Glucosylation of Glycosylphosphatidylinositol Membrane Anchors: Identification of Uridine Diphosphate−Glucose as the Direct Donor for Side Chain Modification in *Toxoplasma gondii* Using Carbohydrate Analogues[†]

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ABSTRACT: *Toxoplasma gondii* is an obligate intracellular parasite of the phylum apicomplexa and a common and often life-threatening opportunistic infection associated with AIDS. A family of parasite-specific glycosylphosphatidylinositols containing a novel glucosylated side chain has been shown to be highly immunogenic in humans (Striepen et al. (1997) *J. Mol. Biol. 266*, 797−813). In contrast to trypanosomes in *T. gondii* side chain modification takes place before addition to protein in the endoplasmic reticulum. The biosynthesis of these modifications was studied in an in vitro system prepared from hypotonically lysed *T. gondii* parasites. Radiolabeled glucose-containing glycosylphosphatidylinositol precursors were synthesized by *T. gondii* membrane preparations upon incubation with uridine diphosphate−[^{3}H]glucose. Synthesis of glucosylated glycolipids took place only in the presence of exogenous uridine diphosphate−glucose and was stimulated by unlabeled uridine diphosphate−glucose in a dose-dependent manner. In contrast to glycosylphosphatidylinositol mannosylation, glucosylation was shown to be insensitive to amphomycin treatment. In addition, the glucose analogue 2-deoxy-D-glucose was used to trace the glycosylphosphatidylinositol glucosylation pathway. Detailed analysis of glycolipids synthesized in vitro in the presence of UDP and GDP derivatives of D-glucose and 2-deoxy-D-glucose ruled out an involvement of dolichol phosphate−glucose and demonstrates direct transfer of glucose from uridine diphosphate−glucose.

Glycosylphosphatidylinositol (GPI)[1] glycolipids have been shown to serve as membrane anchors for a large number of cell surface proteins in various eucaryotic species (*1*, *2*). Detailed structural analysis of several of these protein anchors has led to the proposal of an evolutionary conserved core structure made up by phosphoinositol−lipid linked to a linear core glycan consisting of nonacetylated glucosamine and three mannose residues. This structure is attached to the C-terminal amino acid of the protein via an ethanolamine phosphate bridge. This conserved backbone is modified in different species by a broad spectrum of further peripheral glycosylation, transfer of additional ethanolamine phosphate residues, and hydrophobic modifications (*2*). The precise function of these various modifications is still unknown, but a growing body of evidence is emerging that type and amount of modification clearly affect functional aspects of GPIs such as receptor binding capacity, antigenicity, their role as pathogenicity factors, and interference of GPIs with different signal transduction pathways (*3*−*7*).

Biosynthesis of GPIs shows several parallels to protein N-glycosylation. A preformed glycolipid is transferred to the nascent protein *en bloc* in the endoplasmic reticulum (ER) replacing a short hydrophobic C-terminal signal sequence. Both reactions, the proteolytic cleavage and the glycolipid transfer, are proposed to be performed by a single enzyme in a transamidase-like mechanism (*8*). Biosynthesis of the precursor glycolipid starts with transfer of GlcNAc to phosphatidylinositol from UDP−GlcNAc followed by deacetylation and subsequent mannosylation (*9*). Finally ethanolamine phosphate is transferred using phosphatidylethanolamine as a donor (*10*). In *T. brucei* a complex process of fatty acid remodeling ensuring the lipid moiety to consist exclusively of dimyristoyl glycerol has been described to follow ethanolamine phosphate transfer (*11*).

T. gondii is a well-known pathogen causing congenital infection in man and domestic livestock. More recently *Toxoplasma* has been recognized as a major opportunistic pathogen leading to severe and often lethal encephalitis in AIDS (*12*). A carbohydrate-containing low molecular mass antigen has been described as an antigen with immmunological characteristics suitable for serological diagnosis of acute toxoplasmosis (*13*). We have identified this antigen to be a family of protein-free GPI glycolipids (*14*) and

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[1] Abbreviations: GPI, glycosylphosphatidylinositol; 2deoxyGlc, 2-deoxy-D-glucose; GlcNAc, *N*-acetylglucosamine; Glc, glucose; Man, mannose; dol, dolichol.

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recently elucidated the structures of these GPIs. Two types of core glycans were identified, glycan A modified by GalNAc linked β1−4 to the core mannose adjacent to the nonacetylated glucosamine and glycan B containing a novel Glcα1−4GalNAc side branch (*5*). Subsequent immunological analysis revealed that only glucosylated GPIs containing glycan B were recognized by sera from infected humans suggesting that the unique glucose modification is required for immunogenicity (*5*).

MATERIALS AND METHODS

Materials. Uridine diphosphate-6-[^{3}H]glucose (15 Ci/mmol) was obtained from ARC, guanosine diphosphate-3,4-[^{3}H]mannose from Dupont-New England Nuclear, and 6-[^{3}H]glucosamine hydrochloride (26 Ci/mmol) from Amersham. Ion-exchange resins were analytical grade and purchased from Bio-Rad. Amphomycin was a gift of Tropon Werke, Cologne, Germany.

Culture and in Vivo Metabolic Labeling of Glycolipids. RH-strain *T. gondii* tachyzoites were grown in Vero cells. Confluent cell cultures (175 cm^2) were infected with 5×10^7 tachyzoites in Dulbecco's modified Eagle medium supplemented with 2% fetal calf serum. The 72 h postinfection cultures were washed twice with glucose-free Dulbecco's modified Eagle medium containing 20 mM sodium pyruvate. Labeling was performed in the same medium supplemented with 0.5 mCi [^{3}H]glucosamine for 4 h at 37 °C. After labeling, parasites were liberated from host cells with 20 strokes in a Dounce homogenizer and purified by glass wool filtration. Purified tachyzoites were washed twice with phosphate buffered saline and extracted as described (*15*). Briefly, tachyzoites were extracted twice with 1 mL of 2:1 chloroform/methanol by volume (CM) and the residual CM insoluble pellet was extracted twice with 10:10:3 chloroform/methanol/water by volume (CMW). To remove nonlipid contamination, CMW extracts were dried and glycolipids were partitioned between water and water-saturated *n*-butanol. Glycolipids contained in the butanol phase were analyzed by TLC as described (*5*). Briefly, glycolipids were spotted onto Merck Si 60 HPTLC plates and run in 3:10:10:2:1 hexane/chloroform/methanol/water/acetic acid by volume. Dried plates were scanned for radioactivity using a Berthold LB2842 linear analyzer and Berthold software.

Preparation of a Cell Free System from T. gondii and in Vitro Synthesis of Glycolipids. A cell free system for in vitro labeling was prepared with modifications according to the protocol of Masterson et al. (*16*). Briefly, 2×10^9 tachyzoites purified from host cells as described above were hypotonically lysed in 375 μL of ice-cold water containing 1 μg/mL leupeptin and 0.1 mM tosyl lysylchloromethyl ketone (TLCK) for 5 min on ice and then homogenized by 50 strokes in a 3 mL Dounce homogenizer. The lysate was mixed with an equal volume of incubation buffer (50 mM Na HEPES at pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM TLCK, and 1 μg/mL leupeptin) and again briefly homogenized. A 75 μL volume of tachyzoite cell free membrane preparation representing 2×10^8 tachyzoites was added to each incubation tube. Incubation tubes contained predried 1 mM ATP, CoA, UDP−GlcNAC, UDP−GalNAc and GDP−Man (in the case of label other than GDP−[^{3}H]Man; concentrations are final concentrations in 75 μL), and 2 μCi GDP−^{3}H-Man or 5 μCi UDP−^{3}H-Gluc. Tubes were briefly vortexed and incubated for 2 h at 37 °C. Incubation was terminated by addition of 2 mL of CM, and extraction of GPIs and TLC analysis was subsequently performed as described above.

Generation and Analysis of Core Glycans. In vitro radiolabeled glycolipids contained in the butanol phase of the CMW extract were dephosphorylated, deaminated, and reduced as described (*15*). Briefly, dephosphorylation was performed by incubation with 48% aqueous HF for 60 h at 0 °C and stopped by blowing off HF under a stream of nitrogen. The reaction mixture was deaminated and reduced as described and finally desalted over 1 mL of AG50W-X12 and a tandem-ion exchange column of 0.4 mL of Chelex 100 (Na^+), 0.2 mL of AG50W-X12 (H^+), 0.4 mL of AG3-X4 (OH^-), and 0.2 mL of QAE-Sephadex (OH^-) both equilibrated and eluted with water and filtered through a 0.25 μm filter. Core glycans were analyzed by Dionex HPAEC and aminopropyl HPLC. Aminopropyl HPLC was performed as described (*5*). Briefly, an AsahiPak NH_2 column (250 × 4.9 mm, Shodex) was linked to a Waters 510 HPLC System and equilibrated with 28% water in acetonitrile by volume. Samples were injected in 30 μL of water and eluted isocratically, and 1 mL fractions were taken for scintillation counting.

Generation and Analysis of Delipidated GPI Fragments. In vitro radiolabeled glycolipids were delipidated by incubation in concentrated 1:1 ammonia−methanol by volume at 50 °C for 6 h (*17*). The reaction mixture was dried and flash evaporated twice with methanol, and the residual material was subjected to butanol/water partition. The water phase was filtered, concentrated, and analyzed by HPAEC and BioGel P4 gel filtration.

HPAEC Analysis of Labeled Core Glycans and Delipidated Fragments. Radiolabeled core glycans were analyzed on a Dionex Bio liquid chromatograph. Samples were mixed with partially hydrolyzed dextran prior to injection to a Carbopak PA1 column equilibrated with 100 mM NaOH. Elution was accomplished using 100% buffer A (100 mM NaOH) for 6 min, followed by a linear increase of buffer B (100 mM NaOH, 250 mM NaOAc) from 0 to 30% in 30 min at a flow rate of 1 mL/min. Fractions of 0.4 mL were collected. Delipidated fragments were analyzed on a Carbopak PA1 equilibrated with 85% buffer A and 15% buffer B and eluted by increasing buffer B to 50% in 45 min. Fractions of 0.5 mL were collected.

Bio-Gel P4 Analysis. Delipidated fragments generated from radiolabeled glycolipids were mixed with partially hydrolyzed dextran as internal standard (see below) and applied to Bio-Gel P4 columns (1 × 130 cm, 400 mesh). Columns were eluted with 0.2 M ammonium acetate containing 0.02% sodium azide, and fractions of 24 min (850 μL) were collected. Elution positions of standard glucose oligomers were identified in 25 μL aliquots of each fraction by detection with 2 mg/mL orcinol in sulfuric acid.

Acid Hydrolysis and HPAEC Monosaccharide Analysis. Core glycan B labeled via UDP−[^{3}H]glucose was purified by aminopropyl HPLC as described above. Purified glycan was dried and hydrolyzed with 2 M trifluoracetic acid for 4 h at 100 °C. The hydrolysate was dried and washed 4 times with methanol. The sample was mixed with a monosaccha-

ride standard mixture and injected into an HPAEC system fitted with a CarboPak PA1 column equilibrated and eluted isocratically with 15 mM NaOH. Standard detection was by pulsed amperiometric detection.

Synthesis of UDP− and GDP−2-Deoxy-D-glucose. UDP−2-deoxy-D-glucose and GDP−2-deoxy-D-glucose were prepared using a combination of chemical and enzymatic synthesis as described by Schwarz and Schmidt (*18*). Briefly, 2-deoxy-D-glucose was taken up in water-free pyridine and O-acetylated with acetic anhydride. After acidification, neutralization, and desalting the product was crystallized and separated into α and β anomers mechanically. The β-1,3,4,6-tetra-*O*-acetyl compound was phosphorylated by addition of crystalline phosphoric acid in dry tetrahydrofuran to give the 1-phosphate derivative. After incubation at 50 °C for 30 min the sample was neutralized with ice-cold 1 M LiOH and desalted by passage over a Dowex 50 × 2 (H^+ form) and passed over Dowex WX2 (cyclohexylammonium form). 2-Deoxy-D-glucose 1-phosphate was finally purified by ion-exchange chromatography on a Dowex 1 × 8 (HCO_3^- form) column eluted with a linear gradient of triethylammonium hydrogen carbonate from 0.05 to 0.3 M.

An enriched enzyme preparation was obtained from fresh lactating bovine mammary gland by extraction and ammonium sulfate precipitation (*18*). After dialysis of the ammonium sulfate the preparation was stored at −80 °C. To synthesize UDP−2-deoxy-D-glucose, 4 mL of 0.1 M UTP, 600 μL of 0.1 M 2deoxyGlc-1-P, 6 mL of enzyme preparation in 0.05 M Tris/HCl containing 6 mM $MgCl_2$ at pH 7.8, 100 μL of UDP−Glc pyrophosphorylase, and 10 μL of inorganic pyrophosphatase (yeast) were mixed. GDP−2deoxyGlc was synthesized by mixing 1200 μL of 0.1 GTP, 200 μL of 0.1 M ATP, 600 μL of 0.1 M 2deoxyGlc-1-P, and 10 μL of inorganic pyrophosphatase with 6 mL of the enzyme preparation. Samples were incubated overnight at 25 °C prior to ethanol precipitation of protein. The final product was purified from the supernatant by two consecutive steps of preparative chromatography on Whatman No. 3 paper using *n*-propanol/25% ammonia (6:4 v/v) as solvent.

RESULTS

Synthesis of T. gondii GPIs in Vitro. A cell free system was prepared by hypotonic lysis from purified tachyzoites essentially following the protocol of Masterson et al. (*16*). Parasite lysates were incubated with guanosine diphosphate−[^{3}H]mannose (GDP−[^{3}H]Man) or uridine diphosphate−[^{3}H]-glucose (UDP−[^{3}H]Glc) in the presence of unlabeled UDP−*N*-acetylglucosamine, UDP−*N*-acetylgalactosamine, ATP, and CoA. Following a 2 h incubation glycolipids were isolated by sequential extraction with chloroform/methanol (2:1 CM) and chloroform/methanol/water (10:10:3 CMW). The CMW extract was dried and phase partitioned between water and water saturated *n*-butanol. Radiolabeled glycolipids contained in the butanol phase were analyzed by TLC and compared with glycolipids of known structure obtained by in vivo labeling with [^{3}H]glucosamine. As shown in Figure 1 (panel C) labeling via GDP−[^{3}H]Man resulted in one major peak comigrating with glycolipid III the major nonglucosylated GPI obtained by in vivo labeling with [^{3}H]glucosamine. After incubation with UDP−[^{3}H]Glc several peaks were detected, of which the four major species comigrating with

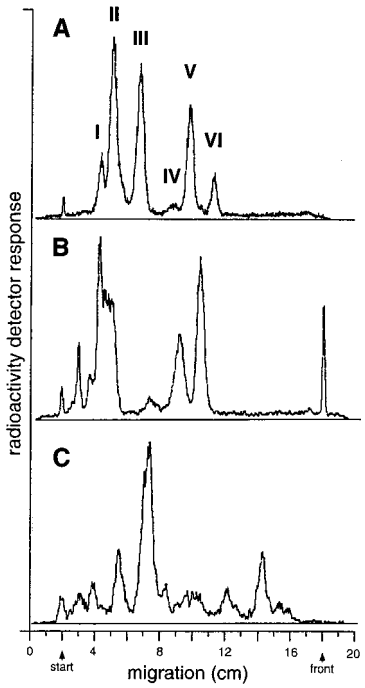

FIGURE 1: TLC analysis of GPI glycolipids synthesized by a cell-free labeling system prepared from *T. gondii* tachyzoites. GPIs were labeled in vivo with [^{3}H]GlcN (A) and in vitro with UDP−[^{3}H]-Glc (B) and GDP−[^{3}H]Man. Glycolipids were isolated by sequential extraction of the parasite pellet or the in vitro incubation respectively with CM and CMW followed by butanol−water partition of the CMW extracted material. An aliquot of the resulting butanolphase was spotted onto Merck Si 60 HPTLC plates. Plates were run in hexane/chloroform/methanol/water/acetic acid (3:10:10:2:1) and scanned for radioactivity. Dol−P-Glc and Dol-P−Man would migrate directly after the solvent front in the TLC system used but are efficiently extracted by CM extraction. We analyzed the CM extract of UDP−[^{3}H]Glc in vitro labeling by TLC and identified two glycolipid species. Both lipids did not comigrate with Dol-P−Glc standards and in addition were not sensitive to mild acid treatment which cleaves the phosphodiester linkage in Dol-P−Glc (data not shown).

glycolipids I, II, IV, and V labeled in vivo which previously have been shown to contain the Glc-GalNAc side branch (panel B; ref *5*). As the TLC spectra obtained from in vitro labeling experiments are complex especially due to the presence of lyso-intermediates (*19*), the glycolipid core glycans were prepared to simplify the interpretation.

Analysis of Core Glycans from GPI Glycolipids. Glycolipids labeled in vitro as described above were subjected to dephosphorylation, deamination, and reduction. The resulting neutral core glycans were analyzed by high-pH anion exchange chromatography (HPAEC) and aminopropyl HPLC in comparison to glycans generated from lipids labeled in vivo (Figure 2). HPAEC analysis of in vivo and in vitro labeled material showed a peak coeluting with the 3 glucose unit internal standard (panels A, C, and E). For glycolipids labeled in *vivo* this peak was previously shown to contain a mixture of two glycans. Core glycan A which was characterized to have the structure Manα1−2Manα1−6-(GalNAcβ1−4)Manα1,4-AHM and core glycan B differing by the presence of an additional glucose residue linked α1−4 to GalNAc (panel A and B; ref *5*). To discriminate between those two structures aminopropyl HPLC was performed. Core glycans derived from glycolipids labeled via GDP−[^{3}H]Man coeluted with core glycan A and Man_3−AHM (panel D).

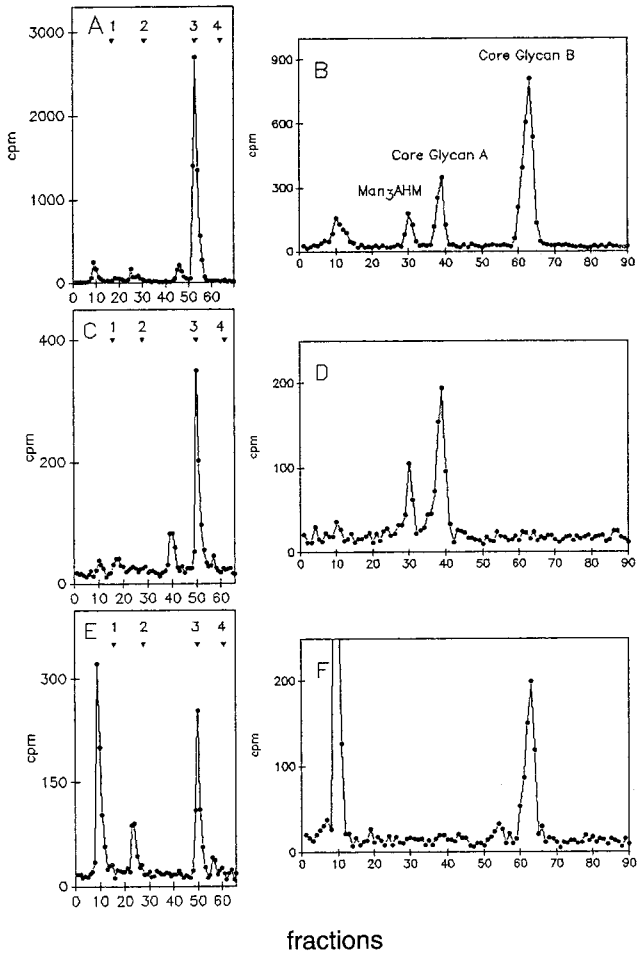

FIGURE 2: Analysis of core glycans derived from GPIs labeled in vitro. Glycolipids were labeled and isolated as described in Figure 1. The respective core glycans were prepared by dephosphorylation, deamination, and reduction of glycolipids recovered in the butanol phase and were analyzed by HPAEC (left column) and aminopropyl HPLC (right column). Core glycans derived from GPIs labeled in vivo with [^{3}H]GlcN are shown in panels A and B; glycans from GPIs labeled in vitro with GDP−[^{3}H]Man and with UDP−[^{3}H]-Glc are given in panels C and D and panels E and F, respectively. The minor peak at 1.5 GU in panel E was consistently found both after UDP−[^{3}H]Glc and in vivo [^{3}H]GlcN labeling in varying amounts and probably represents a nonspecific breakdown product occurring in the course of mild acid treatment and deamination.

This is consistent with earlier findings identifying glycolipids containing core glycan A upon in vitro synthesis and proposing them as the "mature" GPI-anchor precursor (*19*). In contrast, glycolipids labeled via UDP−[^{3}H]Glc showed a major peak coeluting with the glucosylated core glycan B (Figure 2, panel F) and some material with low affinity to the aminopropyl phase (not further characterized). No peaks were detected comigrating with core glycan A or Man_3−AHM after incubation with UDP−[^{3}H]Glc. Core glycan B labeled via UDP−[^{3}H]Glc was purified by aminopropyl HPLC and subjected to acid hydrolysis followed by HPAEC monosaccharide analysis. All radioactivity eluted with an internal glucose standard (data not shown) proving that all radioactivity is incorporated as glucose into core glycan B and no metabolisation of the label has taken place. These data show that glucosylated GPIs are synthesized by *T. gondii* membrane preparations in vitro and can be specifically labeled with UDP−[^{3}H]Glc.

GPI Glucosylation Is Stimulated by Exogenously Added Unlabeled UDP−Glc. The observation of synthesis of glucosylated GPIs in the *T. gondii* cell free system using labeling via UDP−[^{3}H]Glc but not via GDP−[^{3}H]Man alone suggests that the transferase is active under the conditions applied but that an appropriate glucose donor might be absent in the in vitro labeling system. To address this point a stimulation experiment with unlabeled UDP−Glc was performed. Cell free incubations prepared from tachyzoites were labeled with GDP−[^{3}H]Man for 60 min as described above prior to addition of unlabeled UDP−Glc to achieve final concentrations varying from 1 μM to 1 mM. In control experiments UDP−Glc addition was omitted. Preparations were allowed to incubate for further 60 min, and glycolipids were extracted and analyzed by TLC. Without addition of UDP−Glc mainly glycolipid III and a lipid with lower TLC mobility probably lysoglycolipid III are synthesized (*19*). Incubation with rising concentrations of UDP−Glc leads to a decrease of these two peaks and the formation of species comigrating with the glucosylated glycolipids I and II (Figure 3 panels A−E). To simplify interpretation core glycans were prepared from all concentration steps and analyzed by aminopropyl HPLC.

As shown in Figure 3 (panels F−G) aminopropyl HPLC analysis demonstrates that addition of unlabeled UDP−Glc results in the synthesis of glycolipids containing core glycan B. The amount of core glycan B synthesized from the nonglucosylated glycan A shows a clear correlation to the amount of added UDP−Glc. These data show that GPI glucosylation can be stimulated in a dose-dependent fashion by addition of UDP−Glc. The pool of endogenous UDP−Glc in the cell free system might be too small, not accessible, cleaved by phosphodiesterase, or depleted by other enzymes metabolizing UDP−Glc as no glucosylation takes place in the absence of exogenous UDP−Glc. Furthermore this experiment suggests that UDP−Glc or an intermediate formed using UDP−Glc is the donor in the GPI glucosylation reaction.

Transfer of Glucose Is Not Affected by Amphomycin. Transfer of monosaccharides during GPI biosynthesis was shown to be accomplished either by direct transfer from the respective sugar nucleotide (e. g. UDP−GlcNAc as donor of the glucosamine) or via Dol-P intermediates as for the core mannose residues (reviewed in ref *3*).The antibiotic amphomycin is a strong inhibitor of Dol-P−Man and Dol-P−Glc formation (*20*−*22*). Amphomycin was used here to address the question if GPI glucosylation like GPI mannosylation involves a Dol-P intermediate as donor. The observed lack of GPI glucosylation in vitro in the absence of exogenous UDP−Glc was exploited to investigate the effect of this inhibitor (as amphomycin strongly affects earlier steps of GPI biosynthesis such as mannosylation). Lysates were incubated with GDP−[^{3}H]Man for 60 min to allow formation of fully mannosylated glycolipids. After this preincubation amphomycin was added in concentrations ranging from 1 μg to 1 mg/mL final concentration to the cell free system. After additional incubation for 5 min glucosylation was initiated by adding UDP−Glc to a final concentration of 1 mM. Labeling was stopped after a total incubation time of 2 h, and glycolipids were extracted. TLC analysis of glycolipids and aminopropyl HPLC analysis of core glycans derived from these glycolipids showed no

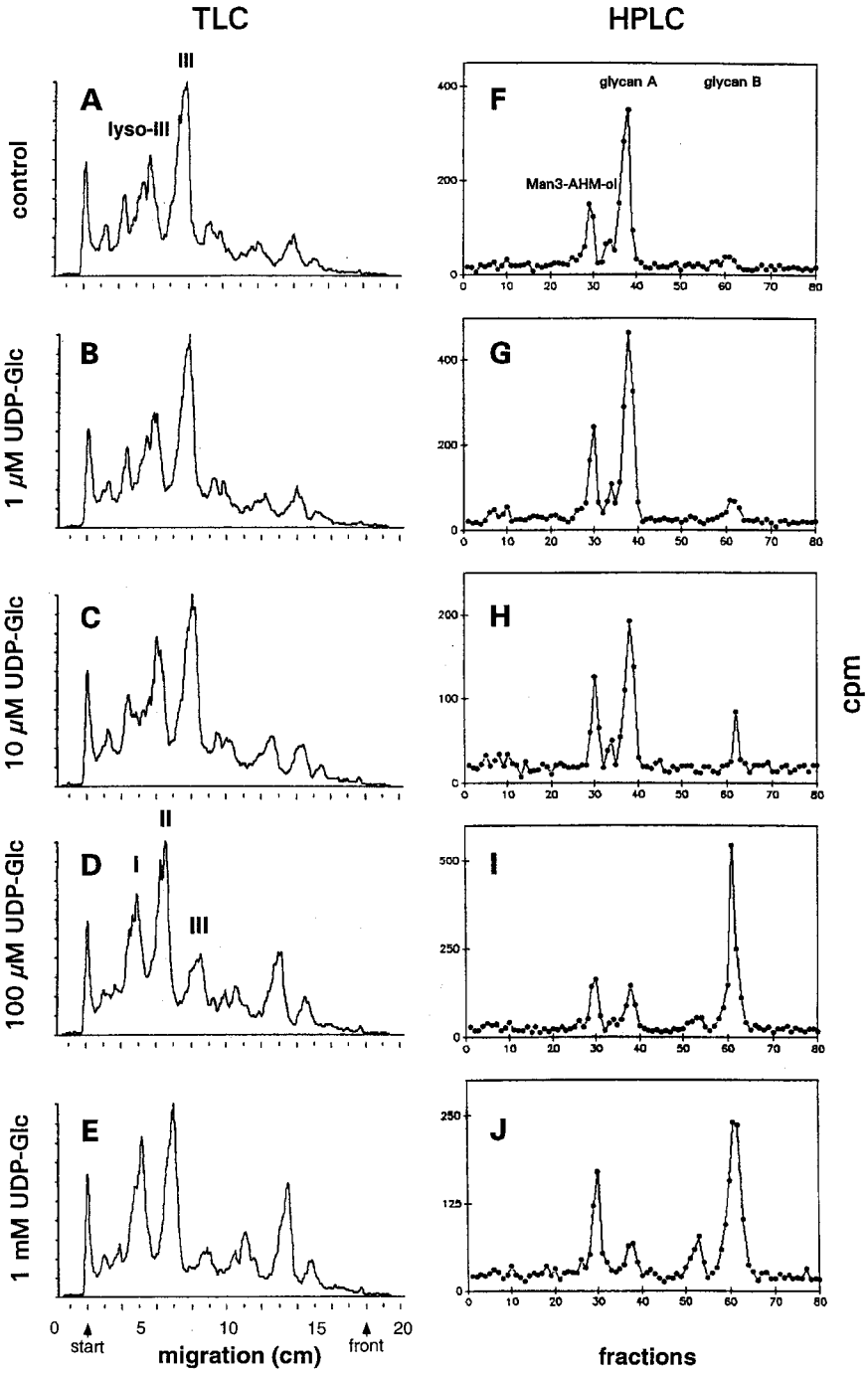

FIGURE 3: Glucosylation of GPIs is stimulated in vitro by UDP−Glc. Cell-free membrane preparations were incubated for 60 min with GDP−[^{3}H]Man. After preincubation unlabeled UDP−Glc was added to final concentrations from 1 μM to 1 mM and samples were incubated for an additional 60 min. Glycolipids were extracted and analyzed by TLC (panels A−E). Core glycans were prepared from the respective glycolipids and were analyzed by aminopropyl HPLC shown in (panels F−J). The respective concentration of unlabeled UDP−Glc is given as the final concentration obtained in the incubation.

significant difference between all incubations. Even at the highest concentration of 1 mg/mL amphomycin glucosylation was not affected as shown in Figure 4.

Direct addition of amphomycin without prelabeling resulted in a complete block of GPI biosynthesis by inhibiting the formation of Dol-P−Man (data not shown), demonstrating the principal activity of the inhibitor used in this experiment. These results strongly suggest that Dol-P−Glc is not an intermediate of GPI glucosylation but transfer is accomplished directly from UDP−Glc. Considering the possibility that the *Toxoplasma* GPI-glucosyl-transferase uses Dol-P−Glc but is insensitive to amphomycin due to structural or kinetic differences to the glucosyl-transferase involved in N-glycosylation, the question was approached in a second independent series of experiments.

2-Deoxy-D-glucose Is Transferred to GPIs via UDP−2-Deoxy-D-glucose but Not via GDP−2-Deoxy-D-glucose. 2-Deoxy-D-glucose (2deoxyGlc) has been extensively used to analyze biosynthetic pathways of protein N-glycosylation (reviewed in ref *21*). 2deoxyGlc is used by eucaryotic cells as analogue of both glucose and mannose. Studies using 2deoxyGlc nucleotides have shown that GDP−2deoxyGlc is a substrate for the Dol-P−Man synthase in the formation of Dol-P−2deoxyGlc. In contrast incubation of cell free

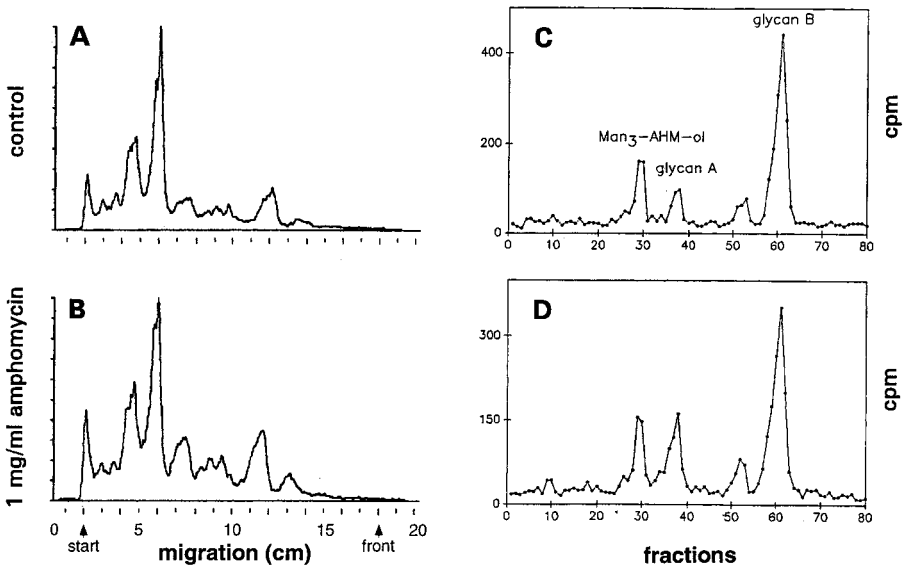

FIGURE 4: Glucosylation of GPIs is not affected by amphomycin. Membrane preparations were prelabeled with GDP−[^{3}H]Man for 60 min before addition of amphomycin. After an additional incubation for 5 min UDP−Glc was added to a final concentration of 1 mM. Glycolipids were extracted after a total labeling time of 2 h. TLC analysis of the glycolipids is shown in panels A and B, and aminopropyl HPLC analysis of the respective core glycans in panels C and D. Panels A and C show incubations without antibiotic, and panels B and D, incubations in the presence of 1 mg/mL amphomycin. Incubations in the presence of 10, 100, and 500 μg/mL are not shown as identical with panels B and D.

systems with UDP−2deoxyGlc does not result in the formation of Dol-P−2deoxyGlc (*18*, *21*). Obviously the Dol-P−Glc synthase which transfers glucose from UDP−Glc to dolicholphosphate is not recognizing 2deoxyGlc as a substrate. Biosynthetic transfer has therefore only been shown to occur from UDP−2deoxyGlc in steps that are characterized by direct transfer from the sugar−nucleotide diphosphate. This different use of 2-deoxyglucose nucleotides was exploited in this study to trace the pathway of GPI glucosylation. UDP and GDP−2deoxyGlc which are not commercially available were prepared by chemical synthesis of 2deoxyGlc 1-phosphate from 2deoxyGlc followed by enzymatic synthesis of the respective nucleotide as described in Material and Methods.

Cell free lysates prepared from tachyzoites were prelabeled as described above for 60 min with GDP−[^{3}H]Man. After addition of UDP−2deoxyGlc, GDP−2deoxyGlc, UDP−Glc, or GDP−Glc to the lysates to a final concentration of 50 μM, samples were incubated for additional 60 min in the presence of the analogues. Glycolipids were extracted and analyzed by TLC. TLC scans obtained from glycolipids synthesized in the presence of GDP−Glc and GDP−2deoxyGlc showed identical peak patterns and were indistinguishable from control incubations with GDP−[^{3}H]Man alone (Figure 5 panel B and D). In contrast GPI in vitro synthesis in the presence of UDP−2deoxyGlc showed clearly different glycolipid patterns. Two new peaks were observed (labeled I* and II* in Figure 5 panel C) with slightly higher TLC mobility proposing reduced polarity compared to glucosylated GPIs obtained after labeling in the presence of UDP−Glc (Figure 5 panel A). For further characterization glycolipids were delipidated by mild base treatment, and the resulting hydrophilic fragments were analyzed by HPAEC and Bio-Gel P4. The preparation of core glycans including HF treatment used in previous experiments to show glucose transfer was avoided with these samples as 2-deoxysugars are known for the acid lability of their 2-deoxyglycosyl bonds (*18*, *23*).

Four HPAEC peaks were detected in samples derived from glycolipids synthesized in the presence of UDP−Glc (Figure 5 panel E). Individual peaks were identified by Bio-Gel P4 in comparison to previously published data (data not shown, ref *5*); fragments labeled B′ contain the glucose-bearing core glycan B, and fragments labeled EtN contain terminal ethanolamine phosphate. In vitro labeling in the presence of GDP−Glc and GDP−2deoxyGlc led only to the formation of fragment A′ and EtN−A′ (both containing core glycan A) showing that no further modification has taken place. In contrast analysis of fragments derived from incubations under UDP−2deoxyGlc identified two previously not observed peaks in addition to A′ and EtN−A′ (Figure 5 panel G). Those components show a reduced chromatographic retention on the HPAEC column in contrast to the glucosylated fragments B′ and EtN−B′. This finding is consistent with the reduced acidity of 2-deoxyglucose compared to glucose due to the lack of a hydroxyl group. To confirm that the modification is indeed 2deoxyGlc further experiments were performed. Delipidated fragments from glycolipids synthesized in the presence of UDP−Glc and UDP−2deoxyGlc were sized on Bio-Gel P4 in parallel. For both incubations four major products with similar sizes were detected suggesting that the number of monosaccharides per oligosaccharides is identical for both incubations and does not account for the different behavior on HPAEC (data not shown).

To investigate if the structures synthesized under UDP−2deoxyGlc show the above-mentioned acid lability typical for 2-deoxy sugars, glycolipids were subjected to mild acid hydrolysis by HF treatment followed by deamination and reduction. The resulting core glycans were analyzed in parallel with core glycans obtained from glycolipids labeled in the presence of UDP−Glc in vitro and with [^{3}H]GlcN in vivo on Bio-Gel P4. Only a 6 glucose unit peak (core glycan

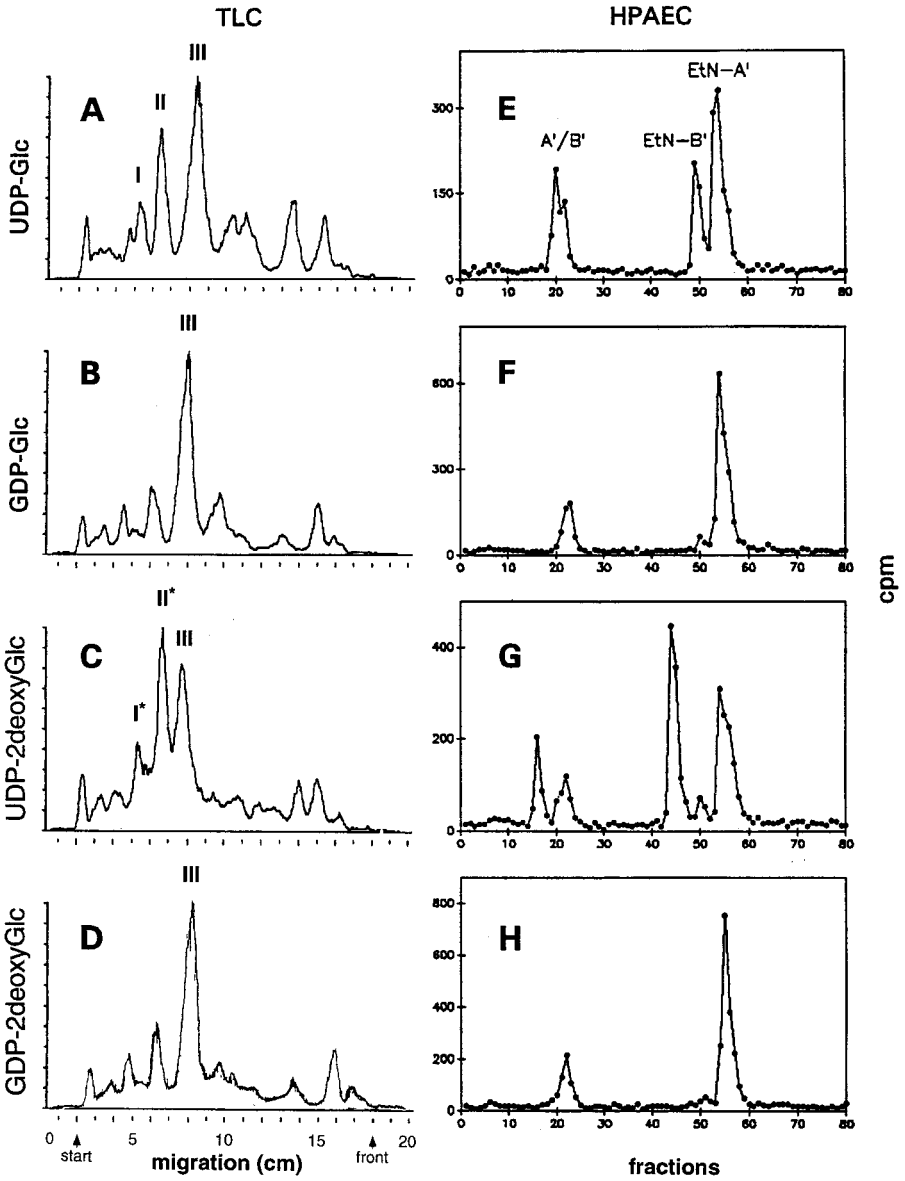

FIGURE 5: Tracing of GPI glucosylation with synthetic 2-deoxy-D-glucose nucleotides. Membrane preparations from *T. gondii* were prelabeled with GDP−[^{3}H]Man for 60 min prior to addition of UDP−Glc (panels A and E), GDP−Glc (panels B and F), UDP−2deoxyGlc (panels C and G), and GDP−2deoxyGlc (panels D and H), respectively, to a final concentration of 50 μM. TLC scans of glycolipids extracted as described in Figure 1 are shown in the left column (panels A−D). Two new glycolipid species synthesized in the presence of UDP−deoxyGlc are indicated in panel C as lipids I* and II*, respectively. The right column (panels E−H) shows HPAEC chromatograms of corresponding hydrophilic fragments generated by alkaline saponification. Fragments labeled B contain glucose; those labeled A do not contain glucose. EtN indicates the presence of a terminal ethanolamine phosphate group and the prime indicates that these fragments in contrast to core glycans still contain the inositol phosphate−glycerol bridge.

A) was observed after this treatment of glycolipids synthesized in the presence of UDP−2deoxyGlc, in contrast to peaks coeluting with the 7 glucose unit standard (core glycan B) obtained from glycolipids synthesized under UDP−Glc (data not shown). This finding shows that the modification obtained by labeling in the presence of UDP−2deoxyGlc is indeed lost under acidic conditions as predicted for 2-deoxy sugars.

The observation of an intact core glycan A after removal of 2deoxyGlc by acid hydrolysis indicates that 2deoxyGlc was not incorporated randomly into the sugar chain but as glucose linked to GalNAc. The detailed analysis of glycolipids labeled in the presence of UDP−2deoxyGlc strongly suggests that the newly formed glycolipids (and their corresponding fragments) are a product of 2deoxyGlc transfer from UDP−2deoxyGlc. Taken together these data show that the in vitro system prepared from *T. gondii* tachyzoites is capable to transfer 2deoxyGlc from UDP−2deoxyGlc but not from GDP−2deoxyGlc to GPIs containing core glycan A in a fashion analogous to glucosylation observed with the natural nucleotide sugar. The use of UDP−2deoxyGlc but not of GDP−2deoxyGlc additionally suggests direct transfer from UDP−Glc and excludes a Dol-P−Glc intermediate.

DISCUSSION

In addition to its importance as a human pathogen *T. gondii* has gained considerable interest as eucaryotic model organism. As a model *T. gondii* provides both the whole array of eucaryotic cell functions and a reduced complexity and minute size forced by its intracellular parasitism (*24*). The major surface proteins of *T. gondii* are linked to the cell membrane via a GPI glycolipid anchor (*25*, *26*). The

structures of the membrane anchor of the major surface protein SAG1 and a family of precursor glycolipids has recently been solved (Zinecker, C. F., Striepen, B., Geyer, R., Geyer, H., and Schwarz, R. T., submitted for publication; ref *5*). The glycan structure of the protein anchor was found to be identical to precursor GPIs arguing for side chain modification in the ER prior to protein attachment in contrast to trypanosomes which transfer side chain galactose to GPIs already attached to the VSG protein in the Golgi apparatus (*27*). In *T. gondii* addition of the GPI anchor has been shown to be crucial to target proteins to the surface of the parasite, analogous to the role GPI anchoring plays for apical membrane targeting of certain proteins in polarized epithelial cells (*28*, *29*). Characterization of the early steps of GPI biosynthesis has shown that pathways in *T. gondii* follow the conserved routes first established in *T. brucei* (*15*, *16*, *19*, *30*). Aside from GPI anchoring, *T. gondii* was shown to modify proteins also by N-glycosylation (*31*) and O-glycosylation (*32*). Labeling of the N-glycosylation precursors $Man_9GlcNAc_2$−PP-Dol with GDP−[^{3}H]Man and Glc_3-$Man_9GlcNAc_2$−PP-Dol with UDP−[^{3}H]Glc in cell free preparations of purified parasites (*31*) has established that *T. gondii* has its own dolichol cycle independent from its mammalian host cell and further substantiated by the recent cloning of the *T. gondii* UDP−GlcNAc::Dol-P−GlcNAc−P-transferase (Striepen, B., and Roos, D. S., unpublished).

In this paper we present a detailed analysis of GPI glucosylation in *T. gondii* in vitro. An in vitro system has been established allowing one to synthesize and radiolabel different subpopulations of *T. gondii* GPI biosynthetic intermediates by varying the sugar nucleotides added to the lysates. Incubation with GDP−[^{3}H]Man leads only to synthesis and labeling of GPIs lacking the "mature" glucose modification, whereas incubation with UDP−[^{3}H]Glc results in exclusive labeling of glucosylated glycolipids as judged by thin-layer chromatography. This finding was confirmed by HPAEC and aminopropyl HPLC analysis of core glycans prepared from the respective glycolipids showing Man_3−AHM and the nonglucosylated glycan A (Manα1−2Manα1−6-(GalNAcβ1−4)Manα1,4-AHM) for GDP−[^{3}H]Man labeling and glycan B (Manα1−2Manα1−6-(Glcα1−4GalNAcβ1−4)Manα1,4-AHM) for UDP−[^{3}H]Glc labeling. These data show that an exogenous glucose donor is required to complete *T. gondii* GPI biosynthesis in vitro. In vitro glucosylation of preformed nonglucosylated GPIs can indeed be stimulated by addition of exogenous unlabeled UDP−Glc in a dose-dependent manner. The pool of UDP−Glc accessible to the GPI glucosyltransferase seems to be either very small and/or exhausted rapidly by other glucosyltransferases as virtually no transfer occurs in the absence of exogenous UDP−Glc whereas in the absence of GDP−Man and UDP−GlcNAc at least a small amount of GPI biosynthesis is observed (B.S. and R.T.S., unpublished observation). This lack of glucosylation in the absence of exogenously added UDP−Glc was exploited in subsequent experiments which used inhibitors and analogues which interfere with early steps in GPI biosynthesis to discriminate between glucose transfer from UDP−Glc and transfer via Dol-P−Glc. GPI glucosylation was found to be insensitive toward amphomycin at concentrations as high as 1 mg/mL. Concentrations of 0.1 mg/mL block GPI synthesis in *T. gondii* and *T. brucei* by inhibiting Dol-P−Man formation (*15*, *19*) and inhibit N-glycosylation in mammalian cells by interference of amphomycin with both the synthesis of Dol-P−Man and Dol-P−Glc (*20*, *22*). These data argue strongly against a transfer of glucose via a dolichol bound intermediate as found for GPI mannosylation or glucosylation of the dolichol bound *N*-glycan precursor $Man_9GlcNAc_2$−PP-Dol. Instead of using Dol-P stimulation assays, which often result in reduced labeling efficiencies due to the need to introduce detergents into the cell free system, we used glucose analogues as a second independent approach to test this result.

2deoxyGlc was previously shown to interfere as an analogue with both mannosylation and glucosylation (reviewed in ref *21*). Insights into the effects of 2deoxyGlc on the cellular glycosylation pathways were obtained from detailed biochemical analysis using viral glycoproteins as models (*18*, *21*, *33*). It was shown that only GDP−2deoxyGlc is an appropriate precursor for the synthesis of Dol-P-2deoxyGlc (*18*); we exploited this specificity here by using synthetic GDP−2deoxyGlc and UDP−2deoxyGlc to trace GPI glucosylation. Both nucleotide deoxysugars in parallel with GDP− and UDP−Glc were added to the *T. gondii in vitro* system. Incubation under UDP−2deoxyGlc resulted in the formation of previously not detected glycolipids. Careful analysis of these glycolipids confirmed that they contain 2deoxyGlc linked to the GPI core glycan analogous to glucose. In contrast GDP−deoxyGlc incubation led only to glycolipids containing Man_3AHM and core glycan A. All data presented here are consistent with a direct transfer of glucose from UDP−Glc by a *T. gondii* specific UDP−Glc::GPI-glucosyltransferase.

The use of Dol-P−Glc as a donor for glucosylation of the *N*-glycan precursor $Man_9GlcNAc_2$−PP-Dol by ER glucosyltransferases is a long established fact (*34*, *35*). More recently a glycoprotein glucosyltransferase using UDP−Glc as direct donor has been identified (*36*, *37*). This enzyme transfers glucose residues to high mannose *N*-glycan chains previously deglucosylated by the action of glucosidase I and II. A growing body of evidence suggests that this reglucosylation reaction is used to tag misfolded glycoproteins to be retained in the ER and to bind the ER chaperone calnexin (*38*, *39*). Whereas Dol-P−Glc-mediated glucosylation is believed to act on the cytoplasmatic face of the ER (*40*), the UDP−Glc-dependent transferase was shown to be a soluble luminal enzyme (*36*).

The topology of GPI biosynthesis is still under investigation. Studies using lectins and GPI specific phospholipases in conjunction with in vitro labeling suggest that the entire biosynthesis of the *T. brucei* anchor precursor P2/glycolipid A (which has no side chain modifications) takes place at the cytoplasmatic face of the ER (*41*, *42*). We propose that *T. gondii* as a model system might give new insights in the topology of GPI biosynthesis. The availability of monoclonal antibodies and lectins specific for *T. gondii* GPIs in different states of biosynthetic completion (*5*) together with a well-characterized and easy to manipulate cell free synthesis system should allow identification of the side of glucose and *N*-acetylgalactosamine transfer to GPI precursors in the ER. Recent advances have provided a wide array of genetic tools for *T. gondii*, including the tagging of genes by insertional mutagenesis and marker rescue (*43*−*45*). Experiments to establish a screen for insertional mutants using the GPI

specific monoclonal antibodies to identify and clone the GPI glucosyl- and the *N*-acetylgalactosamine transferase are under way.

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