Glycosylphosphatidylinositol (GPI) anchors are crucial for the survival of the intraerythrocytic stage *Plasmodium falciparum* because of their role in membrane anchoring of merozoite surface proteins involved in parasite invasion of erythrocytes. Recently, we showed that mannosamine can prevent the growth of *P. falciparum* by inhibiting the GPI biosynthesis. Here, we investigated the effect of isomeric amino sugars glucosamine, galactosamine, and their N-acetyl derivatives on parasite growth and GPI biosynthesis. Glucosamine, but not galactosamine, N-acetylglucosamine, and N-acetylgalactosamine inhibited the growth of the parasite in a dose-dependent manner. Glucosamine specifically arrested the maturation of trophozoites, a stage at which the parasite synthesizes all of its GPI anchor pool and had no effect during the parasite growth from rings to early trophozoites and from late trophozoites to schizonts and merozoites. An analysis of GPI intermediates formed when parasites incubated with glucosamine indicated that the sugar interferes with the inositol acylation of glucosamine-phosphatidylinositol (GlcN-PI) to form GlcN-(acyl)PI. Consistent with the non-inhibitory effect on parasite growth, galactosamine, N-acetylglucosamine, and N-acetylgalactosamine had no significant effect on the parasite GPI biosynthesis. The results indicate that the enzyme that transfers the fatty acyl moiety to inositol residue of GlcN-PI discriminates the configuration at C-4 of hexosamines. An analysis of GPIs formed in a cell-free system in the presence and absence of glucosamine suggests that the effect of the sugar is because of direct inhibition of the enzyme activity and not gene repression. Because the fatty acid acylation of inositol is an obligatory step for the addition of the first mannosyl residue on parasite GPI biosynthesis, our results offer a strategy for the development of novel anti-malarial drugs. Furthermore, this is the first study to report the specific inhibition of GPI inositol acylation by glucosamine in eukaryotes.

Malaria is a major health problem in many countries of the world. Nearly 40% of the global population is vulnerable to this disease. In Africa alone, malaria causes millions of deaths each year, mostly children (1, 2). In recent years, the death toll due to malaria has been rapidly increasing because of the widespread resistance of parasites to chloroquine and other commonly used antimalarial drugs. Therefore, there is an urgent need for the development of novel drugs. Identification of new drugs targeting the metabolic pathways of *Plasmodium falciparum*, the parasite that causes severe malaria, has been the subject of study in several laboratories. The recently studied parasite choline transporter, type II fatty acid biosynthesis, dihydrofolate reductase-thymidylate synthase, and glycosylphosphatidylinositol (GPI) anchor biosynthesis are some potential targets for drug design (3–7).

GPIs are a class of glycolipids involved in anchoring certain functionally important proteins to the outer surfaces of cell membranes (8). GPIs consist of a conserved trimannosylglycosaminyl core linked to the inositol residue of a phosphatidylinositol (PI). GPIs from different species or cell types vary widely in the type of fatty acyl/alkyl residues and with respect to additional sugar residues and/or ethanolamine phosphate attached to the glycan core (8). These variations confer broad structural diversity to GPIs, resulting in a wide range of biological activity (8, 9).

In the case of intraerythrocytic stage *P. falciparum*, several proteins are anchored to the outer leaflet of the plasma membrane through GPIs (10–13). These proteins include functionally important molecules such as merozoite surface protein-1 (MSP-1), MSP-2, MSP-4, a 71-kDa protein of the heat shock family, the 102-kDa transferrin receptor, and a 75-kDa serine protease. Of these, MSP-1 is crucial for recognition, binding, and invasion of erythrocytes (10–13). Furthermore, like many other parasites, *P. falciparum* synthesizes GPIs in severalfold excess than that required for protein anchoring. Accumulated evidence suggests that parasites use free GPIs for the regulation (stimulation/suppression) of the host immune system to assure their survival in the harsh environments of the host (9). Therefore, GPIs are crucial for the survival of *P. falciparum* in the host.

The GPIs of intraerythrocytic *P. falciparum* consist of EtN-P(6-Man)1–2-Man1–2-Man1–6-Man1–4-GlcN linked to the inositol residue of PI. The PI moiety is heterogeneous with regard to fatty acyl substituents at the sn-1 and sn-2 positions and at C-2 of the inositol residue (14, 15). *P. falciparum* syn-

**Glucosamine Inhibits Inositol Acylation of the Glycosylphosphatidylinositol Anchors in Intraerythrocytic Plasmodium falciparum**

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\* The abbreviations used are: GPI, glycosylphosphatidylinositol; MSP, merozoite surface protein; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone; GlcN, glucosamine; N-acetylgalactosamine; PI, phosphatidylinositol; EtN, ethanolamine; HPTLC, high performance thin-layer chromatography; MDCK, Madin-Darby canine kidney; Bicine, N,N-bis(2-hydroxyethyl)glycine.
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thizes GPIs exclusively during the trophozoite stage in a developmental stage-specific manner. We have previously shown that mannose inhibits the growth of *P. falciparum* specifically at the trophozoite stage and thereby causes the death of the parasite (6). In *P. falciparum*, mannose pre- vents the addition of the first mannose to the inositol-acylated GlcN-PI intermediate. The mechanism by which mannose inhibits GPI biosynthesis in *P. falciparum* is different from that observed in other organisms (16–19). In this study, we investigated the effect of two isomeric amino sugars, glucosa- mine and galactosamine, and their N-acetyl derivatives on the parasite growth and GPI biosynthesis. The data presented here show that of these amino sugars, only glucosamine inhibits the growth of *P. falciparum* and parasite GPI biosynthesis. The sugar inhibits fatty acid acylation of the inositol residue of GlcN-PI, a novel inhibition mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—RPMI 1640 culture medium and HEPES were purchased from In Vitrogen. TLCK, leupeptin, hypoxanthine, p-aminobenzoic acid, saponin, galactosamine, glucose, N-acetylglucoamine, N-acetyl galactosamine, and jack bean α-mannosidase (30 units/mg) were pur chased from Sigma. Gentamycin sulfate was from Biofluids (Rockville, MD). O-type human blood was obtained from the Georgetown University Hospital. O-type human serum was from Interstate Blood Bank. O-type human serum was from Interstate Blood Bank.

**Culturing of Parasite—*P. falciparum***

Synchronous cultures of the parasites (1 ml) with 22% parasitemia were harvested at mid-trophozoite stage. Parasites were released through various stages of development. Glucosamine inhibited the growth of parasites treated with 1.25 mM glucosamine also grew normally in the case of 5 mM glucosamine, the rate of parasite growth was significantly reduced during the first cell cycle itself. Approximate 75% of the parasites that entered into the third cell cycle was reduced by 20% compared with that of the untreated culture. The parasite treated with 2.5 mM glucosamine also grew normally in the first cycle, but the growth was significantly reduced during the second cell cycle.

**Glycosylphosphatidylinositol Anchors of P. falciparum**—The synchronous cultures of the parasites at the ring stage 8 h after the invasion of erythrocytes were treated with various concentrations of the test sugars, and the growth of the parasites was monitored through various stages of development. Glucosamine inhibited the parasite growth in a dose-dependent manner (Fig. 1 and Table I). The growth of parasites treated with 1.25 mM glucosamine was similar to control cultures during the first cell cycle. However, the efficiency of erythrocyte invasion of merozoites that entered into the third cell cycle was reduced by 20% compared with that of the untreated culture. The parasites treated with 2.5 mM glucosamine also grew normally in the first cycle, but the growth was significantly reduced during the second cell cycle. The efficiency of erythrocyte invasion of merozoites that entered into the third cell cycle was reduced by 20% compared with that of the untreated culture. The parasites treated with 2.5 mM glucosamine also grew normally in the first cycle, but the growth was significantly reduced during the second cell cycle. The efficiency of erythrocyte invasion of merozoites that entered into the third cell cycle was reduced by 20% compared with that of the untreated culture. The parasites treated with 2.5 mM glucosamine also grew normally in the first cycle, but the growth was significantly reduced during the second cell cycle.

**Isolation of GPIs—** GPIs from parasites were extracted four times with 5 volumes of CHCl3/CH3OH/water (10:10:3, v/v/v). The extract was dried by SpeedVac and then partitioned between water and water-saturated 1-butanol, dried, and analyzed by HPTLC. The glycan moieties were recovered by chromatography on Bio-Gel P-4 (1 × 90 cm) in 100 mM pyridine, and 100 mM HAc, pH 5.2.

**Treatment of GPIs with Aqueous Hydrofluoric Acid—** The GPIs (100,000–200,000 cpm) in 75 μl of 0.2% NaOAc, pH 3.75, 0.1% Nonidet P-40 were treated with 75 μl of 1 M NaNO3 (22, 23). After a 24-h incubation at room temperature, the released lipid moieties were extracted with water-saturated 1-butanol, dried, and analyzed by HPTLC. The glycan moieties were recovered by chromatography on Bio-Gel P-4 (1 × 90 cm) in 100 mM pyridine, and 100 mM HAc, pH 5.2.

**Treatment of GPIs with Aqueous Hydrofluoric Acid—** The GPIs (100,000–200,000 cpm) were treated with 50% aqueous hydrofluoric acid (50 μl) in an ice bath for 48 h (22, 23). The reaction mixture was neutralized with a mixture of formic acid and water-saturated 1-butanol, dried, and analyzed by HPTLC.

**Alkaline Hydrolysis of GPIs—** The GPIs in 100 μl of the methanol-ammonia mixture (methanol, 30% ammonia, 1:1 (v/v), freshly prepared) were incubated at 37 °C for 4 h. At the end of the incubation, the reaction mixture was evaporated to dryness and extracted with water-saturated 1-butanol, dried, and analyzed by HPTLC.

**SDS-PAGE and Fluorography—** The [35S]methionine-labeled parasites were dissolved in 106 μl of SDS-PAGE sample, the lysates were heated in a boiling water bath for 5 min, and then they were electrophoresed under non-reducing conditions with 6–20% SDS-polyacrylamide gradient gels (25). The gels were fixed, washed with water, soaked in 1 M sodium salicylate solution for 30 min, dried, and exposed to x-ray films at −80 °C (10).

**RESULTS**

**Effect of Hexosamines and N-Acetylhexosamines on the Survival of Intracellular *P. falciparum***—The synchronous cultures of the parasites at the ring stage 8 h after the invasion of erythrocytes were treated with various concentrations of the test sugars, and the growth of the parasites was monitored through various stages of development. Glucosamine inhibited the parasite growth in a dose-dependent manner (Fig. 1 and Table I). The growth of parasites treated with 1.25 mM glucosamine was similar to control cultures during the first cell cycle. However, the efficiency of erythrocyte invasion of merozoites that entered into the third cell cycle was reduced by 20% compared with that of the untreated culture. The parasites treated with 2.5 mM glucosamine also grew normally in the first cycle, but the growth was significantly reduced during the second cell cycle. The efficiency of erythrocyte invasion of merozoites that entered into the third cell cycle was reduced by 20% compared with that of the untreated culture. The parasites treated with 2.5 mM glucosamine also grew normally in the first cycle, but the growth was significantly reduced during the second cell cycle.

**Effect of Hexosamines and N-Acetylhexosamines on the Survival of Intracellular *P. falciparum***—The synchronous cultures of the parasites at the ring stage 8 h after the invasion of erythrocytes were treated with various concentrations of the test sugars, and the growth of the parasites was monitored through various stages of development. Glucosamine inhibited the parasite growth in a dose-dependent manner (Fig. 1 and Table I). The growth of parasites treated with 1.25 mM glucosamine was similar to control cultures during the first cell cycle. However, the efficiency of erythrocyte invasion of merozoites that entered into the third cell cycle was reduced by 20% compared with that of the untreated culture. The parasites treated with 2.5 mM glucosamine also grew normally in the first cycle, but the growth was significantly reduced during the second cell cycle. The efficiency of erythrocyte invasion of merozoites that entered into the third cell cycle was reduced by 20% compared with that of the untreated culture. The parasites treated with 2.5 mM glucosamine also grew normally in the first cycle, but the growth was significantly reduced during the second cell cycle. The efficiency of erythrocyte invasion of merozoites that entered into the third cell cycle was reduced by 20% compared with that of the untreated culture.
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**Fig. 1. Effect of hexosamines and N-acetyhexosamines on the growth and development of *P. falciparum*.** Equal aliquots of synchronous cultures of the parasites (4% parasitemia) 8 h after erythrocyte invasion were separated treated with 1.25–10 mM of the indicated sugars in complete medium. An aliquot of untreated parasites was cultured in parallel as controls. Parasitemia was measured by counting the cells in Giemsa-stained thin smears using light microscope. The percent inhibition in parasitemia in cultures treated with various amino sugars with respect to the level of parasitemia in the control culture was plotted against the concentrations of amino sugars in the culture medium. A, percent decrease in parasitemia in cultures after 48-h (8 h after parasites enter into the second cell cycle) treatment with the indicated amounts of sugars. White, gray, striped, and black bars indicate the cultures with 1.25, 2.5, 5, and 10 mM sugars, respectively. B, percent decrease in parasitemia in cultures treated with the 5 mM sugars for various time periods. White, gray, striped, and black bars indicate the cultures treated for 24, 48, 72, and 96 h, respectively.

Parasites treated with 5 mM glucosamine completely arrested parasite growth in the second life cycle, and all parasites died. Parasites treated with 10 mM glucosamine failed to develop into trophozoites, and subsequently all parasites died.

To determine whether the observed effect of glucosamine on parasite growth is the result of the inhibition of specific metabolic pathway or nonspecific cell toxicity, parasites were treated at the ring stage (6 h post-erythrocyte invasion) with 5 and 10 mM glucosamine. At various time intervals, glucosamine was withdrawn and the growth of the parasites was monitored. In this case, treatment with 5 or 10 mM glucosamine during ring stage for 8–10 h had no effect on the growth. At both concentrations, the parasites developed into trophozoites and schizonts and the released merozoites invaded the erythrocytes with the same efficiency as that of the untreated control culture. However, treatment for 24 h (both the ring and trophozoite stages) with 5 mM glucosamine caused a 40–50% reduction in parasitemia in the second cell cycle. The surviving parasites developed normally during the second cell cycle.

In contrast to glucosamine, galactosamine had no effect on the growth and development of the parasites. The growth of the parasites treated with 2.5 or 5 mM galactosamine was similar to that of the untreated culture (Fig. 1 and Table I). The parasite treated with 10 mM galactosamine also developed into late trophozoites and schizonts, but the erythrocyte invasion of the merozoites formed was 73% compared with that of the control culture. Of the parasites that entered into the second cycle in 10 mM galactosamine-treated culture, ~73% developed into trophozoites during the second cell cycle and the erythrocyte invasion efficiency of the merozoites to enter into the third life cycle was markedly reduced. The inhibition of parasite on prolonged treatment with 10 mM galactosamine is probably because of the metabolic conversion of galactosamine into glucosamine 6-phosphate (26), which could inhibit parasite growth in a manner similar to that by glucosamine.

*N-Acetylgalactosamine and N-acetylgalactosamine treatment was significantly less inhibitory on the growth and development of *P. falciparum* compared with the levels of inhibition by glucosamine and galactosamine, respectively. At 2.5 mM concentrations, both N-acetylgalactosamine and N-acetylgalactosamine showed no noticeable inhibitory effect on the parasite growth during the three cell cycles studied. Even at a 5 mM concentration, N-acetylgalactosamine and N-acetylgalactosamine caused no effect on the parasite growth during the first cell cycle. In both cases, the merozoites formed during the first cell cycle invaded the erythrocytes with same efficiency as that of untreated cultures, and the parasite developed to the trophozoite stage normally in the second cell cycle (Table I). However, merozoites formed from cultures treated with 5 mM N-acetylgalactosamine invaded erythrocytes with only ~66% efficiency compared with the control culture to enter the third cell cycle; however, 5 mM N-acetylgalactosamine had no significant effect. At 10 mM, N-acetylgalactosamine markedly inhibited parasite growth and development; the erythrocyte invasion efficiency was ~80% and ~33% for parasite entering the second and third cell cycles, respectively (Table I). In the case of N-acetylgalactosamine, 10 mM had no effect on the parasite in the first cell cycle. The erythrocyte invasion efficiency of merozoites entering second life cycle was comparable to the control culture. However, approximately ~87% of parasites developed into matured schizonts in the second cell cycle, and the merozoites formed successfully invaded the erythrocytes to enter into the third cell cycle.

**Effect of Hexosamines and N-Acetyhexosamines on *P. falciparum* GPI Biosynthesis**—We have previously shown that the intraerythrocitic *P. falciparum* synthesizes all of its GPI pool in the trophozoite stage (6). Therefore, to investigate the effect of amino sugars on GPI biosynthesis, parasites were metabolically labeled with [3H]mannose, [3H]glucosamine, and [3H]inositol in the presence of glucosamine, galactosamine, N-acetylgalactosamine, and N-acetylgalactosamine at the trophozoite stage. Parasites were treated with inhibitory sugars 2 h prior to the addition of radioactive sugars to the culture medium and then labeled for 6 h (12 h in the case of [3H]inositol) in the presence of inhibitors. The GPIs synthesized by the treated and untreated parasites were isolated and analyzed by HPTLC (Figs. 2 and 3). The GPIs and biosynthetic intermediates formed were characterized by specific degradation by HNO2 and hydrofluoric acid, susceptibility to jack bean α-mannosidase, and identification of products by HPTLC (Fig. 3) (data not shown) (see also Refs. 6 and 10). In both labeling procedures tested, glucosamine inhibited the GPI biosynthesis in a dose-dependent manner. The parasites treated with 1.25, 2.5, and 10 mM glucosamine had synthesized 95, 69, 36, and 13% GPIs, respectively, compared with those synthesized by the untreated parasites (Fig. 2 and Table II). At high concentrations, N-acetylgalactosamine also inhibited GPI synthesis. Thus, the parasites treated with 5 and 10 mM N-acetylgalactosamine synthesized 79 and 60% GPIs, respectively (Table II). This finding suggests that the inhibition of the growth and development of *P. falciparum* by N-acetylgalactosamine noted above (Table I) is because of the interference of the sugar in the biosynthesis of GPIs. In contrast, the levels of the mature GPIs and intermediates synthesized by parasites treated with 5 or
Equal aliquots of synchronous cultures of the parasites with 4% parasitemia were treated with the indicated sugars at various concentrations. At the indicated time, the levels of parasitemia and developmental stages were assessed by light microscopic examination of the Giemsa-stained thin smears.

| Sugar treated | Parasite developmental stage and percent parasitemia |
|---------------|------------------------------------------------------|
|               | 32 h⁺ | 56 h⁺ | 80 h⁺ | 104 h⁺ | 128 h⁺ |
| Untreated     | Late trop (4%) | Rings (15%) | Late trop (15%) | Rings (4 x 15%) | Late trop (4 x 15%) |
| GlnN          | Late trop (4%) | Rings (15%) | Late trop (14%) | Rings (4 x 12%) | Late trop (4 x 12%) |
| 1.25 mM       | Late trop (4%) | Rings (15%) | Late trop (12%) | Rings (4 x 9%) | Trops (4 x 7%) |
| 2.5 mM        | Late trop (3%) | Rings (8%) | Midtrops (7%) | Unhealthy trop (6%) | Dead parasites |
| 5 mM          | Late trop (2%) | Rings (11%) | Late trop (8%) | Rings (12%) | Late trop (7%) |
| 10 mM         | Midtrops (1%) | Schizonts (1%) | — | — | — |
| GalN          | Late trop (4%) | Rings (15%) | Late trop (15%) | Rings (4 x 15%) | Late trop (4 x 15%) |
| 1.25 mM       | Late trop (4%) | Rings (15%) | Late trop (15%) | Rings (4 x 15%) | Late trop (4 x 15%) |
| 2.5 mM        | Late trop (4%) | Rings (15%) | Late trop (15%) | Rings (4 x 15%) | Late trop (4 x 15%) |
| 5 mM          | Late trop (4%) | Rings (15%) | Late trop (15%) | Rings (4 x 15%) | Late trop (4 x 15%) |
| 10 mM         | Late trop (2%) | Rings (11%) | Late trop (8%) | Rings (12%) | Late trop (7%) |
| 15%          | Midtrops (1%) | Schizonts (2%) | — | — | — |
| GlcNAc        | Late trop (4%) | Rings (15%) | Late trop (15%) | Rings (4 x 15%) | Late trop (4 x 15%) |
| 2.5 mM        | Late trop (4%) | Rings (15%) | Late trop (15%) | Rings (4 x 15%) | Late trop (4 x 15%) |
| 5 mM          | Late trop (4%) | Rings (15%) | Late trop (15%) | Rings (4 x 15%) | Late trop (4 x 15%) |
| 10 mM         | Late trop (2%) | Rings (11%) | Late trop (8%) | Rings (12%) | Late trop (7%) |
| 15%          | Midtrops (1%) | Schizonts (2%) | — | — | — |
| GalNAc        | Late trop (4%) | Rings (15%) | Late trop (15%) | Rings (4 x 15%) | Late trop (4 x 15%) |
| 2.5 mM        | Late trop (4%) | Rings (15%) | Late trop (15%) | Rings (4 x 15%) | Late trop (4 x 15%) |
| 5 mM          | Late trop (4%) | Rings (15%) | Late trop (15%) | Rings (4 x 15%) | Late trop (4 x 15%) |
| 10 mM         | Late trop (2%) | Rings (11%) | Late trop (8%) | Rings (12%) | Late trop (7%) |
| 15%          | Midtrops (1%) | Schizonts (2%) | — | — | — |

\* The life cycle of intraerythrocytic *P. falciparum* is 48 h: 0–48 h, first cell cycle; 48–96 h, second cell cycle; 96–144 h, third life cycle. The indicated time represents time after the parasite invaded erythrocytes in the first cell cycle. Therefore, 56 and 104 h refers to 8 h after parasites entered the second and third cycles, respectively.

\( \text{Trops, trophozoites.} \)

\( \text{The number in the parenthesis indicates the percent parasitemia, i.e. the number of parasite-infected erythrocytes/100 total erythrocytes (infected plus non-infected) in culture. The indicated increase in parasitemia at 56 and 104 h is because of the multiplication of parasites in the second and third life cycles. 4 x 15% indicates culture multiplices.} \)

10 mM galactosamine were not significantly different from the amounts of GPIs synthesized by the untreated parasites (Fig. 2 and Table II). N-Acetylgalactosamine caused little or no inhibition. Parasites treated with 10 mM sugar synthesized ~92% GPIs compared with untreated parasites (Table II).

When parasites were labeled with \(^{3}H\)mannose in the presence of glucosamine, GPI-biosynthetic intermediates containing 1–3 mannose were barely observed, indicating that the amino sugar inhibits a biosynthetic step prior to the addition of the first mannose residue. We have previously shown by labeling with \(^{3}H\)glucosamine that mannans, an epimer of glucosamine, inhibits GPI biosynthesis in *P. falciparum* by inhibiting the transfer of the first mannose residue to acylated GlcN-Pi intermediate (6). However, inhibition of GPIs by glucosamine cannot be studied using \(^{3}H\) glucosamine, and labeling with \(^{3}H\)mannose will not furnish information regarding inhibition prior to mannose addition. Therefore, the parasites were labeled with \(^{3}H\)inositol in the presence of 5 or 10 mM of either glucosamine or mannansome and the products were analyzed by HPTLC (Fig. 3). The identity of the GPI intermediates was confirmed by specific degradation. HNO\_2 converted GPIs and GPI intermediates into (acyl)PI, and \(\alpha\)-mannosidase converted EtN-P-Man\_n-GPI into EtN-P-Man\_n-PI and converted the intermediates lacking ethanolamine into GlcN-(acyl)PI (Fig. 3B). These results revealed that although inositol-acylated GlcN-PI intermediate is present in significant amounts in parasites labeled with mannansome (Fig. 3A, lanes 4 and 5), the amount of inositol-acylated GlcN-PI is dramatically decreased in glucosamine-treated parasites (Fig. 3A, lanes 2 and 3). In contrast, significant levels of nonacylated GlcN-PI were present in both glucosamine- and mannansome-treated parasites. These results demonstrate that glucosamine inhibits the inositol acylation of GlcN-PI intermediate.

**Inhibition of GPI Synthesis by Glucosamine in the Cell-free System**—To determine whether the effect of glucosamine on GPI biosynthesis by *in vitro* cultured *P. falciparum* is the result of the inhibition of the enzyme activity or interference of the sugar with the gene expression, we studied GPI synthesis in a cell-free system using parasite membranes (21). Membranes were prepared by harvesting the parasite at mid trophozoite
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**Fig. 2.** Analysis of [3H]mannose-labeled GPIs synthesized by *P. falciparum* treated with hexosamines and N-acetylhexasosamines. Equal aliquots of parasite cultures (1 × 10^7 cells) with 15% parasitemia were treated with 1.25, 2.5, 5, or 10 mM amino sugars at 28 h after erythrocyte invasion (trophozoite stage) in complete medium. After 2 h, [3H]mannose (50 μCi/ml) was added to the culture in the presence of the respective amounts of amino sugars and incubated for 6 h. An aliquot of untreated parasites was cultured in parallel as controls. The parasites were harvested, and free GPIs and GPI intermediates were isolated, analyzed by HPTLC, and visualized by fluorography. A, lane 1, GPIs synthesized by untreated parasites. Lanes 2–5, GPIs synthesized by parasites treated with 1.25, 2.5, 5, and 10 glucosamine, respectively. B, lane 1, GPIs synthesized by untreated parasites. Lanes 2 and 3, GPIs synthesized by parasites treated with 5 and 10 mM galactosamine; lanes 4 and 5, GPIs synthesized by parasites treated with 5 and 10 mM N-acetylglucosamine; lanes 6 and 7, GPIs synthesized by parasites treated with 5 and 10 mM N-acetylgalactosamine. The identity of the GPIs and their intermediates are indicated. M_Gn-(A)PI, Man_GlcN-(acyl)PI; EM_Gn-(A)PI, EtN-P-Man_GlcN-(acyl)PI; EM_Gn-(A)PI, EtN-P-Man_GlcN-(acyl)PI.

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Discussion

A previous study from our laboratory showed that mannosamine prevents the growth and development of *P. falciparum* by inhibiting the GPI biosynthesis (6). It has been previously shown that in mammalian cells and other microorganisms that mannosamine inhibits GPI biosynthesis by interfering with the addition of the third mannose residue of the conserved glycan core (17–19). In contrast, we found that in *P. falciparum*, mannosamine blocked the addition of the first mannose residue (6). In this study, we show that glucosamine, an epimer of mannosamine, can also inhibit the growth of *P. falciparum* by specifically blocking the biosynthesis of GPI anchors. However, unlike mannosamine, glucosamine inhibits the parasite GPI biosynthesis by interfering with the fatty acylation of the inositol residue of GlcN-PI intermediate. This is a novel mechanism of inhibition of GPI biosynthesis (see below). Our data also show that galactosamine, a C-4 isomer of glucosamine, and N-acetylgalactosamine and N-acetylgalactosamine do not inhibit either the parasite growth or the GPI biosynthesis.

Several lines of evidence demonstrate that glucosamine can arrest the growth of *P. falciparum* by specifically inhibiting GPI biosynthesis. Glucosamine inhibits the parasite GPI biosynthesis in a dose-dependent manner; however, the sugar had no effect on the protein synthesis. The inhibition of parasite growth by glucosamine is developmental stage-specific, i.e., the sugar specifically inhibits the maturation of trophozoites, the developmental stage at which the intraerythrocytic parasite synthesizes its entire GPI pool. Glucosamine had no effect on the growth of the ring or schizont stage parasites. The parasites treated with 10 mM glucosamine during most of the ring stage developed normally into early trophozoites, which became schizonts and functional merozoites. The parasites also developed into normal schizonts and functional merozoites when treated with 10 mM glucosamine after the formation of late trophozoites. However, treatment with 10 mM glucosamine during the trophozoite stage caused complete growth arrest and death at the mid-trophozoite stage. Treatment of parasite with 5 mM glucosamine during the entire first cell cycle caused a significant reduction in the formation of mature schizonts, and the erythrocyte invasion of merozoite was ~50% compared with that of untreated culture. Continued presence of 5 mM glucosamine caused complete cell death during the second cell cycle. Thus, the disruption of *P. falciparum* trophozoite maturation with concomitant inhibition of GPI biosynthesis by glucosamine clearly indicates that the GPIs are essential for the growth of parasites.

Although glucosamine specifically arrested the growth of *P. falciparum* at the trophozoite stage by inhibiting the GPI biosynthesis, galactosamine did not inhibit either parasite growth or GPI biosynthesis. Unlike glucosamine, which at 5 and 10 mM concentrations inhibited parasite GPI biosynthesis by 36 and 13%, respectively, 5 and 10 mM galactosamine had no noticeable effect on GPI biosynthesis. Galactosamine-treated parasites synthesized similar levels of GPIs compared with untreated culture (see Fig. 2 and Table II). Consistent with these results and in agreement with our finding that GPIs are essential for parasite growth and development, galactosamine had no effect on the parasite growth. Thus, in contrast to parasite culture treated with 5 mM glucosamine, which caused complete cell death during the second cell cycle, the parasites treated with 5 mM galactosamine grew normally through all of the three cell cycles measured. Whereas 10 mM glucosamine completely prevented the trophozoite development in the first cell cycle, parasites treated with 10 mM galactosamine developed into trophozoites and schizonts and entered the second life cycle; however, the merozoite invasion efficiency was somewhat decreased. This moderate inhibitory effect on parasite growth by 10 mM galactosamine could be the result of the metabolic conversion of significant amount of galactosamine to glucosamine-6-phosphate (25), which is likely to cause the inhibition of GPI synthesis similar to that by glucosamine.

Our finding on the inhibitory effect of hexosamines on par-
Asite GPI biosynthesis is in agreement with a previous observation by Pan et al. (17) that glucosamine but not galactosamine inhibits the biosynthesis of GPIs in MDCK cells. Treatment of the MDCK cells with 5 mM glucosamine caused a 67% reduction in the GPI biosynthesis, whereas similar amounts of galactosamine had no effect on the levels of GPI biosynthesis (17). However, the mechanism of inhibition of GPI synthesis by glucosamine in MDCK cells has not been investigated. Based on the results of this study (discussed below), it is likely that in MDCK and other mammalian cells, glucosamine also inhibits the inositol acylation of GlcN-PI.

Compared with glucosamine, N-acetylglucosamine is significantly less inhibitory on parasite growth and GPI biosynthesis. Treatment with 5 and 10 mM N-acetylglucosamine caused 21 and 40% decrease, respectively, in GPI synthesis compared with untreated parasites. Consistent with only modest inhibition of GPI synthesis, 5 mM N-acetylglucosamine had no significant inhibitory effect on parasite growth in the first cell cycle, although there was a low to moderate levels of inhibition in the second and third cell cycles. Treatment with 10 mM N-acetylglucosamine throughout the first cell cycle caused a 25% reduction in parasite growth. The inhibitory effect of N-acetylglucosamine on treatment with 10 mM sugar or prolonged treatment at 5 mM sugar could be attributed to either the formation of a significant amount of glucosamine 6-phosphate (25) or another intermediate from N-acetylglucosamine that may be inhibitory or cause a nonspecific effect.

Table II

| Sugar treated | Inhibition of GPI Synthesis (Percent control) |
|--------------|----------------------------------------------|
|              | 1.25 mm | 2.5 mm | 5 mm | 10 mm |
| GlcN         | 5       | 31     | 64   | 87    |
| GaIN         | NI      | NI     | 3    | 11    |
| GlcNac       | NI      | NI     | 21   | 40    |
| GalNAc       | NI      | NI     | 1    | 8     |

Fig. 3. Analysis of [3H]inositol-labeled GPIs synthesized by P. falciparum treated with glucosamine and mannosamine. Equal aliquots of parasite cultures with 15% parasitemia were treated with 5 and 10 mM sugars 20 h after erythrocyte invasion (late rings and early trophozoites) in complete medium. After 2 h, [3H]inositol (50 μCi/ml) was added to the culture in the presence of glucosamine or mannosamine and incubated for 12 h. An aliquot of untreated parasites was cultured in parallel as controls. The parasites were harvested, and free GPIs and GPI intermediates were isolated, analyzed by HPTLC, and visualized by fluorography. A, lane 1, GPIs synthesized by control parasite culture. Lanes 2 and 3, GPIs synthesized by parasites treated with 5 and 10 mM glucosamine, respectively; lanes 4 and 5, GPIs synthesized by parasites treated with 5 and 10 mM mannosamine, respectively. B, GPIs synthesized by control parasite culture. Lane 1, untreated GPIs; lanes 2 and 3, GPIs treated with nitrous acid and jack bean α-mannosidase, respectively. The identity of the GPIs and their intermediates are indicated. (A)PI, (acyl)PI; Gn(−A)PI, GlcN(−acyl)PI; GlcNAc-PI, N-acetylgalactosamine-PI; M2Gn(−A)PI, Man2-GlcN(−acyl)PI; M2Gn(−A)PI, Man2-GlcN(−acyl)PI; EM2Gn−(A)PI, EtN-P-Man2−GlcN−(acyl)PI; EM3Gn−(A)PI, EtN−P−Man4−GlcN−(acyl)PI; EM4Gn−(A)PI, EtN−P−Man4−GlcN−(acyl)PI; PI*, PI without acyl substituent on the inositol residue.

Fig. 4. Inhibition of GPI synthesis by glucosamine in P. falciparum cell-free system. The freshly prepared parasite membranes were incubated with GDP-[3H]mannose in the presence of glucosamine as described under “Experimental Procedures.” The GPIs formed were isolated by extraction with 1-butanol, washed with water, dried, analyzed by HPTLC, and visualized by fluorography. Lane 1, GPIs synthesized by control parasite membranes. Lane 2 and 3, GPIs synthesized by parasite membranes treated with 5 and 10 mM glucosamine, respectively. The identity of the GPIs and GPI intermediates are indicated in the left margin. DPM, dolichol phosphate mannose; M2Gn−(A)PI, Man2−GlcN−(acyl)PI; EM2Gn−(A)PI, EtN−P−Man2−GlcN−(acyl)PI; EM3Gn−(A)PI, EtN−P−Man4−GlcN−(acyl)PI; PI*, PI without acyl substituent on the inositol residue.
The data presented here show that glucosamine inhibits a specific step in the biosynthetic pathway of GPIs and that the enzyme involved in this step discriminates the configurational structures of hexosamines. As shown in Fig. 3A, inositol-acylated GlcN-PI is barely formed in parasite treated with glucosamine, suggesting that the glucosamine specifically inhibits the parasite GPI-biosynthetic pathway by interfering with the inositol acylation of GlcN-PI intermediate to form GlcN-(acyl)PI. The parasites treated with glucosamine synthesized significant levels of GlcN-PI, suggesting that the effect of glucosamine on P. falciparum biosynthesis is not at or before the N-deacetylation of N-acetylglucosamine-PI. In contrast, galactosamine or mannosamine has no effect on inositol acylation. Thus, the inositol fatty acid-transferring enzyme exhibits strict specificity to the equatorial hydroxyl group at C-4 of glucosamine in GlcN-PI, and the results of this study offer a strategy for the development of specific inhibitors for GPI synthesis.

In summary, the data presented here show that glucosamine but not galactosamine can specifically inhibit the biosynthesis of GPIs in intraerythrocytic P. falciparum. Unlike mannosamine, an epimer that has been previously shown to inhibit GPI biosynthesis in the parasite by interfering with the addition of mannose to GlcN-(acyl)PI intermediate, glucosamine inhibits GPI biosynthesis by preventing the transfer of fatty acyl moiety to GlcN-PI, a novel mode of inhibition. Our data also show that glucosamine directly inhibits the inositol-acylating enzyme activity but does not repress the gene expression. Thus, glucosamine and mannosamine inhibitory steps of parasite GPI biosynthesis can be differential targets for the development of anti-malarial drugs.

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