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Drosophila β1,4-*N*-acetylgalactosaminyltransferase-A synthesizes the LacdiNAc structures on several glycoproteins and glycosphingolipids

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Abstract

The GalNAcβ1,4GlcNAc (LacdiNAc or LDN) structure is a more common structural feature in invertebrate glycoconjugates when compared with the Galβ1,4GlcNAc structure. Recently, β1,4-*N*-acetylgalactosaminyltransferase (β4GalNAcT) 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* β4GalNAcTA 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 dβ4GalNAcTA contributed to the synthesis of the LDN structure *in vivo*. The *dβ4GalNAcTA* mRNA was highly expressed in the developmental and adult neuronal tissues. Thus, these results suggest that dβ4GalNAcTA 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: dβ4GalNAcTA; LacdiNAc; *N*-Glycans; *O*-Glycans; GSLs

The LDN structure is a major structural feature in invertebrate glycoconjugates as compared with the lactosamine Galβ1,4GlcNAc (LacNAc or LN) structure, particularly in many parasitic nematodes and trematodes [1–3]. In *Caenorhabditis elegans*, the abolition of LDN biosynthesis of the *arthro*-series GSLs, such as GalNAcβ1,4GlcNAcβ1,3Manβ1,4Glcβ1Cer, by mutation in *Ceβ4GalNAcT* (*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 β1,4-galactosyltransferases (β4GalTs). Two of these glycosyltransferases, which are encoded by *dβ4GalNAcTA* and *dβ4GalNAcTB*, are considerably closely related to mammalian β4GalT-I to -VI than β4GalT-VII. These two proteins

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have conserved motifs of β4GalTs, however, the position corresponding to Tyr-289 of β4GalT-I is Ile or Leu, which is similar to Ceβ4GalNAcT and *Trichoplusia ni* (Tn) β4GalNAcT [5,6]. Based on the analysis of the crystal structure of bovine β4GalT-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 dβ4GalNAcTA and dβ4GalNAcTB exhibit GalNAcT activity toward GlcNAc. Mutation in *dβ4GalNAcTA* results in behavioral phenotypes in adult flies [8]. On the other hand, mutation in *dβ4GalNAcTB* does not result in any discernible phenotypes, and flies doubly mutated for both genes display only the behavioral phenotypes associated with the mutation in *dβ4GalNAcTA*. Therefore, it has been suggested that dβ4GalNAcTA 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 *dβ4GalNAcTA* exhibit abnormal neuromuscular system [9].

In the present study, we examined the acceptor substrate specificity of dβ4GalNAcTA by using substrates that are expected to be found in *Drosophila* and determined that dβ4GalNAcTA 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 *dβ4GalNAcTA*$^{4.1}$ *and dβ4GalNAcTA*$^{7.1}$ was kindly gifted by Dr. K.D. Irvine [8]. UDP-Gal and UDP-GalNAc were purchased from Sigma. UDP-*N*-acetyl-[6-^{3}H]D-galactosamine (15 Ci/mmol) and UDP-[4,5-^{3}H]galactose (48.3 Ci/mmol) were obtained from American Radiolabeled Chemicals Inc. and Perkin-Elmer Life Sciences, respectively. Pyridyl amino (PA)-labeled *N*-glycan-related acceptor substrates were purchased from Seikagaku Corp. and Takara. GlcNAcβ1,3Fuc-DNS was prepared previously [10]. Glucosylceramide (GlcCer), lactosylceramide (LacCer), gangliotriaosylceramide (Gg_3Cer), and gangliotetraosylceramide (Gg_4Cer) were obtained from Wako. HRP-conjugated *Wisteria floribunda* (WFA) was purchased from EY Laboratories.

Construction and purification of FLAG-tagged soluble dβ4GalNAcTA enzyme. The putative catalytic domain of dβ4GalNAcTA (amino acids 62–403) was expressed as a secreted protein fused with a FLAG tag in *Sf21* insect cells according to the instruction manual of GATEWAY™ Cloning Technology (Invitrogen) as previously reported [11]. pVL1393-FLAG is an expression vector derived from pVL1393 (Pharmingen) 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-*dβ4GalNAcTA* was cotransfected with BacuroGold viral DNA (Pharmingen) into *Sf21* 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 dβ4GalNAcTA and dβ4GalNAcTB 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-μl reaction mixture contained 14 mM Hepes (pH 7.4), 0.2% Triton X-100, 11 mM $MnCl_2$, 0.25 mM UDP-Gal or -GalNAc, 4 μM GlcNAcβ1,3Fuc-DNS, and soluble enzyme. For *N*-glycan-related oligosaccharides as acceptor substrates, 20-μl reaction mixture contained 100 mM sodium cacodylate (pH 7.2), 0.4% Triton X-100, 1 mM UDP-GalNAc, 40 mM $MnCl_2$, 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-μl aliquot was subjected to HPLC on an ODS-80Ts QA column (4.6 × 250 mm; Tosoh) and a PALPAK TypeN amide-adsorption column (Takara) for DNS-labeled and PA-labeled substrates, respectively. Thirty percent acetonitrile/70% H_2O 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′-AAGGCCTACATGGCCGGACCG GCGATATGCTTGTTGCTGGTG-3′; the reverse primer, 5′-AATCTA GAGGTACCTGATGCTGGGCATTGCAGTTT-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 w^{1118} stock as a host to make *UAS-dβ4GalNAcTA-IR* fly lines. Each line was mated with the *Act5C-GAL4* fly line, and the F_1 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 N_2. 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-μl reaction mixture containing 0.1 M sodium cacodylate, 4 mM ATP, 40 mM $MnCl_2$, 0.4% Triton X-100, 0.5 mM UDP-[^{3}H]GalNAc (533.33 mCi/mmol), 10 μg neutral GSLs derived from *Act5C-GAL4/+* third instar larvae, and enzyme solution. Enzyme solution was prepared from full-length *dβ4GalNAcTA*-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 $MgCl_2$ 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 (Millipore). 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 dβ4GalNAcTA 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 *dβ4GalNAcTA* mRNA expression was performed by real-time PCR using the forward primer 5′-CAGAAGGAAA AGGCCAATCCT-3′, and the reverse primer 5′-CGAGTTGATTCCA TCCTGTTCTATT-3′. The probe, 5′-GCTCATGCCATTCTGTAAGT TTTCATAGCGCT-3′, was labeled at the 5′-end with the reporter dye,