Fucosyltransferase substrate specificity and the order of fucosylation in invertebrates

Katharina Paschinger, Erika Staudacher, Ute Stemmer, Gustáv Fabini1, and Iain B. H. Wilson2

Department für Chemie der Universität für Bodenkultur, Muthgas 18, 1190 Wien, Austria

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Core α1,6-fucosylation is a conserved feature of animal N-linked oligosaccharides being present in both invertebrates and vertebrates. To prove that the enzymatic basis for this modification is also evolutionarily conserved, cDNAs encoding the catalytic regions of the predicted Caenorhabditis elegans and Drosophila melanogaster homologs of vertebrate α1,6-fucosyltransferases (E.C. 2.4.1.68) were engineered for expression in the yeast Pichia pastoris. Recombinant forms of both enzymes were found to display core fucosyltransferase activity as shown by a variety of methods. Unsubstituted non-reducing terminal GlcNAc residues appeared to be an obligatory feature of the substrate for the recombinant Caenorhabditis and Drosophila α1,6-fucosyltransferases, as well as for native Caenorhabditis and Schistosoma mansoni core α1,6-fucosyltransferases. On the other hand, these α1,6-fucosyltransferases could not act on N-glycopeptides already carrying core α1,3-fucose residues, whereas recombinant Drosophila and native Schistosoma core α1,3-fucosyltransferases were able to use core α1,6-fucosylated glycans as substrates. Lewis-type fucosylation was observed with native Schistosoma extracts and could take place after core α1,3-fucosylation, whereas prior Lewis-type fucosylation precluded the action of the Schistosoma core α1,3-fucosyltransferase. Overall, we conclude that the strict order of fucosylation events, previously determined for fucosyltransferases in crude extracts from insect cell lines (core α1,6 before core α1,3), also applies for recombinant Drosophila core α1,3- and core α1,6-fucosyltransferases as well as for core fucosyltransferases in schistosomal egg extracts.

Key words: Caenorhabditis/Drosophila/fucosyltransferase/Schistosoma

Introduction

Glycan analyses of the two invertebrate model organisms, Caenorhabditis elegans and Drosophila melanogaster, indicate that paucimannosidic oligosaccharides carrying a core α1,6-linked fucose are among the major N-linked glycan species (Altmann et al., 2001; Cipollo et al., 2002; Fabini et al., 2001; Haslam et al., 2002; Natsuka et al., 2002; Williams et al., 1991). Core α1,6-fucosylation is also a hallmark of many vertebrate complex N-glycans, and indeed cDNAs encoding bovine, human, murine, and porcine core α1,6-fucosyltransferases (Hayashi et al., 2000; Javaud et al., 2000; Uozumi et al., 1996; Yanagidani et al., 1997) have been cloned. Although computer analyses could predict the presence of single genes in nematode and insect species homologous to these mammalian ones, the enzymatic activity of the encoded Caenorhabditis (FUT-8) and Drosophila (FucT6) proteins has previously not been proven.

The substrate specificity of the invertebrate enzymes is of interest because, even though core α1,6-fucosyltransferases transfer fucose to N-glycans carrying nonreducing terminal N-acetylglucosamine (e.g., structures such as GnGn according to the nomenclature of Schachter, 1986), these N-acetylglucosamine residues are largely absent from the core fucosylated oligosaccharides isolated from many invertebrates. Insect core α1,3-fucosyltransferases also require the presence of nonreducing terminal N-acetylglucosamine; however, the Caenorhabditis FUT-1 cannot act when the α1,3-linked mannose is substituted with N-acetylglucosamine (Paschinger et al., 2004). The action of both types of core fucosyltransferase is required to produce N-glycans carrying both core α1,3- and core α1,6-linked fucose residues that are found in Caenorhabditis and Drosophila. These glycans, seemingly absent from vertebrates, are recognized by antibodies raised against plant glycoproteins: particularly anti–horseradish peroxidase has been used to track neuronal pathways in invertebrates (Haase et al., 2001; Jan and Jan, 1982; Siddiqui and Cullotti, 1991; Snow et al., 1987), a cross-reaction probably due to the presence of core α1,3-fucose in both plants and invertebrates (Fabini et al., 2001). However, N-glycans carrying only core α1,3-fucose are relatively rare in invertebrates, a finding that may suggest that either the pools of substrate(s) for core α1,3-fucosyltransferases are already predominantly core α1,6-fucosylated before they encounter core α1,3-fucosyltransferases (due to the relative localization of the enzymes in the Golgi and/or higher core α1,6-fucosyltransferase activity) and/or that core α1,3-fucosyltransferases prefer core α1,6-fucosylated glycans (e.g., GnGnF6) over the non-fucosylated forms (e.g., GnGn). Indeed the K_m values for the only proven Drosophila core α1,3-fucosyltransferase (FucTA) would support the latter hypothesis (Fabini et al., 2001); experiments to address the former await a closer examination of the secretory pathway in those cells that express both types of core fucosyltransferase. On the other hand, previous experiments using enzyme activities in crude extracts suggest that core α1,6-fucosyltransferases cannot

1Present address: Octapharma Pharmazeutika Produktionsges. m.b.H., A-1100 Wien
2To whom correspondence should be addressed: e-mail: iain.wilson@boku.ac.at

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463
use substrates carrying core α1,3-fucose (Staudacher and März, 1998).

In the present study, Caenorhabditis and Drosophila core α1,6-fucosyltransferase cDNAs were cloned and expressed in the yeast Pichia pastoris. Not only was the enzymatic function of the proteins demonstrated, but we could, for the first time using also recombinant enzymes, confirm our previous hypotheses (Staudacher and März, 1998) that there is a strict order of core fucosylation events in insects and that this order is also applicable to a trematode (Schis-tosoma mansoni), whereas the situation in Caenorhabditis is more complex, partly due to the novel specificity of its core α1,3-fucosyltransferase.

**Results**

Cloning of core α1,6-fucosyltransferase cDNAs

Using the sequences of mammalian core α1,6-fucosyltransferases (encoded by FUT8 genes) to probe the genome sequence databanks, we could identify a single homologous gene in both C. elegans and D. melanogaster. The Caenorhabditis gene on chromosome V has been previously listed in Wormbase as fut-8 (hence the protein name is FUT-8) and by Oriol and colleagues (1999) as FucTD. Expressed sequence tag information suggests that fut-8 cDNAs carry an SL1 spliced leader (see GenBank accession numbers BJ773553 and BJ763494). For the Drosophila protein, encoded by chromosome X, we use the existing Flybase name FucT6. A high-throughput in situ hybridization screen of cDNAs encoding transmembrane and soluble proteins has shown that FucT6 is particularly expressed in the anterior and posterior midgut primordium of Drosophila embryos (Kopczynski et al., 1998; clone CK00490).

To verify our gene models, cDNA fragments corresponding to Caenorhabditis and Drosophila α1,6-fucosyltransferases were isolated and sequenced. While this work was in progress, cDNA sequences corresponding to both these genes (AJ512486 for fut-8 and AF441264/AY051451 for FucT6) were deposited in the databanks; our sequences were compatible to these except for some small differences. In the case of fut-8, an A→T substitution resulting in the second codon encoding Phe and not Leu was within the 5′-primer region. With FucT6, eight nucleotide differences were observed in the clone used for yeast expression; however, only one of these (in the 151st codon, GAG→GGG) resulted in an amino acid substitution.

The Caenorhabditis cDNA encodes a protein of 559 amino acids, and its gene consists of nine exons, whereas the Drosophila protein is predicted to be of 619 residues and its gene has four exons; in comparison the bovine, canine, chicken, human, murine, porcine, and rat homologs are all 575 amino acids long and the human FUT8 gene consists of nine coding region exons and a variable number of 5′ untranslated region exons, whereas the bovine FUT8 gene has five coding exons (Javaud et al., 2000; Martinez-Duncker et al., 2004). Among other species, Ciona intestinalis, Drosophila pseudoobscura, Xenopus laevis, and Danio rerio putative α1,6-fucosyltransferases (GenBank accession numbers AJ51451, AJ830720, AJ514872, and AJ781407) have 513, 625, 578, and 580 amino acids, respectively. Thus Drosophila has one of the longest and Caenorhabditis one of the shortest core α1,6-fucosyltransferase sequences determined thus far: The discrepancies in length are mainly within the probable stem region (see Figure 1). Both the Caenorhabditis and Drosophila α1,6-fucosyltransferases have the diarginine motif previously found in the human ortholog to be important for enzymatic activity (Takahashi et al., 2000). They also share with other α1,6-fucosyltransferases a C-terminal SH3 domain; such domains, which bind ligands containing a PXXP motif, are also a feature of...
Sr family kinases (Tatosyan and Mizzenina, 2000). In contrast to the mammalian α1,6-fucosyltransferases, as well as the Xenopus ortholog, which are not N-glycosylated, the Caenorhabditis and Drosophila α1,6-fucosyltransferases have one nonconserved potential N-glycosylation site each; the Anopheles ortholog, indeed, has two such sequons.

**Comparison of recombinant Caenorhabditis and Drosophila core fucosyltransferases**

To engineer the cDNAs to facilitate expression of soluble forms in P. pastoris, polymerase chain reaction (PCR) reamplification using the cDNA encoding the full-length form of the Caenorhabditis enzyme or regular reverse transcriptase (RT)-PCR using Drosophila cDNA was performed. The cDNA fragments were ligated into compatibly cut vectors of the pPICZα series and the resulting plasmids used to transform the Pichia GS115 strain. The Drosophila cDNA fragment for FucT6 encoded the full putative stem and catalytic regions (residues 36–619), whereas in the case of the Caenorhabditis fragment a less conservative truncation was performed (residues 57–559). A shorter form of the Drosophila FucT6 was also engineered (residues 71–619) but was seemingly inactive.

To test the order of core fucosylation with recombinant enzymes, we expressed the previously described Drosophila core α1,3-fucosyltransferase FucTA (Fabini et al., 2001) also in Pichia. The activity of the recombinant insect (FucTA and FucT6) and nematode (FUT-8) enzymes was examined by the measurement of the transfer of radioactive fucose to free nonderivatized oligosaccharides. Preliminary data indicated that the presence of Mn(II) ions enhanced activity as compared to assays in the presence of ethylenediamine tetra-acetic acid (EDTA) but also that EDTA does not completely inhibit any of these enzymes at the concentration used. The Drosophila enzymes, as well as Caenorhabditis FUT-8, were active at room temperature (23°C) and less active at 37°C.

The three enzymes were then incubated with either GnGn, GnGnFβ, and GnGnFδ oligosaccharides in the presence of MnCl₂ at a final concentration of 10 mM (Table I). With respect to the different substrates, Drosophila FucTA was active toward both GnGn and GnGnFδ, whereas Drosophila FucT6 was only significantly active with GnGn. In contrast Caenorhabditis FUT-8 could transfer fucose to either GnGn or GnGnFβ oligosaccharides; the latter (discussed later) being an unexpected result in comparison to other data in the present and in previous studies on α1,6-fucosyltransferases from Caenorhabditis and other sources. There was no difference in the levels of eluted radioactivity when using supernatants of Pichia transformed with vector containing no insert in the absence and presence of an acceptor substrate.

**Further characterization of Caenorhabditis and Drosophila α1,6-fucosyltransferases**

Various dabsyl peptides (dabsyl-MM, dabsyl-GnGnFβ, dabsyl-GalGal, and dabsyl-βGnβGN) were then tested in addition to dabsyl-GnGn as substrates for the Caenorhabditis α1,6-fucosyltransferase. Transfer was only observed to dabsyl-GnGn, as judged by an increase in the m/z by 146 (Figure 2) and not to any other dabsyl-glycopeptide tested. Identical observations were obtained with Drosophila FucT6, whereas supernatants of Pichia transformed with vector lacking insert displayed no fucosyltransferase activity (data not shown). Thus the two recombinant enzymes have an absolute requirement for nonsubstituted nonreducing terminal GlcNAc residues; removal of these residues (as in MM) or their substitution by β1,4-linked Gal or GalNAc (as in GalGal or βGnβGN) prevents transfer. Furthermore, prior incubation of the glycopeptide with Arabidopsis core α1,3-fucosyltransferase FucTA (resulting in formation of GnGnFβ) prevents any further addition of fucose by either Caenorhabditis or Drosophila core α1,6-fucosyltransferases.

The result of the assay with the glycopeptide and the Caenorhabditis enzyme is in apparent contradiction to the data with the free oligosaccharide assay (see Table I), in that free GnGnFβ was a substrate and dabsyl-GnGnFβ was not; however, a second glycopeptide (dabsyl-GnGnFβ), which has a different amino acid sequence) was also not a substrate for either recombinant Caenorhabditis FUT-8 or Drosophila FucT6 (reverse-phase high-performance liquid chromatography [RP-HPLC] data not shown). As will be discussed later, the assays with the glycopeptides probably better reflect the natural attachment of the glycan substrate to the protein and concur with previous data on core α1,6-fucosyltransferases. As another means of characterization, the recombinant Drosophila core α1,6-fucosyltransferase was assayed with dansyl-GnGn and the products were analyzed by RP-HPLC. Shifts to lower or higher retention time on incubation of dansyl-IgG glycopeptides with fucosyltransferases are diagnostic of respectively core α1,3- and core α1,6-fucosylation (Roitinger et al., 1998). As shown in Figure 2, a shift to a higher retention time

### Table I. Radioactive-based assays using underivatized oligosaccharides

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>GnGn-oligosaccharide</th>
<th>GnGnFβ-oligosaccharide</th>
<th>GnGnFδ-oligosaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dm α1, 3-FucT</td>
<td>3098 ± 122</td>
<td>812 ± 49</td>
<td>ND</td>
</tr>
<tr>
<td>Dm α1, 6-FucT</td>
<td>355 ± 31</td>
<td>ND</td>
<td>59 ± 29</td>
</tr>
<tr>
<td>Ce α1, 6-FucT</td>
<td>8146 ± 144</td>
<td>ND</td>
<td>3861 ± 238</td>
</tr>
<tr>
<td>ND</td>
<td>244.4</td>
<td>28.7</td>
<td></td>
</tr>
</tbody>
</table>

Supernatants of Pichia expressing recombinant Caenorhabditis (Ce) and Drosophila (Dm) core α1,6-fucosyltransferases, as well as recombinant Drosophila core α1,3-fucosyltransferase FucTA, were assayed at 23°C in the presence of 10 mM Mn(II) in triplicate for 5 h using GnGn (4 nmol) or preincubated GnGnFβ (2 nmol) and GnGnFδ (4 nmol) oligosaccharide variants. The amounts of supernatant used (corrected for concentration) were 6 μl for the Caenorhabditis and 30 μl for the Drosophila enzymes (see Materials and methods). The listed average counts per minute (± SD) and average pmol transferred per μl unconcentrated supernatant are corrected by subtraction of the values in the absence of acceptor, which were 650 ± 17, 752 ± 18, and 556 ± 65 for Pichia transformed with, respectively, Drosophila α1,3-fucosyltransferase, Drosophila α1,6-fucosyltransferase, and Caenorhabditis α1,6-fucosyltransferase. ND, no detectable activity.
was observed when the Drosophila enzyme was incubated with dansyl-GnGn in the presence of GDP-Fuc (chromatogram C) as compared to the incubation in the absence of the nucleotide sugar (chromatogram D). Identical changes in retention time in the presence of GDP-Fuc were observed when assaying fucosyltransferase activity in a crude chicken liver extract, which contains a core α1,6-fucosyltransferase activity (Struppe and Staudacher, 2000), as well as with the recombinant Caenorhabditis FUT-8 enzyme (data not shown).

Using either of the glycopeptide-based assays, the cation and pH dependence of Caenorhabditis core α1,6-fucosyltransferase FUT-8 was studied (this enzyme was examined due to its superior activity as compared to Drosophila FucT6). In five independent sets of experiments, a broad pH optimum between 6 and 7.5 was determined (Figure 3A), whereas of the different cations tested, Mn(II), Mg(II), and Ca(II) resulted in the highest activity (Figure 3B). These properties are similar to those of core α1,6-fucosyltransferases from a number of mammalian and avian sources (Struppe and Staudacher, 2000), although in the case of the recombinant porcine enzyme lower activity was reported in the presence of Mn(II) ions (Uozumi et al., 1996). The enzyme was also active in the presence of EDTA and in the absence of added cations; comparably, EDTA had only a negligible effect on the activity of the porcine enzyme (Uozumi et al., 1996). The result with Zn(II) chloride should be taken with caution, because Zn(II) can act as a Lewis acid.
Various dabsyl-glycopeptides—dabsyl-MM, dabsyl-GnGn, dabsyl-GnGnF, dabsyl-GnGnF, dabsyl-GalGal, and dabsyl-betaGNbetaGN—were also tested as substrates for fucosyltransferase activities in Caenorhabditis and Schistosoma extracts. In the case of Caenorhabditis, only dabsyl-MM and dabsyl-GnGn were substrates (Figure 4A and B); no transfer to dabsyl-GnGnF, dabsyl-GnGnF, dabsyl-GalGal, and dabsyl-betaGNbetaGN was observed (Figure 4C–F). In contrast, Schistosoma extracts mediated transfer to dabsyl-GnGn, dabsyl-GnGnF, dabsyl-GalGal, and dabsyl-betaGNbetaGN (Figure 4H, I, K, and L) but not to dabsyl-MM or dabsyl-GnGnF (Figure 4G and J). Control incubations lacking GDP-fucose were also performed with both extracts and showed similar patterns of partial degradation (either removal of hexosamine or methyl moieties; data not shown). In particular, Caenorhabditis extract showed a strong beta-hexosaminidase activity removing a single GlcNAc residue as judged by a decrease in m/z of GnGn by 203 in addition to demethylation (probably of the dabsyl moiety), resulting in a decrease in m/z of 14. Schistosoma extracts were also tested with forms of GalGal subject either to prior core or Lewis-type alpha3-fucosylation to generate, respectively, GalGalF and GalFGalF (see Table II). Whereas GalGalF appeared to be a substrate for presumably a Lewis-type fucosyltransferase, the GalFGalF glycopeptide carrying two Lewis groups was not fucosylated further, suggesting that core alpha3-fucosylation is blocked by prior Lewis-type fucosylation of the antennae.

To demonstrate the location of the added fucose residues, glycosidase digestions of the dabsyl-glycopeptides were performed as follows: (1) jack bean alpha-mannosidase with Caenorhabditis generated MMF to check that the fucosylation was indeed on the core and not on the peripheral mannose residues; (2) jack bean beta-hexosaminidase with Caenorhabditis- and Schistosoma-fucosylated forms of GnGn to also indicate core fucosylation; (3) PNGase F with various Caenorhabditis and Schistosoma fucosylation products to distinguish core alpha1,3- and core alpha1,6-fucosylation; (4) Aspergillus beta-galactosidase and jack bean beta-hexosaminidase with Schistosoma-generated GalGalF (F) followed by PNGase F to distinguish antennal and core fucosylation; (5) and jack bean beta-hexosaminidase with Schistosoma-generated betaGNbetaGN(F) followed by PNGase F also to distinguish antennal and core fucosylation. The principle behind the PNGase F digestes is that core alpha3-fucosylated glycans are resistant to this enzyme, whereas core alpha1,6-fucosylated ones are PNGase F-sensitive (Tretter et al., 1991). In the case of the galactosidase and hexosaminidase digestes, an absence of digestion down to the core mannose residues would be indicative of antennal (or Lewis-type) fucosylation. The results of the glycosidase digestions are summarized in Table II.

For the products of the two native Caenorhabditis fucosyltransferases, the data of the glycosidase digestions would indicate that the MMF structure was core alpha1,3-fucosylated because two mannose residues could be removed from it and that it was resistant to PNGase F digestion. The

Fig. 4. MALDI-TOF MS analysis of products of native Caenorhabditis and Schistosoma fucosyltransferases. Caenorhabditis (A–F) and Schistosoma (G–L) extracts were incubated at room temperature with either dabsyl-MM (A, 48 h), dabsyl-GnGn (B, H, 72 h), dabsyl-GnGnF (C, I, 72 h), dabsyl-GnGnF (D, J, 72 h), dabsyl-GalGal (E, K, 48 h), or dabsyl-betaGNbetaGN (F, L, 48 h). Dabsyl-glycopeptides are subject to variable laser-induced degradation (m/z: 132 products indicated by asterisks) as well as, in the case of Caenorhabditis extracts, to demethylation (m/z: 14 products indicated by an M). Major substrate and product peaks not resulting from laser degradation or methylation are indicated with the relevant m/z value: MU due to endogenous mannosidase, 1494; MM, 1656; MMF, 1802; GnM due to endogenous hexosaminidase, 1859; GnMF due to endogenous hexosaminidase and fucosyltransferase, 2005; GnGn, 2062; GnGnF, 2208; GnGnFF, 2354; GalGal, 2386; GalGalF, 2352; GalGalFF, 2678; GnbetaGn due to action of endogenous hexosaminidase on betaGNbetaGN, 2265; betaGNbetaGn, 2468; betaGNbetaGn, 2614; betaGNbetaGnFF, 2700.
GnGnF generated by Caenorhabditis extract, however, was sensitive to both hexosaminidase (MMF as product) and PNGase F treatment (peptide lacking glycan as product). The latter result was also found on digestion of the GnGnF product of the recombinant Caenorhabditis FUT-8 (data not shown) and is consistent with the known sensitivity of core α1,6-fucosylated glycans to PNGase F. The absence of obvious Lewis fucosylation by Caenorhabditis extracts of N-glycan substrates such as GalGal contrasts with previous data indicating that tetrasaccharides containing LacNAc or LacdiNAc motifs, albeit at far higher concentration (5 mM as compared to 80 μM), are acceptors for fucosyltransferase activities in nativeworm extracts (deBose-Boyd et al., 1998).

With the Schistosoma-generated fucosylation products, hexosaminidase removed both GlcNAc residues from Schistosoma-generated GnGnF and GnGnFF, whereas PNGase F treatment of the Schistosoma-generated GnGnF(F), GalGalF(F), and βGnβGnF(F) with or without prior galactosidase or hexosaminidase digestion indicated that the majority of the fucosylated material was resistant, consistent with core α1,3-fucosylation. The presence of GalMFF and GnMFF after digestion of GalGalFF and βGnβGnF(F) is indicative of Lewis-type fucosylation on one arm only and is suggestive that these difucosylated species carry only one fucose on the core. Overall, the data suggest that the Schistosoma core α1,3-fucosyltransferase is capable of using GnGn, GalGal, and βGnβGn glycopeptides but not GalGFaL as substrates.

The assays for core fucosyltransferase activities of Caenorhabditis and Schistosoma extracts were also performed with dansyl-GnGn; the resulting RP-HPLC profiles (Figure 5) show that with Caenorhabditis extracts a product with an elution time comparable to that of recombinant core α1,6-fucosyltransferases was observed, in addition to putative hexosaminidase digestion products of the type also observed with the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) assay. On the other hand, three product peaks (corresponding to the elution times for GnGnF(F), GnGnF(F), and GnGnF(F), respectively) result from the action of Schistosoma core fucosyltransferases. The Schistosoma extract also contained hexosaminidase activity, but this is not as pronounced as in the Caenorhabditis extract.

**Discussion**

**Core α1,6-fucosylation: enzymes and glycans**

Core α1,6-fucosyltransferase activities were first described in mammals by Schachter and colleagues (Longmore and Schachter, 1982; Wilson et al., 1976). Later these enzymes were purified and their cDNAs cloned from human and porcine sources (Uozumi et al., 1996; Yanagidani et al., 1997). As in vertebrates, the genomes of both Caenorhabditis and Drosophila each encode only one homolog of mammalian core α1,6-fucosyltransferases. Using Picha to express recombinant forms of these enzymes, we demonstrate that both these homologs are enzymatically active and use biantennary N-glycans as substrates. To this extent the results are not surprising, but a comparison to other fucosyltransferases (of which there are normally multiple forms with different activities in both vertebrates and invertebrates) shows the importance of verifying the actual biochemical function of these enzymes. In the case of α1,3-fucosyltransferases, they can either transfer to the core region of N-glycans (E.C. 2.4.1.214) or to the antennae (Lewis-type α1,3- or α1,4-fucosyltransferases). For α1,2-fucosyltransferases, transfer to galactose residues of glycan antennae is probably the common feature, but in the

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**Table II. Results of glycosidase digestion of Caenorhabditis and Schistosoma fucosyltransferase products**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Substrate (m/z)</th>
<th>First products (m/z)</th>
<th>Treatment</th>
<th>Final products (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caenorhabditis</td>
<td>MM (1656)</td>
<td>MMF (1802)</td>
<td>α-Man</td>
<td>PNGase F MMF (1802)</td>
</tr>
<tr>
<td></td>
<td>GnGn (2062)</td>
<td>GnPnF (2208)</td>
<td>β-Hex</td>
<td>PNGase F GnPnF (2208)</td>
</tr>
<tr>
<td></td>
<td>GnM (1859)*</td>
<td>GnPnM (2208)</td>
<td>MM</td>
<td>MMF (1802)</td>
</tr>
<tr>
<td></td>
<td>GnPnF (2005)*</td>
<td>GnPnF (2208)</td>
<td>PNGase F</td>
<td>Peptide (764)</td>
</tr>
<tr>
<td>Schistosoma</td>
<td>MM (1656)</td>
<td>No transfer</td>
<td>Not</td>
<td>Not applicable</td>
</tr>
<tr>
<td></td>
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<td>β-Hex</td>
<td>MFF (1802)</td>
</tr>
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<tr>
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<td>(see Figure 5G)</td>
<td>(see Figure 5H)</td>
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<td>MFF (1802)</td>
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<td>then</td>
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<td>(see Figure 5L)</td>
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<tr>
<td>GalGFαL (2678)</td>
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<td>transfer</td>
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<td>Peptide</td>
<td>(Data not shown)</td>
</tr>
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<td>GalGFαL (2532)</td>
<td>GalGalFF (2678)</td>
<td>PNGase F</td>
<td>GalGalFF</td>
<td>(Data not shown)</td>
</tr>
</tbody>
</table>

The MALDI-TOF MS results of the same fucosyltransferase incubations as shown in Figure 4 are summarized here (see first product), except for the substrates that showed no activity with Caenorhabditis extracts. The incubations were then treated with α-mannosidase, β-N-acetylhexosaminidase, β-galactosidase, or PNGase F either singly or successively (see treatment) as described in Materials and methods and again analysed by MALDI-TOF MS to yield the listed final products. Species marked with an asterisk are considered to originate from endogenous hexosaminidase activities.
Invertebrate core fucosyltransferases

The biosynthetic origin of difucosylated glycans in insects

In contrast to the core α1,6-fucosyltransferases, natural and recombinant insect and plant core α1,3-fucosyltransferases are more flexible in their substrate specificities and can utilise GnGn, GnGnF6 and, to a variable extent, galactosylated substrates (Fabini et al., 2001; Leiter et al., 1999; Staudacher et al., 1991; Staudacher and März, 1998). This led us to conclude that, in order to synthesise GnGnF6 (a difucosylated glycan which is the precursor to those found in bee venom or fly extracts), in insect cells there is a strict order of fucosylation events: i.e., α1,6-fucosylation must take place before α1,3-fucosylation (see also Figure 6). The specificities of the recombinant Drosophila FucTA (which utilises GnGn, GalGal, GnGnF6 and GalGalF6) and FucT6 (which utilises only GnGn, but not GnGnF6, GalGal or βGnβGN) demonstrate that this rule is also valid for recombinant insect enzymes. Furthermore, the Drosophila FucT6, as previously determined for the Drosophila FucTA (Fabini et al., 2001), does not transfer fucose to dabsyl-MM. Thus the prior action of N-acetylgalcosaminyltransferase I is necessary, even though the GlcNAc residue transferred by this enzyme is absent from most of the core fucosylated structures in the fly; therefore, the specificity of FucT6 is another piece of evidence to support the notion of a transitory GlcNAc residue removed by a β-N-acetylgalcosaminidase in the insect Golgi (Altmann et al., 1995). We assume, but have not proven, that MGn would also be a substrate for both core α1,3- and core α1,6-fucosyltransferases; since the Golgi β-N-acetylgalcosaminidase only
The biosynthetic origin of difucosylated glycans in Caenorhabditis

In the case of Caenorhabditis FUT-8, a requirement for unsubstituted nonreducing GlcNAc was also observed because neither MM, GalGal, nor βGNβGN were substrates for this enzyme. This requirement appeared to apply to both the native and recombinant forms. Interestingly, the free GnGnF3 oligosaccharide, not tested with the native enzyme, clearly appeared to be a substrate for recombinant FUT-8 (see Table I); however, in assays with both the native and recombinant Caenorhabditis enzymes with two different GnGnF3 glycopeptide substrates (both fibrin-derived dabsylated and IgG-derived dabsylated glycopeptides) the strict order (α1,6 before α1,3) was maintained. We believe that the glycopeptide results are more physiologically relevant because the linkage to protein is mimicked, but the results of the oligosaccharide-based assay with Caenorhabditis FUT-8 do indicate that this enzyme has a degree of flexibility in substrate specificity not previously seen with other α1,6-fucosyltransferases. This may be due to differences in the structure of this enzyme, which is shorter than the other α1,6-fucosyltransferases characterized to date and is still able to efficiently utilize a form of GnGnF3 not constrained by the presence of a peptide.

On the other hand, no difucosylation of dabsyl- or dansyl-GnGn was observed in vitro with Caenorhabditis extract; seemingly uniquely, though, a core fucosyltransferase activity toward MM was observed in the extract. From the data in the present study, in conjunction with other recently published data (Paschinger et al., 2004), we assume that the fucosyltransferase in worm extracts that fucosylates MM and MMF6 is not the native FUT-8 but is the core α1,3-fucosyltransferase FUT-1. Compatible with detection of this activity are recent data indicating that fucosylated N-glycans of the form Fuc1–2Hex4–9HexNAc2 exist in worms with mutations in all three N-acetylglucosaminyltransferase genes (Zhu et al., 2004), thus verifying that GlcNAc-TI-independent fucosylation pathways exist in this organism.

Because in Caenorhabditis, as in Drosophila, there are few N-glycans carrying both fucose and terminal N-acetylglucosamine residues and the core α1,3-fucosyltransferase does not use N-glycans with a GlcNAc on the α1,3-antenna (Paschinger et al., 2004), we also assume that a Golgi β-N-acetylglucosaminidase has a role after α1,6-fucosylation in the biosynthesis of N-glycans in the worm. Indeed, in our fucosyltransferase assays, a rather strong hexosaminidase activity complicated the interpretation of spectra and chromatograms alike (Figures 4 and 5). With crude extracts (Figure 5) and in microsomes (data not shown), HPLC retention time data indicated that this hexosaminidase activity has a specificity akin to that of the aforementioned insect one (i.e., converting GnGn to GnM); however, Zhang et al. (2003) found a microsomal activity acting only on M4Gn and MGn. The reason for this discrepancy is unclear, but based on the presence of N-glycans such as MMF6 and MMF3F6 in Caenorhabditis, we would, in the absence of evidence to the contrary, suppose that MGn is most probably the major core α1,6-fucosyltransferase substrate in vivo. The accumulated data, therefore, lead us to hypothesize that the in vivo route to MMF3F6 is via MGn, MGnF6, and MMnF6 (as shown in Figure 6). Thus the action of the putative Golgi β-N-acetylglucosaminidase is an intermediate (rather than final) step in the biosynthesis of difucosylated paucimannosidic glycans in wild-type Caenorhabditis and so distinguishes the nematode from other invertebrates studied to date.

N-glycan fucosylation pathways in schistosomes

In this study, we also confirmed and extended our previous data on core fucosylation in Schistosoma mansoni egg extracts (Faveeuw et al., 2003). We conclude that in schistosomes there is also a strict order of core fucosylation, as...
in insects, because GnGnF was a substrate for a second core fucosyltransferase, but GnGnF was not (thus we combine insects and schistosomes into the same scheme in Figure 6). Unlike in Caenorhabditis, there was no transfer to MM, but there was activity toward GalGal and βGNβGN. Based on the subsequent glycosidase digestions, we assume that Schistosoma core α,3-fucosyltransferase will transfer to both these substrates and so can accept substitutions of the nonreducing terminal GlcNAc of N-glycans (analogous to the ability of fly FucTA to utilize GalGal); on the other hand, core α,6-fucosylation presumably must occur in vivo before addition of core α,3-fucose, galactose, or GalNAc residues, whereas core α,1,3-fucosylation must occur before Lewis-type fucosylation. It is still uncertain, though, whether the detected core α,1,3-fucosyltransferase activity is encoded by the previously cloned S. mansoni α,3-fucosyltransferase homolog (Trottein et al., 2000).

The second fucose transferred by Schistosoma extracts during the observed difucosylation of GalGal and βGNβGN (Figure 4) is presumably due to Lewis-type fucosyltransferases that generate the fucosylated LacNac and LacdiNac moieties observed on schistosomal N-glycans (Khoo et al., 2001; Srivatsan et al., 1992a,b) and may correspond to those activities previously detected in schistosome extracts with small oligosaccharide substrates (DeBose-Boyd et al., 1996; Marques et al., 2001). Transfer of a third fucose residue, compatible with the generation of Lewis groups on both antennae, was not observed.

Conclusion
In this study, we have shown that as with all other core α,1,6-fucosyltransferases examined to date, the enzymes from Caenorhabditis, Drosophila, and Schistosoma displayed a requirement for the prior action of N-acetylgalactosaminyltransferase I but are unable to fucosylate galactosylated glycans. Furthermore, although the Caenorhabditis α,1,6-fucosyltransferase can transfer fucose to a core α,1,3-fucosylated free oligosaccharide, core α,1,6-fucosyltransferases from three invertebrates, whether native or recombinant, share an inability to use core α,1,3-fucosylated N-glycopeptides as substrates. On the other hand, core α,1,3-fucosyltransferases from insects and schistosomes are more flexible in their substrate specificity because they can use as substrates N-glycans with core α,1,6-fucose or certain nonreducing terminal substitutions, whereas the Caenorhabditis core α,1,3-fucosyltransferase Fut-1 (Paschinger et al., 2004) presents novel properties but still can also use core α,1,6-fucosylated glycan substrates, albeit after the action of a putative Golgi β-hexosaminidase. Therefore, our overall conclusion is that the α,1,6 before α,1,3 rule for core fucosylation applies more widely than previously determined.

Materials and methods
Cloning of Caenorhabditis and Drosophila α,1,6-fucosyltransferase homologs
Homologs were identified by BLAST searching of the genomic databases with gene models being also partly based on relevant expressed sequence tag sequences. For the Caenorhabditis homolog (Wormbase entry C10F3.6; fut-8 gene), cDNA fragments were isolated by RT-PCR of RNA purified from the N2 strain; nematodes were grown in liquid culture using Escherichia coli OP50 as a food source and extracted using Trizol (Invitrogen, Carlsbad, CA). Reverse transcription was performed using ImProm II reverse transcriptase (Promega, Madison, WI) and oligo-d(T)18. For cloning the full open reading frame, the primers CeFUT8/1 (GAAATGTTAAAAATGTATTGCC) and CeFUT8/2 (CCTAATCTAAAAAGAGCTTCG) were used with Expand polymerase (Roche, Mannheim, Germany); the PCR product was purified using the GFX DNA purification kit (Amer sham, Little Chalfont, U.K.) and ligated into the pGEM-T vector (Promega). The sequence of a positive plasmid (pCeFUT8) was verified by sequencing using the BigDye terminator kit (Applied Biosystems, Foster City, CA). For a fragment encoding a soluble form of the enzyme, 1:500 diluted plasmid pCeFUT8 was used as template, CeFUT8/7 (TATTCAAAATTATCAAATAATC) and CeFUT8/2/Kpn1 (CGGGTACCTATCTAAAAAGAGCTTCG) as primers and Pfu polymerase (Promega); the annealing temperature was 54°C. The fragment was purified using the GFX kit and cut with Kpn1, prior to ethanol/acetate precipitation and subsequent ligation into pPICZα B cut with PmlI (which generates a blunt end) and Kpn1. Selected clones were sequenced, and pPIC/CeFUT8/15 was identified as being in-frame and encoded amino acid residues 57–559 of the Caenorhabditis Fut-8 protein. For the Drosophila homolog (CG2448; FucT6), the full-length reading frame and a fragment encoding a soluble form were isolated by RT-PCR of RNA isolated from the adult Canton S flies using Trizol before reverse transcription with MMLV reverse transcriptase and oligo-d(T)18. The following primers were employed: DmFUT8/7 (GAGCAGCATGGGAACG; for the full reading frame), DmFUT8/1/PstI (CGCGCTGCAAGCTGACACCAATCTCAAGG for the soluble form), and DmFUT8/2/Kpn1 (CGCG GTACCTAATGGCAGCATAGAGAG; for all forms); an annealing temperature of 55–56°C was used. The full-length reading frame was purified directly from the PCR mixture prior to sequencing, whereas the encoding sequence of the gel-primed (Qiagen, Valencia, CA) prior to digestion with PstI and Kpn1 and ligation into pPICZα B cut with the same enzymes. The Pichia expression plasmid used in this study encoded amino acid residues 36–619 of the Drosophila FucT6 protein.

Expression of Caenorhabditis and Drosophila α,1,6-fucosyltransferase homologs
Soluble forms of both the Caenorhabditis and Drosophila α,1,6-fucosyltransferases were expressed in P. pastoris under control of the methanol-inducible AOX1 promoter as previously described for other enzymes (Bencuřová et al., 2003). After preculturing in the presence of zeocin at 30°C, induction was performed at 16°C (no activity was detected when the induction was performed at 30°C). Culture supernatants were collected after 3 or 4 days and concentrated using UltraFree centrifugal concentration devices or, for larger volumes, an Amicon concentrator (molecular weight cut-off 10,000). The previously described core
\( \alpha_1,3\)-fucosyltransferases from \textit{Arabidopsis} and \textit{Drosophila} were also expressed in \textit{P. pastoris}, but induction was performed at 30°C (Fabini et al., 2001; Wilson et al., 2001).

**Preparation of Caenorhabditis and Schistosoma extracts**

\textit{C. elegans} N2 wild-type nematodes were grown for 4 days in liquid culture with \textit{E. coli} OP50 and separated from bacteria and debris by 30% (w/v) sucrose gradient centrifugation. A total of 1 g of nematodes (wet weight) were suspended in 3 ml 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 7, containing protease inhibitor cocktail (EDTA-free, Roche) and 1% (w/v) Triton X-100 and disrupted using a hand-held glass homogenizer. After 30 min on ice, the debris was removed by centrifugation at 10,000 \( \times g \) for 10 min at 4°C. The supernatant was aliquoted and stored at -20°C before use. \textit{Schistosoma} egg extracts were prepared as previously described (Faveeuw et al., 2003).

**Assay of core fucosyltransferases**

Radioactive-based assays were performed using the free oligosaccharide form of GnGn (purified after PNGase A digestion of glycopeptides from bovine fibrin) or derivatives thereof. GnGnF\(^3\) and GnGnF\(^6\) were generated by the action of \textit{Drosophila} core \( \alpha_1,3\)- and \( \alpha_1,6\)-fucosyltransferases, respectively. The acceptor substrates, either 4 nmol GnGn or GnGnF\(^3\) and 2 nmol GnGnF\(^6\), were dried in reaction tubes prior to addition of 5 \( \mu l \) 0.4 M MES, pH 6.5, 1 \( \mu l \) 0.2 M MnCl\(_2\) or EDTA, 6 \( \mu l \) water, 5 \( \mu l \) GDP-[\(^{14}\)C]Fuc (total, 5 nmol or 25,000 cpm), and 3 \( \mu l \) 10-fold concentrated recombinant \textit{Pichia} culture supernatant (twofold in the case of \textit{Pichia} transformed with \textit{Caenorhabditis fut-8} due to the higher activity). Reactions were stopped and radioactivity eluted from AG1\( \times 8 \) as previously described (Staudacher et al., 1991).

For assays using MALDI-TOF MS or RP-HPLC, dbsyl- and dansyl-glycopeptides were used as previously described (Fabini et al., 2001; Roitinger et al., 1998). In the case of the dbsyl-glycopeptide from fibrin, the primary glycopeptide products after desialylation was dbsyl-GalGal, whereas after desialylation and degalactosylation the primary glycopeptide was dabsyl-GalGal, whereas dabsyl-glycopeptide from fibrin, the primary glycopeptide (Fabini et al., 2001; Roitinger et al., 1998). In the case of determining the pH dependency, the assays were performed similarly but with only 0.25 \( \mu l \) unconcentrated supernatant, bovine serum albumin as stabilizer, and solutions of MES (pH 5–7.5) or 2-amino-2-methyl-1,3-propanediol (AMPD; pH 6.5–8) as buffers.

**Exoglycosidase digestions**

Aliquots of fucosyltransferase assay mixtures with dabsyl-glycopeptides (1.5 \( \mu l \)) were mixed with 1.5 \( \mu l \) 0.4 M MES, pH 5.5, and 0.5 \( \mu l \) (1 mU) of either \textit{Aspergillus} \( \beta \)-galactosidase, jack bean \( \alpha \)-acetylated-neurosaminidase or jack bean \( \alpha \)-mannosidase and incubated overnight at 37°C. In the case of \( \alpha \)-mannosidase, the incubation was supplemented to contain 10 mM ZnCl\(_2\). In addition, either to undigested fucosylation products or after combined \( \beta \)-galactosidase and \( \beta \)-N-acetylated-neurosaminidase digestion, PNGase F (0.2 U) and 1 \( \mu l \) 0.4 M ammonium carbonate, pH 8, were added with subsequent incubation for overnight at 37°C. Glycosidase digestions were analysed by MALDI-TOF MS.

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**Abbreviations**

AMPD, 2-amino-2-methyl-1,3-propanediol; EDTA, ethylenediamine tetra-acetic acid; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser
desorption ionization time-of-flight; MES, 2-(N-morpholino)ethanesulfonic acid; MS, mass spectrometry; PCR, polymerase chain reaction; RP, reverse-phase; RT, reverse transcriptase. Oligosaccharides are abbreviated according to a system based on that of Schachter (1986).

MM, Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc; GnGnF(F), GnGn carrying one (or two) fucose residues

References


Koschmieder et al.