Evidence for N-linked glycosylation in *Toxoplasma gondii*

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In this paper we report experiments demonstrating the presence of N-linked oligosaccharide structures in *Toxoplasma gondii* tachyzoites, providing the first direct biochemical evidence that this sporozoan parasite is capable of synthesizing N-linked glycans. The tachyzoite surface glycoprotein gp23 was metabolically labelled with [^{3}H]glucosamine and [^{3}H]mannose. Gel-filtration chromatography on Bio-Gel P4 columns produced four radiolabelled N-linked glycopeptides which were sensitive to peptidase-N-glycanase F, but resistant to endoglycosidases H and F. Using chemical analysis and exoglycosidase digestions followed by Dionex-high-pH anion-exchange chromatography and size fractionation on Bio-Gel P4 we show that gp23 has N-linked glycans in the hybrid- or complex-type structure composed of *N*-acetylgalactosamine, *N*-acetylglucosamine and mannose and devoid of sialic acid and fucose residues. In addition, the sensitivity of glycopeptides from glycoprotein extracts to endoglycosidases H and F revealed the *in vivo* synthesis of oligomannose-type structures by *T. gondii* tachyzoites. We have extended these findings by demonstrating the ability of *T. gondii* microsomes to synthesize *in vitro* a glucosylated lipid-bound high-mannose structure ($Glc_3Man_9GlcNAc_2$) that is assumed to be identical with the common precursor for N-glycosylation in eukaryotes.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular coccidian protozoan parasite that belongs to the class Sporozoa, which also includes the malaria parasites (*Plasmodia*) and the animal coccidian parasites *Eimeria* and *Sarcocystis*. *T. gondii* is the etiologic agent of toxplasmosis, which affects humans, a wide variety of birds and mammals. Although toxoplasmosis is generally asymptomatic in healthy adults, primary infection in a pregnant women is associated with severe congenital defects. In recent years, this parasite has emerged as an important opportunistic pathogen [1,2] which can cause the death of immunocompromised individuals such as those with acquired immunodeficiency syndrome (AIDS).

The life cycle of *T. gondii* consists of three major stages, namely sporozoite, tachyzoite and bradyzoite all of which are intracellular parasites. The parasite can invade most nucleated animal cells and replicates within a parasitophorous vacuole. The biological function of this vacuole, the parasite membrane and its surface proteins is not presently known, but they are presumed to play a role in the intracellular growth of *T. gondii* parasites. Most *T. gondii* research has focused on the rapidly growing tachyzoite stage because of its ease of *in vitro* propagation.

Immunochemical analysis of surface proteins of *T. gondii* tachyzoites led to the characterization of five major proteins which could be surface-iodinated [3–6]. These proteins, named P22, P23, P30, P35 and P43 according to their apparent molecular masses on SDS/PAGE, are all anchored to the parasite membrane via glycosyl-phosphatidylinositol (GPI) anchors [7–9].

Many eukaryotic proteins are modified by N-linked glycosylation, a process in which the presynthesized oligosaccharide, $Glc_3Man_9GlcNAc_2$ from a lipid precursor is added to an asparagine residue in the nascent polypeptide. This initial core glycan is processed by the sequential removal and addition of monosaccharides by a series of glycosidase and glycosyltransferase enzymes [10]. N-linked glycosylation influences many properties of proteins, such as intracellular transport [11–14], biological activity [12,13,15,16], stability [17,18], and antigenicity [19–21]. To our knowledge, except for the presence of GPI anchoring, little is known about glycosylation in the Sporozoa parasites.

In *T. gondii*, the existence of glycoproteins has been examined by Handman et al. [3] and Mauras et al. [22], with somewhat contradictory findings. Handman et al. [3] reported that living tachyzoites of *T. gondii* are unable to bind any lectins that are generally used to identify surface glycoproteins of most eukaryotic cells. In contrast, Mauras et al. [22], Sharma et al. [23] and Johnson et al. [24] studies have detected concavalin A binding to both living tachyzoites and isolated proteins. While studying the mode of anchoring of the tachyzoite surface proteins, we showed that these molecules were metabolically labelled with [^{3}H]-glucosamine, -mannose and -galactose [8] and also provided evidence that a portion of these radioactive sugars were incorporated into the GPI anchor of two surface proteins [9,25]. Our data also suggested that the radiolabel was used for the synthesis of carbohydrate structures linked to the core polypeptides. For this reason, we chose to investigate the biosynthesis of N-linked structures in *T. gondii* tachyzoites. This paper describes the first structural biochemical evidence that N-linked glycoproteins, as well as the lipid-linked glycan precursor for N-linked oligosaccharides, are synthesized in *T. gondii* tachyzoites.

Abbreviations used: GPI, glycosyl-phosphatidylinositol; PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C; Endo H and F, endoglycosidases H and F; PNGase F, peptidase-N-glycanase F; h.p.a.e.c., high-pH anion-exchange chromatography; 2D, two-dimensional; i.e.f., electric focusing; C/M, chloroform/methanol; C/M/W, chloroform/methanol/water; DMEM, Dulbecco's modified Eagle medium; NP40, Nonidet P40; GU, glucose unit; Neu5AC, 5-acetylneuraminic acid.

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MATERIALS AND METHODS

Materials

[6-^{3}H]Glucosamine hydrochloride (26.0 Ci/mmol), [2-^{3}H]-mannose (13.6 Ci/mmol), [6-^{3}H]galactose (25.5 Ci/mmol), amino acid mixture ([L-4,5-^{3}H]leucine, 163 Ci/mmol; [L-4,5-^{3}H]lysine, 95 Ci/mmol; [L-2,3,4,5,6-^{3}H]phenylalanine, 129 Ci/mmol; [L-2,3,4,5-^{3}H]proline, 105 Ci/mmol; [L-2,3,4,5,6-^{3}H]-tyrosine, 92 Ci/mmol) were purchased from Amersham. GDP-[3,4-^{3}H]Man (15.1 Ci/mmol) and UDP-[6-^{3}H]Glc were from New England Nuclear. PI-specific phospholipase C (PI-PLC) from *Bacillus cereus*, endoglycosidases H and F, and peptide-N-glycanase F (PNGase F) were purchased from Boehringer Mannheim. *N*-acetyl-β-hexosaminidase from *Aspergillus niger*, jack-bean (*Canavalia ensiformis*) α-mannosidase and β-galactosidase were obtained from Sigma. Sialidase from *Vibrio cholerae* was from Behring. All solvents were of analytical or h.p.l.c. grade.

Metabolic labelling of *T. gondi* tachyzoites

Monolayers of Vero cells (usually 75 cm^2 flasks) were infected 48 h earlier with 5×10^7 *T. gondii* tachyzoites (RH strain) in Dulbecco's modified Eagle medium (DMEM) supplemented with 2% (v/v) fetal-calf serum. The infected cells were washed with appropriate medium (see below) and incubated with radiolabelled precursors.

For amino acid labelling, the infected monolayer cells were washed with RPMI medium lacking the five amino acids cited above. Pulse labelling was performed with the tritiated amino acid mixture at 37 °C for 15 min and chased for 1 h. For sugar labelling we used glucose-free DMEM supplemented with 22 mM sodium pyruvate and either [^{3}H]glucosamine, [^{3}H]mannose or [^{3}H]galactose at a final concentration of 50 μCi/ml. Labelling was carried out at 37 °C for 4 h.

After labelling, the intracellular parasites were released by cell disruption using a Dounce homogenizer equipped with a tight-fitting pestle. Freed parasites were purified from cell debris by filtration on a glass-wool column [26].

Immunopurification of gp23 and p30

Detailed procedures for the production of monoclonal antibodies specific for gp23 (T4 2E12) and p30 (T4 1E5) have been described elsewhere [6]. Labelled parasites were lysed with 0.5% Nonidet P40 (NP40) prepared in PBS containing 2 mM EDTA, 1 mM phenylmethanesulphonyl fluoride, 0.1 mg/ml aprotinin and 0.01 mg/ml leupeptin. After 1 h at 4 °C, insoluble material was removed by centrifugation at 10000 *g* for 10 min. Gp23 and p30 were purified from the supernatant using 50 μl of immunosorbent prepared with purified IgG from monoclonal antibody T4 2E12 or T4 1E5 respectively coupled to CNBr–Sepharose 4B. The beads were washed three times with a buffer containing 0.5 M NaCl, 1 mg/ml BSA, 5 mM EDTA, 0.5% NP40 in Tris/HCl, pH 8.3, and twice with the same buffer without NaCl and BSA. After a final wash with 62.5 mM Tris/HCl, pH 6.8, elutions were performed by boiling beads in electrophoresis sample buffer.

Electrophoresis procedures

Purified gp23 and p30 were electrophoresed in a SDS/12% (w/v)-polyacrylamide gel according to method described by Laemmli [27]. After electrophoresis, the gels were fixed and soaked in En^{3}Hance (New England Nuclear), dried and exposed to Kodak X-Omat AR film at −80 °C.

A modification of the method described by O'Farrel [28] was used for a two-dimensional (2D) electrophoresis. The isoelectric-focusing gels contained 9.5 M urea, 4% acrylamide, 2% Ampholines (pH 5–7 and pH 3–10 in the proportion 9:1, v/v). Tachyzoites (15 μg of protein equivalents/tube) were dissolved in 9.95 M urea/4% NP40/2% ampholines/0.33% SDS. Electrophoresis in the second dimension was performed as described above and proteins were transferred to nitrocellulose using a 'semi-dry' LKB Multiphor II apparatus (Pharmacia). The nitrocellulose sheet was sequentially incubated with five different monoclonal antibodies specific for the major tachyzoite surface proteins of *T. gondii* (p22, gp23, p30, p35 and p43 respectively).

Preparation of radiolabelled glycopeptides from gp23 and p30

After fluorography, areas of polyacrylamide gel containing gp23 and p30 labelled with [^{3}H]sugars were excised and soaked in dimethyl sulphoxide to remove the En^{3}Hance. The gel pieces were washed (three times) with water and dialysed overnight against water. These gel pieces were sliced and mixed with 0.15 M Tris/HCl (pH 7.8)/1.5 mM $CaCl_2$/1 mg/ml Pronase. Digestion was performed at 37 °C for 72 h with addition of fresh enzyme every 24 h. Digestion was terminated by heating to 100 °C for 10 min. The reaction mixtures were centrifuged, and supernatants containing labelled glycopeptides were processed for analysis as described below.

Size fractionation of glycopeptides

Glycopeptides were desalted on a 1 cm × 100 cm Bio-Gel P6 (200–400 mesh) column (10 ml/h by gravity; 0.6 ml/fraction), equilibrated, and eluted in 0.1 M pyridine acetate, pH 5. Blue Dextran and Phenol Red were mixed with probes and served as markers for void volume (V_0) and column included volume (V_i) respectively. An aliquot of each fraction was measured for radioactivity by liquid-scintillation counting. Fractions containing glycopeptides were pooled, dried, resuspended in water and then fractionated on a 1 cm × 150 cm Bio-Gel P4 (−400 mesh) column (2.2 ml/h by gravity; 0.4 ml/fraction) equilibrated and eluted in water containing 0.02% NaN_3. In this case, BSA (50 mg/ml) was used as V_0 marker and the column was pre-calibrated with [^{14}C]oligosaccharides generated from lipid-linked oligosaccharides from mammalian cells [29].

Carbohydrate compositional analysis by Dionex high-pH anion-exchange chromatography (h.p.a.e.c.)

Polyacrylamide-gel pieces containing gp23 or p30 labelled individually with mannose and glucosamine were acid-hydrolysed with 4 M HCl at 100 °C for 4 h. The resulting hydrolysates were dried and the residual materials were flash-evaporated with 200 μl of methanol (three times), then resuspended in bidistilled water and filtered through a 0.4 μm-pore-size filter prior to Dionex h.p.a.e.c. (Carbopac TM PA1 column; 4 mm × 250 mm; Bio-LC, Dionex Co., Sunnyvale, CA, U.S.A.) using 15 mM NaOH as elution solvent at a flow rate of 1.0 ml/min. Non-radioactive internal monosaccharides used as standards were detected by pulsed amperometry. Fractions (200 μl each) were collected into tubes containing 50 μl of 0.5 M acetic acid and radioactivity was measured by liquid-scintillation counting.

Endoglycosidase treatments

[^{3}H]Glucosamine-labelled glycopeptides were resuspended in 120 μl of incubation buffer containing 5 munits of endo-

glycosidase H (endo H), endo F and treated for 16–20 h at 37 °C. Samples were also treated with N-glycanase (PNGase F) for 72 h. Buffers were as follows: endo H, 0.1 M citrate/phosphate, pH 5.5; PNGase F, 0.1 M KH_2PO_4/50 mM EDTA/0.1 M phenanthroline, pH 7.4; endo F, conditions identical with those for PNGase F, except that the pH was adjusted to 4.2.

Exoglycosidase treatments

The sample was resuspended with 100 μl of 50 mM sodium acetate buffer, pH 4.5, containing α-fucosidase (80 munits) and incubated at 37 °C for 48 h, or with 50 mM sodium citrate, pH 5.0, containing *N*-acetyl-β-hexosaminidase (200 munits) and incubated for 48 h at 37 °C, or with 50 mM sodium phosphate pH 4.5, containing β-galactosidase, or with sialidase (100 munits), and incubated for 24 h at 37 °C, or with jack-bean α-mannosidase (1.5 units) in 50 mM sodium acetate/0.2 mM $ZnCl_2$ for 72 h at 37 °C.

Biosynthesis of dolichyl diphosphate-oligosaccharide precursors in *T. gondii* microsomes

Microsomes from *T. gondii* were prepared using a modification of the method described by McDowell and Schwarz [29]. Briefly, purified parasites (7.5×10^9 tachyzoites) were hypotonically lysed in ice-cold 20 mM Tris/HCl, pH 7.5, for 10 min. Parasites were further disrupted by sonication (Branson 3200 sonifier; 47 kHz; 3 min). A 100 μl portion of 1 M $MgCl_2$ and 330 μl of 1 M NaCl were added to the lysate and, after 10 min, a second sonication was performed. Complete lysis was confirmed by microscopic examination. The homogenate was centrifuged at 1000 *g* at 4 °C for 5 min to remove cell debris and nuclei. The microsomes obtained after ultracentrifugation of the supernatant at 100000 *g* at 4 °C for 1 h (Beckman Ti50 rotor) were resuspended in 2.5 ml of 20 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 0.4 mM $MgCl_2$ and 0.4 mM $MnCl_2$. Further homogenization was achieved using a Dounce homogenizer equipped with a loose-fitting pestle. A 50 μl portion of microsomes was preincubated with non-radioactive UDP-GlcNAc (22 mM) for 20 min at 37 °C. Afterwards, 0.05 μCi of GDP-[^{3}H]Man was added to the reaction mixture and incubation was continued for 10 min. Labelling with UDP-[^{3}H]Glc was performed using the same method, but in this case the microsomes were preincubated with non-radioactive GDP-Man (8.3 mM) and UDP-GlcNAc (22 mM) and the duration of incubation was 15 min.

The labelled lipid precursors were extracted twice with 2 ml of chloroform/methanol (C/M; 2:1, v/v) and the pellet was sequentially washed with methanol/water (M/W). The residual pellet was finally extracted twice with 2 ml of chloroform/methanol/water (CMW; 10:10:3, by vol.). The C/M/W extracts were pooled, dried and subjected to mild acid hydrolysis using 1 ml of n-propanol and 1 ml of 2 M HCL for 15 min at 50 °C. After hydrolysis, reaction mixtures were partitioned between chloroform and 4 mM $MgCl_2$, and the radioactive lipid-linked oligosaccharide precursors were recovered from the chloroform phase.

Radiolabelled standards ($Glc_3Man_9GlcNac_2$ and $Man_9GlcNac_2$) generated from lipid-linked oligosaccharides were also prepared from chicken fibroblasts using the same method.

Preparation of glycopeptides from Sindbis virus

Confluent monolayers of BHK cells were infected with Sindbis virus (0.1 plaque-forming unit/cell in 4 ml of PBS). Virus was allowed to adsorb to cells for about 20 min at room temperature and cultured in 10 ml of glucose-free DMEM supplemented with 2% fetal-calf serum for 4 h. A 200 μCi portion of [^{3}H]mannose was then added and labelling was performed for 16 h. Labelling medium containing virions was collected and centrifuged at 3300 *g* for 15 min and the supernatant was loaded on top of a 25% layer of saccharose and centrifuged at 100000 *g* for 1 h. The pellet was resuspended in PBS and virions were pelleted by ultracentrifugation (100000 *g*, 30 min). The pellet was finally resuspended in PBS, and Laemmli buffer [27] was added for SDS/PAGE analysis. After fluorography, the virion glycoproteins designated E1 and E2 [30] were isolated and glycopeptides were prepared as described.

RESULTS

Incorporation of [^{3}H]sugars into gp23 and p30

To evaluate whether *T. gondii* is capable of synthesizing N-linked glycoproteins, we first determined whether the parasite was able to incorporate tritiated monosaccharides into proteins. Intracellular *T. gondii* tachyzoites were metabolically labelled with [^{3}H]sugars, and parasites were purified and lysed with detergent. As shown in Figure 1, at least 20 proteins were strongly labelled with [^{3}H]glucosamine (lane 1), whereas only three proteins were labelled with [^{3}H]manose (lane 4) and no detectable signal was observed for [^{3}H]galactose labelling (lane 7) when the polyacrylamide gel was fluorographed for 10 days. However, using monoclonal antibodies T4 1E5 and 2E12, we showed that both gp23 and p30 were strongly labelled with [^{3}H]glucosamine (lanes 2 and 3) and also p30 was significantly labelled with [^{3}]mannose and [^{3}H]galactose (lanes 5 and 8). gp23 was only faintly labelled with [^{3}H]mannose (lane 6) and on a short exposure did not appear to be labelled by [^{3}H]galactose (lane 9). Faint incorporation of galactose in gp23, however, was observed when the

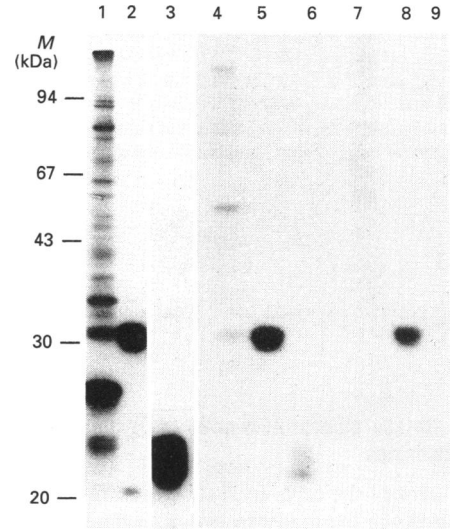

Figure 1 Metabolic labelling of *T. gondii* tachyzoites with [^{3}H]sugars and immunoprecipitations

Fluorogram of an SDS/12%-(w/v) polyacrylamide gel. Lanes 1, 4 and 7 were purified tachyzoite lysates respectively labelled with [^{3}H]glucosamine, -mannose and -galactose. Immunoprecipitations of p30 and gp23 individually labelled with glucosamine, mannose and galactose were performed using monoclonal antibodies, 1E5 (lanes 2, 5 and 8) and 2E12 (lanes 3, 6 and 9). The exposure time was 10 days. The values on the right refer to apparent molecular mass (*M*).

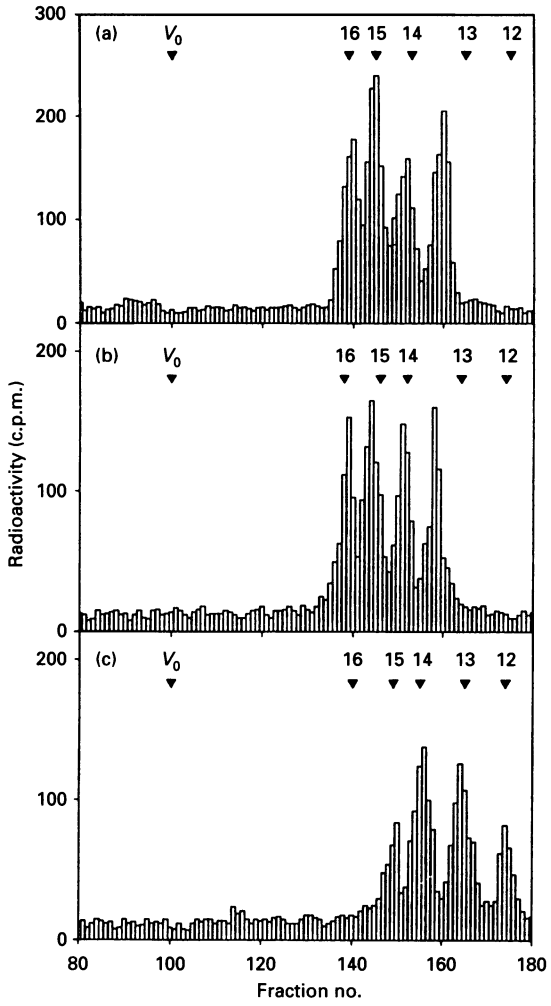

Figure 2 Size fractionation of glycopeptides from [^{3}H]glucosamine-labelled gp23

Purified gp23 labelled with [^{3}H]glucosamine was extensively digested with Pronase for 3 days and the resulting material was desalted on Bio-Gel P6 and then divided in three aliquots. One aliquot (5000 c.p.m.) was sized on a Bio-Gel P4 column (**a**), the second and third aliquots (6000 c.p.m. of each) were respectively incubated with Endo H (**b**) or PNGase F (**c**) and rechromatographed on a Bio-Gel P4 column. The elution position of isomaltose oligosaccharides from dextran hydrolysates (at the top of each panel) and the void volume (V_0) are indicated in this and later Figures.

polyacrylamide gel was exposed for 6 weeks (results not shown; [9]).

The tachyzoite surface glycoprotein gp23 of *T. gondii* contains N-linked carbohydrates

We wished to identify the carbohydrate structures of the surface glycoproteins gp23 individually in order to determine whether they contained the high-mannose-, hybrid- or complex-type oligosaccharides. To address this question, [^{3}H]glucosamine-labelled gp23 proteins were extensively digested with Pronase and the resulting glycopeptides were subjected to gel filtration on a Bio-Gel P4 column. Four glycopeptides co-eluted respectively with 16, 15, 14 and 13.5 glucose units (GU) were identified (Figure 2a). Fractions containing these four peaks were pooled and further analysed using two endoglycosidases. After treatment with PNGase F, an enzyme with broad specificity for N-linked oligosaccharides [31], all four glycopeptides shifted to new elution positions corresponding to 15, 14, 13 and 12 GU (Figure 2c), whereas they showed no changes after treatment with Endo H (Figure 2b), an enzyme with specificity for high-mannose-type oligosaccharides [32]. In addition, these glycopeptides were also resistant to Endo F treatment (results not shown).

Together, the sensitivity to PNGase F and the resistance to Endo H and F indicate that the tachyzoite surface glycoprotein gp23 contains N-linked glycans having hybrid- or complex-type structures.

The tachyzoite major surface protein p30 of *T. gondii* is not N-glycosylated

Specific surface radioidination of *T. gondii* tachyzoites followed by SDS/PAGE reveals four major labelled proteins [3,6]. The most prominent one, p30, represents as much as 3–5% of the total parasite protein [4]. The gene encoding p30 has been sequenced [33] and the predicted amino acid sequence contains one potential N-linked glycosylation sequon: Asn-Ala-Ser. Therefore it was of interest to determine whether this sequon is functional in living parasites. To answer this question, with Pronase glycopeptides were generated from p30 purified from tachyzoites metabolically labelled with [^{3}H]glucosamine. When the radiolabelled glycopeptides from p30 were analysed by gel-filtration chromatography on a Bio-Gel P4 column, two unresolved glycopeptides that were co-eluted with 12 and 11 GU were detected (Figure 3a). The susceptibility of these two glycopeptides to PNGase F, Endo H and F was investigated. As shown in Figures 3(b) and 3(c), digestion of fractions containing these glycopeptides with Endo H (b) or PNGase F (c) yielded radiolabelled peaks that were co-eluted exactly with the undigested material (a), indicating that the two glycopeptides from p30 do not contain N-linked carbohydrates. Under the same experimental conditions these two endoglycosidases were active on $Man_9GlcNAc_2$ standards generated from lipid-linked oligosaccharide of chicken fibroblasts (Endo H; *d* versus *e*) and on glycopeptides generated from well-characterized N-linked glycoproteins of Sindbis virus (PNGase F; *f* versus *g*). Moreover, we have identified the glucosamine-labelled glycopeptides generated from p30 as the C-terminal GPI anchor fragments based on their sensitivity to PI-PLC and nitrous acid deamination (C. F. Zinecker, S. Tomavo, J. F. Dubremetz and R. T. Schwarz, unpublished work).

We conclude that the unique putative N-glycosylation site of the tachyzoite major surface protein p30 is not functional *in vivo*, whereas the surface glycoprotein gp23 (which has not yet been cloned and sequenced) has N-glycosylation sites which are utilized *in vivo*.

Biosynthesis of tachyzoite surface glycoprotein gp23 and protein p30

We performed pulse–chase experiments to determine the precise biosynthetic relationship of the multiple polypeptide forms of gp23. Parasites were pulsed with a mixture of tritiated amino acids (see the Materials and methods section) and chased in the presence of unlabelled medium. As shown in Figure 4(a), the lower band of gp23 was the unique form recovered after a 15 min pulse (lane 4). After 60 min of chase, this major species was converted into two other upper bands (lane 5). However, this conversion was not complete after 60 min. Even if the chase was done up to 6 h, these three polypeptides corresponding to the pattern of gp23 after continuous labelling for 4 h (lane 6), were

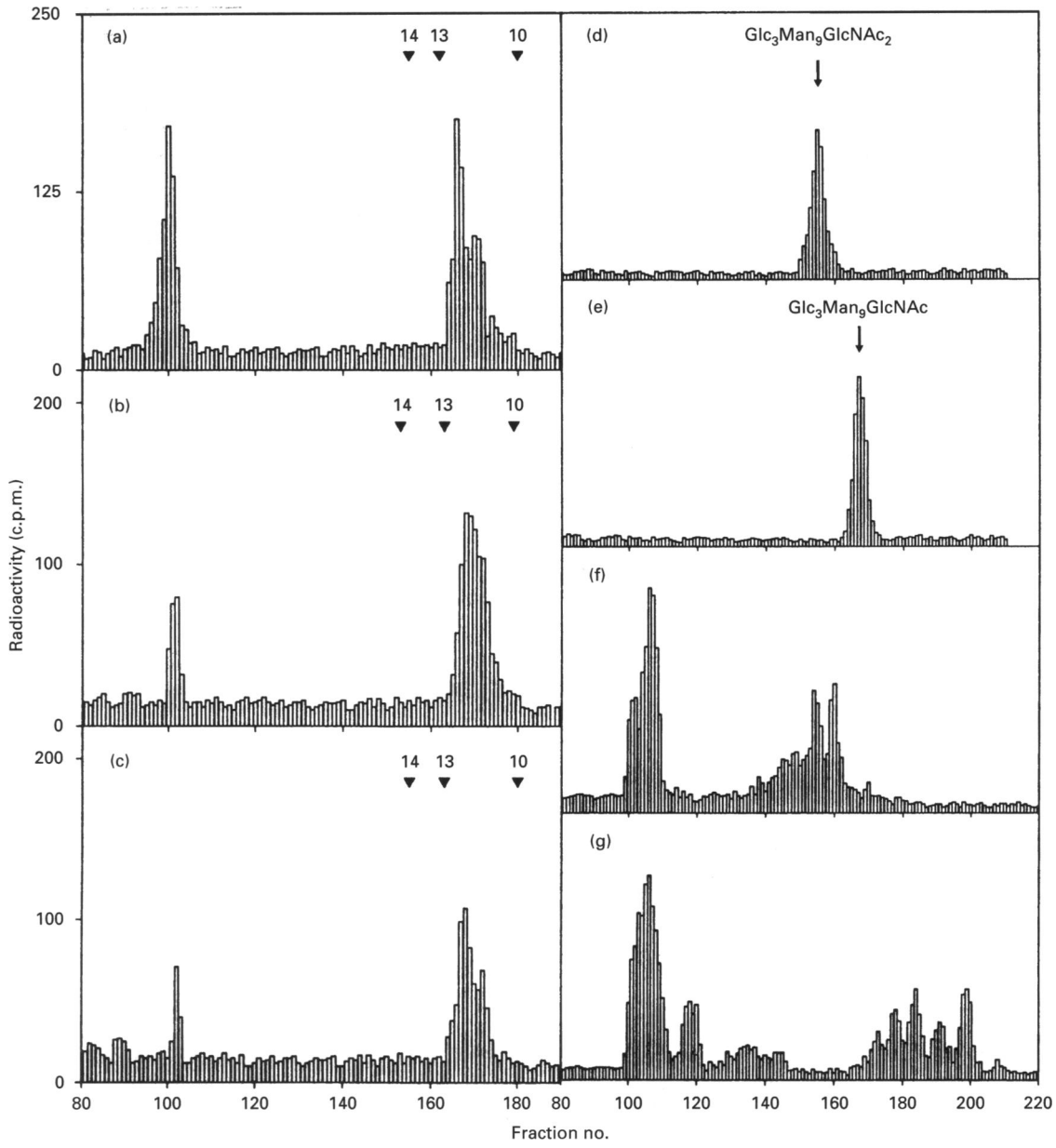

Figure 3 Endoglycosidase digestions of glycopeptides from the major surface protein, p30, of *T. gondii* tachyzoites and those of N-linked oligosaccharide standards

[^{3}H]Glucosamine-labelled glycopeptides from p30 (6000 c.p.m.) were fractionated on a Bio-Gel P4 column without (**a**) or with Endo H (**b**) and PNGase F (**c**) treatments as described in Figure 2. (**d**) Untreated $Man_9GlcNAc_2$ standard generated from lipid-linked oligosaccharides isolated from the chicken fibroblast microsomes. (**e**) Oligosaccharide from (**d**) treated with Endo H. (**f**), untreated glycopeptides derived from Sindbis-virus N-linked glycoproteins. (**g**) Glycopeptides from (**f**) digested with PNGase F.

not further processed. In contrast with gp23, no processing was observed for p30 during the pulse–chase experiments (lanes 1–3).

To characterize further gp23 and p30, 2D isoelectric focusing (i.e.f.)–SDS/PAGE was performed. In this system, proteins are resolved on the basis of their charge in a pH gradient in the first dimension and on the basis of their molecular mass in SDS/polyacrylamide gel in the second dimension [28]. After electrophoresis, the polyacrylamide gel was transferred to nitrocellulose which was sequentially subjected to five different monoclonal antibodies specific for tachyzoite surface proteins [6]. As shown in Figure 4(b), gp23 showed the same broad bands of polypeptides which revealed no charge heterogeneity (pI 6.1), whereas p30 ran as two closely migrating bands resolved in two isoforms with pI values of 6.75 and 6.95. Since the pulse–chase experiment reveals only a unique band for p30 (Figure 4a: lanes 1, 2 and 3), we assume that the presence of two polypeptides in the 2D i.e.f.–SDS/PAGE system is probably an artefact. However, we cannot exclude the possibility of co- and/or post-translational events that we are unable to identify under our pulse–chase conditions. Indeed, we have recently found that the serine residues in the surface protein p30 were phosphorylated [34.].

Comparison with the other tachyzoite surface proteins showed that p35 behaves as gp23, with no detectable charge heterogeneity (pI 5.9), whereas p43 and p22 also gave several distinct isoforms.

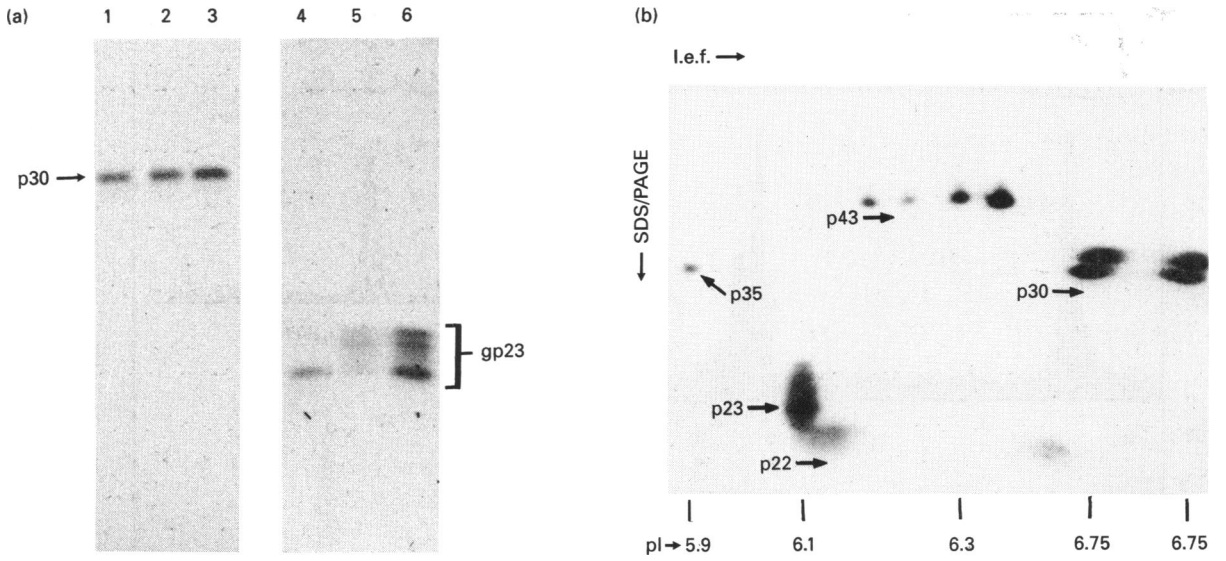

Figure 4 Biosynthesis and processing of gp23 and p30

(**a**) Pulse–chase analysis of gp23 and p30. Intracellular parasites were pulsed (lanes 1 and 4) with a tritiated-amino-acid mixture (see the Materials and methods section) for 15 min and then chased (lanes 2 and 5) with non-radioactive medium for 1 h. After labelling, the infected cells were solubilized with detergent, and gp23 or p30 was immunoprecipitated using monoclonal antibodies T4 1E5 and 2E12. Lanes 3 and 6, controls gp23 and p30 immunoprecipitated from parasites which were continuously labelled for 4 h. (**b**) 2D i.e.f.–SDS/PAGE analysis of the surface proteins of *T. gondii* tachyzoites. Ampholytes of pI ranges 3–10 and 5–7 in a ratio of 9:1 were used in the first dimension, and 12%-polyacrylamide gel was used for the second. After electrophoresis, the gel was transferred to nitrocellulose which was developed successively with five monoclonal antibodies specific for surface proteins indicated on the Figure.

The processing of gp23 during the pulse–chase experiments is consistent with the presence of N-linked glycans on this molecule. Interestingly, 2D i.e.f.–SDS/PAGE analysis of gp23 showed no charge heterogeneity, suggesting that its N-linked glycans are composed only of neutral monosaccharides. This assumption is confirmed below.

Carbohydrate compositional analysis of gp23

Gp23 purified from parasites metabolically labelled with [^{3}H]sugars was acid-hydrolysed (4 M HCl; 100 °C; 4 h) and analysed by Dionex h.p.a.e.c. Analysis of [^{3}H]glucosamine-labelled gp23 (Figure 5a) showed that the supplied radioactive glucosamine was metabolically converted into galactosamine, which represented 87% of the N-linked oligosaccharides of this molecule. In addition, the N-linked glycans of gp23 were sensitive to β-hexosaminidase treatment, indicating that galactosamine was in its N-acetylated form. In contrast, the radiolabel of [^{3}H]mannose remained unchanged (Figure 5b).

To evaluate further the monosaccharide compositions of gp23, the four glycopeptides were digested with exoglycosidases. The glycopeptides were insensitive to neuraminidase, and no 5-acetylneuraminic acid (Neu5Ac) was detected on Dionex h.p.a.e.c. after mild acid hydrolysis. Moreover, the absence of Neu5Ac was confirmed by the fact that no mannosamine (the precursor for Neu5Ac) was detected after mild hydrolysis of gp23 metabolically labelled with [^{3}H]glucosamine. The absence of Neu5Ac in gp23 is also consistent with its electrophoretic pattern seen in 2D i.e.f.–SDS/PAGE (Figure 4b). Furthermore, the N-linked glycans of gp23 were also insensitive to fucosidase treatment, and no fucose was incorporated in this molecule after metabolic labelling (S. Tomavo and J. F. Dubremetz, unpublished work).

On the basis of the endo-/exo-glycosidase results, monosaccharide composition analysis and the metabolic labelling data, we conclude that the tachyzoite surface glycoprotein, gp23 of *T. gondii* contains N-linked hybrid-type glycans composed of at least *N*-acetylgalactosamine, *N*-acetylglucosamine and mannose.

Biochemical analysis of glycopeptides from crude protein extracts of *T. gondii* tachyzoites

To examine whether *T. gondii* tachyzoites are capable of synthesizing other types of glycan structures, parasites were metabolically labelled with [^{3}H]glucosamine. After labelling, parasites were purified as described in the Materials and method section, and glycolipids were removed by organic solvents. The residual material corresponding to the crude protein extract was digested with Pronase to give glycopeptides. Analysis of the Pronase digestion products on a Bio-Gel P6 column yielded only in two major radiolabelled peaks (Figure 6a), named P1 and P2. Fractions containing these glycopeptides were individually pooled and further characterized using endoglycosidase digestions, followed by size fractionation. On the Bio-Gel P4 column, P1 chromatographed near to the void volume (b). The treatment of P1 with Endo F and Endo H released 30 and 40% of N-linked oligosaccharides from P1 (c and d), indicating that *T. gondii* tachyzoites are also capable of synthesizing N-linked oligosaccharides having the high-mannose-type structure. However, a majority of the radiolabel eluted as P1 appeared to be resistant to Endo H and F treatments as well as to PNGase F. The same situation was observed for P2, which was also resistant to these endoglycosidases (results not shown). Our preliminary studies suggest that the undigested material of P1 are *O*-linked glycopeptides (M. Odenthal-Schnittler, S. Tomavo, J. F. Dubremetz and R. T. Schwarz, unpublished work).

Isolation and purification of *T. gondii* from Vero cells was done according to standard procedures [26]. A minor contamination with Vero-cell material might be possible, but up to

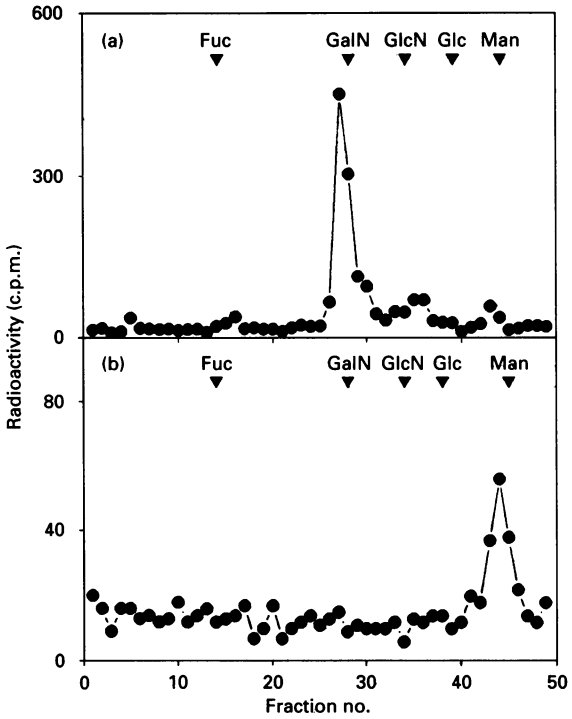

Figure 5 Analysis of the monosaccharide composition of metabolically labelled gp23 on Dionex h.p.a.e.c.

[^{3}H]glucosamine (**a**)- or [^{3}H]mannose (**b**)-labelled gp23 was hydrolysed with 4 M HCl (100 °C for 4 h). The resulting hydrolysates were mixed with unlabelled monosaccharide standards and then subjected to Dionex chromatography. The elution positions of monosaccharide standards are shown at the top of each panel.

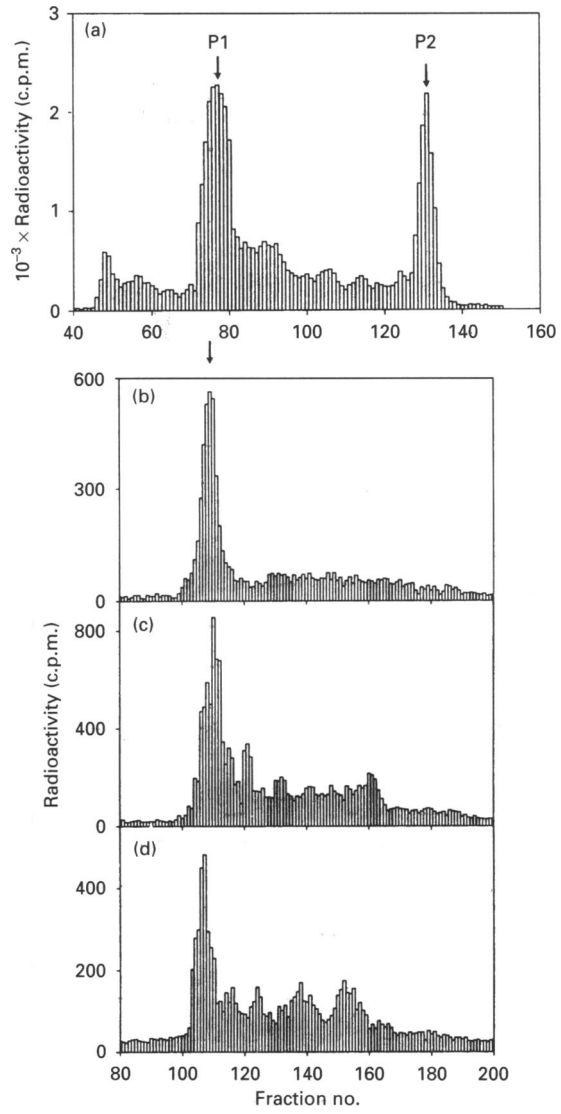

Figure 6 Size fractionation of glycopeptides from crude protein extracts of tachyzoites metabolically labelled with [^{3}H]glucosamine

Purified parasites metabolically labelled with [^{3}H]glucosamine were extracted with C/M and C/M/W to remove glycolipids. The residual pellet containing total proteins was digested with Pronase, and the resulting glycopeptides were sized on a Bio-Gel P6 column (**a**). Radioactive fractions were pooled according to radioactive peaks P1 and P2. Each sample of material was dried and resuspended in water. (**b**) Shows the Bio-Gel P4 chromatogram of P1. Other aliquots of P1 were treated with Endo F (**c**), Endo H (**d**) and rechromatographed on the Bio-Gel P4 column.

now no purification method resulting in 100% pure *T. gondii* parasites has been described in the literature. Interestingly, and confirming the results that the analysed glycopeptides are derived from the parasites themselves, exactly the same peak profiles (P1 and P2) were found after labelling extracellular parasites. Living parasites were isolated and purified on a glass-wool column and taken up in PBS and centrifuged to remove a possible contamination by Vero-cell debris (microsomes etc.). This washing procedure was repeated twice. Subsequently the still-living parasites were labelled with [^{3}H]glucosamine. With both intracellular or extracellular labelling most of the glucosamine label of peak P1 was converted into N-acetyl-galactosamine (80–90%; results not shown). A substantial part of the labelled material of peak P2 consists presumably of amino acids (about 90%). The labelled material from peak P2 analysed by Dionex h.p.a.e.c. after hydrolysis shows the same elution characteristics as amino acids (results not shown).

Detection of the lipid-linked oligosaccharide precursor for *T. gondii* N-linked glycoproteins

It has been established in mammalian cells that the major pathway of N-linked glycosylation involves the synthesis and the transfer of the precursor oligosaccharide, $Glc_3Man_9GlcNAc_2$, attached to dolichol, a long-chain isoprenoid alcohol. The precursor oligosaccharide is processed to give the mature carbohydrate structures of glycoproteins [10]. We have investigated the presence of such lipid-linked oligosaccharides in *T. gondii* microsome preparations which were incubated with [^{3}H]sugar nucleotides, and the lipid-linked glycans were extracted with organic solvent. When *T. gondii* microsomes were labelled with UDP-[^{3}H]glucose and the labelled oligosaccharides were analysed after mild acid hydrolysis of the lipid-linked molecules, one peak, which was co-eluted with the $Glc_3Man_9GlcNAc_2$ standard, was obtained (Figure 7c). Furthermore, the oligosaccharide intermediate, $Man_9GlcNAc_2$ was also isolated from glycolipid fractions (Fig. 7a) when GDP-[^{3}H]mannose was introduced in the *T. gondii* microsomes. Both the $Glc_3Man_9GlcNAc_2$ and $Man_9GlcNAc_2$ species were sensitive to Endo H (Figures 7b and 7d), releasing the first *N*-acetylglucosamine, as expected for high-mannose-type structures. In addition, the introduction of tunicamycin in the *T. gondii* microsomes completely abolished

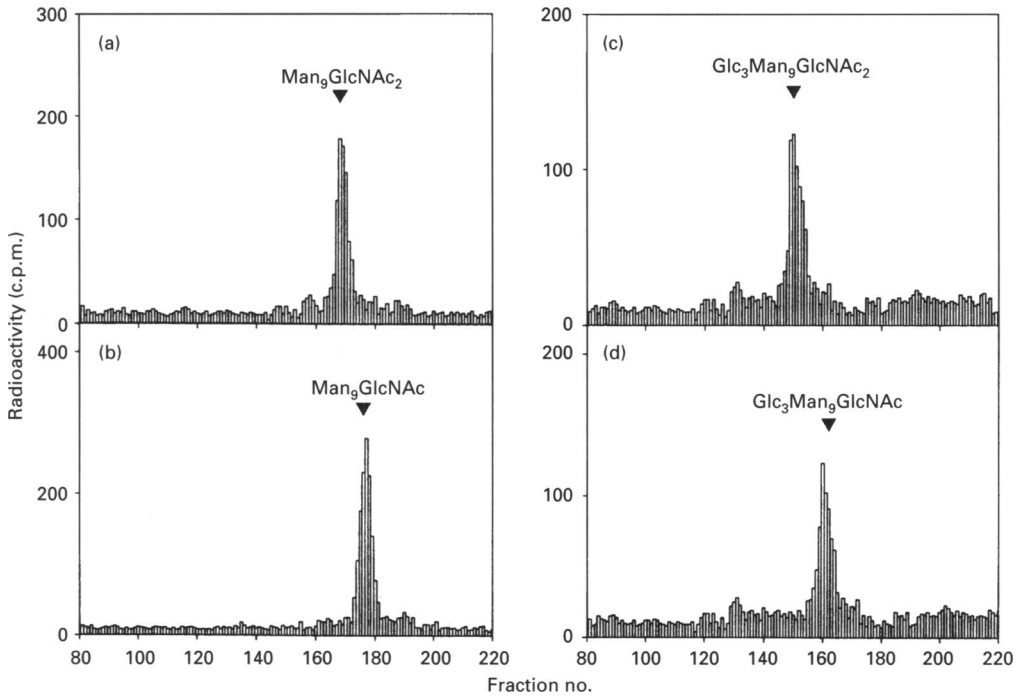

Figure 7 Bio-Gel P4 elution profile of radiolabelled glycans from lipid-linked oligosaccharides synthesized in *T. gondii* microsomes

T. gondii microsomes were incubated with labelled sugar nucleotides. After incubation, oligosaccharides derived from the lipid-extracts were sized on Bio-Gel P4 columns (**a** and **c**). The elution positions of oligosaccharides ($Man_9GlcNAc_2$ and $Glc_3Man_9GlcNAc_2$) from the lipid precursors for the N-linked protein glycosylation in chicken fibroblasts are indicated by an arrow. (**b**) and (**d**) Bio-Gel P4 analysis of $Man_9GlcNAc_2$ and $Glc_3Man_3GlcNAc_2$ of *T. gondii* tachyzoites after digestion with endo H.

the synthesis of these lipid-linked oligosaccharides (results not shown).

Together, these results show that *T. gondii* tachyzoites are indeed able to synthesize the lipid-linked oligosaccharide precursors for the N-linked glycoproteins, as would be expected given the discovery of such post-translational modifications in this parasite.

DISCUSSION

The tachyzoite surface glycoprotein gp23 contains four N-linked oligosaccharides which can be released after PNGase F treatment. The N-linked oligosaccharides of gp23 were resistant to endoglycosidases H and F. The monosaccharide compositional analysis of the N-glycans of gp23 indicated that they contain at least *N*-acetylgalactosamine, *N*-acetylglucosamine and mannose. It is of interest to note that some common substituents of mammalian N-linked carbohydrates, such as fucose and sialic acid, were absent from N-glycans of gp23, as were certain classes of hybrid and multi-antennary complex-type N-linked oligosaccharides.

We have also investigated the presence of N-linked glycan structures on the major tachyzoite surface protein p30, given that its amino acid sequence shows the presence of one putative N-linked glycosylation sequon, namely Asn-Ala-Ser. Radiolabelled glycopeptides were prepared from purified p30 and then subjected to gel-filtration analysis. Unexpectedly, the glycopeptides generated from p30 were resistant to PNGase F, Endo H and F, indicating that the putative N-linked glycosylation site in the primary sequence of p30 is not functional in living tachyzoites of *T. gondii*. However, we have demonstrated the presence of N-linked glycans on another surface glycoprotein, gp23. In addition, we performed pulse–chase experiments to determine the biosynthetic relationship between the three detectable bands of gp23 consistently seen after SDS/PAGE. The lower band appears to be the earliest detectable form which gives rise to the upper bands. This behaviour is consistent with the processing of N-linked glycoproteins. We have also analysed this molecule by 2D i.e.f.–SDS/PAGE and showed that the different bands of gp23 have no charge heterogeneity which is also consistent of the co-ordinate insensitivity to sialidase.

We conclude that the N-linked carbohydrates have features typical of hybrid-type species or multi-antennary complex type without sialic acid and fucose residues. However, we have identified high-mannose-type glycans in *T. gondii* tachyzoites, as demonstrated by the Endo H- and F-sensitivity of glycopeptides generated from crude protein extracts. We do not know the identity and the localization of the oligomannose-rich glycoproteins, but it is of interest to note that de Carvallo et al. [35] have recently described a panel of lectins which bind to the rhoptries, apical organelles of *T. gondii* playing a key role during the invasion process. Furthermore, we were able to show that *T. gondii* microsomes are capable of synthesizing the precursors of N-glycans, the dolichol-linked oligosaccharides ($Glc_3Man_9GlcNAc_2$ and $Man_9GlcNac_2$; Figure 7).

The most unusual features of the N-glycosylated structures of *T. gondii* are the absence of both sialic acid and fucose and the predominent presence of *N*-acetylgalactosamine. This latter monosaccharide has been found in terminal position in very few animal cell glycoproteins [36–38] and in the glycoproteins of *Schistosoma mansoni* [39]. The biological significance of terminal GalNAc residues on carbohydrate chains is unknown and it also remains to be determined whether this monosaccharide or the entire N-linked carbohydrates are involved in any biological function of this parasite.

Conclusion

We show that one of the five major surface proteins of *T. gondii* tachyzoites gp23 is N-glycosylated. Previous reports have indicated that lectins are unable to bind the pellicle of living parasites. We speculate that this difference may be explained by steric reasons, which thereby prevent the accessibility of the carbohydrate structures present on the parasite surface. To our knowledge this study represents the first report of N-linked glycans in a sporozoan parasite. Indeed, the inability of *Plasmodium falciparum* to synthesize N-linked glycans has been recently reported [40]. It also remains to be determined whether the other parasites belonging to the Sporozoa class (*Eimeria*, *Sarcocystis*, *Babesia* etc.) are capable of producing such post-translation modifications.

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