The N-Terminal Domain and Glycosomal Localization of Leishmania Initial Acyltransferase LmDAT Are Important for Lipophosphoglycan Synthesis

Gada K. Al-Ani¹, Nipul Patel², Karim A. Pirani¹, Tongtong Zhu², Subbhalkashmi Dhalladoo², Rachel Zufferey¹,²*

¹Department of Biochemistry, Kansas State University, Manhattan, Kansas, United States of America, ²Department of Biological Sciences, St. John's University, Jamaica, New York, United States of America

Abstract

Ether glycerolipids of Leishmania major are important membrane components as well as building blocks of various virulence factors. In L. major, the first enzyme of the ether glycerolipid biosynthetic pathway, LmDAT, is an unusual, glycosomal dihydroxyacetonephosphate acyltransferase important for parasite’s growth and survival during the stationary phase, synthesis of ether lipids, and virulence. The present work extends our knowledge of this important biosynthetic enzyme in parasite biology. Site-directed mutagenesis of LmDAT demonstrated that an active enzyme was critical for normal growth and survival during the stationary phase. Deletion analyses showed that the large N-terminal extension of this initial acyltransferase may be important for its stability or activity. Further, abrogation of the C-terminal glycosomal targeting signal sequence of LmDAT led to extraglycosomal localization, did not impair its enzymatic activity but affected synthesis of the ether glycerolipid-based virulence factor lipophosphoglycan. In addition, expression of this recombinant form of LmDAT in a null mutant of LmDAT did not restore normal growth and survival during the stationary phase. These results emphasize the importance of this enzyme’s compartmentalization in the glycosome for the generation of lipophosphoglycan and parasite’s biology.

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* E-mail: zufferer@stjohns.edu

Introduction

Worldwide Leishmania parasites cause important human and animal diseases collectively called leishmaniasis. Disease transmission occurs upon biting by an infected female sand fly. The parasite develops extracellularly as flagellated promastigotes in the phagosome of the vertebrate host’s macrophages. L. major is responsible for the cutaneous form of leishmaniasis which manifests in a local self-healing skin lesion and affects approximately 1–1.5 million patients every year [1]. Ninety percent of cases of cutaneous leishmaniasis are found in Afghanistan, Pakistan, Syria, Saudi Arabia, Algeria, Iran, Brazil, and Peru [1].

Ether glycerolipids are major components of Leishmania membranes, representing approximately 20% of total cellular lipids [2,3]. In Leishmania major, they are found primarily in the phosphatidylethanolamine and phosphatidylglycerol ether lipids [2,3,4,5]. They are of particular importance for this parasite because ether glycerolipid based virulence factors such as lipophosphoglycan (LPG) and glycosylphosphatidylglycerol-anchored proteins play critical roles throughout its life cycle [reviewed in [6,7,8,9,10]]. Structurally, LPG is a complex glycolipid that is anchored to the plasma membrane via an ether lysophosphatidylglycerol anchor [11]. The salient feature of LPG is the conserved domain consisting of the Galβ1,4Manα1-PO4 backbone of repeat units that in L. major are branched with galactose and arabinose residues [6,9,12,13,14].

In Leishmania, ether lipid biosynthesis initiates with the acylation of dihydroxyacetonephosphate (DHAP) by the DHAP acyltransferase (DHAPAT) LmDAT, an obligatory step for the biosynthesis of ether lipids ([4,5,16]; Fig. 1). The product of this first acylation reaction, 1-acyl-DHAP, is then converted to 1-alkyl-DHAP by the alkyl DHAP synthase ADS1, which is further reduced to 1-alkyl-glycerol-3-phosphate (1-alkyl-G3P) by a NADPH-dependent alkyl/acyl-DHAP reductase [2,17]. The intermediate 1-alkyl-G3P serves as the obligate precursor for all other ether glycerolipids. Alternatively, in the absence of the G3P acyltransferase LmGAT, 1-acyl-DHAP can be reduced to 1-acyl-G3P by a NADPH-dependent alkyl/acyl-DHAP reductase, which is subsequently used for the biosynthesis of ester glycerolipids [18]. The DHAPAT and alkyl-DHAP synthase are sequestered in the peroxisome-like organelle, called glycosome in Leishmania and related parasites [2,16,19], while the alkyl/acyl-DHAP reductase is associated with the glycosomes but its active site faces the cytoplasm [17].
Leishmania Initial Acyltransferase LmDAT

LmDAT is a unique DHAPAT that bears a very large N-terminal extension of approximately 650 amino acids that is absent in mammalian orthologs [16]. Our previous studies demonstrated that LmDAT is important for growth, survival during stationary phase, the synthesis of ether lipids that includes the ether lipid based LPG, and for virulence, but is dispensable for raft formation [2,4,15]. All together, these data support the notion that LmDAT may represent a potential target for anti-leishmanial chemotherapy. In the present work, a rational deletion approach was applied i) to address the role of the N-terminal extension of LmDAT in enzyme stability and activity, and ii) to investigate the significance of LmDAT glycosomal localization in the synthesis of the ether lipid based LPG. Last, point mutation analysis was carried out to assess whether a catalytically active LmDAT enzyme is required to support normal growth and survival during the stationary phase of the parasite.

Materials and Methods

Strains and growth conditions

Promastigotes of L. major Friedlin V1 strain (MHOM/IL/80/Friedlin) were propagated in liquid and semi-solid M199-derived medium [2]. The null mutant Abn/dt/Abn/dt and complemented line Abn/dt/Abn/dt (LmDAT NEO) were described in [4]. Transfection was performed according to Ngo and colleagues [20] and selection was applied as appropriate in the presence of 20–40 μg/ml G418 or 25–50 μg/ml of hygromycin. To follow parasite proliferation, mid log phase parasites were diluted to 5×10⁵/ml and enumerated with a hemacytometer as a function of time.

Plasmids

Deletion constructs of LmDAT were created by polymerase chain reaction (PCR) using pL-BSD.LmDAT [16] as a template, and the primer pairs O33 (5′-CCGGGATCCCATATGAGCTTCCCACCACCTC-GG-3′) and O116 (5′- CGGGATCCCTCATATGAGCTTCCCACCACCTC-GG-3′) and O41 (5′-CCGGATCCCTCATATGAGCTTCCCACCACCTC-GG-3′) and O111 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′) and O111 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′) and O41 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′) and O111 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′) and O111 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′) and O111 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′) and O111 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′) and O111 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′) and O111 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′) and O111 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′) and O111 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′) and O111 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′) and O111 (5′-CCGGATCCATGAGCTTCCCACCACCTC-GG-3′). The resulting amplified DNA fragments were digested with BamHI and cloned in sense orientation into the respective sites of pXG.HV-LmDAT to yield pXG.HV-LmDAT-AC₄ (Ec440).

The plasmids pXG.HV-LmDAT [16], pXG.HV-LmDAT-AC₇₃₃₃, pXG.HV-ΔN₅₄₆₋LmDAT, pXG.HV-ΔN₅₄₆₋LmDAT, pXG.HV-LmDAT-AC₄₆₉₁, and pXG.HV-LmDAT-AC₄ were transformed into the null mutant Abn/dt/Abn/dt to give the strains Abn/dt/Abn/dt (HV-LmDAT NEO), Abn/dt/Abn/dt (HV-LmDAT-AC₇₃₃₃ NEO), Abn/dt/Abn/dt (HV-LmDAT-AC₄₆₉₁ NEO), and Abn/dt/Abn/dt (HV-LmDAT-AC₄ NEO), respectively. In addition, pXG.HV-LmDAT and pXG.HV-LmDAT-AC₄ were also transformed into the wild type to yield FV1 (HV-LmDAT NEO) and FV1 (HV-LmDAT-AC₄ NEO), respectively.

Enzymatic assays

Leishmania protein extracts were prepared as described previously [16,18]. Protein concentration was determined by the bichinchoninic acid assay using bovine serum albumin as a standard. DHAPAT activity was assessed by measuring the acylation rate of DHAP produced by catabolism of fructose-1,6-biphosphate by the action of an aldolase and a triose phosphate isomerase, based on a protocol established by Bates and Saggerson as described in [16]. The specificity of [U-14C]D-fructose-1,6-biphosphate (MP Biomedicals) was 395 cpm/mmol.

Digitonin fractionation and electrophoresis

For digitonin treatment fresh end-log cells were harvested, washed once in phosphate buffered saline (PBS), and resuspended in 20 mM TrisHCl (pH 8.0), 1 mM EDTA, 1 mM DTT containing a protease inhibitor cocktail (Roche) at a cell density of 2×10⁹/ml. Aliquots of 100 μl were made and supplemented with increasing [0 to 0.6 mg/ml] concentrations of digitonin (stock solution of 15 mg/ml in PBS) and incubated at 26°C for 10 min. Cells were then centrifuged at 20,800 g for 2 min. Supernatants were immediately removed and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Western blot analyses in the presence of monoclonal WIC79.3 (specific to β-galactoside sile-chains; generous gift from S. Turco; [21]) and V5 (Invitrogen) antibodies, and polyclonal immuno-
globsulins specific to hypoxanthine guanine phosphoribosyltransferase (generous gift from A. Jardim; [22]), phosphomannomutase (generous gift from L. Kedzierski; [23]), and arginase (generous gift from B. Ullman; [24]) were carried out as described previously [2,4].

**Immunofluorescence assay**

Immunofluorescence assay was performed with wild-type parasites expressing pXG, HV-LmDAT-ΔC3, as described previously [16]. The recombinant His6-V5 (HV) tagged HV-LmDAT-ΔC3 was revealed with V5 monoclonal antibodies (Invitrogen) and hypoxanthine guanine phosphoribosyltransferase was visualized with specific rabbit polyclonal immunoglobulins [22]. Both antibodies were used at a 1:500 dilution. Images were taken with a Leica fluorescence microscope.

**Results and Discussion**

**The N-terminal extension is important for LmDAT activity**

Amino acid sequence alignment of DHAPAT enzymes from human, rat, Caenorhabditis elegans, Trypanosoma brucei and Trypanosoma cruzi shows that orthologs of parasites of the trypanosomatidae family bear a very large N-terminal extension of approximately 650 amino acids that is absent in higher eukaryotic orthologs ([16]; Fig. 2A; data not shown). Curiously, this domain fails to exhibit any similarity to known proteins and thus, is parasite specific. The function of this N-terminal extension was first investigated by creating truncated LmDAT versions that were N-terminally tagged with a hexahistidine fused to a V5 epitope (HV) for visualization with the V5-specific monoclonal antibody. Plasmids coding for truncated proteins lacking the N-terminal 546 and 686 amino acids (HV-ΔN546-LmDAT and HV-ΔN686-LmDAT), respectively, were constructed, as well as a plasmid coding for a recombinant protein missing the 733 C-terminal amino acids (HV-LmDAT-ΔC733). In the latter case, the LmDAT C-terminal tripeptide SKM was fused to the C-terminal portion of the truncated protein to ensure glycosomal targeting [25,26,27]. These recombinant proteins, as well as a wild-type HV tagged version (HV-LmDAT), were expressed in the null mutant Abndat/Abndat background [16].

We verified first that the HV tag did not affect the function of LmDAT; the recombinant protein HV-LmDAT was expressed as an approximately 150 kDa band as shown by Western blot analysis using the monoclonal antibody specific to the V5 epitope (Fig. 2C). Bands present at lower molecular weights very likely represent degradation products of the full length tagged protein. In addition, HV-LmDAT was enzymatically active, and supported normal growth and survival during the stationary phase (Fig. 2B, 3A; [16]). The presence of ether lipids was assessed by investigating the migration behavior of the ether lipid anchored virulence factor LPG; it has been established that ether lipids are dispensable for growth and survival during the stationary phase [2]. Thus, LmDAT may be a bipartite protein with an N-terminal domain involved in growth and survival during the stationary phase, and a C-terminal domain functioning in catalysis. To assess whether a catalytically active LmDAT enzyme is important for normal growth and survival during the stationary phase, a mutant enzyme was created that bears a leucine instead of a lysine at position 852 (K852L), based on the information that replacement of the corresponding conserved amino acid by a histidine in the human DHAPAT abrogated its enzymatic activity [28]. As described above, a HV-tagged version (HV-LmDAT-K852L) was expressed in the null mutant background. Western blot analysis was performed to verify its expression level that was similar to HV-tagged wild-type LmDAT (Fig. 2C). HV-LmDAT-K852L enzyme displayed no DHAPAT activity and led to the production of slow migrating LPG glycolipids similar to that of the null mutant (Fig. 2B, 2D). In addition, HV-LmDAT-K852L failed to support normal growth and survival during the stationary phase (Fig. 3B).

Altogether, these results demonstrate that the conserved lysine K852 is important for substrate recognition or catalysis of LmDAT similar to the human ortholog. Together with the fact that the N-terminal domain alone is unable to support normal growth and survival during the stationary phase, our results suggest that the acyltransferase activity of LmDAT itself is critical for normal growth and survival during the stationary phase. Our data also exclude the idea that LmDAT is a bipartite enzyme with an N-terminal domain responsible for growth and/or survival during the stationary phase, and a C-terminal part implicated in acyltransferase activity.
Glycosomal expression of \( Lm \) DAT is critical for LPG synthesis

\( Lm \) DAT resides in glycosomes consistent with the presence of a typical type 1 C-terminal glycosomal targeting signal sequence (SKM), that is sufficient for targeting proteins to this organelle [16,19]. Hence, the importance of the glycosomal subcellular localization of \( Lm \) DAT for ether lipid biosynthesis was assessed by expressing a HV-tagged \( Lm \) DAT recombinant enzyme lacking the C-terminal glycosomal targeting tripeptide SKM (HV-\( Lm \) DAT-\( DC_3 \)) in the null mutant background [25,27,29]. Western blot analysis was performed in the presence of anti-V5 monoclonal antibodies demonstrated that the levels of HV-\( Lm \) DAT-\( DC_3 \) protein were similar to that of wild-type tagged HV-\( Lm \) DAT expressed in the null mutant background (Fig. 2C). Subcellular localization of HV-\( Lm \) DAT-\( DC_3 \) was assessed by immunofluorescence assay. HV-\( Lm \) DAT-\( DC_3 \) was revealed in the presence of V5-specific (upper; V5) and hypoxanthine guanine phosphoribosyltransferase specific (lower; HGPRT; loading control) antibodies. Equivalent of 5 x 10⁷ cells were loaded in each lane. The apparent molecular weight is shown on the left. (D) Western blot analysis in the presence of WIC79.3 antibody to detect LPG. Equivalent of 10⁶ cells were loaded in each lane. (B, C, D): 1, \( Dlmdat/Dlmdat \); 2, \( Dlmdat/Dlmdat \) [HV-\( Lm \) DAT NEO]; 3, \( Dlmdat/Dlmdat \) [HV-\( DC_3 \) Lm DAT NEO]; 4, \( Dlmdat/Dlmdat \) [HV-\( DC_3 \) Lm DAT NEO]; 5, \( Dlmdat/Dlmdat \) [HV-\( Lm \) DAT-\( AC_733 \) NEO]; 6, \( Dlmdat/Dlmdat \) [HV-\( Lm \) DAT-\( AC_3 \) NEO]; 7, \( Dlmdat/Dlmdat \) [HV-\( Lm \) DAT-\( DC_3 \) NEO]; WT, wild type.

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Figure 2. Characterization of mutant forms of \( Lm \) DAT. (A) Schematic representation of human DHAPAT (hDHAPAT) and mutant forms of \( Lm \) DAT. The grey rectangle, the black rectangle and the hatched area depict the HV tag, the conserved domain, and the C-terminal glycosomal targeting tripeptide, respectively, and the asterisk depicts the point mutation. (B) DHAPAT activity was quantified as described in Materials and Methods. Equivalent of 0.5 mg protein extracts were applied for the assay. Null mutant alone or expressing HV-tagged wild-type and mutant forms of \( Lm \) DAT were used as a source of protein extracts. Activity is expressed as percentage of the positive control, the wild type (WT). The assay was performed twice in duplicate, and the graph depicts one representative experiment. Standard deviations are shown. (C) Western blot analyses in the presence of V5-specific (upper; V5) and hypoxanthine guanine phosphoribosyltransferase specific (lower; HGPRT; loading control) antibodies. Equivalent of 5 x 10⁷ cells were loaded in each lane. The apparent molecular weight is shown on the left. (D) Western blot analysis in the presence of WIC79.3 antibody to detect LPG. Equivalent of 10⁶ cells were loaded in each lane. (B, C, D): 1, \( Dlmdat/Dlmdat \); 2, \( Dlmdat/Dlmdat \) [HV-\( Lm \) DAT NEO]; 3, \( Dlmdat/Dlmdat \) [HV-\( DC_3 \) Lm DAT NEO]; 4, \( Dlmdat/Dlmdat \) [HV-\( DC_3 \) Lm DAT NEO]; 5, \( Dlmdat/Dlmdat \) [HV-\( Lm \) DAT-\( AC_733 \) NEO]; 6, \( Dlmdat/Dlmdat \) [HV-\( Lm \) DAT-\( AC_3 \) NEO]; 7, \( Dlmdat/Dlmdat \) [HV-\( Lm \) DAT-\( DC_3 \) NEO]; WT, wild type.

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Glycososomal expression of \( Lm \) DAT is critical for LPG synthesis

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Subcellular localization of HV-\( Lm \) DAT-\( DC_3 \) was assessed by immunofluorescence assay. HV-\( Lm \) DAT-\( DC_3 \) was revealed in the presence of V5 monoclonal antibodies and gave a signal that was partially overlapping with that obtained with antibodies specific to the glycosomal resident hypoxanthine guanine phosphoribosyltransferase suggesting a glycosomal association ([22]; Fig. 4). However, digitonin fractionation provided evidence for HV-\( Lm \) DAT-\( DC_3 \) localizing outside the glycosomes. Digitonin specif-
ically permeabilizes the cytoplasmic membrane at low concentrations while high doses of this detergent are needed to solubilize the glycosomal membrane [30]. HV-LmDAT required a minimal concentration of digitonin of 0.45 mg/ml in order to be properly released in the cell supernatant and behaved similarly as the glycosomal resident arginase ([31]; Fig. 5), In contrast, HV-LmDAT-DC3 readily fractionated in the cell supernatant at a low digitonin concentration of 0.075 mg/ml, as the cytosolic enzyme phosphomannomutase [23]. Our data suggest that HV-LmDAT-DC3 is partially associated with glycosomes and possibly other organelles but resides on the cytosolic side of the organellar membrane. This is consistent with previous studies that demonstrated that abrogation of the type 1 C-terminal glycosomal targeting signal sequence resulted in a cytoplasmic localization [25,27,29].

In vitro DHAPAT assays showed that HV-LmDAT-ΔC3 was enzymatically active as the wild-type HV-tagged version of LmDAT (Fig. 2B), demonstrating that the C-terminal glycosomal targeting sequence was dispensable for enzymatic activity. Surprisingly, Western blot analysis performed in the presence of WIC79.3 demonstrated that, in contrast to HV-LmDAT, HV-LmDAT-ΔC3 expression failed to restore the synthesis of normal migrating LPG (Fig. 2D). Consistent with this result, expression of HV-LmDAT-ΔC3 did not ameliorate the slow growth and survival during the stationary phase of the null mutant (Fig. 3B). These data suggest that the proper acyl donor for LmDAT, palmitoyl-CoA, may not be available in the cytosol [16]. This is unlikely because fatty acyl-CoAs are made either in the endoplasmic reticulum by elongases or in the mitochondria by type II fatty acyl-CoA synthases, and have to be transported via the cytosol to the endoplasmic reticulum and glycosomes where lipid biosynthesis occurs [16,32,33,34]. Alternatively, the role of the glycosomal compartmentalization of

**Figure 3. Growth curves.** Cells were inoculated at a cell density of 5 × 10⁵/ml and were enumerated with a hemacytometer as a function of time. The assay was performed twice and the graphs represent a typical experiment. Standard deviations are shown. (A) Black circles, wild type; grey circles, complemented line Δlmdat/Δlmdat [HV-LmDAT NEO]; white circles, Δlmdat/Δlmdat; white triangles, Δlmdat/Δlmdat [HV-LmDAT ΔC3 NEO]; grey triangles, Δlmdat/Δlmdat [HV-LmDAT-DC3 NEO]; black triangles, Δlmdat/Δlmdat [HV-LmDAT ΔC3 NEO]. (B) Black circles, wild type; grey circles, complemented line Δlmdat/Δlmdat [HV-LmDAT NEO]; white circles, Δlmdat/Δlmdat; white triangles, Δlmdat/Δlmdat [HV-LmDAT ΔC3 NEO]; grey triangles, Δlmdat/Δlmdat [HV-LmDAT-DC3 NEO]; black triangles, Δlmdat/Δlmdat [HV-LmDATK852L NEO].

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**Figure 4. HV-LmDAT-ΔC3 does not localize in the glycosomes.** Wild type expressing recombinant HV-LmDAT-ΔC3 was analyzed by phase contrast (panel 1) or immunofluorescence microscopy using anti-V5 antibody (panel 2) or polyclonal antiserum specific to hypoxanthine guanine phosphoribosyltransferase (panel 3). Panel 4 shows the merge of panels 2 and 3.

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**Figure 5. Digitonin fractionation followed by Western blot analysis.** FV1 [HV-LmDAT NEO] and FV1 [HV-LmDAT-ΔC3 NEO] were fractionated in the presence of digitonin as described in Materials and Methods. Cell supernatants were then subjected to Western blot analysis in the presence of monoclonal anti-V5 antibodies (V5), and of polyclonal immunoglobulins specific to arginase (ARG) and phosphomannomutase (PMM). Equivalent of 10⁷ cell supernatants were loaded in each lane. The apparent molecular weight markers are shown.

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Leishmania Initial Acyltransferase LmDAT is to sequester its product, 1-acyl-DHAP, in this organelle for conversion into 1-alkyl-DHAP by the glycososomal alkyl DHAP synthase ADS1 rather than being metabolized to 1-acyl-G3P by the cytosolic alkyl/acyl-DHAP reductase for the synthesis of ether glycerolipids [2,17]. Last, DHAP produced in the glycosome may not be available in the cytosol where HV-LmDAT-AC2 accumulates [19]. These results are also in accordance with the idea that the glycosomal membrane does not allow transport of 1-acyl-DHAP from the cytosol into the glycosome for conversion to 1-alkyl-DHAP by the alkyl-DHAP synthase ADS1.

**Author Contributions**

Conceived and designed the experiments: RZ NP. Performed the experiments: GKA NP KAP TZ SD RZ. Analyzed the data: GKA NP RZ. Wrote the paper: RZ.

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