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Identification of a novel lipin homologue from the parasitic protozoan *Trypanosoma brucei*

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Abstract

Background: Arginine methylation is a post-translational modification that expands the functional diversity of proteins. Kinetoplastid parasites contain a relatively large group of protein arginine methyltransferases (PRMTs) compared to other single celled eukaryotes. Several *T. brucei* proteins have been shown to serve as TbPRMT substrates *in vitro*, and a great number of proteins likely to undergo methylation are predicted by the *T. brucei* genome. This indicates that a large number of proteins whose functions are modulated by arginine methylation await discovery in trypanosomes. Here, we employed a yeast two-hybrid screen using as bait the major *T. brucei* type I PRMT, TbPRMT1, to identify potential substrates of this enzyme.

Results: We identified a protein containing N-LIP and C-LIP domains that we term TbLpn. These domains are usually present in a family of proteins known as lipins, and involved in phospholipid biosynthesis and gene regulation. Far western and co-immunoprecipitation assays confirmed the TbPRMT1-TbLpn interaction. We also demonstrated that TbLpn is localized mainly to the cytosol, and is methylated *in vivo*. In addition, we showed that, similar to mammalian and yeast proteins with N-LIP and C-LIP domains, recombinant TbLpn exhibits phosphatidic acid phosphatase activity, and that two conserved aspartic acid residues present in the C-LIP domain are critical for its enzymatic activity.

Conclusions: This study reports the characterization of a novel trypanosome protein and provides insight into its enzymatic activity and function in phospholipid biosynthesis. It also indicates that TbLpn functions may be modulated by arginine methylation.

Keywords: Kinetoplastid, Lipin, Arginine methylation, Phosphatidic acid phosphatase

Background

Arginine methylation is a post-translational modification whose importance and widespread impact has recently begun to be fully appreciated [1-4]. In yeast and mammals, arginine methylation has been associated with a diversity of cellular processes including signal transduction [5,6], RNA transport [7,8] and processing [9-12], protein localization [13-15], and transcription [16]. The effects of arginine methylation on these processes are exerted primarily through the modulation of protein-protein and, less often, protein-nucleic acid interactions [17-20]. Common sites of arginine methylation within proteins include RGG, RG, or RXR motifs [21-23], although methylation of arginine also occurs within other sequence contexts [24]. Catalysis of arginine methylation is carried out by a family

of enzymes termed protein arginine methyltransferases [PRMTs]. While these enzymes are apparently absent from prokaryotes, putative PRMTs have been identified in the genomes of all eukaryotes examined with the exception of *Giardia lamblia* [1,25,26].

PRMTs are classified into four types. Both type I and II PRMTs catalyze the formation of ω -N^G monomethylarginine (MMA). Type I enzymes subsequently synthesize ω -N^G,N^G asymmetrical dimethylarginine (ADMA), while the type II enzymes form ω -N^G,N^G symmetrical dimethylarginine (SDMA). Type III and type IV enzymes catalyze the formation of only ω -N^G monomethylarginine (MMA) or δ -N^G monomethylarginine, respectively. In humans, nine PRMTs have been confirmed, most of them being type I enzymes [3]. In contrast to what has been described in humans, only three PRMTs have been described in *Saccharomyces cerevisiae*, one each of type I type II, and the apparently fungal-specific type IV [1]. Most

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protozoa with the exception of *Giardia* who lacks putative PTMTs, are predicted to possess at least one type I and one type II PRMTs [26].

Trypanosoma brucei is a parasitic protozoan and the causative agent of African sleeping sickness in humans and nagana in African livestock. The genome of *T. brucei* predicts the presence of five PRMTs [26], a relatively large number for a single celled organism [1]. These PRMTs, with the exception of the putative type I TbPRMT3, have previously been characterized. TbPRMT1 is the major type I PRMT in *T. brucei*, analogous to its role in yeast and mammals [27]. TbPRMT5 is a type II enzyme homologous to human PRMT5 [28]. TbPRMT7 is a novel, kinetoplastid-specific type III PRMT [29]. Finally, the recently characterized TbPRMT6 is a type I PRMT capable of automethylation [30]. To date, only a few arginine methylproteins have been reported in *T. brucei*. These include the mitochondrial RNA binding proteins RBP16, TbRGG1, TbRGG2, and MRP2. The effects of RBP16 methylation have been characterized. RBP16 is a TbPRMT1 substrate, as shown by *in vitro* methylation assays and the hypomethylated state of RBP16 in TbPRMT1 knockdown cells [31]. Arginine methylation affects the ability of RBP16 to stabilize specific mitochondrial RNAs and exerts both positive and negative impacts on the interaction of RBP16 with different classes of RNAs and ribonucleoprotein complexes [18,31]. In addition, a large number of proteins harboring arginine/glycine rich regions likely to undergo methylation are predicted by the *T. brucei* genome, and several *T. brucei* RNA binding proteins serve as TbPRMT substrates *in vitro* [26-29,32]. This indicates that a large number of proteins whose functions are modulated by arginine methylation await discovery in trypanosomes.

To gain insight into functions of arginine methylation in trypanosome gene regulation, we set out to identify substrates of the major *T. brucei* type I PRMT, TbPRMT1. We performed a yeast two-hybrid screen using the entire TbPRMT1 open reading frame as bait, exploiting the propensity of PRMTs to associate in a relatively stable manner with their substrates [33]. Using this approach, we identified a protein containing two conserved domains found in a family of proteins known as lipins. Lipins are involved in adipocyte development and phospholipid biosynthesis in mammalian and yeast cells. We termed this protein TbLpn. While these two domains, known as N-LIP and C-LIP domains, are found in several mammalian and yeast lipin proteins [34,35], TbLpn possesses no homology to known proteins outside these two domains and is, thus, a kinetoplastid-specific protein. Consistent with the yeast-two-hybrid data, we show that TbLpn interacts *in vivo* with TbPRMT1, and that it is methylated on arginine residues *in vivo*. We also show that, as predicted by the

presence of conserved domains, TbLpn displays phosphatidic acid phosphatase activity *in vitro*, and that the two conserved aspartic acid residues present in the C-LIP domain, are essential for enzymatic activity.

Results

Identification of TbLpn as a TbPRMT1-interacting protein

To begin to understand the functions of protein arginine methylation in trypanosomes, we sought to identify proteins that interact with the major type I PRMT in *T. brucei*, TbPRMT1. PRMTs tend to associate in a relatively stable manner with their substrates, and several mammalian methylproteins have been identified through protein-protein interaction screens with PRMTs [36,37]. To identify TbPRMT1-interacting proteins, we screened a yeast-two-hybrid library comprised of mixed procyclic (PF) and bloodstream form (BF) *T. brucei* cDNA [38] using the entire TbPRMT1 ORF as bait. Approximately 800 colonies that grew under moderate selection on SD medium (-Trp, -Leu, -His) were selected for more stringent screening on SD medium (-Trp, -Leu, -His, -Ade).

One of the colonies isolated from this screen contained a 1,071-nucleotide insert, which we identified as a fragment of *T. brucei* gene Tb927.7.5450 (<http://www.genedb.org>) (Figure 1A). The predicted protein encoded by this gene contains an N-LIP domain at its amino terminus, as well as a C-LIP domain extending from amino acid 441–593. These 2 domains are found in a family of proteins known as lipins (Figure 1B). Lipin-1, the first member of this family, was identified in the mouse by positional cloning of the mutant gene responsible for fatty liver dystrophy (*fld*) [39]. In addition, the *fld* mice also exhibit hypertriglyceridemia, increased susceptibility to atherosclerosis, insulin resistance, and peripheral neuropathy [39-41]. Lipin proteins are present in organisms from a wide evolutionary spectrum, including protozoa, yeast, *Drosophila*, fish, and mammals (Figure 1B) [39,42-45]. TbLpn homologues can be identified in other trypanosome genomes such as *Trypanosoma cruzi* and *Leishmania major*, and these proteins display between 32–43.5% amino acid identity with TbLpn [46]. The members of the lipin family serve two major cellular functions: as an enzyme necessary for phospholipid and triacylglycerol biosynthesis, and as a transcriptional cofactor involved in the regulation of lipid metabolism genes [34]. In addition, lipin homologues have been shown to play an essential role in nuclear membrane biogenesis in yeast [47].

To begin to characterize TbLpn, we amplified the complete predicted ORF from PF cDNA. Sequence analysis revealed six nucleotide differences from the Tb927.7.5450 sequence reported in GeneDB, three of which result in amino acid changes (Glu-157 → Gly-157, Lys-675 → Thr-675, Val-715 → Ala-715). The predicted TbLpn

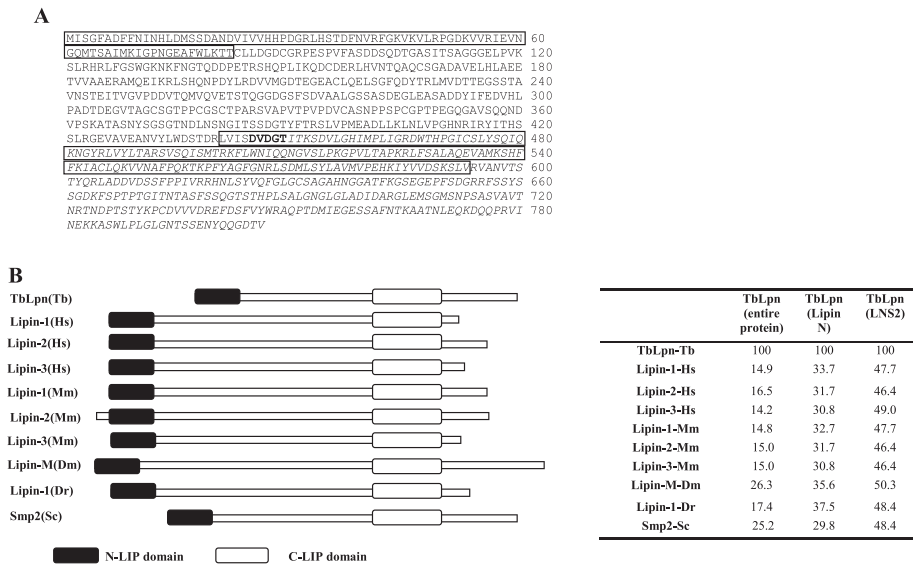


Figure 1 TbLpn sequence analysis. **A**) Shown is the predicted amino acid sequence of TbLpn. The region shown to interact with TbPRMT1 by yeast-two hybrid analysis is shown in italics. The N-LIP and C-LIP domains are boxed. The conserved DxDxT domain is shown in bold. **B**) A schematic representation of TbLpn amino acid sequence aligned with members of the lipin family is shown in the left panel. The degree of amino acid sequence identity between TbLpn and members of the lipin family is shown on the right panel. TbLpn [*T. brucei*, (Tb), accession number AAX78871], Lipin-1 [Human, (Hs), AAH30537], Lipin-2 [Human, (Hs), AA152449], Lipin-3 [Human, (Hs), CA142978], Lipin-1 [Mouse, (Mm), NP_766538], Lipin-2 [Mouse, (Mm), AAH39698], Lipin-3 [Mouse, (Mm), EDL06298], Lipin-M [*Drosophila melanogaster*, (Dm), NP_001188884], Lipin-1 [*Danio rerio*, (Dr), AAX19945], Smp2 [*Saccharomyces cerevisiae*, (Sc), BAA00880].

protein is 806 amino acids in length (Figure 1A) with a predicted molecular mass of 86.7 kDa. The N-LIP domain of TbLpn displays 30–37.5% amino acid identity with the corresponding domains from lipin proteins (Figure 1B and Figure 2A). In addition, the C-LIP domain of TbLpn exhibits 46–50% amino acid identity with the corresponding domains from members of lipin family, such as mammalian lipin-1, lipin-2, lipin-3, and yeast Smp2 (Figure 1B and Figure 2B). Most interesting, the motif (DXDXT) shown to confer phosphatidic acid phosphatase activity to mammalian and yeast lipins, is present within the C-LIP domain of TbLpn (⁴⁴⁵DVDGT) [43]. In addition, a conserved glycine residue shown to be essential for the mouse Lipin-1 function is also present in the predicted amino acid sequence of TbLpn (Gly-74) [39]. Apart from this domain, no significant homology is observed between TbLpn and other members of the lipin family. For instance, although lipin proteins share the LXXIL motif, which has been shown to be essential for interaction of Lipin-1 with the nuclear cofactors involved in the regulation of fatty acid metabolism, TbLpn lacks that conserved LXXIL motif, suggesting that TbLpn might have a different function than other lipins [48]. Although TbLpn may not possess co-transcriptional activity, it might still act as a phosphatidic acid phosphatase. In addition, the conserved nuclear localization sequence, usually found in almost all species [34], is absent in TbLpn.

Subcellular localization of TbLpn

To determine the subcellular localization of TbLpn, PF *T. brucei* cells were fractionated into cytosolic and nuclear extracts, and the presence of TbLpn within these compartments assessed by western hybridization. The efficiency of the fractionation procedure was confirmed by using antibodies directed against cytosolic Hsp70 and nuclear RNA polymerase II. As shown in Figure 3, a band of the expected size for TbLpn (~ 83 kDa) was present exclusively in the cytoplasm of the parasite. This is in contrast to all previously characterized mammalian and yeast lipins which display cytoplasmic as well as nuclear localization [34,39,49–51]. In addition, SMP2, the yeast lipin homologue, has been shown to be present in the cytosol as well as associated with the membrane [43]. We did however detect the presence of a protein band with decreased electrophoretic mobility (~120 kDa) in the nuclear extract. This strongly suggests that TbLpn is present in both cytosol and nucleus and, in the nucleus, is heavily modified by post-translational modifications such as arginine methylation and/or phosphorylation.

TbLpn interacts with TbPRMT1 *in vitro* and *in vivo*

We further confirmed the TbPRMT1/TbLpn interaction identified by yeast-two-hybrid first by Far Western hybridization. To this end, recombinant His-TbLpn was electrophoresed and transferred to PVDF, and the membrane was incubated with recombinant His-TbPRMT1.

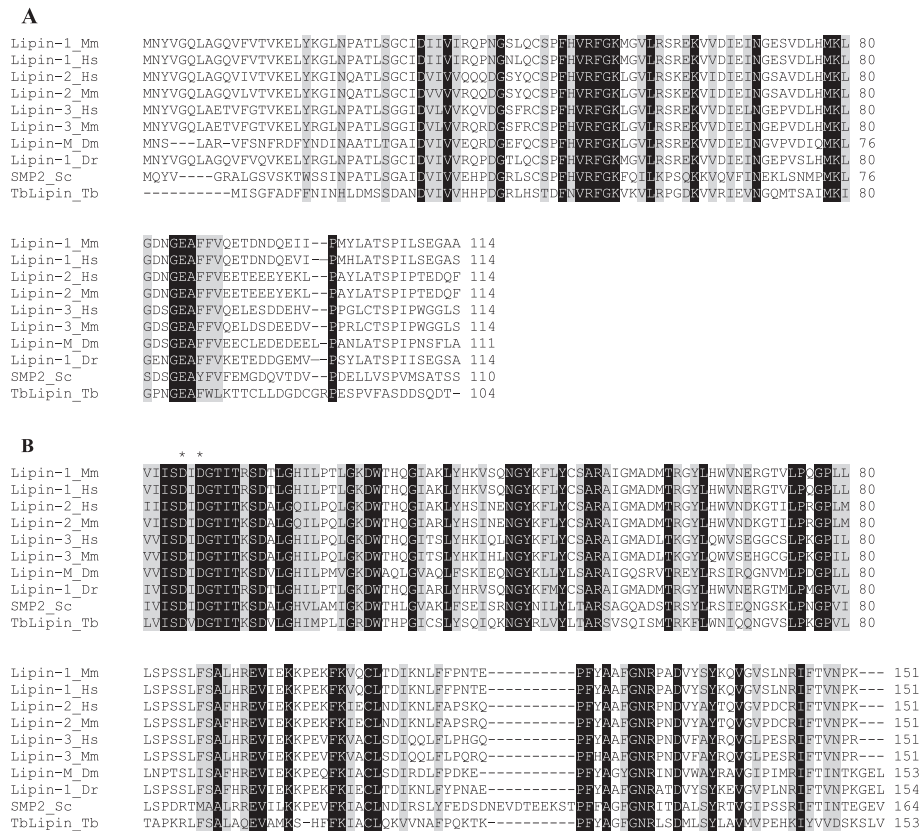


Figure 2 Amino acid sequence alignment of TbLpn conserved domains and other lipin family members. A) Amino acid sequence alignment of N-LIP domains. Sequences were aligned using CLUSTALW. Identical and conserved amino acids are shown in black and grey boxes, respectively. **B)** Amino acid sequence alignment of C-LIP domains. Sequences were aligned using CLUSTALW. Identical and conserved amino acids are shown in black and grey boxes, respectively. The conserved aspartic acid residues shown to be essential for enzymatic activity in yeast and mammalian lipins are indicated by asterisks (*).

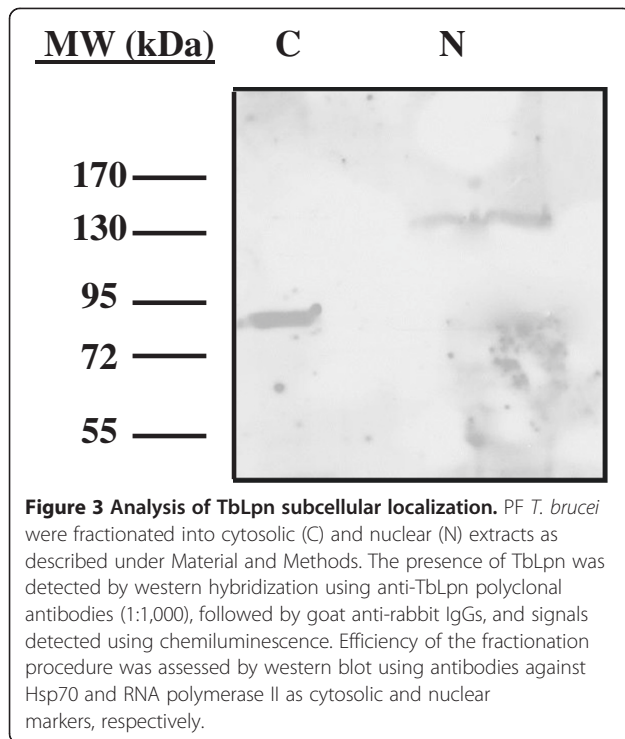
Detection of His-TbPRMT1 with polyclonal anti-TbPRMT1 antibodies revealed the presence of a band at 105 kDa, which is the predicted size of His-TbLpn, thereby demonstrating direct binding of His-TbPRMT1 to His-TbLpn (Figure 4A). As a negative control, His-RBP16, expressed and purified using the same protocol as for the purification of His-TbLpn, was used. Using this negative control, no band was detected. The data indicate that TbLpn and TbPRMT1 interact directly.

In order to examine the interaction between TbPRMT1 and TbLpn *in vivo*, we performed a co-immunoprecipitation. As shown above, TbLpn is located in the cytosol of the parasite. For this reason, TbLpn was immunoprecipitated from PF *T. brucei* cytosolic extracts using purified polyclonal anti-TbLpn antibodies. Proteins that were immunoprecipitated along with TbLpn were separated by electrophoresis and transferred onto PVDF. The presence of TPRMT1 in association with TbLpn was determined by using purified polyclonal anti-TbPRMT1 antibodies to probe the membrane by western hybridization. The results

shown in Figure 4B clearly show that a band of approximately the size of TbPRMT1 (38.9 kDa) co-precipitates exclusively with TbLpn, and is not present in the negative control.

TbLpn is methylated *in vivo*

The physical association of TbPRMT1 with TbLpn suggests that TbLpn may serve as a substrate for methylation by TbPRMT1. In support of this hypothesis, several arginine residues throughout the TbLpn sequence are located within preferred motifs for methylation, such as RG or RXR. To evaluate whether TbLpn is methylated *in vivo*, an immunoprecipitation was performed from PF *T. brucei* cytosolic extracts using purified anti-TbLpn polyclonal antibodies. The presence of methylated arginine residues was then determined by western hybridization using anti-mRG polyclonal antibodies. These antibodies were raised against a peptide containing 7 asymmetric dimethylarginine residues alternating with 8 glycine residues. This motif is found most prevalently among verified

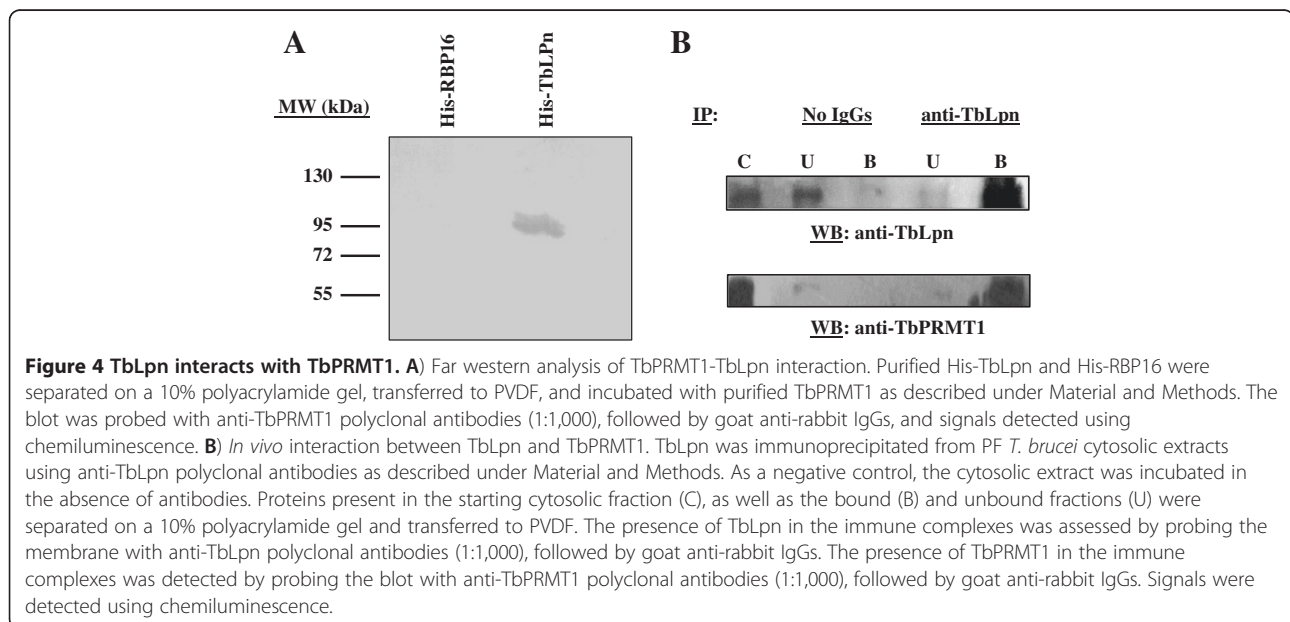


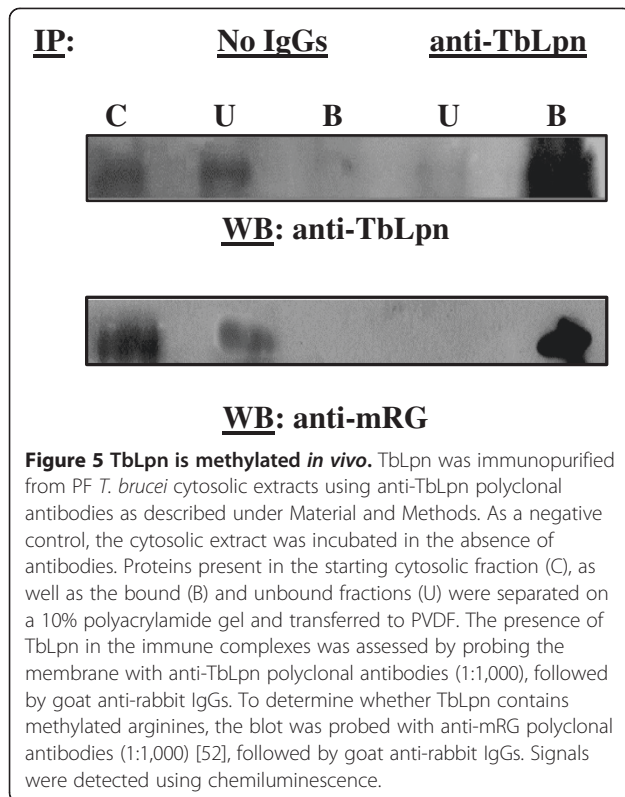
dimethylarginine- containing proteins. The antibodies have been shown to specifically recognize methylated arginine residues [52]. Using these antibodies to probe the blot, a protein band was observed at 85 kDa, which is the predicted size of TbLpn, in the bound but not the unbound fraction (Figure 5). This clearly indicates that native TbLpn contains methylated arginine residues. This result, in conjunction with the ability of TbLpn

to interact with TbPTMT1 *in vivo*, suggests that one or several arginine residues within TbLpn might be asymmetrically dimethylated by TbPRMT1. Further experiments are underway to identify the enzyme(s) responsible for TbLpn methylation.

TbLpn displays phosphatidic acid phosphatase activity *in vitro*

Lipin proteins are known to exhibit Mg²⁺-dependent phosphatidic acid phosphatase activity, catalyzing dephosphorylation of phosphatidic acid (PA) into diacylglycerol. The predicted amino acid sequence of TbLpn contains two conserved domains found in all lipins. In addition, two aspartic acid residues that have been shown to be essential for enzymatic activity of yeast and mammalian lipins are also found in TbLpn. To determine whether recombinant TbLpn could catalyze dephosphorylation of phosphatidic acid, enzymatic assays were performed using the substrate 1,2-dioctanoyl-sn-glycero-3-phosphate (DiC8 PA), Mg²⁺, and increasing amount of His-TbLpn. Following incubation at 30°C, the amount of Pi released was measured by reading the absorbance at 620 nm following the addition of PiBlue reagent. Figure 6 shows that recombinant TbLpn exhibits phosphatidate phosphatase activity, suggesting that TbLpn may play a role in the synthesis of phospholipids. From our data, we calculated that recombinant TbLpn has a specific activity of 200–225 nmol/min/mg. In contrast, the recombinant mutant in which the two conserved aspartic acid residues (Asp-445, Asp-447) were changed to alanines (His-DEAD) shows significantly less phosphatase activity. The calculated specific activity of 11–12 nmol/min/mg calculated for the mutant protein clearly implies

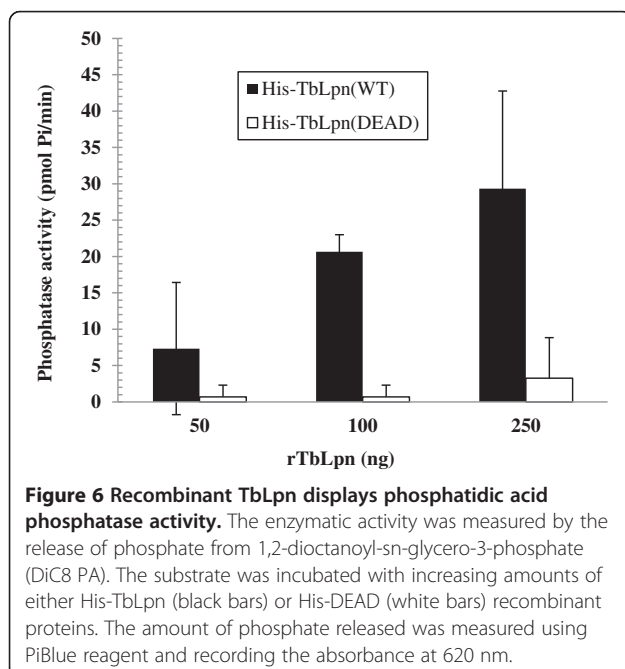




that the two conserved aspartates are essential for this enzymatic activity.

Discussion

In an effort to discover trypanosome substrates of PRMTs, we utilized a yeast two-hybrid screen to identify proteins



that associate with the major type I protein arginine methyltransferase in *T. brucei*, TbPRMT1 [27]. Of particular interest to us are proteins whose functions might be affected by arginine methylation. Here, we report that TbPRMT1 directly interacts in both Far Western and co-immunoprecipitation assays with a novel protein. We termed this protein TbLpn, based on the presence of two conserved (N-LIP and C-LIP) domains found in a family of proteins called lipins. We further demonstrate that, like TbPRMT1, TbLpn is cytoplasmic in PF *T. brucei*, consistent with a function in TbLpn methylation. Together, these data point to TbLpn as a candidate protein whose post-transcriptional gene regulatory functions are affected by arginine methylation.

We demonstrated that, as predicted from the amino acid sequence, recombinant TbLpn, as other members of the lipin family, exhibits phosphatidic acid phosphatase enzymatic activity. Mutation of the conserved aspartic acid residues (Asp-445 and Asp-447) to alanines results in a significant reduction in the enzymatic activity of TbLpn. These two aspartic acid residues are part of the conserved DxDxT motif found in lipin proteins and other members of the haloacid dehalogenase (HAD)-like superfamily [53,54]. Based on the crystal structure of L -2-haloacid dehalogenase from *Pseudomonas*, it is likely that Asp-445 in TbLpn acts as a nucleophile in the phosphoryl transfer reaction.

Compared to the recombinant yeast PAH1 (3000 nmol/min/mg) and human Lipin-1 (1,600 nmol/min/mg), His-TbLpn displays a lower but still significant specific activity [43]. One possible explanation for this lower specific activity is the fact that the recombinant protein may not contain the same post-translational modifications as those found in the native protein. It is of interest that several lipin homologues are highly modified at the post-translational level. In rat and in mouse adipocytes, Lipin 1 contains at least 19 and as many as 23 sites that are phosphorylated in response to insulin [49,55,56]. Although it does not affect its intrinsic phosphatidic acid phosphatase activity, phosphorylation of Lipin-1 decreases the association with intracellular membranes, thus the active lipin fraction [49]. In addition, the lipin homologue SMP2 is phosphorylated by the cyclin-dependent kinase Cdc28/Cdk1 in budding yeast [57]. The authors have shown that phosphorylation of SMP2 by Cdc28/Cdk1 enhances its association with promoters of lipid biosynthetic genes, which leads to their transcriptional down-regulation. Careful analysis of TbLpn amino acid sequence revealed the presence of 5 conserved amino acid residues shown to be phosphorylated in either mouse (Mm) Lipin-1 or yeast (Sc) Smp2. These residues are Ser-102 (Ser-110 in Sc), Thr-239 (Thr-282 in Mm), Thr-255 (Thr-298 in Mm), Ser-282 (Ser-328 in Mm), and Ser-343 (Ser-392 in Mm). In addition, a previous

analysis of the cytosolic phosphoproteome of BF *T. brucei* identified TbLpn as containing two phosphorylated residues (Thr-211 and Ser-221) [58]. Whether additional or different amino acids are phosphorylated in the PF is still unclear.

Phosphorylation of TbLpn may also impact its association with other proteins, as it has been demonstrated for at least one other member of the lipin family. In adipocytes, Lipin-1 interacts directly with 14-3-3 proteins [51]. 14-3-3 proteins are known to regulate the subcellular localization of a wide variety of proteins through interaction with phosphoserine residues. In adipocytes, Lipin-1 is mostly localized to the cytosol and translocate to the endoplasmic reticulum membrane where it catalyzes dephosphorylation of phosphatidic acid. Stimulation of adipocytes by insulin promotes phosphorylation of Lipin-1 and enhances binding by 14-3-3 proteins. This results in cytoplasmic retention of Lipin-1.

Additionally, we showed that TbLpn is methylated on arginine residues *in vivo*. To our knowledge, this is the first instance of any lipin or phosphatidic acid phosphatase being methylated. The demonstration that TbLpn is methylated *in vivo* suggests that methylation could directly modulate TbLpn enzymatic activity or protein-protein interactions, or both. Arginine methylation has been shown to generally alter protein function by modulating protein-protein interactions, protein-nucleic acid interactions, and protein trafficking [11,21,59,60]. Arginine residues that serve as substrates for PRMTs are usually found within glycine/arginine rich (GAR) domains [61-63]. Based on this, arginine residues throughout TbLpn, including both the N-LIP and C-LIP domains are predicted to undergo methylation. Thus, it will be of great future interest to determine whether TbPRMT1 and/or other TbPRMTs are responsible for TbLpn methylation *in vivo*, and to determine whether TbLpn methylation has any effect on its ability to interact with other proteins and whether it modulates its enzymatic activity.

In yeast and mammals, lipin proteins enable the cell to generate diacylglycerol (DAG) by catalyzing the dephosphorylation of phosphatidic acid. In addition to serving as a precursor for triacylglycerol (TAG), DAG is also used to synthesize phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [64]. In mammalian and yeast cells, the bulk of the PC pool is synthesized by the CDP-choline branch of the Kennedy pathway [64]. In addition, a small fraction of PC is generated by sequential methylation of PE [64]. In eukaryotes, PE can be synthesized by decarboxylation of phosphatidylserine (PS), by head group exchange with PS, by acylation of lyso-PE, or by the CDP-ethanolamine branch of the Kennedy pathway [65,66]. As for other eukaryotes, PC and PE constitute the majority of phospholipids in trypanosomes [67]. Of great importance is the fact that, as opposed to other

parasitic organisms, trypanosomes synthesize phospholipids *de novo* [68]. Although the pathways for phospholipids biosynthesis have not been very well characterized, recent data have helped to better understand how trypanosomes are able to assemble phospholipids. In *T. brucei*, PC is synthesized solely by the CDP-choline branch of the Kennedy pathway, while PE is produced exclusively via the CDP-ethanolamine branch of the Kennedy pathway [67,69,70]. Disruption of the enzymes of the CDP-ethanolamine pathway by RNA interference have shown that this branch of the Kennedy pathway is essential for both procyclic and bloodstream form *T. brucei* cell growth [69,71].

PE and phosphatidylinositol (PI) are key phospholipids involved in the biosynthesis of glycosylphosphatidylinositol (GPI). In trypanosomes, a large number of surface proteins with critical role in virulence surface proteins are anchored to the plasma membrane via GPI molecules. One of these proteins is the variant surface glycoprotein (VSG), a major virulence factor that undergoes antigenic variation and enables the parasite to evade the immune system of its mammalian host [70]. The steps involved in the biosynthesis of GPI, a process essential for *T. brucei* bloodstream form survival, have been well studied. This synthesis differs in certain aspects from the pathway in mammalian cells and yeast. In *T. brucei*, the pool of PI used for GPI synthesis is supplied from glucose-6-phosphate by the action of PI synthase, an enzyme shown to be essential in both bloodstream and procyclic form trypanosomes [68,70,71]. A crucial step in the GPI synthesis pathway is the transfer of phosphoethanolamine (PEtN) to mannose residues on the growing GPI. In this reaction, the ethanolamine moiety is provided by PE [72]. As described earlier, synthesis of PE in *T. brucei* is carried out via the CDP-ethanolamine branch of the Kennedy pathway using DAG as the initial substrate. It has been demonstrated that inhibition of PE synthesis prevents *de novo* GPI biosynthesis [73]. As we demonstrated in the current paper that TbLpn catalyzes the dephosphorylation of PA into DAG, it is attractive to speculate that TbLpn plays an important role in GPI biosynthesis, and thus in the expression of this major virulence factor.

Conclusion

The results clearly identify TbLpn as a new member of the lipin family of proteins. The presence of the conserved N-LIP and C-LIP domains, and especially the ability of recombinant TbLpn to dephosphorylate phosphatidic acid indicate that this enzyme is likely to be involved in phospholipid biosynthesis in trypanosomes. Finally, the observation that, *in vivo*, TbLpn contains methylated arginine residues is very significant, as it is the only lipin or phosphatidic acid phosphatase to date to exhibit such a post-translational modification.

Methods

Trypanosome growth

Procyclic form *T. brucei brucei* clone IsTaR1 stock EATRO 164 was grown as described in SDM-79 medium supplemented with 15% fetal bovine serum (FBS) [74].

Identification of TbLpn by yeast two-hybrid screening

For two-hybrid screening, the TbPRMT1 open reading frame (ORF) was amplified by 35 cycles of PCR from pMal-TbPRMT1 [27] using primers PRMT1-Y2H/myc-5' (5'-GCTCTAGACATATGACGGTGGACGCAAATGCG-3') and PRMT1-Y2H/myc-3' (5'-GCGGATCCCTACTAGACCGCAGCCGAAAATCCTGGTGC-3') which allowed introduction of *NdeI* and *BamHI* restriction sites respectively (underlined). The PCR product was then cloned into *NdeI* and *BamHI* sites of pAS2-1 (CLONTECH Laboratories), and transformed into *Escherichia coli* DH5 α competent cells (Invitrogen).

The bait plasmid pAS2-TbPRMT1 was co-transformed into the competent yeast strain AH109, along with a mixed procyclic and bloodstream form *T. brucei* cDNA library (a generous gift from George Cross, Rockefeller Univ. and Vivian Bellofatto, UMDNJ) cloned into pGADT7 (CLONTECH Laboratories) using the LiAc/PEG method [75]. Transformed cells were plated onto synthetic dextrose medium (SD) supplemented with an amino acid dropout solution lacking histidine (His), leucine (Leu), and tryptophan (Trp) and incubated at 30°C. Resultant colonies were then streaked onto SD medium lacking His, Leu, Trp, and adenine (Ade). Colonies that grew on this medium were grown overnight at 30°C in 3 ml of SD broth lacking Leu. Cells were collected by centrifugation at 14,000 \times rpm for 5 min in a Biofuge centrifuge. The pellet was resuspended in about 50 μ l of residual liquid, and 10 μ l of a 10 units/ μ l lyticase solution was added and thoroughly mixed. Cell lysis was allowed to proceed at 37°C for 60 min with shaking at 250 rpm. Twenty μ l of 10% SDS was then added and the tube vortexed for 1 min. The samples were then put to a freeze/thaw cycle (at -20°C) and vortexed one more time. The plasmid was purified using a GFX DNA purification column (GE Healthcare) following the manufacturer's instructions, and eluted with 50 μ l of deionized water. Five μ l of the purified plasmid was used to transform 20 μ l of ELECTROMAX DH10B cells (Invitrogen). Briefly, electroporation was carried out on ice in 2-mm cuvettes using a Bio-Rad electroporator with the following settings: 2,000 V, 25 μ F, 200 Ω . Following electroporation, 1 ml of SOC was added and the cells were transferred to a 15-ml snap cap tube, and incubated for 60 min at 37°C with shaking (250 rpm). Fifty and 500 μ l were then plated onto LB plates containing 0.1 mg/ml ampicillin, and cells were allowed to grow at least 18 hours at 37°C. Colonies with pGADT7 containing a DNA fragment were identified by PCR using primers GAL4AD5' (5'-CAGGGATGTT

TAATACCACTA-3') and GAL4AD3' (5'-GCACAGTTG AAGTGAACCTGC-3'), and sequenced.

Production of recombinant TbLpn

C-terminally his-tagged TbLpn was generated as follows. Total PF cDNA was generated by reverse transcription primed with [dT]-RXS. The entire TbLpn ORF was amplified using Deep Vent DNA polymerase (New England Biolabs), and using oligonucleotides his10-lipin-5' (5'-CGG GATCCATGATATCTGGTTTTGCAGATTTC-3') and his10-lipin3' (5'-CCCAAGCTTCCGCTCGAGTCACA CAGTGTACCTTGTTGATA-3') (restriction sites are underlined) which were constructed based on the genomic sequence. The PCR product was then digested with *BamHI* and *XhoI*, ligated into the pET26-His₁₀Smt3 expression vector, giving rise to pHis10-TbLpn, and transformed into *E. coli* BL21 competent cells (Invitrogen). A mutant version of TbLpn, in which the two conserved aspartic acid residues in the DVDGT motif (Asp-445, Asp-447) are changed to alanine (pHis-TbLpn(DEAD)), was generated by PCR amplification from pHis10-TbLpn using the QuikChange II XL™ Site-Directed Mutagenesis Kit (Agilent Technologies) and the mutagenic primers TbLpn-DEAD-5' (5'-CTTGTCATTAGTGAAGTGGA AGGCACGATCACGAAAAG-3') and TbLpn-DEAD-3' (5'-CTTTTCGTGATCGTGCCTTCCACTTCACTAAT GACAAG-3').

Protein expression was induced with 1 mM isopropyl β -thiogalactopyranoside (IPTG) and 2% ethanol for 20 h at 17°C. Cells were resuspended in lysis buffer (10 mM Tris [pH 8.6], 10 mM glycine, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 10% ethanol, 4% Tween-20, and 3% Triton X-100) containing 0.05 mg/ml lysozyme, 0.01 mg/ml DNase I, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin A, and lysed by 3 freeze/thaw cycles. Each cycle consisted of incubation at 37°C for 15 minutes, followed by incubation at -80°C for another 15 minutes. The lysed cell suspension was centrifuged at 17,000 \times g for 15 min at 4°C, and the supernatant was mixed with Probond Ni²⁺ resin (Invitrogen) for 12 h at 4°C. The mixture was poured into a column and the column washed with 40 volumes of wash buffer (10 mM Tris [pH 7.0], 200 mM NaCl, 30 mM imidazole, 10% glycerol). His-tagged proteins were eluted with 10 volumes of wash buffer (pH 6.0) containing 200 mM imidazole.

Polyclonal antibody production

Affinity purified polyclonal anti-TbLpn antibodies were obtained from Bethyl Laboratories, Inc. using a peptide corresponding to amino acids 791–806 (GLCNTSSENYQQGDTV).

Far western analysis

His-tagged TbLpn was electrophoresed on a denaturing 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane at 50 V for 45 min in 10 mM 3-[Cyclohexylamino]-1-propanesulfonic acid (CAPS) buffer (pH 11.0) containing 10% methanol. As a negative control, his-tagged RBP16 was expressed as described [76] and purified using the same protocol used for the purification of His-TbLpn described above. The membrane was blocked in TBS buffer containing 5% nonfat dry milk for 1 hour, washed twice for 5 min in TBS buffer containing 0.05% Tween-20 (TBS-T), and then incubated with 0.5-1.0 µg of purified TbPRMT1 [27] in TBS-T containing 2% nonfat dry milk overnight at 4°C. After two 15 minute washes in TBS-T, the membrane was probed with anti-TbPRMT1 polyclonal antibodies (1:1,000) for 2 hours, washed in TBS-T twice for 15 min, and incubated with goat anti-rabbit IgGs coupled to horseradish peroxidase. Reactive proteins were detected using enhanced chemiluminescence (GE Healthcare).

Preparation and fractionation of trypanosome cellular extracts

Log-phase PF *T. brucei brucei* clone IsTaR1 stock EATRO 164 were harvested by centrifugation at 6,090 × g for 10 min at 4°C. Fractionation of trypanosome cellular extracts was performed as described previously [77]. The integrity of the cellular compartment was confirmed by using antibodies directed against the cytosolic protein Hsp70 or the nuclear RNA polymerase II [78].

Immunoprecipitation of TbLpn from *T. brucei* cytosolic extracts

As it was previously determined that TbLpn is localized in the cytosol, immunoprecipitation of TbLpn was performed using PF form *T. brucei* cytosolic extracts. Ten µg of purified anti-TbLpn antibodies or 10 µl of IP buffer (for mock immunoprecipitations) (20 mM Hepes [pH 7.9], 150 mM sucrose, 150 mM KCl, 3 mM MgCl₂, 0.5% Nonidet- P40, 1 µg/ml of pestatin A, 1 µg/ml of leupeptin, 5 mM PMSF) were added to 200 µl of cytosolic extract in a final volume of 300 µl of IP buffer. The samples were incubated at 4°C for at least 12 h with gentle rotation. Ten µl of Protein A-Sepharose (GE Healthcare) was then added, and the samples incubated 1 hour at 4°C with gentle rotation. Immune complexes were recovered by centrifugation at 3,000 × g for 30 s and washed five times, each time for 5 min, with 1 ml of IP buffer.

Phosphatidic acid phosphatase assays

The standard reaction contained 50 mM Tris-HCl buffer (pH 7.5), 1 mM MgCl₂, and 0.4 mM 1,2-dioctanoyl-*sn*-glycero-3-phosphate (DiC8 PA) (Avanti Polar Lipids) in a total volume of 50 µl. Reactions were initiated by the

addition of recombinant proteins (50–250 ng), and carried out in triplicate at 30°C for 30 min. The reaction was terminated by the addition of 100 µl of PiBlue reagent (BioAssay Systems), and the color allowed to develop at room temperature for 30 minute. The absorbance was measured with a spectrophotometer at 620 nm. The amount of phosphate produced was quantified from a standard curve using 0.5–4 nmol of potassium phosphate. The reactions were linear with time and protein concentration. The enzymatic activity was expressed as the number of pmol of phosphate released per minute.

Competing interest

The authors declare that they have no competing interest.

Authors' contribution

MP designed the study. MP performed the yeast-two hybrid screening and analysis. JMW performed the subcellular fractionation and localization assays. JSS and DNM expressed and purified wild type His ~ TbLpn. ARK performed the site-directed mutagenesis, expressed, and purified the His ~ DEAD mutant. ASF contributed by performing immunoprecipitation and western hybridization analyses. The *in vitro* phosphatidic acid phosphatase assays were performed by MP, DNM, and ARK. MP wrote the manuscript. All authors read and approved the final manuscript.

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