Glycosylphosphatidylinositols Synthesized by Asexual Erythrocytic Stages of the Malarial Parasite, *Plasmodium falciparum*

**CANDIDATES FOR PLASMODIAL GLYCOSYLPHOSPHATIDYLINOSITOL MEMBRANE ANCHOR PRECURSORS AND PATHOGENICITY FACTORS**

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*Plasmodium falciparum* is the causative agent of malaria tropica in man. Biochemical studies were focused on the asexual, intraerythrocytic stages of *P. falciparum*, because of their role in the clinical phase of the disease and the possibility of propagation in a cell culture system. In this report, we describe the in-culture labeling of malarial glycolipids and the analysis of their hydrophilic moieties. They were identified as glycosylphosphatidylinositol (GPI) moieties by 1) labeling with $[^3]H$mannose, $[^3]H$glucosamine, and $[^3]H$ethanolamine and 2) sensitivity toward glycosylphosphatidylinositol-specific phospholipase D, phospholipase A$_2$, and nitrous acid. Malarial GPIs are shown to be unaffected by treatment with phosphatidylinositol-specific phospholipase C, regardless of prior treatment with mild base commonly used for inositol deacylation. Two candidates for putative GPI-anchor precursors to malarial membrane proteins with the structures ethanolamine-phosphate-$\alpha$(Man$_1\alpha$2)Man$_1\alpha$2Man$_1\alpha$2GlcN-PI (P$_{\alpha}$) and ethanolamine-phosphate-$\beta$(Man$_1\alpha$2Man$_1\alpha$2Man$_1\alpha$2GlcN-PI (P$_{\beta}$) were identified.

Malaria is the disease caused by parasitic protozoa of the genus *Plasmodium, Plasmodium falciparum*, the most dangerous of the four species that are naturally infectious to man, still remains the cause of one of the most important diseases in tropical and subtropical areas. In 1985, the WHO reported 4.8 million documented cases of malaria, and more than 50% of the world’s population live in areas where malaria is endemic (Lockyer and Holder, 1989).

Glycosylphosphatidylinositols (GPIs) are a recently discovered class of glycolipids that anchor proteins and sugars into the plasma membrane, in a wide range of organisms. A comparison of the glycan structures of GPI membrane anchors indicates that the glycans parts of GPI-anchors contain a conserved structure consisting of ethanolamine-P$_{\alpha}$-6Man$_1\alpha$2Man$_1\alpha$2GlcN-C (for review see Cross (1990)).

A huge variety of modifications on the periphery of the core glycan structure have been observed. However, the core glycan of protein-linked GPIs is highly evolutionarily conserved throughout mammalian and protozoan cells. Therefore, it is likely that identical or similar biosynthetic pathways are used in all eukaryotes.

The biosynthesis of the trypanosomal GPI has been elucidated using a cell-free system from *Trypanosoma brucei* (Masterson et al., 1989; Doering et al., 1990; Menon et al., 1990a) and was also elucidated for some other biological systems (Puoti et al., 1991; Stevens and Raetz, 1991; Tomavo et al., 1992). GPI synthesis starts with the transfer of GlcNac from UDP-GlcNAc to PI, followed by de-N-acetylation. Three mannose residues, each derived from dolichol-phosphate mannose, are then added, yielding Man$_3$GlcN-PI. Phosphoethanolamine, derived from phosphatidylethanolamine (Menon et al., 1993), is added to the terminal mannose to complete the GPI-anchor precursor which is thought to be added to the protein in exchange for a C-terminal hydrophobic domain in a transamidase reaction (Cross, 1990).

Like many parasitic protozoa, *P. falciparum* has a complex life cycle involving an insect vector (*Anopheles spp.*). During the development in man, the intraerythrocytic, asexual stages produce the merozoite surface protein 1 (MSP1), the merozoite surface protein 2 (MSP2), and other proteins (Holder et al., 1995; Haldar et al., 1985; Haldar et al., 1986; Smythe et al., 1988; Braun-Breton et al., 1988), which were shown to be membrane-anchoring involving diacylglycerol. These data lead to the proposition that some *P. falciparum* surface proteins are anchored by a glycosylphosphatidylinositol (GPI) membrane anchor to the parasite membrane (Haldar et al., 1985; Haldar et al., 1986; Schwarz et al., 1986; Schwarz et al., 1987).

It has recently been demonstrated that the GPI moiety may also play an important role in the pathology of severe malaria, in addition to its role in membrane anchoring of surface proteins. Purified C-terminal GPI derived from MSP1 and MSP2 by pronase hydrolysis induces tumor necrosis factor and interleukin 1 production by macrophages and regulates metabolism in adipocytes. When administered to mice in vivo, it induces cytokine release, transient pyrexia, and hypoglycemia and under some conditions elicits cachexia (Schofield and Hackett, 1993).

In this report we provide evidence that the biosynthesis of GPIs in the parasitic protozoan *P. falciparum* involves a hydrophobically modified inositol ring and therefore shows a similarity to the pathways described for mammalian cells (for a
review see Englund (1993)). The malarial pathway leads to two putative GPI-anchor precursors (ethanolamine-phosphate-6Manα1→2Manα1→6Manα1→4GlcN-PI and ethanolamine-phosphate-6α1→2Manα1→2Manα1→6Manα1→4GlcN-PI) whose glycan structures differ only in the number (3 versus 4) of mannose residues.

EXPERIMENTAL PROCEDURES

Materials—[6-3H]Glucosamine hydrochloride (26.0 Ci/mmol), [12-3H]mannose (13.6 Ci/mmol), [9,10-3H]myristic acid (50.0 Ci/mmol), [9,10-3H]palmitic acid (53.4 Ci/mmol), [3H]inositol (108.4 Ci/mmol), and [1-3H]ethanol-1-amine (31.7 Ci/mmol) were purchased from Amersham. Tunicamycin was purchased from Calbiochem. Phosphatidylinositol-specific phospholipase C (PI-PLC) from Bacillus cereus and phospholipase A2 (PLA2) from bee venom were from Boehringer Mannheim. Jack bean α-mannosidase was from Sigma, and Aspergillus saitoi α-nanoseidosidase was obtained from Oxford GlycoSystems. Ion exchange resins were analytical grade and purchased from Bio-Rad. All sugar and lipid standards were obtained from Sigma. All solvents were analytical or high performance liquid chromatography grade.

Parasite—The P. falciparum strain FC8 was obtained from Dr. B. Enders, Behring Co., Marburg, Germany. It was maintained in RPMI 1640 medium supplemented with 25 m units hepes, 21 m sodium bicarbonate, 0.37 m hypoxanthine, 0.1 mg/ml neomycin, and 10% defibrinated (v/v) fresh human serum (modified from Trager and Jensen (1976)). The cultures contained human A-erythrocytes (leukocyte-depleted erythrocyte concentrate, Blood Donation Center, University of Marburg, Germany) at 5% hematocrit and were incubated at 37 °C in gas-tight containers (modular incubation chamber, Flow Laborato- ries) being gassed with a mixture of 90% nitrogen/5% oxygen/5% carbon dioxide. Development and multiplication of the cultures were followed by microscopic examination of Giemsa-stained smears. Synchronous development of the parasite was achieved by the sorbitol method (Lambris and Vanderberg, 1976).

Extraction and Purification—Washed parasites were harvested by centrifugation at 4 °C (Goman et al., 1982). Less polar glycolipids were precipitated by addition of ice-cold 48% aqueous HF for 60 h at 0 °C. After neutralization with 4 M NaOH and 4 M Na2CO3, the glycolipids were washed twice with 2 m chloroform/methanol (CM 2:1, v:v) to remove neutral lipids and phospholipids. This extraction was carried out for 30 min at room temperature under sonication in a water bath sonicator (Branson 3200, 47 MHz). The residual pellet was then extracted twice with 1 ml of chloroform/methanol (CM 10:3, v:v) to obtain the more polar glycolipids. The CM extracts were pooled and subjected to repeated Folch washes (Figure 1). Chloroform/methanol (25:15:4:2, by volume); B, chloroform/methanol/water (10:10:2.5, by volume); C, chloroform/

methanol/0.1% KCl (10:10:3, by volume).

Analysis and Purification of Glycolipids—The Folch-washed CM extracts and the butanol components were analyzed on Silica Gel 60 TLC plates (Merek) using solvent systems A and B, respectively. After chromatography, the plates were dried and scanned for radioactivity using a Benchold LB 2842 automatic TLC scanner. The areas corresponding to the bands of radioactivity were excised, and the glycolipids were extracted twice with chloroform/methanol (2:1, v:v), twice with chloroform/methanol/water (10:10:3, v:v), and then with methanol/pyridine/water (2:1:1, v:v:v) for 30 min each under sonication.

The yield of purified glycolipids was estimated to be approximately 40%.

Acid Hydrolysis for Component Analysis—TLC-purified glycolipids were dried to the bottom of the glass tubes (Duran) and hydrolyzed for 4 h in 4 M HCl at 100 °C. HCl was removed from the samples by repeated flash evaporation with 100 μl of methanol. The released monosaccharides were analyzed by Dionex-HPAEC (Dionex, Sunnyvale, CA) using elution program 2 (see below).

Alkaline Saponification of Ester-like Bound Fatty Acids—Malarial glycolipids were dried and saponified in 200 μl of 0.5 × KOH/MeOH (1:1; v:v) for 4 h at 100 °C. The reaction was terminated by drying the reaction mixture in a Speed-Vac evaporator. After phase partition between water and water-saturated 1-butanol, the cleavage rate was estimated by liquid scintillation counting of aliquots of the organic and aqueous phases.

Enzymatic Cleavage of Glycolipids—The radiolabeled glycolipid mixtures or TLC-purified glycolipids were dried and redisolved in detergent-containing buffer for the corresponding enzymatic reactions. Phosphatidylinositol-specific phospholipase C (PI-PLC) digestions were carried out in 100 μl of 0.1 m Tris-Cl (pH 7.4), containing 0.1% sodium deoxycholate and 1 unit of B. cereus PI-PLC (Boehringer Mannheim). In some cases, TLC-purified glycolipids were treated with tunicamycin (325 μg/ ml NH4/MeOH (1:1; v:v), 1 h, 37 °C) prior to PI-PLC digestion (Mayor et al., 1990). This treatment is supposed to selectively cleave palmitic acid at the inositol ring. Glycosphatidylskyl-specific phospholipase D (GPI-PLD) treatment was performed under identical conditions, but in this case the incubation buffer contained 2 m CaCl2, 10 μl of normal rabbit serum was used as a source of GPI-PLD (Davitz et al., 1988). Phosphodiesterase A2 (PLA2) reactions were performed in the same incubation buffer but with 1 m CaCl2 and 50 units of the bee venom enzyme. After incubation for 14 h (PI-PLC, PI-PLA2) or 3 h (GPI-PLD) at 37 °C, the reactions were terminated by heating at 100 °C for 1 min, and the reaction products were extracted twice (with 100 μl of water-saturated 1-butanol. The radioactivity in aliquots of the aqueous and organic phases were counted. The organic phase was analyzed by TLC using solvent system B.

Nitrous Acid Deamination—Glycolipid mixtures or TLC-purified glycolipids were dried and resuspended in 100 μl of freshly prepared 0.1 m sodium acetate (pH 4.0), containing 0.05% SDS, 0.25 m NaNO2, and incubated for 1 h at ambient temperature. The reaction was terminated by the addition of 5 μl of 6 × HCl and extracted as described above. The organic phases were analyzed on TLC. The radioactivity released into the aqueous phases was analyzed on Bio-Gel P4 as described below.

Bio-Gel P4 Gel Filtration Analysis—Water-soluble products generated by enzymatic or nitrous acid treatment of radiolabeled glycolipids were sized on Bio-Gel P4 columns (1 × 130 cm, 400 mesh) in 0.2 m ammonium acetate, 0.02% sodium azide (Masterson et al., 1989). Fractions (about 850 μl) were collected at a rate of 1 fraction per 24 min. Glucose oligomers from partially hydrolyzed dextran were included as internal standards and detected by oxidation with 2 mg/ml orcinol in concentrated sulfuric acid.

Generation and Analysis of Core Glycans from Glycolipids—TLC-purified glycolipids were dephosphorylated, deaminated, and reduced according to a modification of the method described by Mayor and Menon (1990). Briefly, the glycolipids were dephosphorylated by treatment with ice-cold 48% aqueous HF for 60 h at 0 °C. After neutralization of the frozen solution, the reaction mixture was deamylated, deaminated by HNO3 treatment for 3 h, and reduced by sodium borohydride for 5 h. The reduced material was desalted on a tandem ion exchange column and filtered through a 0.4-μm and 0.25-μm filter. The desalted and filtered core glycans were analyzed by Dionex-HPAEC using gradient elution program 1 (Mayor and Menon, 1990).

Exchange HPAEC Analysis of Labeled Glycans—Desalted glycans were analyzed by anion exchange chromatography on a Dionex Basic Chromatography System. The separation was accomplished by gradient elution (program 1) using a Carbopak PA1 (4 × 250 mm) column. Monosaccharides were analyzed using isocratic conditions (pro-
gram 2). Mono- and oligosaccharide standards were detected by pulsed amperometric detection. The elution programs for Dionex HPAEC were: 1) 100% buffer A (0.1 M NaOH), 0% buffer B (0.1 M NaOH, 0.25 M NaOAc) up to 6 min after injection, then an increase of buffer B to 30% at 36 min, at a flow rate of 1 ml/min and 2) isocratic 15 ml NaOH at a flow rate of 1 ml/min.

Exoglycosidase Digestion of Glycolipids—For malarial digestion, TLC-purified glycolipids were deaminated and desalted as described above. The resulting water-soluble material was resuspended in 100 µl of 50 mM sodium acetate (pH 4.5) containing 0.2 mM ZnCl₂, 0.02% sodium azide, and 1.7 units of jack bean α-mannosidase and incubated at 37 °C for 24 h. Additional enzyme (0.85 unit) was added 14 h after the beginning of the incubation. For A. saitoi α-mannosidase digestion of dephosphorylated, deaminated, and reduced glycolipids, the glycan samples were resuspended in 30 µl of 0.1 M sodium acetate (pH 5) containing 10 µimicronits of A. saitoi α-mannosidase and incubated for 14 h at 37 °C.

All digestions were stopped by heating at 100 °C for 1 min. The reaction mixtures were deasalted, filtered, and analyzed by Bio-Gel P4 or by Dionex HPAEC chromatography.

Acetylation of Neutral Glycans—Radiolabeled glycolipids were cleaved by a modification of the method described by Mayor and Menon (1990). This procedure preferentially cleaves Man₆-6Man linkages (Rosenfeld and Ballou, 1974). Briefly, the glycan samples were dissolved in 30 µl of acetic anhydride/pyridine (1:1, v/v) and incubated for 30 min at 100 °C under an acetylate. After drying, the samples were resuspended in 50 µl of acetic acid/acetic anhydride/H₂SO₄ (10:10:1, v/v) and treated for 6 h at 37 °C. After neutralization, the samples were partitioned between 250 µl of chloroform and 1 ml of water. The chloroform phases were dried and resuspended in 200 µl of methanol/32% ammonia (1:1, v/v) for 16 h at 37 °C to de-O-acetylate the glycans. The de-O-acetylated material was deasalted and analyzed by Dionex HPAEC chromatography.

Neutral Glycan Standards—Glucose oligomer standards were prepared according to Yamashita et al. (1992) by partial acid hydrolysis of Dextran (Sigma). For oligosaccharide analysis on Bio-Gel P4 and Dionex HPAEC, these glucose oligomers were incorporated as internal standards. Neutral glycan analysis on Dionex HPAEC was correlated by TLC analysis on Bio-Gel P4 and TLC standards. Neutral glycan analysis on Dionex HPAEC was correlated by Dionex HPAEC, these glucose oligomers were incorporated as internal standards. Neutral glycan analysis on Dionex HPAEC was correlated by TLC analysis on Bio-Gel P4 and TLC standards. Neutral glycan analysis on Dionex HPAEC was correlated by Dionex HPAEC, these glucose oligomers were incorporated as internal standards.

RESULTS

Extraction and Analysis of Glycolipids—We metabolically labeled trophozoites (33–43 h after infection), an intraerythrocytic, asexual stage of P. falciparum, with different radioactive precursors, such as [3H]GlcN and [3H]Man. The presence of tunicamycin had no effect on the glycolipid pattern irrespective of the radioactive precursor used (Gerold et al., 1992b; Dieckmann-Schuppert et al., 1992a). Parasites were purified and extracted as described under "Experimental Procedures." Uninfected erythrocytes showed no (<1%) incorporation of radioactivity into glycolipids. TLC analysis of the "Folch"-washed CM extracts and the butanol component of the CMW extracts (butanol phases) showed qualitatively identical glycolipid patterns in TLC systems A and B. Therefore, the Folch-washed CM extracts and the butanol components were pooled and analyzed together using solvent system B (Fig. 1). TLC analysis of these pooled glycolipids (termed glycolipid extract) revealed eight and seven glycolipids labeled with [3H]GlcN (Fig. 1A) and [3H]Man (Fig. 1B), respectively. The relative migration (R₂) values of these glycolipids were 0.25 (P₁₂ α, 0.33 (P₂₂ β), 0.50 (P₃₂ γ), 0.56 (P₄₂ δ), 0.62 (P₅₂ ε), 0.66 (P₆₂ ζ), 0.71 (P₇₂ η), 0.85 (P₈₂ θ), and 0.9 (dolichol(?) phosphate-mannose)² (Table 1). We have used infected erythrocytes rather than isolated parasites for the following experiments. The glycolipid pattern is conserved throughout all experiments. However, we have observed differences in the relative amount of labeling of single malarial glycolipids in various labeling experiments (up to 30 times). The obtained glycolipid pattern is different from that described²

²(?) indicates that chain length and saturation are unknown.
TABLE I
Characterization of malarial glycolipids

<table>
<thead>
<tr>
<th>Glycolipid species</th>
<th>Proposed structure</th>
<th>Rf values</th>
<th>Labeled via</th>
<th>Sensitivity to</th>
<th>Susceptibility to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfgl α</td>
<td>EtN-P-Man, GlcN-Ino(x)-PA</td>
<td>0.25</td>
<td>L</td>
<td>L</td>
<td>+</td>
</tr>
<tr>
<td>Pfgl β</td>
<td>EtN-P-Man, GlcN-Ino(x)-PA</td>
<td>0.33</td>
<td>L</td>
<td>L</td>
<td>+</td>
</tr>
<tr>
<td>Pfgl γ</td>
<td>Man-GlcN-Ino(x)-PA</td>
<td>0.50</td>
<td>L</td>
<td>NL</td>
<td>-</td>
</tr>
<tr>
<td>Pfgl δ</td>
<td>Man-GlcN-Ino(x)-PA</td>
<td>0.56</td>
<td>L</td>
<td>NL</td>
<td>-</td>
</tr>
<tr>
<td>Pfgl ε</td>
<td>Man-GlcN-Ino(x)-PA</td>
<td>0.62</td>
<td>L</td>
<td>NL</td>
<td>-</td>
</tr>
<tr>
<td>Pfgl η</td>
<td>GlcN-Ino-PA</td>
<td>0.66</td>
<td>L</td>
<td>NL</td>
<td>-</td>
</tr>
<tr>
<td>Pfgl θ</td>
<td>GlcN-Ino(x)-PA</td>
<td>0.85</td>
<td>L</td>
<td>NL</td>
<td>-</td>
</tr>
<tr>
<td>(Dol(P-Man))</td>
<td></td>
<td>0.90</td>
<td>NL</td>
<td>NL</td>
<td>-</td>
</tr>
</tbody>
</table>

The results summarized in this table correspond to the average of two [3H]ethanolamine and four [3H]Man, [3H]GlcN radiolabeling experiments. NL, not labeled; +, sensitive; -, insensitive.

Parasites were labeled with [3H]GlcN, [3H]Man, or [3H]ethanolamine, and glycolipids were sequentially extracted with CM and CMW. TLC-purified glycolipids were subjected to different treatments. After butanol-water phase separation, cleavage was measured by scintillation counting. The comparison of the GPI-PLD-generated hydrophobic fragments of other PI-PLC-resistant malarial glycolipids (corresponding to 6.7 and 0.9 glucose units) carry a hydrophobic modification at the inositol ring (Menon et al., 1988). Pfgl θ is totally susceptible to PLA2 treatment, consistent with the assumption that this glycolipid has no modification at the inositol.

After PI-PLC, GPI-PLD, or HNO2 treatment of malarial glycolipids, the released oligosaccharide moieties were analyzed on Bio-Gel P4 (Fig. 2). In contrast, the radioactive release by PI-PLC elutes as one peak on the Bio-Gel P4 columns. This is consistent with the observation that only Pfgl θ disappeared from the organic phase after PI-PLC treatment. Determination of the Size and Presence of Terminal Mannosidase-blocking Residues—The presence of the terminal ethanolamine in GPI-anchor precursors renders the structure resistant to jack bean α-mannosidase which requires an unmodified terminal mannose residue (Li and Li, 1972). Glycolipids Pfgl α, β, γ, δ, ε, η, and θ were subjected to mild alkaline hydrolysis by ammonia prior to PI-PLC treatment. Only glycolipid θ became 45% susceptible, whereas the glycolipids Pfgl α, β, γ, δ, ε, η, and θ remained insensitive.

The glucosamine-inositol fragment of Pfgl α, generated by dephosphorylation and treatment with jack bean α-mannosidase, was completely (>90%) recovered in the organic phase after phase partition. Therefore, Pfgl α (and presumably all other PI-PLC-resistant malarial glycolipids) carry a hydrophobic modification at the inositol ring. The treatment of the glycolipids with GPI-PLD and HNO2 leads to recovery of >90% (only <10% in the case of Pfgl θ) and 60% of the radioactivity, respectively, in the organic phases. The comparison of the GPI-PLD-generated hydrophobic fragments of Pfgl α and the putative GPI-anchor precursor P3 of T. brucei in the TLC system C showed significant differences in the chromatographic behavior (Rf = 0.07 and Rf = 0.75, respectively) although both GPIs share the same hydrophilic moieties (see below).

However, after HNO2 treatment, we found one major and several minor products in the organic phases. The generation of alternative deamination products appears to be a common feature of nitrous acid deamination (Krakow et al., 1986; Mayor et al., 1990). Based on the results that the efficiency of deamination in our experiments is similar to that of the deamination of the GPI-anchor precursors P2 and P3 of T. brucei, we conclude that the malarial glycolipids are sensitive to deamination, indicating that they have a nonacylated hexosamine, as is described for GPIs.

The treatment of the malarial glycolipids with phospholipase A2 (PLA2) leads to the formation of lyso-forms of each malarial glycolipid with the exception of dolichol(?)-phosphate-mannose (Dol(?)-P-Man; Table I). However, only about 30–40% of each glycolipid, except Pfgl θ, ε, was converted to its lyso-form, consistent with the results described for T. brucei GPI-anchor precursor P3, which was described to have a palmitoylated inositol.
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FIG. 2. Characterization of the [3H]GlcN-labeled glycolipids. [3H]GlcN-labeled glycolipids were treated with PI-PLC, GPI-PLD, and HNO₂ as described under “Experimental Procedures.” After incubation, the reaction mixture was partitioned between water and water-saturated butanol. The organic phases were analyzed on silica TLC plates using solvent system B (left panel). The aqueous phases were analyzed on Bio-Gel P4 columns (right panel) with internal dextran standards (see “Experimental Procedures”). Positions of the standards are shown at the top. The glycolipids marked with * were not further characterized.

A1) show that the side chain mannose is preferentially labeled.

The HNO₂-generated fragments of the [3H]mannose-labeled glycolipids Pf₁α, δ, ε, and η (which elute at 4.7, 3.7, 2.7, and 1.8 glucose units, respectively; Fig. 3B) are sensitive to α-mannosidase treatment (i.e. elution at 0.9 glucose unit after treatment), confirming that their terminal mannose residues are not blocked.

The glycolipids Pf₁ζ and Pf₁θ, labeled only with [3H]glucosamine, are not affected by digestion with jack bean α-mannosidase (Fig. 3B, panels F and H). Together with the failure to label these two glycolipids with [3H]mannose and their elution size (1.2 glucose units), these results indicate that Pf₁ζ and θ do not contain mannose residues.

Based on the labeling experiments with [3H]ethanolamine, the sensitivity toward dephosphorylation with aqueous HF (see below), the α-mannosidase digestions, and the conserved structure of other GPI-anchor precursors indicate that the terminal mannose residues of Pf₁α and β are blocked by ethanolamine-phosphate.

Analysis of the Glycans by Dionex-HPAEC—For further characterization, the [3H]GlcN-labeled glycolipids Pf₁α, β, γ, δ, ε, ζ, η, and θ were dephosphorylated by treatment with aqueous HF. This procedure selectively cleaves phosphodiester linkages. The HF-generated glycan fragments were deaminated and reduced (as described under “Experimental Procedures”), then analyzed by Dionex-HPAEC using elution program 1 (Fig. 4, A and B).

The neutral glycans derived from glycolipids Pf₁α and γ (Fig. 4A, panels A and C) co-eluted with a Man₄-2,5-anhydromannitol (AHM) standard generated from the Thy-1 GPI-anchor (a generous gift from Dr. M. Ferguson). After treatment of the neutral glycans of Pf₁α and γ with A. saitoi α-mannosidase (an exo-mannosidase specific for Man₁–2Man linkages), the resulting fragments were analyzed on the Dionex-HPAEC system and found to co-elute with a Man₃-AHM standard generated from T. brucei (Fig. 4A, panels A1 and C1). This indicates the removal of two α1–2-linked mannoses from the glycans of Pf₁α and γ.

Recent experiments show that the malarial surface proteins MSP1 and MSP2 possess GPI-anchors with the same neutral
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Fig. 3, A, Bio-Gel P4 chromatography of HNO₂-generated hydrophilic moieties before and after jack bean α-mannosidase treatment. TLC-purified, [³H]Man-labeled glycolipids P₅₆α, P₅₆β, P₅₆γ, and P₅₆δ were deaminated, and an aliquot (1000–1500 cpm for each control; A–D) of the HNO₂-generated hydrophilic fragment was loaded on a Bio-Gel P4 column. A second aliquot (1500 cpm) was incubated with jack bean α-mannosidase and analyzed on the same column (panels A₁–D₁). Glucose oligomers were added to the samples as internal standards, and their elution positions are indicated on the top of each profile. Radioactivity (y-axis) in the fractions (850 pl) was plotted against the fraction number. B, Bio-Gel P4 chromatography of HNO₂-generated hydrophilic moieties before and after jack bean α-mannosidase treatment. TLC-purified, [³H]Man-labeled glycolipids P₅₆ε and P₅₆η, and [³H]GlcN-labeled P₅₆ζ and P₅₆θ were deaminated, and an aliquot (1000–1500 cpm for each control; E–H) of the HNO₂-generated hydrophilic fragment was loaded on a Bio-Gel P4 column. A second aliquot (1500 cpm) was incubated with jack bean α-mannosidase and analyzed on the same column (panels E₁–H₁). Glucose oligomers were added to the samples as internal standards, and their elution positions are indicated on the top of each profile. Radioactivity (y-axis) in the fractions (850 pl) was measured and plotted against the fraction number.

The neutral glycans of the glycolipids P₅₆α and δ both co-eluted with a Man₂-AHM standard prepared from T. brucei (Fig. 4A, panels B1 and D1). When the malarial neutral glycans were treated with A. saitoi α-mannosidase, the resulting fragments co-eluted on Dionex-HPAEC with a Man₂-AHM standard (Fig. 4A, panels B1 and D1), confirming the removal of one terminal α1-2-linked mannose residue, as described for the neutral glycan of the T. brucei GPI-anchor precursors P2 and P3 (Mayor et al., 1990).

The HF-generated, deaminated, and reduced glycan fragments from P₅₆ε and η co-eluted with Man₂-AHM and Man₃-AHM standards, respectively (Fig. 4B, panels E and G). Consistent with the linkages in the corresponding GPI-anchor biosynthesis intermediates from T. brucei, the two malarial

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Fig. 4. A, Dionex-HPAEC analysis of the HF-generated, dephosphorylated, deaminated, and reduced core glycans. TLC-purified, [3H]GlcN-labeled glycolipids Pfα, Pfβ, Pfγ, and Pfδ were dephosphorylated, deaminated, and reduced as described under "Experimental Procedures." The neutral glycans were desalted and divided into aliquots. One aliquot (1200-1500 cpm) of each sample was analyzed by Dionex-HPAEC using gradient elution program 1 (profiles A–D). A second aliquot (1500 cpm) was treated with A. saitoi α1–2-specific mannosidase and desalted prior to analysis on Dionex-HPAEC (profiles A′–D′). The flow rate was 1 ml/min, and fractions were collected every 0.4 min. B, Dionex-HPAEC analysis of the HF-generated dephosphorylated, deaminated, and reduced core glycans. TLC-purified, [3H]GlcN-labeled glycolipids Pfε, Pfζ, Pfη, and Pfθ were dephosphorylated, deaminated, and reduced as described under "Experimental Procedures." The neutral glycans were desalted and divided into aliquots. One aliquot (1200-1500 cpm) of each sample was analyzed by Dionex-HPAEC using gradient elution program 1 (profiles E–H). A second aliquot (1500 cpm) was treated with A. saitoi α1–2-specific mannosidase and desalted prior to analysis on Dionex-HPAEC (profiles E′–H′). The standards indicated on the top of each profile are generated from Thy-1 GPI-anchor and GPI-anchor precursors of T. brucei. The flow rate was 1 ml/min, and fractions were collected every 0.4 min.

Dionex-HPAEC analysis of the neutral glycans derived from the [3H]GlcN-labeled glycolipids Pfε and θ (which were not labeled by [3H]mannose, Fig. 1A) showed that both fragments were identical and co-eluted with 2,5-anhydromannitol (Fig. 4B, panels F and H). Treatment with A. saitoi α-mannosidase had no effect on the elution behavior of the glycans of both
glycolipids, indicating that they have no terminal α1–2 mannose residue (Fig. 4B, panels F1 and H1).

**Jack Bean α-Mannosidase Digestion of Neutral Glycans**
—Digestion of HF-generated neutral glycans of the glycolipids Pfgl α, Pfgl β, and Pfgl γ with jack bean α-mannosidase produced single glycans for each malarial neutral glycan (Fig. 5, panels A1, B1, and C1). These single glycans co-elute in each case with a 2,5-anhydroxymanitol standard. These data show that the neutral glycans of Pfgl α, β, and γ consist of α-mannose residues without additional non-mannose components.

**Acetolysis of the Neutral Glycans of Malarial Glycolipids**—To test for Manα1–6Man linkages, we made use of the relatively selective acetolysis conditions described by Rosenfeld and Baldues of the malarial glycolipids (1988). The HF-generated deaminated and reduced neutral glycans of [3H]GlcN-labeled Pfgl α, Pfgl β, and Pfgl γ were subjected to partial acetolysis and then analyzed on Dionex HPAEC (Fig. 5, panels A2, B2, and C2). Besides the unhydrolyzed neutral glycans of these three malarial glycolipids, we found in all cases material co-eluting with Manα1-AHM and AHM standards. The identification of a Manα1-AHM fragment generated by acetolysis is consistent with the presence of a Manα1–6Man bond between the first and second mannose residues of the malarial glycolipids Pfgl α, Pfgl β, and Pfgl γ. The observation of under- and overdigestion products in acetolysis studies is confirmed by the results of Mayor et al. (1990).

**DISCUSSION**

In this report we provide evidence that the asexual, intraerythrocytic late stages of the malaria parasite *P. falciparum* produce two putative glycosylphosphatidylinositol membrane anchor precursors: ethanolamine-phosphate-6α1–2Man) Manα1–2Manα1–6Manα1–4GlcN-PI (Pfgl α) and ethanolamine-phosphate-6Manα1–2Manα1–6Manα1–4GlcN-PI (Pfgl β) and a series of minor glycosylphosphatidylinositol standards (GPIs) showing a lesser degree of glycosylation.

The glycolipid extracts contain Dol(α1–2Man, six [3H]Man and eight [3H]GlcN-labeled glycolipids which are shown to be GPIs by different chemical and enzymatic cleavages. All glycolipids, except Dol(α1–2Man, are sensitive to GPI-PLD, PLAs, HNO₂, and NaOH. Only the glucosamine-labeled Pfgl α is sensitive to PI-PLC. All other glycolipids are insensitive to PI-PLC treatment suggesting that these glycolipids might have a modified inositol structure, which is described to hinder PI-PLC cleavage (Roberts et al., 1988). Additional evidence for a modified inositol structure is provided by the only partial sensitivity of the malarial GPIs toward PLAs which is similar to that of the *T. brucei* GPI-anchor precursor P3 (Menon et al., 1988, 1990). Different protein-bound GPIs (Roberts et al., 1988; Walter et al., 1990; Clayton and Mowatt, 1989) and GPI-lipids (Krakov et al., 1989; Mayor et al., 1990; Field et al., 1991) are described which have inositol structures modified by palmitic acid. In these cases, pretreatment with ammonia prior to PI-PLC treatment leads to partial susceptibility toward PI-PLC (Krakov et al., 1989; Mayor et al., 1990). Malarial glycolipids, however, could not be rendered PI-PLC-sensitive by pretreating with ammonia. Partition of the glucosamine-inositol-X fragment of Pfgl α into the organic phase and the different chromatographic behavior of the GPI-PLD-generated fragment of Pfgl β and the
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respective fragment of P3 indicate that all malarial GPIs, except Pfgl ε, carry a hydrophobic modification on the inositol ring different from palmitic acid which had been described for trypanosomal P3 (Menon et al., 1988).

We describe two malarial glycolipids with a single glucosamine as their hydrophilic head group (Pfgl ε and η). Both glycolipids differ in their chromatographic behavior and their sensitivity toward PI-PLC. Therefore, we postulate an additional hydrophobic modification at the inositol of Pfgl ε, η, and ι. This would be consistent with a biosynthetic pathway beginning with glucosamine-PI (Pfgl ε, η, and ι) followed by the addition of a hydrophobic modification (Pfgl ε, η, and ι) prior to the addition of the first mannose residue. A similar biosynthetic pathway has been described for mammalian GPIs (Urakaze et al., 1992; Hirose et al., 1992). At this stage, it is difficult to assess the exact location of this modification on the inositol ring, but it is assumed to be an ester-bound hydrophobic moiety because of its sensitivity to treatment with NaOH.

One glycolipid releases mannose upon mild acid hydrolysis specific for Dol-P-Man (McDowell and Schwarz, 1988). This glycolipid is presumably Dol-P-Man as judged by its chromatographic properties. It co-chromatographs with a mannolipid synthesized in the cell-free system (Gerold et al., 1992a) showing properties of Dol-P-Man (alkali stability, chromatographic behavior on DEAE-cellulose), the synthesis of which is inhibited in vitro by amphotericin. The donor of the mannose residues to the core glycan of T. brucei GPIs has been shown to be Dol-P-Man (Schwarz et al., 1989; Menon et al., 1990b). Therefore, by analogy, an isoprenoid-P-Man can be postulated to play a similar role for at least some of the mannose residues of the malarial GPIs. The lack of Dol-PP-(GlcNAc), Dol-PP-(GlcNAc)₆, and higher glycosylated intermediates is consistent with the findings that the asexual, intraerythrocytic stages of plasma gondii, and its biosynthetic intermediates. Labeling with [³H]ethanolamine and the analysis of the neutral glycans after HF dephosphorylation strongly suggest Pfgl ε, η, and ι are potential inducers of cytokine release. Together with findings that purified GPIs derived from protein hydrolysis of MSP1 and MSP2 induce cytokine release, transient pyrexia, hypoglycemia, and lethal cachexia in mice (Schofield and Hackett, 1993) and with other evidence (Kwiatkowski et al., 1990), these results point to an important role of this class of molecules in the pathology of malaria.

Elucidation of the structures and biosynthetic pathways of malarial GPIs may provide a basis for the development of a glycolipid-based "antisick" vaccine (Playfair et al., 1990; Schofield and Hackett, 1993).

Additional studies in progress using a cell-free system will provide more detailed information of the biosynthetic pathway of malarial GPIs and help to determine whether Pfgl ε is an additional possible GPI-anchor precursor. Also, the design and testing of inhibitors of GPI biosynthesis should be facilitated by studying some of the reactions involved in the cell-free system.

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