Synthesis of paucimannose N-glycans by *Caenorhabditis elegans* requires prior actions of UDP-*N*-acetyl-D-glucosamine:α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I, α3,6-mannosidase II and a specific membrane-bound β-*N*-acetylglucosaminidase

Wenli ZHANG*, Pinjiang CAO*, Shihao CHEN*¹, Andrew M. SPENCE†, Shaoxian ZHU†, Erika STAUDACHER‡ and Harry SCHACHTER*§²

*Department of Structural Biology and Biochemistry, The Hospital for Sick Children, 555 University Avenue, Toronto, Ont. Canada, M5G 1X8, †Department of Molecular and Medical Genetics, University of Toronto, 1 King's College Circle, Toronto, Ont., Canada M5S 1A8, ‡Institut für Chemie, Universität für Bodenkultur, Muthgasse 18, A-1190 Vienna, Austria, and §Department of Biochemistry, University of Toronto, 1 King's College Circle, Toronto, Ont., Canada M5S 1A8

We have previously reported three *Caenorhabditis elegans* genes (*gly-12*, *gly-13* and *gly-14*) encoding UDP-*N*-acetyl-D-glucosamine:α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I (GnT I), an enzyme essential for hybrid and complex N-glycan synthesis. GLY-13 was shown to be the major GnT I in worms and to be the only GnT I cloned to date which can act on [Manα1,6(Manα1,3)Manα1,6](Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc-R, but not on Manα1,6(Manα1,3)Manβ1-*O*-R substrates. We now report the kinetic constants, bivalent-metal-ion requirements, and optimal pH, temperature and Mn²⁺ concentration for this unusual enzyme. *C. elegans* glycoproteins are rich in oligomannose (Man₆₋₉GlcNAc₂) and 'paucimannose' Man₃₋₅GlcNAc₂(± Fuc) N-glycans, but contain only small amounts of complex and hybrid N-glycans. We show that the synthesis of paucimannose Man₃GlcNAc₂ requires the prior actions of GnT I, α3,6-mannosidase II and a membrane-bound β-*N*-acetylglucosaminidase similar to an enzyme

previously reported in insects. The β-*N*-acetylglucosaminidase removes terminal *N*-acetyl-D-glucosamine from the GlcNAcβ1,2Manα1,3Manβ- arm of Manα1,6(GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc-R to produce paucimannose Man₃GlcNAc₂ N-glycan. *N*-acetyl-D-glucosamine removal was inhibited by two *N*-acetylglucosaminidase inhibitors. Terminal GlcNAc was not released from [Manα1,6(Manα1,3)Manα1,6](GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc-R nor from the GlcNAcβ1,2Manα1,6Manβ- arm. These findings indicate that GLY-13 plays an important role in the synthesis of N-glycans by *C. elegans* and that therefore the worm should prove to be a suitable model for the study of the role of GnT I in nematode development.

Key words: *N*-acetylglucosaminidase, *N*-acetylglucosaminyltransferase I, *Caenorhabditis elegans*, N-glycan biosynthesis, mannosidase II, paucimannose N-glycans.

INTRODUCTION

Caenorhabditis elegans is a nematode worm which has been used extensively as a model organism to study animal development and behaviour [1–4]. It is now the most completely understood metazoan in terms of anatomy, genetics and development. Although glyobiological studies in *C. elegans* are still in their infancy, several groups have recently used MS to study N-glycans released from *C. elegans* glycopeptides by peptide N-glycanase or hydrazinolysis [5–9]. Many of the N-glycans found in *C. elegans* differ either qualitatively or quantitatively from those present in vertebrates. Worms contain a predominance of oligomannose (Man₆₋₉GlcNAc₂) N-glycans, but only small amounts of the hybrid N-glycans Manα1,6(GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4(± Fuc)-

GlcNAc (MGn and MGnFuc) and complex N-glycans with two to four antennary GlcNAc residues. Sialic acid has to date not been detected in *C. elegans*.

C. elegans contains relatively large amounts of paucimannose Man₃₋₅GlcNAc₂Fuc₀₋₁ N-glycans [5–8], not usually seen in vertebrates, in which the fucose may be linked either α1,6 or α1,3 to the core [8]. Other *C. elegans* structures not found in vertebrates are paucimannose Man₃₋₅GlcNAc₂Fuc₂₋₄ N-glycans (some of the fucose residues are attached to mannose residues) [5,7], O-methylated fucose and mannose residues [5,7], and terminal phosphocholine groups [6,7]. Since core fucosylation requires the prior action of UDP-*N*-acetyl-D-glucosamine:α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I (GnT I, EC 2.4.1.101) in vertebrates [10], plants [11,12] and insects [13], it has been suggested [5,8,14] that the synthesis of the

Abbreviations used: GlcNAc, *N*-acetyl-D-glucosamine; GnGn, (GlcNAcβ1,2Manα1,6)(GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc; GnM, (GlcNAcβ1,2Manα1,6)(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc; GnT I, UDP-*N*-acetyl-D-glucosamine:α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I (EC 2.4.1.101); GnT II, UDP-*N*-acetyl-D-glucosamine:α-6-D-mannoside β1,2-*N*-acetylglucosaminyltransferase II (EC 2.4.1.143); M1Gn2, Manβ1,4GlcNAcβ1,4GlcNAc; M2Gn3, GlcNAcβ1,2Manα1,3Manβ1,4GlcNAcβ1,4GlcNAc; M₃-octyl, Manα1-6(Manα1-3)Manβ-octyl; M4Gn2, (Manα1,6)(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc; M4Gn2Fuc1, (Manα1,6)(Manα1,3)Manβ1,4GlcNAcβ1,4(Fuc)GlcNAc; M4Gn3, (Manα1,6)(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc; M5Gn2, [Manα1,6(Manα1,3)Manα1,6](Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc; M5Gn3, [Manα1,6(Manα1,3)Manα1,6](GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc; MALDI-TOF, matrix-assisted laser-desorption-ionization time-of-flight; MGn, Manα1,6(GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc; MGnFuc, Manα1,6(GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4(Fuc)GlcNAc; MM, Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc; MMFuc, Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4(Fuc)GlcNAc; NGM, nematode growth medium; PA, pyridylamino; S2 cells, Schneider 2 cells.

¹ Present address: The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, U.S.A.

² To whom correspondence should be addressed (e-mail harry@sickkids.on.ca).

core fucosylated paucimannose N-glycan $\text{Man}\alpha 1,6(\text{Man}\alpha 1,3)\text{-Man}\beta 1,4\text{GlcNAc}\beta 1,4(\text{Fuc})\text{GlcNAc}$ (MMFuc) in *C. elegans* requires the prior actions of GnT I and a β -N-acetylglucosaminidase.

We have previously reported the cloning and expression of cDNAs for three *C. elegans* genes (*gly-12*, *gly-13* and *gly-14*) homologous with the gene for mammalian GnT I, an enzyme essential for hybrid and complex N-glycan synthesis [15]. Recombinant GLY-12 and GLY-14, but not GLY-13, were shown to have GnT I activity with the synthetic substrate $\text{Man}\alpha 1,6(\text{Man}\alpha 1,3)\text{Man}\beta$ -octyl (M_3 -octyl) [15]. However, all three *C. elegans* GnT I proteins are active with the physiological substrate $[\text{Man}\alpha 1,6(\text{Man}\alpha 1,3)\text{Man}\alpha 1,6](\text{Man}\alpha 1,3)\text{-Man}\beta 1,4\text{GlcNAc}\beta 1,4\text{GlcNAc-X}$ ($\text{M}_5\text{Gn}_2\text{-X}$), where X is either asparaginyl-ribonuclease B (RNase B), asparaginyl-peptide or pyridylamino (PA), and GLY-13 is, in fact, the major GnT I in normal worms [16,17]. A double-isotope quantitative MS method for the measurement of N-glycans was applied to extracts of worm lines with single-null mutations in the *gly-12*, *gly-13* and *gly-14* genes and a double-null mutation in the *gly-12* and *gly-14* genes (*gly-14;gly-12*) [9,16,17]. The results showed that the absence of GLY-13 leads to significant decreases in $\text{Man}\alpha 1,6(\text{Man}\alpha 1,3)\text{Man}\beta 1,4\text{GlcNAc}\beta 1,4(\pm \text{Fuc})\text{GlcNAc}$ (MM and MMFuc) and increases in $\text{Man}_5\text{-}_7\text{GlcNAc}_2$, thereby proving that GLY-13 action plays an important role in the synthesis of the paucimannose N-glycans [16]. Deletion of *gly-12* and/or *gly-14* had no effect on the N-glycan pattern in the presence of normal amounts of GLY-13.

In the present study we determined the kinetic properties of GLY-13, the only GnT I discovered to date with an absolute specificity for M_5Gn_2 acceptors, and we provide direct evidence that the synthesis of the paucimannose structure MM requires the prior actions of GnT I, $\alpha 3,6$ -mannosidase II and a membrane-bound β -N-acetylglucosaminidase.

EXPERIMENTAL

Molecular-biological procedures

Unless otherwise stated, standard molecular-biological procedures were used [18,19] and *C. elegans* technology was as described by Epstein and Shakes [4]. Oligonucleotides were synthesized on an automated DNA synthesizer and purified by a cartridge method at the Hospital for Sick Children Biotechnology Center, Toronto, Canada. All DNA constructs were sequenced in both directions by the double-strand dideoxy method [20].

C. elegans culture, heat shock, worm lysis and microsome preparation

The standard laboratory wild-type strain N2 (Bristol), or a *smg-1* ($\epsilon 1228$) mutant derived from N2 nematodes, were grown on NGM (nematode growth medium) agar plates (3 g of NaCl, 2.5 g of peptone and 17 g of agar dissolved in 975 ml of water) [21] seeded with *Escherichia coli* strain OP50 (a 'leaky' uracil-requiring strain) at 20 °C [1]. DNA injection into the *C. elegans* germ line and establishment and maintenance of transgenic lines were carried out as previously described [15,22]. To measure the activity of overexpressed GLY-13, heat shock was carried out on the transgenic worm [15,22]. Heat-shocked worms from 10–15 10-cm-diameter agar plates were harvested, bacteria were removed by centrifugation in 30 % (w/w) sucrose, and worms were suspended in 1 ml of buffer A [20 mM Tris/HCl (pH 7.5)/250 mM sucrose/protease inhibitor cocktail (Boehringer

Mannheim)] [15]. Worms were stored at -70 °C and lysed by sonication [15] as required. All subsequent procedures were carried out at 4 °C. The sonicated material was centrifuged at 1500 g in a Beckman JA17 rotor for 10 min, the supernatant was centrifuged at 126 000 g for 1 h (Beckman 100.3 rotor), and the resulting microsomal pellet was resuspended in buffer A containing 0.05 % Triton X-100. The solution was re-centrifuged as above, the supernatant was discarded, the pellet was resuspended in lysis buffer [25 mM Mes (pH 6.1)/1 % Triton X-100/protease inhibitor cocktail], and the resulting microsomal extract was used as soon as possible for enzyme assays.

Enzyme substrates and inhibitors

RNases A and B were obtained from Sigma. RNase B is a good substrate for GnT I [23]. RNase B substrate concentrations were calculated on the basis of a single N-glycosylation site and about 60 % M_5Gn_2 content [24]. M_5Gn_2 -peptide was kindly provided by Dr Lukas Mach and Dr Jan Mucha (Centre for Applied Genetics, University of Agricultural Sciences, Vienna, Austria). $\text{M}_5\text{Gn}_2\text{-PA}$, $[\text{Man}\alpha 1,6(\text{Man}\alpha 1,3)\text{Man}\alpha 1,6](\text{GlcNAc}\beta 1,2\text{Man}\alpha 1,3)\text{Man}\beta 1,4\text{GlcNAc}\beta 1,4\text{GlcNAc-PA}$ ($\text{M}_5\text{Gn}_3\text{-PA}$), MGn-PA , $(\text{GlcNAc}\beta 1,2\text{Man}\alpha 1,6)(\text{GlcNAc}\beta 1,2\text{Man}\alpha 1,3)\text{Man}\beta 1,4\text{GlcNAc}\beta 1,4\text{GlcNAc-PA}$ (GnGn-PA) and $(\text{GlcNAc}\beta 1,2\text{Man}\alpha 1,6)(\text{Man}\alpha 1,3)\text{Man}\beta 1,4\text{GlcNAc}\beta 1,4\text{GlcNAc-PA}$ (GnM-PA) were prepared as previously described [25–29]. M_3 -octyl and the N-acetylglucosaminidase inhibitors 2-acetamido-1,2-dideoxy-2-deoxynojirimycin and O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino N-phenyl carbamate were purchased from Toronto Research Chemicals, Toronto, Canada.

Expression of recombinant GLY-13 in *Drosophila* Schneider 2 (S2) cells

Truncated *gly-13* cDNA was inserted behind an inducible metallothionein promoter and used for transient transfection of *Drosophila* S2 cells [30]. The *gly-13* cDNA segment (1.3 kb) encoding 421 amino acids (the first 28 amino acids at the N-terminus were deleted) was prepared by PCR using pD49.83/B-Myc [15] as a template and primers 5'-CGGCCTCGAGAGAACCAGAAATACGGATA-3' (forward) and 5'-ATGTCGTTTAACTTAGAAGTCAGAGGCACGGGC-3' (reverse). The protein expression vector pMT/Bip/His (kindly provided by Dr David Rose and Dr Doug Kuntz, Ontario Cancer Institute, Toronto, Canada) was modified from Invitrogen vector pMT/Bip/V5-His so that a His₆ sequence is expressed at the N-terminus of secreted recombinant protein rather than at the C-terminus. The PCR product was subcloned into the pMT/Bip/His vector to produce pMT/Bip/His/*gly-13*, and the latter was expressed in *Drosophila* S2 cells following the Invitrogen protocol (Invitrogen *Drosophila* Expression System, DES®). S2 supernatant was used as the source of GLY-13 protein.

Expression of recombinant GLY-13 in transgenic *gly-14;gly-12* double-null worms

The *gly-13* gene was expressed by injecting *gly-13* transgene into *gly-14;gly-12* double-null worms [17,31]. This procedure reduced the background GnT I activity caused by the presence of the *gly-12* and *gly-14* genes in the wild-type worms previously used to create *gly-13* overexpressing worm lines [15]. Roller plasmid pRF4 (50 ng/ μ l) and plasmids p78B-Myc (25 ng/ μ l) and p83B-Myc (25 ng/ μ l) carrying the *gly-13* gene were co-injected into *gly-14;gly-12* double-null worms as described previously for

wild-type worms [15]. The *gly-13*-overexpressing transgenic lines carrying the injected DNA on extra-chromosomal arrays were established from F₂ Rol progeny of the injected *gly-14;gly-12* animals.

GnT I enzyme assays

Recombinant GnT I preparations were either microsomal fractions prepared from lysates of heat-shocked transgenic worms overexpressing GLY-13, or supernatants from cultures of metal-induced *Drosophila* S2 cells transfected with a *gly-13* cDNA construct. GnT I activity was measured using as acceptor substrate either 0.25 mM M₃-octyl, 0.25 mM M5Gn2-PA, 0.25 mM M5Gn2-peptide or 5.4–7.2 mM RNase B, and as donor substrate 0.5 mM UDP-[³H]GlcNAc (New England Nuclear) diluted with non-radioactive UDP-GlcNAc (Sigma) to a specific activity of 7000–10 000 d.p.m./nmol. The assay mixture also contained 75–100 mM Mes buffer, pH 7.0, 5 mM AMP, 0.2 M GlcNAc, 10 mM MnCl₂, 0.5–1.0% Triton X-100 and enzyme, in either 0.020 or 0.040 ml total volume. Incubations were carried out for 15–60 min at 26 °C. Products containing the hydrophobic octyl or PA groups were purified by the SepPak C₁₈ cartridge method [32,33]. Glycopeptide and glycoprotein products were purified by passage through columns of AG 1-X8 resin (100–200 mesh; Cl[−] form) equilibrated with water [23]. Protein concentration was determined by the bicinchoninic acid ('BCA') assay method (Pierce Biotechnology). Kinetic parameters were determined on recombinant GLY-13 expressed in *Drosophila* S2 cells by a series of reciprocal velocity-versus-substrate-concentration plots (0.7, 1.4, and 2.9 mM RNase B and 0.02, 0.04, 0.06, 0.08 and 0.10 mM UDP-GlcNAc) [33,34]. Control assays for endogenous acceptors were carried out with no added acceptor substrate, or with RNase A instead of RNase B; these values were subtracted from the standard incubation values. Endogenous GnT I activity controls were lysates of wild-type worms (both heat-shocked and non-heat-shocked) and of non-heat-shocked transgenic worms overexpressing GLY-13; we also assayed supernatants from non-induced *gly-13*-transfected S2 cells and metal-induced S2 cells transfected with a control cDNA construct not encoding GnT I activity. The endogenous GnT I activity of worms and S2 cells was negligible relative to the activity of recombinant GnT I after heat-shock or metal induction respectively. Product formation was proportional to time of incubation and volume of enzyme under the conditions of the standard enzyme assay. Bivalent-cation requirements (at 5 mM), and optimum Mn²⁺ concentration, pH and temperature were determined by standard methods [35].

Metabolic fate of the product of GLY-13 action

Microsome suspensions from heat-shocked transgenic worms overexpressing GLY-13 were incubated with 0.25 mM M5Gn2-PA and 0.5 mM non-radioactive UDP-GlcNAc at 26 °C for 1.5, 3, 6, 12, 24 and 72 h, as described above for the standard GnT I assay. The incubations were subjected to the SepPak C₁₈ cartridge assay [32,33] and material adhering to the C₁₈ resin was eluted with methanol, dried, and analysed by HPLC and MALDI-TOF (matrix-assisted laser-desorption-ionization time-of-flight) MS. Size fractionation on normal-phase HPLC was modified from the procedure of Khoo et al. [36] using a Palpak type N column (4.6 mM internal diameter × 250 mM long; TaKaRa, Otsu, Shiga Prefecture, Japan) at a flow rate of 1 ml/min. Solvent A was 75 : 25 (v/v) acetonitrile/stock solution [3% (w/v) acetic acid/triethylamine buffer, pH 7.3, with 10% (v/v) acetonitrile].

Solvent B was 50 : 50 (v/v) acetonitrile/stock solution. The run was started with 5 min at 10% solvent B followed by a linear gradient of 2.8%/min to 80% solvent B, and terminated by 8 min at 80% solvent B. Fluorimetric detection was carried out using 320 nm as excitation wavelength and 400 nm as emission wavelength. MALDI-TOF MS was carried out as previously described [37]. The sample (1 µl; 0.2–0.8 pmol) was spotted on to the target and dried, followed by the addition of 0.8 µl of matrix [2% (w/v) 2,5-dihydroxybenzoic acid in water containing 30% (v/v) acetonitrile]. The platen was immediately transferred to a desiccator, and a vacuum was applied until all solvent had evaporated. Spectra were recorded on a DYNAMO linear MALDI-TOF mass spectrometer (Thermo BioAnalysis, Hemel Hempstead, Herts., U.K.) operated with a dynamic extraction setting of 0.1. External mass calibration was performed with pyridylaminated N-glycan standards derived from bovine fibrin. About 20 individual laser shots were summed.

GLY-13 product identification by NMR spectrometry

Recombinant GLY-13 (0.26 ml of S2 supernatant) was incubated with 0.25 mM M5Gn2-peptide and 0.5 mM non-radioactive UDP-GlcNAc in a total volume of 0.32 ml at 26 °C under standard GnT I assay conditions. Fresh GLY-13 (0.16 ml of S2 supernatant) was added at 24, 48 and 72 h and the reaction was stopped at 96 h. A similar incubation with radioactive UDP-GlcNAc showed essentially 100% conversion of substrate into product. The incubation mixture was passed through AG 1-X8 resin (100–200 mesh; Cl[−] form) equilibrated with water to remove UDP-GlcNAc [23], the eluate was purified by adsorption to, and elution from, concanavalin A–Sepharose [38], and the methyl α-D-glucopyranoside used for elution from concanavalin A–Sepharose was removed by chromatography on a Sephadex G-10 column equilibrated with water. The product (≈ 60 nmol) was dried by vacuum evaporation at 50–60 °C, resuspended in 1.5 ml of ²H₂O (99 atom % ²H), exchanged repeatedly in 1.5 ml of ²H₂O (99.9 atom % ²H) and finally dissolved in 0.8 ml of ²H₂O (99.96 atom % ²H). One-dimensional proton-NMR spectra were recorded at 500 MHz at 20, 25 and 50 °C using a Varian Utility Plus 500 MHz narrow-bore Fourier-transform NMR spectrometer (University of Toronto Nuclear Magnetic Resonance Spectroscopy Laboratory, Molecular Medicine Research Centre, Toronto, Canada). Chemical shifts (δ) were expressed in p.p.m. relative to an internal acetone standard (δ 2.225). NMR spectra of M5Gn2-peptide were also obtained.

N-Acetylglucosaminidase assays

Recombinant rabbit GnT I was incubated with M₃-octyl and UDP-[³H]GlcNAc (6800 d.p.m./nmol) to prepare Manα1,6-([³H]GlcNAcβ1,2Manα1,3)Manβ1-O-octyl as previously described [39]. Microsomes from wild-type N2 worms were incubated with ≈ 0.1 mM Manα1,6([³H]GlcNAcβ1,2Manα1,3)-Manβ1-O-octyl, 75 mM Mes buffer, pH 7.0, 25 mM MnCl₂ and 0.5% Triton X-100 in a total volume of 0.020 ml at 37 °C for 0, 3 and 6 h (duplicate assays were run). Control assays were carried out without addition of microsomes. Identical incubations were carried out in duplicate for 6 h in the presence of either 3.3 mM 2-acetamido-1,2-dideoxynojirimycin or 12.5 mM O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino N-phenyl carbamate. The incubations were subjected to the SepPak C₁₈ cartridge assay [32,33] and the radioactivity adhering to C₁₈ (non-degraded substrate) was eluted with methanol and counted for radioactivity. The radioactivity obtained in the presence of microsomes was

subtracted from the control values obtained in the absence of microsomes to determine the amount of radioactive free GlcNAc released during the incubations.

Identical incubations were carried out for 5 h using the same microsome suspension from N2 worms and the following non-radioactive substrates (0.13 mM): GnGn-PA, MGn-PA, GnM-PA and M5Gn3-PA. The incubations were subjected to the SepPak C₁₈ cartridge assay [32,33] and material adhering to the C₁₈ resin was eluted with methanol, dried, and analysed by HPLC using a fluorimetric detector and MALDI-TOF MS, as described above.

RESULTS

Kinetic properties of GLY-13

The endogenous GnT I activity of supernatants from various control *Drosophila* S2 cells not expressing recombinant GLY-13 was negligible relative to the activity of recombinant enzyme. S2 cell supernatant was therefore used as a source of recombinant GLY-13. Kinetic analysis of recombinant GLY-13 with RNase B as acceptor gave linear 1/*v*-versus-1/[S] plots for UDP-GlcNAc and RNase B (Figures 1A and 1B respectively).

Figure 1 Reciprocal velocity-[substrate] plots using recombinant GLY-13 as enzyme, RNase B as acceptor substrate and UDP-GlcNAc as donor substrate

(A) Concentrations of RNase B (mM): □, 0.7; △, 1.4; ●, 2.9. (B) Concentrations of UDP-GlcNAc (mM): □, 0.02; △, 0.04; ○, 0.06; +, 0.08; ●, 0.10. Rate of product formation is expressed as nmol/h per 0.044 ml of S2 supernatant at 26 °C. Duplicate determinations are shown.

These findings are consistent with an ordered sequential Bi Bi mechanism if one assumes steady-state rather than rapid-equilibrium kinetics [34]. The kinetic constants for GLY-13 were 0.15, 0.08, 1.3 and 0.6 mM for K_{acceptor} , K_{donor} , $K_{i(\text{acceptor})}$ and $K_{i(\text{donor})}$ respectively. For comparison, we have carried out similar kinetic studies with recombinant GLY-14 and rabbit GnT I (see [35] for enzyme preparations) using M5Gn2-peptide as acceptor. GLY-14 gave values of 0.32 and 0.03 mM and rabbit GnT I gave values of 0.44 and 0.005 mM for K_{acceptor} and K_{donor} respectively. The data indicate that the three enzymes have similar kinetic properties using M5Gn2 as acceptor, but GLY-13 differs markedly from the other two enzymes in its inability to utilize M₃-octyl [16].

Recombinant GLY-13 showed a pH optimum of 7.0 (Figure 2A) and a temperature optimum of 20–22 °C (Figure 2B). GLY-12 and GLY-14 also have relatively low temperature optima (20–30 °C) [35]. GLY-13 enzyme activity was zero in the absence of Mn²⁺ and increased with Mn²⁺ concentration to 20 mM (Figure 2C). There was no significant difference in GLY-13 enzyme activity between Mn²⁺ concentrations from 20 to 50 mM (Figure 2C). GLY-13 activity was most activated by Mn²⁺, moderately activated by Co²⁺, Mg²⁺, Cd²⁺ and Fe²⁺ (< 34 % of maximum activity), and very poorly activated by Cu²⁺ (3 % of maximum activity), while Ni²⁺, Ba²⁺, Zn²⁺ and Ca²⁺ had no effect (Figure 2D). The rabbit and GLY-14 enzymes showed very similar metal-ion requirements [35], an absolute requirement for Mn²⁺ and little (< 20 % of maximum activity) or no activity with Mg²⁺, Ni²⁺, Ba²⁺, Ca²⁺, Cd²⁺, Fe²⁺ or Cu²⁺.

The product obtained when microsomal suspensions from heat-shocked transgenic worms overexpressing GLY-13 act on M5Gn2-PA was shown to be M5Gn3-PA by HPLC and MS analysis (Figures 3–6; discussed below). The product obtained with recombinant GLY-13 expressed in S2 cells and M5Gn2-peptide as substrate gave proton-NMR spectra similar to the M5Gn3-peptide spectra previously reported [40]. A chemical shift of δ 4.54 p.p.m. at 20 °C (coupling constant = 8.3 Hz), due to the anomeric C-1 hydrogen atom of a GlcNAc residue in β 1, 2 linkage to mannose, was present in the product spectrum, but absent in the M5Gn2-peptide spectrum. Chemical shifts at 50 °C for the anomeric C-1 hydrogen atoms of five mannose residues in the product were seen at δ 5.13, 5.11, 4.92, 4.88 and 4.78 p.p.m. (α 3-linked internal, α 3-linked terminal, α 6-linked terminal, α 6-linked internal and β 4-linked respectively).

Metabolic fate of the product of GLY-13 action

Microsomal suspensions from heat-shocked transgenic worms overexpressing GLY-13 were incubated with 0.25 mM M5Gn2-PA and 0.5 mM non-radioactive UDP-GlcNAc at 26 °C for 1.5, 3, 6, 12, 24 and 72 h, and PA-containing material adhering to the SepPak C₁₈ cartridges was analysed by HPLC on Palpak (Figures 3 and 4) and MALDI-TOF MS (Figures 5 and 6). The following structures were identified: M5Gn3-PA, MGn-PA, (Man α Man α 1,6)(GlcNAc β 1,2Man α 1,3)Man β 1,4GlcNAc β 1,4GlcNAc-PA (M4Gn3-PA), (Man α Man α 1,6)(Man α 1,3)Man β 1,4GlcNAc β 1,4GlcNAc-PA (M4Gn2-PA) and Man α 1,6(Man α 1,3)Man β 1,4GlcNAc β 1,4GlcNAc-PA (MM-PA). Treatment of the products of the 72 h incubation with endoglycosidase H showed that this enzyme did not cleave MM-PA and MGn-PA; the enzyme cleaved M4Gn2-PA, M4Gn3-PA and M5Gn3-PA with resulting accumulation of Gn-PA (Figure 4C and MS results not shown). Jack-bean (*Concanavalia ensiformis*) α -mannosidase (Sigma) cleaved MM-PA and M4Gn2-PA to Man β 1,4GlcNAc β 1,4GlcNAc-PA (M1Gn2-PA), and cleaved M5Gn3-PA and M4Gn3-PA to MGn-PA (Figure 4D and MS data

Figure 2 Dependence of recombinant GLY-13 activity (enzyme volumes as indicated) on (A) pH (0.018 ml), (B) temperature (0.020 ml), (C) Mn²⁺ concentration (0.016 ml) and (D) bivalent cations at 5 mM (0.009 ml)

Rate of product formation is in nmol/h using the designated enzyme volume. Duplicate determinations are shown.

not shown). At 1.5 h of incubation, none of the original M5Gn2-PA was seen, indicating complete conversion into M5Gn3-PA (Figures 3C and 5A). Small amounts of M4Gn3-PA appeared at 3 h (Figures 3D and 5B) and MGn-PA was first clearly seen at 24 h (Figures 3G and 5E). Neither M4Gn2-PA nor MM-PA were seen at 24 h (Figures 3G and 5E). It is concluded that GLY-13 converted M5Gn2-PA into M5Gn3-PA within 1.5 h. This was followed by relatively slow α 3,6-mannosidase II-catalysed conversion of M5Gn3-PA into M4Gn3-PA and MGn-PA.

A membrane-bound β -N-acetylglucosaminidase is responsible for the synthesis of paucimannose N-glycans

Although there was no indication that any paucimannose N-glycans (M4Gn2-PA and MM-PA) were formed after 24 h of incubation of microsomal suspensions from heat-shocked transgenic worms overexpressing GLY-13 with M5Gn2-PA and UDP-GlcNAc (Figure 5E), the largest peak seen on HPLC at 72 h was MM-PA (Figure 4B). The M4Gn2-PA peak could not be separated from MGn-PA by HPLC, but this compound was identified in the 72 h incubation by MALDI-TOF (Figure 6). It is concluded that M4Gn2-PA and MM-PA were formed from M4Gn3-PA and MGn-PA respectively by the action of a β -N-acetylglucosaminidase.

In order to obtain more direct evidence for such a β -N-acetylglucosaminidase, a microsomal suspension prepared from wild-type N2 worms was incubated with Man α 1,6-([³H]GlcNAc β 1,2Man α 1,3)Man β 1-O-octyl. [³H]GlcNAc was released during the incubation, and this release was markedly inhibited by two N-acetylglucosaminidase inhibitors (Figure 7).

The same microsome preparation was incubated for 5 h with GnGn-PA, MGn-PA, GnM-PA and M5Gn3-PA, and PA-containing material was analysed by HPLC on Palpak (Figure 8) and MALDI-TOF MS (Figure 9). The results show that the microsome preparation removes GlcNAc from MGn-PA to form MM-PA (Figures 8C and 9D), but not from GnM-PA (Figures 8E and 9C) and GnGn-PA (Figures 8H and 9A). Since there is no formation of M5Gn2-PA from M5Gn3-PA (Figures 8J and 9B), the microsomes were unable to remove GlcNAc from M5Gn3-PA. The incubation shows instead the action of α 3,6-mannosidase II, which converts M5Gn3-PA into M4Gn3-PA and MGn-PA (see Figures 3–6 for similar results). There is also formation of GlcNAc β 1,2Man α 1,3Man β 1,4GlcNAc β 1,4GlcNAc-PA (M2Gn3-PA) (Figure 8J), presumably by the action of an α -mannosidase on MGn-PA, and of MM-PA (Figure 9B) due to the action of β -N-acetylglucosaminidase on MGn-PA. The small peak of M5Gn2-PA seen in the incubation is due to a contamination of the starting M5Gn3-PA preparation (Figure 8I). The data prove the presence of a membrane-bound

Figure 3 HPLC analysis on a Palpak column of PA-oligosaccharides

Microsome suspensions from heat-shocked transgenic worms overexpressing GLY-13 were incubated with 0.25 mM M5Gn2-PA and 0.5 mM non-radioactive UDP-GlcNAc at 26 °C for (C) 1.5 h, (D) 3 h, (E) 6 h, (F) 12 h and (G) 24 h. (A) Isomaltose standard; (B) a mixture of M5Gn2-PA and M5Gn3-PA standards. M4Gn3-PA and M5Gn2-PA run together on this HPLC system; however, only M4Gn3 was formed during the incubation as determined by MS analysis (Figure 5). None of the starting M5Gn2-PA was detected at 1.5 h (C) indicating complete conversion of M5Gn2-PA into M5Gn3-PA at that time point. See the Abbreviations footnote for the identification of structures.

β -N-acetylglucosaminidase which is specific for removal of GlcNAc from the GlcNAc β 1,2-Man α 1,3-Man β 1,4- arm of MGn-PA, but which cannot act on GnM-PA, GnGn-PA and M5Gn3-PA. A similar enzyme has been described in insects [28]. The failure of the microsome preparation to remove GlcNAc from M5Gn3-PA explains why no M5Gn2-PA was detected at any time point between 1.5 and 72 h of incubation with GLY-13 overexpressing worm microsomes (Figures 5 and 6). Alternatively, this may be due in part to rapid re-conversion of M5Gn2-PA into M5Gn3-PA by the high levels of GLY-13 in these experiments. We detected some M4Gn2-PA at 72 h (Figure 6), suggesting that the β -N-acetylglucosaminidase can act on M4Gn3-PA.

It is concluded that α 3,6-mannosidase II converts M5Gn3-PA into M4Gn3-PA and M4Gn3-PA into MGn-PA; M4Gn3-PA and MGn-PA are then respectively converted into M4Gn2-PA and MM-PA by a membrane-bound β -N-acetylglucosaminidase. There is no evidence of a direct conversion of M5Gn2-PA into M4Gn2-PA and MM-PA by a GnT I-independent α 3,6-mannosidase, at least in the presence of GLY-13.

DISCUSSION

GnT I is essential for the synthesis of hybrid and complex N-glycans [41]. Studies on 'knock-out' mice with a null mutation in the GnT I gene [42,43] and on humans with congenital disorder of glycosylation type IIa [44] have indicated that interference with complex N-glycan synthesis is associated with severe defects in embryonic development, particularly of the nervous system. We have initiated studies on the expression of GnT I in *C. elegans* in the hope that this relatively simple and thoroughly

Figure 4 HPLC analysis on a Palpak column of PA-oligosaccharides

(B) The incubation mixture was identical with that in Figure 3 and the incubation was run for 72 h. (A) Isomaltose standard; (C) products treated with endoglycosidase H; (D) products treated with jack-bean α -mannosidase. Some of the M5Gn3-PA formed at 1.5 h (Figure 3C) is still present after 72 h of incubation, and some has been converted into M4Gn3-PA and MGn-PA by α 3,6-mannosidase II. At 72 h, the incubation also contains M4Gn2-PA and MM-PA, indicating the action of a β -N-acetylglucosaminidase (see the text). Although M4Gn2-PA and MGn-PA run together on this HPLC system, MS analysis Figure 6 and results not shown) was used to distinguish M4Gn2-PA and MGn-PA in the various incubations shown in (B), (C) and (D). Endoglycosidase H (C) degraded M5Gn3-PA, M4Gn3-PA and M4Gn2-PA, but not MGn-PA and MM-PA, to Gn-PA (the cleaved oligosaccharides do not contain a PA moiety and are not detected by fluorimetry). Jack-bean α -mannosidase (D) degraded M4Gn2-PA and MM-PA to M1Gn2-PA, and M5Gn3-PA and M4Gn3-PA to MGn-PA. See the abbreviations footnote for the identification of structures.

characterized organism will provide important information on the role of complex N-glycans in eukaryote development.

The activity of GnT I with M5Gn2 relative to MM acceptor substrates varies between species, e.g., the following M5Gn2/MM ratios, shown in face ({}), have been reported: bovine colostrum {2.0} [23]; human hepatoma cell line HepG2 {1.4} [27]; four insect cell lines [27], *Spodoptera frugiperda* (fall armyworm) Sf9 {14} and Sf21 {14}, *Mamestra brassicae* (cabbage moth) Mb-0503 {12} and *Bombyx mori* (silkworm) Bm-N {8.6}; *Nicotiana tabacum* (tobacco) { $\approx$ 4} [45]; *C. elegans* GLY-12 {1.3}, GLY-13 {> 575} and GLY-14 {2.6} [16]. All species therefore show a preference for M5Gn2 substrates, especially insects and plants. *C. elegans* GLY-12 and GLY-14 have relatively low M5Gn2/MM ratios, similar to those in vertebrates. However, GLY-13 is unique in that it has essentially zero activity with M₃-octyl and acts only on M5Gn2 substrates [16]. GLY-13 has a temperature optimum at 20–22 °C, similar to the temperature optima (20–30 °C) of GLY-12 and GLY-14 [35] and of other *C. elegans* glycosyltransferases [46], while rabbit and *Drosophila* GnT I have a temperature optimum at 37 °C [35]. The *C. elegans* enzyme optimum probably reflect the temperature of the animal's normal habitat.

GnT I enzyme assays on the various GnT I-null worms show clearly that GLY-13 is the major GnT I in *C. elegans*. The *gly-14;gly-12* double-null mutant has the same activity with M5Gn2 acceptor substrate as wild-type worms (4.1–11.1 nmol/h per mg of protein) [16] and the *gly-13* null worm has the same

Figure 5 MALDI-TOF MS spectra of PA-oligosaccharides

The incubations were identical with those in Figure 3. **(A)** 1.5 h, **(B)** 3 h, **(C)** 6 h, **(D)** 12 h and **(E)** 24 h. The five peaks marked with an asterisk in **(B)** are due to the MALDI-TOF procedure and are present in all samples. The MS data for the peaks are shown only in **(E)**. See the abbreviations footnote for the identification of structures.

Figure 6 MALDI-TOF MS spectra of PA-oligosaccharides

The incubation was identical with that in Figure 4 (26 °C for 72 h). See the abbreviations footnote for the identification of structures.

Figure 7 Assay of β -N-acetylglucosaminidase activity

Microsomes (0.015 ml) from wild-type N2 worms were incubated with ≈ 0.1 mM $\text{Man}\alpha 1,6([\text{H}]\text{GlcNAc}\beta 1,2\text{Man}\alpha 1,3)\text{Man}\beta 1$ -O-octyl at 37 °C for 0, 3 and 6 h. The Figure shows nmol of free $[\text{H}]\text{GlcNAc}$ released: $\bullet$, no inhibitors; $\blacksquare$, 3.3 mM 2-acetamido-1,2-dideoxyribose added; $\blacktriangle$, 12.5 mM O-(2-acetamido-2-deoxy-2-glucopyranosylidene)amino N-phenyl carbamate added. Duplicate determinations are shown.

low activity (≈ 0.1 nmol/h per mg) with both M3-octyl and M5Gn2 (W. Zhang, S. Chen, A.M. Spence and H. Schachter, unpublished work). This low activity is due to the presence of GLY-12 and GLY-14 [16]. Deletion of GLY-12 and GLY-14 appears to have no effect on worm phenotype [17,31]. The mass spectra for N-glycans from *gly-12* and *gly-14* single-null mutants and the *gly-14;gly-12* double-null mutant are identical with that of the wild-type animals [16]. The role of GLY-12 and GLY-14 in normal N-glycan biosynthesis and worm metabolism is therefore not clear. The enzymes may be required for glycosylation of a few specific glycoproteins or may have unknown tissue-specific functions (*gly-14* expression is restricted to intestinal tissue [15]). The effects of GLY-12 and GLY-14 action may therefore be masked by GLY-13 when studying total N-glycans from the normal animal.

N-glycan synthesis in vertebrates [41] differs from the pathway in insects [13], plants [47,48] and *C. elegans* in that the latter three species accumulate appreciable amounts of the paucimannose N-glycans MM, MMFuc, M4Gn2 and $(\text{Man}\alpha 1,6)(\text{Man}\alpha 1,3)\text{Man}\beta 1,4\text{GlcNAc}\beta 1,4(\text{Fuc})\text{GlcNAc}$ (M4Gn2Fuc1). Several plant species have been shown to accumulate over 30 % of their N-glycans as the paucimannose structure $\text{Man}\alpha 1,6(\text{Man}\alpha 1,3)(\text{Xyl}\beta 1,2)\text{Man}\beta 1,4\text{GlcNAc}\beta 1,4(\text{Fuc}\alpha 1,3)\text{GlcNAc}$ -Asn-protein

Figure 8 β -N-Acetylglucosaminidase assays using HPLC analysis on a Palpak column of PA-oligosaccharides

Microsomes (0.015 ml) from wild-type N2 worms were incubated as in Figure 7 with MGn-PA, GnM-PA, GnGn-PA and M5Gn3-PA (all at 0.13 mM) at 37 °C for 5 h. The Figure shows PA-oligosaccharides formed during the incubation. (A) Isomaltose standard; (B) MGn-PA standard; (C) MGn-PA incubation; (D) GnM-PA standard; (E) GnM-PA incubation; (F) isomaltose standard; (G) GnGn-PA standard; (H) GnGn-PA incubation; (I) M5Gn3-PA standard; (J) M5Gn3-PA incubation. MGn-PA is partially degraded to MM-PA (C). GnM-PA (E) and GnGn-PA (H) are not degraded under these conditions. M5Gn3-PA (J) is degraded to M4Gn3-PA, MGn-PA and M2Gn3-PA, but not to M5Gn2-PA (the small peak of M5Gn2-PA in the incubation is due to a contamination in the M5Gn3-PA starting compound, as seen in I). MS analyses have verified these findings (Figure 9). See the abbreviations footnote for the identification of structures.

Figure 9 β -N-Acetylglucosaminidase assays using MALDI-TOF MS spectra of PA-oligosaccharides formed during the incubations analysed in Figure 8

(A) GnGn-PA; (B) M5Gn3-PA; (C) GnM-PA; (D) MGn-PA. The conclusions are identical with those found by HPLC analysis (Figure 8). M5Gn3-PA (B) is degraded to M4Gn3-PA, MGn-PA and M2Gn3-PA (a small peak not marked on the spectrum), but not to M5Gn2-PA (the small peak of M5Gn2-PA is due to a contamination in the M5Gn3-PA starting compound, Figure 8). (B) and (D) show peaks of MM-PA indicating conversion of MGn-PA into MM-PA. See the abbreviations footnote for the identification of structures. The four peaks marked with an asterisk in (D) are due to the MALDI-TOF procedure and are present in all samples.

[47,48]. Since both the β 1,2-xylosyltransferase and the core α 1,3-fucosyltransferase require prior action of GnT I [49], the formation of these paucimannose compounds indicates that a β -N-acetylglucosaminidase, presumably located in the Golgi apparatus, removes the GlcNAc β 1,2 residue while the nascent glycoprotein traverses the lumen of the endoplasmic reticulum–Golgi assembly line [48,50].

A β -N-acetylglucosaminidase activity has been described in the lepidopteran insect cell line Sf21 which can remove GlcNAc from pyridylaminated oligosaccharides and chromogenic synthetic glycosides [28]. With the biantennary substrate GnGn-PA, the β -N-acetylglucosaminidase removed GlcNAc β 1,2 exclusively from the Man α 1,3Man β - arm of the core. M5Gn3, the primary product of GnT I, was not perceptibly hydrolysed. β -N-Acetylglucosaminidases with the same branch specificity were also found in the lepidopteran cell lines Bm-N and Mb-0503 [28] and Sf9 [51]. The addition of the N-acetylglucosaminidase inhibitor 2-acetamido-1,2-dideoxyojirimycin to baculovirus-infected insect cells decreased the synthesis of MM and MMFuc and allowed the accumulation of sialylated N-glycans [52]. Those authors suggested that insect-specific, Golgi-associated

β -N-acetylglucosaminidase-mediated removal of GlcNAc residues from the precursor N-glycans prevented galactosylation and terminal sialylation.

The accumulation of paucimannose N-glycans in *C. elegans* suggests that worms have a β -N-acetylglucosaminidase similar to the enzyme in insects ('GlcNAcase' in Scheme 1). In order to provide evidence for this hypothesis, we have carried out MS analyses on N-glycans isolated from a *C. elegans* strain with a null mutation in the *gly-13* gene encoding the major GnT I enzyme in the wild-type worm [16]. N-glycans from wild-type and *gly-13*-null worms were quantitatively compared by differential methylation with 2 H-labelled and non- 2 H-labelled iodomethane respectively. The results showed that absence of GLY-13 results in significant decreases in the amounts of N-glycans downstream of the GLY-13 block and increases in upstream N-glycans, and that therefore synthesis of at least some of the paucimannose N-glycans MM and MMFuc depends on prior GLY-13 action. This finding supported the suggestion that *C. elegans* has a β -N-acetylglucosaminidase which acts on N-glycans during *in vivo* biosynthesis. In the present study we have provided *in vitro* evidence that, at least in the presence of GLY-13, conversion of M5Gn2 into paucimannose MM requires prior actions of GnT I (converting M5Gn2 into M5Gn3), α 3,6-mannosidase II (M5Gn3 into M4Gn3 and MGn) and β -N-acetylglucosaminidase (M4Gn3 and MGn into M4Gn2 and MM respectively) (Figures 3–6 and Scheme 1). We have proved that *C. elegans* microsomes contain a specific β -N-acetylglucosaminidase which can remove GlcNAc from MGn-PA and M4Gn3-PA, but is unable to act on GnM-PA, GnGn-PA and M5Gn3-PA (Figures 6–9). This enzyme therefore has properties very similar to the insect enzyme [28], except that the insect enzyme converted GnGn-PA into GnM-PA, while the *C. elegans* microsomes did not act on GnGn-PA. The proposed pathway for paucimannose N-glycan synthesis in *C. elegans* is summarized in Scheme 1.

Human and Chinese-hamster α 3, 6-mannosidase IIx [53–56] and mouse α 3, 6-mannosidase III [57] are able to convert M5Gn2 into MM without prior GnT I action. However, the kinetics of formation of MM from M5Gn2 in the presence of GLY-13 (Figures 3–6) indicate that prior GnT I action is required for synthesis of MM. We have not tested whether M5Gn2 can be converted into MM by microsomes from *gly-13*-null worms, owing to the difficulty of growing the sickly survivors [17].

Truncated bi-, tri- and tetra-antennary complex N-glycans [6,7] are presumably formed by the action of *C. elegans* UDP-N-acetyl-D-glucosamine: α -6-D-mannoside β 1,2-N-acetylglucosaminyltransferase II (GnT II) [17,58] on MGn and MGnFuc followed by the actions of other branching N-acetylglucosaminyltransferases [41]. GnM has been reported in *C. elegans* [8]. Since GnT II action depends on prior GnT I action [17,58], this suggests that there may be a *C. elegans* β -N-acetylglucosaminidase capable of degrading GnGn to GnM. The usefulness of *C. elegans* in studying the role of complex N-glycans in development and other biological processes depends on the worm's ability to make reasonable amounts of these compounds. Our demonstration that the large amounts of MM and MMFuc seen in *C. elegans* are indeed footprints of prior GnT I action provide additional evidence that GnT I may play essential roles in *C. elegans* development.

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REFERENCES

- for gifts of materials, and Dr. Ranjith Muhanidiram, Director of the University of Toronto Nuclear Magnetic Resonance Laboratory, for running NMR spectra.
- ## REFERENCES
- Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71–94
 - Riddle, D. L., Blumenthal, T., Meyer, B. J. and Priess, J. R. (1997) *C. elegans* II. Cold Spring Harbor Monograph Series, vol. 33, Cold Spring Harbor Laboratory Press, Plainview, NY
 - Wood, W. B. (1988) The nematode *Caenorhabditis elegans*. Cold Spring Harbor Laboratory Press, Plainview, NY
 - Epstein, H. F. and Shakes, D.C. (1995) *Caenorhabditis elegans*: modern biological analysis of an organism. *Methods Cell Biol.* **48**
 - Altmann, F., Fabini, G., Ahorn, H. and Wilson, I. B. (2001) Genetic model organisms in the study of N-glycans. *Biochimie* **83**, 703–12
 - Cipollo, J. F., Costello, C. E. and Hirschberg, C. B. (2002) The fine structure of *Caenorhabditis elegans* N-glycans. *J. Biol. Chem.* **277**, 49143–49157
 - Haslam, S. M., Gerns, D., Morris, H. R. and Dell, A. (2002) The glycomes of *Caenorhabditis elegans* and other model organisms. *Biochem. Soc. Symp.* **69**, 117–134
 - Natsuka, S., Adachi, J., Kawaguchi, M., Nakakita, S.-I., Hase, S., Ichikawa, A. and Ikura, K. (2002) Structure analysis of N-linked glycans in *Caenorhabditis elegans*. *J. Biochem. (Tokyo)* **131**, 807–813
 - Rosa, J. C., Schachter, H., Reinhold, B. and Reinhold, V. N. (2001) The glycome of *Caenorhabditis elegans*, a pathway to function. *Glycobiology* **11**, 884–885

- 10 Wilson, J. R., Williams, D. and Schachter, H. (1976) The control of glycoprotein synthesis. I. *N*-acetyl- β -glucosamine linkage to mannose residue as a signal for the attachment of L-fucose to the asparagine-linked *N*-acetyl- β -glucosamine residue of glycopeptide from α_1 -acid glycoprotein. *Biochem. Biophys. Res. Commun.* **72**, 909–916
- 11 Staudacher, E., Dalik, T., Wawra, P., Altmann, F. and Marz, L. (1995) Functional purification and characterization of a GDP-fucose: β -*N*-acetyl- β -glucosamine (Fuc to Asn linked GlcNAc) α 1,3-fucosyltransferase from mung beans. *Glycoconj. J.* **12**, 780–786
- 12 Leiter, H., Mucha, J., Staudacher, E., Grimm, R., Glössl, J. and Altmann, F. (1999) Purification, cDNA cloning, and expression of GDP-L-Fuc:Asn-linked GlcNAc- α 1,3-fucosyltransferase from mung beans. *J. Biol. Chem.* **274**, 21830–21839
- 13 März, L., Altmann, F., Staudacher, E. and Kubelka, V. (1995) Protein glycosylation in insects. *Glycoproteins* **29a**, 543–563
- 14 Chen, S., Spence, A. M. and Schachter, H. (2001) Complex N-glycans – when, why? *Trends Glycosci. Glycotechnol.* **13**, 447–462
- 15 Chen, S. H., Zhou, S. H., Sarkar, M., Spence, A. M. and Schachter, H. (1999) Expression of three *Caenorhabditis elegans* *N*-acetylglucosaminyltransferase I genes during development. *J. Biol. Chem.* **274**, 288–297
- 16 Schachter, H., Chen, S., Zhang, W., Spence, A. M., Zhu, S., Callahan, J. W., Mahuran, D. J., Fan, X., Bagshaw, R. D., She, Y. M. et al. (2002) Functional post-translational proteomics approach to study the role of N-glycans in the development of *Caenorhabditis elegans*. *Biochem. Soc. Symp.* **69**, 1–21
- 17 Chen, S., Tan, J., Reinhold, V. N., Spence, A. M. and Schachter, H. (2002) UDP-*N*-acetyl- β -glucosamine: α -3- β -mannoside β -1,2-*N*-acetylglucosaminyltransferase I and UDP-*N*-acetyl- β -glucosamine: α -6- β -mannoside β -1,2-*N*-acetylglucosaminyltransferase II in *Caenorhabditis elegans*. *Biochim. Biophys. Acta* **1573**, 271–279
- 18 Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1993) *Current Protocols in Molecular Biology*, John Wiley and Sons, New York
- 19 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 20 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- 21 Church, D. L., Guan, K. L. and Lambie, E. J. (1995) Three genes of the MAP kinase cascade, *mek-2*, *mpk-1/sur-1* and *let-60 ras*, are required for meiotic cell cycle progression in *Caenorhabditis elegans*. *Development* **121**, 2525–2535
- 22 Mello, C. and Fire, A. (1995) DNA transformation. *Methods Cell Biol.* **48**, 451–482
- 23 Vella, G. J., Paulsen, H. and Schachter, H. (1984) Control of glycoprotein synthesis. IX. A terminal Man α 1–3Man β 1-sequence in the substrate is the minimum requirement for UDP-*N*-acetyl- β -glucosamine: α - β -mannoside (GlcNAc to Mana1–3-) β 2-*N*-acetylglucosaminyltransferase I. *Can. J. Biochem. Cell Biol.* **62**, 409–417
- 24 Fu, D., Chen, L. and O'Neill, R. A. (1994) A detailed structural characterization of ribonuclease B oligosaccharides by ^1H NMR spectroscopy and mass spectrometry. *Carbohydr. Res.* **261**, 173–186
- 25 Staudacher, E., Kubelka, V. and März, L. (1992) Distinct N-glycan fucosylation potentials of three lepidopteran cell lines. *Eur. J. Biochem.* **207**, 987–993
- 26 Kubelka, V., Altmann, F., Staudacher, E., Tretter, V., März, L., Hård, K., Kamerling, J. P. and Vliegenter, J. F. G. (1993) Primary structures of the N-linked carbohydrate chains from honeybee venom phospholipase A₂. *Eur. J. Biochem.* **213**, 1193–1204
- 27 Altmann, F., Kornfeld, G., Dalik, T., Staudacher, E. and Glossl, J. (1993) Processing of asparagine-linked oligosaccharides in insect cells – *N*-acetylglucosaminyltransferase I and II activities in cultured lepidopteran cells. *Glycobiology* **3**, 619–625
- 28 Altmann, F., Schwihla, H., Staudacher, E., Glossl, J. and Marz, L. (1995) Insect cells contain an unusual, membrane-bound β -*N*-acetylglucosaminidase probably involved in the processing of protein N-glycans. *J. Biol. Chem.* **270**, 17344–17349
- 29 Altmann, F. and Marz, L. (1995) Processing of asparagine-linked oligosaccharides in insect cells: evidence for α -mannosidase II. *Glycoconj. J.* **12**, 150–155
- 30 van den Elsen, J. M., Kuntz, D. A. and Rose, D. R. (2001) Structure of Golgi α -mannosidase II: a target for inhibition of growth and metastasis of cancer cells. *EMBO J.* **20**, 3008–3017
- 31 Chen, S., Spence, A. M. and Schachter, H. (2003) Isolation of null alleles of the *Caenorhabditis elegans* *gly-12*, *gly-13* and *gly-14* genes, all of which encode UDP-GlcNAc: α -3- β -mannoside β 1,2-*N*-acetylglucosaminyltransferase I activity. *Biochimie*, in the press
- 32 Palčić, M. M., Heerze, L. D., Pierce, M. and Hindsgaul, O. (1988) The use of hydrophobic synthetic glycosides as acceptors in glycosyltransferase assays. *Glycoconj. J.* **5**, 49–63
- 33 Sarkar, M. (1994) Expression of recombinant rabbit UDP-GlcNAc: α 3- β -mannoside β -1,2-*N*-acetylglucosaminyltransferase I catalytic domain in Sf9 insect cells. *Glycoconj. J.* **11**, 204–209
- 34 Dixon, M. and Webb, E. C. (1964) *Enzymes*, Longmans, London
- 35 Sarkar, M. and Schachter, H. (2001) Cloning and expression of *Drosophila melanogaster* UDP-GlcNAc: α -3- β -mannoside β 1,2-*N*-acetylglucosaminyltransferase I. *Biol. Chem.* **382**, 209–217
- 36 Khoo, K. H., Huang, H. H. and Lee, K. M. (2001) Characteristic structural features of schistosome cercarial N-glycans: expression of Lewis X and core xylosylation. *Glycobiology* **11**, 149–163
- 37 Kolarich, D. and Altmann, F. (2000) N-glycan analysis by matrix-assisted laser desorption/ionization mass spectrometry of electrophoretically separated nonmammalian proteins: application to peanut allergen Ara h 1 and olive pollen allergen Ole e 1. *Anal. Biochem.* **285**, 64–75
- 38 Narasimhan, S., Wilson, J. R., Martin, E. and Schachter, H. (1979) Structural basis for four distinct elution profiles on concanavalin A–Sepharose affinity chromatography of glycopeptides. *Can. J. Biochem.* **57**, 83–96
- 39 Reck, F., Springer, M., Paulsen, H., Brockhausen, I., Sarkar, M. and Schachter, H. (1994) Synthesis of tetrasaccharide analogues of the N-glycan substrate of β -(1 $\rightarrow$ 2)-*N*-acetylglucosaminyltransferase II using trisaccharide precursors and recombinant β -(1 $\rightarrow$ 2)-*N*-acetylglucosaminyltransferase I. *Carbohydr. Res.* **259**, 93–101
- 40 Allen, S. D., Tsai, D. and Schachter, H. (1984) Control of glycoprotein synthesis. X. The *in vitro* synthesis by hen oviduct membrane preparations of hybrid asparagine-linked oligosaccharides containing 5 mannose residues. *J. Biol. Chem.* **259**, 6984–6990
- 41 Schachter, H. (1991) The “yellow brick road” to branched complex N-glycans. *Glycobiology* **1**, 453–461
- 42 Metzler, M., Gertz, A., Sarkar, M., Schachter, H., Schrader, J. W. and Marth, J. D. (1994) Complex asparagine-linked oligosaccharides are required for morphogenic events during post-implantation development. *EMBO J.* **13**, 2056–2065
- 43 Ioffe, E. and Stanley, P. (1994) Mice lacking *N*-acetylglucosaminyltransferase I activity die at mid-gestation, revealing an essential role for complex or hybrid N-linked carbohydrates. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 728–732
- 44 Tan, J., Dunn, J., Jaeken, J. and Schachter, H. (1996) Mutations in the *MGAT2* gene controlling complex N-glycan synthesis cause carbohydrate-deficient glycoprotein syndrome type II, an autosomal recessive disease with defective brain development. *Am. J. Hum. Genet.* **59**, 810–817
- 45 Strasser, R., Mucha, J., Schwihla, H., Altmann, F., Glössl, J. and Steinkellner, H. (1999) Molecular cloning and characterization of cDNA coding for β 1,2-*N*-acetylglucosaminyltransferase I (GlcNAc-TI) from *Nicotiana tabacum*. *Glycobiology* **9**, 779–785
- 46 Hagen, F. K. and Nehrke, K. (1998) cDNA cloning and expression of a family of UDP-*N*-acetylglucosamine:polypeptide *N*-acetylglucosaminyltransferase sequence homologs from *Caenorhabditis elegans*. *J. Biol. Chem.* **273**, 8268–8277
- 47 Wilson, I. B., Zeleny, R., Kolarich, D., Staudacher, E., Stroop, C. J., Kamerling, J. P. and Altmann, F. (2001) Analysis of Asn-linked glycans from vegetable foodstuffs: widespread occurrence of Lewis x, core alpha1,3-linked fucose and xylose substitutions. *Glycobiology* **11**, 261–274
- 48 Sturm, A. (1995) N-glycosylation of plant proteins. *Glycoproteins* **29a**, 521–541
- 49 Johnson, K. D. and Chrispeels, M. J. (1987) Substrate specificities of *N*-acetylglucosaminyl-, fucosyl-, and xylosyltransferases that modify glycoproteins in the Golgi apparatus of bean cotyledons. *Plant Physiol.* **84**, 1301–1308
- 50 Vitale, A. and Chrispeels, M. J. (1984) Transient *N*-acetylglucosamine in the biosynthesis of phytohemagglutinin: attachment in the Golgi apparatus and removal in protein bodies. *J. Cell Biol.* **99**, 133–140
- 51 Wagner, R., Geyer, H., Geyer, R. and Klenk, H. D. (1996) *N*-acetyl- β -glucosaminidase accounts for differences in glycosylation of influenza virus hemagglutinin expressed in insect cells from a baculovirus vector. *J. Virol.* **70**, 4103–4109
- 52 Watanabe, S., Kokuho, T., Takahashi, H., Takahashi, M., Kubota, T. and Inumaru, S. (2002) Sialylation of N-glycans on the recombinant proteins expressed by a baculovirus–insect cell system under β -*N*-acetylglucosaminidase inhibition. *J. Biol. Chem.* **277**, 5090–5093
- 53 Akama, T. O., Nakagawa, H., Sugihara, K., Narisawa, S., Ohya, C., Nishimura, S., O'Brien, D. A., Moremen, K. W., Millan, J. L. and Fukuda, M. N. (2002) Germ cell survival through carbohydrate-mediated interaction with Sertoli cells. *Science* **295**, 124–127
- 54 Misago, M., Liao, Y. F., Kudo, S., Eto, S., Mattei, M. G., Moremen, K. W. and Fukuda, M. N. (1995) Molecular cloning and expression of cDNAs encoding human α -mannosidase II and a previously unrecognized α -mannosidase II isozyme. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11766–11770
- 55 Ogawa, R., Misago, M., Fukuda, M. N., Kudo, S., Tsukada, J., Morimoto, I. and Eto, S. (1996) Structure and transcriptional regulation of human α -mannosidase II (α -mannosidase II isotype) gene. *Eur. J. Biochem.* **242**, 446–453

- 56 Oh-Eda, M., Nakagawa, H., Akama, T. O., Lowitz, K., Misago, M., Moremen, K. W. and Fukuda, M. N. (2001) Overexpression of the Golgi-localized enzyme α -mannosidase IIx in Chinese hamster ovary cells results in the conversion of hexamannosyl-*N*-acetylchitobiose to tetramannosyl-*N*-acetylchitobiose in the N-glycan-processing pathway. *Eur. J. Biochem.* **268**, 1280–1288
- 57 Chui, D., Oh-Eda, M., Liao, Y. F., Panneerselvam, K., Lal, A., Marek, K. W., Freeze, H. H., Moremen, K. W., Fukuda, M. N. and Marth, J. D. (1997) α -Mannosidase-II deficiency results in dyserythropoiesis and unveils an alternate pathway in oligosaccharide biosynthesis. *Cell* **90**, 157–167
- 58 Tan, J., Chen, S., Spence, A. M. and Schachter, H. (2000) Cloning and expression of *Caenorhabditis elegans* UDP-GlcNAc: α -6-D-mannoside β -1,2-*N*-acetylglucosaminyl-transferase II (GnT II). *Glycobiology* **10**, 1115
- 59 Verbert, A. (1995) From Glc₃Man₉GlcNAc₂-protein to Man₅GlcNAc₂-protein: transfer 'en bloc' and processing. *Glycoproteins* **29a**, 145–152
- 60 Schachter, H. (1995) Glycosyltransferases involved in the synthesis of N-glycan antennae. *Glycoproteins* **29a**, 153–199
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