The Plasmodium falciparum PfGatp is an Endoplasmic Reticulum Membrane Protein Important for the Initial Step of Malarial Glycerolipid Synthesis*

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During its 48-h asexual life cycle within human erythrocytes, Plasmodium falciparum grows to many times its own size and divides to produce 16–32 new parasites. This rapid multiplication requires active synthesis of new membranes and is fueled by phospholipid precursors and fatty acids that are scavenged from the human host. Plasmodium membrane biogenesis relies heavily on the expression of parasite enzymes that incorporate these precursors into phospholipids. However, little is known about the genes involved in membrane biogenesis or where this process takes place within the parasite. Here, we describe the analysis in P. falciparum of the first step of phospholipid biosynthesis that controls acylation of glycerol 3-phosphate (GPAT) at the sn-1 position. We show that this activity is of parasite origin and is specific for glycerol 3-phosphate substrate. We have identified the gene, PfGAT, encoding this activity in P. falciparum and reconstituted its codon composition for optimal expression in the yeast Saccharomyces cerevisiae. PfGAT complements the lethality of a yeast double mutant gat1Δgat2A, lacking GPAT activity. Biochemical analysis revealed that PfGatp is a low affinity GPAT enzyme with a high specificity for C16:0 and C16:1 substrates. PfGatp is an integral membrane protein of the endoplasmic reticulum expressed throughout the intraerythrocytic life cycle of the parasite but induced mainly at the trophozoite stage. This study, which describes the first protozoan GPAT gene, reveals an important role for the endoplasmic reticulum in the initial step of Plasmodium membrane biogenesis.

Plasmodium species are obligate intraerythrocytic protozoan parasites that undergo a number of developmental stages in the vertebrate host. In humans, they annually cause clinical illness in 300–500 million people with 1.5–2.7 million deaths, mainly caused by Plasmodium falciparum (1). Drug resistance is widespread, and the need for more efficacious and less toxic agents that exploit pathways and targets unique to the parasite is acute.

In the 48 h after invasion of human red blood cells, P. falciparum grows to many times its original size and then divides to produce 16–32 daughter parasites. This high rate of growth and multiplication requires synthesis of new membranes. Accordingly, the phospholipid content of malaria-infected erythrocytes increases by up to 5-fold during parasite maturation, with 85% of the newly synthesized phospholipids being either phosphatidylcholine or phosphatidylethanolamine (2). Parasite infection is also accompanied by a marked increase in neutral lipid species like fatty acids, diacylglycerol (DAG) and triacylglycerol (TAG) (3). This synthesis of parasite phospholipids and neutral lipids relies upon transport of choline, inositol, and fatty acids from host plasma (2, 4–10) and de novo synthesis of fatty acids by type II fatty acid-synthesizing enzymes (11). The finished malaria genome sequence revealed the presence in P. falciparum of type II fatty acid and phospholipid genes (12, 13). Whereas all of the known and predicted enzymes of the type II fatty acid-synthesizing enzyme pathway contain a signal peptide and a targeting sequence for apicoplast (14, 15), a plastid-like organelle in Apicomplexa parasites, most known and predicted enzymes for the synthesis of phosphatidylycerine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol lack these signals, suggesting that the synthesis of the malarial phospholipids does not occur in the apicoplast and that this process takes place in other cellular organelles, the identity of which is not yet known.

Because of their importance for parasite development, the pathways of synthesis of phospholipids have long been considered attractive targets for chemotherapy. Accordingly, quaternary ammonium compounds, analogs of choline, have been shown to interfere with phospholipid metabolism, to inhibit parasite growth in vitro, and to clear malaria infection in mice and monkeys (16). In eukaryotes, the initial step of phospholipid synthesis involves acylation of glycerol 3-phosphate at the sn-1 position by glycerol-3-phosphate acyltransferases (GPAT) to form lysophosphatidic acid (17–21). Lysophosphatidic acid acyltransferases then catalyze the acylation of lysophosphatidic acid at the sn-2 position to generate phosphatidic acid (19, 20). Phosphatidate phosphatase and CDP-DAG synthase en-

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§§§ These abbreviations used are: DAG, diacylglycerol; DHAPAT, dihydroxyacetone phosphate acyltransferase; FITC, fluorescein isothiocyanate; GPAT, glycerol-3-phosphate acyltransferase; PBS, phosphate-buffered saline; PGATcOD, codon-optimized PGAT; TAG, triacylglycerol.

1 The abbreviations used are: DAG, diacylglycerol; DHAPAT, dihydroxyacetone phosphate acyltransferase; FITC, fluorescein isothiocyanate; GPAT, glycerol-3-phosphate acyltransferase; PBS, phosphate-buffered saline; PGATcOD, codon-optimized PGAT; TAG, triacylglycerol.
zymes convert the phosphatidic acid formed into DAG and CDP-DAG, respectively. DAG subsequently enters the zymases for synthesis of phosphatidylinositol and phosphatidylethanolamine, respectively. CDP-DAG enters the

**EXPERIMENTAL PROCEDURES**

**Parasite Culture**—All reagents were from Sigma unless otherwise specified. *P. falciparum* clones were grown using the method developed by Trager and Jensen (29). Serum was replaced with 0.5% Albumax (Invitrogen). All reagents were from Sigma unless otherwise specified.

**PfGAT Construction—**Codon-optimized PfGAT _co_ was synthesized using the forward and reverse primers shown in Fig. 5 and those small fragments that were subsequently used as templates to amplify the full-length

**Plasmid Construction—**Codon-optimized PfGAT _co_ was synthesized using the forward and reverse primers shown in Fig. 5 and those small fragments that were subsequently used as templates to amplify the full-length

**Strain and Genotype**

| Strain | Genotype | Source |
|--------|----------|--------|
| BY4741 (ScCHO1) | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Research Genetics |
| ScCHO92 | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gat1::KanR | This study |
| ScCHO93 | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gat1::KanR [pYES2.1 GAL1::PfGAT URA3] | This study |
| ScCHO101 | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gat1::KanR [pYES2.1 GAL1::PfGAT _co_ URA3] | This study |
| CMY228 | MATα his3Δ-11,15 leu2Δ-3,112 trp1Δ-1 ade2Δ-1 can1Δ-100 gat1Δ-1 TRP1 gat2Δ-1 HIS3 ura3-1 [pGAL1::GAT1 URA3] | This study |
| ScCHO88 | MATα his3Δ-11,15 leu2Δ-3,112 trp1Δ-1 ade2Δ-1 can1Δ-100 gat1Δ-1 TRP1 gat2Δ-1 HIS3 ura3-1 [pGAL1::GAT1 URA3] | This study |
| ScCHO89 | MATα his3Δ-11,15 leu2Δ-3,112 trp1Δ-1 ade2Δ-1 can1Δ-100 gat1Δ-1 TRP1 gat2Δ-1 HIS3 ura3-1 [pGAL1::GAT1 URA3] | This study |
| ScCHO104 | MATα his3Δ-11,15 leu2Δ-3,112 trp1Δ-1 ade2Δ-1 can1Δ-100 gat1Δ-1 TRP1 gat2Δ-1 HIS3 ura3-1 [pGAL1::GAT1 URA3] | This study |

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**Table I**

| Strain | Genotype | Source |
|--------|----------|--------|
| BY4741 (ScCHO1) | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Research Genetics |
| ScCHO92 | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gat1::KanR | This study |
| ScCHO93 | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gat1::KanR [pYES2.1 GAL1::PfGAT URA3] | This study |
| ScCHO101 | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gat1::KanR [pYES2.1 GAL1::PfGAT _co_ URA3] | This study |
| ScCHO88 | MATα his3Δ-11,15 leu2Δ-3,112 trp1Δ-1 ade2Δ-1 can1Δ-100 gat1Δ-1 TRP1 gat2Δ-1 HIS3 ura3-1 | This study |
| ScCHO89 | MATα his3Δ-11,15 leu2Δ-3,112 trp1Δ-1 ade2Δ-1 can1Δ-100 gat1Δ-1 TRP1 gat2Δ-1 HIS3 ura3-1 | This study |
| ScCHO104 | MATα his3Δ-11,15 leu2Δ-3,112 trp1Δ-1 ade2Δ-1 can1Δ-100 gat1Δ-1 TRP1 gat2Δ-1 HIS3 ura3-1 | This study |
mM imidazole, 0.2% Triton X-100, pH 8. Histidine-tagged proteins were purified by Ni²⁺-NTA chromatography, washed with 20 volumes of buffer A, and eluted in the presence of 50 mM NaH₂PO₄, 200 mM NaCl, 250 mM imidazole, and 0.2% Triton X-100, pH 8.

**GPAT and DHAPAT Assays**—Parasites from 3D7 asynchronous and synchronous cultures (2% hematocrit, 10% parasitemia) were isolated from infected erythrocytes by treatment with 0.07% saponin for 15 min at 0 °C followed by centrifugation at 2,061 × g for 15 min. The pellet was washed in PBS and resuspended in 500 μl of 50 mM Tris·HCl, pH 7.5, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA. After sonication followed by centrifugation at 1,500 × g, the supernatant was recovered and used for GPAT and DHAPAT assays. Yeast extracts were obtained as described previously (28). For the GPAT assay, 200 μg of protein extracts was added to 200 μl of GPAT buffer (75 mM Tris·HCl, pH 7.5, 1 mM dithiothreitol, 2 mM MgCl₂, 45 μM fatty acyl-CoA, 1 mg/ml bovine serum albumin, and 0.4 mM [14C]glycerol 3-phosphate (2.5 μCi/μmol) and incubated at 37 °C for 1 h, except as otherwise mentioned. The reaction was stopped by the addition of 600 μl of 1% HClO₄. The DHAPAT assay was performed as described by Athenstaedt et al. (30). For both activities, lipid extraction was performed by adding 3 ml of chloroform:methanol (1:2, v/v) to the mixture followed by adding 1 ml of chloroform and 1 ml of 1% HClO₄. After centrifugation at 1,250 × g for 5 min, the organic phase was recovered and washed with 2 ml of 1% HClO₄ followed by centrifugation at 1250 × g for 5 min. The chloroform phase was transferred to a scintillation vial, dried, and counted. Aqueous and organic phases were also analyzed by thin layer chromatography (TLC) using silica gel plates (Whatman). The hydrophilic phase of the first extraction was dried in a SpeedVac and resuspended in chloroform for loading on TLC. A solvent made of chloroform:methanol:water:acetic acid (70:30:4:2) was used to separate radiolabeled products and to confirm their identity. The main product detected in the organic phase of the GPAT assay was phosphatidic acid. No phosphatidic acid or lysophosphatidic acid could be detected in the aqueous phase. For the DHAPAT assay, low counts could be measured from the organic phase, and no radiolabeled acyl-acyldihydroxyacetone phosphate could be measured in the water phase after TLC separation. Standards used were 1-oleoyl-sn-glycerol 3-phosphate and 1,2-dioleoyl-sn-glycerol 3-phosphate (Sigma).

**Gel Filtration Assay**—Infected red blood cells from 84 ml of P. falciparum asynchronous culture (2% hematocrit, 10% parasitemia) were treated with 0.07% saponin for 15 min at 0 °C. After centrifugation at 1,875 × g for 10 min the pellet was washed in PBS, resuspended in PBS containing a mixture of protease inhibitors, sonicated, incubated in 1% Triton X-100 for 30 min at 0 °C, and centrifuged at 16,300 × g for 15 min. The supernatant was concentrated and separated on a Superose 12 HR 10/30 column at a flow rate of 0.2 ml/min. Fractions of 1 ml were collected and trichloroacetic acid precipitated. The precipitates were resuspended in 20 μl of SDS-PAGE loading buffer, neutralized and separated by electrophoresis on 10% SDS-polyacrylamide gels, and analyzed by Western blotting, using affinity-purified anti-PfGatp antibodies.

**Analysis of PfGatp Membrane Association**—Infected red blood cells from a 36-ml asynchronous culture (2% hematocrit, 10% parasitemia) were treated with 0.07% saponin for 15 min at 0 °C. After centrifugation at 1,875 × g for 10 min, the pellet was washed in PBS and resuspended in PBS. After sonication, the extract was subjected to various treatments followed by a 10-min centrifugation at 100,000 × g. Treatments included a 30-min incubation at 0 °C with buffer alone, 1% Triton X-100, 1% Triton X-114, 1% n-decyl-β-D-maltoside (Anatrace), 0.5 mM potassium acetate or 0.1 mM Na₂CO₃ at pH 11. Supernatant and pellet fractions were separated by SDS-PAGE and immunoblotted with affinity-purified anti-PfGatp and anti-PfNT1 antibodies (31). Bound antibodies were visualized by ECL.

**Immunofluorescence Microscopy**—Synchronous cultures of P. falciparum-infected erythrocytes were washed twice in PBS, placed onto coverslips, and dried at room temperature. Fixation, washes, and mounting were performed as described by Rager et al. (31). Coverslips were incubated simultaneously with affinity-purified anti-PfGatp antibodies (diluted 1:10) and either mouse monoclonal antibodies (Sigma) to the red blood cell band 3 protein (diluted 1:500) or rat polyclonal antibodies (MBL) to the P. falciparum endoplasmic reticulum marker BiP (32) at 37 °C with gentle shaking for 1 h. The coverslips were washed and then incubated with anti-rabbit fluorescein isothiocyanate (FITC) conjugate and anti-mouse conjugated to Texas Red (Molecular Probes) or anti-rabbit rhodamine and anti-rat FITC-conjugated secondary antibodies for 1 h at 37 °C. Nuclei were stained by incubating the coverslips in PBS containing 3 μg/ml Hoechst stain (Molecular Probes) for 5 min at room temperature. Mitochondrial staining was performed by incubating infected red blood cells with 250 nM MitoTracker Red CMXRos (Molecular Probes) for 5 min prior to fixation and incubation with affinity-purified PfGatp antibodies (diluted 1:10). The coverslips were washed and then incubated with goat anti-rabbit conjugated to FITC (Molecular Probes) secondary antibodies for 1 h at 37 °C. Images were analyzed by high resolution fluorescence and confocal microscopy.

**RESULTS**

**GPAT and DHAPAT Activities in P. falciparum**—To examine the presence of GPAT and/or DHAPAT activities in P. falcipa-
rum-infected erythrocytes and to determine their origin (i.e., red blood cell or parasite), hemolysate and parasite fractions were prepared from red blood cells infected with an asynchronous culture of the 3D7 clone of *P. falciparum* and analyzed for their GPAT and DHAPAT activities using glycerol 3-phosphate and dihydroxyacetone phosphate substrates, respectively. Parasite extracts were able to catalyze the acylation of glycerol 3-phosphate substrate and dihydroxyacetone phosphate substrates (Fig. 1, A and B). However, the *P. falciparum* DHAPAT represented less than 1% of the GPAT activity (Fig. 1B). No GPAT or DHAPAT activities could be detected in red blood cell hemolysates from infected as well as control uninfected red blood cells (Fig. 1, A and B). To determine the GPAT activity during *P. falciparum* intraerythrocytic development, extracts were prepared from a highly synchronous culture of 3D7-infected erythrocytes and analyzed for GPAT activity. Equal amounts of proteins from each developmental stage resulted in relatively similar acylation activities (not shown). However, determination of the total activity per developmental stage indicated a 1.4- and 2.3-fold increase in the acylation activity during trophozoite and schizont stages (1.267 nmol of glycerol 3-phosphate/10^8 trophozoites and 2.052 nmol of glycerol 3-phosphate/10^8 schizonts), respectively, compared with the ring stage (0.88 nmol of glycerol 3-phosphate/10^8 rings) (Fig. 2).

**Table II**

| Protein     | Motif I     | DBM | Motif II | DBM | Motif III | DBM | Motif IV | Accession no. |
|-------------|-------------|-----|----------|-----|-----------|-----|----------|---------------|
| PfGatp      | HNNQFID     | 36  | SVKR     | 102 | FPEGG     | 29  | SIHPGGLSY | AY007373      |
| Gat1p       | HANQFDID    | 47  | PPVR     | 120 | FPEGG     | 31  | VVPCGLHY  | P36148        |
| Gat2p       | HANQFDID    | 40  | GVVR     | 109 | FPEGG     | 31  | IVPCGMYN  | Z35773        |
| Hs.GAT      | HRSHID      | 38  | FFIIR    | 35  | FLEGT     | 30  | ULPVGSY   | NP065969      |
| Mm.GAT      | HRSHID      | 38  | FFIIR    | 35  | FLEGT     | 30  | ULPVGSY   | AAA19201      |
| Ec.GAT      | HRSHID      | 38  | FFIIR    | 35  | FLEGT     | 30  | ULPVGSY   | AAA19201      |
| At.GAT      | HRSEAD      | 22  | AGDR     | 57  | WIAPSGG   | 36  | IYPMSL    | Q43397        |
| Ps.GAT      | HRSEAD      | 22  | AGDR     | 57  | WIAPSGG   | 36  | IYPMSL    | P80796        |

DBM, distance between motifs in number of amino acid residues.
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Fig. 3. PfGatp expression during P. falciparum intraerythrocytic life cycle. A, Western blot analysis was performed using protein extracts from supernatant (S), hemolysate (H), and parasite (P) fractions from an asynchronous culture of P. falciparum 3D7 clone. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by immunoblot using affinity-purified PfGatp-antibodies as described under “Experimental Procedures.” B, Western blot analysis was performed using protein extracts prepared from a highly synchronous culture of P. falciparum 3D7 clone at different times after P. falciparum invasion of red blood cells. C, Western blot analysis was performed using soluble (S) and pellet fractions (P) of parasite extracts treated or not with 1% Triton X-100, 1% Triton X-114, 1% n-decyl-β-D-maltopyranoside (DM), 0.5 M potassium acetate or 0.1 M carbonate, pH 11, followed by 100,000 × g ultracentrifugation. Immunoblot analysis was performed using anti-PfGatp and anti-PfNT1 antibodies.

1C). Consistent with data from asynchronous parasites, only residual DHAPAT activity (~0.15% of the cellular GPAT activity) could be detected from ring, trophozoite, and schizont extracts (data not shown). These results indicate that in P. falciparum the first step of glycerolipid biosynthesis is directed mostly toward the acylation of glycerol 3-phosphate substrate. As a control, yeast extracts were used, and both activities were detected (Fig. 1, A and B) in agreement with data published previously (20, 28, 33).

PfGatp Is a Yeast-like GPAT Protein—To identify the enzyme(s) responsible for the malarial GPAT activity, we searched for GPAT-like proteins in the P. falciparum genome data bases using known GPAT proteins as query. A gene that we named PfGAT was identified based on its homology with yeast GPAT enzymes. PfGAT was cloned from the P. falciparum 3D7 clone (The Netherlands) and was found to encode a polypeptide of 583 amino acids which exhibits ~28% identity and 49% similarity to S. cerevisiae Gat1p and Gat2p (Fig. 2A) and other putative GPAT enzymes from the fission yeast Schizosaccharomyces pombe and the filamentous yeast Candida albicans (not shown). In contrast, PfGatp shares little or no homology with bacterial, mammalian, and plant known and putative GPAT proteins. The four motifs known to play a crucial role in GPAT activity are present in PfGatp and are highly homologous to those found in yeast GPAT proteins (34). Interestingly, these motifs are different from the previously characterized or predicted human, mouse, bacterial, and plant GPAT enzymes (Table II). Furthermore, whereas PfGatp and its yeast homologs Gat1p and Gat2p have a long stretch of 102, 120, and 109 amino acid residues between motifs II and III, respectively, this linker is much shorter in the human, mouse, bacterial, and plant known and putative GPAT enzymes with, respectively, 35, 35, 29, and 57 amino acid residues only (Table II). The derived amino acid sequence of PfGatp was analyzed to determine the hydrophobic character of the protein, using the TMHMM program (35). PfGatp is predicted to possess three hydrophobic membrane spanning domains with a long N-terminal stretch (1–382) exposed outside the membrane (Fig. 2B). This topology is similar to that predicted for the yeast Gat1p and Gat2p proteins (28).

PfGatp Is an Endoplasmic Reticulum Membrane Protein Expressed throughout the Intraerythrocytic Life Cycle—We have expressed and purified the C-terminal region of PfGatp and used it to immunize rabbits and produce polyclonal antibodies. These antibodies were affinity purified over a PfGatp maltose-binding protein affinity matrix and used in Western blot assays to monitor PfGatp temporal and spatial expression during the P. falciparum intraerythrocytic life cycle. Although no immunoreaction could be detected in the culture supernatant or the hemolysate fractions of uninfected or P. falciparum-infected erythrocytes, a single band with a molecular mass of 67 kDa was detected in the parasite fraction (Fig. 3A). This size is consistent with that predicted from the PfGatp translation product. Analysis of PfGatp expression during the intraerythrocytic life cycle of the parasite showed that it is expressed in all the stages (ring, trophozoite, and schizonts), but its level increases during the trophozoite stage (24–36 h) (Fig. 3B). As a positive control, expression of the P. falciparum elongation factor 1β was regulated, with higher expression observed during the later stages of the parasite development (data not
shown), as we have reported previously (31, 36). To determine whether PfGatp is an integral or peripheral membrane protein, parasite lysates prepared from *P. falciparum* at the schizont stage of the parasite intraerythrocytic development with PfGatp- and Band 3-specific antibodies. In red, Band 3 conjugated to the Texas Red-conjugated anti-mouse secondary antibody. In green, PfGatp conjugated to the FITC-conjugated goat anti-rabbit secondary antibody. In yellow, FITC-conjugated goat anti-rabbit secondary antibody. In red, MitoTracker. In green, BiP conjugated to the FITC-conjugated goat anti-rabbit secondary antibody. DNA was counterstained with Hoechst (Fig. 4, A–E). Similar results were obtained using confocal microscopy (data not shown).

Yeast Complementation and PfGatp-mediated GPAT Activity—For functional analysis of PfGatp, we have used yeast as a model system to characterize the protein at the biochemical and genetic levels. In *S. cerevisiae*, two genes, *GAT1* and *GAT2*, encode GPAT activities (27, 28). Single disruption of *GAT1* or *GAT2* causes no discernible growth defects; however, disruption of both genes is lethal (27, 28). To overcome expression problems caused by the high A+T content of *PfGAT*, we used a PCR-based approach to synthesize a codon-optimized version of *PfGAT*, *PfGAT* 

**Fig. 4.** Immunofluorescence microscopy of *P. falciparum*-infected red blood cells using PfGatp antibodies. A, double-labeling immunofluorescence of erythrocytes infected with *P. falciparum* at the schizont stage of the parasite intraerythrocytic development with PfGatp- and Band 3-specific antibodies. In green, PfGatp conjugated to the FITC-conjugated goat anti-rabbit secondary antibody. In red, Band 3 conjugated to the Texas Red-conjugated anti-mouse secondary antibody. B, double-labeling immunofluorescence of erythrocytes infected with *P. falciparum* at the schizont stage of the parasite intraerythrocytic development with PfGatp-specific antibodies and MitoTracker. In green, PfGatp conjugated to the FITC-conjugated anti-rabbit secondary antibody. In red, MitoTracker. DNA was counterstained with Hoechst. C–E, double-labeling immunofluorescence of erythrocytes infected with *P. falciparum* at the ring (C), trophozoite (D), and schizont (E) stages of the intraerythrocytic development with PfGatp- and BiP-specific antibodies. In red, PfGatp conjugated to the rhodamine-conjugated anti-rabbit secondary antibody. In green, BiP conjugated to the FITC-conjugated anti-rat secondary antibody. DNA was counterstained with Hoechst (blue). Yellow represents regions of overlap between red and green.
Gat1p GPAT activity, \( P_{GAT}^{CO} \) was expressed under the regulatory control of the \( ADH1 \) constitutive promoter (\( pBEVY-L ADH1::PfGATCO \)) in the yeast strain CMY228, which contains the plasmid \( pGAL1::GAT1 URA3 \), which harbors the yeast \( GAT1 \) gene under the regulatory control of the inducible \( GAL1 \) promoter (27). The CMY228 strain is not viable on medium containing glucose and grows only on galactose (27). CMY228 cells expressing \( PfGATCO \) were able to grow on glucose (Fig. 6B), whereas CMY228 control cells expressing the empty vector \( pBEVY-L ADH1 LEU2 \) resulted in clones that were unable to grow on glucose (Fig. 6B). Furthermore, because the endogenous \( pGAL1::GAT1 URA3 \) plasmid contains the \( URA3 \) positive/negative marker, we applied a negative selection using 5-fluorotic acid to eliminate this plasmid. The strain Sc-CHO104, which harbors the plasmid \( pBEVY-L ADH1::PfGATCO \) and therefore relies solely on PfGatp expression for survival, was obtained and confirmed further for the loss of the endogenous \( pGAL1::GAT1 URA3 \) plasmid (Fig. 6C). These studies thus provide genetic evidence that PfGatp plays the same cellular function as the yeast Gat1p and Gat2p. Protein extracts from ScCHO104 strain were prepared and used to characterize further the PfGatp-mediated GPAT activity in the presence of glycerol 3-phosphate and dihydroxyacetone phosphate substrates. Similar to our results in \( P. falciparum \), PfGatp activity was specific for glycerol 3-phosphate substrate.

![Codon optimization of the PfGAT gene.](image)

**Fig. 5.** Codon optimization of the PfGAT gene. Nucleotide and protein sequences of \( PfGAT \) and \( PfGATCO \) are shown. Arrows indicate the oligonucleotides used for assembly and amplification of \( PfGATCO \) as described under “Experimental Procedures.”
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The PfGatp GPAT activity measured at 37 °C was linear during the first 6 min, after which it reached a plateau (Fig. 7A). No significant activity could be detected at 0 °C (Fig. 7A). We have measured the kinetic parameters of the GPAT activity using increasing concentrations of glycerol 3-phosphate substrate. PfGatp displayed an apparent affinity \(K_m\) for glycerol 3-phosphate of 2.55 ± 0.58 mM and a maximum velocity \(V_{max}\) of 55.1 ± 7.2 nmol × mg\(^{-1}\) × min\(^{-1}\) (Fig. 7B). The substrate specificity of PfGatp was measured using unsaturated and saturated fatty acyl-CoA substrates with different chain lengths (C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, and C20:0). PfGatp displayed a major preference for palmitoyl-CoA (C16:0) and palmitoleoyl-CoA (C16:1), low preference for C14:0, C18:0, C18:1, and C20:0 substrates, and no specificity for C20:0 (Fig. 7C). Unlike its GPAT activity, PfGatp-mediated DHAPAT was found to be very low and represented only 0.5–2.5% of its GPAT activity (Fig. 7D).

PfGatp Exists as a Large Multimeric Protein Complex in the Endoplasmic Reticulum Membrane—To examine whether the native PfGatp exists as a monomer or is part of protein complex, native proteins were separated under native conditions and analyzed by Western blot using anti-PfGatp-specific antibodies. Native PfGatp migrated as a high molecular mass polypeptide of an estimated >450 kDa (Fig. 8A). Cross-linking studies using increasing concentrations of the alkylation agent ethylene glycol bis(succinimidylsuccinate) followed by SDS-PAGE analysis showed a shift of PfGatp from a monomeric form to higher molecular masses (500 kDa) (Fig. 8B). As a control, the fractions collected by gel filtration were analyzed by Western blot using antibodies against P. falciparum elongation factor 1α and showed, as expected, the presence of this protein both as a monomer (free PfEF-1α) and as a large protein complex (EF-1 complex) as described previously (Fig. 8, C and D) (36).

DISCUSSION

During its asexual 48-h development and multiplication cycle within human erythrocytes P. falciparum produces between 16 and 32 new merozoites that subsequently invade new red blood cells. This rapid multiplication of the parasite within human erythrocytes is accompanied by a marked increase in phosphatidicholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, fatty acids, DAG, and TAG content (3). This increased metabolic need of P. falciparum to generate new membranes has stimulated efforts to identify compounds that can interfere with parasite membrane biogenesis and block malaria proliferation. Analysis of the available P. falciparum genomic sequences points to the presence of the genes of the major pathways for synthesis of glycerolipids (12). The few of those genes that have been characterized thus far show major structural and catalytic differences from their human counterparts, thus opening future avenues for lipid-based therapeutic strategies to fight malaria.

Here, we have characterized the initial step of glycerolipid synthesis in Plasmodium-infected erythrocytes. We found that P. falciparum catalyzes the acylation of glycerol 3-phosphate into 1-acylglycerol 3-phosphate, which is the main precursor for phosphatidic acid and subsequently for the phospholipid precursors DAG and DCP-DAG. P. falciparum also catalyzes the acylation of dihydroxycetone phosphate, although less efficiently compared with the GPAT substrate. This low DHAPAT activity suggests that malaria parasites may not require specialized DHAPAT enzymes and may not synthesize ether lipids. This idea is supported further by the lack in the finished genomic sequence of P. falciparum of homologs of...
DHAPAT and alkylidihydroxyacetone phosphate synthase genes, which are important for ether lipid synthesis.

Our studies revealed that the *P. falciparum* PfGAT gene encodes a GPAT enzyme. To our knowledge, this is the first GPAT gene to be identified in protozoa. Affinity-purified polyclonal antibodies against PfGatp indicated that this protein is expressed throughout the asexual life cycle of the parasite but induced mainly during the trophozite stage during which an active synthesis of phospholipids takes place, likely to provide membranes for the newly formed parasites. A similar regulation pattern was observed for the PfGAT transcript using large scale microarray analyses (41, 42). Our characterization of the native PfGatp demonstrated that it is an integral membrane protein of the endoplasmic reticulum and suggests that this organelle plays an important role in phospholipid biosynthesis in *P. falciparum*. Furthermore, we found that native PfGatp exists as a high molecular mass protein. We do not know at this stage whether this high molecular complex is composed solely of PfGatp or whether this enzyme associates with other parasite proteins.

Analysis of the sequence of PfGatp protein suggests that it is a yeast-like GPAT enzyme. The four motifs known to be important for GPAT catalysis are present in PfGatp and are highly similar in residue composition as well as in their spatial distribution to those of the yeast GPAT proteins, Gat1p and Gat2p. Interestingly, these motifs are highly divergent from those of mammalian and bacterial GPAT enzymes. Motifs II and III in PfGatp are separated by 102 amino acid residues, whereas the human and mouse GPATs have only 35 residues between these two motifs. The fact that yeast possesses two genes *GAT1* and *GAT2* that catalyze the GPAT activity and that disruption of both genes is lethal has made it possible for us to functionally characterize PfGAT at the genetic and the biochemical levels using yeast as a surrogate system. The finished sequence of the *P. falciparum* nuclear genome indicated that its overall A+T composition is 80.6% and rises to ~90% in...
introns and intergenic regions, making it the most A+T-rich genome sequenced to date (12). This unusual property of P. falciparum genes has hampered efforts to perform straightforward expression and complementation analyses in heterologous systems. Because of low expression levels in yeast, initial attempts to express PfGAT gene in gat1Δ or gat2Δ single knock-outs to measure activity or in the double knock-out gat1Δgat2Δ to complement its lethal phenotype were not successful. To overcome this problem, we synthesized a codon-optimized version, PfGAT CO. This resulted in an increase in the G+C composition of PfGAT from 26.6 to 34.5% and a dramatic increase in the expression of PfGAT in yeast. Furthermore, expression of PfGAT CO in the double knock-out gat1Δgat2Δ mutant could complement its lethal phenotype. In accordance with our results in P. falciparum, PfGatp was found to be specific for glycerol 3-phosphate and showed a very low activity toward dihydroxyacetone phosphate substrate. Kinetic studies revealed that PfGatp is a low affinity GPAT enzyme that displays high substrate specificity. PfGatp activity was higher when the acyl-CoA substrates, C16:0 and C16:1, were used. Interestingly, C16:0 has been shown to be transported by the parasite from host plasma and is essential for parasite growth and survival (43).

In summary, the work reported here supports the conclusion that PfGatp encodes an unusual yeast-like GPAT enzyme of P. falciparum expressed throughout the asexual life cycle (ring, trophozoite, and schizont stages) of the parasite within the host red blood cells. The identification of PfGatp is a critical step toward understanding membrane biogenesis in this parasite. The finished genome sequence of P. falciparum has also revealed a second gene, PfPLSB, encoding a polypeptide that shares homology with plant GPAT enzymes. Future studies are needed to determine whether the encoded protein catalyzes the acylation of glycerol 3-phosphate and/or dihydroxyacetone phosphate. Our attempts to target PfGAT gene for disruption have resulted in integration events to different loci in the genome but not to PfGAT locus, suggesting that PfGAT might be essential for parasite survival and that PfGatp and PfPlsB functions might not be redundant. Future complementation studies in P. falciparum to confirm the essential role of PfGAT are warranted and could provide useful information for the rational design of compounds that could specifically inhibit PfGatp activity and block parasite membrane biogenesis and multiplication.

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