BBA - General Subjects 1862 (2018) 2191–2203

Contents lists available at ScienceDirect

BBA - General Subjects

journal homepage: www.elsevier.com/locate/bbagen

Ablation of *N*-acetylglucosaminyltransferases in *Caenorhabditis* induces expression of unusual intersected and bisected N-glycans

Shi Yan (闫石)[a,b], Huijie Wang (王慧捷)[a], Harry Schachter[c], Chunsheng Jin (金春生)[d], Iain B.H. Wilson[a,*], Katharina Paschinger[a]

[a] Department für Chemie, Universität für Bodenkultur, 1190 Wien, Austria
[b] Institut für Parasitologie, Veterinärmedizinische Universität Wien, 1210 Wien, Austria
[c] Hospital for Sick Children, University of Toronto, Toronto, ON M5G 1X8, Canada
[d] Institutionen för Biomedicin, Göteborgs universitet, 405 30 Göteborg, Sweden

ARTICLE INFO

Keywords:
N-glycans
Nematode
Glycomics

ABSTRACT

The modification in the Golgi of N-glycans by *N*-acetylglucosaminyltransferase I (GlcNAc-TI, MGAT1) can be considered to be a hallmark of multicellular eukaryotes as it is found in all metazoans and plants, but rarely in unicellular organisms. The enzyme is key for the normal processing of N-glycans to either complex or paucimannosidic forms, both of which are found in the model nematode *Caenorhabditis elegans*. Unusually, this organism has three different GlcNAc-TI genes (*gly-12*, *gly-13* and *gly-14*); therefore, a complete abolition of GlcNAc-TI activity required the generation of a triple knock-out strain. Previously, the compositions of N-glycans from this mutant were described, but no detailed structures. Using an off-line HPLC-MALDI-TOF-MS approach combined with exoglycosidase digestions and MS/MS, we reveal that the multiple hexose residues of the N-glycans of the *gly-12;gly-13;gly-14* triple mutant are not just mannose, but include galactoses in three different positions (β-intersecting, β-bisecting and α-terminal) on isomeric forms of $Hex_{4-8}HexNAc_2$ structures; some of these structures are fucosylated and/or methylated. Thus, the N-glycomic repertoire of *Caenorhabditis* is even wider than expected and exhibits a large degree of plasticity even in the absence of key glycan processing enzymes from the Golgi apparatus.

1. Introduction

N-glycans probably represent the most diverse range of post-translational modifications of proteins, whereby the linkage of oligosaccharides to asparagine residues is known in many bacteria or archaea and in almost all eukaryotes [1]. The heterogeneity of N-glycan structures in most eukaryotes is due to processing events in the Golgi apparatus. In multicellular organisms, three glucose and up to four α1,2-mannose residues are removed prior to the 'rebuilding' of the oligosaccharide by glycosyltransferases; a key enzyme, thereby, is *N*-acetylglucosaminyltransferase I (GlcNAc-TI, EC 2.4.1.101, encoded in mammals by *mgat1* genes), which appears to be absent from most unicellular eukaryotes [2]. The biological phenotypes caused by ablation of *mgat1* genes vary from altered susceptibility to stress in plants or to bacteria in *Caenorhabditis elegans* through to cell migration or neuronal defects in invertebrates and, most severely, embryonic lethality in mice [3–8]. The glycomic changes in *mgat1* mutants are always significant, as $Man_5GlcNAc_2$ is no longer normally processed. In plants, flies and mammals, this also means that core fucosylation is heavily reduced in keeping with assays showing that the relevant enzymes are dependent, at least *in vitro*, on the prior action of GlcNAc-TI [9–11].

Unusually, *C. elegans* possesses three GlcNAc-TI genes (*gly-12, gly-13* and *gly-14*), all of which have been proven to be enzymatically active and all of which need to be deleted in order to abolish GlcNAc-TI activity *in vivo* [12,13]. The three products of the differentially-expressed genes have slightly different substrate specificities in that they can either accept $Man_3GlcNAc_2$ or $Man_5GlcNAc_2$, in contrast to the homologues from most other organisms which can transfer GlcNAc to both. A previous MALDI-TOF MS-based screen of the *gly-12; gly-13;gly-14* triple knock-out did indeed indicate fucosylation of glycans with compositions suggestive of oligomannosidic structures [13]; however, our recent off-line MALDI-TOF MS data demonstrate that not all hexose residues in *C. elegans* N-glycans are mannose, as bisecting β-galactose on the core β-mannose and an α-galactose modification of the α1,3-mannose have been found [14]. As part of our continuing studies on the diversity of wild-type and mutant *C. elegans* N-glycomes, we have

* Corresponding author.
E-mail address: iain.wilson@boku.ac.at (I.B.H. Wilson).

https://doi.org/10.1016/j.bbagen.2018.07.002
Received 20 April 2018; Received in revised form 30 June 2018; Accepted 4 July 2018
Available online 05 July 2018

reappraised that of the *gly-12; gly-13;gly-14* triple knock-out and reveal that also this mutant expresses unusual N-glycans containing up to three galactose substitutions of mannose residues.

2. Methods

2.1. Biological material

The *C. elegans gly-12;gly-13;gly-14* strain (*gly-14* (III);*gly-12 gly13* (X)) was previously prepared as described by crossing the *gly-12(id47)*, *gly-13(ok712)* and *gly-14(id48)* alleles [13] and was maintained under standard conditions; mixed stages were cultivated at 20 °C (160 rpm; 4–6 days) in liquid culture with *E. coli* OP50 in standard S complete medium prior to harvesting and purification by sucrose density centrifugation [15]. Harvested worms were boiled in water for 10 min to heat-inactivate proteases prior to homogenisation. The homogenates were transferred in glass flasks and pepsinised overnight at 37 °C. Glycopeptides were purified by cation exchange chromatography (Dowex 50 W × 8; elution with 0.5 M ammonium acetate, pH 6) followed by G25 gel filtration.

2.2. N-glycan release, purification and labelling

Enzymatic release of N-glycans from worm peptic glycopeptides was done sequentially using two different peptide:N-glycosidases: first recombinant PNGase F (Roche) was employed under alkaline conditions (pH 8.0) and the released glycans were separated from the remaining glycopeptides on Dowex 50 W × 8 (10 ml resin, with glycans in the 2% acetic acid 'wash' and glycopeptides in the 0.5 M ammonium acetate eluate). The glycopeptide fraction was desalted, lyophilised and taken up in pH 5.0 buffer prior to treatment with native almond PNGase A (Roche; at pH 5.0) and another round of Dowex 50 W × 8 chromatography. Both N-glycan pools were separately purified by solid-phase extraction using non-porous graphitised carbon (nPGC; elution with 40% acetonitrile) and LiChroprep® RP-18 (C18; elution with water) prior to pyridylamination as previously described [16–18]. For a fuller description, refer to the Supplement.

2.3. Mass spectrometric analysis

The N-glycomes of the different strains were profiled by MALDI-TOF MS (Autoflex Speed, Bruker Daltonics, Germany) in positive ion mode using FlexControl 3.4 software. PA-labelled N-glycans were fractionated by 2D-HPLC as described below and all HPLC peaks were collected and examined by MALDI-TOF MS, using 6-aza-2-thiothymine (ATT) as matrix; MS/MS to confirm the composition of all proposed structures was performed by laser-induced dissociation (the typical precursor ion selector window of ± 0.6% being adjusted as considered appropriate for samples containing multiple glycans). The detector voltage was generally set at 1977 V for MS and 2133 V for MS/MS; 1000–3000 shots from different regions of the sample spots were summed. Spectra were processed with the manufacturer's software (Bruker Flexanalysis 3.3.80) using the SNAP algorithm with a signal/noise threshold of 6 for MS (unsmoothed) and 3 for MS/MS (four-times smoothed). In total approximately 3500 MS and MS/MS spectra were manually interpreted on the basis of the mass, fragmentation pattern and results of chemical and enzymatic treatments; isomeric structures present in different 2D-HPLC fractions were defined on the basis of comparisons of the aforementioned parameters. At least five MS/MS fragment ions were used to aid definition of each of the structures. LC-MS^n was performed on a $Gal_3Man_4GlcNAc_2$ structure as well as two $Man_7GlcNAc_2$ isomers as previously described [14].

2.4. HPLC purification of N-glycans

Separation of PA-labelled glycans was carried out on a Shimadzu HPLC system equipped with a fluorescence detector (RF 10 AXL). First the glycans were fractionated by NP-HPLC using a Tosoh Amide-80 column (4.6 × 250 mm); dried samples were taken up in 50 µl of a 25:75 mixture of buffer A (10 mM ammonium formate, pH 7) and buffer B (95% acetonitrile) prior to injection and the following gradient was applied: 0–5 min, 75% B; 5–15 min, 75–65% B; 15–40 min, 65% B; 40–55 min, 65–57% B; followed by a return to the starting conditions. Selected fractions were then subject to RP-HPLC on a Hypersil ODS column (5 µm, 4 × 250 mm; Agilent), whereby buffer C was 0.1 M ammonium acetate, pH 4, and buffer D was 30% (*v*/v) methanol. Gradients of increasing methanol (1% buffer D per minute) were applied. Fluorescence was recorded at 320 nm (excitation) and 400 nm (emission). The columns were calibrated daily in terms of glucose units (g.u.) with a pyridylaminated partial dextran hydrolysate. Various modifications of N-glycans have different effects on retention time as compared to the 'parent' structure: α1,3-fucose and α1,6-fucose resulting in either 2 g.u. earlier or 3 g.u. later RP-HPLC elution; bisecting galactose in 3 g.u. earlier elution on RP-HPLC; intersecting galactose in 1 g.u. earlier elution on RP-HPLC; methylation in 0.3 g.u. earlier on NP-HPLC, but later elution on RP-HPLC.

2.5. Structural elucidation using exoglycosidases and chemical treatment

In general, a 1 µl aliquot of a previously dried and redissolved HPLC fraction was mixed with 0.2 µl exoglycosidase and 0.8 µl 100 mM ammonium acetate solution, pH 5.0; after an overnight incubation at 37 °C, 0.5 µl aliquot of the mixture was analysed by MALDI-TOF MS. Exoglycosidases employed were: α-galactosidase from green coffee beans (Sigma, 11 mU), recombinant β-galactosidase from *Aspergillus niger* (produced in-house, 144 µU), *Aspergillus* α1,2-specific mannosidase (Prozyme, 4 µU), jack bean α-mannosidase (either from Sigma-Aldrich, 6.25 mU, New England Biolabs, 400 mU, or Prozyme, 30 mU), recombinant *Xanthomonas manihotis* α1,2/3- or α1,6-mannosidases (New England Biolabs, 6–8 U), microbial α1,2-specific fucosidase (*E*-FUCM from Megazyme, 3 mU) and α-L-fucosidase from bovine kidney (Sigma-Aldrich, 10 mU). Specificities of these glycosidases have been previously described by us or others [19–25]; as discussed below, release of some α-mannose and β-galactose residues is limited by mutual steric hindrance. For removal of α1,2/3-linked fucose or methylfucose, glycan samples were dried in a Speed-Vac and then incubated with 3 µl of 48% (*w*/*v*) hydrofluoric acid (HF) on ice for 24 h, before evaporation in a SpeedVac. Chemically or enzymatically treated glycans were generally reanalysed, unless otherwise stated, by MALDI-TOF MS and MS/MS without further purification.

3. Results

3.1. Overall glycome of a N-acetylglucosaminyltransferase I null mutant

Previously, the N-glycome of the *C. elegans gly-12;gly-13;gly-14* triple GlcNAc-TI knock-out strain was profiled by mass spectrometry, but only compositions of the hydrazine-released structures were described [13]; since then, it has become obvious that nematode glycomes are not as simple as was thought. In order to resolve specific structural details, glycans were released serially with PNGase F and PNGase A; such a procedure is necessary as only use of the latter enzyme, and not the former, can yield core α1,3-fucosylated structures. The pools were separately labelled with 2-aminopyridine as a fluorescent tag, screened by MALDI-TOF-MS and then subject to 2D-HPLC using NP-HPLC followed by RP-HPLC of selected fractions; alternatively, in another experiment, only PNGase A and a single round of RP-HPLC was employed (see Fig. 1 as well as Supplementary Figs. 1–3). The major N-glycan in the glycome was $Hex_5HexNAc_2$ as defined by its mass (*m*/*z* 1313 or 1335 as either $[M + H]^+$ or $[M + Na]^+$) as opposed to $Hex_3HexNAc_2Fuc_{0–1}$ in the wild-type [26]. The expectation that the major glycan would be $Man_5GlcNAc_2$ when GlcNAc-TI activity is