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Activation of β1,3-*N*-Acetylglucosaminyltransferase-2 (β3Gn-T2) by β3Gn-T8

*POSSIBLE INVOLVEMENT OF β3Gn-T8 IN INCREASING POLY-N-ACETYLLACTOSAMINE CHAINS IN DIFFERENTIATED HL-60 CELLS**

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Enzymatic activities of some glycosyltransferases are markedly increased via complex formation with other transferases or cofactor proteins. We previously showed that β1,3-*N*-acetylglucosaminyltransferase-2 (β3Gn-T2) and β3Gn-T8 can form a heterodimer *in vitro* and that the complex exhibits much higher enzymatic activity than either enzyme alone (Seko, A., and Yamashita, K. (2005) *Glycobiology* 15, 943–951). Here we examined this activation and the biological significance of complex formation in differentiated HL-60 cells. β3Gn-T2 and -T8 were co-immunoprecipitated from the lysates of both-transfected COS-7 cells, indicating their association *in vivo*. We prepared inactive mutants of both enzymes by destroying the DXD motifs. The mixture of mutated β3Gn-T2 and intact β3Gn-T8 did not exhibit any activation, whereas the mixture of intact β3Gn-T2 and mutated β3Gn-T8 had increased activity, indicating the activation of β3Gn-T2 via complex formation. Next, we compared expression levels of β3Gn-T1-T8 in HL-60 cells and DMSO-treated differentiated HL-60 cells, which produce larger poly-*N*-acetyllactosamine chains. The expression level of β3Gn-T8 in the differentiated cells was 2.6-fold higher than in the untreated cells. Overexpression of β3Gn-T8, but not β3Gn-T2, induced an increase in poly-*N*-acetyllactosamine chains in HL-60 cells. These results raise a possibility that up-regulation of β3Gn-T8 in differentiated HL-60 cells increases poly-*N*-acetyllactosamine chains by activating intrinsic β3Gn-T2.

Glycosyltransferases are present in the endoplasmic reticulum/Golgi membranes, cytoplasm, cell surface, and body fluids. In the presence of appropriate sugar donors, they work for the biosynthesis of various glycoconjugates. Recently, it has been shown that some glycosyltransferases form protein complexes with other glycosyltransferases and/or non-glycosyltransferase proteins (reviewed in Ref. 1). Complex formation contributes to enzymatic activation, stable expression in the Golgi apparatus, correct localization in intracellular vesicles, efficient biosynthesis of glycan chains, and modification of substrate specificities. Enzymatic activation has been proven for protein *O*-mannosyltransferases (2–4), *N*-acetylglucosaminyltransferases and glucuronyltransferases involved in heparan sulfate biosynthesis (5–9), ST8Sia-I (GD_3 synthase) and β4GalNAc-T1 (GM_2/GD_2 synthase) (10), and chondroitin synthase (11–13). In these cases, glycosyltransferases exhibit little enzymatic activity when expressed alone, but their catalytic activities emerge if their respective cofactor proteins are simultaneously expressed. Because the activation does not occur by *in vitro* mixing of the glycosyltransferases and cofactor proteins, the process of complex formation appears to involve intermolecular disulfide bond formation or complicated interactions during early stages of polypeptide synthesis. In contrast, we previously found that β1,3-*N*-acetylglucosaminyltransferase-2 (β3Gn-T2)[2] and β3Gn-T8 can form a heterodimer *in vitro* and that the enzymatic activity of the dimer is much higher than the sum of the individual activities (14). Because *in vitro* mixing of individually expressed fractions of β3Gn-T2 and -T8 is sufficient for enzymatic activation, dimer formation and enzymatic activation should occur with the completely folded proteins. However, it has been unclear whether the two enzymes associate with each other *in vivo* and which of the one or more enzymes are catalytically activated in the complex.

Poly-*N*-acetyllactosamine (polyLacNAc) is a linear glycan chain consisting of repeating *N*-acetyllactosamine units $(Gal\beta1-4GlcNAc\beta1-3)_n$. The glycan chains occur in glycosphingolipids and *N*-linked/*O*-linked glycan chains of specific glycoproteins. In some cases, the 3-OH and/or 6-OH of galactose (Gal) and *N*-acetylglucosamine (GlcNAc) residues are modified by sialic acids, fucose (Fuc), and/or sulfate residues, which serve as determinants for various carcinoembryonic antigens and ligands for various cell recognition-associated lectins (15). In HL-60 cells, polyLacNAc chains exist primarily on lysosomal membrane glycoproteins (lamps) (16). HL-60 cells can differentiate to granulocytic cells in the presence of DMSO.

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[2] The abbreviations used are: β3Gn-T, β1,3-*N*-acetylglucosaminyltransferase; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GnT, β-*N*-acetylglucosaminyltransferase; lamps, lysosomal membrane glycoproteins; Man, mannose; polyLacNAc, poly-*N*-acetyllactosamine; tetraGP, $Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow2(Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow4)Man\alpha1\rightarrow3[Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow2(Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow6)Man\alpha1\rightarrow6]Man\beta1\rightarrow4GlcNAc$.

Lee *et al.* (17) showed that polyLacNAc chains increase in the DMSO-treated differentiated HL-60 cells. They also showed that β3Gn-T activities in the differentiated HL-60 cells are 1.5-fold higher than those in the undifferentiated cells, suggesting that β3Gn-T is a rate-limiting enzyme for the biosynthesis of longer polyLacNAc chains. This linear glycan is biosynthesized by the repeating action of β1,4-galactosyltransferase and β3Gn-T. β3Gn-T1, -T2, -T3, -T4, -T7, and -T8 have been shown to possess the ability to synthesize polyLacNAc chains (14, 18–21). However, it is unclear which of the one or more enzymes are responsible for the increase in polyLacNAc chains in the differentiated HL-60 cells.

In this study, we have addressed the following issues: whether β3Gn-T2 and -T8 associate with each other *in vivo*; whether β3Gn-T2 and/or -T8 are catalytically activated in a complexed state; and which one or more β3Gn-Ts are associated with the increase in polyLacNAc chains in differentiated HL-60 cells.

EXPERIMENTAL PROCEDURES

Materials—UDP-[6-^{3}H]GlcNAc (2.2 TBq/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). A tetra-antennary oligosaccharide, Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→4)Manα1→3[Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→6)Manα1→6]Manβ1→4GlcNAc (tetraGP), was obtained from the urine of G_{M1} gangliosidosis patients (22).

Expression of Tagged and Mutated Human β3Gn-T2 and β3Gn-T8—An expression vector for C-terminally FLAG-tagged β3Gn-T2 was prepared from the p3XFLAG-CMV™-14 vector (Sigma-Aldrich) and the cDNA fragment for full-length β3Gn-T2 without a stop codon. The fragment was amplified by PCR from pcDNA3-β3Gn-T2 (21). The oligonucleotide primers used were 5′-tttaagcttGAGAAATGAGTGTTGGA-3′ (forward primer) and 5′-ttttctagaGCATTTTAAATGAGCACT-3′ (reverse primer). The cDNA was cloned into p3XFLAG-CMV™-14 between the HindIII and XbaI sites. The resulting plasmid (pFLAG-T2) was sequenced with a Prism 310 Genetic Analyzer. C-terminally myc-tagged β3Gn-T8 (pMyc-T8) was prepared similarly. The cDNA fragment was amplified by PCR from pcDNA3-β3Gn-T8 (14). The oligonucleotide primers used were 5′-tttaagctt-GGGTCATGCGCTGCCCCAAGTG-3′ (forward primer) and 5′-ttttctagaGCACTGGAGCCTTGGGT-3′ (reverse primer). The cDNA was cloned into the pcDNA4/myc-His® expression vector (Invitrogen) between the HindIII and XbaI sites. Expression vectors for C-terminally FLAG-tagged β3Gn-T8 (pFLAG-T8) and C-terminally myc-tagged β3Gn-T2 (pMyc-T2) were obtained from pMyc-T8 and pFLAG-T2, respectively, by HindIII and XbaI digestion and ligation into the respective plasmids.

Pichia pastoris expression vectors for the truncated forms of human β3Gn-T2 and β3Gn-T8, lacking cytoplasmic and transmembrane domains, were prepared previously (14). β3Gn-T2 and β3Gn-T8 mutated in their D*X*D motifs were prepared using a QuikChange™ site-directed mutagenesis kit (Stratagene). The mutagenic oligonucleotide primers used were 5′-CTGAGTTTGTTTTCAAGGGCGCTGACGATGTTTTTGTG-3′ (forward primer for β3Gn-T2), 5′-CACAAAAACATCGTCAGCGCCCTTGAAAACAAACTCAG-3′ (reverse primer for β3Gn-T2), 5′-GAGTTTTGTCTTGCGAGCTGCGGACGATGCCTTTGTAC-3′ (forward primer for β3Gn-T8), and 5′-GTACAAAGGCATCGTCCGCAGCTCGCAAGACAAAACTC-3′ (reverse primer for β3Gn-T8). Mutated plasmids were used for the transformation of *P. pastoris* KM71 cells, and the recombinant proteins were purified from the culture media as described previously (14). The yields for recombinant human β3Gn-T2, β3Gn-T8, mutated β3Gn-T2 (T2-DA), and mutated β3Gn-T8 (T8-QA) from 100 ml of buffered methanol-complex media were 17, 130, 2.1, and 94 μg, respectively.

The cDNA fragments for the truncated forms of murine β3Gn-T2 and β3Gn-T8 were amplified by PCR from the genomic DNA from NIH 3T3 cells, because both were encoded in each single exon. The oligonucleotide primers used were 5′-TTTGAATTCTCCAAAAACAGTAGCCAAGAC-3′ (forward primer for β3Gn-T2), 5′-TTTGCGGCCGCAGCTCATGTCTATTTCAGCA-3′ (reverse primer for β3Gn-T2), 5′-TTTGAATTCAAAAAGGCTGAACCCCGGGGC-3′ (forward primer for β3Gn-T8), and 5′-TTTGCGGCCGCCGGTCAGCACTGGAGCTC-3′ (reverse primer for β3Gn-T8). The cDNA was cloned into pPIC9 expression vector (Invitrogen). The recombinant proteins were prepared as described previously (14). The yields for recombinant murine β3Gn-T2 and β3Gn-T8 from 100 ml of buffered methanol-complex media were 5.6 and 8.6 μg, respectively. Peptide:*N*-glycosidase F (Takara Bio Inc., Otsu, Japan) digestion was performed according to the manufacturer's instructions.

Assay of β3Gn-T Enzymatic Activity—The assay conditions were as described previously (21). Briefly, the reaction mixtures (20 μl) consisted of 50 mM HEPES-NaOH (pH 7.2), 10 mM $MnCl_2$, 0.1% (w/v) Triton X-100, 0.3 mM tetraGP, 2.5 μM UDP-[^{3}H]GlcNAc (6.7×10^6 dpm), 50 μg/ml protamine chloride, 0.5 mM spermine, and appropriately diluted enzyme fractions. The mixtures were incubated at 37 °C for 1 h. The ^{3}H-labeled products were separated by paper electrophoresis (pyridine:acetic acid:water, 3:1:387, pH 5.4) and then by paper chromatography (pyridine:ethyl acetate:acetic acid:water, 5:5:1:3). After drying, the paper was monitored for radioactivity with a radiochromatogram scanner.

Co-immunoprecipitation—The plasmids (5 μg) pFLAG-T2 and pFLAG-T8 were transfected into semi-confluent COS-7 cells on 10-cm dishes using 20 μg of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stable transfectants were isolated by selection using 400 μg/ml G418 sulfate (Calbiochem, Darmstadt, Germany). A second transfection for transient expression was performed with 10 μg of the appropriate plasmids and Lipofectamine 2000. The cells were harvested after 24 h and washed twice with phosphate-buffered saline. After adding 1 ml of lysis buffer (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% (v/v) Nonidet P-40, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml pepstatin, and 1 μg/ml leupeptin), the cell pellets were suspended and lysed on ice for 20 min. After centrifugation, the supernatants were collected and incubated with 1 μg of anti-myc antibody (Invitrogen) and 15 μl of Protein G-Sepharose™ 4 Fast Flow (GE Healthcare, Buckinghamshire, England) at 4 °C for 1 h. The resins were washed with the lysis buffer four times. Equal aliquots of the resins were used for SDS-PAGE, and the proteins were trans-

ferred onto a nitrocellulose membrane (Bio-Rad Trans-Blot® Transfer Medium, Hercules, CA). Tag-conjugated proteins on the membranes were treated with horseradish peroxidase)-conjugated anti-FLAG-M2 antibody (0.3 μg/ml) (Sigma-Aldrich) or horseradish peroxidase-conjugated anti-myc antibody (1 μg/ml, Invitrogen) and detected with ECL Western blotting Detection Reagents (GE Healthcare).

Reverse Transcription-PCR for β3Gn-Ts—HL-60 cells (Health Science Research Resources Bank, Sennan, Japan) were differentiated by 1.5%-DMSO treatment for 3 days. The cDNAs were synthesized from total RNA using SuperScript III reverse transcriptase (Invitrogen). Oligonucleotide primers used for PCR were 5′-AGGTCTTTGACAAGCTAGCCAGG-3′ (forward primer for β3Gn-T1), 5′-TCCTGCCACGTAGAATGGCTCC-3′ (reverse primer for β3Gn-T1), 5′-CCACCCCGACCTTTCAGATATGC-3′ (forward primer for β3Gn-T2), 5′-ACCTGGTCAGTGATATGGTACAGC-3′ (reverse primer for β3Gn-T2), 5′-CACGAGGCCCGCAAGGTCAAC-3′ (forward primer for β3Gn-T3), 5′-GACAGGCGTTGCGATGGAGCC-3′ (reverse primer for β3Gn-T3), 5′-GATCCGCTCCCCCAGCCCAG-3′ (forward primer for β3Gn-T4), 5′-CATCTTCCATGATAGCCTGGAGG-3′ (reverse primer for β3Gn-T4), 5′-TCCACTGGAGGGAGAAGAACTAC-3′ (forward primer for β3Gn-T5), 5′-GCAGGGATGATAAGGAGTTTTACC-3′ (reverse primer for β3Gn-T5), 5′-GGGGCAAGAGCGCAGCTACGG-3′ (forward primer for β3Gn-T6), 5′-CTGTGCGCCAGGCAGCTGCAC-3′ (reverse primer for β3Gn-T6), 5′-AGGAGCGCACGCACTACCAGC-3′ (forward primer for β3Gn-T7), 5′-CAGGGTGTCGCAGGCATGGTG-3′ (reverse primer for β3Gn-T7), 5′-GGCCTGACCTAGACTCACTAGTG-3′ (forward primer for β3Gn-T8), and 5′-CGCAGTGCGGTCTGCTGGCCAG-3′ (reverse primer for β3Gn-T8). PCR was performed under following conditions: 28 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and elongation at 72 °C for 2 min. After agarose-gel electrophoresis, the amplified products were quantified using an FLA-2000 multi-imager (Fuji Photo Film, Japan).

Tomato-lectin Blotting—The plasmids (10 μg) were transfected into HL-60 cells (1×10^6) using 20 μg of Lipofectamine 2000 according to the manufacturer's instructions. After 48 h, the cells were collected and washed with phosphate-buffered saline three times. Aliquots of cell homogenates (3 μg of protein) were used for SDS-PAGE, and the proteins were transferred onto a nitrocellulose membrane. Parts of membranes were digested with *Escherichia freundii* endo-β-galactosidase (Seikagaku Co., Tokyo, Japan) (50 milliunits of enzyme in 0.1% bovine serum albumin-50 mM sodium acetate buffer (pH 5.5) at 25 °C for 16 h). After blocking with 1% bovine serum albumin/phosphate-buffered saline, the membrane was treated with 10 μg/ml biotin-conjugated lectin from *Lycopersicon esculentum* (tomato, Sigma-Aldrich) in phosphate-buffered saline/0.1% Tween 20 at 4 °C for 2 h. After washing, the membrane was treated with horseradish peroxidase-conjugated streptavidin (GE Healthcare) at 4 °C for 1 h. The detection was performed using ECL Western blotting Detection Reagents. Chemiluminescence was quantified using a LAS-1000 multi-imager (Fuji Photo Film, Japan).

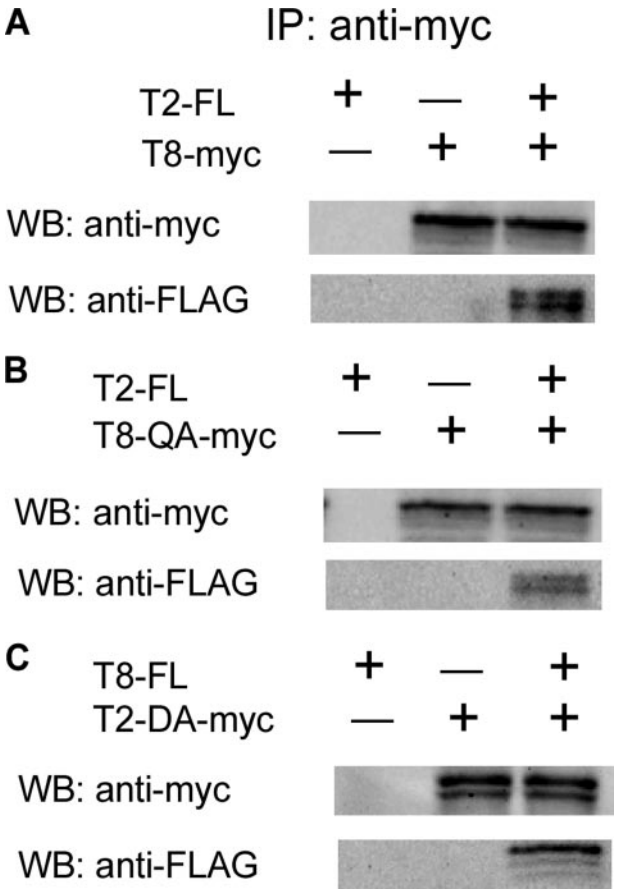

FIGURE 1. ***In vivo* interaction of wild-type and D*X*D-mutated β3Gn-T2 and β3Gn-T8.** Co-immunoprecipitation of β3Gn-T2 and β3Gn-T8 (*A*), β3Gn-T2 and T8-QA (*B*), and T2-DA and β3Gn-T8 (*C*). COS-7 cells were transfected with expression vectors for the FLAG- or myc-tagged enzymes. Cell lysates were immunoprecipitated (*IP*) with anti-myc antibody. Equal aliquots of the pellets were analyzed by Western blotting (*WB*) with the antibodies indicated on the *left*.

RESULTS

β3Gn-T2 Associates with β3Gn-T8 in Vivo—We previously found that the mixture of β3Gn-T2 and β3Gn-T8 exhibited much higher enzymatic activity than either enzyme alone and that the two enzymes could form a heterodimer *in vitro* (14). However, it remained unclear whether the two enzymes interacted with each other *in vivo*. To assess this, co-immunoprecipitation was performed. β3Gn-T2 FLAG tagged at the C terminus (T2-FL) and β3Gn-T8 myc tagged at the C terminus (T8-myc) were simultaneously expressed in COS-7 cells, and the cell lysates were immunoprecipitated by anti-myc antibody. The precipitates were analyzed by Western blotting (Fig. 1*A*). T2-FL co-immunoprecipitated with T8-myc and was detected by anti-FLAG antibody, indicating that β3Gn-T2 associates with β3Gn-T8 *in vivo*.

β3Gn-T2 Is Activated by β3Gn-T8—Increased enzymatic activity in the mixture of β3Gn-T2 and -T8 indicated that the enzyme(s) were activated in the complex. To assess which β3Gn-T was activated, we prepared mutated proteins that were enzymatically inactive, but could form the complex. Glycosyltransferases generally have a D*X*D motif (23), which is involved in binding to divalent cations and sugar nucleotides and is thus essential for their catalytic activities. β3Gn-T2 and -T8 contain

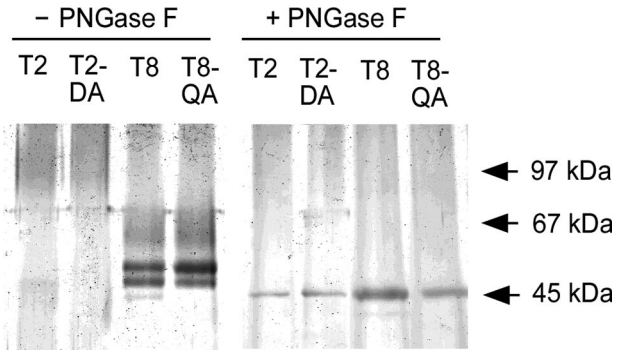

FIGURE 2. **SDS-PAGE of soluble forms of β3Gn-T2 (*T2*), β3Gn-T8 (*T8*), and the mutated proteins, *T2-DA* and *T8-QA*, produced by *P. pastoris*.** Purified proteins were applied to the gel. After electrophoresis, the proteins were detected by Sypro Orange staining. The digests by peptide:*N*-glycosidase F (*PNGase F*) are also shown in the *right four lanes*.

^{245}DDD^{247} and ^{246}QDD^{248}, respectively, as D*X*D motifs. We constructed expression vectors with mutated β3Gn-T2 (T2-DA) and -T8 (T8-QA), which had ^{245}ADD^{247} and ^{246}ADD^{248}, respectively. The ability of T2-DA and T8-QA to form a complex *in vivo* was examined by co-immunoprecipitation as above. Fig. 1 (*B* and *C*) shows that the substitutions in their D*X*D motifs did not affect their ability to interact with their wild-type counterpart. Next, we examined the enzymatic activities of T2-DA, T8-QA, and 1:1 mixtures with wild-type β3Gn-T2 and -T8. Soluble forms of $(His)_6$-tagged T2-DA and T8-QA were produced using with the *P. pastoris* protein expression system, as previously described (14), and purified by Ni-NTA agarose chromatography (Fig. 2). Both mutant proteins electrophoresed as smeared bands, similar to the wild-type proteins. Peptide:*N*-glycosidase F treatment showed that the polypeptide moieties of T2-DA and T8-QA had the same molecular masses (~45 kDa) as the wild-type enzymes (Fig. 2). The smeared profiles were attributed to heterogeneity in yeast large *N*-linked glycans. The enzymatic activities are shown in Fig. 3. Neither T2-DA nor T8-QA had any enzymatic activity, indicating that the D*X*D motifs are required for catalytic activity. The mixture of wild-type β3Gn-T2 and T8-QA had 6.3-fold higher enzymatic activity (1.9 ± 0.05 pmol/min) than β3Gn-T2 alone (0.30 ± 0.04 pmol/min), whereas the mixture of wild-type β3Gn-T8 and T2-DA had the same level of activity as β3Gn-T8 alone (0.014 ± 0.002 pmol/min). The same results were obtained from another independent experiment using individually prepared enzymes. These results indicate that β3Gn-T2 is activated by β3Gn-T8, and that the mutant T8-QA, which has no enzymatic activity, is also able to activate β3Gn-T2.

Next, we examined whether murine β3Gn-T8 could activate murine and human β3Gn-T2. Amino acid similarities for catalytic domains of β3Gn-T2 and β3Gn-T8 between human and murine were 87 and 75%, respectively. As shown in Table 1, the V_{max}/K_m value of the mixture, murine β3Gn-T2 and murine β3Gn-T8, was approximately twice higher than that of murine β3Gn-T2 alone. Interestingly, murine β3Gn-T8 could activate human β3Gn-T2, although the degree of the activation by murine β3Gn-T8 was lower than that by human β3Gn-T8. In converse, human β3Gn-T8 could also activate murine β3Gn-T2. These results indicate that the ability of β3Gn-T8 to activate β3Gn-T2 is conserved between human and murine.

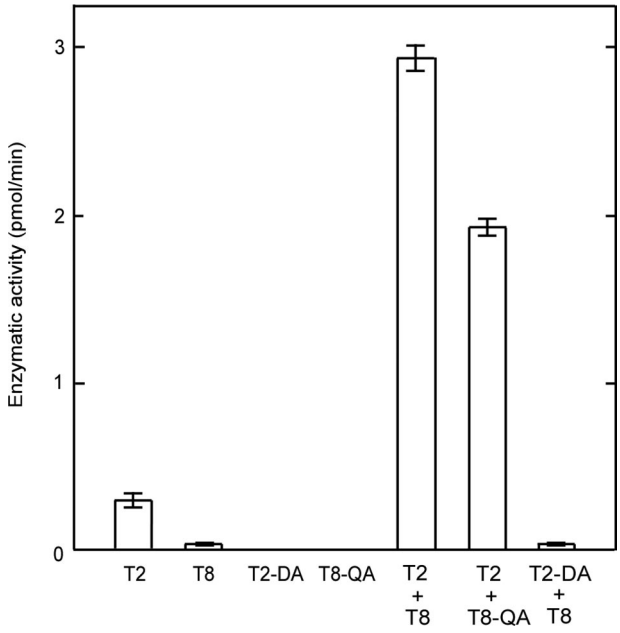

FIGURE 3. **Specific activities of β3Gn-T2 (*T2*), β3Gn-T8 (*T8*), *T2-DA*, *T8-QA*, and their combinations.** Each protein (7 ng) was assayed for enzymatic activity as described under "Experimental Procedures." Tetra-antennary *N*-linked oligosaccharide was used as an acceptor substrate. The enzymatic activities are the means of five independent experiments. The minimal detectable amount of the activity was 0.005 pmol/min.

TABLE 1

Kinetic analysis of human β3Gn-T2 (H2), human β3Gn-T8 (H8), murine β3Gn-T2 (M2), murine β3Gn-T8 (M8), and the mixtures of each two enzyme

The acceptor substrate used was 2,6-branched triGP, Galβ1→4GlcNAcβ1→2Manα1→3[Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→6)Manα1→6]Manβ1→4GlcNAc. It was prepared as in a previous study (14).

	H2	H2+H8	H2+M8	M2	M2+M8	M2+H8
K_m (mM)	0.42	0.15	0.25	0.33	0.071	0.18
V_{max} (nmol/min/mg of protein)	150	610	390	170	74	360
V_{max}/K_m	360	4100	1600	520	1000	2000

Furthermore, to assess whether β3Gn-T8 could stabilize β3Gn-T2 or not, the stability of the enzymatic activities of human β3Gn-T2 in the presence or absence of human β3Gn-T8 was examined. As shown in Fig. 4, the enzymatic activities of β3Gn-T2 alone and the mixture of β3Gn-T2 and β3Gn-T8 decreased in the same manner. This suggests that β3Gn-T8 does not have the ability to stabilize the enzymatic activity of β3Gn-T2 at least *in vitro*.

β3Gn-T8 Is Involved in Increasing PolyLacNAc Chains in the Differentiated HL-60 Cells—By structural studies for *N*-linked glycan chains of lamps, Lee *et al.* (17) showed that the amount of polyLacNAc chains in HL-60 cells increased with differentiation by DMSO treatment. They also assayed the enzymatic activities of β3Gn-Ts, β1,6-*N*-acetylglucosaminyltransferase-V, and β,4-galactosyltransferases, and found that only β3Gn-T activity increased 1.6-fold in the differentiated HL-60 cells using a linear substrate Galβ1→4GlcNAcβ1→6Manα1→6Manβ1→R. We assayed β3Gn-T activity in these cells using tetraGP, which is a good substrate for all β3Gn-Ts (14, 20, 21). As shown in Table 2, the β3Gn-T activities in HL-60 cells and in DMSO-treated HL-60 cells were 6.5 ± 0.7

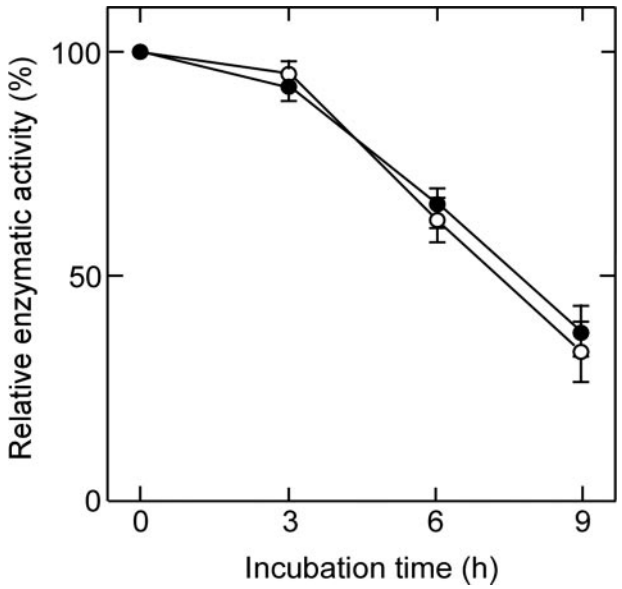

FIGURE 4. **Stability of the enzymatic activities of human β3Gn-T2 (*closed circle*) and the mixture of human β3Gn-T2 and β3Gn-T8 (*open circle*).** Equal amounts of enzyme proteins were incubated in 10 mM HEPES-NaOH, 0.15 M NaCl (pH 7.2) at 37 °C, and at the indicated time, aliquots of the enzyme solution were assayed for the β3Gn-T activity. Four independent experiments were performed.

TABLE 2

β3Gn-T activity in HL-60 cells and in DMSO-treated differentiated HL-60 cells

TetraGP was used as an acceptor substrate. The enzymatic activity was the mean of five independent experiments.

	β3Gn-T activity	Ratio
	pmol/min/mg protein	
HL-60 cells	6.5 ± 0.7	1
DMSO-treated HL-60 cells	35 ± 1.8	5.3

and 35 ± 1.8 pmol/min/mg of protein, respectively, indicating that the activity increased 5.3-fold in the course of differentiation. To assess which β3Gn-Ts were up-regulated in the process of differentiation of HL-60 cells, we compared expression levels of transcripts for β3Gn-Ts in HL-60 cells and DMSO-treated HL-60 cells. It has been shown previously that β3Gn-T1, -T2, -T3, -T4, -T7, and -T8 are able to synthesize polyLacNAc chains (14, 18–21). As shown in Fig. 5, substantial expression was detected for β3Gn-T1, -T2, -T4, -T5, -T7, and -T8 in HL-60 cells (Fig. 5, *U*). Among these, transcript levels increased for only β3Gn-T2 and -T8 (1.5- and 2.6-fold, respectively) after DMSO treatment. This result suggests that the increased expression level of either β3Gn-T2 or -T8 is responsible for the increase in β3Gn-T activity. Next we examined whether the expression level of polyLacNAc chains increased with overexpression of β3Gn-T2 or -T8 in HL-60 cells. Expression vectors for β3Gn-T2 and -T8 were transiently transfected into HL-60 cells, and the cell homogenates were subjected to tomato-lectin blotting analysis. Because this lectin recognizes three or more linear units of repeating LacNAc (24), it is suitable for detection of rather long polyLacNAc chains. In HL-60 cells, polyLacNAc moieties are carried primarily on lamp proteins corresponding to 100–150 kDa (16). As shown in Fig. 6, overexpression of β3Gn-T2 or T2-DA did not affect the amount of polyLacNAc moieties on lamps, whereas overexpression of β3Gn-T8 and T8-QA increased the polyLacNAc moieties 1.7 ± 0.2- and 2.1 ± 0.3-fold, respectively. The lectin binding was completely lost when the blotting membrane was digested with *E. freundii* endo-β-galactosidase, which hydrolyzes polyLacNAc moieties (data not shown), confirming the detection of polyLacNAc chains by this lectin. These results suggest that up-regulation of β3Gn-T8 is primarily responsible for the increase in polyLacNAc moieties in the differentiated HL-60 cells.

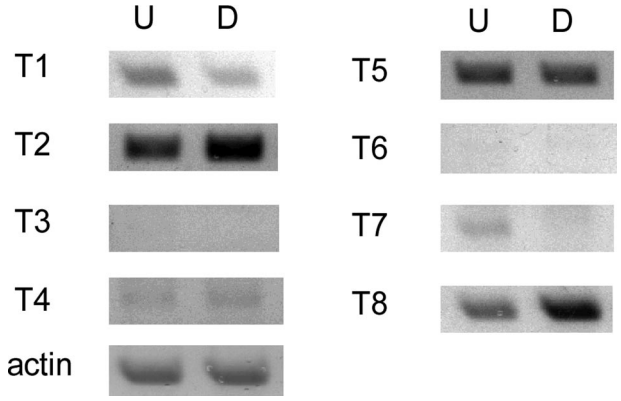

FIGURE 5. **Reverse transcription-PCR analysis of expression of eight β3Gn-Ts (*T1–T8*) in HL-60 cells (*U*), and DMSO-treated differentiated HL-60 cells (*D*).** The amounts of cDNAs were normalized by the expression levels of β-actin. cDNAs were detected and quantified by ethidium-bromide staining.

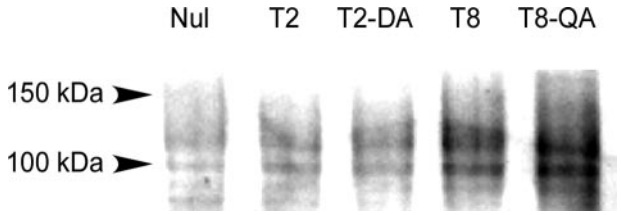

FIGURE 6. **Tomato-lectin blotting analysis of crude extracts of HL-60 cells (*Nul*) and HL-60 cells transfected with plasmids carrying β3Gn-T2 (*T2*), *T2-DA*, β3Gn-T8 (*T8*), and *T8-QA*.** Cell-extract proteins (3 μg) were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. Three or more repeating units of LacNAc were recognized by biotin-conjugated tomato lectin. One representative blot of four depicted is shown.

DISCUSSION

We have clearly demonstrated in this study that (i) β3Gn-T2 and -T8 associate *in vivo*, (ii) β3Gn-T2 is activated by β3Gn-T8, and (iii) the increase in polyLacNAc chains in differentiated HL-60 cells is due primarily to up-regulation of β3Gn-T8. We previously showed that soluble forms of β3Gn-T2 and -T8, lacking transmembrane and cytoplasmic regions, could form an activated heterodimer *in vitro* (14). This result suggested that catalytic domains and/or stem regions are necessary for the complex formation and enzymatic activation. However, it remained unclear which regions of the two enzymes interacted with each other. Because activation occurs between β3Gn-T2 and D*X*D-mutated T8-QA, the binding of β3Gn-T8 to UDP-GlcNAc is not necessary for the complex formation, and therefore the two enzymes may interact in a polypeptide region outside of the catalytic region. Their interaction seems to change the conformation of the catalytic region of β3Gn-T2 and elevate its catalytic activity. Several studies have examined the

molecular mechanism of complex formation by glycosyltransferases: stem regions are important for the interaction of β1,2-*N*-acetylglucosaminyltransferase-I and α-mannosidase II (25) and for the oligomerization of β1,3-glucuronosyltransferase (26), β1,6-*N*-acetylglucosaminyltransferase-V (27), heparan sulfate 6-*O*-sulfotransferases (28), and GlcNAc 6-*O*-sulfotransferase-1 (29). Transmembrane regions are also important for oligomerization of β1,4-galactosyltransferase-I (30), α1,3-fucosyltransferase VI (31), and α2,6-sialyltransferase-I (32). Catalytic domains are involved in oligomerization of α2,6-sialyltransferase-I (33) and dimerization of GM_2 synthase (34). Some glycosyltransferases form oligomer/multimer complexes with rather high molecular weights, whereas other complexes are formed via intermolecular disulfide bond(s), most likely in the process of polypeptide biosynthesis. The heterodimer between β3Gn-T2 and -T8 may be suitable for tertiary structural studies of glycosyltransferase complexes, because this complex can be formed *in vitro*. Such studies will lead to a better understanding of the molecular mechanism of complex formation by glycosyltransferases.

The V_{max}/K_m value of the human T2/T8 complex is 9.3-fold higher than that of β3Gn-T2 alone (14). By complex formation with β3Gn-T8, the K_m value of human β3Gn-T2 decreases 2.4-fold, and the V_{max} value increases 3.9-fold (14). In contrast, the substrate specificity of the complex is almost the same as that of β3Gn-T2 (14), and β3Gn-T8 cannot stabilize the enzymatic activity of β3Gn-T2 (in this study), indicating that β3Gn-T8 can augment turnover velocity of β3Gn-T2. Interestingly, murine β3Gn-T8 can also activate murine β3Gn-T2 (Table 1). In this case, the K_m value of β3Gn-T2 decreases 4.6-fold by complex formation, and the V_{max} value also decreases 2.3-fold. In murine T2/T8, the K_m value is altered by the complex formation more than the V_{max} value. In contrast, the V_{max} value is altered more than the K_m value in human T2/T8. The biological significance of this difference is unclear, but it may be possible that the availability of acceptor substrates is different between in human and murine cells. It should be noted that murine and human β3Gn-T8 can activate human and murine β3Gn-T2, respectively. Human β3Gn-T8 is more effective for the activation of murine β3Gn-T2 than murine β3Gn-T8. This result suggests that putative binding sites between β3Gn-T2 and -T8 could be conserved in human and murine.

What is the biological significance of complex formation between β3Gn-T2 and -T8 and the resulting enzymatic activation? It should be noted that β3Gn-T2 alone has substantial activity. In fact, when tetra-antennary *N*-linked glycan is used as a substrate, β3Gn-T2 has the highest specific activity of β3Gn-T1, -T2, -T3, -T4, -T7, and -T8, all of which are able to synthesize polyLacNAc chains (14, 18–21). Moreover, although tetra-antennary glycan is the best substrate for β3Gn-T2, this enzyme can also efficiently act on tri-, bi-, and mono-antennary *N*-linked glycans (14). Both β3Gn-T2 and -T8 are expressed in various human tissues, but their relative expression levels differ between tissues. In particular, β3Gn-T8 is poorly expressed in colon, prostate, and brain, whereas β3Gn-T2 is substantially expressed in those tissues (19, 20). Considering these facts, we speculate that β3Gn-T2, even in the absence of β3Gn-T8, usually synthesizes rather shorter

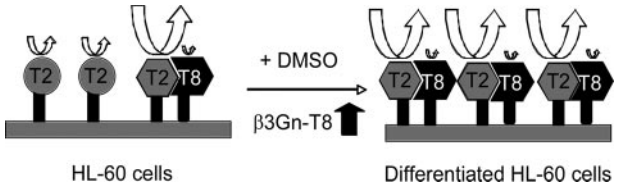

FIGURE 7. **A hypothetical schematic model for the increase in polyLacNAc chains during differentiation of HL-60 cells.** In HL-60 cells, β3Gn-T2 is more highly expressed than β3Gn-T8, and some β3Gn-T2 (*circles*) is free from the complex with β3Gn-T8. After DMSO treatment, β3Gn-T8 is up-regulated. Newly synthesized β3Gn-T8 forms a complex with free β3Gn-T2 and activates β3Gn-T2 (*hexagons*). The *arrow sizes* indicate the intensity of the relative enzymatic activities.

polyLacNAc chains or elongates lower branching *N*-linked glycans. In contrast, expression of β3Gn-T8 may be required for elongated-polyLacNAc-chain synthesis in some specific tissues, at specific developmental stages, and in carcinogenesis. Ishida *et al.* (20) have reported that expression of β3Gn-T8 is quite low in normal colon, but increases markedly in colon cancer tissues. Although it has been unclear whether expression of polyLacNAc chains $[\rightarrow\underline{3Gal\beta1}\rightarrow4GlcNAc\beta1\rightarrow]_n$ increases in colon cancer, Terada *et al.* (35) have recently reported that novel fucosylated polyLacNAc chains, $[\rightarrow\underline{3Gal\beta1}\rightarrow3(Fuc\alpha1\rightarrow4)GlcNAc\beta1\rightarrow]_n$, occur in colon cancer SW1116 cells and serve as ligands for mannan-binding protein. These results suggest that β3Gn-T8 is responsible for the biosynthesis of this type of polyLacNAc chain in malignant tumor cells.

Recently Togayachi *et al.* (36) reported on β3Gn-T2 knock-out mice, in which the expression of polyLacNAc chains detected by tomato lectin is markedly reduced, at least in thymus, spleen, lymphocytes, and macrophages, suggesting that β3Gn-T2 is predominantly involved in the synthesis of polyLacNAc chains in these tissues. Their results are in accordance with our results showing that the increased enzymatic activity is attributable to the β3Gn-T2 portion of the β3Gn-T2/T8 complex.

Lee *et al.* (17) performed quantitative analysis of *N*-linked glycan chains of lamps in HL-60 cells and HL-60 differentiated cells and clearly showed the increase of polyLacNAc chains in the latter. Because the level of *N*-linked glycans binding to tomato lectin increases only 1.5- to 1.6-fold, this may not seem so remarkable. However, our results are in agreement, because the polyLacNAc chains detected by tomato-lectin staining in β3Gn-T8-transfected HL-60 cells increased ~2-fold (Fig. 6). Because the molecular weights of lamps derived from β3Gn-T8- or T8-QA-transfected cells are comparable with those from untreated HL-60 cells, the increase in LacNAc units in the transfected cells does not seem particularly large. Actually, Lee *et al.* (17) showed that the molecular masses of lamp proteins increase from 110–150 kDa to 130–170 kDa in the differentiation of HL-60 cells. We observed the same results as them (data not shown). This increase seems to be due to the increase of polyLacNAc chains, because Lee *et al.* (17) showed by structural studies that levels of other sugar modifications such as sialylation and fucosylation in lamp proteins don't change between the differentiated and undifferentiated HL-60 cells. This fact suggests that the expression level of β3Gn-T8 in the differentiated HL-60 cells may be higher than those in the transfectants for wild and mutated β3Gn-T8 in Fig. 6. On the

other hand, transfection with β3Gn-T2 did not increase the number of polyLacNAc chains (Fig. 6). One explanation for these results is that there is more β3Gn-T2 protein than β3Gn-T8 protein in HL-60 cells and that β3Gn-T8 is saturated as a complex with β3Gn-T2 (Fig. 7). In this case, the introduction of exogenous β3Gn-T8 or T8-QA activates pre-existing free β3Gn-T2, leading to an increase in polyLacNAc chains.

PolyLacNAc is known to be expressed in specific cells/tissues associated with development and carcinogenesis and to serve as a cell-recognition molecule by binding to several lectin proteins. The next step is to determine whether these biological phenomena are related to β3Gn-T2/T8 complex formation.

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