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Molecular & Biochemical Parasitology 118 (2001) 49–59

MOLECULAR & BIOCHEMICAL PARASITOLOGY

www.parasitology-online.com.

Arginine methylation of a mitochondrial guide RNA binding protein from *Trypanosoma brucei*

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Received 18 April 2001; accepted in revised form 17 August 2001

Abstract

RBP16 is a mitochondrial Y-box protein from the parasitic protozoan *Trypanosoma brucei* that binds guide RNAs and ribosomal RNAs. It is comprised of an N-terminal cold-shock domain and a C-terminal domain rich in glycine and arginine residues, resembling the RGG RNA-binding motif. Arginine residues found within RGG domains are frequently asymmetrically dimethylated by a class of enzymes termed protein arginine methyltransferases (PRMTs). As Arg-93 of RBP16 exists in the context of a preferred sequence for asymmetric arginine dimethylation (G/FGGRGGG/F), we investigated whether modified arginines are present in native RBP16 by MALDI-TOF and post-source decay analyses. These analyses confirmed that Arg-93 is dimethylated. In addition, Arg-78 exists as an unmodified or as a monomethylated derivative, and Arg-85 is present in forms corresponding to the unmodified, di-, and tri-methylated state. While Arg-93 is apparently constitutively dimethylated, the methylation of Arg-78 and Arg-85 is mutually exclusive. Furthermore, whole cell extracts from procyclic form *T. brucei* are able to methylate bacterially expressed RBP16 (rRBP16), as well as endogenous proteins, in the presence of S-adenosyl-L-[*methyl*-^{3}H]methionine. While assays of mitochondrial extracts suggest a small amount of PRMT may also be present in this subcellular compartment, the majority of trypanosome PRMT activity is extramitochondrial. We show that rRBP16 is methylated in trypanosome extracts through the action of a type I methyltransferase as well as serving as a substrate for heterologous mammalian type I PRMTs. In addition, we demonstrate the presence of type II PRMT activity in trypanosome cell extracts. These results suggest that protein arginine methylation is a common posttranslational modification in trypanosomes, and that it may regulate the function of RBP16.

Keywords: *Trypanosoma*; Guide RNA; Arginine methylation; RNA editing; RNA binding protein

1. Introduction

RBP16 is a mitochondrial Y-box family protein from the parasitic protozoan *Trypanosoma brucei* [1]. It was first identified as a protein capable of forming multiple, stable complexes with small RNA molecules known as guide RNAs (gRNAs) in vitro, primarily via the gRNA oligo(U) tail. Immunoprecipitation and *in organello* UV cross-linking experiments subsequently demonstrated an association between RBP16 and gRNAs within *T. brucei* mitochondria [1,2]. gRNAs are involved in kinetoplastid RNA (kRNA) editing, a remarkable RNA processing mechanism characterized by the site-specific insertion and deletion of uridylate residues into pre-mRNAs that is required for the creation of functional mRNA molecules [3]. Demonstration of an RBP16-gRNA interaction in vitro and in vivo suggests a role for this protein in kRNA editing. In addition, RBP16 binds rRNAs in vivo, presumably via the oligo(U) tails present on these molecules [1]. This, in addition to the multifunctional nature of many Y-box proteins [4], suggests that RBP16 may also be involved in mitochondrial RNA translation or may couple the editing and translation processes.

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0166-6851/01/$ - see front matter
PII: S0166-6851(01)00367-X

RBP16 contains an N terminal cold shock domain a C-terminal domain rich in glycine and arginine residues, resembling the RGG RNA-binding motif [1,5]. In vitro assays indicate that the RBP16 RGG domain functions to increase the overall affinity of RBP16-gRNA binding through interactions with the encoded portion of the gRNA ([6], Miller and Read, unpublished results). Arginine residues found within RGG RNA-binding domains are frequently asymmetrically dimethylated [7–9]. This posttranslational modification is carried out by a class of enzymes known as protein arginine methyltransferases (PRMTs). In mammalian cells, two distinct types of PRMTs have been identified [8]. The type I enzymes catalyze the formation of both ω-N^G-monomethylarginine (MMA) and asymmetric ω-N^G,N^G-dimethylarginine (ADMA) residues. Substrates for the type I PRMTs are predominantly RNA binding proteins including hnRNP A1 and A2 [7,10], nucleolin [11], fibrillarin [12], poly(A)-binding protein II [13], and the yeast Npl3 protein [14]. Type II enzymes form both MMA and symmetric ω-N^G,N'^G-dimethylarginine (SDMA). Myelin basic protein [15] and the Sm ribonucleoproteins D1 and D3 [16] are the only known substrates for the type II PRMTs. Although methylation of arginine residues was first discovered over 30 years ago, its functions remain poorly understood. Recently, this class of posttranslational modifications has become increasingly implicated in the regulation of protein–protein interactions [8,9]. Evidence has also revealed a role for protein arginine methylation in the regulation of protein sorting, transcription, and signal transduction [9].

Although protein methylation in trypanosomes has been reported, these studies focused on the carboxymethylation of aspartate and glutamate [17,18]. To date, no arginine-methylated protein has been identified in trypanosomes, and the overall extent of protein arginine methylation and the fate of methyl groups in trypanosomes remain virtually unknown. As a sequence identified as a preferred site for asymmetric arginine methylation (G/FGGRGGG/F) is found within the Arg/Gly-rich C-terminal domain of RBP16 [1], we examined RBP16 purified from mitochondria of procyclic form *T. brucei* by MALDI-TOF and post-source decay analyses to determine whether modified arginines were present. These analyses confirmed that Arg-93 is dimethylated. They showed, in addition, that Arg-78 can be monomethylated, and that Arg-85 is present in forms corresponding to the unmodified, di-, and trimethylated state. To our knowledge, this is the first report of a native trimethylated arginine residue. *T. brucei* procyclic form cellular extracts are able to carry out the methylation of bacterially expressed RBP16 (rRBP16), as well as that of several endogenous proteins, in the presence of S-adenosyl-L-[*methyl*-^{3}H]methionine (^{3}H-AdoMet). We further demonstrate that the trypanosome methyltransferase acting on rRBP16 is a type I PRMT, and that rRBP16 can be methylated in vitro by mammalian type I PRMTs. Although RBP16 is not a substrate for type II PRMTs, this class of enzyme is also detectable in trypanosome cellular extracts. Our results constitute the first demonstration of a specific protein arginine methylation event in any protozoan. The identification of multiple arginine methylation events in a primitive organism such as *T. brucei* suggests a widespread importance of methylation as a regulatory mechanism in eukaryotes. Moreover, our results suggest that the mitochondrial function of RBP16 may be regulated by specific arginine methylation events.

2. Materials and methods

2.1. Cell culture, extract preparation, and mitochondrial vesicle isolation

Procyclic form *Trypanosoma brucei brucei* clone IsTar1 stock EATRO 164 was grown in SDM-79 medium as described [19]. Mitochondrial extracts were obtained as described [20]. Whole cell extracts were prepared as follows. Cells were harvested by centrifugation at $6090 \times g$ for 10 min at 4 °C, resuspended in SBG buffer (20 mM sodium phosphate (pH 7.9), 150 mM NaCl, 20 mM glucose), and centrifuged at $6090 \times g$ for 10 min at 4 °C. The pellet was then resuspended in DTE buffer (1 mM Tris (pH 8.0), 1 mM EDTA) at 1.2×10^9 cells per ml. The suspension was quickly dounced (five strokes), the cells were lysed by passage through a 26 gauge needle, and sucrose was adjusted to a final concentration of 0.25 M. After centrifugation at $15\,800 \times g$ for 10 min at 4 °C, the pellet was resuspended in DTE.

PC12 cells were cultured as described [21,22]. In the 16 h prior to harvest, nerve-growth factor (NGF) was added to 50 ng ml^{-1} to some cultures. The cells were harvested in 20 mM Tris buffer (pH 8.0) containing 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg ml^{-1} benzamidine, and 0.1% Triton X-100. After vortexing, the nuclei were isolated by centrifugation at $800 \times g$ for 5 min at 4 °C. To generate a nuclear fraction, the nuclei were washed in 20 mM Tris buffer (pH 8.0) containing 2 mM PMSF and 10 μg ml^{-1} benzamidine. After removing the wash buffer, nuclei were resuspended in 20 mM Tris buffer (pH 8.0) containing 2 mM PMSF, 10 μg ml^{-1} benzamidine, and 300 mM NaCl. Following gentle agitation for 10 min, the nucleoplasmic extracts were separated from the insoluble nuclear matrix material by centrifugation at $12\,000 \times g$ for 5 min at 4 °C.

2.2. Bacterial expression and purification of recombinant RBP16 (rRBP16) and rat PRMT1 (rPRMT1)

RBP16 was expressed as a maltose-binding fusion protein in *Escherichia coli*, and purified by amylose and poly(U)-Sepharose chromatography as previously described [1]. Rat PRMT1 was expressed as a glutathione S-transferase (GST) fusion from pGEX-PRMT1 (a gift from Dr Harvey R. Herschman, UCLA) [23]. *E. coli* BL-21 cells carrying the pGEX-PRMT1 plasmid were grown in LB medium at 37 °C to an optical density of 0.6. Cells were induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 2 h and harvested by centrifugation at $5000 \times g$ for 10 min at 4 °C. Cells were resuspended in PBS buffer (containing 0.4 mM PMSF and 2 μM benzamidine) and lysed by sonication on ice (3 pulses of 30 s each). The cells were then centrifuged at $14\,000 \times g$ for 20 min at 4 °C. The supernatant was mixed with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) for 10 min at room temperature and the mixture was subsequently diluted with PBS buffer. The mixture was poured into a column and the column washed with 30 column volumes of PBS buffer. The GST-PRMT1 fusion was then eluted with 10 volumes of PBS buffer containing 5 mM reduced glutathione.

2.3. Mass spectrometry analysis

RBP16 from procyclic form *T. brucei* was purified by poly(U) affinity chromatography as described [1]. The RBP16-containing fractions were pooled and further fractionated by SDS-PAGE on a 12.5% polyacrylamide gel. The band corresponding to RBP16 was excised from the gel and subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Borealis Biosciences Inc, Toronto, Ont., Canada). Briefly, the gel slices were prepared by reducing the gel with DTT and alkylating cystines with iodoacetamide. The protein was digested with trypsin and peptides were extracted. The extracted peptides were purified, desalted and concentrated by reverse phase micro-chromatography. The sample was mixed with the matrix α-cyano-4-hydroxycinnamic acid and analyzed by a Perceptive Biosystems Voyager STR MALDI-TOF mass spectrometer. The sample was internally calibrated and the calibrated and experimental masses were searched using Profound (Proteometrics Ltd, New York, NY) software for correlative mass mapping of experimental masses to calculated masses. Some of the peptides showing mass shifts correlating to methylation were analyzed by post-source decay (PSD) to induce fragmentation of the peptide. The fragment data was analyzed using the Pepfrag software (Proteometrics Ltd, New York, NY). This matched the induced and measured fragments with all possible calculated fragments. The matching parameters were changed to account for possible methylation of arginine residues.

2.4. In vitro methylation assays

The 50 μl standard in vitro methylation reaction mixture contained 80 mM Tris (pH 8.0), 0.4 mM PMSF, 2 μM benzamidine, 1.7 μg rRBP16, and 4 μCi of S-Adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet) (Amersham Pharmacia Biotech, 88 Ci mmol^{-1}). Reactions were initiated by the addition of whole cell or mitochondrial extract (10–100 μg) and incubated for 40 min at 36 °C and stopped by the addition of SDS-PAGE sample buffer. In some experiments, samples were treated prior to addition of sample buffer as follows: 5 U DNAse I on ice for 30 min, 1 μg RNAse A at 37 °C for 30 min, or 1 μg proteinase K at 37 °C for 30 min. Competition reactions were performed by addition of increasing amount of either myelin basic protein (MBP) from bovine brain (Sigma) or nucleolin-derived peptide (^{334}GRGGFGGRGGFRGGRG^{350}G) prior to the addition of whole cell extract (100 μg). For in vitro assays using purified GST-PRMT1 as the methyltransferase, rRBP16 was incubated with 1–3 μg of purified GST-PRMT1 for 60 min at 36 °C. When using PC12 cell nuclear extract, 10 μg of nuclear extract from cells grown in the presence or absence of nerve growth factor (50 ng ml^{-1}) was used and the reactions incubated for 60 min at 36 °C. Reactions were stopped by the addition of 12.5 μl of 5× SDS-PAGE loading buffer and boiled for 5 min. Samples were resolved on 12.5% polyacrylamide (2.7% bis-acrylamide) gels, and the gels were then stained for 1 h with 0.1% Coomassie Brilliant Blue in a 50% methanol/10% acetic acid solution in water. For fluorography, the gels were destained for at least 1 h in a 5% methanol/10% acetic acid solution in water, then soaked for 1 h in EN^{3}HANCE (NEN-Dupont), and finally soaked in water for 30 min. The gels were then dried and exposed to film (Kodak X-OMAT Blue XB-1).

2.5. Cross-linking of antibodies to protein A-sepharose and immunoprecipitation of methylated RBP16

One hundred micrograms of affinity purified polyclonal anti-RBP16 antibodies or 100 μl pre-immune serum was incubated with 30 μl of protein A-Sepharose (Amersham Pharmacia Biotech) (previously equilibrated in phosphate-buffered saline (PBS; pH 7.2)) for 1 h at 4 °C with gentle mixing. The beads were washed three times with 10 volumes (300 μl) of 0.1 M sodium borate (pH 9.0) by centrifugation at $10\,000 \times g$ for 30 s and resuspended in 1 ml of sodium borate in the presence of 5 mg of dimethylpimelimidate. After incu-

bation at room temperature for 30 min, the antibody–protein A-Sepharose complexes were recovered by centrifugation at 10 000 × *g* for 30 s and the pellet washed once with 10 volumes of 0.2 M triethanolamine (pH 9.0) for 30 s and once with 1 ml of the same buffer for 2 h at room temperature with gentle mixing. After centrifugation at 10 000 × *g* for 30 s, non-covalently bound antibodies were removed by washing with 1 ml of 0.1 M glycine (pH 2.5) for 1 min at room temperature. The final pellet was resuspended in 100 μl of 80 mM Tris (pH 8.0).

Immunoprecipitation of [^{3}H]AdoMet-labeled RBP16 was performed as follows. Following in vitro methylation of *T. brucei* whole cell extract as described above, the reaction was diluted 2-fold with assay buffer and 50 μl of either pre-immune IgGs or anti-RBP16 cross-linked to protein A-Sepharose was added. After incubating for 6 h at 4 °C, the immune complex was recovered by centrifugation at 10 000 × *g* for 30 s. The supernatant and pellet were then electrophoresed on a 15% polyacrylamide gel. The gel was treated for fluorography as described above.

2.6. Western blot analysis

Proteins (2.5 μg) from either whole cell or mitochondrial extract were separated by 15% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane (Schleicher and Schuell) at 50 V for 30 min in 10 mM 3-[Cyclohexylamino]-1-propanesulfonic acid (CAPS) buffer (pH 11.0) containing 10% methanol. After transfer, the membrane was blocked for 1 h at room temperature with 5% (w/v) dry milk in Tris-buffered saline (TBS). The membrane was then incubated for 2 h at room temperature with either anti-RBP16 or anti-poly(A)-binding protein I (PABI; a generous gift from Dr Noreen Williams), at a 1:1000 and 1:5000 dilution, respectively, in TBS containing 2% (w/v) dry milk and 0.05% (v/v) Tween 20, followed by incubation with goat anti-rabbit antibody coupled to horseradish peroxidase (Pierce Endogen) for 1 h at room temperature. The detection was performed by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

3. Results

3.1. Identification of arginine methylation sites in RBP16 by mass spectrometry

Arginine residues found within RGG RNA-binding domains are frequently asymmetrically dimethylated [8,9]. Asymmetric dimethylation of arginines has been reported to be involved in a number of processes such as signal transduction, intracellular trafficking, and modulation of protein–protein and protein–nucleic acid interactions [24–28]. Arg-93 in the RGG domain of RBP16 is found within a sequence corresponding to the asymmetric arginine methylation motif of hnRNP A1 (G/FGGRGGG/F) [7]. This observation prompted us to determine whether arginine modified forms of RBP16 exist. Toward this end, native RBP16 from procyclic form *T. brucei* was purified by poly(U)-Sepharose affinity chromatography [1] and subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) following tryptic digestion (Fig. 1A). Four peaks whose observed masses deviated from the expected values were observed (Fig. 1B, peptides 7, 9, 10, and 11) (theoretical mass difference for a methylated amino acid = 14.5 Da/methyl group). The observed mass of peptide 11 (848.350 Da) corresponding to amino acids 88–96 was found to deviate from the theoretical expected mass for a unmodified peptide by 28.944 Da, thereby indicating the presence of a dimethylated arginine residue within this peptide. Since trypsin does not cleave after a methylated arginine [7,15], we conclude that Arg-93, not Arg-96, is dimethylated, as predicted from the sequence context of Arg-93. No peptide containing an unmethylated Arg-93 was isolated. This suggests that, in vivo, all RBP16 molecules are dimethylated at that position. It is possible that a fraction of the RBP16 population is unmethylated at Arg-93 and that, due to difference in nucleic acid binding properties, unmethylated protein did not bind to the poly(U)-Sepharose column used for the purification of the native RBP16. However, this is unlikely since western blot analysis using anti-RBP16 antibodies indicated that all RBP16 molecules bound to the column (not shown).

The observed mass of peptide 7 is 14.926 Da greater than the expected mass for an unmodified peptide, in agreement with the presence of a monomethylated arginine. Since no tryptic cleavage occurs after either mono- or dimethylated arginine [7,15], the presence of two arginine residues (Arg-78 and Arg-85) in this tryptic peptide ending with an arginine indicates that the internal Arg-78 rather than the C-terminal Arg-85 is monomethylated. Three peptides corresponding to amino acids 73–87 were isolated: peptides 8, 9, and 10. While peptide 8 showed no modification, the observed masses of peptides 9 and 10 deviated from the theoretical expected mass for an unmodified peptide by 28.969 and 42.981 Da, respectively. These differences indicate the presence of two and three methyl groups within peptide 9 and 10, respectively. Since there are two potential methylated arginines in these peptides (Arg-78 and Arg-85), we utilized post-source decay (PSD) to precisely determine which residues were modified. PSD analysis indicated that Arg-85 is present in forms corresponding to the di- and tri-methylated state, and that Arg-78 is unmodified within peptides 8, 9, and 10. The

presence of Arg-78 within the sequence X-Arg-Pro, which is known to inhibit trypsin [29], may explain the absence of cleavage at this site. No peptide corresponding to amino acids 97–106 was isolated, and thus we cannot ascertain whether Arg-102 is methylated in vivo. Interestingly, the methylation of Arg-78 and Arg-85 appears to be mutually exclusive. That is, PSD analysis indicates that Arg-78 is unmodified in all peptides containing modified Arg-85 (peptides 9 and 10). Conversely, cleavage of peptide 7 after Arg-85 indicates that this residue is always unmodified while Arg-78 is monomethylated. While the dimethylation of RBP16 at Arg-93 appears to be constitutive, the presence of either unmodified or methylated Arg-78 and Arg-85 suggests a regulatory function for methylation at these sites. Finally, the presence of a trimethylated form of Arg-85 in some peptides is of particular interest. Although the in vitro synthesis of a trimethylarginine had been described [30], this is, to our knowledge, the first evidence of the existence of an arginine trimethylated in vivo.

3.2. Protein methylation activity in T. brucei cellular extract

We next investigated whether *T. brucei* cellular extracts possess protein methyltransferase activities capable of methylating RBP16. Since RBP16 is translated in the cytosol and subsequently imported into the mitochondrion, the potential for methylation of this protein exists in both cellular compartments. Thus, we tested whole cell and mitochondrial extracts from procyclic form trypanosomes as a source of methyltransferase for the methylation of bacterially expressed RBP16 (rRBP16) in the presence of [^{3}H]AdoMet. As shown in Fig. 2A, extracts from both whole cells and mitochondria are able to support methylation of rRBP16. Maltose-binding protein alone was not methylated (not shown). Comparison of the [^{3}H]AdoMet-labeled rRBP16 signals obtained with equivalent amounts of whole cell and mitochondrial extracts clearly indicates that the bulk of methylating activity in procyclic trypanosomes is extramitochondrial. To determine whether the small amount of activity present in mitochondrial extracts could be accounted for by contamination of the mitochondrial preparation, we performed Western blot analysis with antibodies against the cytosolic/nuclear PABI [31,32] and mitochondrial RBP16 [1] (Fig. 2B). As expected, mitochondrial extracts were almost 10-fold enriched for the mitochondrial marker (Fig. 2B, anti-RBP16). Slight contamination of the mitochondrial extract preparation with PABI was observed (Fig. 2B, anti-PABI). Densitometry indicated that the anti-PABI signal in the mitochondrial extract preparation was 6% of that obtained in the equivalent amount of whole cell extract. Quantification of the rRBP16 methylation activity in Fig. 2A (lanes 1 and 4) indicates that the level of mitochondrial activity is 8% of that observed in whole cell extract. As the level of methylating activity is slightly above the level of cytoplasmic/nuclear contamination, these results suggest

A

^{1}NKGK$^{\downarrow}$VISWMSGR$^{\downarrow}$GFGFIEDDADKK$^{\downarrow}$QHFVHFSALQTETGGFR$^{\downarrow}$ALTVGQEVEFEVASQDGR$^{\downarrow}$TRAENVTSPGGAK$^{\downarrow}$LPSGPRPPE
^{82}GAGR$^{\downarrow}$GR$^{\downarrow}$GFGGGRGGR$^{\downarrow}$DFGGDRNSGR$^{\downarrow}$GR$^{\downarrow}$NDNQQGGQHQSFSDDF

B

Peptide #	Sequence	Expected mass (Da)	Observed mass (Da)	Δ mass (Da)	Modification
1	^{5}VISWMSG^{12}R	934.469	935.394	0.925	None
2	^{13}GFGFIEDDADK^{24}K	1340.620	1341.531	0.911	None
3	^{25}QHFVHFSALQTETGGF^{41}R	1960.954	1961.927	0.973	None
4	^{42}ALTVGQEVEFEVASQDG^{59}R	1933.938	1934.896	0.958	None
5	^{60}TRAENVTSPGGA^{72}K	1286.653	1287.541	0.888	None
6	^{73}LPSGPRPPEGAG^{85}R	1289.684	1290.600	0.916	None
7	^{73}LPSGP**R**PPEGAG^{85}R	1289.684	1304.610	14.926	Monomethyl
8	^{73}LPSGPRPPEGAGRG^{87}R	1502.802	1503.010	0.208	None
9	^{73}LPSGPRPPEGAG**R**G^{87}R	1502.802	1531.771	28.969	Dimethyl*
10	^{73}LPSGPRPPEGAG**R**G^{87}R	1502.802	1545.783	42.981	Trimethyl*
11	^{88}GFGGG**R**GG^{96}R	819.406	848.350	28.944	Dimethyl
12	^{107}GRNDNQQGGQHQSFSDD^{124}F	2035.832	2036.805	0.973	None
13	^{109}NDNQQGGQHQSFSDD^{124}F	1822.714	1823.657	0.943	None

Fig. 1. Identification of arginine methylation sites in RBP16. (A) Amino acid sequence of RBP16. Note that the N-terminal amino acid of the protein is an asparagine, due to cleavage of the encoded mitochondrial import sequence [1]. The arrows indicate the trypsin cleavage sites that generated the analyzed peptides. (B) Characterization of the peptides obtained after digestion of RBP16 with trypsin by MALDI-TOF mass spectrometry. Modified arginines are indicated in bold. The asterisks indicate that these peptides were further characterized by post-source decay analyses to precisely map the modified arginine residues.

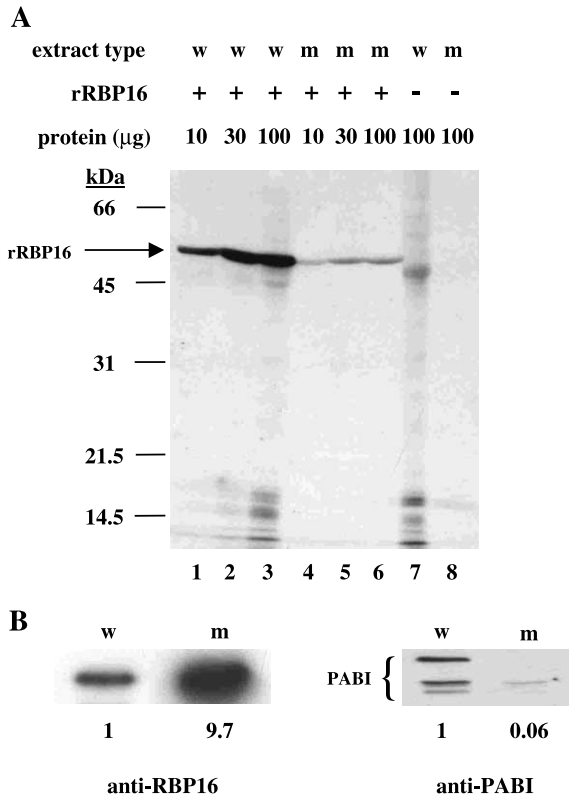

Fig. 2. Detection of rRBP16 methylating activity in trypanosome extracts. (A) In vitro methylation of rRBP16 by *T. brucei* whole cell and mitochondrial extracts. Methylation reactions were performed as described in Section 2 in the presence (lanes 1–6) or absence (lanes 7 and 8) of 1.7 μg of maltose binding protein-RBP16 fusion protein (rRBP16). Reactions were initiated by the addition of increasing amounts of whole cell (w) or mitochondrial (m) extract. Samples were resolved on a 12.5% polyacrylamide gel. (B) Western blot analysis of proteins from whole cell (w) or mitochondrial (m) extracts. *T. brucei* whole cell and mitochondrial extracts (2.5 μg each) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against the cytoplasmic and nuclear PABI protein, and the mitochondrial RBP16 protein. Hybridizing proteins were detected by ECL and signals from non-saturated autoradiograms determined by densitometry (values are indicated below the blots). The bracket indicates the PABI signal consisting of both full length PABI and its characteristic degradation products [31]. The absence of any full length PABI in the mitochondrial sample is presumably due to enhanced protein degradation that took place during the mitochondrial isolation procedure.

that mitochondria may possess a distinct PRMT activity, albeit very low compared with that in other cellular compartments. It should also be kept in mind that while whole cell extracts are rapidly produced and frozen, the mitochondrial isolation procedure takes several hours. Thus, it remains possible that mitochondria possess a more robust PRMT activity that does not withstand the isolation procedure.

In addition to the methylation of rRBP16, several endogenous labeled bands became visible when large amounts of whole cell extracts were incubated with [^{3}H]AdoMet (Fig. 2A, lanes 3 and 7). Since both proteins and nucleic acids can serve as substrates for methyltransferases, the nature of these bands was unclear. To evaluate the overall extent of protein methylation in *T. brucei*, we sought to determine whether the in vitro labeled endogenous bands represented DNA, RNA, or protein. In vitro methylation of whole cell extract was performed in the absence of added rRBP16 to allow optimal labeling of the endogenous bands (Fig. 3). Reactions were subsequently treated with either DNAse, RNAse, or proteinase. DNAse I and RNAse A treatment following the in vitro methylation did not alter the methylation pattern, while labeling of all but the smallest (<6 kDa) band was abolished by proteinase K treatment (Fig. 3A). This indicates that the majority of labeled bands are indeed methylated proteins. Since the smallest labeled band is resistant to DNAse, RNAse, and protease treatment, and unreacted [^{3}H]AdoMet migrates at a similar position (not shown), this band most likely represents free [^{3}H]AdoMet. Eight major protein bands whose apparent molecular weights range from 16 to approximately 75 kDa were observed. The ability of these proteins to be methylated in vitro indicates that they are not completely methylated in vivo. Immunoprecipitation of in vitro labeled endogenous proteins with anti-RBP16 polyclonal antibodies demonstrated that one of the major endogenous [^{3}H]AdoMet-labeled proteins is RBP16 (Fig. 3B). As indicated by MALDI-TOF mass spectrometry, Arg-93 is dimethylated in vivo on every RBP16 molecule. Since Arg-93 would thus not be available for incorporation of [^{3}H]AdoMet, this indicates that native RBP16 is methylated in vitro on arginine residues other than Arg-93, possibly Arg-78 and/or Arg-85.

3.3. rRBP16 is a type I PRMT substrate

PRMTs are classified as type I or type II based on their ability to catalyze synthesis of asymmetric or symmetric dimethylation of arginine residues, respectively. To identify the type(s) of methyltransferase(s) catalyzing the methylation of rRBP16, we performed the in vitro methylation of rRBP16 using whole cell extracts in the presence of increasing amounts of either myelin basic protein (MBP) or of a nucleolin-derived peptide. Since nucleolin is a type I PRMT substrate, the nucleolin-derived peptide would be expected to compete only for type I PRMTs [33]. Likewise, as MBP is a substrate of only type II methyltransferases, competition of methylation activity by this protein indicates the action of a type II PRMT. The results presented in Fig. 4 show that the methylation of rRBP16 was substantially competed by the nucleolin-derived peptide at 25-fold molar excess over rRBP16, and that it was almost totally competed at 50-fold molar excess. On the other hand, MBP had no significant effect on the methylation of rRBP16, even at 50-fold molar excess.

The slight inhibition observed in the presence of 50-fold excess of MBP was also observed when an identical amount of BSA was used as a control (data not shown). This indicates that the *T. brucei* enzyme responsible for rRBP16 methylation in vitro is a type I PRMT. Moreover, the endogenous 16 kDa methylated protein identified by immunoprecipitation as RBP16 (see Fig. 3B), is also competed exclusively by the nucleolin-derived peptide (Fig. 4), reinforcing our conclusion. The inability of MBP, a type II PRMT substrate, to compete the methylation of RBP16 indicates that RBP16 is not a substrate for type II PRMTs. However, this class of enzymes is present in trypanosome extracts. When high levels of MBP were used in this competition experiment, a 14 kDa methylated band became gradually visible (Fig. 4, asterisk). As this band was only visible in samples containing large amounts of exogenous MBP, we concluded that it represents the methylated form of MBP. Furthermore, an endogenous protein of about 15 kDa is competed by MBP as well as the nucleolin-derived peptide (Fig. 4). Taken together, these results indicate that *T. brucei* possesses both type I and type II PRMT activities.

To confirm that RBP16 is a substrate for type I PRMTs, we examined whether rRBP16 could be methylated by known type I PRMTs. PRMT1, the major type I PRMT in mammalian cells, methylates arginine residues present in RGG and RXR motifs of several RNA-binding proteins [34]. Recombinant rat PRMT1 was expressed as a GST fusion protein and its ability to methylate rRBP16 was tested. As shown in Fig. 5, purified GST-PRMT1 catalyzes methylation of RBP16, supporting our conclusion that RBP16 is a substrate for type I PRMTs. rRBP16 can also be methylated by recombinant CARM1 (data not shown), a PRMT involved in the transcriptional activation by the estrogen receptor [35]. Protein methylation in rat pheochromocytoma (PC12) cells has been extensively studied, and the presence of at least 50 endogenous methylated proteins has been reported [36]. Furthermore, the methylating activity of the PC12 nuclear extract is enhanced when the cells are cultured in the presence of NGF [21], and the majority of the protein methylation in both untreated and NGF-treated cells appears to be catalyzed by type I PRMTs (Cimato and Aletta, unpublished). Fig. 5 clearly shows that rRBP16 is methylated by PC12 nuclear extract, and that this methylation activity is increased by NGF treatment. Thus, RBP16 serves as a substrate for both trypanosome and mammalian type I PRMTs.

4. Discussion

In this report, we demonstrate that the *T. brucei* mitochondrial gRNA binding protein RBP16 is post-translationally modified by methylation on at least three arginine residues through the action of a type I

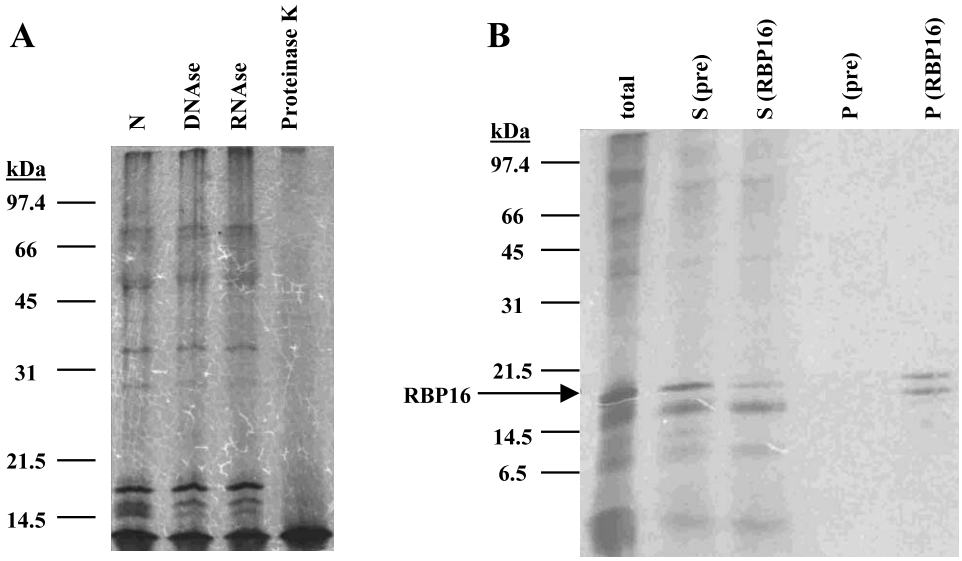

Fig. 3. Characterization of endogenous PRMT substrates. (A) Identification of in vitro methylated products as proteins. *T. brucei* procyclic form whole cell extract (100 μg) was incubated in the presence of 4 μCi of [^{3}H]AdoMet as described in Section 2. Reactions were either stopped by the addition of 5 × SDS-PAGE loading buffer (N) or incubated with either 5 U of DNAse I on ice for 30 min, 1 μg of RNAse A at 37 °C for 30 min, or 1 μg of proteinase K at 37 °C for 30 min. Samples were resolved on a 12.5% polyacrylamide gel. (B) RBP16 is a major endogenous PRMT substrate. *T. brucei* procyclic form whole cell extract (250 μg) was incubated in the presence of 4 μCi of [^{3}H]AdoMet as described in Section 2. Immune complexes were then formed using either pre-immune IgGs (pre) or affinity purified anti-RBP16 (RBP16) cross-linked to protein A-Sepharose, and recovered by centrifugation. The supernatant (S) (30% of the total volume) and the pellet (P) (100% of the total volume) were then electrophoresed on a 15% polyacrylamide gel. Total: in vitro methylation of 250 μg of whole cell extract from *T. brucei* procyclic form prior to immunoprecipitation. The arrow indicates the position of in vitro labeled endogenous RBP16.

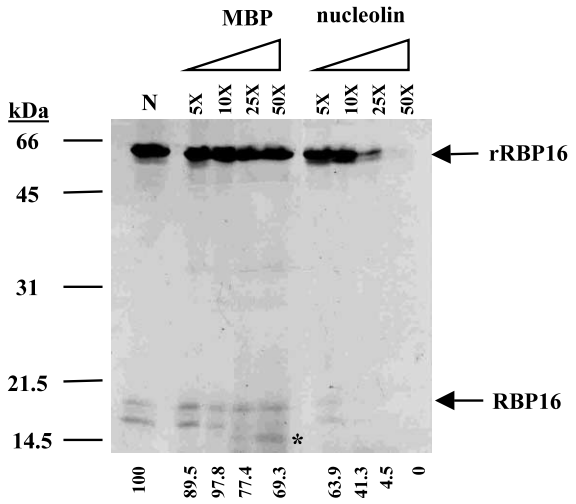

Fig. 4. RBP16 is a type I PRMT substrate. In vitro methylation reactions were performed in the presence of increasing amounts of either the type II PRMT substrate myelin basic protein (MBP) or the type I PRMT substrate nucleolin-derived peptide (nucleolin). MBP or nucleolin were used at molar excesses of 5, 10, 25, and 50-fold as compared with rRBP16. N indicates methylation in the absence of competitor. The numbers below the figure indicate the signal intensity corresponding to rRBP16 (as determined by densitometry) relative to the signal in the absence of competitor. The arrows indicate the position of in vitro labeled rRBP16 and endogenous RBP16. * indicates the position of labeled MBP.

PRMT. In addition, we present results indicating the presence of several additional type I PRMT substrates as well as at least one substrate for a type II PRMT in *T. brucei*. The present study is the first demonstration of arginine methylation in *T. brucei*. Indeed, this is the first report of this type of posttranslational modification in any protozoan. *T. brucei* is an ancient organism, evolutionarily further removed from yeast than yeast is

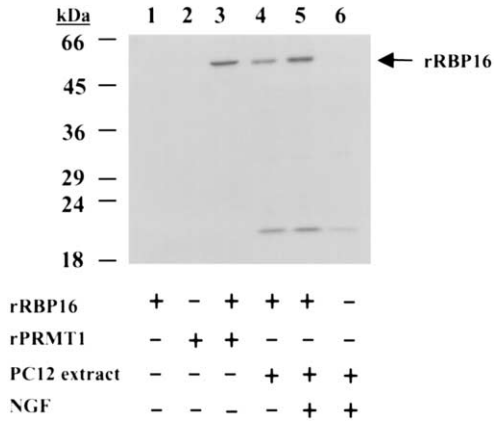

Fig. 5. In vitro methylation of rRBP16 by heterologous type I PRMTs. In vitro methylation reactions were performed either in the presence (lanes 1, 3–5) or absence (lanes 2 and 6) of rRBP16. Reactions were initiated by the addition of 3 μg of purified GST-PRMT1 (lane 2–3), 10 μg of untreated PC12 nuclear extract (lane 4), or 10 μg of nuclear extract from PC12 cells previously treated with nerve growth factor (50 ng ml^{-1}) (lanes 5–6).

from humans [37]. Thus, the presence of both type I and type II PRMTs in such a primitive organism highlights the importance of arginine methylation as a regulatory mechanism in eukaryotes.

The two arginine residues found to be dimethylated on RBP16 in vivo, Arg-85 and Arg-93, are located within a preferred sequence context for asymmetric arginine dimethylation [7]. That is, both of these modified arginines are flanked by a C-terminal as well as an N-terminal glycine. Although we have not directly demonstrated that Arg-85 and Arg-93 are asymmetrically dimethylated, it is strongly suggested by their presence within the preferred sequence for asymmetric dimethylation, and by the ability of both trypanosome and mammalian type I PRMTs to methylate RBP16. The sequence context of the monomethylated Arg-78 residue is more unusual in that it is not flanked by glycines, but by proline residues. However, as additional methylated proteins are reported, it is becoming clear that arginines in numerous sequence contexts can undergo this modification (e.g. [13,38]). For example, in the poly(A)-binding protein II (PABP2), only three of the thirteen identified methylated arginines are flanked by a C-terminal glycine [13]. Twelve of the modified residues are present within an RXR motif, where the two arginines are asymmetrically dimethylated, and X represents a small amino acid such as glycine, alanine, serine, or proline. Although Arg-78 is monomethylated and is not present specifically within an RXR motif, it is flanked by two proline residues. This may represent a context for arginine methylation similar to that observed in PABP2.

Unexpectedly, we found, by MALDI-TOF mass spectrometry and post-source decay analyses, that one residue, Arg-85, is trimethylated in vivo. This report constitutes the first evidence for the occurrence of this type of arginine derivative in vivo. The precise structure of trimethylated Arg-85 of RBP16 is unknown. However, as discussed above, it is likely that this residue exists in an asymmetrically dimethylated form, with two of the methyl groups present on the same terminal nitrogen. The in vivo methylation of the δ (internal) nitrogen of the guanidino group of arginine residues has been reported in yeast proteins [39]. Thus, the third methyl group within Arg-85 of RBP16 may be present on the δ nitrogen of that residue. Alternatively, it is possible that the third methyl group is present on the ω nitrogen of Arg-85. The structure of this novel methylated arginine derivative awaits detailed structural analysis.

In addition to rRBP16, eight major labeled bands whose apparent molecular weights range from 15 to approximately 75 kDa were observed when whole cell extract from the procyclic life stage were incubated with [^{3}H]AdoMet. We have identified one of these proteins as RBP16. The in vitro labeled proteins detected in this

manner are likely to represent only a subset of the total methylated proteins in trypanosomes, as some proteins were presumably not available for [^{3}H]AdoMet incorporation due to the complete methylation of specific arginine residues in vivo. Since *T. brucei* possesses both type I and type II PRMTs (Fig. 4), the in vitro labeled endogenous proteins may contain either asymmetric or symmetric dimethylarginines. While the identities of these proteins are not known, several RGG domain-containing proteins that would be predicted to be asymmetrically dimethylated, based on the presence of arginine residues within the preferred sequence for this modification [7], have been described in trypanosomes. They are the snoRNA-associated fibrillarin [40], the mitochondrial oligo(U) binding protein TBRGG1 [41], and the nucleolar RNA binding protein Nopp44/46 [42]. It will be important in the future to determine the full range of PRMT substrates in trypanosomes and the roles of arginine methylation in RNA processing. The propensity for RNA binding proteins to act as PRMT substrates [8,9] and the heightened importance of post-transcriptional gene regulation in trypanosomatids [43] suggests that arginine methylation is likely to be of significance in regulating gene expression in these organisms.

The functional significance of RBP16 methylation is currently unknown, but several possible roles can be envisioned. Arginine methylation plays a role in intracellular protein trafficking in both yeast and mammalian cells [10,26]. As RBP16 is nuclearly encoded but transported into the mitochondrion via a cleaved signal peptide [1], methylation of RBP16 could conceivably be involved in mitochondrial import. Since all RBP16 molecules isolated from purified mitochondrial vesicles are dimethylated at Arg-93, asymmetric dimethylation of Arg-93 in the cytosol may be a prerequisite for the import of RBP16 into the mitochondria, and thus may be a signal for mitochondrial import. Moreover, our demonstration that the majority of PRMT activity in *T. brucei* is extramitochondrial suggests that at least a subset of RBP16 methylation events may take place prior to mitochondrial import. The role of Arg-93 dimethylation in mitochondrial import will be the subject of future experiments.

The purification of RBP16-derived peptides containing unmodified Arg-78 and Arg-85 indicates that methylation of these two residues is not essential for mitochondrial import. The presence of mitochondrially localized RBP16 that is either unmodified or methylated at Arg-78 and Arg-85 suggests a regulatory function for methylation at these sites. Indeed, methylation of Arg-78 and Arg-85 appears to be mutually exclusive, possibly reflecting exclusive functions of these methylated RBP16 derivatives. That is, methylation on Arg-78 may play a role functionally incompatible with methylation on Arg-85. Arginine methylation at these sites has the potential to regulate the interaction of RBP16 with both nucleic acids and proteins. Introduction of a methyl group to the guanidino nitrogen of arginine is expected to weaken the hydrogen bonding between proteins and RNA. The RGG domain of RBP16, in which all three methylated arginine residues reside, is involved in stabilizing the RBP16-gRNA interaction through non-specific interactions with the encoded region of the gRNA ([6], Miller and Read, unpublished). Furthermore, we have shown, by modification of arginine residues with phenylglyoxal, that arginines are important for RBP16-RNA binding [6]. Thus, methylation of one or several arginines within the RGG domain of RBP16 may modulate its affinity for gRNA, thereby regulating gRNA usage during RNA editing. In an analogous fashion, methylation of hnRNP A1 was shown to decrease its affinity for single stranded DNA [25]. A growing body of evidence indicates that arginine methylation is also critically important in modulating protein–protein interactions [8,9,24,44]. We have identified by affinity chromatography several RBP16-interacting proteins in *T. brucei* mitochondria (Hayman and Read, unpublished). In particular, RBP16 specifically binds a 22 kDa protein (p22), which exhibits homology to the multifunctional human p32 [45]. Future experiments will address whether arginine methylation of RBP16 modulates its interaction with p22 or other mitochondrial proteins. Conversely, p22 may regulate the methylation of RBP16 in a manner analogous to the role of human p32 in preventing phosphorylation of the splicing factor ASF/SF2 [45]. A combination of in vitro and in vivo experiments utilizing RBP16 mutated at each of the three modified arginine residues will be required to fully determine the effects RBP16 methylation on mitochondrial gene expression.

Acknowledgements

We thank Dr Harvey R. Herschman for providing recombinant rat PRMT1, and Dr Michael R. Stallcup for providing the recombinant CARM1. We are also grateful to Dr Noreen Williams for providing antisera against *T. brucei* PABI. We thank Dr Thomas Melendy, Melissa Miller, and Christopher Ryan for critical reading of the manuscript. This work was supported by NIH grants GM53502 and AI47329 to Laurie K. Read, who is also a recipient of the Burroughs Wellcome Fund New Investigator Award in Molecular Parasitology, and NS40533 to John M. Aletta.

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