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UDP-Gal/UDP-GlcNAc chimeric transporter complements mutation defect in mammalian cells deficient in UDP-Gal transporter

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ABSTRACT

The role of UDP-galactose transporter (UGT; SLC35A2) and UDP-*N*-acetylglucosamine transporter (NGT; SLC35A3) in glycosylation of macromolecules may be coupled and either of the transporters may partially replace the function played by its partner. The aim of this study was to construct chimeric transporters composed of the N-terminal portion of human NGT and the C-terminal portion of human UGT1 or UGT2 (NGT-UGT1 or NGT-UGT2, respectively), as well as of the N-terminal portion of UGT and C-terminal portion of NGT (UGT-NGT), overexpress them stably in UGT-deficient CHO-Lec8 and MDCK-RCA^r cells, and characterize their involvement in glycosylation. Two chimeric proteins, NGT-UGT1 and NGT-UGT2, did not overexpress properly. In contrast, UGT-NGT chimeric protein was successfully overexpressed and localized properly to the Golgi apparatus. UGT-NGT chimeric transporter delivered UDP-Gal to the Golgi vesicles of UGT-deficient cells, which resulted in the increased content of galactosylated structures to such an extent that the wild-type phenotypes were completely restored. Our data further support our hypothesis that UGT and NGT cooperate in the UDP-Gal delivery for glycosyltransferases located in the Golgi apparatus. In a wider context, the results gained in this study add to our understanding of glycosylation, one of the basic posttranslational modifications, which affects the majority of macromolecules.

1. Introduction

Glycosylation is one of the most important 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). Sugars activated in the cytosol or nucleus are subsequently transported into the Golgi apparatus and/or ER by nucleotide sugar transporters (NSTs). NSTs are hydrophobic proteins with a molecular weight of 30–45 kDa [1,2], which function in the form of dimers or higher oligomers [3–8].

One of the best characterized NSTs is the UDP-galactose transporter (UGT; SLC35A2) [e.g., 8–16]. Two splice variants of UGT (UGT1 and UGT2) have been identified in human tissues, in the Chinese hamster ovary (CHO) and Madin-Darby canine kidney II (MDCK) cell lines [9–12,14]. Nonsense mutations identified in mutant cells, namely in MDCK cells resistant to *Ricinus communis* agglutinin (MDCK-RCA^r), CHO-Lec8 cells, and Had-1 cells, cause inhibition of UGT production, resulting in macromolecules enriched in terminal *N*-acetylglucosamine (GlcNAc) and deficient in terminal galactose (Gal) and sialic acid [11,13,16–21].

Within known UDP-*N*-acetylglucosamine transporters, NGT assigned as SLC35A3 is assumed to play a main role in glycosylation of macromolecules [22,23], while the function of SLC35D1 [24], SLC35D2 [25], and SLC35B4 [26] multi-specific transporters appears to be less important. Compared with SLC35A3, which is ubiquitously expressed, the above-mentioned NSTs are less common and rather tissue-specific.

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. This assumption has been based on the following findings: (i) both transporters are evolutionarily related [1,27,28], (ii) overexpression of NGT in UGT-deficient cells partially restores galactosylation [15], and (iii) NGT and UGT form heterologous complexes in the Golgi membrane [8]. To gain additional knowledge of the function played by N- and C-terminal domains of both transporters, we constructed chimeric proteins composed of the N-terminal portion of human NGT and the C-terminal portion of human UGT1 or UGT2 (NGT-UGT1 or NGT-UGT2, respectively), as well as of the N-terminal portion of UGT and the C-terminal portion of NGT (UGT-NGT), and analyzed their involvement in glycosylation in UGT-deficient cells.

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

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2. Materials and methods

2.1. Construction of chimeric transporters

Chimeric cDNAs constructed between human UGT1 (NM_001042498.2) or UGT2 (NM_005660.1) and NGT (NM_005660) were prepared using HsUGTGolgi2 [14], HsUGTER [14], and HA-HsNGT plasmids [8]. First, mutagenesis (QuikChange Multi Site-Mutagenesis Kit, Stratagene) with HsGlcNAcMut-Afl (5′GGTTTACTTTGAGAAAATCCTTAAGGAAACAAAACAATCAGTGTG-G3′) and HsGalMut-Afl (5′CTACTTTGAGAAGATCCTTAAGGGCAGCT CAGGCTCCG3′) primers was carried out to introduce the unique restriction site *cttaag* for the AflII in the original *ctcaaa* sequence in the human UGT1 and UGT2 or the original *ttaaaa* sequence in the human NGT in the conserved internal motif encoding Leu-Lys in these transporters (Fig. 1F). This procedure did not result in

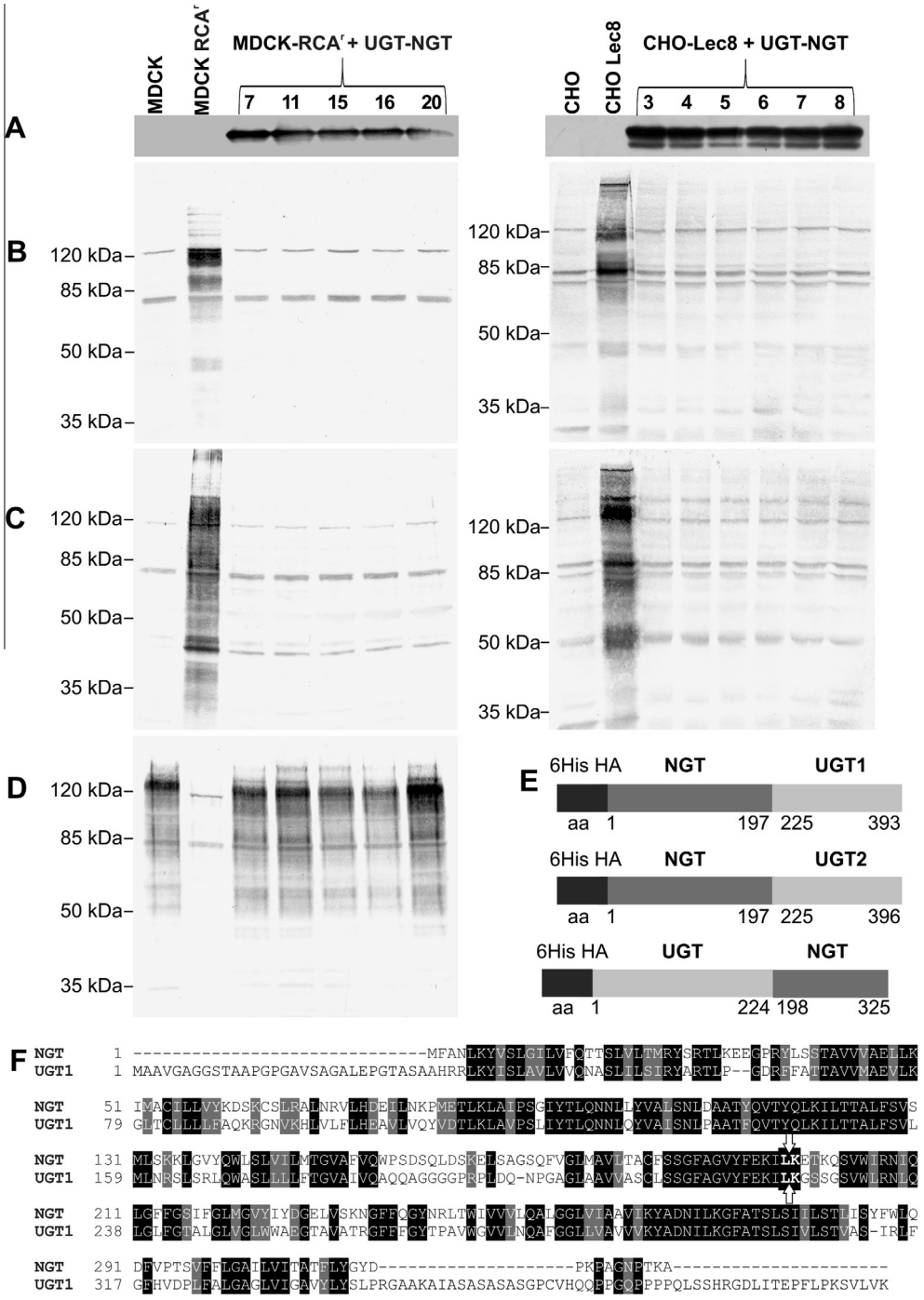

Fig. 1. Construction and overexpression of UGT-NGT chimeric transporter, and analysis of phenotypic correction of MDCK-RCA^r and CHO-Lec8 cells. (A) Western blotting of 6His-HA-tagged UGT-NGT chimeric transporter in mutant cells stably transfected with expression plasmid. Phenotypic correction determined using (B) GSL II (*Griffonia simplicifolia* lectin II specific for terminal *N*-acetylglucosamine), (C) VVL (*Vicia villosa* lectin specific for terminal *N*-acetylgalactosamine), and (D) MAL I (*Maackia amurensis* lectin I specific for terminal galactose and α2,3-sialic acid-galactose). Proteins present in cell lysates were separated by SDS–PAGE (20 μg) and transferred onto nitrocellulose membranes. Overexpressed UGT-NGT chimeric transporter was visualized with HRP-conjugated anti-HA antibody. Biotinylated lectins attached to glycoproteins were detected using alkaline phosphatase (AP)-conjugated avidin D. (E) Schematic diagram demonstrating construction of chimeric transporters. (F) Amino acid alignment of NGT and UGT1. Arrows indicate region used to ligate cDNAs encoding the N-terminal portion of UGT and the C-terminal portion of NGT. UGT, UDP-Gal transporter; NGT, UDP-*N*-acetylglucosamine transporter.

amino acid changes. To overexpress UGT-NGT chimeric protein, respective regions were digested with BamHI and AflII (UGT) or AflII and NheI (NGT), ligated and cloned into MCS1 of the pVitro1-neo vector (Invivogen) as described previously [8,14]. After final ligation, all constructs were sequenced (Genomed). A similar approach was employed to construct NGT-UGT1 and NGT-UGT2 chimeric transporters. The entire procedure allowed overexpression of the 6His-HA-tagged chimeric proteins (Fig. 1E). All restriction enzymes and the Rapid Ligation Kit were purchased from Fermentas.

2.2. Cell maintenance and transfection

MDCK, MDCK-RCA^r, CHO, and CHO-Lec8 cells were grown and transfected with expression plasmids as described previously [14]. Stable transfectants overexpressing chimeric proteins were selected in complete media containing 600 μg/ml (MDCK-RCA^r) or 500 μg/ml (CHO-Lec8) G-418 sulfate (InvivoGen).

2.3. Immunoreactivity and reactivity with lectins

Proteins present in cell lysates were subjected to SDS–PAGE using 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Whatman) as described previously [14]. 6His-HA-tagged fusion proteins were detected using horseradish peroxidase (HRP)-conjugated anti-HA antibody (Roche), and visualized with an enhanced chemiluminescence system (PerkinElmer). Reactivity of glycoproteins with lectins was performed as described previously [14,15].

2.4. 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 [15]. 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 purified, fluorescently labeled with 2-aminobenzamide (2-AB), and separated on a GlycoSep N column (Glyko) as described previously [15]. MALDI-TOF MS analysis was carried out in positive ion mode with Na^+ excess as previously reported using *N*-glycans subjected to neuraminidase treatment [15].

2.5. Subcellular fractionation and transport assay

The Golgi fraction was isolated from CHO, CHO-Lec8, and CHO-Lec8 cells overexpressing UGT-NGT chimeric transporter and UDP-Gal transport into Golgi vesicles was subsequently determined as described previously [14,15].

2.6. Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously [29] using MDCK-RCA^r cells overexpressing UGT-NGT chimeric transporter except that HA epitope was detected using a 1:100 dilution of rat anti-HA monoclonal antibody (Roche) followed by incubation with a 1:100 dilution of goat anti-rat Cy3-conjugated antibody (Abcam).

3. Results and discussion

3.1. UGT-NGT chimeric transporter localizes to the Golgi apparatus

UGT and NGT display significant homology and partial functional redundancy [1,8,15,27,28]. To gain further insight into their

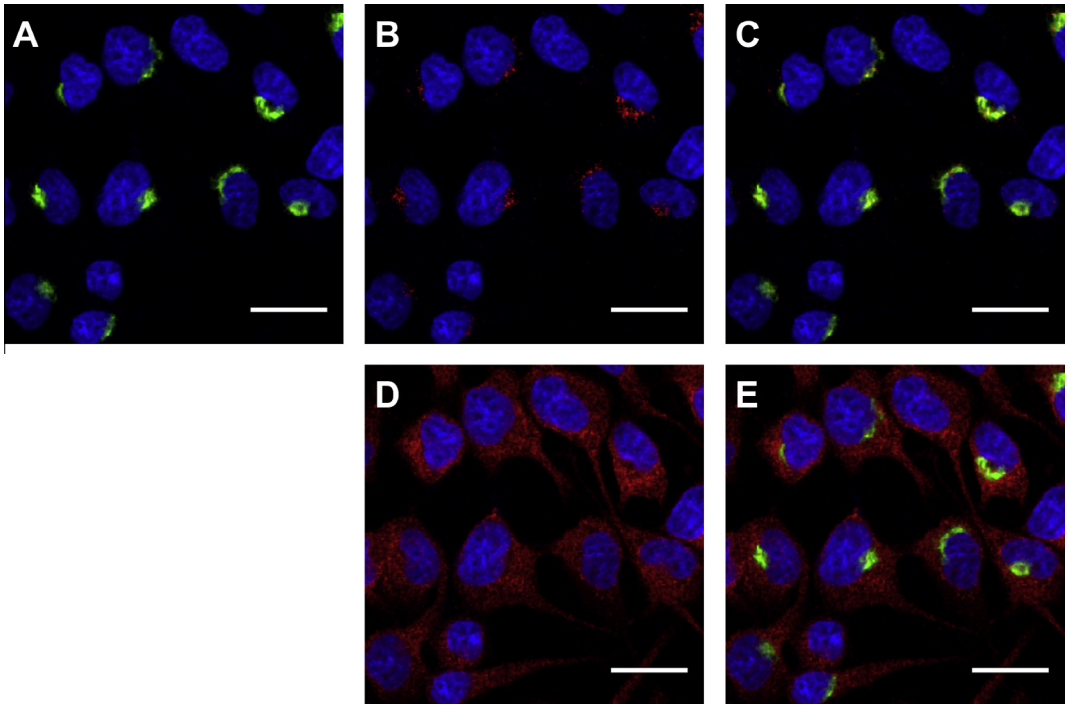

Fig. 2. Subcellular localization of UGT-NGT chimeric transporter in MDCK-RCA^r cells by indirect immunofluorescence. MDCK-RCA^r cells were stably transfected with an expression construct encoding 6His-HA-tagged UGT-NGT chimeric transporter and treated with antibodies as described in Section 2. (A) Reactivity with HA-specific antibodies, (B) reactivity with Golgi marker (GM130) antibodies, (C) overlay of A and B, (D) reactivity with ER marker (calnexin) antibodies, (E) overlay of A and D. Cell nuclei were counterstained with Hoechst 33342 dye. Bar = 20 μm.

function, we constructed three chimeric transporters (Fig. 1E) and overexpressed them stably in UGT-deficient cells. UGT-NGT chimeric transporter was successfully overexpressed in several clones (Fig. 1A) and localized properly to the Golgi apparatus as shown by immunofluorescence microscopic analysis (Fig. 2). In contrast, NGT-UGT1 and NGT-UGT2 chimeric transporters failed to overexpress, as confirmed by the lack of reactivity with antibodies directed to the tag examined by Western blotting and immunofluorescence microscopy (data not shown). It is possible that these chimeric proteins had not been properly folded.

3.2. UGT-NGT chimeric transporter delivers UDP-Gal to the Golgi vesicles

It has been shown by others that some regions of UGT could be substituted by CMP-sialic acid transporter (CST) counterparts or even deleted without loss of the UDP-Gal transporting activity [24,30]. It should be noted however that the majority of chimeric transporter (up to 265 amino acid residues) must be derived from UGT to preserve its activity [31,32]. In addition, it has been shown that helix 8 of UGT is crucial for its UDP-Gal transporting activity [30–32]. The specificity of those chimeras was confirmed by transport measurements in yeast vesicles and the lectin binding test. In our studies we first employed a transport assay in mammalian Golgi vesicles and found that the replacement of the C-terminal region of UGT (225–393 amino acid residues) by NGT (198–325 amino acid residues) did not destroy its UDP-Gal transporting activity. Compared with the UDP-Gal transport carried out in the CHO-Lec8 mutant cells (0.512 ± 0.111 pmol/mg/min), overexpression of UGT-NGT chimeric transporter in the mutant cells resulted in significantly increased UDP-Gal transport activity (1.814 ± 0.072 pmol/mg/min), comparable with that observed in the wild type CHO cells (2.244 ± 0.165 pmol/mg/min). Our data demonstrated that UGT-NGT chimeric transporter constructed in this study exhibits UDP-Gal transporting activity.

3.3. Overexpression of UGT-NGT chimeric transporter corrects mutant phenotype in UGT-deficient cells

Additionally we examined whether and to what extent UGT-NGT chimeric transporter would be able to correct mutant phenotypes of UGT-deficient cells. Therefore we compared glycosylation of cellular glycoproteins synthesized by the mutant cells alone or mutant cells overexpressing UGT-NGT chimeric transporter with glycoproteins produced by the wild-type cells. First we applied GSL II and VVL lectins, which are specific towards terminal GlcNAc and *N*-acetylgalactosamine (GalNAc), respectively. Due to defective galactosylation, glycoproteins synthesized by the MDCK-RCA^r and CHO-Lec8 mutant cells are significantly enriched in terminal GlcNAc in *N*-glycans and GalNAc in *O*-glycans, resulting in increased reactivity with GSL II and VVL lectins, respectively. As shown in Fig. 1B and C, overexpression of UGT-NGT chimeric transporter in both mutant cell lines decreased reactivity with GSL II and VVL lectins to the level typical of the wild-type cells, suggesting complete correction of the galactosylation defect in terms of both *N*- and *O*-glycosylation. An increase of Gal-specific MAL I lectin reactivity with glycoproteins synthesized by the MDCK-RCA^r cells overexpressing UGT-NGT chimeric transporter further confirmed restoration of galactosylation (Fig. 1D).

MDCK cells are not the best model for glycosylation analysis since they produce mostly *N*-glycans of high-mannose type [15]. Therefore, in this study CHO and CHO-Lec8 cells were used to perform detailed oligosaccharide analysis. Complete correction of the mutant phenotype was clearly visible in *N*-glycan profiles obtained after separation on a GlycoSep N column (Fig. 3). In an attempt to determine the extent of phenotypic correction more precisely, we performed a detailed MALDI-TOF MS analysis of desialylated *N*-glycans. Surprisingly, overexpression of UGT-NGT chimeric transporter increased the content of galactosylated structures in both UGT-deficient cell lines to such an extent that the wild-type phenotypes were completely restored (Fig. 4).

3.4. Biological implications

Taking into account our previous data concerning phenotypic effects of UGT and NGT overexpression in UGT-deficient cells [14,15], it can be concluded that UGT-NGT chimeric transporter could be as effective as UGT and much more effective than NGT in delivering UDP-Gal to the Golgi apparatus. It is worth noting that replacement of the C-terminal portion of UGT by the NGT counterpart may suggest that the C-terminal domain present in both transporters may play a similar function in UDP-Gal delivery to the Golgi apparatus and subsequent galactosylation. It should also be emphasized that, in contrast to UGT-NGT chimeric transporter, NGT overexpression in UGT-deficient cells partially restored galactosylation only in terms of *N*-glycans [15], while *O*-glycans remained significantly

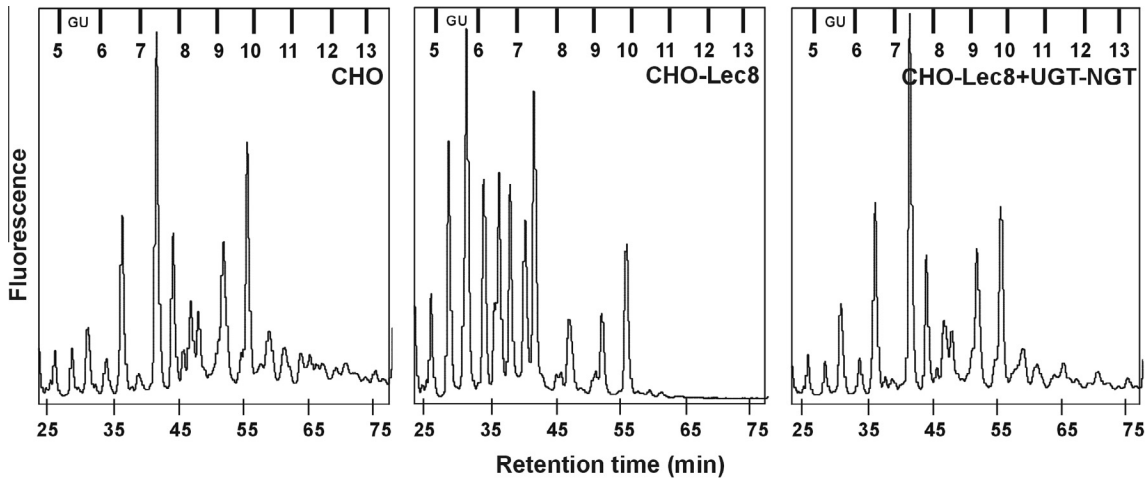

Fig. 3. Analysis of *N*-glycan profiles. *N*-Glycans were enzymatically released from glycoproteins produced by the cells, fluorescently labeled with 2-AB, purified and separated on the GlycoSep N column using HPLC [15]. Representative data out of 2 independent measurements with a similar tendency from selected clones are shown. CHO, wild-type Chinese hamster ovary cells; CHO-Lec8, CHO mutant cells lacking functional UDP-Gal transporter; CHO-Lec8 + UGT-NGT, CHO-Lec8 cells overexpressing UGT-NGT chimeric transporter; GU, glucose units.

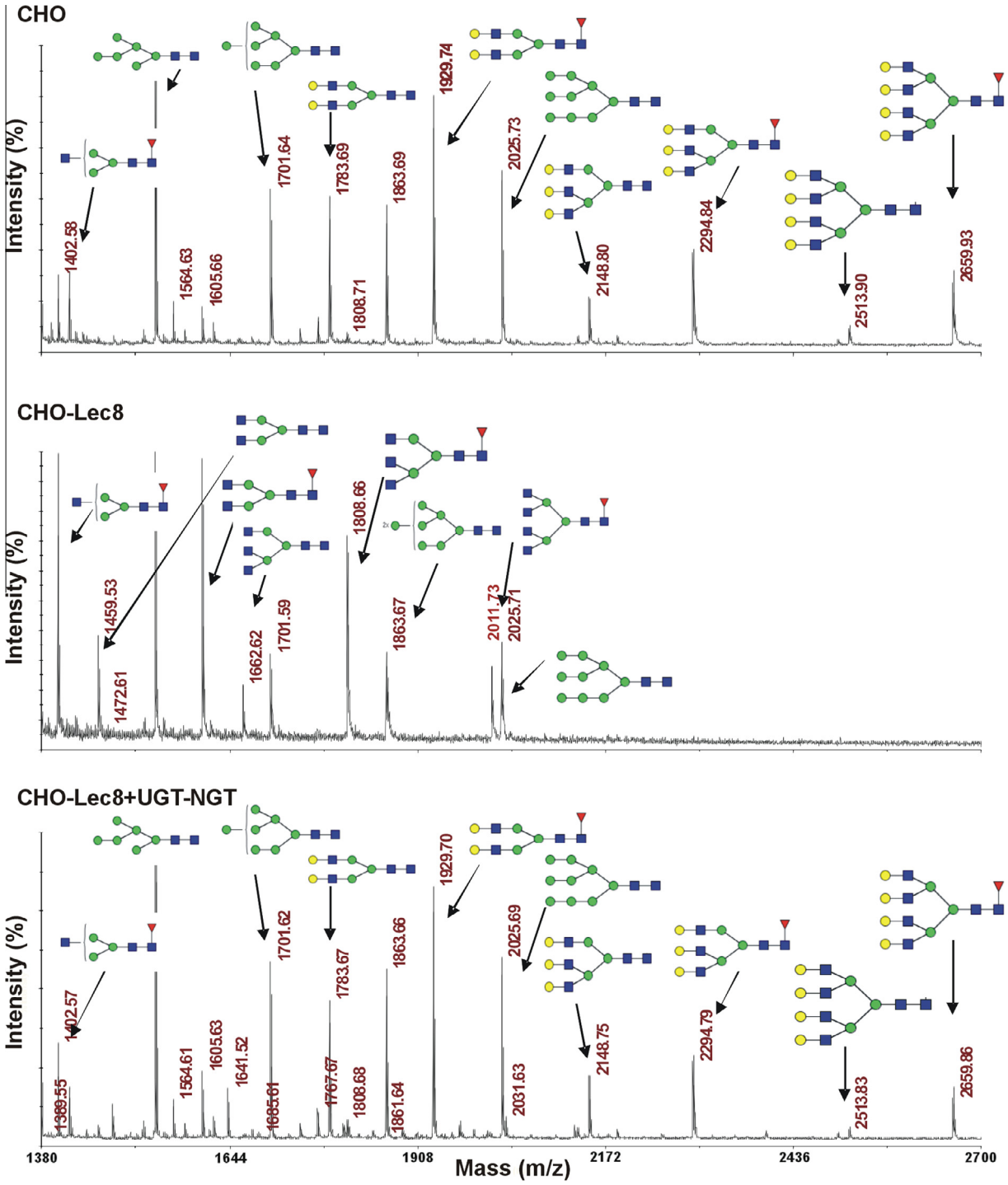

Fig. 4. Structure analysis of *N*-glycans. 2-AB labeled *N*-glycans derived from the cells were treated with neuraminidase and subjected to MALDI-TOF MS analysis carried out in positive ion mode with Na^+ excess. Glycans were identified by comparing experimental data expressed in GU resulting from separation 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 [15]. Representative data out of 2 independent measurements with a similar tendency at the mass region exhibiting the most profound differences from selected clones are shown. Blue squares, *N*-acetylglucosamine (GlcNAc); green circles, mannose (Man); yellow circles, galactose (Gal); red triangles, fucose (Fuc). CHO, wild-type Chinese hamster ovary cells; CHO-Lec8, CHO mutant cells lacking functional UDP-Gal transporter; CHO-Lec8 + UGT-NGT, CHO-Lec8 cells overexpressing UGT-NGT chimeric transporter. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this book.)

undergalactosylated (our unpublished data). One possible explanation for this phenomenon may be that the N-terminal portion of UGT corresponding to UGT-NGT chimeric transporter might be involved in some as yet unidentified interactions with galactosyltransferases mediating *O*-glycan biosynthesis.

Data obtained from the overexpression of UGT-NGT chimeric transporter further supports our hypothesis that UGT and NGT cooperate in the UDP-Gal delivery for glycosyltransferases located in the Golgi apparatus. In a wider context, the results gained in this study add to our understanding of glycosylation, one of the basic

posttranslational modifications, which affects the majority of macromolecules.

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