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Characterization of a mutation and an alternative splicing of UDP-galactose transporter in MDCK-RCA^r cell line

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Abstract

The UDP-galactose (UDP-Gal) transporter present in the Golgi apparatus is a member of a transporter family comprising hydrophobic proteins with multiple transmembrane domains. Co-immunoprecipitation experiments showed that the full-length UDP-Gal transporter protein forms oligomeric structures in the MDCK cell. A ricin-resistant mutant of the MDCK cell line (MDCK-RCA^r) is deficient in galactose linked to macromolecules because of a lower UDP-Gal transport rate into the Golgi apparatus. We cloned this mutated protein and found that it contains a stop codon close to the 5′ terminus of its open reading frame. We also detected a shorter splicing variant of the UDP-Gal transporter which contains a 183-nt in-frame deletion in both the wild-type and the mutant mRNA. We showed that the protein, when overexpressed, is localized in the Golgi apparatus and could partially correct the phenotype of the MDCK-RCA^r and CHO-Lec8 mutant cell lines. The level of mRNA of the UDP-Gal transporter is much lower (25–30 copies per cell) than those of the CMP-sialic acid transporter (100 copies per cell), UDP-N-acetylglucosamine transporter (80 copies per cell), and GDP-fucose transporter (65 copies per cell). The transcript level of the shorter splicing variant of the UDP-Gal transporter is extremely rare in wild-type MDCK cells (a few copies per cell), but it is significantly increased in the mutant, RCA-resistant cells.

Keywords: UDP-galactose transporter; MDCK; Golgi apparatus; Membrane protein

1. Introduction

Nucleotide-sugar transporters (NSTs) are proteins which provide essential components of the glycosylation pathways in eukaryotic cells (for reviews, see [1–4]). They function to translocate activated sugars from the cytosol into the lumen of the endoplasmic reticulum and the Golgi apparatus. The best-characterized proteins include the mammalian transporters for UDP-galactose (UDP-Gal) [5,6], CMP-sialic acid (CMP-SA) [7,8], UDP-N-acetylglucosamine (UDP-GlcNAc) [9], the yeast transporters for UDP-GlcNAc [10] and UDP-Gal [11,12], and the *Leishmania* GDP-mannose (GDP-Man) transporter [13,14]. In recent years, the genes encoding nucleotide-sugar transporters have been cloned from several species [15–23]. These genes encode structurally related hydrophobic membrane proteins. Hydropathy analysis predicted eight or nine transmembrane domains, but Eckhardt et al. [24] determined by an epitope-insertion analysis that the CMP-SA transporter has ten transmembrane domains with both the N- and C-termini facing the cytoplasm. Several reports suggest that NSTs function as oligomers. The formation of homodimers has been reported for the rat Golgi UDP-GlcNAc and GDP-Fuc transporters [25,26] and the yeast Golgi GDP-Man transporter [27,28]. There is also evidence of a hexameric complex in the *Leishmania* GDP-Man transporter [29]. Because the PAPS transporter appears to be a homodimer *in situ* [30], it is possible that the leucine-zipper motif present in this protein participates in its oligomerization. The *Kluyveromyces lactis* UDP-GlcNAc [20] and murine CMP-sialic acid [7] transporters also contain leucine-zipper motifs, suggesting a possibility of oligomeric structure formation. The homodimer structure may permit the formation of translocation membrane channels, although its presence has not been demonstrated in all NSTs.

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NSTs show considerable similarity with each other but have distinct substrate specificities. They were thought to be specific to one substrate; however, in humans, *Caenorhabditis elegans*, and *Drosophila* both monospecific and multispecific transporters have been cloned and characterized [17,18,31,32]. Although some multisubstrate nucleotide-sugar transporters can be considered orthologs, they differ in the kind and number of UDP-activated sugars that are transported. Most of the information concerning nucleotide-sugar transporters structure–function comes from data involving chimeras of human UDP-Gal and CMP-SA transporters and shows that different transmembrane domains are involved in substrate recognition [33,34]. It has been shown that NSTs involved in the transport of nucleotide-sugars from the cytosol into the Golgi apparatus or endoplasmic reticulum lumen act as antiporters [35,36], exchanging the nucleotide-sugar with the corresponding nucleoside monophosphate, which is a product of the glycosylation reaction.

Several Golgi NSTs have been cloned by phenotypic correction of the corresponding transport mutants and by in vitro characterization of the transport activity using microsomal fractions [5–7,11,13,20]. Yeast, *Leishmania*, and mammalian cell line mutants impaired in the transport of specific nucleotide-sugars into the Golgi lumen have severe deficiencies of the corresponding sugar in their macromolecules [1], demonstrating the essential role of the transport process in glycosylation [1,10,37–39]. In addition, fibroblasts from a patient with a clinical phenotype resembling that of leukocyte adhesion deficiency II are defective in GDP-fucose transport into the Golgi [40]. These mutants were isolated based on their resistance to plant lectins.

The Madin–Darby canine kidney (MDCK) mutant cell line (MDCK-RCA^r) was isolated based on its resistance to *Ricinus communis* agglutinin (RCA). The cells tolerate a 10 times higher concentration of the lectin than do the wild-type cells [41]. Ricin toxicity requires binding to terminal galactosyl residues, and the ricin-resistant phenotype of MDCK-RCA^r cells correlates with a pleiotropic deficiency in galactosylation of glycoproteins and glycosphingolipids [37]. The MDCK-RCA^r [37] and the Chinese hamster ovary (CHO) CHO-Lec8 [42–44] mutant cell lines have a 70–90% deficiency of galactose in their glycoproteins, glycosphingolipids, and selective proteoglycans. Glycoproteins and glycosphingolipids are also deficient in sialic acid because this sugar is most often attached to galactose. The altered phenotype of these cells was determined to have a 95–98% deficiency in the rate of transport of UDP-Gal into the Golgi vesicles, while the transport of other nucleotide-sugars, including uridine derivatives and CMP-SA, was similar to that of the wild-type cells [37,45]. The levels of activity of galactosyltransferases and sialyltransferases were the same in the mutant and the wild-type cells. The defect resulted in the enrichment of cell surface glycoconjugates bearing terminal N-acetylglucosamine and of glucosylceramides, both endogenous acceptors for galactose, while polymers with galactose in the linkage region between the protein and the polymer, such as chondroitin and heparan sulfate, were unaltered [38]. Keratan sulfate, which has galactose in its polymer, was almost absent in the mutant cells.

In an attempt to further characterize the UDP-Gal transporter, we identified the mutation present in this gene in the MDCK-RCA^r mutant cell line. We found that both the wild-type and the mutant cells, in addition to full-length UDP-Gal transporter mRNA, contain a shorter splicing variant of this mRNA. We determined the level of mRNA encoding both splicing variants of the UDP-Gal transporter as well as four other nucleotide-sugar transporters in MDCK cells. We also confirmed that the UDP-Gal transporter, similar to other NSTs, forms oligomeric structures in the Golgi apparatus.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. MEM and alpha-MEM media, fetal bovine serum (FBS), media-grade streptomycin and penicillin, PCR Platinum Supermix, Thermoscript cDNA Synthesis Kit, and G418 were from Gibco BRL (Gaithersburg, MD) and molecular biology grade agarose and CAPS were from ICN (Costa Mesa, CA). Superfect transfection reagent, DNase, DNeasy, RNeasy, and Qiagex nucleic acid isolation and purification kits were from Qiagen (Valencia, CA), NothernMax Kit and RT-PCR Competitor Construction Kit were from Ambion (Austin, TX), and all organic solvents were from Fisher (Pittsburgh, PA). Nitrocelulose membranes, prestained protein standards, acrylamide/bis-acrylamide solutions, ammonium persulfate, TEMED, non-fat milk, and Tween 20 were purchased from BioRad (Hercules, CA), anti-mouse monoclonal antibodies against HA, c-*myc* and FLAG tags from Babco (Richmond, CA), anti-mouse, and HRP-labeled secondary antibodies were from Promega (Madison, WI), Renaissance Western blot Reagent Plus from Perkin Elmer, pcDNA TOPO and pCR2.1 vectors, and Hygromycin B were from Invitrogen (Carlsbad, CA), and the Complete protease inhibitor set, MAA lectin, NBT phosphatase substrate, and Fast Ligation Kit from Roche (Indianapolis, IN). Protein Assay Reagent was purchased were from Pierce (Rockford, IL).

2.2. Mammalian cell lines

MDCK wild-type cells, CHO wild-type cells and CHO-Lec8 mutant cells were from ATCC (Manassas, VA), and the *Riccinus communis* agglutinin-resistant strain of MDCK-RCA^r cells was provided by Dr. Enrique Rodriguez-Boulan (Department of Cell Biology and Anatomy, Cornell University Medical College, New York).

MDCK cell lines were grown in 5% CO_2 at 37 °C in MEM medium, containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. The CHO cell lines were grown in 37 °C under 5% CO_2 in alpha-MEM medium containing 10% FBS and penicillin/streptomycin.

2.3. SDS-PAGE and immunoblotting analysis

SDS-PAGE was performed on 10% polyacrylamide gels [46]. After electrophoresis, the proteins were transferred at 400 mA for 90 min onto nitrocellulose membrane in 30 mM 3-cyclohexylamino-1-propane sulfonic acid (CAPS, pH 11) containing 10% methanol. The membranes were blocked in PBS containing 5% non-fat milk for 12 h at room temperature. The primary antibodies were diluted 1:1000 (anti-HA, anti-c-*myc*, anti-FLAG) or 1:5000 (anti-V5) in PBS, containing 1% non-fat milk and 0.5% Tween 20. Secondary HRP-conjugated antibodies were used as 1:10,000 diluted solution. The reaction was developed using Renaissance Western Blot Reagent Plus.

2.4. Detection of terminal α2–3-linked sialic acid in cell lysates

Cell extract proteins (20 μg) were separated on 10% polyacrylamide gels [46] and then transferred onto nitrocellulose membranes at 600 mA for 1 h in cold CAPS buffer. The membranes were blocked in 3% BSA in TBS and then incubated with digoxygenin-labeled MAA lectin (5 μg/ml) in TBS, washed

several times in TBS and then incubated with anti-digoxygenin antibodies conjugated with alkaline phosphatase (0.2 μg/ml) in TBS. Color reaction was developed using NBT phosphatase substrate.

2.5. RNA purification, PCR and RT-PCR analyses

The RNeasy Mini Kit and RNeasy Blood Mini Kit were used to purify total RNA from cell cultures of mammalian MDCK cells and dog blood (PelFreeze), respectively. To avoid nuclear splicing variants in the preparation and genomic DNA contamination in purified RNA, the cytosolic protocol was chosen and RNA was treated with DNase. The quality of RNA was checked on 1% formaldehyde agarose gel. The concentration of purified RNA was determined spectrophotometrically at 260 nm. Random hexamers or Oligo dT_{18} were used as primers for reverse transcriptase reaction. The cDNA synthesis was performed at 55 °C for 1 h according to the manufacturer's instructions using 2 μg of total RNA as a template.

2.6. Sequencing of canine CMP-SA and UDP-Gal transporters

The cDNA library was constructed as previously described [9]. The average insert was 2.0 kb (±0.6 kb). The library was searched with radioactive probes specific to the murine CMP-SA transporter (the probe encompassed positions 171–454 of the ORF) and human UDP-Gal transporter (the probe encompassed positions 608–941 of the ORF). The positive clones were collected and fragments of the library were sequenced. One of the clones from CMP-SA transporter screening contained the complete ORF of the canine transporter (EMBL accession number AY064407). Five positive clones containing UDP-Gal transporter sequences lacked the 5′ end of the ORF. Therefore, we employed the 5′RACE Kit (Gibco) to identify the full-length ORF of the transporter (EMBL accession number AY064406).

2.7. Construction of mammalian expression plasmids

The ORF of UDP-Gal transporter was amplified from cDNA synthesized as described above. The forward primer (MO1, see Table 1) contained a Kozak sequence at the 5′ end, and the reverse primer lacked the stop codon (MO2). The PCR product was cloned into a pcDNA3.1 TOPO vector encoding the V5 epitope and 6His tag at the C-terminus of the resulting recombinant protein. To obtain the protein with an HA tag at the C-terminus, the PCR product was amplified using the same forward primer and reverse primer containing the nucleotide sequence of the epitope sequence and stop codon at the 3′ end and then cloned into the pcDNA3.1 TOPO vector. To clone the transporter with c-*myc* and FLAG epitopes, the forward primer contained a *Hin*dIII restriction site at the 5′ end and the reverse primer contained an *Xba*I restriction site at the 5′ end. The PCR product was cloned into a pCDNA3.1 Hygro vector containing the *Hin*dIII and *Xba*I sites in a polylinker region. All plasmids used in this study are listed in Table 2.

2.8. Transfection of mammalian cells

To transfect CHO and MDCK cells, SuperFect lipid-mediated reagent (Qiagen) was used. The transfection was done in 6-well plates for 3 h. After transfection, the cells were incubated in medium without a selecting antibiotic for 48 h and then trypsinized, diluted 1:50 and transferred to new plates containing 500 μg/ml of G418 and/or 200 μg/ml of Hygromycin B. After several days of selection, twelve colonies were chosen and transferred to new plates. The lysates from one set of plates were analyzed for the presence of tagged proteins. To obtain double transfectants for immunoprecipitation experiments, the stable transfectants with HA-tagged UDP-Gal transporter and G418 resistance were transfected with the same type of construct (pCDNA3.1 plasmid) but containing a Hygromycin-resistance gene and c-*myc* or FLAG epitopes.

2.9. Ricin resistance of MDCK cells

The MDCK cells were trypsinized and transferred to 24-well plates (about 10^4 cells per well). Increasing amounts of ricin resuspended in PBS were added to each well. The cells were grown for one week at standard conditions, then washed once with PBS, fixed and stained for 30 min in 50% methanol, containing 1 mg/ml methylene blue.

2.10. Immunoprecipitation of UDP-Gal transporter overexpressed in CHO-Lec8 mutant cells

Confluent double-transfected CHO-Lec8 cells cultured in 100-mm plates were placed on ice, washed once with cold PBS, and then scraped in 1 ml of RIPA buffer (50 mM Tris/HCl, pH 8.0, 0.15 M NaCl, 1% Nonidet NP-40, 0.5% sodium deoxycholate, 0.1% SDS and Complete protease inhibitor cocktail). After 20 min of incubation on ice, the lysates were transferred to microcentrifuge tubes and centrifuged for 20 min at 20,000 × *g* at 2 °C. Supernatants were precleared with protein G-Sepharose beads (60 μl, 30% solution in RIPA buffer). After centrifugation, the supernatants were placed in new tubes and incubated with 5 μl of monoclonal anti-HA antibodies. The mixture was rotated for 2 h at 4 °C, followed by addition of 60 μl of Protein G-Sepharose beads. After 1 h of rotation, the beads were spun down for 2 min at 20,000 × *g* and the supernatants were discarded. The beads were washed 3 times with 1 ml of ice-cold RIPA buffer, resuspended in 50 μl of 2×Laemmli sample buffer and incubated at 60 °C for 15 min. Twenty-μl samples were loaded onto 10% SDS polyacrylamide gel.

2.11. Estimation of mRNA levels of various nucleotide-sugar transporters in the MDCK cell line

In order to determine the transcript level of four nucleotide-sugar transporters, competitive RNA templates were designed. First, DNA templates of about 300 bp in length but with a deletion of 10% compared with the original sequences were synthesized by PCR. The forward primer contained the T7 promoter sequence (Table 1). These templates were used to produce RNA in vitro. Because the sequence of GDP-Fuc transporter from canine cells (MDCK) was not known, before starting the experiment we performed PCR using primers designed for the conservative sites of the human GDP-Fuc transporter (EMBL accession number AF323970). The discrete product was sequenced (EMBL accession number AY064408). The fragment encompassing 119 amino acids of the putative canine GDP-Fuc transporter showed 94% identity compared with the human GDP-Fuc transporter (amino acid positions 63–182). Based on this sequence, primers for the GDP-Fuc competitive assay were designed.

The RNA synthesis reaction was done using a RT-PCR Competitor Construction Kit according to the manufacturer's instructions (Ambion). To detect the full-length RNA competitor product and to determine the amount of synthesized RNA, 0.4 μl of [α-^{32}P]-GTP [800 Ci/mM] was added to the reaction mixture. The competitive RNA was separated on 5% polyacrylamide/urea gel. After autoradiography, gel slices containing full-length products were cut out and RNA was eluted from the gel. The RNA was purified by precipitation in 70% ethanol and then resuspended in water. The competitor RNA was stored in siliconized microtubes at −70 °C.

The competitive reactions were performed according to the Ambion manual. Briefly, cDNA from known amounts of RNA competitors were synthesized. Different volumes from this reaction were mixed with cDNA obtained from a constant amount (1 μg) of total RNA from MDCK cells. The mixtures were used as a template in PCR reactions and the intensity of two products (the full-length original sequence and the shorter competitor sequence) were analyzed. To obtain bands of the same intensity, a second round of PCR reactions was performed. In the final experiment we used the original RNA competitors mixed with total RNA from MDCK cells. These mixtures were used as templates in RT-PCR reactions. The tubes containing equal amounts of amplified products from original and competitor RNA were chosen to calculate the transcript level in MDCK cells.

2.12. Northern blotting of UDP-Gal transporter

Total RNA was purified from MDCK cells using a Qiagen RNeasy Mini Kit and cytosolic RNA protocol according to the manufacturer's instructions. RNA was separated on 1% formaldehyde agarose gel and transferred onto nylon membranes. As a probe, the antisense RNA (183 bp) fragment was used. The probe was synthesized using the transcription reaction with the appropriate DNA

Table 1
Primers used in this study

Primer	Sequences of forward (F) and reverse (R) primers	Description
MO1	CACATGGCAGCGGTGGGGTCCGGC (F)	Amplify the ORF of UDP-Gal transporter
MO2	CTTCACCAGCACTGACTTCGGCAG (R)	
MO1	CACATGGCAGCGGTGGGGTCCGGC (F)	Amplify the ORF of UDP-Gal transporter with HA tag
MO3	TCAGAGTGATGCGTAGTCTGGTACGTCGTATGGGTACTTCACCAGCACTGACTTCGGCAG (R)	
MO4	GCGAAGCTTCACATGGCAGCGGTGGGGTCCGGC (F)	Amplify the ORF of UDP-Gal transporter with FLAG tag
MO5	GCTCTAGACGTCACTTATCGTCATCGTCCTTGTAGTCCTTCACCAGCACTGACTTCGGCAG (R)	
MO4	GCGAAGCTTCACATGGCAGCGGTGGGGTCCGGC (F)	Amplify the ORF of UDP-Gal transporter with c-*myc* tag
MO6	GCTCTAGACGTCAAAGATCCTCCTCGGAGATAAGCTTCTGTTCCTTCACCAGCACTGACTTCGGCAG (R)	
MO7	GCTCACCGGCGCCTCAAATAC (F)	Amplify the template for antisense RNA probe specifc to the full-length UDP-Gal transporter used in Northern Blotting
MO8	TAATACGACTCACTATAGGCCTCTTTTGTGCGAAGAGCAG (R)	
Fuc-Hum-F	CTGGTGGTCTCCCTCTACTGG (F)	Amplify the fragment of canine GDP-Fuc transporter
Fuc-Hum-R	CTGGTCCACACCAAGCCAGAA (R)	
T7-Short	GCGTAATACGACTCACTATAGGGAGAGGAGGGAACCGCTAGTGCGGGTAACCTGGTGCAGTATGTGGACAC (F)	Primers used in competitive experiments to estimate the transcript level of the short, splicing variant of UDP-Gal transporter in MDCK cells
P1-Short	GGAACCGCTAGTGCGGGTAAC (F)	
P2-Short	GACAGACACGAGGCCACGAC (R)	
P4-Short	AGGTTGCGGAGCCACACCGA (R)	
T7-Gal	GCGTAATACGACTCACTATAGGGAGAGGAGATGGCAGCGGTGGGGTCCGGGTCTCCGCGGGGTCGTTGGAG (F)	Primers used in competitive experiments to estimate the transcript level of the full-length UDP-Gal transporter in MDCK cells
P1-Gal	ATGGCAGCGGTGGGGTCCGG (F)	
P2-Gal	GTCCACATACTGCACCAGGAC (R)	
P4-Gal	CCTCTTTTGTGCGAAGAGCAGC (R)	
T7-Glc	GCGTAATACGACTCACTATAGGGAGAGGAGATGTCTACCAACCTAAAATACCCAGTTTGGTTCTAACAATGCG (F)	Primers used in competitive experiments to estimate the transcript level of UDP-GlcNAc transporter in MDCK cells
P1-Glc	ATGTCTACCAACCTAAAATACC (F)	
P2-Glc	CTGAAGAGTATATATCCCTGATG (R)	
P4-Glc	GATATGTAACCTGATACGTAG (R)	
T7-SA	GCGTAATACGACTCACTATAGGGAGAGGAGATGGCTGCCCCAAGAGAAAATGTCCTGGTGGCTGCAGCTTATACC (F)	Primers used in competitive experiments to estimate the transcript level of CMP-SA transporter in MDCK cells
P1-SA	ATGGCTGCCCCAAGAGAAAATGTC (F)	
P2-SA	GATGGTACACTTAACTTCATCAG (R)	
P4-SA	TACTGCTGCATCCAGATTGCTA (R)	
T7-Fuc	GCGTAATACGACTCACTATAGGGAGAGGAGAAGAGCCTCAGCACACTGGCCTTTCCCACCCTGCGCCTGGAC (F)	Primers used in competitive experiments to estimate the transcript level of GDP-Fuc transporter in MDCK cells
P1-Fuc	AAGAGCCTCAGCACACTGGC (F)	
P2-Fuc	CTGCTTGAGCAGCAGGTAGC (R)	
P4-Fuc	AGAAGCCACCAATGATGATGC (R)	

Table 2
Plasmids used in this study

Plasmid	Description	Source
pCR2.1 TOPO	*E. coli* plasmid used for sequencing of DNA inserts	Invitrogen
pcDNA3.1 TOPO	Mammalian expression plasmid containing G418-resistance gene	Invitrogen
pcDNA3.1 Hygro	Mammalian expression plasmid containing hygromycin-resistance gene	Invitrogen
pMAR1	pcDNA3.1 TOPO containing the ORF of UDP-Gal transporter with V5 and 6His epitopes	This study
pMAR2	pcDNA3.1 Hygro containing the ORF of UDP-Gal transporter with HA tag	This study
pMAR3	pcDNA3.1 TOPO containing the ORF of UDP-Gal transporter with FLAG tag	This study
pMAR4	pcDNA3.1 TOPO containing the ORF of UDP-Gal transporter with c-*myc* tag	This study
pMAR5	pcDNA3.1 Hygro containing the ORF of the short, splicing variant of UDP-Gal transporter with HA tag	This study

template containing the T7 promoter sequence and a fragment of the antisense sequence from the canine UDP-Gal transporter (Table 1). The probe was labeled with radioactive CTP. The procedure was performed according to Ambion's NorthernMax Kit manual.

2.13. Immunofluorescence microscopy

CHO-Lec8 cells stably transfected with tagged UDP-Gal transporter sequence or a splicing variant of the transporter, were grown to confluence on glass 8-chamber glass slides (Fisher). The cells were washed twice with PBS, fixed for 2 min in a methanol/acetone solution (1:1 v/v), washed again with PBS, and permeabilized for 15 min in PBS containing 1% Triton X-100. The fixed cells were incubated overnight with primary anti-HA monoclonal antibodies (1:200) and anti-GOS28 rabbit serum (1:200) in permeabilization buffer at 4 °C. After washing, the cells were stained in permeabilization buffer containing a mixture of secondary anti-mouse goat antibodies labeled with Texas Red marker (Molecular Probes, Eugene, OR) and anti-rabbit goat antibodies labeled with Oregon Green (Molecular Probes). The slides were secured in mounting media (BioRad) and analyzed using a Nikon fluorescence microscope.

2.14. Protein assay

The protein concentration in the cell lysates was determined by the bicinchoninic acid method (BCA) [47].

3. Results

3.1. Sequence of canine UDP-Gal transporter: identification of the mutation, evidence for a full-length transporter and its shorter splicing variant

The primary defect observed in MDCK-RCA^r cells is impaired transport of UDP-Gal into the Golgi apparatus. To understand the nature of this mutation in the UDP-Gal transporter, Northern blotting analysis using a highly specific antisense RNA probe was performed in the MDCK wild-type and mutant cell lines. The size of mRNA of the transporter in both the wild-type and the mutant cells was about 2450 nt in length (Fig. 1). Sequencing of cDNA from the wild-type and the mutant cells showed that the ORF of the wild-type transporter was 1203 bp long, similar in length to that of the human and murine transporters. The mutant cells contained full-length mRNA with a point mutation at position 136, which changes a CAG codon (Gln) to a TAG stop codon. Sequencing of the mutated region in the genomic DNA showed that only one of the two alleles has this stop codon. The cDNA from these cells contained only the mutated mRNA. PCR amplification of the UDP-Gal transporter ORF from the wild-type and the mutant cells showed an additional, shorter product (Fig. 1). Interestingly, although the product has a 183-bp deletion in the 5′region from position 91 to 273, the ORF was in frame (EMBL accession number AM110117). To confirm that both products are alternatively spliced we sequenced the 5′ end of the genomic DNA (Fig. 1D) (EMBL accession number AM087559). The mRNA of the shorter splicing variant does not contain exactly exon 2. Because the point mutation of the full-length ORF of the UDP-Gal transporter is localized in this region (position 136), the shorter version of the mRNA of the transporter has the same sequence in the wild-type MDCK and mutant MDCK RCA^r cells. In an important study, we found that the shorter splicing variant of the UDP-Gal transporter mRNA can also be detected in leukocytes of canine blood. The sequences of the shorter variant of the transporter isolated from the blood cells and from the MDCK cell line were identical (data not shown), confirming that this is not a specific feature only for MDCK cells. The hydrophobicity plot [48] of both transporters supported their highly hydrophobic properties and detected that in the shorter splicing variant the first two transmembrane domains were not present (data not shown).

3.2. mRNA levels of nucleotide-sugar transporters in the wild-type MDCK and MDCK RCA^r mutant cells

To gain information concerning the abundance of nucleotide-sugar transcripts in the cell, competitive RT-PCR was employed. Primers designed for unique regions were synthesized to produce DNA templates specific to UDP-GlcNAc, CMP-SA, GDP-Fuc, and UDP-Gal transporters as well as the shorter splicing variant of the GDP-Gal transporter. The incorporation of radioactivity was high (3–5%) and the concentration of the purified product was about 5×10^{11} copies per μl. The concentration of the competitors did not decrease when stored in siliconized tubes at −70 °C for several weeks. As shown in Fig. 2, the transcript levels of the CMP-SA, UDP-GlcNAc, and GDP-Fuc transporters were similar (70–100 copies per cell), but the transcript level of the UDP-Gal transporter seemed to be much lower (20–30 copies per cell). Although the transcript level of the shorter splicing variant of the UDP-Gal transporter was very low in the wild-type cell line (a few copies per cell), we observed a significant increase in this product in RCA^r mutant cells. We also detected a decrease in the full-length transporter mRNA (which is not functional as it has a

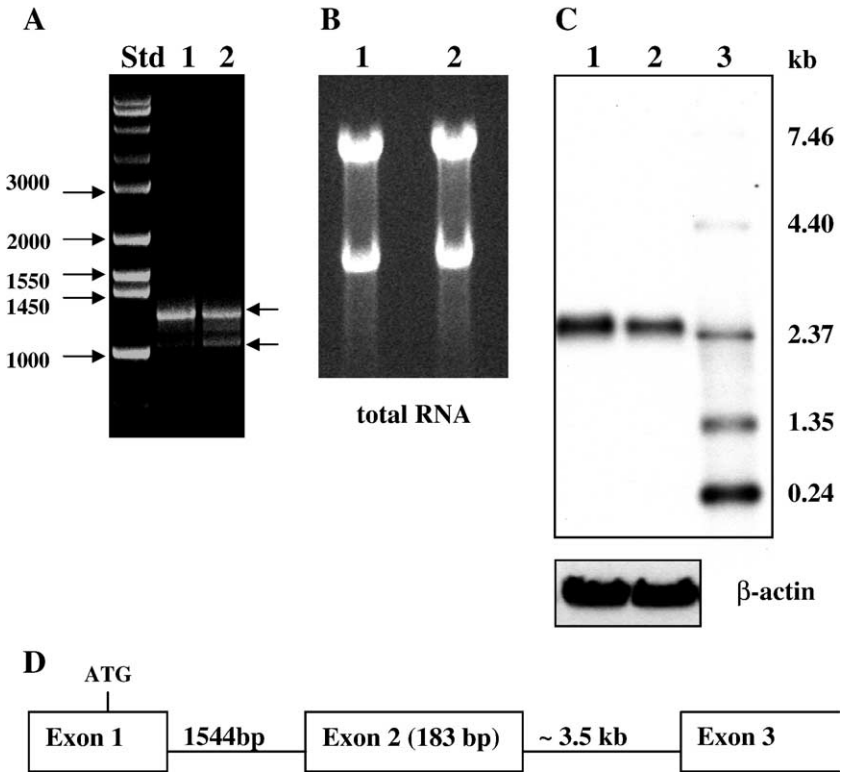

Fig. 1. Identification of UDP-Gal transporter in MDCK cells. (A) RT-PCR of UDP-Gal transporter from the wild-type MDCK and mutant MDCK-RCA^r cells. The arrows indicate two major products amplified. Std, molecular weight marker; lane 1, MDCK wild-type RNA; lane 2, MDCK-RCA^r RNA. (B) Agarose electrophoresis of total RNA isolated from the wild-type (lane 1) and RCA-resistant (lane 2) MDCK cells. Two μg of total RNA was loaded on each lane. (C) Northern blotting. The 183-nt ^{32}P-labeled antisense RNA probe specific to the full-length UDP-Gal transporter only. Lane 1, MDCK wild-type RNA; lane 2, MDCK-RCA^r RNA; lane 3, molecular weight marker. The 414-nt antisense RNA probe derived from a sequence of canine β-actin was used as a control. (D) Schematic representation of the 5′ end of genomic DNA encoding the UDP-Gal transporter in MDCK cells.

stop codon close to the 5′ end of the ORF) in the MDCK-RCA^r mutant cells, which confirmed the Northern blotting results.

3.3. The full-length of the UDP-Gal transporter and its shorter, splicing variant correct the mutant phenotype of MDCK-RCA^r and CHO-Lec8 cells

To demonstrate that the mutation detected in the UDP-Gal transporter mRNA was indeed responsible for the RCA^r phenotype, the full-length PCR product of the transporter was overexpressed in these mutant cells (Fig. 3). Phenotypic correction was measured by detecting terminal α2–3-linked sialic acid with MAA lectin, since glycoproteins with lower levels of galactosylation are supposed to be less sialylated. As expected, the wild-type UDP-Gal transporter corrected the phenotype of RCA^r cells (Fig. 3). In addition, we found that a low overexpression level caused more efficient recovery of the wild-type phenotype compared with clones expressing higher level of the recombinant protein. It is likely that higher expression levels might be toxic to MDCK cells.

The effect of overexpression of the shorter splicing variant of the UDP-Gal transporter was also examined using the lectin-resistance test (Table 3). MDCK RCA^r cells were grown to confluence in the presence of 2.7 ng/ml of ricin, which is about 30 times higher than that used for the wild-type cells. Mutant cells corrected by overexpression of the wild-type UDP-Gal transporter were sensitive to ricin in a manner similar to that of the wild-type cells. We also observed partial correction of the sensitivity to ricin when the shorter splicing variant of UDP-Gal transporter was overexpressed. The cells were resistant to up 0.4 ng/ml of ricin present in the medium. This suggested that higher amounts of this splicing variant could partially compensate the lack of a full-length transporter.

The CHO-Lec8 mutant cell line does not produce UDP-Gal transporter mRNA [49]. Therefore we used these cells to overexpress the canine UDP-Gal transporter. Fig. 4 shows overexpression of this transporter and its shorter splicing variant in CHO-Lec8 cells. Although the level of the shorter protein was lower compared with the full-length UDP-Gal transporter, both transcripts were stable in CHO cells. Fig. 4 shows the levels of terminal α2–3-linked sialic acid in CHO-Lec8 glycoproteins after overexpression of both splicing variants. The full-length canine transporter could correct the defect, but the shorter version of the transcript (the splicing variant) of the transporter from MDCK cells also caused a slight increase in sialylation.

3.4. Localization of canine UDP-Gal transporter

CHO-Lec8 mutant cells were also used to overexpress the canine full-length UDP-Gal transporter and its shorter

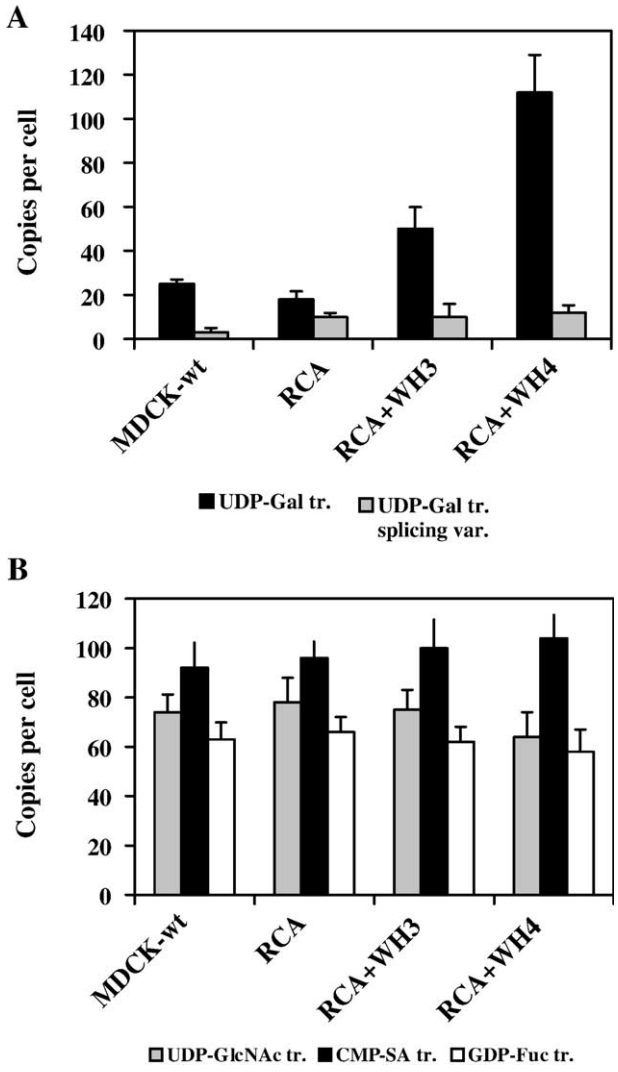

Fig. 2. mRNA levels of the Golgi transporters in MDCK cells. The final calculations were done based on RNA competitor experiments. Constant amounts of total RNA isolated from MDCK cells were mixed with known amounts of competitive RNA. The error bars indicate standard deviation (SD) from four independent experiments.

splicing variant and to detect the proteins using immunofluorescence microscopy. The MDCK cells were very sensitive to the expression of the tagged shorter splicing variant probably because of the high toxic effect of the protein produced at high concentration (the endogenous protein is also present in RCA^r mutant cells). Fig. 5 shows that both proteins are located in the cells with the GOS28 Golgi apparatus protein marker.

3.5. UDP-Gal transporter forms oligomers in the Golgi apparatus

To examine the putative oligomeric structure of the UDP-Gal transporter, we employed co-immunoprecipitation experiments performed in CHO-Lec8 mutant cells. The cells were stably transfected with pcDNA3.1 plasmid containing the ORF of the canine UDP-Gal transporter with the HA tag sequence at the C-terminus and the G418-resistance gene. After detection of the expression of the tagged protein, one clone was chosen and transfected with the same type of plasmid containing the same ORF but with FLAG or c-*myc* at the C-terminal region and the hygromycin-resistance gene. Twelve clones were chosen to determine the expression levels of the tagged transporters in the cells (Fig. 6). Fig. 7 shows Western blotting of lysates containing HA/c-*myc* and HA/FLAG-tagged proteins immunoprecipitated with anti-HA monoclonal antibodies and detected on the membrane with anti-c-*myc* antibodies. Our results showed that UDP-Gal transporter forms oligomeric structures in the cell. We were

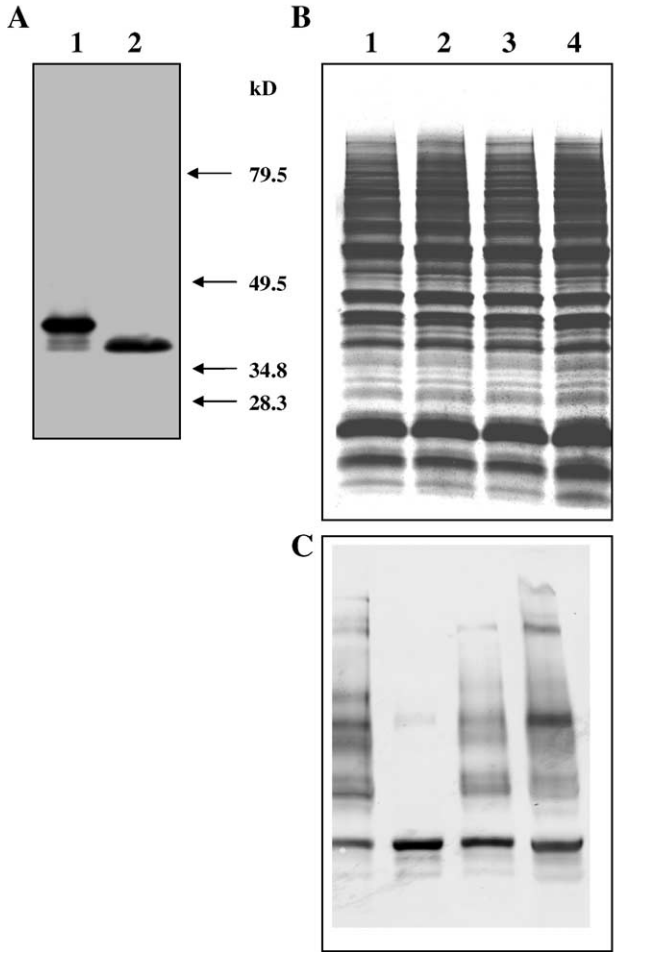

Fig. 3. Correction of the MDCK-RCA^r mutant phenotype. (A) Overexpression of canine UDP-Gal transporter and its shorter splicing variant in MDCK-RCA^r mutant cells. Proteins (30 μg) were separated on 10% SDS-PAGE and after transfer probed with anti-V5 antibodies and anti-mouse antibodies conjugated with horseradish peroxidase. Lane 1, MDCK-RCA^r cells overexpressing the full-length UDP-Gal transporter with the C-terminal V5/6His fusion peptide; lane 2, MDCK-RCA^r cells overexpressing the shorter splicing variant of the UDP-Gal transporter with the C-terminal V5/6His fusion peptide. (B) Silver staining of proteins (20 μg per lane) in cell lysates. (C) Western blotting of cell lysates after staining with MAA lectin, specific to terminal α2–3-linked sialic acid. Lane 1, wild-type MDCK cells; lane 2, MDCK-RCA^r cells; lane 3, MDCK-RCA^r cells overexpressing the full-length MDCK UDP-Gal transporter (clone WH3); lane 4, MDCK-RCA^r mutant cells overexpressing the full-length MDCK UDP-Gal transporter (clone WH4). Clone WH4 overexpressed more protein than clone WH3 (data not shown).

Table 3
Ricin-resistance of the wild type MDCK, mutant MDCK-RCA^r and MDCK-RCA^r mutant cells overexpressing two splicing variants of canine UDP-Gal transporter

MDCK cell line	Minimal lethal concentration of ricin in the medium (ng/ml)
Wild type	0.19
RCA^r with empty vector pDNA3.1	2.80
RCA^r with full-length splicing variant	0.10
RCA^r with short splicing variant	0.66

not able to prove this using anti-FLAG antibodies in Western blotting of HA-immunoprecipitated UDP-Gal transporter (data not shown). We showed that the protein has lower molecular weight when overexpressed with FLAG tag (about 35 kDa). In contrast, c-*myc*-tagged protein as well as V5–6His-tagged protein both showed the predicted molecular weight of 46 kDa. It is likely that the FLAG-tagged protein is truncated and may not form stable oligomers *in vivo*.

4. Discussion

To gain more information regarding various NSTs, first we identified the sequence encoding the entire UDP-Gal transporter from the MDCK cell line. The predicted protein is highly homologous to other UDP-Gal transporters present in the mouse [2,3] and hamster [49]. In these cells only one form of the transporter was found. In contrast, two UDP-Gal isoforms (UGT1 and UGT2), resulting from alternative splicing, were detected in humans [5,6]. The UDP-Gal transporter identified in MDCK cells is homologous to the human UGT1 splicing variant. We showed that it localized to the Golgi-like structures. Recently, other investigators reported on different locations of the two human UGT isoforms [49,50]. They found that the UGT1 form was localized in the Golgi apparatus, whereas the UGT2 form was present in both the Golgi apparatus and the endoplasmic reticulum. The dilysine motif in the C-terminal region of the transporter seems to be responsible for these differences [50]. In agreement with these studies, UDP-Gal from MDCK cells, which does not possess this motif, was also found in the Golgi apparatus. However, we cannot rule out the possibility that in these cells a UGT2-like variant is also present.

Further, we identified the entire sequence of the CMP-SA transporter and the fragment of the GDP-Fuc transporter and determined the transcript levels encoding four major Golgi NSTs. We found that the levels of mRNA of all the transporters analyzed in the MDCK cell are quite low. The UDP-Gal transporter mRNA level is much lower than the mRNAs encoding other transporters. RNA encoding the CMP-SA transporter is the most abundant, whereas the levels of UDP-GlcNAc and GDP-Fuc transporters transcripts are lower.

The MDCK-RCA^r [37] and the CHO-Lec8 [42–44] mutant cell lines belong to the same genetic complementation group. The altered phenotype of these cells is caused by an impaired rate of UDP-Gal transport into the Golgi vesicles. To date, molecular defects in several CHO-Lec8 isolates have been very well characterized [49]. Out of nine independent clones, six expressed truncated UDP-Gal transporter transcripts, whereas three mutants possessed the transporter with a single amino acid replacement. Although the RCA-resistant mutant of the MDCK cell line has been very well known to be deficient in galactose attached to macromolecules, the nature of this mutation has not yet been identified. In this study we characterized a mutation in the gene encoding canine UDP-Gal transporter protein. We detected this defect as a point mutation at position 136 of the ORF, which replaces the Gln codon with a stop codon. Although the mutation is present in one allele only (the second allele has a wild-type genotype), the MDCK-RCA^r mutant cell line produces the non-functional, mutated mRNA. Therefore it is not clear why cells lacking the UDP-Gal transporter are still

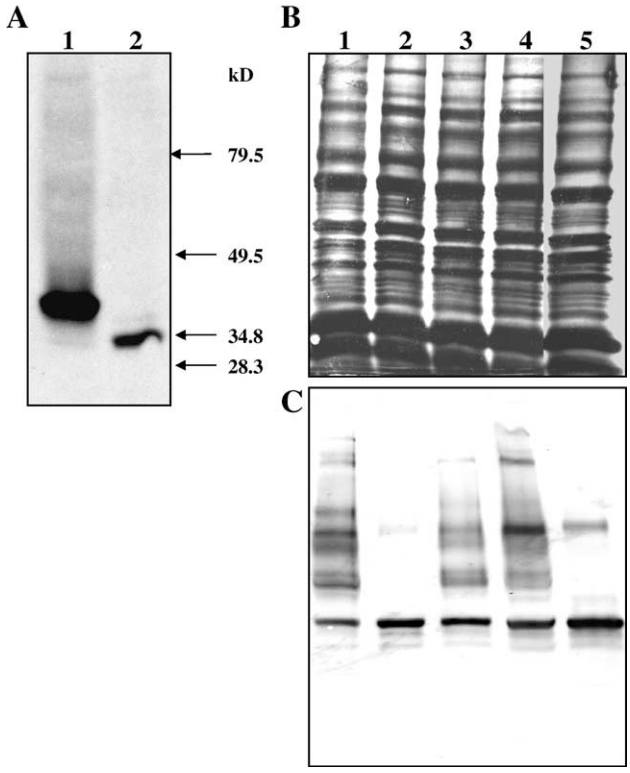

Fig. 4. Correction of the CHO-Lec8 mutant phenotype. (A) Overexpression of canine UDP-Gal transporter and its shorter splicing variant in CHO-Lec8 mutant cells. Proteins (60 μg) were separated on 10% SDS-PAGE and after transfer probed with anti-V5 antibodies and anti-mouse antibodies conjugated with horseradish peroxidase. Lane 1, CHO-Lec8 cells overexpressing the full-length UDP-Gal transporter with the C-terminal V5/6His fusion peptide; lane 2, CHO-Lec8 cells overexpressing the shorter splicing variant of the UDP-Gal transporter with the C-terminal V5/6His fusion peptide. (B) Silver staining of proteins (20 μg per lane) in cell lysates. (C) Western blotting of cell lysates after staining with MAA lectin, specific to terminal α2–3-linked sialic acid. Lane 1, wild-type CHO cells; lane 2, CHO-Lec8 cells; lane 3, CHO-Lec8 cells overexpressing the full-length MDCK UDP-Gal transporter (clone H1); lane 4, CHO-Lec8 mutant cells overexpressing the full-length MDCK UDP-Gal transporter (clone H3); lane 5, CHO-Lec8 mutant cells overexpressing the shorter splicing variant of the UDP-Gal transporter from MDCK cells. Clone H1 overexpressed more protein than clone H3 (data not shown).

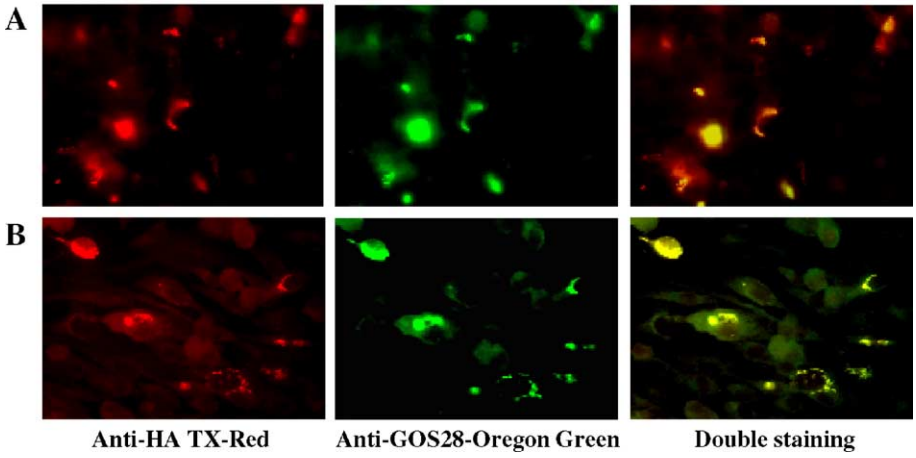

Fig. 5. Localization of the shorter splicing variant of the UDP-Gal transporter (A) and the full-length UDP-Gal transporter (B). CHO-Lec8 mutant cells were grown on chamber glass slides in standard conditions. Anti-HA monoclonal antibodies were used to detect transfectant proteins. Secondary anti-mouse antibodies were conjugated with Texas Red dye. Polyclonal antibodies raised against Golgi protein GOS28 were used as the Golgi apparatus marker. Secondary anti-rabbit antibodies were conjugated with Oregon Green dye. Both overexpressed products, UDP-Gal full-length transporter (panel B) and its shorter splicing variant (panel A), are localized in the Golgi-like structures.

able to produce some macromolecules containing galactose [37]. This effect may be explained, at least in part, by the presence of another transporter for UDP-Gal localized in the Golgi apparatus or transporters specific to other nucleotide-sugars, which can also transfer UDP-Gal at lower rates. Oelmann et al. [49] found a similar mutation in CHO-Lec8 cells caused by a deletion of 100 bp in the coding region of the UDP-Gal transporter, introducing a premature stop codon. This truncated protein (about 10 kDa) was stable but localized to the endoplasmic reticulum. Although we did not overexpress and did not examine the localization of the truncated (about 4.5 kDa) protein which could be produced by the MDCK-RCA^{r} mutant cells, we suspect that such a short protein would be not functional.

In this study, we also detected a second splicing variant of the UDP-Gal transporter in MDCK cells. The predicted

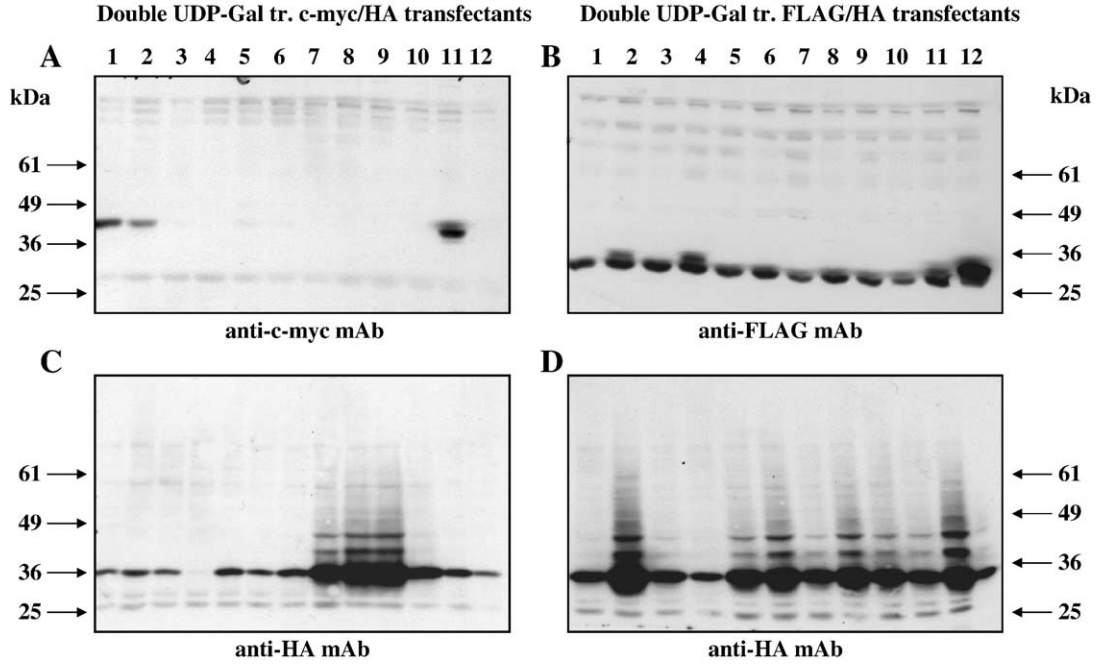

Fig. 6. Western blotting of 12 clones of CHO-Lec8 cells stably transfected with UDP-Gal transporter with HA tag and UDP-Gal transporter with c-*myc* tag (left side of the picture; panels A and C) and 12 clones of CHO-Lec8 cells stably transfected with UDP-Gal transporter with FLAG tag and UDP-Gal transporter with HA tag (right side of the picture; panels C and D). Clone #11 of the HA/c-*myc* double transfectants and clone #1 of the FLAG/HA double transfectants were chosen for the co-immunoprecipitation experiment. Panel A, staining with anti-c-*myc* antibodies; panel B, staining with anti-FLAG antibodies; panel C and D, staining with anti-HA antibodies.

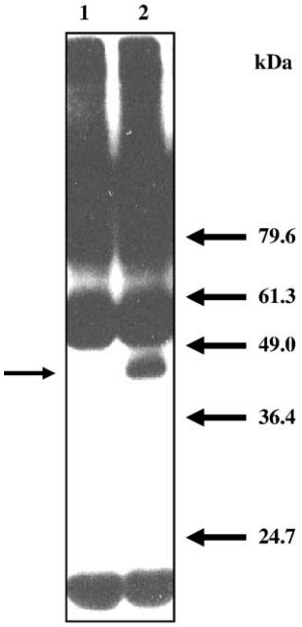

Fig. 7. Co-immunoprecipitation of canine UDP-Gal transporter. The lysates from CHO-Lec8 cells transfected with both c-*myc*-tagged and HA-tagged UDP-Gal transporter fusion proteins (clone #1, Fig. 6A and C) were immunoprecipitated with anti-HA antibodies and then bound to Protein G-Sepharose beads. Proteins were separated by SDS-PAGE and after transfer probed with anti-c-*myc* monoclonal antibodies. The HA/FLAG double transfectant lysate (clone #1, Fig. 6B and D), treated as described above, was used as a negative control. Lane 1, control sample; lane 2, immunoprecipitated sample.

protein produced from this mRNA is 61 amino acids shorter than the full-length transporter. Compared with the levels of the transcripts encoding other transporters examined in this study, mRNA encoding the shorter splicing variant version is extremely rare. The sequences of shorter splicing variant of the UDP-Gal transporter from wild-type MDCK and mutant RCA^r cells are identical, but their mRNA levels are higher in the mutant cells. This may be a kind of compensatory effect in the cells which probably do not produce the functional protein. The shorter splicing variant of the UDP-Gal transporter could partially correct the galactosylation defect in the mutant CHO-Lec8 cells. We also observed partial phenotype correction of MDCK-RCA^r cells after overexpression of this splicing variant. It might be possible that the presence of this splicing variant in RCA-resistant cells could explain the fact that some proteins and proteoglycans still contain galactose. We also showed that the shorter splicing variant of the UDP-Gal transporter detected in the MDCK cell line is also present in canine leukocytes, suggesting that its occurrence is not only limited to the MDCK cell line.

Several investigators demonstrated that NSTs function as dimers or higher order oligomers [25–29]. However, no report showing oligomers of UDP-Gal transporter have been published. Using co-immunoprecipitation experiments we confirmed that UDP-Gal transporter forms oligomeric structures. Compared with ultracentrifugation, a method used previously by other investigators [25–27,29], the detection of oligomers using co-immunoprecipitation suggests that protein monomers interact with high affinity. We also observed that overexpressed UDP-Gal transporter protein tagged with FLAG epitope migrates differentially in SDS-PAGE compared with the protein with V5–6His, HA or c-*myc* epitopes. This may suggest different susceptibility to proteolysis of these proteins, but also posttranslational modification of the transporter depending on the C-terminal fusion protein attached. Since the kind of the fusion protein attached to the recombinant protein may influence its functionality, it should be taken into consideration that various transporters with different tags should be examined.

In conclusion, in this study we reported on canine UDP-Gal transporter sequence identification and characterization of the mutation responsible for defective UDP-galactose transport in MDCK-RCA^r cells. In addition, the copy number per MDCK cell of UDP-Gal transporter in comparison with other NSTs was presented. We also identified the shorter splicing variant of the UDP-Gal transporter present in canine cells. Further studies will focus on detailed analysis of the glycoproteins produced in MDCK-RCA^r mutant cells as well as on the characterization of the putative proteins involved together with the UDP-Gal transporter in UDP-galactose transport into the Golgi apparatus.

References

[1] C.B. Hirschberg, P.W. Robbins, C. Abeijon, Transporters of nucleotide sugars, ATP, and nucleotide sulfate in the endoplasmic reticulum and Golgi apparatus, Annu. Rev. Biochem. 67 (1998) 49–69.
[2] M. Kawakita, N. Ishida, N. Miura, G.H. Sun-Wada, S. Yoshioka, Nucleotide sugar transporters: elucidation of their molecular identity and its implication for future studies, J. Biochem. (Tokyo) 123 (1998) 777–785.
[3] R. Gerardy-Schahn, S. Oelmann, H. Bakker, Nucleotide sugar transporters: biological and functional aspects, Biochimie 83 (2001) 775–782.
[4] I. Martinez-Duncker, R. Mollicone, P. Codogno, R. Oriol, The nucleotide-sugar transporter family: a phylogenetic approach, Biochimie 85 (2003) 245–260.
[5] N. Ishida, N. Miura, S. Yoshioka, M. Kawakita, Molecular cloning and characterization of a novel isoform of the human UDP-galactose transporter, and of related complementary DNAs belonging to the nucleotide-sugar transporter gene family, J. Biochem. (Tokyo) 120 (1996) 1074–1078.
[6] N. Miura, N. Ishida, M. Hoshino, M. Yamauchi, T. Hara, D. Ayusawa, M. Kawakita, Human UDP-galactose translocator: molecular cloning of a complementary DNA that complements the genetic defect of a mutant cell line deficient in UDP-galactose translocator, J. Biochem. (Tokyo) 120 (1996) 236–241.
[7] M. Eckhardt, M. Muhlenhoff, A. Bethe, Expression cloning of the Golgi CMP-sialic acid transporter, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7572–7576.
[8] M. Eckhardt, R. Gerardy-Schahn, Molecular cloning of the hamster CMP-sialic acid transporter, Eur. J. Biochem. 248 (1997) 187–192.
[9] E. Guillen, C. Abeijon, C.B. Hirschberg, Mammalian Golgi apparatus UDP-N-acetylglucosamine transporter: molecular cloning by phenotypic correction of a yeast mutant, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 7888–7892.
[10] C. Abeijon, E.C. Mandon, P.W. Robbins, C.B. Hirschberg, A mutant yeast deficient in Golgi transport of uridine diphosphate N-acetylglucosamine, J. Biol. Chem. 271 (1996) 8851–8854.

[11] M. Tabuchi, N. Tanaka, S. Iwahara, K. Takegawa, The *Schizosaccharomyces pombe* gms1+ gene encodes an UDP-galactose transporter homologue required for protein galactosylation, Biochem. Biophys. Res. Commun. 232 (1997) 121–125.
[12] H. Segawa, N. Ishida, K. Takegawa, M. Kawakita, *Schizosaccharomyces pombe* UDP-galactose transporter: identification of its functional form through cDNA cloning and expression in mammalian cells, FEBS Lett. 451 (1999) 295–298.
[13] A. Descoteaux, Y. Luo, S.J. Turco, S.M. Beverly, A specialized pathway affecting virulence glycoconjugates of *Leishmania*, Science 269 (1995) 1869–1872.
[14] D.Q. Ma, D.G. Russell, S.M. Beverly, S.J. Turco, Golgi GDP-mannose uptake requires *Leishmania* LPG2: a member of a eukaryotic family of putative nucleotide-sugar transporters, J. Biol. Chem. 272 (1997) 3799–3805.
[15] S.K. Roy, Y. Chiba, M. Takeuchi, Y. Jigami, Characterization of yeast Yea4p, a uridine diphosphate-N-acetylglucosamine transporter localized in the endoplasmic reticulum and required for chitin synthesis, J. Biol. Chem. 275 (2000) 13580–13587.
[16] T.C. Baldwin, M.G. Handford, M.I. Yuseff, A. Orellana, P. Dupree, Identification and characterization of GONST1, a Golgi-localized GDP-mannose transporter in *Arabidopsis*, Plant Cell 13 (2001) 2283–2295.
[17] M. Muraoka, M. Kawakita, N. Ishida, Molecular characterization of human UDP-glucuronic acid/UDP-N-acetylgalactosamine transporter, a novel nucleotide sugar transporter with dual substrate specificity, FEBS Lett. 495 (2001) 89–93.
[18] P. Berninsone, H.Y. Hwang, I. Zemtseva, H.R. Horvitz, C.B. Hirschberg, SQV-7, a protein involved in *Caenorhabditis elegans* epithelial invagination and early embryogenesis, transports UDP-glucuronic acid, UDP-N-acetylgalactosamine, and UDP-galactose, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 3738–3743.
[19] H. Segawa, M. Kawakita, N. Ishida, Human and *Drosophila* UDP-galactose transporters transport UDP-N-acetylgalactosamine in addition to UDP-galactose, Eur. J. Biochem. 269 (2002) 128–138.
[20] C. Abeijon, P.W. Robbins, C.B. Hirschberg, Molecular cloning of the Golgi apparatus uridine diphosphate-N-acetylglucosamine transporter from *Kluyveromyces lactis*, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 5963–5968.
[21] T. Lubke, T. Marquardt, A. Etzioni, E. Hartmann, K. von Figura, C. Korner, Complementation cloning identifies CDG-IIc, a new type of congenital disorders of glycosylation, as a GDP-fucose transporter deficiency, Nat. Genet. 28 (2001) 73–76.
[22] N. Ishida, S. Yoshioka, Y. Chiba, M. Takeuchi, M. Kawakita, Molecular cloning and functional expression of the human Golgi UDP-N-acetylglucosamine transporter, J. Biochem. (Tokyo) 126 (1999) 68–77.
[23] N. Ishida, S. Yoshioka, M. Iida, K. Sudo, N. Miura, K. Aoki, M. Kawakita, Indispensability of transmembrane domains of Golgi UDP-galactose transporter as revealed by analysis of genetic defects in UDP-galactose transporter-deficient murine had-1 mutant cell lines and construction of deletion mutants, J. Biochem. (Tokyo) 126 (1999) 1107–1117.
[24] M. Eckhardt, B. Gotza, R. Gerardy-Schahn, Membrane topology of the mammalian CMP-sialic acid transporter, J. Biol. Chem. 274 (1999) 8779–8787.
[25] L. Puglielli, E. Mandon, D.M. Rancour, A.K. Menon, C.B. Hirschberg, Identification and purification of the rat liver Golgi membrane UDP-N-acetylgalactosamine transporter, J. Biol. Chem. 274 (1999) 4474–4479.
[26] L. Puglielli, C.B. Hirschberg, Reconstitution, identification and purification of the rat liver Golgi membrane GDP-fucose transporter, J. Biol. Chem. 274 (1999) 35596–35600.
[27] M. Abe, H. Hashimoto, K. Yoda, Molecular characterization of Vig4/Vrg4 GDP-mannose transporter of the yeast *Saccharomyces cerevisiae*, FEBS Lett. 458 (1999) 309–312.
[28] X.D. Gao, N. Dean, Distinct protein domains of the yeast Golgi GDP-mannose transporter mediate oligomer assembly and export from the endoplasmic reticulum, J. Biol. Chem. 275 (2000) 17718–17727.
[29] K. Hong, D. Ma, S.M. Beverly, S.J. Turco, The *Leishmania* GDP-mannose transporter is an autonomous, multi-specific, hexameric complex of LPG2 subunits, Biochemistry 39 (2000) 2013–2022.
[30] E.C. Mandon, M.E. Milla, E. Kempner, C.B. Hirschberg, Purification of the Golgi adenosine 3′-phosphate 5′-phosphosulfate transporter, a homodimer within the membrane, Proc. Natl. Acad. Sci. 91 (1994) 10707–10711.
[31] E.M. Selva, K. Hong, G.H. Baeg, S.M. Beverly, S.J. Turco, N. Perrimon, U. Hacker, Dual role of the fringe connection gene in both heparan sulphate and fringe-dependent signalling events, Nat. Cell Biol. 3 (2001) 809–815.
[32] A. Ashikov, F. Routier, J. Fuhlrott, Y. Helmus, M. Wild, R. Gerardy-Schahn, H. Bakker, The human solute carrier gene SLC35B4 encodes a bifunctional nucleotide sugar transporter with specificity for UDP-Xylose and UDP-N-Acetylglucosamine, J. Biol. Chem. 280 (2005) 27230–27235.
[33] K. Aoki, N. Ishida, M. Kawakita, Substrate recognition by UDP-galactose and CMP-sialic acid transporters. Different sets of transmembrane helices are utilized for the specific recognition of UDP-galactose and CMP-sialic acid, J. Biol. Chem. 276 (2001) 21555–21561.
[34] K. Aoki, G.H. Sun-Wada, H. Segawa, S. Yoshioka, N. Ishida, M. Kawakita, Expression and activity of chimeric molecules between human UDP-galactose transporter and CMP-sialic acid transporter, J. Biochem. 126 (1999) 940–950.
[35] J.M. Capasso, C.B. Hirschberg, Effect of nucleotides on translocation of sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate into Golgi apparatus vesicles, Biochim. Biophys. Acta 777 (1984) 133–139.
[36] B.C. Waldman, G. Rudnick, UDP-GlcNAc transport across the Golgi membrane: electroneutral exchange for dianionic UMP, Biochemistry 29 (1990) 44–52.
[37] A.W. Brandli, G.C. Hansson, E. Rodriguez-Boulan, K. Simons, A polarized epithelial cell mutant deficient in translocation of UDP-galactose into the Golgi complex, J. Biol. Chem. 263 (1988) 16283–16290.
[38] L. Toma, M.A.S. Pinhal, C.P. Dietrich, H.B. Nader, C.B. Hirschberg, Transport of UDP-galactose into the Golgi lumen regulates the biosynthesis of proteoglycans, J. Biol. Chem. 271 (1996) 3897–3901.
[39] P. Berninsone, J.J. Miret, C.B. Hirschberg, The Golgi guanosine diphosphatase is required for transport of GDP-mannose into the lumen of *Saccharomyces cerevisiae* Golgi vesicles, J. Biol. Chem. 269 (1994) 207–211.
[40] T. Lubke, T. Marquardt, K. von Figura, C. Korner, A new type of carbohydrate-deficient glycoprotein syndrome due to a decreased import of GDP-fucose into the Golgi, J. Biol. Chem. 274 (1999) 25986–25989.
[41] H.K. Meiss, R.F. Green, E.J. Rodriguez-Boulan, Lectin-resistant mutants of polarized epithelial cells, Mol. Cell. Biol. 2 (1982) 1287–1294.
[42] P. Stanley, Lectin-resistant CHO cells: selection of new mutant phenotypes, Somatic Cell Genet. 9 (1983) 593–608.
[43] P. Stanley, Membrane mutants of animal cells: rapid identification of those with a primary defect in glycosylation, Mol. Cell. Biol. 5 (1985) 923–929.
[44] P. Stanley, Chinese hamster ovary cell mutants with multiple glycosylation defects for production of glycoproteins with minimal carbohydrate heterogeneity, Moll. Cell. Biol. 9 (1989) 377–383.
[45] S.L. Deutscher, C.B. Hirschberg, Mechanism of galactosylation in the Golgi apparatus. A Chinese hamster ovary cell mutant deficient in translocation of UDP-galactose across Golgi vesicle membranes, J. Biol. Chem. 261 (1986) 96–100.
[46] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[47] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, M.N. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[48] J. Kyte, R.F. Doolittle, A simple method for displaying the hydropathic character of a protein, J. Mol. Biol. 157 (1982) 105–132.
[49] S. Oelmann, P. Stanley, R. Gerardy-Schahn, Point mutations identified in Lec8 Chinese hamster ovary glycosylation mutants that inactivate both the UDP-galactose and CMP-sialic acid transporters, J. Biol. Chem. 276 (2001) 26291–26300.
[50] R. Kabuß, A. Ashikov, S. Oelmann, R. Gerardy-Schahn, H. Bakker, Endoplasmic reticulum retention of the large splice variant of the UDP-galactose transporter is caused by a dilysine motif, Glycobiology 15 (2005) 905–911.