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Short N-terminal region of UDP-galactose transporter (SLC35A2) is crucial for galactosylation of *N*-glycans

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ABSTRACT

UDP-galactose transporter (UGT) and UDP-*N*-acetylglucosamine transporter (NGT) form heterologous complexes in the Golgi apparatus (GA) membrane. We aimed to identify UGT region responsible for galactosylation of *N*-glycans. Chimeric proteins composed of human UGT and either NGT or CMP-sialic acid transporter (CST) localized to the GA, and all but UGT/CST chimera corrected galactosylation defect in UGT-deficient cell lines, although at different efficiency. Importantly, short N-terminal region composed of 35 N-terminal amino-acid residues of UGT was crucial for galactosylation of *N*-glycans. The remaining molecule must be derived from NGT not CST, confirming that the role played by UGT and NGT is coupled.

1. Introduction

Glycosylation of macromolecules is one of the most important posttranslational modifications of proteins [1]. The substrates required for glycosylation are sugars activated by the addition of mono- or diphosphonucleotides (UDP, GDP or CMP). To be delivered into the endoplasmic reticulum (ER) and the Golgi apparatus (GA), they need to be transported across their membranes by nucleotide sugar transporters (NSTs). NSTs are highly conserved type III multitransmembrane proteins with molecular weight of 30–45 kDa that function as antiporters delivering nucleotide sugars into the ER or GA and transferring corresponding nucleoside monophosphate into the cytosol [1–4].

One of the best characterized NSTs is UDP-galactose transporter (UGT; SLC35A2). Two splice variants of UGT (UGT1 and UGT2) have been identified in human tissues, the Chinese hamster ovary (CHO) and Madin–Darby canine kidney II (MDCK) cell lines [5–8]. MDCK cells also possess an additional, shorter UGT isoform [9]. Characterization of UGT was possible after mammalian cell lines deficient in UGT activity, such as CHO-Lec8, MDCK cells resistant to *Ricinus communis* agglutinin (MDCK-RCA^r) and murine Had-1 cells, have been identified [6,10,11]. Glycans produced in these cells are enriched in terminal *N*-acetylglucosamine (GlcNAc) and exhibit significantly reduced terminal galactose and sialic acid. Although UGT is not active in mutant cells, some galactosylation still occurs [8,10–14]. Recently, it has been shown that *SLC35A2* gene mutation can result in early-onset epileptic encephalopathy and developmental delay [15,16].

In contrast to UDP-galactose transporter, little is known about mammalian UDP-*N*-acetylglucosamine transporter (NGT; SLC35A2), which is assumed to play the main role in UDP-GlcNAc transport. Multi-specific transporters SLC35D1, SLC35D2 and SLC35B4 appear to be involved in glycosylation of other macromolecules and seem to be rather tissue specific [17–20]. It is worth noting that silencing of the gene encoding NGT results in significant decrease of highly branched *N*-glycans as well as keratan sulfate, but do not affect mono- and diantennary oligosaccharide structures [21]. Recently, it has been reported that impaired UDP-GlcNAc transport into the Golgi vesicles leads to serious disorders. Point mutations in the gene encoding NGT result in complex vertebral malformation in cattle [22] and autism spectrum disorders, epilepsy as well as arthrogryposis in humans [23].

We hypothesize that the role played by NGT and UGT in galactosylation is coupled. We have shown that overexpression of NGT may partially restore galactosylation of *N*-glycans in the mutant cells defective in UGT activity [24]. Moreover, using immunoprecipitation and FLIM-FRET technique we proved that NGT, UGT1 and UGT2 form heterologous complexes in the GA membrane [25]. Our recently published [21] and unpublished data also

Abbreviations: UGT, UDP-galactose transporter; NGT, UDP-*N*-acetylglucosamine transporter; NST, nucleotide sugar transporter; ER, endoplasmic reticulum; GA, Golgi apparatus.

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suggest that NGT and UGT1 are in the close proximity to *N*-acetylglucosamine glycosyltransferases (Mgats).

In our previous study we have demonstrated that UGT/NGT chimeric transporter is able to correct galactosylation defect in UGT-deficient mammalian cells at better degree compared with the NGT overexpression [26]. The chimeric protein was composed of 224 N-terminal amino-acid residues of UGT fused to C-terminal region of NGT. Even though almost half of the protein was derived from the different NST, the chimera localized to the GA membrane, was fully functional in respect to UDP-galactose transport and restored galactosylation in UGT-deficient cells. This phenomenon convinced us to study the role of UGT N-terminal region in galactosylation in more details. Therefore, we constructed six novel chimeric proteins and analyzed their involvement in galactosylation in UGT-deficient mammalian cell lines.

2. Materials and methods

2.1. Construction of chimeric transporters

Chimeric constructs 2–4 were prepared by introducing unique AflII restriction site into plasmids containing sequences encoding UGT and NGT, without changing of protein sequences (Fig. 1), as previously described for UGT/NGT chimera [27]. Chimeric construct 5 was prepared using primers which amplify N-terminal region of UGT and C-terminal region of NGT. After purification, the blunt-ends of DNA fragments were phosphorylated using T4 kinase (Thermo Scientific) and ligated (Fast Ligation Kit, ThermoScientific). The ligation reaction mixture was used as a template to perform PCR using primers specific for 5′ sequence of UGT and 3′ sequence of NGT, respectively. Finally, the PCR product was cloned to polilinker #1 of the modified pVitro1 vector, containing 6His-HA N-terminal tag, as previously described [26]. The same strategy was used in the case of UGT/CST chimera, with appropriate primers designed to amplify N-terminal region of UGT and C-terminal region of CST. Sequence encoding NGT/UGT1 chimera was amplified using forward primer containing additional, short sequence encoding 5 N-terminal amino-acid residues derived from NGT. The cDNA was synthesized using total RNA purified from HeLa cells as a template (Superscript III, Invitrogen). All mutagenesis primers and primers used in PCR are listed in Supplementary Table 1.

2.2. Cell maintenance and transfection

CHO, CHO-Lec8, MDCK and MDCK-RCA^r cells were grown and transfected with expression plasmids as described previously [7]. Stable transfectants expressing chimeric proteins were selected in complete media containing either 500 μg/ml (CHO-Lec8) or 600 μg/ml (MDCK-RCA^r) G-418 sulfate (InvivoGen).

2.3. Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously [26] using MDCK-RCA^r cells overexpressing all analyzed chimeric proteins except that GA was visualized using a 1:200 dilution of rabbit polyclonal antibody against giantin (Abcam) followed by incubation with a 1:100 dilution of goat anti-rabbit Cy5-conjugated antibody (Abcam).

2.4. Analysis of glycoproteins with lectins

Proteins present in cell lysates were subjected to SDS–PAGE using 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Whatman) [7]. Reactivity of glycoproteins with lectins was performed as described previously [7,24].

2.5. Isolation and separation of fluorescently labeled N-glycans and MALDI-TOF MS analysis

N-glycans were isolated, purified, fluorescently labeled with 2-aminobenzamide (2-AB) and separated on a GlycoSep N column (Glyko) as described previously [24]. Prior mass spectrometry (MS) analysis, *N*-glycans were subjected to neuraminidase treatment. MALDI-TOF MS analysis was carried out in positive ion mode with Na^+ excess as previously reported [24].

3. Results

3.1. Localization of chimeric proteins

We constructed six novel chimeric transporters (Fig. 1) and overexpressed them stably in UGT-deficient mammalian cell lines. All proteins were successfully overexpressed in several clones and localized in the GA membrane as shown by immunofluorescence microscopic analysis using MDCK-RCA^r cells (Supplementary Fig. 1). The expression levels of chimeras 2–5 and UGT/CST chimera were significantly higher compared to NGT/UGT1 chimera (Supplementary Fig. 2).

3.2. Reactivity of glycoproteins with lectins

To determine whether and to what extent all analyzed chimeric proteins would be able to restore galactosylation of CHO-Lec8 and MDCK-RCA^r cells, analysis of lectin reactivity was employed using GSLII (*Griffonia simplicifolia* lectin II) and VVL (*Vicia villosa* lectin) lectins. Glycoproteins produced in CHO-Lec8 and MDCK-RCA^r cells are significantly enriched in terminal GlcNAc in *N*-glycans and *N*-acetylgalactosamine (GalNAc) in *O*-glycans, resulting in increased reactivity with GSLII and VVL lectins, respectively. Here we demonstrate that only UGT/CST chimera did not correct the mutant phenotype of the cells examined (Supplementary Figs. 3 and 4). Overexpression of the other five transporters (chimeras 2–5 and NGT/UGT1 chimera) both in CHO-Lec8 and MDCK-RCA^r resulted in decreased reactivity with GSLII and VVL lectins to the level similar to the wild-type cells. These data show that in contrast to UGT/CST chimera, correction of galactosylation defect, in terms of both *N*- and *O*-glycosylation was clearly visible.

MALI (*Maackia amurensis* lectin I) lectin is specific for terminal galactose and alpha 2,3-sialic acid-galactose, structures characteristic for the wild-type cells. An increase of galactose-specific MALI lectin reactivity with *N*-glycosylated proteins synthesized by MDCK-RCA^r cells expressing chimeras 2–5 as well as NGT/UGT1 chimera confirms correction of galactosylation defect. This effect was not visible in the case of UGT/CST chimera (Supplementary Fig. 3). In contrast to MDCK and MDCK-RCA^r cells, no difference in MALI reactivity was observed between CHO and CHO-Lec8 cells.

3.3. Identification of glycan structures

To identify more specific structural changes, *N*-glycans isolated from glycoproteins derived from the cells overexpressing chimeric transporters were characterized. Although correction of the mutant phenotype was visible for MDCK-RCA^r and CHO-Lec8 cells, we demonstrate detailed data for CHO and CHO-Lec8 cells analysis only, because MDCK and MDCK-RCA^r cells are not the best model for glycosylation analysis since they produce mostly *N*-glycans of high-mannose type [24]. Correction of the mutant phenotype was clearly visible for CHO-Lec8 cells overexpressing chimera 2