Bisecting Galactose as a Feature of N-Glycans of Wild-type and Mutant Caenorhabditis elegans*

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The N-glycosylation of the model nematode Caenorhabditis elegans has proven to be highly variable and rather complex; it is an example to contradict the existing impression that “simple” organisms possess also a rather simple glycomic capacity. In previous studies in a number of laboratories, N-glycans with up to four fucose residues have been detected. However, although the linkage of three fucose residues to the N,N’-diacetylated core has been proven by structural and enzymatic analyses, the nature of the fourth fucose has remained uncertain. By constructing a triple mutant with deletions in the three genes responsible for core fucosylation (fut-1, fut-6 and fut-8), we have produced a nematode strain lacking products of these enzymes, but still retaining maximally one fucose residue on its N-glycans. Using mass spectrometry and HPLC in conjunction with chemical and enzymatic treatments as well as NMR, we examined a set of α-mannosidase-resistant N-glycans. Within this glycomic subpool, we can reveal that the core β-mannose can be trisubstituted and so carries not only the ubiquitous α1,3- and α1,6-mannose residues, but also a “bisecting” β-galactose, which is substoichiometrically modified with fucose or methylfucose. In addition, the α1,3-mannose can also be α-galactosylated. Our data, showing the presence of novel N-glycan modifications, will enable more targeted studies to understand the biological functions and interactions of nematode glycans. Molecular & Cellular Proteomics 14: 10.1074/mcp.M115.049817, 2111–2125, 2015.

Nematodes represent, along with arthropods, one of the largest groups of animals to exist on the planet; 25,000 species are described, but the existence of up to one million has been estimated (1, 2). They have various ecological niches and include free-living “worms” in the soil, fungivorous, entomopathogenic, and necromenic species as well as parasites of plants and mammals, which share the basic conserved body plan (more-or-less a digestive tube surrounded with muscle, whether larger or smaller). There are five major clades (Rhabditina, Enoplia, Spirurina, Tylenchina, and Dorylaimia) (2), yet the glycosylation of only a few nematode species has been studied with an inevitable focus on the model nematode Caenorhabditis elegans and parasitic species (3). Thereby, the use of C. elegans mutants has been highly valuable in dissecting aspects of nematode N-glycan biosynthesis and revealing the in vivo substrates for certain glycosyltransferases (4).

As many nematodes are parasites, their interactions with the immune systems of their hosts have attracted attention; particularly, there are relationships between autoimmunity, allergy, vaccination, and helminth infections. The “old friends” hypothesis seeks to understand the evolutionary factors that have shaped the immune system and to explain correlations between lifestyles in the developed world and modern “epidemics,” which are due to immunological imbalance (5–7). Promising data have suggested that “worm therapy” may bring advantages to some patients with Crohn’s disease or allergies (8, 9); however, such approaches are controversial. Nevertheless, crude extracts even of Caenorhabditis elegans were shown to induce a glycan-dependent Th2 response (10), whereas the excretory-secretory products of some nematodes also have immunomodulatory activity (11). Furthermore, the native glycoproteins of some nematodes have proven effective in vaccination trials, whereas recombinant forms are not, which is suggestive that post-translational modifications may have a role in an efficacious immune response (12).

As at least some of the molecules relevant to nematode immunomodulation or vaccination are glycoproteins, a proper understanding of nematode glycosylation is of biomedical and veterinary relevance. Over the years, it has become apparent
that the core chitobiaryl region of nematode N-glycans is subject to a range of modifications, with up to three core fucose residues being present (α1,3- and α1,6-linked on the reducing-terminal “proximal” GlcNAC and α1,3-linked on the second “distal” GlcNAC). However, up to four fucose residues have been detected on C. elegans N-glycans and the exact nature of the linkage of the fourth fucose has remained obscure despite work in our own and other laboratories (3, 13–15). Combined with the latest knowledge regarding the specificity of C. elegans core fucosyltransferases (13, 16, 17) as well as our recent data regarding the exact structures of N-glycans from the C. elegans double hexosaminidase mutant and other nematodes (18–20), we concluded that some models for the tri- and tetrafucosylated N-glycans were incorrect. By preparing a triple mutant unable to core fucosylate its N-glycans, we generated a C. elegans strain containing maximally one fucose residue on the N-linked oligosaccharides. Thereby a pool of unusual mannosidase-resistant N-glycans was identified and, using mass spectrometry (MS) and NMR, we reveal their modification with bisecting galactose frequently capped with fucose or methylfucose.

EXPERIMENTAL PROCEDURES

Preparation of the C. elegans Triple Mutant—Wild-type C. elegans (N2) and single mutants fut-1(ok892), fut-6(ok475) and fut-8(ok2558) were obtained from the Caenorhabditis Genetics Centre (CGC), University of Minnesota, USA. All C. elegans strains were cultured under standard conditions at 20 °C (21). A fut-1;fut-6 double mutant was generated by standard crossings (22). Briefly, the ok892 single mutant was first crossed with N2 wild-type males in order to produce male progeny for subsequent fertilization of the ok475 single mutant. Hermaphrodites from the F2 generation were isolated and allowed to produce eggs prior to examination of the maternal genotypes by PCR. F3 progeny from the heterozygotes carrying both mutations were grown in liquid culture with 0.05‰ (w/v) of ethidium bromide and visualized on a UV transilluminator (Bio-Rad, Hercules, CA). 0.5 μg of 100 bp DNA ladder (New England Biolabs, Ipswich, MA) were used as a reference to estimate the sizes of the DNA amplicons.

N-glycan Preparation and MALDI TOF MS Analysis—C. elegans were grown in liquid culture with E. coli OP50 in standard S complete medium, harvested after cultivation at room temperature (20 °C) for 4–6 days and purified by sucrose density centrifugation (in two independent preparations, the yield was 5 and 9 g of worms respectively). N-glycans were released from worm peptic peptides using peptide/N-glycosidase F as previously described, with a subsequent digestion of remaining glycopeptides using peptide/N-glycosidase A (23). The N-glycome of the mutant was profiled by MALDI-TOF MS (Autoflex Speed, Bruker Daltonics, Bremen, Germany) in positive ion mode using FlexControl 3.4 software. Free glycans were labeled with 2-aminopyridine prior to fractionation by normal phase high pressure liquid chromatography (NP-HPLC)1 and reversed-phase HPLC (RP-HPLC; see below). All the HPLC peaks were collected and examined by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) MS, using 6-aza-2-thiothymine as matrix; tandem MS (MS/MS) to confirm the composition of all proposed structures was performed by laser-induced dissociation (precursor ion selector was generally set to >0.6%). 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 six for MS (unsmoothed) and three for MS/MS (four-times smoothed). In total ~1500 MS and MS/MS spectra were manually interpreted on the basis of the mass, fragmentation pattern, and results of chemical and enzymatic treatments; isoobaric structures present in different RP–HPLC fractions were defined on the basis of comparisons in the aforementioned parameters. At least five MS/MS fragment ions were used to aid definition of each of the structures.

HPLC Purification of N-glycans—Separation of PA-labeled glycans was carried out on a Shimadzu HPLC system equipped with a fluorescence detector (RF 10 AXL). In case of NP-HPLC, a TSKgel Amide-80 column (Tosoh Bioscience, Tokyo, Japan) was used with 10 mM ammonium formate, pH 7.0 (buffer A) and 95% (v/v) acetonitrile (buffer B). The gradient of buffer B was applied as follows: 0–5 min, 75% B; 5–10 min, 75–70% B; 10–15 min, 70–65% B; 15–55 min, 65–55% B. In case of RP-HPLC, a Hypersil ODS column (Agilent, Santa Clara, CA) was used with 100 mM ammonium acetate, pH 4.0 (buffer C) and 30% (v/v) methanol (buffer D); a gradient of increasing buffer D (1% per minute) was programmed. The columns were calibrated daily in terms of glucose units (g.u.) with a pyridylaminated partial dextran hydrolysate.

Structural Elucidation Using Exoglycosidases and Chemical Treatment—In general, a 1 μl aliquot of a HPLC fraction was mixed with 0.2 μl exoglycosidase and 0.8 μl 50 mM ammonium acetate solution, pH 5.0; after an overnight incubation at 37 °C, 0.5 μl aliquot of the mixture was analyzed by MALDI-TOF MS. Exoglycosidases employed were: α-galactosidase from green coffee bean (Sigma, 11 μU), recombinant β-galactosidase from Aspergillus niger (24), jack bean α-mannosidase (Sigma-Aldrich, 6.25 μU) and recombinant Xan-

1 The abbreviations used are: MALDI-TOF, matrix-assisted laser desorption-ionization time-of-flight; NMR, nuclear magnetic resonance; NP-HPLC, normal-phase HPLC; PA, pyridylamin; PC, phosphorylcholine; RP-HPLC, reversed-phase HPLC; TOCSY, total correlation spectroscopy.
thomonas manihotis α1,2/3-mannosidase (NEB, 6.4 U). Also, digestions were attempted with α-L-fucosidases from bovine kidney (Sigma-Aldrich, 10 mU), Xanthomonas (α1,2-specific; NEB, 4 mU) and Corynebacterium (α1,2-specific; Takara, 4 μU). For removal of fucose or methylfucose, glycan samples were dried in a SpeedVac and then incubated with 3 μl of 48% (w/v) hydrofluoric acid (HF) on ice for 24 h. The HF was allowed to evaporate overnight. Chemically or enzymatically treated glycans were reanalyzed by MALDI-TOF MS and MS/MS without further purification.

**NMR Spectroscopy**—The isolated oligosaccharides have been lyophilized, dissolved in D$_2$O (99.996%; Sigma-Aldrich) in concentrations of ~150 μg in 600 μl and transferred into 5 mm high precision NMR sample tubes (Pramochem, Wesel, Germany). All spectra have been recorded on a Bruker AV III-600 AVANCE spectrometer (Bruker, Rheinstetten, Germany) at 600.13 MHz (1H) equipped with a CryoProbe™. Prodigy and were performed using the Bruker Topspin 3.1 software. The 1D proton spectra were recorded with presaturation, acquisition of 32 k data points and a relaxation delay of 1.0 s. After zero filling to 64 k data points and Fourier transformation spectra were obtained by RP-HPLC (Fig. 1A) and transferred into 5 mm high precision NMR sample tubes (Promochem, Wesel, Germany). All spectra have been recorded on a Bruker AV III-600 AVANCE spectrometer (Bruker, Rheinstetten, Germany) at 600.13 MHz (1H) equipped with a CryoProbe™. Prodigy and were performed using the Bruker Topspin 3.1 software. The 1D proton spectra were recorded with presaturation, acquisition of 32 k data points and a relaxation delay of 1.0 s. After zero filling to 64 k data points and Fourier transformation spectra were performed with a range of 7200 Hz. 2D homonuclear DOY-COSY and TOCSY (100 ms mixing time) spectra have been measured with standard Bruker programs; 128 experiments, each with 2048 data points, were recorded with an appropriate number of scans. Linear forward prediction to 256 data points in the f$_2$ dimension and sinusoidal multiplication in both dimensions and Fourier transformation led to 2D-spectra with a range of 6000 Hz in both dimensions. All measurements have been made at 298.1 K and chemical shifts were referenced to external acetone (δ$_{CH$_3$} = 2.225$ ppm).

**LC-MS/MS of HPLC-purified PA-labeled N-glycans**—PA-labeled N-glycans were analyzed by LC-MS/MS using a 10 cm × 150 μm i.D. column, prepared in-house, containing 5 μm porous graphitized carbon (PGC) particles (Thermo Scientific, Waltham, MA). Glycans were eluted using a linear gradient from 0–40% acetonitrile in 10 mM ammonium bicarbonate over 40 min at a flow rate of 10 μl/min. The eluted N-glycans were detected using a LTQ ion trap mass spectrometer (Thermo Scientific) in negative-ion mode with an electrospray voltage of 3.5 kV, capillary voltage of −33.0 V and capillary temperature of 300 °C. Air was used as a sheath gas and mass ranges were defined dependent on the specific structure to be analyzed. The data were processed using the Xcalibur software (version 2.0.7, Thermo Scientific). Glycans were identified from their MS/MS spectra by manual annotation; the nomenclature of Domon and Costello for fragment annotation was employed (25).

**RESULTS**

**Impact of a Triple Fucosyltransferase Knock-out on the C. elegans Glycome**—Previously, N-glycans with up to four fucose residues have been detected in C. elegans (13, 15, 26–30), whereas only three fucosyltransferases (FUT-1, FUT-6 and FUT-8) required for the trifucosylation of the core chitobiosyl region of N-glycans in C. elegans have been identified (13, 16, 17). With the goal of restricting the N-glycome of this organism to glycans with maximally one fucose, a triple mutant with deletions in the three corresponding genes (fut-1; fut-6; fut-8) was constructed. Deletions of all three genes were confirmed by genomic PCR (supplemental Fig. S1), but no overt major phenotypic defects were detected under laboratory conditions. The N-glycans were prepared after large-scale liquid cultivation of the worms and were subsequently fluorescently labeled with 2-aminopyridine; the overall mass spectrometric profile indicated the presence of a range of N-glycans (m/z 665–1961 as [M+H]+; Fig. 1A and Table I). More exact examination of the spectra showed mass differences of 2 or 14 Da (e.g. m/z 1135, 1149 and 1151 or 1297, 1311 and 1313 as [M+H]+); thereby, some Δm/z values between glycans were of 146 (deoxyhexose), 160 (methyl and deoxyhexose), or 162 (hexose). This was an indication for the presence of fucose on a number of glycans and suggested compositions of Hex$_3$-HexNAc$_2$-Fuc$_{0–1}$Me$_{0–2}$-PA. In addition to the fucosylated and/or methylated glycans, masses corresponding to oligomannosidic and phosphorylcholine-modified oligosaccharides were detected (Table I); the latter modification is defined because of the diagnostic m/z 369 MS/MS fragments (HexNAcPC$_1$; supplemental Fig. S2). However, as such glycans have been previously described in nematodes (14, 15, 18–20), their analysis was not the focus of the current study.

Upon incubating the complete N-glycome with jack bean α-mannosidase, the disappearance of the oligomannosidic glycans was accompanied by a large increase in the peak at m/z 665 (Hex, HexNAc$_2$-PA). Nevertheless, a number of glycans were not shifted by this treatment, including the one at m/z 1151, which is predicted to have the composition of Hex$_3$-HexNAc$_2$-PA. On the other hand, the glycan with the composition Hex$_3$-HexNAc$_2$-PA ([M+H]+ ion of m/z 1313) was now absent (Fig. 1B). Comparable data regarding mannosidase-resistance of some glycans of the mutant strain was obtained by RP-HPLC (Fig. 1C and 1D), which is well-established to also separate isomers of many N-glycans. Compatible with the overall mass spectrometric profiles, the major HPLC peak of 7.2 g.u. containing Man$_3$GlcNAc$_2$-PA and Man$_3$GlcNAc$_2$-PA was replaced, after mannosidase digestion, by a new peak of 6.5 g.u. containing a glycan of m/z 665 corresponding to Man$_3$GlcNAc$_2$-PA; the retention time for this “trimmed-down” product of the oligomannosidic glycan is in keeping with literature values (31). More significantly, a set of glycans eluting between 4.0 and 5.5 glucose units (g.u.) were seemingly resistant to this treatment because their elution position did not shift after incubation with the mannosidase. MALDI-TOF MS of the relevant fractions indicated that they contained glycans with the composition of Hex$_3$-HexNAc$_2$-Fuc$_{0–1}$Me$_{0–2}$-PA ([M+H]+ ion of m/z 989 to m/z 1311). As expected from having deletions in all three known core fucosyltransferase genes, MS/MS of these glycans strongly suggested that core fucosylation (which would be shown by a fragment of m/z 446, i.e. Fuc$_1$GlcNAc$_1$-PA) was absent, whereas in general MS/MS spectra of the various glycans primarily confirmed composition (Fig. 2). Treatment of the glycopeptides remaining after PNGase F digestion with PNGase A merely resulted in release of residual glycans of the same m/z as those in the PNGase F pool and that also displayed no sign of core fucosylation as judged by MS/MS (data not shown).

**Structure of Mannosidase-resistant Paucihexosidic N-glycans**—As a number of glycans in the triple mutant were...
Fig. 1. Total N-glycome of the fut-1;fut-6;fut-8 triple knockout and its sensitivity to jack bean α-mannosidase. PA-derivatised N-glycans before and after mannosidase digestion were profiled by MALDI-TOF MS (A and B) and by reversed phase HPLC (C and D); the region of panel B above m/z 900 is magnified fivefold because of the dominance of the m/z 665 mannosidase-digestion product. The major glycan structures identified in this mutant were detected as [M+H]^+ and are annotated on the HPLC chromatograms according to the nomenclature of the Consortium for Functional Glycomics. The intensity is in arbitrary units (a.u.).
Hex3–4HexNAc2-PA (C fraction eluting at 4.2 g.u. (see Fig. 1) containing glycans of these more closely, including the fucosylated forms. First, a positions normally associated with paucimannosidic N-glycans were confirmed to be mannosidase-resistant, but the C. elegans fut-1;fut-6; glcNAc-TIII has no homolog in nematodes and that bisecting GlcNAc is known to cause mannosidase-resistance of the two core α-mannose residues (32, 33), we postulated that a bisecting position (C-4) for β-galactose prevented removal of the nonsubstituted α-mannose residues. A C-2 modification of the core β-mannose is unlikely as glycans carrying a substitution on the C-2 position, such as β1,2-xylate, display a different mannosidase sensitivity to the glycans studied here (34).

In contrast, the fucose-containing glycan of m/z 1297 (m/z 1319 as [M+Na]⁺ in the 4.2 g.u. fraction was resistant to both α-mannosidase and β-galactosidase. A closer examination of the MS/MS spectrum shows that the fucose is two hexose residues distant from the distal GlcNAc as shown by the fragment ions at m/z 973 (or 987 in case of the methylated structures; Fig. 2). To explore the structure of Hex₄HexNAc₂Fuc₁-PA more thoroughly, we performed selected treatments on a 2D-HPLC fraction containing solely this glycan. Initially, we tried a number of fucosidase treatments, but no removal of fucose was observed (data not shown). However, the glycans is partially sensitive to hydrofluoric acid and the defucosylated portion was sensitive to β-galactosidase, but resistant to α-galactosidase (Fig. 4). Subsequently, one mannose could be released from the β-galactosidase-sensitive portion when incubating the glycan with α1,2-mannosidase; because of the initial galactosidase resistance, the pattern of digestion after hydrofluoric acid treatment suggests that the glycan has a fucose cap on the β-galactose of a structure the same as the aforementioned Hex₄HexNAc₂-PA. Regarding the type of linkage, we found in control experiments that hydrofluoric acid can remove α1,2-fucose residues, but not so efficiently as it cleaves α1,3-fucose (supplemental Fig. S3); however, longer incubations resulted in artifacts. Therefore, considering also previous GC-MS data showing the presence of 2-substituted galactose (27) and the multiplicity of α1,2-fucosyltransferase homologs (35) in C. elegans, we propose that the Hex₄HexNAc₂Fuc₁-PA contains an α1,2-fucose linked to the bisecting β-galactose. Further evidence came from NMR and LC-MS° experiments (see below).

Methylated Fucose as a Component of Paucihexosidic N-glycans—We also investigated the nature of N-glycans putatively containing methyl groups. The fraction of 5.2 g.u. (see Fig. 1C) containing glycans of Hex₃,₄HexNAc₂-PA (m/z 989 and 1151) and one of Hex₄HexNAc₂Fuc₁-PA (m/z 1297) was analyzed. All three glycans were confirmed to be mannosidase-resistant, but the two glycans lacking fucose lost one hexose upon treatment with recombinant Aspergillus β-galactosidase (Fig. 3). Subsequent incubation with α1,2/3-specific mannosidase resulted in primary products of Hex₁,₂HexNAc₂-PA. Considering previous GC-MS data that 3,4,6-trisubstituted mannose exists in C. elegans (27), whereas on the other hand the relevant

| Observed m/z | Calculated m/z | Predicted composition |
|--------------|----------------|----------------------|
| 665          | 665.29         | HN2                  |
| 827          | 827.34         | H2N2                 |
| 841          | 841.36         | H2N2Me               |
| 987          | 987.41         | H2N2FMe              |
| 989          | 989.39         | H3N2                 |
| 1135         | 1135.45        | H3N2F                |
| 1149         | 1149.47        | H3N2FMe              |
| 1151         | 1151.45        | H4N2                 |
| 1163         | 1163.48        | H3N2FMe2             |
| 1192         | 1192.47        | H3N3                 |
| 1297         | 1297.50        | H4N2F                |
| 1311         | 1311.52        | H4N2FMe              |
| 1313         | 1313.50        | H5N2                 |
| 1325         | 1325.54        | H4N2FMe2             |
| 1338         | 1338.53        | H3N3F                |
| 1339         | 1339.55        | H4N2FMe3             |
| 1357         | 1357.53        | H3N3PC               |
| 1395         | 1395.55        | H3N4                 |
| 1459         | 1459.56        | H5N2F                |
| 1473         | 1473.57        | H5N2FMe              |
| 1475         | 1475.55        | H6N2                 |
| 1560         | 1560.61        | H3N4PC               |
| 1598         | 1598.63        | H3N5                 |
| 1637         | 1637.60        | H7N2                 |
| 1725         | 1725.56        | H3N4PC2              |
| 1763         | 1763.69        | H3N5PC               |
| 1799         | 1799.66        | H8N2                 |
| 1928         | 1928.74        | H3N5PC2              |
| 1961         | 1961.71        | H9N2                 |
| 2093         | 2093.80        | H3N5PC3              |
| 2131         | 2131.82        | H3N6PC2              |
hydrofluoric acid-treated fraction was then incubated sequentially with β-galactosidase and α1,2/3-mannosidase, which resulted in products of m/z 827 and 665. The portion of Hex₃HexNAC₂Fuc₁Me₁-PA, which had not lost the methylfucose residue was, however, resistant to both hexosidases.

Among other fractions, we examined the β-galactosidase and hydrofluoric acid sensitivity of glycans of m/z 1135 (Hex₃HexNAC₂Fuc₁-PA) and 1163 (Hex₃HexNAC₂Fuc₁Me₂-PA) as well as two isomers of m/z 1311 (Hex₄HexNAC₂Fuc₁Me₁-PA) separated in the two fractions of 4.8 and 5.5 g.u.; the earlier fraction also contains an m/z 989 structure, which is a putative reducing-terminal epimer of standard Man₃GlcNAc₂ (epimerization of up to 10% of the reducing-terminal GlcNAc to ManNAC has been previously reported (36)). Whereas the m/z 1311 glycan (Hex₃HexNAC₂Fuc₁-PA (m/z 446) and Hex₃HexNAC₂Fuc₁Me₂-PA (m/z 608) indicated that there is no core fucosylation in this triple knockout. Other key ions such as Hex₃HexNAC₂Me-PA (m/z 841) and Hex₃HexNAC₂Fuc₁Me-PA (m/z 987) are indicative of the methyl group.

Fig. 2. MALDI-TOF MS/MS spectra of pyridylamino-labeled N-glycans carrying bisecting β-galactose. N-glycans from the fut-1;fut-6;fut-8 triple mutant separated by RP-HPLC (4.2–5.5 g.u.) were subject to MS/MS. Nonfucosylated forms (A and B), fucosylated forms (C and D) and methyl-fucosylated forms (E–H) displayed sequential “loss” of hexose and (methyl-) fucose residues or methylhexose (the latter only in G). For all (methyl-) fucosylated structures, the absence of key ions such as HexNAC₂Fuc₁-PA (m/z 446) and Hex₃HexNAC₂Fuc₁-PA (m/z 608) indicated that there is no core fucosylation in this triple knockout. Other key ions such as Hex₃HexNAC₂Me-PA (m/z 841) and Hex₃HexNAC₂Fuc₁Me-PA (m/z 987) are indicative of the methyl group.
both the fucose and the α1,3-mannose residues. This is compatible with the MS/MS spectrum indicating an m/z 841 fragment (putatively Man2GlcNAc2Me1-PA; Fig. 2G). Due to their similar fragmentation and digestion properties, it is concluded that the basic structures of the Hex3–4HexNAc2Fuc1 and Hex3–4HexNAc2Fuc1Me1 glycans are the same. Indeed, the fragmentation patterns of the isobaric hydrofluoric acid digestion products of m/z 1297 and m/z 1311 were highly similar suggestive of a common basic structure (supplemental Fig. S4C and S4D).

**NMR Analysis Indicative of Bisecting Galactose**—As the data from chemical and enzymatic digestions were indicative for an unusual location of galactose residues, which blocked the action of jack bean α-mannosidase, we sought further confirmation for our model of bisecting galactose being a feature of the mannosidase-resistant glycans of *C. elegans*. Because of the relatively low amount of glycan material available, we considered use of a nondestructive method of glycan analysis and so turned to NMR to yield further insights. To aid definition of the novel features of the putatively bisected glycans from the *C. elegans*, 1H NMR and homonuclear 2D NMR spectra of “classical” Man3GlcNAc2-PA and a pool of mannosidase-resistant paucihexosidic glycans from the triple mutant were compared (Fig. 7). Data on related N-glycan structures were used for comparative analysis of the interglycosidic bonds (37–39).

For the pool of putatively bisected glycans, the structural variants led to broadening or doubling of some proton NMR signals; certainly, the modification of the core β-D-Man (residue 3; see Fig. 7), as compared with standard Man3GlcNAc2-PA, can be surmised by the alterations in a number of the chemical shifts for this residue (e.g. 3.77, 3.80 and 3.85 ppm rather than 3.82, 3.69 and 3.77 ppm for the H-3, H-4 and H-6b protons; see Table II). The strong shift for H-4 can be taken as confirmation for a bisecting residue on C-4 of the core β-Man.
nose and so shares a trend observed with data on a bisected mammalian glycan (40), whereas the data are not compatible with a C-2 modification. The presence of \( \text{H}_{9252}^{-}\text{D-Galp} (\text{residue 4}) \) and some \( \text{H}_{9251}^{-}\text{d-Galp} (\text{residue C}) \) linked to mannose as well as some \( \text{H}_{9251}^{-}\text{L-Fucp} \) linked to galactose is shown by relevant chemical shifts typical for such residues (4.6–4.3 (38, 40), 5.5–5.1 (41), and 5.3–5.2 ppm (42) respectively for the anomeric H-1); for the latter, it is noteworthy that older NMR data was interpreted as showing \( \text{H}_{9251}^{-}1,2\text{-fucosylation of mannose} \) (43), but our data overall indicate the presence of a bisecting \( \text{Fucp} \) \( \text{1,2Gal} \) motif. Indeed, the proton signals of the \( \text{H}_{9252}^{-}\text{D-Galp} 4 \) show the most pronounced shift variations, which may be caused by the fucose or methylfucose bound in substoichiometric amounts to this unit, whereas the \( \alpha\)-galactose is assumed to be linked to position 2 of the \( \text{H}_{9251}^{-}\text{D-Manp} \) (residue 4). In conclusion, the NMR data support the presence of 3,4,6-trisubstituted bisected core \( \beta\)-mannose as well as the presence of galactose and fucose residues.

**LC-MS Analyses Confirm the \( \text{Fucp}1,2\text{Galp}1,4 \) Modification**—As a final analytical method, purified N-glycans were also applied to LC-MS/MS in negative ion mode (Fig. 8). All MS/MS spectra were dominated by an ion resulting from 2,4A cross-ring cleavage of penultimate GlcNAc, which are diagnostic for N-glycans. In the case of the singly-charged precursors, we observed \( Y_3 \) ions indicative of loss of one antenneral hexose ([M-H]^- ions such as m/z 825, 985 or 987 corresponding to the positive mode MALDI-TOF MS/MS [M+H]^+ fragment ions at m/z 827, 987 or 989 shown in Fig. 2) and B ions resulting from loss of GlcNAc-PA (e.g. m/z 688 in Fig. 8A and m/z 850 in Fig. 8B) together with 2,4A cross-ring cleavage of the penultimate GlcNAc suggested that the reducing-terminal GlcNAc-PA was not modified. Lower in-

![Figure 8](image_url)

**Fig. 8.** Structural analysis of a novel methylated Hex$_3$HexNAc$_2$Fuc$_1$Me-PA N-glycan. This \( \alpha\)-mannosidase resistant structure was separated by RP-HPLC, eluting at 5.2 glucose unit (A). HF treatment was first applied to the fraction and resulted in a partial conversion to Hex$_3$HexNAc$_2$-PA (m/z 989.4) (B). This product was incubated with either jack bean \( \alpha\)-mannosidase or \( \beta\)-galactosidase, but only the latter resulted in loss of a hexose residue (C and D). Further digestion of the \( \beta\)-galactosidase product using \( \alpha1,2/3\)-mannosidase resulted in removal of the “lower arm” mannose and formation of a final product with m/z 665.3.
intensity MS² ions, though, were more informative in terms of the linkage of the bisecting galactose and of the fucose residues.

To confirm the linkage of bisecting Gal, a glycan with composition of \( \text{Hex}_4\text{HexNAc}_2\text{Fuc}_1\text{Me}_1\)-PA \( ([\text{M}-\text{H}^-] \) ions at \( m/z \) 987) was analyzed (Fig. 8A). Fragment ions at \( m/z \) 627 \( (Z_{3,2}/Z_{1,0}) \) suggest bi-substitution by hexose residues of the \( \beta\)-Man. In comparison to a typical \( \text{Man}_9\text{GlcNAc}_2 \) N-glycan, “D ions” \( (m/z \) 323 and 305, mass of 6-antenna plus \( \beta\)-Man \( (44) \)) were absent indicating that one of the hexoses should be \( \alpha\)1,3-linked Man.

**Fig. 6. Structural characterization of two isomeric N-glycans by \( \alpha\)-galactosidase digestion and HF treatment.** Two different RP-HPLC fractions containing Hex\(_4\text{HexNAc}_2\text{Fuc}_1\text{Me}_1\)-PA with \( m/z \) 1311.6 (4.8 g.u. and 5.5 g.u.; A and D) were treated with \( \alpha\)-galactosidase (B and E); complete loss of one hexose was observed only for the late eluting fraction, resulting in a product of \( m/z \) 1149.5. Hydrofluoric acid treatment resulted in partial removal of either fucose or methylfucose (FMe) residues from Hex\(_4\text{HexNAc}_2\text{Fuc}_1\text{Me}_0\text{-PA} \) (C and F); thus, the fucose is not \( \alpha\)1,3-linked (which would be fully removed by this treatment) and not \( \alpha\)1,6-linked (which would be resistant), but the degree of release is compatible with the proposed Fuc\(_{1,2}\) linkage.
**FIG. 7.** $^1$H NMR and TOCSY of a standard trimannosyl N-glycan and the pool of bisected N-glycans. Separated signals in the $^1$H NMR spectra of the 7.2 g.u. fraction containing Man$_3$GlcNAc$_2$-PA (A) and of the 4.0–5.5 g.u. fractions (B, lacking the glycan of 4.8 g.u.) from the triple mutant are indicated according to the numbering shown on the structures. In the TOCSY spectra, separated cross peaks of the involved nuclei in according spin systems are marked. The chemical shifts of both compounds are listed in Table II.

**TABLE II**

$^1$H NMR chemical shifts (parts per million) of the mannosidase-resistant N-glycan pool. The nomenclature for the numbering of the saccharide units is according to that of Halbeek et al. (37). Structures and spectra are shown in Figure 7; numbers in brackets refer to the corresponding chemical shifts for the Man$_3$GlcNAc$_2$ structure. Significant differences in the chemical shifts (0.08–0.12 ppm) can be observed for H-3, H-4, and H-6b of the $\beta$-mannose 3 and the H-1 and H-2 of the $\alpha$1,3-linked mannose 4. nd not determined

| Unit | H-1 | H-2 | H-3 | H-4 | H-5 | H-6a | H-6b | Ac |
|------|-----|-----|-----|-----|-----|------|------|----|
| PA   | –   | –   | 6.69 (6.69) | 7.60 (7.60) | 6.75 (6.75) | 8.01 (8.00) | –   | –  |
| 1 (GlcNAc) | 3.61/3.43 (3.64/3.44) | 4.39 (4.38) | 3.94 (3.94) | 3.86 (3.86) | 3.91 (3.92) | 3.60 (3.60) | 3.47 (3.48) | 1.95 (1.96) |
| 2 (GlcNAc) | 4.67 (4.68) | 3.75 (3.76) | 3.83 (3.82) | 3.74 (3.75) | 3.52 (3.53) | 3.84 (3.84) | 3.74 (3.76) | 2.01 (2.10) |
| 3 ($\beta$Man) | 4.81 (4.81) | 4.22 (4.20) | 3.77 (3.82) | 3.80 (3.69) | 3.68 (nd) | 3.90 (3.90) | 3.85 (3.77) | –  |
| 4 ($\alpha$3Man) | 5.25 (5.13) | 4.21 (4.10) | 3.89 (3.92) | nd (3.59) | nd (3.64) | 3.84 (3.85) | 3.80 (3.79) | –  |
| 4’ ($\alpha$6Man) | 4.99/4.96 (4.95) | 4.11 (4.07) | 3.95 (3.93) | 3.64 (3.63) | 3.64 (nd) | 3.85 (3.90) | 3.75 (3.70) | –  |
| 4” ($\beta$4Gal) | 4.48–4.40 | 3.90–3.82 | 3.85–3.68 | 3.85–3.68 | – | – | – | – |
| C ($\alpha$Gal) | 5.57 | 3.57 | 3.86 | 3.50 | 4.16 | 3.78 | nd | – |

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FIG. 8. Detection of fucose-substituted and nonsubstituted bisecting Gal by LC-MS/MS. LC-MS/MS spectra of N-glycans from the 4.0–5.5 g.u. fut-1;fut-6;fut-8 pool: A, a paucihexosidic N-glycan containing a bisecting galactose (Hex$_3$HexNAc$_2$-PA, [M-H] of m/z 987), B, a Man$_3$GlcNAc$_2$ N-glycan with a bisecting Gal (Hex$_4$HexNAc$_2$-PA, [M-H] of m/z 1150), C, an N-glycan in which a methylated Fuc is linked to the bisecting Gal (Hex$_3$HexNAc$_2$Fuc$_1$Me$_1$-PA, [M-H] of m/z 1147), (D) an bisectet Man$_3$GlcNAc$_2$ N-glycan in which Fuc is linked to the bisecting Gal (Hex$_4$HexNAc$_2$Fuc$_1$-PA, [M-2H] of m/z 647). Schematic representations explaining the Domon and Costello nomenclature of indicative glycosidic or cross-ring cleavages are shown in the panels on the right.
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This was further confirmed by the presence of Z₃/₄₅CH₂O ions at m/z 597, which indicated a C-3 and C-4 bi-substitution of the β-Man (Fig. 8A, right panel); this is similar to the Z₅/₇CH₂O fragmentation pattern of a Lewis type structure (45). In addition, fragment ions at m/z 443 were concluded to result from 0.2A cleavage of β-Man, which would be absent if the C-2 of the core β-mannose is substituted; together with the β-galactosidase sensitivity and NMR data, the MS/MS results are compatible with this glycan carrying a bisecting β1,4-linked Gal. Furthermore, the MS/MS spectrum of a glycan with a 6-antenna (composition of Hex₅HexNAc₂-PA, [M-H⁻] ions at m/z 1150; Fig. 8B) shows triple Z ions (Z₃/₄₅Z₃/₄₅) at m/z 609 indicative that the β-Man is tri-substituted with two Man and one Gal. D ions at m/z 485 and 323 (with or without bisecting Gal) were also consistent with the presence of tri-substituted β-Man. Thus, this structure was assigned as Man₃GlcNAc₂ modified with a bisecting Gal.

To confirm that fucose could modify the bisecting Gal, a glycan with composition of Hex₅HexNAc₂FucMe₁-PA was analyzed (m/z 1147; Fig. 8C). The 2,4A cleavage of the penultimate GlcNAc (ion at m/z 705) and B₂ ions at m/z 848 suggested that the fucose was on the nonreducing end of the N-glycan; the Y₃/₄₅ ions at m/z 985 and B₂/C₃/₄₅ ions m/z 321/339 are compatible with the presence of one terminal hexose and one terminal Me-Fuc-Hex motif (322 Da). The Z₃/₄₅CH₂O ions at m/z 597 indicate, as discussed for Fig. 8A, the C3 and C4 bi-substitution of the β-Man (i.e. by 1,3-linked mannose and a methylfucose-capped β1,4-linked galactose). The 0.2A cleavage of β-Man (0.2A ions at m/z 603) and dominant fragment ions at m/z 423 (0.2A₃/₄₅H₂O/Y₃/₄₅) suggest that the methylated Fuc is linked to the bisecting Gal. A further variant is exemplified by a structure with an additional α,1,6-linked Man (Hex₅HexNAc₂FucMe₁-PA, m/z 1310; Fig. 8D) displaying an 0.2A₃/₄₅H₂O ion at m/z 747 and an 0.2A₃/₄₅Y₃/₄₅ ion at m/z 603. As for the linkage between Fuc and the bisecting Gal, MS/MS of a doubly charged ion (Hex₅HexNAc₂FucMe₁-PA, [M-2H]²⁻ ions at m/z 647; Fig. 8E) resulted in fragment ions at m/z 205 (which could be because of either 0.2A₃/₄₅Gal, 1.3A₃/₄₅, or 2.4A₃/₄₅Gal cleavage) but not at m/z 265 (0.2A₃/₄₅Gal); this is suggestive of a 1.3A cleavage of bisecting Gal, which would correlate with Fuc substituting C-2 of the bisecting Gal. Fragmentation ions at both m/z 205 and m/z 247 (FucGal₁-C₂H₂O₂) are diagnostic for an H type structure (45). Thus, consistent with the partial sensitivity toward hydrofluoric acid, we conclude that the bisecting Gal is modified with α,1,2-linked Fuc.

Galactose as a Component of Paucihexosidic N-glycans in Wild-type C. elegans—Considering the results with the triple mutant, we sought for glycans of the same RP-HPLC retention time (4.0–5.5 g.u.) as the mannosidase-resistant forms in other strains. As part of an earlier study, extensive 2D-HPLC fractionation (normal phase followed by reversed phase) had been performed on the pmk-1 strain, which is defective in a p38 MAP kinase homolog and is hypersensitive to some fungal galectins (46), but in glycosylation terms is “pseudowild-type”. We found one 2D-HPLC fraction containing a glycan with the composition Hex₅HexNAc₂-PA (m/z 1313), which did not co-elute with standard Man₉GlcNAc₂-PA (7.2 g.u.) on a standard RP-HPLC column, but instead eluted at 4.6 g.u., i.e. within the elution range for the mannosidase-resistant glycans from the triple mutant. The MS/MS spectrum of this structure was also different as compared with that of the standard Man₉GlcNAc₂ (i.e. the dominance of the m/z 827 fragment in the latter was not apparent; supplemental Fig. S4E and S4F).

Considering the hexosidases available and data indicative for the presence of galactose on C. elegans N-glycans, we attempted both α-mannosidase and α- or β-galactosidase treatments of this glycan. Indeed, the Hex₅HexNAc₂-PA from the pmk-1 strain was resistant to α-mannosidase, but sensitive to the recombinant fungal β1,4-galactosidase (Fig. 9). After this treatment, a further hexose could then be removed with jack bean α-mannosidase, but further digestion was still not possible. We observed that α-galactosidase could remove one hexose and that a final hexose is cleaved by an α,1,3,2-specific mannosidase. Thus, the Hex₅HexNAc₂-PA glycan was concluded to have the composition Gal₅Man₃GlcNAc₂; the digestion data is compatible with a bisecting position for β-galactose and α-galactosylation of the α,1,3-mannose residue. Furthermore, in the wild-type N2 embryo N-glycome, an Hex₅HexNAc₂ glycan (4.2 g.u.) displayed a similar α-mannosidase resistance, but β-galactosidase sensitivity, as for the co-eluting m/z 1151 glycan from the triple mutant (data not shown).

DISCUSSION

Studies over the past 15 years have revealed a huge diversity in the N-glycome of the “simple” multicellular nematode Caenorhabditis elegans (3, 14). Some of the features are shared with other parasitic and nonparasitic nematode species (18–20, 47, 48). Examples include trifucosylation of the core chitobiose (including fucosylation of the distal core GlcNAc), galactosylation of core fucose residues and the antennal modification with phosphorylcholine. On the other hand, although antennal α,1,3-fucosylation (Lewis and Lewis-type epitopes) is known from some parasitic nematode species (49), this feature is lacking from C. elegans. However, a fourth fucose residue is apparent in a subset of N-glycans of C. elegans (13, 15, 26–30) and structural models have suggested that this is attached either directly or indirectly (via galactose) to α-mannose residues of the trimannosylchitobiosyl core (15, 26, 50, 51). However, tetrafucosylation of a trimannosylchitobiosyl core is a structural proposition contradicting not only the known specificity of the enzyme (the FUT-6 α,1,3-fucosyltransferase), which modifies the distal core GlcNAc (17), but also GC-MS data indicating that a portion of the core β-mannose residues is 3,4,6-trisubstituted and that 2-substituted galactose is also present in wild-type C. elegans (27).
yield the core trisaccharide structure (\(m/z\) 665.2). In order to simplify the N-glycome, we constructed a triple mutant containing maximally one fucose residue. Focusing on a range of HPLC-enriched N-glycans, which possess up to five hexose residues, but which were \(\alpha\)-mannosidase resistant (hence “pauchihexosidic”), we reveal, using chemical and enzymatic treatments in conjunction with off-line MALDI-TOF MS and on-line LC-MS as well as NMR, that the fucose residue on such glycans is \(\alpha\)1,2-linked via a bisecting \(\beta\)1,4-galactose to the core \(\beta\)-mannose. Thereby, some of these glycans either lack an \(\alpha\)1,6-mannose or carry an \(\alpha\)-galactose on the \(\alpha\)1,3-mannose; variants of these glycans are also methylated.

Preliminary data, as well as the aforementioned “old” GC-MS data (27), lead us to believe that the antennal fucose is also significantly present on core fucosylated glycans in the wild-type. Furthermore, we have previously shown that the “GalFuc” epitope can also carry a fucose residue (18), which yields a fifth attachment point for fucose on C. elegans N-glycans; nevertheless, only maximally four fucose residues have ever been detected on oligosaccharides of this organism. The difficulty in digesting these bisected N-glycans with glycosidases may be explained by predicted 3D-conformations suggesting that the bisecting modification “folds back” onto the core of the glycan (supplemental Fig. S5).

Thus, after some fifteen years of work on C. elegans glycans by various laboratories, we offer a reinterpretation of a number of previously-published studies regarding the N-glycome of this organism, which may have also repercussions for the understanding of data regarding glycan-binding nematotoxic proteins. As a double hexosaminidase worm mutant, which apparently lacks bisecting galactose (18), is completely or partially resistant to tectonin and Mpl (51, 56), it would be attractive to propose that the binding sites on N-glycan antennae for these nematoxins are indeed the methylated or nonmethylated forms of the \(\alpha\)1,2-fucose attached to the bisecting galactose. Armed with the exact structure of C. elegans N-glycans, the binding specificities of such proteins can be reassessed; furthermore, such glycomic knowledge is a prerequisite to fine-tune approaches to understand the biological significance of the seemingly endless variation of the C. elegans glycome in order to make this otherwise well-understood organism into a system truly suitable for examination of glycobiological paradigms.

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**Fig. 9.** Sequential enzymatic digestions of an Hex\(_5\)HexNAc\(_2\) isofrom. A structure ([M+H]+) produced by the pmk-1 strain was isolated on HPLC via a two-dimensional approach (NP-HPLC followed by RP-HPLC); this glycan elutes unusually early (4.6 g.u.) on the reversed phase column (A). Jack bean \(\alpha\)-mannosidase digestion resulted in no loss of mannose (B), whereas \(\beta\)-galactosidase removed one hexose residue (C). Mannosidase digestion of the \(\beta\)-galactosidase product resulted in additional loss of one hexose (D) yielding a glycan ([M+H]+ 989.3), which was further trimmed down to Hex\(_4\)HexNAc\(_2\)-PA with green coffee bean \(\alpha\)-galactosidase (E). Finally, the Hex\(_3\)HexNAc\(_2\)-PA was digested by \(\alpha\)1,2/3-mannosidase (F) to yield the core trisaccharide structure ([M+H]+ 665.2).
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