Keywords: Galβ1,4Fuc epitope; planaria; N-glycan; methylhexose; mass spectrometry

INTRODUCTION

Glycans cover the surfaces of all cells and, therefore, it is expected that many cell–cell interactions, whether between the cells of an individual organism or between symbionts or pathogens and their hosts, are glycan dependent.[1] As some of these glycan-based cell–cell interactions are of developmental relevance, it is of interest to identify paradigmatic examples of simple and/or tractable developmental systems, examples being those of slime moulds, nematodes or insects.[2] The planaria represent another developmental model, whose properties are remarkable in the context of cell programming, as by means of pluripotent neoblasts, a large degree of regeneration of a wounded animal is possible.[3,4] However, despite the general biological knowledge about planaria and the identification of some differentially expressed potential C-type lectins in one species, Girardia tigrina, and the identification of some differentially expressed potential of these organisms. Therefore, we examined the N-glycans of one of them, Dugesia japonica; such data are a pre-requisite before embarking on studies examining whether the glycome has a role in the special regenerative properties of planarian species. By the use of MS/MS and exoglycosidase digestion, we extend data recently published on the N-glycans of this organism.[5]

EXPERIMENTAL PROCEDURES

Sample preparation

The planarians (D. japonica) were harvested from local fresh water streams in Japan and cultured in water at 15 °C in the dark. They were occasionally fed with chicken liver. Before harvesting, they were starved for a week, washed with phosphate-buffered saline (pH 7.4) several times and subjected to homogenisation (dissected and then sonicated) in 10 mm phosphate buffer, pH 7.0. The pellet was isolated by centrifugation (3000 g, 15 min, 4 °C) and suspended in acetone/water (1 : 1, v/v) several times. The final pellet suspended in water was subjected to lyophilisation. Glycopeptides were prepared from the lyophilised material (6 mg) using pepsin, basically as previously described[8]; after initial ion-exchange (Dowex 50 W × 8, Sigma–Aldrich) and gel filtration (Sephadex G25, GE Healthcare) chromatography, the sample was dissolved in 50 μl of 5% (v/v) ammonia. In order to avoid later fluorescent labelling of residual non-N-glycan free oligosaccharides in the mixture (see also Section on Results), the sample was pre-reduced with 50 μl of 1% (w/v) sodium borohydride at room temperature for 2 h prior to the addition of 2 μl of acetic acid and subsequent lyophilisation. The N-linked glycans were released using peptide:N-glycosidase A (Roche). Thereafter, the sample was subject to Dowex 50 W × 8 cation exchange chromatography; the unbound fraction was pyridylaminated at pH 7[9] and excess reagent was removed by gel filtration (Sephadex G15, GE Healthcare).

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HPLC and MALDI-TOF MS analyses

The labelled N-glycans were analysed by reversed-phase and normal-phase HPLC using, respectively, an MZ Analytik ODS Hypersil and a Takara Palpak type N column on a Shimadzu HPLC system equipped with a fluorescence detector. In the case of RP-HPLC, a linear gradient of 0.3%/min methanol in 0.1 m ammonium acetate, pH 4, was applied. For NP-HPLC, buffer A was a 25:75 mixture of 3% acetic acid adjusted with triethylamine and acetonitrile, whereas buffer B was a 50:50 mixture. The gradient of buffer B was applied as follows: 0–5 min, 10% B; 5–45 min, 10–100% B; 45–50 min, 100% B; followed by a return to the starting conditions. The columns were calibrated with an pyridylaminated oligoglucose standard as well as with N-glycans prepared from Drosophila melanogaster S2 cells. Glycans were detected by fluorescence (excitation, 310 or 320 nm; emission, 380 or 400 nm). In the case of the RP-HPLC, each fraction was lyophilised and dissolved in 10 µl; 1 µl thereof was dried on a steel sample plate under vacuum before applying either 2,5-dihydroxybenzoic acid or 6-aza-2-thiothymine as matrix, which was again dried under vacuum. The samples were then analysed in positive mode by MALDI-TOF MS using a Bruker Ultraflex I equipped with a nitrogen laser (337 nm; laser frequency of 50 Hz and pulse length of 200 ns); typically 400–1000 shots were summed. Selected species were further examined by MS/MS (post-source decay).

Exoglycosidase digestion

An aliquot of fraction X (1 µl) was mixed with 0.5 µl of 0.1 m ammonium acetate, pH 5, buffer in a PCR tube together with either bovine α-fucosidase (10 mU), Aspergillus oryzae β1,4-galactosidase (70 mU) or a combination of the two enzymes and incubated overnight at 37 °C, prior to MALDI-TOF MS and MS/MS analysis with 6-aza-2-thiothymine as the matrix.

RESULTS

Overall N-glycomic analysis of Dugesia japonica

The basic procedures for the analysis of the N-glycans of the planaria D. japonica were performed as with other organisms; however, as initial studies indicated that a polyhexose series of unknown origin was present, a subsequent preparation was subject to reduction prior to the release of the N-glycans, so that only the released glycans and not the polyhexose compounds could be later labelled by the reductive pyridylamination method. Subsequent NP-HPLC analysis, using an isomalto-oligosaccharide series and a sample of N-glycans of insect S2 cells as standards, indicated that D. japonica expressed a range of glycans co-eluting, in part, with an oligomannosidic series (Fig. 1(A)). However, the major fraction of five glucose units (g.u.) did not co-elute with any of the S2 glycans.

Figure 1. Chromatographic analysis of pyridylaminated Dugesia N-glycans. Normal phase (NP) and reverse phase (RP) HPLC of planaria glycans were performed in comparison to an isomalto-oligosaccharide series (3–10 g.u.) and a preparation of N-glycans from Drosophila S2 cells. The ten collected RP-HPLC fractions are indicated in roman numerals, while the major fractions of S2 cells are annotated according to the glycans detected by MALDI-TOF MS: MM/MMF (Man3GlcNAc2Fuc0–1) and M5/M6/M7/M8/M9 (Man5–9GlcNAc2).
MS analysis of planaria N-glycans

As the triethylamine buffer used for Palpak NP-HPLC is problematic in terms of later MALDI-TOF MS, RP-HPLC was also performed, using also isomalto-oligosaccharides and S2 N-glycans as calibrants, in order to separate fractions prior to mass spectrometry. Ten fractions (I–X; Fig. 1(B)) containing obvious glycans were isolated and the subsequently acquired spectra (Supporting Information Fig. 1) indicated that Hex5–1 0 HexNAc2 were present in a number of fractions with retention times compatible with those of the S2 glycans. In addition, a number of putatively methylated species were detected. Other than for the unmodified oligomannosidic glycans, MS/MS was performed in order to verify the putative compositions (Table 1).

MS/MS analysis of methylated oligomannosidic glycans of Dugesia

The major fraction (VIII) was found to contain predominantly a glycan with an m/z value of 1355; under consideration of the biosynthetic pathway for N-glycans in eukaryotes, the MS/MS data suggest that this corresponds to a Man5GlcNAc2 glycan with methylation of the three terminal mannose residues as indicated by three losses of m/z 176 and two of m/z 162 from the parent ion (Fig. 2). The significance of the presence of a glycan with the same composition and similar fragmentation in fraction V is unclear; epimerisation of the core GlcNAc during derivatisation may be an explanation, as an earlier retention time for ManNAc-PA as opposed to GlcNAc-PA has been previously reported.12

A number of other glycans are also predicted by interpretation of MS/MS spectra (Supporting Information Fig. 2) to have terminal methylation of mannose residues: trimethylation of putative Man6–9 GlcNAc2 is apparent as is dimethylation of putative Man3,6,8,9GlcNAc2. For instance, the major components of fractions Table 1. Summary of MALDI-TOF MS analysis of RP-HPLC fractions

| m/z [M + H+] | m/z [M + Na+] | Composition | Fraction no. | Notes |
|--------------|--------------|-------------|-------------|------|
| 1017         | 1039         | H13N2Me2    | Fr. IX      | MS/MS 176 |
| 1313         | 1335         | H13N2       | Fr. IV      | Co-elution |
| 1325         | 1347         | H13N2F1Me2  | Fr. X       | MS/MS 607 |
| 1341         | 1363         | H13N2Me2    | Fr. VII     | Prediction |
| 1355         | 1377         | H13N2Me3    | Fr. V/VIII  | MS/MS 176 |
| 1475         | 1497         | H13N2       | Fr. III     | Co-elution |
| 1500         | 1522         | H13N2F1     | Fr. IX      | MS/MS 607 |
| 1501         | 1523         | H13N2F1Me3  | Fr. X       | MS/MS 783 |
| 1503         | 1525         | H13N2Me2    | Fr. VI      | MS/MS 176 |
| 1517         | 1539         | H13N2Me3    | Fr. V/VIII  | MS/MS 176 |
| 1531         | 1553         | H13N2Me4    | Fr. IX      | MS/MS 176 |
| 1637         | 1659         | H13N2       | Fr. III     | Co-elution |
| 1679         | 1701         | H13N2Me3    | Fr. VI/IX   | MS/MS 176 |
| 1799         | 1821         | H13N2       | Fr. I       | Co-elution |
| 1827         | 1849         | H13N2Me2    | Fr. V       | MS/MS 176 |
| 1841         | 1863         | H13N2Me3    | Fr. VI      | MS/MS 176 |
| 1961         | 1983         | H13N2       | Fr. II      | Co-elution |
| 1989         | 2011         | H13N2Me2    | Fr. VI      | MS/MS 176 |
| 2003         | 2025         | H13N2Me3    | Fr. VII     | MS/MS 176 |
| 2123         | 2145         | H13N2       | Fr. III     | Prediction |

F, fucose; H, hexose; N, HexNAc.

The m/z values and predicted compositions of pyridylaminated Dugesia N-glycans observed in the collected fractions I–X. As appropriate, further data aiding identification of the glycan are noted: either MS/MS with an indication of a key diagnostic fragment (see Supporting Information Data for all MS and MS/MS spectra) or co-elution with an oligomannosidic glycan from S2 cells. The m/z [M + Na+] indicated to facilitate comparison with data from the literature.

![Figure 2. Mass spectrometric analysis of the dominant Dugesia N-glycan.](image-url)

The major RP-HPLC fraction (VIII) was analysed by MALDI-TOF MS and the dominant species with m/z 1355 (see inset) and putative composition Hex5HexNAc2Me3-PA was further examined by MS/MS. The key diagnostic fragments are those of m/z 176 (methylhexose), 203 (internal HexNAc), 299 (HexNAc-PA), 365 (Hex;HexNAc; putatively Manβ1,4GlcNAc), 502 (HexNAc-PA), 541 (Hex;HexNAc;Me1; putatively MeManα1,3Manβ1,4GlcNAc), 664 (Hex;HexNAc;Me1; putatively MeManα1,3Manβ1,4GlcNAc), 841 (Hex;HexNAc;Me1;PA), 1003 (Hex;HexNAc;Me1;PA; loss of two terminal methylhexose residue), 1055 (Hex;HexNAc;Me3;PA; loss of HexNAc-PA) and 1179 (Hex;HexNAc;Me3;PA; loss of one terminal methylhexose residue). The proposed structure is shown according to the nomenclature of the Consortium for Functional Glycomics (Man, circles; GlcNAc, squares). Of the remaining annotated molecular ions in this fraction, those with m/z 1188 and 1222 have MS/MS spectra indicating that they are not glycans (present in fractions VI–IX; see also Supporting Information Fig. 1), whereas the ion with m/z 1517 corresponds to Hex4HexNAc2Me3.
VI (Hex$_4$HexNAc$_2$Me$_2$; m/z 1841) and IX (Hex$_3$HexNAc$_2$Me$_2$; m/z 1017) are also, on the basis of MS/MS data predicted to contain, respectively, three and two terminal methylhexose residues each. Another glycan in fraction IX has the predicted composition Hex$_4$HexNAc$_2$Me$_2$; the fragment of this tetramethylated glycan with m/z 1017 would be compatible with the presence of two methylated mannose residues linked in series to the mannosylchitobiosyl core.

**Analysis of a late-eluting fraction of Dugesia N-glycans**

Among the fractions putatively containing methylated glycans was fraction X; two species with m/z 1325 and 1501 were detected which could correspond to Hex$_4$HexNAc$_2$Fuc$_1$Me$_2$ and Hex$_5$HexNAc$_2$Fuc$_1$Me$_3$, respectively (Fig. 3). In order to examine these glycans further, fraction X was subjected to exoglycosidase digestions with either bovine $\alpha$-fucosidase, which cleaves core $\alpha$1,6-fucose linkages 20 times more rapidly than core $\alpha$1,3-fucose,[13] or a fungal galactosidase, previously shown to be $\beta$1,4-specific.[14] Fucosidase alone did not alter the MALDI-TOF MS spectrum; however, in the case of the galactosidase, loss of the m/z 1325 glycan was accompanied by the appearance of a glycan of m/z 1163. A provisional conclusion was that a $\beta$1,4-galactose residue ‘blocks’ the action of the fucosidase towards Hex$_4$HexNAc$_2$Fuc$_1$Me$_2$, indicative of the presence of a Gal$\beta$1,4Fuc moiety on the reducing-terminal GlcNAc; this supposition was confirmed by using a combination of both enzymes, which resulted in digestion to a species with m/z 1017. The putative Hex$_5$HexNAc$_2$Fuc$_1$Me$_3$ glycan was not affected by either treatment; considering also the MS/MS data (see below),

![Figure 3](image-url)

**Figure 3.** Exoglycosidase digestion of a *Dugesia* galacto-fucosylated N-glycan. The RP-HPLC purified fraction X was incubated with either no enzyme, $\alpha$1,6-fucosidase, $\beta$1,4-galactosidase or a combination of fucosidase and galactosidase; the products were analysed by MALDI-TOF MS. Treatment with fucosidase alone resulted in no digestion; however, in the presence of galactosidase, the m/z 1325 species (Hex$_4$HexNAc$_2$Fuc$_1$Me$_2$-PA) was converted to a species with m/z 1163, consistent with the removal of one hexose. A combination of both galactosidase and fucosidase resulted in a product with m/z 1017, consistent with the loss of both a galactose and a fucose residue; the m/z 1501 species (Hex$_5$HexNAc$_2$Fuc$_1$Me$_3$-PA) was resistant to this treatment. The m/z 1054 and 1379 species, marked with an asterisk, are components present in the galactosidase preparation. Proposed structures for both the m/z 1325 and 1501 glycans, based also on MS/MS data shown in Fig. 4, are depicted according to the nomenclature of the Consortium for Functional Glycomics (Gal, yellow circles; undefined Hex, white circle; Man, green circles; Fuc, red triangle; GlcNAc, blue squares).
it is concluded that, on this glycan, the Gal\(^{\beta}1,4\)Fuc modification is ‘capped’ with a methylhexose residue; a precedent for such a modification is the hexose capping of ‘GalFuc’ observed on glycans from keyhole limpet hemocyanin\(^{[15]}\) and nematodes.\(^{[16]}\) The RP-HPLC elution time of the original fraction (ca 30 min) is also compatible with the presence of the ‘GalFuc’ modification, as glycans from Caenorhabditis elegans carrying this moiety display a late retention time.\(^{[17]}\)

The undigested and digested forms of fraction X were examined by MS/MS. For the two major species in the undigested sample, major fragmentation products of \(m/z\) 607 (Hex\(_2\)HexNAc\(_1\)Fuc\(_1\)PA) and 783 (Hex\(_2\)HexNAc\(_1\)Fuc\(_1\)Me\(_1\)PA) were observed, the former being reminiscent of a fragment from nematode glycans carrying galactosylated core fucose residues.\(^{[18]}\) Upon galactosidase and combined galactosidase/fucosidase digestion, the \(m/z\) 607 fragment was no longer observed; indeed, MS/MS of the product of galactosidase digestion resulted in a fragment of \(m/z\) 445 (Fuc\(_1\)HexNAc\(_1\)PA), which was no longer apparent in the product of combined galactosidase/fucosidase treatment (Fig. 4).

It is also concluded, by analogy to other structures in this species and from the fragmentation pattern, that the two putative terminal mannose residues are also methylated in these two glycans. The \(m/z\) 607 fragment was also observed when analysing a glycan in fraction IX whose composition is probably Hex\(_2\)HexNAc\(_2\)Fuc\(_1\) (\(m/z\) 1500; see Supporting Information Fig. 2); however, the amount of this glycan was too low for further analysis.

**DISCUSSION**

As shown by MALDI-TOF MS analyses of RP-HPLC fractions, D. japonica expresses an unusual range of glycans including standard oligomannosidic, methylated oligomannosidic and at least three galacto-fucosylated N-glycans. As this manuscript was in preparation, a study verifying the presence of the former two glycan categories was published; the authors also showed the

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**Figure 4.** Mass spectrometric analysis of Dugesia galacto-fucosylated N-glycans. The late-eluting RP-HPLC fraction (X) was analysed by MALDI-TOF MS either without (A and D) or with digestion with galactosidase (B) or a combination of fucosidase and galactosidase (C) (Fig. 3) and the major species were subject to MS/MS. Diagnostic fragments include those of \(m/z\) 203 (internal HexNAc), 299 (HexNAc\(_1\)PA), 445 (Fuc\(_1\)HexNAc\(_1\)PA), 502 (HexNAc\(_2\)PA), 607 (Hex\(_1\)Fuc\(_1\)HexNAc\(_1\)PA), 718 (Me\(_1\)Hex\(_1\)HexNAc\(_1\)), 783 (Me\(_1\)Hex\(_1\)Fuc\(_1\)HexNAc\(_1\)PA), 840 (Me\(_1\)Hex\(_2\)HexNAc\(_2\)PA, loss of the ‘GalFuc’ modification and loss of one terminal methylhexose) and 1017 (Me\(_2\)Hex\(_3\)HexNAc\(_2\)PA, loss of ‘GalFuc’).
presence of a glycan with the same mass as one of the galacto-
fucosylated glycans described here, but neither exoglycosidase
digestions nor MS/MS analyses were apparently performed and
the presence of a ‘GalFuc’ epitope was not postulated.[7] On
the other hand, they suggested the presence of a pentose
and a fucose or two fucose residues on one of the glycans;
however, masses compatible with such modifications, which are
conceivable for invertebrates, were not revealed in our analysis.
Certainly, both their report and ours agree that the major glycan is
a trimethylated species, most probably with the composition
Man3GlCNAC3M6Me3, whereby monosaccharide analyses would
indicate 3-O-methylation of mannose, a modification conferring
resistance to α-mannosidase digestion[17]; our MS/MS data are
compatible with a terminal location of the methyl residues.
Methylation of terminal mannose residues is known, for instance,
on some mollusc N-glycans.[10,19,20]

Our data indicating modification of planaria glycans by
Galβ1,4Fuc indicate that this epitope is more wide- 
previously thought; until now the only reports of this moiety
were on glycans from molluscs, such as squid,[21] octopus[22] and
keyhole limpet, [15] as well as from the nematode
C. elegans.[16] Recently, Galβ1,4Fuc was demonstrated to be the target of
a fungal nematotoxic lectin, CGL2, and nematode mutants
with defects in either the core α1,6-fucosyltransferase FUT-8 or a novel
β1,4-galactosyltransferase GALT-1 were resistant to this lectin.[18]
Therefore, it will be interesting to observe whether platyhelminths
other than planaria express this epitope, particularly as some
trematode and cestode species (e.g. Schisotoma spp. and
Echinococcus spp.) are parasitic and that new therapeutic strategies
are being sought.

Another aspect of our data is that we can predict some as-
psects of the glycogenomic capabilities of planaria. For instance,
unlike some parasitic protозoa,[23] we expect that planaria contain
the full complement of mannosyltransferases required for syn-
thesis of the dolichol-linked precursor for N-glycosylation as, at
least, a Hexα1HexNAc2 glycan, putatively Glc1Manα1GlCNAC2 was
detected; also, there should be a core α1,6-fucosyltransferase and
a fucoside-modifying β1,4-galactosyltransferase as well as sugar
methyltransferases (see Fig. 5 for a potential biosynthetic scheme).
Depending on the processing of glycans in the planaria Golgi
apparatus, mannosidases and an N-acetylgalcosaminyltransferase I
should be encoded by the genome. However, considering the
almost complete absence of non-reducing terminal GlcNAc from
the observed glycans, we would postulate that a Golgi hex-
osaminidase is present, as is the case in insects and nematodes.[17]

Preliminary homology-based searching of expressed sequence
tags and draft partial genome sequences available from
S. mediterranea would indeed suggest that homologues of
N-acetylgalcosaminyltransferase I, core α1,6-fucosyltransferase
and a fucoside-modifying β1,4-galactosyltransferase are present
in at least one related planaria species (data not shown). The
detection of a glycan with the composition Hexα1HexNAc3Fuc1
would indeed suggest that homologues of
N-acetylgalcosaminyltransferase I being a pre-requisite for core
fucosylation and subsequent capping with galactose, as is the
case with recombinant C. elegans FUT-8 and GALT-1.[24]

Considering that the Galβ1,4Fuc epitope is a target of some
galactins, it is relevant to consider the biological roles that
these proteins have in other organisms. In mammals, other
types of terminal galactose residues are recognised by galectins,
which have a number of roles, e.g. in immunity,[25] infection[26]
and cellular regulation,[27] whereas in C. elegans, LEC-6 is an
endogenous ‘GalFuc’ receptor.[28] Therefore, one question is
whether galectins of planaria also have such roles and whether,
in the absence of obvious endogenous N-acetyllactosamine
modifications on N-glycans, they are capable of interacting with
the Galβ1,4Fuc epitope. It is naturally quite another question
to determine whether this modification or any other, such as
methylation, has a relevance to the regenerative ability of these
organisms, but identification of their N-glycans is a first step in
order to design strategies to do so.

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Supporting information

Supporting information may be found in the online version of this
article.
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