TbPRMT6 is a Type I protein arginine methyltransferase that contributes to cytokinesis in Trypanosoma brucei

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Running title: Characterization of a Type 1 PRMT in T. brucei

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ABSTRACT

Arginine methylation is a widespread posttranslational modification of proteins catalyzed by a family of protein arginine methyltransferases (PRMTs). In yeast and mammals, this modification impacts multiple cellular processes such as chromatin remodeling leading to transcriptional regulation, RNA processing, DNA repair, and cell signaling. The protozoan parasite, *Trypanosoma brucei*, possesses five putative PRMTs in its genome. This is a large number of PRMTs relative to other unicellular eukaryotes, suggesting an important role for arginine methylation in trypanosomes. Here, we present the *in vitro* and *in vivo* characterization of a *T. brucei* enzyme homologous to human PRMT6, which we term TbPRMT6. Like human PRMT6, TbPRMT6 is a Type I PRMT, catalyzing the production of monomethylarginine and asymmetric dimethylarginine residues. In *in vitro* methylation assays, TbPRMT6 utilizes bovine histones as a substrate, but it does not methylate several *T. brucei* glycine/arginine rich proteins. As such, it exhibits a relatively narrow substrate specificity compared to other *T. brucei* PRMTs. Knockdown of TbPRMT6 in both procyclic form and bloodstream form *T. brucei* leads to a modest but reproducible effect on parasite growth in culture. Moreover, upon TbPRMT6 depletion, both PF and BF exhibit aberrant morphologies indicating defects in cell division, and these defects differ in the two life cycle stages. Mass spectrometry of TbPRMT6 associated proteins reveals histones, components of the nuclear pore complex, and flagellar proteins that may represent TbPRMT6 substrates contributing to the observed growth and morphological defects.
INTRODUCTION

Post-translational methylation of proteins on arginine residues has multiple roles in a wide array of cellular functions, such as chromatin remodeling leading to transcription activation or repression, RNA processing, DNA repair, and various forms of cell signaling (5, 6, 8, 9, 52, 70, 98). The process of arginine methylation involves the transfer of methyl groups from S-adenosyl-methionine (AdoMet) to arginine residues of proteins and is catalyzed by a group of enzymes known as protein arginine methyltransferases (PRMTs). PRMTs themselves are further divided into four classes, depending on the type of methylated arginine generated. The largest PRMT class comprises the Type I enzymes, as characterized by the first discovered PRMT, PRMT1. Type I PRMTs initially catalyze the formation of monomethylated arginine (MMA) on the terminal ω-nitrogen, followed by addition of a second methyl group on the same ω-nitrogen, which yields asymmetric dimethylarginine (ADMA). The Type II PRMTs are a smaller group, presently consisting only of PRMT5 and its homologues. These enzymes also catalyze the synthesis of MMA on the terminal ω-nitrogen, but in contrast to the Type I enzymes, Type II PRMTs add a second methyl group to the adjacent terminal ω-nitrogen, resulting in symmetric dimethylarginine (SDMA). Almost all known eukaryotic cells possess at least one Type I PRMT and one Type II PRMT in the form of PRMT1 and PRMT5 homologues, respectively (3). Less is understood about the Type III and Type IV PRMTs. Type III PRMTs catalyze the production of only MMA. Thus far, the T. brucei homologue of human PRMT7, TbPRMT7, is the only enzyme thought to be exclusively Type III, and the specificity of the mammalian homologue PRMT7 is controversial (27, 54, 64). Finally, the Type IV PRMTs catalyze MMA on the δ-nitrogen of arginine, but to date have only been described in fungi (63, 69).
PRMT substrates are varied and include chromatin associated proteins, signaling proteins, and a large number of RNA binding proteins (RBPs) (5). RBPs are usually methylated within glycine/arginine rich (GAR) regions (68), often within canonical RGG motifs. However, methylation of arginine residues in non-canonical regions is becoming more apparent, suggesting a more complex specificity than initially thought (6, 97). Thus, a large number of PRMT substrates cannot be identified based on their sequences, and so must be empirically defined.

The homologues of PRMT6 in humans and other higher eukaryotes comprise a family of Type I PRMTs involved in transcription and DNA repair (28, 53). PRMT6 exhibits a relatively narrow substrate specificity, with the currently known substrates being HMG1A (66, 87, 106), histone subunits (32, 37, 38), DNA polymerase beta (20), and several components of the HIV virus (10, 39, 40), as well as PRMT6 itself (28). The human enzyme is reported to display an exclusively nuclear localization pattern (28), consistent with its known roles in nuclear processes. Detailed in vitro studies showed that human PRMT6 catalyzes methyltransfers in a distributive manner, depositing the first methyl group and creating MMA, dissociating from the substrate, then rebinding to the methyl mark and forming ADMA (53). Homologues of PRMT6 are apparently absent from the genomes of most single-celled eukaryotes, with the exception of Trypanosoma brucei and, possibly, Dictyostelium (3).

The kinetoplastid protozoan T. brucei is the causative agent of African sleeping sickness. Kinetoplastid parasites, including T. brucei, T. cruzi and Leishmania spp., exhibit several unique features, one of the most striking of which is the absence of gene regulation at the level of transcription (13, 14). Instead, these parasites regulate several post-transcriptional processes including RNA stability, translation, and RNA editing to control gene expression. This unusual mode of gene regulation necessitates the involvement of a large number of RBPs, a few of which
have been identified (26, 35, 48, 61, 83, 91, 92). Correspondingly, the *T. brucei* genome encodes a large number of RBPs. Because many of these RBPs contain GAR motifs, they are in turn proposed targets of regulation by arginine methylation ((17) and Read, L. K., unpublished results).

 Previously, we identified five putative PRMTs in the *T. brucei* genome, which is, to our knowledge the highest number in a single-celled eukaryote (3, 73). In this study, we present an *in vitro* and *in vivo* characterization of the *T. brucei* homologue of the human PRMT6, which we term TbPRMT6. TbPRMT6 is a Type I PRMT with a relatively narrow substrate specificity compared to other *T. brucei* PRMTs. Knockdown of TbPRMT6 in both procyclic form (PF) and bloodstream form (BF) *T. brucei* leads to a modest but reproducible effect on parasite growth in culture, as well as differential defects in cell division. Mass spectrometry of TbPRMT6 associated proteins reveals several potential substrates that may contribute to these growth and morphological defects.
MATERIALS AND METHODS

Cloning and expression of TbPRMT6. The gene encoding the TbPRMT6 open reading frame (Tb927.5.3960) was PCR amplified from oligo(dT)-primed cDNA extracted from procyclic form (PF) T. brucei (strain 927 Eatro 1.1) RNA using the primers PRMT6-5'-BamHI 5'-GAGGATCCATGGAGTCCGGAGGGTTTG-3' and PRMT6-3'-HindIII 5'-GGAAGCTTTTAACTCGAGCTCAATG-3'. The restriction enzyme cut sites are underlined. The resultant product was cloned into pJET (CloneJet cloning kit, Fermentas). TbPRMT6 was excised from pJET-TbPRMT6 and ligated into the BamHI and HindIII sites of both the pET21a and pET42a vectors (Novagen). The resultant plasmids were then transformed into Rosetta strain Escherichia coli cells (Novagen) for expression. GST-tagged TbPRMT6 (from pET42a-TbPRMT6) was purified using single step glutathione-agarose (Invitrogen) and a standard GST purification protocol. TbPRMT6 with a C-terminal 6x-Histidine tag was produced from pET21a-TbPRMT6 and was purified by a standard purification protocol using TALON resin (Clontech).

Antibodies. Polyclonal antibodies against TbPRMT6 and TbPRMT7 were raised by injecting rabbits with full-length GST-TbPRMT6 or GST-TbPRMT7 (27) (Proteintech, Inc.). Affinity purified polyclonal anti-peptide antibodies against TbPRMT1 were purchased from Bethyl Laboratories. Monoclonal antibodies against Protein C were purchased from Sigma. Antibodies against Hsp70 and the CTD of RNA polymerase II were generously provided by James Bangs (University of Wisconsin) and Vivian Bellafatto (University of Medicine and Dentistry of New Jersey), respectively. Antibodies against MRP2 were previously described (1).

Cell culture and RNA interference. PF T. brucei strain 29-13 (from Dr. George A.M. Cross, Rockefeller University), which contains integrated genes for the T7 RNA polymerase and
the tetracycline repressor, were grown in SDM-79 media supplemented with 15% fetal bovine serum (FBS) as indicated previously (75), unless otherwise noted. Bloodstream form (BF) single marker $T. brucei$ cells (also provided by Dr. George A.M. Cross) were cultured in HMI-9 media supplemented with 10% FBS and 10% Serum Plus (SAFC) (75). For creation of cells expressing dsRNA targeting TbPRMT6 for RNA interference (RNAi), the full-length TbPRMT6 open reading frame was excised from pET42a-TbPRMT6 using BamHI and HindIII and ligated into the BamHI-HindIII sites of the tetracycline inducible RNAi vector p2T7-177 (95) creating p2T7-177-TbPRMT6. NotI-linearized p2T7-177-TbPRMT6 was transfected into PF and BF cells, and cells harboring this construct were selected with 2.5 $\mu$g/mL phleomycin. Clones were obtained by serial dilution and grown over the indicated time periods in the absence or presence of 2.5 $\mu$g/mL tetracycline. For exogenous expression of TbPRMT6, we engineered the vector pLEW79-MH-TAP to express TbPRMT6 with a C-terminal myc-6x histidine TAP tag (43). Full-length TbPRMT6 was amplified using the primers PRMT6 5’ HindIII 5’ -
GGAAGCTTATGGAGTCCGGAGGGTTTG-3’ and TbPRMT6 3’ BamHI 5’ -
GAGGATCCTTTAAAACTCGAGCTCAATG-3’ (restriction sites are underlined), cloned in to the cloning vector pJET, and finally cloned in to the HindIII-BamHI sites of pLEW79-MH-TAP. The resultant vector pLEW79-TbPRMT6-MH-TAP was transfected in to 29-13 cells and clones selected by limiting dilution. Expression of tetracycline inducible TbPRMT6-MH-TAP was verified by Western blotting against both TbPRMT6 and the myc tag, which confirmed expression of the native 41 kDa TbPRMT6 as well as the approximately 69 kDa tagged TbPRMT6-MH-TAP.

**In vitro methylation assays.** $S$-adenosyl-[methyl-3H]methionine ([3H]AdoMet) (65-82 Ci/m mole) was purchased from Amersham or Perkin Elmer. Methylation assays were performed...
essentially as in (73, 74), with the following modifications. Purified TbPRMT6-His (3µg) was incubated in 50 µL of PBS buffer with specified substrate (typically 3 µg; 10 µg for histones) and 2 µCi [3H]AdoMet for 14 hours at room temperature. The substrates used in this study are as follows: the synthetic peptide H-CGRGRGGRGRRGRG-NH2 (12), myelin basic protein (MBP) (Sigma), bovine mixed histones (IIAS, Sigma), His-RBP16 (35), GST-TbRGG1 (74), and GST-TbRGG2 (26).

**High resolution amino acid analysis of acid hydrolysates of TbPRMT6 products.** Reactions were carried out overnight as indicated above using either 3 µg of GST-TbPRMT6 alone or GST-TbPRMT6 with 10 µg bovine histones. The reactions were precipitated with 50% trichloroacetic acid (TCA), washed with ice-cold 100% acetone, and resultant TCA pellet was acid hydrolyzed to component amino acids under vacuum for 20 hours at 110 °C. The samples were dried and resuspended in 50 microliters of water, followed by addition of 500 microliters of sodium citrate buffer (pH 2.2), and 1.0 µmol each of unlabeled standards of ω-N\(^{\text{G}}\)-monomethylarginine (acetate salt) (MMA), ω-N\(^{\text{G}}\), N\(^{\text{G}}\)-dimethylarginine (hydrochloride) (ADMA), and ω-N\(^{\text{G}}\), N\(^{\text{G}}\)-dimethylarginine (di(p-hydroxyazobenzene-\(p'\)-sulfonate salt) (SDMA) (all purchased from Sigma) in a total of 1 mL. The reaction was then analyzed by cation exchange chromatography as detailed previously (82).

**Subcellular fractionation of TbPRMT6-PTP cells.** PF 29-13 cells were fractionated into cytoplasmic and nuclear fractions using methods described previously (102). Mitochondria were purified by the method of Harris, *et al.* (33). To establish the efficiency of fractionation, western blots were performed with antibodies specific for cytoplasmic Hsp70, nuclear RNA polymerase II CTD, and mitochondrial TbRGG2 (26).
Microscopy of TbPRMT6 RNAi cells. TbPRMT6 RNAi cells (1 x 10^6) in PBS were spread on poly-L-lysine coated slides (Electron Microscope Sciences) and adhered at room temperature for 20 minutes. Cells were then fixed in 4% paraformaldehyde for 15 minutes. Fixation was stopped by washing the slides twice for 5 minutes each in PBS with 0.1M glycine (pH 8.5). Cells were permeabilized for 5 minutes by addition of PBS with 0.025% weight/volume Triton X-100 and then washed with PBS for 5 minutes. Slides were then immersed in methanol at -20°C overnight. Cells were then rehydrated by three washes of PBST (PBS with 0.1% Tween-20) for 5 minutes each, followed by staining with 0.1 µg/mL DAPI in PBS for 5 minutes, and two final washes with PBS. Slides were mounted using a cover slip and Vectashield (Vector Laboratories) as antifade. Cells were visualized using a Zeiss Axio Observer Inverted Microscope and Zeiss Axiovision software.
RESULTS

Identification and sequence analysis of TbPRMT6. Of the five putative PRMTs in the annotated *T. brucei* genome database, three have been previously characterized in our laboratory. This includes canonical homologues of the human Type I PRMT1 and Type II PRMT5 (73, 74), as well as a unique Type III enzyme known as TbPRMT7 (27). A fourth enzyme (Tb927.10.3560), as yet uncharacterized, appears to be the homologue of human PRMT3. The fifth putative PRMT (Tb927.5.3960) shares overall amino acid sequence identities with human PRMT1, but appears to be most similar to human PRMT6 (HsPRMT6) when sequences outside the catalytic core are analyzed. To determine the relationship of Tb927.5.3960 to human PRMTs, we used CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), to align the amino acid sequences of three human Type I PRMTs, HsPRMT1, HsPRMT3, and HsPRMT6 with putative Type I PRMTs from *T. brucei*. A cladogram of this alignment (Fig. 1A) demonstrates that Tb927.5.3960 is most similar to HsPRMT6. Based on this alignment and analyses described below, we coined this protein TbPRMT6. All active PRMTs share several common motifs required for binding to AdoMet and substrate proteins and catalysis of the methyl transfer (3, 6, 49). In Fig. 1B, we align the amino acid sequences of HsPRMT6 and TbPRMT6, demonstrating that TbPRMT6 possesses all of these motifs (motifs I, post I, II, and III, and the double E and THW loops; Fig. 1), although motif III of TbPRMT6 diverges from the canonical motif. Across the regions of motif I through the THW loop, the human and *T. brucei* enzymes share 31% identity and 45.7% similarity. While the proteins are generally less conserved outside this region, we identified several shared motifs between TbPRMT6 and HsPRMT6 that are absent in other Type I PRMTs (“PRMT 6”, Fig. 1B), and which presumably contribute to the homology of the two proteins as
shown in Fig. 1A. TbPRMT6 appears to lack the N-terminal nuclear localization signal present
in HsPRMT6. Whereas human PRMT6 is exclusively nuclear (28), the absence of an evident
NLS in TbPRMT6 suggested that its localization may differ from that of its human counterpart
(see below). The homologous proteins in the related kinetoplastid parasites *T. cruzi* (two distinct
contigs, Tc00.1047053507057.30 and Tc00.1047053506947.80) and *L. major* (LmjF16.0030),
which are 57.1% and 47.3% identical to TbPRMT6 at the amino acid level, respectively, also
lack an evident N-terminal NLS. Based on the primary sequence analysis, the TbPRMT6 gene
appears to encode an active PRMT enyzme.

**Characterization of TbPRMT6 activity.** To determine whether TbPRMT6 possesses
PRMT activity, we conducted *in vitro* methyltransferase assays using methyl-[H$^3$] labeled
AdoMet, recombinant 6x histidine-tagged TbPRMT6, and several potential substrate proteins.
Because HsPRMT6 methylates the tail of histone H3 (37, 38), we tested a mixture of bovine
histones as an *in vitro* substrate for TbPRMT6. Additionally, we tested three *T. brucei* proteins
involved in RNA metabolism, RBP16 (75), TbRGG1 (34), and TbRGG2 (26). All three of these
proteins have glycine/arginine rich (GAR) regions that are common sites of protein arginine
methylation, and all three are *in vitro* substrates for other trypanosome PRMTs (27, 73, 74).
Surprisingly, as shown in Fig. 2, we observed no methylation of RBP16, TbRGG1, or TbRGG2
by TbPRMT6, even after a one month exposure to film. Other common PRMT substrates, such
as an RG-containing peptide and myelin basic protein, were also not methylated by TbPRMT6 in
these assays (data not shown). In contrast, TbPRMT6 did demonstrate PRMT activity towards
proteins in the bovine mixed histone preparation, with the specific substrates appearing to be
histone H3 and H4 according to their migration on SDS-PAGE (Fig. 2). These data demonstrate
that TbPRMT6 is an active PRMT with a narrow substrate range, similar to HsPRMT6.
TbPRMT6 differs from the other trypanosome PRMTs characterized to date in this regard, since those enzymes all methylate a wide range of substrates (27, 73, 74).

HsPRMT6 is a Type I PRMT that synthesizes MMA followed by ADMA in a distributive enzymatic manner (28, 53). To determine whether TbPRMT6 similarly exhibits Type I methyltransfer, we performed in vitro methyltransferase reactions in the presence or absence of bovine histones under the same conditions shown in Fig. 2. Following the reactions, proteins were TCA precipitated and acid hydrolyzed to single amino acids. The products were separated by high resolution chromatography and compared to known standards for MMA, ADMA, and SDMA. As shown in Fig. 3C, TbPRMT6 catalyzed the formation of both MMA and ADMA reactions on the histone substrates, typical of a Type I PRMT. There was no evidence of SDMA production by TbPRMT6. It is important to note that, in these experiments, the peaks of radioactivity eluted approximately 1 minute earlier than the MMA and ADMA standards detected by ninhydrin analysis. Because of their mass and pKa differences, tritiated amines and amino acids elute slightly earlier on high resolution chromatography than their hydrogen counterparts (30, 44, 46, 99). The elution of radioactivity here agrees with that observed previously for tritiated MMA and ADMA (55, 58, 65). Thus, we conclude that MMA and ADMA are the major methyl species detected. The absence of any methylated amino acids in a reaction without enzyme (Fig. 3A) demonstrates that TbPRMT6 catalyzes the production of these methyarginine derivatives on bovine histones. Notably, when TbPRMT6 was analyzed in the absence of substrate, we also observed MMA and ADMA production (Fig. 3B). This indicates that, like human PRMT6 (28), TbPRMT6 is also able to catalyze automethylation. In summary, these data demonstrate that TbPRMT6 is a Type I PRMT.
TbPRMT6 subcellular localization and expression through the life cycle. Having shown that TbPRMT6 is an active PRMT, we next wanted to characterize the *in vivo* properties of the enzyme. In humans, HsPRMT6 is the only PRMT that displays an exclusively nuclear localization. Because the HsPRMT6 NLS is not conserved in TbPRMT6 (Fig. 1), it was of interest to determine the subcellular localization of the trypanosome enzyme. To this end, we analyzed equal protein amounts (20 µg) of nuclear, cytoplasmic and mitochondrially enriched fractions from PF *T. brucei* by immunoblotting with anti-TbPRMT6 antibodies. Antibodies against Hsp70, TbRGG2, and RNA Polymerase II CTD were used as markers for cytoplasm, mitochondria, and nuclei, respectively (26, 31, 45). As seen in Fig. 4A, TbPRMT6 appears almost exclusively cytoplasmic. No signal was detected in mitochondrially enriched fractions. We did, however, observe a faint signal for TbPRMT6 in nuclear extract. The TbPRMT6 signal in the nuclear fraction may result from a small amount of cytoplasmic contamination as suggested by faint nuclear signal observed for the cytoplasmic Hsp70. However, when we analyzed the localization of TbPRMT1 and TbPRMT7 in the same fractions, these enzymes were clearly absent from the nucleus and appeared entirely cytoplasmic as shown previously ((27) and Pelletier, M., manuscript submitted). Thus, TbPRMT6 is primarily cytoplasmic, although we cannot rule out that a small amount of enzyme is also present in the nucleus. The predominant cytoplasmic localization of TbPRMT6 is in direct contrast to the exclusively nuclear localization of its human counterpart.

We next asked whether TbPRMT6 is expressed in both insect and human infective stages of the *T. brucei* life cycle. Equal protein amounts of PF and BF whole cell extracts were analyzed by immunoblot with anti-TbPRMT6 antibodies. Loading was normalized using antibodies against MRP2, a protein that is equally expressed in both life cycle stages (92). Fig.
4B shows that TbPRMT6 is expressed at essentially equal levels in the PF and BF life cycle stages, suggesting that there is no life cycle dependent regulation of TbPRMT6 levels.

**Effects of RNAi-mediated knockdown of TbPRMT6.** Previous studies from our laboratory demonstrated that targeted depletion of TbPRMT1, TbPRMT5, and TbPRMT7 individually has no effect on the growth of PF cells *in vitro* (27, 73, 74). Whether this is due to redundancy between PRMTs or the fact that RNAi does not completely eliminate the protein of interest is currently unknown. Here, we analyzed the effect of RNAi-mediated depletion of TbPRMT6 on the growth of PF and BF *T. brucei*. We utilized the p2T7-177 RNAi vector (96) to generate clonal PF and BF lines expressing TbPRMT6 RNAi. We then induced TbPRMT6 RNAi using tetracycline and monitored cell growth for twelve days (PF, Fig. 5A) or ten days (BF, Fig. 5B). Depletion of TbPRMT6 protein was confirmed on several days during the time course by immunoblotting with anti-TbPRMT6 antibodies (Fig. 5A and B). In both PF (Fig. 5A) and BF (Fig. 5B) we observed a modest, but highly reproducible, slow growth phenotype beginning on day 6 upon induction of TbPRMT6 RNAi under normal culture conditions. This growth phenotype was also evident in multiple analyzed clones of both PF and BF lineages (data not shown). To further investigate the effect of TbPRMT6 downregulation on *T. brucei* growth, we cultured PF cells under nutrient deprivation (1% FBS), and induced the RNAi under these “stressed” conditions (Fig. 5A). Uninduced cells grew at similar rates in 10% and 1% serum. However, when TbPRMT6 RNAi was induced, cells grown in 1% FBS displayed a significantly slower growth rate than did uninduced cells or induced cells in 10% FBS (Fig. 5A). Thus, TbPRMT6 is essential for optimal growth of both PF and BF *T. brucei*. These data suggest that...
TbPRMT6 has a role in cellular growth that is not shared by the other trypanosome PRMTs investigated to date.

In addition to the evident growth effect following loss of TbPRMT6, we also observed some striking morphological effects in the induced PF TbPRMT6 RNAi cells, even when grown under normal conditions (10% FBS) (Fig. 6A). Starting at about day 4 following induction of RNAi and carrying into days 6 and 8, cells began displaying an unusual morphology which we termed “hydra”. These cells display a single body with multiple half-formed cell “heads” that protrude from the central, often nuclei containing, body. The heads of the hydra occasionally contained DAPI staining kinetoplast DNA or nuclear DNA, but not normal numbers of both, and they were frequently devoid of DNA (Fig. 6B, white arrows indicate nuclei, white arrowheads indicate kinetoplasts). PF cells with the hydra morphology were most abundant on day 4, where they comprised almost 5% of the induced cells counted, but they also persisted in days 6 and 8 following tetracycline induction. We next analyzed the morphology of BF TbPRMT6 RNAi cells on day 6 following tetracycline addition (Fig. 7A). While some hydra-like cells were observed in TbPRMT6 depleted BF cells, these cells generally exhibited a different morphology that was characterized by giant rounded cells containing large numbers of both nuclei and kinetoplasts (Fig. 7B). Apparent detachment of the flagella was also evident in several of these giant cells. Giant cells comprised approximately 10% of the BF cells fixed to the microscopy slide. In contrast, no PF or BF cells with these distinct morphologies were ever observed in the uninduced TbPRMT6 RNAi cell cultures. In an attempt to further define a defect in cell division, we counted nuclei and kinetoplasts in at least 200 PF cells for days 4 and 6, or at least 300 BF cells for day 6, (i.e., the approximate time frame at which the growth effect reproducibly manifested) following induction in uninduced and induced cultures. However, we did not detect
any significant deviance from the normal 1N1K to 2N2K distribution of nuclei and kinetoplasts in the cells exhibiting normal morphologies (data not shown). Together, these results suggest that the PF hydra morphology and the BF giant cells are the result of a defect in cell division in a population of cells depleted of TbPRMT6, and that this in turn contributes to the slowing of growth observed at day 6 of the RNAi induction.

Identification of TbPRMT6 associated proteins. Because the associations between PRMTs and their substrates are often relatively stable, some PRMT substrates, as well as upstream activators, have been identified using various protein-protein interaction strategies (29, 89, 90). To begin to understand the molecular basis of the growth and morphological defects in TbPRMT6 RNAi cells, we identified TbPRMT6 interacting proteins using affinity purification followed by mass spectrometry. To this end, we generated a PF *T. brucei* cell line expressing TbPRMT6 with a C-terminal Myc-His-TAP tag (TbPRMT6-MHT) in a tetracycline-inducible manner, a strategy that often yields relevant *in vivo* complexes (34, 72, 84). We purified TbPRMT6-MHT over sequential IgG and calmodulin columns, analyzed the resultant eluted proteins by silver stain (Fig. 8), and subjected the proteins to LC-MS/MS analysis (Seattle Biomedical Research Institute). By silver stain, the primary protein in the eluate was the expected size of TbPRMT6-MHCBP (TbPRMT6 with Myc-His-Calmodulin Binding Protein tag), and only one other sub-stoichiometric protein at approximately 45 kDa was apparent (Fig. 8). However, using mass spectrometry, forty-three proteins, in addition to TbPRMT6, were identified by two or more peptides. Of these, almost half were presumed to be common contaminants present in TAP-based purifications, based on mass spectrometry studies from other laboratories (85, 101, 104, 105) and our previous experience (Table S1). It was striking that the remaining TbPRMT6-associated proteins could be readily classified into three major groups:
nucleocytoplasmic transport, histones, and flagellar proteins (Table 1). Of the seven proteins involved in nucleocytoplasmic transport, six were identified in a recent proteomic study of nuclear pore complexes (NPCs) (18). TbPRMT6 associated NPC proteins were diverse, including members of the B-propeller, α-solenoid, B-sandwich, and FG-repeat classes. Together, these proteins are predicted to be present on either the outer rings of the NPCs on both the nuclear and cytoplasmic faces (TbSec13, TbNup82, TbNup96, TbNup 158) or to line the NPC channel (TbNup98, TbNup149) (18). The seventh protein listed in Table 1 under nucleocytoplasmic transport, NUP-1, is thought to be a major component of the trypanosome nuclear lamina (81). The second major group of proteins that co-purified with TbPRMT6 were histones, including histones H2A, H2B and an H2B variant, H3 and an H3 variant, and two alleles of H4 (Table 1). This finding, together with the ability of TbPRMT6 to methylate bovine histones and the established function of PRMT6-catalyzed histone methylation in humans (32, 37, 38), suggests that trypanosome histones may be a bone fide substrate of TbPRMT6. The third class of TbPRMT-interacting proteins comprised proteins involved in flagellar function and structure (Table 1). Interestingly, several flagellar proteins have been reported to play a role in cell division in T. brucei (11, 50, 78, 79), including the TbPRMT6 associated KMP-11, depletion of which was shown to cause inappropriate nuclear division (57). Finally, TbPRMT6 also co-purified with two proteins reported or suggested to be involved in RNA metabolism: the RNA binding protein, TbRBD3 (21), and the putative RNA helicase, TbDed1, which also associated with TbPRMT5 (73). The low stoichiometry of these proteins to TbPRMT6 itself (Fig. 8) is consistent with their acting as substrates for TbPRMT6 rather than as stable components of a TbPRMT6-containing complex. Overall, these data suggest multiple mechanisms by which TbPRMT6 catalyzed arginine methylation could affect cell growth and morphology, including...
modulation of nucleocytoplasmic transport of macromolecules, chromatin modification, and modulation of RNA processing.
DISCUSSION

In this manuscript, we report the *in vitro* and *in vivo* characterization of a PRMT from the protozoan parasite, *T. brucei*. This enzyme, which we term TbPRMT6, is most similar to PRMT6 in the family of mammalian protein arginine methyltransferases. The mammalian PRMT6 enzymes have been shown to function in DNA repair and transcriptional control (20, 37, 38). However, PRMT6 homologues appear to be absent from yeast and most other protozoa (3). Despite the evolutionary distance between humans and trypanosomes, HsPRMT6 and TbPRMT6 are similar in that they exhibit Type 1 PRMT activity and catalyze automethylation. The consequences of this automethylation are currently unknown. TbPRMT6 also resembles human PRMT6 in its apparently narrow substrate range, and it differs from the other more promiscuous *T. brucei* PRMTs in this regard (27, 73, 74). *In vitro*, TbPRMT6 methylated a mixture of bovine histones, but did not methylate several other substrates, including three trypanosome GAR proteins. TbPRMT6 histone methylation may be of functional significance since histones are an *in vivo* substrate of human PRMT6 and we found that TAP-tagged TbPRMT6 expressed in PF *T. brucei* co-purified with several histones (see below). In contrast to the properties listed above, HsPRMT6 and TbPRMT6 differ in their subcellular localization, with the human enzyme exhibiting an exclusively nuclear localization, while TbPRMT6 is predominantly cytoplasmic. It is important to note, however, that the cytoplasmic steady state localization of TbPRMT6 does not preclude its having nuclear functions. Several human PRMTs shuttle between the cytoplasm and nucleus, and human PRMT5 appears predominantly cytoplasmic at steady state despite several well-documented roles in transcription and cell cycle progression (23, 41, 51, 80, 86, 94, 103). While experiments using leptomycin B, an inhibitor of crm1/exportin1 dependent nuclear export, did not provide evidence for nucleocytoplasmic shuttling of TbPRMT6 (data not shown),
we cannot rule out that TbPRMT6 shuttles through a different pathway or that a small amount of
nuclear enzyme is sufficient for its nuclear functions.

Of the four *T. brucei* PRMTs that have been characterized to date, TbPRMT6 is the only
enzyme whose downregulation leads to a growth defect. While this defect is modest under
normal culture conditions, it becomes pronounced when PF cells are nutrient starved. Thus,
TbPRMT6 performs a non-redundant function that is critical for cell growth. TbPRMT6
depletion also leads to morphological defects in a subset of both PF and BF cells. Aberrant PF
cells appeared to have initiated, but not completed, cell division. In addition, there often
appeared to be multiple nuclei at the junction of partially divided cells, suggesting that nuclear
segregation was impaired in cells depleted of TbPRMT6. In BF, giant rounded cells with large
numbers of nuclei and kinetoplasts accumulated, often with detached flagella, again indicating
defects in cytokinesis in cells with decreased TbPRMT6 levels.

Mass spectrometry analysis of TbPRMT6 associated proteins highlighted several cellular
pathways that could contribute to an essential function of the enzyme. Notably, histones were a
major class of proteins that were associated with TbPRMT6-MHT in PF cells. We obtained
multiple peptide hits for seven different trypanosome histone proteins. This finding, coupled
with the ability of HsPRMT6 to methylate histones *in vivo* and our demonstration that TbPRMT6
methylates bovine histones *in vitro* (Fig. 2), suggests that histones might be an *in vivo* substrate
for TbPRMT6. The identification of histones as putative TbPRMT substrates was somewhat
surprising, as analyses of histone modifications in *T. brucei* to date have identified acetylation,
phosphorylation, and lysine methylation, but no methylation of arginine residues (25). Previous
analyses of histone modifications were performed using Edman degradation (42, 62). Recently
developed mass spectrophotometric techniques for detection of methylarginines (93) may be
useful for identification of previously overlooked methylarginine residues in histones of *T. brucei*. If such modifications are detected in wild type cells, it will be of interest to analyze histone modifications in TbPRMT6 depleted cells to reveal whether this enzyme catalyzes histone arginine methylation and whether it, in turn, affects chromatin structure and gene regulation.

A second family of proteins that was highly represented in TbPRMT6-MHT purification was that comprising components of the nuclear pore complex (NPC). There are two different possibilities to account for the association of TbPRMT6 with the NPC, and these scenarios are not mutually exclusive. First, as suggested above, TbPRMT6 may itself be a shuttling protein. As such, NPC components may have been isolated in combination with TbPRMT6 that was itself undergoing nucleocytoplasmic transport at the time of cell lysis. Alternatively, some NPC components may be substrates for TbPRMT6. Arginine methylation is well known to affect the subcellular localization of many proteins and RNAs (2, 8, 60, 76, 88). In some instances, methylation-induced alterations in nucleocytoplasmic transport have been directly attributed to modification of specific shuttling proteins, but a role for methylation of NPC components can easily be envisioned.

We also identified four flagellar proteins in association with TbPRMT6, although no defects in cell motility were evident in those TbPRMT6 RNAi cells with normal morphology. However, several flagellar proteins of *T. brucei* are reported to affect both the cell cycle and cellular division. While we did not detect specific cell cycle defects in the majority TbRMT6 depleted cells, up to 5% of PF cells exhibited the dramatically altered morphology described above that we termed “hydra”. A similar morphology was observed when the paraflagellar rod protein pfr2 was depleted in PF cells (79). As stated above, the aberrant forms present in
cultures of TbPRMT6 depleted BF cells were even more striking. These altered morphologies indicate that cells depleted for TbPRMT6 exhibit defects in cytokinesis, and these defects are more pronounced in the BF stage. Interestingly, depletion of various flagellar components or flagellar interacting proteins leads to aberrant cytokinesis and/or abnormal numbers of nuclei/kinetoplasts, and these defects are often more severe in BF cells compared to PF cells (11, 47, 50, 78). Loss of another TbPRMT6 interacting protein KMP-11, leads to altered cytokinesis, attachment of flagella, and multiple nuclei (57). Together, these data suggest a model whereby TbPRMT6 catalyzed methylation of a subset of flagellar proteins is essential for proper cell division in T. brucei.

Finally, two proteins that have been shown or are presumed to function in RNA metabolism, TbRBD3 and TbDed1, were isolated with TbPRMT6-MHT. In general, RBPs constitute a major class of arginine methylproteins in both mammals and yeast (4, 36, 59, 70, 89). Methylation of RBPs generally leads to dramatic alterations in protein-protein interactions (7, 15, 19, 24, 56, 67, 100) and, less often, in protein-RNA interactions. Estevez (21) recently reported that TbRBD3 binds to a subset of developmentally regulated transcripts and promotes their stabilization. Because TbRBD3 is equally expressed in PF and BF T. brucei, it was proposed that its role in developmentally modulated RNA stabilization may result from life cycle specific posttranslational modifications. Thus, it will be interesting to determine the methylation status of TbRBD3 in PF and BF. TbDed1 is a DEAD box protein and putative RNA helicase that has been reported in TAP pulldowns of RNA polymerase II and a non-canonical nuclear poly(A) polymerase in T. brucei (16, 22), although its functions have not been elucidated. Notably, we also identified TbDed1 in association with the SDMA-producing TbPRMT5 from T. brucei cytoplasm (73). In addition, the human homologue of TbDed1, DDX3, associates with
the Type I PRMT, PRMT8 (71). Recent analyses in our laboratory suggest that TbDed1 contains both ADMA and SDMA modifications (S.G. Menon, J.C. Fisk, and L.K. Read, unpublished observations). Future studies will be focused on the roles of arginine methylation in modulating the actions of this apparently multi-functional DEAD box protein in trypanosome gene regulation.

Collectively, the studies presented here identify TbPRMT6 as a Type I PRMT with a key function in both PF and BF *T. brucei*. The enzyme exhibits a narrow substrate range, which must include proteins that are critical for cell growth. Analysis of the methylation status and function of the TbPRMT6 associated proteins reported here will provide insight into the essential roles this enzyme and its methylarginine function in trypanosomes.

**Acknowledgements**

We would like to thank Jay Bangs and Vivian Bellofatto for antibodies, George Cross for cell lines, and David Campbell for leptomycin B. Thanks are due to Yuko Ogata at SBRI for mass spectrophometric analysis of TbPRMT6 complexes. LKR was supported by NIH grant #R01 AI060260, and SGC was supported by NIH grant #R37 GM026020. CZL was supported by a Ruth L. Kirschstein National Research Service Award (NRSA) from the NIH (GM078761). JCF was supported in part by NIH NRSA postdoctoral fellowship #F32 AI07718501.
methyltransferase 1 regulates its binding to proliferating cell nuclear antigen. FASEB Journal 21:26-34.


FIGURE LEGENDS

FIGURE 1. **Sequence analysis of TbPRMT6.** A, CLUSTALW-generated cladogram of the human PRMT1, PRMT3, and PRMT6 with the trypanosome TbPRMT1, TbPRMT6 (Tb927.5.3960), and Tb927.10.3560. B, Sequence comparison of human PRMT6 (HsPRMT6) and TbPRMT6. Horizontal lines indicate conserved PRMT domains required for AdoMet binding and enzymatic activity (see text), as well as the nuclear localization signal (NLS) present only in the human enzyme. Regions typically found in mammalian PRMT6 enzymes are also labeled as “PRMT6” regions. The asterisks indicate the position of the conserved “double E” loop amino acids. Black background with white text indicates identical amino acids; gray background with white text indicates conserved amino acids.

FIGURE 2. **Enzymatic activity of recombinant TbPRMT6.** Three micrograms of TbPRMT6-His was incubated in the presence of $^3$H-AdoMet and the indicated substrate in PBS for fourteen hours at 22°C. Products were resolved by SDS-PAGE and Coomassie stained (bottom panels). Gels were then treated with EnH$^3$ance, dried, and exposed to film for 1 month (top panels; Fluorograph).

FIGURE 3. **Amino acid analysis of methylarginine derivatives formed by TbPRMT6.** A, Ten micrograms of the bovine histone substrate alone was incubated in the presence of $^3$H-AdoMet in PBS for fourteen hours at 22°C. The proteins were precipitated with 50% trichloroacetic acid and digested into amino acids by acid hydrolysis. Amino acids were analyzed by cation exchange chromatography in the presence of unlabeled ADMA, SDMA, and MMA standards (dotted lines). 200 µl of each fraction (1/5 of the total fraction) was removed.
for radioactivity analysis and 100 µL was removed for ninhydrin analysis, and the fractions were counted three times for three minutes each (solid lines).  

B, Three micrograms of TbPRMT6-His in the absence of additional substrate was incubated and analyzed as in A.  

C, Ten micrograms of bovine histones was incubated with three micrograms of TbPRMT6-His and incubated and analyzed as in A.

FIGURE 4. Subcellular localization of TbPRMT6.  

A, PF cells were separated into cytoplasmic and nuclear fractions. Mitochondrial extracts were also prepared in a separate fractionation scheme. An equal amount of protein (20 µg) from whole cell lysates (WCL), cytoplasm, mitochondria, and nuclei were fractionated on SDS-PAGE. Antibodies against Hsp70 (cytoplasmic), TbRGG2 (mitochondrial), and RNA polymerase II CTD (nuclear) were used to monitor fractionation efficiency. TbPRMT1 and TbPRMT7 are two cytoplasmic PRMTs that were also used as controls.  

B, Determination of TbPRMT6 levels in the procyclic form (PF) and bloodstream form (BF) life cycle stages of *T. brucei*. Total cell extract (equivalent of 5 x 10^6 cells) from 29-13 PF cells and single marker BF cells were resolved by SDS-PAGE and immunoblotted using an antibody against TbPRMT6. As a loading control, levels of MRP2 (a protein known to be equivalent between PF and BF life cycles (92)) were also analyzed by Western blot.

FIGURE 5. Effect of TbPRMT6 depletion on procyclic form (PF) and bloodstream form (BF) *T. brucei* cell growth.  

A, PF 29-13 cells harboring the tetracycline regulatable RNAi vector p2T7-177-TbPRMT6 were treated in the absence of tetracycline (solid lines) or in the presence of 5 µg/mL tetracycline (dotted lines) and counted every two days. RNAi induction...
and cell growth analysis was performed in both 10% fetal bovine serum (FBS; diamonds) and 1% FBS (squares). The lower panel shows Western blotting of TbPRMT6 on days 2, 4, and 6 following tetracycline induction of RNAi. Hsp70 was used as a loading control. B, BF single marker cells transfected with the RNAi vector p2T7-177-TbPRMT6 were uninduced (solid line) or induced in A (dotted line). The lower panel shows the Western blotting of TbPRMT6 and Hsp70 on days 2, 4, and 6 following tetracycline induction.

FIGURE 6. Microscopy of procyclic form cells exhibiting aberrant morphologies upon TbPRMT6 depletion. A, Morphological changes evident in PF TbPRMT6 RNAi cells. Cells grown in the presence (+TET) or absence (-TET) of tetracycline for 6 days were fixed to slides, stained with DAPI, and analyzed by microscopy. B, Images of PF “hydra” cells, characterized by a central body containing nuclei (white arrows) with multiple heads with or without kinetoplasts (white arrowheads).

FIGURE 7. Microscopy of bloodstream form cells exhibiting aberrant morphologies upon TbPRMT6 depletion. A, Morphological changes evident in BF TbPRMT6 RNAi cells. Cells grown in the presence (+TET) or absence (-TET) of tetracycline for 6 days were fixed to slides, stained with DAPI, and analyzed by microscopy as in Fig. 6. B, Images of the giant cells with detached flagella evident in BF TbPRMT6 RNAi cells. Note the BF cell with normal morphology (black arrow) for scale reference.

FIGURE 8. Silver stain of TAP tagged purified TbPRMT6 complexes. TbPRMT6-Myc-His-TAP was expressed in PF 29-13 cells and purified using standard tandem affinity purification.
The eluate was resolved using SDS-PAGE and silver stained. In addition to the major band representing TbPRMT6-MHCBP (TbPRMT6-Myc/His/Calmodulin Binding Protein; bold arrow), a protein of approximately 45 kDa was observed (thin arrow).

TABLE 1. Proteins identified in TbPRMT6-MHT eluates. TbPRMT6-MHT was purified by tandem affinity chromatography and resulting eluates were analyzed by mass spectrometry. Peptides identified were compared to known sequences in the annotated trypanosome database GeneDB (http://www.genedb.org). Identified proteins were further categorized based on their known or putative functions. Number of unique peptides identified for each protein is shown. Proteins for which only one peptide was identified were excluded from the analysis.

SUPPLEMENTAL TABLE 1. Proteins identified in TbPRMT6-MHT eluates that are common contaminants of tandem affinity purifications in T. brucei.
Figure 1, Fisk, et.al.
Figure 3, Fisk, et.al.
Figure 4, Fisk, et.al.
A

Proyclic

B

Bloodstream

Figure 5, Fisk, et.al
Fig 6, Fisk et al
Figure 7, Fisk, et. al.
Figure 8, Fisk, et al.
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Table 1, Fisk, et.al.