The N-glycans of *Trichomonas vaginalis* contain variable core and antennal modifications

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Trichomonad species are widespread unicellular flagellated parasites of vertebrates which interact with their hosts through carbohydrate–lectin interactions. In the past, some data have been accumulated regarding their lipo(phospho)glycans, a major glycoconjugate on their cell surfaces; on the other hand, other than biosynthetic aspects, few details about their N-linked oligosaccharides are known. In this study, we present both mass spectrometric and high-performance liquid chromatography data about the N-glycans of different strains of *Trichomonas vaginalis*, a parasite of the human reproductive tract. The major structure in all strains examined is a truncated oligomannose form (Man₅GlcNAc₂) with α1,2-mannose residues, compatible with a previous bioinformatic examination of the glycomonomic potential of *T. vaginalis*. In addition, dependent on the strain, N-glycans modified by pentose residues, phosphate or phosphoethanolamine and terminal N-acetyllactosamine (Galβ1,4GlcNAc) units were found. The modification of N-glycans by N-acetyllactosamine in at least some strains is shared with the lipo(phospho)glycan and may represent a further interaction partner for host galectins, thereby playing a role in binding of the parasite to host epithelia. On the other hand, the variation in glycosylation between strains may be the result of genetic diversity within this species.

**Keywords:** mass spectrometry / N-glycan / pentose / phosphoethanolamine / trichomonads

Introduction

The glycoconjugates of eukaryotic parasites play important roles in host–pathogen interactions, whether it be in terms of binding, invasion, immune recognition or immunomodulation, and are often necessary for virulence. The major categories of these molecules in unicellular protozoal parasites include N-glycans, O-glycans and lipophosphoglycans (Guha-Niyogi et al. 2001). In the case of trichomonads, a number of studies have demonstrated the presence of a lipo(phospho)glycan containing a range of monosaccharide units, such as xylose and rhamnose (Singh et al. 2009; Ryan et al. 2011), as well as poly-N-acetyllactosamine moieties which interact with host galectins (Okumura et al. 2008). Indeed, lipo(phospho)glycan-mediated binding of trichomonads to mucosa is required for adherence and proinflammatory epithelial activation, which are features of *Trichomonas vaginalis* infections affecting large percentages of the human population worldwide (Schwebke and Burgess 2004). In cattle and cats, another sexually transmitted trichomonad, *Trichomonas foetus*, has also been studied as regards its cell surface lipo-phosphoglycans, which, in contrast to that of *T. vaginalis*, is characterized by large numbers of fucose residues (Singh 1993). An oligoglucose O-glycan has also been identified from *T. vaginalis* (Grabińska et al. 2008).

In the present study, we have focused on the N-linked oligosaccharides of one of these organisms, *T. vaginalis*; to date, these glycans have not been highly characterized. As part of a bioinformatically driven study on the enzymes required for the synthesis of N-glycans, it was noted that the major structure of *T. vaginalis* has the composition Man₅GlcNAc₂, but further features of the N-glycome were not described (Samuelson et al. 2005; Banerjee et al. 2007); also, the presence of xylose on its N-glycans was mentioned in a publication describing potential xylosyltransferase genes, but no structural details were shown (Hwa et al. 2007). Here, using high-performance liquid chromatography (HPLC) and matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), we compare the N-glycans of different strains of *T. vaginalis*, thereby demonstrating strain-specific modification of their N-glycans.

Results

**General analytical approach**

In order to analyze the N-glycomes of different *T. vaginalis* strains, glycans were released using PNGase A and/or...
PNGase F. The glycans from the first sample of the C1 strain to be analyzed (C1/1A) were fractionated first by NP-HPLC (Supplementary data, Figure S1), prior to subsequent refractionation by RP-HPLC, analysis by MALDI-TOF MS and MS/MS and selected exoglycosidase digestions (see Table I for a summary of these data). In addition, considering a cautionary tale regarding data on another parasite grown in a medium containing bovine serum, *Giardia intestinalis* (Robbins and Samuelson 2005), glycans were also prepared from the C1 strain after starvation of serum for 2 days prior to harvesting (samples C1/2F and C1/3FA). The subsequent isomeric variation. Multiple forms of glycans are possible due to isomeric variation. Values are shown; the differences in these values vary between −0.2 and 0.31. Multiple forms of glycans are possible due to isomeric variation. Fractions marked with an asterisk (*) are considered to be derived from a serum in the cultivation medium, whereas those retention times in g.u. marked with a double dagger (‡) are similar to those described in the literature (Tomiyama et al. 1988, 1991).

Table I. Summary of *T. vaginalis* PNGase A-released glycans

<table>
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<tr>
<th>Fraction</th>
<th>m/z [M + H]+ (observed)</th>
<th>m/z [M + H]+ (calculated)</th>
<th>RP-HPLC (g.u.)</th>
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</tbody>
</table>

Glycans from sample C1/1A were initially subject to NP-HPLC (Tosoh Amide 80; fractions A-L) prior to refractionation of each NP-HPLC fraction by RP-HPLC (Hypersil, MZ Analytik) and MALDI-TOF MS using DHB as matrix (see Supplementary data, Figure S2). Both calculated and observed m/z values are shown; the differences in these values vary between −0.2 and 0.31. Multiple forms of glycans are possible due to isomeric variation. Fractions marked with an asterisk (*) are considered to be derived from a serum in the cultivation medium, whereas those retention times in g.u. marked with a double dagger (‡) are similar to those described in the literature (Tomiyama et al. 1988, 1991).

Analysis of the major pentamannosidic glycan

In its pyridylaminated form, the major glycan in three strains (C1, G3 and TV2) and the second-most dominant glycan in the IR-78 strain has a predicted composition of Hex₂HexNAc₂ (m/z 1313) and an RP-HPLC retention time of 5.8 glucose units (g.u.; Figures 1 and 2). This is unlike a typical Man₆GlcNAc₂ commercial standard which has a retention time corresponding to around 7 g.u.; in a mass spectrometry assay using an α1,2,3-specific mannosidase, these three mannoside residues were removed (Supplementary data, Figure S2A). Thereby, our data are compatible with the major N-glycan in *T. vaginalis* strains being Man₆1,2Man₆1,3(Man₆1,6)Manβ1,4GlcNAcβ1,4GlcNAc.

Plant-like xylosylation of N-glycans from the C1 strain

The initial mass spectrometric data were suggestive of the presence of pentose residues in three strains (C1, TV2 and IR-78). Considering the presence of a pentose (xylose) on the core β-linked mannose residue is a feature of plants and of trematodes (Paschinger et al. 2009), a preliminary western blotting experiment with anti-horseradish peroxidase (HRP), which recognizes both core α1,3-fucose and β1,2-xylose, was performed; indeed, reactivity was observed and could be reduced if the antibody was pre-incubated with a bovine serum albumin conjugate carrying a plant-type N-glycan (data not shown). As apparently no core α1,3-fucosylated glycans were released by PNGase A, which unlike PNGase F can release such structures (Tretter et al. 1991), we presumed that the reactivity with anti-HRP was due to the presence of xylose. This supposition is supported by the presence of a series of glycans in the C1/1A sample whose fragmentation patterns indicate the presence of differences of 132 (Figure 3). In particular, a fragment of m/z 796.7 is compatible with a Hex₂HexNAc₂Pent₁ motif and is present in the glycans found in 2D-HPLC fractions E2 (m/z: 1283; Hex₂HexNAc₂Pent₁-PA) and H2 (m/z: 1445; Hex₂HexNAc₂Pent₁-PA). In the case of the IR-78 strain, the major peak in all preparations analyzed had the predicted composition Hex₂HexNAc₂Pent₁-PA (m/z: 1121); the RP-HPLC elution time (7.5 g.u.; fraction 7) corresponded to that of a standard “MMX” plant glycan, prepared from beans and sharing a similar fragmentation pattern upon MS/MS. This glycan was also sensitive to combined α1,2,3-mannosidase and β1,2-xylosidase digestion (Supplementary data, Figure S2B). The differences in the degree of the presence of the plant-like core xylosylation in the four strains tested is also apparent as judged by a comparison of anti-horseradish peroxidase reactivity (Supplementary data, Figure S3).

Pentosylation of the distal core N-acetylglucosamine residue

In other putatively mono-pentosylated glycans from the C1 strain, fragments of m/z 335.0 (Hex₆HexNAc₂Pent₁-) and m/z 634.2 (Hex₆HexNAc₂Pent₁-) are observed in the case of a glycan with the same m/z value as for that in the aforementioned 2D-HPLC fraction E2, but which elutes earlier on the RP-HPLC column (fraction F1; Figure 3B); these fragments are also found in the MS/MS spectrum of the putatively.
doubly pentosylated glycan found in fraction H1 (m/z 1415; Hex4HexNAc2Pent2-PA, see Figure 3C). In contrast to the C1/1A sample, the other C1 samples were hallmarked by an absence of double pentosylation; only in the case of two low abundance glycans in the C1/3FA sample (m/z 1618 and 1780; Hex4-5HexNAc3Pent2) is such a modification apparent (Table II). Thereby, in samples C1/2F, C1/4A and C1/4F, modification by pentose residues of the core mannose and distal (second) core GlcNAc appeared to be, in general, mutually exclusive; based on the aforementioned fragmentation patterns, the pentosylation of mannose is predominantly predicted for Hex3-5HexNAc2Pent1-PA, whereas the pentosylation of distal core GlcNAc is presumed for the larger Hex4-6HexNAc3-4Pent1-PA structures in these samples.

Pentosylation was not detectable among the glycans present in the G3 strain, but was obvious in low amounts in HPLC fractions of glycans from the TV2 strain (Figure 3E; m/z 1445); in the latter case, the pentose would appear also to be on the distal core GlcNAc residue. As the amounts of the glycans displaying this type of pentosylation are very low, there was insufficient material for compositional and linkage analyses to resolve which type of pentose was bound to the core region in which linkage.

**T. vaginalis N-glycans can be modified with N-acetyllactosamine units**

Another feature of the *T. vaginalis* C1 and G3 glycomes were masses compatible with the presence of three or more N-acetyllactosamine residues. Indeed, neutral loss of 203 mass units was observed in MS/MS spectra of a number of glycans (some with pentose residues in the case of the C1 strain) as was the serial loss of 162 and 203; the latter pattern was suggestive of the presence of terminal N-acetyllactosamine (LacNAc) units (Supplementary data, Table II).
strain (glycans released by PNGase A and sample C1/4 released by PNGase F), G3 single hexose when incubated with either A. oryzae E4 and G5 from sample C1/1A) with and then β spectra —— the shift to the left suggesting the presence of the Glc1Man5GlcNAc3 or Gal1Man5GlcNAc3. Other than a trace of a probable serum-derived component (Hex3HexNAc3), no modification of N-glycans by N-acetyllactosamine was obvious in the TV2 strain.

Distinguishing parasite-derived from serum-derived glycans

The presence of significant levels of larger oligosaccharides in the first sample (C1/1A), with compositions of Hex5,6HexNAc4-Fuc0-1 was noted; however, our hypothesis was that these larger structures were in part derived from serum components such as fetuin. These peaks were indeed less conspicuous or absent in the spectra of glycans prepared from T. vaginalis C1 maintained for 2 days in the absence of any serum. However, “serum-free” maintenance was not conducive to good cell viability (unpublished observation) and appeared, as summarized in Table II, to result in a loss of glycomic complexity; thus, the majority of our analyses were performed on glycans of the C1/1A and C1/4A samples (grown in the presence of serum). Thereby, it was important to distinguish glycans of parasites and the serum origin. Larger glycans containing pentose residues (Hex5-6HexNAc4-5Fuc0-1) presumably originate from the parasite. However, glycans in low amounts containing fucose (m/z 1865, Hex4HexNAc4Fuc; e.g. fraction C1/1A/J1, see Table I) or sialic acid (e.g. m/z 2010, NeuAc1HexHexNAc4; detected in negative mode) are considered to be of the mammalian origin. These glycans are, in addition to triantennary NeuAc0,3HexHexNAc5, observed when glycans released from fetal calf serum proteins were directly analyzed (Supplementary data, Figure S5). The presence of bi- and triantennary structures in serum is consistent with previous data on the N-glycans of bovine fetuin, one of the major components of fetal calf serum (Green et al. 1988).

In the case of the C1/4A RP fraction 11, a portion of the Hex3HexNAc4 (m/z 1719; Supplementary data, Figure S6) species was sensitive to β-hexosaminidase and to α1,2-mannosidase digestion, indicative of a terminal GlcNAc and a terminal α1,2-mannose residue: such a structure is, based on our understanding of parasite and mammalian N-glycosylation, most probably of the T. vaginalis origin. The other portion of the glycans with m/z 1719 was β-galactosidase sensitive in a manner predicted for biantennary mammalian glycans and so suggestive of a trace of glycans derived from the medium in this fraction. On the other hand, the next two glycans in the same fraction (Hex3HexNAc4, m/z 1881, and Hex-HexNAc4, m/z 2246) are sensitive to α1,2-mannosidase and to β1,4-galactosidase and in both cases only a single hexose is removed; once β-hexosaminidase is added to the galactosidase digestion mixture, the structures “collapse” down to Hex3HexNAc2. Particularly, the mannosidase sensitivity of these two glycans is indicative of their parasitic origin.

Somewhat similar to the detection of multiple forms of Hex3HexNAc4 in C1/4A RP fraction 11 is the situation with the 2D-HPLC-separated fractions C1/1A/L1 and L2; in this
Fig. 2. RP-HPLC analysis of *T. vaginalis* N-glycans. (Top panel) Pyridylaminated N-glycans from the same samples as shown in Figure 1 were subject to RP-HPLC fractionation (Hypersil ODS, Agilent); the column was calibrated using an isomalto-oligosaccharide series (2–14 g.u.). Fractions were collected as indicated and subject to MALDI-TOF MS analysis; for simplicity and due to their similarity, the G3/A, TV2/A and IR-78/A chromatograms, with the same fraction numbering, are not shown. (Bottom panel) The major RP-HPLC fraction of *T. vaginalis* C1 glycans was analyzed by RP-HPLC (5.8 g.u.) before and after digestion with *Aspergillus α*1,2-mannosidase. In comparison, commercial Man3GlcNAc2-PA (ca. 7 g.u.; Takara, Shiga, Japan) and Man5GlcNAc2-PA (ca. 5.5 g.u.; Takara) as well as an isomaltose standard were run. The identity of both substrate and product was verified by MALDI-TOF MS. Based on the previous data, the Man3GlcNAc2 digestion product is expected to have a retention time approximately the same as that of the commercial Man5GlcNAc2. The glycans are drawn according to the nomenclature of the Consortium for Functional Glycomics (gray circles, mannose; black squares, GlcNAc).
case, two forms of Hex$_6$HexNAc$_3$ can be resolved by RP-HPLC (Supplementary data, Figure S1). Galactosidase digestion of L1 results in loss of up to three hexose residues, whereas L2 only loses one residue (data not shown); these data suggest that approximately one half of the Hex$_6$HexNAc$_3$ is a triantennary glycan derived from the medium, whereas the other half is a parasite glycan carrying three N-acetyllactosamine residues in series.

Differential expression of zwitterionic glycans in T. vaginalis strains

Initial negative ion mode MALDI-TOF MS data of N-glycans from G3 and TV2, but not C1 and IR-78, indicated the presence of relatively strong signals not accounted for by the major species in the positive spectra. Specifically, the negative spectrum acquired with glycans from the G3 strain was particularly striking considering the signals at $m/z$ 1637 and 1799. In the case of TV2, signals with $m/z$ 1110, 1272 and 1434 are present, which suggested that a typical series of Man$_3$,GlcNAc$_2$ glycans may be modified by a moiety of 123 mass units (Supplementary data, Figure S7). Indeed, a modification of this mass was previously found, e.g. to be present on mannose residues of glycosylphosphatidylinositol (GPI) anchors and was identified as phosphoethanolamine (EtNP; Fukushima et al. 2003). Therefore, in order to test this assumption, dephosphorylation with HF acid (Schneider and Ferguson 1995) was performed on the aliquots of these two N-glycomes. Changes in both the negative and the positive mode spectra were observed (Supplementary data, Figure S7). The use of HF acid resulted in the respective loss of, e.g. the $m/z$ 1637 and 1434 $[M-H]^{-}$ species from the G3 and TV2 negative mode spectra; the loss of the corresponding $m/z$ 1639 and 1436 $[M+H]^+$ species from the positive mode spectra was also observed. Therefore, their compositions were assumed to be Hex$_3$HexNAc$_3$(EtNP)$_1$ and Hex$_3$HexNAc$_2$(EtNP)$_1$.

In order to examine the nature of the putative Hex$_3$HexNAc$_3$(EtNP)$_1$ glycan from the G3/A sample further,
an early RP-HPLC fraction (3.7 g.u.) enriched in this species was subject to treatment with HF as well as with exoglycosidasies (Figure 5A). Dephosphorylation with HF resulted in the appearance of a species with $m/z$ 1516 in positive mode (a loss of 123 mass units), whereas galactosidase digestion resulted in the loss of a single hexose residue (the product having respective $m/z$ values of 1475 and 1477 in the negative and positive modes). On the other hand, jack bean $\beta$-hexosaminidase or Aspergillus $\alpha$1,2-mannosidase digestion of the native glycan resulted in no change in the mass spectra; however, digestion by $\alpha$1,2-mannosidase (to $m/z$ 1354) was successful after HF treatment and combined hexosaminidase/galactosidase treatment resulted in the removal of one hexose and one $N$-acetylhexosamine (data not shown).

The positive mode MS/MS spectra of this glycan of $m/z$ 1639 (also in its galactosidase and galactosidase/hexosaminidase-digested forms; Figure 5B) indicated fragments of, on average, $m/z$ 285.7, 447.7 and 813.9, which would correspond to Hex$_1$, Hex$_2$ and Hex$_3$HexNAc$_1$ modified by EtNP. Thus, the smallest common fragment containing an early RP-HPLC fraction (3.7 g.u.) enriched in this species was subject to treatment with HF as well as with exoglycosidasies (Figure 5A). Dephosphorylation with HF resulted in the appearance of a species with $m/z$ 1516 in positive mode (a loss of 123 mass units), whereas galactosidase digestion resulted in the loss of a single hexose residue (the product having respective $m/z$ values of 1475 and 1477 in the negative and positive modes). On the other hand, jack bean $\beta$-hexosaminidase or Aspergillus $\alpha$1,2-mannosidase digestion of the native glycan resulted in no change in the mass spectra; however, digestion by $\alpha$1,2-mannosidase (to $m/z$ 1354) was successful after HF treatment and combined hexosaminidase/galactosidase treatment resulted in the removal of one hexose and one $N$-acetylhexosamine (data not shown).

The positive mode MS/MS spectra of this glycan of $m/z$ 1639 (also in its galactosidase and galactosidase/hexosaminidase-digested forms; Figure 5B) indicated fragments of, on average, $m/z$ 285.7, 447.7 and 813.9, which would correspond to Hex$_1$, Hex$_2$ and Hex$_3$HexNAc$_1$ modified by EtNP. Thus, the smallest common fragment containing
both EtNP and N-acetylmuramidase can be proposed to correspond to EtNP-Manα1,2Manα1,3Manβ1,4GlcNAc. The EtNP moiety is thereby presumed to be linked to the terminal α1,2-mannose of the α1,3-arm; the presence of this residue would in turn indicate that the N-acetyllactosamine unit (Galβ1,4GlcNAc) is on the α1,6-arm.

Another variant of an EtNP-modified glycans in the G3 strain is represented by a glycan of m/z 1801 with a predicted composition of Hex5HexNAc3(EtNP)1. When analyzed by positive mode tandem MS, a fragment of 285.9 was also apparent (data not shown); the difference between the two zwitterionic G3 glycans is predicted to be an α1,2-mannose residue. The overall compositions of the m/z 1639 and 1801 [M + H]+ species are thereby predicted to be Gal1Manα5,GlcNAc5(EtNP)1-PA. The “parent” glycans lacking the charged moiety are present in later-eluting RP-HPLC fractions (8 and 9 g.u. as compared to 3.7 g.u.; Supplementary data, Figure S4).

In addition to the analysis of the EtNP-modified glycans from the G3 strain, MS/MS was also performed on a number of glycans carrying putative phosphate or phosphodiester in three RP-HPLC fractions from the TV2 strain. Again, fragments of m/z ~286 were observed and, in one case, a glycan carrying two EtNP moieties was detected; fragments of m/z 651 (Hex5HexNAc3EtNP) and 733 (Hex5[EtNP]2) indicate that one or both α-linked mannose residues are, respectively, modified in the glycans with m/z 1112 and 1235 (Figure 6).

Furthermore, the positive ion MS/MS spectrum for the m/z 1393 molecular ion indicated the presence of a phosphorylated hexose (fragment of m/z 242.9) and so is compatible with a composition of Man5,GlcNAc3,Phos1-PA. It is noteworthy that the types of N-glycans carrying EtNP in G3 and TV2 contrast in terms of the basic structure (either N-acetyllactosamine-containing in the former or paucimannosidic in the latter).

Discussion

Key features of N-glycans from T. vaginalis

In this most exhaustive analysis of the N-glycan structures of T. vaginalis to date, a number of key findings can be summarized (Figure 7): first, as predicted from previous studies (Samuelson et al. 2005; Banerjee et al. 2007), the major glycan in all three T. vaginalis strains examined is Man5GlcNAc2 in its “biosynthetic” form. This is, in part, the result of a lack of genes encoding the four luminal mannosyltransferases required for the formation of the full Glc3Man9GlcNAc2 dolichol-linked precursor generally found in eukaryotes. Indeed, genes encoding putative Alg7, Alg13 and Alg14 [the presence of Alg13 and Alg14 homologs (TVAG_183240 and TVAG_038800) has been confirmed by our own database searches], Alg1, Alg2, Alg11 and Rft1 homologs can be found in the T. vaginalis genome.

Second, the C1 and IR-78 strains produce pentosylated glycans: whereas the pentose attached to the core β-linked mannose can be assumed to be xylose on the basis of anti-HRP staining and xylosidase sensitivity, the pentosylation of the distal GlcNAc is reminiscent of the xylosylation of microalgal N-glycans (Levy-Ontman et al. 2011).

Furthermore, the functionality of a recombinant form of a putative UDP-xylose synthase has been demonstrated (Rosenberger et al. 2012) and a number of β1,2-xylosyltransferase homologs have been found (Hwa et al. 2007). [In a conference abstract, the same authors also suggest the presence of a novel core structure and xylose as a minor component of T. vaginalis N-glycans (Hwa et al. 2006).] On the other hand, there is no evidence for the presence of deoxyhexose in the N-glycans of this organism. However, both pentose (xylose) and deoxyhexose (rhamnose) residues are found in its lipo(phospho)glycan (Bastida-Corcuera et al. 2005); the probable absence of fucose would be consistent with the lack of genes predicted to encode enzymes involved in GDP-fucose synthesis. Indeed, the absence of fucose from the lipo(phospho)glycan and N-glycans of T. vaginalis is compatible with the inability to label its cell surface with a fucose-specific lectin (Mirhaghani and Warton 1998). In contrast, T. foetus lipo(phospho)glycan contains both fucose and xylose (Singh 1993; Singh et al. 1994); however, there is no information available to allow a genomic comparison of these species.

Third, as observed for the lipo(phospho)glycan of UR1, B7RC2 and CD-C85 strains (Singh et al. 1994;
Bastida-Corcuera et al. 2005; Singh et al. 2009; Ryan et al.
2011), the C1 and G3 strains produce N-glycans modified
with N-acetyllactosamine units. This moiety is an epitope for
*Ricinus communis* agglutinin and strains resistant to this lectin
have a lower galactose content which correlates with lower
adhesion to mammalian cells and a loss of the ability to bind
human galectin-1 (Bastida-Corcuera et al. 2005; Okumura
et al. 2008), a putative “receptor” for a wild-type strain; we
have also observed the activity of a recombinant
UDP-galactose epimerase (Rosenberger et al. 2012). The presence
of single LacNAc units (or in the C1 strain also up to at
least three repeats) on the N-glycans would indicate that these
are also candidates for mediating binding to host cells; however, information as to the N-glycan structures or the rele-
vant mutation(s) in the lectin-resistant strains is yet to be pub-
lished. [Data have been presented at a recent conference
which indicate the absence of LacNAc units from the
*N*-glycans of ricin-resistant strains of *T. vaginalis* (Carpentieri
et al. 2010).] Another point when considering the glycans of
this organism is that, despite the well-known production of a
range of glycosidases (e.g. galactosidase and hexosaminidase)
by trichomonads (Lockwood et al. 1988; Costa e Silva Filho
et al. 1989; Connaris and Greenwell 1997), larger
N-glycans can still be isolated from the cultivated cells; thus, at least in
part, the N-acetyllactosamine units survive exposure to these
hydrolases.

Fourth, charged glycans from the G3 and TV2 strains are
modified with a moiety with 123 mass units, putatively EtNP,
which is sensitive to HF acid treatment and results in earlier
elution on RP-HPLC. The C1 strain, in contrast, displays only
a minor degree of such modifications. There are precedents
for the presence of this moiety on other categories of glyco-
conjugates; for instance, EtNP is a component of GPI anchors
(Fukushima et al. 2003; Paulick and Bertozzi 2008), insect

Fig. 7. Proposed major N-glycan processing pathways and glycan classes in *T. vaginalis*. A non-exhaustive selection of potential N-glycosylation modification reactions are shown (A) based on the determined glycan structures, which can be classified into, for example, paucimannosidic, pentosylated, phosphoethanolaminated, simple pseudohybrid and extended pseudohybrid types (B). Variations between strains in terms of the degree of these modifications are obvious; the identity of the pentose residue as xylose is proposed on the basis of preliminary data from others and from its presence in the lipoglycan. Enzymes, such as Manl (class I mannosidase), UGGT (UDP-Glc:glycoprotein glucosyltransferase), GlcII (glucosidase II), Gn-T (N-acetylgalactosaminyltransferase), Gal-T (galactosyltransferases), Xyl-T (xylosyl- or other pentosyltransferases) and EtNP-T (phosphoethanolaminyltransferase), are either proven or proposed, but may have different or more substrate specificities than those shown. The glycans are drawn according to the nomenclature of the Consortium for Functional Glycomics (green circles, mannose; yellow circles, galactose; blue squares, GlcNAc; stars, pentose).
and nematode glycolipids (Seppo et al. 2000; Friedl et al. 2003), insect O-glycans (Gareniaux et al. 2011) and some bacterial lipopolysaccharides (Cox et al. 2002). To our knowledge, there is only one previous report of a similar modification of an N-glycan, specifically that of locust apolipoporphin with 2-aminoethylphosphonate (Hård et al. 1993).

Although present on phospholipids and GPI anchors, EtNP is not a component of mammalian N-glycans and so its presence on the glycans of a parasite may potentially result in a response by the adaptive or innate immune systems. Indeed, EtNP is a ligand for the pentraxin, serum amyloid P (Schwalbe et al. 1992), which is a mediator of a classical complement pathway (Deban et al. 2009), whereas glycans modified with its methylated form, phosphorylcholine, are associated with immunomodulation by nematode parasites (Harnett and Harnett 2001). It is therefore of interest in the future to consider whether the modification of trichomonad glycans by EtNP has any repercussions for host–parasite interactions or the host immune system. Significantly, our preliminary data from T. foetus (K. Paschinger, preliminary data) indicate the modification of some N-glycans with phosphorylcholine; therefore, the modification of T. vaginalis glycans by EtNP is not so unexpected.

Analytical lessons from the study of T. vaginalis glycans

The unambiguous analysis of heterogeneous glycans from relatively low amounts of parasite material is a challenge for a number of reasons, including the presence of unusual modifications and traces of non-parasite glycans which are, in this case, derived from serum glycoproteins. Therefore, we adopted a mixed approach not just on MS alone, but supported by exoglycosidase digestions and HPLC analyses of glycans released by PNGase A or F: the precaution of using both enzymes was in retrospect not necessary, due to the lack of core α1,3-fucosylated species which cannot be cleaved by PNGase F (Tretter et al. 1991). The typical elution behavior of some pyridylaminated glycans when analyzed by RP-HPLC before and after exoglycosidase digestion is an aid in defining isomers; for instance, in our study and in accordance with previous data (Tomiya et al. 1991), the major trichomonad Man3GlcNAc2 glycan elutes at 5.8 g.u. and contains two α1,2-linked mannose residues. In contrast, the typical protein-bound Golgi-processed Man4GlcNAc2 in multicellular eukaryotes (represented in our study by a commercial sample) elutes at around 7 g.u.

In another instance, comparison with another study of Tomiya et al. (1988) is also useful in order to indicate that the glycan with a composition of Gal1Man3GlcNAc3 is actually “GalM” [Schachter nomenclature for Manα1,3Galβ1,4GlcNAcβ1,2Manα1,6Manβ1,4GlcNAcβ1,4GlcNAc-P; see Schachter 1986] as opposed to the MGal isomer; these have respective literature retention times of 8.0 and 9.9 g.u.. GalM is predicted to shift to 9.5 and 7.4 g.u. upon serial treatment with galactosidase (to GnM, as opposed to the MGN isomer) and hexosaminidase (to MM). Our results (Figure 4) are compatible with these values and indicate that, for example, the glycan in fraction C1/1A/E4 carries an LacNAc unit on the α1,6-antenna; the definition of the galactose as being β1,4-linked on trichomonad N-glycans is based on the specificity, as quoted in the literature (Zeleny et al. 1997) or the manufacturer’s information, of the two galactosidases employed. As core β1,2-xylene has relatively little effect on the RP-HPLC retention time (Wilson and Altmann 1998), the values for the putatively xylosylated glycan in fraction C1/1A/G5 (“GalMX”) are similar to those with the non-xylosylated C1/1A/E4 glycan. The presence of a residual α1,2-mannose on other structures containing N-acetylactosamine units (Supplementary data, Figure S4) is also compatible with presence of Galβ1,4GlcNAc on the α1,6-arm of T. vaginalis glycans, as the α1,3-arm is thereby “blocked” by the mannose residue, leaving only the other antenna free for modification by an N-acetylglucosaminyltransferase.

Despite the advantages of using diagnostic HPLC shifts, in a number of cases, we rely on MS/MS data in order to propose some of the structures. Regarding pentosylated species, some care was required as a fragment of m/z 431 (HexNAc4Pent4-PA) of low abundance is apparent in a number of MS/MS spectra (Figure 3); this was also observed in a glycan prepared by incubation with a recombinant plant core β1,2-xylosyltransferase as well as a glycan purified from beans (data not shown). However, the MS/MS of these same glycans also showed a loss of 299 from the parent molecular ion, which is compatible with the loss of GlcNAc-PA alone, but not of HexNAc4Pent4-PA. Therefore, we conclude that the m/z 431 fragment is an artifact resulting from an internal rearrangement similar to that proposed for some fucosylated glycans (Wuhrer et al. 2006).

Inter-strain differences in glycosylation

In this study, we have included data on four different strains; samples of one of these strains were also obtained from three different laboratories using three different media. We chose one typical laboratory strain (C1) as well as the strain used to sequence the genome (G3) and two locally isolated strains (TV2 and IR-78), one of which displays metronidazole resistance. The glycosylation of these various samples indicates that the glycome of T. vaginalis is not uniform but has variations in extent and type of pentosylation and also differences in the degree of modification with charged moieties and with N-acetylactosamine. Thus, it may be that glycosylation is affected by a number of different factors, including the strain, cultivation method or period of time in continuous culture; indeed, the loss of gene expression has been reported for other protozoa in long-term cultivation (Köhlsler et al. 2009). The effect of the cultivation of C1 for 2 days in the absence of serum suggests some loss of complexity, but the N-glycome of the TV2 strain cultivated in the presence of serum is even less heterogeneous. The variation between the two C1 samples tested with anti-HRP (Supplementary data, Figure S3) would suggest a higher abundance of xylosylation of the core mannose in sample C1/1 as opposed to C1/4; certainly a lower number of mono- and dipentosylated structures (Table II) were identified in the latter sample. Hex1HexNAc4Pent4, for instance, is distinctly obvious in the complete C1/1 spectrum (Figure 1), but is only a minor structure in the other C1 spectra.

Without further data regarding the virulence, genomic differences or metabolic status of the different strains in the...
different laboratories, we do not speculate as to the significance of our comparative N-glycomic data. Certainly, those studying the glycosylation of this organism should be aware of such differences; however, it can be argued that variance between the glycoconjugate patterns of these four T. vaginalis strains reflects the considerable genetic diversity within this species (Conrad et al. 2011). In this context, it is interesting to note that also the protein expression patterns of strains C1, G3, TV2 and IR-78, as revealed by 2D gel electrophoresis, also display numerous and substantial differences (D. Leitsch, unpublished results).

Biosynthetic conclusions from glycomic data

As summarized above, the major N-glycan of T. vaginalis corresponds to the Man5GlcNAc2 isomer found during the biosynthesis of the dolichol-linked precursor prior to flipping from the cytoplasmic to the lumenal face of the endoplasmic reticulum. Whereas in most eukaryotes this pentamannosyl structure is elongated by a further four mannose and three glucose residues, T. vaginalis lacks the relevant glucosyltransferase genes. Therefore, as is also the case in Entamoeba histolytica, Man5GlcNAc2-PP-Dol is the primary oligosaccharidyltransferase “donor” substrate for protein N-glycosylation in this organism. The oligosaccharyltransferase complex of T. vaginalis apparently consists of fewer subunits than is generally the case for eukaryotes and displays a preference for non-glucosylated glycan donors (Kelleher et al. 2007); interestingly, in Trypanosoma brucei, there are at least two types of oligosaccharyltransferase which accept either Man5GlcNAc2-PP-Dol or Man9GlcNAc2-PP-Dol as the substrate (Izquierdo, Schulz, et al. 2009).

After transfer to protein, the presence of a UDP-Glc:glycoprotein glucosyltransferase gene in the T. vaginalis genome (Banerjee et al. 2007) indicates that glycosylation can take place as part of a quality-control system for glycoproteins (D’Alessio et al. 2010); the occurrence of a glucosyl, as noted in this and an earlier study, with the composition HexαHexNAc2 (m/z 1475) would be compatible with terminal glucose attached to ManαGlcNAc2 (Banerjee et al. 2007). There is, though, no commercially available glucosidase II which can be used to further analyze this structure. The occurrence of Manα4GlcNAc2 is also compatible with the presence of an active class I mannosidase (Banerjee et al. 2007). However, other than these glucosyltransferase and mannosidase genes, there is a complete lack of knowledge regarding the genes required for the subsequent processing events in T. vaginalis.

From our data, we can predict that N-acetylgalactosaminyltransferases, galactosyltransferases, xylosyltransferases and phosphoethanolaminyltransferases should be encoded by the T. vaginalis genome—while xylosyltransferase homologs have been identified (Hwa et al. 2007), no obvious homologs for either GlcNAc-TI or -TII or β1,4-galactosyltransferase can be detected in this genome. Perhaps, these activities are covered by members of other glycosyltransferase families; for instance, as lateral gene transfer from bacteria has been postulated (Carlton et al. 2007), homologs of bacterial glycosyltransferases are candidates for being responsible for these activities. It also should be noted that a classical GlcNAc-TI or -TII cannot be expected in this organism: the partial presence of an α1,2-mannose remaining on the α1,3-arm of its N-glycans means that a typical GlcNAc-TI cannot act. As summarized in Figure 7A, although the putative location (the α1,6-arm) for the extension by the single non-reducing terminal GlcNAc residue or LacNAc repeats on some glycans is the same as that modified in other organisms by GlcNAc-TII, the inability of a classical GlcNAc-TI to act beforehand means that any GlcNAc-TII-like enzyme will have a non-classical GlcNAc-TII-independent specificity.

In summary, the glycans present in the various strains studied are somewhat akin to hybrid oligosaccharides from multicellular eukaryotes, but are different due to the presence of the “complex” modifications on the α1,6-arm and of the remaining mannose residues on the α1,3-arm; thus, we propose that these glycans from T. vaginalis be defined as “pseudo-hybrid” structures in either “simple” or “extended” forms (Figure 7B). From the analytical data, we surmise that the glycomic potential of different strains varies and that, for instance, the C1 strain has significant LacNAc polymerization activity as well as pentosylation activities, whereas in TV2, the galactosyltransferase expression is low and in IR-78 the xylosylation of the β1,4-mannose is high. Finally, the phosphoethanolaminyltransferase activities in G3 and TV2 are probably higher than those present in C1. Our data mean that, although there are generalized structural trends regarding the N-glycosylation of T. vaginalis, there is no “species-specific” glycosylation signature.

Comparisons between T. brucei and T. vaginalis

The general biosynthetic situation we propose for T. vaginalis is akin to that observed in T. brucei: even in glucosidase II mutants in which glucose is retained on the α1,3-arm, the presence of LacNAc units on the α1,6-arm has been demonstrated (Jones et al. 2005; Izquierdo, Atrih, et al. 2009); indeed, in T. brucei, very long poly-LacNAc chains can be present (Zamze et al. 1991; Atrih et al. 2005). However, also no obvious GlcNAc-TI or -TII or β1,4-galactosyltransferase homologs have been identified in T. brucei (Izquierdo, Nakanishi, et al. 2009); to date, potentially relevant β-galactosyltransferase and dolichol-independent N-acetylgalactosaminyltransferase activities have been detected only in T. brucei microsomes (Rovis and Dubé 1982; Grab et al. 1984; Pingel and Duszenko 1992). As the α1,6-arm may be the first target for modification by an N-acetylgalactosaminyltransferase, prior to galactosylation, in both T. brucei and T. vaginalis, it may be attractive to propose that the relevant LacNAc-synthesizing enzymes may either be related phylogenetically or at least display the same substrate specificity. However, the evolutionary distance between trypanosomes and trichomonads may mean that a set of “convergent” evolutionary events has occurred so that the structural “result” of these non-classical glycan processing pathways is based on disparate phyloglycogenic mechanisms.

Experimental procedures

Cultivation of parasites

In this study, four different strains of T. vaginalis grown in three different laboratories were employed: C1 (ATCC
Medizinische Universität Wien (Blaha et al. 2006), whereas Professor A Stary, Universitätsklinik für Dermatologie, metronidazole-susceptible trichomoniasis kindly provided by was isolated in 2002 from material from a patient with 30001), G3 (A TCC PRA-98), TV2 and IR-78. The TV2 strain

\[ fi \]

fion of formic acid [up to 5% (v/v)] and 1 mg porcine

\[ fi \]

was 0.1 M ammonium acetate, pH 4, and buffer B was 30%

\[ fi \]

lowness, whereas G3, the strain used for the genome sequence (Carlton et al. 2007), and IR-78 were non-adherent.

Preparation of glycans
Parasite cells (2 g if grown in serum or 0.4 g if cultivated “serum-free”) or lyophilisates (50 mg) were suspended in 10 mL boiling water and denatured for 5 min, prior to the addition of formic acid [up to 5% (v/v)] and 1 mg porcine pepsin. Proteolysis was allowed to proceed for 2 days at 37°C and the samples were centrifuged to remove insoluble material. The supernatant of the proteolyse was then incubated with 10 mL prewashed Dowex AG50 W · 2 (Sigma-Aldrich) for 1 h at 23°C. The material was then poured into a column and the flowthrough fraction was reapplied; the column was then washed with 2% (v/v) acetic acid to remove unbound material and glycopeptides were eluted with 0.5 M ammonium acetate, pH 6, and lyophilized prior to gel filtration (Sephadex G25; GE Healthcare, Vienna, Austria). Orcinol-positive fractions were pooled, heat treated for 5 min and subject to digestion with either PNGase A (peptide: N-glycosidase from almonds; Roche, Vienna, Austria) in 50 mM ammonium acetate, pH 5, or PNGase F (peptide: N-glycosidase from Flavobacterium; Roche) in 100 mM ammonium carbonate, pH 8, overnight at 37°C (in the case of the C1/4, G3, TV2 and IR-78 samples, the pooled gel filtration fractions were separated into two halves prior to glycan release, whereas the C1/3 preparation was subject to PNGase F then PNGase A treatment in series). A second round of Dowex chromatography was performed and the unbound glycans were analyzed, without further purification, by MALDI-TOF MS.

Pyridylamination was subsequently performed basically as described (Hase et al. 1984). In brief, 100 mg 2-aminopyridine (Sigma-Aldrich) was dissolved in 76 µL concentrated HCl and 152 µL water; 80 µL of this solution was added to the dried glycan sample, prior to incubation in boiling water for 15 min. Then, a solution of 4.4 mg of sodium cyanoborohydrde (Sigma-Aldrich) in a mixture of 9 µL of the aforementioned 2-aminopyridine solution and 13 µL of water was prepared; 4 µL of this cyanoborohydrde–2-aminopyridine solution was added to the sample and the incubation was continued overnight at 90°C prior to gel filtration (Sephadex G15; GE Healthcare). Fluorescence (excitation/emission 320/400 nm) of the fractions was measured using a Tecan microtiter plate reader.

The glycan samples were named on the basis of the numbering of the original culture (see Cultivation of parasites and Supplementary data, Table) and the enzymes used for release: for example, C1/1A refers to PNGase A-released glycans from sample C1/1; C1/2F refers to PNGase F released glycans from sample C1/2; C1/3FA to combined PNGase F and A release of glycans from sample C1/3. In the case of G3 and IR-78, two independent cultures of each strain were used prior to being divided for PNGase A and F digestion; only data from one culture are presented here.

**MALDI-TOF mass spectrometry**
Free glycans or pyridylaminated glycans (0.8 µL) were dried under vacuum onto a ground steel sample plate prior to application of 0.8 µL of either 2,5-dihydrobenzoic acid (DHB) or 6-aza-2-thiobothymine (ATT) as matrices and vacuum drying once more. The samples were analyzed in positive or negative ion modes 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. Predicted glycan species were examined by MS/MS (post-source decay) and all spectra were evaluated manually. A key diagnostic MS/MS fragment is that corresponding to the core reducing-terminal GlcNAc-PA (expected m/z 300.15, observed m/z values were generally in the range 299.7–300.2).

**HPLC of N-glycans**
Pyridylaminated N-glycans were analyzed by either normal-phase (NP) or reverse-phase (RP) HPLC using a Shimadzu HPLC system equipped with a fluorescence detector (RF 10 AXL). For NP-HPLC, a Tosoh Amide-80 column (4.6 × 250 mm) was used and calibrated daily using a pyridylaminated isomaltose series. Prior to injection, 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). The gradient was applied as follows: 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. For RP-HPLC, Hypersil ODS columns (5 mm, 4 × 250 mm; purchased from either MZ Analytik or Agilent) were used. For both columns, buffer A was 0.1 M ammonium acetate, pH 4, and buffer B was 30% (v/v) methanol. Gradients of increasing methanol (1% buffer B per minute) were applied. Fluorescence was recorded at 320 nm (excitation) and 400 nm (emission). For “2D-HPLC” (two-dimensional HPLC) of sample C1/1A, glycans were first fractionated by NP-HPLC, collected, lyophilized and analyzed by MALDI-TOF MS prior to being subject to RP-HPLC. All collected fractions were subject to MALDI-TOF MS and the compositions of glycan species (in terms of hexose, N-acetyhexosamine, pentose) were verified by MS/MS. Only selected spectra and chromatograms are presented here.
Enzymatic or chemical digestion of N-glycans

Aliquots of isolated 2D-HPLC fractions were, based on the results of MALDI-TOF MS, subject to targeted exoglycosidase digestions. Depending on the peak intensity, between one-twentieth and one-half of a fraction was incubated with either 0.2 μL Streptococcus β-hexosaminidase (0.01 U; Calbiochem, Darmstadt, Germany) using the manufacturer’s buffer, 0.1 μL Aspergillus saitoi α,1,2-mannosidase (2 μU; Prozyme, Hayward, CA) with the manufacturer’s buffer, 0.1 μL repurified Aspergillus oryzae β1,4-galactosidase (0.027 U) with 50 mM sodium citrate, pH 4.5 (Zeleny et al. 1997), or 0.5 μL jack bean α-mannosidase (0.5 U; Sigma-Aldrich) with 50 mM sodium citrate, pH 4.5. The incubations were subject to a second round of RP-HPLC and MALDI-TOF MS.

For glycans solely fractionated by RP-HPLC, one-tenth of each redissolved fraction (1 μL) was mixed with 0.5 μL of 0.2 M ammonium acetate, pH 5, buffer and either 0.5 μL A. saitoi α,1,2-mannosidase (10 μU; Prozyme), 0.5 μL Xanthomonas α,1,2/3-mannosidase (32 U; New England Biolabs, Ipswich, MA), 0.25 μL Xanthomonas β1,2-xylosidase (2.5 μU, Merck Calbiochem), 0.5 μL recombinant Bacteroides fragilis β,1,4-galactosidase (2 U; New England Biolabs), 0.25 μL jack bean α-mannosidase (Sigma-Aldrich) or 0.25 μL jack bean β-hexosaminidase (0.06 U; Sigma-Aldrich); incubations were performed overnight at 37°C and analyzed directly by MALDI-TOF MS using ATT as a matrix.

For dephosphorylation, either whole N-glycames or selected fractions were dried and incubated overnight at 0°C with 3 μL of 48% (v/v) hydrofluoric (HF) acid prior to evaporation; the samples were diluted in water and re-evaporated, prior to redissolving once again and analysis by MALDI-TOF MS.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

ATT, 6-aza-2-thiothymine; 2D, two-dimensional; DHB, 2,5-dihydrobenzoic acid; EtNP, phosphoethanolamine; g.u., glucose units; GPI, glycosylphosphatidylinositol; HF, hydrofluoric; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; LacNAc, N-acetyllactosamine; MALDI, matrix assisted laser-desorption/ionization; MS, mass spectrometry; NP, normal phase; RP, reverse phase; TOF, time-of-flight; TYM, trypsinicpeptone/yeast extract/maltose.

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