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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