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A novel fluorescent assay for T-synthase activity

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Loss of T-synthase (uridine diphosphate galactose:*N*-acetylgalactosaminyl-α1-Ser/Thr β3galactosyltransferase), a key enzyme required for the formation of mucin-type core 1 *O*-glycans, is observed in several human diseases, including cancer, Tn syndrome and IgA nephropathy, but current methods to assay the enzyme use radioactive substrates and complicated isolation of the product. Here we report the development of a novel fluorescent assay to measure its activity in a variety of tumor cell lines. Deficiencies in T-synthase activity correlate with mutations in the gene encoding the molecular chaperone Cosmc that is required for folding the T-synthase. This new high-throughput assay allows for facile screening of tumor specimens and other biological material for T-synthase activity and could be used diagnostically.

Keywords: Cosmc / fluorescent assay / glycosyltransferase assay / 4-methylumbelliferone / T-synthase

Introduction

Protein glycosylation provides glycans important in many biological processes, including signaling, cell–cell interaction, growth regulation, differentiation, angiogenesis and adhesion (Varki 1993; Leppanen et al. 1999; Xia et al. 2004; Ohtsubo and Marth 2006; Fu et al. 2008; Marth and Grewal 2008). Thus, it is not surprising that acquired and inherited mutations in glycosylation pathways, as seen in Tn syndrome, congenital muscular dystrophies and congenital disorders of glycosylation (CDG), are associated with disease and pathology (Berger 1999; Ju and Cummings 2005; Endo and Manya 2006; Freeze 2007; Jaeken and Matthijs 2007; Reed 2009a, 2009b). Particular emphasis has been directed to *O*-glycans based on sequences deriving from the precursor Tn antigen (GalNAcα1-Ser/Thr), since this antigen is expressed abnormally in several diseases and disorders (Cartron and Nurden 1979; Springer 1984; Springer et al. 1985; Allen et al. 1997; Berger 1999; Blanchard et al. 2008).

The Tn antigen is a normal endogenous substrate for several enzymes, most notably the T-synthase (uridine diphosphate galactose:*N*-acetylgalactosaminyl-α1-Ser/Thr β3galactosyltransferase), a β3-galactosyltransferase that transfers D-galactose (Gal) from uridine diphosphate galactose (UDP-Gal) to the Tn antigen to form the core 1 *O*-glycan Galβ1-3GalNAcα1-Ser/Thr (Ju, Brewer et al. 2002; Ju and Cummings 2005; Ju, Lanneau et al. 2008). A unique feature of vertebrate T-synthase is its requirement for a specific molecular chaperone, termed Cosmc, to promote correct folding in vivo (Ju and Cummings 2002; Ju, Aryal et al. 2008). Deficiencies of the T-synthase activity have been linked to acquired, somatic mutations in the X-linked *Cosmc* (Ju and Cummings 2005; Ju, Lanneau et al. 2008).

Typical assays of glycosyltransferases are difficult, since they commonly utilize radioactive nucleotide sugar donors. T-synthase activity is commonly assayed using radiolabeled UDP-Gal by measuring the ^{3}H- or ^{14}C-Gal incorporated into acceptor glycopeptides, phenyl-α-GalNAc or benzyl-α-GalNAc, followed by separation techniques to remove unreacted nucleotide sugars (Mendicino et al. 1982; Furukawa and Roth 1985; Granovsky et al. 1994; Ju, Cummings et al. 2002; Ju and Cummings 2010). Such approaches are time-consuming and impractical, especially for high-throughput screening. To address this problem, we have developed a sensitive fluorescent method for assessing T-synthase activity and show its utility to quantify T-synthase activity in a variety of biological samples. We also genetically characterize several human Jurkat leukemic cell lines with regard to mutations in the *Cosmc* gene and the effects on T-synthase activity.

Results

GalNAc-α-(4-methylumbelliferone) as acceptor for T-synthase

The potential assay method for T-synthase utilizes GalNAc-α-(4-methylumbelliferone) (GalNAc-α-(4-MU)) as its acceptor substrate and UDP-Gal as a donor to form Galβ1-3GalNAc-α-(4-MU) (Figure 1A). The reaction product is cleaved by endo-α-*N*-acetylgalactosaminidase (*O*-glycosidase) to release free 4-MU, which is highly fluorescent (Figure 1A). This enzyme is specific for the release of the Galβ1-3GalNAc disaccharide from *O*-glycans linked to aglycones (Kobata

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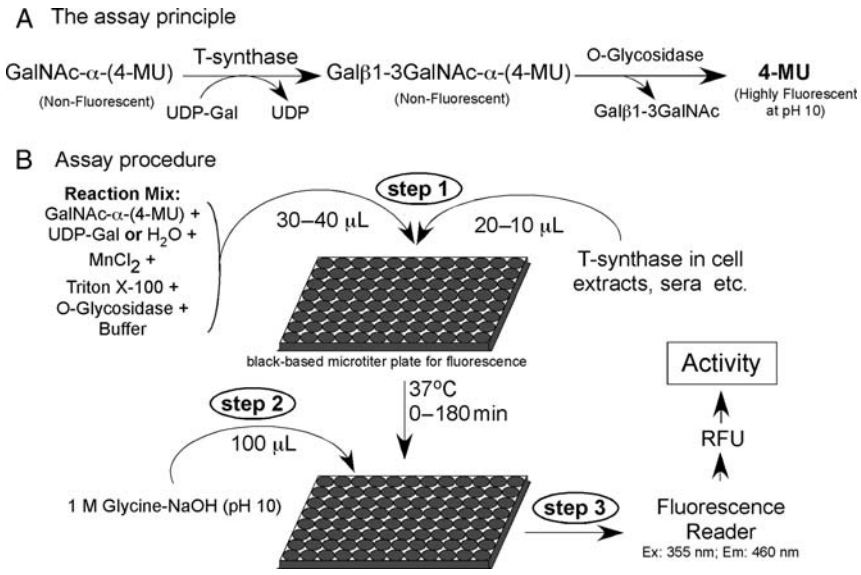

Fig. 1. Schematic illustration of the fluorescent assay for T-synthase activity. (**A**) The principle of the method: T-synthase utilizes GalNAc-α-(4-MU) as its acceptor substrate and UDP-Gal as a donor to form Galβ1-3GalNAc-α-(4-MU), which is subsequently quantitatively cleaved by *O*-glycosidase to release highly fluorescent 4-MU. The fluorescence intensity of 4-MU represents the amount of T-synthase product. (**B**) The procedure of the fluorescent T-synthase assay: reaction mixtures including the acceptor, donor or H_2O, divalent cation, detergent, buffer and *O*-glycosidase are prepared and aliquot into the 96-well black plate, and cell extracts or sera are added to the corresponding wells. The reaction is incubated at 37°C for a certain period of time, the stop solution was added, the fluorescence of 4-MU is measured and the activity of T-synthase is directly calculated in terms of pmol product over time and per protein concentration.

1979). The experimental procedure requires three simple steps (Figure 1B).

To define the feasibility of this assay, we tested reaction mixtures containing GalNAc-α-(4-MU) as an acceptor and either purified human recombinant T-synthase co-expressed with Cosmc in Hi-5 cells or extracts from these cells (Figure 2A). The fluorescent intensity in the reaction is high in the presence of all four critical components (acceptor, donor, T-synthase and *O*-glycosidase) and in the presence of ~1500 pmol/h purified enzyme, as expected, generated much higher relative fluorescence unit (RFU; ~900,000) than that in the presence of ~500 pmol/h enzyme (~300,000) from the cell extracts. Importantly, the signal from the blank (no UDP-Gal) is very low and the background fluorescence levels (reactions without *O*-glycosidase, without enzyme or with enzyme alone) are also very low. These results show that GalNAc-α-(4-MU) is an acceptor for T-synthase and that the signal of 4-MU is derived from the enzymatic hydrolysis of the T-synthase product.

To further characterize the reaction, all compounds from the overnight reactions in the absence of *O*-glycosidase were purified on C18 cartridges, and then analyzed on LC-MS. The standard GalNAc-α-(4-MU) was eluted as a single peak at 9.14 min (Figure 2B). The C18-purified material from the blank reaction without UDP-Gal gave a major peak with elution time at 9.14 and minor peak at 10.75 min, representing the acceptor substrate and 4-MU, respectively (Figure 2C), as confirmed by the 4-MU standard (Figure 2D). The free 4-MU likely results from the hydrolysis of GalNAc-α-(4-MU) by endogenous lysosomal α-*N*-acetylhexosaminidase overnight incubation, which is consistent with the observation (Figure 2A) that the reaction using Hi-5 cell extracts gave a higher background than the purified enzyme. The monosaccharide GalNAc [*N*-acetylgalactosamine (2-acetamido-2-deoxy-Gal)] was lost during purification using C18 cartridge, because it does not bind to the C18 cartridge. Since those compounds were detected by LC-MS, it is difficult to precisely quantify each of them; therefore, we use the terms "major" or "minor" to refer to their abundance. These results indicate that the acceptor GalNAc-α-(4-MU) is stable except for very minor hydrolysis by the presumed endogenous lysosomal glycosidase (Figure 2C). *O*-Glycosidase treatment did not change the profile of this material, indicating that this *O*-glycosidase lacks significant exoglycosidase activity (Figure 2E). The material purified from the reaction in the presence of UDP-Gal gave a major peak at elution time 8.88 min, which was the expected product of T-synthase, Galβ1-3GalNAc-α-(4-MU) (Figure 2F), and two other peaks, the unreacted acceptor and 4-MU (Figure 2C). Furthermore, after treatment with *O*-glycosidase, the product peak Galβ1-3GalNAc-α-(4-MU) disappeared, and one new peak emerged at 0.63 min corresponding to Galβ1-3GalNAc, which is hydrophilic, and was eluted in the void volume, along with increased free 4-MU and unchanged acceptor (Figure 2G). *O*-Glycosidase specifically cleaves core 1 *O*-glycan to release Galβ1-3GalNAc from glycopeptides, glycoproteins and artificial carriers (Kobata 1979; Fujita et al. 2005; Goda et al. 2008; Suzuki et al. 2009; Willis et al. 2009). Thus, the disaccharide structure Galβ1-3GalNAc of the product is based on both the co-chromatography with standard disaccharide and the specificity of the *O*-glycosidase. Taken together, these results demonstrate that GalNAc-α-(4-MU) is an acceptor substrate for T-synthase and the assay is specific for T-synthase.

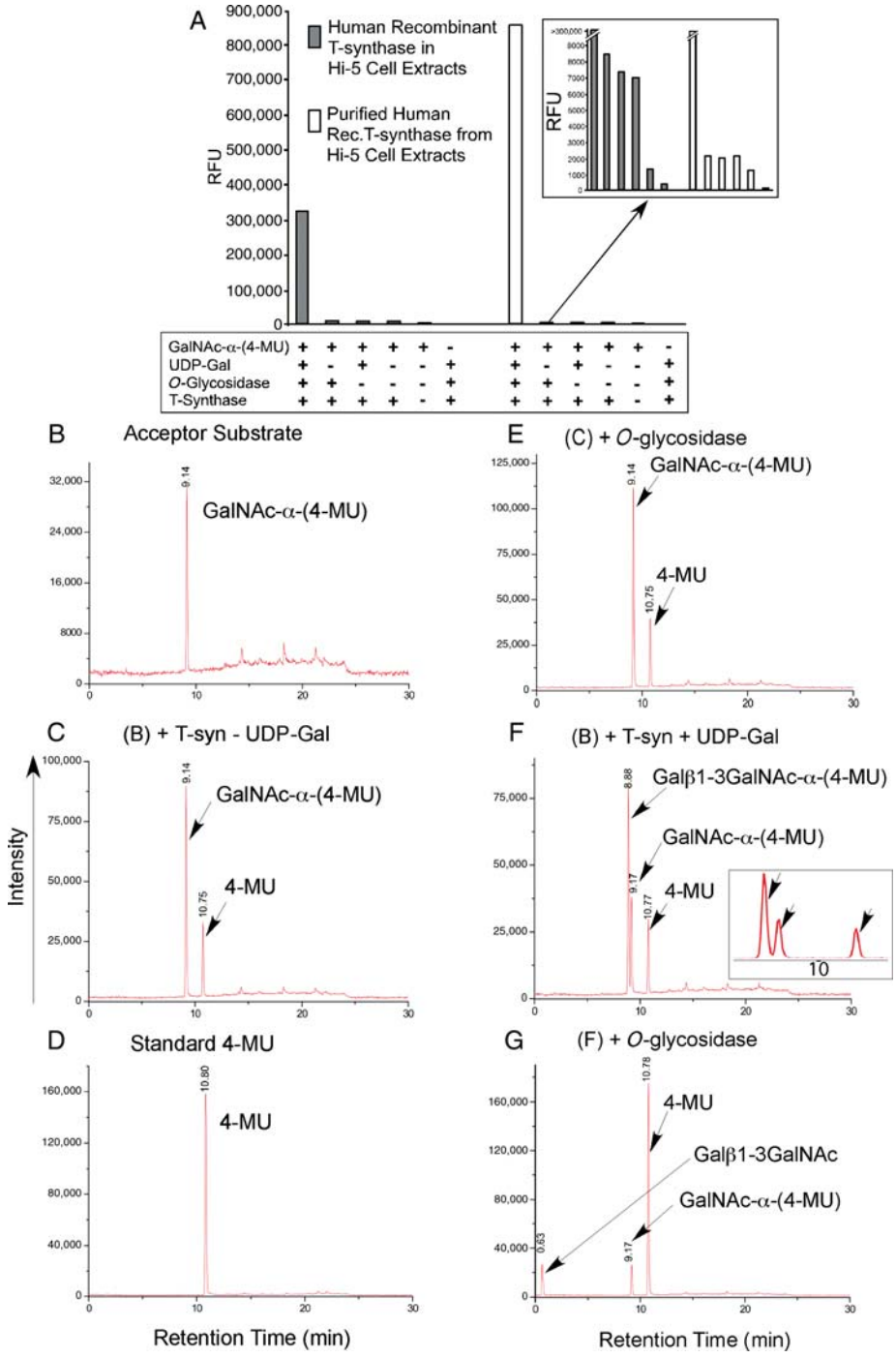

Fig. 2. GalNAc-α-(4-MU) can serve as an acceptor for T-synthase. (**A**) Fluorescent intensity: 4-MU fluorescence from the reactions, blanks and backgrounds using both Hi-5 cell extracts containing 500 pmol/h human recombinant T-synthase and 1500 pmol/h purified recombinant T-synthase as enzyme source. The experiments were performed in duplicate. The inset shows the graph of RFU up to 9000. (**B**–**G**) LC-MS data of the acceptor and the T-synthase product: T-synthase reactions with and without the donor UDP-Gal were set-up in the absence of *O*-glycosidase and incubated overnight at 37°C. The reactions were chromatographed on C18 cartridges, and the bound material was eluted and dried, then redissolved in 50 μL of H_2O and analyzed on LC-MS equipped with a C18 column. (B) Acceptor GalNAc-α-(4-MU) standard; (C) the C18 cartridge-purified material from the blank reaction (without UDP-Gal); (D) the standard 4-MU; (E) sample from (C) treated with *O*-glycosidase; (F) the C18 cartridge-purified material from the T-synthase reaction (with all of the components); the inset shows an expansion of the three major peaks; (G) sample from (F) treated with *O*-glycosidase.

Product characterization

Although the results from Figure 2 showed that the product from the T-synthase reaction was Galβ1-3GalNAc-α-(4-MU), as indicated by the LC-MS profile and specific cleavage by *O*-glycosidase, we also sequenced the products by ESI-MS/MS. The profile of standard GalNAc-α-(4-MU) displayed a

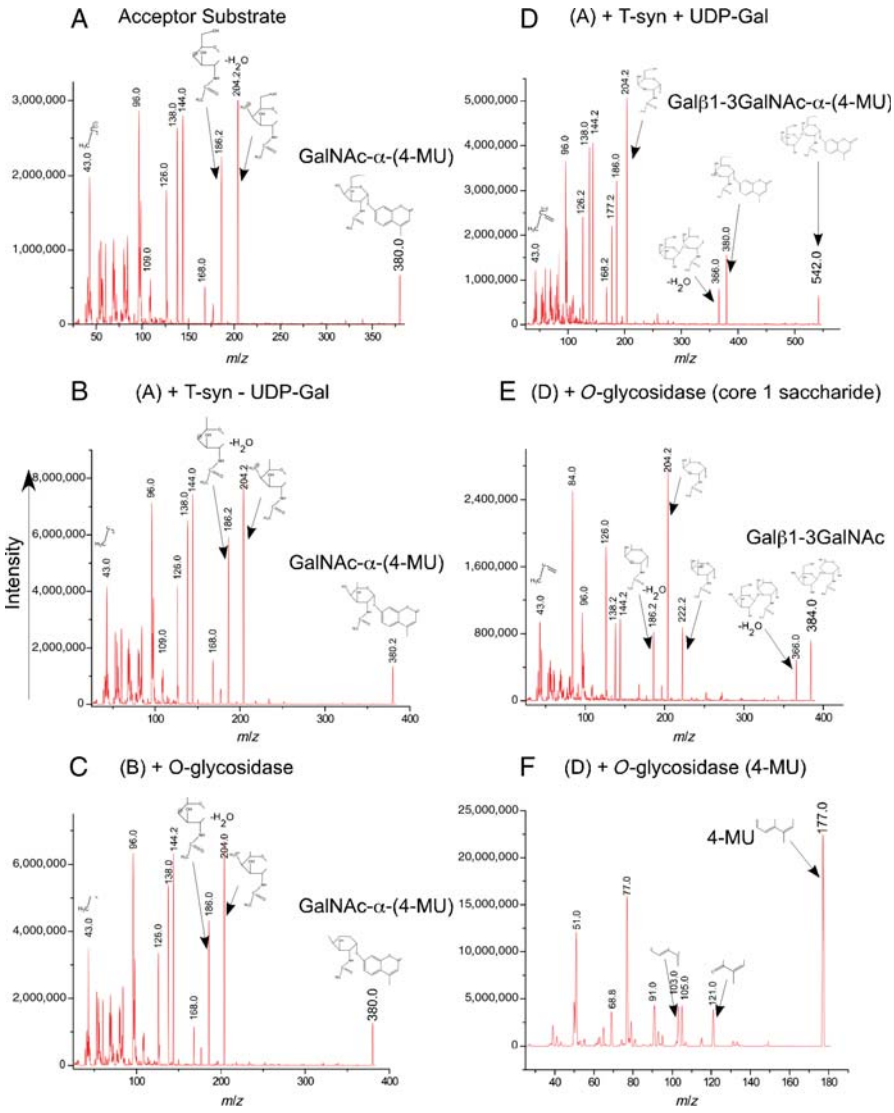

Fig. 3. Characterization of the product by ESI-MS/MS: the standard GalNAc-α-(4-MU) and purified material from T-synthase reactions as in Figure 2C, D, F and G were subjected to ESI-MS/MS analyses. (**A**) The fragmentation of standard acceptor GalNAc-α-(4-MU) (380.0 Da); (**B**) The fragmentation profile of the C18 cartridge-purified material (380.2 Da) from the blank reaction (without UDP-Gal); (**C**) the profile of sample in (B) treated with *O*-glycosidase; (**D**) the fragmentation profile of C18 cartridge-purified material from the T-synthase reaction (with all of the components); (**E**) the MS/MS data of disaccharide (384.0 Da) from the treatment of the product (542.0 Da) with *O*-glycosidase; (**F**) the MS/MS data of 4-MU (177.0 Da) from the product (542.0 Da) treated with *O*-glycosidase.

peak of 380 Da representing the intact molecule, as well as fragments with smaller mass such as 43 Da (acetyl group), 204.2 Da (acetylgalactosaminyl group) and others (Figure 3A). The material from the blank reaction (without UDP-Gal) regardless of treatment with *O*-glycosidase showed the same profiles (Figure 3B and C) as the standard (Figure 3A). The product purified from T-synthase reaction appeared with the expected size of 542.0 Da, corresponding to Galβ1-3GalNAc-α-(4-MU) (Figure 3D). The MS/MS profile gave 380, 366.0 and 177.0 Da fragments, which are presumably the acceptor, dehydrated disaccharide and 4-MU respectively. All other fragments from the GalNAc-α-(4-MU) were also seen, comparable with Figure 3A–C. After treatment of *O*-glycosidase, the 542.0 Da product disappeared, and the disaccharide (384 Da) and 4-MU (177.0 Da) appeared. The MS/MS confirmed that this 384 Da peak was Galβ1-3GalNAc with the correct fragmentation pattern, including the GalNAc residue (222.2 Da), dehydrated GalNAc residue (204.2 Da) and even double dehydrated GalNAc (186.2 Da; Figure 3E). The 177.0 Da peak corresponds to 4-MU (Figure 3F) with the expected fragmentation pattern. The galactose residue from the product was not seen by MS/MS, probably because free galactose is not ionized well in MS, unlike its amino sugar galactosamine. These results demonstrate that T-synthase synthesizes Galβ1-3GalNAc-α-(4-MU) from the acceptor GalNAc-α-(4-MU) and donor UDP-Gal and that *O*-glycosidase specifically cleaves the product, but not GalNAc-α-(4-MU).

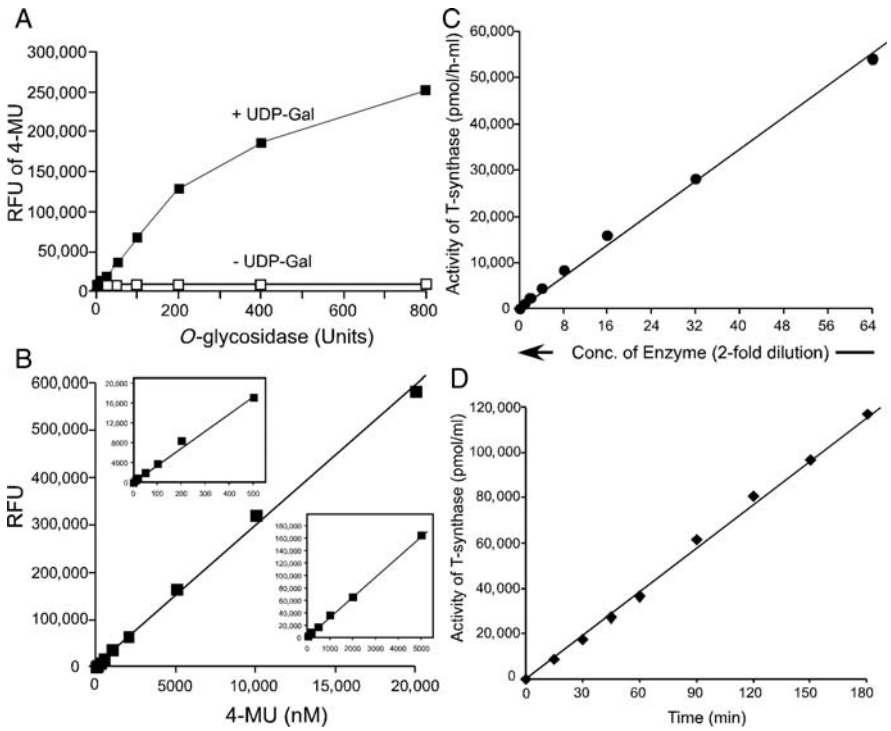

Fig. 4. Characterization of the method: (**A**) *O*-glycosidase curve: the T-synthase reactions were set-up in the presence of different amounts (0–800 U) of *O*-glycosidase. The reactions were incubated at 37°C for 60 min, and the stop solution was added and the fluorescence united was measured. RFU of 4-MU was plotted versus the *O*-glycosidase concentration. (**B**) Standard curve of 4-MU: 50 µL of a serial concentration of standard 4-MU solutions ranging from 10 to 20,000 nM in triplicate were pipetted into plates and incubated at 37°C for 60 min and 100 µL of stop solution was added. The RFU were measured and plotted with corresponding concentrations of 4-MU. (**C**) Enzyme concentration curve: a serial dilution of the cell extracts from Hi-5 cell co-expressing human recombinant T-synthase and wild-type Cosmc were incubated with the reaction mixture. The 4-MU fluorescence was measured after incubation at 37°C for 60 min and plotted with the enzyme concentration. (**D**) Time course: the reaction with Hi-5 cell extracts containing human recombinant T-synthase was set up in the same tube and 50 µL of reaction mixtures were aliquot into the fluorescent plate. Reactions were incubated at 37°C from 0 to 180 min until addition of stop solution as indicated. The fluorescence of 4-MU was measured at the same time and plotted with the incubation time (min). All of the experiments were performed in duplicates or triplicates.

Determining amount of O*-glycosidase required in the assay*

The T-synthase activity is quantified by the fluorescent intensity of 4-MU released from its product by *O*-glycosidase; thus, the accuracy of this assay relies on the sufficiency of the *O*-glycosidase in the reaction. In the presence of different amounts of *O*-glycosidase, free 4-MU intensity was linear within a range of 0–200 units of *O*-glycosidase, indicating that 200 units *O*-glycosidase is insufficient to cleave all product of T-synthase (Figure 4A). With the amount of *O*-glycosidase above 400 units per reaction, the 4-MU intensity approached a plateau, which reflects the activity (~400 pmol/h, calculated based on 1 pmol 4-MU = 600 RFU as determined below) of T-synthase. Therefore, 800 units of *O*-glycosidase were used as the standard assay, in which the T-synthase, range of 0–800 pmol/h can be measured accurately. Within the preparation of *O*-glycosidase, there are only traces of exo-α-*N*-acetylhexosaminidase activity, which does not disturb this assay. These results not only define the amount of *O*-glycosidase required for this method, but also demonstrate that the assay specifically reflects the activity of T-synthase.

4-MU standard curve

To determine the specific activity of 4-MU, the sensitivity of the fluorescent method, and the linear range of 4-MU, serial concentrations of 4-MU were measured for their RFU. The lowest detectable concentration of 4-MU in the current assay condition was 20 nM (1 pmol), and the RFU was linear with 4-MU concentration up to 20,000 nM (1000 pmol), giving a specificity of 4-MU around 600 RFU/pmol (Figure 4B, insets).

Kinetics of T-synthase reaction

We also determined the linear range for the time- and enzyme concentration. Under the defined assay conditions, the reaction product formation was linear with respect to enzyme concentration from 500 to 60,000 pmol/h-mL (Figure 4C), through 3 h of incubation (Figure 4D).

Comparison to UDP-[^{3}*H]-Gal method and sequencing* Cosmc *in Jurkat I 2.1 and I 9.2 cells*

To substitute the common radioactive methods with this new fluorescent assay, we sought to compare the sensitivity of these two approaches using purified T-synthase, renatured and refolded T-synthase by Cosmc in vitro and T-synthase in cell extracts by the two methods in parallel. The purified recombinant T-synthase had comparable activity measured by both methods (Figure 5A). We observed that the radioactive

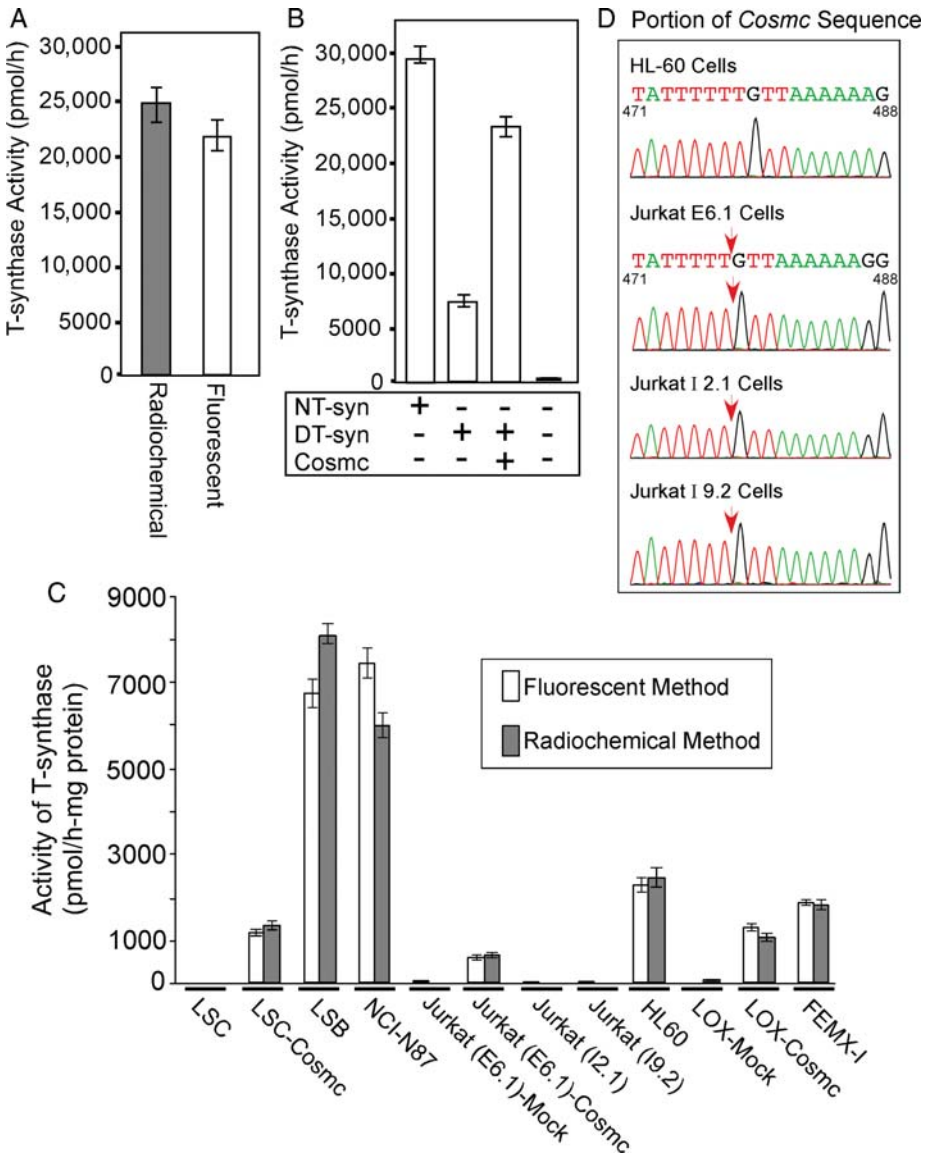

Fig. 5. Application of the fluorescent method for assaying T-synthase activity: (**A**) purified recombinant T-synthase: approximately 0.25 μg of purified T-synthase co-expressed with Cosmc in Hi-5 insect cells was assayed for its activity by using the 4-MU fluorescent method and the radioactive method. The experiments were carried out in five replicates and averaged, and the activity of T-synthase (mean ± SD) obtained from both methods was compared. (**B**) The 4-MU method was used for studying in vitro reconstitution of denatured T-synthase: purified recombinant T-synthase was thermally denatured at 54°C for ~2 min. Reconstitution was carried out with or without addition of Cosmc and T-synthase activity was measured. The experiment was performed in four replicates and averaged. (**C**) The activity of T-synthase in the cell extracts from cell lines: the cell extracts were made from the cell lines indicated and assayed for T-synthase activity in triplicates by the 4-MU fluorescent method and the radioactive method. The specific activity of T-synthase from the same cell lines by those two methods was compared side-by-side on bar graphs. (**D**) Comparison of the Sequence of *Cosmc*: the genomic DNA from HL60, Jurkat E6.1 and Jurkat clones I 2.1 and I 9.2 were isolated from the cells and the open reading frame of *Cosmc* was amplified by PCR. The PCR product was purified and subjected to direct sequencing. The mutated region within the *Cosmc* sequence is shown.

method gave slightly higher activity of this recombinant T-synthase. This difference could be due to the high amount of enzyme or activity that was used in these assays in which any technical variation could result in this difference. Furthermore, by means of this fluorescent assay, thermally denatured recombinant T-synthase lost more than 60% activity; after renaturation by incubating with purified Cosmc, the T-synthase activity was recovered significantly (Figure 5B), consisting with our earlier observation (Aryal et al. 2010). These results indicate that the fluorescent method is a suitable replacement method for the radiochemical approach for assaying T-synthase activity.

To evaluate the utilization of this fluorescent method for assaying T-synthase activity in mammalian cell extracts, we chose a variety of cell lines with different levels of T-synthase activity. There was good agreement in the results between the two assay approaches, especially for cells containing a moderate level of T-synthase activity, such as Cosmc-transfectants

LSC-Cosmc, Jurkat-Cosmc and LOX-Cosmc cells, as well as HL60 and FEMX-I cells (Figure 5C). Mock-transfected Jurkat E6.1, human colorectal carcinoma LSC and human melanoma LOX cells have little to no T-synthase activity due to mutations in *Cosmc* resulting in an inactive T-synthase (Ju and Cummings 2002; Ju, Lanneau et al. 2008). Both methods gave similar sensitivity, detecting as low as 1–2 pmol/h of enzyme. Introduction of wild-type *Cosmc* into these cells restored the T-synthase activity, shown in both methods. However, there were some differences noted in cell lines with very high activity of T-synthase, such as the human colorectal carcinoma cells, LSB and NCI-87. Since the radioactive assays by necessity are performed in lower nucleotide sugar concentrations compared with the fluorescent assay, differences between these cell lines in nucleotide sugar stability and/or product stability could account for some of these differences. In any case, it is important when attempting to define that total T-synthase activity by either method to re-measure activities after an appropriate dilution.

Interestingly, both methods revealed that Jurkat clones I2.1 and I9.2 had very low T-synthase activity, as seen in Jurkat clone E6.1 which has a mutated *Cosmc* resulting in an inactive T-synthase in these cells in our previous studies (Ju and Cummings 2002). To explore whether clones I2.1 and I9.2 had a mutation in *Cosmc* and whether the mutation was congruent to the E6.1 clone, we performed PCR and sequencing of *Cosmc*. Although HL60 cells contain wild-type *Cosmc*, both Jurkat I2.1 and I9.2 cells contained the same mutation in *Cosmc* as in Jurkat E6.1 (mock-transfected), which has a T-deletion at 478 in its nucleotide sequence (Figure 5D). This mutation results in truncated Cosmc with little chaperone activity for T-synthase as shown in our earlier study (Ju and Cummings 2002). This is the first evidence that all three Jurkat cell clones tested here carry the identical mutation in *Cosmc*.

Discussion

The T-synthase is an essential glycosyltransferase in the mucin-type *O*-glycan biosynthesis. *O*-Glycans play important roles in many biological processing, such as leukocyte recruiting (McEver et al. 1995; McEver and Cummings 1997; Leppanen et al. 1999; Sperandio 2006), lymphocyte homing (Yeh et al. 2001; Leppanen et al. 2003), T cell differentiation (Fukuda and Carlsson 1986), angiogenesis (Xia et al. 2004) and lymphatic vessel development (Fu et al. 2008). Deficiency of T-synthase activity is directly associated with some human diseases, such as Tn syndrome (Cartron and Nurden 1979; Berger 1999), IgA nephropathy (Allen et al. 1997) and cancer (Springer 1984; Springer et al. 1985; Blanchard et al. 2008). Being able to easily assess T-synthase activity by the method described here will be beneficial not only to glycobiology research laboratories, but also to clinical labs that may be able to utilize this assay for diagnostic purposes. Furthermore, this method will make it possible to screen for inhibitors and activators of T-synthase to modulate *O*-glycan structures and functions and to discover chemical chaperones that might mimic Cosmc function for T-synthase for novel therapeutics. The latter is especially important, since several significant human diseases are caused by somatic mutations in *Cosmc* or alterations in *Cosmc* expression that results in the loss of T-synthase activity (Ju and Cummings 2002, 2005; Schietinger et al. 2006; Crew et al. 2008; Qin et al. 2008; Inoue et al. 2010; Wang et al. 2010; Yamada et al. 2010).

To readily enable such measurements, we have developed the fluorescent method for measuring T-synthase activity, which can replace the cumbersome and the expensive radioactive assays. Our results show that T-synthase efficiently utilizes GalNAc-α-(4-MU) as its acceptor substrate and transfers Gal from UDP-Gal to form Galβ1-3GalNAc-α-(4-MU), which can then be specifically and quantitatively hydrolyzed by *O*-glycosidase to release the highly fluorescent 4-MU (Figure 1A and B). This method is linear over a wide range of enzyme concentrations and reaction time and its sensitivity is comparable with the assay using the radiochemical method and can be used to assay the activity of the enzyme in cell extracts.

Several different methods for T-synthase activity have been used by many groups, but most involve radioactive substrates or donors and/or HPLC or complex separation technologies (Mendicino et al. 1982; Furukawa and Roth 1985; Granovsky et al. 1994; Leppanen et al. 1999; Ju, Cummings et al. 2002; Ju and Cummings 2010). Compared with other methods for measuring T-synthase activity, this fluorescent assay has many significant advantages. The fluorescent assay is more accurate, since the activity is reflected by the enzyme product; although the radiochemical method also measures the product, the calculation is based on the concentration of donor UDP-Gal; therefore, the accuracy of the donor concentration and the quality of both UDP-Gal and UDP-[^{3}H]-Gal are important. This new assays is also much simpler and only requires three steps (Figure 1B). Other methods, such as radioactive methods, require numerous steps involving manipulating the radiolabeled compounds. This new assay is cheaper, since GalNAc-α-(4-MU) is a relatively inexpensive chemical compound, and *O*-glycosidase is a recombinant bacterial enzyme. In contrast, UDP-[^{3}H or ^{14}C]-Gal is expensive, and the assay requires C18 columns, scintillation vials, scintillation cocktail and radioactive waste disposal. This new assay is also comparable in sensitivity to the radioactive assays, generates little waste, utilizes a plate-based format that is suitable for high-through assays and can be easily performed in typical clinical laboratory conditions. Finally, this new assay might be useful for enzymatically quantifying UDP-Gal in biological fluids, if recombinant T-synthase is provided in the reaction system and endogenous T-synthase is inactivated, e.g. boiling or chemical treatment.

A unique finding in our study was the evidence that several different clones of Jurkat cells, which are commonly used in many types of immunological studies (Binstadt et al. 2000; Abraham and Weiss 2004), all lack T-synthase activity. In particular, we examined Jurkat clones I2.1 (FADD deficient) and I9.2 (caspase-8 deficient), which are cell lines originally established from a wild-type subclone termed Jurkat A3, used for apoptosis studies (Juo et al. 1998, 1999). Using this new assay, as well as the traditional radiochemical assay method, we showed that both I2.1 and I9.2 clones have very low T-synthase activity, similar to what is seen in the originally

ATCC-deposited Jurkat clone E6.1. The study of T-synthase activity and Cosmc in these Jurkat clones is important on several levels. Cosmc is encoded by a single exon gene on Xq24 and functions as an endoplasmic reticulum (ER) localized molecular chaperone to prevent aggregation and subsequent proteasomal degradation of newly synthesized T-synthase (Ju, Aryal et al. 2008). We have shown that tumor antigens Tn and STn arise from the mutations in *Cosmc* in human tumor cell lines, including Jurkat E6.1 and primary human cervical cancer samples (Ju, Lanneau et al. 2008). However, we considered the possibility that the mutation in *Cosmc* from Jurkat cell clone E6.1 might have resulted spontaneously over the years of in vitro cell culturing, rather than being a mutation in cells from the original patient. Our results demonstrate that all Jurkat cell clones have similar low T-synthase activity and carry the same mutation in *Cosmc*. The results strongly suggest that the mutation in *Cosmc* seen in all Jurkat clones may have been present originally in the first established leukemic cell line from the patient. The new assay described here will facilitate rapid screening of all T cell lines, including Jurkat, for T-synthase activity and potential mutations in *Cosmc*.

Sugar derivatives of 4-MU have been used to assay many exoglycosidases, such as α-glucosidase A (Fensom et al. 1976), α-L-iduronidase (Isemura et al. 1978; Minami et al. 1980), α-galactosidase (Hultberg et al. 1975), α-mannosidase (Ockerman 1969) and α-L-fucosidase (Gramer et al. 1994). 4-MU is advantageous because it is highly fluorescent at pH 10, but derivatives with modified 7-OH groups are nonfluorescent, including derivatives having sugars linked via a glycosidic bond. There have been several assays that utilize 4-MU derivatives as acceptors, such as the use of 4-MU-xylose as an acceptor for galactosyltransferase-I activity assay (Higuchi et al. 1994). However, such assays have not exploited the highly fluorescent nature of free 4-MU. In our study, we show that GalNAc-α-(4-MU) serves as an excellent acceptor for T-synthase to form the product Galβ1-3GalNAc-α-(4-MU) (Figure 2). The *O*-glycosidase (Fujita et al. 2005; Goda et al. 2008; Suzuki et al. 2009; Willis et al. 2009) specifically and efficiently cleaves the product of T-synthase, which makes the fluorescent assay ideal for assessing T-synthase activity. Interestingly, the recombinant *O*-glycosidase cloned from *Enterococcus faecalis* and expressed in *Escherichia coli* (Koutsioulis et al. 2008) can hydrolyze both unsubstituted core 1 and 3 (GlcNAcβ1-3GalNAcα1-Ser/Thr) *O*-glycans. However, UDP-Gal is the only donor substrate supplied in our assay system, Galβ1-3GalNAc-α-(4-MU), the product of T-synthase will be specifically synthesized and subsequently hydrolyzed by the *O*-glycosidase. Nevertheless, this same assay system could be used to assay the core 3 β1-3 *N*-acetylglucosaminyltransferase (Core 2 GnT), if UDP-GlcNAc, rather than UDP-Gal is supplied. Our assay may presage the development of additional fluorescent-based assays for other hydrolytic enzymes, such as *N*-glycanase, and other glycosyltransferases, such as GnT-I through -VI, fucosyltransferases and sialyltransferases, if specific endoglycosidases can be identified to cleave the products and release free 4-MU. In fact, the production of disaccharide products from sugar-4-MU acceptors could be useful in identifying such unique endoglycosidases from various microbes. Moreover, this assay could be adapted to include recombinant, exogenous T-synthase along with the acceptor GalNAc-α-(4-MU), so that the assay could be used to measure *O*-glycosidase activity and could also be used to measure UDP-Gal levels in biological fluids, such as cytoplasm, Golgi apparatus, ER and sera.

The specificity of the assay for the T-synthase using UDP-Gal as the donor is remarkable. This conclusion is supported by the results from Figure 5C in which three cell lines, LOX, LSC and Jurkat cell extracts were lacking T-synthase activity due to the mutations in *Cosmc* in these cells. Human colorectal carcinoma LSC cells contain a mutated *Cosmc* with a T-insertion at position 53 resulting in a 28 amino acid polypeptide with no chaperone function, whereas the melanoma LOX cell line lacks the transcript for *Cosmc* because of the deletion of its promoter. The activity in those cell lines with a dysfunctional *Cosmc* was restored only by transfection of wild-type *Cosmc* (Ju, Lanneau et al. 2008). Conveniently, the *O*-glycosidase works efficiently during the reaction of the T-synthase at nearly neutral pH, without further purifying the product, changing buffer pH or adding other components to the reaction, which made this assay method even easier, simpler and suitable for high-throughput assays of large quantities of samples. To our knowledge, this is the first report on a fluorescent assay of glycosyltransferase activity that uses a sugar-(4-MU) as an acceptor to generate a fluorescent product. This work will not only facilitate the research and clinical application of assessing the T-synthase activity in biological samples for potential diagnostic purpose, but also promote the development for high-throughput assays using fluorescence measurements for other glycosyltransferases for screening and diagnosis of CDG.

Materials and methods

Materials

UDP-Gal was purchased from Calbiochem (San Diego, CA). GalNAc-α-(4-MU) was purchased from Carbosynth Limited (Berkshire, UK). 7-Hydroxy-(4-MU) was obtained from Acros Organics (Geel, Belgium). Benzyl-α-GalNAc was purchased from Sigma-Aldrich (St Louis, MO). C18 cartridges were obtained from Millipore Corp. (Billerica, MA). Flexigene DNA Kit and QIAquick Gel Extraction Kit were purchased from Qiagen Inc. (Valencia, CA). *O*-Glycosidase (40,000,000 units/mL) and the Phusion™ High Fidelity PCR Kit were obtained from New England Biolabs (New England, MA).

Cell lines and culture

Human T-lymphoid Jurkat cells [Clone E6-1 (ATCC TIB-152™), I 2.1 (CRL-2572™) and I 9.2 (CRL-2571™)], NCI-N87 and Hi-5 insect cells were originally purchased from ATCC. Human melanoma LOX cells were kindly provided by the group of Dr. Oystein Fodstad at the Norwegian Radium Hospital Research Foundation (Oslo, Norway). Human colorectal carcinoma cells, LSC and LSB, were a gift from Dr. Steve Itzkowitz (Mount Sinai School of Medicine, New York). Mammalian cells were cultured in RPMI1640 media containing 10% FBS in 5% CO_2 at 37°C. Jurkat, LOX, LSC and LSB cells were transfected with either empty vector

pcDNA3.1(+) or pcDNA3.1(+) expressing human wild-type Cosmc. Cosmc-transfected LOX cells were sorted with FITC-labeled peanut agglutinin (PNA) (Sigma-Aldrich) after desialylation on a FACSorter (Becton Dickinson) and maintained in complete media in 5% CO_2 at 37°C.

Reaction system

The acceptor GalNAc-α-(4-MU) was dissolved in DMSO at 40 mM concentration and then adjusted to 10 mM in 0.5 M MES-NaOH (pH 6.8) as a stock stored at 4°C. The UDP-Gal was dissolved in water at 10 mM as a stock at −20°C. The *O*-glycosidase was diluted 1:50 in 25 mM MES-NaOH (pH 6.8) at concentration of 800 units/μL. The 50 μL reaction system containing 1000 μM GalNAc-α-4-(MU), 500 μM UDP-Gal, 20 mM $MnCl_2$, 0.2% Triton X-100, 800 units of *O*-glycosidase, in 50 mM MES-NaOH buffer (pH 6.8), and an appropriate amount of enzyme was place in a 96-well black plate suitable for fluorescence assay. The blank reaction was set-up by replacing the donor UDP-Gal with H_2O in the 50 μL reaction system. The background was set up with everything except *O*-glycosidase and/or enzyme. The reactions were incubated at 37°C for 60 min or the time indicated. Then, 100 μL of 1.0 M glycine-NaOH (pH10.0) was added to each well to stop the reaction, and the relative fluorescence intensity or RFU were measured on a Victor Multiple-Label Counter (PerkinElmer) using umbelliferone mode, e.g. Ex 355 nM and Em 460 nm. For the concentration of enzyme, the cell extracts from Hi-5 cells expressing human recombinant T-synthase and wild-type Cosmc were serially diluted with TBS containing 0.5% Triton X-100 and protease inhibitor cocktail. For the time course experiment, the reaction was set up in the same tube and 50 μL reactions were aliquotted into the plate. Stop solution was added to the time zero wells immediately, and the plate was incubated at 37°C. At time points of 15, 30, 45, 60, 90, 120, 150 and 180 min, the stop solution was added to the corresponding wells, and the RFU for all time points was determined.

O-*Glycosidase concentration dependence*

The T-synthase reactions were set up as above in the time course, except for the different amounts of *O*-glycosidase (0–800 units) added. The reactions were incubated at 37°C for 60 min, the stop solution was added and the fluorescence was measured.

Standard curve for 4-MU

4-MU was dissolved in DMSO at 1.0 mM, and the concentrations of 10, 50, 100, 200, 500, 1000, 2000, 5000 10,000 and 20,000 nM were made by dilution with 50 mM MES-NaOH (pH 6.8). Then, 50 μL of 4-MU of each concentration was transferred into plates in triplicates and 100 μL of stop solution was added. The fluorescence units were measured on the Victor Multiple-Label Reader.

Preparation of cell extracts

Cell pellets of Hi-5 cells coexpressing human recombinant T-synthase and Cosmc, mock-transfected LSC, Cosmc-transfected LSC, LSB and NCI-N87 cells, mock-transfected and Cosmc-transfected LOX cells, mock-transfected and Cosmc-transfected Jurkat cells (Clone E6-1) and Jurkat (clones I2.1 and I9.2) cells were suspended in an appropriate volume (1:8, v:v) of TBS containing Complete-Mini protease inhibitor cocktail and sonicated on ice using the micro-tip for 3 s five times. The cell post-nuclear supernatant was obtained by centrifugation at 1000 × *g* at 4°C for 5 min. The cell extracts were prepared by adding Triton X-100 0.5% (final concentration), vortexed well and solubilized on ice for 20 min. The extracts were ready for T-synthase activity and protein concentration assay.

Preparation of the product Galβ1-3GalNAc-α-(4-MU)

Standard reactions for T-synthase activity assay in 1.5 mL microtubes with and without UDP-Gal were set up with 10 nmol/h recombinant T-synthase and incubated for 16 h. Then, 0.5 mL of H_2O was added into the reactions, which were loaded onto two C18 cartridges (50 mg) pre-activated with methanol and washed with H_2O. After washing with 1.2 mL of H_2O six times, the bound materials were eluted with 80% acetonitrile and dried in a speed-vac system. The dried materials were dissolved in 50 μL of H_2O. A 10 μL aliquot was transferred into 500-μL microtubes, 1 μL of *O*-glycosidase (800 units) in 25 mM MES was added and 1 μL of 25 mM MES was added to the untreated tube. After incubation at 37°C for 8 h, the materials were analyzed by LC-MS and MS/MS.

Characterization of the T-synthase product by ESI-MS/MS

The substrate and the product were analyzed in a positive mode by LC-MS/MS, QTRAP 5500 from Applied Biosystems (Foster City, CA). For the LC-MS experiment, the sample was diluted using solution (water/acetonitrile/formic acid, 98/2/0.1, v/v/v) to 50 fmol/μL and 10 μL for analysis was injected on an Ultra Aqueous C18 (3 μm, 50 × 2.1 mm) from Resteck (Bellefonte, PA). The mobile phase was methanol with 0.05% formic acid. The sample was eluted using a gradient from 2% methanol to 92% methanol over 20 min at a flow rate of 0.25 mL/min. For the MS/MS, the samples were dissolved or diluted in acetonitrile/water/formic acid (50/50/0.25, v/v/v) at a final concentration of 1 pmol/μL by infusion. The mass spectrometry parameters are as follows: the infusion flow rate is 10 μL/min; curtain gas: 25.0; ion spray: 5500.00; temperature: 350°C; nebulizer gas: 30.0; heater gas: 30.0; collisionally activated dissociation: medium; declustering potential: 90.0; entrance potential: 10.0. The peak of interest on the first MS was subjected to secondary MS and the data were collected.

Additional methods

T-synthase activity assays using UDP-[^{3}H]-Gal were also conducted as described previously (Ju, Cummings et al. 2002). A soluble version of HPC4-tagged recombinant human T-synthase was expressed by coexpressing wild-type Cosmc and was directly purified from the media as described previously (Ju, Aryal et al. 2008). A soluble version of recombinant Cosmc was purified. Heat denaturing and reconstitution experiments were carried out as described (Aryal et al. 2010).

The genomic DNA preparation and PCR of *Cosmc* from cell lines were performed as reported previously (Ju and Cummings 2002). The protein concentration in cell extracts was determined by the BCA method (Pierce, Rockford, IL) following the manufacturer's instructions with bovine serum albumin as a standard. PCR was carried out with Phusion™ High Fidelity PCR Kit (New England Biolabs) as described previously (Ju, Lanneau et al. 2008). The PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's protocol and sequenced.

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Conflict of interest

None declared.

Abbreviations

CDG, Congenital Disorders of Glycosylation; ER, endoplasmic reticulum; 4-MU, 4-methylumbelliferone; Gal, D-galactose; GalNAc, *N*-acetylgalactosamine (2-acetamido-2-deoxy-D-galactose); *O*-glycosidase, endo-α-*N*-acetylgalactosaminidase; PNA, peanut agglutinin; RFU, relative fluorescence unit; Ser/Thr, serine/threonine; T-synthase, UDP-Gal: *N*-acetylgalactosaminyl-α1-Ser/Thr β3galactosyltransferase; Tn antigen, GalNAcα1-Ser/Thr; UDP-Gal, uridine diphosphate galactose.

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