The Drosophila melanogaster brainiac Protein Is a Glycolipid-specific β1,3N-Acetylglucosaminyltransferase*

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Mutations at the Drosophila melanogaster brainiac locus lead to defective formation of the follicular epithelium during oogenesis and to neural hyperplasia. The brainiac gene encodes a type II transmembrane protein structurally similar to mammalian β1,3-glycosyltransferases. We have cloned the brainiac gene from D. melanogaster genomic DNA and expressed it as a FLAG-tagged recombinant protein in Sf9 insect cells. Glycosyltransferase assays showed that brainiac is capable of transferring N-acetylglucosamine (GlcNAc) to β-linked mannose (Man), with a marked preference for the disaccharide Man(β1,4)Glc, the core of arthro-series glycolipids. The activity of brainiac toward arthro-series glycolipids was confirmed by showing that the enzyme efficiently utilized glycolipids from insects as acceptors whereas it did not with glycolipids from mammalian cells. Methylation analysis of the brainiac reaction product revealed a β,1,3 linkage between GlcNAc and Man, proving that brainiac is a β,1,3GlcNAc-transferase. Human β,1,3GlcNAc-transferases structurally related to brainiac were unable to transfer GlcNAc to Man(β1,4)Glc-based acceptor substrates and failed to rescue a homozygous lethal brainiac allele, indicating that these proteins are paralogous and not orthologous to brainiac.

The importance of glycoconjugates in regulating developmental processes is continually being supported by studies performed in various model organisms like Caenorhabditis elegans (1), Drosophila melanogaster (2), and the mouse (3). The Drosophila genes sugarless, sulfatless, pipe, tout-velu, and daily participate in the formation of proteoglycans. Loss of function mutations in some of these genes produce polarity defects whereas it did not with glycolipids from mammalian cells. Methylation analysis of the brainiac reaction product revealed a β,1,3 linkage between GlcNAc and Man, proving that brainiac is a β,1,3GlcNAc-transferase. Human β,1,3GlcNAc-transferases structurally related to brainiac were unable to transfer GlcNAc to Man(β1,4)Glc-based acceptor substrates and failed to rescue a homozygous lethal brainiac allele, indicating that these proteins are paralogous and not orthologous to brainiac.

The signaling proteins wingless and hedgehog (4–6). The rotated abdomens locus, whose disruption is associated with a helical rotation of the body, has been found to encode a potential O-mannosyltransferase (7), and fringe, which modulates the interaction of the Notch receptor with its ligands (8), has recently been demonstrated to be a β,1,3N-acetylglucosaminyltransferase (GlcNAcT) (9, 10).

The Drosophila gene brainiac (brn) encodes a protein that shares structural motifs with β1,3glycosyltransferases (12, 13). The brn gene is localized on the X chromosome. brn was shown to cooperate with the epidermal growth factor receptor and one of its ligands, the Drosophila TGFα homologue gurken (11) during oogenesis. Mutant brn alleles exhibit altered morphology of the follicular epithelium (11), female sterility (14), and germ line loss (15). Furthermore, brn embryos develop neural hyperplasia and epidermal hypoplasia (11) as encountered with Notch hypomorphic alleles and other neurogenic mutants, suggesting implications of brn in Notch signaling (16, 17).

While the relationships between brn and specific signaling pathways have been examined genetically, the nature of these interactions remained elusive as long as the biochemical function of brn was unclear. In the present study, we show that brn has a β,1,3N-acetylglucosaminyltransferase (GlcNAcT) activity directed toward the Man(β1,4)Glc core structure of arthro-series glycolipids.

EXPERIMENTAL PROCEDURES

Cloning and Expression—The brn gene was amplified by PCR from D. melanogaster OregonR genomic DNA during 30 cycles at 95 °C for 45 s, 55 °C for 30 s, 72 °C for 60 s using the primers 5′-TTTGGATCC-GTGGCCATGCAAAGT-3′ and 5′-CCCTGTTCATGATCAGCGTAA-T-3′. The resulting 1.0-kb fragment was ligated with BamHI and XhoI and subcloned into the pFastbac-FLAG(a) vector (Invitrogen) linearized at the BamHI and XhoI sites. The FLAG-tagged brn gene was expressed as a recombinant baculovirus in insect cells as described previously (13). Infected cells (108) were lysed at 72 h post-infection in 600 µl of 50 mM Tris/HC1, pH 7.4, 150 mM NaCl, 1% Triton X-100, and a protein inhibitor mixture (complete, EDTA free, Roche Diagnostics) on ice. Post-nuclear supernatants were incubated with 240 µl of anti-FLAG M2-agarose beads (Sigma) under rotation for 2.5 h at 4 °C. Beads were washed three times with Tris-buffered saline and used as enzyme source for assays.

Glycosyltransferase Assays—All donor and acceptor substrates were from Sigma except Man(β1,4)Glc(β1,6)OPNP (pNP = p-nitrophenyl), which was purchased from Toronto Research (North York, Canada). Glycosyltransferase activity was assayed for 60 min at 25 °C with 15 µl of beads, 5% Me3SO, 20 mM MnCl2, 0.08 mM UDP-GlcNAc including 5 × 104 cpm of UDP-[14C]GlcNAc (Amersham Biosciences), and various acceptors (see Table I). Reaction products were purified over C18 Sep-Pak cartridges (Waters) (18) and quantified in a Tri-Carb 2900TR liquid scintillation counter (Packard) with luminescence correction. Glycolipid Extraction—D. melanogaster Schneider 2 cells, Spodoptera frugiperda Sf9 cells, and human colon carcinoma Caco-2 cells were washed three times in phosphate-buffered saline and extracted in iso-propanol:hexane:H2O (55:25:20). Extracts were spun twice at 500 g, and supernatants were dried under N2. Phospholipids were removed by saponification in 0.2 M NaOH in methanol for 24 h at 37 °C. After neutralization with HCl, the extracts were expanded to theoretical upper phase (methanol:water:chloroform, 47:48:3), applied on C18-Sep-

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The abbreviations used are: GlcNAcT, N-acetylglucosaminyltransferase; brn, brainiac; TLC, thin-layer chromatography; pNP, p-nitrophenyl; Cer, ceramide; Lc2, Gal(β1,4)Glc-Cer; Lc3, GlcNAc(β1,3)Gal(β1,4)Glc-Cer; HPLC, high performance liquid chromatography.

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Pak cartridges, and eluted with 5 ml of methanol. Eluates were dried under N₂ and resuspended in 500 μl of methanol. The procedure yielded about 120 μg of mannose equivalents for 10⁶ S2 and Sf9 cells and 20 μg of mannose equivalents from 10⁷ Caco-2 cells as determined by the phenol sulfuric acid assay (19).

**Thin-layer Chromatography (TLC)—**Glycolipids (5 μg of mannose equivalents per assay) were dried under N₂ and incubated together with 10 μl of beads-bound enzyme in 50 μl of 50 mM cadoxolute buffer, pH 7.1, 20 mM MnCl₂, 0.06% Triton X-100, 2.5 × 10⁻⁴ cmol of UDP-[¹⁴C]GlcNAc for 90 min at 25 °C. Reaction products were separated to theoretical upper phase and purified over C₁₈ Sep-Pak cartridges as described above. After drying over N₂, the eluates were taken up in 100 μl of methanol/chloroform (1:1) and separated on aluminum high-performance thin-layer chromatography plates (Merck, Darmstadt, Germany) using a solvent system of chloroform:methanol:0.25% CaCl₂ (5:4:1). Plates were stained with orcinol sulfuric acid (Sigma). The [¹⁴C]GlcNAcβ₁,3Galβ₁,4Glc-ceramide (Lc3) standard was produced enzymatically with the Lc3 synthase β₁,3 GlcNAcT protein (20) using Galβ₁,4Glc-ceramide (Lc2) (Sigma) as acceptor substrate.

**bnr Complementation in Drosophila—**Human (β₃GnT1 (21), β₃GnT4 (22) and β₃GnT5 (20)) cDNAs and the Drosophila bnr gene were subcloned into the pUAST vector (23). The rescue constructs pUAST-β₃GnT1, pUAST-β₃GnT4, pUAST-β₃GnT5, and pUAST-bnr were injected together with the pUc8sp2Δ-3 F-element helper plasmid (Flybase accession FBme0050938) into yellow white Drosophila embryos. The progeny examined for males carrying the forked mutation for 8 days after eclosion of the first flies. At least two independent lines of each transgene were used for the complementation assay, which were repeated four times.

**Structural Analysis—**A mixture of substrate and of 10 nmol of product was separated by reversed phase HPLC on a 3 μm column filled with 5 μM ODS Hypersil (Shandon) at a flow rate of 0.6 ml/min. The column was eluted with a linear gradient from 6 to 24% of methanol during 18 min in 0.1 M ammonium acetate, pH 4.0. p-Nitrophenylglycosides were monitored at 245 nm. The mixture was also analyzed after incubation with N-acetyl-β-D-hexosaminidase from jack beans (Sigma) (25). The fraction of interest was collected in a screw capped glass vial and dried in a speed-vac concentrator. A small aliquot was used for the analysis of the sample was dried over phosphorus pentoxide and permethylated using NaOH (26). Partially permethylated alditol acetates were prepared using NaBD₄ as the reducing agent and analyzed by gas chromatography/mass spectrometry using a 60 m SP2330 column (Restek) (27) and a Finnigan Ion Trap ITD800. Derivatives of terminal Fuc(1-OpNP) 5.4 5.7
Gal(1-OpNP) 6.9 7.2
GalNAc(1-OpNP) 7.6 4.5
Fuc(1-OpNP) 6.6 5.5
Man(1-OpNP) 4.6 16.1
Man(β₁,3Glc(1-OpNP) 5.6 400.0
Man(β₁,4Glc(1-OpNP) 3.0 855.1
Gal(β₁,4Glc(1-OpNP) 4.5 24.9

| Acceptor substrate (20 nm) | Mocka | bna² |
|--------------------------|-------|------|
| Glc(1-OpNP)              | 7.2   | 5.2  |
| Glc(β₁,3GnT)             | 5.4   | 5.7  |
| Gal(1-OpNP)              | 8.4   | 11.1 |
| Gal(β₁,3GnT)             | 6.2   | 12.6 |
| GalNAc(1-OpNP)           | 6.7   | 7.2  |
| GalNAc(β₁,3GnT)          | 5.3   | 8.9  |
| Fuc(1-OpNP)              | 7.6   | 4.5  |
| Fuc(β₁,3GnT)             | 6.6   | 5.5  |
| Man(1-OpNP)              | 4.6   | 16.1 |
| Man(β₁,3GnT)             | 5.6   | 400.0|
| Man(β₁,4Glc(1-OpNP)      | 3.0   | 855.1|
| Gal(β₁,4Glc(1-OpNP)      | 4.5   | 24.9 |

* Anti-FLAG bead bound lysate from Sf9 cells infected with mock baculovirus.

**RESULTS**

We have cloned the D. melanogaster bnr gene by PCR amplification and expressed it as an N-terminally FLAG-tagged full-length protein in Sf9 insect cells. The recombinant bnr protein was bound to anti-FLAG-agarose beads, and cellular contaminants such as possible endogenous acceptor substrates were washed out before assaying for enzymatic activity. A GlcNAcT activity was only detected toward the Man(β₁,3GnT) acceptor when monosaccharide substrates were assayed (Table I). Highest activity was measured toward the disaccharide acceptor Man(β₁,4Glc(β₁,3GnT)) acceptor whereas a slight activity was also detected toward Gal(β₁,4Glc(β₁,3GnT)) (Table I). The Man(β₁,4Glc) structure represents the core of arthro-series glycolipids found in nematodes (28) and insects (29) among others.

In Drosophila, the arthro-series Man(β₁,4Glc) core is elongated with a β₁,3-linked GlcNAc (30), suggesting that bnr may represent the enzyme catalyzing this step. To test this hypothesis, we have isolated neutral glycolipids from Drosophila S2 and Spodoptera Sf9 cells and assayed these glycolipids as acceptors for the anti-FLAG beads-bound bnr enzyme. A significant GlcNAc-transferase activity was detected when incubating bnr together with insect glycolipids, whereas only a low activity was measured with glycolipids extracted from mammalian Caco-2 cells, likely reflecting the low specificity of bnr for lactosylceramide. The reaction products were separated by TLC and plates were autoradiographed, revealing a [¹⁴C]GlcNAC-label band at the size of a trihexoside ceramide in S2 and Sf9 cells (Fig. 1).

The nature of the linkage between GlcNAc and the underlying β-linked Man residue was investigated by methylation analysis of the bnr reaction product GlcNAc-Man(β₁,3GnT). In reversed phase HPLC, the presumed disaccharide product eluted slightly ahead of the substrate Man(β₁,3GnT). The disaccharide peak disappeared upon incubation with N-acetyl-β-D-hexosaminidase (Fig. 2A). The purified fraction corresponding to the disaccharide peak exhibited a pseudomolecular ion of m/z 513.5. Linkage analysis of the GlcNAc-Man(β₁,3GnT) disaccharide product gave a peak at the relative retention time of 0.597, which suggests a 2- or a 3-substituted mannosyl residue (27). The fragment spectrum clearly identified the derivative as substituted in the 3-position (Fig. 2B), thus confirming the identity of bnr as a β₁,3 GlcNAcT.

The bnr protein is structurally related to human β₁,3 glycosyltransferase enzymes (12, 13). The acceptor specificity of bnr for the arthro-series glycolipid core suggested that it represents a paralogous enzyme to the mammalian β₁,3 glycosyltransferases, including β₁,3 galactosyltransferases (13, 31, 32), β₁,3 GlcNAcT (20), and a β₁,3-N-acetylgalactosaminyltransferase (33) acting on GlcNAcβ₋₃Galβ₋₃, Galβ₋₃, and GalNAcβ₋₃-based acceptors. Although no mammalian β₁,3 GlcNAcT has been described to act on β-linked Man acceptors, we have investigated whether the three human β₁,3 GlcNAcT structurally closest to bnr can complement the lethal phenotype of bnr deficient Drosophila flies. To this end, we have expressed the human β₃GnT-I (21), IV (22), and V (20) in bnr¹⁶⁶⁷ mutants flies (34) using the UAS-GAL4 transgene system (23).

The human β₃GnT transgenes and a bnr transgene were expressed in flies carrying the allele bnr¹⁶⁶⁷, which causes lethality at the late pupal stage. The transgenes were expressed ubiquitously using armadillo GAL4 transactivator (16) (Table I). The bnr transgene did rescue bnr¹⁶⁶⁷ mutant flies from their hemizygous late pupal lethality, whereas the human β₃GnT transgenes did not (Table II). The rescue of bnr¹⁶⁶⁷ males was confirmed by detection of the forked marker, whose gene is located besides the bnr¹⁶⁶⁷ allele on the X chromosome. Control crosses of females carrying bnr¹⁶⁶⁷ with yellow white males did not yield any living bnr¹⁶⁶⁷ forked/N males either. The inability of human β₃GnT enzymes to compensate for the
loss of brn activity in mutant flies suggested that the former enzymes cannot elongate the arthro-series glycolipid core in vivo. This was confirmed in vitro by showing that the human β3GnT-V enzymes did not exhibit significant activity toward the Man(β1,4)Glc(β1-OpNP) acceptor (Table II).

DISCUSSION

We have shown that Drosophila brn, a member of the β1,3 glycosyltransferase family, encodes a β1,3 GlcNAcT enzyme with a specificity for the Man(β1,4)Glc disaccharide found in arthro-series glycolipids (29). Several mammalian enzymes structurally related to brn have been suggested to represent homologues (35–37). However, the specificity of brn for Man(β1,4)Glc, a disaccharide that has never been described in vertebrates, rather indicates that brn and mammalian β1,3 glycosyltransferases are paralogous proteins derived from a common ancestor gene.

The functional disparity between the β1,3 GlcNAcT brn and mammalian β1,3 GlcNAcT enzymes is further supported by the inability of the latter to complement the lethal phenotype of the Man(β1,4)Glc-1,4)Glc-Cer (Gb3), and GalNAc(1,3)GalNAc(β1,3)Gal(β1,4)Glc-Cer (FS). Lc2/Lc3, Lc2 was elongated to Lc3 by incorporation of [14C] GlcNAc catalyzed by the human β3GnT-V enzyme (20).

FIG. 1. TLC separation of brn-modified glycolipids. The top panel shows a plate stained with orcinol reagent and the bottom panel the autoradiogram of the same plate. brn-bound beads (brn) or beads preincubated with mock-infected Sf9 cells (mock) were incubated with neutral glycolipids from Drosophila S2 cells (S2), Spodoptera Sf9 cells (Sf9), human Caco-2 cells (Caco), or without added glycolipids (no GL). The neutral glycolipid standard (GL Std) contained: GalCer; Gal(β1,4)Glc-Cer (Lc2); Gal(α1,4)Gal(β1,4)Glc-Cer (Gb3); Gal(α1,4)Gal(β1,4)Glc-Cer (Gb4), and GalNAcα1,3GalNAcβ1,3Gal(α1,4)Glc-Cer (FS). Lc2/Lc3, Lc2 was elongated to Lc3 by incorporation of [14C] GlcNAc catalyzed by the human β3GnT-V enzyme (20).

FIG. 2. Structural analysis of brn product. A, HPLC separation of product and substrate of β1,3GlcNAcT brn. p-Nitrophenyl-β-D-mannopentose was incubated with brn in the presence of UDP-GlcNAc. The mixture was prepurified over Sep-Pak C18 cartridges and subjected to reversed phase chromatography (trace a). A small product peak (P) eluted ahead of the substrate (S). The product disappeared upon incubation with β-β-acyclohexosaminidase (trace b) which indicates it to contain a β-linked GlcNAc residue. B, methylation analysis of p-nitrophenyl disaccharide. The electron impact mass spectrum of the partially methylated monodeuterated alditol acetate derived from the mannosyl residue of the disaccharide product shows several fragments indicative of a substitution in position 3 as depicted by the insert. Especially the presence of mass 118 and the absence of mass 190 exclude a 2-substitution, which could not be ruled out from the reten- tion time alone (27).

TABLE II

Complementation of Drosophila brn<sup>1</sup> <sup>+,4</sup>

Rescue of the brn<sup>1</sup> <sup>+,4</sup> late pupal lethal phenotype by ubiquitous expression of Drosophila brn and human β1,3GlcNAcT transgenes.

| β1,3GlcNAcT gene<sup>a</sup> | Lines<sup>b</sup> | brn<sup>1</sup> <sup>+,4</sup> rescue<sup>c</sup> | GlcNAc -> Man(β1,4)Glc activity<sup>d</sup> |
|----------------------------|-----------------|-------------------------------|------------------|
| Drosophila brn              | 2               | 2                             | 100%             |
| Human β3GnT-I               | 2               | 0                             | 6.3              |
| Human β3GnT-IV              | 2               | 0                             | 1.6              |
| Human β3GnT-V               | 6               | 0                             | 8.6              |

<sup>a</sup> β3GnT-I (21), β3GnT-IV (22), and β3GnT-V (20).

<sup>b</sup> Number of independent lines per transgene tested.

<sup>c</sup> Number of independent lines per transgene rescuing brn<sup>1</sup> <sup>+,4</sup> (34).

<sup>d</sup> In vitro GlcNAc-transferase activity towards Man(β1,4)Glc(β1-OpNP) given in percentage of the activity measured with brn.

interactions. On the other hand, arthro-series glycolipids may modulate specific signaling proteins in a way similar to gangliosides affecting the epidermal growth factor receptor (41, 42), insulin receptor (43), and platelet-derived growth factor receptor (44) signaling cascades. The notion that brn glycolipid products interact with adhesion or signaling proteins implies
that other mutant genes with phenotypes similar to those encountered in brn mutant flies may encode partner lectin/signaling proteins. Along this line, Drosophila egghead mutants have similar and non-additive phenotypes to brn (17). Experiments aimed at characterizing the biochemical and functional relation between brn products and the egghead protein should reveal the mechanisms how arthro-series glycolipids regulate morphogenetic events during Drosophila development.

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