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Endoplasmic reticulum retention of the large splice variant of the UDP-galactose transporter is caused by a dilysine motif

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Nucleotide-sugar transporters supply mainly the Golgi glycosyltransferases with substrates. Some glycosyltransferases in the endoplasmic reticulum (ER), however, also use activated sugars. Recent studies have demonstrated that UDP-galactose (UDP-Gal) is the substrate for the ER resident ceramide-galactosyltransferase (cer-GalT) and cells expressing cer-GalT are able to retain the UDP-Gal transporter (UGT) by physical contacts formed between the two proteins. Here, we describe a second active mechanism for ER localization of the UGT. The UGT is produced in two splice forms UGT1 and UGT2. The proteins vary only at their extreme C-termini but show strikingly different intracellular distribution. Although N-terminally epitope tagged forms of UGT1 localize exclusively to the Golgi, similar constructs of UGT2 show both ER and Golgi localization. The dilysine motif KVKGS contained in UGT2 can be demonstrated to be responsible for the dual localization because: (1) disturbance of the signal via site specific mutation or C-terminal extension completely shifts the transporter to the Golgi, (2) transfer of the dilysine motif is sufficient to redistribute the Golgi CMP-sialic acid transporter to the ER, and (3) replacement of KVKGS by the strong ER retention signal KKNT is sufficient to completely retain UGT2 in the ER.

Key words: dilysine/ER retention/Golgi/nucleotide sugar transporter/UDP-galactose transporter

Introduction

Nucleotide-sugar transporters (NSTs) supply the glycosyltransferases involved in the biosynthesis of glycoconjugates along the secretory pathway of eukaryotic cells with activated sugars that are synthesized in the cytoplasm or cell nucleus (Hirschberg *et al*., 1998). Most glycosyltransferases that use nucleotide activated sugars are active in the Golgi, whereas the majority of ER resident glycosyltransferases depends on the presentation of activated sugars by dolichol phosphate (Burda and Aebi, 1999). Accordingly, nucleotide sugar transport activities could be measured in Golgi membrane preparations (Sommers and Hirschberg, 1982; Capasso and Hirschberg, 1984; Hirschberg *et al*., 1998) and after molecular cloning, the corresponding NSTs could be localized to the Golgi membranes (Eckhardt *et al*., 1996; Dean *et al*., 1997; Yoshioka *et al*., 1997; Ishida *et al*., 1999; Lühn *et al*., 2001). Transport of some specific nucleotide sugars into ER vesicles must, however, exist, because a panel of nucleotide sugar requiring glycosylation reactions takes place in the ER. The UDP-glucose : glycoprotein glucosyltransferase (Zuber *et al*., 2001) uses a nucleotide sugar as substrate, evidence has been provided that proteoglycan biosynthesis is initiated in the ER (Kearns *et al*., 1993), and some members of the UDP-glycosyltransferase gene superfamily (Mackenzie *et al*., 1997) involved in glycosylation of lipophilic compounds (Meech and Mackenzie, 1997; de Wildt *et al*., 1999) are located in the ER lumen. Indeed, nucleotide sugar transport activities corresponding to these reactions could be measured in ER vesicles (Bossuyt and Blanckaert, 1997; Hirschberg *et al*., 1998; Castro *et al*., 1999), and, by indirect immunostaining methods, the yeast UDP-*N*-acetylglucosamine transporter (Roy *et al*., 2000) and the human UDP-glucuronic acid/UDP-*N*-acetylgalactosamine transporter (Muraoka *et al*., 2001) have been localized to the ER. Interestingly, the yeast UDP-*N*-acetylglucosamine transporter has a C-terminal dilysine motif (Nilsson *et al*., 1989; Zerangue *et al*., 2001), potentially responsible for ER retention.

UDP-galactose (UDP-Gal) transport has been exclusively assigned to the Golgi (Perez and Hirschberg, 1985; Hirschberg *et al*., 1998), and, after it was cloned, the UDP-Gal transporter (UGT) has been localized to the Golgi (Ishida *et al*., 1996; Yoshioka *et al*., 1997; Oelmann *et al*., 2001). However, in a more recent study, Sprong *et al*. (1998) have shown that the ER-resident ceramide-galactosyltransferase (cer-GalT) is inactive, if expressed in chinese hamster ovary (CHO) cells of the complementation group Lec8, which exhibit a genetic defect in the UDP-Gal transport protein (Deutscher and Hirschberg, 1986). The lack of functionality could be complemented by cotransfecting the UGT cDNA (Sprong *et al*., 1998). The presented data clearly indicate that the lack of substrate in the ER prevented enzymatic activity. The same group has meanwhile shown that cer-GalT is able to make physical contacts to the UGT and thus can retain the protein in the ER (Sprong *et al*., 2003).

The existence of an alternative splice site in the human UGT gene enables the translation of two protein variants (Ishida *et al*., 1996). As shown in Figure 1, translation of the longer message results in the expression of UGT1 having the C-terminal sequence SVLVK, whereas translation of the shorter mRNA generates UGT2, in which the sequence SVLVK is replaced by LLT**K**V**K**GS. UGT2 contains a

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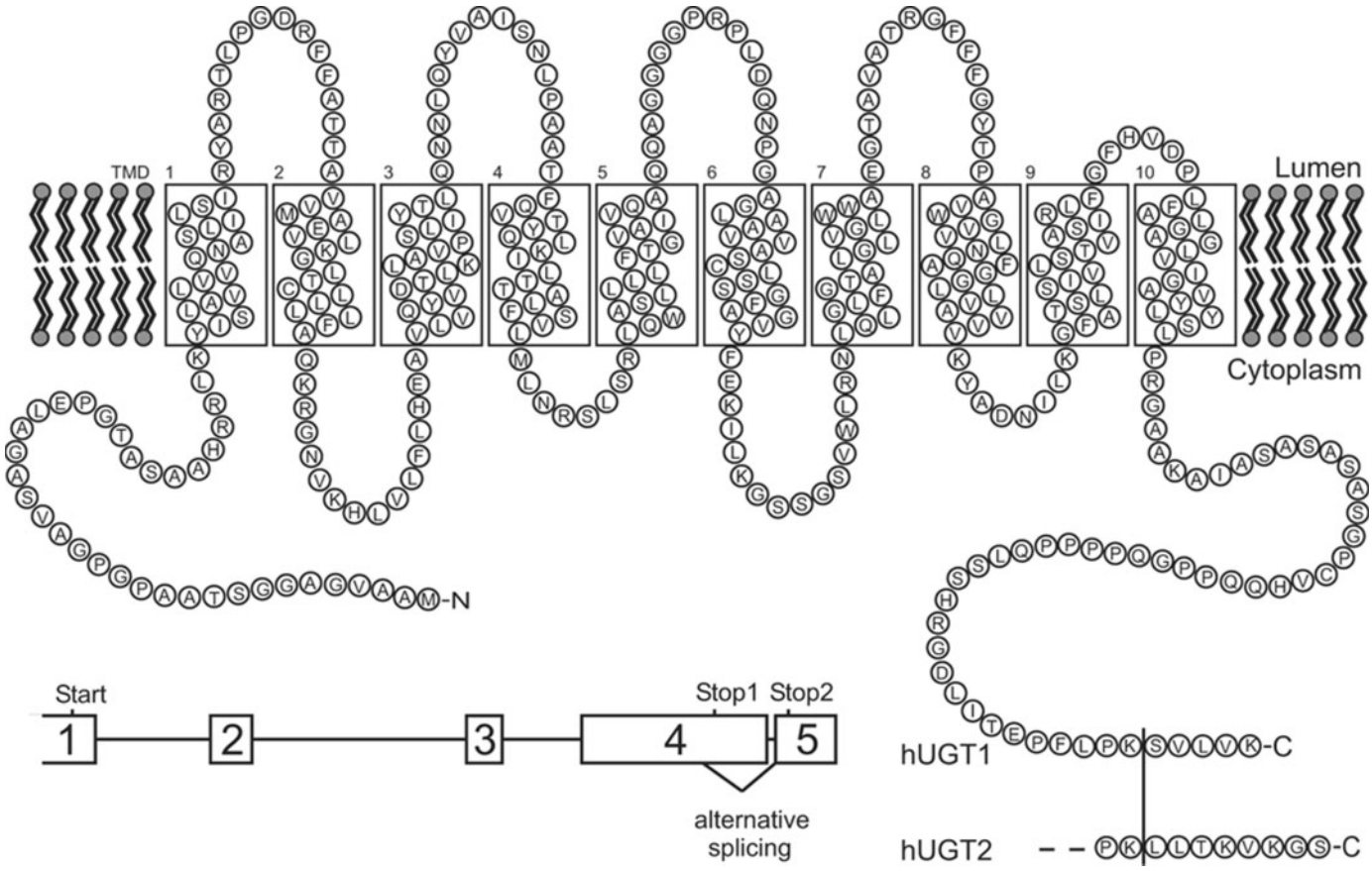

Fig. 1. Topology prediction of the human UDP-galactose transporter (UGT) and display of the alternative splice site. The topology model is based on prediction software (Tusnady and Simon, 2001) and homology to the CMP-sialic acid transporter for which the topology has been experimentally defined (Eckhardt *et al*., 1999). The alternative splice variant UGT2 (Ishida *et al*., 1996) occurs from utilization of a splice site located in exon 4. The shorter mRNA resulting from the use of the alternative splice site encodes a protein in which the last five amino acids SVLVK (UGT1) are replaced by the dilysine motif containing sequence LLT**K**V**K**GS (UGT2).

dilysine motif, shown in other proteins to limit exit from the ER (Nilsson *et al*., 1989; Zerangue *et al*., 2001).

In this study, we present a set of experiments, which conclusively demonstrates that the dilysine motif present in UGT2 is an autonomously active ER retention signal. We, therefore, suggest that a second, cer-GalT independent mechanism exists, by which the UGT can be retained in the ER.

Results

Differences in the subcellular localization of human UDP-Gal transporters 1 and 2

The first indication that the dilysine motif in UGT2 functions as an ER retention signal came from the observation that a C-terminally tagged hamster UGT2 localized exclusively to the Golgi, whereas addition of an N-terminal tag changed the staining pattern. Golgi staining remained and an additional signal, suggesting ER retention of the N-terminally tagged protein, became visible.

To directly show the difference in localization between human UGT1 and UGT2, we transiently expressed N-terminally FLAG-tagged constructs of both splice variants in CHO Lec8 cells and analyzed by intracellular immunofluorescence. Although the FLAG-tagged UGT1 clearly colocalized with the Golgi marker α-mannosidase II and showed no overlap with the ER marker calnexin (Figure 2), FLAG-tagged UGT2 displayed, in addition to the Golgi signal, a signal that could be addressed to the ER by colocalization with calnexin. Therefore, the difference in localization pattern between N-terminally FLAG-tagged UGT1 and UGT2 seems to be exclusively determined by the respective 5 or 8 C-terminal amino acids of the transport proteins.

*The **K**x**K**xx motif is responsible for ER retention*

As UGT2 contains the putative ER retaining dilysine motif **KVKGS**, we examined whether these five amino acids autonomously function as ER retention signal when added to a related protein like the CMP-sialic acid transporter (CST), which is solely Golgi localized (Eckhardt *et al*., 1999). Results shown in Figure 3 clearly demonstrate that the natural CST stained as a Golgi resident protein, whereas the **KVKGS**-extended form of the protein was distributed over ER and Golgi (Figure 3B and C).

In contrast, mutation of the lysine at the –3 position to alanine (**KVKGS** to **KVAGS**) in UGT2 resulted in transport of the protein to the Golgi (Figure 3F and G). These

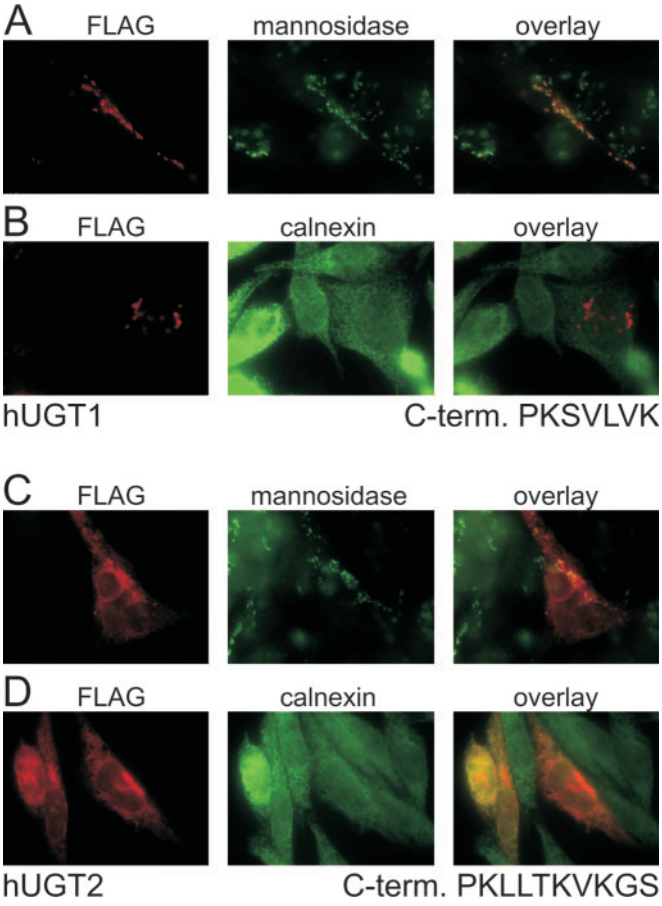

Fig. 2. Intracellular localization of human UGT1 and UGT2. hUGT1 and hUGT2 were transiently expressed in chinese hamster ovary (CHO) Lec8 cells, and the intracellular localization was compared with a Golgi (α-mannosidase II) and an ER (calnexin) marker. UGT1 localizes exclusively in the Golgi (panel **A**) and shows no overlap with an ER marker (**B**) while UGT2 shows dual expression in both Golgi (**C**) and ER (**D**).

experiments clearly demonstrate that the dilysine motif contained in UGT2 acts as an autonomous ER-retention signal and confirm earlier studies, in which the crucial role of the lysine-residue in position –3 has been demonstrated (Zerangue *et al.*, 2001). Obviously, the observation that N- and C-terminally tagged hamster UGT2 behaved different could also be explained by the fact that a C-terminal tag is masking the dilysine motif.

The dilysine motif in UGT2 is a weak ER retention signal

It is known that not all C-terminal **KK**XX and **KX**K**X**X sequences efficiently retain proteins in the ER (Itin *et al.*, 1995; Andersson *et al.*, 1999; Zerangue *et al.*, 2001). The fact that also UGT2 and CST carrying the **KV**K**G**S motif localized to ER and Golgi leaves open the question if this is a property of the nucleotide sugar transporters or the dilysine motif. To investigate this, we replaced the **KV**K**G**S sequence in UGT2 by **KK**NT, a strong ER retention signal (Zerangue *et al.*, 2001). Similarly, we tagged CST with the **KK**NT motif. Both transporters now showed exclusive ER localization (Figure 4). In combination with the above results these data demonstrate that the **KV**K**G**S sequence in

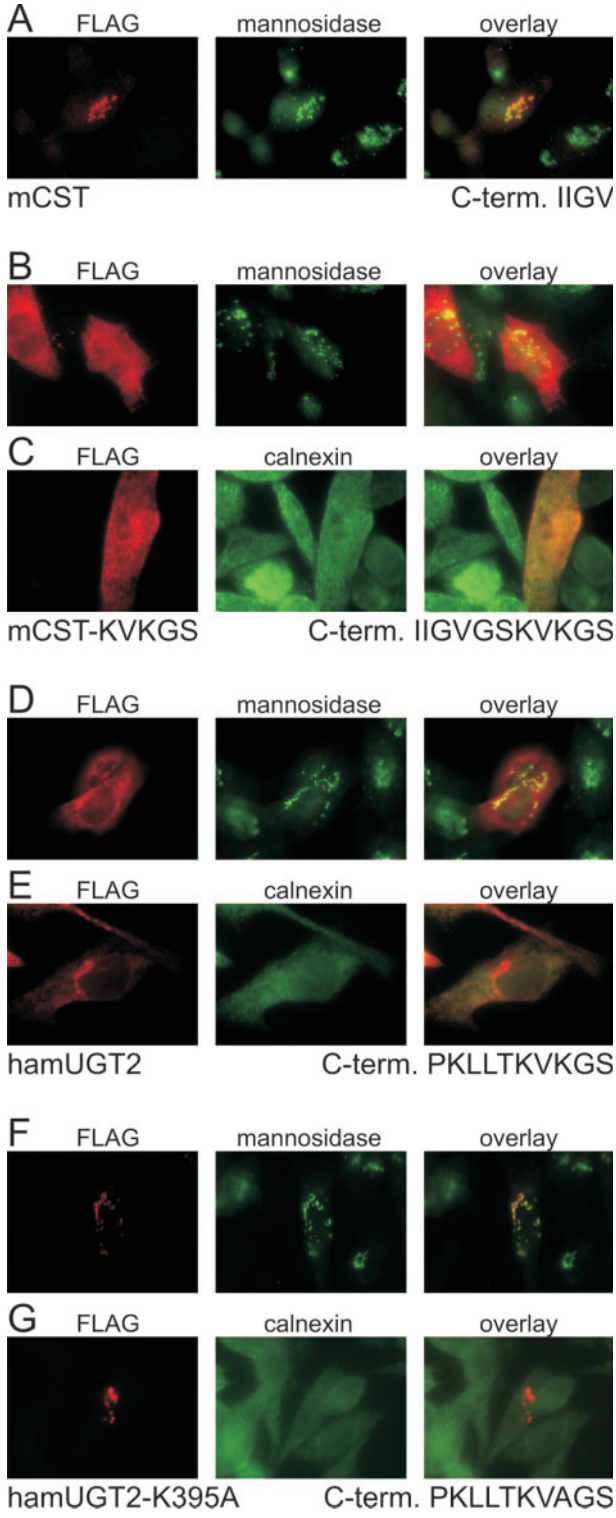

Fig. 3. The **KV**K**G**S motif is responsible for ER retention. mouse CST is a Golgi resident protein (panel **A**), but behaves like UGT2 (both ER and Golgi localization) when the last five amino acids from UGT2 are transferred to the C-terminus (mCST-**KV**K**G**S, **B** and **C**). The hamUGT2 shows dual localization in ER and Golgi (**D** and **E**) but the K396A mutant (**F** and **G**) is exclusively found in the Golgi.

UGT2 functions as an ER retention signal, but the destination signal is weak.

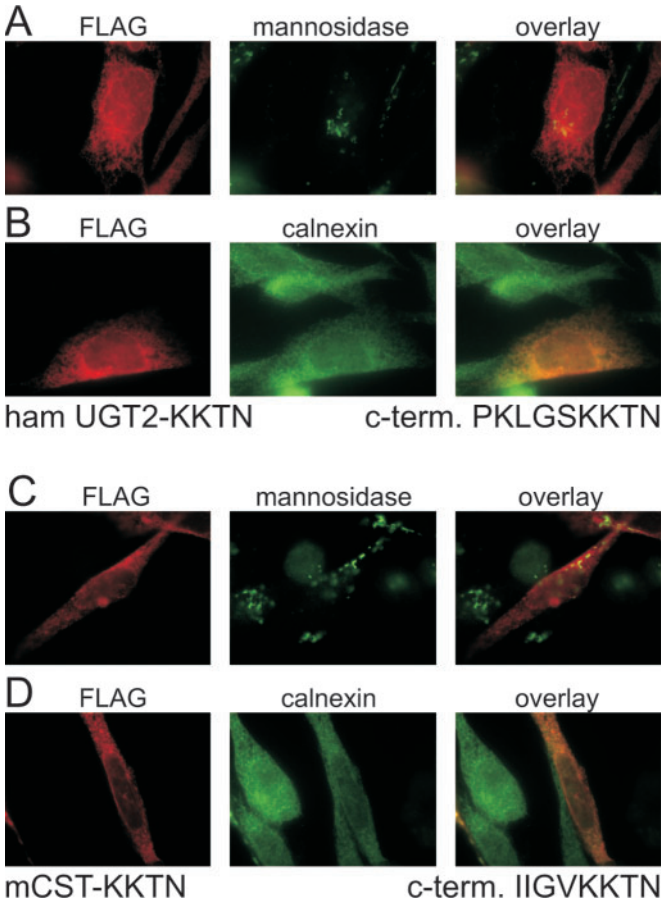

Fig. 4. **KK**TN is a stronger ER retention signal than **KV**K**G**S. In hamUGT2 and mCST-**KV**K**G**S the dilysine motif was replaced by **KK**TN. Both hamUGT2-**KK**TN (panel **A** and **B**) and mCST-**KK**TN (**C** and **D**) are found exclusively in the ER.

Asking whether the functionality of UDP-Gal and CMP-sialic acid transport proteins is altered by changes in their subcellular localization, we used constructs made in this study (listed in Table I) to test complementation activity in CHO Lec8 (Deutscher and Hirschberg, 1986) or Lec2 (Stanley and Siminovitch, 1977) cells. Cells were transiently transformed with the respective cDNAs, and reconstitution of wild type was determined in flow cytometry analyses using the polysialic acid specific monoclonal antibody 735 (Frosch *et al.*, 1985) in the case of Lec2 cells (Eckhardt *et al.*, 1996) and the HNK-1 antibody L2-412 (Kruse *et al.*, 1984) in the case of Lec8 cells (Bakker *et al.*, 2005). To induce the formation of the HNK-1 epitope in Lec8 cells, we cotransfected the UGT cDNAs with a cDNA encoding the β1,3glucuronyltransferase (Terayama *et al.*, 1997). The results shown in Figure 5 indicate that all constructs, independent on their subcellular localization, are able to restore the wild type glycosylation patterns.

Discussion

We have demonstrated that the dilysine motif in the human and hamster UGT functions as a weak ER retention signal.

Table I. Used constructs in this study

Construct	C-terminus
hUGT1	PKSVLVK
hUGT2	PKLLTKVKGS
hamUGT2	PKLLTKVKGS
hamUGT2-K396A	PKLLTKV**A**GS
hamUGT2-BamHI	PKL**GS**KVKGS
hamUGT2-KKTN	PKL**GSKKTN**
mCST	IIGV
mCST-KVKGS	IIGV**GSKVKGS**
mCST-KKTN	IIGV**GSKKTN**

All constructs were made in pcDNA3, and the expressed proteins have a FLAG tag (MDYKDDDDK) at the N-terminus and the indicated sequence at the C-terminus. Amino acid changes form the original sequences are indicated in boldface. h, human; ham, hamster; m, mouse.

In dilysine motifs, the lysine at the –3 position is essential, whereas the second lysine can be at the –4 or –5 position. But not all sequences that fulfil these criteria will target a protein to the ER, indicating that also surrounding amino acids play a role. Moreover, sequences with a lysine at –4 are more likely to function as a retention signal than those with a lysine at the –5 position (Teasdale and Jackson, 1996; Zerangue *et al.*, 2001). Based on this information, the dilysine motif in UGT2 could be marked as a questionable retention signal. However, a clear difference in the localization of UGT1 and UGT2 has been observed in all our experiments, if proteins were expressed with N-terminal tags, whereas the use of C-terminally tagged proteins let to unequivocal Golgi staining. The putative ER retention signal **KV**K**G**S present in UGT2 was mutated to destroy the essential position –3. This experiment shown in Figure 3 resulted in a pure Golgi localized mutant. In a number of systematic analytical steps we than identified the **KV**K**G**S motif as a weak ER-retention signal, which retains activity also after transplantation to the C-terminus of CST.

Still it seems difficult to categorize dilysine motifs as retaining or nonretaining because earlier studies, using combinatorial variants of the dilysine motif, demonstrated that a cell surface protein, used as a model substance, could be targeted to virtually every compartment between ER and cell surface by the artificially made dilysine motifs (Zerangue *et al.*, 2001). Our results, demonstrating that also a naturally occurring membrane protein can show dual destination as a result of the presence of an ER retention signal, provides important evidence for the physiological relevance of the motif.

Surprisingly, all constructs used in this study were able to complement the defect in Lec8 or Lec2 cells. No differences were visible between the complementation activities of mutant and natural proteins. Even NST variants with a strong ER retention signal, which did not show any Golgi localization (Figure 4), were able to fully restore the wild type phenotype of mutant cell lines (Figure 5). This observation suggests that activated sugars can be passively transported throughout ER and Golgi. On the other hand, one cannot rule out the possibility that small quantities of the

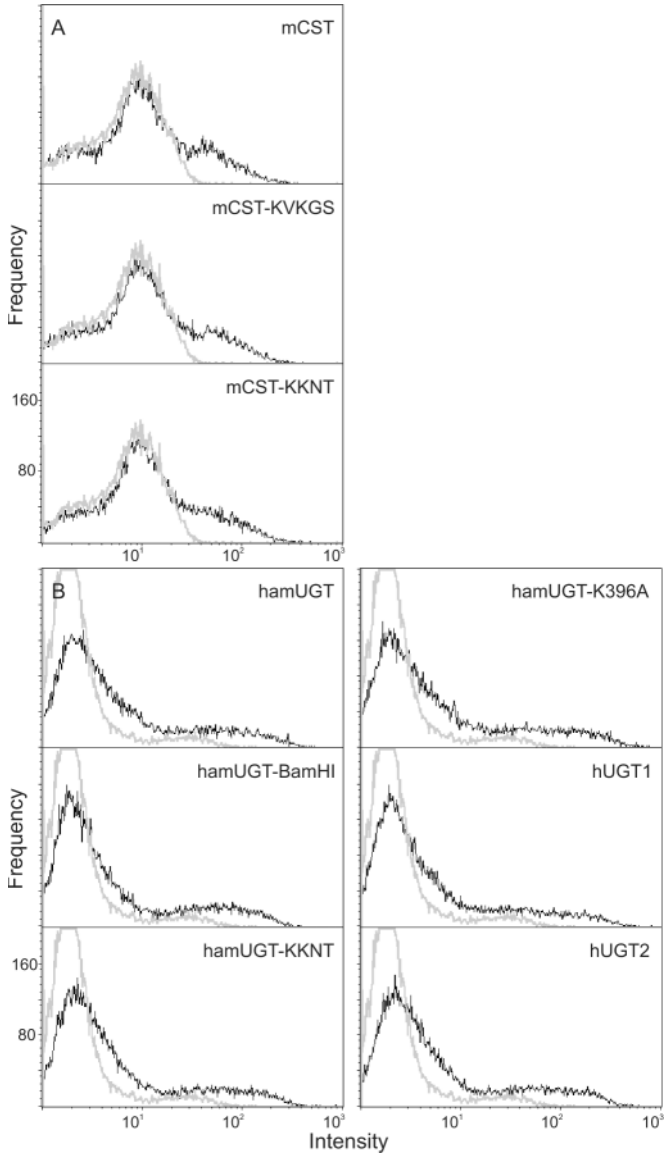

Fig. 5. Complementation of Lec8 and Lec2 cells by all used construct in this study. Transiently transfected cells were immunolabelled and analyzed by flow cytometry. **A**: Lec2 cells transfected with the indicated constructs (black), each time compared to the empty vector control (grey), and labelled using an antibody (735) against polysialic acid. **B**: Lec8 cells transfected with the indicated constructs (black), each time compared to the empty vector control (grey), both cotransfected with a β1,3glucuronyltransferase to allow the detection of the GlcAβ1,3Gal epitope by an HNK-1 antibody (L2-412). Note that expression of the glucuronyltransferase alone (grey) already results in a shift of the transfected population of cells because of the presence of low amounts of galactose still present in Lec8 cells. Complementation results in an additional shift of the transfected population (black).

recombinant proteins reach the Golgi apparatus and thus drive the glycosylation machinery. In transient expression systems recombinant proteins are drastically overexpressed and may overstress the systems that survey cellular targeting processes. In fact, the dilysine motifs are known to be involved in both ER retention and retrieval (Andersson *et al.*, 1999). Thus, the system *per se* may, to a certain extend, allow cycling of proteins between ER and Golgi, and this process may be more pronounced in the transformed cells.

There are other members in the NST family that contain dilysine motifs (Martinez-Duncker *et al.*, 2003). One of these is the yeast UDP-*N*-acetylglucosamine transporter, which has in fact been localized to the ER (Roy *et al.*, 2000). In contrast to UGT2, the addition of a C-terminal tag was not sufficient to destroy ER-retention. Also the human UDP-glucuronic acid/UDP-*N*-acetylgalactosamine transporter (Muraoka *et al.*, 2001) has been localized to the ER even so no dilysine or other signal is present that could explain ER retention. Together these data underline that other mechanisms exist for the retention of NSTs in the ER.

One of these alternative mechanisms may be protein complex formation as it has been demonstrated recently in the case of UGT1 (Sprong *et al.*, 2003). Although UGT1 normally is localized exclusively in the Golgi, it can be detected in the ER when coexpressed with cer-GalT, a member of the UDP-glycosyltransferase gene superfamily (Mackenzie *et al.*, 1997), which by itself harbors a C-terminal dilysine motif (Nilsson *et al.*, 1989; Zerangue *et al.*, 2001) and localizes to the ER (Sprong *et al.*, 1998). Using coimmunoprecipitation experiments the authors found physical interaction between UGT1 and cer-GalT. Obviously, the cer-GalT, retained in the ER via the intrinsic dilysine motif, binds to the UDP-Gal transport protein and thus retains the protein in the ER. Because cer-GalT is not expressed in CHO cells (Sprong *et al.*, 2003), and recombinant UGT1 fully migrates to the Golgi, it can be excluded that this second mechanism of retention exists in CHO cells.

So far, both splice variants of the UGT have only been cloned from man (Ishida *et al.*, 1996), but EST sequences of both forms are also found in mouse, and the encoded proteins have identical C-termini as human UGT1 and 2. Moreover, northern blots of different mouse tissues and developmental stages always show two signals corresponding to the two splice variants (Ishida *et al.*, 1999). Although human expression data have not been published, Kawakita and Ishida (2002) mentioned that northern blot analysis revealed that both forms are ubiquitously expressed in every human tissue so far examined. Also on a northern blot from CHO cells (Oelmann *et al.*, 2001), the two mRNA signals can be distinguished, indicating that both forms can even be expressed within one cell line. Thus, there is until now no indication of differential expression of the two splice forms.

Materials and methods

Antibodies

Monoclonal anti-mouse antibody (mAb) M5 directed against the FLAG sequence MDYKDDDDK was from Sigma (St. Louis, MO). A rabbit antiserum against the catalytic domain of α-mannosidase II was a kind gift of Dr. K. Moremen, University of Georgia, Athens, and anti-calnexin, directed against the cytoplasmic domain of the calnexin, was a kind gift of Dr. A. Helenius (Swiss Federal Institute of Technology, Zurich). Anti-mouse Ig-Cy3-conjugate was from Sigma and anti-rabbit Ig-ALEXA 488-conjugate from Molecular Probes (Eugene, OR).

Cell lines and plasmids

All transient transfections for imunostaining were done in the CHO mutant Lec8 (Deutscher and Hirschberg, 1986) (ATCC CRL 1737) a cell line with a defect in the UGT gene. Cells were maintained in alpha medium (Biochrome, Berlin, Germany) supplemented with 10% fetal calf serum, 2 mM L-glutamin, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were grown in a humidified atmosphere at 37°C and 5% CO_2. Complementation experiments were carried out in Lec8 cells for UGTs and in CHO Lec2 (ATTC CRL 1736; Stanley and Siminovitch, 1977) for CST constructs.

Generation of N-terminally FLAG-tagged constructs of the hamster UGT and mouse CST in pcDNA3 has been described (Eckhardt *et al*., 1998; Oelmann *et al*., 2001) and named hamUGT2 and mCST in this study. cDNA clones for human UGT1 (IMAGp998D17289Q2, IMAGE:163552) and UGT2 (IMAGp998H0411479Q2, IMAGE:5191731) were obtained from RZPD (Berlin), a distributor of the I.M.A.G.E. Consortium [LLNL] cDNA clones (Lennon *et al*., 1996) and cloned into pcDNA3-FLAG (pCDNA3 [Invitrogen, Carlsbad, CA] with the FLAG sequence tag [GGTACCGCCACCATGGACTACAAGGATGATGATGATAAGGGATCC] cloned into the KpnI-BamHI sites). The fragments were amplified with oligonucleotide HB22 and HB23 for UGT1 and HB22 and HB24 for UGT2, digested with BamHI-XbaI and ligated in the corresponding sites of pcDNA3-FLAG. The resulting constructs have the N-terminal sequence MDYKDDDDKGSN (FLAG tag underlined) followed by the complete open reading frames of human UGT1 and UGT2 and named in this article hUGT1 and hUGT2. To introduce a point mutation to create K396A, hamUGT2, which has an N-terminal FLAG tag followed by an EcoRI site, was amplified using the primers SO23 and RK001 and subcloned in the same vector using EcoRI and XbaI (hamUGT2-K396A). A BamHI site was introduced in hamUGT2 by site directed mutagenesis using the oligonucleotides RK007 and RK008. In the resulting protein the C-terminus is changed from *LT*KVKGS into *GS*KVKGS (hamUGT2-BamHI). To create mCST-KVKGS, we the mCST was amplified with the oligonucleotide primers ME71 and ME42, and the resulting product was cloned via EcoRI and BamHI into hamUGT2-BamHI. The sequence KVKVS was replaced in hamUGT2-*Bam*HI and mCST-KVKGS for KKNT by digesting with *Bam*HI-*Xba*I and ligation of a linker composed of the oligonucleotides RK024 and RK025 (hamUGT2-KKNT and mCST-KKNT). All constructs were confirmed by sequencing. Table I summarizes the obtained constructs and highlights the amino acid sequences of the respective C-termini.

Used oligonucleotides: CTA TGG ATC CAA CAT GGC AGC GGT TGG GGC T(HB22), GTA GTC TAG AAT TGC TGC CAG CCC TCA CT(HB23), GTA GTC TAG AAT CCC AGC GGC TAG GAA C(HB24), CGT CTA GAC TAC GAA CCC GCC ACC TTG GTG(RK001), GCG AAT TCG CAG CGG TTG GGG TTG(SO23), CTG CCA AAG TTG GGA TCC AAG GTG AAG GG(RK007), CCC TTC ACC TTG GAT CCC AAC TTT GGC AG(RK008), GCG AAT TCG CTC AGG CGA GAG AA (ME71), GCG GAT CCC ACA CCA ATG ATT CTC TCT TTT(ME42), GAT CCA AGA AGA CTA ACT AGT(RK024), CTA GAC TAG TTA GTC TTC TTG(RK025). Restriction sites are underlined.

Indirect immunofluorescence and complementation studies

For immunofluorescence analysis, we seeded cells onto glass cover slips. Plasmids were transfected using Metafectene (Biontex, Munich, Germany). Cells were analyzed by immunofluorescence 2 days post transfection. Therefore, cells were fixed in 4% paraformaldehyde and permeabilized for 30 min with 0.1% saponin in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin. After permeabilization, samples were incubated with the respective primary antibodies (anti-FLAG mAb M5 and anti-α-mannosidase II or anti-calnexin) followed by incubation with anti-mouse-Cy3 and anti-rabbit-ALEXA 488. Slides were mounted in Mowiol (Calbiochem, Darmstadt, Germany) and analyzed under a Leica DM IRBE.

Complementation of Lec8 and Lec2 cells were essentially done as described (Eckhardt *et al*., 1996; Bakker *et al*., 2005) with the exception that the cells have been analyzed by flow cytometry instead of cell surface staining of adherent cells. For this, plasmids were transfected using Metafectene (Biontex, Munich, Germany). Two days post transfection cells were released from the plates by incubation with PBS/2 mM ethylenediamine tetra-acetic acid and incubated with monoclonal antibody L2-412 (Kruse *et al*., 1984) and anti-Rat-FITC as secondary antibody for Lec8 cells and monoclonal antibody 735 (Frosch *et al*., 1985) and anti-Mouse-FITC as secondary antibody for Lec2 cells. Per construct 30,000 cells were counted.

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Abbreviations

cer-GalT, ceramide-galactosyltransferase; CHO, chinese hamster ovary; CST, CMP-sialic acid transporter; ER, endoplasmic reticulum; NST, nucleotide-sugar transporter; UDP-galactose, UDP-Gal; UGT, UDP-gal transporter.

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