The Role of Inositol Acylation and Inositol Deacylation in the *Toxoplasma gondii* Glycosylphosphatidylinositol Biosynthetic Pathway

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*Toxoplasma gondii* is a ubiquitous parasitic protozoan that invades nucleated cells in a process thought to be in part due to several surface glycosylphosphatidylinositol (GPI)-anchored proteins, like the major surface antigen SAG1 (P30), which dominates the plasma membrane. The serine protease inhibitors phenylmethylsulfonyl fluoride and diisopropyl fluoride were found to have a profound effect on the *T. gondii* GPI biosynthetic pathway, leading to the observation and characterization of novel inositol-acylated mannosylated GPI intermediates. This inositol acylation is acyl-CoA-dependent and takes place before mannosylation, but uniquely for this class of inositol-acyltransferase, it is inhibited by phenylmethylsulfonyl fluoride. The subsequent inositol deacylation of fully mannosylated GPI intermediates is inhibited by both phenylmethylsulfonyl fluoride and diisopropyl fluoride. The use of these serine protease inhibitors allows observations as to the timing of inositol acylation and subsequent inositol deacylation of the GPI intermediates. Inositol acylation of the non-mannosylated GPI intermediate D-GlcNAc1-6-d-myoinositol-1-HPO4-sn-lipid precedes mannosylation. Inositol deacylation of the fully mannosylated GPI intermediate allows further processing, i.e. addition of GalNAc side chain to the first mannose. Characterization of the phosphatidylinositol moieties present on both free GPIs and GPI-anchored proteins shows the presence of a diacylglycerol lipid, whose sn-2 position contains almost exclusively an C18:1 acyl chain. The data presented here identify key novel inositol-acylated mannosylated GPI intermediates, allowing the formulation of an updated *T. gondii* GPI biosynthetic pathway along with identification of the putative genes involved.

"The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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3 The abbreviations used are: GPI, glycosylphosphatidylinositol; Dol-P-Man (DPM), dolichol-phosphate-mannose; Glc, glucose; GlcN, glucosamine; EtNP, ethanolamine phosphate; GalNAc, N-acetylgalactosamine; GlcN-PI, D-GlcN1-6-d-myoinositol-1-HPO4-sn-lipid; Gu, glucose unit; PI-PLC, PI-PLD, phosphatidylinositol-specific phospholipase C and D, respectively; GPino, phosphatidylinositol; HPTLC, high performance thin layer chromatography; JBU-M, jack bean α-mannosidase; EtNP-Man3GlcN-PI, NH2CH2CH2PO4H-6Man1-2Manol-6Manol-1-4GlcNa1-6-D-myo-inositol-1-HPO4-sn-lipid; ES-MS, electrospray mass spectrometry; ER, endoplasmic reticulum; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluoride; Pam, pamityl.
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T. gondii a side chain consisting of glucose α1-4N-acetylgalactosamine linked to the first mannose has been identified (15). It has been shown that free GPs (the so-called “low molecular weight antigen” (16, 17)) present in large amounts on the T. gondii surface are highly immunogenic in humans (18), some able to induce tumor necrosis factor-α production in macrophages (19, 20).

The sequence of events underlying GPI biosynthesis has been studied in several organisms including Trypanosoma brucei (21–29), Trypanosoma cruzi (30), Plasmodium falciparum (31), Leishmania (32, 33), Saccharomyces cerevisiae (34, 35), and mammalian cells (36–38) and to a minor extent in T. gondii (16, 39–41). In all cases, GPI biosynthesis involves the initial addition of GlcN-PI from UDP-GlcNac to phosphatidylinositol (Gplno) to form GlcNac-PI, which is then de-N-acetylated to GlcN-PI (43–44). De-N-acetylation is a prerequisite for mannosylation of GlcN-PI to form later GPI intermediates (45–46). These early events occur on the cytoplasmic face of the endoplasmic reticulum, whereas the subsequent steps of mannosylation take place on the luminal face (10), this followed by the transfer of ethanolamine phosphate to the third mannose (24, 27). Uniquely, the subsequent addition of an ethanolamine phosphate to the third mannose (24, 27). The transfer of GalNAc and Glc to the first mannose takes place on the cytoplasmic face of the ER (49). The pre-assembled glycolipid GPI precursor is then flipped inside the ER where it is transferred en bloc to a protein via a transamidase reaction involving the cleavage of a hydrophobic C-terminal GPI signal sequence (for review, see Refs. 50 and 51).

Nevertheless, significant differences in the timing of certain biosynthetic steps occur between organisms, including the common but sometimes transient addition of an acyl chain linked to the 2-hydroxyl of the myo-inositol of GPI-anchor precursors. In P. falciparum as well as mammalian and yeast cells this inositol acylation is dependent upon acyl-CoA and is required before the addition of the first mannose (52–53). In contrast, T. brucei inositol acylation only occurs after the addition of the first mannose (24, 27), where it is a prerequisite for the subsequent addition of an ethanolamine phosphate to the third mannose (24, 27). Uniquely, T. brucei inositol acylation is not acyl-CoA-dependent, and both the inositol acylation and inositol deacylation are inhibited by the serine protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and diisopropylfluoride (DFP) respectively.

In this paper we show that the serine protease inhibitors PMSF and DFP have a drastic effect on the T. gondii GPI pathway. Using a cell-free system and in vivo labeling allowed the characterization of novel mannosylated inositol-acylated T. gondii GPI intermediates. These allowed the elucidation of the roles of inositol acylation and inositol deacylation in the T. gondii GPI biosynthetic pathway.

EXPERIMENTAL PROCEDURES

Materials—D-[6-3H]GlcNac, dithiothreitol (1 mM) in the absence or presence of ATP (1 mM) and CoA (1 mM) or Acyl-CoA (100 μM). Reactions were brief and incubated for 90 min at 37 °C, and the radiolabeled glycolipids were extracted and analyzed as described below.

In Vitro Labeling of Early, Non-mannosylated GPI Biosynthesis Intermediates—In vitro labeling was performed as described (16, 39–41). Hypotonically permeabilized parasites were pelleted (1000 × g, 10 min, 4 °C) and washed twice with incubation buffer A (50 mM Na-HEPES at pH 7.4, 25 mM KCl, 5 mM MgCl2, 0.1 mM 1-chloro-3-tosylamido-7-amino-2-heptanone, 1 μg/ml leupeptin, 0.2 μg/ml tunicamycin). Labeling experiments were performed in the presence of 8 mM EDTA and absence of Mn2+, as Mn2+ is required for dolichol-phosphate-mannose synthase activity, therefore preventing the formation of Dol-P-Man (38). Aliquots of 1 × 10⁸ tachyzoites were labeled in buffer A supplemented with 2 μCi of GDP-[2-3H]GlcpNac, dithiothreitol (1 mM) in the absence or presence of ATP (1 mM) and CoA (1 mM) or Acyl-CoA (100 μM). Reactions were briefly vortexed and incubated for 90 min at 37 °C, and the radiolabeled glycolipid products were extracted and analyzed as described below.

Labeling of Mannosylated Glycolipids—Aliquots (100 μl) of 0.5 × 10⁸ tachyzoites were labeled in buffer A with 1 μCi of GDP-[2-3H]Man, dithiothreitol (1 mM), and MnCl2 (5 mM) were incubated with either UDP-[6-3H]GlcNac (1 mM) or GlcN-PI (500 μM)² with n-octyl-glucopyranoside (0.3%) in the absence or presence of the following supplements: ATP (1 mM), CoA (100 μM), pamitoyl-CoA (100 μM), UDP-GalNac (1 mM), and UDP-Glc (1 mM). Some reactions were preincubated with PMSF (1 mM) or DFP (1 mM) for 5 min before the addition of supplements. The cell-free system reactions were briefly sonicated and incubated for 120 min at 37 °C. The radiolabeled glycolipids were extracted and analyzed as described below.

Extraction of Glycolipids—All in vivo and in vitro labeled glycolipid products were extracted three times in chloroform/methanol/water (10:10:3, v/v), dried, and recovered from a butan-1-ol partitioning, as previously described (20).
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HPTLC—Samples as well as glycolipid standards were applied to 10-cm aluminum-backed silica gel 60 HPTLC plates that were developed using solvent system A, hexane, chloroform, methanol, water, acetic acid (3:10:10:2:1, v/v), or solvent system B, chloroform, methanol, 1 M ammonium hydroxide (10:10:3, v/v), both before and after enzymatic and chemical digests. Radiolabeled components were detected using either a Berthold LB2842 or a Bioscan AR-200 linear analyzer or by fluorography at −70 °C after soaking in EA-Wax and using Kodak XAR-5 film with an intensifying screen.

Enzymatic and Chemical Treatments of Radiolabeled Glycolipids—Digestions with β-N-acetylhexosaminidase, β-N-acetylglucosaminidase, and α-glucosidase were performed following the manufacturer’s instructions.

Glycan Head-group Analysis—The HPTLC-purified radiolabeled glycolipids were delipidated, deaminated, reduced, dephosphorylated with aqueous HF, and desalted by passage through AG50X12 (H+) and AG3X4 (OH−) ion-exchange resins. The resulting neutral glycan head groups were analyzed before and after various glycosidic digests each by Bio-Gel P4 gel filtration (Ref. 16 and references therein). For these analyses the radiolabeled glycans were detected by scintillation counting and correlated with the elution positions of the co-injected individual glucose oligomer standards.

Exoglycosidase Digestions of Glycans—Glycans obtained from HPTLC-purified radiolabeled glycolipids were digested with either β-N-acetylhexosaminidase, β-N-acetylglucosaminidase, or α-glucosidase. All enzymatic digests were terminated by heating at 100 °C for 5 min. The samples were desalted by passing through 0.25 ml of AG50X12 (H+), dried, and flash co-evaporated with toluene to remove residual acetic acid.

Identification of the Phosphatidylinositol Moieties from Purified Free GPs and GPI-anchored Proteins—T. gondii free GPs and GPI-anchored proteins were purified as previously described (54). Aliquots (2–10 nmol) were dried and dissolved in 15 µl of sodium acetate (0.3 M, pH 4) followed by the addition of 7.5 µl of freshly prepared sodium nitrite (1 M) and incubated for 1 h at room temperature. An additional 15 µl of sodium acetate (0.3 M, pH 4) and 7.5 µl of freshly prepared sodium nitrite (1 M) were added and incubated for a further 2 h at 37 °C. The GPIno moiety released by deamination was partitioned into butan-1-ol (3 × 100 µl). The pooled butan-1-ol extracts were dried and suspended in chloroform/methanol (1:2) and analyzed by negative ion electrospray mass spectrometry (ES-MS) on a Quattro Ultima triple quadrupole instrument. Samples were introduced into the mass spectrometer using nanospray tips. GPIno species were observed in negative ion mode with a capillary voltage of 0.9 kV and a cone voltage of 40–60 V. Daughter ion ES-MS-MS spectra were obtained with a collision voltage of 35–50 V using argon at 3 × 10−3 torr as the collision gas. MassLynx was used to record and process the data.

Identification of Toxoplasma GPI Biosynthetic Genes—Genes for GPI biosynthesis were identified by using sequences of homologue genes for the GPI biosynthesis of different species as probes to systematically search for the homologous genes in data provided by the Toxoplasma Genome resource. In all cases putative homologous genes were identified and analyzed as to their suitability to be true homologues. The homologues were checked for Pfam motifs and conserved residues as well as topology and hydrophobicity comparisons, after which they were submitted to EMBL and GenBank (see Table 2).

RESULTS

Formation of GlcN-(acyl)PI—A cell-free system was utilized to study the Toxoplasma GPI biosynthetic pathway. Priming of the endogenous GPI pathway with UDP-[3H]GlcNAc in the presence of dithiothreitol and EDTA (Fig. 1A) resulted in the formation of two [3H]GlcN-labeled glycolipids. Identification by various digests (Table 1) revealed them to be [3H]GlcN-(acyl)PI and [3H]GlcN-PI, presumably formed via the UDP-GlcNAc:PI GlcNAc transferase and the subsequent GlcNAc-PI de-N-acetylase. The addition of ATP and CoA (Fig. 1B) or Pam-CoA...
The characterization of glycolipids II, III, IV, V, and VI are consistent with previously published findings (16, 39–41). The sizes of the neutral glycans terminating in 2,5-anhydromannitol were determined as described under “Experimental Procedures” and are expressed in glucose units (GU).

Consistent with all three glycans having a Man$_3$-anhydromannitol head group, but two of the three [3H]mannosylated glycolipids were resistant to JBAm, suggesting a group on the non-reducing Man, probably an ethanolamine phosphate group. These data taken together with their Rf suggest that they are glycolipids similar to the T. brucei GPI intermediates Man$_3$GlcN-PI (M3), EtNP-Man$_3$GlcN-PI (A'), and EtNP-Man$_3$GlcN-(lyso)PI (lyso-A').

Replacement of UDP-GlcNAc with an exogenous synthetic acceptor d-GlcNal–6-d-myo-inositol-1-HPO$_4^{2-}$sn-1,2-dipalmitylglycerol (GlcN-PI) in the presence of Pam-CoA (Fig. 2C) resulted in the formation of [3H]mannosylated glycolipids, whose Rf and characterization are the same to those formed with UDP-GlcNAc (Fig. 2B), including an extra minor [3H]mannosylated glycolipid identified as lyso-M3 (Table 1). The incorporation of [3H]mannose into the [3H]glycolipids formed utilizing the exogenous acceptor GlcN-PI (Fig. 2C) is about double that of the endogenously UDP-GlcNAc-primed (Fig. 2B), probably because GlcN-PI only has to be inositol-acylated before [3H]mannosylation as opposed to endogenous priming UDP-GlcNAc, having to form GlcN-PI, which undergoes de-N-acylation to form GlcN-PI. The Rf values of the [3H]mannosylated glycolipids from the exogenous acceptor (Fig. 2C) coincided with those of the endogenously primed glycolipids (Fig. 2B), suggesting that not only do they have the same mannosyltransferase head group but also the total lipid hydrophobicity of the endogenous glycolipids must be similar to those formed from the exogenous synthetic acceptor d-GlcNal–6-d-myo-inositol-1-HPO$_4^{2-}$sn-1,2-dipalmitylglycerol.

Precolumnation of the cell-free system with amphotericin and CaCl$_2$ prevented the formation of Dol-P-[3H]Man from GDP-[3H]Man and in turn prevented the formation of any [3H]mannosylated GPI intermediates (data not shown). This suggests that at least the first mannosyltransferase is Dol-P-Man-dependent, consistent with other GPI pathways where the man-
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![Diagram](image)

**FIGURE 2. Inositol acylation is required before mannosylation of T. gondii GPIs.** Washed parasite membranes were incubated with GDP-[3H]Man and UDP-GlcNAc only (A) or in the presence of ATP and CoA (B). Alternatively, membranes were incubated with GDP-[3H]Man in the presence of GlcN-PI and Pam-CoA (C) or with the addition of UDP-GalNAc (D) or UDP-GalNAc and UDP-Glc (E). The [3H]mannosylated glycolipids were partitioned into butanol and analyzed by HPTLC using solvent system B. The [3H]mannosylated glycolipids were characterized (see Table 1). The identities of the bands are Dol-P-Man (DPM), Man₅GlcN-PI (M3), EtNP-Man₂(GalNAc)ManGlcN-PI (A’), and Man₆GlcN-PI (B’). The bands were compared to the A’ and B’ bands that were used as standards.

The addition of UDP-GalNAc to the cell-free system containing GDP-[3H]Man, GlcN-PI, Pam-CoA, and UDP-GalNAc gave rise to two new mannosylated glycolipids II and V (Fig. 2E). Both of these glycolipids were sensitive to GPI-PLD and PI-PLC (Table 1), showing that they were non-inositol-acylated GPI intermediates. JBoM treatment showed glycolipid II was resistant, although glycolipid V was partially sensitive. The desalted 2,5-anhydromannitol-containing glycan head groups obtained from the glycolipids II–V were analyzed by Bio-Gel P4 gel filtration before and after α-mannosidase and β-N-acetylhexamannidase (Table 1). The glycans obtained from glycolipids II and V had a size of 6.8–7.0 Gu and were sensitive to α-mannosidase and α-glucosidase, giving digested glycan products of 4.9 and 5.9 Gu corresponding to Glcα1–4GalNAcβ1–4Manα1–4anhydromannitol and Manα1–2Manα1–6Manα1–4anhydromannitol, respectively. The desalted 2,5-anhydromannitol-containing glycan head groups obtained from the glycolipids II–V were analyzed by Bio-Gel P4 gel filtration before and after α-mannosidase and β-N-acetylhexamannidase (Table 1). The original neutral glycans obtained from all three glycolipids eluted from the column at 5.9/6.0 Gu and were sensitive to α-mannosidase and β-N-acetylhexamannidase, giving glycan products of 4.4 and 4.2 Gu, respectively, corresponding to GalNAcβ1–4Manα1–4anhydromannitol and Manα1–2Manα1–6Manα1–4anhydromannitol. Thus, the original neutral glycan obtained from both glycolipids III and IV is Manα1–2Manα1–(6GalNAcβ1–4)Manα1–4anhydromannitol. The difference in RF of these glycolipids and the resistance of III but sensitivity of VI to α-mannosidase suggest that the former structure has an ethanolamine phosphate group on the terminal mannose; thus the structures of glycolipid III and VI are EtNP-Man₂(GalNAc)ManGlcN-PI and glycolipid VI Man₅(GalNAc)ManGlcN-PI, respectively.

The addition of UDP-Glc to the cell-free system containing GDP-[3H]Man, GlcN-PI, Pam-CoA, and UDP-GalNAc gave rise to two new mannosylated glycolipids II and V (Fig. 2E). Both of these glycolipids were sensitive to GPI-PLD and PI-PLC (Table 1), showing that they were non-inositol-acylated GPI intermediates. JBoM treatment showed glycolipid II was resistant, although glycolipid V was partially sensitive. The desalted 2,5-anhydromannitol-containing glycan head groups obtained from the glycolipids II–V were analyzed by Bio-Gel P4 gel filtration before and after α-mannosidase and β-N-acetylhexamannidase (Table 1). The glycans obtained from glycolipids II and V had a size of 6.8–7.0 Gu and were sensitive to α-mannosidase and α-glucosidase, giving digested glycan products of 4.9 and 5.9 Gu corresponding to Glcα1–4GalNAcβ1–4Manα1–4anhydromannitol and Manα1–2Manα1–(6GalNAcβ1–4)Manα1–4anhydromannitol, respectively. Thus, the original neutral glycan would correspond to Manα1–2Manα1–6(GalNAcβ1–4)Manα1–4anhydromannitol. The difference in RF of the glycolipids and resistance of II but sensitivity of V to α-mannosidase suggest the former has an ethanolamine-phosphate group on the terminal mannose; thus, glycolipid II has the structure EtNP-Man₂(Glc-GalNAc)ManGlcN-PI, and V has the structure Man₅(Glc-GalNAc)ManGlcN-PI.

**Inhibition of Inositol Acylation and Inositol Deacylation in the T. gondii Cell-free System—Washed Toxoplasma membranes were preincubated with either PMSF (Fig. 3A) or DFP (Fig. 3B) for 5 min on ice before incubation with GDP-[3H]Man, GlcN-PI, and Pam-CoA. In the presence of PMSF only Dol-P-[3H]Man is formed; no additional [3H]mannosylated glycolipids were observed as compared with no PMSF pretreatment (Fig. 2C). The formation of only Dol-P-[3H]Man even in the presence of GlcN-PI and Pam-CoA is similar to that of Fig. 2A, where there is no inositol acylation or mannosylation; thus, PMSF seems to inhibit inositol acylation of GlcN-PI, preventing subsequent mannosylation.

However, when the alternative serine protease inhibitor DFP was preincubated with the cell-free system, different [3H]mannosylated intermediates are observed (Fig. 3B); Dol-P-[3H]Man is formed as well as three novel [3H]mannosylated GPI intermediates. Analysis revealed them all to be resistant to PI-PLD, deamination, and base treatment (Table 1). This indicates that the [3H]glycolipids are inositol-acylated diacylglycerol GPI anchors containing a non-N-acetylated glucosamine component. The [3H]mannosylated neutral glycan head groups were obtained and analyzed by Bio-Gel P4 gel filtration as described earlier. The neutral [3H]glycans obtained from all three glycolipids eluted from the column at
Inhibition of Inositol Deacetylation Prevents Further Processing of Mannosylated Intermediates—A possible consequence of not being able to inositol-deacetyl these intermediates, i.e. (aM3, C') may prevent further processing, i.e. the addition of the Glc-GalNAc side chain. This hypothesis was confirmed by preincubating Toxoplasma membranes with GDP-[3H]Man, GlcN-PI, and Pam-CoA for 5 min before the addition of either PMSF or DFP followed by the addition of either UDP-GalNAc or UDP-GalNac and UDP-Glc (Fig. 4C). The resulting inositol-acylated glycolipids formed (aM3 and C') did not undergo further modification by the addition of GalNAc or GalNac and Glc as compared with no PMSF present (Figs. 2, D and E).

In Vivo Inhibition of Inositol Deacetylation in T. gondii Using PMSF—T. gondii tachyzoites (48–72 h post-infection) were labeled in vivo with [3H]glucosamine for 6 h at 37 °C, after which the GPI glycolipids were extracted and analyzed by thin layer chromatography. The [3H]GlcN-labeled glycolipids II–VI (Fig. 5A) were characterized using various enzyme and chemical digests as well as forming their neutral [3H]GlcN glycan head groups from HPTLC-purified glycolipids. The desalted 2,5-[3H]anhydromannitol-containing glycan head groups obtained from the HPTLC-purified glycolipids II, III, V, and VI, by deacetylation, deamination, reduction, and dephosphorylation were analyzed by Bio-Gel P4 gel filtration before and after various glycosidases; that is, α-mannosidase, α-glucosidase, and β-N-acetylhexosaminidase (Table 1). The released GPI core glycans of Manα1–2Manα1–6(GalNAcβ1–4)Manα1–4anhydromannitol and Manα1–2Manα1–6(Glcα1–4GalNAcβ1–4)Manα1–4anhydromannitol corresponded to previously characterized glycans generated by similar treatments from other in vivo T. gondii labeling (44). Thus, glycolipids II, III, V, and VI have the same structures as characterized earlier (Fig. 2E and Table 1) and are in accordance with previously described T. gondii GPI intermediates (44).

In the presence of DFP (Fig. 5B), in vivo labeling with [3H]GlcN showed no significant difference from the control (Fig. 5A). However, in the presence of PMSF, only one [3H]glycolipid was observed (Fig. 5C). Characterization of this [3H]glycolipid and its corresponding neutral glycan head group as well (Table 1) suggests that the glycolipid could be Manα1,GlcN-PI, and Pam-CoA. The [3H]mannosylated glycolipids were partitioned into butan-1-ol and analyzed by HPTLC using solvent system B. The [3H]mannosylated glycolipids were characterized (Table 1), and the identities of the bands are Dol-P-Man (aM3), Manα1,GlcN(acyl)PI (aM3), and EtN-PI (C').
Thus, PMSF but not DFP is able to inhibit inositol deacylation of the GPI intermediate in vivo, preventing further maturation of the intermediate by the subsequent addition of either the ethanolamine-phosphate or the GalNAc side chain.

Lipid Structure of T. gondii Free GPIs and GPI-anchored Proteins—Purified samples of free GPIs and GPI-anchored proteins, previously shown to be free of phospholipids (54), were treated with nitrous acid causing deamination and the release of the GPIno portion of the GPI structures. The GPIno moieties were extracted into butan-1-ol and analyzed by negative ion ES-MS. Several GPIno species were clearly observed when analyzed by ES-MS-MS using parent ion scanning of the collision-induced daughter ion, inositol 1–2 cyclic phosphate ion (m/z 241). The GPIno species released from both free GPIs and GPI-anchored proteins (Figs. 6, A and B, respectively) were very similar and were diacylglycerol species. Collision-induced dissociation daughter ion spectrum of the two major ions at m/z 835 (Fig. 6C) and m/z 861 (Fig. 6D) further defined them as 1-O-(C16:0)acyl-2-O-(C18:1)acyl glycerol-3-P-inositol and 1-O-(C18:1)acyl-2-O-(C18:1)acyl glycerol-3-P-inositol, respectively. The assignments of the major daughter ions observed for m/z 835 (Fig. 6C) are shown in Fig. 6E. The other minor GPIno species were also characterized by collision-induced dissociation and identified as 1-O-(C14:0)acyl-2-O-(C18:1)acyl glycerol-3-P-inositol (m/z 807), 1-O-(C14:0)acyl-2-O-(C16:0)acyl glycerol-3-P-inositol (m/z 805), 1-O-(C14:0)acyl-2-O-(C18:1)acyl glycerol-3-P-inositol (m/z 805), and 1-O-(C20:1, C20:2, and C20:3)acyl-2-O-(C18:1)acyl glycerol-3-P-inositol (m/z 885, 887, and 889) (data not shown).

DISCUSSION

A family of Parasite-specific glycosylphosphatidylinositol-s containing a novel glucosylated side chain has been shown to be highly immunogenic in humans (41). The following structures were identified: (ethanolamine-PO4)-Manα1–2Manα1–6(Gal-NAcβ1–4)Manα1–4GlcNAcα1–6-D-myoinositol-PO4-lipid and (ethanolamine-PO4)-Manα1–2Manα1–6(Glcα1–4GalNAcβ1–4)Manα1–4GlcNAcα1–6-D-myo-inositol-PO4-lipid both with and without terminal ethanolamine phosphate. T. gondii GPIs bearing a unique glucose-N-acetylgalactosamine as a side branch were shown to be immunogenic in humans.

Synthesis of glucosylated glycolipids is mediated by uridine-diphosphate-glucose (16). The direct donor for the GalNAc moiety has not been defined so far, although the involvement of a lipid intermediate has not been ruled out. Using hypotonically permeabilized T. gondii tachyzoites, the topology of the

FIGURE 5. Effect of DFP or PMSF on T. gondii GPIs synthesized in vivo. Tachyzoites were labeled in vivo with [3H]GlcN for 6 h (A) or chased with either DFP (B) or PMSF (C) for a further 5 min. [3H]GlcN-labeled glycolipids were partitioned into butan-1-ol and analyzed by HPTLC using solvent system A. The [3H]glycolipids were characterized (Table 1); see Figs. 2 and 3 for identity of bands.

FIGURE 6. Negative ion ESI-MS and EI-MS-MS analysis of the PI moieties released from free GPIs and GPI-anchored proteins. ESI-MS spectrum of the PI released by deamination from purified free GPIs (A) and purified GPI-anchored proteins (B), ESI-MS-MS daughter ion spectrum of the m/z 835 ion (C), ESI-MS-MS daughter ion spectrum of the m/z 861 ion (D), Assignment of the principal daughter ions from m/z 835 observed in panel C (E).
free GPls within the endoplasmic reticulum membrane was recently investigated (49). They demonstrate that the higher mannansylation and side chain (Glc-GalNAc)-modified GPl intermediates are preferentially localized on the cytoplasmic leaflet of the ER (49). A new early intermediate with an acyl modification on the inositol was identified indicating that inositol acylation also occurs in T. gondii. However, many details of the biosynthesis of Toxoplasma GPls remained unresolved. Here we provide a detailed study of the biosynthesis of T. gondii GPls with an emphasis on the roles of inositol acylation and subsequent inositol deacylation.

The data presented in this paper support the following conclusions about the GPl biosynthetic pathway in T. gondii. (a) Inositol-acyltransferase acts on GlcN-PI to form GlcN-(acyl)PI. (b) Inositol acylation is a prerequisite for mannansylation. (c) PMSF inhibits both the inositol acylation and the inositol deacylation. (d) DFP does not inhibit inositol acylation but does inhibit inositol deacylation. (e) Inositol deacylation is not a prerequisite for ethanolamine-phosphate addition; hence, the possible formation of glycolipid C'. (f) Inositol deacylation is a prerequisite for the addition of the GalNAc side chain to the first mannose of the GPl intermediates Man3 or A'. (g) Inositol deacylation maintains an equilibrium that favors non-inositol-acylated over inositol-acylated tri-mannansylated GPl intermediates. (h) Formation of mature lyso-GPl intermediates is either an initial step of remodeling of the sn-2 position with a C18:1 fatty acid or the initial stage of catabolism.

These features (described in detail below) allow the formation of a model for the T. gondii GPl biosynthetic pathway (Fig. 7). Putative T. gondii GPl biosynthetic genes for steps 1 (PIG-A, PIG-C, and PGI1), 2 (PIG-L), 3 (PIG-W), 4 (PIG-M), 5 (PIG-V), 6 (PIG-B), 7 (PIG-O and PIG-F), and 11 (PIG8 and GAA1–3) and for the formation of Dol-P-Man (DPM1) were identified by searching the Toxoplasma Genome for homologues of known T. gondii inositol acyltransferase (PIG-W) encodes a 558-amino acid protein and is more similar to homologous from Schizosaccharomyces pombe (~60% similarity), Cryptococcus neoformans, and Cryptococcus albicans than the human PIG-W (supplemental Fig. 1). Unfortunately, no homologue could be identified in the T. brucei genome.

The process of inositol acylation (left to right) is catalyzed by a PMSF-sensitive inositol acyltransferase, and the process of inositol deacylation (right to left) is catalyzed by an inositol deacylase that it sensitive to both PMSF and DFP (the point of inhibitory action are marked with an X). The numbers next to each biosynthetic step refer to those described in Table 2. The T. gondii inositol acyltransferase (PIG-W) encodes a 558-amino acid protein and is more similar to homologous from Schizosaccharomyces pombe (~60% similarity), Cryptococcus neoformans, and Candida albicans than the human PIG-W (supplemental Fig. 1). Unfortunately, no homologue could be identified in the T. gondii genome.

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The observation that preincubation with PMSF inhibits T. gondii inositol acylation indicates that inositol acylation is required before mannansylation (Step 4, Fig. 7 and Table 2). This is in contrast to T. brucei where inhibition of inositol acylation does not prevent mannansylation but prevents further post-mannansylation processing, i.e. the addition of the ethanolamine phosphate to the third mannose, to form glycolipid C', which in turn is a prerequisite for inositol deacylation to glycolipid A' (28).

Unlike Plasmodium, the T. gondii mature cell-surface GPls and GPl-anchored proteins are not inositol-acylated, suggesting that the inositol acylation is only transient. Thus, inositol deacylation must take place at some point after mannansylation (Steps 4–6, Fig. 7 and Table 2). Thus, one could speculate that acylation and deacylation of the inositol ring of T. gondii GPl intermediates play a role in controlling further processing as well as ethanolamine-phosphate addition in T. brucei as described above (28). Preincubation of the T. gondii cell-free system with a different serine protease inhibitor DFP reveals the presence of previously unseen inositol-acylated and man-
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TABLE 2

Identification of the *T. gondii* genes for the GPI biosynthetic pathway using homologues from *P. falciparum*, human, and yeast

| Step | Enzyme | Donor | Mammals | Yeast | *T. gondii* | *P. falciparum* |
|------|--------|-------|---------|-------|-------------|----------------|
| 1    | GlcNAc transferase | UDP–GlcNAc | PIG-A | GPI3/SPT14/CHW6 | PIG-A | PIG-A |
| 2    | De-N-acetylase | | | | | |
| 3    | Inositol-acetylase | Palmitoyl-CoA | PIG-W | (Pig-W) YJ091C | PIG-W | CAH18663 | PIG-W |
| 4    | α1–4 Mannosyltransferase (MT-I) | Dol-P–Man | PIG-M | GPI11 (a) | PIG-M | CAH18667 | PIG-M |
| 5    | α1–6 Mannosyltransferase (MT-II) | Dol-P–Man | PIG-V | | | |
| 6    | α1–2 Mannosyltransferase (MT-III) | Dol-P–Man | PIG-B | GPI10 | PIG-B | |
| 7    | Ethanolamine-P-transferase | PE | PIG-F | GPI11 | PIG-F | |
| 8    | Inositol-deacylase | | | | | |
| 9    | β1–4GlcNAc transferase | UDP–GalNAc | GAA1 | GAA1 | CAI91277 | GAA1 |
| 10   | α1–4Glc transferase | UDP–Glc | GP18 | GP18 | CAD44992 | GP18 |
| 11   | Phospholipase A2 | | GP16 | | |
| 12   | Transamidase | GDP–Man | GAA1 | GAA1 | CAI91277 | GAA1 |
| 13   | Dol-P–Man synthase | GDP–Man | PIG-T | GPI17 | PIG-T | |

| Donor | Mammals | Yeast | *T. gondii* | *P. falciparum* |
|-------|---------|-------|-------------|----------------|
| GDP–Man | DPM1 | DPM1 | DPM1 | CAI84648 | DPM1 |

*Genes are missing.

nosylated *T. gondii* GPI intermediates i.e. aM3, C’, suggesting that DFP does not inhibit inositol acylation but inhibits the inositol deacylation of mannosylated GPI intermediates. These mannosylated intermediates are similar to those described before in the *T. gondii* cell-free system (16, 39–41), except they are inositol-acylated, i.e. M3 becomes aM3, and A’ becomes C’.

These inositol-acylated and mannosylated intermediates also accumulate when the *T. gondii* cell-free system is preincubated with the necessary substrates before the addition of PMSF, causing inositol deacylation inhibition. This leads to the conclusion that inositol deacylation occurs after the addition of all three mannoses, but it is not a prerequisite for ethanolamine phosphate addition (Step 7, Fig. 7 and Table 2); hence, the formation of glycolipid C’.

Inositol deacylation of mannosylated GPI intermediates in *T. brucei* is a prerequisite for fatty acid remodeling and is also inhibited by DFP but not PMSF (25, 28). Yeast and mammalian inositol deacylases normally act on mature GPls or GPI-anchored proteins and are not inhibited by serine protease inhibitors, suggesting a similarity between the *T. gondii* and *T. brucei* inositol deacylases; however, no obvious homologue to the *T. brucei* inositol deacylase (55) could be identified from the *T. gondii* genome.

Further processing of the GPI intermediate was then investigated to ascertain if the side-chain addition of GalNAc and subsequent Glc (Steps 9 and 10, Fig. 7 and Table 2) could take place on the inositol-acylated species. After initiating the GPI cell-free system, PMSF was added to inhibit inositol acylation followed by the addition of either UDP-GalNAc or UDP-GlcnA and UDP-Glc. No further modification to the inositol-acylated glycolipids was observed, suggesting the GalNAc transferase does not act on inositol-acylated species. The inhibition of inositol deacylation preventing further processing of GPI intermediates was also observed in *vivo* in the presence of PMSF, but not DFP, as determined by the accumulation of Man₃GlcN-(acyl)PI. The reason why no inhibition was observed with DFP is unclear but may simply be due to insufficient accessibility or being targeted by other unrelated serine protease-like enzymes elsewhere in the cell. The accumulation of Man₃GlcN-(acyl)PI in the presence of PMSF may suggest either inhibition of the ethanolamine phosphate transferase or more likely inositol deacylation is a prerequisite for ethanolamine-phosphate addition. This is not so defined in the cell-free system by the very nature of the somewhat scrambled and disrupted membranes. Similar results were observed where *in vivo* inhibition by PMSF of *T. brucei* inositol acylation prevents ethanolamine-phosphate addition leading to an accumulation of Man₃GlcN-PI (25).

ES-MS analysis of the GPIno moiety of both the free GPls and GPI-anchored proteins were very similar, with the vast majority of the heterogeneous GPIno species containing an sn-2 C18:1 acyl chain; the two major species accounting for ~80% are 1-O-(C16:0)acyl-2-O-(C18:1)acyl glycerol-3-P-inositol and 1-O-(C18:1)acyl-2-O-(C18:1)acyl glycerol-3-P-inositol. The presence of sn-2 C18:1 fatty acid on the immunogenic GPI anchors of *T. gondii* shown here draws obvious parallels to the highly immunogenic *T. cruzi* trypomastigote GPI anchors, which contain C16:0 (37%), C18:1 (31%), and C18:2 (21%) at the sn-2 position of their alkylacylglycerol-lipid component (56), and *P. falciparum* GPI anchors containing C18:1 (88%) and C18:2 (12%) at the sn-2 position of their diacylglycerol-lipid component (47).

Characterization of the predominant GPIno species present in *T. gondii* membranes, 1-O-(C16:0)acyl-2-O-(C18:1)acyl glycerol-3-P-inositol and 1-O-(C18:1)acyl-2-O-(C18:1)acyl glycerol-3-P-inositol, are the same major species as described for the GPI anchors, suggesting no fatty acid remodeling in *T. gondii*, which

[^5]: T. K. Smith, unpublished information.
has been observed in the GPI pathways of other organisms (20, 21, 33). Thus, the formation of lyso-mature GPI intermediates (Step 11, Fig 7 and Table 2) may be an initial step in a catabolic pathway to remove/recycle excess GPI anchors, as suggested for T. brucei (25).

The characterization of the GPIno moieties of the GPI anchors explains the almost identical Rf values observed for the endogenously (UDP-GlcNAc) primed GPI intermediates, with a total hydrophobicity of C34:1 or C36:2, compared with primed endogenously (UDP-GlcNAc) primed GPI intermediates, with observed differences in the RF of T. gondii GPI intermediates formed by a cell-free system and those formed by an in vivo labeling (40, 44, 49) are not due to a difference in the lipid moiety, leaving the obvious conclusion that in an in vivo labeling there is an extra, as yet uncharacterized labile component attached to the glycan core that is causing the observed lower RF. The identification of this elusive component is presently being investigated.

To conclude, the role of inositol acylation in the T. gondii GPI pathway (Fig. 7) is to ensure full mannosylation of GPI intermediates before the addition of the GalNAc side chain (25). Early GPI intermediates are formed on the cytosolic face of the ER, after which translocation to the cell surface.

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