Fabini, Gustáv, and Iain B.H. Wilson. 2002. Stage and tissue specific expression of a sialyltransferase-like gene (CG4871) in *Drosophila melanogaster*. *Dros. Inf. Serv*. 85: 45-49

Stage and tissue specific expression of a sialyltransferase-like gene (CG4871) in *Drosophila melanogaster*.

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Abstract

Ten years ago the detection by antibodies and lectins of polysialic acid in the *Drosophila* neural system was the first suggestion that this mammalian-like glycoprotein modification may be present in the fly. Exact structural data, though, is still absent. The homology of one *Drosophila* gene (CG4871) to mammalian sialyltransferases, however, increases the possibility that the insects are indeed capable of sialylation, although the enzymatic activity of the CG4871 gene product remains to be determined. In our studies we have found that this gene is transcribed throughout development, but with a maximum in 24 hour embryos. *In situ* hybridisation of embryos indicates the presence of transcripts only in the developing salivary gland.

Introduction

Sialic acids are common components of the cell surfaces of vertebrates and bacteria and are also frequently found on serum glycoproteins of medical interest. The use of insect cells for the expression of recombinant glycoproteins of potential therapeutic or other commercial interest has led to the examination of whether insects can produce mammalian-type sialylated oligosaccharides. However simple the problem may at first sight seem, a significant controversy has developed in insect glycobiology as to whether sialylation does or does not naturally occur in lepidopteran cell lines or in *Drosophila melanogaster* (Fabini and Wilson, 2001). Typical approaches for detecting sialic acids in insects have included lectin blots or sugar composition analyses (Schauer, 2001), although these do not yield structural data and can only be considered suggestive. Specifically in *Drosophila*, binding of *Limax flavus* lectin to developing neural tissue and endosialidase-sensitive recognition of a high-molecular weight protein in 12-18 hour embryos by an antibody apparently directed against polysialic acid was reported (Roth *et al*., 1992). In other insect species, neuraminidase-sensitive ruthenium red binding to sections and EI-MS composition analysis of the prothoracic glands of *Galleria mellonella* (Karacaili *et al*., 1997) and lectinohistochemistry of the Malpighian tubules of *Philaenus spumaris* (Malykh *et al*., 1999) was taken as evidence for the presence of sialic acid. It is safe to say that, to date, no mass spectrometric or NMR data on isolated glycans have been published which demonstrate the presence of sialylated glycoproteins or glycolipids in flies or in other insects. It is probable that, if such compounds are present, they are so in only low amounts or have highly novel characteristics so as to result in their not being detected.

As an alternative approach, a number of researchers have turned to the fly genome in the hope of finding homologues of genes with known functions in sialic acid metabolism. The apparent absence of

homologues to sialic-binding lectins (Siglecs) and one enzyme, UDP-GlcNAc 2-epimerase/*N*-acetylmannosamine kinase, involved in mammalian sialic acid biosynthesis was, though, concluded by one group to mean that flies have an incomplete 'sialic acid toolkit' and that the ability to synthesize and bind sialic acids is limited to animals of the deuterostome lineage (Angata and Varki, 2000, 2002). However, another group recently reported the expression of a functional *Drosophila N*-acetylneuraminic acid phosphate synthase gene (Kin *et al*., 2002). Interestingly, transfection of this gene into Sf-9 cells resulted, when *N*-acetylmannosamine was not added to the medium, in production of 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid (KDN, a deaminosialic acid) and not of *N*-acetylneuraminic acid (Neu5Ac). Whether KDN is a low-level natural component of insect cells (*i.e.*, present when an insect *N*-acetylneuraminic acid phosphate synthase gene is not overexpressed) is unclear. The same article also highlights that genes encoding potential sialyltransferase and a CMP-sialic acid transporter homologues are present in the fly genome, observations that probably intrigued many glycobiologists. As yet no report has appeared to indicate that the fly sialyltransferase homologue is active *in vitro* or *in vivo*. Also no data have been published on the stage- or tissue-specificity of the transcription of this gene. We have, therefore, used *in situ* hybridisation and RT-PCR to examine the expression of this gene in more detail.

Experimental Procedures

Isolation of Drosophila *ST6Gal like cDNA:* The full-length *Drosophila melanogaster* sialyltransferase-like protein cDNA (Flybase entry CG4871 located on arm 2R, 60D14) was initially amplified by RT-PCR from total RNA (isolated by Trizol reagent; Invitrogen) prepared from TF24 wild-type flies using primer pair ST6I-fw: ATGAGGCAAGTGACACGCG and ST6I-rev: TCAGTAGCCAAAGAAGTTGAAG. The PCR fragment was subcloned into the pCR 2.1 vector (Invitrogen) and its sequence was confirmed by DNA sequencing using the ABI PRISM Big Dye Terminator Sequencing Ready Mix and an ABI PRISM 310 Genetic analyser (Perkin Elmer, Applied Biosystems). Nucleic acid sequences were analysed using the DNAStar suite of programs and NCBI BLAST and NCBI BLAST2 programs (www.ncbi.nlm.nih.gov).

Developmental stages and RT-PCR: TF24 wild type *D. melanogaster* flies were maintained at 25°C on a 12hr light/dark cycle. After 60 min pre-collection, eggs were collected for 90 min on standard apple-juice petri dishes and different developmental stages were generated by aging for appropriate times as indicated. Total RNAs from various stages were isolated by TRIzol reagent (Invitrogen) followed by a first strand cDNA synthesis using IMPROM-II reverse transcriptase (Promega) and oligo $(dT)_{18}$ as a primer. Expression of the *Drosophila* sialyltransferase-like gene (CG4871) was analysed by performing a 35 cycle PCR reaction [55°C 30"; 72°C, 2´; 94°C 30"] using stage-specific cDNAs, PCR MasterMix (Promega) and primers producing a 566 bp fragment: Dm_Sia-fw: CTGCGCCATCGTCTCCAG and Dm_Sia-rev: GCCAGGAGCCGAATGTGC. The cDNAs were normalised against the RP49 transcript, coding for a ribosomal protein. The primers used, producing a 440 bp fragment, were as follows: rp49-fw: GACCATCCGCCCAGCATAC and rp49-rev: TCCGACCAGGTTACAAGAAC.

In situ *hybridisation of* Drosophila *whole-mount embryos*: After dechorionation in 50% chlorox for 3 min the embryos were well rinsed in water and fixed for 20 min in 5% paraformaldehyde in PBS with *n*-heptane (1:1) followed by devitellinization in *n*-heptane:methanol (1:1). Fixed embryos were washed three times with methanol and ethanol and stored at –20°C until use. For *in situ* hybridisation the embryos were rinsed three times with methanol, then rehydrated successively in methanol/5%

paraformaldehyde in PBT (0.1% Tween 20 in PBS) and equilibrated in PBT. The embryos were post-fixed for 20 min in 5% paraformaldehyde in PBT, washed four times with PBT and 10 min in 1:1 PBT/Hybe (50% formamide, 5× standard saline citrate [SSC], 100 µg/ml sonicated, boiled salmon sperm DNA [Sigma], 100 µg/ml tRNA [Sigma, type X from yeast], 50 µg/ml heparin and 0.1% Tween 20). After equilibration in Hybe the embryos were pre-hybridised for 1 hr at 55°C without rotation and hybridised overnight at the same temperature using a typical anti-sense RNA DIG probe 1:50 (see below). Excess of the DIG probe was removed by washing the embryos four times with Hybe over 1.5 hr at 55°C, once with Hybe/PBT (1:1) for 10 min and four times in PBT for 1 hr at room temperature. The DIG labelled RNA probe was detected with the aid of a preabsorbed anti-DIG antibody coupled to alkaline phosphatase (Roche) and visualised using NBT/BCIP as substrate (Sigma). The colour reaction was stopped by extensive ethanol washes.

The DNA fragment for the generation of the digoxigenin (DIG)-labelled RNA probe was amplified from the pCR2.1 plasmid containing the entire open reading frame of the sialyltransferase-like gene by PCR using the forward gene-specific primer AAAGCTCAGCCGGGACGC and a reverse primer consisting of a 27 nt 5´ T7 RNA polymerase binding site (GAATTAATACGACTCACTATAGGGAGA) followed by the gene-specific sequence CACCTCGTGTCCTTGCGT. After purification (Qiagen spin columns) the PCR products (1 µg) were used as templates using a T7 transcription kit (Ambion, Austin, TX) combined with a RNA labelling mix containing DIG-UTP (Roche). The DIG labelled RNAs were ethanol precipitated, resuspended in a minimal volume of water and stored at –20°C until use.

Results and Discussion

In the course of our studies on *Drosophila* glycosylation, we found by homology searches a sialyltransferase-like gene (CG4871) and cloned the relevant cDNA from adult flies. The cDNA sequence we found was the same as that later deposited by Kim *et al*. (Genbank accession AF397532), but different from that sequenced by Farkas *et al*. (Genbank accession AF218237). Like the reading frame predicted from the gene sequence, the AF218237 form encodes a protein of 474 amino acids, whereas the AF397532 form would encode a protein lacking residues 57-79 – thus suggesting that CG4871 transcripts are subject to alternative splicing with the predicted 69 nt intron between the first and second introns not always being removed. The predicted *Drosophila* sialyltransferase-like protein has 41% homology over 246 amino acids to human ST6GalII (Takashima, *et al*., 2002), 38% over 280 amino acids to human ST6GalI (Grundmann *et al*., 1990), 26% over 206 amino acids to ST8SiaIV (Nakayama *et al*., 1995) and 49% over 370 amino acids to the predicted *Anopheles* protein EAA04038. Kim *et al.* (2002) note that the fly sialyltransferase homologue shares conservation also with residues in α2,8-sialyltransferases which are absent from α2,6-sialyltransferases. It can then be speculated that the fly enzyme is a bifunctional α2,6/α2,8-sialyltransferase such as the *cst-II* gene product of *Campylobacter jejuni* OH4384 (Gilbert *et al*., 2000). Indeed if there is only one sialyltransferase, no other hypothesis is compatible with the proposed presence of polysialic acid in the fly (Schauer, 2001). The mosquito and fly genes are not the only sialyltransferase homologues in non-vertebrate eukaryotes. There are three such homologues in *Arabidopsis thaliana* (IBHW, unpublished data); however, in contrast to insects, there is no modern report even suggesting the presence of sialic acids in plants.

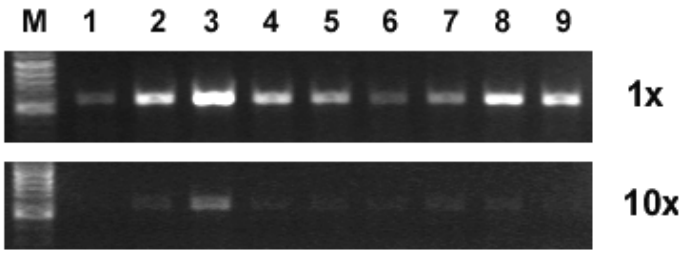

Figure 1. RT-PCR analysis of sialyltransferase-like gene expression during *Drosophila* development. After pre-collection, the embryos were allowed to develop at 25°C. The lanes 1-9 represent the collection times: 8hr embryo, 16hr, 24hr, 48hr, 72hr, 96hr, early pupa, late pupa, adult fly. After isolation of total RNAs first strand cDNAs were analysed by PCR using specific primer set for *Drosophila* sialyltransferase-like gene. The lower panel (10×, represents the ten times diluted first strand cDNAs used in the PCR reactions.

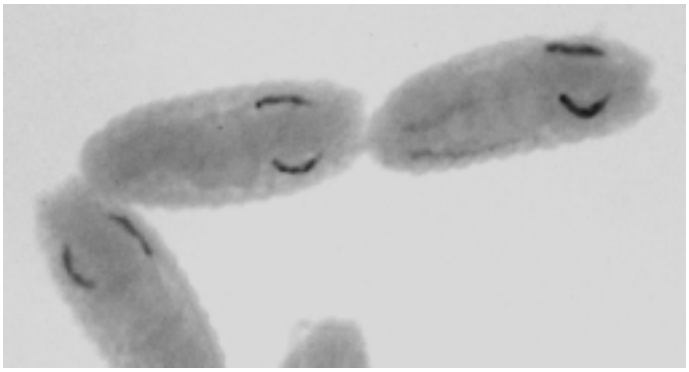

Figure 2. CG4871 *in situ* hybridisation in whole-mount *Drosophila* embryos. A specific digoxigenin labelled anti-sense RNA probe against CG4871 stained intensively salivary glands in the whole-mount preparations of *Drosophila* embryos.

The expression of this gene was first examined by performing PCR with cDNA prepared from various developmen-tal stages. The cDNA samples were normalised with respect to the amount of detected *rp49* transcript. The results (Figure 1) indicate one major peak of expression (24 hour em-bryos), raising the possi-bility that this gene is mainly required during certain key stages of development and can be compared to the previously-determined apparent peak of neural anti-polysialic acid staining at 12-18 hours (Roth *et al.*, 1992). When we then performed *in situ* hybridisation of whole-mount embryos using an RNA probe specific for the CG4871 gene (Figure 2) only a rather discreet expression limited to the embryonic salivary glands was found. Since RNA probes for other transcripts we have tested (data not shown) gave different results, we assume this result is no artefact. It would then seem possible that sialic acid is a component of glue proteins or other mucins, rather than being in the neural system.

Conclusion

We have determined that the sialyltransferase-like gene (CG4871**)** of *Drosophila melanogaster* is transcribed primarily in a stage-specific manner suggestive of a role in development. The results of *in situ* hybridisation indicating the restriction of transcript expression to embryonic salivary glands are, though, somewhat at variance with previous lectin-binding data suggesting that sialic acids are present within the fly neural system. However, in the three insect species examined, sialic acids have been claimed to be present in quite different tissues – and the possibility that binding to lectins or antibodies is due to attached bacteria or sialic acid-like molecules other than Neu5Ac cannot be ruled out. Firm structural and enzymatic data on sialylation is required in order to resolve the apparent ambiguities.

Acknowledgments: This work was funded by a grant from the Fonds zur Förderung der wissenschaftlichen Forschung (P13810) to IBHW.

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