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Subcellular localization of UDP-GlcNAc, UDP-Gal and SLC35B4 transporters

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The mechanisms of transport and distribution of nucleotide sugars in the cell remain unclear. In an attempt to further characterize nucleotide sugar transporters (NSTs), we determined the subcellular localization of overexpressed epitope-tagged canine UDP-GlcNAc transporter, human UDP-Gal transporter splice variants (UGT1 and UGT2), and human SLC35B4 transporter splice variants (longer and shorter version) by indirect immunofluorescence using an experimental model of MDCK wild-type and MDCK-RCA^r mutant cells. Our studies confirmed that the UDP-GlcNAc transporter was localized to the Golgi apparatus only and its localization was independent of the presence of endogenous UDP-Gal transporter. After overexpression of UGT1, the protein colocalized with the Golgi marker only. When UGT2 was overexpressed, the protein colocalized with the endoplasmic reticulum (ER) marker only. When UGT1 and UGT2 were overexpressed in parallel, UGT1 colocalized with the ER and Golgi markers and UGT2 with the ER marker only. This suggests that localization of the UDP-Gal transporter may depend on the presence of the partner splice variant. Our data suggest that proteins involved in nucleotide sugar transport may form heterodimeric complexes in the membrane, exhibiting different localization which depends on interacting protein partners. In contrast to previously published data, both splice variants of the SLC35B4 transporter were localized to the ER, independently of the presence of endogenous UDP-Gal transporter.

Keywords: endoplasmic reticulum, Golgi apparatus, MDCK-RCA^r, nucleotide sugar transporter

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INTRODUCTION

The glycan moiety of glycoproteins is synthesized and modified by glycosyltransferases located in the lumen of the endoplasmic reticulum (ER) and Golgi apparatus. The substrates required by these enzymes are sugars activated by the addition of a nucleoside mono- or diphosphate (UDP, GDP, or CMP). Nucleotide sugars are synthesized in the cytosol (Coates *et al.*, 1980), except for CMP-sialic acid (CMP-Sia), which is synthesized in the nucleus (Munster *et al.*, 1989). To be available for glycosyltransferases, nucleotide sugars must be transported into the ER or Golgi apparatus by nucleotide sugar transporters (NSTs) (for reviews see Hirschberg *et al.*, 1998; Gerardy-Schahn *et al.*, 2001), hydrophobic transmembrane proteins with a molecular mass of 30–45 kDa. Most predictions determine an even number of spans, which results in the N- and C-termini being directed to the cytosolic side of the membrane, but the membrane topology has been experimentally determined for the murine CMP-Sia transporter only (Eckhardt *et al.*, 1999). NSTs function as dimers (Eckhardt *et al.*, 1999; Puglielli & Hirschberg, 1999; Puglielli *et al.*, 1999; Gao & Dean, 2000) or higher oligomers (Hong *et al.*, 2000). It has been proposed that they act as antiporters, exchanging the nucleotide sugar with the corresponding nucleoside monophosphate, which is a product of the glycosylation reaction (Hirschberg *et al.*, 1998; Gerardy-Schahn *et al.*, 2001).

The first characterized NSTs were specific for the translocation of a single nucleotide sugar (for review see Hirschberg *et al.*, 1998; Gerardy-Schahn *et al.*, 2001). Recently, multisubstrate transporters of nucleotide sugars have been described in several organisms, including humans (Muraoka *et al.*, 2001; Suda *et al.*, 2004; Ashikov *et al.*, 2005). Although NSTs have been mainly identified in the Golgi apparatus, those located in the ER have also been characterized, e.g., UDP-*N*-acetylglucosamine (UDP-GlcNAc) transporter of *Saccharomyces cerevisiae* (Roy *et al.*, 2000) or UDP-galactose (UDP-Gal) transporters of *Schizosaccharomyces pombe* and *S. cerevisiae* (Nakanishi *et al.*, 2001).

Although the biosynthesis of nucleotide sugars is well understood, the mechanisms of their transport and distribution in the cell remain unclear. In an attempt to further characterize NSTs, we determined the subcellular localization of overexpressed epitope-tagged canine UDP-GlcNAc transporter, human UDP-Gal transporter splice variants (UGT1 and UGT2), and human SLC35B4 transporter splice variants (longer and shorter version) by indirect immunofluorescence using an experimental model of MDCK wild-type and MDCK-RCA^r mutant cells.

MATERIALS AND METHODS

Construction of mammalian expression plasmids. Open reading frames (ORFs) of canine UDP-GlcNAc transporter, human UDP-Gal transporter splice variants (UGT1 and UGT2), and human SLC35B4 transporter splice variants (long and short) with appropriate restriction sites at both ends were amplified using cDNA synthesized from 5 μg of total RNA as a template. RNA was isolated from $3–5 \times 10^6$ MDCK cells or $3–5 \times 10^6$ HL-60 cells using NucleoSpin RNA II Kit (Macherey-Nagel). The concentration of purified RNA was deter-

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Abbreviations: PBS, phosphate-buffered saline, ER, endoplasmic reticulum; NSTs, nucleotide sugar transporters; UGT1 and UGT2, splice variants of UDP-Gal transporter

Table 1. Plasmids constructed for generation of stably transfected cell lines

Plasmid used for transfection	Original vector (source)	NST sequences cloned in MCS
HsUGTGolgi1[a]	pSelect-zeo (InvivoGen)	Human UGT1 splice variant with HA-6His epitope at N-terminus
HsUGTGolgi2[b]	pVitro1-neo (InvivoGen)	MCS1: human UGT1 splice variant with HA-6His epitope at N-terminus
HsUGTER[a,b]	3×FLAG-myc-CMV-26 (Sigma)	Human UGT2 splice variant with FLAG epitope at N-terminus
CfNGl[b]	pVitro1-neo (InvivoGen)	MCS1: canine UDP-GlcNAc transporter with HA-6His epitope at N-terminus
HsSLC35B4[b]	3×FLAG-myc-CMV-26 (Sigma)	Human SLC35B4 transporter (longer splice variant) with FLAG epitope at N-terminus
HsSLC35B4sh[b]	3×FLAG-myc-CMV-26 (Sigma)	Human SLC35B4 transporter (shorter splice variant) with FLAG epitope at N-terminus

[a]Plasmids used for cotransfection; [b]Plasmids used for single transfection

mined spectrophotometrically at 260 nm. The quality of purified RNA was examined using an Agilent 2100 Bioanalyzer equipped with an RNA Chip (Agilent Technologies). For the reverse transcription reaction, ThermoScript First-Strand Synthesis Kit and oligo $dT_{(20)}$ were used as recommended by the manufacturer (Invitrogen). Constructs containing FLAG epitope at the N-terminus were prepared by ligation of sequences encoding respective transporters (Table 1) into 3×FLAG-myc-CMV-26 (Sigma). For pVitro1-neo and pSelect plasmids (InvivoGen), an adaptor encoding 6His-HA epitope was ligated. The adaptor sequence was prepared from sense (5'-ACCATGGCACATCACCACCACCATCACGCATCTTACCCATACGACGTACCAGACTACGCA-3') and antisense (5'-TGCGTAGTCTGGTACGTCGTATGGGTAAGATGCGTGATGGTGGTGGTGATGTGCCATGGT-3') oligonucleotides, purified using denaturing polyacrylamide gel electrophoresis and annealed at an initial temperature of 80 °C with slow cooling down (1 °C per min) to 35 °C in 0.5 M NaCl. The double-stranded adaptor was phosphorylated with T4 kinase according to the manufacturer's recommendations (Fermentas). Original plasmids were digested with *Bam*HI (for adaptor ligation to pSelect) or *Kpn*2I (for adaptor ligation to pVitro, MCS1). Sticky ends of *Bam*HI or *Kpn*2I linear products were filled with Klenow polymerase according to the manufacturer's instructions (Fermentas). All linear plasmids were dephosphorylated using calf intestine alkaline phosphatase (Fermentas). Phosphorylated adaptors were ligated to linear pSelect or pVitro plasmids using Rapid DNA Ligation Kit (Fermentas). Resulting plasmids containing a 6His-HA epitope at the start of each polylinker were used for ligation of sequences encoding respective transporters. Sequences of primers for amplification of ligated ORFs are available upon request. All ligations were performed using Rapid DNA Ligation Kit. Additional sequences attached to the N-terminus of analyzed proteins contained the respective epitopes and amino-acid residues resulting from the addition of sequences for respective restriction enzymes (3×FLAG: MDYKDHDGDYKDHDIDYKDDDDKL, 6His-HA: MAHHHHHHASYPYDVPDYAPEYTDPN). All plasmids constructed in this study are listed in Table 1.

Cell maintenance and transfection. MDCK wild-type and MDCK-RCA^r mutant cells were grown in minimum essential medium (MEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich) in a humidified incubator (37 °C, 5% CO_2). Cells were transfected with expression plasmids (Table 1) using ESCORT™ IV Transfection Reagent according to the manufacturer's instructions (Sigma-Aldrich). Stable transfectants were selected in complete media containing 200 μg/ml Zeocin and/or 600 μg/ml G-418 sulfate (InvivoGen).

Subcellular fractionation, Western blotting, and lectin reactivity. Subcellular fractionation was performed using discontinuous gradient as described by Balch *et al.* (1984). After final ultracentrifugation, 0.1-ml fractions were collected. Proteins present in respective fractions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) in 8% gels and transferred onto nitrocellulose membranes (Whatman). For the detection of HA epitope, membranes were blotted with a 1:1 000 dilution of mouse anti-HA antibody conjugated with horseradish peroxidase (HRP, Roche). For the detection of FLAG epitope, membranes were blotted with a 1:1 000 dilution of mouse anti-FLAG monoclonal antibody (Sigma-Aldrich), followed by incubation with a 1:10 000 dilution of HRP-conjugated goat anti-mouse antibody (Promega). For the detection of ER or Golgi markers, rabbit antibody specific for calnexin (Abcam) or GM130 (Sigma-Aldrich) at 1:2 000 or 1:5 000 dilution was used, respectively, followed by incubation with a 1:10 000 dilution of HRP-conjugated goat anti-rabbit antibody. Immunoreactive bands were visualized using the Western Lightning Chemiluminescence Reagent Plus system (Perkin Elmer).

For lectin reactivity, cells adapted to serum-free culture conditions were collected and lysed using Complete Lysis-M reagent (Roche) supplemented with protease inhibitor cocktail and 1 mM EDTA according to the manufacturer's instructions. Aliquots containing 20 μg of total protein were separated by SDS/PAGE and transferred onto nitrocellulose membranes. For the detection of glycans bound to glycoproteins, a lectin specific for terminal *N*-acetylglucosamine present in both N- and O-glycans (GSL II) was used. After blocking with Carbo-Free Blocking Solution (Vector Laboratories) membranes were incubated with biotinylated lectin (Vector Laboratories) in 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl (TBS), 0.2% Tween-20, 1 mM Mg^{2+}, 1 mM Ca^{2+} and 1 mM Mn^{2+}. Lectin bound to specific glycans was subsequently detected using alkaline phosphatase-conjugated avidin D (Vector Laboratories) and visualized with NBT/BCIP solution (Roche) according to the manufacturer's instructions.

Nucleotide sugars transport assay. Golgi fraction derived from MDCK, MDCK-RCA^r or MDCK-RCA^r cells overexpressing UDP-Gal transporter splice variants was isolated as described above. UDP-Gal transport into the Golgi vesicles was determined according to the

procedure described by Sturla *et al.* (2001) with modifications. Assays were performed using Golgi vesicles (200 μg of protein) suspended in 10 mM Tris/HCl buffer, pH 7.4, containing 0.14 M KCl, 1 mM $MgCl_2$, 0.25 M sucrose (STKM), supplemented with 0.5 mM 2,3-dimercaptopropanol. Reactions were carried out for 10 min at 30°C in 0.2-ml samples containing 20 μM cold UDP-Gal (Sigma-Aldrich) and 5 μCi tritium-labeled UDP-Gal (American Radionucletide Chemicals, 20 Ci/nmol). Then, reactions were stopped with 1 ml of ice-cold STKM buffer, placed immediately on ice, and centrifuged at $60\,000 \times g$ for 15 min at 4°C. Pellets were washed twice with STKM at the same conditions and dissolved in 0.6 ml of 1 M NaOH. Golgi lysates were neutralized with 0.2 ml of 4 M HCl and mixed with 20 ml of Rotiszint Eco Plus scintillation liquid (Roth). Beckman LS 6500 scintillation counter was used to measure radioactivity of UDP-Gal transported into Golgi vesicles.

Immunofluorescence microscopy. MDCK wild-type and MDCK-RCA^r mutant cells overexpressing respective transporters were grown for 24 h using Lab-Tek Chamber Slide™ System (Nalgene Nunc Int.). Cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 3 min, and non-specific binding sites were blocked with 10% normal goat serum for 30 min. After blocking, cells were incubated overnight with a 1:100 dilution of mouse monoclonal anti-HA antibody (Abcam), a 1:500 dilution of rabbit polyclonal anti-HA antibody (Abcam), or a 1:100 dilution of mouse monoclonal anti-FLAG antibody (Sigma-Aldrich) combined with a 1:100 dilution of rabbit monoclonal antibody against GM130 Golgi protein (Abcam) or a 1:250 dilution of rabbit polyclonal antibody against ER calnexin (Abcam). After washing with PBS, cells were incubated for 1 h with a 1:100 dilution of goat anti-rabbit Cy5-conjugated antibody and/or goat anti-mouse Cy2-conjugated antibody (Abcam). Cell nuclei were counterstained with Hoechst 33342 dye (Sigma-Aldrich). After washing with PBS, slides were mounted onto glass coverslips using Dako Fluorescence Mounting Medium (Dako) and examined using a ZEISS LSM 510 confocal microscope.

RESULTS AND DISCUSSION

Identification of the localization of individual NSTs may be helpful in clarifying their biological role in glycosylation of macromolecules. Therefore in this study, an experimental model of a Madin-Darby canine kidney (MDCK) wild-type and MDCK mutant cells resistant to *Ricinus communis* agglutinin (MDCK-RCA^r) were used to further characterize selected NSTs. The subcellular localization of the overexpressed epitope-tagged NSTs was determined by indirect immunofluorescence. All sequences of interest were labeled with a specific tag attached at the N-terminus (FLAG or HA fusions), since the N-terminus is not required for either ER export or Golgi localization of NSTs (Zhao *et al.*, 2006). To detect marker proteins specific for the ER or Golgi apparatus, antibodies directed against calnexin or GM130 were used, respectively. Unlike wild-type MDCK cells, MDCK-RCA^r mutant cells do not express functional UGT1 and UGT2 splice variants of the UDP-Gal transporter, but they express a short version of UGT (Olczak & Guillen, 2006; Maszczak-Seneczko *et al.*, 2011). Transport of UDP-Gal into the Golgi vesicles in the mutant cells is residual (about 2%) (Brandli *et al.*, 1988). The mutant cells are enriched in glucosyl ceramide and cell surface glycoconjugates possessing terminal *N*-acetylglucosamine and significantly lower amounts of sialic acid attached to glycoproteins and glycosphingolipids. In addition, they possess a still unidentified defect in the keratan sulfate biosynthesis pathway (Toma *et al.*, 1996) which is not corrected by complementation with the UDP-Gal transporter (Maszczak-Seneczko *et al.*, 2011). To assess the functional activity of overexpressed NSTs, we analyzed phenotypic correction using GSL II (*Griffonia simplicifolia* lectin II) and determined the transport of UDP-Gal to Golgi vesicles derived from wild-type MDCK, mutant MDCK-RCA^r and mutant cells overexpressing UDP-Gal transporter splice variants. This confirmed the functionality of this transporter (Fig. 1 and data not shown). Based on the results of the UDP-Gal transporter analysis, our preliminary experiments performed with UDP-GlcNAc transporter (unpublished), and the assumption that all the transporters examined in this study are highly homologous to one another, we assume that the UDP-GlcNAc and SLC35B4 transporters are also functional and properly localized.

UDP-GlcNAc transporter

NSTs transporting UDP-GlcNAc selectively (Abeijon *et al.*, 1996; Guillen *et al.*, 1998; Ishida *et al.*, 1999a) or multi-specific NSTs (Suda *et al.*, 2004; Cipollo *et al.*, 2004) have been identified. Recently, it has been reported that a point mutation of a UDP-GlcNAc transporter causes complex vertebral malformation (CVM) in animals (Thomsen *et al.*, 2006). However, a detailed analysis of this transporter is difficult since mammalian mutant cells defective in this activity have not been isolated. An earlier immunofluorescence microscopy analy-

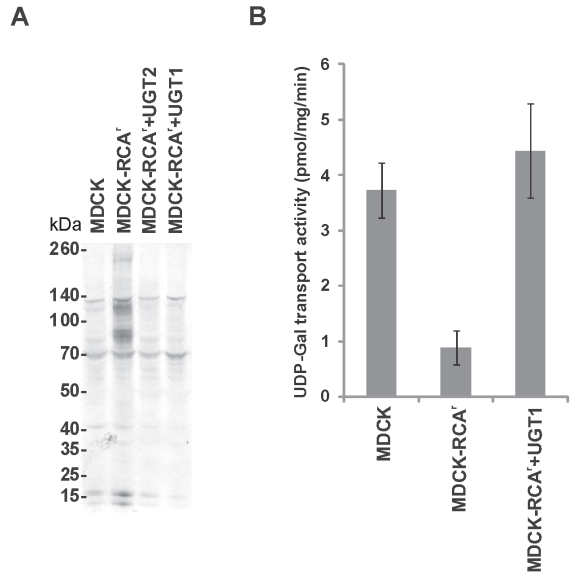

Figure 1. Functional activity of UDP-Gal transporter
(A) Lectin staining of glycoproteins from wild-type MDCK, mutant MDCK-RCA^r, and mutant cells overexpressing UDP-Gal transporter splice variants UGT1 or UGT2. Phenotypic correction was analyzed using GSL II (*Griffonia simplicifolia* lectin II), specific for terminal *N*-acetylglucosamine present in both N- and O-glycans. Results of one of three experiments with a similar pattern are shown. **(B)** UDP-Gal transport into Golgi vesicles derived from wild-type MDCK, mutant MDCK-RCA^r, and mutant cells overexpressing UDP-Gal transporter splice variant UGT1. Results are shown as mean ±standard deviation from three independent experiments performed in duplicate.

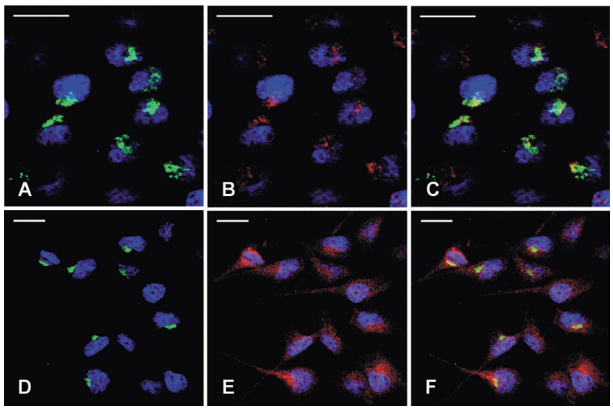

Figure 2. Subcellular localization of UDP-GlcNAc transporter in MDCK-RCA^r cells by indirect immunofluorescence
MDCK-RCA^r cells were stably transfected with expression constructs, cultured and treated with antibodies as described in Materials and Methods. **(A** and **D)** Reactivity with HA-specific antibodies (green, Cy2), (**B**) reactivity with Golgi marker (GM130) antibodies (red, Cy5), **(E)** reactivity with ER marker (calnexin) antibodies (red, Cy5), **(C)** overlay of A and B, **(F)** overlay of D and E. Cell nuclei were counterstained with Hoechst 33342 dye. Bar, 20 μm.

sis of human UDP-GlcNAc transporter overexpressed in CHO cells demonstrated its localization in the Golgi membrane (Ishida *et al*., 1999a). In this study we found that canine UDP-GlcNAc transporter colocalizes with a Golgi marker in both MDCK wild-type (not shown) and MDCK-RCA^r mutant cells (Fig. 2). Based on these results it seems that the transporter is localized exclusively to the Golgi apparatus and this localization is not affected by the presence of endogenous UDP-Gal transporter.

UDP-Gal transporter

One of the best characterized NSTs is the UDP-Gal transporter (Ishida *et al*., 1996; Miura *et al*., 1996; Yoshioka *et al*., 1997; Sun-Wada *et al*., 1998; Berninsone *et al*., 2001; Oelmann *et al*., 2001; Olczak & Guillen, 2006). A detailed characterization of this protein was possible after mutant cell lines, such as MDCK-RCA^r (Brandli *et al*., 1988; Toma *et al*., 1996), CHO Lec-8 (Oelmann *et al*., 2001; Stanley, 1983; 1989), and Had-1 (Ishida *et al*., 1999b), had been generated. Previously, two splice variants of UDP-Gal transporter (UGT1 and UGT2) have been identified in humans (Miura *et al*., 1996; Ishida *et al*., 1996; Muraoka *et al*., 2001), but only single orthologs have been found in the CHO cell line (Oelmann *et al*., 2001), the MDCK cell line (Olczak & Guillen, 2006), and in mouse (Hara *et al*., 1989). The mouse and MDCK UDP-Gal transporter resembles human UGT1, while the CHO gene encodes a protein most homologous to human UGT2. Recently, we observed the presence of a UGT1-like isoform in the CHO cell line and a UGT2-like isoform in the MDCK cell line (Maszczak-Seneczko *et al*., 2011).

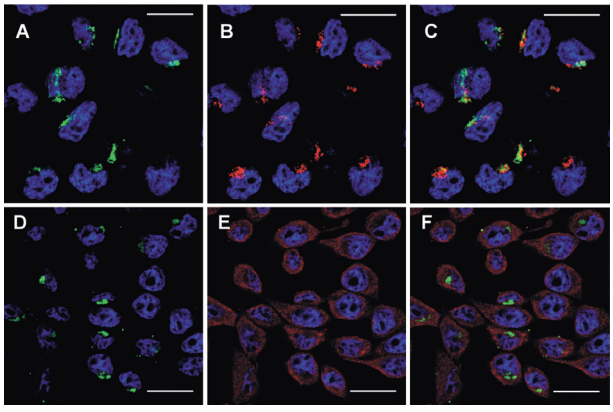

Figure 3. Subcellular localization of UGT1 splice variant of UDP-Gal transporter in MDCK-RCA^r cells by indirect immunofluorescence
MDCK-RCA^r cells were stably transfected with expression constructs, cultured and treated with antibodies as described in Materials and Methods. **(A** and **D)** Reactivity with HA-specific antibodies (green, Cy2), **(B)** reactivity with Golgi marker (GM130) antibodies (red, Cy5), **(E)** reactivity with ER marker (calnexin) antibodies (red, Cy5), **(C)** overlay of A and B, **(F)** overlay of D and E. Cell nuclei were counterstained with Hoechst 33342 dye. Bar, 20 μm.

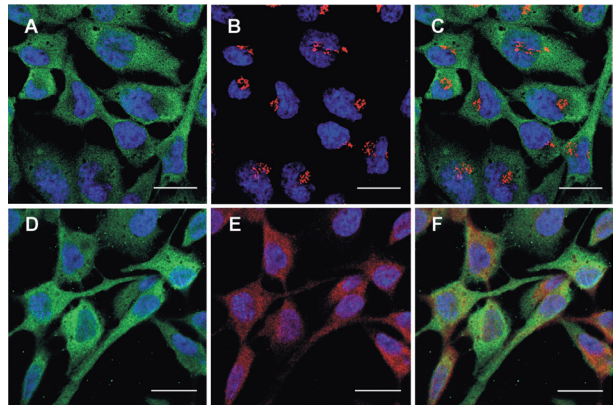

Figure 4. Subcellular localization of UGT2 splice variant of UDP-Gal transporter in MDCK-RCA^r cells by indirect immunofluorescence
MDCK-RCA^r cells were stably transfected with expression constructs, cultured and treated with antibodies as described in Materials and Methods. **(A** and **D)** Reactivity with FLAG-specific antibodies (green, Cy2), **(B)** reactivity with Golgi marker (GM130) antibodies (red, Cy5), **(E)** reactivity with ER marker (calnexin) antibodies (red, Cy5), **(C)** overlay of A and B, **(F)** overlay of D and E. Cell nuclei were counterstained with Hoechst 33342 dye. Bar, 20 μm.

It has been demonstrated that translation of the longer mRNA results in the synthesis of UGT1 possessing the C-terminal sequence SVLVK, whereas translation of the shorter mRNA generates UGT2 possessing the LLTKVKGS sequence. Kabuss *et al.* (2005) reported that the dilysine motif functions as an ER retention signal and directs human and hamster UGT2 also to the ER, resulting in dual localization to both the ER and Golgi apparatus. For murine CMP-Sia transporter only, it has been shown that a C-terminal IIGV motif is responsible for its Golgi localization (Zhao *et al*., 2006). However, it

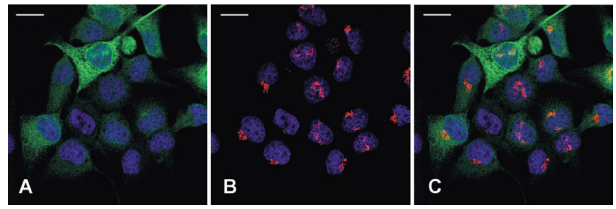

Figure 5. Subcellular localization of UGT2 splice variant of UDP-Gal transporter in MDCK-RCA^r cells overexpressing also UGT1 splice variant of UDP-Gal transporter by indirect immunofluorescence
MDCK-RCA^r cells were stably transfected with expression constructs, cultured and treated with antibodies as described in Materials and Methods. **(A)** Reactivity with FLAG-specific antibodies (green, Cy2), **(B)** reactivity with Golgi marker (GM130) antibodies (red, Cy5), **(C)** overlay of A and B. Cell nuclei were counterstained with Hoechst 33342 dye. Bar, 20 μm.

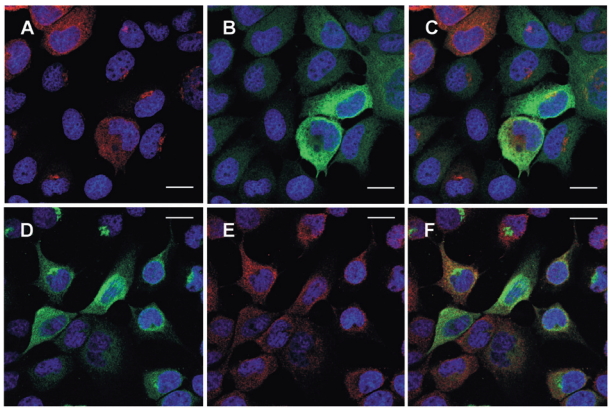

Figure 6. Subcellular localization of UGT1 splice variant of UDP-Gal transporter in MDCK-RCA^r cells overexpressing also UGT2 splice variant of UDP-Gal transporter by indirect immunofluorescence
MDCK-RCA^r cells were stably transfected with expression constructs, cultured and treated with antibodies as described in Materials and Methods. **(A** and **D)** Reactivity with HA-specific antibodies (A – red, Cy5; D – green, Cy2), **(B)** reactivity with FLAG-specific antibodies (green, Cy2), **(E)** reactivity with ER marker (calnexin) antibodies (red, Cy5), **(C)** overlay of A and B, **(F)** overlay of D and E. Cell nuclei were counterstained with Hoechst 33342 dye. Bar, 20 µm.

has been suggested that not all C-terminal KKXX and KXKXX sequences efficiently retain proteins in the ER (Itin *et al.*, 1995; Andersson *et al.*, 1999; Zerangue *et al.*, 2001). The evidence that also CMP-Sia transporter carrying the KVKGS motif localized to the ER and Golgi (Kabuss *et al.*, 2005) leaves open the question of whether this is a characteristic feature of the nucleotide sugar transporter or the dilysine motif. In this study, we found that after overexpression of UGT1 in MDCK-RCA^r mutant cells, the protein colocalized with a Golgi marker only (Fig. 3), which is in agreement with published data (Yoshioka *et al.*, 1997; Kabuss *et al.*, 2005). When UGT2 was overexpressed, the protein colocalized with an ER marker only (Fig. 4). In contrast to published data (Yoshioka *et al.*, 1997; Kabuss *et al.*, 2005), when UGT1 and UGT2 were overexpressed in parallel, UGT1 colocalized with the ER and Golgi markers and UGT2 with the ER marker only (Fig. 5 and 6). This is surprising, because UGT2 may correct, at least in part, the N-glycosylation defect in CHO-Lec8 and MDCK-RCA^r mutant cells (Oelmann *et al.* 2001; Maszczak-Seneczko *et al.*, 2011). The process of N-glycan galactosylation occurs in the Golgi apparatus and one may suspect that UGT2 may be transferred at very low, not easily detectable levels into this organelle in a complex with other proteins, e.g., galactosyltransferases.

It has been previously shown that, although UGT1 localized exclusively to the Golgi apparatus (Yoshioka *et al.*, 1997; Kabuss *et al.*, 2005), it could be detected in the ER when coexpressed with ceramide-galactosyltransferase 1 (cer-GalT 1) (Sprong *et al.*, 1998) which harbors a C-terminal dilysine motif and localizes to the ER. Sprong *et al.* (1998) reported that the ER-resident cer-GalT 1 is inactive if expressed in CHO-Lec8 cells which exhibit a genetic defect in the UDP-Gal transporter. However, the lack of functionality could be complemented by cotransfecting the cells with UDP-Gal transporter cDNA (Sprong *et al.*, 1998). It has been suggested that cer-GalT 1 is able to make physical contact with UGT and thus can retain it in the ER (Sprong *et al.*, 2003). Based on our results it is likely that also another protein partner, such as a splice variant of the same transporter, may be responsible for ER localization of UGT1.

SLC35B4 transporter

Human NSTs exhibiting dual activity and transporting UDP-GlcNAc and a second nucleotide sugar have been characterized by several laboratories. It has been demonstrated that vesicles from yeast cells expressing the human *SLC35B4* gene showed specific uptake of UDP-GlcNAc and UDP-xylose (UDP-Xyl) (Ashikov *et al.*, 2005), whereas the yeast homologue identified by Roy *et al.* (2000) transported UDP-GlcNAc alone. In the case of the human *SLC35B4* gene, two splice variants, a longer version (encoding a protein of 331 amino acids) (Ashikov *et al.*, 2005; Kobayashi *et al.*, 2006) and a shorter version (encoding a protein of 231 amino acids) (Kobayashi *et al.*, 2006), have been reported. Microsomes from V79 cells (Chinese hamster lung fibroblasts) over-

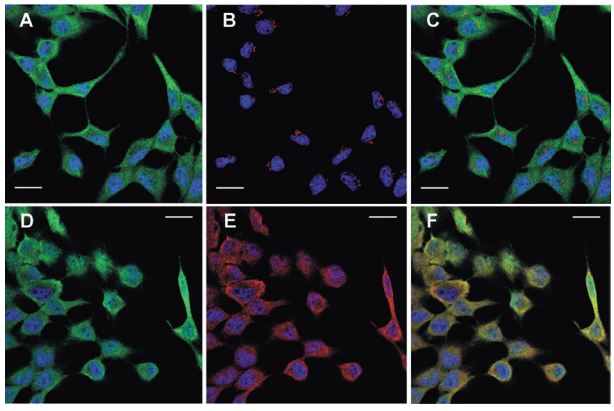

Figure 7. Subcellular localization of longer splice variant of SLC35B4 transporter in MDCK-RCA^r cells by indirect immunofluorescence
MDCK-RCA^r cells were stably transfected with expression constructs, cultured and treated with antibodies as described in Materials and Methods. **(A** and **D)** Reactivity with FLAG-specific antibodies (green, Cy2), **(B)** reactivity with Golgi marker (GM130) antibodies (red, Cy5), **(E)** reactivity with ER marker (calnexin) antibodies (red, Cy5), **(C)** overlay of A and B, **(F)** overlay of D and E. Cell nuclei were counterstained with Hoechst 33342 dye. Bar, 20 µm.

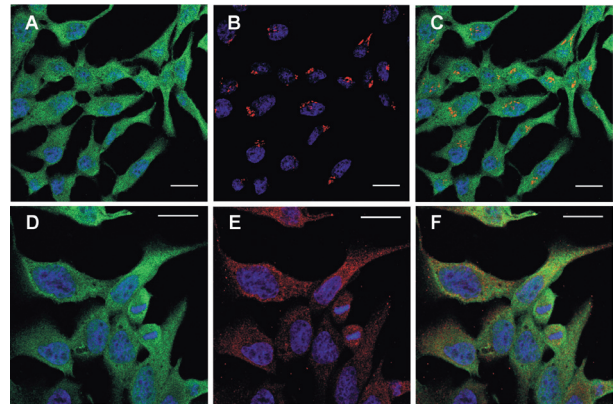

Figure 8. Subcellular localization of shorter splice variant of SLC35B4 transporter in MDCK-RCA^r cells by indirect immunofluorescence
MDCK-RCA^r cells were stably transfected with expression constructs, cultured and treated with antibodies as described in Materials and Methods. **(A** and **D)** Reactivity with FLAG-specific antibodies (green, Cy2), **(B)** reactivity with Golgi marker (GM130) antibodies (red, Cy5), **(E)** reactivity with ER marker (calnexin) antibodies (red, Cy5), **(C)** overlay of A and B, **(F)** overlay of D and E. Cell nuclei were counterstained with Hoechst 33342 dye. Bar, 20 µm.

expressing both splice variants of the SLC35B4 transporter showed specific uptake of UDP-glucuronic acid (UDP-GlcA) only after preloading microsomes with UDP-GlcNAc (Kobayashi *et al*., 2006). Both the yeast UDP-GlcNAc transporter and the human longer version of the UDP-GlcNAc/UDP-GlcA transporter have a C-terminal dilysine motif, potentially responsible for ER retention. However, the human transporter (longer splice variant) has been shown to localize in CHO cells to the Golgi apparatus (Ashikov *et al*., 2005). In contrast, homologous proteins from yeast and *Drosophila* have been found in the ER (Roy *et al*., 2000; Ishikawa *et al*., 2010). The localization of the shorter splice variant has not been examined. Our studies demonstrated that both stably overexpressed SLC35B4 splice variants colocalized in MDCK wild-type (not shown) and MDCK-RCA^r mutant (Fig. 7 and 8) cells with the ER marker only, demonstrating different localization compared with the data obtained by Ashikov *et al.* (2005). Previously published data showing that SLC35B4 is localized in the Golgi apparatus suggested its involvement in delivering UDP-Xyl and UDP-GlcA for, e.g., proteoglycan synthesis. Our results demonstrating localization of SLC35B4 in the ER argue against this hypothesis since the glycosaminoglycan moieties of proteoglycans are not synthesized in this organelle.

CONCLUSIONS

Elucidation of the differences in localization of NSTs may contribute to the understanding of the role of these transporters in cellular glycosylation. Our studies confirmed that the UDP-GlcNAc transporter is localized in the Golgi apparatus and its localization is independent of the presence of endogenous UDP-Gal transporter. In the case of the UDP-Gal transporter, its localization may depend on the presence of the partner splice variant (this study) or glycosyltransferase (Sprong *et al*., 1998; 2003). This suggests that proteins involved in these interactions could form heterodimeric complexes in the membrane, exhibiting different localization compared with single splice variants. Both splice variants of the SLC35B4 transporter, when overexpressed singly, are found in the ER. Based on literature data (Sprong *et al*., 1998; 2003) and the results of this study, we cannot exclude the possibility that localization of NSTs may depend not only on a C-terminal ER/Golgi retention signal, but also on the presence of a partner protein such as glycosyltransferase, other NST/NSTs or a splicing variant of the same transporter. In conclusion, our data suggest that localization of NSTs may be caused by complex mechanisms.

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