Choline Induces Transcriptional Repression and Proteasomal Degradation of the Malarial Phosphoethanolamine Methyltransferase

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During its intraerythrocytic life cycle, the malaria parasite *Plasmodium falciparum* undergoes dramatic metabolic and morphological changes and multiplies to produce up to 36 new daughter parasites. This rapid multiplication of the parasite requires an active synthesis of new membranes. The major component of these membranes, phosphatidylcholine, is synthesized via two metabolic routes, the CDP-choline pathway, which uses host choline as a precursor, and the plant-like serine decarboxylase-phosphoethanolamine methyltransferase (SDPM) pathway, which uses host serine as a precursor. Here we provide evidence indicating that the activity of the SDPM pathway is regulated by the CDP-choline precursor, choline. We show that the phosphoethanolamine methyltransferase, Pfpmt, a critical enzyme in the SDPM pathway, is down-regulated at the transcriptional level as well as targeted for degradation by the proteasome in the presence of choline. Transcript analysis revealed that *PfPMT* transcription is repressed by choline in a dose-dependent manner. Immunoblotting, pulse-chase experiments, and immunoprecipitation studies demonstrated that Pfpmt degradation occurs not only in wild-type but also in transgenic parasites constitutively expressing Pfpmt. The proteasome inhibitor bortezomib inhibited choline-mediated Pfpmt degradation. These data provide the first evidence for metabolite-mediated transcriptional and proteasomal regulation in *Plasmodium* and will set the stage for the use of this system for conditional gene and protein expression in this organism.

The most severe form of human malaria is caused by the protozoan parasite *Plasmodium falciparum*, causing over 2 million deaths annually (23). Within human erythrocytes, *P. falciparum* undergoes dramatic metabolic and morphological changes and multiplies to produce up to 36 new daughter parasites in 48 h (11). This rapid propagation of the parasite requires active synthesis of new membranes, among other components, and is fueled by lipid precursors such as choline, serine, and fatty acids derived from the host. Phosphatidylcholine accounts for the majority (about 50%) of cellular membrane phospholipids in *P. falciparum* (21). Recently, we demonstrated that the biosynthesis of phosphatidylcholine in *P. falciparum* occurs through two metabolic pathways, namely, the de novo CDP-choline pathway and the serine decarboxylase-phosphoethanolamine methyltransferase (SDPM) pathway (16). In the CDP-choline pathway, host choline is taken up by the parasite and phosphorylated to phosphocholine by a parasite choline kinase. The phosphocholine is subsequently converted to CDP-choline by a CDP-choline cytidyltransferase (PfCCT). The CDP-choline formed is then converted to phosphatidylcholine by a CDP-diacylglycerol-choline phosphotransferase (PfCEPT). On the other hand, the biosynthesis of phosphatidylcholine via the SDPM pathway utilizes host serine as a precursor (16). Serine, which is readily available in the parasite cytoplasm either via active degradation of host hemoglobin or transport from host plasma, is first decarboxylated into ethanolamine by a parasite serine decarboxylase enzyme and then phosphorylated to phosphoethanolamine by a parasite ethanolamine kinase. Phosphoethanolamine is methylated to phosphocholine by a three-step S-adenosyl-L-methionine-dependent reaction, utilizing a parasite-specific enzyme, phosphoethanolamine methyltransferase (Pfpmt). The phosphocholine formed is then converted into phosphatidylcholine by the consecutive reactions of PfCCT and PfCEPT. Cell biological as well as genome-wide expression analyses suggest that Pfpmt is expressed throughout the intraerythrocytic cycle as well as during the gametocyte and sporozoite stages of the parasite (12, 16, 22). Pfpmt possesses a single catalytic domain solely responsible for the three-step S-adenosyl-L-methionine-dependent methylation of phosphoethanolamine to form phosphocholine (16). Thus, *P. falciparum* uses two routes for the synthesis of the phosphatidylcholine precursor, phosphocholine (15). However, whether these two routes are coregulated remains unknown. Previous in vitro studies showed that Pfpmt activity is inhibited by phosphocholine (16), suggesting that exogenous choline, which is rapidly transported and phosphorylated by the parasite, could regulate Pfpmt and thus affect the contribution of the SDPM pathway to the biosynthesis of phosphatidylcholine.

In this study we investigated the effects of exogenous choline on the expression of Pfpmt in wild-type and transgenic parasites constitutively expressing this enzyme. We show that exogenous choline induces Pfpmt down-regulation at the transcriptional level as well as via proteasome-mediated degradation.

**MATERIALS AND METHODS**

*Parasite culture.* Synthetic RPMI 1640 basal medium was prepared following the recipe of Invitrogen Gibco Cell Culture Systems (catalog no. 23400), except that choline chloride and L-methionine were not included. Complete liquid medium was prepared using 16.9 g/liter of the basal medium supplemented with 50 mg/liter hypoxanthine, 15 mg/liter L-methionine, 10 mg/liter gentamicin, 0.225% NaHCO₃, and 0.5% Albumax I (Life Technologies). *Plasmodium falcip-
3D7 strain parasites were propagated in human erythrocytes at 2% hematocrit by the method of Trager and Jensen (20).

**Real-time PCR and Northern blot analyses.** Wild-type 3D7 and transgenic parasites constitutively expressing PIPMT under the calmodulin promoter (TCPAM-PIPMT) were synchronized with 5% D-sorbitol and grown to early trophozoite stage in medium without choline. The parasites were resuspended in fresh medium, split into groups with equal starting parasitemia (2%), and cultured for one generation in complete RPMI 1640 medium with 0, 25, 50, 100, 200, 500, or 1,000 μM choline chloride. When the parasitemia was about 10% at the mid-trophozoite stage, the parasites were harvested by saponin treatment. For real-time reverse transcription-PCR (RT-PCR) analysis, total RNA was extracted from the parasite pellets by using the TRIzol reagent (Invitrogen) following the manufacturer’s instructions. Specifically, 1 μg of total RNA was treated with DNase I (Invitrogen) to remove any contaminating genomic DNA and reverse transcribed. Serial dilutions of recombinant PCR 2.1 plasmids carrying a 470-bp, 540-bp, and 500-bp fragment of the PIPMT, PTPX1 (PF14_0368 in PlasmoDB 4.4), and PIRAB6 (PF11_0461 in PlasmoDB 4.4) genes, respectively, were used as quantification standards. PIRAB6 and PTPX1 genes encode *P. falciparum* rabs GTase and Cys peroxiredoxin proteins, respectively (8, 18). The PIPMT, PTPX1, and PIRAB6 fragments were amplified from total cDNA using a LightCycler (Roche Diagnostics, Mannheim, Germany) with the Fast Start DNA Master SYBR Green I kit according to the manufacturer’s protocol. Briefly, 1 μl cDNA sample was added to a reaction mixture (1.6 μl of MgCl₂, 3 mM; 2 μl of Fast Start Master SYBR; 0.5 μl of primer mix, 2.5 pmol each; real-time PCR water to 20 μl). The PCR conditions included an initial denaturation for 10 min at 95°C, 40 cycles of 95°C for 10 s, 65°C for 5 s, and 68°C for 10 s, and a final cooling to 40°C. Transcript levels were determined from the standard curve established using the LightCycler software. The relative concentration of transcripts was determined by dividing the PIPMT band by that of PTPX1 to correct for loading. Relative values of the PTPX1 transcripts were determined using the PIRAB6 values.

For Northern blot analysis, 5 μg of parasite total RNA was fractionated on a 1.2% formaldehyde–agarose gel and transferred onto Hybond-N nylon membranes (Amersham Life Science). As probes, fragments of PIPMT and PIRAB6 were PCR amplified and radiolabeled with [³²P]dCTP using a High Prime DNA labeling kit (Roche). The blots were hybridized overnight at 42°C using the ultrasonesensitive hybridization buffer (Ambion) and washed twice in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 5 min each, followed by two washes in 0.1× SSC, 0.1% SDS, 15 min each. The blots were exposed to X-ray films to generate signals.

**Degradation assay.** To monitor the effect of exogenous choline on the degradation of Pfpmt protein, synchronized wild-type and transgenic parasites at early ring stage (2% parasitemia, 2% hematocrit) were cultured in medium lacking choline until they reached a parasitemia of 10% with 90% of parasites at the early trophozoite stage. Fresh medium containing cycloheximide at a 5-μg/ml concentration and 0.1% sodium deoxycholate, with Complete Mini EDTA-free protease inhibitor cocktail (1×). The cultures were incubated at 37°C for an additional 8 h of incubation. Following saponin treatment and sonication, protein extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and used for immunoblotting with antibodies against Pfpmt and translation elongation factor 1α (PFEF-1α) as previously described (3, 22).

**Pulse-chase analysis.** A culture of synchronized early ring stage wild-type 3D7 parasites (2% parasitemia, 90% early rings, 2% hematocrit) was incubated at 37°C for about 2 days in medium lacking choline until it reached a parasitemia of 5%. The culture was resuspended in fresh medium lacking choline and t-methionine but containing [³⁵S]methionine (0.36 ng/ml; specific activity, 1,175.0 Ci/mmol; Perkin-Elmer, Inc.), mixed thoroughly, and split into fresh culture plates. The parasites were cultured until they reached early trophozoite stage (−18 h). Thereafter, excess cold t-methionine (50 μg/ml final concentration) and 0 or 1 μM (final concentration) choline were added to the cultures, and samples were collected at 0, 2, 4, 6, and 8 h following choline supplementation. Parasites were extracted by saponin treatment and sonicated in lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, with Complete Mini EDTA-free protease inhibitor cocktail). Equal amounts of the soluble extracts were immunoprecipitated using anti-Pfpmt and anti-PFEF-1α polyclonal antibodies. The immunoprecipitated samples were resolved by SDS-PAGE, and the gels were dried and analyzed by autoradiography. The signal intensities were quantified using the ImageJ version 1.37v software (NIH).

**Inhibition of proteasome activity.** Synchronized wild-type 3D7 parasites at early ring stage were cultured in medium without choline until they reached the trophozoite stage. The culture was resuspended in fresh medium without choline, but with 5 μg/ml cycloheximide, and split into three groups. The groups were treated with either 30 nM bortezomib, 1 mM choline, or a combination of both and placed at 37°C for an additional 8 h of incubation. Following saponin treatment and sonication, protein extracts were separated by SDS-PAGE and analyzed by immunoblotting using anti-Pfpmt and anti-PFEF-1α antibodies.

**RESULTS**

Choline down-regulates the intracellular levels of Pfpmt in a dose-dependent manner. To determine the possible regulation of the SDPM pathway by choline, we examined the intracellular levels of Pfpmt in *P. falciparum* in the absence or presence of increasing concentrations of exogenous choline. Treatment of cultures with different concentrations of choline over a period of 48 h showed a dose-dependent reduction in Pfpmt expression, as determined by Western blot analysis using anti-Pfpmt antibodies (Fig. 1A). As a control, the level of PFEF-1α was unaffected by choline supplementation (Fig. 1A).
To determine the level of Pfpmt relative to that of the control, PfEF-1α, at different concentrations of choline in the medium, the signal intensities obtained from the Western blots were quantified by densitometry (Fig. 1B). The level of Pfpmt diminished significantly at choline concentrations equal to or higher than 100 μM, with almost complete depletion at 1 mM (Fig. 1B).

Choline mediates transcriptional and posttranscriptional down-regulation of Pfpmt. To assess whether the choline-induced down-regulation of Pfpmt expression occurred at the transcriptional level, we determined the levels of PfPMT transcripts by quantitative real-time RT-PCR and Northern blotting. Expression levels of the PfTPX1 and PfRAB6 genes were used as controls. Whereas the relative transcript levels of PfTPX1 and PfRAB6 remained the same in the absence or presence of increasing concentrations of choline (Fig. 2B), PfPMT transcription decreased significantly (up to sixfold) as the concentration of choline increased (Fig. 2A). This real-time PCR analysis result was corroborated by Northern blot analysis (Fig. 2C). The 1.2-kb PfPMT transcript was much more abundant in the absence of choline than when choline was added. To decipher the molecular mechanism of choline-induced repression of Pfpmt, the transgenic parasites TP-CAM-PfPMT, expressing PfPMT driven by the calmodulin promoter, which is strongly induced during the parasite’s intraerythrocytic life cycle, were also examined by real-time RT-PCR and Northern blot analyses. Similar levels of PfPMT transcripts in the presence or absence of choline were found in these transgenic parasites (Fig. 2D). Together these results suggest that the PfPMT promoter is repressed by choline. Remarkably, immunoblot analysis showed a dramatic decrease in Pfpmt protein level in the TP-CAM-PfPMT transgenic parasites cultivated in the presence of choline (Fig. 3). This shows that choline also acts to down-regulate Pfpmt independently of transcriptional repression. Altogether, these data demonstrate that choline regulates Pfpmt at both the transcriptional and posttranscriptional levels.

Choline induces degradation of Pfpmt. To gain further insights into the mechanism of posttranscriptional repression of Pfpmt by choline, wild-type parasites cultured in the presence or absence of choline were treated with cycloheximide to block protein translation, and Pfpmt expression was monitored by Western blotting. Although the Pfpmt protein level was significantly higher in TP-CAM-PfPMT transgenic parasites than in wild-type parasites, addition of choline resulted in a similar level of down-regulation of the enzyme in each case, even upon cycloheximide treatment (Fig. 4). This indicated, therefore, that choline-induced regulation of Pfpmt occurs independently of protein synthesis.

To further examine the effect of choline following de novo
synthesis of Pfpmt during the parasite intraerythrocytic progression, parasites were grown in the absence of choline for one generation to the trophozoite stage, during which active expression of Pfpmt occurs (16). The parasites were then treated with cycloheximide in the absence or presence of increasing concentrations of choline for 6 h, after which proteins were isolated and analyzed for Pfpmt levels using anti-Pfpmt antibodies. As shown in Fig. 5, Pfpmt levels decreased significantly as the concentration of choline increased. By contrast, the level of the translation elongation factor PfEF-1α, probed as the control, remained the same in the absence or presence of choline (Fig. 5). These results demonstrate clearly that choline induces degradation of the Pfpmt protein.

To monitor the rate of degradation of Pfpmt protein, pulse-chase experiments were performed in wild-type parasites following transfer from medium lacking choline to medium supplemented with choline. Wild-type 3D7 parasites precultured in the absence of choline and collected at the ring stage were cultured in medium without choline, labeled with L-[35S]methionine, and incubated at 37°C for 18 h until they reached the trophozoite stage, at which time nonradioactive methionine was added alone or in combination with choline. Parasites were harvested at different time points following the chase with unlabeled methionine, and Pfpmt was immunoprecipitated. This analysis revealed a more rapid decline in the level of Pfpmt over time in the samples supplemented with choline (Fig. 6B) compared to the unsupplemented samples (Fig. 6A). As a control, PfEF-1α was immunoprecipitated from equal aliquots of the samples, and no changes were detected among choline-treated and untreated samples (Fig. 6A and B, lower panels). Densitometric analysis of the protein band intensities showed a rapid decline in Pfpmt signal density in samples treated with 1 mM choline compared to the untreated samples (Fig. 6C). By Student’s t test statistical analysis, Pfpmt protein levels in the presence of choline were found to be significantly lower ($P < 0.01$) than those in the absence of choline after 4, 6, and 8 h of culture (Fig. 6C). Quantitative analysis revealed a significant difference ($P < 0.01$) in the half-life of Pfpmt, which was 8 h in the absence of choline and only 5 h in the presence of 1 mM choline (Fig. 6E).
Choline-induced degradation of Pfpm1 is inhibited by the proteasome inhibitor bortezomib. Degradation of many short-lived cellular proteins in eukaryotic cells is mediated by the ubiquitin-proteasome pathway (6). Bortezomib, a dipeptidyl boronic acid, and its analogs are specific and selective inhibitors of the 26S proteasome (1), which inhibits proteasome-mediated degradation of proteins in vitro and in vivo. Interestingly, the bortezomib analog MLN-273 has been demonstrated to specifically inhibit protein degradation mediated by the proteasome, resulting in the accumulation of ubiquitinated proteins in *P. falciparum* and *Plasmodium berghei* (13). To determine whether choline-induced degradation of Pfpm1 is mediated by the proteasome, wild-type 3D7 parasites were grown from early ring to the trophozoite stage in medium without choline and treated with bortezomib alone or in combination with choline for 8 h, and Pfpm1 levels were monitored using an anti-Pfpm1 antibody. Whereas the addition of choline alone resulted in a dramatic reduction of Pfpm1 levels, addition of bortezomib inhibited this choline-mediated repression and resulted in Pfpm1 levels similar to those of the control samples containing bortezomib alone.

**FIG. 6.** Analysis of Pfpm1 degradation by pulse-chase. Synchronized wild-type 3D7 parasites grown without choline to early ring stage and 7% parasitemia were resuspended in fresh medium without choline and t-methionine but containing l-[35S]methionine and grown to the trophozoite stage. Excess cold t-methionine and 0 or 1 mM choline were added and the cultures harvested at time points of 0, 2, 4, 6, and 8 h postaddition. Parasite soluble extracts were immunoprecipitated with anti-Pfpm1 and anti-PfEF-1α antibodies and resolved by SDS-PAGE, and the gels were dried and analyzed by autoradiography. (A and B) Signals from samples without and with 1 mM choline, respectively. PfEF-1α was used to check for loading. (C) The relative Pfpm1 protein values were derived by dividing the Pfpm1 band intensity values by the corresponding intensity values for PfEF-1α at each time point. The relative Pfpm1 values in the absence and presence of 1 mM choline are depicted as white and black columns, respectively, with the level of statistical significance of the difference (P < 0.01) between the two shown by an asterisk. (D and E) The Pfpm1 relative values were expressed as percent protein relative to the protein concentration at time zero, and the half-life of the Pfpm1 protein was derived from the log plot of the percent protein level over time of incubation in the absence (D) and in the presence (E) of 1 mM choline.
We conclude that choline induces the degradation of Pfpmt protein and that this degradation is mediated by the proteasome.

**DISCUSSION**

Genomic and proteomic analyses of *Plasmodium falciparum* have revealed a highly coordinated process of gene and protein expression during the parasite’s development and multiplication within human erythrocytes (2, 4, 9, 12). Unlike many other eukaryotes, only a limited number of transcription factors and regulators are found in the parasite’s genome. This suggests that posttranscriptional regulations may play a crucial role in defining the temporal expression of proteins and the metabolic machineries they control during the parasite’s intraerythrocytic cycle. This study provides the first demonstration that protein degradation plays an important role in this process. Our results show that in addition to transcriptional repression, the posttranslational activity in the possible degradation of the Pfpmt protein.

The data presented here provide evidence that exogenous choline leads to repression of transcription and induction of the proteasomal degradation of Pfpmt. Supplementation of parasite cultures with exogenous choline produced a dose-response reduction in the amount of both *PfPMT* transcript and protein in the parasite. Whereas choline induced a dose-dependent down-regulation of Pfpmt transcription driven by the endogenous promoter, it had no effect on the transcription of Pfpmt driven by the calmodulin promoter. This indicates that the PfPMT promoter might contain repression sites that are activated by choline or phosphocholine, the immediate product of choline kinase and Pfpmt enzyme activities. Alternatively, choline may activate a specific repressor that binds these sites. Future work must uncover promoter elements responsible for choline-induced repression of PfPMT transcription and identify the protein factors involved.

By monitoring the steady-state levels of Pfpmt protein, we found that choline also induced the down-regulation of Pfpmt protein in a dose- and time-dependent manner when the gene was expressed from a strong heterologous promoter. This suggests that choline regulates Pfpmt posttranscriptionally.

We investigated the involvement of the proteasomal proteolytic activity in the possible degradation of the Pfpmt protein. The proteasome is a large complex composed of two multisubunit structures, the 20S and the 19S complexes, that combine to form the 26S particles in which protein degradation occurs (6). Bortezomib is a dipeptidyl boronic acid that specifically and selectively inhibits the activity of the 26S proteasome (1). Recent studies have shown that this compound inhibits the growth of chloroquine- and pyrimethamine-sensitive and -resistant strains of *P. falciparum* with an approximate 50% inhibitory concentration of 30 nM (J. M. Reynolds et al., unpublished data). Interestingly, bortezomib significantly inhibited choline-induced repression of the Pfpmt protein, implying that the proteasome activity is responsible for Pfpmt degradation in the presence of choline.

In conclusion, our findings show that exogenous choline, a substrate utilized by the CDP-choline pathway to synthesize phosphocholine, induces a dose-dependent repression of PfPMT transcription and activates proteasome-mediated degradation of the enzyme. We postulate that as the parasite actively transports and phosphorylates host choline, a buildup of phosphocholine occurs which in turn initiates degradation of Pfpmt. The physiological importance of this regulation during malaria infection remains to be investigated.

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