Insect cell glycosylation pathway branch point

Substrate Specificities and Intracellular Distributions of Three N-glycan Processing Enzymes Functioning at a Key Branch Point in the Insect N-glycosylation Pathway*

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Background: Insect cells have a branched protein N-glycosylation pathway.

Results: Substrate specificities and intracellular distributions of three insect N-glycan processing enzymes were determined.

Conclusion: SIGNT-I, SF-GNT-II, and SF-FDL function around the branch point and determine the net outcome of the insect protein N-glycosylation pathway.

Significance: Deeper understanding of the insect protein N-glycosylation pathway.

SUMMARY

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\text{Man(\(\alpha_1-6\))GlcNAc(\(\beta_1-2\)Man(\(\alpha_1-3\)\)ManGlcNAc}_2 \text{ is a key branch point intermediate in the insect N-glycosylation pathway because it can be either trimmed by a processing \(\beta-N\)-acetylglucosaminidase (FDL) to produce paucimannosidic N-glycans or elongated by \(N\)-acetylglucosaminyltransferase II (GNT-II) to produce complex N-glycans. \(N\)-acetylglucosaminyltransferase I (GNT-I) contributes to branch point intermediate production and can potentially reverse the FDL trimming reaction. However, there has been no concerted effort to evaluate the relationships among these three enzymes in any single insect system. Hence, we extended our previous studies on \textit{S. frugipera} (Sf) FDL to include GNT-I and -II. Sf-GNT-I and -II cDNAs were isolated, the predicted protein sequences were analyzed, and both gene products were expressed and their acceptor substrate specificities and intracellular localizations were determined. Sf-GNT-I transferred \(N\)-acetylglucosamine to Man\(_5\)GlcNAc\(_2\), Man\(_3\)GlcNAc\(_2\), and GlcNAc(\(\beta_1-2\)Man(\(\alpha_1-3\)\)ManGlcNAc\(_2\), demonstrating its role in branch point intermediate production and its ability to reverse FDL trimming. Sf-GNT-II only transferred \(N\)-acetylglucosamine to Man(\(\alpha_1-6\))GlcNAc(\(\beta_1-2\)Man(\(\alpha_1-3\)\)ManGlcNAc\(_2\), demonstrating that it initiates complex \(N\)-glycan production, but cannot use Man\(_3\)GlcNAc\(_2\) to produce hybrid or complex structures. Fluorescently tagged Sf-GNT-I and -II co-localized with an endogenous Sf Golgi marker and Sf-FDL co-localized with SF-GNT-I and -II, indicating that all three enzymes are Golgi resident proteins. Unexpectedly, fluorescently tagged \textit{D. melanogaster} FDL also co-localized with SF-GNT-I and an endogenous \textit{Drosophila} Golgi marker, indicating that it is a Golgi resident in insect cells. Thus, the substrate specificities and physical juxtapositioning of GNT-I, GNT-II, and FDL support the idea that these enzymes function at the \(N\)-glycan processing branchpoint and are major factors determining the net outcome of the insect cell \(N\)-glycosylation pathway.}

The early steps in the insect and mammalian \(N\)-glycosylation pathways are highly similar or identical and culminate with production of the same \(N\)-glycan processing intermediate, Man(\(\alpha_1-6\))GlcNAc(\(\beta_1-2\)Man(\(\alpha_1-3\)\)ManGlcNAc\(_2\).
These pathways subsequently diverge, as the two cell types process MGn in directly opposite ways and ultimately produce distinctly different major N-glycan structures. In insect cells, MGn is trimmed to produce paucimannosidic N-glycans, such as Man$_2$GlcNAc$_2$, or MM (Fig. 1). This trimming reaction is performed by a processing β-N-acetylglucosaminidase known as fused lobes (FDL; 3-5), which specifically removes the N-acetylglucosamine residue introduced by N-acetylglucosaminyltransferase I (GNT-I). In contrast, mammalian cells elongate MGn to produce hybrid and complex N-glycans. These elongation reactions are performed by a diverse set of glycosyltransferases, which transfer various monosaccharides from nucleotide sugar donor substrates to the nonreducing end of MGn in a sequential fashion. N-acetylglucosaminyltransferase II (GNT-II) is a key enzyme in this process, as it initiates elongation of the α1,6-branch mannose in MGn (Fig. 1).

Surprisingly, recent work has shown that this simple comparison of the insect and mammalian N-glycosylation pathways is accurate, but incomplete. It is now clear that at least some insects encode the glycosyltransferases and other machinery needed to produce hybrid and complex, mammalian-type N-glycans (6-12). Furthermore, it is clear that these genes are expressed and their products contribute to the production of hybrid and complex N-glycans in at least some insect tissues and cells (13-16). Thus, while it is true that the insect N-glycosylation pathway produces mainly paucimannosidic N-glycans, it is also true that this pathway has the capacity to produce mammalian-like hybrid and complex N-glycans. Accordingly, MGn is a key branchpoint intermediate and this raises an interesting question: what factors determine the fate of this intermediate and the types of N-glycans ultimately produced by the branched N-glycosylation pathway in insect systems?

Previous results obtained with Spodoptera frugiperda (Sf) cells, which are lepidopteran insect cells that are widely used as hosts for baculovirus expression vectors, indicate that GNT-I, FDL, and GNT-II are key determinants. Over-expression of GNT-I in Sf cells co-expressing influenza virus hemagglutinin yielded a recombinant product with MGn in place of MM, demonstrating that the relative levels of GNT-I and FDL directly influence the outcome of the N-glycosylation pathway (17). This result was consistent with the known substrate specificities of human GNT-I (18) and Sf-FDL (3, 5). Reduced levels of FDL activity also correlated with higher levels of hybrid and complex N-glycans in other insect cell types (19, 20). Finally, overexpression of human GNT-II led to the production of biantennary, complex N-glycans in Sf cells, which were not detected when GNT-II was expressed at endogenous levels (21).

Considering that human GNT-I and -II are Golgi resident enzymes (22, 23), the functional relationships observed between these human enzymes and the endogenous FDL in Sf cells suggest that the latter probably also resides in the Golgi apparatus. This would place these three enzymes in close physical proximity, which would be consistent with the idea that the fate of the branch point intermediate is determined, at least in part, by their relative activities. However, it has been shown that Drosophila melanogaster FDL (Dm-FDL) is found mainly on the cell surface and in late endosomes, or multivesicular bodies, and it was suggested that this enzyme is not a Golgi resident, but rather, only transits through the Golgi apparatus en route to these other compartments (4). Thus, the close physical proximity of GNT-I, GNT-II, and FDL suggested above is not supported by currently available data.

Another factor limiting our understanding of the enzymes functioning around the N-glycosylation branch point in the Sf system is that the endogenous GNT-I and GNT-II genes have not been isolated. Thus, we have not had the tools needed to determine the intracellular distributions of the endogenous Sf GNTs or to express and purify recombinant forms of these enzymes to determine their substrate specificities. The only molecularly cloned insect GNT-I and -II genes reported to date are Drosophila mgat-1 and -2 and only the former was used to produce a purified recombinant insect GNT-I for enzyme activity assays (24). Analysis of Sf GNT-I and -II has been limited to
the use of crude Sf cell microsome fractions, which have low levels of endogenous GNT and competing β-N-acetylglucosaminidase activities (3).

Thus, while previous studies have provided a large amount of useful information, they have not completely illuminated the functional relationships among GNT-I, FDL, and GNT-II, nor have they provided a concerted analysis of all three enzymes in any single insect system. In this study, we addressed this issue by extending our previous efforts to characterize Sf-FDL and gathering a more comprehensive data set that included all three Sf enzymes assayed in the Sf system. We started by isolating Sf cDNAs encoding GNT-I and -II and using bioinformatic tools to analyze the predicted amino acid sequences of the gene products. We subsequently used the cloned genes to express, purify, and characterize recombinant forms of the endogenous Sf enzymes. Finally, we analyzed the relative intracellular distributions of Sf-GNT-I, Sf-GNT-II, and Sf-FDL in relation to a previously described Sf class I processing α-mannosidase (Sf-ManI; 25-27), which is an established endogenous Sf Golgi marker. The substrate specificities and intracellular distributions of the three Sf processing enzymes supported the idea that they function at the N-glycan processing branchpoint and, therefore, that their relative activities are, indeed, major factors determining the net outcome of the insect cell N-glycosylation pathway.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except where otherwise indicated. Restriction enzymes were from New England Biolabs (Ipswich, MA) and DNA oligonucleotides were from Integrated DNA Technologies (San Diego, CA). The sequences of all the oligonucleotide primers used in this study are given in Table S1.

**Cells and cell culture**—Sf9 cells were routinely maintained as shake flask cultures in complete TNM-FH (TNM-FH medium supplemented with 10% fetal bovine serum) as described previously (28). S2 cells were routinely maintained as shake flask cultures in ESF 921 medium (Expression Systems, Woodland, CA) supplemented with 10% FBS.

Molecular cloning of Sf-GNT-I and -II cDNAs—The *A. mellifera*, *A. aegypti*, *B. mori*, and *T. castaneum* genomic databases were searched using tBLASTn (29) with the derived *D. melanogaster* GNT-I (24) Genbank accession number AAF70177) and GNT-II (30) Genbank accession number AAL17663) amino acid sequences as the query. Exons encoding putative insect GNT fragments were joined in silico using a splice site prediction algorithm available on the NetGene2 Server (31) and the predicted amino acid sequences were aligned using ClustalX version 2.0.10 (32) with the default settings. Highly conserved amino acid sequences were then used to design degenerate oligonucleotide primers biased towards the coding sequences of the predicted lepidopteran insect (*B. mori*) GNT’s. These primers (50 pmoles each) were used for PCRs containing Sf cell cDNA, Phusion DNA polymerase (New England Biolabs), dNTPs and the manufacturer’s HF buffer, as detailed below.

The primers SF-GNT-I SPDEG and ASPDEG or SF-GNT-II SPDEG and ASPDEG were used in touchdown PCRs with Sf cell cDNA as the template to amplify portions of the SF-GNT-I and -II open reading frames, respectively, as described previously (5). These degenerate PCRs yielded amplification products in the expected size range (data not shown), which were recovered, purified, and directly sequenced using the same degenerate primers used for the respective PCRs. The resulting DNA sequences were assembled using ContigExpress (Vector NTI Advance 10.3.1; Invitrogen). Gene-specific primers were then designed from the assembled DNA sequences and used for primary and nested 5'- and 3'-RACE reactions using Sf cell cDNA prepared for 5'- and 3'-RACE with the Generacer™ kit (Invitrogen) as described previously (5). Primary and nested 3'-RACE reactions were performed using the primers provided with the kit and the SF-GNT-I and -II SP1 and SP2 primers, respectively, for SF-GNT-I and -II. Primary and nested 5'-RACE reactions were performed using the primers provided with the kit and the SF-GNT-I and -II ASP1 and ASP2 primers, respectively, for SF-GNT-I and -II. The nested 5'- and 3'-RACE reactions yielded strong PCR amplifiers observed by agarose gel.
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electrophoresis, which were not produced in control reactions lacking either primer (data not shown). These reaction products were recovered, purified, and directly sequenced using the primers Sf-GNT-I and -II SP3 for 3'RACE reactions and the 5'RACE nested primer and Sf-GNT-I and -II ASP3 and ASP4 for 5'RACE reactions for Sf-GNT-I and -II, respectively. Sequences obtained from these reactions were used to extend the degenerate amplimer sequences and obtain the putative Sf-GNT-I and -II sequences shown in Figs. S1-S2. Finally, the open reading frames were amplified from Sf cell cDNA using the Sf-GNT-I and Sf-GNT-II FL CLO SP and ASP primers and the resulting amplification products were purified and transferred into pENTR™/D-TOPO® (Invitrogen), yielding pENTR/D-TOPO-SfGNT-I-FL and pENTR/D-TOPO-SfGNT-II-FL. Three clones of each were identified and sequenced to verify the accuracy of the coding sequences assembled from the degenerate PCR and RACE products.

Expression and purification of recombinant Sf-GNT's—Baculovirus expression vectors encoding soluble, secreted forms of Sf-GNT-I and -II with N-terminal affinity purification tags were designed and isolated as described in Supplemental Methods. Titered stocks of these viruses, which were designated AcSfGNT-I-Sol and AcSfGNT-II-Sol, were used to infect mid-log Sf9 cell cultures in complete TNM-FH at a multiplicity of infection of 2 plaque-forming units per cell. The cultures were then incubated for 72 h at 28 °C and 125 rpm in a model 4580 rotary platform shaker-incubator (Forma Scientific, Inc., Marietta, OH). Subsequently, the infected cells were pelleted at 1500 rpm in a Beckman Model GPR centrifuge for 5 min at 4°C. The supernatant was decanted and further clarified at 3750 rpm in a GPR centrifuge for 10 min at 4°C. Finally, the supernatant was cleared of virus by centrifugation at 30,000 rpm in a Beckman Ti45 rotor for 20 min at 4°C in a Beckman Optima XL-100K ultracentrifuge. The cleared culture supernatant was then dialyzed overnight in 55 kDa molecular weight cutoff tubing (Spectrum Medical Industries Inc.; Laguna Hills, CA) against 20 volumes of phosphate buffer (50 mM Na2HPO4, 300 mM NaCl, pH to 8.0) at 4°C. One ml of Probond Nickel-chelating resin slurry (Invitrogen) was applied to a Biorad Poly-Prep 10 ml chromatography column (BioRad; Hercules, CA), drained and equilibrated by washing 3 times with 10 mls of phosphate buffer. The equilibrated resin was resuspended in the dialyzed supernatant and the suspension was transferred to a 250 ml conical bottle and shaken for 30 min at 4°C. The supernatant was transferred to the chromatography column and the resin was drained, washed 3 times with 10 mls of phosphate buffer, and the bound protein was eluted with 2.5 mls of elution buffer (phosphate buffer supplemented with 200 mM histidine, pH 8.0). The elution buffer was exchanged for storage buffer (50 mM MES, 250 mM NaCl, 1% Triton X-100, pH 6.3) by washing a PD10 desalting column (Amersham Biosciences, Piscataway, NJ) with 25 mls of storage buffer, applying the eluate and allowing it to flow through, and eluting the protein by applying 3.5 mls of storage buffer and collecting the eluate. The purity of the recombinant GNT protein preparations was assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Approximately 5 µg of purified proteins were electrophoresed and stained by Coomassie brilliant blue before or after treatment with peptide N-glycosidase F (New England Biolabs) according to the manufacturer's instructions. An identical set of samples was transferred to an Immobilon-P PVDF membrane (Millipore, Bedford, MA), which was blocked and then probed with mouse anti-6xHis and rabbit anti-mouse alkaline phosphatase-conjugated antiserum, and signals were developed using an established method (33).

N-acetylglucosaminyltransferase assays—Enzyme reactions were performed in the presence of 50 mM buffer (acetate: pH 3.65, 4.15, 4.65, 5.15; MES: pH 5.65, 6.15, 6.65; HEPES: pH 7.15; Tris: pH 7.65, 8.15, 8.65, 9.15; glycine: pH 9.65, 10.15, 10.65), 150 mM NaCl, 1 mM UDP-GlcNAc, 10 mM divalent cofactor (CoCl2, MnCl2, NiCl2, MgCl2, ZnCl2, FeCl2, CaCl2, or EDTA instead), 0.5% Triton X-100, enzyme prepared as described above, and 50 pm PA-labeled acceptor glycan prepared as described in Supplemental Methods. The reactions were incubated at 37°C for 15 minutes...
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and frozen until separated by RP-HPLC, which was performed essentially as described previously (34). For pH optima, cofactor requirement, and acceptor substrate specificity determinations, the enzymes were added to achieve approximately 50% conversion (compared to MM for Sf-GNT-I and M Gn for Sf-GNT-II), and efficiencies were calculated by dividing the amount of product formed compared to the amount of input glycan compared to the reference conversion.

Intracellular distribution of fluorescently tagged enzymes—For Sf cell experiments, ~0.5 x 10⁶ Sf9 cells were seeded in 2 ml of complete TNM-FH medium in 35 mm glass bottom culture dishes (No. 1.5, MatTek, Ashland, MA) and allowed to attach for 1 h at 28°C. The cells were then washed twice with Grace’s medium supplemented with 10% FCS and covered with 0.5 ml of the same medium. Six µg of plasmid DNAs encoding the various fluorescently tagged enzymes described in Supplemental Methods were sterilized in TE buffer at 65°C for 10 min, after which 500 µL of transfection buffer (140 mM NaCl, 125 mM CaCl₂, 25 mM HEPES, pH 7.1) were added. The DNA solutions were then added to the cells with gentle rocking for 2 h at 28°C. The transfection mixtures were then removed and the cells were washed twice with complete TNM-FH, covered with 2 ml of complete TNM-FH and incubated for 24 h at 28°C prior to examination.

For nuclear staining, 10 µg/ml of Hoechst 33342 dye (Invitrogen) was added to the transfected cell culture media and the cells were incubated for 10 minutes prior to examination.

For cell surface labeling, transfected cells were incubated in HBS (150 mM NaCl, 10 mM HEPES, 1 mM each of CaCl₂, MgCl₂ and MnCl₂, 0.08% W/V NaN₃, pH 7.5) for 10 min at 4°C. The cells were then washed twice with HBS, and incubated in HBS containing 10 µg/ml of biotinylated ConA (Vector laboratories, Burlingame, CA) for 5 min at 4°C. The cells were once again washed twice with HBS, incubated in HBS containing 10 µg/ml of streptavidin-Texas Red (Vector laboratories) for 5 min at 4°C. Finally, the cells were washed twice with HBS and covered with HBS prior to examination.

For lysosomal labeling, transfected cells were incubated in 50 nM LysoTracker™ Red DND-99 (Invitrogen) in Grace’s medium adjusted to pH 7.4 for 15 minutes at 28°C prior to examination.

For S2 cell experiments, ~5 x 10⁶ S2 cells were seeded in serum-free ESF 921 in a T-25 flask and allowed to attach for 2 h at 28°C. Three µg of plasmid DNAs encoding the various fluorescently tagged enzymes described in Supplemental Methods were brought to 40 µl in TE buffer, sterilized in a 65°C water bath for 10 min, and added to 300 µl of ESF-921. This mixture was added to 300 µl of ESF-921 containing 45 µl Cellfectin (Invitrogen), gently mixed, incubated at RT for 10 min, and 2.5 ml of ESF 921 were added. The medium was replaced with 2 ml of fresh ESF921, the DNA mixture was added to the cells, and the cells were incubated for 12 h at 28°C. After this incubation period, 0.5 ml of FCS was added and the cells were incubated at 28°C for another 24 h. Subsequently, the cells were counted and 1 x 10⁶ cells were pelleted, resuspended in 2 ml of ESF-921, seeded in glass-bottom dishes treated essentially as described previously (35), and allowed to attach for 2 h prior to examination.

Prior to microscopic examination, all cells except those that were surface-stained or stained for lysosomes were washed twice and covered with serum-free Grace’s medium. Cells that were surface-stained or stained for lysosomes were examined directly. Photomicrographs were taken using an Olympus FSX100 microscope. Channels were separated using ImageJ 1.410 and individual channels were enhanced to improve contrast and brightness using Adobe® Photoshop® CS3. The same enhancement was applied uniformly to the entire panel so that no specific feature within any individual panel was enhanced, obscured, moved, removed, or introduced.

Supplemental Methods—The methods used for transmembrane domain predictions, isolation of recombinant baculoviruses encoding Sf-GNT-I and Sf-GNT-II, isolation of expression vectors encoding fluorescently tagged proteins, and production of fluorescently labeled N-glycan substrates are described in Supplemental Methods.
RESULTS

Isolation of genes encoding Sf-GNT-I and -II—Degenerate PCRs were performed with primers directed against highly conserved amino acid sequences in known and putative animal GNT-I and -II proteins and Sf cell cDNA as the template. These reactions yielded specific PCR amplimers of approximately the expected sizes (data not shown), which were not obtained in negative controls with just one primer or no template. Direct sequencing of the amplification products revealed partial open reading frames encoding putative Sf-GNT fragments. These sequences were extended using 3' and 5'-RACE to produce cDNA sequences with complete open reading frames encoding the putative full-length Sf-GNT-I and -II proteins, which were deposited in Genbank under accession numbers HQ888863 and HQ888864, respectively.

Analysis of the predicted Sf-GNT-I and -II gene products—The nucleotide and predicted protein sequences of Sf-GNT-I and -II are shown in Figs. S1 and S2, respectively. Both proteins have DxD motifs, a hallmark feature of the glycosyltransferases, which conform to the hhhxDxxh consensus, where h is a hydrophobic amino acid residue, x is any amino acid residue, and D is an acidic amino acid residue (36). Both were predicted to be type II transmembrane proteins, another hallmark feature of the glycosyltransferases (37), when analyzed by TMHMM (38). The N-terminal transmembrane domains of both proteins were about the same length as those of N-glycan processing enzymes known to reside in the Golgi apparatus (Table 1). Furthermore, each had a dibasic motif in its cytoplasmic tail, proximal to the predicted transmembrane domain (Table 1), which functions as an endoplasmic reticulum exit signal in known Golgi resident glycosyltransferases (39). Finally, both proteins had several consensus N-glycosylation sites, predicting they are N-glycosylated, when analyzed by NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/).

A multiple sequence alignment was constructed with Sf-GNT-I and other known or predicted animal GNT-I’s using ClustalX (32). The other sequences used for this alignment included human GNT-I (40), the major C. elegans GNT-I (Gly-13; 41), D. melanogaster GNT-1, which is the only other insect GNT-I cloned and characterized to date (24), and rabbit GNT-I (42), for which detailed structural information has been obtained (43, 44). The alignment showed that most of the amino acid residues known to interact with the UDP-GlcNAc donor substrate, N-glycan acceptor substrate, and Mn\(^{2+}\) cofactor, as well as four cysteine residues that form disulfide bonds, are strictly conserved in Sf-GNT-I (marked by arrows in Fig. S3; 44). Sf-GNT-I also had two notable conservative substitutions (asterisks in Fig. S3), including a serine for the threonine at position 315 of rabbit GNT-I, which is involved in coordinating the Mn\(^{2+}\) (44), and a lysine for the arginine at position 303 of the Chinese hamster enzyme, which is inactivated when replaced by a tryptophan residue (45). The only identifiable non-conservative substitution in Sf-GNT-I was a phenylalanine for the tryptophan at position 290 of the rabbit enzyme (dagger in Fig. S3), which interacts with the UDP-GlcNAc donor substrate (44). The phenylalanine in Sf-GNT-I is unlikely to accommodate this interaction because it lacks the requisite indole nitrogen of the tryptophan residue in the rabbit enzyme.

An analogous multiple sequence alignment was constructed with Sf-GNT-II and other known or predicted animal GNT-II’s (Fig. S4), including the well characterized human (46) and rat (47) enzymes, as well as the D. melanogaster enzyme predicted from the only other cloned insect GNT-II gene to date (30), and the predicted C. elegans Gly-20 gene product (48). Neither of the latter two gene products has been expressed and characterized. We could not directly assess the conservation of functional amino acids in Sf-GNT-II because no GNT-II structure has been reported. However, GNT-I and -II catalyze extremely similar transferase reactions. Thus, we indirectly assessed amino acid conservation in Sf-GNT-II in context of the structure of rabbit GNT-I. This analysis indicated that amino acid residues likely to interact with the Mn\(^{2+}\) cofactor and three of the four amino acid residues likely to interact with the UDP-GlcNAc donor substrate in rabbit GNT-I are strictly conserved in Sf-GNT-II (arrows in Fig. S4). Three other amino acid
residues known to be required for GNT-II function by mapping human mutations (49, 50) are also strictly conserved in Sf-GNT-II (asterisks in Fig. S4).

Interestingly, the alignment shown in Fig. S4 revealed that Sf-GNT-II has a 44 amino acid insert (residues 296-339) relative to the other GNT-II’s. A protein encoded by a splice variant from the D. melanogaster GNT-II (mgat-2) gene, which was not included in our multiple sequence alignment, has an even larger, 120 amino acid insert at this same position (30). By searching various EST databases, we found several other predicted insect GNT-II’s with amino acid insertions at this position, including those from the monarch butterfly D. plexippus, silkworm B. mori, honeybee A. mellifera, and mosquito A. gambiae. Interestingly, none of the deuterostome GNT-II sequences included in Genbank had this insertion. This major difference between the insect and deuterostome GNT-II sequences raised the possibility that the insect and mammalian enzymes might have different acceptor substrate specificities. A particularly intriguing possibility in context of our focus on a branched model of the insect N-glycosylation pathway (Fig. 1) was that insect GNT-II’s might be able to transfer paucimannosidic N-acetylglucosamine to the α6 branch mannose of their acceptor substrate specificities. The pH optima were determined for Sf-GNT-I and -II in enzyme activity assays containing UDP-N-acetylglucosamine as the donor and MM or Gm labeled with 2-aminopyridine (MM-PA or Gm-PA) as the acceptor substrates, respectively. These assays also included 10 mM Mn2+, which had been shown to support high levels of N-acetylglucosamine transfer in previous assays (11). We found that Sf-GNT-I has high activity over a broad pH range, from 6.6 - 8.1, with an optimal pH of 7.1 (Fig. 3A). Sf-GNT-II had an optimal pH of 6.7, with a broad shoulder of high activity from pH 7.1 - 8.1 (Fig. 3B). The Sf-GNT-I and -II metal requirements were examined in assays that included the same donor and acceptor substrates at the optimal pH for each enzyme and all activities were expressed as a percentage of the level observed with Mn2+. Sf-GNT-I had higher, equal, and lower levels of activity when assayed in the presence of Co2+, Ni2+, and a group of other metals, respectively (Fig. 3C). Sf-GNT-II had lower levels of activity when assayed in the presence of all other metals tested, relative to Mn2+ (Fig. 3D). Both enzymes had an absolute metal requirement, as evidenced by the absence of detectable activity in assays containing EDTA (Figs. 3C-3D).

Expression and purification of recombinant Sf-GNT-I and -II—Initially, we examined the pH and metal requirements of Sf-GNT-I and -II to establish the conditions needed to determine their acceptor substrate specificities. The pH optima for Sf-GNT-I and -II in all enzyme activity assays were expressed as a percentage of the level observed with Mn2+. Sf-GNT-I had higher, equal, and lower levels of activity when assayed in the presence of Co2+, Ni2+, and a group of other metals, respectively (Fig. 3C). Sf-GNT-II had lower levels of activity when assayed in the presence of all other metals tested, relative to Mn2+ (Fig. 3D). Both enzymes had an absolute metal requirement, as evidenced by the absence of detectable activity in assays containing EDTA (Figs. 3C-3D).

After establishing the assay conditions for purified Sf-GNT-I and -II, as described above,
we examined their acceptor substrate specificities under those conditions. SF-GNT-I specificity was assessed using M5-PA, MM-PA, and GnM-PA as potential acceptor substrates. We found that this enzyme could transfer N-acetylglucosamine to the free α3-branch mannose residue of all three substrates, indicating that it does not qualitatively discriminate among paucimannosidic acceptors with or without substituents on the α6-branch mannose (Figs. 4A-4C). These results demonstrated that SF-GNT-I can directly reverse FDL trimming by converting MM to MGn (Fig. 4B), which was consistent with previous results obtained with an analogous form of the D. melanogaster gene product (24). Our results also demonstrated that SF-GNT-I can reverse FDL trimming by converting GnM to GnGn (Fig. 4C). SF-GNT-II specificity was assessed using MM-PA and MGn-PA as potential acceptor substrates. We found that this enzyme could transfer N-acetylglucosamine to MGn-PA (Fig. 4D), but not to MM-PA (Fig. 4F), indicating that it discriminates among paucimannosidic acceptors and can only utilize those with an N-acetylglucosamine linked to the α3-branch mannose. Thus, the 44 amino acid insertion in SF-GNT-II, which was discussed above, does not relax specificity and allow this enzyme to initiate a reversal of FDL trimming by converting MM to GnM. Finally, by extending the assay incubation time 100-fold, we found that SF-GNT-II could transfer a second N-acetylglucosamine residue to GnGn-PA, the product of the first transferase reaction (Fig. 4E). However, because this was an extremely slow reaction, it is unlikely to be biologically relevant.

Intracellular distribution of SF-GNT-I, SF-GNT-II, SF-FDL and SF-ManI—The roles of SF-GNT-I and SF-GNT-II (11), as well as SF-FDL (3, 5) in transferase or trimming reactions around the branch point intermediate in the insect cell N-glycosylation pathway supported the idea that these enzymes have a key role in determining the net outcome of this pathway. Considering these functional relationships, one might logically expect all three enzymes to occupy the same intracellular compartment. Moreover, previous work on human GNT-I and -II (22, 23, 51), as well as the lengths of the transmembrane domains and the presence of adjacent dibasic motifs in the SF enzymes (Table 1), predicted that each of the SF enzymes would reside in the Golgi apparatus. However, there were no published studies on the intracellular distributions of any insect GNT’s, and the only published observations on the intracellular distribution of an insect FDL (4) were inconsistent with this prediction. Hence, we took advantage of the opportunity to directly examine the intracellular distributions of all three enzymes, each derived from one insect system, in their native cellular environment.

SF cells were co-transfected with various combinations of expression plasmids encoding the full-length SF enzymes fused to C-terminal fluorescent protein tags, and then the intracellular fluorescence patterns were examined in living cells. A previously characterized, endogenous SF class Iα-mannosidase I (SF-ManI) was used as a known Golgi marker (25, 26). The results obtained in a variety of permutations, with various SF enzymes tagged with either GFP or RFP, are shown in Fig. 5. First, it is important to note that the Golgi apparatus has a distinct, punctate distribution throughout the cytoplasm of SF and other insect cells, which contrasts to the perinuclear appearance of the Golgi apparatus in mammalian cells (8, 10, 25, 52-54). In addition, it should be noted that the fluorescence patterns observed in SF cells expressing the same protein with either GFP or RFP tags overlapped significantly (Figs. 5A-5B). Given that the addition of a GFP tag is generally considered to preserve the normal intracellular distribution of protein fusion partners (55), this result suggested that the addition of RFP also preserves their normal intracellular distributions.

SF cells expressing GFP- or RFP-tagged SF-GNT-I or -II had a fluorescence pattern characterized by the presence of cytoplasmic punctae (Figs. 5A-5B), which was consistent with the idea that both enzymes reside in the Golgi apparatus. SF cells co-expressing SF-GNT-I and -II with reciprocal tags had tightly overlapping, punctate, cytoplasmic red and green fluorescence (Figs. 5C-5D), which supported the idea that these two enzymes reside in the same subcellular compartment, probably the Golgi apparatus. SF cells co-expressing RFP-
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tagged Sf-GNT-I or -II and GFP-tagged Sf-ManI, the endogenous Golgi marker, also had tightly overlapping, punctate, cytoplasmic red and green fluorescence (Figs. 5E-5F), which strongly supported the idea that SF-GNT-I and -II reside in the Golgi apparatus. Finally, Sf cells co-expressing RFP-tagged SF-GNT-I or -II and GFP-tagged SF-FDL had tightly overlapping punctate patterns of cytoplasmic red and green fluorescence (Figs. 5G-5H), indicating that SF-GNT-I, SF-FDL, and SF-GNT-II are all found in close proximity to each other, in the Golgi apparatus, together with SF-ManI.

Because a previous analysis had indicated that Dm-FDL localizes mainly to late endosomes (multivesicular bodies) and the plasma membrane, with only low levels found in the endoplasmic reticulum (ER) and Golgi apparatus (4), we compared the distribution of Sf-FDL with several different subcellular organelles that might harbor this protein (Fig. 6). The results showed that there was substantial overlap between Sf-FDL and two different Golgi markers (Figs. 6E-6F), but virtually no overlap between Sf-FDL and the plasma membrane (Con A; Fig. 6A), nucleus (Hoechst 33342; Fig. 6B), late endosomes (Rab7a; Fig. 6C), lysosomes (LysoTracker Red; Fig. 6D), or ER (ER-RFP; Fig. 6E). The staining patterns obtained for each organelle were distinct from each other and similar to those observed in previous studies of these organelles in insect cells (8, 10, 25, 52-54, 56-59). Thus, these results showed that the resolution of our light microscopy was adequate to distinguish the distributions of the major subcellular organelles that might harbor Sf-FDL and also showed that this protein resides in the Golgi apparatus of Sf cells.

Intracellular distribution of Dm-FDL—This conclusion was obviously inconsistent with the previous conclusion that Dm-FDL is not a Golgi resident protein, but only transits through this compartment and accumulates on the plasma membrane, in multivesicular bodies, and is ultimately secreted (4). This prompted us to examine the intracellular distribution of Dm-FDL in our system. Sf cells co-expressing GFP-tagged Dm-FDL and RFP-tagged Sf-GNT-I had mainly punctate, cytoplasmic green and red fluorescence (Fig. 7A) with substantial overlap. These results indicated that Dm-FDL, like SF-FDL, resides mainly in the Golgi apparatus of Sf cells. However, it was possible that this result was specific to Sf cells, as Léonard et al. (4) used a Drosophila cell line, S2, for their study. Hence we examined the distribution of GFP-tagged Dm-FDL in S2 cells co-expressing RFP-tagged SF-GNT-I. The results showed that these cells also had mainly punctate, cytoplasmic green and red fluorescence (Fig. 7B) with substantial overlap, as observed in Sf cells. Given that SF-GNT-I was a heterologous Golgi marker for S2 cells, we also compared the intracellular distribution of GFP-tagged Dm-FDL in S2 cells co-expressing an RFP-tagged, truncated form of Drosophila Golgi mannosidase II (ΔdGMII), which is an established endogenous Drosophila Golgi marker (54). Again, the results showed that S2 cells co-expressing these two proteins had mainly punctate, cytoplasmic green and red fluorescence (Fig. 7C) with substantial overlap. Thus, both SF-FDL and DM-FDL both appear to be Golgi-localized proteins when expressed in their native host cell systems.

DISCUSSION

The purpose of this study was to further evaluate the relationships among three N-glycan processing enzymes, GNT-I, FDL, and GNT-II, which function around a key branch point in the insect cell N-glycosylation pathway. Our approach was to assemble data on the biochemical activities and intracellular distributions of all three enzymes in a single insect system. The results directly confirmed the expected biochemical roles of these enzymes in producing or processing MGn, which is the key branch point intermediate in the pathway, and newly demonstrated that they all function in close physical proximity to each other. Thus, our results were consistent with the idea that the concerted action of these three enzymes determines, at least in part, the net outcome of the insect N-glycosylation pathway.

The roles of some of these enzymes in the insect N-glycosylation pathway had been surmised from the substrate specificites observed in previous assays of crude microsomal fractions isolated from Sf cells (3, 11) and, in the case of GNT-I and -II, from their
established roles in the mammalian pathway (1). Thus, it was likely that insect GNT-I was involved in the production of MGn, which was subsequently trimmed by FDL or, at a much lower frequency, elongated by insect GNT-II. It also was clear that the relative levels of GNT-I, FDL, and GNT-II strongly influenced the types of N-glycans produced by insect cells (4, 17, 19-21). Finally, there was reason to believe that insect GNT-I was involved in the production of MGn, which was conserved in the putative Sf enzymes (Figs. S1-S2 and Table 1). In addition, all but one of the amino acids identified as crucial residues in structural and/or functional studies were conserved in the putative Sf enzymes (Figs. S3-S4). We subsequently expressed and purified the gene products and performed enzyme activity assays analogous to those performed with purified recombinant SF-FDL (Fig. 3). The optimal pH of Sf-GNT-I was 7.1, which is similar to that of the lepidopteran insect M. brassicae (11), Chinese hamster (61), rat (62), and human (63) enzymes. The optimal pH of Sf-GNT-II was 6.7, which is similar to that of porcine GNT-II (64). The optimal pH values for both enzymes were close to the reported pH of the Golgi apparatus, which was consistent with the expectation that these enzymes reside and function in this compartment (55). Both enzymes had an absolute requirement for a metal cofactor. Sf-GNT-I was most active in the presence of Co2+, highly active in the presence of Mn2+ and Ni2+, and had little or no activity in the presence of Mg2+, and had almost no activity in the presence of other metals, similar to the Drosophila (24), rabbit (18), rat (65), and human (62) enzymes. Sf-GNT-II was most active in the presence of Mn2+, like the mammalian enzyme (66), and had little or no activity in the presence of any of the other metals tested. The metal ions presumably coordinate the donor substrate through an interaction with the conserved DxD motifs in these enzymes (67).

Subsequently, we showed that Sf-GNT-I has broad substrate specificity, as it transferred N-acetylglucosamine to each of the three N-glycans tested, though it preferred M5 to Gmn or MM (Fig. 4A-4C). The same results were obtained in previous assays of GNT-I in crude microsomal membrane preparations from Sf cells (11) and with purified mammalian GNT-I (18, 62, 65, 68). Perhaps the most illuminating result from these studies was that Sf-GNT-I can transfer N-acetylglucosamine to MM, like the
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*Drosophila* enzyme (24), and also to GnM, indicating that the insect GNT-I’s can reverse the FDL trimming reaction.

In contrast to Sf-GNT-I, Sf-GNT-II had a narrow substrate specificity, as it only transferred N-acetylgalactosamine to MGn, not MM (Fig. 4D-4F), like other animal GNT-II’s (66, 68, 69). We had initially hypothesized that Sf-GNT-II and other insect GNT-II’s might have relaxed acceptor substrate specificities due to the presence of a large amino acid insert absent in the deuterostome enzymes (Fig. S4). The ability to use MM as an acceptor would have suggested that insect GNT-II’s can redirect paucimannosidic products into the hybrid/complex N-glycan product pool by creating a new substrate for GNT-I (see Fig. 4C). It also would have explained the presence of GnM in the N-glycan profiles of some glycoproteins produced by insect cells (70, 71). However, because Sf-GNT-II failed to transfer N-acetylgalactosamine to MM, we concluded that it has the conventional substrate specificity and that the GnM observed in insect N-glycan profiles is due to the sequential action of GNT-II and FDL on the key branchpoint intermediate, MGn (Fig. 1). The latter conclusion is supported by the fact that the N-glycan profiles of wild type, but not *fdl* mutant flies, include GnM (4). Similarly, *Drosophila mgat1* mutants fail to produce either GnM or MGn (72). Finally, in addition to being able to produce GnGn from MGn, we found that Sf-GNT-II can transfer a second N-acetylgalactosamine residue to the initial GnGn reaction product, but the extremely slow reaction rate indicated it is unlikely to be biologically relevant.

While there were no published data on the intracellular distributions of any insect GNT’s, it seemed highly likely that Sf-GNT-I and -II would reside in the Golgi apparatus because human GNT-I and -II are known Golgi resident enzymes (22, 23, 51) and the Sf enzymes had transmembrane domains with similar lengths and adjacent dibasic amino acid motifs, which are believed to direct protein egress from the endoplasmic reticulum and Golgi residence (Table 1; 39). Sf cells expressing fluorescently tagged versions of Sf-GNT-I and -II had strongly overlapping patterns of punctate, cytoplasmic fluorescence, which mirrored the distribution of the Golgi apparatus in insect cells (Fig. 5). This pattern also overlapped strongly with the punctate, cytoplasmic fluorescence obtained with Sf-Man-I, an endogenous Sf cell Golgi marker (25), which strongly supported the conclusion that Sf-GNT-I and -II both reside in the Golgi apparatus.

Considering the functional relationships among these three enzymes, the newly determined Golgi localization of Sf-GNT-I and -II, and the presence of a transmembrane domain with adjacent dibasic motifs, it seemed likely that Sf-FDL also would be a Golgi resident protein. In support of this prediction, we found that Sf cells expressing fluorescently tagged FDL had punctate, cytoplasmic fluorescence, which overlapped strongly with the pattern observed in cells co-expressing Sf-GNT-I or -II (Fig. 5). Despite our predictions, this result was somewhat unexpected because a previous study concluded that Dm-FDL was present mainly in late endosomes and on the plasma membrane of *Drosophila* S2 cells, with only a small minority localized in the Golgi apparatus and ER (4). Hence, we also compared the Sf-FDL staining pattern to those obtained with markers of various subcellular organelles. These experiments indicated that we could adequately resolve these organelles and that Sf-FDL failed to substantially co-localize with plasma membrane, nuclear, late endosomal, lysosomal, or ER markers. In contrast, there was substantial co-localization between Sf-FDL and the Golgi apparatus (Fig. 6). Finally, we examined the intracellular distribution of Dm-FDL in both S2 and *Drosophila* S2 cells (Fig. 7). In both cases, Dm-FDL exhibited punctate, cytoplasmic fluorescence that substantially overlapped with Sf-GNT-I. As in Sf cells, the S2 Golgi apparatus is known to have punctate cytoplasmic staining patterns (73), suggesting that the observed punctae were, in fact, Golgi vesicles. The observation that GNT-I and FDL reside in the same subcellular compartment is corroborated by biochemical evidence (3, 20). Finally, our finding that fluorescently tagged *Drosophila* FDL accumulates in the Golgi apparatus was confirmed in a homologous test system using S2 cells and an endogenous *Drosophila* Golgi marker protein (Fig. 7C). Thus, we conclude that FDL accumulates and functions in the Golgi.
apparatus of insect cells and should be regarded as a Golgi-resident enzyme. The close physical proximity and enzyme activities of GNT-I, FDL, and GNT-II are consistent with the idea that these enzymes have key roles in producing and processing the branchpoint intermediate, MGn, and determining the net outcome of the protein N-glycosylation pathway in insect systems.

REFERENCES

Insect cell glycosylation pathway branch point

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FOOTNOTES

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1To whom correspondence should be addressed. Tel.: +1-307-766-4282; Fax: +1-307-766-5098; e-mail: dljarvis@uwyo.edu
2The abbreviations used are: ER, endoplasmic reticulum; FDL, fused lobes, the insect N-glycan processing β-N-acetylglucosaminidase; GFP, green fluorescent protein; GNT-I, UDP-N-acetylglucosamine: α-3-D-mannoside β1,2-N-acetylglucosaminyltransferase I; GNT-II, UDP-N-acetylglucosamine: α-6-D-mannoside β1,2-N-acetylglucosaminyltransferase II; PA, 2-aminopyridine; RACE: rapid amplification of cDNA ends; RFP, red fluorescent protein; RP-HPLC, reverse phase high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sf, Spodoptera frugiperda.
### TABLE 1
Cytoplasmic tails and transmembrane domains of Golgi resident N-glycan processing enzymes

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<th>TM&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup>Predicted N-terminal cytoplasmic tails are indicated with the dibasic ER export motifs in bold, and arginine and lysine residues underlined.

<sup>b</sup>Predicted lengths of the transmembrane domains are averages of the lengths predicted by several different algorithms, as described in Experimental Procedures.
FIGURE LEGENDS

FIGURE 1. A key N-glycosylation pathway branchpoint. The initial steps in the insect and mammalian cell N-glycosylation pathways are similar or identical and produce the same N-glycan processing intermediate, MGn (boxed). GNT-I contributes to MGn production by adding N-acetylglucosamine to the mannose residue on the α3 branch of the M5 precursor, as shown. In insect cells, MGn is typically trimmed by FDL, which yields paucimannosidic N-glycan products. In mammalian cells, MGn is elongated by GNT-II and other glycosyltransferases, which yield hybrid and complex N-glycan products. However, recent studies have shown that insect cells also have the biochemical machinery required to produce hybrid and complex, as well as paucimannosidic N-glycans. Thus, insect cells have a branched N-glycosylation pathway in which MGn is the key branchpoint intermediate for alternative processing and production of structurally distinct N-glycans. Previous studies suggest that GNT-I, FDL, and GNT-II are major factors determining the outcome of this branched pathway.

FIGURE 2. Expression and purification of recombinant Sf-GNT-I and -II. The 6xHis-tagged ectodomains of Sf-GNT-I and -II were expressed in recombinant baculovirus-infected Sf cells and purified from the extracellular fraction by nickel affinity chromatography, as described in Experimental Procedures. Samples of the purified proteins were treated with buffer alone (-) or PNGase-F (+), and then equal amounts of the untreated or treated proteins were analyzed by SDS-PAGE with Coomassie Brilliant Blue staining (CBB, left panel) or immunoblotting with an antiserum specific for 6xHis (α-6xHis; right panel). The calculated molecular weights of the recombinant 6xHis-tagged Sf-GNT-I and -II ectodomains minus the signal peptides are 49.4 and 57.1 kDa, respectively.

FIGURE 3. Enzymatic characterization of recombinant Sf-GNT-I and -II. The pH and metal requirements of Sf-GNT-I and -II were determined using an HPLC assay with MM-PA and MGn-PA as the acceptor substrates, respectively, as described in Experimental Procedures. Sf-GNT-I (A) and -II (B) activities at various pH values are expressed as percentages of the optima. Sf-GNT-I (C) and -II (D) activities in the presence of various metals are expressed as a percentage of the levels observed in the presence of Mn²⁺ (100%).

FIGURE 4. Substrate specificities of recombinant Sf-GNT-I and -II. Purified recombinant Sf-GNT-I was incubated with M5-PA (A), MM-PA (B), or GnM-PA (C) in the presence of 10 mM Co²⁺ and 1 mM UDP-GlcNac at pH 7.5. Purified recombinant Sf-GNT-II was incubated with MGN-PA (D, E) or MM-PA (F) in the presence of 10 mM Mn²⁺ and 1 mM UDP-GlcNac at pH 6.7. In (E), Sf-GNT-II was incubated with MGN-PA as in (D), except the incubation time was extended 100-fold. Each panel shows the RP-HPLC profile obtained with the indicated reaction products.

FIGURE 5. Intracellular distributions of GFP- or RFP-tagged Sf gene products. Sf cells were co-transfected with plasmids encoding various C-terminally GFP- or RFP-tagged Sf N-glycan processing enzymes, and then phase contrast and fluorescence images were taken, as described in Experimental Procedures. GFP-tagged and RFP-tagged Sf-GNT-I (A), GFP-tagged and RFP-tagged Sf-GNT-II (B), GFP-tagged Sf-GNT-I and RFP-tagged Sf-GNT-II (C), GFP-tagged Sf-GNT-II and RFP-tagged Sf-GNT-I (D), GFP-tagged SfMan-I and RFP-tagged Sf-GNT-I (E), GFP-tagged SfMan-I and RFP-tagged Sf-GNT-II (F), GFP-tagged SfFDL and RFP-tagged Sf-GNT-I (G), GFP-tagged SfFDL and RFP-tagged Sf-GNT-II (H). Phase contrast images are shown in the left-hand column, GFP fluorescence in the second column, RFP fluorescence in the third column, merged GFP and RFP fluorescence pattern in the fourth column, and merged fluorescence patterns overlaid on phase contrast images are shown in the right-hand column.

FIGURE 6. Intracellular distribution of GFP-tagged Sf-FDL and various organelles. Sf cells were transfected with a plasmid encoding GFP-tagged SfFDL and either co-transfected with plasmids encoding
RFP-tagged marker proteins or otherwise labeled with red fluorescent markers, and then phase contrast and fluorescence images were taken, as described in Experimental Procedures. Cell surface (A), nuclei (B), late endosomes/multivesicular bodies (C), lysosomes (D), ER (E), Golgi (F). The columns are arranged as described in the Legend to Fig. 5.

**FIGURE 7.** Intracellular distribution of GFP-tagged Dm-FDL and RFP-tagged Golgi markers. Sf9 or S2 cells were co-transfected with plasmids encoding GFP-tagged Dm-FDL and either RFP-tagged Sf-GNT-I or RFP-tagged Dm-ΔGMII, and then phase contrast and fluorescence images were taken, as described in Experimental Procedures. GFP-tagged Dm-FDL and RFP-tagged Sf-GNT-I in Sf9 cells (A), GFP-tagged Dm-FDL and RFP-tagged Sf-GNT-I in S2 cells (B), GFP-tagged Dm-FDL and RFP-tagged Dm-ΔGMII in S2 cells (C). The columns are arranged as described in the Legend to Fig. 5.
FIGURE 1
FIGURE 3
FIGURE 4
FIGURE 5
FIGURE 6
FIGURE 7
Substrate specificities and intracellular distributions of three N-glycan processing enzymes functioning at a key branch point in the insect N-glycosylation pathway

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