Developmental Stage-specific Biosynthesis of Glycosylphosphatidylinositol Anchors in Intraerythrocytic Plasmodium falciparum and Its Inhibition in a Novel Manner by Mannosamine*

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Glycosylphosphatidylinositol (GPI) anchors represent a distinct class of glycolipids found covalently attached to proteins in almost all eukaryotic cells (1, 2); they are particularly abundant in parasites, in which they occur as free lipids or linked to proteins (3, 4). Although the primary function of GPIs is to anchor proteins to cell surfaces, they have been implicated in a number of biological responses including transmembrane signaling, apical targeting of proteins in polarized cells, insulin mimetic activity, and endocytic mechanisms (1, 5, 6).

Plasmodium falciparum, the most virulent parasite among the four species of plasmodial apicomplexan protozoa that infect man, causes millions of deaths each year in tropical and subtropical countries (7). In the past decades, chloroquine and other drugs have been very useful in reducing mortality due to malaria. Although these conventional drugs are still widely used for the treatment of parasite infection, the parasite is rapidly becoming drug-resistant, raising the death toll from malaria (7). Therefore, malaria is once again a global concern, and novel antimalarial drugs are urgently needed.

In P. falciparum, GPIs are the major carbohydrate moieties, whereas N- and O-linked carbohydrates or other glycolipids are either absent or present only at very low levels (8, 9). More than 15 proteins of the intraerythrocytic stage P. falciparum including functionally important proteins such as merozoite surface protein-1, -2, and -4, a 71-kDa heat shock family protein, a 102-kDa transferrin receptor, and a 75-kDa serine protease are modified with GPI anchors (8, 10, 11). These observations suggest that GPI biosynthesis is vital to the parasite.

The P. falciparum GPIs contain a conserved core structure, ethanolamine-phosphate-Manα1–2Manα1–6Manα1–4GlcN, that is α-(1–6)-linked to the inositol residue of PI (8, 13, 14). The glycan core has an additional Man residue linked to the third Man at O-2. The parasite GPIs differ significantly from those of the host, particularly with respect to the absence of additional ethanolamine substituents on the glycan core, the presence of a fourth Man residue, the absence of an alkyl substituent at the sn-1 position, and the type of acyl substituents on the glycerol and inositol moieties (15).

During the 48-h intraerythrocytic life cycle of P. falciparum, the parasite undergoes several morphologically distinct developmental stages, rings, trophozoites, schizonts, and finally differentiates into merozoites, which upon release invade other red blood cells. In this study, we show that intraerythrocytic P. falciparum synthesizes GPI anchors exclusively at the trophozoite stage in a developmental stage-specific manner. We also investigated the effect of the inhibition of GPI anchor biosynthesis on the growth and development of the parasite using ManN, a known inhibitor of GPI biosynthesis in eukaryotic
cells (16–21). In eukaryotes, ManN can also inhibit the assembly of N-linked oligosaccharides (17), and it is a precursor for the synthesis of siatic acid (22). Because P. falciparum contains little or no N-glycosylation capacity, and the parasite does not synthesize siatic acid, the effect of ManN on the parasite is because of the inhibition of GPI biosynthesis. In the presence of ManN, the growth of the parasite was arrested specifically at the trophozoite stage, a developmental stage at which the parasite synthesizes all of its GPls; this resulted in the death of the parasite. Detailed study demonstrated that the lethal effect of ManN on the parasite is because of the inhibition of GPI biosynthesis, and that ManN interferes with the addition of the first mannose residue to the inositol-acylated GlcN-P1, a mechanism of inhibition that is different from that observed in other organisms.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 culture medium and HEPES were purchased from Life Technologies, Inc. Hypoxanthine, p-amino benzoic acid, saponin, and ManN were purchased from Sigma. Aspergillus saitoi α-mannosidase (400 units/mg) and jack bean α-mannosidase (30 units/mg) were from Oxford Glycosystems (Rosedale, NY). Gentamycin sulfate was from Biofluids, Inc. (Rockville, MD). [6-3H]GlcN (25 Ci/mmol), [35S]Met (1000 Ci/mmol), and 14C-labeled protein molecular weight markers were from Amersham Pharmacia Biotech. Silica gel 60 HPTLC plates were from either EM Science (Gibbstown, NJ) or Whatman, Inc. (Clifton, NJ). EnHance™ fluorography spray for TLC plates was from NEN Life Science Products. O-type human serum was from the Georgetown University Hospital. O-type human serum was from Interstate Blood Bank, Inc. (Memphis, TN).

Culturing of the Parasite—P. falciparum (FCR-3 strain) were cultured in RPMI 1640 medium supplemented with 22 mM HEPES, 29 mM NaHCO3, 0.05% hypoxanthine, p-amino benzoic acid (2 mg/liter), gentamycin sulfate (50 mg/liter), and 10% human serum at 3–4% hematocrit (8). Cultures were maintained in an atmosphere of 90% N2, 5% O2, and 5% CO2. The parasites were synchronized between water and water-saturated 1-butanol. The organic phase was removed, and the aqueous phase was washed extensively with an equal volume of water until the glycan cores of the glycans were free of 1-butanol. The aqueous phase was then washed twice with water and lyophilized. The glycans were then digested with 1-propanol/acetonewater (10:6:4, v/v/v), and the GPs were identified by comparison of the Rf values of the glycan cores with those of a standard 2,5-anhydromannitol to Man2,5-anhydromannitol ladder (8).

SDS-PAGE and Fluorography—The [3H]GlcN- or [35S]Met-labeled parasites were dissolved in nonreducing SDS-PAGE sample buffer, and the lysates were heated in a boiling water bath for 5 min and then electrophoresed under nonreducing conditions on 6–20% SDS-polyacrylamide gradient gels (28). The gels were fixed, washed with water, soaked in 1 M sodium salicylate in water for 30 min, dried, and exposed to x-ray films at ~80 °C.

RESULTS

Effect of ManN on the Survival of P. falciparum at Different Stages of the Intraerythrocytic Development—We investigated the developmental stage-specific biosynthesis of GPls in intraerythrocytic P. falciparum and the effect of inhibition of the GPl biosynthetic pathway on the survival of the parasite. Synchronous cultures of the parasites were treated with 1.25–10 mM ManN at the early ring stage (8 h after invasion of erythrocytes), and the growth of parasites was monitored through various stages of intraerythrocytic development. ManN inhibited parasite growth in a dose-dependent manner (Fig. 1 and Table I). Parasites treated with 10 mM ManN developed nor-

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**Fig. 1. Effect of ManN on the growth and development of P. falciparum.** Parasites, 8 h after erythrocyte invasion, were separately treated with 0.25–10 mM ManN in complete medium; untreated parasites were cultured in parallel as controls. Parasitemia was measured by counting the cells in Giemsa-stained thin smears using light microscopy. Shown is the percent decrease in parasitemia compared with untreated culture 50 h after ManN treatment (second cell cycle). NaBH4 in 100 mM NaOH at room temperature for 6 h. Excess NaBH4 was destroyed by acidification with 2 M H2SO4 to pH 5 in an ice bath, and the solutions were deionized with AG 50W-X16 (H+) and AG 4-X4 (base) resins, dried, and analyzed by HPLC.

Treatment with Mannosidase—The [3H]GlcN-labeled GPls (25,000–50,000 cpm) were treated with jack bean α-mannosidase (30 units/ml) in 40 μl of 100 mM NaOAc, 2 mM ZnCl2, pH 5.0, containing 0.1% sodium taurocholate at room temperature for 2 h and then at 37 °C for 22 h (27). The solutions were heated in a boiling water bath for 5 min, extracted with water-saturated 1-butanol, and analyzed by HPLC. For digestion of the GPl glycan cores (10,000 cpm) with jack bean α-mannosidase, the above buffer without the detergent was used; products were deionized with AG 50W-X16 (H+) and lyophilized. The glycan cores (5000 cpm) were also digested with A. saitoi α-mannosidase (0.5 milliunits/ml) in 100 mM NaOAc, pH 5.6, at 37 °C for 20 h (8), deionized, and lyophilized.

Thin Layer Chromatography—The [3H]GlcN-labeled GPls were applied onto HPTLC plates, developed with chloroform/methanol/water (10:1:2, v/v/v), and dried. The GPl bands were viewed by fluorography using EnHance™ (8). The glycan cores obtained by treatment of GPls with HF and nitrous acid/NaBH4 and their mannosidase digestion products were analyzed by HPTLC using 1-propanol/acetonewater (10:6:4, v/v/v). The GPs were identified by comparison of the Rf values of the glycan cores with those of a standard 2,5-anhydromannitol to Man2,5-anhydromannitol ladder (8).

Nitrous Acid Treatment—The [3H]GlcN-labeled GPls or their intermediates isolated by preparative TLC (20,000–100,000 cpm), in 75 μl of 0.2 M NaOAc, pH 3.75, 0.1% Nonidet P-40, were treated with 75 μl of 1 M NaNO2 (25, 26). After a 24-h incubation at room temperature, the released lipid moieties were extracted with water-saturated 1-butanol, dried, and analyzed by HPTLC.

Treatments with Aqueous HF—The [3H]GlcN-labeled GPls (30,000–50,000 cpm) were treated with 50% aqueous HF (50 μl) in an ice bath for 48 h (25, 26). The reaction mixture was neutralized with frozen saturated LiOH, extracted with water-saturated 1-butanol, dried, and analyzed by HPTLC.

Characterization of GPl Glycan Core—The [3H]GlcN-labeled GPls or GPl intermediates (50,000–100,000 cpm) were treated with HF and then with HNO2 (25, 26). The samples were reduced with 100 μl of 1 M NaBH4 in 100 mM NaOH at room temperature for 6 h. Excess NaBH4 was destroyed by acidification with 2 M H2SO4 to pH 5 in an ice bath, and the solutions were deionized with AG 50W-X16 (H+) and AG 4-X4 (base) resins, dried, and analyzed by HPTLC.
Parasites were assessed by examining Giemsa-stained thin smear of cultures under light microscope. When treated with 10 mM ManN for 24 h, the growth was then grew at the same efficiency as that of the control culture.

In parasitemia in the second cell cycle; the surviving parasites period to the trophozoite stage, caused about a 60% reduction in merozoites formed during 5 mM ManN treatment invaded red blood cells with significantly lower efficiency compared with the untreated parasites. Continued treatment with 5 mM ManN through the second cell cycle completely arrested parasite growth at the trophozoite stage and all parasites died. The parasites treated with 1.25 mM or 2.5 mM ManN grew similar to that of the untreated parasites. However, in the continued presence of 10 mM ManN, the growth of the parasites was not affected. The parasites treated with 10 mM ManN remained unhealthy mid-trophozoites or dead parasites.

Analysis of GPI Biosynthesis at Different Developmental Stages of Intraerythrocytic P. falciparum—The level of GPI biosynthesis at various stages of intraerythrocytic P. falciparum was determined by measuring the incorporation of $^{3}$H[GlcN into GPIs. Synchronous cultures of the parasites were treated with $^{3}$H[GlcN 10 h after invasion, and then at 6 h intervals, the parasites were harvested, and the GPIs formed were analyzed. The parasites did not synthesize GPIs during the ring stage (until 16 h after invasion); low levels of GPIs were synthesized by early trophozoites (16–28 h after invasion) (Fig. 2). However, the parasites synthesized almost all of their GPIs during development from early trophozoites to late trophozoites (28–40 h of the parasite erythrocytic life cycle). Little or no GPI synthesis occurred during the differentiation of trophozoites to schizonts (40–48 h of the cell cycle). These results, taken together with mannose inhibition data demonstrate that GPIs are critically required for the development of trophozoites to schizonts.

The $^{3}$H[GlcN-labeled GPIs and their intermediates were identified by their susceptibility to nitrous acid and HF and by analysis of their glycan cores before and after digestion with α-mannosidase. The results are summarized in Table II. All the major $^{3}$H[GlcN-labeled bands on HPTLC corresponded to GPIs and their intermediates; glycolipids other than GPI were not present at significant amounts.

In P. falciparum, EtN-P-Man$_{4}$GlcN-PI is the major GPI, and it is exclusively found in GPI-anchored parasite proteins (8, 14). Among various GPI intermediates formed during biosynthesis, the formation of Man$_{5}$-GlcN-PI that lacks the ethanolamine phosphate substituent (see also Ref. 13), in addition to EtN-P-Man$_{4}$GlcN-PI, the conserved GPI structure in all eukaryotes, is noteworthy. The presence of both these GPI species in significant amounts suggests that the formation of the matured GPI, EtN-P-Man$_{5}$-GlcN-PI, occurs through two different pathways: (i) by transfer of a mannose residue to EtN-P-Man$_{4}$-GlcN-PI, and (ii) by the addition of mannose to Man$_{5}$-GlcN-PI and then substitution with EtN-P. As far as we know, the latter reaction sequence is novel. The operation of this pathway is evident by nearly complete conversion of the $^{3}$H[GlcN-labeled intermediate to the matured GPI when $^{3}$H[GlcN-containing medium was replaced with regular medium at the midtrophozoite stage and the parasites were grown to schizonts.$^3$

To determine the time required for the incorporation of ex-

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3 R. S. Naik and D. C. Gowda, unpublished data.
with jack bean α-mannosidase treatment, by comparison of the constituent on the inositol residue.

Addition of \([3H]\text{GlcN}\) to the culture medium, with maximum incorporation reached at 4 h (Fig. 3). Therefore, the observed absence of GPI synthesis during ring stage development is not because of any delay in the uptake or utilization of \([3H]\text{GlcN}\) by the parasite.

**Effect of ManN on P. falciparum GPI Biosynthesis**—Because, in intracellularly cultured parasites, the GPIs are synthesized only at the trophozoite stage, the parasites were treated with varying concentrations of ManN at the early trophozoite stage (28 h after invasion), and 2 h later, they were labeled with \([3H]\text{GlcN}\) for 6 h in the continued presence of ManN. ManN inhibited GPI synthesis in a dose-dependent manner (Fig. 4). The parasites treated with 1.25, 2.5, 5, and 10 mM ManN synthesized -30, 18, 12, and 6% of GPIs, respectively, compared with the amount of GPIs synthesized by the untreated parasites. This dose-dependent inhibition of parasite GPI biosynthesis by ManN parallels the concentration-dependent inhibition of parasite growth by ManN. Whereas significant proportions of both the GlcN-PI and inositol-acylated GlcN-PI intermediates were observed in untreated parasites, inositol-acylated GlcN-PI was the major intermediate in ManN-treated parasites (Fig. 4). However, whereas untreated parasites contain a small amount of Man1-GLcN-PI, this GPI intermediate is below the detectable level in the ManN-treated parasites (compare Fig. 2C with Fig. 4). Furthermore, both untreated and ManN-treated parasites contain small but similar proportions of Man1-GlcN-PI. Together these results suggest that although inositol-acylated GlcN-PI formed in a significant amount, this intermediate is not utilized rapidly for mannosylation. Thus, ManN inhibits the addition of the first mannose residue to the inositol-acylated GlcN-PI intermediate. This is in contrast to the mechanism of inhibition of GPI biosynthesis by ManN in vivo.

**TABLE II**

The structures of \([3H]\text{GlcN}\)-labeled GPIs and their intermediates synthesized by *P. falciparum*

| GPI species               | Structure                      | \(R_2\) values | Glycan core identified |
|--------------------------|--------------------------------|----------------|-----------------------|
| EM,GnPl                  | EtN-P-Man1,GlcN-PI              | 0.19           | Man,GlcN              |
| EM,GnPI                  | EtN-P-Man1,GlcN-PI              | 0.29           | Man,GlcN              |
| M1GnPI                   | Man1,GlcN-PI                   | 0.61           | Man,GlcN              |
| M1GnPI                   | Man1,GlcN-PI                   | 0.74           | Man,GlcN              |
| M1GnPI                   | Man1,GlcN-PI                   | 0.84           | Man,GlcN              |
| GnPl*                    | GlcN-PI*                       | 0.87           | GlcN                  |
| GnAcPI*                  | GlcNAc-PI*                     | 0.88           | GlcNAc                |
| M1GnPI                   | Man1,GlcN-PI                   | 0.88           | Man,GlcN              |
| GnPl                     | GlcN-PI                        | 0.90           | GlcN                  |

*GPI species without an acyl substituent on the inositol residue.

Glycosylphosphatidylinositol Anchors of *P. falciparum*
other organisms, where either the accumulation of Man$_2$-GlcN-PI or the formation of Man$_N$-Man-GlcN-PI was observed (5, 17–21).

The matured GPIs synthesized by parasites treated with ManN appear to be utilized efficiently for theanchoring of proteins. SDS-PAGE fluorography of the parasite lysates showed significant amounts of GPI-modified proteins in parasites treated with 1.25 or 2.5 mM ManN (Fig. 5); this agrees with the growth of the treated parasites through several cycles. The parasites treated with 5 mM ManN contained low levels of GPI-anchored proteins, and those treated with 10 mM ManN almost completely lacked GPI-anchored proteins. These results suggest the essential role of GPI-anchored proteins in parasite survival.

**Effect of ManN on P. falciparum Protein Synthesis**—To examine whether ManN treatment also affected protein synthesis, the parasites were labeled with $[^{35}S]$Met, and the cell lysates were analyzed by SDS-PAGE fluorography. The results show that the parasites treated with 1.25–10 mM ManN for 5–6 h all synthesized similar levels of proteins compared with the untreated parasites (not shown). Protein synthesis, however, was significantly reduced when parasites were treated with 10 mM ManN during the entire trophozoite stage because of slow growth or growth arrest (not shown).

**DISCUSSION**

Four important findings emerge from this study: 1) intraerythrocytic *P. falciparum* synthesizes GPI anchors in a developmental stage-specific manner exclusively at the trophozoite stage; 2) GPI biosynthesis is critical to the differentiation of trophozoites into schizonts, and thus the inhibition of GPI biosynthesis causes parasite growth arrest at the trophozoite stage; 3) in *P. falciparum*, ManN inhibits GPI biosynthesis by interfering with the attachment of first Man to the inositol-acylated GlcN-PI intermediate, a mechanism that is distinctively different from those in other organisms, including man; and 4) The matured GPI, EtN-P-Man$_4$-GlcN-PI, is formed by two different reaction sequences: transfer of a mannose residue to EtN-P-Man$_4$-GlcN-PI and to Man$_N$-GlcN-PI; Man$_N$-GlcN-PI is then substituted with ethanalamine phosphate.

This study shows that ManN inhibits the growth of *P. falciparum* specifically at the trophozoite stage by inhibiting GPI biosynthesis. 1) The parasite synthesizes almost all of its GPI pool at the trophozoite stage. 2) ManN treatment does not affect the growth of the parasite at either the ring or the schizont stage. Rings treated with even 10 mM ManN developed into early trophozoites, and late trophozoites became schizonts and functional merozoites even in the continued presence of 10 mM ManN. 3) When ManN treatment was withdrawn at the late ring stage, the parasites developed normally to schizonts despite having been treated during most of the ring stage. However, continued treatment with 5 or 10 mM ManN either significantly reduced the growth rate or caused complete growth arrest and death of parasites at the midtrophozoite stage. 4) ManN inhibits GPI biosynthesis and the GPI-anchor modification of proteins in a dose-dependent manner. 5) ManN does not significantly affect protein synthesis in the parasite. In several eukaryotic systems, ManN has been shown to be a specific inhibitor of GPI and N-linked oligosaccharide biosynthesis (14). Because intraerythrocytic *P. falciparum* has little or no N-glycosylation capacity and the parasite treated with tunicamycin (an inhibitor of N-glycosylation) during the trophozoite stage, synthesized normal levels of GPIs, and developed into schizonts, the inhibition of ManN is not because of the inhibition of N-glycosylation of parasite proteins. These observations conclusively establish that the effect of ManN on the growth of trophozoite stage *P. falciparum* is specifically because of the inhibition of GPI biosynthesis.
The data presented here demonstrate that the biosynthesis of GPIs in *P. falciparum* occurs exclusively at the trophozoite stage of the parasite. Metabolic labeling of the parasites with $[^3]$HGlCn at various stages of intraerythrocytic development and analysis of the labeled GPIs indicated that the GPIs are not synthesized during the ring stage. As shown in Fig. 2, all of the GPIs were synthesized during the trophozoite stage (between 28 and 38 h after erythrocytic invasion), and little or no GPIs were synthesized during the schizont stage. The absence of GPI biosynthesis during the ring stage, however, is not because of a delay in the passage of $[^3]$HGlCn through erythrocyte membranes and its entry into the parasites, because trophozoites synthesize significant amounts of GPIs within 30 min after the addition of $[^3]$HGlCn to the culture medium.

Although GPI structures of all eukaryotes contain EtN-P-Man$_3$-GlcN-PI, a conserved core structure, the sugar backbone is variously modified with additional sugars and oligosaccharide moieties, which are usually attached to the third and/or first mannose residues (12, 15). Among known substrates, the presence of a fourth mannose residue appears to be the common feature of many eukaryotic GPIs (15). The biosynthetic pathway by which the fourth mannose is added is not clear. It is believed that the formation of EtN-P-Man$_3$-GlcN-PI occurs through the transfer of a mannose residue to EtN-P-Man$_3$-GlcN-PI. However, the results of this study suggest that, in *P. falciparum*, EtN-P-Man$_3$-GlcN-PI is formed via two distinct reaction sequences: 1) by transfer of a mannose residue to EtN-P-Man$_3$-GlcN-PI, and 2) by the addition of mannose to Man$_3$-GlcN-PI followed by the transfer of an ethanolamine phosphate moiety. If the latter sequence is unique to *P. falciparum* GPIs, then understanding of the relative regulation of the two reaction sequences may provide a parasite-specific target for the development of antimalarial agents.

The inhibition of parasite growth at the trophozoite stage is not because of nonspecific ManN toxicity. Parasites treated with 10 mM ManN after the midtrophozoite stage develop normally to schizonts, and the formed merozoites successfully invaded erythrocytes. Withdrawal of ManN from the parasite cultures treated up to late ring stage also allowed normal parasite development and protein synthesis. The data establish that the deleterious effect of ManN on the trophozoite stage of *P. falciparum* is specifically because of the inhibition of GPI biosynthesis and agrees with the synthesis of GPIs occurring exclusively during trophozoite development.

The effect of ManN specifically at the trophozoite stage of *P. falciparum*, i.e. the failure of the parasite to develop into schizonts with concomitant inhibition of GPI biosynthesis, strongly suggests that the GPIs are essential for the differentiation of trophozoites into schizonts. Complex carbohydrates in the forms of glycoproteins and/or glycolipids are present in almost all eukaryotic organisms where they have critical roles in development and differentiation. In animals, GPIs have been shown to be vital for normal development (11), although they are present at markedly lower levels compared with those in parasites.

Analysis of the GPI intermediates formed in *P. falciparum* treated with ManN suggested that ManN affects the parasite biosynthetic pathway by a mechanism distinctly different from those in other organisms. In several eukaryotic systems including trypansomes, *Leishmania*, Madin-Darby canine kidney cells, and HeLa cells, ManN has also been shown to inhibit GPI anchor biosynthesis without affecting protein synthesis. In HeLa cells, ManN was shown to inhibit the α-(1→2)-mannosyltransferase responsible for the transfer of the third Man residue, causing the accumulation of the Mana1–6Mana1–4GlcN-PI intermediate (17–19). In Madin-Darby canine kidney cells, *Trypanosoma brucei*, and *Leishmania mexicana*, the inhibition of mature GPI formation was shown to be because of the incorporation of ManN into Man-GlcN-PI. This results in the formation of an aberrant GPI intermediate, ManN-Man-GlcN-PI, which prevents further addition of Man residues because of the lack of a hydroxyl group on C2 (5, 20, 21). In *P. falciparum*, however, ManN inhibits GPI biosynthesis by inhibiting the addition of the first mannose to the inositol-acylated GlcN-PI, suggesting significant differences in the specificity of the parasite and mammalian enzymes.

In conclusion, the data presented here show that intraerythrocytic *P. falciparum* synthesizes GPIs in a developmental stage-specific manner, exclusively at the trophozoite stage. The inhibition of *P. falciparum* GPI biosynthesis by mannosamine prevented the differentiation of trophozoites to schizonts. Mannosamine inhibits the GPI biosynthesis by a novel mechanism, which suggests that the enzymes of the parasite GPI biosynthetic pathway can be exploited as parasite-specific targets for the development of antimalarial drugs.

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