# Expression of a functionally active human hepatic UDP-glucuronosyltransferase (UGT1A6) lacking the N-terminal signal sequence in the endoplasmic reticulum

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**Abstract UDP-glucuronosyltransferase 1A6 (UGT1A6) is a membrane glycoprotein of the endoplasmic reticulum playing a key role in drug metabolism. It is synthesized as a precursor with an N-terminal cleavable signal peptide. We demonstrate that deletion of the signal peptide sequence does not prevent membrane targeting and integration of this human isoform when expressed in an in vitro transcription-translation system, as shown by *N*-glycosylation, resistance to alkaline treatment and protease protection. Furthermore, UGT1A6 lacking the signal peptide (UGT1A6Δsp) was targeted to the endoplasmic reticulum in mammalian cells as shown by immunofluorescence microscopy and was catalytically active with kinetic constants for 4-methylumbelliferone glucuronidation similar to that of the wild-type. These results provide evidence that the signal peptide is not essential for the membrane assembly and activity of UGT1A6 suggesting that additional topogenic element(s) mediate(s) this process.**

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*Key words:* UDP-glucuronosyltransferase; Signal peptide; Endoplasmic reticulum targeting; Membrane translocation; Human

## 1. Introduction

Most eukaryotic proteins which are translocated across the endoplasmic reticulum (ER) membrane are synthesized as precursors with a N-terminal signal sequence (for review see [1]). Integral membrane proteins possess in addition a 'stop transfer' sequence which prevents complete translocation and stably anchors the protein into the membrane [2]. Single membrane-spanning proteins with a cleavable signal peptide usually have an N-terminal side of the transmembrane domain localized in the lumen and the C-terminal portion exposed on the cytoplasmic side. UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17) are predicted to present such a type I topology with the amino-terminus and the majority of the protein located at the lumenal face of the ER. These proteins are anchored to the membrane via a single C-terminal transmembrane domain followed by a short cytoplasmic tail. The proposed transmembrane topology of UGTs has been essentially constructed on computer-based predictions. Experimental evidence using rat and human liver microsomes as well as recombinant enzymes confirm the general features of this model, but in the absence of available X-ray crystallography, this model remains hypothetical [3,4].

UGTs are members of a superfamily of glycosyltransferases that catalyze the conjugation of a variety of hydrophobic aglycone substrates with glucuronic acid donated by UDP-glucuronic acid [5]. This reaction results in the formation of a hydrosoluble β-D-glucuronide that is easily excreted into bile or urine. Thus, these enzymes are centrally involved in the metabolism and detoxication of drugs, pollutants and toxic substances [6]. UGT1A6 is a human isoform encoded by the *UGT1* locus, which is expressed predominantly in the liver but also in kidney and other extrahepatic tissues [7]. It is responsible for the conjugation of planar phenols such as 4-methylumbelliferone (4-MU), drugs (paracetamol, naftazone [8,9]) or environmental carcinogens such as chrysene and benzo[*a*]pyrene derived phenols [10]. The individual specificity of each UGT isoform towards aglycone substrates is provided by the N-terminal domain that represents the major region of dissimilarity between UGTs [11]. The high degree of sequence conservation in the C-terminal half of UGT proteins suggests that this domain is involved in the binding of the common cosubstrate, UDP-glucuronic acid.

Towards a detailed understanding of the mechanisms of membrane assembly of UGTs which are critical for the structure and the function of these enzymes, we decided to investigate the role of the cleavable N-terminal signal peptide sequence of UGT1A6. A previous study designed to address the influence of exchanging the eukaryotic signal peptide with bacterial counterparts suggested that the enzyme deprived of its signal peptide (UGT1A6Δsp) was nevertheless associated with the membrane fraction of recombinant *Escherichia coli* [12]. In this report, we investigate the role of the cleavable N-terminal signal peptide in the ER membrane targeting of UGT1A6 in an in vitro transcription-translation system and in recombinant mammalian cells. Interestingly, we found that the signal peptide of UGT1A6 is not required either for the ER subcellular localization or for the functional integrity of the enzyme.

## 2. Materials and methods

### *2.1. Materials*

UDP-[U-$^{14}$C]glucuronic acid (300 mCi/mmol) and [$^{35}$S]methionine

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*Abbreviations:* DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; 4-MU, 4-methylumbelliferone; PBS, phosphate buffer saline; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; UGT, UDP-glucuronosyltransferase

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(100 mCi/mmol) were obtained from NEN. Restriction enzymes, T4 DNA ligase, RNAsin and pGEM-3Z were purchased from Promega. The plasmid pcDNA3.1 was from Invitrogen. *Vent* DNA polymerase was provided by Biolabs. All other reagents, including Geneticin (G418), proteinase K, 4-MU (free acid) and 4-MU-glucuronide were purchased from Sigma. The acceptor tripeptides *N*-benzoyl-Asn-Asn-Thr-*N*-methylamide and *N*-benzoyl-Asn-Ala-Ser-*N*-methylamide were synthesized by Dr. G. Bloomberg (Recognition Centre Peptide Synthesis Facility, Bristol, UK) and used as competitive inhibitors of asparagine-linked glycosylation as previously described [13].

*2.2. Plasmid constructions*

Cloning and sequencing of full-length UGT1A6 cDNA (GenBank data base access number M84130) have been reported previously [14]. For the in vitro transcription-translation experiments, UGT1A6 cDNA was subcloned into the *Sma*I site of pGEM-3Z under the control of the T7 promoter. For the expression in mammalian cells, this sequence was subcloned into *Hin*dIII-*Xba*I sites of the pCDNA3.1 eukaryotic expression vector as described [7].

UGT1A6 cDNA encoding the protein lacking the 26 N-terminal amino acids corresponding to the signal peptide sequence (UGT1A6Δsp) was generated by polymerase chain reaction (PCR) using a 5′ primer corresponding to amino acid residues 27–33 of the preprotein with an attached Kozak sequence and a *Hin*dIII site. The 3′ primer was complementary to the sequence encoding the last five amino acid residues and the stop codon with an *Xba*I attached site. PCR amplification was carried out using UGT1A6 cDNA as template. The resulting PCR product was ligated into the *Sma*I site of pGEM-3Z for in vitro transcription-translation studies.

A second construct in which 62 bp of the 5′ untranslated region of UGT1A6 cDNA was attached upstream to the sequence encoding the protein lacking the signal peptide (UGT1A6Δsp) was generated by simple overlapping ends PCR amplification using appropriate oligonucleotides [15]. The resulting fragment was subcloned into the *Sma*I site of pGEM-3Z under the control of the T7 promoter for in vitro transcription-translation studies. Finally, the latter sequence, encoding UGT1A6Δsp, was subcloned into *Hin*dIII-*Xba*I sites of pcDNA3.1 expression vector and the recombinant plasmid was used for expression in mammalian cells. All mutant clones were screened for *Taq*-introduced errors by dideoxy sequencing [16].

*2.3. In vitro protein synthesis*

The recombinant pGEM-3Z plasmids encoding the full-length UGT1A6 and its counterpart lacking the signal peptide (UGT1A6Δsp) were linearized at the *Xba*I restriction site localized downstream of the TAG stop translation codon. These linearized plasmids served as templates for in vitro transcription by T7 RNA polymerase coupled to translation in a rabbit reticulocyte lysate using the TNT Coupled Reticulocyte Lysate kit (Promega). The reactions were carried out at 30°C for 60 min in the presence of [$^{35}$S]methionine. The radiolabeled transcription-translation products were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [17]. For membrane insertion tests, canine pancreas microsomal membranes (Promega) were added to the transcription-translation mixture before the addition of T7 RNA polymerase according to the suggestions of the supplier. The canine pancreas microsomal membranes used in this study were assayed for signal peptidase and *N*-glycosylation activities using two different control mRNAs, the precursor for β-lactamase from *E. coli* and the precursor for α-mating factor from *Saccharomyces cerevisiae*, respectively, as described by the supplier (Promega). *N*-Glycosylation of newly synthesized polypeptides (full-length UGT1A6 and UGT1A6Δsp) was tested by addition of competitive glycosylation acceptor tripeptides (0.2 mM) in the transcription-translation mixture. These tripeptides mimic each of the two potential asparagine-linked glycosylation sites of UGT1A6 (asparagine 293 and 345). For alkaline extraction, the incubation mixture containing the microsomes was diluted two times in buffer A (50 mM potassium acetate, 200 mM sodium carbonate, 20 mM triethanolamine, 1 mM magnesium acetate, pH 11.5). The reaction mixture was kept in ice for 20 min and layered over 250 μl buffer B (0.25 M sucrose, 50 mM triethanolamine, 140 mM potassium acetate, 2.5 mM magnesium acetate) containing 100 mM sodium carbonate, pH 11.5. Microsomes were sedimented at 100 000 × *g* in a Sorvall RC M120 micro-ultracentrifuge for 20 min. The pellet was washed with 25 μl of buffer B and suspended in gel loading buffer. Supernatant proteins were precipitated with trichloroacetic acid (10% (v/v)) and the samples were electrophoresed on 10% (w/v) SDS-polyacrylamide gels [17]. The protease sensitivity of the expressed polypeptides was analyzed using proteinase K (0.2 mg/ml) at 0°C for 30 min as described by Connolly et al. [18]. The samples were heated at 100°C in Laemmli sample buffer prior to SDS-PAGE. Dried gels were exposed directly to Kodak Biomax film for visualization.

*2.4. Heterologous expression in mammalian cells*

V79 cells (Chinese hamster lung fibroblasts) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 8% (v/v) Nu-Serum (Becton-Dickinson) and antibiotics. Transfection and stable expression were carried out as previously described [19]. For each transfection, 30 colonies were screened for in situ 4-MU-glucuronide production, as described below. The colony expressing the highest level of 4-MU glucuronidation activity was selected and cultured in the conditions described above.

*2.5. Immunofluorescence microscopy*

Immunofluorescence was carried out on V79 cells stably expressing UGT1A6 or UGT1A6Δsp. Cells were cultured for 24 h in 30 mm diameter wells on coverslips, fixed with 3% (w/v) *para*-formaldehyde in phosphate buffer saline (PBS, Gibco-BRL) and permeabilized with 0.4% (w/v) saponin/PBS followed by blocking with 0.2% (w/v) gelatin/PBS. Permeabilized cells were then incubated for 1 h with polyclonal anti-UGT1A6 antibodies [7]. These antibodies were raised against the 120 N-terminal amino acid residues of UGT1A6 protein expressed in *E. coli*, and have been shown to be highly specific towards the UGT1A6 isoform [7]. The cells were then washed with PBS and incubated for 20 min with fluorescein isothiocyanate-conjugated secondary antibodies (Sigma). For double immunofluorescence staining, the same procedure was repeated using monoclonal anti-calnexin primary antibodies provided by Affinity Bioreagents Inc. This antibody was raised against a human calnexin protein, a typical ER resident protein classically used as a marker for this organelle [20]. In this case, polyclonal anti-mouse IgG rhodamine-conjugated antibodies (Sigma) were used as secondary antibodies. Finally, cells were washed in PBS, mounted on microscopy slides and examined by fluorescence microscopy using a Zeiss Axiophot microscope with a 100× objective.

*2.6. Preparation of membrane enriched fraction of recombinant cells and enzymatic assays*

The measurement of in situ 4-MU-glucuronide formation in recombinant V79 cells was performed as previously described [21]. The amount of 4-MU-glucuronide released into the culture medium was quantitated by fluorescence spectroscopy [22].

The preparation of the membrane enriched fraction of recombinant V79 cells was performed as previously described [21]. Protein concentration was measured according to Bradford [23] with Bio-Rad reagent (Bio-Rad). Glucuronidation of 4-MU in membrane fraction and in supernatant of the 100 000 × *g* centrifugation was assayed as previously reported [22]. Apparent kinetic constants for the glucuronidation of 4-MU in membrane fractions were calculated by linear least squares regression analysis of values from double-reciprocal plots corresponding to at least six different concentrations of substrate (0.05–5 mM) at a constant concentration of UDP-glucuronic acid (5 mM).

## 3. Results

*3.1. In vitro translocation and processing of UGT1A6 expressed with and without signal peptide*

In vitro transcription-translation in the presence of pancreas microsomal membranes is a powerful tool routinely used to study the targeting, translocation, post-translational modifications and membrane insertion of proteins [24–26]. This system was thus used to study the cleavage of the N-terminal signal peptide and asparagine-linked glycosylation, which are the only post-translational modifications described for UGTs as well as the membrane insertion of UGT1A6 [27].

In vitro transcription of UGT1A6 coupled to translation in a reticulocyte lysate resulted in the synthesis of a single 54 kDa

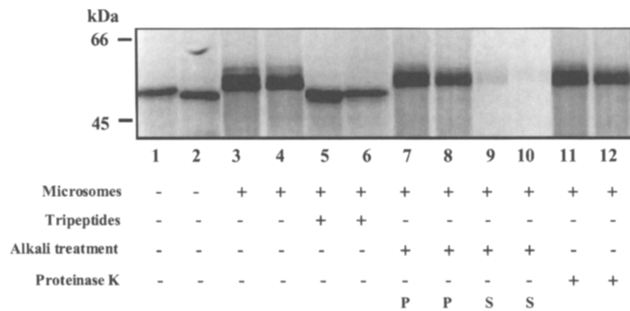


Fig. 1. In vitro expression of the cDNAs encoding the precursor and mature form of UGT1A6. UGT1A6 and UGT1A6Δsp were transcribed and translated in a reticulocyte lysate system in the absence (lanes 1 and 2, respectively) or in the presence of dog pancreas microsomes (lanes 3 and 4, respectively). Acceptor tripeptides were used as inhibitors of *N*-linked glycosylation of UGT1A6 and UGT1A6Δsp synthesized in the presence of membranes (lanes 5 and 6, respectively). Microsomal membranes containing UGT1A6 and UGT1A6Δsp were subjected to carbonate extraction and fractionated by centrifugation on a sucrose cushion to yield pellet (P, lanes 7 and 8, respectively) and supernatant (S, lanes 9 and 10, respectively) fractions. Microsomal membranes containing UGT1A6 and UGT1A6Δsp were subjected to treatment by proteinase K as described in Section 2 (lanes 11 and 12, respectively). The $^{35}$S-radiolabelled products were analyzed by SDS-PAGE and subjected to autoradiography.

polypeptide corresponding to the expected apparent molecular mass for the preprotein UGT1A6 (Fig. 1, lane 1). The addition of canine pancreas microsomes to the reaction mixture produced slower-migrating polypeptides of about 55–56 kDa corresponding to the cleavage of the signal peptide followed by *N*-asparagine linked glycosylation (Fig. 1, lane 3). Consistently, the addition to the reaction mixture of tripeptides that prevent the *N*-glycosylation abolished the observed shift, yielding a single polypeptide band of 52 kDa corresponding to the mature unglycosylated form of UGT1A6 (Fig. 1, lane 5).

An attempt was made to express UGT1A6Δsp from the UGT1A6 sequence deleted from both the 5′ untranslated region and the signal peptide coding sequence. However, a very small amount of the corresponding polypeptide was yielded. We therefore designed a second construct in which the UGT1A6Δsp sequence was placed downstream of the 5′ untranslated region of the wild-type UGT1A6 cDNA. In this case, in vitro expression was successful, giving rise to a polypeptide of the expected apparent molecular mass of about 52 kDa (Fig. 1, lane 2). Interestingly, in the presence of microsomal membranes, the apparent molecular mass of the neosynthesized polypeptide was increased by about 2 kDa (Fig. 1, lane 4). This decrease of mobility was abolished by addition of glycosylation inhibitory tripeptides (Fig. 1, lane 6), indicating that the deletion mutant was translocated and *N*-glycosylated in the lumen of the ER. Furthermore, UGT1A6 lacking the signal peptide as well as the wild-type protein were found to be stably integrated into the microsomal membranes. Indeed, the majority of UGT1A6 and of UGT1A6Δsp polypeptides was recovered in the pellet fraction (Fig. 1, lanes 7, 8, respectively) whereas tiny amounts of protein could be detected in the supernatant (Fig. 1, lanes 9, 10, respectively) following treatment with sodium carbonate. Finally, UGT1A6 and UGT1A6Δsp polypeptides were treated with proteinase K. As shown in Fig. 1, both the wild-type UGT1A6 (lane 11) and the deletion mutant (lane 12) were protected against proteolysis. Based on these criteria, our results established that UGT1A6 deprived of its N-terminal cleavable signal peptide was targeted, translocated and integrated into the ER membrane in an apparently identical orientation, compared to the wild-type protein.

### 3.2. *Targeting of UGT1A6 expressed with or without signal peptide in mammalian cells*

We next investigated the subcellular localization of UGT1A6Δsp compared to the wild-type protein by immunofluorescence microscopy after stable expression in V79 cells. In cells expressing UGT1A6, a typical ER fluorescence pattern (Fig. 2A), similar to that of calnexin, an integral membrane protein used as ER marker (Fig. 2B), was observed. Interestingly, like the wild-type protein, UGT1A6Δsp was found to be localized in the ER (Fig. 2C) with an immunofluorescence pattern overlapping that of calnexin (Fig. 2D). These observations show that, despite the absence of signal peptide, UGT1A6 was addressed to the ER in mammalian cells.

### 3.3. *UGT1A6 lacking the signal peptide is functionally expressed in mammalian cells*

The ex vivo assay was based on the measurement of the amount of glucuronides produced and excreted after addition of the substrate 4-MU into the culture medium. After 24 h, about 44 ± 6 nmol and 39 ± 4 nmol of 4-MU-glucuronide were produced per $10^6$ cells in the extracellular medium of cultured UGT1A6 and UGT1A6Δsp V79 cells, respectively, while less than 1 nmol was produced by non-transfected cells during the same time period.

Evaluation of the glucuronidation of 4-MU in the membrane enriched fraction and in the $100\,000 \times g$ supernatant of UGT1A6 and UGT1A6Δsp V79 cells showed that the enzymatic activity was exclusively found in the membrane fraction of both recombinant cells. The apparent $K_m$ and $V_{max}$ values for 4-MU catalyzed by UGT1A6Δsp (0.55 ± 0.17 mM, 8.39 ± 1.23 nmol/min/mg protein, respectively) in the membrane enriched fractions were in the same range as those obtained for the native recombinant protein (0.61 ± 0.19 mM, 11.14 ± 2.11 nmol/min/mg protein, respectively). Similar observations were found when the kinetics of 1-naphthol was considered (results not shown).

## 4. Discussion

We report, for the first time, that a type I integral ER membrane protein, the human UGT1A6, was targeted, translocated into and retained in the ER in the absence of its N-terminal cleavable signal peptide. This result was based on several lines of evidence. (1) UGT1A6 lacking the signal peptide expressed in vitro in a transcription-translation system was glycosylated as the wild-type protein, indicating that in both cases the *N*-glycosylation sites have been translocated across the microsomal membranes. (2) UGT1A6Δsp remained associated with the pelleted fraction following alkaline extraction, suggesting that, like the wild-type protein, the polypeptide was stably integrated into the microsomal membranes. (3) Both the deletion mutant and the wild-type UGT1A6 synthesized in vitro in the presence of membranes were protected from protease degradation suggesting a lumenal orientation of both polypeptides. (4) Immunofluorescence studies illustrated that, despite the lack of signal peptide, UGT1A6 stably ex-

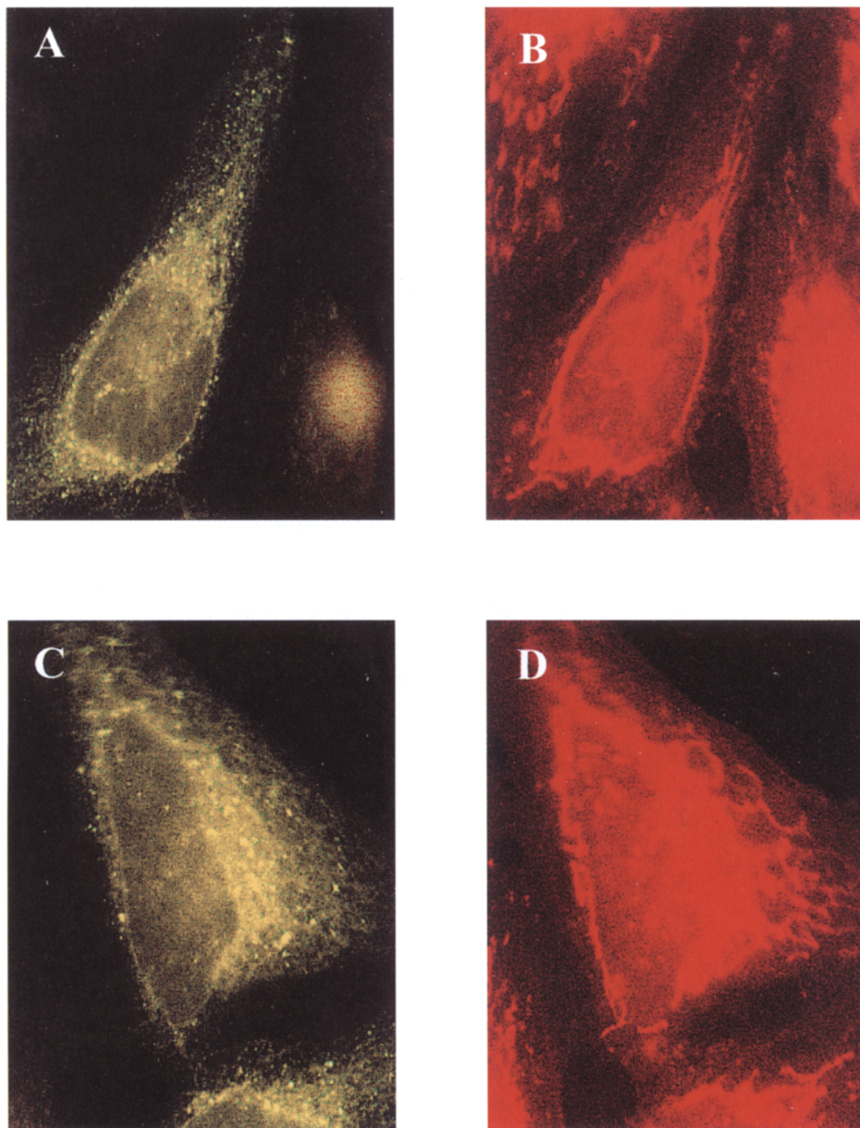


Fig. 2. Intracellular localization of UGT1A6 expressed with and without signal peptide in V79 cells. Immunofluorescence detection of UGT1A6 (A) and UGT1A6Δsp (C) was performed in cells stably expressing each polypeptide using a primary polyclonal anti-UGT1A6 antibody and a FITC-conjugated secondary antibody. Immunofluorescence detection of calnexin was subsequently performed on the same V79 UGT1A6 (B) and V79 UGT1A6Δsp cells (D), using anti-calnexin primary antibodies and rhodamine-conjugated secondary antibodies.

pressed in mammalian cells was addressed to and retained in the ER.

Interestingly, UGT1A6Δsp was fully active as shown by the in situ production of 4-MU-glucuronide, which was not significantly different from that measured with the wild-type protein. Furthermore, the glucuronidation activity with 4-MU as substrate was associated with the microsomal fraction and exhibited apparent kinetic constants similar to UGT1A6. These results indicate that the mutant was correctly folded and retained a tertiary structure allowing a normal substrate recognition and catalytic activity.

The role of the signal peptide in the initiation of the translocation process has been well documented [1]. In general, mutations and deletions within the signal peptide sequence result in a strong inhibition of the translocation process leading to the accumulation of the protein in the cytosol or to its degradation [28]. However, the observation that the natural cleavable N-terminal signal sequence can be entirely removed without precluding the translocation of secretory or membrane proteins across the ER, as for UGT1A6, is not without precedent. It has been clearly shown that despite the absence of its N-terminal signal sequence, the yeast alkaline phosphatase could be translocated across the ER membrane in vivo or in vitro [29]. In the same way, Loo et al. [30] recently reported for the human $Na^{+}$-$Ca^{2+}$ exchanger, an integral plasma membrane polytopic protein, that despite the lack of a signal peptide sequence, the protein was addressed to its normal subcellular localization and fully functional, when expressed in mammalian cells.

For UGT1A6 and other proteins that tolerate a deletion of the signal peptide, the role of this sequence may therefore be questioned. One possibility is that it may enhance membrane insertion. In fact, it has been reported that the deletion of the signal peptide reduced the efficiency of secretion of alkaline phosphatase in whole cells [31]. In the same way, it has been demonstrated that the fusion of a cleavable signal peptide to

the human $\beta_2$-adrenergic receptor increased the translocation of the receptor into the ER [32]. It can be suggested that such an increase would enhance the expression of the protein in cell types which express low levels of the protein, but would not be detectable in the high levels of expression achieved in V79 cells. Similarly to our data, Loo et al. [30] did not observe any reduced efficiency in the expression of $Na^{+}$-$Ca^{2+}$ exchanger expressed without signal peptide in mammalian cells.

Xu et al. [33] recently demonstrated that bacteriorhodopsin made without a 13 residue leader peptide was properly assembled in the membrane. However, deletion of the leader region resulted in an unstable mRNA and almost no bacteriorhodopsin production. It is noteworthy that in the absence of the untranslated region, UGT1A6Δsp was inefficiently expressed in vitro in contrast to the wild-type protein. It is therefore possible to speculate that the signal peptide could contribute to the efficiency of translation and/or mRNA stability.

In summary, data obtained from expression of UGT1A6 in vitro as well as in mammalian cells clearly showed that the signal peptide sequence is not essential for ER targeting and functionality of this protein. The most likely explanation for these observations is the presence of redundant topogenic elements capable of directing the protein deprived of signal peptide to the ER membrane. In fact, cooperativity between topogenic elements has recently been described for several polytopic plasma membrane protein such as band 3 anion exchanger [26], or sodium calcium exchanger [30]. Computer prediction of secondary structures reveals the presence of hydrophobic stretches in the N-terminal half of UGT1A6 that may interact with the membranes. The topogenic role of these putative signal-like sequences is under investigation.

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