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In vitro and in vivo analysis of the major type I protein arginine methyltransferase from *Trypanosoma brucei*

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Abstract

In mammals and yeasts, arginine methylation, catalyzed by protein arginine methyltransferases (PRMTs), has been implicated in regulation of diverse processes such as protein–protein interaction, protein localization, signal transduction, RNA processing, and transcription. A large number of PRMT substrates are RNA binding proteins. In trypanosomes, gene regulation is controlled primarily at the levels of RNA processing, stability, and translation, and likely involves numerous RNA binding proteins. Thus, arginine methylation may be especially important in controlling gene expression in this evolutionarily ancient group of organisms. To begin to understand the role of arginine methylation in trypanosomes, we identified and characterized a type I PRMT from *Trypanosoma brucei*, termed TbPRMT1. TbPRMT1 displays 51% amino acid identity to human PRMT1. It possesses an S-adenosylmethionine binding site and double E and THW loops, common and absolute features associated with other PRMTs. Recombinant TbPRMT1 methylates both an artificial RG-rich peptide and the *T. brucei* mitochondrial RNA binding protein, TBRGG1, and it exhibits differences in substrate specificity compared to rat PRMT1. TbPRMT1 is constitutively expressed during the *T. brucei* life cycle. Disruption of TbPRMT1 gene expression by RNA interference did not result in a significant growth defect in procyclic form *T. brucei*. Finally, we observe a dramatic decrease in the cellular level of asymmetric dimethylarginine upon TbPRMT1 knock down, indicating that TbPRMT1 is the predominant type I PRMT in *T. brucei*. The strong conservation of PRMT1 homologs between protozoa and humans highlights the importance of arginine methylation as a regulatory mechanism in eukaryotes.

Keywords: Arginine methylation; Asymmetrical dimethylarginine; RNA binding proteins; Gene regulation; Kinetoplastid

1. Introduction

Protein arginine methylation is an irreversible post-translational modification resulting in the addition of methyl groups from *S*-adenosylmethionine (AdoMet) to the nitrogen of arginine residues in proteins (reviewed in [1,2]). Arginine methylation has been described in a variety of organisms including mammals [3–5], yeast [6,7], filamentous fungi [8], *Drosophila* [9], *Xenopus* [10], trypanosomes [11], and during infection by adenovirus [12]. Recent reports have established a role for arginine methylation in the control of signal transduction [13–15], RNA transport [16,17], RNA processing [18,19], protein localization [20,21], and transcription (reviewed in [22]). However, the functional significance of many arginine methylation events is currently unknown. Arginine methylation is catalyzed by enzymes known as protein arginine methyltransferases (PRMTs). In mammalian cells, two major and distinct types of PRMTs have been identified [1]. Type I enzymes catalyze the formation of both monomethylarginine (MMA) and asymmetrical dimethylarginine (ADMA), while the type II enzyme forms MMA and symmetrical dimethylarginine (SDMA). Asymmetrical dimethylation catalyzed by type I PRMTs often occurs within glycine-arginine-rich domains [1,23]. RNA binding proteins, particularly those containing RGG RNA binding motifs, are common type I PRMT substrates. Known type I substrates include hnRNP Al and A2 [20,23], nucleolin [24], fibrillarin [25,26], the yeast Npl3 [27], Ewing sarcoma protein [28], SAM68 [29], and the *Trypanosoma brucei* RNA editing accessory factor, RBP16 [11]. Transcriptional regulatory proteins such as p300 [30] and STAT1 [31], as well as histones [32,33] are also asymmetrically methylated by type I PRMTs. Sm proteins Dl, D3, and B/B′, which were originally

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reported to contain only SMDA [18,34], have also recently been shown to contain ADMA, indicating that some proteins may be substrate for both type I and type II PRMTs, or that certain PRMTs carry both activities [35]. Mammals contain multiple type I PRMTs, while budding yeast possess only one type I enzyme, a homolog of mammalian PRMT1, termed HMT1 [2].

T. brucei is the etiological agent of African sleeping sickness in humans and nagana in African livestock. It is an evolutionarily ancient organism, further removed from yeast than yeast is from humans [36]. In this parasitic protozoan, transcriptional gene regulation is essentially absent. Rather, gene expression is controlled primarily at the levels of RNA processing, stability, and translation, and likely involves a substantial number of RNA binding proteins [37–39]. Interestingly, a large percentage of type I PRMT substrates are RNA binding proteins, suggesting that arginine methylation is likely to play a key role in the control of gene expression in *T. brucei*. To understand the roles of arginine methylation in trypanosome gene expression, we began by identifying PRMT activities and PRMT encoding genes in *T. brucei*. We have previously reported the presence of both type I and type II PRMT activities in *T. brucei* cell extracts [11]. In addition, we demonstrated that the mitochondrial RNA editing accessory protein, RBP16, is methylated on at least three arginine residues in vivo [11]. A type I PRMT activity present in *T. brucei* whole cell extracts methylates RBP16, as well as multiple unidentified endogenous proteins [11].

Here, we identify and characterize a type I PRMT from *T. brucei*, which we term TbPRMT1. TbPRMT1 is 51% identical to human PRMT1 at the amino acid level and possesses common and absolute features associated with other PRMTs. Recombinant TbPRMT1 is able to methylate both an artificial RG-rich peptide and the *T. brucei* mitochondrial RNA binding protein, TBRGG1, in vitro. Despite the high sequence similarity between TbPRMT1 and rat PRMT1, these enzymes display somewhat different substrate specificities. Disruption of TbPRMT1 gene expression by RNA interference (RNAi) had no significant effect on the growth of procyclic form *T. brucei*. Finally, TbPRMT1 down-regulation leads to a dramatic decrease in cellular ADMA levels, indicating that TbPRMT1 is the major PRMT in vivo in *T. brucei*.

2. Materials and methods

2.1. Trypanosome growth

Procyclic form *T. brucei brucei* clone IsTaR1 stock EATRO 164 was grown as described [40]. Bloodstream form *T. brucei* strain 427 (a generous gift from Dr. George A.M. Cross, Rockefeller University) was cultured in HMI-9 medium as described [41]. Procyclic *T. brucei* strain 29-13 (provided by Dr. George A.M. Cross, Rockefeller University), which contains integrated genes for T7 RNA polymerase and the tetracycline repressor, were grown in SDM-79 supplemented with 15% fetal bovine serum (FBS) as described [40,42], in the presence of G418 (15 μg/ml) and hygromycin (50 μg/ml).

2.2. Transfection and induction of TbPRMT1 RNAi

To achieve down-regulation of TbPRMT1 expression by RNAi, a 503-bp fragment of the TbPRMT1 coding region corresponding to nucleotides 4–507 from the start codon was amplified by 35 cycles of PCR from the pMal-TbPRMT1 plasmid (see below) using primers PRMT1-5′ST (5′-CCCAAGCTTACGCGTACGGTGGACGCAAATGCCGCCT-3′) and PRMT1-3′ST (5′-GCTCTAGAGTCAGTAATGCCGC-ATACGTGCAT-3′) which allowed introduction of *Mlu*I and *Hin*dIII (PRMT1-5′ST) and *Xba*I (PRMT1-3′ST) restriction sites (underlined). Cloning of the PCR product into the "stem-loop" vector (a generous gift from Drs. Christian Tschudi and Elisabetta Ullu, Yale University Medical School) was achieved as described [43]. For transfection, cells (1.1×10^7) were washed once in 1.5 ml ice-cold EM buffer [44] and resuspended in 0.45 ml of EM buffer containing 100 μg of plasmid linearized with *Eco*RV. Electroporations (two pulses) were carried out on ice in 2-mm cuvettes using a Bio-Rad electroporator with the following settings: 800 V, 25 μF, and 40 Ω. Following electroporation, 0.25 ml of the cell suspension was transferred into 4 ml SDM-79 supplemented with 15% fetal bovine serum, in the presence of G418 and hygromycin and allowed to recuperate for 20 h. Selection was then applied by the addition of 2.5 μg/ml phleomycin, and the cells were grown for 4 weeks to obtain stable transfectants. Phleomycin-resistant cultures were then cloned by limiting dilution in 96-well microtiter plates. For induction of dsRNA, cells were cultured in the presence of 2.5 μg/ml tetracycline. Growth curves were obtained by plotting the cell densities (represented as the product of the cell number and the total dilution).

2.3. Production of recombinant proteins

TbPRMT1 was identified by an in silico database search (Wellcome Trust Sanger Centre and the Institute for Genomic Research *T. brucei* databases) for proteins displaying homology to the yeast HMT1 enzyme. The search revealed the presence of a gene encoding a putative PRMT1 enzyme located on chromosome I (locus Tb927.1.4690). To clone TbPRMT1, total procyclic form cDNA was generated by reverse transcription primed with [dT]-RXS (5′-GAGAATTCTCGAGTCGACTTTTTTTTTTTTTTT-3′). The entire TbPRMT1 ORF was amplified using oligonucleotides PRMT1-5′ exp (5′-GCGGATCCGCTAGCATGACGGTGGA-CGCAAATGCCGGC-3′) and PRMT1-3′ exp (5′-CCCAAGC-TTCTACTCGAGCCGCAGCCGAAAATCCTGGTCA-3′) (restriction sites are underlined) which were constructed based on the genomic sequence. The PCR product was then digested with *Bam*HI and *Hin*dIII, ligated into the pMal-C2 expression vector (New England Biolabs), and transformed into *E. coli* DH5α competent cells (Invitrogen). MBP-TbPRMT1 expression was then induced with isopropyl β-D-thiogalactopyranoside (IPTG) for 2 h at 30 °C. Cells were resuspended in amylose column wash buffer (20 mM Tris [pH 7.5], 200 mM NaCl, and 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 0.12 mg/ml lysozyme, and

25 units/ml DNase I, and lysed by sonication (four pulses of 30 s each). The lysed cell suspension was centrifuged at 14,000 × *g* for 20 min at 4 °C, and the supernatant was mixed with amylose resin (New England Biolabs) for 2 h at 4 °C. The mixture was poured into a column and the column washed with 15 volumes of amylose column buffer. The MBP-TbPRMT1 fusion was eluted with 4 volumes of amylose column buffer containing 10 mM maltose. The MBP moiety of MBP-TbPRMT1 was cleaved with factor Xa (20 μg/ml of recombinant protein) for 18 h at 25 °C. PMSF was then added at a final concentration of 0.3 mM to stop digestion, and the incubation was allowed to proceed for an additional 30 min. Following dialysis against 1000 volumes of amylose column buffer, the sample was mixed with amylose resin for 2 h at 4 °C. The mixture was poured into a column, and the cleaved TbPRMT1 recovered in the flow through. This protein was used in all subsequent experiments.

For expression of MBP-TBRGG1, the nucleotide sequence corresponding to amino acid 30–268 from TBRGG1 [45] was amplified from [dT]-RXS primed cDNA using oligonucleotides Rgg-5′ exp (5′-CGGGATCCGCTAGCATGCGTGGCCAGTGGGGAAAT-3′) and Rgg-3′ exp (5′-ACGCGTCGACCTACTCGAGCGCTCGCCGCACGATGCGGC-3′). The PCR product was cloned into pMal-C2, and MBP-TBRGG1 was expressed and purified as described above for TbPRMT1 except that the MBP moiety was not removed. His-tagged RBP16, CSD, and RGG were obtained as described [46]. Rat GST-PRMT1 was expressed and purified as published [11].

2.4. Nucleic acid analyses

For Northern analysis, total RNA from procyclic form *T. brucei* clone IsTaR1 stock EATRO 164, bloodstream form *T. brucei* 427, and procyclic form 29-13 cells transfected with the TbPRMT1-RNAi vector (either uninduced or induced with tetracycline), was purified from ∼1 × 10^9 cells using the Purescript RNA Isolation Kit (Gentra Systems). Total RNA (10 μg) was electrophoresed on a 1.5% agarose/formaldehyde gel and transferred to Nytran. The membrane was pre-hybridized in 5× SSPE, 1× Denhardt's, 50% formamide, 1% SDS, 0.15 mg/ml denatured salmon sperm DNA for 6 h at 65 °C. An antisense riboprobe for TbPRMT1 was generated by in vitro transcription with incorporation of [α-^{32}P]UTP and added to the pre-hybridization buffer, and the hybridization was carried out for 18 h at 65 °C. The membrane was then washed 2 × 15 min in 2× SSPE, 0.1% SDS at 65 °C, and 2 × 15 min in 0.1× SSPE, 0.1% SDS at 65 °C. An antisense α-tubulin riboprobe was generated as described for the TbPRMT1 probe using pZJM [43] as a template.

2.5. UV cross-linking of [^{3}H]AdoMet to TbPRMT1

TbPRMT1 (4 μg) was incubated with 0.4 μM [^{3}H]AdoMet (Amersham Pharmacia Biotech, 1 μCi/μl, 80 Ci/mmol) in 50 mM sodium phosphate [pH 7.4] in a total volume of 50 μl. In competition experiments, unlabelled AdoMet (ICN) or ATP (Sigma) were added to a final concentration of 0.4 mM. Samples were added to 96-well plates and exposed to UV irradiation at 254 nm for 30 min at 4 °C using a UV Stratalinker 2400 (Stratagene). Reactions were stopped by the addition of 5× SDS-PAGE sample buffer and boiled for 5 min. Samples were resolved by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 for 30 min and destained in 10% methanol and 5% acetic acid overnight. For fluorography, gels were treated with EN^{3}HANCE (Perkin-Elmer Life Sciences). Gels were dried at 70 °C in vacuo and exposed to Kodak X-Omat AR scientific imaging film at −80 °C.

2.6. Glutaraldehyde cross-linking assay

Recombinant TbPRMT1 (0.15 nmol) was incubated for 20 min at 25 °C in 50 mM sodium phosphate [pH 7.4] in a total volume of 30 μl. Glutaraldehyde was then added to a final concentration of 0.2%, and reactions were allowed to proceed for an additional 10 min at 25 °C. Reactions were stopped by the addition of 5× SDS-PAGE sample buffer and resolved on a 12.5% SDS-PAGE gel. Proteins were visualized by silver staining.

2.7. In vitro methylation assays

The in vitro methylation activity of recombinant TbPRMT1 was assayed as follows. Purified MBP-TBRGGl (5 μg) or an artificial peptide (H-CGRGRGRGRGRGRG-NH_2) [15; a generous gift from Dr. John M. Aletta, Department of Pharmacology and Toxicology, SUNY at Buffalo] (0.5 μg) was incubated with 4 μg of purified recombinant TbPRMT1, in the presence of 2 μCi [^{3}H]AdoMet in either PBS [pH 7.4] or 40 mM Tris [pH 8.0], at 25 or 37 °C, in a total volume of 30 μl. All buffers contained 0.4 mM PMSF and 2 μM benzamidine. Reactions were carried out for 2 h and stopped by the addition of 5× SDS-PAGE loading buffer, and boiled for 5 min. Proteins were resolved by SDS-PAGE on 10% (for reactions containing MBP-TBRGGl) or 20% (for reactions containing the RG-rich peptide) acrylamide gels. For in vitro methylation of his-RBP16, his-CSD, his-RGG, or maltose binding protein (New England Biolabs), 0.2 nmol of protein was incubated with 1.5–3.0 μg of purified recombinant TbPRMT1, in the presence of 2 μCi [^{3}H]AdoMet in PBS [pH 7.4] or 40 mM Tris [pH 8.0], in a total volume of 50 μl for 18 h at 25 °C. Reactions were stopped as above, and proteins were resolved on 20% SDS-PAGE gels. Gels were stained and treated for fluorography as described above.

2.8. Thin layer chromatography (TLC) analysis of in vivo methylated proteins

TbPRMT1 RNAi expressing cells were diluted to 5 × 10^5 cells/ml in SMD-79 media supplemented with 10% FBS, 15 μg/ml G418, 50 μg/ml hygromycin B, and 2.5 μg/ml phleomycin. The culture was split and incubated at 27 °C in the presence (induced) or absence (uninduced) of 2.5 μg/ml tetracycline. On day 4 post-induction, uninduced and induced cells were harvested by centrifugation (6000 × *g*/10 min/4 °C), washed in 50 ml PBSG (PBS supplemented with 7 mM glucose), and resuspended to 1 × 10^8 cells/ml in PBSG. Cells were then incubated in PBSG in a final volume of 130 μl

for 1 h at room temperature in the presence of 1×10^7 cells, 0.5 μM [^{3}H]AdoMet, and 0.8 μM unlabeled AdoMet. For the preparation of whole cell extract, cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C, washed with 500 μl PBS, and resuspended in 3 volumes buffer/1 g cell mass [volume (ml) = 3 × cell mass (g)] low salt hypotonic lysis buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 1 μM leupeptin, and 2 mM benzamidine) containing 0.2% NP-40. Lysates were incubated on ice for 10 min before an additional 12 volumes cell mass low salt hypotonic lysis buffer containing 0.5% NP-40 was added. Cells were then disrupted with a dounce homogenizer and passed through a 26-gauge syringe 10 times. Protein levels in whole cell extracts were quantified by Bradford assay. Sixty-five micrograms of uninduced and induced whole cell extract was then precipitated with cold TCA (10% final) on ice for 30 min, centrifuged at 13,000 × *g* for 10 min, and washed with 500 μl acetone. The TCA precipitated pellets were then resuspended in 200 μl of 6 M constant boiling HCl, and acid hydrolyzed under vacuum at 110 °C for 20 h. HCl was evaporated in a speed vac at medium speed for 1 h, and pellets resuspended in 10 μl distilled water. Separation of methylated amino acids was performed as previously described [47]. Briefly, hydrolysates were loaded onto a LK6DF silica gel 60 TLC plate (Whatman) alongside 30 nmol each of the following amino acid standards: ADMA (Calbiochem), MMA, SDMA, L-methionine (Sigma), and AdoMet iodide salt (MP Biomedicals). Samples were separated with ammonia hydroxide:chloroform:methanol:water (2:0.5:4.5:1). Amino acid standards were visualized with spray ninhydrin reagent (AllTech). For the analysis of tritiated arginine residues, the TLC plate was treated with EN^{3}HANCE spray reagent and visualized by fluorography. A second in vivo analysis of methylation activity in TbPRMT1 RNAi cells was carried out essentially as above, but with the following modifications: (1) cells were labeled in vivo for 4 h on day 5 post-induction; (2) 100 μg whole cell extract was acid hydrolyzed and analyzed by TLC. Non-saturated autoradiographs of the TLC plates were analyzed by densitometry.

3. Results

3.1. TbPRMT1 possesses all the characteristic features of a protein arginine methyltransferase

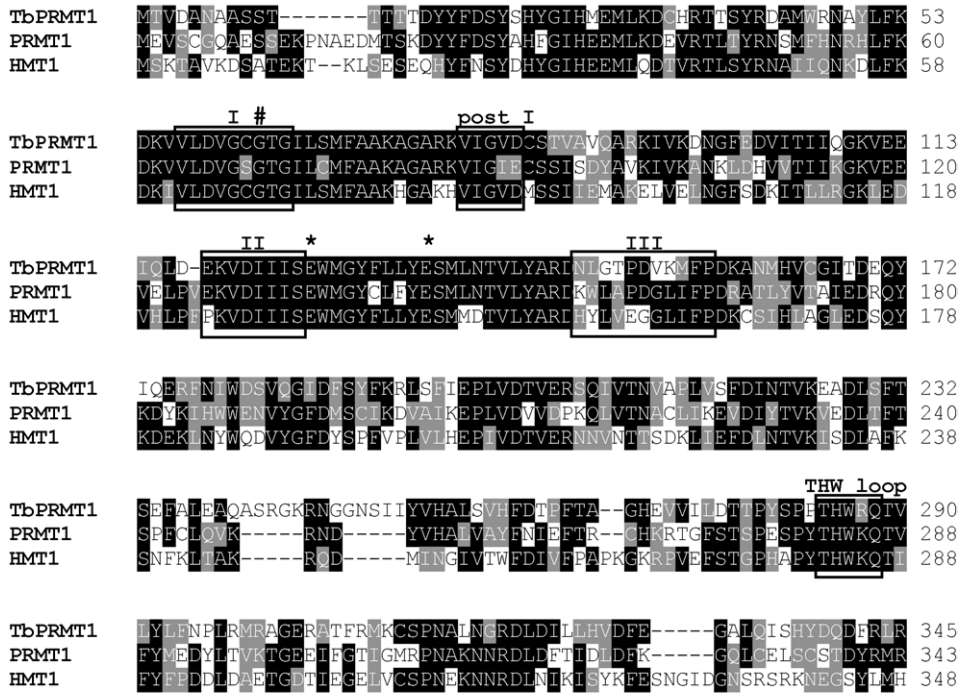
In an effort to identify *T. brucei* homologs of protein arginine methyltransferases, we used the BLAST algorithm to search the Wellcome Trust Sanger Centre and the Institute for Genomic Research *T. brucei* databases for proteins with homology to the yeast HMT1 enzyme (accession number CAA84976.1). The search revealed a gene encoding a putative PRMT1 enzyme located on chromosome I (locus Tb927.1.4690). The complete predicted ORF was amplified from procyclic form oligo(dT)-primed cDNA, cloned into the pMalC-2 vector, and sequenced in both directions. Five different clones were sequenced and they all displayed the same minor differences from the sequence present in the database. Four nucleotide substitutions result in four amino acid changes (E93 → K93, R96 → K96, N182 → S182, S324 → L324). The gene encodes a predicted 345 amino acid protein, that we termed TbPRMT1. The predicted protein has a molecular weight of 39.2 kDa and p*I* of 5.38, and displays 51 and 49% identity at the amino acid level to human PRMT1 and yeast HTM1, respectively. Alignment of the amino acid sequence of these three enzymes emphasizes the high degree of identity between TbPRMT1 and its human and yeast homologs throughout the protein (Fig. 1A). Importantly, TbPRMT1 contains the conserved methyltransferase motifs I, post-I, II, and III, including G63 (# in Fig. 1A), which has been shown to be essential for the activity of the yeast enzyme [48]. In addition, two conserved glutamate residues, E126 and E135 (* in Fig. 1A), part of the arginine binding active site, are also found in TbPRMT1. Searches of several protozoan parasite databases revealed the presence of genes encoding a predicted TbPRMT1 homolog in *Trypanosoma cruzi*, *Leishmania major*, *Leishmania infantum*, *Plasmodium falciparum*, and *Plasmodium berghei*. All of these predicted proteins display a very high amino acid identity to TbPRMT1, ranging from 42% for *P. falciparum* to 76% for *T. cruzi* (Fig. 1B). Remarkably, TbPRMT1 shows a higher degree of identity to the human PRMT1 (51%) than it does to the PRMT1 homologs of the protozoan parasites, *P. falciparum* and *P. berghei*.

Southern blot analysis indicates that the *TbPRMT1* gene is present as a single copy in the *T. brucei* genome (data not shown). Northern analysis reveals that TbPRMT1 is expressed as an approximately 1800-nucleotide transcript (Fig. 2). Since the ORF predicts a 1042-nucleotide long transcript, this indicates the presence of 5′ and 3′ untranslated regions of approximately 750 nucleotides combined. Northern analysis also indicates that TbPRMT1 RNA is expressed at similar levels in both procyclic and bloodstream form *T. brucei* (Fig. 2).

As the amino acid sequence of TbPRMT1 revealed the presence of a conserved AdoMet binding domain located from amino acids 35 to 158, we first asked if TbPRMT1 could bind to the methyl donor AdoMet. Recombinant TbPRMT1 was produced as described under Section 2 and exposed to UV irradiation at 254 nm in the presence of [^{3}H]AdoMet [49]. The reaction mixture was then separated by SDS-PAGE and analyzed by fluorography. As shown in Fig. 3A, a radiolabeled band was observed at 40 kDa, the size of TbPRMT1. To confirm the binding specificity for AdoMet, we included a 1000-fold excess of unlabeled AdoMet or ATP in the cross-linking reaction. Addition of the unlabeled AdoMet severely inhibited the cross-linking reaction, whereas the addition of the UV-absorbing non-substrate ATP had no effect. The Coomassie stained gel shown in Fig. 3B demonstrates that equal amounts of TbRMT1 were present in all reactions. From these results, we conclude that TbPRMT1 specifically binds AdoMet as predicted by its amino acid sequence.

Crystal structures have been solved for the human PRMT1 and PRMT3 enzymes. The structures are similar, and indicate that these type I PRMTs form both dimers and tetramers [50,51]. Similarly, the human type II PRMT5 is present as dimeric and tetrameric forms. Oligomerization is critical for the catalytic activity of both PRMT3 and PRMT5 [50,52]. To determine

(A)



(B)

Amino acid identity (%)

	T.b.	*T.c.*	*L.m.*	*L.i.*	*P.f.*	*P.b.*
T.b.	100	76	71	71	42	46
T.c.	76	100	74	74	42	46
L.m.	71	74	100	93	44	46
L.i.	71	74	93	100	44	46
P.f.	42	42	44	44	100	73
P.b.	46	46	46	46	73	100

Fig. 1. (A) Amino acid sequence alignment of TbPRMT1 with human PRMT1 and yeast HMT1. Sequences were aligned using CLUSTALW. Identical and conserved amino acids are shown in *black* and *grey boxes*, respectively. Signature PRMT motifs I, post-I, II, III, and the THW loop are *boxed*. The glutamate residues conserved in all PRMTs are indicated by *asterisks* (*). The conserved glycine residue, shown to be essential for the activity of the yeast HMT1, is indicated by the (#) symbol. (B) Amino acid sequence identity between PRMT1 homologs from protozoan parasites. *T. brucei* (*T.b.*) (locus Tb927.1.4690), *T. cruzi* (*T.c.*) (locus 00.1047053508593.110), *L. major* (*L.m.*) (locus LmjF12.1270), *L. infantum* (*L.i.*) (locus LinJ12.0850), *P. falciparum* (*P.f.*) (locus PF14_0242), and *P. berghei* (*P.b.*) (locus PB001663.02.0).

whether TbPRMT1 forms homo-oligomers, purified recombinant TbPRMT1 was incubated in the presence of the protein cross-linking agent glutaraldehyde. Untreated and cross-linked proteins were then analyzed by SDS-PAGE (Fig. 4). While the untreated TbPRMT1 migrated at the size predicted for a monomeric form (~40 kDa) (Fig. 4, lane 1), TbPRMT1 that had been cross-linked with glutaraldehyde formed species corresponding to the sizes of homo-dimers, homo-trimers, or homo-tetramers (Fig. 4, lane 2). No difference was observed when the cross-linking was performed in the presence of AdoMet. These results suggest that TbPRMT1 is able to assemble into multimeric forms.

3.2. TbPRMT1 displays protein arginine methyltransferase activity and differs in substrate specificity compared to its mammalian homolog

We next wanted to demonstrate that TbPRMT1 has intrinsic protein arginine methyltransferase activity and assess its substrate specificity. To this end, we incubated purified recombinant TbPRMT1 with [^{3}H]AdoMet and one of several potential protein and peptide substrates. One putative substrate for TbPRMT1 is the RNA editing accessory factor, RBP16. RBP16 is the only identified methylprotein in *T. brucei*, and we previously demonstrated that this protein is methylated by a type I PRMT activity in *T. brucei* cell extracts [11]. RBP16 is comprised entirely of two distinct RNA binding domains: an N-terminal cold-shock domain (CSD), and a C-terminal RGG domain that contains three methylated arginine residues in vivo [11]. We first tested whether RBP16 could serve as a substrate for TbPRMT1 in vitro by incubating either recombinant full length his-RBP16 or its constituent domains, his-CSD and his-RGG, with purified TbPRMT1 and [^{3}H]AdoMet in PBS [pH 7.4] for up to 24 h at 25 °C. Surprisingly, neither full length RBP16 nor its RGG domain was methylated under these conditions (Fig. 5A, lanes 1 and 3). The RBP16 CSD was also not methylated in vitro (Fig. 5A, lane 2), as predicted, since arginine residues within the CSD are not methylated in vivo [11]. The inability of TbPRMT1

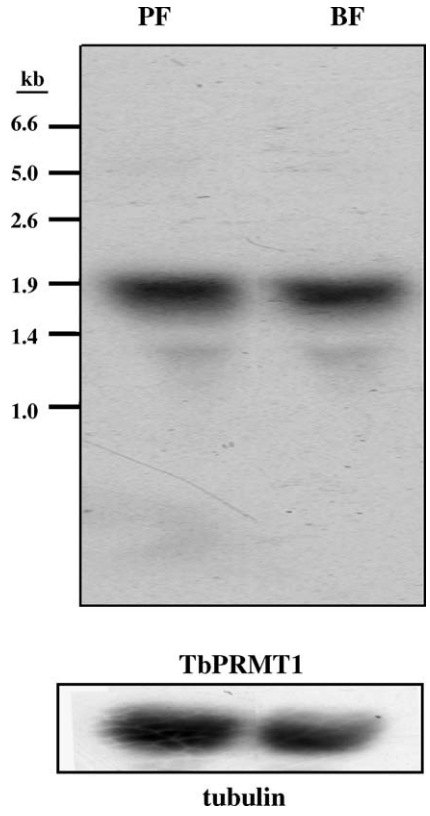


Fig. 2. Analysis of TbPRMT1 expression in *T. brucei* procyclic and bloodstream forms. TbPRMT1 mRNA levels from procyclic form *T. brucei brucei* clone IsTaR1 stock EATRO 164 (PF) and bloodstream form *T. brucei* strain 427 (BF) were analyzed by Northern hybridization. Ten micrograms of total RNA was subjected to 1.5% agarose/formaldehyde electrophoresis, transferred to Nytran and probed with [^{32}P]-labeled antisense RNA probe to TbPRMT1. α-Tubulin RNA levels were assessed as a loading control.

to methylate RBP16 was unexpected. We repeated this reaction using several different buffer conditions and temperatures, and extended incubation times up to 24 h, but never observed methylation of RBP16 or its RGG domain by TbPRMT1. These results may indicate that TbPRMT1 requires a cofactor for this reaction or that another enzyme performs this function in vivo.

To further examine the potential enzymatic activity of TbPRMT1, we utilized two additional RG-rich potential substrates: (1) an artificial peptide consisting of seven RG repeats (Fig. 5A, lane 5) [15] and (2) the N-terminal RGG domain of the *T. brucei* TBRGG1 protein (Fig. 5A, lane 6) [45]. The artificial RG-rich peptide has been shown to block methylation of PC12 cell proteins in vitro, and represents a substrate for mammalian type I PRMT [15]. TBRGG1 is a *T. brucei* mitochondrial RNA binding protein whose N-terminal RGG RNA binding domain contains several arginines found within the preferred site for asymmetric arginine dimethylation [45]. The in vivo methylation status of TBRGG1 is unknown, but the presence of an RGG domain suggests that it is likely to serve as a PRMT substrate. Following incubation of the RG peptide with purified TbPRMT1 in the presence of [^{3}H]AdoMet in PBS buffer [pH 7.4] at 25 °C, a labeled band was detected at ∼1.7 kDa, the predicted size for the RG-rich peptide (Fig. 5A, lane 5). Similarly, the RGG domain of TBRGG1 expressed as a MBP fusion protein (MBP-TBRGGl) also served as a substrate for TbPRMT1 as evidenced by the presence of a labeled band at ∼68 kDa, the predicted size for the MBP-TBRGGl protein (Fig. 5A, lane 6). TbPRMT1 did not methylate MBP (Fig. 5A, lane 4), indicating that the labeled band observed at ∼68 kDa was due to methylation of the TBRGG1 moiety. These data conclusively show that TbPRMT1 possesses protein arginine methyltransferase activity, and that it displays activity towards at least one native trypanosome protein, TBRGG1.

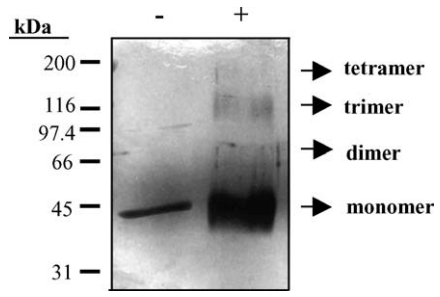


Fig. 4. TbPRMT1 forms homo-oligomers. TbPRMT1 (0.15 nmol) purified as described under Section 2 was incubated in the absence (−) or the presence (+) of 0.2% glutaraldehyde for 20 min at 25 °C prior to SDS-PAGE and silver staining. Stained bands corresponding to monomers and potential dimers, trimers, and tetramers are indicated.

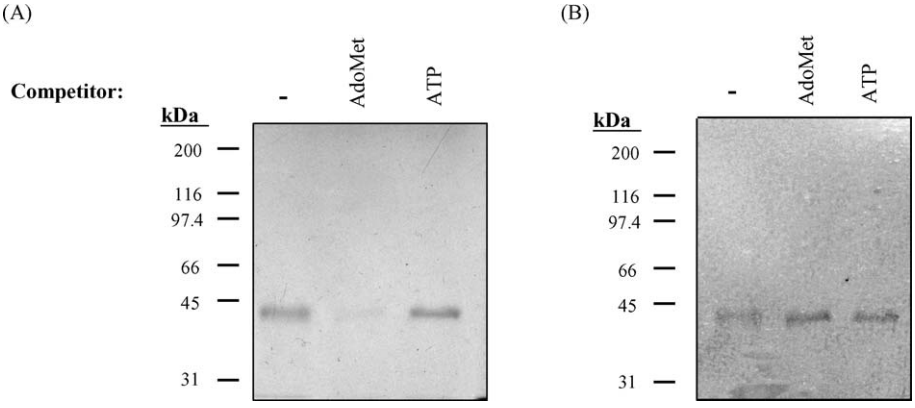


Fig. 3. TbPRMT1 specifically binds [^{3}H]AdoMet in vitro. (A) Recombinant TbPRMT1 (0.1 nmol) was incubated with [^{3}H]AdoMet in the absence of competitor (–) or in presence of a 1000-fold molar excess of unlabeled AdoMet or ATP, and irradiated at 254 nm. Proteins were separated by SDS-PAGE and analyzed by fluorography. (B) Coomassie stain of the gel prior to fluorography.

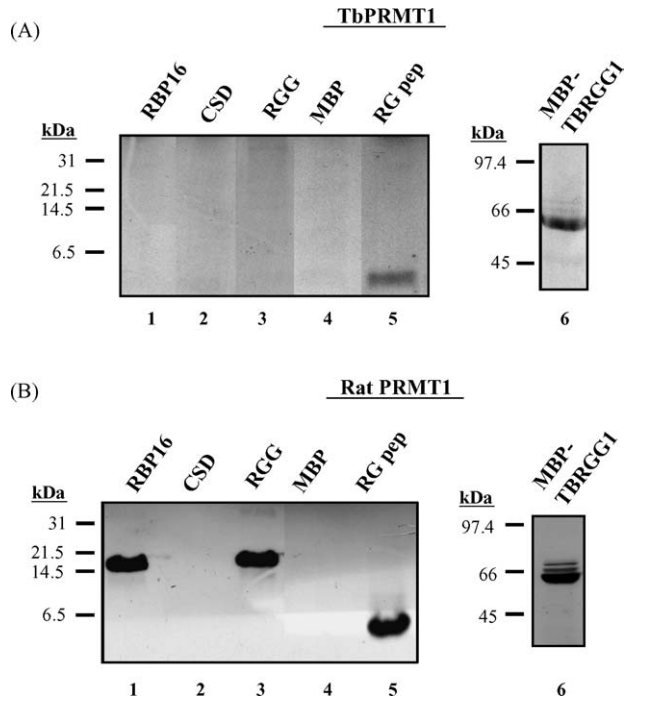


Fig. 5. TbPRMT1 displays protein arginine methyltransferase activity with a substrate specificity different from that of mammalian PRMT1. (A) In vitro protein arginine methyltransferase activity of purified TbPRMT1 (3 μg) was assayed with 0.2 nmol of the following substrates: his-RBP16 (lane 1), his-CSD (lane 2), his-RGG (lane 3), maltose binding protein (lane 4), RG-rich peptide (lane 5), and MBP-TBRGG1 (lane 6). (B) The same substrates as in panel A were incubated with the rat GST-PRMT1 (1 μg).

Because the trypanosome and mammalian PRMT1 enzymes are highly homologous (Fig. 1), we next wanted to compare the biochemical properties of the trypanosome and mammalian enzymes. Rat PRMT1 was expressed as a GST fusion protein for comparison to TbPRMT1 in in vitro assays [11]. We first compared the substrate specificities of rat PRMT1 and TbPRMT1, using the substrates described above. Trypanosome and rat PRMT1 were similar in their abilities to catalyze methyl transfer to the RG peptide and MBP-TBRGGl (compare Fig. 5A and B, lanes 5 and 6). However, the two enzymes differed strikingly in their abilities to methylate RBP16 or its RGG domain. While TbPRMT1 could not utilize these substrates, both RBP16 and its RGG domain were robustly methylated in vitro by recombinant rat PRMT1 (compare Fig. 5A and B, lanes 1 and 3). As predicted, neither MBP nor the RBP16 CSD was methylated by rat PRMT1 (Fig. 5B, lanes 2 and 4). We note that in vitro methylated his-RGG migrates at ∼16 kDa, while recombinant his-RGG migrates with an apparent molecular mass of ∼9 kDa under denaturing conditions [46], indicating that methylation significantly affects the electrophoretic mobility of this small protein. We conclude from these experiments that, despite their sequence similarity, trypanosome and rat PRMT1 possess different substrate specificities.

Tris buffer has been reported to inhibit the activities of several mammalian PRMTs in vitro, presumably due to its ability to act as a structural analog of the arginyl side chain methyl-accepting substrate [53]. To compare the inhibitory effect of Tris on TbPRMT1 and its mammalian homolog, we assayed the activities of these enzymes in reactions buffered with either 40 mM Tris or with PBS. Methylation of both the RG peptide and MBP-TBRGGl substrates was assessed. The trypanosome and rat enzymes differed dramatically in their sensitivity to Tris when the RG peptide was used as a substrate (Fig. 6, left panel). Methylation of the RG-rich peptide by TbPRMT1 was decreased 12-fold when the assay was performed in Tris buffer [pH 8.0] compared to assays performed in PBS (Fig. 6, left panel). This inhibition is due to the buffer composition and not the pH, as a similar decrease in RG-rich peptide methylation was observed when the reaction was carried out in Tris buffer at pH 7.4, the pH of the PBS buffer used (data not shown). In contrast, 40 mM Tris buffer inhibited the activity of the rat PRMT1 towards the RG peptide only slightly (∼2-fold) (Fig. 6, left panel), in keeping with previous results [53]. Remarkably, unlike the RG peptide, in vitro methylation of the MBP-TBRGGl substrate by TbPRMT1 was essentially unaffected by the buffer composition (Fig. 6, right panel). This suggests that Tris competes more efficiently with some substrates than with others for TbPRMT1 binding. Overall, these experiments demonstrate that TbPRMT1 sensitivity to inhibition by Tris is substrate specific. Moreover, the ability of Tris to differentially inhibit trypanosome and rat enzymes further highlights the divergence between the trypanosome and rat PRMT1 enzymes regarding their substrate preferences.

3.3. *TbPRMT1 is non-essential in procyclic form T. brucei*

To gain insight into TbPRMT1 function in vivo, we generated clonal procyclic form *T. brucei* cell lines in which TbPRMT1 mRNA levels were down-regulated by RNAi. A 503-bp fragment of the TbPRMT1 coding region corresponding to nt 4–507 from the start codon was cloned into a "stem-loop" plasmid, allowing production of dsRNA as a stem-loop from a tetracycline-regulated T7 promoter [43]. The linearized plasmid was transfected into *T. brucei* strain 29-13, which harbors integrated genes encoding T7 RNA polymerase and tetracycline repressor protein [42]. Cultures containing cells in which the TbPRMT1 RNAi plasmid had integrated into the non-transcribed rDNA spacer were selected by addition of phleomycin to the growth medium. Clonal lines were subsequently obtained by limiting dilution, and one of these clones was further characterized. Northern blot analysis showed that by days 3–4 following addition of tetracycline (2.5 μg/ml) to the medium, TbPRMT1 RNA was almost completely absent (Fig. 7A, left panel). The level of a control mRNA, α-tubulin, did not change following tetracycline induction, indicating that the mRNA down-regulation was specific to TbPRMT1 (Fig. 7A, right panel). We next assessed the effect of TbPRMT1 disruption on cell growth. Although the TbPRMT1 mRNA levels were dramatically reduced by day 3 following tetracycline induction, we did not observed a significant effect on the growth rate of these cells compared to uninduced cells (Fig. 7B). Together, these experiments indicate that TbPRMT1 is not an essential protein for survival of procyclic form *T. brucei*. As anti-TbPRMT1 antibodies are currently unavailable, we could not determine the TbPRMT1 protein level in cells induced for TbPRMT1 RNAi

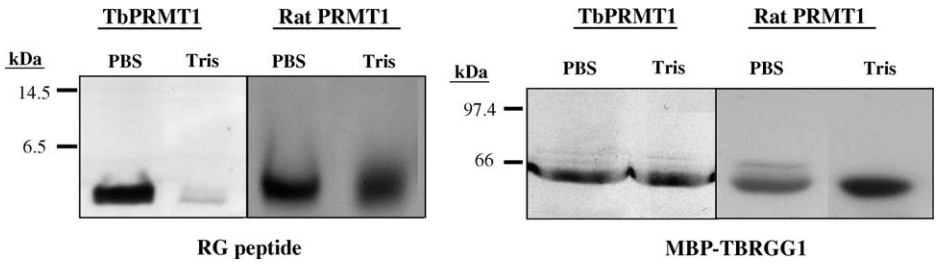


Fig. 6. Tris inhibits TbPRMT1 activity in a substrate specific manner. Left panel: RG-rich peptide (0.2 nmol) was incubated with purified TbPRMT1 (4 μg) or rat GST-PRMT1 (1 μg) in PBS or 40 mM Tris buffer for 2 h at 25 °C in the presence of 2 μCi [^{3}H]AdoMet. Right panel: the activity of purified TbPRMT1 or rat GST-PRMT1 was determined as described above using MBP-RGG1 (0.2 nmol) as a substrate. Proteins were resolved by SDS-PAGE on 10% (right panel) or 20% (left panel) polyacrylamide gels. Gels were stained with Coomassie, and labeled bands detected by fluorography.

versus that in uninduced cells. Therefore, it remains possible that the protein level in TbPRMT1 RNAi cells exceeds the RNA level measured by Northern blot, and that this amount of residual TbPRMT1 protein is sufficient to allow the cell to survive.

3.4. TbPRMT1 disruption results in a dramatic decrease in asymmetric dimethylarginine in vivo

In order to confirm that TbPRMT1 catalyzes ADMA formation in vivo and determine whether the enzyme constitutes a predominant type I PRMT in *T. brucei* procyclic form cells, we assessed the effect of TbPRMT1 disruption on total cellular ADMA levels. To this end, we down-regulated TbPRMT1 expression by induction of RNAi with tetracycline over a period of 4 days. Cells were then labeled in vivo in the presence of [^{3}H]AdoMet for 1 h, followed by acid hydrolysis and separation of MMA, ADMA and SDMA by thin layer chromatography [47]. SDMA is significantly less abundant than ADMA in these cells and was not detected in the experiments presented in Fig. 8. However, we observed a very significant decrease in the level of ADMA (∼90%) in cells in which TbPRMT1 expression was disrupted by RNAi, as opposed to uninduced cells as well as the parental strain (Fig. 8, Experiment 1, and data not shown). Thus, TbPRMT1 possesses type I PRMT activity in vivo, consistent with the high degree of homology to mammalian and yeast type I PRMTs. Moreover, TbPRMT1 is responsible for synthesis of the majority of total cellular ADMA in *T. brucei*.

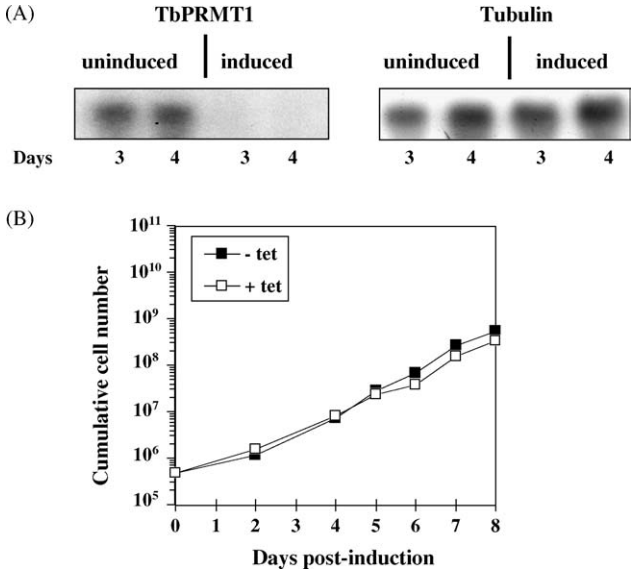


Fig. 7. Down-regulation of TbPRMT1 expression in *T. brucei* procyclic forms. (A) Northern blot analysis of TbPRMT1 (left panel) or α-tubulin (right panel) RNA from TbPRMT1 RNAi transfectants uninduced or induced with tetracycline (2.5 μg/ml) for 3 or 4 days. Total RNA (10 μg) was separated on a 1.5% agarose/formaldehyde gel, transferred to Nytran, and probed with a [^{32}P]-labeled antisense RNA probe against TbPRMT1 (left panel) or α-tubulin (right panel). (B) Growth of procyclic form *T. brucei* TbPRMT1 RNAi cells either uninduced (closed squares) or induced with 2.5 μg/ml tetracycline (open squares). Growth curves were obtained by plotting the cell densities as the product of the cell number and the total dilution. The values shown are the average of two independent determinations.

In addition to the decrease in ADMA, we observed that the levels of MMA were increased approximately 2-fold in TbPRMT1-disrupted cells. This suggests TbPRMT1 may catalyze sequential formation of MMA and ADMA on a given arginine residue, but when present at low enzyme to substrate ratios the enzyme acts distributively, producing primarily MMA. Alternatively, TbPRMT1 may catalyze ADMA production on arginine residues that have been monomethylated by another enzyme, again leading to MMA buildup when TbPRMT1 is down-regulated. To confirm the effect of TbPRMT1 knock down on cellular ADMA levels, we performed a second experiment in which tetracycline induction was performed for 5 days and cells were labeled in vivo in the presence of [^{3}H]AdoMet for 4 h instead of 1 h (Fig. 8, Experiment 2). While the decrease in ADMA levels in induced versus uninduced cells was not as dramatic following a 4 h labeling as compared to a 1 h labeling, we again observed a substantial decrease in ADMA levels upon TbPRMT1 down-regulation (compare Fig. 8, Experiments 1 and 2). Comparison of the 1 and 4 h labeling experiments (Experiments 1 and 2, Fig. 8, respectively) suggests that residual TbPRMT1 catalyzes ADMA formation in the knock down cells and that proteins containing this modification are relatively stable over the 4 h labeling period. In summary, in vivo labeling of methylated proteins in TbPRMT1 knock down cells demonstrates that TbPRMT1 comprises the major type I PRMT in *T. brucei*.

4. Discussion

In this report, we describe the major type I PRMT in *T. brucei*, a homolog of mammalian PRMT1 that we have termed

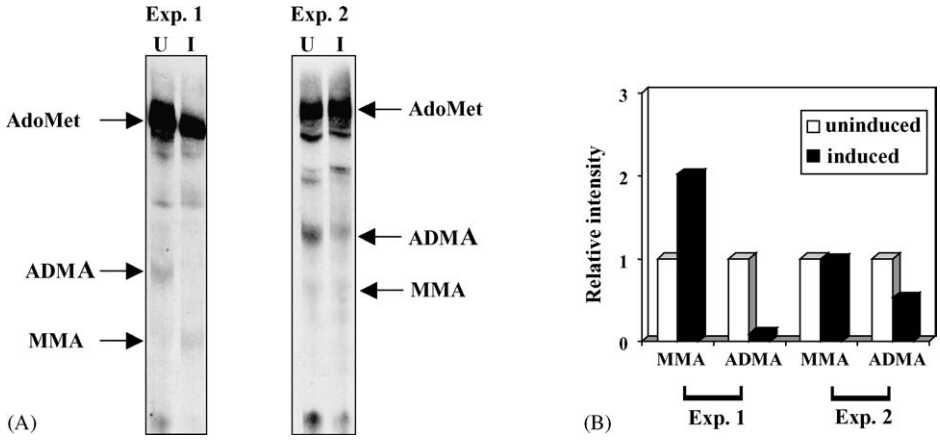


Fig. 8. TbPRMT1 disruption results in a decrease in asymmetrical dimethylarginine in vivo. (A) *T. brucei* TbPRMT1 RNAi cells either uninduced (U) or induced (I) with tetracycline (2.5 μg/ml) for 4 days (Experiment 1) or 5 days (Experiment 2) were labeled in vivo with a mixture of 0.5 μM [^{3}H]AdoMet and 0.8 μM unlabeled AdoMet for 1 h (Experiment 1) or 4 h (Experiment 2). Cells were harvested and labeled amino acids were analyzed as described under Section 2. (B) Non-saturated autoradiographs obtained from the TLC plates were analyzed by densitometry. The intensity of the ADMA and MMA signals are represented as the relative intensity of the radioactive signal between tetracycline-induced and uninduced cells (the intensity of the ADMA and MMA residues obtained from the uninduced cells was arbitrarily fixed at 1).

TbPRMT1. TbPRMT1 is the first PRMT identified in a protozoan. Although *T. brucei* is a very early branching eukaryote, TbPRMT1 displays strikingly high amino acid conservation with its mammalian and yeast homologs (∼50% in both cases). Homology is evident in each of the four distinct PRMT domains that were identified upon crystallization of rat PRMT1: the N-terminal domain, the AdoMet binding domain, the dimerization arm, and the β-barrel domain [50]. The N-terminal domain, extending from amino acids 1 to 41 and 38 for the human and yeast PRMT1, respectively, is the least conserved region among PRMTs. Interestingly, TbPRMT1 and human PRMT1 display high amino acid sequence identity in this region (64%) compared to the human versus yeast enzymes (43%). The AdoMet binding domain of TbPRMT1 is extremely well conserved and contains the methyltransferase motifs I, post-I, II, and III, including residues G61 and G63R, as well as E126 and E135, which have all been shown to mediate interaction with the substrate AdoMet in rat PRMT1 [50]. In accordance with this sequence conservation, we show that TbPRMT1 specifically binds AdoMet. The dimerization arm of TbPRMT1, which extends from residues 170 to 196 in TbPRMT1, is also well conserved. While the oligomerization status of TbPRMT1 in vivo is unknown, our results demonstrating that TbPRMT1 can form multimers upon incubation with the cross-linking agent glutaraldehyde suggest that the enzyme may form homo-oligomers in its native environment. Finally, the C-terminal regions of PRMTs typically form β-barrel structures. This region of TbPRMT1 is the least conserved. Nevertheless, prediction of TbPRMT1 secondary structure using the GOR4 tool suggests that the C-terminus is likely to form a β-barrel structure (data not shown). The high level of overall amino acid identity between the trypanosome and human PRMT1 homologs strongly suggests that processes in which arginine methylation participates are universally important to eukaryotes.

Disruption of TbPRMT1 expression by RNAi demonstrated that the enzyme is not essential for survival of procyclic form *T. brucei*. The fact that TbPRMT1 is dispensable is reminiscent of the yeast HMT1, which is not required for cell survival [54]. Likewise, the murine PRMT1 and the fission yeast type I PRMT3 have been shown to be non-essential for cell viability [55,56]. However, mouse PRMT1 null mutations result in embryonic lethality [56]. It remains possible that TbPRMT1, although not essential for procyclic form *T. brucei*, may be more critical for the growth of other developmental stages.

Acid hydrolysis of the in vivo labeled proteins from *T. brucei* cells disrupted in TbPRMT1 expression clearly showed a significant decrease in the levels of ADMA when compared to uninduced cells. This unequivocally demonstrates that TbPRMT1 exhibits a type I PRMT activity in vivo and that it constitutes the predominant type I PRMT in trypanosomes, similar to its role in yeast and mammals [6,14]. The reduction of ADMA levels in TbPRMT1 knock down cells is accompanied by a significant increase in the MMA, which is an intermediate in the synthesis of both ADMA and SDMA [1]. Accumulation of MMA in TbPRMT1 knock down cells suggests that TbPRMT1 acts primarily in a distributive manner at low cellular concentrations, performing only the first step in ADMA synthesis (i.e., MMA formation) before dissociating from its substrate. In this case, primarily MMA would be synthesized, and this residue would accumulate because it is not efficiently converted to ADMA. This model is consistent with the properties of human PRMT7, which catalyzes increased synthesis of MMA and severely decreased synthesis of SDMA at increasing substrate to enzyme ratios [57]. It is also possible that other PRMTs present in TbPRMT1-depleted cells are able to catalyze the formation of MMA, which then accumulates due to the reduced levels of conversion to ADMA by TbPRMT1. Predicted proteins that could catalyze MMA formation in *T. brucei* include homologs of human PRMT3 and PRMT5 that are identifiable in the genome sequence [M. Pelletier and L. Read, unpublished results]. Double RNAi knock downs of each of these proteins in combination with TbPRMT1 would reveal whether the PRMT3

or PRMT5 homologs can provide monomethylated substrates for TbPRMT1 in vivo.

Despite the high sequence identity between TbPRMT1 and mammalian PRMT1 homologs, a comparison of the properties of recombinant rat PRMT1 and TbPRMT1 revealed a difference in substrate specificities. While the RG peptide and MBP-TBRGG1 could serve as substrates for both enzymes, full length RBP16 or its RGG domain were only modified by the rat PRMT1. Moreover, the two enzymes differed markedly in their ability to be inhibited by Tris. TbPRMT1 activity towards the RG-rich peptide was much more dramatically inhibited by Tris than was the activity of the rat enzyme, whereas neither enzyme was inhibited by Tris in the presence of the MBP-TBRGG1 substrate. It has been suggested that Tris acts as a competitive inhibitor of some PRMTs by acting as a structural analog of the arginyl side chain on the substrate [53]. Our results indicate that some substrates are more successful at competing with Tris than others, particularly for binding to TbPRMT1. TbPRMT1 may recognize the RGG motif that is present in multiple copies in MBP-TBRGG1 much more efficiently than the RG motif that is repeated in the RG peptide substrate, leaving the latter open to Tris competition. The differences in the biochemical properties of rat and trypanosome PRMT1s suggest that comparison of these enzymes will be useful for future studies defining motifs involved in substrate recognition by PRMTs.

The inability of TbPRMT1 to methylate the RNA binding protein, RBP16, in vitro was somewhat surprising. RBP16 is methylated in vivo on three different arginines, and at least some of these events are mediated by a type I PRMT in *T. brucei* cellular extracts [11]. It is possible that RBP16 methylation in vivo does not involve TbPRMT1, perhaps being mediated by the PRMT3 homolog described above. Alternatively, TbPRMT1 may have a relatively low affinity for RBP16, preventing the efficient methylation of RBP16 by TbPRMT1 under the conditions used in of our in vitro assay. It is particularly intriguing that, in contrast, RBP16 is a robust substrate for rat PRMT1 in vitro. The inability of TbPRMT1 to methylate RBP16 in vitro may suggest that cellular cofactors are required for TbPRMT1 to properly and efficiently methylate certain substrates, thereby conferring specificity to these methylation events. In terms of RBP16 methylation, potential cofactors include RNA and/or the RBP16-associated protein, p22 [58]. Stimulation of TbPRMT1 activity by a protein binding partner would be similar to reported PRMT modulation in other systems. For example, two immediate-early/primary response proteins, TIS21 and BTG1, have been shown to stimulate the methylation activity of both endogenous and recombinant human PRMT1 [3]. In addition, the interleukin enhancer binding factor 3 (ILF3) both serves as a PRMT1 substrate and enhances the methylation activity of a recombinant PRMT1 [14]. To sort out these possibilities, we are currently attempting to determine the precise methylation status of RBP16 in TbPRMT1-depleted cells. In addition, we are performing in vitro assays of TbPRMT1 activity towards RBP16 in the presence of potential cofactors.

Since TbPRMT1 constitutes the major type I PRMT in *T. brucei*, it is likely that this enzyme plays a role in multiple physiological and gene regulatory processes. In *T. brucei*, transcriptional gene regulation is essentially absent. Instead, polycistronic transcripts are resolved by 5′ *trans*-splicing and 3′ cleavage and polyadenylation, and gene expression is controlled primarily at the levels of RNA processing, stability, and translation. Each of these processes likely involves multiple RNA binding proteins [37–39]. As a large percentage of type I PRMT substrates are glycine-arginine-rich RNA binding proteins [11,20,23–29], it is tempting to envision a role for arginine methylation in multiple facets of gene regulation in *T. brucei*. Indeed, a large number of proteins containing RGG-type RNA binding domains are present in the *T. brucei* genome [M. Pelletier and L. Read, unpublished results]. In this study, we identified one such protein, TBRGG1, as a TbPRMT1 substrate in vitro. It has been suggested that TBRGG1 functions in mitochondrial RNA editing [45], although this has not been conclusively demonstrated. Future investigations of RNA processing, stability, and translation in TbPRMT1 RNAi cells will shed light on the functions of this enzyme in *T. brucei*, and may provide insight into early evolving roles of arginine methylation.

Acknowledgements

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