BBA - Gene Regulatory Mechanisms 1860 (2017) 1047–1057

Contents lists available at ScienceDirect

BBA - Gene Regulatory Mechanisms

journal homepage: www.elsevier.com/locate/bbagrm

DNA methyltransferase homologue TRDMT1 in *Plasmodium falciparum* specifically methylates endogenous aspartic acid tRNA

Gayathri Govindaraju, CA. Jabeena, Devadathan Valiyamangalath Sethumadhavan, Nivethika Rajaram, Arumugam Rajavelu*

Department of Tropical Disease Biology Group, Rajiv Gandhi Centre for Biotechnology, Thycaud PO, Thiruvananthapuram, Kerala 695 014, India

ARTICLE INFO

Keywords:
Plasmodium
RNA
Epigenetics
tRNA methylation and cytosine methyltransferase

ABSTRACT

In eukaryotes, cytosine methylation regulates diverse biological processes such as gene expression, development and maintenance of genomic integrity. However, cytosine methylation and its functions in pathogenic apicomplexan protozoans remain enigmatic. To address this, here we investigated the presence of cytosine methylation in the nucleic acids of the protozoan *Plasmodium falciparum.* Interestingly, *P. falciparum* has TRDMT1, a conserved homologue of DNA methyltransferase DNMT2. However, we found that TRDMT1 did not methylate DNA, *in vitro*. We demonstrate that TRDMT1 methylates cytosine in the endogenous aspartic acid tRNA of *P. falciparum*. Through RNA bisulfite sequencing, we mapped the position of 5-methyl cytosine in aspartic acid tRNA and found methylation only at C38 position. *P. falciparum* proteome has significantly higher aspartic acid content and a higher proportion of proteins with poly aspartic acid repeats than other apicomplexan pathogenic protozoans. Proteins with such repeats are functionally important, with significant roles in host-pathogen interactions. Therefore, TRDMT1 mediated C38 methylation of aspartic acid tRNA might play a critical role by translational regulation of important proteins and modulate the pathogenicity of the malarial parasite.

1. Introduction

Nearly 300 million malaria infections occur annually resulting in over half a million deaths [1]. The severe form of malaria evolves through interplay among capillary sequestration of parasitized erythrocytes, a deregulated inflammatory response and hemostasis dysfunction. *P. falciparum* is an obligate intracellular parasite, which grows in two different hosts and requires both human and vector mosquito to complete its life cycle [2]. The female anopheles mosquito injects 20–100 sporozoites to human dermis to initiate the infection. These sporozoites reach liver *via* blood stream. Further, the parasite multiplies in RBCs and typical malaria symptoms occur. The parasite has a complex genetic makeup, an AT rich genome and contains diverse epigenetic modifications including the presence of unusual histone variants and a unique set of histone modifications [3–5]. However, the role of various epigenetic modifications in the regulation of gene expression in *P. falciparum* is poorly understood. Recent reports suggest that *P. falciparum* under goes massive changes in transcriptional activity during RBC cycle (clinical phase) and antigenic variation processes, indicating an important role of epigenetic players at different stages of the parasite life cycle [6,7]. In addition, the parasite has several unique features in its epigenome such as the absence of linker histone H1, RNA interference machinery and presence of various unusual histone variants with unique set of modifications [8]. The malarial parasite genome is highly acetylated and majority of its chromosomes constitute euchromatin with several short heterochromatic regions [8–10]. DNA methylation and repressive chromatin modifications are essential for heterochromatin structure formation and gene repression in many eukaryotes [11,12]; but the roles of the various chromatin modifications and DNA methylation in *P. falciparum* have remained obscure.

Although the *P. falciparum* genome encodes a protein with C5 methyltransferase motif, there is a lack of clarity on DNA methylation in the malarial parasite [13]. Importantly, DNA methylation is absent in other apicomplexan group of protozoan parasites such as Toxoplasma and Cryptosporidium [14]. While the absence of 5-methyl cytosines in the genome of *P. falciparum* [13], few studies have shown the presence of DNA methylation in the *P. falciparum* genome by restriction digestion based [15] and mass spectrometry based assays [16].

Understanding the nucleic acid methylation of an important human pathogen *P. falciparum* is paramount in elucidating the mechanisms of gene regulation that affect parasite survival and pathogenicity. The genome sequencing of the parasites revealed the presence of a putative C5 methyltransferase, a homologue of DNMT2 called TRDMT1 (tRNA Aspartic acid Methyltransferase 1) (PlasmoDB: PF3D7_0727300) which

* Corresponding author at: Tropical Disease Biology Group, Rajiv Gandhi Centre for Biotechnology, Thycaud Post, Thiruvananthapuram, Kerala 695 014, India.
E-mail address: arajavelu@rgcb.res.in (A. Rajavelu).

http://dx.doi.org/10.1016/j.bbagrm.2017.08.003
Received 23 June 2017; Received in revised form 17 August 2017; Accepted 21 August 2017
Available online 26 August 2017
1874-9399/

carries a characteristic CFT motif required for tRNA specific methylation activity and also contains the conserved motifs specific for DNA methyltransferases [17,18]. Phylogenetic analysis revealed that *P. falciparum* TRDMT1 clustered into tRNA specific methyltransferase family [19]. It is still unclear whether *P. falciparum* TRDMT1 methylates DNA and/or tRNA.

To address this, here we have characterized the functions of Pf-TRDMT1. We found that the Pf-TRDMT1 methylates tRNA aspartic acid of the parasite and does not methylate DNA *in vitro*. RNA bisulfite sequencing analyses confirmed the presence of cytosine methylation at C38 position of endogenous aspartic acid tRNA, close to anti codon loop of aspartic acid tRNA.

Strikingly, *P. falciparum* proteome is enriched for aspartic acid and proteins with polyAsp repeats that are functionally important. Thus, methylation of aspartic acid tRNA by TRDMT1 might be an important step in translational regulation of such important proteins which could influence the pathogenicity of *P. falciparum*.

2. Experimental procedures

2.1. P. falciparum culture maintenance and isolation

The *P. falciparum* 3D7 strain was maintained under laboratory conditions as described previously [20]: The parasites were cultivated in 5% O + hematocrit in RPMI1640 (GIBCO) supplemented with 10% of O + human plasma. Parasite cycle was synchronized and the late trophozoite stage parasites were released from infected RBCs by 0.15% saponin treatment. Lysed RBCs were pelleted at 10,000 RPM at 4 °C for 10 min to separate intact parasites and the parasites were washed with ice cold PBS for 3 times and stored at − 80 °C for RNA and DNA preparation.

2.2. Cloning, expressions and purification of Pf-TRDMT1

Full length Pf-DNMT2 construct was prepared by PCR amplification from genomic DNA of *P. falciparum* using gene specific primers (FP 5′GCGGCGGATCCATGCACAAAATAAAGGTACTGGAA3′, RP 5′CGCGC CTCGAGTTATATGTGTATATGCTCAAATA3′) and cloned into pET28a (+) (Novagen) vector using *Bam*HI and *Xho*I sites. The sequence encoding amino acids 144 to 706 of Pf-TRDMT1 was cloned into pET28a using gene specific internal forward primer (FP 5′-GCGGC GGATCCGAAAAGAATAATATTTTTAATAT-3′) and above mentioned reverse primer to generate a Pf-NΔTRDMT1 construct. All the clones were sequenced to confirm the frame alignment. The Pf-TRDMT1 protein was expressed in Rosetta2 DE3 cells: The cells were grown till OD_{600} 0.8 and the cells were shifted to 18 °C. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) (sigma) and incubated further over night with shaking. The cells were collected and suspended with sonication buffer (30 mM Sodium phosphate buffer pH 7.5, 300 mM NaCl, 0.2 mM DTT and 10% glycerol), containing protease inhibitor cocktail (Sigma) and lysed with sonicator using 36 amps with 1 s on and 1 s off. The soluble fraction was collected by high-speed centrifugation and passed to the column packed with His60 Ni Super flow resin (Takara-Clontech). The column was washed with sonication buffer containing 20 mM imidazole and the bound proteins were eluted using sonication buffer containing 300 mM imidazole. The proteins were dialyzed against the buffer (30 mM sodium phosphate pH 7.5, 150 mM NaCl, 0.2 mM DTT and 10% glycerol) for 2 h at 4 °C and the proteins were aliquoted as small fraction and snap frozen. The concentration of the proteins was measured using nanodrop and purity was analyzed on SDS-PAGE gel, stained with coomassie brilliant blue stain. Protein identities were confirmed by Western blot using anti-His antibody. The mouse Dnmt3a was used as positive control for DNA methylation assays, was overexpressed and purified as described earlier [21].

2.3. Synthesis of tRNAs by in vitro transcription

The aspartic acid and valine tRNAs of *P. falciparum* were synthesized by *in vitro* transcription using substrate DNA containing T7 RNA polymerase promoter. The primer sequences and template sequences used for *in vitro* transcription are given in Table S1. Transcription was carried out in 200 μL reactions using transcription buffer containing NTPs (Thermo Scientific) and T7 RNA polymerase (Thermo Scientific) in the presence of RNasin (Promega) and reaction mixture was incubated at 37 °C for 3 h. After 3 h incubation, transcribed products were treated with the DNase for 1 h at 37 °C. The products were then separated on 8 M urea-acrylamide gel. The bands were precisely cut from the gel and eluted using elution buffer (50 mM Tris pH 8.0, 300 mM Sodium acetate, 0.5% SDS). The eluted tRNAs were precipitated in 100% ethanol at − 80 °C for 30 min and − 20 °C for 2 h followed by 80% ethanol wash at 4 °C. Precipitated tRNA pellets were dissolved with nuclease free water and concentration of tRNAs were measured using nano drop, while the quality of transcribed tRNAs were analyzed on 8 M urea gel (Fig. S3). The mutant C38U aspartic acid tRNA and human aspartic acid tRNAs were synthesized as mentioned above and the quality was analyzed on 8 M urea gel.

2.4. DNA and RNA methylation assays for Pf-NΔTRDMT1

DNA methylation activity for purified Pf-NΔTRDMT1 and mouse DNMT3A–C was carried with CpG rich DNA substrates as described earlier [21]. Briefly, the reaction mix was prepared in methylation buffer (20 mM HEPES pH 7.6, 50 mM KCl, 1 mM EDTA) containing 600 nM of CpG rich DNA, 2 μM of Pf-NΔTRDMT1 and the reaction was initiated by addition of 0.76 μM tritium labeled S-Adenosyl-L-methionine (SAM) (PerkinElmer). The reaction was incubated at 37 °C. Methylation reaction was stopped in buffer containing 10 mM non-radiolabelled SAM at various time points. Methylated DNA was bound to glass fiber membrane (GE healthcare) and unreacted SAM and proteins were washed out using 70% ethanol followed by 5% TCA. The filter paper was air dried and mixed with scintillation cocktail (PerkinElmer) and the counts were measured using a scintillation counter. Experiments were repeated at least thrice. The CPM values were subtracted from the background counts and average values were plotted. Linear regression was calculated using $y = mx + c$ formula and the slope values were calculated to obtain the initial rate of enzyme activity. The tRNA methylation activity for Pf-NΔTRDMT1 was measured as described elsewhere [18,22]. Briefly methylation reaction was prepared in 20 μL volume containing methylation buffer (20 mM Tris pH 8.0, 20 mM Ammonium acetate, 2 mM $MgCl_2$, 2 mM DTT and 0.02 mM EDTA), 25 μg/mL BSA, 600 nM of each tRNA substrates and 2 μM Pf-NΔTRDMT1. Methylation reaction was initiated by addition of 0.76 μM tritium labeled SAM and incubated at 37 °C. The methylation reaction was stopped at different time points by addition of 2.5 μL from the reaction to the 40 μL of stop buffer (5% TCA). As described above, the methylated tRNAs were bound to glass fiber membrane and the unreacted radiolabeled SAM and proteins were washed out using 70% ethanol followed by 5% TCA. The counts were measured, plotted as described above and the standard error bar was calculated from atleast three experimental replicates.

2.5. tRNA methylation assay by denatured urea gel

Methylation of various tRNAs by Pf-NΔTRDMT1 was characterized *in vitro* by urea gel assay. The assay for Pf-NΔTRDMT1 was carried out with CpG rich DNA substrate, aspartic acid DNA substrate as control samples and *in vitro* transcribed *P. falciparum* aspartic acid tRNA as test sample. Methylation reactions were carried out with methylation buffer (20 mM Tris pH 8.0, 20 mM ammonium acetate, 2 mM MgCl2, 2 mM DTT and 0.02 mM EDTA), 25 μg/mL of BSA; Reaction was initiated by addition of 0.76 μM tritium labeled S-Adenosyl-L-methionine. Reaction