Genetic Interactions Between Drosophila sialyltransferase and β1,4-N-acetylgalactosaminyltransferase-A Genes Indicate Their Involvement in the Same Pathway

Michiko Nakamura, Dheeraj Pandey, and Vladislav M. Panin
Department of Biochemistry & Biophysics, Texas A&M University, College Station, Texas 77843-2128

ABSTRACT Sialylated glycans play a prominent role in the Drosophila nervous system where they are involved in the regulation of neural transmission. However, the functional pathway of sialylation in invertebrates, including Drosophila, remains largely unknown. Here we used a combination of genetic and behavioral approaches to shed light on the Drosophila sialylation pathway. We examined genetic interactions between Drosophila sialyltransferase (DSiaT) and β1,4-N-acetylgalactosaminyltransferase (β4GalNAcT) genes. Our results indicated that β4GalNAcT and DSiaT cooperate within the same functional pathway that regulates neural transmission. We found that β4GalNAcT is epistatic to DSiaT. Our data suggest an intriguing possibility that β4GalNAcT may participate in the biosynthesis of sialylated glycans.

Sialylation is a common type of protein glycosylation in vertebrate organisms (Schauer 2009; Varki and Schauer 2009). In mammals, sialylated glycans affect a plethora of protein interactions in the extracellular milieu, play a variety of important biological roles in development, and in influenza virus infection (Varki and Schauer 2009). In mammals, sialylation is a common type of protein glycosylation in vertebrate organisms, it regulates neural transmission. We found that β4GalNAcT is epistatic to DSiaT. Our data suggest an intriguing possibility that β4GalNAcT may participate in the biosynthesis of sialylated glycans.

The structure of Drosophila N-linked glycans indicates that ganglioside residues (Gal) of LacNAc termini (Galβ1,4GlcNAc) serve as acceptors for sialylation (Aoki et al. 2007; Koles et al. 2007). Therefore, a galactosyltransferase attaching β1,4-linked Gal to N-glycans should be required for sialylation, and this enzyme is expected to cooperate with DSiaT in the regulation of neural transmission. However, so far no β1,4 galactosyltransferase (β4GalT) of this type has been identified in invertebrates. In mammalian cells, the corresponding Gal residues are added by one of the six β4GalTs (β4GalT1–6), the enzymes that function with apparent redundancy in modifying N-glycans (Hennet 2002). In Drosophila, the family of most closely related homologs of these β4GalTs consists of two enzymes, β1,4-N-acetylgalactosaminyltransferases A and B (β4GalNAcTA and β4GalNAcTB) (Haines and Irvine 2005). However, when assayed in vitro, these two glycosyltransferases exhibit substrate specificity different from that of vertebrate β4GalTs. Both of them prefer to transfer N-acetylgalactosamine (GalNAc) and synthesize LacdiNAc (GalNAcβ1,4GlcNAc) instead of LacNAc, whereas their ability to transfer Gal is low (Chen et al. 2007; Haines et al. 2007; Aoki et al. 2007; Koles et al. 2007). Here we used a genetic strategy, combined with the knowledge of glycan structures identified on fly glycoproteins, to shed light on the sialylation pathway in Drosophila.
and and Irvine 2005; Ramakrishnan and Qasba 2007). Despite the fact that β4GalNAcTA and β4GalNAcTB have similar in vitro activities, they have non-redundant functions in vivo (Chen et al. 2007; Haines and Irvine 2005; Haines and Stewart 2007; Stolz et al. 2008). The β4GalNAcTB enzyme modifies glycosphingolipids, and its function affects EGFR signaling during oogenesis (Chen et al. 2007; Stolz et al. 2008). Because β4GalNAcTA is capable of elongating βGalNAc-termini of glycosphingolipids by adding β1,4-linked GaINAc in vivo, this glycosyltransferase may also have some role in glycosphingolipid biosynthesis in vivo (Chen et al. 2007; Johswich et al. 2009). However, this role is likely to be minor because β4GalNAcTA mutants have no discernable defects of glycosphingolipids, and the endogenous targets of β4GalNAcTA remain largely elusive (Chen et al. 2007; Johswich et al. 2009; Stolz et al. 2008). Mutations in β4GalNAcTA result in behavioral phenotypes, ultrastructural defects of muscles, and neuromuscular junction abnormalities (Chen et al. 2007; Haines and Irvine 2005; Haines and Stewart 2007).

Considering the close evolutionary relationship between β4GalNAcTA/B and vertebrate β4GalTs, we reasoned that these Drosophila enzymes might participate in the biosynthesis of N-linked glycans in vivo. This scenario entails a possibility that β4GalNAcTA/B is involved in the generation of glycan acceptors for DSiaT, and therefore, the mutations in these genes would affect DSiaT-mediated processes. Here we test this hypothesis using genetic and behavioral approaches.

**MATERIALS AND METHODS**

**Drosophila rearing and genetic stocks**

Flies were reared in a temperature-controlled (25°C) and humidity-controlled (37%) environment at diurnal light conditions. We used the following mutant alleles for DSiaT and β4GalNAcTA/B genes: DSiaT(w1118 Canton-S), β4GalNAcTA41, and β4GalNAcTB47, designated here as DSiaT-, β4GalNAcTA-, and β4GalNAcTB-, respectively. These mutants represent loss-of-function alleles and were previously described (Haines and Irvine 2005; Repnikova et al. 2010). Double mutants DSiaT- β4GalNAcTA- were generated by recombination. The DSiaT- and β4GalNAcTA- single and double mutants were confirmed by genomic PCR and sequencing for the presence of corresponding mutations: the DSiaT- allele includes two stop codons within the DSiaT coding region that truncate the encoded DSiaT protein sequence at positions Cys18 and Leu377 (Repnikova et al. 2010); the β4GalNAcTA- allele includes a 609 bp deletion that removes 113 bp upstream of the start codon along with the downstream region encoding the first 143 amino acids of β4GalNAcT (Haines and Irvine 2005). The following PCR primers were used for genomic PCR reactions for DSiaT-28, St-gen-up (5'-TTAAGTGGGAGCTAAGGTTCAAT3') and Sia-spe (5'-CACTAGATAATGGCCTCTCTTAGAT3'); for β4GalNAcTA41, TA-P2 (5'-TGCGGCTGTGCTAGGAT3') and TA-P3 (5'-AACGAGGGATGACTTTGTAAT3'). The β4GalNAcTB47 mutation was confirmed by genomic PCR reactions with two sets of primers that amplify the genomic region of β4GalNAcT disrupted by gene targeting, as described in Haines and Irvine (2005). The presence of β4GalNAcTB- was also corroborated by scoring the dorsal appendage fusion phenotype in homozygous mutants (Chen et al. 2007). The ectopic expression of β4GalNAcTA was induced using the UAS-GALA system (Brand et al. 1994) specifically in neurons (β4GalNAcTANeuro) or in muscles (β4GalNAcTAMuscle) with CI55-Gal4 and Dmef2-Gal4 drivers, respectively (Lin and Goodman 1994; Ranganayakulu et al. 1996). We used w1118 Canton-S as a wild-type control in our experiments.

**Heat-induced paralysis assays**

We assayed five-day-old males for heat-induced paralysis using the previously described protocol (Repnikova et al. 2010). At least 20 flies were assayed for each data point. Unless indicated otherwise, the heat-shock assays were performed with individual flies at 38°C.

**Statistical analysis**

We used Student unpaired t-test with two-tailed distribution to assess the statistically significant differences between groups of related data.

**RESULTS AND DISCUSSION**

To test the possibility that β4GalNAcTA/B glycosyltransferases could be involved in the functional pathway mediated by sialylation, we examined genetic interactions between DSiaT and β4GalNAcTA/B genes. DSiaT mutations cause a characteristic temperature-sensitive paralysis phenotype (TS paralysis) (Repnikova et al. 2010). We used the TS paralysis assay to characterize genetic interactions between DSiaT and β4GalNAcTA/B. Whereas DSiaT mutants were consistently paralyzed within 7–10 min, neither β4GalNAcTA nor β4GalNAcTB mutants showed TS paralysis (Figure 1). Strikingly, the analysis of double mutants revealed that the β4GalNAcTA mutation suppressed the paralysis phenotype of DSiaT mutants. At the same time, no significant interaction was observed between DSiaT and β4GalNAcTB (Figure 1). To characterize the interaction between DSiaT and β4GalNAcTA/B in more detail, we assayed the “kinetics” of paralysis by counting the number of paralyzed flies in a 3-min interval after transferring them to the restrictive temperature (38°C). We found that β4GalNAcTA mutation semi-dominantly suppressed the phenotype of DSiaT mutants, which indicates that the DSiaT phenotype is very sensitive to the level of β4GalNAcTA activity (Figure 2). It was previously shown that β4GalNAcTA plays separate roles in neural and muscle cells (Haines and Stewart 2007). Thus, we investigated whether the neural or muscle-specific function of β4GalNAcTA is responsible for the interaction with DSiaT. We used a rescue strategy with the UAS-GALA ectopic expression system (Brand et al. 1994) to induce the expression of β4GalNAcTA specifically in neurons or muscle cells of DSiaT- β4GalNAcTA double mutants. These experiments revealed that the neuronal expression of β4GalNAcTA could suppress the effect of β4GalNAcTA mutation on the paralysis of DSiaT mutants, whereas the expression in muscles...
did not influence the phenotype of the double mutants (Figure 3). Therefore, we concluded that it is the neuron-specific function of \( \beta 4GalNAcTA \) that affects the paralysis of \( DSiaT \) mutants. Moreover, we found that the ectopic expression of \( \beta 4GalNAcTA \) in the neurons of \( DSiaT \) mutants could further enhance the phenotype (Figure 4A), which again highlighted that the TS paralysis of \( DSiaT \) mutants depends on the neural activity of \( \beta 4GalNAcTA \). The involvement of neural activity of \( \beta 4GalNAcTA \) in the \( DSiaT \)-mediated pathway is consistent with the fact that \( DSiaT \) function is restricted to neurons at all developmental stages (Koles et al. 2004; Repnikova et al. 2010). Collectively, our results indicate that \( \beta 4GalNAcTA \) and \( DSiaT \) cooperate within the same functional pathway that regulates neural excitability and that \( \beta 4GalNAcTA \) is epistatic to \( DSiaT \).

The \( \beta 4GalNAcTA \) protein is the closest \( Drosophila \) homolog of vertebrate \( \beta 4GalT1 \)–6 (Haines and Irvine 2005). These \( \beta 4GalT \)s are thought to originate from invertebrate \( \beta 4GalNAcT \)s during evolution (Haines and Irvine 2005; Ramakrishnan and Qasba 2007). Interestingly, the donor substrate specificity of \( \beta 4GalT \) and \( \beta 4GalNAcT \) enzymes, including \( \beta 4GalNAcTA \), can be changed between Gal and GalNAc just by a single amino acid substitution in the active site (Ramakrishnan and Qasba 2002, 2007). Moreover, the donor and acceptor specificities of mammalian \( \beta 4GalT1 \) can be modified through the binding of a protein cofactor, \( \alpha \)-lactalbumin (Do et al. 1995; Henret 2002). Such “flexibility” of the \( \beta 4GalT/\beta 4GalNAcT \) catalytic pocket capable of adjusting to different substrates suggests that the \( \beta 4GalNAcTA \) specificity may be modified in vivo by a co-factor to synthesize LacNAc termini of N-linked glycans. This possibility is further supported by the fact that the ability to bind a co-factor is an evolutionarily ancient feature of \( \beta 4GalT/GalNAcT \) enzymes, and that this feature is also preserved for \( Drosophila \) \( \beta 4GalNAcT \) (Neeleman and van de Eijnden 1996; Ramakrishnan and Qasba 2007). The scenario that \( \beta 4GalNAcTA \) may synthesize LacNAc termini, the potential acceptors for sialylation, is also consistent with the epistatic interaction between \( \beta 4GalNAcTA \) and \( DSiaT \) that was revealed in our experiments. However, this scenario does not rule out that \( \beta 4GalNAcTA \) has other functions that are not limited to its role in the \( DSiaT \)-mediated pathway. This is supported by the fact that \( \beta 4GalNAcTA \) mutants exhibit other phenotypes apparently unrelated to the function of \( DSiaT \), such as muscle abnormalities and the defects of neuromuscular junctions at muscle 6 during the larval stage (Haines and Stewart 2007; Repnikova et al. 2010).

The hypothesis that \( \beta 4GalNAcTA \) may be involved in the biosynthesis of \( DSiaT \) acceptors predicts that the overexpression of \( \beta 4GalNAcT \) in the nervous system of wild-type flies, in the presence of endogenous \( DSiaT \) activity, may result in increased resistance to heat. This possibility is based on the fact that a limiting factor of the insect biosynthesis of complex N-glycans, including sialylated structures, is the high activity of the GlcNAcase \( \beta 4GalNAcTA \). The upregulation of \( \beta 4GalNAcTA \) might outcompete GlcNAcase, while protecting glycan termini from trimming by converting them to LacNAc, the substrate for further sialylation. Thus, in the presence of \( DSiaT \), the overexpression of \( \beta 4GalNAcTA \) may result in a more efficient biosynthesis of sialylated glycans, which in turn would increase the stability of neural transmission at elevated temperatures. Indeed, we observed that wild-type flies become more resistant to heat when \( \beta 4GalNAcTA \) was ectopically overexpressed using a neuronal driver (Figure 4B).

Taken together our results demonstrated that \( \beta 4GalNAcTA \) genetically interacts with \( DSiaT \), indicating that these genes cooperate in the...
same functional pathway affecting neural transmission. Our data also suggest an intriguing possibility that β4GalNAcTA may participate in vivo in the biosynthesis of LacNAc termini of N-glycans, including sialylated glycans. In the light of the fact that the loss of β4GalNAcTA activity suppresses the mutant phenotype of DSiaT, it is tempting to speculate that sialic acids may play a masking role, capping LacNAc structures and thus regulating their interactions in the nervous system. However, other scenarios are also possible, and the mechanism of the interplay between β4GalNAcTA and sialylation pathway awaits further investigation using biochemical and in vivo approaches.

**ACKNOWLEDGMENTS**

We thank Nicola Haines and Ken Irvine for mutant and transgenic β4GalNAcTA/B strains, Michael Tiemeyer and Pradman Qasba for useful discussions, and Naosuke Nakamura for help with the experiments. We also thank Daria Panina and Courtney Caster for comments on the manuscript. This work was supported by NIH grants GM-069952 and NS-075534 (to V.M.P.).

**LITERATURE CITED**

Aoki, K., M. Perlman, J. M. Lim, R. Cantu, L. Wells et al., 2007 Dynamic developmental elaboration of N-linked glycan complexity in the Drosophila melanogaster embryo. J. Biol. Chem. 282: 9127–9142.

Brand, A. H., A. S. Manoukian, and N. Perrimon, 1994 Ectopic expression in Drosophila. Methods Cell Biol. 44: 635–654.

Chen, Y. W., J. W. Pedersen, H. H. Wandall, S. B. Levery, S. Pizette et al., 2007 Glycosphingolipids with extended sugar chain have specialized functions in development and behavior of Drosophila. Dev. Biol. 306: 736–749.

Do, K. Y., S. I. Do, and R. D. Cummings, 1995 Alpha-lactalalbumin induces bovine milk beta 1,4-galactosyltransferase to utilize UDP-GalNAc. J. Biol. Chem. 270: 18447–18451.

Haines, N., and B. A. Stewart, 2007 Functional roles for beta1,4-N-acetylgalactosaminyltransferase-A in Drosophila larval neurons and muscles. Genetics 175: 671–679.

Haines, N., and K. D. Irvine, 2005 Functional analysis of Drosophila beta1,4-N-acetylgalactosaminyltransferases. Glycobiology 15: 335–346.

Hennet, T., 2002 The galactosyltransferase family. Cell. Mol. Life Sci. 59: 1081–1095.

Johns, A., B. Kraft, M. Wuhrer, M. Berger, A. M. Deelder et al., 2000 Golgi targeting of Drosophila melanogaster beta4GalNAcT by requirements of a DHHC protein-family-related protein as a pilot. J. Cell Biol. 184: 173–183.

Klee, R., and M. Schachner, 2004 Glycans and neural cell interactions. Nat. Rev. Neurosci. 5: 195–208.

Koles, K., K. D. Irvine, and V. M. Panin, 2004 Functional characterization of Drosophila sialyltransferase. J. Biol. Chem. 279: 4346–4357.

Koles, K., J. M. Lim, K. Aoki, M. Porterfield, M. Tiemeyer et al., 2007 Identification of N-glycosylated proteins from the central nervous system of Drosophila melanogaster. Glycobiology 17: 1388–1403.

Leonard, R., D. Rendic, C. Rabouille, I. B. Wilson, T. Preat et al., 2006 The Drosophila fused lobes gene encodes an N-acetylgalactosaminidase involved in N-glycan processing. J. Biol. Chem. 281: 4867–4875.

Lin, D. M., and C. S. Goodman, 1994 Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. Neuron 13: 507–523.

Neeleman, A. P., and D. H. van de Eijnden, 1996 Alpha-lactalumitins affects the acceptor specificity of Lymnaea stagnalis albumen gland UDP-GalNAc: GlcNAc beta-R beta 1→4-N-acetylgalactosaminyltransferase: synthesis of GalNAc beta 1→4Glc. Proc. Natl. Acad. Sci. USA 93: 10111–10116.

Ramakrishnan, B., and P. K. Qasba, 2002 Structure-based design of beta 1,4-galactosyltransferase I (beta 4Gal-T1) with equally efficient N-acetylgalactosaminyltransferase activity: point mutation broadens beta 4Gal-T1 donor specificity. J. Biol. Chem. 277: 20833–20839.

Ramakrishnan, B., and P. K. Qasba, 2007 Role of a single amino acid in the evolution of glycans of invertebrates and vertebrates. J. Mol. Biol. 365: 570–576.

Ranganayakulu, G., R. A. Schulz, and E. N. Olson, 1996 Wingless signaling induces nautilus expression in the ventral mesoderm of the Drosophila embryo. Dev. Biol. 176: 143–148.

Repnikova, E., K. Koles, M. Nakamura, J. Pitts, H. Li et al., 2010 Sialyltransferase regulates nervous system function in Drosophila. J. Neurosci. 30: 6466–6476.

Schafer, R., 2009 Sialic acids as regulators of molecular and cellular interactions. Curr. Opin. Struct. Biol. 19: 507–514.

Stolz, A., N. Haines, A. Pich, K. D. Irvine, C. H. Hokke et al., 2008 Distinct contributions of beta 4GalNAcT and beta 4GalNAcTA to Drosophila glycosphingolipid biosynthesis. Glycoconj. J. 25: 351–359.

Varki, A., 2007 Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. Nature 446: 1023–1029.

Varki, A., 2008 Sialic acids in human health and disease. Trends Mol. Med. 14: 351–360.

Varki, A., and R. Schafer, 2009 Sialic acids, pp. 199–219 in Essentials of Glycobiology, edited by A. R. D. Varki, J. D. Cummings, H. H. Esko, P. Freeze, and Stanley et al. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Watanabe, S., T. Kokuho, H. Takahashi, M. Takahashi, T. Kubota et al., 2002 Sialylation of N-glycans on the recombinant proteins expressed by a baculovirus-insect cell system under beta-N-acetylgalactosaminidase inhibition. J. Biol. Chem. 277: 5090–5093.

Communicating editor: J. A. Birchler