Characterization of PRMT1 from *Plasmodium falciparum*

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Arginine methylation is a post-translational modification that affects many cellular processes in eukaryotes. The malaria parasite *Plasmodium falciparum* encodes three conserved PRMTs (protein arginine N-methyltransferases). We have determined that PfPRMT1 (*P. falciparum* PRMT1) has authentic type I PRMT activity to form monomethylarginines and asymmetric dimethyl-arginines. Compared with mammalian PRMT1s, PfPRMT1 possesses a distinctive N-terminal sequence that is ∼50 amino acids longer and is essential for enzyme activity. Recombinant PfPRMT1 methylated histones H4 and H2A and several conserved substrates involved in RNA metabolism, including fibrillarin, poly(A)-binding protein II, ribosomal protein S2 and a putative splicing factor. Using synthetic peptides and MS, we determined target arginines in several substrates and studied the enzyme kinetics. Whereas the kinetic parameters of recombinant PfPRMT1 on an H4 peptide and S-adenosylmethionine were similar to those of mammalian PRMT1s, PfPRMT1 had much higher substrate-turnover rates. In the histone H4 N-terminus, PfPRMT1 could methylate only Arg3, a mark for transcription activation. Western blotting detected dynamic dimethylation of H4_Arg3 during parasite development, suggesting that histone-arginine methylation may play a conserved role in chromatin-mediated gene regulation. Consistent with the presence of potential substrates in both the cytoplasm and nucleus, green fluorescent protein-tagged PfPRMT1 and untagged PfPRMT1 were localized in both cellular compartments, with the majority in the cytoplasm. *In vitro* assays showed that PfPRMT1 could be inhibited by several small-molecule inhibitors, with IC₅₀-values in the sub-micromolar range. Most of these compounds also effectively inhibited parasite growth, suggesting that parasite PRMTs are promising targets for developing antiparasitic drugs.

Key words: arginine methylation, enzyme activity, histone, protein arginine methyltransferase (PRMT), small-molecule inhibitor, transcription regulation.

INTRODUCTION

Arginine methylation is a widespread post-translational modification in eukaryotes and its significance in regulating many cellular processes, such as signal transduction, RNA processing and transport, and transcription, has been increasingly recognized [1–3]. Arginine methylation is catalysed by a family of PRMTs (protein arginine N-methyltransferases), which add one or two methyl groups from AdoMet (S-adenosylmethionine) to the guanidine-nitrogens of arginine, resulting in three major forms of methylated arginines: MMA (ω-N⁺-monomethylarginine), ADMA [asymmetric ω-N⁺,N°-DMA (dimethylarginine)], or SDMA (symmetric ω-N⁺,N°-DMA). Four types of PRMT have been described, with type I and type II enzymes as the main types in mammalian cells. Type I enzymes catalyse the formation of MMA and ADMA, whereas type II enzymes catalyse the formation of MMA and SDMA [3]. The human genome encodes at least 11 PRMTs, and PRMT1 (type I) and PRMT5 (type II) are evolutionarily conserved in most, if not all, eukaryotes [4,5].

The many substrates identified for the type I enzymes often contain GAR (glycine- and arginine-rich) motifs in RGG, RG, or RXXG sequences [2,6]. Among them, the best known RNA-binding proteins include heterogeneous nuclear ribonucleoproteins, fibrillarin, nucleolin and PABPII (poly(A)-binding protein II), which are involved in various aspects of RNA processing and transport [3]. Recently, histone-arginine methylation has been recognized as an important means of transcription regulation. Three mammalian PRMTs have been identified to catalyse histone methylation. PRMT1 methylates Arg3 in histone H4 and, to a lesser extent, Arg2 in H2A [7]. Another type I enzyme, PRMT4 [also known as CARM1 (coactivator-associated arginine methyltransferase 1)] methylates Arg2, Arg17, Arg26 and several arginines in the C-terminus of H3 [8]. The type II enzyme PRMT5 methylates Arg8 in H3 and Arg4 in H4 [9]. In vivo, PRMTs often interact with other cofactors, such as histone acetyltransferases and chromatin remodelers, to regulate transcription [10,11]. In mammals, the asymmetric arginine dimethylation of histones that is catalysed by PRMT1 and CARM1 is associated with active genes, whereas PRMT5-mediated symmetric dimethylation of histones is often linked to transcription repression [12].

The malaria parasite *Plasmodium falciparum* is responsible for more than one million deaths annually. Like other eukaryotes, *P. falciparum* genomic DNA is organized into nucleosomes involving both core and variant histones [13,14]. In addition to general conservation of the basal transcription machinery in the parasite [15], the presence of a large number of chromatin-modifying proteins suggests a prominent role of chromatin-mediated mechanisms in transcription regulation [16]. Although a deficiency of specific transcription factors in the parasite genome seemed to support this [17], the recent identification of a large
family of AP2-domain proteins as transcription activators or repressors in several apicomplexan genomes has changed this view [18,19]. Therefore, it is very likely that the malaria parasite uses both chromatin- and DNA-binding transcription factors to regulate gene expression [20,21].

The *P. falciparum* histones have many covalent modifications that play evolutionarily conserved roles in transcription regulation [14,22]. Histone acetylation is generally associated with active transcription, whereas the H3Lys\(^3\) trimethylation is negatively correlated with global gene expression [23]. Specifically, in the control of monoallelic expression of the *var* gene families, H3Lys\(^4\) di- and trimethylation, and trimethylation of the heterochromatin marker H3Lys\(^9\), are associated with active and silent *var* genes respectively [24,25]. Tandem-MS analysis of *P. falciparum* histones has revealed the presence of arginine methylation on H3 and H4 [14]. Besides, several PRMTs have been characterized recently in the protozoan parasites *Trypanosoma brucei* and *Toxoplasma gondii* [26–29]. In particular, H3Arg\(^17\) methylation, catalysed by the CARMA-like PRMT in *T. gondii*, has been attributed to gene activation [27]. These findings have prompted us to characterize the PRMTs in the malaria parasite and study their functions in transcription.

Here, we report the identification and characterization of the PRMT1 homologue in *P. falciparum*, referred to as PfPRMT1. PfPRMT1 is an evolutionarily conserved protein with type I PRMT activity towards histone H4 and a number of GAR-motif proteins as potential substrates. PfPRMT1 is expressed in all asexual erythrocytic stages of the parasite, and the protein is localized in both the cytoplasm and the nucleus. The inhibitory effect of general methyltransferase inhibitors on the parasite asexual erythrocytic stages of the parasite, and the protein is localized in both the cytoplasm and the nucleus. The inhibitory effect of general methyltransferase inhibitors on the parasite asexual erythrocytic stages of the parasite, and the protein is localized in both the cytoplasm and the nucleus. The inhibitory effect of general methyltransferase inhibitors on the parasite asexual erythrocytic stages of the parasite, and the protein is localized in both the cytoplasm and the nucleus. The inhibitory effect of general methyltransferase inhibitors on the parasite asexual erythrocytic stages of the parasite, and the protein is localized in both the cytoplasm and the nucleus. The inhibitory effect of general methyltransferase inhibitors on the parasite asexual erythrocytic stages of the parasite, and the protein is localized in both the cytoplasm and the nucleus. The inhibitory effect of general methyltransferase inhibitors on the parasite asexual erythrocytic stages of the parasite, and the protein is localized in both the cytoplasm and the nucleus.

**EXPERIMENTAL**

**Identification of potential PfPRMTs and substrates in the parasite genome**

To identify PRMTs in the *Plasmodium* genome, we used the BLASTP (protein-BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search the malaria database PlasmoDB (http://www.plasmodb.org/plasmo) using the protein sequences of human PRMTs [4]. Protein pattern and architecture were examined further using an HMM (hidden Markov model) embedded in the SMART (simple modular architecture research tool) program (http://smart.embl.de). To identify the protein homologues for known PRMT substrates, we used the human fibrillarin (Genbank\(^{®}\) accession number CAA39935), PABPII (Genbank\(^{®}\) accession number AAH10939) and rpS2 (ribosomal protein S2; Genbank\(^{®}\) accession number NP_002943), and Saccharomyces cerevisiae Gar1p (Genbank\(^{®}\) accession number AAB68929) to search PlasmodB using BLASTP.

**Protein expression and purification**

Full-length cDNA encoding PfPRMT1 was amplified by PCR with the primer pair PfPRMT1-F1/PfPRMT1-R1 (Supplementary Table S1 at http://www.BiochemJ.org/bj/421/bj4210107add.htm) and cloned at the BamHI and SalI sites of the vectors pMAL-c2x (New England Biolabs) and pGEX-6P-1 (GE Healthcare) according to the manufacturers’ instructions to produce MBP (maltose-binding protein)–PfPRMT1 and GST (glutathione transferase)–PfPRMT1 respectively. Recombinant protein was expressed in Bacterial Strain BL21 (GE Healthcare) after induction with 0.3 mM isopropyl \(\beta\)-D-thiogalactoside for 3 h at 37°C. For the purification of recombinant MBP–PfPRMT1, bacteria were resuspended in amnylose column buffer [20 mM Tris/HCl, pH 7.5, 200 mM NaCl, 2 mM DTT (dithiothreitol) and 1 mM PMSF] and lysed by sonication (four pulses of 40 s each). The lysate was clarified by centrifugation at 14000 \(g\) for 20 min at 4°C. The supernatant was diluted (1:5, v/v, with column buffer) and loaded on to an amnylose resin column (New England Biolabs). The column was washed with 20 column vol. of amnylose column buffer. The MBP–PfPRMT1 fusion protein was eluted with column buffer containing 10 mM maltose. Similarly, GST–PfPRMT1 was purified using a glutathione Sepharose 4B column (GE Healthcare) as described previously [22]. PfPRMT1 truncations corresponding to amino acids 35–401, 56–401 and 1–348 were amplified with primer pairs PfPRMT1-F7/PfPRMT1-R1, PfPRMT1-F4/PfPRMT1-R1 and PfPRMT1-F1/PfPRMT1-R7 respectively (Supplementary Table S1) and cloned into pGEX-6P-1 at the BamHI and SalI sites to produce GST fusion proteins. Tag-free PfPRMT1 was cleaved off the column using PreScission\(^{TM}\) protease (GE Healthcare). All purified recombinant proteins were dialysed twice in 1 litre of 50 mM sodium phosphate buffer, pH 8.0, containing 2 mM DTT and 10% (v/v) glycerol at 4°C. When necessary, recombinant proteins were concentrated using Microcon\(^{®}\) YM-10 centrifugal filter units (Millipore). Purified recombinant PfPRMT1 was kept at −70°C for binding and enzyme-activity assays. For antibody production, full-length PfPRMT1 was cloned into the pET28a (+) vector (Novagen) at BamHI and SalI sites. Recombinant PfPRMT1 was purified with Ni-NTA (Ni\(^{2+}\)-nitrilotriacetate) resin (Qiagen) and dialysed overnight in 1 litre of PBS at 4°C. The purified recombinant PfPRMT1 was used to produce polyclonal rabbit anti-PfPRMT1 antiserum (Proteintech Group Inc.) according to the manufacturer’s instructions. This antiserum was used directly in subsequent experiments without purification of the antibodies.

To identify potential methylation substrates for PfPRMT1, we selected nine GAR-motif-containing *P. falciparum* genes and expressed the full-length or truncated proteins fused to either GST or MBP, as described above, using primers listed in Supplementary Table S1. These are PABPII (PFH1175c), fibrillarin (PF14_0068), putative PABP (MAL13P1.303), Garl1-like homologue (PF13_0051), rpS2 (PF14_0448), and four splicing-factor-like proteins (PF1135w, PF10_0068, PF10_0217 and PFE0865c) (http://www.plasmodb.org/plasmo). Purified recombinant proteins were used in methylation assays with MBP–PfPRMT1.

**In vitro dimerization and AdoMet binding of recombinant PfPRMT1**

To determine whether PfPRMT1 forms dimers and oligomers, purified recombinant protein (1 \(\mu\)M) was incubated with or without 0.025% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer, pH 8.0, at room temperature (25°C) for 3 or 5 min [30]. The reactions were stopped by the addition of an equal volume of 2x Laemml sample buffer (4% (v/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (v/v) Bromophenol Blue and 0.125 M Tris/HCl, pH 6.8], and the proteins were separated by SDS/PAGE (10% gel), and then detected by immunoblotting with the polyclonal rabbit anti-PfPRMT1 antiserum (diluted 1:5000) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (diluted 1:3000).

To determine whether PfPRMT1 binds to AdoMet as the substrate, ~3 \(\mu\)g (1.2 \(\mu\)M) of recombinant MBP–PfPRMT1 or PfPRMT1 were incubated with 1.6 \(\mu\)M \(^{[3H]}\)AdoMet (82.0 Ci/mmol, GE Healthcare) in 30 \(\mu\)l of cross-linking buffer (50 mM sodium phosphate, pH 8.0, 5 mM MgCl\(_2\) and 2 mM DTT) at 4°C overnight [26]. For competition experiments, a 1000-fold
excess of unlabelled AdoMet or ATP (Sigma) was included in the reaction. The reactions were placed on ice and exposed to UV radiation (254 nm) for 60 min at a distance of 5 cm from the UV light. Afterwards, the proteins were resolved by SDS/PAGE (10 % gel), stained with Coomassie Brilliant Blue, and processed for fluorography using an autoradiographic enhancer (Perkin Elmer). The gel was dried and exposed to X-ray film for five weeks.

**In vitro methylation assay**

In vitro methylation assays of PfPRMT1 activity were carried out in 20 μl of methylation-assay buffer (50 mM sodium phosphate, pH 8.0, 5 mM MgCl₂ and 2 mM DTT) containing 1 μCi of [³H]AdoMet, 2–4 μg of substrates and 1.5 μg (0.6 μM) of recombinant MBP–PfPRMT1 at 30 °C for 1 h. The reactions were stopped by the addition of 2× Laemmli sample buffer and boiled for 5 min. Proteins were resolved by SDS/PAGE (10 % or 15 % gels), stained and processed for fluorography as described above.

**MS analysis of substrate peptides and enzyme kinetics**

To further identify the methylated arginines in potential substrates, two *P. falciparum* histone H4 peptides, PHH4-21 (Ac-SGRKGGKGLGKGAGKHKRI and PfH4-21R3K (Ac-SGKGGKGLGKGAGKHKRI), and two GAR-motif peptides, PF11175c-16 (amino acids 156–171) (KISPFRRGRMK-SALGV) and PF14_0068-15 (amino acids 44–58) (GRGGGGG-GRGGGGG) (http://www.plasmodb.org/plasmo) were synthesized (GenScript Corp.). Methylation reactions were performed in 10 μl of methylation buffer with the addition of 250 μM AdoMet, 20 μM of each peptide and 3 μg (1.2 μM) of recombinant MBP–PfPRMT1 at 37 °C. Reactions were stopped at different time points by adding 0.5 % (v/v) TFA (trifluoroacetic acid), and methylation products were detected by MS using the Micromass MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) mass spectrometer (Waters Corp.).

Enzyme kinetics were determined for the recombinant PfPRMT1 using a similar method to that described in [31]. To determine the kinetic parameters for the PHH4-21 peptide, the methylation reaction was performed with 1.1 μM of MBP–PfPRMT1, a constant amount of [³H]AdoMet (50 μM), and varying concentrations of PHH4-21 (2–20 μM) at 37 °C for 5 min. For the substrate AdoMet, the methylation reaction was performed with a constant amount of PHH4-21 peptide (50 μM) and varying concentrations of [³H]AdoMet (0.2–2 μM) at 37 °C for 10 min. All reactions were performed in duplicate in a final volume of 10 μl, stopped by the addition of 0.5 % (v/v) TFA and spotted on to Whatman P-81 cation-exchange filter papers. The radiolabelled products were processed for quantification using a liquid scintillation counter as described previously [32]. Data for each sample were fitted to the Michaelis–Menten plot to obtain the binding constant (Kₘ) and catalytic turnover (kₗ) values.

**Parasite culture**

The *P. falciparum* 3D7 clone was cultured as described in [33]. For time-course studies, parasites were synchronized by two rounds of treatment of ring-stage parasites with 5 % (v/v) sorbitol for 5 min, then collected at ring, early trophozoite, late trophozoite and schizont stages [34]. Parasites were released by lysing the erythrocyte membrane with 0.05 % (v/v) saponin for 10 min and washed twice with PBS.

**In vivo PfPRMT1 expression and activity**

Total RNA was extracted from the parasites using TRIzol® (Invitrogen) and treated with RNase-free DNase I [35]. The sequences of the 5’- and 3’-ends of the *PfPRMT1* mRNA were determined by the FirstChoice™ RLM-RACE (RNA ligase-mediated rapid amplification of cDNA ends) kit (Ambion) with *PfPRMT1*-specific primers (Supplementary Table S2 at http://www.BiochemJ.org/bj/421/bj4210107add.htm) as described in [36]. RACE products were cloned in TOPO® cloning vector (Invitrogen) and sequenced. Relative levels of *PfPRMT1* expression were studied by real-time RT–PCR (reverse transcription PCR) analysis at four stages of the *P. falciparum* asexual erythrocytic cycle using HotStart-IT® SYBR® Green One-Step qRT–PCR Master Mix Kit (USB) with primers *PfPRMT1*-F3 and *PfPRMT1*-R3 (Supplementary Table S2). The constitutively expressed seryl-tRNA synthetase gene (PF07_0073; http://www.plasmodb.org/plasmo) was used as the internal reference. Data analysis and determination of the Cₛ-values (threshold cycle values) were performed as described previously [14].

To study *PfPRMT1* protein expression during the IDC (intraerythrocytic developmental cycle), synchronized parasites were lysed by sonication (three pulses of 10 s each). Equal amounts of the parasite lysates (30 μg) at each developmental stage were separated by SDS/PAGE (10 % gel) and transferred to nitrocellulose membranes. Western blotting was performed using a standard procedure [37] with rabbit anti-PfPRMT1 antiserum (diluted 1:2000) as the primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:3000) as the secondary antibody. Blots were developed using DAB (diaminobenzidine) substrate (Roche).

To determine PfPRMT1 activity *in vivo*, endogenous PfPRMT1 was obtained by an IP (immunoprecipitation) assay as described in [40]. Briefly, 50 μl of protein A agarose (Sigma) was washed three times with an IP buffer [150 mM NaCl, 1 % (v/v) Triton X-100, 50 mM Tris/HCl, pH 8.0, 1 mM DTT and 0.2 mM PMSF], and then bound with 50 μl of either preimmune or anti-PfPRMT1 antiserum. After washing with the IP buffer, the agarose beads were mixed with 300 μl of precleared lysate from 2 × 10⁴ parasites for 1 h at 4 °C. Afterwards, the beads were washed three times with the IP buffer, twice with the methylation-assay buffer, and resuspended in methylation buffer to a total of 50 % (v/v) slurry. The agarose beads were directly assayed for methylation activity using recombinant PHH4 as the substrate.

**Plasmid construction and parasite transfection**

To generate GFP (green fluorescent protein)-tagged PfPRMT1, a construct containing PfPRMT1 amino acids 1–401 fused with a downstream GFP tag and pDT3 terminator was made in pBluescript (Stratagene). The resulting PfPRMT1–GFP-pDT3 cassette was then cloned into pHDD22Y [38] at the BamHI and NotI sites to obtain the final transfection construct pHDD22Y-PfPRMT1–GFP. Parasite transfection was carried out by culturing the late-stage schizonts with RBCs (red blood cells) preloaded with 100 μg of plasmid DNA [39]. Parasites containing the plasmid were selected with 2.5 mM WR99210, a dihydrofolate reductase inhibitor, for approx. 3 weeks and replenished weekly with fresh RBCs. Parasites with stable chromosomal integration of the plasmid were enriched by two cycles of drug on–off selection, and single clones of transformed parasites were obtained by limiting dilution [40]. Integration of the plasmid at the PfPRMT1 locus was screened by integration-specific PCR and Western blotting. Primary antibodies used were either anti-PfPRMT1
antiserum at 1:2000 dilution or monoclonal anti-GFP antibody at 1:1000 dilution (Roche).

Histone-arginine methylation in parasites

To study histone-arginine methylation, histones were isolated from the three IDC stages of synchronized parasites as described previously [14]. Equal amounts of proteins (~3 μg/lane) were separated by SDS/PAGE (18% gel) analysis. Western blotting was performed using anti-dimethylated H4-Arg[1] antibody and anti-dimethylated H3-Arg[3] antibody (Millipore). These antibodies do not react with recombinant \textit{P. falciparum} histones expressed in \textit{Escherichia coli} (results not shown). As a loading control, the blot was probed with anti-H4 antibodies.

Subcellular localization of PIIPRMT1

Immunofluorescence assays were performed as described in [41]. Briefly, infected RBCs were washed once with PBS and the cell pellet (~100 μl) was fixed with 1 ml of 4% (v/v) paraformaldehyde and 0.0075% (v/v) glutaraldehyde in PBS for 30 min. Fixed cells were washed twice with PBS and treated with 0.5% (v/v) Triton X-100 in PBS for 10 min. Then, cells were washed twice with PBS and blocked in 3% (v/v) BSA for 1 h at room temperature. The polyclonal anti-PIIPRMT1 antiserum (1:300 dilution) was added and incubated for another 2 h. After washing the cells three times with PBS, FITC-conjugated anti-rabbit IgG antibodies (Sigma) were added at 1:100 dilution in 3% (v/v) BSA containing DAPI (4',6-diamidino-2-phenylindole) and incubated for 1 h. Images were captured using a Nikon Eclipse E600 epifluorescent microscope. Localization of GFP-tagged PIIPRMT1 was determined similarly.

To estimate the distribution of PIIPRMT1 in the cytoplasmic and nuclear compartments of the parasite, ~100 μl of parasite pellet was resuspended in 300 μl of a hypotonic buffer [10 mM Hepes, pH 7.9, 1.5 mM MgCl\(_2\), 10 mM KCl, 0.5 mM DTT, 0.5 mM EDTA and 1% (v/v) protease-inhibitor cocktail (Roche)] and incubated on ice for 10 min. The parasites were mechanically lysed by 30–40 strokes in a Dounce homogenizer with a type-B pestle and then centrifuged at 2700 g for 10 min at 4°C. The supernatant was used as the cytoplasmic extract and the pellet was resuspended in 300 μl of PBS as the nuclear extract. Equal ratios (1:15) of the protein extracts (20 μl) were resolved by SDS/PAGE (15% gel), stained by Coomassie Blue and detected by immunoblotting using the anti-PIIPRMT1 antiserum (1:2000 dilution) and anti-histone H4 antibodies (1:1000 dilution; Millipore) as a control. For a cytoplasmic protein, we used a rabbit antiserum against the recombinant histidine-tagged \textit{P. falciparum} histone-like protein (PIPHL, PF10230c; http://www.plasmodb.org/plasmo), which specifically reacts with PfHLP in the parasite (results not shown).

Small-molecule inhibitors

To evaluate the potential effect of small-molecule inhibitors on recombinant PIIPRMT1 and parasite growth, the following compounds were selected: sinefungin, MTA (5′-deoxy-5′-methylthioadenosine), AMI-5 (eosin Y), chaetocin and AdOx (adenosine-2′,3′-dialdehyde). To estimate the IC\(_{50}\) values of these compounds for PIIPRMT1, 0.8 μM MBP–PIIPRMT1 and different concentrations of the inhibitors were pre-incubated at room temperature for 15 min in methylation buffer. Subsequently, 6 μM recombinant PfH4 and 0.3 μM \[^{3}H\]AdoMet were added, and the mixture was incubated at 30°C for 30 min. Reactions were stopped by incubating at 80°C for 10 min and spotted on to P-81 filters for liquid scintillation counting. Assays were performed in duplicate. To determine the effect of these chemicals for parasite growth, a luciferase-expressing strain, 3D7-luc, was used [42]. Late ring-stage parasites (100 μl) were seeded in triplicate in 96-well plates at 2% parasitaemia and 2% haematocrit and incubated at 37°C for 48 h with different concentrations of the chemicals. IC\(_{50}\) values were calculated by linear regression analysis [42].

RESULTS

\textit{Plasmodium} encodes conserved PRMTs

A BLASTP search of the \textit{P. falciparum} genome in PlasmoDB identified three PRMT candidates (Figure 1). Putative PIIPRMT1 (PF14_0242) and PIIPRMT5 (PF13_0323) have been identified previously [4,26]. PF08_0092 encodes a putative PRMT with all conserved methyltransferase motifs; it is probably a homologue of the mammalian CARM1 protein, because its homologue in \textit{T. gondii} could methylate Arg[7] in histone H3 [27]. No additional domains were identified in the PIIPRMT proteins. Phylogenetic analysis clearly identified PF14_0242 and PF13_0323 as PRMT1 and PRMT5 homologues respectively (Figure 1B). These three PRMTs are conserved in all \textit{Plasmodium} genomes sequenced so far.

PIIPRMT1 has 401 amino acids with a predicted molecular mass of 47.4 kDa. Compared with Rn (\textit{Rattus norvegicus}) PRMT1 and Sc (\textit{Saccharomyces cerevisiae}) HMT1 (heterogeneous nuclear ribonucleoprotein methyltransferase 1), the structures of which have been solved [43,44], PIIPRMT1 is ~50 amino acids longer in the N-terminus (Figure 1A). PIIPRMT1 contains the conserved methyltransferase motifs I, post-I, II, III and the THW loop. Most of the residues (10 of 13) involved in AdoMet binding are conserved [44]. In addition, Gly\(^{68}\), which is essential for the SchHMT1 activity, and RnPRMT1 Glu\(^{44}\) and Glu\(^{153}\), which are involved in binding the guanidine group in the arginine substrate, are also conserved in PIIPRMT1. The dimerization domain shows a lesser degree of conservation, with 62% amino acid identity.

Microarray data showed that PIIPRMT1 is expressed throughout the intraerythrocytic stages. Real-time RT–PCR analysis using RNA from synchronized parasites yielded a similar result, with the highest PIIPRMT1 mRNA level being detected in early trophozoites and the lowest level in schizonts (Figure 1C). To determine the transcription-initiation site and polyadenylation sites of PIIPRMT1 mRNA, RLM–RACE and 3′-RACE analyses were performed with RNA from asexual blood-stage parasites. From a total of 18 clones sequenced for the 5′-RLM–RACE, the 5′-ends were mapped to −560 (8), −528 (6), −527 (1), −529 (1), −533 (1) and −537 bp (1) upstream of the putative ATG codon (numbers in parentheses indicate the number of clones sequenced), indicating that PIIPRMT1 has a relatively long 5′-untranslated region (5′-UTR). Sequencing of 13 clones from 3′-RACE analysis detected two clusters of polyadenylation sites at 37 (1), 40 (6) and 49 bp (1), and at 363 (1), 364 (2) and 365 bp (2) downstream of the stop codon. Therefore the predicted PIIPRMT1 mRNA is approx. 1.8–2.1 kb in size.

PIIPRMT1 has conserved type I PRMT activity

As PIIPRMT1 has the conserved AdoMet-binding and dimerization motifs, we first tested whether these motifs are functional. Recombinant PIIPRMT1 was expressed in Bacterial Strain BL21 and used in UV-cross-linking experiments with radiolabelled AdoMet. The result showed that recombinant PIIPRMT1 could bind to AdoMet (Figure 2A). This binding was
**Figure 1**  Sequence conservation, phylogeny and gene expression of *PfPRMT1*

(A) Alignment of *PfPRMT1* with *RnPRMT1* (GenBank® accession number NP_077339) and *ScHMT1* (GenBank® accession number NP_009590). Identical and similar amino acids are shaded in black and grey respectively. The methyltransferase signature motifs I, post-I, II, III and the THW loop are boxed. Borders of the cofactor-binding domain and dimerization domain are marked by black arrows. Asterisks below the sequences indicate residues involved in AdoMet binding. Downward arrows indicate the sites of the truncation in *PfPRMT1* for activity analysis. 

(B) Phylogenetic analysis of the human proteins *HsPRMT1* (NP_001527), *HsPRMT3* (AAH37544), *HsCARM1* (Q86×55), *HsPRMT5* (O14744), *HsPRMT6* (AAH73866), *HsPRMT7* (NP_061896) and *HsPRMT8* (AAH22458). *ScHMT1*, *RnPRMT1* and three *P. falciparum* PRMTs (all accession numbers are from GenBank®). Shown is the the UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) tree constructed using the MEGA (Molecular Evolutionary Genetics Analysis) program (http://www.megasoftware.net). 

(C) Real-time RT–PCR determination of the relative expression levels of *PfPRMT1* during the IDC of *P. falciparum*. IDC stages: R, ring; ET, early trophozoite; LT, late trophozoite; S, schizont.

**Figure 2**  UV-cross-linking to detect specific binding of *PfPRMT1* to AdoMet

(A) Upper panel: Coomassie-Blue-stained gel. Either recombinant MBP–*PfPRMT1* (upper bands) or *PfPRMT1* (lower band) was used in the binding reactions. A 1000-fold excess of unlabelled ATP or AdoMet was included as the competitor. Lower panel: fluorograph. 

(B) Oligomerization of recombinant *PfPRMT1*. Recombinant *PfPRMT1* was incubated without glutaraldehyde (lane 1), or with 0.025 % glutaraldehyde for 3 min (lane 2) and 5 min (lane 3) respectively. After cross-linking, the proteins were separated by SDS/PAGE (10 % gel) and probed with the anti-*PfPRMT1* antiserum. Monomers, dimers, tetramers and multimers are indicated. The asterisk indicates a bacterial protein present in the purified *PfPRMT1* that does not show detectable cross-linking under these conditions.
PfPRMT1 has broad substrate specificity

In model eukaryotes, PRMT1 methylates a wide variety of substrates, including histones, transcription factors and a number of proteins involved in RNA processing, transport and translation [1]. Among them, fibrillarin [47], PABPII [48,49], Gar1p homologue [50] and rpS2 [51] are evolutionarily conserved in P. falciparum. In addition, we have also identified several potential splicing factors, which have putative RGG, RG or RXG boxes. In order to determine whether these P. falciparum proteins could serve as the PfPRMT1 substrates in vitro, these proteins or their GAR-motif-containing domains were expressed in E. coli as either GST- or MBP-fusion proteins (Supplementary Table S1), and were tested in methylation reactions using [3H]AdoMet. The results showed that fibrillarin (PF14_0068), PABPII (PF11175c), IP of parasite lysates using preimmune serum or polyclonal antiserum against recombinant PfPRMT1. Proteins precipitated with the PfPRMT1 antiserum had authentic methyltransferase activity to methylate recombinant PfH4, whereas this activity was not observed with proteins precipitated with the preimmune serum (Figure 3C).

PRMT1 exhibits important sequence variations in the first 40 amino acids at the N-terminus. In the rat PRMT1, deletion of the first 14 amino acids does not affect the enzymatic activity, whereas truncation to amino acid 38 abolishes enzymatic activity [44]. In the yeast HMT1, the first 15 amino acids are not essential, but deletion of the first 20 amino acids results in markedly reduced enzyme activity [43]. PfPRMT1 has ~50 extra amino acids in the N-terminus compared with the yeast and rat homologues (Figure 1A). To determine whether this sequence is important for methyltransferase activity, we generated several truncations of PfPRMT1 and tested their enzyme activity using recombinant H4 as the substrate. We found that deletion of the first 34 amino acids had no effect on methyltransferase activity (Figure 4). However, truncation to amino acid 56 completely abolished the enzyme activity. Similarly, the C-terminal 54 amino acids were also critical for the enzyme activity (see the next section).
Figure 5  PIPRMT1 activity on potential substrates

Nine *P. falciparum* proteins were expressed in *E. coli* as either GST- or MBP-fusion proteins, purified and incubated with MBP–PIPRTM1 in methylation assays. Upper panel: Coomassie-Blue-stained gel. Lower panel: fluorograph. GST-fusion proteins include PABPII (1–202), fibrillarin (1–100), putative PABP (1–383), Gar1-like (1–209), PF10_0068 (1–246), PFF1135w (128–347) and PF10_0217 (391–538). MBP-fusion proteins include PFE0865c (1–298), PFE0865c-C (199–298), PFE0865c-N (1–112) and rpS2 (1–60).

rpS2 (PF14_0448) and a putative splicing factor (PFE0865c) (http://www.plasmodb.org/plasmo) could be methylated in vitro, indicating that they are potential physiological substrates for PIPRMT1 (Figure 5). It is noteworthy that PIPRMT1 methylated the N-terminal polypeptides of fibrillarin (amino acids 1–100) and rpS2 (amino acids 1–60), which have extensive GAR motifs. Among the putative splicing-factor-like proteins, PFE0865c could be methylated by PIPRMT1, suggesting that PIPRMT1 may affect pre-mRNA splicing events. PFE0865c is an arginine- and serine-rich protein with two RNA-recognition motifs. One typical RGG motif and three RG motifs are located in the N-terminus between the two RNA-recognition motifs, while the C-terminal 100 amino acids are highly enriched with serine (36 of 100 amino acids). To determine which region was methylated by PIPRMT1 in PFE0865c, we expressed the N-terminal RG-containing fragment (amino acids 1–112) and the C-terminal serine-rich domain (amino acids 199–298) separately. The results showed that both regions could be methylated by PIPRMT1. Since the C-terminal domain contained only one RSG sequence, which was shown to be a very poor substrate for PRMT1, this result supported the notion that PRMT1 could methylate arginine residues beyond the RG motifs [52].

**PIPRMT1 enzyme kinetics with substrate peptides**

Having determined that *P. falciparum* histone H4, PABPII and fibrillarin were in vitro substrates of recombinant PIPRMT1, we wanted to identify the substrate arginines, and determine their methylation status and dynamics. To confirm that H4-Arg3 is the only arginine in the N-terminus that is methylated by PIPRMT1, we synthesized the wild-type H4 peptide (amino acids 1–21) and H4 peptide with the R3K mutation (PH4-21R3K). Despite the fact that PH4-21 contains three arginines, only Arg3 is in the RG context. The results showed that only the wild-type PH4-21 peptide, but not PH4-21R3K, was methylated by PIPRMT1, demonstrating the specificity of PIPRMT1 for Arg3 (Figure 6A). In addition, the PABPII peptide (PF11175c-16) with two RG motifs was also methylated by PIPRMT1 (Figure 6A). To determine the methylation status and dynamics of the peptides, time-course studies were performed with these peptides, and the methylation products were analysed by MS. The spectra showed that the two RG motifs in the PABPII peptide and the two RGG motifs in fibrillarin were both methylated by PIPRMT1 (Figure 6B). For both peptides, the predominant forms of the methylation products at 60 min contained two methyl groups. For the H4 peptide, the presence of only one arginine for methylation enabled us to analyse the dynamics of the methylation reactions. By 20 min of the reaction, approximately equimolar amounts of MMA and DMA were detected (Figures 6B and 6C), which conforms to the partially processive mechanism of the reaction proposed for PRMT1, and suggests that monomethylation and dimethylation occur sequentially without the release of the monomethylated product [31]. By 2 h, the Arg3 in the H4 peptide was predominantly dimethylated (Figure 6C).

The kinetics of the H4-Arg3 methylation in the PH4-21 peptide was characterized and compared with the mammalian homologues. Although the kinetic parameters were generally agreeable with those of the rat PRMT1 [31], PIPRMT1 had a >5 times higher $k_{cat}/K_m$ value and an approx. 2 times higher $k_{cat}/K_m$ value than the mammalian homologue for the peptide and AdoMet substrates respectively (Table 1).
Figure 6  PIPRMT1 activity on peptides

(A) Recombinant MBP–PIPRT1 activity on peptides. Upper panel: Peptides PfH4-21, PfH4-21R3K, and PfI1175c-16 were incubated with MBP–PIPRT1, separated by SDS/PAGE (20% gel) and stained by Coomassie Blue. Lower panel: Methylation of the peptides was detected by fluorography. (B) Spectra of the MALDI–TOF analysis of methylation of peptides PfH4, PF14_0068, and PFI1175c by recombinant MBP–PIPRT1 to indicate the unmodified and methylated (MMA, DMA) peptides (with a mass increment of 14). H4 peptide has only one substrate arginine, while the other two peptides both contain two substrate arginines. (C) A time-course analysis of methylation of PfH4-21 by MBP–PIPRT1. The graph is a representative of two experiments to show the relative quantities of the unmodified substrate and the products with MMA (mono-R) and ADMA (di-R).

Table 1  Characterization of the kinetics for the recombinant PIPRMT1

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*Parameters for the rat PRMT1 (rPRMT1) were from [31].
Plasmodium PRMT1

Figure 7 PfPRMT1 expression in the parasite

(A) Right panel: Time-course expression of PfPRMT1 during the IDC. Equal amounts of parasite lysate (∼30 μg) were separated by SDS/PAGE (10% gel) and probed with polyclonal anti-PfPRMT1 antiserum. Left panel: the Coomassie-Blue-stained gel to indicate approximately equal loading. The asterisk indicates the cross-reacting protein. Stages: R, ring; ET, early trophozoite; LT, late trophozoite; S, schizont.

(B) Confirmation of the C-terminal GFP-tagging of the endogenous PfPRMT1 locus. Two clones (GFP-1 and GFP-2) were probed with the anti-PfPRMT1 antiserum (left panel) or the anti-GFP antibody (right panel). Lysate from wild-type 3D7 parasite was included as a GFP-negative control.

PfPRMT1 protein expression in the parasite

To detect PfPRMT1 expression in the parasite, polyclonal rabbit antiserum was produced against recombinant PfPRMT1. First, PfPRMT1 expression was assessed by Western blotting using equal amounts of protein lysates from synchronized parasites. Western blotting detected a protein band of ∼47 kDa, consistent with the predicted molecular mass of PfPRMT1 (Figure 7A). PfPRMT1 was detected throughout the IDC, with higher levels detected in trophozoites. In addition, a protein band of ∼70 kDa cross-reacted with the PfPRMT1 antiserum (left panel) or the anti-GFP antibody (right panel). Lysate from wild-type 3D7 parasite was included as a GFP-negative control.

Histone-arginine methylation during the IDC of the parasite

Histones from synchronized parasites were purified, separated and probed with anti-dimethylated H4-Arg^3 (H4R3m2) and anti-dimethylated H3-Arg^17 (H3R17m2) antibodies. Immunoblotting with anti-H4 antibodies was included to indicate equal loading. Stages: R, ring; ET, early trophozoite; LT, late trophozoite; S, schizont.

Histone-arginine methylation during parasite development

With H4 being determined as a PfPRMT1 substrate in vitro, we next wanted to determine the presence and dynamics of H4-Arg^3 dimethylation during the IDC. The result showed that H4-Arg^3 dimethylation was present in all asexual erythrocytic stages, with much higher levels found in late trophozoites and schizonts (Figure 8). In comparison, the level of dimethylated H3-Arg^17, possibly modified by the CARM1-like PRMT in the parasite, was prevalent throughout the IDC.

Subcellular localization of PfPRMT1

In the parasite cell line with GFP-tagged PfPRMT1, strong GFP fluorescence was detected in both the cytoplasm and the nucleus (Figure 9A). Similar results were obtained with wild-type 3D7 parasites by immunofluorescence assay using anti-PfPRMT1 antiserum (results not shown). Further analysis of the nuclear–cytoplasmic distribution of PfPRMT1 was performed using fractionated parasite lysates. Parasites were fractionated into nuclear and cytoplasmic extracts, and the proteins from each fraction were resolved by SDS/PAGE and subjected to immunoblotting. The correct fractionation of parasites was demonstrated by the predominant detection of histone H4 in the nuclear fraction and the apicoplast-specific protein PfHLP in the cytoplasmic fraction [53]. The results showed the presence of PfPRMT1 in both cellular fractions, with the majority localized in the cytoplasm (Figure 9B). Only a minor fraction of PfPRMT1 was present in the nucleus, which is similar to the cellular distribution of human PRMT1 [54]. This is consistent with the presence of nuclear (e.g. fibrillarin and histones) and cytoplasmic (e.g. rpS2) substrates.

The effect of small-molecule inhibitors

AdoMet-dependent methyltransferases are inhibited by the reaction product, SAH (S-adenosyl-L-homocysteine). Among the two structural analogues of SAH, sinefungin was a potent inhibitor of PfPRMT1, with an IC₅₀ of <1 μM, whereas MTA was a poor inhibitor (Table 2). These values are compatible with those for recombinant human PRMT1 [31]. AMI-5, recently identified as a general inhibitor for arginine and lysine methyltransferases.
Figure 9 Subcellular localization of PfPRMT1 in the ring, trophozoite and schizont stages

(A) Localization of GFP–PfPRMT1. Representative images of GFP–PfPRMT1 in a ring-, trophozoite-, and schizont-stage parasite. Nuclei were stained with DAPI. ‘Triple’ indicates merging of the light, GFP and DAPI images of the same cells. (B) Western blots of the parasite nuclear (N) and cytoplasmic (C) fractions, separated by SDS/PAGE (10 % gel), and probed with anti–PfPRMT1 antiserum (upper panel), anti–H4 antibodies (middle panel) and anti–PfHLP antiserum (lower panel).

[55], also displayed strong inhibitory activity for PfPRMT1. In comparison, the fungal metabolite chaetocin, an inhibitor for the lysine-specific histone methyltransferase SU(VAR)3-9 [56], displayed no obvious inhibition of PfPRMT1 at a lower micromolar concentration. When tested on the intraerythrocytic stages of the malaria parasites, all compounds except MTA had potent parasite-growth-inhibition activities, with IC₅₀ values below 15 μM (Table 2). AdOx, an inhibitor for SAH hydrolase, also showed similar inhibitory activity on parasite growth. AdOx treatment may have affected all AdoMet-dependent methyltransferases, thus leading to parasite-growth inhibition, because it can elevate the cellular level of SAH [57].

DISCUSSION

In the present study, we present biochemical evidence to show that PfPRMT1 has intrinsic type I PRMT activity, catalysing the formation of MMA and ADMA. In particular, PfPRMT1 shares significant sequence homology and similar enzymatic activity on potential substrates to PRMT1 homologues in higher eukaryotes. Although the methyltransferase motifs in PfPRMT1 are highly conserved, its extra 50-amino-acid N-terminal sequence is not only highly divergent from the mammalian orthologues, but also essential for its enzymatic activity. Furthermore, despite the observation that enzyme-kinetic parameters of the recombinant PfPRMT1 are similar to those of its mammalian enzymes, PfPRMT1 has much higher turnover rates. These and other differences from the mammalian enzymes may provide the basis for developing specific inhibitors for the parasite enzyme.

Methylation of arginines in histones has profound effects on gene expression [12]. We showed that recombinant PfPRMT1 methylates H4 predominantly at Arg³, forming MMA and ADMA. Besides, both forms of H4-Arg³ methylation are present during the entire IDC. Furthermore, both PfPRMT1 and asymmetric H4-Arg³ dimethylation are dynamic during parasite development, exhibiting similar patterns of increases as the parasites mature (Figures 7 and 8). We have also confirmed the presence of H3-Arg¹⁷ methylation in the parasite, a potential substrate of the CARM1-like PRMT (Figure 8). Although the methylation of H4-Arg³ and H4-Arg¹⁷ is known to be involved in transcription activation in mammals [11,58,59], its significance in the epigenetics of the malaria parasite awaits further elucidation.

Besides histones, a large number of cellular proteins have been identified as physiological substrates of PRMT1s [1]. PfPRMT1 could methylate four of the nine selected P. falciparum proteins, including the conserved PRMT1 substrates fibrillarin and PABPII. Fibrillarin is an evolutionarily conserved ribose-2′-O-methylase that is associated with C/D-class small nucleolar RNAs, and involved in rRNA maturation and ribosome assembly [60]. PABPII is a nuclear protein involved in mRNA polyadenylation [49,61]. In addition, PfPRMT1 could methylate a new GAR-motif-containing splicing-factor-like protein (PFE0865c; http://www.plasmodb.org/plasmo) in vitro. These results strongly indicate conserved functions for PfPRMT1 in RNA processing and metabolism. In higher eukaryotes, the evolution of many more PRMTs allows functional divisions of PRMTs. Malaria parasites have only three PRMTs; thus each of these protozoan PRMTs may have a broader substrate range, which may represent their ancestral functions. Interestingly, we also found that PfPRMT1 could methylate rpS2, a physiological substrate of PRMT3 [62], in vitro, suggesting that PfPRMT1 might affect ribosome biogenesis and the translational machinery. In trypanosomes, PRMT1 also methylates proteins that affect the metabolism of specific mitochondrial mRNAs [29]. Therefore investigations into the in vivo substrates of protozoan PRMT1s may reveal novel pathways in which these enzymes play regulatory roles.

PRMTs have been considered promising pharmaceutical targets [4]. We have shown that the inhibitors sinefungin and AMI-5 both had lower micromolar IC₅₀ values for the recombinant PfPRMT1 and the parasite growth. This result indicates that parasite methyltransferases could serve as potential targets for
the development of antimalarials. As most of the compounds tested presumably affect all methyltransferase activities non-specifically, it is highly desirable to screen for specific inhibitors for different PRMTs [55]. The robust PRMT activity of the recombinant PiPRMT1, and its divergence from the mammalian PRMT1s, may provide such a tool for screening specific inhibitors.

**AUTHOR CONTRIBUTION**
Qi Fan designed and performed most of the experiments and participated in writing the manuscript. Jun Miao participated in the cellular localization studies, Long Cui performed the drug assays in parasites, and Liwang Cui supervised this research and participated in writing the manuscript.

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We thank Dr Hesheng Zhang (Department of Entomology, The Pennsylvania State University, PA, U.S.A.) for providing the PHEL P antiserum and Ms Xiaolian Li for technical assistance.

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**SUPPLEMENTARY ONLINE DATA**

Characterization of PRMT1 from *Plasmodium falciparum*

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Department of Entomology, The Pennsylvania State University, 501 AG Sciences & Industries Building, University Park, PA 16802, U.S.A.

Table S1 Primers used for cloning PIPRMT1 and potential substrates

Restriction sites are underlined.

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Table S2 Primers used for studying PIPRMT1 expression

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