UDP-N-acetylglucosamine transporter (SLC35A3) regulates biosynthesis of highly branched N-glycans and keratan sulfate

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Running title: SLC35A3 regulates N-glycan and keratan sulfate synthesis

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Keywords: UDP-N-acetylglucosamine transporter, branched N-glycans, glycosylation, keratan sulfate, Golgi apparatus

Background: Knowledge regarding UDP-N-acetylglucosamine transporter (NGT; SLC35A3) is incomplete due to the lack of NGT-deficient model cell lines.

Results: The siRNA approach showed that NGT silencing reduces branching of complex N-glycans and keratan sulfate synthesis.

Conclusion: NGT function may be coupled to the specific glycosylation pathway/s of particular macromolecules.

Significance: Our results add to the understanding of glycosylation, one of the basic posttranslational modifications.

SUMMARY

SLC35A3 is considered the main UDP-N-acetylglucosamine transporter (NGT) in mammals. Detailed analysis of NGT is restricted since mammalian mutant cells defective in this activity have not been isolated. Therefore, using the siRNA approach, we developed and characterized several NGT-deficient mammalian cell lines. CHO, CHO-Lec8 and HeLa cells deficient in NGT activity displayed a decrease in the amount of highly branched tri- and tetraantennary N-glycans, while monoantennary and diantennary ones remained unchanged or even were accumulated. Silencing the expression of NGT in MDCK cells resulted in a dramatic decrease in the keratan sulfate content, whereas no changes in biosynthesis of heparan sulfate were observed. We also demonstrated for the first time close proximity between NGT and mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetylglucosaminyltransferase (Mgat5) in the Golgi membrane. We conclude that NGT may be important for the biosynthesis of highly branched, multiantennary complex N-glycans and keratan sulfate. We hypothesize that NGT may specifically supply beta-1,3-N-acetylglucosaminyltransferase 7 (β3GnT7), Mgat5 and possibly mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase (Mgat4) with UDP-GlcNAc.

Glycosylation is one of the most frequent posttranslational modifications of macromolecules. The glycan moiety is synthesized and modified by glycosyltransferases acting in the lumen of the endoplasmic reticulum (ER) and Golgi apparatus. The substrates required by glycosyltransferases are sugars activated by the addition of a nucleoside mono- or diphosphate (UDP, GDP, or CMP). Nucleotide sugars are synthesized in the cytosol (1), except for CMP-sialic acid, which is synthesized in the nucleus (2), and transported by nucleotide-sugar transporters (NSTs) into the ER and/or Golgi apparatus. NSTs are hydrophobic, multi-transmembrane proteins with a molecular weight of 30-45 kDa (3,4). Several data demonstrated that NSTs function in the form of dimers or higher oligomers (5-11).
One of the best characterized NSTs is the UDP-Gal transporter (UGT; SLC35A2) (6,12-16). 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 (6,12-14,16). Detailed characterization of UGT was possible after mutant cell lines, such as MDCK cells resistant to *Ricinus communis* agglutinin (MDCK-RCA17,18), CHO-Lec8 cells (14,19), and Had-1 cells (15,20), had been generated. Nonsense mutations identified in the mutant cells cause inhibition of UGT production, resulting in macromolecules enriched in terminal N-acetylglucosamine (GlcNAc), deficient in terminal galactose (Gal) and sialic acid (17,21,22).

Compared with mammalian UGT, knowledge regarding mammalian UDP-N-acetylglucosamine transporter is limited. Within known UDP-GlcNAc transporters, the protein assigned as SLC35A3 (NGT) is assumed to play a main role in glycosylation of macromolecules (23,24), while the function of SLC35D2 (25,26), SLC35B4 (27), and SLC35D1 (28) multi-specific transporters appears to be less important. Recently, it has been reported that a point mutation of the SLC35A3 gene causes complex vertebral malformation (CVM) in animals, resulting from impaired UDP-GlcNAc transport into the Golgi vesicles (29).

We hypothesize that the role of NGT and UGT in glycosylation of macromolecules may be coupled and both transporters may partially replace the function played by its partner. Firstly, both transporters are evolutionarily related (4,10,30-32). Secondly, we showed that overexpression of NGT in UGT-defective cells partially restores galactosylation (30), and UGT-NGT chimeric transporter complemented the mutation defect (33). Finally, recently we demonstrated that NGT and UGT form complexes in the Golgi membrane (10).

Although NGT is considered the main UDP-GlcNAc transporter in mammals, its biological role awaits further attention. However, detailed analysis of this transporter is restricted since mammalian mutant cells defective in this activity have not been isolated. Therefore, using the siRNA approach we developed and characterized several NGT-deficient mammalian cell lines.

**EXPERIMENTAL PROCEDURES**

**Molecular cloning of hamster NGT and canine β4GalT4 - cDNA clones containing the complete coding regions for hamster UDP-N-acetylglucosamine transporter (NGT) and canine beta-1,4-galactosyltransferase 4 (β4GalT4) were generated and sequenced using degenerate primers designed based on known homologous mammalian sequences and the modified RACE technique as described previously (16).**

**Construction of NGT- and β4GalT4-targeting siRNA plasmids – siRNA sequences targeting human NGT (NM_005660), canine NGT (NM_001003385.1), hamster NGT (FN825777.1), and canine β4GalT4 (AM989461.1) were selected using InvivoGen’s siRNA Wizard™ online tool (http://www.sirnawizard.com/). A pair of control sequences (scrambled siRNA; Scr) was also designed. Based on selected siRNA sequences, pairs of complementary (sense and antisense) oligonucleotides were designed (Table S1) using the above-mentioned program. Complementary oligonucleotide pairs were PAGE-purified and annealed by incubation at the 50 µM concentration in 0.1 M NaCl at 80°C (2 min) followed by slow (1°C per min) cooling down to 35°C. The resulting double-stranded DNA fragments were cloned into the psiRNA-DUO plasmid according to the manufacturer’s instructions using a two-step procedure (InvivoGen).**

**Construction of eGFP and mRFP expression plasmids - ORFs of human mannosyl (alpha-1,3-)glycoprotein beta-1,4-N-acetylgalosaminyltransferase, isozyme A (Mgat4A; NM_012214.2) and mannosyl**
(alpha-1,6-)glycoprotein beta-1,6-N-acetylgalcosaminyltransferase (Mgat5; NM_002410.3) with appropriate restriction sites at both ends were amplified using cDNA synthesized from total RNA isolated from HeLa cells and subsequently cloned into pTagGFP2-C vector (Evrogen). We assumed that among known Mgat4 isozymes (A, B and C), Mgat4A is the main isozyme accounting for Mgat4 activity, because the amount of the corresponding mRNA in HeLa cells was more abundant compared with other isozymes (data not shown). Sequence encoding human UDP-N-acetylglucosamine transporter (NGT; NM_012243.2) with N-terminal mRFP fusion was subcloned from the expression plasmid previously constructed in pTagRFP-C vector (10) into pSELECT-zeo plasmid (InvivoGen) and the resulting construct was used instead of the initial one due to superior fluorescence properties of the resulting mRFP fusion protein. Briefly, insert encoding mRFP-NGT fusion protein was cut out from the parent vector (10) using NheI and BamHI restriction enzymes end both 3’- and 5’-ends were blunted using Mung Bean Nuclease (New England Biolabs). pSELECT-zeo plasmid (InvivoGen) was digested with BamHI restriction enzyme, blunt-ended as described above and dephosphorylated using calf intestine alkaline phosphatase (Fermentas). ORF of human UDP-xylose/N-acetylgalacosamine transporter (SLC35B4; NP_116215.1) with BglII and BamHI restriction sites at both ends was amplified using cDNA synthesized from total RNA isolated from HeLa cells and subsequently cloned into pTagGFP2-C vector (Evrogen). All ligations were performed using Rapid DNA Ligation Kit (Fermentas). Expression plasmid containing mRFP-UGT2 was constructed previously (10). Primers used for amplifications are listed in Table S3. The obtained eGFP and mRFP expression plasmids are listed in Table S2.

**RT-PCR evaluation of the NGT and β4GalT4 silencing efficiency** – The efficiency of NGT and β4GalT4 silencing was determined at the mRNA level using a semiquantitative RT-PCR approach. For this purpose, total RNA was isolated from selected NGT- or β4GalT4-deficient clones as well as from cells transfected with control Scr plasmid and a fragment of NGT- or β4GalT4-encoding sequences was subsequently amplified using Titan One Tube RT-PCR Kit according to the manufacturer’s instructions (Roche). Fragments of either CMP-sialic acid (CST; SLC35A1) or GDP-fucose transporter (GFT; SLC35C1) sequences were amplified in parallel as a reference. In addition, transcript levels of SLC35D1, SLC35D2, and SLC35B4 transporters were examined. Amplification products were separated in 0.8% agarose gel and visualized with ethidium bromide. Primer sequences and length of the amplified fragments are listed in Table S3.

**Cell maintenance and transfection** – MDCK, MDCK-RCA’, CHO and CHO-Lec8 cells were grown as described previously (16). For the detection of keratan sulfate, MDCK and MDCK-RCA’ cells were grown in a serum-free medium as reported previously (16). HeLa and A375 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 IU penicillin/ml and 100 µg streptomycin/ml. Cells were transfected with shRNA expression plasmids listed in Table S2. Stable transfectants were selected in complete media containing 200 µg/ml (MDCK), 150 µg/ml (CHO and CHO-Lec8) or 100 µg/ml (HeLa) Zeocin (InvivoGen).

**Immunoreactivity and reactivity with lectins** – Cell lysates were subjected to SDS-PAGE using 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Whatman) as described previously (16). Detection of keratan sulfate and reactivity of glycoproteins with lectins were performed as reported previously (16,30). Heparan sulfate was detected using mouse anti-heparin/heparan sulfate antibody (1:5 000; Millipore; clone T320.11, cat. no. MAB2040), followed by anti-mouse IgG antibody conjugated with HRP (1:10 000; Promega). Selected Golgi and ER proteins were detected with rabbit anti-GM130 and anti-calnexin antibodies (1:2 000; Abcam), followed by antirabbit IgG antibody conjugated with HRP (1:10 000; Sigma).

**Isolation and separation of fluorescently labeled N-glycans and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis** – N-glycans were isolated as described previously (30). Briefly, cell lysates...
were diluted to 2 mg/ml using the lysis buffer, and 500-µl aliquots were precipitated overnight at −20°C with an equal volume of cold acetone. After centrifugation at 10 000×g for 10 min, precipitates were air-dried and resuspended in glycoprotein denaturation buffer (N-glycosidase F deglycosylation enzyme pack, New England Biolabs). Deglycosylation was performed using 1 µl of the enzyme (500 U) for 18 h at 37°C in deglycosylation buffer. Released N-glycans were isolated, fluorescently labeled with 2-aminobenzamide (2-AB), purified and separated on a GlycoSep N column (Glyko) as described previously (30). In addition, enzymatic sequencing was performed to confirm particular structures and type of linkages in branched N-glycans (34). MALDI-TOF MS analysis was carried out in positive ion mode with Na+ excess as previously reported (30).

Subcellular fractionation and transport assay – The Golgi fraction was isolated from mammalian cells and UDP-Gal or UDP-GlcNAc transport into Golgi vesicles was subsequently determined as described previously (16,30).

Confocal and fluorescence lifetime imaging microscopy (FLIM) – Confocal and FLIM microscopy was performed as described previously (10) except that A375 cells were used in some experiments and cells transiently transfected with the expression plasmids listed in Table S2 were seeded onto the 35 mm CELLview™ Glass Bottom Dishes (Greiner Bio-One) prior to imaging. Analysis of FLIM-FRET experiments was carried out as reported previously (10).

RESULTS

Molecular cloning and sequence analysis of the hamster NGT and canine β4GalT4 – In contrast to the human and canine NGT-encoding sequences, a hamster ortholog has not been identified so far. We therefore cloned its sequence using the RACE strategy (16), thus enabling silencing of NGT expression in CHO and CHO-Lec8 cells. The obtained ORF corresponded to a 326-amino-acid protein sharing almost 95% identity with human NGT (data not shown). The sequence was deposited in the EMBL Nucleotide Sequence Database with accession number FN825777.1. A similar approach was applied for identification of the canine β4GalT4-encoding sequence, which resulted in the ORF corresponding to a 344-amino-acid protein sharing 86% identity with human β4GalT4 (data not shown). The sequence was deposited in the EMBL Nucleotide Sequence Database with accession number AM989461.1.

Construction of mammalian NGT- and β4GalT4-deficient cells – To produce shRNA sequences targeting NGT- or β4GalT4-specific mRNAs, the psiRNA-DUO plasmid was used, which allowed generation of two different siRNAs in parallel, thus increasing inhibition of a desired target. Preliminary data showed that after stable transfection, production of transcripts encoding NGT or β4GalT4 in several clones was significantly decreased (Fig. 1A). In contrast, no significant changes in transcript levels were observed in the case of SLC35D1, SLC35D2 and SLC35B4 transporters (Fig. 1B). In preliminary experiments, several clones were examined and those subjected to further analyses, from which results are shown in this study, are marked with asterisks.

NGT silencing reduces keratan sulfate biosynthesis in mammalian cells – MDCK-RCAi cells are completely devoid of keratan sulfate due to a mutation in the UGT-encoding gene. As shown in Fig. 2A, also NGT silencing caused a dramatic decrease in keratan sulfate production in MDCK cells. As expected, significantly decreased production of keratan sulfate was also observed in the β4GalT4-deficient cells. In contrast, no changes in heparan sulfate synthesis were observed after NGT silencing (Fig. 2E), as demonstrated by heparan sulfate proteoglycan analysis. Similar results were demonstrated in CHO, CHO-Lec8 and HeLa cells (data not shown).

NGT silencing reduces N-glycan branching in mammalian cells – Since all complex N-glycans synthesized in the Golgi apparatus contain GlcNAc, it was reasonable to assume that NGT silencing would alter this type of oligosaccharides. Therefore we performed a detailed, structural analysis of N-glycan structures using chromatographic separation of fluorescently labeled oligosaccharides released from cellular glycoproteins. Surprisingly, in all examined cells a reduction in the relative amount of highly branched tri- and tetraantennary
complex N-glycans was mainly observed. As shown in Fig. 3, in the case of CHO wild-type cells a decrease in tri- and tetraantennary oligosaccharide structures with accompanying accumulation of diantennary N-glycans was shown in analyzed oligosaccharide profiles obtained after separation on a GlycoSepN column. Differences in specific oligosaccharide structures were more visible after MALDI-TOF MS identification (the most notable differences are marked with red arrows in Figs 4A and 4B). The NGT silencing effect was even more profound in CHO-Lec8 mutant cells, because they are significantly deficient in terminal Gal and sialic acid residues, thus allowing better observation of N-glycans terminated with GlcNAc. Differences in N-glycan profiles were mostly expressed in a decrease in tri- and tetraantennary oligosaccharides (Fig. 3), comprising a considerable part of the total pool of N-glycans in non-silenced cells. These results were confirmed in detail by MALDI-TOF MS analysis (the most notable differences are marked with red arrows in Figs 4C and 4D). In HeLa cells, a similar effect as in CHO wild-type cells was observed, although at lower intensity (Fig. 5 and data not shown). Analysis of several clones demonstrated that this effect may be explained by lower efficiency of NGT silencing (Fig. 1).

NGT silencing reduces both UDP-GlcNAc and UDP-Gal transport in mammalian cells — Since UDP-GlcNAc is considered the main NGT substrate, we isolated the Golgi fraction from NGT-deficient CHO and CHO-Lec8 cells and measured UDP-GlcNAc transport across the Golgi membrane. UDP-GlcNAc transport activity was decreased in NGT-deficient cells compared with the wild-type cells (Fig. 6), but the effect was not as dramatic as we expected. In the CHO-Lec8 mutant cells defective in UDP-Gal transport and deficient in NGT synthesis no significant difference was observed compared with mutant CHO-Lec8 cells. Our previous data showed that NGT is also involved in UDP-Gal delivery to the Golgi apparatus (10,30). In accordance with those data, here we demonstrated that in NGT-deficient CHO cells UDP-Gal transport was severely diminished (Fig. 6). This effect was not profound in CHO-Lec8 cells. UDP-Gal transport into Golgi vesicles of previously developed CHO-Lec8 cells stably overexpressing UGT1 (16) was measured as an additional reference.

FLIM-FRET analysis demonstrates interaction between NGT and Mgat5 — Since NGT silencing resulted in a decrease in the amount of tri- and tetraantennary complex N-glycans, we attempted to investigate putative interactions between NGT and transferases mediating biosynthesis of these structures using the FLIM-FRET approach. For this purpose, A375 cells were transiently transfected with plasmids enabling expression of NGT and Mgat4A or Mgat5 in fusion with mRFP and eGFP, respectively (Table S2). However, overexpression of Mgat4A in fusion with eGFP resulted in protein mislocalization (data not shown), so that FLIM-FRET experiments could not be conducted. In contrast, both NGT and Mgat5 localized properly to the Golgi apparatus when overexpressed in fusion with respective fluorescent proteins (Figs 7A, 7C and 7D). In the FLIM-FRET approach, a reduction in the fluorescence lifetime of the donor fluorophore in the presence of the acceptor fluorophore is indicative of the interaction between analyzed fusion proteins (fluorescence lifetime of the donor fluorophore in the absence of acceptor fluorophore is considered as a reference). We demonstrated that in the absence of an acceptor fluorophore, the mean lifetime of eGFP-tagged Mgat5 was $2.63 \pm 0.03$ ns (n = 19; Figs 7B and K), while coexpression with mRFP-tagged NGT reduced it significantly to $2.22 \pm 0.18$ ns (n = 24; Figs 7E and 7K). A reduction in the fluorescence lifetime was best described by means of a bi-exponential model, allowing for the distinguishing of two lifetime components: a longer one ($2.56 \pm 0.1$ ns), corresponding to that of the control and resulting from the presence of a non-interacting donor fraction, and a shorter one ($1.06 \pm 0.19$ ns), reflecting donor fraction involved in the energy transfer (differential contribution of this fraction accounts for a slightly higher SD that accompanies the mean lifetime value of the donor fluorophore in the presence of the acceptor fluorophore). These data strongly demonstrate very close proximity of NGT and Mgat5 in the Golgi membrane of living cells. One may assume that extensive overexpression of two transmembrane proteins may trigger their non-specific aggregation. In
order to prove that close proximity of Mgat5 and NGT in the Golgi membrane does not result from such artificial aggregation we employed a negative control comprising two multi-transmembrane fusion proteins, namely eGFP-B4 (human UDP-xylose/N-acetylglucosamine transporter) and mRFP-UGT2 (human UDP-galactose transporter, splice variant 2). As we expected, both fusion proteins were properly overexpressed in the ER membrane of UGT-deficient MDCK-RCA cells and displayed significant colocalization (Figs 7H and 7I). In this case, however, we did not observe any reduction in the mean lifetime of the donor fluorophore (i.e. eGFP-B4) in the presence of the acceptor fluorophore (i.e. mRFP-UGT2; Figs 7G, 7J and 7L). This remark allowed us to conclude that the interaction between Mgat5 and NGT might occur.

**DISCUSSION**

It has been reported that missense mutation (G to T transversion) of evolutionarily conserved valine at position 180 to phenylalanine in the *SLC35A3* gene is responsible for congenital disorder identified in cattle (29). This defect resulted in glycosylation changes of glycoproteins derived from calf cardiac and muscle tissues, and probably in defective glycosaminoglycan synthesis causing severe malformations in animals. In addition, the wild-type gene, but not the mutated one, complemented the *Kluyveromyces lactis* mutant deficient in UDP-GlcNAc transport. However, no detailed phenotypic analysis of those cells has been performed and, to date, mammalian mutant cell lines defective in NGT activity have not been isolated. Therefore, we developed model studies using wild-type (CHO, MDCK, HeLa) and mutant mammalian cell lines defective in UGT activity (CHO-Lec8), in which expression of endogenous NGT was significantly decreased. For this purpose, the siRNA approach was successfully employed, resulting in several NGT-deficient mammalian cell lines, which were subsequently analyzed in terms of synthesized N-glycan structures and keratan sulfate production.

Detailed structural analysis of N-glycans was carried out using CHO- and HeLa-derived NGT-deficient cells. It is worth noting that most commonly used lectins did not clearly discriminate between NGT-deficient and wild-type cells (data not shown), which may explain why mutant cell lines lacking NGT activity have not been isolated so far. GlcNAc-containing N-glycans of complex type are synthesized in the Golgi apparatus by subsequent action of respective mannoside GlcNAc transferases (Mgats). Mono- and diantennary glycans are formed by action of mannosyl (alpha-1,3-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase (Mgat1) and mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase (Mgat2), while further branching is performed by Mgat4 and Mgat5, resulting in respective formation of tri- and tetraantennary structures (Fig. 8). Here we demonstrated that NGT silencing caused a decrease in the amount of tri- and tetraantennary N-glycans, while diantennary ones remained mainly unaffected or even were accumulated. Based on these findings we hypothesize that NGT might supply UDP-GlcNAc as a substrate for Golgi-resident glycosyltransferases generating branching of diantennary oligosaccharides, namely Mgat4 and Mgat5. It is possible that UDP-GlcNAc may be delivered as a substrate for Mgat1 and Mgat2 by another transporter because we did not observe decreased activity of these transferases, which was demonstrated by similar mono- and diantennary N-glycan structures produced after silencing of NGT. However, this hypothesis should be viewed with caution and several reasons should be taken into consideration. One cannot exclude possibility that differences between activity of Mgat1/Mgat2 and Mgat4/Mgat5 may result from different affinities for UDP-GlcNAc. For example, Mgat1 and Mgat2 were reported to display lower *Km* values for UDP-GlcNAc than Mgat4 and Mgat5, which both are believed to be limited by concentrations of this nucleotide sugar (35). It should be noted, however, that after significant silencing of NGT (inhibition of transcript production higher than 90%) one may expect a decrease in the number of mono- or diantennary structures containing GlcNAc. Instead we observe a similar amount or even accumulation of such structures in NGT-deficient CHO wild-type and HeLa cells, most likely due to inhibition of further branching. It is also possible that higher structural complexity of N-glycans might affect the rate of biosynthesis, with
higher complexity structures requiring higher concentrations of the donor.

In this study, we demonstrated for the first time that NGT and Mgat5 are in close proximity in the Golgi membrane, which might significantly facilitate direct supplementation of the latter with UDP-GlcNAc. Unlike in the case of interaction between NGT and UGT (10), we were not able to confirm NGT and Mgat5 association using coimmunoprecipitation analysis. This effect may be caused by technical difficulties in finding proper conditions allowing both presence of two membrane proteins in solution and preservation their native interactions. One may also suspect weaker interaction between NGT and Mgat5 compared with the two nucleotide sugar transporter molecules, because transferases contain only one transmembrane domain that could mediate NGT binding, which may be insufficient for maintaining an intact protein complex during coimmunoprecipitation. To date, putative interactions between NSTs and glycosyltransferases have been suggested, but the only confirmed data have been presented for the interaction between UDP-galactose transporter (UGT) and UDP-galactose:ceramide galactosyltransferase in the ER (36). Here we demonstrate for the first time the close proximity of UDP-GlcNAc transporter with mannoside GlcNAc transferase in the Golgi membrane of living cells.

NGT silencing in the wild-type CHO cells resulted in slightly decreased UDP-GlcNAc transport to the Golgi vesicles, whereas in the mutant CHO-Lec8 cells slight increase of the transporting activity was demonstrated. In both cases observed differences were different than we expected and not profound. Determination of transport activity performed in this study is based on analysis of tritium-labeled monosugars which are transported by nucleotide sugar transporters into the Golgi apparatus and subsequently incorporated into glycoconjugates. Our structural studies (Fig. 4) demonstrated that NGT silencing result in decreased amount of multiantennary oligosaccharides and accumulation of mono- and diantennary oligosaccharides, which may at least in part, explain this phenomenon. We suspect that this compensation may be caused by activity of another nucleotide sugar transporter/s of UDP-GlcNAc, which deliver the substrate to mono- and di-antennary oligosaccharides. Surprisingly, in NGT-deficient CHO wild-type cells we observed a significant reduction in UDP-Gal transport. This effect was not profound in CHO-Lec8 cells since they do not possess UGT activity and the observed phenomenon, at least in part, may explain our hypothesis that NGT may play a role in UDP-Gal transport and NGT might be required for the efficient delivery of both UDP-GlcNAc and UDP-Gal to the Golgi apparatus. Recently, we demonstrated that UGT-NGT chimeric transporter restores galactosylation defect in UGT-deficient cells (33). We also showed that UGT and NGT are able to form heterologous complexes in the Golgi membrane (10). NGT silencing would disrupt such functional complexes, thus impairing delivery of both nucleotide sugars to the Golgi apparatus. This hypothesis is now being confirmed by our detailed studies which are underway.

Silencing NGT activity did not completely abolish attachment of GlcNAc to synthesized oligosaccharides. Based on our data we cannot exclude the possibility of existence of another NST, which delivers UDP-GlcNAc as a substrate mostly for glycosyltransferases active at early stages of N-glycan synthesis in the Golgi apparatus, resulting in mono- and diantennary structures (Fig. 8). It is highly unlikely that this role may be played by known transporters. Compared with SLC35A3, which is ubiquitously expressed, SLC35D1, SLC35D2 and SLC35B4 are less common and are rather tissue-specific (25-28). Another issue is their differential subcellular localization. Immunofluorescence microscopic analysis of human SLC35A3 overexpressed in CHO (24) and MDCK-RCA' (37) cells and human SLC35D2 overexpressed in CHO cells (25) demonstrated their localization in the Golgi apparatus. On the other hand, analysis of SLC35D1 showed its ER localization (28). Although SLC35B4 has been shown to be located in the Golgi apparatus (27), recently we demonstrated its ER localization (37). Therefore, another UDP-GlcNAc transporter, not yet identified, should be taken into consideration.
NGT function is not only limited to N-glycan synthesis but is also crucial for keratan sulfate synthesis. MDCK cells are not the best model for oligosaccharide studies since they produce mostly N-glycans of high-mannose type (30). However, in contrast to CHO cells, which do not produce keratan sulfate, MDCK cells synthesize significant amounts of this glycosaminoglycan (16,18), which is mainly composed of repeating disaccharide units containing Gal and GlcNAc, sulfated to a various degree. Elongation of the keratan sulfate molecule is performed by the sequential action of β4GalT4 and beta-1,3-N-acetylglucosaminyltransferase 7 (β3GnT7) (38). We showed that NGT-deficient MDCK cells exhibit a dramatic decrease in keratan sulfate production compared with the wild-type cells, suggesting high dependence of β3GnT7 activity on NGT function. Apart from GlcNAc, Gal is another main constituent of keratan sulfate chains. A reduction of UDP-Gal delivery to the Golgi vesicles in NGT-deficient cells may therefore additionally contribute to a decrease in keratan sulfate production. In contrast to keratan sulfate, it seems that NGT does not participate in heparan sulfate synthesis since comparable levels of this compound present in heparan sulfate proteoglycans were detected before and after NGT silencing, even in the mutant CHO-Lec8 and MDCK-RCA1 cell lines after NGT silencing, which are deficient in both UGT and NGT. It is likely that other transporters, such as SLC35D2 (25), may participate in heparan sulfate synthesis.

The results gained in this study add to the understanding of glycosylation, one of the basic posttranslational modifications, which affects the majority of macromolecules. Our data suggest that NGT function might be coupled to the specific glycosylation pathway/s of particular macromolecules. We also hypothesize that interaction between NSTs and glycosyltransferases might constitute a general mechanism utilized in the glycosylation process.

ACKNOWLEDGEMENTS

This work was supported by grant no. 2011/03/B/NZ1/02084 from the National Science Center (NCN), Krakow, Poland.

Abbreviations used: NST, nucleotide sugar transporter; UGT, UDP-galactose transporter; NGT, UDP-N-acetylglucosamine transporter.
REFERENCES


Legends to figures

FIGURE 1. Analysis of nucleotide sugar transporters and β-1,4-galactosyltransferase 4 expression in NGT-deficient mammalian cells. Total RNA was isolated from several stable transfectants and the RT-PCR reaction was performed using primers designed to (A) NGT and β4GalT4 or (B) NGT and SLC35D1, SLC35D2, SLC35B4. Sequences encoding fragments of either CMP-sialic acid (CST) or GDP-fucose (GFT) transporters were amplified in parallel as a reference. RT-PCR products were separated in 0.8% agarose gels and visualized with ethidium bromide. Representative data are shown and clones subjected to further analyses (A), from which results are shown in this study, are indicated with asterisks. (B) Numbers indicate NGT-deficient clones. NGT, UDP-GlcNAc transporter (SLC35A3); β4GalT4, beta-1,4-galactosyltransferase 4; MDCK, wild-type Madin-Darby canine kidney II cells; CHO, wild-type Chinese hamster ovary cells; CHO-Lec8, CHO cells lacking functional UDP-Gal transporter; HeLa, human cervical carcinoma cell line; siNGT, cells deficient in NGT production; siβ4GalT4, cells deficient in β4GalT4 production; Scr, scrambled siRNA.

FIGURE 2. Analysis of keratan sulfate and heparan sulfate synthesis in NGT-deficient mammalian cells. Proteins present in cell lysates derived from MDCK wild-type cells or cells deficient in NGT (MDCK-siNGT), cells deficient in β4GalT4 (MDCK-siβ4GalT4), or cells deficient in UGT (MDCK-RCA′) were separated by SDS-PAGE (20 µg) and transferred onto nitrocellulose membranes. Keratan sulfate (A) and heparan sulfate proteoglycans (E) were subsequently visualized with specific antibodies followed by incubation with HRP-conjugated secondary antibody. To demonstrate equal loading, proteins were stained with Coomassie Brilliant Blue G-250 (D) or selected Golgi (GM130; B) and ER (calnexin; C) marker proteins were detected with specific antibodies. Representative data out of three sets with a similar pattern are shown. MDCK, wild-type Madin-Darby canine kidney II cells; MDCK-RCA′, Madin-Darby canine kidney II cells resistant to Ricinus communis agglutinin lacking functional UDP-Gal transporter; NGT, UDP-GlcNAc transporter; UGT, UDP-Gal transporter; β4GalT4, beta-1,4-galactosyltransferase 4; siβ4GalT4, cells deficient in β4GalT4 production; siNGT, cells deficient in NGT production.

FIGURE 3. N-glycan profiles of NGT-deficient mammalian cells. N-glycans were enzymatically released from glycoproteins produced by CHO (left panel) or CHO-Lec8 (right panel) cells, fluorescently labeled with 2-AB, purified and separated on the GlycoSep N column using HPLC (30). Arrows indicate glycan fractions, comprising oligosaccharides demonstrating the most profound differences. Changes in levels of sialylated three- and tetraantennary oligosaccharides are shown with brackets. Asterisks indicate peaks comprising increased levels of disialylated and diantennary fucosylated oligosaccharides after NGT silencing. These peaks also comprise high-mannose oligosaccharides, which levels are not influenced by NGT silencing, as examined by detailed structural analysis. Blue squares, N-acetylgalactosamine (GlcNAc); green circles, mannose (Man); yellow circles, galactose (Gal); red triangles, fucose (Fuc); purple rhombs, sialic acid (Sia). Glycan indicated in the left panel: retention time 48.4 min, GU=8.46; glycan indicated in the right panel: retention time 36.5 min, GU=6.43 and 39.0 min, GU=6.83, top and bottom, respectively. Results for two selected clones are shown. CHO, wild-type Chinese hamster ovary cells; CHO-Lec8, CHO cells lacking functional UDP-Gal transporter; siNGT, cells deficient in NGT production; GU, glucose units.

FIGURE 4. Structure analysis of N-glycans from NGT-deficient mammalian cells. 2-AB labeled N-glycans derived from CHO (A and B) or CHO-Lec8 (C and D) cells were subjected to MALDI-TOF MS analysis carried out in positive ion mode with Na⁺ excess. Glycans were identified by comparing experimental data expressed in GU resulting from separation on a GlycoSep N column with data deposited in GlycoBase 3.0, followed by comparing these data with molecular weights of respective structures, experimentally determined by MALDI-TOF MS. N-glycan composition was subsequently estimated using the GlycoMod tool (30). Representative data out of 2-4 independent measurements with a similar tendency are shown. Blue squares, N-acetylglucosamine (GlcNAc);
green circles, mannose (Man); yellow circles, galactose (Gal); red triangles, fucose (Fuc). Black arrows, respective peaks; red arrows, peaks corresponding to glycans demonstrating the most profound differences. CHO, wild-type Chinese hamster ovary cells; CHO-Lec8, CHO cells lacking functional UDP-Gal transporter; siNGT, cells deficient in NGT production.

FIGURE 5. N-glycan profiles of NGT-deficient HeLa cells. N-glycans were enzymatically released from glycoproteins produced by HeLa cells, fluorescently labeled with 2-AB, purified and separated on the GlycoSep N column using HPLC (30). Arrows indicate glycan fractions, comprising oligosaccharides, demonstrating the most profound differences. Changes in levels of sialylated three- and tetaantennary oligosaccharides are shown with brackets. Blue squares, N-acetylglucosamine (GlcNAc); green circles, mannose (Man); yellow circles, galactose (Gal); red triangles, fucose (Fuc), purple rhombs, sialic acid (Sia). Glycan indicated in the figure: retention time 48.4 min, GU=8.46. HeLa, human cervical carcinoma cell line; siNGT, cells deficient in NGT production; GU, glucose units.

FIGURE 6. Nucleotide sugar transport assay. Transport of UDP-GlcNAc (A) and UDP-Gal (B) into mammalian Golgi vesicles. Data are shown as mean±standard deviation from 3-5 independent experiments performed in duplicate. CHO, wild-type Chinese hamster ovary (CHO) cells; CHO-siNGT, wild-type UDP-GlcNAc transporter (NGT)-deficient cells; CHO-Lec8, mutant cells defective in UDP-Gal transport activity; CHO-Lec8-siNGT, NGT-deficient mutant cells defective in UDP-Gal transport activity; CHO-Lec8+UGT, mutant cells defective in UDP-Gal transport activity overexpressing UDP-Gal transporter (UGT).

FIGURE 7. In vivo FLIM–FRET analysis of interaction between mannosyl (alpha-1,6-)glycoprotein beta-1,6-N-acetylglucosaminyltransferase (Mgat5) and UDP-N-acetylglucosamine transporter (NGT). Confocal intensity-resolved (A, C and D) and time-resolved (B and E) imaging of eGFP-Mgat5 interaction with mRFP-NGT (C, D and E) in A375 cells in comparison with cells expressing eGFP-Mgat5 only (A and B). B and E show typical pseudocolored FLIM data representing average donor lifetimes across images. Red-to-blue color changes reflect shortening of the fluorescence lifetime. The rainbow scale bar placed next to time-resolved images (B and E) represents fluorescence lifetime range between 2.3 ns (blue) and 2.9 ns (red). Confocal intensity-resolved (F, H and I) and time-resolved (G and J) imaging of a control combination comprising non-interacting eGFP-B4 and mRFP-UGT2 (C, D and E) in MDCK-RCA′ cells in comparison with cells expressing eGFP-B4 only (F and G). The rainbow scale bar placed next to time-resolved images (G and J) represents fluorescence lifetime range between 2.5 ns (blue) and 3.5 ns (red). Mean GFP lifetime values in the absence and the presence of the acceptor are also presented (K and L). Data are shown as mean±standard deviation from several measurements of the indicated cell number. Statistically significant (Student’s t-test, p<0.001) reduction of GFP lifetime upon coexpression of fluorophore-tagged Mgat5 and NGT was demonstrated comparing with eGFP-Mgat5 alone (K). No difference in GFP lifetime was found when fluorophore-tagged B4 and UGT2 were coexpressed (L). Bar = 20 μm. GFP, green fluorescent protein; RFP, red fluorescent protein; τ, fluorescence lifetime; Mgat5, mannosyl (alpha-1,6-)glycoprotein beta-1,6-N-acetylglucosaminyltransferase; NGT, UDP-GlcNAc transporter; B4, UDP-xyllose/N-acetylglucosamine transporter; UGT2, UDP-galactose transporter (splice variant 2); A375, human melanoma cell line; MDCK-RCA′, Madin-Darby canine kidney II cells resistant to Ricinus communis agglutinin.

FIGURE 8. Schematic diagram showing selected pathways of N-glycosylation in the Golgi apparatus in mammalian cells. Blue squares, N-acetylglucosamine (GlcNAc); green circles, mannose (Man); red triangle, fucose (Fuc); Mgat1, mannosyl (alpha-1,3-)glycoprotein beta-1,2-N-acetylglucosaminyltransferase; Mgat2, mannosyl (alpha-1,6-)glycoprotein beta-1,2-N-acetylglucosaminyltransferase; Mgat4, mannosyl (alpha-1,3-)glycoprotein beta-1,4-N-acetylglucosaminyltransferase; Mgat5, mannosyl (alpha-1,6-)glycoprotein beta-1,6-N-acetylglucosaminyltransferase; Fut8, fucosyltransferase 8, alpha (1,6) fucosyltransferase, NGT, UDP-N-acetylglucosamine transporter (SLC35A3) delivering UDP-GlcNAc.
Figure 1A

A

MDCK – siNGT

1 2 3 4 5 6* Scr
NGT
CST

HeLa – siNGT

1 2* 4 5 6 Scr
NGT
CST

CHO – siNGT CHO-Lec8 – siNGT

Scr 1* 2* Scr 9* 19*
NGT
GFT

MDCK – siβ4GalT4

7* 14 18 Scr
β4GalT4
CST
Figure 1B
Figure 2
Figure 3
Figure 6
Figure 7
Figure 8
UDP-N-acetylg glucosamine transporter (SLC35A3) regulates biosynthesis of highly branched N-glycans and keratan sulfate
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J. Biol. Chem. published online June 13, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.460543

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