Arginine Methylation of TbLpn, a Trypanosome Lipin Homologue, by TbPRMT Enzymes

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Arginine Methylation of TbLpn, a Trypanosome Lipin Homologue, by TbPRMT Enzymes

A Senior Honors Thesis

Submitted in Partial Fulfillment of the Requirements for Graduation in the Honors College

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Abstract

Phospholipids biosynthesis, particularly phosphatidylcholine (PC) and phosphatidylethanolamine (PE) plays a major role in the survival of *T. brucei*. Of great importance is the fact that, as opposed to other parasitic organisms, trypanosomes synthesize phospholipids de novo. 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. Previous work in our lab has shown that a protein, termed TbLpn, is a phosphatidic acid phosphatase potentially involved in phospholipid biosynthesis in *T. brucei*. In addition, TbLpn contains methylated arginine residues and interacts with *T. brucei* major Protein Arginine Methyltransferases, TbPRMT7. The major focus of my project is to identify the effect of TbLpn methylation by TbPRMT7 on its enzymatic activity and cellular localization.
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Introduction

*Trypanosoma brucei* is a single celled, parasitic, protozoan organism that is responsible for causing “African trypanosomiasis,” or more commonly known as, “African sleeping sickness.” African sleeping sickness is transmitted when a person is bitten by a tsetse fly. These flies are native to Sub-Saharan Africa, and harbor *T. brucei*, and inject it into their human or animal target (1). This illness initially causes fever, headache, muscle pain, joint pain, and enlarged lymph nodes in the initial stages of the sleeping sickness, and eventually experience severe mental deterioration, paralysis, disturbances in the sleep cycle, and death within a few months of infection (2).

Cellular Structure of *Trypanosoma brucei*

The cellular make-up of *T. brucei* is very unique. This protozoan contains a kinetoplast, a compact disc that houses mitochondrial DNA, to store its genetic material, and a singular flagellum for locomotion (1). In order to maintain its shape, *T. brucei* uses a corset-like cytoskeleton made of microtubules, and is aptly named the “microtubule-corset.” This protects the organism from damage and may possibly increase its chances of survival in adverse chemical or physical conditions (1). The flagellum gains its shape by the interactions between the flagellar axoneme, paraflagellar rod, and the flagellum attachment zone. The flagellar axoneme consists of several tubules in a circular formation spanning the entire length of the flagellum, giving it a long, cylindrical structure. The paraflagellar rod’s precise function is still unknown, but it spans the majority of the flagellar length, and is important in maintaining the movement of the flagellum. Four microtubules and a specialized filament compose the flagellum attachment zone. This structure is located just beneath the flagellum on the microtubule corset. The flagellum
exits the Trypanosome through the flagellar pocket, which allows for free movement of
the flagellum (1).

**T. brucei Life Cycle**

Understanding the life cycle of *Trypanosoma brucei* helps researchers to understand the complex relationship between *T. brucei* and its vector host, the tsetse fly. After initial human infection caused by a bite from an infected tsetse fly, metacyclic trypomastigotes are inserted into the human bloodstream (3) from the salivary glands of the tsetse fly (1). These metacyclic trypomastigotes become bloodstream trypomastigotes and can then be carried to other areas within the human body (3). Asexual reproduction occurs through binary fission once the bloodstream trypanomastigotes enter body fluids including the blood, lymph, and spinal fluid (3).

If the infected mammalian host is bitten by an uninfected tsetse fly, this fly will become a carrier of *T. brucei*. Once the fly ingests infected blood, the parasite will infect the host fly’s mid-gut. Here, they will transform into procyclic trypomastigotes (3). In the mid-gut, this type of trypomastigotes will divide, again, by means of binary fission. Once the procyclic trypomastigotes leave the gut, they will evolve into epimastigotes. In this stage, the parasite may enter the tsetse fly salivary glands and transform into metacyclic trypomastigotes. Now, the parasite may exit the tsetse fly vector and enter a new mammalian host, starting the life cycle over again, as shown in Figure 1 (3).

Once the host is infected by *T. brucei*, the host will begin the development of African Trypanosomiasis, or African sleeping sickness. This illness develops in two distinct stages, defined by location of *T. brucei*. In stage I, the parasite can be found in general circulation throughout the body, in locations such as the blood and lymph. During
this stage, symptoms include: fever, headache, muscle pain, joint pain, swollen lymph nodes, weight loss, and swelling or itchiness of the insect bite, or a “chancre (2).” This stage can last anywhere from one to two weeks, to several years. The completion of stage I is marked by the entrance of *T. brucei* into the cerebrospinal fluid. Stage II begets more complicated and dangerous symptoms than stage I. Stage II can cause severe mental deterioration, personality changes, difficulties in staying awake during the day, inability to sleep through the night, paralysis, difficulties in balancing or walking, hormonal imbalances, coma, and, eventually, death (2). Diagnosis of African Trypanosomiasis can be made through microscopic analysis of the lymph node aspirate. To determine whether the illness had progressed to stage II, the cerebrospinal fluid must be examined (4).

Figure 1. The life cycle of *Trypanosoma brucei* (3)
Recorded Epidemics of Human African Trypanosomiasis

There are two different species of *Trypanosoma brucei* that can cause African sleeping sickness in humans (5). *Trypanosoma brucei rhodesiense* is most commonly found in Tanzania, Uganda, Malawi, and Zambia. This strain of *T. brucei* primarily affects cattle, but can affect hunters who visit East Africa (5). *Trypanosoma brucei gambiense* most commonly affects the Democratic Republic of Congo, Angola, Sudan, Central African Republic, Chad, and northern Uganda (5). This strain is the most common cause of Human African Trypanosomiasis (HAT) in human subjects, causing 7,000 to 10,000 infections each year (5). Most commonly, disease transmission occurs through tsetse fly bite, but can be transmitted through mechanisms of blood contact including shared needles and blood transfusions, and can be transmitted sexually, or from pregnant mother to unborn fetus (5).

During the years of 1889-1896, Africa experienced its first recorded African sleeping sickness epidemic. As a result of increased colonization of African countries, death of 90% of the livestock population, and a series of natural disasters, the tsetse fly began targeting humans as their primary food source. The World Health Organization estimates that from areas along the Congo River to Lake Victoria, 500,000 people died (6). In 1901, an epidemic occurred in Uganda, wiping out an estimated 200,000 people in four years (7). African sleeping sickness outbreaks also occurred in Tanzania and Sudan from 1904-1905, Northern Rhodesia and Nyasaland in 1908 (7). As a result of these epidemics, imperialistic countries sent scientists to Africa to study pathogen causing “nagana,” the livestock variation of African Trypanosomiasis. David Bruce discovered this pathogen, and *T. brucei* was named for him (7). These scientific missions trips
started the initial research into treatment for African sleeping sickness, and generated interest in understanding the trypanosome itself.

Uganda experienced a prolonged outbreak of African Trypanosomiasis from 1900 to 1920, and again from 1976-1989 (8). The most recent outbreak was linked to degradation in sociopolitical stability, and this epidemic’s downfall was linked with a return to stability and re-establishment of public health awareness measures (8).

Because of the preventative measures some African governments have taken against African sleeping sickness, the incidence of this illness had decreased significantly in once heavily affected areas (9). For the first time in over 50 years, the number of reported cases per year has dropped below 10,000 (9). This does not include unreported cases, but the World Health Organization estimates that the overall incidence of this illness has still dropped based on the sample of the populations they have tested. *T. b. gambiense* is responsible for 97% of the African Trypanosomiasis cases that have been recorded. It typically affects areas of West Africa, Central Africa, and, most notably, the Democratic Republic of the Congo. West Africa and Central Africa both showed low to very low prevalence and transmission of *T. b. gambiense* from 2001 to 2010. The Democratic Republic of the Congo produces over 80% of the total Human African Trypanosomiasis cases (9) and has some areas of very low prevalence of HAT, and some areas that still exhibit very high prevalence of HAT. The areas that exhibit the most cases of African sleeping sickness are the areas of African countries that have the greatest amount of poverty, least advanced healthcare, lowest health priority, social instability, and the greatest geographical isolation (10). There is a high concentration of these areas
in the Democratic Republic of the Congo, which has lead to their higher incidence of 
HAT in certain areas.

*Trypanosoma brucei rhodesiense* accounts for less that 3% of all Human 
African Trypanosomiasis (9). This strain usually only affects areas of East Africa, and 
mainly affects cattle and wild animals. While there is very low risk of human 
transmission of this virus, it cannot be disregarded when studying the epidemiology of 
HAT (9).

**Current Treatment Methods for HAT**

There are few affective treatments currently in use to treat both forms of HAT. The blood 
stream and lymph stage, or “Hemolymphatic” stage, of *T. b. gambiense* can be treated 
using Pentamidine (Figure 2). This drug should be administered through an intravenous 
infusion (IV) or intramuscular injection (IM). Four milligrams of Pentamidine should be 
administered per kilogram the patient weight, per day for seven to ten days (11). If the 
medication is administered using an IV, the IV should be given over two hours (11). Side 
effects of this medication include pain at injection site, low blood sugar, diarrhea, nausea, 
and vomiting (11).

![Figure 2. Pentamidine](image-url)
Once this parasite crosses into the central nervous system, Eflornithine (Figure 3) becomes the most affective treatment. This drug should be administered 4 times each day for 14 days using an IV. 400 milligrams should be administered per kilogram the patient weights each day. This treatment can cause bone marrow suppression, gastrointestinal abnormalities, and seizures (11).

![Eflornithine](image)

**Figure 3. Eflornithine**

Two different drugs are used to treat the two different stages of *T. b. rhodesiense*. Suramin (Figure 4) is used to fight the hemolymphatic stage. One gram of Suramin should be administered to adults affected by the East African form on HAT over a 21 day period, on days 1,3,7,14, and 21. For children who contract this form of HAT, the dosage is decreased to 20 milligrams per kilogram, on the same dosage schedule. Side effects include a rash, nephrotoxicity, and peripheral neuropathy (11). These side effects are common, but are usually mild and reversible (11).
Melarsoprol (Figure 5) is an arsenic based drug, and is the only effective treatment for stage two *T. b. rhodesiense*. This treatment can cause encephalitis in 5-10% of patients, and as a result produces a 50% fatality rate in patients who experience this side effect. In some patients, the use of corticosteroids can be effective in reducing the risk of encephalitis (11). Melarsoprol can also cause skin reactions, gastrointestinal irritation, peripheral neuropathy, and phlebitis at the site of intravenous injection (11). Because this drug has so many devastating side effects, the dosage schedule is very complicated. On the first day of treatment, the patient will receive two 3.6 milligram per kilogram doses via an IV. This dosage will be repeated for the first three days of treatment. After seven days, another 3.6 milligram per kilogram per day dose will be administered every day for three days. Then, after another seven days, the patient must receive a final 3.6-milligram per kilogram dose of Melarsoprol (11).
Antigenic Variation of *T. brucei*

A unique immune system evasion adaptation that *Trypanosoma brucei* has developed is termed “antigenic variation.” This method involves a switch of VSGs, or, Variant Surface Glycoproteins. These proteins coat the cell in a protective layer and allow for antigenic variation to occur (12). The region where VSGs exist on the surface of the cell is only about 15 nanometers thick and are both synthesized and cleaved in the endoplasmic reticulum (13). VSGs are secured to the surface of the trypanosome by glycosylphosphatidylinositol (GPI), which is also synthesized in the endoplasmic reticulum (14).

Once the host immune system recognizes the trypanosome; it is very effective at eliminating 99% of the parasites present. Approximately 1% of the remaining cells will contain a different type of VSG. The remaining 1% of the cells will reproduce and will contain a different, but singular, type of glycoprotein on the cell membrane. The host immune system will have built a defense against this new strain on *T. brucei*, so it is able
to continue the progression of African Trypanosomiasis (14). Expression of VSG occurs at polycistronic telomere expression sites. Each VSG is expressed in a monoallelic fashion, meaning that only one of approximately 15 VSG expression sites is transcribed at a time (15). All other telomeric expression sites are silenced when one singular site is being transcribed. It has been estimated that 20-30% of the trypanosome genome is devoted to storing over 2,000 different VSG genes (15). This strategy of randomly varying surface proteins makes it impossible to create a vaccine against African Trypanosomiasis.

**Arginine Methylation**

Arginine methylation is an important posttranscriptional modification made to certain proteins, resulting in an extra methyl carbon group on the N-terminus of certain proteins. This adjustment aids in several essential processes, including cell signaling, RNA processing, DNA repair, and chromatin remodeling (16). There are four general types of PRMTs that have been identified thus far. These types are characterized by the amount of modification performed on an individual arginine residue from a methyl donor, usually S-adenosylmethionine, or “AdoMet,” on a terminal side (17). If the enzyme only catalyzes the addition of one methyl group to the terminal nitrogen side of an arginine residue, the enzyme is classified as type III. If the PRMT may transfer a second methyl carbon group to the same terminal nitrogen, resulting in the formation of omega N°-N°-asymmetric dimethylarginine (ADMA), the enzyme is classified as type I. If a third methyl group is added by an enzyme to the nitrogen adjacent to the already dimethylated arginine residue, leading to the production of omega N°-N°-symmetric dimethylarginine (SDMA), the enzyme is classified as type II. While type IV PRMTs
have only been found in certain yeasts, they are still relevant. Type IV PRMTs have the ability to transfer a single methyl group to an internal nitrogen on an arginine residue (17).

In *Trypanosoma brucei*, there are five types of protein arginine methyltransferases (PRMT). TbPRMT1, TbPRMT3, and TbPRMT6 are type I PRMTs, while TbPRMT5 is a type II enzyme. As for TbPRMT7, it is classified as a type III PRMT as it has been shown to catalyze the addition of only one methyl group. TbPRMT1 is responsible for the vast majority of AMDA formation in vivo, similar to what is observed in mammalian cells (17). TbPRMT3 has not been associated with a specific function in *T. brucei* yet. In humans, PRMT3 facilitates ribosome assembly (17). Conversely, the enzyme TbPRMT5 has several known functions. It is able to methylate DEAD-box RNA helicase in vitro, has a very broad substrate range, and may affect RNA metabolism (17). TbPRMT6 lacks the ability to methylate certain targets however, it may have the ability to modify trypanosomal histones (17). TbPRMT7 is where I focused my research. This enzyme has been found to be 30 times more active than its human counterpart based on methyl groups transferred per hour per microgram of the enzyme (17). Experimentally, TbPRMT7 has been shown to catalyze the methyl transfer of bovine histones, myelin basic proteins, and several *T. brucei* RNA binding proteins (17). While the exact function of this enzyme is still unknown, these findings suggest that TbPRMT7 may serve a function in several cellular processes (17).

**The Importance of Lipins in *T. brucei***
The lipin family of proteins is important in the production of lipids. These proteins function as phosphatidate phosphatase enzymes that catalyze the dephosphorylation of phosphatidic acid to diacylglycerol. Diacylglycerol is a cell signaling molecule that aids in the synthesis of triacylglycerol; an energy storage molecule that plays a key role in the maintenance of energy homeostasis (18). Lipin-1 is the most recognized and studied of the lipin family of proteins. Mutations in this lipin have presented with insulin resistance, neonatal fatty liver, elevated triglyceride levels, and abnormal distribution of fats in mice (18). These lipin proteins have also been shown to be dependent on Mg\(^{2+}\) for proper enzymatic activity. Without it, binding sites on these lipins is inactive (18).

Mammalian lipin has orthologs present in many different species, such as trypanosomes. The protein TbLpn was first uncovered during a yeast two hybrid screen of proteins interacting with TbPRMT1, the major protein arginine methyltransferase in T. brucei (14). TbLpn displays the conserved N-LIP and C-LIP domains that are present in all lipin proteins. These were the only two domains found on TbLpn and may be useful in finding a drug target against T. brucei in mammals (19). TbLpn mainly interacts with TbPRMT1 and is mainly localized in the cytosol. In vivo, TbLpn displays phosphatidic acid phosphatase (PAP) activity. This enzymatic activity controls the balance of phosphatidic acid, diacylglycerol (20). This activity demonstrates, again, that TbLpn is a lipin that should be further studied in T. brucei elimination within a mammalian host.
Materials and Methods

Preparation of trypanosome cell extracts

Procyclic form *Trypanosoma brucei brucei* 29-13 cells that have been engineered to allow disruption of TbPRMT7 by RNAi were grown in SM medium supplemented with 10% fetal bovine serum (FBS), 15 µg/ml G418, 50 µg/ml hygromycin B, and 2.5 µg/ml phleomycin were incubated at 27 °C in the presence (induced) or absence (uninduced) of 2.5 µg/ml tetracycline for 2 days. Cells were first centrifuged at 3,750 RPM for 10 minutes at 4 °C in an accuSpin 3R centrifuge (Fisher Scientific). The cell pellet was washed twice in 25ml of Buffer A [150 mM sucrose, 20 mM KCl, 2 mM MgCl₂, 20 mM Hepes (pH to 7.9)]. The final pellet was resuspended in 2 ml of buffer A to which NP-40 was added at a final concentration of 0.2%. Cells were lysed by three passages through a 26-gauge needle, being careful to avoid excess foaming.

Phosphatidic Acid Phosphatase Assay

A Phosphatidic Acid Phosphatase Assay is used to measure the activity of TbLpn when it is methylated by any of the five TbPRMT species that have been identified. I studied TbPRMT7. As stated in the introduction, TbLpn has the ability to catalyze the dephosphorylation of phosphatidic acid to diglycerol. Phosphate is a second product of this reaction and may be used to measure the effect of TbPRMT methylation on TbLpn on its ability to catalyze this process. Ten separate samples were created under different
conditions. 10 milliliters of a 10X Buffer was added to each tube. This buffer consisted of 0.5 M Tris (pH 7.5), and 10 mM MgCl. The first and second samples contained 10 microliters (µl) of buffer and 2 µl of 1,2-dioctanoyl-sn-glycero-3-phosphate (DiC8 PA), a cell permeable phosphatidic acid (5), and 88 µl of water. The third and fourth samples contained 10 µl of the 10X buffer, 2 µl of DiC8 PA, 78 µl of water, and 10 µl of cell extract of TbPRMT7-tet, i.e. cells that have not been induced for RNAi (contains wild type levels of TbPRMT7 enzyme). Samples five and six were very similar to samples 3 and 4. In samples five and six, cell extract TbPRMT7 +tet was used rather than cell extract TbPRMT7-tet. This means that RNA interference was induced, and the cells contain less TbPRMT7, and thus less TbLpn should be methylated in the cell sample. Samples seven and eight contained 10 µl of the 10X Buffer, 10 µl of cell extract TbPRMT7-tet, and 80 µl of water. The final set of samples, nine and ten, each contained 10 µl of the 10X buffer, 10 µl of cell extract +tet, and 80 µl of water.

These ten samples were incubated at 30 °C for 30 minutes. After this incubation period, 200 µl of ammonium molybdate-malachite green reagent (1 volume of 34 mM ammonium molybdate in 5M HCl + 3 volumes of 2.16 mM Malachite Green oxalate) was added to each of the tubes. This reagent allows the color of the sample to develop based on the amount of phosphate produced. If more phosphate is developed as a waste product, then the color will be darker. These samples were then mixed by inversion, and allowed to incubate for 30 minutes at room temperature. After this period, 300 µl of water was added to each of the samples. The absorbance values were then measured at 660 nm.
Results

Through the analysis of a Phosphatidic Acid Phosphatase Assay, we were able to see a visual representation of TbLpn activity. I studied the effect of down regulating the activity of TbPRMT7 on TbLpn activity. A sample of un-RNAi-induced TbPRMT7 was analyzed along with a sample of tet-induced TbPRMT7. The goal of the tet-induced sample was to show the effects of down regulating the action of the TbPRMT7 enzyme on TbLpn. This experiment resulted in an uninduced sample with a phosphatase activity level of 17 nanomoles of Pi (phosphate) released per minute per milligram of protein. The tet-induced sample resulted in a phosphatase activity level of 12 nanomoles of Pi released per minute per milligram of protein, as shown in figure 6. Another student working on this project analyzed the activity of uninduced TbPRMT1 versus tet-induced TbPRMT1 using the same method used in this project.
The main goal of this experiment was to understand how TbPRMT7 affects the activity of TbLpn. TbPRMT7 is an enzyme found naturally in *T. brucei*. Unlike TbPRMT1, little is known about TbPRMT7 function *in vivo*. We knew from previous experimentation that TbPRMT enzymes may aide in arginine methylation of TbLpn. TbLpn is a lipin homologue that has been shown to undergo arginine methylation as a posttranscriptional modification. It has been previously demonstrated that TbLpn is able to carry out the dephosphorylation of phosphatidic acid into diacylglycerol with the release of inorganic phosphate. In order to address the effect of TbLpn methylation by TbPRMT7 on its enzymatic activity, we measured the release of inorganic phosphate from the substrate DiC8 PA using *T. brucei* cells expressing wild-
type levels of TbPRMT7, as well as cells whose TbPRMT7 expression has been downregulated by RNAi.

The uninduced sample showed a slightly higher phosphatidic acid phosphatase activity compared to the tet-induced sample. Our initial hypothesis was, “If TbPRMT7 plays a significant role in the arginine methylation and enzymatic activity of TbLpn, then down-regulation of TbPRMT7 expression by tet-induced RNAi will result in a decrease in the amount of phosphatase activity compared to the uninduced cells.” However, because of the calculated error that is shown in Figure 2, we cannot say for sure that methylation by TbPRMT7 plays a significant role in TbLpn phosphatidic acid phosphatase activity.

As previously stated in this paper, TbPRMT7 is a type III TbPRMT. This means that it only transfers only methyl carbon group onto the lipin before its activity ceases (17). It is the only type III protein methyltransferase that has shown any significant activity in T. brucei in vivo (17). TbPRMT1 has been shown to be important in AMDA formation in vivo, classifying it as a type I PRMT. Recent studies have shown that TbPRMT1 interacts with TbLpn in vivo (17).

In the future this enzyme, along with the other three TbPRMT enzymes that have known actions within this protozoan species, should be analyzed further. Future research should work to understand exactly what the mechanism of action is for these enzymes. Researchers should also study whether these protein methyltransferases have any function in phospholipid bilayer synthesis. It has been indicated that TbLpn is involved in the production of a phospholipid bilayer in T. brucei (17). If any of these enzymes regulate phospholipid bilayer synthesis, they may aid in the production of the
typanosomal cell membrane. Many treatments for bacterial, and fungal pathogens target the cell wall or cell membrane to destroy the cell. If future research can prove that TbLpn is involved in the production of a phospholipid bilayer and VSG synthesis, then it, and the TbPRMT enzymes that regulate it, will be excellent candidates for a new method of treatment against African Trypanosomiasis.

References


