The Drosophila fused lobes Gene Encodes an N-Acetylglucosaminidase Involved in N-Glycan Processing*

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Most processed, e.g. fucosylated, N-glycans on insect glycoproteins terminate in mannose, yet the relevant modifying enzymes require the prior action of N-acetylglucosaminyltransferase I. This led to the hypothesis that a hexosaminidase acts during the course of N-glycan maturation. To determine whether the Drosophila melanogaster genome indeed encodes such an enzyme, a CDNA corresponding to fused lobes (fdl), a putative β-N-acetylglucosaminidase with a potential transmembrane domain, was cloned. When expressed in Pichia pastoris, the enzyme exhibited a substrate specificity similar to that previously described for a hexosaminidase activity from Sf-9 cells, i.e. it hydrolyzed exclusively the GlcNAc residue attached to the α,1,3-linked mannose of the core pentasaccharide of N-glycans. It also hydrolyzed p-nitrophenyl-N-acetyl-β-glucosaminide, but not chitooligosaccharides; in contrast, Drosophila HEXO1 and HEXO2 expressed in Pichia cleaved both these substrates but not N-glycans. The localization of recombinant FDL tagged with green fluorescent protein in Drosophila S2 cells by immunoelectron microscopy showed that this enzyme transits through the Golgi, is present on the plasma membrane and in multivesicular bodies, and is secreted. Finally, the N-glycans of two lines of fdl mutant flies were analyzed by mass spectrometry and reversed-phase high-performance liquid chromatography. The ratio of structures with terminal GlcNAc over those without (i.e. paucimannosidic N-glycans) was drastically increased in the fdl deficient flies. Therefore, we conclude that the fdl gene encodes a novel hexosaminidase responsible for the occurrence of paucimannosidic N-glycans in Drosophila.

Insect cells are considered to be an interesting alternative to mammalian, yeast, or bacterial cells for the expression of recombinant proteins (1, 2), because the potentially high levels of expression are associated with the ability to perform many eukaryotic post-translational modifications. Nevertheless, glycoproteins produced in insects and insect cells have been mainly found to carry paucimannosidic N-glycans, i.e. glycans consisting of the pentasaccharide core with or without α1,6- and/or α1,3-linked fucose (1, 3–5). In addition to the possible presence of the immunogenic core α1,3-linked fucose, the lack of complex type N-glycans with complete, sialylated antennae as found on mammalian glycoproteins makes insect cells unsuitable for the production of many therapeutic glycoproteins (6).

Only in a few cases have substitutions of the terminal mannose residues of insect N-glycans been described. The most common substitution is the presence of terminal GlcNAc on the α1,3-linked mannose residue (GlcNAc-1) added by GlcNAc-transferase I, although in a few cases further modifications such as galactose, N-acetylgalactosamine, fucose, and aminoethylphosphonate (i.e. phosphorylthanolamine) have been found to link this GlcNAc residue (7–11). The occurrence of larger and sialylated N-glycans has been reviewed elsewhere (1, 4). In most instances, however, the α1,3-linked mannose residue was found as terminal residue, even though the enzymes involved in the biosynthesis of other features of the glycan structures found in insects require the presence of GlcNAc-1. Indeed in Sf-9 cells, in the same way as previously found in mammals (12), all the activities of relevant enzymes such as α-mannosidase II (13), α1,3-fucosyltransferase (14), α1,6-fucosyltransferase (14, 15), and GlcNAc-transferase II (15) were found to depend upon this GO signal; similar results have been obtained with recombinant forms of the Drosophila core α1,3- and α1,6-fucosyltransferases FucTA and FucT6 (5, 16). Furthermore, the presence of significant levels of the GlcNAc-transferase I has been demonstrated in several insect cell lines (15), whereas the Drosophila GlcNAc-transferase I cDNA has been cloned and shown to encode an active enzyme (17).

It was therefore hypothesized that in insect cells, as in plant cells, the GlcNAc-1 is cleaved off at some stage. In the case of phytohemagglutinin, it appears that removal of GlcNAc residues occurs after the arrival of the protein in the vacuoles of the plant cell (18). Indeed, it appears that in plants only proteins resident in the glycosidase-rich vacuolar compartment lack the GlcNAc-1 residue, whereas in insects this residue is absent from the vast majority of glycoproteins, including those that are secreted (19). Thus hexosaminidase trimming could constitute a regular and deliberate event during glycoprotein maturation occurring within the insect secretory pathway.

In fact, in 1995, Sf-9 cells (as well as Bm-N and MB0503 cells) were reported to contain a membrane-bound N-acetylglucosaminidase possibly residing in the Golgi apparatus (20). It is noteworthy that this enzyme could not hydrolyze Man5Gn6, but acted only further “downstream” on M Gn and GnGn where it specifically removed GlcNAc-1 (20). Subsequent cell culture experiments in the presence of hex-
osaminidase inhibitors (7, 11, 21) or of recombinant β1,4-galactosyltransferase (22, 23) corroborated the putative existence of a processing hexosaminidase. It is obvious, therefore, that this enzyme interferes in strategies to produce glycoproteins with human-like glycosylation in insect cells.

Various attempts have been made to overcome this problem. For instance, the heterologous overexpression of mammalian GlcNAc-transferase I was performed, but, because of the competition with the endogenous hexosaminidase, most of the N-glycans still did not carry GlcNAc-1 (24). Another approach is to overexpress β1,4-galactosyltransferase (25), which has the effect of “capping” the substrate for the hexosaminidase, or to inhibit the latter using inhibitors (26). However, the most elegant approach would be to specifically knock down or knock out the hexosaminidase gene.

To perform any specific ablation of the hexosaminidase, it is necessary to identify the relevant gene. To this end, we have identified and recombinantly expressed the three obvious homologues of family 20 glycohydrolases in the fruit fly. Furthermore, we have determined the substrate specificity, molecular function, and subcellular localization of one of them, which we conclude to be the D. melanogaster processing β-N-acetylglucosaminidase.

**EXPERIMENTAL PROCEDURES**

*Fly Strains*—The wild-type flies used in this study were of the Canton Special (CS) strain. The hypomorphic *fdl* mutant, due to the insertion of a transposable P-element, was recovered after a screen for adult brain anatomical mutants (27). The *Df(2R)achi* strain, which lacks six genes, including *fdl* (28), was a gift from Dr. Robert A. H. White.

*Cloning and Expression of cDNAs Encoding N-Acetylglucosaminidases from Drosophila*—The sequences encoding the putative Drosophila N-acetylglucosaminidases were found by searching for homologues of human lysosomal hexosaminidase within assembled gene products of the Drosophila genome data base (www.flybase.org/). The predictions of the subcellular location and transmembrane topology were performed using, respectively, WoLF PSORT (wolfpsort.seq.cbc.jp) and TMPRED (www.ch.embnet.org/software/TMPRED_form.html) (29).

RNA was extracted from *Drosophila* S2 cells using TRIzol reagent, and RT-PCR was performed using Expand polymerase (Roche Applied Science). For the soluble form of FDL (amino acids 30–660), the primers 5’-GGAATTCAGCTGGAGTCAAGG-3’ and 5’-GGGTTACCTCAATGTCG-3’ were used; for *HEXO*1 and *HEXO*2, the primer pairs 5’-GGAATTCAGCTGGAGTCAAGG-3’ and 5’-GGGTTACCTCAATGTCG-3’ or 5’-GGATTCTGAGCTGGAGTCAAGG-3’ and 5’-TCCCCCGCAGGATTAGGGTACCTCAATGTCG-3’, respectively, were employed. The PCR products were digested with the relevant restriction enzymes and cloned into the pPICZaA expression vector (Invitrogen). The integrity and reading frames of the constructs, as well as 5’- and 3’-ends of full-length RT-PCR products, were confirmed by DNA sequencing using an ABI PRISM BigDye Terminator Sequencing Ready Mix and ABI PRISM 310 genetic analyzer (Applied Biosystems). Nucleic acid sequences were analyzed using NCBI BLAST and BLAST2 programs (www.ncbi.nlm.nih.gov/). Predicted protein sequences were aligned and phylogenetically analyzed using Multalin (prodes.toulouse.inra.fr/multalin/multalin.html) (30).

The hexosaminidase homologues were then expressed in *Drosophila* S2 cells using TRIzol reagent, Dye Terminator Sequencing Ready Mix and ABI PRISM 310 genetic analyzer. The integrity and reading frames of the constructs, as well as 5’- and 3’-ends of full-length RT-PCR products, were confirmed by DNA sequencing using an ABI PRISM BigDye Terminator Sequencing Ready Mix and ABI PRISM 310 genetic analyzer (Applied Biosystems). Nucleic acid sequences were analyzed using NCBI BLAST and BLAST2 programs (www.ncbi.nlm.nih.gov/). Predicted protein sequences were aligned and phylogenetically analyzed using Multalin (prodes.toulouse.inra.fr/multalin/multalin.html) (30).

**RESULTS**

**Identification and Cloning of Drosophila N-Acetylglucosaminidases**—Our hypothesis was that the putative insect Golgi hexosaminidase would be homologous to known hexosaminidases. Thus we performed tBLASTn searches of the Drosophila genome and found three obvious fly homologues (CG1318, CG1787, and CG8824) of the human lysosomal hexosaminidases HEXA and HEXB. Indeed, these homologues have been previously assigned to the glycohydrolase family 20 in the CAZy data base (35).

**Asay of Recombinant N-Acetylglucosaminidase Activity**—The supernatants of yeast expressing soluble forms of the *N*-acetylglucosaminidases were incubated with the different substrates at 37°C for 1–20 h. For experiments with *p*-nitrophenyl-GlcNAc, the substrate concentration was 5 mM in a total volume of 0.04 ml of 0.1 M citrate-phosphate buffer at pH 3–8. The reactions were terminated by the addition of 0.26 ml of 0.4 M glycine/NaOH buffer at pH 10.4, and absorbance at 405 nm was measured with a microtitr plate reader.

Pyridylaminated oligosaccharides were used at a final concentration of 0.1 mM in a total volume of 0.02 ml of 0.1 M citrate/phosphate buffer at pH 3–8. Incubation was terminated by the addition of 0.18 ml of 20 mM ice-cold sodium borate. Aliquots of 0.05 ml were analyzed by reverse-phase HPLC as described previously (15, 20).

**Analysis of N-Glycan of Wild-type and Mutant Drosophila**—Adult flies, either Canton S wild-type (0.65 g), *fdl* mutant (0.41 g), or *Df(2R)achi* (0.107 g), were collected and anesthetized with CO2 before addition of 4 ml of water and boiling for 10 min. The preparation of N-glycans was then performed basically as previously described (32) using pyridine-N-glycosidase digestion of peptic peptides.

Enzymatic digestion of peptic peptides was performed using 2,5-dihydroxybenzoic acid as matrix.

**Immunoelectron Microscopy**—The full-length *fdl* cDNA was cloned in-frame with a gfp sequence at the 3’-end into a derivative of the pRMHA3 vector, which includes a metallothionein promoter. *Drosophila* S2 cells were then transfected using Effectene reagent (Qiagen) followed by a 2-h chase. Medium was then aspirated, and the cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and processed for immunoelectron microscopy as described earlier (34). 60- to 80-nm cryosections were cut on an UltraS (Leica) and immunolabeled using the anti-GFP rabbit polyclonal antibody (Molecular Probes) followed by protein A conjugated to 10 nm gold particles.
between the PCR product and the data bank sequences. The fdl cDNA contains a predicted open-reading frame encoding a protein of 660 amino acids (\(M_r 75,485\)). The comparison of the cDNA sequence with that of the corresponding genome sequence indicates that the fdl open reading frame spans five exons. On the other hand, hexo1 and hexo2 share a common open reading frame organization with only three exons and both encode proteins of around \(M_r 70,000\).

At the amino acid level, the Drosophila FDL sequence, which is predicted to be a type II transmembrane protein, shares 33% amino acid sequence identity and 64% sequence similarity with the fly HEXO1 and 31% sequence identity and 62% sequence similarity with the fly HEXO2 (Fig. 1). The predictions by WoLF PSORT would suggest that both HEXO1 and FDL are plasma membrane proteins, whereas HEXO2 is most likely to be secreted. A phylogenetic analysis suggests that amongst insect hexosaminidases there are two major groups. The first is composed of enzymes related to the aforementioned \(N\)-acetylglucosaminidase from \(B.\) \(mori\) (36) and includes DmHEXO1 as well as DmHEXO2. The second group contains the homologues of DmFDL for which we could find predicted sequences from honeybee \(A.\) \(mellifera\), tsetse fly \(G.\) \(morsitans\) \(morsitans\), and the mosquito \(A.\) \(gambiae\) (Fig. 2). Of all the available putative insect \(N\)-acetylglucosaminidase sequences, FDL is most similar to the incomplete mosquito sequence, AgFDL, sharing 64% sequence identity and 90% sequence similarity over a stretch of 449 amino acids. All insect hexosaminidase sequences are phylogenetically distant from the lysosomal mammalian hexosaminidases (38) and even more distant from the group of \(N\)A hexosaminidases previously characterized in fungi (39).

Characterization of Recombinant Drosophila-processing \(N\)-Acetylglucosaminidase—A soluble form of the Drosophila processing \(N\)-acetylglucosaminidase lacking the first 29 amino acid residues (i.e. lacking the putative cytoplasmic and transmembrane domains) was amplified by RT-PCR, and the resulting DNA fragment was

![FIGURE 1. Alignment of Drosophila FDL with other insect \(N\)-acetylglucosaminidases.](image)
Drosophila N-Glycan Hexosaminidase

cloned into the pPICZαA vector prior to transformation into P. pastoris. Similarly, the two other Drosophila N-acetylglucosaminidase homologues, HEXO1 and HEXO2, were also expressed as soluble forms in P. pastoris lacking, respectively, the N-terminal 22 and 35 amino acids. In an initial screen, the culture supernatants of transformed yeast were tested for their hexosaminidase activity using p-nitrophenyl-β-N-acetylglucosaminide and indeed all three homologues utilized this substrate. A Myc-tagged form of FDL (estimated Mr, 70,000) was also active when expressed in insect cells, as judged by a 4-fold increase in the cleavage of pNP-GlcNAc and GnGn-PA in comparison to mock-transfected cells (data not shown).

To determine the specificities toward natural substrates, the enzymatic activities of HEXO1, HEXO2, and FDL were subsequently evaluated by reversed-phase HPLC with N-glycans, such as GnGn-PA (see Fig. 3 for structure). Based on previous analyses (40), the removal of the GlcNAc residue from the α1,3-arm to generate Gm causes a reduction in retention time, whereas removal of the residue from the α1,6-arm to generate MGn causes a reduction in retention time as compared with GnGn.

After 1 h of incubation of FDL with this substrate, a small peak of greater retention time could be observed that co-eluted with a GnM standard (Fig. 4C); after 16 h the reaction was complete (Fig. 4D). On the other hand, the formation of GnGn-PA was not observed when the supernatant of untransformed P. pastoris was used in the reaction (Fig. 4A), indicating that the conversion observed is due to the recombinantly expressed protein. Furthermore, there is no formation by FDL of either MM-PA or MGn-PA from GnGn-PA, which illustrates its branch specificity and its ability to remove exclusively the GlcNAc linked to the α1,3-mannose residue of the core structure. A mass spectrometric assay was used to demonstrate that a single GlcNAc residue was removed by FDL; using a dabsylated GnGn glycopeptide as substrate, a reduction in m/z of 203 Da, which is equivalent to one GlcNAc residue, was observed (data not shown). Taken together, these results indicate that the recombinant Drosophila FDL N-acetylglucosaminidase specifically removed the GlcNAc residue linked to the α1,3-mannose from the biantennary oligosaccharide. This is consistent with the previous data on the insect Golgi hexosaminidase (20).

However, in contrast to data on the Sf-9 microsomal hexosaminidase activity, FDL did not digest chitotriose, as judged by an HPLC-based assay using chitotriose-PA (GlcNAc3-PA) as substrate. This discrepancy might be due to the presence of a distinct hexosaminidase activity in the Sf9 microsome preparation. Our present results, therefore strongly indicate that FDL is not involved in chitin degradation. On the other hand, both HEXO1 and HEXO2 displayed chitotriose-degrading activity. All assays performed consistently indicated that N-glycans are not substrates for either HEXO1 or HEXO2 (data not shown). This result was expected considering their close relationship to the B. mori hexosaminidase (36).

As a result of the data showing that only FDL possessed the activity most reminiscent of the Golgi hexosaminidase from Sf9 cells, we concentrated our subsequent studies on this protein. The recombinant FDL was, therefore, tested for its ability to degrade an extended range of...
cells under control of an inducible promoter. Preliminary results with confocal microscopy showed a punctuated pattern but no colocalization of the labeling with the Golgi marker BODIPY ceramide; therefore, the localization of the fusion protein was assessed by immunoelectron microscopy. The transfected cells were fixed and processed, and ultrathin cryosections were immunolabeled using an anti-GFP rabbit polyclonal antibody followed by protein A conjugated to gold particles. The fusion protein is found in all compartments of the secretory pathway, the endoplasmic reticulum, and the Golgi apparatus but at a much higher level on the plasma membrane and in multivesicular bodies representing late endosomes (Fig. 6, A and B). In addition, the high level of labeling observed extracellularly shows that the protein is also, to a large extent, sequestered into the medium. This is consistent with the notion that FDL only transits through the Golgi apparatus en route to the plasma membrane and is not a resident enzyme of this organelle. Even in cells showing a very high level of expression, the Golgi apparatus did not exhibit an exaggerated labeling (Fig. 6, C and C’); this contrasts with observations on Golgi glycosyltransferases such as Fringe.5 The FDL localization in the late endosomes might result from either direct targeting to the endosomal compartment after the transit through the Golgi apparatus or from the endocytosis of FDL after its transport to both the plasma membrane and the medium.

Comparison of N-Glycans from Wild-type and Mutant Flies—To investigate whether FDL was responsible for the conversion of Drosophila N-glycans to the paucimannosidic type in vivo, N-glycan analyses were conducted on flies with either a hypomorphic (fd1) or complete (Df(2R)ach2 mutant) deficiency in the fd1 gene. The N-glycans released by peptide N-glycosidase A from glycopeptides obtained after peptic digestion of the fly samples were subjected to mass spectrometry analysis (Fig. 7 and Table 1) and, in the case of fd1, to HPLC (data not shown). The N-glycan patterns obtained with these mutants were compared with the profile of the wild-type flies (Canton S). As previously described (5, 43), the dominant structures present in normal adult flies are of paucimannosidic or oligomannosidic type and indeed MMF6 accounts for more than 40% of the total N-glycans. On the other hand, in the hypomorphic fd1 mutant, the occurrence of paucimannosidic N-glycans decreases from 62% to 30%, although there is no change in the percentage of oligomannosidic structures. At the same time, the amount of N-glycans carrying at least one elongated antenna increases dramatically in the fd1 flies, from ~1% to 33% of the structures. Among these,

5 C. Rabouille, unpublished observations.

Drosophila N-Glycan Hexosaminidase
the main structure is MGnF6 (as assessed by its HPLC retention time; data not shown). Furthermore, in this mutant, complex glycans (with two GlcNAc residues), which were not detectable in Canton S flies, represent ~4% of the total amount of N-glycans (Table 1). These effects are accentuated in the Df(2R)achi2 mutant, which lacks the entire fdl gene as well as five others, achintya, vismay, CG30044, CG12370, and CG13156, none of which are predicted to have a role in N-glycan modification. In addition to a very strong overrepresentation of the oligomannosidic structure Man9, the abundance of paucimannosidic structures in this mutant is reduced 7-fold as compared with wild-type flies (Table 1). Thus, the glycan analysis indicates that FDL has an important role in the biosynthesis of the major wild-type N-glycans in Drosophila.

DISCUSSION

The class 20 glycosidase family encompasses a range of β-hexosaminidases from bacteria, plants, fungi, and animals normally associated with catabolism of glycoconjugates. Probably the two most well known examples are the mammalian lysosomal β-hexosaminidases, encoded by the HEXA and HEXB genes, mutations of which are associated with the Tay-Sachs and Sandhoff diseases and result in severe neurological symptoms (44). In Drosophila, there are three obvious homologues of this glycosidase class and, remarkably, mutations in one of the corresponding genes, fdl, have been previously demonstrated (27) to affect normal development of the fly brain; the major anatomical changes noted concerned the mushroom body β lobes, which were fused in homozygous adults. Another mutant for the same gene

FIGURE 6. Immunoelectron localization of FDL-GFP in Drosophila S2 cells. Ultrathin cryosections of Drosophila S2 cells were labeled with anti-GFP antibody followed by protein A coupled to 10 nm gold. As shown, gold labeling was found at a low level in the Golgi apparatus (A) and at a much higher level on the plasma membrane and in the multivesicular bodies (B, C, and C). In the cells where the multivesicular bodies and the plasma membrane are strongly labeled, no accumulation of gold particles was observed in the Golgi (C and C). FDL is also secreted into the cell medium (C). G, Golgi stacks; mvb, multivesicular bodies; ER, endoplasmic reticulum; PM, plasma membrane. Bars, 200 nm.

FIGURE 7. MALDI-TOF MS of neutral N-glycans of wild type and mutant adult D. melanogaster. MALDI-TOF spectra of underivatized N-glycans from Canton S wild-type (A), fdl (B), and Df(2R)achi2 flies (C). The spectra are annotated with the m/z values for [M + Na]+ ions and the corresponding structure names (refer to Fig. 3 for relevant structures). The structures marked with an asterisk correspond to [M + K]+ adducts as assessed by comparison to spectra obtained in the presence of 10 mM potassium chloride (data not shown); as revealed by this method, the peak labeled “MMF*” also contains a small amount of Man6.
TABLE 1

N-Glycans of adult wild-type and fdl mutant flies

The m/z values of underivatized glycans from the spectra of the complete N-glycan pool were determined by external calibration that was refined by internal calibration relative to the MMF and Man9 peaks. Percentage occurrences were calculated based on peak areas from MALDI-TOF-MS data. The hypothetical glycan structures are given in parentheses (cf. Fig. 3); however other isobaric structures cannot be entirely ruled out.

| Structure                          | [M+Na]+ | Wild-type CS | fdl mutant | Df(2R)achi2 mutant |
|------------------------------------|---------|--------------|------------|-------------------|
| Paucimannosidic glycans            |         |              |            |                   |
| Hex3HexNAc2Fuc (MUF)               | 771.7   | 5.5          | 3.4        | ND*               |
| Hex3HexNAc2 (MMF)                  | 917.8   | 5.4          | 2.8        | ND                |
| Hex3HexNAc2 (MMF)*                 | 933.8   | 11.1         | 3.1        | 2.6               |
| Hex5HexNAc2 (MUF)*                 | 1080.0  | 40.6         | 21.0       | 6.5               |
| Total                              | 62.6    | 30.3         | 9.1        |                   |
| Oligomannosidic glycans            |         |              |            |                   |
| Hex5HexNAc2 (Man5)*                | 1258.1  | 13.1         | 12.2       | 5.5               |
| Hex5HexNac2 (Man6)*                | 1420.2  | 5.9          | 6.3        | 8.4               |
| Hex5HexNac2 (Man7)*                | 1582.4  | 3.9          | 4.4        | 7.0               |
| Hex5HexNac2 (Man8)*                | 1744.5  | 3.8          | 4.3        | 9.5               |
| Hex5HexNac2 (Man9)*                | 1906.7  | 9.6          | 9.4        | 24.3              |
| Total                              | 36.3    | 36.6         | 54.7       |                   |
| Hybrid and complex glycans         |         |              |            |                   |
| HexNacHexHexNac2 ( MGnF 6 )*       | 1137.2  | n.d          | 0.6        | 3.0               |
| HexNacHexHexNac2 ( MGnF 6 ) and (GnMF 6 )* | 1283.2 | 1.1        | 28.7       | 25.6              |
| HexNacHexHexNac2 (GnGn)            | 1340.2  | ND           | ND         | 2.0               |
| HexNacHexHexNac2 (GnGnF)           | 1486.4  | ND           | 3.8        | 5.6               |
| Total                              | 1.3     | 33.1         | 36.2       |                   |

* ND, not determined.

a Structure based on HPLC data for fdl.

b Based on previously published data for wild-type CS (5).

(Df(2R)achi2) has been generated (26). According to the authors, the severe phenotypic changes observed for this mutant can be mainly attributed to another gene (Achi), and no conclusion can be drawn from this mutant concerning the morphological effect of an fdl deletion. Nevertheless, as shown in the present study, FDl is a hexosaminidase with a profound effect on oligosaccharide structure; as judged by the types of oligosaccharide structures present in fdl flies, we hypothesize it has a primary role during the biosynthesis, rather than the catabolism, of N-glycans. The plant Drosophila N-glycan Hexosaminidase (Drosophila N-Glycan Hexosaminidase) is an established approach in such studies (56–59). The localization of FDl was examined by use of C-terminal GFP and Myc fusions, this being an established approach in such studies (56–59). Although we cannot rule out some artificial localization, both fusion proteins showed the same distribution, i.e. that FDl mainly localizes to the plasma membrane, the late endosomes and the extracellular medium, and only to a minor extent in the Golgi apparatus. Although, these data deviate from the conclusions from the previous biochemical study (20), we expect that, other than perhaps some resident Golgi proteins, any glycoprotein leaving the Golgi apparatus is either localized in the vacuole itself or in a prevacuolar compartment distinct from the Golgi apparatus. On the contrary, in insects, the N-glycan-trimming hexosaminidase activity previously characterized (20), which we now have identified as FDl, is not localized in the lysosomal compartment.
**Drosophila N-Glycan Hexosaminidase**

The capacity of insect cells to synthesize complex N-glycans has been a matter of some controversy. Indeed, recent studies show that the genome of *Drosophila* encodes two N-acetylglactosaminyltransferases (62) and a sialyltransferase (63); whereas *Trichoplusia ni* has a proven GalNAc-transferase that could in theory participate in the synthesis of complex N-glycans (64). However, to date there is no published evidence for such structures in adult flies (although some complex structures such as fucosylated LacdiNAc are found on bee venom glycoproteins (91)). Thus, to overcome the difference in N-glycosylation of recombinant glycoproteins produced in insect cells and those derived from mammalian sources, several approaches to making insect cell systems suitable for the production of therapeutic glycoproteins have been attempted. One of the main targets in this quest has been to increase the amount of structures carrying a GlcNAc residue linked to the α1,3-mannose of the N-glycan core, which would, in turn, allow further elongation of this antenna. To this end, several approaches have been explored, ranging from the use of N-acetylglucosaminidase inhibitors (11) to the expression of mammalian glycosyltransferases capable of transfer of galactose and sialic acid to the nascent antennae to prevent the ablation of FDL expression could prove to be an elegant way toward facilitating the synthesis of mammalian-like N-glycans in insect cells. The severely reduced amounts of MM and MMα in *fdl* mutant flies would mean that it is not necessary to introduce additional GlcNAc-transferase I into insect cells; the absence of FDL is also expected to increase the efficiency of GlcNAc-transferase II and Gal-transferase/GalNAc-transferase enzymes in insect cells. Therefore, we conclude that identification of the molecular basis for the removal of GlcNAc-1 opens new perspectives in obtaining insect cell lines with humanized N-glycans.

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ila S2 cells were kindly provided by Gerald Aichinger from Intercell AG, Therefore, we conclude that identification of the molecular basis for the
of whole flies, the complete absence of the enzyme mainly responsible for the occurrence of paucimannosidic structures does not lead to the obvious synthesis of N-glycan structures with mammalian-like extended antennae, the considerable increase in structures carrying GlcNAc-1 indicates that the ablation of FDL expression could prove to be an elegant way toward facilitating the synthesis of mammalian-like N-glycans in insect cells. The severely reduced amounts of MM and MMα in *fdl* mutant flies would mean that it is not necessary to introduce additional GlcNAc-transferase I into insect cells; the absence of FDL is also expected to increase the efficiency of GlcNAc-transferase II and Gal-transferase/GalNAc-transferase enzymes in insect cells. Therefore, we conclude that identification of the molecular basis for the removal of GlcNAc-1 opens new perspectives in obtaining insect cell lines with humanized N-glycans.

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