***Drosophila* β 1,4-*N*-acetylgalactosaminyltransferase-A Synthesizes the LacdiNAc Structures on Several Glycoproteins and Glycosphingolipids**

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Abstract

The GalNAc β 1,4GlcNAc (LacdiNAc or LDN) structure is a more common structural feature in invertebrate glycoconjugates when compared with the Gal β 1,4GlcNAc structure. Recently, β 1,4-*N*-acetylgalactosaminyltransferase (β 4GalNAcT) was identified in some invertebrates including *Drosophila*. However, the LDN structure has not been reported in *Drosophila*, and the biological function of LDN remains to be determined. In this study, we examined acceptor substrate specificity of *Drosophila* β 4GalNAcTA by using some *N*- and *O*-glycans on glycoproteins and neutral glycosphingolipids (GSLs). GalNAc was efficiently transferred toward *N*-glycans, *O*-glycans, and the *arthro*-series GSLs. Moreover, we showed that d β 4GalNAcTA contributed to the synthesis of the LDN structure *in vivo*. The *d β 4GalNAcTA* mRNA was highly expressed in the developmental and adult neuronal tissues. Thus, these results suggest that d β 4GalNAcTA acts on the terminal GlcNAc residue of some glycans for the synthesis of LDN, and the LDN structure may play a role in the physiological or neuronal development of *Drosophila*.

Keywords: d β 4GalNAcTA; LacdiNAc; *N*-glycans; *O*-glycans; GSLs

Introduction

The LDN structure is a major structural feature in invertebrate glycoconjugates as compared with the lactosamine Gal β 1,4GlcNAc (LacNAc or LN) structure, particularly in many parasitic nematodes and trematodes [1-3]. In *C.elegans*, the abolition of LDN biosynthesis of the *arthro*-series GSLs, such as GalNAc β 1,4GlcNAc β 1,3Man β 1,4Glc β 1Cer, by mutation in *Ce β 4GalNAcT* (*bre-4*) confers resistance to *Bacillus thuringiensis* (Bt) toxin. This effect is similar to the mutations in *bre-2* and *bre-3*, which are homologues of *egh* and *brn*, respectively [4]. However, the function of the LDN of the *N*- and *O*-glycans in invertebrates remains to be clarified.

To date, only three predicted *Drosophila* glycosyltransferases have been identified to be homologous to mammalian β 1,4-galactosyltransferases (β 4GalTs). Two of these glycosyltransferases, which are encoded by *d β 4GalNAcTA* and *d β 4GalNAcTB*, are considerably closely related to mammalian β 4GalT-I to -VI than β 4GalT-VII. These two proteins have conserved motifs of β 4GalTs, however, the position corresponding to Tyr-289 of β 4GalT-I is Ile or Leu, which is similar to *Ce β 4GalNAcT* and *Trichoplusia ni* (Tn) β 4GalNAcT [5,6]. Based on the analysis of the crystal structure of bovine β 4GalT-I, it has been reported that Tyr-289 is essential for donor binding and the mutation of Tyr-289 to Leu, Ile, or Asn switches the donor substrate preference [7]. Thus, both

d β 4GalNAcTA and d β 4GalNAcTB exhibit GalNAcT activity toward GlcNAc. Mutation in *d β 4GalNAcTA* results in behavioral phenotypes in adult flies [8]. On the other hand, mutation in *d β 4GalNAcTB* does not result in any discernible phenotypes, and flies doubly mutated for both genes display only the behavioral phenotypes associated with the mutation in *d β 4GalNAcTA*. Therefore, it has been suggested that d β 4GalNAcTA is essential for neuromuscular physiology or development but is not strictly required for viability, fertility, or external morphology. Very recent study has reported that mutants for *d β 4GalNAcTA* exhibit abnormal neuromuscular system [9].

In the present study, we examined the acceptor substrate specificity of d β 4GalNAcTA by using substrates that are expected to be found in *Drosophila* and determined that d β 4GalNAcTA transferred GalNAc to some substrates, including a trihexoside of the *arthro*-series GSLs. These results suggest that the LDN structure is present on not only GSLs but also the *N*- and *O*-glycans in *Drosophila*, and LDN-containing glycans may play an important role in neuromuscular physiology or development.

Materials and methods

Materials. The *Drosophila* expressed sequence tag clone LD02648 was obtained from Research Genetics, Inc. The *Drosophila* deletion mutant $d\beta 4GalNAcTA^{4.1}$ and $d\beta 4GalNAcTA^{7.1}$ were kindly gifted by K. D. Irvine [8]. UDP-Gal and UDP-GalNAc were purchased from Sigma. UDP-*N*-acetyl-[6-³H]D-galactosamine (15 Ci/m mol) and UDP-[4,5-³H]galactose (48.3 Ci/m mol) were obtained from American Radiolabeled Chemicals Inc. and PerkinElmer Life Sciences, respectively. Pyridyl amino (PA)-labeled *N*-glycan-related acceptor substrates were purchased from Seikagaku Corp. and Takara. GlcNAc β 1,3Fuc-DNS was prepared previously [10]. Glucosylceramide (GlcCer), lactosylceramide (LacCer), gangliotriaosylceramide (Gg₃Cer), and gangliotetraosylceramide (Gg₄Cer) were obtained from Wako. HRP-conjugated *Wistaria floribunda* (WFA) was purchased from EY Laboratories.

Construction and Purification of FLAG-tagged Soluble d β 4GalNAcTA Enzyme. The putative catalytic domain of d β 4GalNAcTA (amino acids 62-403) was expressed as a secreted protein fused with a FLAG tag in *Sf21* cells according to the instruction manual of GATEWAY™ Cloning Technology (Invitrogen) as previously reported [11]. pVL1393-FLAG is an expression vector derived from pVL1393 (Pharmlngen) and contains a fragment encoding the signal peptide of the human

immunoglobulin κ , the FLAG tag, and a conversion site for the GATEWAY system.

Constructed pVL1393-FLAG-*d β 4GalNAcTA* was cotransfected with BaculoGold viral DNA (Pharmingen) into *Sf21* insect cells according to the manufacturer's instructions, and incubated to produce recombinant viruses. *Sf21* cells were infected with recombinant viruses and incubated for 72 h at 27 °C. The culture supernatants were harvested, and purified using anti-FLAG M1 AFFINITY GEL (Sigma). These purified enzymes were quantified by Western blot analysis as previously reported [10].

The full-length d β 4GalNAcTA and d β 4GalNAcTB proteins were also expressed in *Sf21* cells using pVL1393 vector.

Assay for Glycosyltransferase Activity. In the case of the assay for enzyme activity toward *O*-glycan related disaccharide, the 20- μ l reaction mixture contained 14 mM HEPES (pH 7.4), 0.2% Triton X-100, 11 mM MnCl₂, 0.25 mM UDP-Gal or -GalNAc, 4 μ M GlcNAc α 1,3Fuc-DNS, and soluble enzyme. For *N*-glycan related oligosaccharides as acceptor substrates, 20- μ l reaction mixture contained 100 mM sodium cacodylate (pH 7.2), 0.4% Triton X-100, 1 mM UDP-GalNAc, 40 mM MnCl₂, 4 mM ATP, 0.125 mM acceptor substrates, and soluble enzyme. The reaction was performed at 25 °C for 3, 6, 24 or 48 h. After the mixture was filtrated, a 15- μ l aliquot was subjected to HPLC on an ODS-80Ts QA column (4.6 x 250 mm; Tosoh) and a PALPAK TypeN amide-adsorption column (Takara) for DNS-labeled and PA-labeled substrates, respectively. 30% acetonitrile/70% H₂O and 38%

1 M acetic acid-triethylamine (pH 7.3)/62% acetonitrile were used as the running solution at a flow rate of 1.0 ml/min at 30 °C for the analyses of DNS-labeled and PA-labeled substrates, respectively.

The assay for LacCer synthase activity is shown in online supplemental material.

RNAi Fly. A 500-bp cDNA fragment of *dβ4GalNAcTA* was amplified by PCR (the forward primer, 5'-AAGGCCTACATGGCCGGACCGGCGATATGCTTGTTGCTGGTG-3'; the reverse primer, 5'-AATCTAGAGGTACCTGATGCTGGGCATTGCAGTTT-3'), and inserted as an inverted repeat (IR) with a head-to-head orientation into a modified Bluescript vector, pSC1. The cloning of *dβ4GalNAcTA* into the transformation vector pUAST was done as previously reported [11]. The transformation of *Drosophila* embryos was carried out according to Spradling [12] with *w¹¹¹⁸* stock as a host to make *UAS-dβ4GalNAcTA-IR* fly lines. Each line was mated with the *Act5C-GAL4* fly line, and the F₁ progeny was raised at 28 °C.

Neutral Glycosphingolipids Extraction. Neutral GSLs were isolated as described previously [13]. Third instar larvae were extracted with chloroform:methanol (2:1) by homogenization, and the residue was then re-extracted with chloroform:methanol:water (30:60:8). The combined extracts were spun, and supernatants were dried under N₂. The crude lipid fraction was dissolved in chloroform:methanol (8:2), and applied to the PROSEP[®]-PB (Millipore) column. Neutral lipids and alkaline-resistant phospholipids were washed with chloroform:methanol (8:2). After elution with

chloroform:methanol:water (5:4:1), the eluates, neutral GSLs, were dried, and dissolved in chloroform:methanol (2:1).

Assay for GalNAcT Activity toward Neutral Glycosphingolipids. GalNAcT activity was assayed for 3 h at 25 °C in a 25- μ l reaction mixture containing 0.1 M sodium cacodylate, 4 mM ATP, 40 mM MnCl₂, 0.4% TritonX-100, 0.5 mM UDP-[³H]GalNAc (533.33 mCi/m mol), 10 μ g neutral GSLs derived from *Act5C-GAL4/+* third instar larvae, and enzyme solution. Enzyme solution was prepared from full-length *d β 4GalNAcTA* transfected *Sf21* cells as mentioned above. The products were separated by Sep-PakC18 and the radioactivity was measured by liquid scintillation. The residual eluates were dried, dissolved in chloroform/methanol (2:1), applied to an HPTLC plate (Merck), and developed in chloroform/methanol/water (60:40:10). The bands of reaction products incorporating radioactivity were detected with BAS2000 Imaging Analyzer system.

Lectin Blot Analysis. Protein extract was prepared from third instar larvae with a buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂ 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.4% sodium deoxycholate, and protease inhibitors. The extract was subjected to 2-15% gradient SDS-PAGE, and transferred to a Hybond-P membrane. The membrane was probed with HRP-WFA or anti- α -Tubulin antibody (Sigma) and stained with Konica Immunostaining HRP-1000. For enzyme treatment, the membrane was incubated with α -1,3/4-L-Fucosidase (Takara) for 24 h at 37 °C, and followed with

N-glycanase (Sigma) for 24 h at 37 C°.

Quantitative Analysis of d β 4GalNAcTA mRNA by Real Time PCR. Total RNA was extracted by TRIZOL Reagent (Invitrogen), and first-strand cDNA was synthesized using a Superscript II first strand synthesis kit (Invitrogen). Quantitation of *d β 4GalNAcTA* mRNA expression was performed by real time PCR using the forward primer 5'-CAGAAGGAAAAGGCCAATCCT-3', and the reverse primer 5'-CGAGTTGATTCCATCCTGTTCTATT-3'. The probe, 5'-GCTCATGCCATTCTGTAAGTTTTCATAGCGCT-3', was labeled at the 5'-end with the reporter dye, 3FAM, and at the 3'-end with the quencher dye TAMRA (Applied Biosystems).

Results and Discussion

GalNAcT Activity of d β 4GalNAcTA Toward GSLs

We determined the activity of d β 4GalNAcTA toward GSLs. LacCer is a common synthetic precursor of the structure of several major GSLs such as globo-GSLs in mammals. However, *Drosophila* LacCer synthase has not yet been identified. Both d β 4GalNAcTA and d β 4GalNAcTB did not exhibit LacCer synthase activity (data not shown). Recently, it has been reported that the *Drosophila* glycosyltransferase (CG17223) that is homologous to mammalian Gb₃ synthase exhibited α 4GalNAcT, and may contribute to the biosynthesis of the *arthro*-series GSLs [14]. Therefore, the structures synthesized on LacCer, including globo-GSLs, may not occur in *Drosophila*. In *Drosophila*, the core structure of the *arthro*-series GSLs, GalNAc α 1,4GalNAc β 1,4GlcNAc β 1,3Man β 1,4Glc β 1Cer, is present in most of the GSLs [15]. To examine whether d β 4GalNAcTA contributes to the biosynthesis of this structure, we determined the GalNAcT activity of d β 4GalNAcTA toward the neutral GSLs isolated from the third instar larvae of *Drosophila*. Significant GalNAcT activity was detected when d β 4GalNAcTA was incubated along with the neutral GSLs (Fig. 1A). Furthermore, an autoradiogram of the TLC plate revealed a [³H]GalNAc-labeled band corresponding to the size of a

tetrahexoside ceramide, which was subsequently synthesized to penta-hexoside presumably by an endogenous enzyme (Fig. 1B).

Since d β 4GalNAcTA exhibited GalNAcT activity toward the neutral GSLs (Fig. 1), we examined whether d β 4GalNAcTA acts on GSLs *in vivo*. We attempted to produce RNAi mutant flies by using the GAL4-UAS-IR system. We used *Act5C-GAL4* as a GAL4 driver fly to induce *d β 4GalNAcTA* gene silencing in all the cells and at all the developmental stages of the fly. The third instar larvae of inducible *d β 4GalNAcTA* RNAi flies (*Act5C-GAL4/UAS-d β 4GalNAcTA-IR*) and control flies (*Act5C-GAL4/+*) were collected. Then, we determined the mRNA levels of the third instar larvae by the real time PCR. The *d β 4GalNAcTA* mRNA level of the *d β 4GalNAcTA* RNAi flies was 41% of that of the control flies (Fig. 2A). The neutral GSLs were extracted from the third instar larvae. As observed in TLC separation, in *d β 4GalNAcTA* RNAi flies, the intensity of the band corresponding to the size of a tetrahexoside ceramide was less than half that of the control (Fig. 2B). These results confirm that d β 4GalNAcTA plays a role in the biosynthesis of a tetrahexoside of the *arthro*-series GSLs.

It has been reported that in *C. elegans* the abolition of the biosynthesis of the core *arthro*-series GSLs by mutation in *bre-4* confers resistance to the Bt toxin, similar to the mutations in *bre-2* and *bre-3* [4]. Therefore, in *Drosophila*, mutation in *d β 4GalNAcTA* may also confer resistance to the Bt

toxin.

GalNAcT Activity of d β 4GalNAcTA Toward N- and O-glycans

To identify other possible acceptor substrates, we determined the specificity for acceptor substrates by using some *N*- and *O*-glycans containing GlcNAc at their non-reducing terminal. Some of the *O*-glycans from *Drosophila* have been characterized. In a previous report, only the simple core 1 Gal β 1,3GalNAc type has been described as mucin-type *O*-glycans [16], while no β 1,6-*N*-acetylglucosaminyltransferase, which is a core 2 synthase, is absent. Moreover, only the single mannosyl-modified glycans may be present as *O*-mannosyl glycans because the protein *O*-mannose β 1,2-*N*-acetylglucosaminyltransferase is absent [17]. Therefore, the remaining candidate of *O*-glycans containing terminal GlcNAc residue is *O*-fucosylated glycans, which is a modification of epidermal growth factor-like domains found in Notch. To date, only the disaccharide structure modified by *fringe*, a β 1,3-*N*-acetylglucosaminyltransferase, is known as *O*-fucosylated glycan in *Drosophila*, and an extended structure has not been reported. Therefore, to determine whether the *O*-fucosylated glycans showed an extended structure, we examined the activity of d β 4GalNAcTA toward the disaccharide, GlcNAc β 1,3Fuc. As shown in Table I, d β 4GalNAcTA efficiently transferred

only GalNAc toward the disaccharide. As previously reported [8,9], flies with mutation in *dβ4GalNAcTA* are viable and morphologically normal, and they show only behavioral phenotypes. On the other hand, null mutants of *fringe* are lethal in *Drosophila*, and even weak alleles have obvious morphological phenotypes in adult flies [18]. This confirms that dβ4GalNAcTA is not required for Notch signaling and in the essential developmental stage. However, it is suggested that the novel trisaccharide structure GalNAcβ1,4GlcNAcβ1,3Fuc may occur in *Drosophila*, and it may contribute to some physiological functions.

In the case of *N*-glycans, dβ4GalNAcTA efficiently transferred GalNAc, and the PA-2 structure accepted GalNAc with approximately 5-fold higher efficiency than the PA-1 structure (Table II). This suggests that a β4-linked GlcNAc may be more efficient than a β2-linked GlcNAc. Then, we performed WFA lectin blot analysis to examine whether dβ4GalNAcTA acts on *N*-glycans *in vivo*. WFA specially recognizes terminal GalNAc residue on *N*-glycans, and has been demonstrated to bind strongly to LDN structure [19,20]. As shown in Fig. 3A, the positive staining of smear bands (above 83 kDa) recognized by WFA was reduced significantly after treatment with both α-1,3/4-L-Fucosidase and *N*-glycanase, indicating that those regions contain *N*-glycans carrying core α3Fuc residue. Such WFA-recognized *N*-glycans were significantly reduced in *dβ4GalNAcTA*^{4.1} and *dβ4GalNAcTA*^{7.1} (Fig.3B). Thus, these results demonstrate that dβ4GalNAcTA synthesizes LDN structure on *N*-glycans.

In invertebrates, LDN is a more common structure than LN, and has been found in many parasitic nematodes and trematodes [1-3]. However, the function of LDN in invertebrates remains to be clarified. Recently, Tn β 4GalNAcT has been identified [6], suggesting the presence of an LDN structure on *N*-glycans. However, LDN has only been reported on the *N*-glycans of honeybee venom glycoproteins in insects [21]. In *Drosophila*, LDN structure on *N*-glycans is not yet to be identified. As shown in Table II, d β 4GalNAcTA efficiently transferred GalNAc toward *N*-glycans. A previous study reported that *Drosophila* sialyltransferase (d.SiaT) is very active toward the LDN-containing structures, and the strongest expression of d.SiaT was detected during the late embryonic stages in presumably neurons within the developing central nervous system (CNS) [22]. The position of d.SiaT-expressing cells within CNS has been mapped to the region of specified motoneurons [23]. It is conceivable that d.SiaT functions within these cells to modulate the adhesive properties of motoneuronal processes by sialylating cell-surface *N*-glycans. d β 4GalNAcTA synthesizes LDN structure on *N*-glycans *in vivo* (Fig. 3B), and flies with mutation in *d β 4GalNAcTA* show behavioral phenotypes and abnormalities of neuromuscular junctions [8,9]. Recently, the activity of the muscle-specific receptor tyrosine kinase, a key mediator of neuromuscular synapse formation, has been found to be mediated by sialylated *N*-glycans in mammalian cells [24]. Thus, it is suggested that d β 4GalNAcTA contribute to the synthesis of the LDN structure on *N*-glycans, and the sialylated LDN

may be important for the establishment of neuromuscular junctions in neuronal development.

Expression Patterns of $d\beta 4GalNAcTA$ mRNA

We determined the expression patterns of $d\beta 4GalNAcTA$ mRNA by the real time PCR. The results showed that $d\beta 4GalNAcTA$ mRNA was highly expressed in 0-2 h, suggesting a strong maternal expression. Further, the $d\beta 4GalNAcTA$ mRNA was expressed throughout the life cycle of the fly, i.e., from the embryo stage. In the larval stage, $d\beta 4GalNAcTA$ mRNA was expressed to a greater extent in the eye disc and larval brain than in the wing disc and leg disc (Fig. 4).

Conclusion

We determined that $d\beta 4GalNAcTA$ plays an important role in LDN synthesis on not only the *arthro*-series GSLs but also *N*- and *O*-glycans. Further, there is a possibility that LDN occurs in *Drosophila* and exhibits some physiological functions. In invertebrates, including *Drosophila*, LDN may occur instead of LN and subsume the biological function of LN in vertebrates. Further studies will be required to reveal the significance of LDN present on the *N*- and *O*-glycans on glycoproteins

and GSLs in physiological functions.

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Figure legends

Fig. 1. GalNAcT activity toward the *Drosophila* neutral GSLs (A) and TLC separation (B). *A*,

GalNAcT activity toward the neutral GSLs extracted from *Drosophila* was measured. An enzyme solution was prepared from *Sf21* cells infected with either recombinant full-length d β 4GalNAcTA baculovirus (*solid bar*) or wild-type virus (*hatched bar*). *B*, The reaction products were separated by TLC, and radioactivity of the incorporated product was detected using BAS2000 radioimage analyzer. The *arrow* shows [3 H]GalNAc-incorporated tetrahexoside ceramide, and the *arrowhead* shows subsequently synthesized pentahexoside. The plate was developed in chloroform/methanol/water (60:40:10). The migration of the standard GSLs is indicated.

Fig. 2. Quantitative analysis of *d β 4GalNAcTA* mRNA (A) and the neutral GSLs in *d β 4GalNAcTA*

RNAi flies (B). *A*, *d β 4GalNAcTA* mRNA levels in the third instar larvae of *d β 4GalNAcTA* RNAi flies were determined by the real time PCR. *Act5C-GAL4/+* corresponds to the control flies. The actual amounts of *d β 4GalNAcTA* mRNA were normalized to those of the *RpL32* mRNA, which were measured using the same cDNAs. Two independent experiments were conducted, and the representative results are shown. *B*, The neutral GSLs were extracted from the third instar larvae of

dβ4GalNAcTA RNAi flies or control flies. TLC separation is shown in upper (the *arrow* indicates tetrahexoside ceramide). The plate was developed in chloroform/methanol/water (60:40:10) and stained with copper (II) acetate/8% phosphoric acid. The migration of the standard GSLs is indicated. The intensity of the tetrahexoside ceramide band in upper was measured using densitometer and is indicated in lower.

Fig. 3. WFA lectin blot analysis. *A*, WFA lectin blot analysis was performed on protein extracts from *wild type* third instar larvae. The extract was subjected to 2-15% gradient SDS-PAGE and transferred to a Hybond-P membrane. After treatment with or without α-1,3/4-L-Fucosidase or/and *N*-glycanase, the membrane was probed with HRP-WFA and stained. *B*, Protein extract from *wild type*, *dβ4GalNAcTA^{4.1}* or *dβ4GalNAcTA^{7.1}* was subjected to SDS-PAGE and transferred as like *A*. The membrane was probed with HRP-WFA or anti-α-Tubulin antibody as an internal control and stained.

Fig. 4. The expression patterns of *dβ4GalNAcTA* mRNA. Quantitative analysis was performed by the real time PCR for various *Drosophila* mRNA, which were derived from 0-2, 2-4, 4-8, 8-12, 12-16, and 16-22 h old embryos, the first, second, and third instar larvae, male and female bodies, male and female heads, wing discs, leg discs, eye discs, and larval brains. The actual amounts of *dβ4GalNAcTA*

mRNA were normalized to those of the *RpL32* mRNA, which were measured using the same cDNAs.

Two independent experiments were conducted, and the representative results are shown.

Fig.1

A)

B)

Fig.2

A)

B)

Fig.3

A)

B)

Fig.4

Table I

Activity of d β 4GalNAcTA toward GlcNAc β 1-3Fuc disaccharide

Acceptor substrate	UDP donor	Enzyme activity ^a
		<i>pmol/nmol/h</i>
GlcNAc β 1-3Fuc-DNS	UDP-Gal	ND ^b
GlcNAc β 1-3Fuc-DNS	UDP-GalNAc	284

^a Assays were performed in duplicate as described in the Experimental Procedures. The concentrations of acceptor substrate and UDP donor were 4 μ M and 0.25 mM, respectively.

^b ND, not detected.

Table II

Activity of d β 4GalNAcTA toward N-glycans

Acceptor substrate		Enzyme activity ^a
		<i>pmol/nmol/h</i>
PA-1	$ \begin{array}{c} \text{GlcNAc}\beta 1-2\text{Man}\alpha 1-6 \\ \diagdown \quad \diagup \\ \text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3 \quad \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc-PA} \end{array} $	3.03
PA-2	$ \begin{array}{c} \text{GlcNAc}\beta 1-2\text{Man}\alpha 1-6 \\ \diagdown \quad \diagup \\ \text{GlcNAc}\beta 1-4 \quad \text{Man}\alpha 1-3 \quad \text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc-PA} \\ \diagdown \\ \text{GlcNAc}\beta 1-2 \end{array} $	15.63

^a Assays were performed in duplicate as described in the Experimental Procedures. The concentrations of acceptor substrate and UDP donor were 125 nM and 1 mM, respectively.

The assay for LacCer synthase activity was performed as previously reported [T. Nomura, M. Takizawa, J. Aoki, H. Arai, K. Inoue, E. Wakisaka, N. Yoshizuka, G. Imokawa, N. Dohmae, K. Takio, M. Hattori, and N. Matsuo, Purification, cDNA cloning, and expression of UDP-Gal: glucosylceramide beta-1,4-galactosyltransferase from rat brain, J. Biol. Chem. 273 (1998) 13570-13577.]. LacCer synthase activity was assayed for 3 h at 25 °C in the 25- μ l reaction mixture contained 100 mM sodium cacodylate (pH 7.2), 40 mM MnCl_2 , 76 mM dioleoylphosphatidylcholine, 30 mM CDP-choline, 10 μ g GlcCer or GalCer, 0.5 mM UDP-[^3H]Gal (10 Ci/ m mol), and full-length enzyme. In this assay, enzyme was prepared from *d β 4GalNAcTA* or *d β 4GalNAcTB* transfected *Sf21* cells by lysis with a buffer containing 20 mM HEPES (pH 7.4), 154 mM NaCl, 1% Triton X-100, and protease inhibitors. Preparation of the products was carried out using a Sep-PakC18 column. The unreacted UDP-Gal was washed out with water, and the products were eluted with chloroform:methanol (2:1). The products were assayed for incorporation of radioactivity by liquid scintillation.