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Glycosyltransferases in *Drosophila melanogaster*.

Fabini, Gustáv, and Iain B.H. Wilson[1]. Glycobiology Division, Institut für Chemie, Universität für Bodenkultur, Muthgasse 18, A-1190 Wien, Austria. [1]Corresponding author: iwilson@edv2.boku.ac.at; Fax: +43-1-36006-6059

It is now some fifteen years since the first sequences of glycosyltransferases related to eukaryotic glycoconjugate biosynthesis were published (*e.g.* α2,6-sialyltransferase and β1,4-galactosyltransferase) [1]. Before this time what is now called glycobiology focussed on studying N- and O-linked oligosaccharide structures; in the past few years, though, the number of glycosyltransferase genes and cDNAs cloned has vastly increased - primarily human, bovine and murine sequences have been defined - partly due to the high biomedical interest and partly that those glycosyltransferases purified to homogeneity, in order to sequence peptides, have been usually mammalian. Now, though, the realisation that glycosyltransferases are often members of conserved families allows the mining of the wholly sequenced lower eukaryotic genomes for the identification of genes of glycobiological interest - the primary drawback being in defining the substrate specificity of the encoded enzymes, which is a result of a lack of structural knowledge. For instance, in the case of Drosophila some structures of N- and O-linked glycans were identified, but none were found which would account for the neural anti-horseradish peroxidase epitope that was presumed to be of carbohydrate origin [2].

In the past two years a small number of fly glycoenzyme cDNAs have been cloned and the function of the encoded enzymes determined. Four are of importance for N-glycans: α-mannosidases I and II [3, 4], β1,2-*N*-acetylglucosaminyltransferase I [5] and a core α1,3-fucosyltransferase [6]. Two others, Fringe [7, 8] and Brainiac [9] have respective roles in modifying the Notch protein and in glycolipid biosynthesis. Some years before, the glycoprotein glucosyltransferase, which has a role in the glucosylation-deglucosylation cycle relevant to protein folding, was also identified [10]. The biosynthesis of proteoglycans should also not be forgotten with the cloning of Tout-velu (*in vitro* function as yet to be verified) [11], heparan sulphate 6-*O*-sulphotransferase [12] and the proteoglycan core *O*-xylosyltransferase (IBHW, manuscript in preparation).

N-glycan biosynthesis

The early parts of the pathway for the synthesis of N-glycans in flies probably follow that previously well-studied in mammals and yeast: *i.e.*, dolichylpyrophosphate acts as the anchor for the building-up of a tetradecasaccharide precursor ($Glc_3Man_9GlcNAc_2$) [13], which is transferred en bloc to nascent polypeptide chains in the endoplasmic reticulum by the oligosaccharyltransferase complex, the cDNA of one of whose subunits (encoding the OST48/WBP1 subunit) has been cloned from fly [14].

Once attached to the protein removal of glucose residues (with temporary reglucosylation as part of the folding pathway) and of one mannose residue proceeds to often yield an intermediate $Man_8GlcNAc_2$. Here the yeast diverge from other eukaryotes in that they tend to add many mannose residues to their N-glycans, whereas 'higher' eukaryotes tend to remove further mannose residues. This results initially in a range of oligomannose structures ($Glc_{0-1}Man_{5-9}GlcNAc_2$). The stepwise cleavage of $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ is mediated by α-mannosidase I, the fly gene (MAS-1) having been identified [3]. Interestingly *mas-1* mutants can still process their oligomannosidic oligosaccharides

suggesting that, like mammals, multiple α-mannosidase homologues may be active [15]. At this point, β1,2-*N*-acetylglucosaminyltransferase I (GlcNAc-TI) can act: the action of this enzyme opens the way for complex and hybrid N-glycans in vertebrates, while in plants and invertebrates it is necessary for core fucosylation. In plants and animals, α-mannosidase II can then act to generate $GlcNAc_1Man_3GlcNAc_2$ (MGn), the substrate for β1,2-*N*-acetylglucosaminyltransferase II (GlcNAc-TII). The α-mannosidase II from fly has been identified [4] and localised to the Golgi [16]; even the crystal structure of this fly enzyme was recently determined [17].

It is at this point that plants and invertebrates begin to diverge from vertebrates in terms of N-glycan biosynthesis. In particular, the types of complex oligosaccharides differ: in vertebrates, a wide range of further branching, galactosylation, sialylation and other events occur. In plants the most complex N-glycans found to date are biantennary and can carry two outer arm Galβ1,3(α1,4-Fuc)GlcNAc (Lewis-a) modifications, in addition to core β1,2-xylose and α1,3-fucose [18]. In insects the most complex N-glycan found thus far is from bee venom phospholipase and carries one outer arm GalNAcβ1,4(α1,3-Fuc)GlcNAc as well as both core α1,3- and α1,6-linked fucose [19].

In contrast to mammalian complex-type N-glycans, though, plant and invertebrate glycans that have been processed by GlcNAc-TI (as judged by being, for example, core fucosylated) often have mannose as the non-reducing terminal sugar residue. This means that the *N*-acetylglucosamine residue added by GlcNAc-TI has been later removed. Indeed, such a specific 'processing' Golgi β-*N*-acetylhexosaminidase has been described in insects, which has properties distinct from those of the 'degradative' lysosomal β-*N*-acetylhexosaminidase [20]. In flies this enzyme would appear to be highly active since a large percentage of the N-glycans are core α1,6-fucosylated, yet lack terminal *N*-acetylglucosamine residues, while α1,6-fucosyltransferases require such residues on their substrates.

The fly GlcNAc-TI (Mgat1; AF251495) has recently been identified due to its homology to the mammalian form of this enzyme and is a protein of 456 amino acids with 53% identity to residues 46-445 of the human sequence [5]. The enzyme was successfully expressed using the baculovirus system in a form lacking the N-terminal transmembrane domain and was found to be active. The GlcNAc-TII cDNA has also been cloned (Mgat2; AY055120), but no details as to its activity have yet been published.

Another indication that GlcNAc-TI is a key enzyme in the fly is to be deduced from the substrate specificity of the core α1,3-fucosyltransferase (FucTA; AJ302045) [6]. The presence of such an enzyme was presumed, since the neural tissue of the fly can be stained using a polyclonal antiserum raised against the plant glycoprotein horseradish peroxidase [21-23], which carries core β1,2-xylose and α1,3-fucose on its N-glycans, and since core α1,3-fucose has been found on bee venom glycoproteins [19, 24]. Using plant core and mammalian Lewis-type α1,3-fucosyltransferase sequences as 'probes' in computer searches, we identified up to four α1,3-fucosyltransferase homologues. Of these, one (CG9169) lacked parts of the key fucosyltransferase motifs and also did not seem to have a clearly-definable transmembrane region. The other three were expressed in *Pichia pastoris* and tested with various substrates. Of these three, FucTA showed demonstrable activity towards N-glycans carrying either non-reducing terminal *N*-acetylglucosamine or Galβ1,4GlcNAc groups (*i.e.*, N-glycans of the GnGn or GalGal types) and showed a preference for glycans that were previously core α1,6-fucosylated ($GnGnF^6$ or $GalGalF^6$). Furthermore, FucTA was used to convert human transferrin to a form capable of being recognised by anti-horseradish peroxidase. It remains to be seen whether any of the mutants, *nac* [25, 26], TM3 [21] and Brd^{15} [27], that affect the neural epitope mediate their effects through FucTA. Interestingly, in the latter two cases the genomic region that includes the core α1,3-fucosyltransferase gene is, together with other genes such as Tollo, predicted to be missing.

A current controversy in insect glycobiology exists as to whether insects can modify their N-glycans with galactose and sialic acid. New studies suggest that some insect cell lines may be able recycle sialic acid from sialylated glycoproteins in culture media [28] and sialylate proteins when they are engineered to contain a mammalian sialyltransferase [29], suggesting that there is some sort of sialic acid biosynthetic machinery in insects. Also, lepidopteran Sf9 cells apparently produced sialylated recombinant glycoproteins upon inhibition of the Golgi β-*N*-acetylhexosaminidase [30]. Whether flies have similar capabilities remains to be elucidated, although one sialyltransferase cDNA has been cloned from flies (AF218237 and AF397532) and an apparently functional *N*-acetylneuraminic acid (Neu5Ac) phosphate synthase gene is the subject of a paper in press [31].

Mucin-type O-glycans

In insects, glycans as large as Galα1,4Galβ1,3GalNAc have been found [32, 33], although the major structure found on a mucin specifically from *Drosophila* was only Galβ1,3GalNAc [34]. It could, therefore, be anticipated that there would be α1,4-galactosyltransferase(s), core 1 β1,3-galactosyltransferase(s) and polypeptide *N*-acetylgalactosaminyltransferase(s) in *Drosophila*. Since the Galα1,4Galβ1,3GalNAc trisaccharide is absent in mammals, no direct homologues for such an α1,4-galactosyltransferase can be identified. However, a family of enzymes that create α1,4-glycosidic linkages does exist, including the human α1,4-galactosyltransferase required to synthesise the P^k (CD77) antigen glycolipid (Gb_3 synthetase) [35] and a human α1,4-*N*-acetylglucosaminyltransferase involved in the biosynthesis of some O-glycans [36]: up to two homologues of these can be found in the *Drosophila* genome and so, in theory, could be α1,4-galactosyltransferases.

The sequences of mammalian and worm core 1 β1,3-galactosyltransferases, which create Galβ1,3GalNAc, have recently been determined [37] and the fly also has a homologue of these. Recently, data on the cloning and successful expression of a number of polypeptide *N*-acetylgalactosaminyltransferases has been presented in poster form [38].

Other O-glycans

A number of other types of O-glycans have been described in the fly or are deduced by the presence of relevant genes: O-fucosylation of EGF domains (such as present on Notch), collagen glycosylation, cytosolic *N*-acetylglucosaminyltransferase and O-mannosylation (such as found either in yeast or on mammalian proteins such as dystroglycan).

In the case of O-fucosylation, fucose is directly attached to the polypeptide in a highly sequence-dependent manner by *O*-fucosyltransferase, the mammalian form of which has been purified and also examined in recombinant form [39]. The fucose residue can be modified by β1,3-linked *N*-acetylglucosamine or glucose. The former type of modification is catalysed by fringe [7, 8], resulting - upon subsequent galactosylation and perhaps sialylation - in modulation of Notch signalling. In mammals, the ubiquitous β1,4-galactosyltransferase I can modify the GlcNAcβ1,3Fuc disaccharide prior to subsequent sialylation [40]. It remains to be determined whether any of the three fly β1,4-galactosyltransferase homologues and the single fly α2,6-sialyltransferase homologue actually perform the same reactions.

It appears that *Drosophila* has two protein-*O*-mannosyltransferases, one of which was originally designated the rotated abdomen (rt) gene: this gene apparently has a role in muscle development and the *rt* mutants have, as the name suggests, a clockwise-twisting of the body [41]. As judged by homologies, nuclear and cytoplasmic O-linked GlcNAc transferase is also present in both fly [42], as well as one homologue of the lysyl hydroxylase 3 protein [43], which has now been shown to also possess collagen glucosyltransferase activity [44].

Glycolipids

The glycosphingolipids of *Drosophila* are more akin to those of Caenorhabditis than to those of mammals in that the core is GalNAcβ1,4(*P*Etn-6)GlcNAcβ1,3Manβ1,4GlcβCer. Brainiac, a member of the β1,3-galactosyltransferase family [45] and required for epithelial morphogenesis, has been described in a recent patent application to catalyse the formation of GlcNAcβ1,3Man linkages [9] and so is a candidate for the third step of glycolipid biosynthesis in the fly.

Proteoglycans

Due to their roles in development, the proteoglycans seem particularly interesting, not just because they are also conserved between animals in general [46, 47]. Recently many mammalian enzymes related to proteoglycan biosynthesis have been cloned and, in particular the cloning of the mammalian xylosyltransferases I and II responsible for initiation of proteoglycan cores [48] allows us to conclude that there exists only one homologue in the fly. We have recently expressed the xylosyltransferase in *Pichia pastoris* and found it to be active (manuscript in preparation).

Of other genes relevant to proteoglycan biosynthesis, *tout-velu* (*ttv*) encoding an *EXT* co-polymerase homologue [11], *sugarless* (*sgl*; also known as *suppenkasper* or *kiwi*) encoding a demonstrated UDP-Glc dehydrogenase [49-51], and *sulphateless* (*sfl*) encoding homologue of the heparin/heparan sulphate *N*-deacetylase/*N*-sulphotransferase [52, 53] are relevant to Hedgehog, Wingless and fibroblast growth factor signalling. Analysis of the glycosaminoglycan chains of these three mutants [54] show alterations in structure compatible with the protein sequence homologies. Amongst other fly genes potentially relevant to proteoglycan formation, however, it is not clear which of the three β1,4-galactosyltransferase homologues has the same function as the worm SQV3 in the biosynthesis of the proteoglycan Galβ1,4Xyl core, while the enzymatic activity of only one sulphotransferase has been proven *in vitro*: the single heparan sulphate 6-*O*-sulphotransferase. RNAi experiments with this gene suggest that this enzyme is necessary for viability, particularly due to a role in primary branching of the tracheal system [12]. Two other potential sulphotransferase homologues are PIPE, a uronyl 2-sulphotransferase homologue, for which ten different transcripts are known and which has a role in the definition of embryonic dorsal-ventral polarity [55, 56], and segregation distorter (Sd) protein, a heparan sulphate 2-*O*-sulphotransferase homologue with a role in male meiosis [57].

Conclusion

Even in the past two years since a review appeared on *Drosophila* glycans [58], there has been continuing progress on studying the glycobiology of the fly. However, there is a long way still to go to transform identifying the many glycosyltransferase homologues into demonstrating actual enzymatic activity and biological function.

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