In Vivo Evidence for the Specificity of Plasmodium falciparum Phosphoethanolamine Methyltransferase and Its Coupling to the Kennedy Pathway*

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Unlike humans and yeast, Plasmodium falciparum, the agent of the most severe form of human malaria, utilizes host serine as a precursor for the synthesis of phosphatidylethanolamine via a plant-like pathway involving phosphoethanolamine methylation. The monopartite phosphoethanolamine methyltransferase, Pfpmt, plays an important role in the biosynthetic pathway of this major phospholipid by providing the precursor phosphoethanolamine methyltransferase gene. The monopartite phosphoethanolamine methyltransferase gene, Pfpmt, has strong specificity for phosphoethanolamine methylation. The in vitro studies showed that Pfpmt has strong specificity for phosphoethanolamine. However, the in vivo substrate (phosphoethanolamine or phosphatidylethanolamine) is not yet known. We used yeast as a surrogate system to express Pfpmt and provide genetic and biochemical evidence demonstrating the specificity of Pfpmt for phosphoethanolamine in vivo. Wild-type yeast cells, which inherently lack phosphoethanolamine methylation, acquire this activity as a result of expression of Pfpmt. The Pfpmt restores the ability of a yeast mutant pem1∆pem2∆ lacking the phosphatidylethanolamine methyltransferase genes to grow in the absence of choline. Lipid analysis of the Pfpmt-complemented pem1∆pem2∆ strain demonstrates the synthesis of phosphatidylcholine but not the intermediates of phosphatidylethanolamine transmethylation. Complementation of the pem1∆pem2∆ mutant relies on specific methylation of phosphoethanolamine but not phosphatidylethanolamine. Interestingly, a mutation in the yeast choline-phosphate cytidylyltransferase gene abrogates the complementation by Pfpmt thus demonstrating that Pfpmt activity is directly coupled to the Kennedy pathway for the de novo synthesis of phosphatidylethanolamine.

With more than 1 million deaths and 300 million clinical cases annually, malaria is a major worldwide health concern and a major economic burden to the developing world (1). The illness is caused by intraerythrocytic protozoan parasites of the genus Plasmodium. Plasmodium falciparum, which is responsible for the most severe form of the disease, has developed resistance to almost all the available drugs in the antimalarial armamentarium (2). New chemotherapeutic strategies are therefore urgently needed to combat this disease. One strategy is to target the ability of the parasite to synthesize new membranes and inhibit its replication within host erythrocytes. Lipid inhibitors such as choline and phosphocholine analogs have been shown to inhibit P. falciparum membrane biogenesis and to block parasite proliferation (3–7). Some of these compounds are currently being evaluated for treatment of drug-resistant malaria (5).

Biochemical studies have indicated that the synthesis of phosphatidylcholine (PtdCho), 1 the major phospholipid in P. falciparum membranes can occur via two metabolic pathways, the de novo choline → phosphocholine → CDP-choline pathway, also named the Kennedy pathway, and a newly identified route that we named the serine decarboxylation-phosphoethanolamine methylation (SDPM) pathway (6, 8). The Kennedy pathway initiates with the transport of choline from host serum into the parasite cytoplasm by unidentified erythrocyte and P. falciparum choline transporters. Choline is then converted into PtdCho by the sequential action of choline kinase, choline-phosphate cytidylyltransferase, and CDP-diacylglycerol-cholinephosphotransferase (9–12). The SDPM pathway initiates from serine that is either transported from the host or obtained from active degradation of host proteins (13, 14). Serine is first decarboxylated to form ethanolamine by a serine decarboxylase and then phosphorylated to produce phosphoethanolamine (P-Etn) by an ethanolamine kinase (8). P-Etn is subsequently methylated to generate phosphocholine (P-Chol) by a phosphoethanolamine methyltransferase (Pfpmt) (6). The P-Chol enters the Kennedy pathway to form PtdCho. The SDPM pathway has thus far been characterized only in P. falciparum and plants (6, 15–17), and available genomic data suggest that it might also take place in Caenorhabditis elegans, Caenorhabditis briggsae, and Anopheles gambiæ (6).

Pfpmt is expressed throughout the intraerythrocytic cycle as well as during the gametocyte and sporozoite stages of the parasite (6, 18, 19). Pfpmt does not share homology with phosphatidylethanolamine (PtdEtn) methyltransferases from lower and higher eukaryotes, and no other homologs of this protein...
could be found in human or other mammalian data bases (6). Unlike the bipartite structure of plant phosphoethanolamine methyltransferases with two AdoMet-dependent catalytic domains, the malarial Pfpm is only half the size of plant phosphoethanolamine methyltransferases and possesses a single catalytic domain solely responsible for the three-step AdoMet-dependent methylation of P-Etn into P-Cho (6). Although biochemical studies in vitro showed that Pfpm and plant phosphoethanolamine methyltransferases were specific for P-Etn and did not catalyze the methylation of PtdEtn (6), one cannot exclude that these phosphoethanolamine methyltransferases (PMT) also catalyze the methylation of PtdEtn in vivo. The difficulty in genetically manipulating *P. falciparum*, and the fact that yeast cells lack the ability to convert P-Etn into P-Chol make yeast an attractive system to assess the specificity of Pfpm in vivo. In yeast grown in the absence of choline, the synthesis of PtdCho occurs primarily via the transmethylation of PtdEtn by two methyltransferases encoded by the *PEM1* (*CHO2*) and *PEM2* (*OPI3*) genes (20–22). Individual deletions of *PEM1* or *PEM2* cause no discernable phenotypes, whereas disruption of both genes is lethal unless choline is provided exogenously and transported via the choline transporter Hnm1 (20, 21). Here we have taken advantage of the extensive biochemical and genetic knowledge of phospholipid metabolism in yeast to examine the in vivo substrate specificity of Pfpm. Our data provide biochemical and genetic evidence demonstrating that in vivo Pfpm does not catalyze the transmethylation of PtdEtn to form PtdCho but instead catalyzes the methylation of P-Etn into P-Chol. Furthermore, we show that Pfpm plays an important role in PtdCho biosynthesis by coupling its activity to the Kennedy pathway.

**MATERIALS AND METHODS**

**Strain Construction, Growth Conditions, and Media**—The *Saccharomyces cerevisiae* strains used in this study are described in Table I. Standard methods for yeast culture and manipulation were used (23). Yeast was cultivated at 30°C in YPD (yeast extract/potato/dextrose) (24) or in synthetic minimal media containing 2% glucose (SD medium) or lactose. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by immunoblot using anti-V5 primary (Invitrogen, 1:5000 dilution) and anti-mouse secondary (1:10 000 dilution) antibodies.

**Enzyme Assays**—Yeast cells were grown in 50 ml of SD or SG media to an optical density of 1 at 30°C and lysed in 50 mM Tris-HCl buffer, pH 8.0, 10% glycerol, 1 mM diithiothreitol, and protease inhibitor mix by agitation with acid-washed glass beads. The 10,000 × g super- nate was dialyzed against 5 mM Hepes, pH 7.8, and 0.5 mM diithiothreitol. Pfpm was then affinity-purified as recommended by the manufacturer. Pfpm activity was determined by measuring the incorporation of radioactivity from [methyl-14C]AdoMet into P-Etn as described previously (6). A 1-ml incubation mixture containing 100 μM adenosylmethionine and 100 μM PtdEtn as described previously (6). For determination of PtdEtn methyltransferase activity, a PtdEtn emulsion was prepared by sonication in the presence of 0.02% Triton X-100 as described previously (16, 28), and the reaction was performed in Tris buffer, pH 8.5, using 50 μg of crude extract.

**RESULTS**

**Malarial Phosphoethanolamine Methyltransferase**

**Acquire Phosphoethanolamine Methyltransferase Activity**—The inability of the yeast *S. cerevisiae* to catalyze the three-step methylation of P-Etn for synthesis of P-Chol (Fig. 1) made it possible to assess biochemically and genetically the substrate specificity of *P. falciparum* Pfpm in vitro. Because of the high A+T content of Pfpm (72.5% A+T-rich) and to allow its successful expression in yeast, the full codon sequence of the cDNA was reconstructed in vitro to augment
its G+C content. Thirty-two 50-nucleotide overlapping primers were first assembled, and the resulting assembly product was used as a template for PCR amplification to create a codon-optimized gene, PfPMT\textsubscript{CO}, with 51.3% A+T content (Fig. 2). PfPMT\textsubscript{CO} was then cloned downstream of the GAL1-inducible promoter in the yeast expression vector pYES2.1, which allowed addition of a C-terminal V5-His\textsubscript{6} epitope tag to monitor protein expression. As shown in Fig. 3A, expression of Pfpmt could be detected in wild-type yeast cells grown in the presence of galactose, which induces GAL1 promoter-regulated genes but not in the presence of glucose, which represses GAL1 promoter-regulated expression.

To determine whether the expression of Pfpmt can provide yeast with the ability to catalyze the three-step methylation of P-Etn into P-Cho, protein extracts were prepared from wild-type yeast with the ability to catalyze the three-step AdoMet-dependent methylation of P-Etn to form P-Cho, whereas the same cells grown in presence of glucose or wild-type cells expressing the empty vector failed to catalyze this reaction (Fig. 3B). Pfpmt was further purified from wild-type cells grown in the presence of galactose by nickel affinity chromatography, and its activity and substrate specificity were analyzed. Similar to the PMT activity measured in P. falciparum extracts or that of the Escherichia coli recombinant-purified enzyme (6), Pfpmt purified from yeast catalyzed the three-step AdoMet-dependent methylation of P-Etn to form P-Cho (Fig. 4, A and B). The enzyme preparation showed only background activity when ethanolamine or PtdEtn were used as substrates (Fig. 4C).

**PfPMT\textsubscript{CO} Suppresses the Choline Auxotrophy of a Yeast pem1\textsuperscript{Δ}pem2\textsuperscript{Δ} Mutant**—Yeasts lack PMT activity. However, they express two genes PEM1 and PEM2, which encode two enzymes required for the three-step transmethylation of PtdEtn into PtdCho. Deletion of either PEM1 or PEM2 encodes viable strains, whereas deletion of both genes is lethal unless external choline is provided (20, 21). To determine whether expression of Pfpmt can complement the loss of PtdEtn transmethylation, a yeasty strain, pem1\textsuperscript{Δ}pem2\textsuperscript{Δ}, lacking PEM1 and PEM2 was constructed and transformed with the GAL1-PfPMT\textsubscript{CO} plasmid or the empty vector. The resulting transformants were confirmed to express Pfpmt by immunoblotting using anti-V5 monoclonal antibodies (Fig. 5A) and were further tested for their ability to grow in the absence of choline under inducing (in presence of galactose) and repressing (in presence of glucose) conditions (Fig. 5B). Because of residual choline in agar containing media, the assays were performed in both solid and liquid media. As expected pem1\textsuperscript{Δ}pem2\textsuperscript{Δ} cells expressing the empty vector required choline for growth under both glucose and galactose conditions. In contrast, pem1\textsuperscript{Δ}pem2\textsuperscript{Δ} cells expressing GAL1-PfPMT\textsubscript{CO} were able to grow in the absence of choline when galactose was the sole carbon source but required choline when grown in the presence of glucose (Fig. 5B). These data demonstrate that Pfpmt suppresses the choline auxotrophy of the pem1\textsuperscript{Δ}pem2\textsuperscript{Δ} mutant.

**PtdCho Biosynthesis by Pfpmt Is Independent of PtdEtn Transmethylation**—To confirm that Pfpmt suppression of the choline auxotrophy of pem1\textsuperscript{Δ}pem2\textsuperscript{Δ} is via restoration of the synthesis of PtdCho, phospholipids were prepared from wild-type and pem1\textsuperscript{Δ}pem2\textsuperscript{Δ} GAL1-PfPMT\textsubscript{CO} cells, grown in absence of choline, analyzed by TLC, and quantified. PtdCho could be detected in both strains (Fig. 6A), although the level of this phospholipid was lower in mutants expressing Pfpmt than in wild-type strains. Interestingly, Pfpmt expression did not affect growth rates of either strain (Fig. 6B).
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The activity was measured at 30 °C in the presence of 50 μM of P-Etn and [14C]AdoMet as substrates. To further validate this interpretation we assessed the activity was grown in SD (wild-type medium supplemented with 1 mM choline and containing glucose (Glu) or galactose (Gal) as described under “Materials and Methods.” B. S. cerevisiae was grown in SD (Glu) or SG (Gal) media, and the PMT activity was measured in absence (WT) or presence (WT+Pfpm1) of the malarial Pfpm1. PMT activity in S. cerevisiae extracts was determined using phosphoethanolamine (P-Etn) and [14C]AdoMet as substrates. The activity was measured at 30 °C in the presence of 50 μg of S. cerevisiae protein extract, and the product phosphocholine (P-Cho) was purified using AG-50(W) ion exchange resin. Each value is the mean of duplicate experiments.

![Image](89x622 to 275x738)

**FIG. 4.** Substrate specificity of Pfpm1 in *S. cerevisiae.* A, PMT activity was determined after affinity purification of Pfpm1 from an extract of pem1Δ pem2Δ-GAL1-PfPMTCO cells grown at 30 °C in SD (Glu) or SG (Gal) media. B, TLC analysis of the reaction product P-Cho after purification using AG-50(W) ion exchange resin. Lane S1, standard [14C]PCho. C, substrate specificity of affinity purified Pfpm1, in the presence of 100 μM P-Etn, Etn, and PtdEtn as described under “Materials and Methods.” Yeast cells were grown at 30 °C in the presence of glucose or galactose. Each datum represents an average of a duplicate.

phospholipid in the pem1Δpem2Δ-GAL1-PfPMTCO strain constituted only 32% of what is synthesized by wild-type cells (Fig. 6B). In addition, the PtdEtn level in the pem1Δpem2Δ-GAL1-PfPMTCO strain was almost double that of the wild-type cells (Fig. 6). Together these results suggest that Pfpm1-mediated synthesis of PtdCho is not via methylation of PtdEtn but rather via the methylation of P-Etn. To further validate this interpretation we assessed the specificity of protein extracts prepared from wild-type, pem1Δpem2Δ, and pem1Δpem2Δ-GAL1-PfPMTCO cells for P-Etn versus PtdEtn substrates. Protein extracts from wild-type cells catalyzed the transmethylation of PtdEtn into PtdCho, but showed no specificity toward P-Etn substrate (Fig. 7A). On the other hand, protein extracts from pem1Δpem2Δ-GAL1-PfPMTCO cells catalyzed the three-step methylation of P-Etn into P-Cho but showed no specificity for PtdEtn substrate (Fig. 7). As expected protein extracts from pem1Δpem2Δ grown in the presence of choline lacked both P-Etn and PtdEtn methylation activities (Fig. 7).

**FIG. 5.** Functional complementation of Pfpm1 in *S. cerevisiae.* A, Western blot analysis was performed using crude extracts from pem1Δ pem2Δ-pYES2.1 (pemΔ) and pem1Δ pem2Δ-GAL1-PfPMTCO (pemΔ Pfpm1) grown at 30 °C in minimal medium supplemented with 1 mM choline and containing glucose or galactose as described under “Materials and Methods.” B and C, the strains BY4741-pYES2.1 (wild type, 1), pem1Δ pem2Δ-pYES2.1 (2), and pem1Δ pem2Δ-GAL1-PfPMTCO (3) were grown in solid (B) or liquid (C) minimal medium galactose (Gal) or glucose (Glu) with (+ Cho) or without (− Cho) 1 mM choline for 24 h. The growth measurements were performed in both agar medium (left panels) and liquid medium (right panels).

**FIG. 6.** Phospholipid analysis. A, wild type (WT) harboring the pYES2.1 vector and complemented (pemΔ pem2Δ-GAL1-PfPMTCO) strains were grown in minimal medium plus galactose containing 2 mM ethanolamine until an A600 was 1. Lipids were extracted and separated by a two-dimensional TLC as described under “Materials and Methods.” The position of the lipids after iodine staining is shown. B, phospholipid composition. Each lipid was recovered from the TLC plate and quantified by measuring phosphorus. The results are shown as the percentage of total lipid phosphorus in each phospholipid fraction. PtdEtn(Me), dimethyl-PtdEtn; Ptd2Gro, phosphatidylglycerol; PtdOH, phosphatidic acid. Data are means ± S.D. for three independent experiments.
Membrane biogenesis is essential for *P. falciparum* development and multiplication with human erythrocytes. Understanding this process is an important step toward designing better therapeutic strategies to block parasite replication and eliminate the pathological symptoms associated with the intraerythrocytic cycle of the parasite. Upon infection of human erythrocytes, the phospholipid content of *P. falciparum* increases by at least 5–6-fold (30). Available data suggest that the synthesis of PtdCho, the major phospholipid in human erythrocytes, the phospholipid content of which is to catalyze the three-step AdoMet-dependent methylation of P-Etn (6). The resultant P-Cho represents an average of a duplicate.

**DISCUSSION**

Membrane biogenesis is essential for *P. falciparum* development and multiplication with human erythrocytes. Understanding this process is an important step toward designing better therapeutic strategies to block parasite replication and eliminate the pathological symptoms associated with the intraerythrocytic cycle of the parasite. Upon infection of human erythrocytes, the phospholipid content of *P. falciparum* increases by at least 5–6-fold (30). Available data suggest that the synthesis of PtdCho, the major phospholipid in *P. falciparum* membranes, can occur via two metabolic pathways: the Kennedy pathway, thus resulting in a growth defect at 37 °C. Expression of Pfpmt function is directly coupled to the Kennedy pathway, further demonstrating the specificity of this enzyme for P-Etn in vivo.

Because no homologs of PtdEtn methyltransferases could be found in the finished genome sequence of *P. falciparum*, it remained unclear whether in vivo Pfpmt might also catalyze the three-step methylation of PtdEtn. In the present study, we provide genetic and biochemical evidence demonstrating that the primary function of Pfpmt in vivo is to catalyze the methylation of P-Etn to form P-Cho. Six lines of evidence support this important conclusion. First, expression of Pfpmt in wild-type *S. cerevisiae* cells, which inherently lack P-Etn methyltransferase activity, resulted in their acquiring this activity. Second, PMT activity of wild-type yeast expressing Pfpmt was specific for P-Etn, and no methylation of ethanolamine or PtdEtn could be detected. Third, expression of Pfpmt in a yeast mutant, pem1Δpem2Δ, lacking the two genes PEM1 and PEM2, which encode the enzymes Pem1 and Pem2 essential for the synthesis of PtdCho from PtdEtn, resulted in a complete suppression of their requirement for exogenous choline. Fourth, lipid analysis of the pem1Δpem2Δ cells expressing Pfpmt demonstrated the synthesis of PtdCho in the absence of choline, but unlike the wild-type strain, no intermediates of the transmethylation of PtdEtn could be detected in the complemented strain. Fifth, the PtdEtn level in the pem1Δpem2Δ strain expressing Pfpmt was almost double that of the wild-type cells, suggesting continuous accumulation of this phospholipid as a result of synthesis via phosphatidylserine decarboxylation and the CDP-ethanolamine pathway in conjunction with the elimination of PtdEtn methylation. Finally, complementation of pem1Δpem2Δ choline auxotrophy by Pfpmt required a functional Kennedy pathway, because alteration of this pathway by mutation in the PCT1 gene abolishes this complementation.

The ability of Pfpmt to complement pem1Δpem2Δ mutant depends on the availability of the P-Etn substrate. In fact, the growth of the complemented strain was much better when the medium was supplemented with ethanolamine (not shown). In the absence of exogenous ethanolamine, this precursor could be obtained either via degradation of sphingosine-1-phosphate by the lyase encoded by the *DPL1* gene, or via hydrolysis of PtdEtn by the Ca2+-dependent phospholipase D activity. Interestingly, although the complemented and wild-type strains grew equally well in the absence of choline, the level of PtdCho (as percent total phospholipid) in the complemented strain was only ~4% in the absence (not shown) and ~12% in the presence (Fig. 6) of ethanolamine. In contrast PtdCho was ~40% total phospholipid present in wild-type cells. These data suggest that although PtdCho is the major phospholipid in yeast membranes, its level in wild-type cells far exceeds what is needed for growth and survival, at least under laboratory conditions.

To enhance expression of Pfpmt in yeast, we completely redesigned its cDNA by reducing its A+T content from 72.5 to 51.3% to prevent early transcriptional termination. Successful expression of Pfpmt was demonstrated by inserting a V5-His6 tag in the C-terminal region of the encoded protein and monitoring expression levels using anti-V5 monoclonal antibodies.
Pfpmt was further purified from yeast cells by affinity chromatography and used in enzymatic assays. Similar success in complementing yeast after codon optimization was achieved with PfGAT gene of \textit{P. falciparum}, which encodes a glycerol-3-phosphate acyltransferase of the endoplasmic reticulum important for the initial step of malarial glycerolipid metabolism (25). Pfpmt purified from yeast exhibited the same biochemical properties as the native PMT activity obtained from \textit{P. falciparum} or recombinant Pfpmt purified from \textit{E. coli} (6).

Our present results are concordant with previous biochemical analysis using recombinant Pfpmt purified from \textit{E. coli} and provide strong evidence that Pfpmt exhibits high specificity toward the P-Etn substrate and does not catalyze the methylation of PtdEtn in vivo. Thus it seems that the most critical precursor for the synthesis of PtdCho in \textit{P. falciparum} is P-Chol, which can be obtained either via Pfpmt methylation of serine-derived P-Etn or by phosphorylation of choline transported by the parasite choline transporter from the host. The relative contribution of the two pathways to the total cellular pool of PtdCho is not yet clear. Recent studies have provided new information about the biochemical properties of the parasite choline transporter (31, 32). However, the genes encoding choline transport activity have not yet been identified in \textit{Plasmodium} or any other protozoan parasite. More detailed genetic studies in \textit{P. falciparum} are now needed to evaluate the importance of choline transport and Pfpmt in phospholipid synthesis as it relates to parasite development and survival.

In summary, we have shown that Pfpmt is a highly specific methyltransferase enzyme acting exclusively on P-Etn substrate in vivo. The essential dependence of the pem1\Delta pem2\Delta mutant on Pfpmt for growth and survival makes this strain an ideal system to screen for inhibitors that can specifically inhibit Pfpmt activity.

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