed in HepG2 cells

To investigate the subcellular localization of URG7, a C-terminal flagged URG7 construct was expressed in HepG2 cells. URG7 was detected on immunoblots of cell lysates collected 48 h after transfection, by using an anti-Flag tag antibody (Fig. 1A, lane 2). Two URG7 protein bands migrated between the 7 and 17 kDa markers. The size of the lower band fits well with the predicted molecular weight of the flag-tagged URG7 protein (12 kDa), while the upper band could correspond to a glycosylated form of URG7. This hypothesis was tested by treating the cell lysate with the deglycosylation enzyme PNGase F (Fig. 1B) and tunicamycin treatment of the HepG2 cells (Fig. 1C), which both resulted in the disappearance of the upper URG7 protein band, indicating that it corresponds to an N-glycosylated form of URG7. Because the sequence of URG7 contains a single N-linked consensus glycosylation site, $N_{15}Q_{16}T_{17}$, located N-terminally of the predicted transmembrane segment (residues 34–56 according to TMHMM [17]), we deduced that this particular site is modified and that this fraction of 30–35% of URG7 is targeted to the ER where N-glycosylation exclusively takes place.

To investigate if URG7 is localized to the ER or continues through the secretory pathway, URG7flag in HepG2 cells was followed by using immunofluorescent microscopy. URG7 was found around the nucleus and streaked out along the cellular body (green signal), which coincided with the localization of the ER marker calnexin (red signal, Fig. 2A). URG7 did not enter the Golgi as seen by analyzing potential co-localization with the Golgi marker TGN46 (red signal, Fig. 2B) and nor is it associated with mitochondria marked with Mitotracker (red signal, Fig. 2C). These results clearly demonstrate that URG7 has a distinct localization within the cells at the level of the ER.

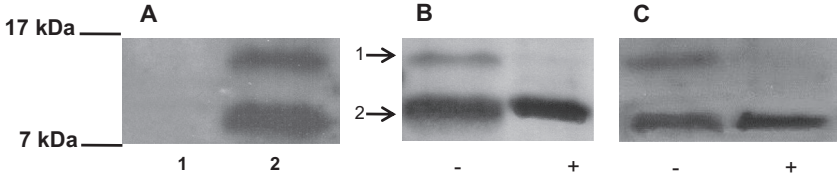

Fig. 1. Immunoblot analysis. (A) HepG2 cells transfected with empty vector pcDNA3 (lane 1) and with pcDNA3/URG7flag (lane 2). (B) URG7 enzymatic deglycosylation. Whole lysate from URG7flag expressing cells was incubated in the presence or absence of PNGase F (+/−). (C) Tunicamycin treatment. Whole lysate from URG7flag transfected cells grown in the presence or absence (+/−, respectively) of tunicamycin (5 μg/ml). Arrows 1 and 2 mark the N-glycosylated and the deglycosylated URG7 proteins, respectively.

3.2. Targeting and topology of URG7 expressed in vitro

To further investigate the ER targeting and membrane topology of URG7, wild-type URG7 (WT) was expressed in the reticulocyte lysate *in vitro* system in the absence and presence of pancreatic canine rough microsomes (CRM). The radioactively labeled protein products were analyzed by SDS–PAGE (Figs. 3 and 4) and quantified (Table 1). In the absence of CRM, URG7 WT migrated close to the 15 kDa marker (Fig. 3A, lane 1), which corresponds well to its theoretical molecular weight of 10.8 kDa. In the presence of CRM, about 54% of URG WT was in an endoglycosidase H (Endo H) sensitive fraction (Fig. 3A, lanes 2–4) that migrated about 2.5–3 kDa above the protein in the absence of CRM. Thus, the only N-linked glycosylation site possibly modified in URG7 ($N_{15}Q_{16}T_{17}$) is glycosylated *in vitro* to a similar level as found in HepG2 cells. The results indicate that at least 54% of URG7 expressed *in vitro* is targeted to the ER (Fig. 1).

To address whether URG7 is translocated across the ER membrane and forms a soluble lumenal protein or if its predicted transmembrane segment is inserted into the ER membrane forming an integral membrane protein, a set of constructs with engineered N-linked glycosylation sites were designed. An additional N-linked glycosylation site with the acceptor asparagine in position 73 (N73) on the C-terminal side of the predicted transmembrane segment was made and the expressed protein was 9% doubly glycosylated (Fig. 3B), indicating that 9% of URG7 is totally translocated across the ER membrane. 50% of URG7 N73 was singly glycosylated, which suggests that the transmembrane segment is inserted in the membrane with either the N- or C-terminal tail glycosylated in the lumen of the ER.

To verify the topology of the ER membrane inserted fraction of URG7, double glycosylation sites were introduced in either the N- or C-terminal tail in the constructs URG7 P7A/N8 and URG7 N73/N85, respectively (Fig. 3C and D). 11% of URG7 P7A/N8 was glycosylated once and 40% twice (Fig. 3C), whichcorresponds to the fraction of the protein product with the N-terminal tail in the lumen of the ER. In agreement with the above results, the URG7 N73/N85 construct (Fig. 3D) was doubly glycosylated to 18% (URG7 inserted in the membrane with the C-terminal tail in the lumen) and 46% singly glycosylated (URG7 inserted in the membrane with the N-terminal in the lumen). The possible triple glycosylated product of this construct could not be detected because it would co-migrate with an Endo H insensitive product.

To increase the signal and the size of the expressed URG7, the soluble P2 domain of *Escherichia coli* leader peptidase (Lep) with one glycosylation site ($N_{97}S_{98}T_{99}$) was introduced as a fusion protein at the C-terminus of URG7 (Fig. 4A–C). URG7-P2 displayed 5% doublyglycosylated product and 51% singly glycosylated (Fig. 4A), which implies that 5% of the whole protein is in the lumen and 51% with either N- or C-terminal region in the lumen. An additional glycosylation site was inserted in the N- (P7A/N8-P2) and the C- (N73-P2) terminal regions of this construct to determine the fractions of the protein with a topology that had the N- and C-terminal region in the lumen, respectively. The URG7 N73-P2 construct displayed 2% triply, 9% doubly and 50% singly glycosylated product, indicating that 2% is totally translocated,

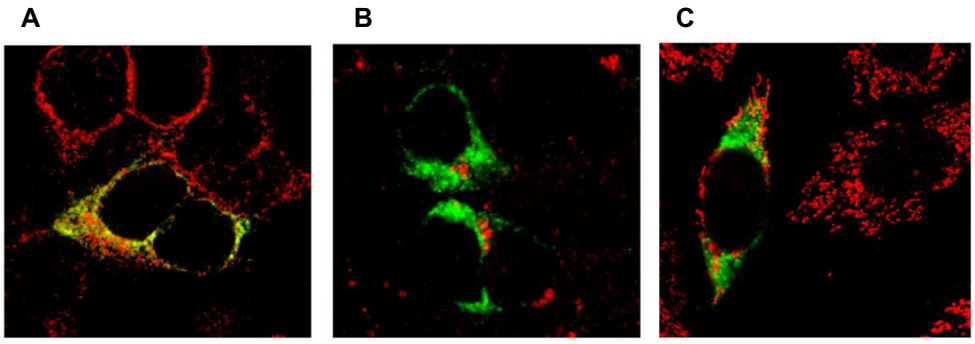

Fig. 2. Immunofluorescence analysis of cells expressing URG7. HepG2 cells, transiently transfected with pcDNA3/URG7flag, were assessed for colocalization (yellow) after staining with anti-URG7flag (green), anti-calnexin (red, A) and anti-TGN46 (red, B) antibodies as well as MitoTracker (red, C). Planar XY confocal images were depicted.

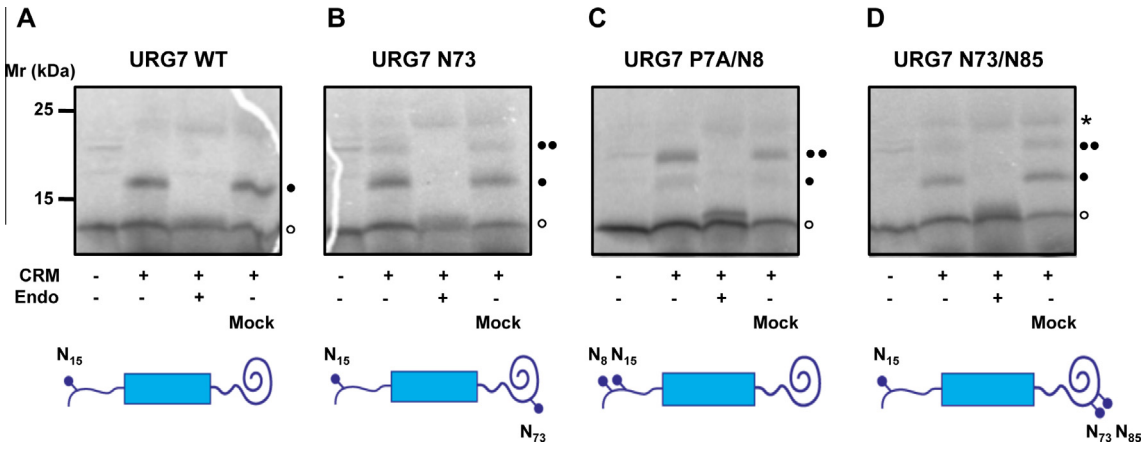

Fig. 3. SDS–PAGE analysis of the URG7 constructs expressed *in vitro*. (A) URG7 WT, (B) URG7 N73, (C) URG7 P7A/N8 and (D) URG7 N73/N85. The constructs were expressed in the absence and presence of CRM, and were treated with Endo H or untreated but in the same reaction conditions (Mock). The unglycosylated and glycosylated forms of the protein are indicated as open and filled circles, respectively. The number of filled circles indicates the number of glycosylations. The star is indicating the Endo H insensitive background band. The models of the constructs indicate the positions of the N-linked glycosylation sites (N_8, N_{15}, N_{73} and N_{85}).

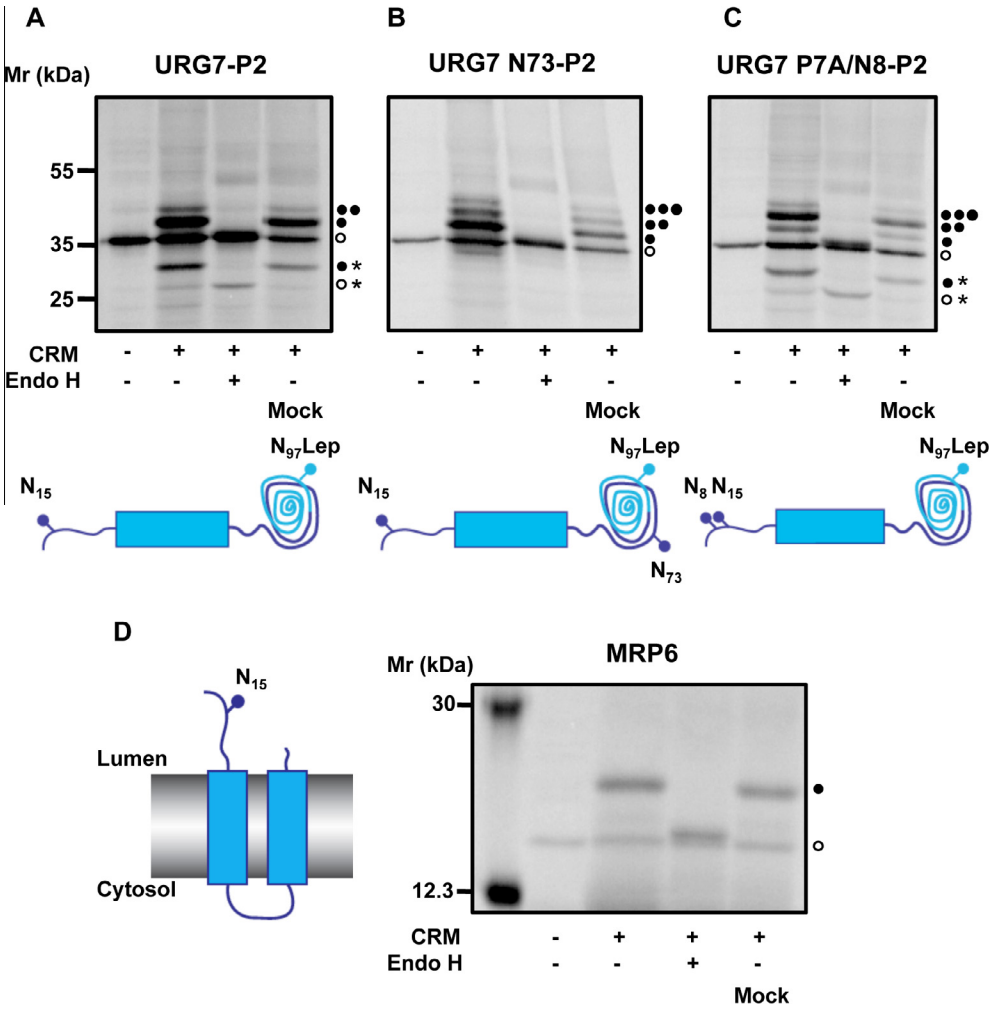

Fig. 4. SDS–PAGE analysis of the URG7-P2 and MRP6 (TM1-TM2) constructs expressed *in vitro*. (A) URG7-P2, (B) URG7 N73-P2, (C) URG7 P7A/N8-P2 and (D) MRP6 (TM1-TM2). Key as in Fig. 3. The stars indicate cleaved products. The P2 domain of Lep is indicated in light blue.

9% has the C-terminal in the lumen and 50% has the N-terminal in the lumen. In good agreement with this, the URG7 P7A/N8-P2 construct exhibited 4% triply, 36% doubly and 10% singly glycosylated product which implies that 4% is totally translocated, 36% has the N-terminal and 10% the C-terminal in the lumen.

It should be noted that degradation productswere detected for the URG7-P2 and URG7 P7A/N8-P2 constructs (Fig. 4A and C) but not for the URG7 N73-P2 construct, which suggests that the glycosylation in N73 (Fig. 4B) protects the protein from cleavage by signal peptidase [10].

As a positive control for ER targeting, a construct with the first 102 amino acids of MRP6, MRP6 (TM1–TM2), containing the first two predicted transmembrane α-helices (residues 34–56 and 73–92 according to TMHMM) [17], was expressed in the *in vitro* system. As for URG7 WT (Fig. 3A), also in this case an Endo H-sensitive product was detected in the presence of CRM (Fig. 4D). For MRP6 (TM1–TM2) the protein was glycosylated to 80%, indicating that it is inserted in the ER membrane with the N-terminus in the lumen.

Table 1
Quantification of the URG7 protein products presented in Figs. 3 and 4. The numbers are given as percentage of total protein products from at least two independent experiments. Key as in Figs. 3 and 4.

URG7 constructs	Figure	○ (%)	• (%)	•• (%)	••• (%)	∗ (%)
WT	3A	46	54			
N73	3B	41	50	9		
P7A/N8	3C	49	11	40		
N73/N85	3D	36	46	18		
–P2	4A	37	51	5		7
N73-P2	4B	39	50	9	2	
P7A/N8-P2	4C	42	10	36	4	8
MRP6 (TM1–TM2)	4D	20	80			

4. Discussion

We have investigated the cellular localization of URG7 in HepG2 cells and its membrane topology in an *in vitro* expression system. The expression of URG7 in HepG2 cells lead to the production of two distinct protein products of which the one of higher molecular weight was sensitive to PNGase F treatment and tunicamycin (Fig. 1). These results indicate that about 35% of URG7 is N-glycosylated in HepG2 cells suggesting that it is at least initially targeted to the ER. These findings were corroborated by immunolocalization experiments showing a co-localization of URG7 with the ER marker calnexin (Fig. 2A). The fact that URG7 does not enter the Golgi apparatus (Fig. 2B) or mitochondria (Fig. 2C) strongly supports that it remains in the ER.

The topology and ER targeting of URG7 was determined by *in vitro* expression of URG7 WT and as P2 fusions with engineered N-linked glycosylation sites (Fig. 3A–D and Fig. 4A–C and Table 1). These results indicate that 50–64% of URG7 is targeted to the ER as seen from the percentage of protein glycosylated at least once

(Fig. 3B and D and Fig. 4A–C). The topological studies of URG7 can be summarized as follows: 2–9% resides in a soluble form in the lumen as indicated by the doubly glycosylated (Fig. 3B and Fig. 4A) and triply glycosylated products (Fig. 4B and C); 9–18% spans the membrane with the C-tail facing the lumen as suggested by the doubly glycosylated product (Fig. 3D and Fig. 4B) and singly glycosylated products (Fig. 4C); 36–50% of URG7 spans the membrane with the N-tail facing the lumen as shown by the doubly (Fig. 3C and Fig. 4C) and singly glycosylated products (Fig. 3D and Fig. 4B). In conclusion, the dominant topology of URG7 expressed *in vitro* is the N_{lumen}–$C_{cytosol}$ orientation, although it displays minor topological variations.

MRP6 (TM1–TM2) was also expressed *in vitro* and it displayed 80% glycosylated product (Fig. 4D) close to the maximum observed in this system [15,14] corroborating previous reports suggesting that full-length MRP6 is glycosylated [6]. Thus the MRP6 residues that differ from URG7 (75–102 of MRP6) improve ER targeting and insertion, which could be explained by the higher hydrophobicity of the second transmembrane segment (ΔG-value of -0.922 kcal mol^{-1}, http://dgpred.cbr.su.se/) found in that region, compared to the first transmembrane segment (ΔG-value of 0.707 kcal mol^{-1}). Another difference is that URG7, unlike MRP6, is most likely targeted post-translationally to the ER membrane because its C-tail contains only 43 residues, which is just about enough to span the ribosome tunnel [18,19]. Although MRP6 and URG7 share targeting signals for the ER, MRP6 has been suggested to be localized to the plasma membrane or mitochondria-associated membranes [6,7]. Our results therefore further suggest that the ER retention signal for URG7 is found among residues 74–99 that are different from those of MRP6.

URG7 has been shown to inhibit TNFα-mediated apoptosis by blocking caspase 8 and downstream caspase 3 activity. It was hypothesized that URG7 acts byactivating β-catenin or PI3K/Akt [3]. According to the localization, targeting and topology analysis in this study, the majority of the N-terminal 74 residues of URG7, that are also identical to those of MRP6, either faces the ER lumen or spans ER membrane. It is therefore unlikely that this protein portion plays a role in apoptosis. The C-terminal 25 residue region of URG7 was found to face the cytosol, which makes it a more likely player interfering with apoptosis. It should be noted that the residues 82–95 in the C-terminal cytosolic region of URG7 contain a sequence motif that is highly homologous to the extreme C-terminal residues 269–283 of the human synoviolin isoform CRA_b (EAW74358.1).

URG7	82	EP-GNV–RGRQGTGWN	95	(99)
		EP-G-V–RGRQ–GWN		
CRA_b	269	EPLGTVTARGRQ–GWN	283	(285)

Another longer isoform of synoviolin, also known as HRD1, has been shown to be located to the ER membrane with its C-terminal domains in the cytoplasm [20], where it sequesters and facilitates degradation of p53 as a part of an anti-apoptotic mechanism [21]. The CRA_b isoform has not yet been characterized so it is not known if it plays a role in apoptosis and the homologous sequence motif found in URG7 and CRA_b is not found in HRD1. However, we would like to speculate that the C-terminal cytosolic tails of URG7 and CRA_b could be involved in an analogous anti-apoptotic mechanism by binding a pro-apoptotic component, anchoring it to the ER membrane and thereby inhibiting the apoptosis signaling pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.07.042.

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