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***Leishmania* parasites possess a platelet-activating factor acetylhydrolase important for virulence**

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Abstract

Leishmania parasites are intracellular protozoans capable of salvaging and remodeling lipids from the host. To understand the role of lipid metabolism in *Leishmania* virulence, it is necessary to characterize the enzymes involved in the uptake and turnover of phospholipids. This study focuses on a putative phospholipase A2 (PLA2)/platelet-activating factor acetylhydrolase (PAF-AH) in *L. major*. In mammals, PAF-AH is a subgroup of PLA2 catalyzing the hydrolysis/inactivation of platelet-activating factor (PAF), a potent mediator of many leukocyte functions. By immunofluorescence microscopy, *L. major* PLA2/PAF-AH is predominantly localized in the ER. While wild type *L. major* parasites are able to hydrolyze PAF, this activity is completely absent in the PLA2/PAF-AH-null mutants. Meanwhile, deletion of PLA2/PAF-AH had no significant effect on the turnover of common glycerophospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol. PLA2/PAF-AH is not required for the growth of *L. major* parasites in culture, or the production of GPI-anchored virulence factors. Nonetheless, it does play a key role in the mammalian host as the PLA2/PAF-AH null mutants exhibit attenuated virulence in BALB/c mice. In conclusion, these data suggest that *Leishmania* parasites possess a functional PAF-AH and the degradation of PAF or PAF-like lipids is an important step in infection.

Keywords

Phospholipase; virulence; *Leishmania*; platelet-activating factor; PAF-AH; macrophage

1. Introduction

Leishmania parasites are Trypanosomatid protozoans which cause a range of diseases from self-limiting cutaneous skin lesion to lethal, visceral infection that damages the spleen, liver, and bone marrow [1]. It is estimated that 350 million people in 88 countries are at risk and 12 million people are already infected by *Leishmania* [2]. Current prevention and treatment strategies are often ineffective with harmful side effects [3, 4]. To develop new drugs and vaccines, it is necessary to understand the molecular interaction between host cells and parasites.

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In the vector host (sandfly), *Leishmania* parasites proliferate extracellularly as flagellated promastigotes. In the mammalian host, they grow and replicate inside the parasitophorous vacuole of macrophages as non-flagellated amastigotes [5]. The parasitophorous vacuole is formed after phagocytosis and constitutes a permissive environment for *Leishmania* to gain nutrients and interact with the host [6]. It is known that *Leishmania* amastigotes can salvage and remodel host lipids [7–9]. Recently, we characterized an inositol phosphosphingolipid phospholipase C-like protein (ISCL) in *L. major* which is responsible for the degradation of both sphingomyelin (a prevalent sphingolipid in mammals) and inositol phosphorylceramide (the dominant sphingolipid in *Leishmania*) [10]. Deletion of ISCL led to a mild defect in morphology during the promastigote phase [10]. Importantly, these ISCL-null mutants were hypersensitive to acidic pH [11] and lacked virulence in mice [10]. Infectivity was restored when ISCL-null mutants were complemented with a functional sphingomyelinase [10, 12]. These findings indicate that the turnover of host sphingolipids is essential for *Leishmania* survival in mammals.

In addition to sphingomyelin, other types of recycled lipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylglycerol (PG) should also be plentiful in the parasitophorous vacuole. Given the importance of ISCL in *Leishmania* virulence, it is of interest to investigate the enzymes responsible for the salvage and turnover of these lipid molecules. In most organisms, a diverse family of phospholipases including phospholipase A1 (PLA1), phospholipase A2 (PLA2), phospholipase C, and phospholipase D are involved in the degradation and remodeling of glycerophospholipids. These enzymes have a broad range of functions from membrane repair to generation of signaling molecules. In mammals, PLA1 (EC 3.1.1.32) and PLA2 (EC 3.1.1.4) can produce bioactive lipids such as lysophospholipids and arachidonic acid [13–17]. PLA1 hydrolyzes the *sn*-1 acyl esters from glycerophospholipids and has been detected in many cell types. PLA2 specifically catalyzes the release of fatty acid at *sn*-2 position to generate a free fatty acid and a lysophospholipid. There are four major types of PLA2: secreted PLA2, cytosolic PLA2, calcium-independent PLA2, and platelet-activating factor acetylhydrolase (PAF-AH) [18]. Secreted PLA2s are characterized by a low molecular weight (13–19 KDa) and a large number of disulfide bonds [13]. Intracellular and calcium-independent PLA2s (85–88 KDa) are responsible for cell signaling by generating arachidonic acid, a precursor of eicosanoids (including prostaglandins and leukotrienes) which regulate inflammation and immunity [19, 20].

Another type of PLA2 is PAF-AH (EC 3.1.1.47), which removes the *sn*-2 acetyl group from platelet-activating factor (PAF) to generate lysophosphocholine and acetate. In animals, PAF (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) [21] is a bioactive molecule produced by activated platelets, leukocytes, and endothelial cells [22, 23]. In addition to promoting platelet aggregation, PAF is a powerful pro-inflammatory signal involved in many leukocyte functions [22, 23]. By removing the *sn*-2 acetyl group, PAF-AH eliminates the biological activity of PAF and therefore regulates the homeostasis of PAF [24].

Genes encoding putative phospholipases are present in the *L. major* genome with syntenic homologues in other *Leishmania* species and related Trypanosomatids such as *Trypanosoma brucei* and *Trypanosoma cruzi* (Table 1). In *T. brucei*, a PLA1 has been cloned and biochemically characterized as a cytosolic protein (35 KDa) that prefers PC as a substrate and is crucial for the production of lysophosphatidylcholine [25–27]. PLA2 activity has also been described in *T. brucei* which can be stimulated by Ca^{2+} and regulates the release of arachidonic acid [28, 29]. In *T. cruzi*, PLA activities have been detected in whole cell lysate and potential substrates include phosphatidylinositol and inositol phosphorylceramide [30]. In *Toxoplasma gondii*, an Apicomplexan parasite, a secreted PLA2 is involved in the host

cell invasion process [31–33]. Reports on the role of PLA (both endogenous and exogenous) in *Leishmania* species have also begun to emerge [34–36].

In this study, we focused on a putative PLA2 from *L. major* which shows significant homology to the cytosolic PAF-AH in mammals. We tested whether this PLA2/PAF-AH possessed any activity against PAF or common phospholipids (such as 1,2-diacylglycerophospholipids with C16–18 in fatty acyl chain length). Previous reports suggest that PAF may inhibit *Leishmania* survival in macrophages by activating nitric oxide (NO) synthesis [37, 38]. The leishmanial PLA2/PAF-AH may thus serve as the degradative enzyme to inactivate PAF. Alternatively, this enzyme may be responsible for the degradation and/or remodeling of glycerophospholipids which are important membrane components and anchors for surface glycoconjugates. Results from this study should provide new insight into the role of lipid metabolism in *Leishmania*-host interaction.

2. Materials and methods

2.1 Materials

1-O-hexadecyl-2-acetyl-³H[N]-*sn*-glycero-3-phosphocholine ([³H]-PAF, 10–30 Ci/mmol) was purchased from Perkin Elmer Inc. Purified bovine pancreas PLA2 was purchased from Sigma-Aldrich (P 8913). The following fluorescently labeled lipids were purchased from Avanti Polar Lipids: 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl]-*sn*-glycero-3-phosphoethanolamine (NBD-PE); 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl]-*sn*-glycero-3-phosphocholine (NBD-PC); 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl]-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] ammonium salt (NBD-PG); 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl]-*sn*-glycero-3-phosphoserine ammonium salt (NBD-PS); and 12-N-methyl-(7-nitrobenz-2-oxa-1,3-diazo)aminostearic acid (NBD-fatty acid or NBD-FA). These NBD-labeled lipids were used as substrates or TLC standards in the PLA2 assay described in 2.7. All other chemicals were purchased from Fisher Scientifics unless specified otherwise.

2.2 Molecular cloning

The entire open reading frame of *PLA2/PAF-AH* (1362 bp) was PCR amplified from *L. major* genomic DNA using primers #37 (GTATCGAGGATCCACCATGCACCCAATCTTCGACTAC) and #38 (ATTCACGGATCCTTACCCCTCAGCGCAGCGGC). The resulting DNA fragment was digested with *Bam*HI and cloned into the expression site of pXG vector [39] to generate pXG-PLA2 (B118). To generate knockout constructs, the 5'- and 3'-untranslated regions of *PLA2/PAF-AH* were PCR amplified and cloned into pUC18 vector. Primers #39 (TACGCAGAATTCTTGTGACGCATGGCCTCTAC) and #40 (TCGATCGGGATCCGTGCAATCTAGATGTGCGCGATGCGTGTGTGC) were used to produce the 5'-flanking region (~0.6 Kb). Primers #41 (ATGCTCGGATCCAGGTTGTTTGCCACTTTAC) and #42 (CATCGTAAGCTTGGCGACGGCGTCTCTGTGCGCC) were used to produce the 3'-flanking region (~1.0 Kb). Subsequently, genes conferring resistance to puromycin (*PAC*) and blasticidin (*BSD*) were cloned between the 5'- and 3'-flanking regions to generate pUC18-KO-PLA2:PAC (B120) and pUC18-KO-PLA2:BSD (B121), respectively.

For localization studies, *PLA2/PAF-AH* was fused in frame with a green fluorescence protein (GFP) gene at the C-terminus (to preserve the predicted N-terminal signal peptide sequence) (Fig. 1) to generate PLA2-GFP. To do so, a modified form of *PLA2/PAF-AH* without stop codon was PCR amplified using primers #37 and #259

(GATCATGGATCCCCCCTCAGCGCAGCGGCGAGCGC) and cloned into pXG-‘GFP⁺ [39] to generate pXG-PLA2-GFP (B271). All molecular constructs were confirmed by restriction enzyme digestion and DNA sequencing.

2.3 Leishmania promastigote culture and transfection

L. major LV39 clone 5 (Rho/Su/59/P) promastigotes were cultured at 26 °C in M199 media with 10% heat-inactivated fetal bovine serum and other supplements as previously described [40]. To generate *PLA2/PAF-AH*-null mutants, DNA knockout constructs (B120 and B121) were linearized by restriction enzymes to sequentially replace the *PLA2/PAF-AH* alleles in wild type (WT) *L. major* promastigotes [41]. The resulting *pla2⁻* cells ($\Delta PLA2::PAC/\Delta PLA2::BSD$) were selected based on resistance to puromycin and blasticidin. To confirm the loss of *PLA2/PAF-AH*, genomic DNA from promastigotes was digested with *Bgl*II and *Hind*III, separated on a 0.7% agarose gel, transferred to a nitrocellulose membrane, and hybridized with a [³²P]-labeled DNA probe that hybridized to a ~ 400-bp upstream flanking region of *PLA2/PAF-AH*. To complement the mutant, pXG-PLA2 or pXG-PLA2-GFP was transfected into *pla2⁻* by electroporation and the resulting cells were referred to as *pla2⁻/+PLA2* ($\Delta PLA2::PAC/\Delta PLA2::BSD/+pXG-PLA2$) or *pla2⁻/+PLA2-GFP* ($\Delta PLA2::PAC/\Delta PLA2::BSD/+pXG-PLA2-GFP$). To prevent the loss of virulence during *in vitro* culture and genetic manipulation, stationary phase promastigotes of WT, *pla2⁻* and *pla2⁻/+PLA2* were passed through BALB/c mice at $1-5 \times 10^7$ cells/mouse and recovered one month later [42]. These parasites were then converted back to promastigotes and used in the virulence study described in 2.6.

To determine *in vitro* growth rates, promastigotes of WT, *pla2⁻*, and *pla2⁻/+PLA2* were inoculated in M199 medium at 1.0×10^5 cells/ml and cell densities were measured every 24 hours using a hemacytometer. To measure the percentage of dead cells during stationary phase, parasites were stained with 5.0 µg/ml of propidium iodide and analyzed by flow cytometry [43]. Infective metacyclics were isolated from stationary phase promastigotes using the lectin agglutination method [44] or density centrifugation method [45].

2.4 Immunofluorescence microscopy

In general, immunofluorescence microscopy was performed as previously described [46]. To determine the localization of PLA2-GFP in log phase promastigotes, *pla2⁻/+PLA2-GFP* parasites were permeabilized with 100% ethanol on ice and labeled with a rabbit anti-*T. brucei* BIP polyclonal antibody (1:10,000)[47], followed by a goat anti-rabbit IgG-Texas Red labeled secondary antibody (Life Technologies, 1:2000). Cells were then stained with Hoescht 33342 (2.5 µg/ml) and visualized using an Olympus BX51 Upright Fluorescence Microscope.

The distribution of PLA2-GFP was also examined in amastigotes. Briefly, bone marrow cells were isolated from the femur of female BALB/c mice and differentiated into macrophages (MΦs) using 30 ng/ml of macrophage colony stimulating factor [11]. Stationary phase promastigotes of *pla2⁻/+PLA2-GFP* were opsonized with 4% C57BL6 mouse serum and applied to MΦs at a ratio of 10 parasites per MΦ. After incubation at 33 °C for 48 hours, MΦs were fixed with 3.7% formaldehyde and permeabilized with 100% ethanol on ice. PLA2-GFP was then detected using a rabbit anti-GFP serum (Life Technologies, 1:1000) followed by a goat anti-rabbit IgG-Texas Red labeled secondary antibody (Life Technologies, 1:2000).

To label mitochondrion, log phase *pla2⁻/+PLA2-GFP* promastigotes were incubated in 250 nM of Mitotracker Red 580 (Life Technologies) in DMEM for 30 minutes at 26 °C and fixed in 3.7% formaldehyde. Parasites were then washed with phosphate buffered saline

(PBS), attached to polylysine-coated coverslips, and washed again with 50% cold ethanol (prepared in PBS). Cells were then stained with Hoescht 33342 and visualized as described above.

To determine the localization of LPG or GP63, log phase promastigotes were probed with monoclonal antibodies against LPG (WIC79.3) [48] or GP63 (Cedarlane labs clone 96/26) as previously described [43]. In all immunofluorescence microscopy experiments, we included negative controls which were cells stained by secondary antibody only (to ensure a low background).

2.5 Western blotting

Leishmania promastigotes were inoculated at 1.0×10^5 cells/ml and cultured to log phase ($<1.0 \times 10^7$ cells/ml) or stationary phase ($2.5\text{--}3.5 \times 10^7$ cells/ml); Cells were harvested by centrifugation (a minimum of 1×10^8 cells were collected for log phase parasites and 4×10^8 cells were collected for stationary phase parasites); culture supernatants were then transferred to new tubes and boiled in $2 \times$ SDS sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 10% 2-mercaptoethanol); and cells were boiled in $1 \times$ SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 5% 2-mercaptoethanol) at 5.0×10^7 cells/ml. Boiled cell lysates or culture supernatants were then separated by SDS-PAGE (10% polyacrylamide gel) and transferred to PVDF membranes, followed by blocking with 5% dry milk. To detect PLA2-GFP, the membrane was probed with a rabbit anti-GFP antiserum (Life Technologies, 1:1000), followed by a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Life Technologies, 1:2000). To examine the expression level of GPI-anchored molecules, the membrane was probed with antibodies against LPG or GP63 as previously described [49]. To ensure equal loading, the membrane was also probed with a monoclonal antibody against α -tubulin (Sigma-Aldrich) (1:5000), followed by a HRP-conjugated goat anti-mouse IgG secondary antibody (1:2000). Western blot signals were detected using autoradiography or a FluorChem E imager (Protein Simple).

2.6 Use of mice in virulence study and ethics statement

BALB/c mice (female, 7–8 weeks old) were purchased from Charles River Laboratories International. The use of mice in this study was approved by the Animal Care and Use Committee at Texas Tech University (US PHS Approved Animal Welfare Assurance NO. A3629-01). Mice were housed and cared for in the facility operated by the Animal Care and Resources Center at Texas Tech University. The facility was inspected monthly and animals were monitored daily by staff members. A complete range of clinical veterinary services was available on a 24-hour basis and includes consultation, diagnostic work-up and clinical care. To minimize the pain and distress on animals without compromising the quality of research, mice were under anesthesia (through the peritoneal injection of ketamine hydrochloride/xylazine) during recurring procedures including the injection of *Leishmania* parasites into footpads, the recovery of parasites from infected mice, and the measurement of lesion size using a caliper. Usually, no more than one procedure was performed on any mouse within a week. Mice were monitored carefully (twice a week for appearance, size, movement, and general health condition) and euthanized by carbon dioxide asphyxiation when footpad lesions became over 2.5 mm or when secondary infections occurred. Mice were also euthanized prior to the isolation of femur cells and limiting dilution assay (to determine parasite load in infected tissue).

Virulence study of *pla2⁻* mutants was performed using the mouse footpad infection model as previously described [50]. Parasites that were recently recovered from mice were cultured *in vitro* for 3 passages and metacyclics were purified from late stationary phase promastigotes

using the density centrifugation method [45]. Metacyclics were then resuspended in DMEM and injected into the footpads of 8-week old, female BALB/c mice (1.0×10^5 metacyclics/mouse, 5 mice per group). Footpad thickness was measured weekly using a Vernier caliper. Parasite numbers in infected footpads were determined by limiting dilution assay [50]. Proper restraining and injection techniques were employed by trained lab personnel to reduce pain and distress of animals.

2.7 PAF-AH assay and PLA2 assay

Promastigotes were cultured to the second day in stationary phase and collected by centrifugation. Cells were washed once in PBS and lysed at 4.0×10^8 cells/ml in a PBS-based lysis buffer containing 5 mM of $MgCl_2$, 0.1 % Triton X-100, and 5 mM of dithiothreitol (5 min on ice with sonication). Protein concentrations in parasite lysates were determined using a BCA Protein Assay Kit-Reducing Agent Compatible (Thermo Scientific). The PAF-AH assay was performed as previously described with minor modifications [51]. Briefly, 50 μ l of parasite lysate was combined with 60,000 cpm of [3H]-PAF to a final volume of 250 μ l containing 50 mM of Tris-HCl and 5 mM of EDTA (pH 7.5). After one hour incubation at 37 °C, the reaction was terminated by adding 250 μ l of water and 2.5 ml of chloroform:methanol (4:1, v/v). [3H]-acetate was collected in the aqueous phase after extraction and analyzed by scintillation counting. The amounts of [3H]-acetate (product), [3H]-PAF (substrate), and protein were used to calculate the PAF-AH activity in each sample (pmol/[mg $\times$ hour]). Negative control: 50 μ l of boiled WT lysate (100 °C for 10 minutes). Positive Control: 10 μ l BALB/c mouse serum diluted in lysis buffer to a final volume of 50 μ l.

The PLA2 assay on glycerophospholipids was adopted from a previous report on the Ca^{2+} -dependent cytosolic PLA2 from zebrafish [52]. Briefly, 50 μ l of parasite lysate was combined with 0.5 nmole of NBD-labeled phospholipid substrate (PE, PC, PS, or PG; stocks for phospholipids were prepared in methanol) to a final volume of 250 μ l containing 13.7 mM of NaCl, 0.537 mM of KCl, 0.025 mM of Na_2HPO_4 , 0.044 mM of KH_2PO_4 , 1.30 mM of $CaCl_2$, 1 mM of $MgCl_2$, and 4.2 mM of $NaHCO_3$ (pH 7.2). After one hour incubation at 37 °C, the reaction was stopped by the addition of 250 μ l of water and 2.5 ml of chloroform:methanol (4:1, v/v), followed by vortex for 30 seconds. The aqueous phase was then removed after centrifugation (1500 g for 10 minutes). The organic phase was dried under a steady stream of air and dissolved in 15 μ l of chloroform: methanol (2:1, v/v). Lipids in the organic phase were then spotted onto a thin layer chromatography (TLC) plate (silica gel 60) and separated using toluene:diethyl ether:ethanol:acetic acid (50:40:2:0.2, v/v/v/v) as solvent. Fluorescent lipids on TLC plates were detected and quantified using a Storm 860 phosphoimager. The ratio of NBD-fatty acid/(NBD-FA + NBD-phospholipid) was used to calculate the amount of NBD-FA in each reaction. The apparent PLA2 activity was then calculated based on the amount of NBD-FA and total protein in each sample (nmol/[mg $\times$ hour]). Negative control: 50 μ l of boiled WT lysate (100 °C for 10 minutes) or lysis buffer only. Positive control: 1.0 unit of purified bovine PLA2 diluted in lysis buffer to a final volume of 50 μ l.

2.8 Statistical analysis

One-way ANOVA and pair-wise comparisons using the Holm-Sidak correction were used to compare PAF-AH activity levels.

3. Results

3.1 Identification and targeted deletion of a putative PLA2/PAF-AH in *L. major*

In addition to the previously described ISCL [10], *Leishmania* parasites possess other phospholipases. Mining the *L. major* genome revealed ortholog genes for two phospholipase C, one lysophospholipase, one PLA1, and one PLA2/PAF-AH (Table 1). As the focus of this study, *L. major* PLA2/PAF-AH (LmjF35.3020) is a 453-amino acid protein with 27–29% identity and 43–49% similarity to the cytoplasmic isoform 2 of mammalian PAF-AH (Fig. 1). Its N-terminus has a predicted signal peptide sequence followed by a transmembrane helix (the shaded portion in Fig. 1). Along with its syntenic orthologs in *T. brucei* and *T. cruzi*, *L. major* PLA2/PAF-AH possesses a GX SXG motif (underlined in Fig. 1) that is characteristic of serine-dependent neutral lipases [20] including the human intracellular PAF-AH2 (Fig. 1) [53].

To evaluate its role, we generated the PLA2/PAF-AH-knockout mutant in *L. major* (referred to as *pla2*[−]) through targeted gene deletion. Southern blot analysis confirmed the loss of *PLA2/PAF-AH* alleles in the homozygote mutants (Fig. 2A–B). Two independent knockout clones (*pla2*[−] #6-1 and *pla2*[−] #6-2, Fig. 2A) exhibited very similar phenotypes and the data for *pla2*[−] #6-1 and its complemented control (referred to as *pla2*[−]/+*PLA2*) are presented here. In culture, *pla2*[−] and *pla2*[−]/+*PLA2* parasites were fully viable and grew at a nearly identical rate as WT *L. major* (Fig. 2C). We also measured the differentiation of *pla2*[−] parasites into infective metacyclics during stationary phase using the peanut agglutinin method [54] and results showed no significant difference from WT promastigotes (data not shown). Therefore, PLA2/PAF-AH is not essential for the survival, replication, or differentiation of *L. major* in culture.

3.2 PLA2/PAF-AH is not required for the degradation of glycerophospholipids

Because *L. major* PLA2/PAF-AH is annotated as a putative phospholipase, we examined whether it was involved in the degradation of glycerophospholipids such as PE, PC, PG and PS. To validate our PLA2 assay, a purified bovine PLA2 was incubated with a fluorescent PC (the fatty acyl chain at the *sn*-2 position was conjugated to NBD) to generate an unlabeled lyso-PC and a NBD-labeled fatty acid. Thin layer chromatography (TLC) was then used to separate the NBD-labeled PC (substrate) from the NBD-labeled fatty acid (product) because PC migrated much slower than fatty acid (the right 2 lanes in Fig. S1). Clearly, the purified bovine PLA2 caused the release of fatty acid from the *sn*-2 position of PC whereas reaction buffer alone did not (the left 2 lanes in Fig. S1), indicating that this assay could detect PLA2 activity.

Next, whole cell lysates from WT, *pla2*[−] and *pla2*[−]/+*PLA2* promastigotes were assessed for PLA2 activity against NBD-labeled PE, PC, PS, or PG under the same condition as described for the purified PLA2. As shown in Fig. 3A–D, *L. major* parasites possessed a detectable level of PLA2 activity against PE, PC, PS, and PG. As expected, negative controls using lysis buffer only in Fig. 3A–C) or boiled WT lysate (in Fig. 3D) failed to show any PLA2 activity. The apparent PLA2 activity in *Leishmania* cell lysates against various phospholipid substrates was calculated based on the amount of fatty acid produced and the amount of protein in each reaction (Fig. 3E). Importantly, no significant difference in PLA2 activity was found among WT, *pla2*[−] and *pla2*[−]/+*PLA2* parasites (Fig. 3E), suggesting that *L. major* PLA2/PAF-AH is not involved in the degradation of regular glycerophospholipids. Therefore, other enzyme(s) must be responsible for these activities. Meanwhile, parasite lysates exhibited a higher level of PLA2 activity towards PS and PG than PE and PC (Fig. 3E). It is not clear whether this is due to the substrate specificity or expression level of specific leishmanial lipases.

3.3 PLA2/PAF-AH is responsible for the PAF-AH activity in *L. major*

Because *L. major* PLA2/PAF-AH is similar to the PAF-AH2 in mammals (Fig. 1), we examined whether it can hydrolyze PAF, a special type of phospholipid (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) synthesized by immune cells to activate inflammation and platelet aggregation. To measure PAF-AH activity, *Leishmania* whole cell lysates were incubated with radiolabeled PAF (the acetyl-group at the *sn*-2 position was labeled with [³H]). After extraction, the amount of radioactive acetate in the aqueous phase was determined in each sample. As summarized in Fig. 4 and Table S1, WT and *pla2*⁻/*PLA2* parasites exhibited detectable levels of PAF-AH activity, which was absent in *pla2*⁻ or boiled WT cells. These results suggest that PLA2/PAF-AH is responsible for the degradation of PAF in *L. major*.

3.4 PLA2/PAF-AH is important for *L. major* infection in BALB/c mice

The lack of pleiotropic defects from *pla2*⁻ promastigotes allowed us to examine whether PLA2/PAF-AH was required for parasite proliferation and pathogenesis in the mammalian host. To do so, metacyclic parasites of WT, *pla2*⁻, or *pla2*⁻/*PLA2* were inoculated into the footpads of BALB/c mice, which are susceptible to *L. major* infection. As shown in Fig. 5A, mice infected by WT parasites developed lesions that grew rapidly in size. In comparison, mice infected by *pla2*⁻ parasites showed a significant delay in lesion development (Fig. 5A). With restored PLA2/PAF-AH expression, *pla2*⁻/*PLA2* parasites exhibited much improved virulence than *pla2*⁻ (Fig. 5A). We also examined the number of parasites in the footpads of mice infected by WT, *pla2*⁻, or *pla2*⁻/*PLA2*. As illustrated in Fig. 5B, lesion sizes showed a positive correlation with parasite numbers in infected footpads. Together, these data indicate that PLA2/PAF-AH is important for *L. major* to efficiently establish infection and proliferate in mice.

3.5 PLA2/PAF-AH is not required for the synthesis and targeting of GPI-anchored molecules

In *Leishmania* promastigotes, the dominant surface virulence factor is a heterogeneous glycoconjugate called lipophosphoglycan or LPG [55, 56]. Because LPG has a 1-alkyl-2-lyso-phosphatidylinositol anchor which could be generated from the activity of a PLA2, we tested whether the cellular abundance of LPG was altered in *pla2*⁻ or *pla2*⁻/*PLA2* parasites by western blot using a LPG-specific monoclonal antibody [48]. As illustrated in Fig. S2 (results from two experiments were shown), deletion of *PLA2/PAF-AH* had no obvious effect on the cellular level of LPG or GP63, a GPI-anchored metalloprotease with 1-alkyl-2-acyl phosphatidylinositol anchor [57, 58]. We also examined the localization of LPG and GP63 by immunofluorescence microscopy. In WT promastigotes, LPG was predominantly found on the plasma membrane while GP63 was detected both on the plasma membrane and at intracellular locations which probably represent the Golgi (Fig. S3A–B). Very similar results were observed in *pla2*⁻ or *pla2*⁻/*PLA2* parasites (Fig. S3A–B). Therefore, PLA2/PAF-AH is probably not required for the synthesis or targeting of GPI-anchored virulence factors.

3.6 Localization of PLA2/PAF-AH in promastigotes and amastigotes

To determine the localization of PLA2/PAF-AH, we introduced a PLA2-GFP chimera into *pla2*⁻ mutants. Western blot (using an anti-GFP antibody) revealed a ~69 KDa protein in the whole cell lysate but not the culture supernatant of *pla2*⁻/*PLA2-GFP* promastigotes (Fig. S4), suggesting that PLA2-GFP is not secreted. As shown in Fig. 4 and Table S1, *pla2*⁻/*PLA2-GFP* parasites had a near WT-level of PAF-AH activity, indicating that the PLA2-GFP fusion protein was functional. In promastigotes, we observed an uneven, intracellular distribution of PLA2-GFP extending from the peri-nuclear region (Fig. 6A–C). Co-staining

with an anti-*T. brucei* BIP antibody (an endoplasmic reticulum or ER marker) [47] revealed significant overlap (Fig. 6D–E), suggesting that PLA2-GFP is primarily located at the ER. We also labeled promastigotes with Mitotracker Red 580 but did not detect any significant overlap between PLA2-GFP and mitochondrion (Fig. S5).

Finally, we examined the localization of PLA2/PAF-AH in the mammalian stage of *L. major* (Fig. 6F–J). Bone marrow-derived MΦs (from BALB/c mice) were infected with *pla2*⁻/*+PLA2-GFP* parasites for 48 hours. The distribution of PLA2-GFP in intracellular amastigotes was then determined by immunofluorescence microscopy using an anti-GFP antibody. As illustrated in Fig. 6H–J, the pattern of anti-GFP staining was quite similar to what was observed in *pla2*⁻/*+PLA2-GFP* promastigotes (Fig. 6A–C). Together, our results suggest that *L. major* PLA2/PAF-AH is an intracellular enzyme that was mainly targeted to the ER.

4. Discussion

Recent studies have highlighted the essentiality of ISCL, a developmentally regulated neutral sphingomyelinase, in *L. major* virulence [10, 12]. Based on these reports, it may be of interest to characterize other enzymes involved in the degradation of phospholipids synthesized by the host or *Leishmania*. In light of this, a group of putative phospholipases were identified from the *L. major* genome and among them is a PLA2/PAF-AH. Despite the lack of a canonical ER retention sequence, PLA2/PAF-AH is primarily found in the ER of promastigotes and amastigotes (Figs. 1 and 6), suggesting that this enzyme may utilize a novel signal. To characterize PLA2/PAF-AH, we first examined the PLA2 activity in *L. major* cell lysates on several glycerophospholipids using an assay previously developed for the intracellular PLA2 in zebra fish [52]. Results clearly showed that *L. major* parasites can degrade PC, PE, PS and PG by releasing fatty acids from the *sn*-2 position, indicating that they possess at least one functional PLA2 (Fig. 3). Since our assay was performed using whole cell lysate and diluted phospholipid substrates in an aqueous environment which might limit the performance of certain phospholipases, the actual PLA2 activity in *L. major* may be significantly higher than the observed value. Nonetheless, deletion of PLA2/PAF-AH has little impact on this activity (Fig. 3), suggesting that other lipases are responsible for the degradation and remodeling of glycerophospholipids. In addition, *pla2*⁻ promastigotes exhibit no apparent defects in the synthesis or targeting of GPI-anchored virulence factors such as LPG (Figs. S2–3). One possibility is that PLA2/PAF-AH is not required for the generation of the 1-alkyl-2-lyso phosphatidylinositol anchor of LPG, i.e. phosphatidylinositol is not a substrate of PLA2/PAF-AH. Alternatively, the LPG anchor in *pla2*⁻ parasites may be somewhat altered which may be determined by mass spectrometry-based analysis.

Besides glycerophospholipids, *Leishmania* parasites can also hydrolyze PAF and this activity is clearly dependent on PLA2/PAF-AH (Fig. 4). In culture, *pla2*⁻ mutants grow and differentiate normally (Fig. 2C) so PAF degradation is not essential for the promastigote stage of *L. major*. Effects of PAF on *Leishmania* infection of MΦs (their definitive host cells) have been investigated by several groups. When MΦs were treated with PAF, *L. amazonensis* infection was inhibited (by ~3 folds) [37, 38]. Mechanism of PAF's action is not well understood. It may induce NO production from MΦs and therefore inhibit *Leishmania* infection and intracellular multiplication [37, 38]. The reduced virulence of *pla2*⁻ mutants in BALB/c mice (Fig. 5) is consistent with previous reports on the anti-*Leishmania* effect of PAF [37, 38]. It is possible that PLA2/PAF-AH can regulate the level of PAF and/or lyso-PC during *Leishmania* infection to facilitate parasite survival by reducing inflammatory responses.

In addition to its effect on PAF, PLA2/PAF-AH may possess other unidentified functions that contribute to *Leishmania* infection. It is known that mammalian PAF-AHs (including the cytoplasmic isoform II that shares significant homology to PLA2/PAF-AH; Fig. 1) can hydrolyze substrates with longer chains (>2) at the *sn*-2 position including some oxidized phospholipids [53, 59, 60]. Some of these PAF-like lipids can induce inflammation and may act via the PAF receptor [61, 62]. In this study, the release of PLA2-GFP by intact promastigotes is undetectable (Fig. S4), raising the question of how an intracellular enzyme may gain access to host PAF or PAF-like lipids. One possibility is that endocytosed host lipids may interact with PLA2/PAF-AH via the endosome-ER network [63]. Alternatively, PLA2/PAF-AH released from lysed parasites may facilitate the survival of viable parasites in mammals. Another possibility is that this enzyme has endogenous targets. It is not known whether *Leishmania* parasites can synthesize PAF or PAF-like lipids, although it has been reported that promastigotes pre-treated with PAF infect macrophages more efficiently than untreated promastigotes [64, 65]. The mechanism of such an infection-enhancing effect (which is contrary to the effect of PAF treatment on macrophages) is not clear, although PAF could stimulate the activity of protein kinases secreted by *Leishmania* [64].

In summary, we have shown that *L. major* possesses a functional PAF-AH which is important for virulence in mammals. Future study may include the characterization of active, recombinant enzyme and identification of its endogenous target. Since homologues of PLA2/PAF-AH are present in other trypanosomatids, a better understanding of its function will likely shed new light into the role of lipid metabolism in this group of medically important pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PLA2	phospholipase A2
PAF-AH	platelet-activating factor acetylhydrolase
PAF	platelet-activating factor
ISCL	inositol phosphosphingolipid phospholipase C-like protein
NBD	7-nitro-2-1,3-benzoxadiazol-4-yl
PAC	puromycin resistance gene
BSD	blastocidin resistance gene
TLC	thin layer chromatography
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PS	phosphatidylserine

PG	phosphatidylglycerol
LPG	lipophosphoglycan

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Research highlights

- *L. major* parasites possess a functional PAF-AH.
- PAF-AH is not required for the degradation of common glycerophospholipids.
- PAF-AH is not required for parasite growth or differentiation in culture.
- Deletion of PAF-AH leads to attenuated virulence in mice.

Tbg972.9.7760	MVTWALKYFVRVVRWSTEAFLIWPTPLFDYATSLHCVPISGTFITSVLL	50
Tc00.1047053510743.60	-----MSATAGFFAGAPNSFWDYVISPHLILLFAFFPLSMIL	37
LmjF.35.3020	-----MHPIDFYILSIHYLPAGASVLLSCLA	26
Human PAF-AH2	-----	
Tbg972.9.7760	CFYGFPLLWSTAMVAVGFIISGVLFVSPPLPKPIGGRYSVGLVHMNGC	100
Tc00.1047053510743.60	RIFGLGAVFFLPPIIPGLIIGAAMFILLVPVPIEPVGGHYHVGVHVS	87
LmjF.35.3020	AAWGV ^u DWKVCIMMTCVGS ^u LVTCAVFYVQPLQCFSP ^u LGGSFNVGTREVCGE	76
Human PAF-AH2	-----MGVNQSVGFPPVTGPHLVGCGDVMG	26
	: : : * : * : *	
Tbg972.9.7760	RSQSIPPVAVFYPTNMVPEKKGLPYVPFGDDRLRGVAA ^u YANVPFFIIRD	150
Tc00.1047053510743.60	HSQTMPLAVYYPTNETPRRKGIIQYIPFNDVRFMSGLSSRAGVPLYLMRD	137
LmjF.35.3020	RGAMKPPVTIVYPTASGTPRSGIQYIPFGERSYLVGIASYSKVPYALVKD	126
Human PAF-AH2	QNLQGSFRLFYPCQKAETMEQP-LWIPRYEYCTGLAEYLQFNKRCGGL	75
	: . . . : ** : : : * : :	
Tbg972.9.7760	FSFVRISASRNAVPAA-LLNQYERVPPIVVF ^u SHGLAGYHLYSCFALDLA	199
Tc00.1047053510743.60	FLFVRLRATEGAKPIP-LINSSGIPLPVII ^u FSHGLYGYHRLYSALQADLA	186
LmjF.35.3020	LWLLRKKMRPNAEPAP-LFQRDGM ^u PRMIVFSHGLGGFPHLYSTLLMDLA	175
Human PAF-AH2	LFNLAVGSCRLPVSWNGPFKTKDSGYPLII ^u FSHGLGAFRTLYSAFCMELA	125
	: : . . : : * : : * : : * : : *	
Tbg972.9.7760	ARGAIVICLGHCDNSAS---FMRDSSGKES-----EVPLKDYG	234
Tc00.1047053510743.60	SRGAVVSVGHCDGSAS---FMRDSE ^u RGK-----EFFLRQVD	221
LmjF.35.3020	VRGAVVFALSHMDGSAA---FCRDAEREIR-----IPLNTQVG	210
Human PAF-AH2	SRGFVVAVPEHRDRSAATTYFCQQAPEENQPTNESLQEEWIPFRRVEEGE	175
	** : * * * * : * : :	
Tbg972.9.7760	WEVPAREAQVAQRVSEVRGTLQRLTEK----DFWTTTLGYSNSDIDKFLS	279
Tc00.1047053510743.60	WDSPVCEEALAQRMETRRTLKRLSEK----EFWKELGFADLDAEQYLR	266
LmjF.35.3020	WTS ^u EDRAPQLEVRIRETLNTIKRIRSG----ELLALGYDKETVDKYIE	255
Human PAF-AH2	KEFHVRNPQVHQRVSECLRVLKILQEV ^u TAGQTVFNILPGLDLMTLK ^u GNI	225
	: * : * : : . : : * : :	
Tbg972.9.7760	KPLQVHLAGHSFGGATVLA ^u AAL ^u ENQNP--VKGVS-VKSVYTFDPWMLPI	326
Tc00.1047053510743.60	QSPRVHLSGHSFGGATALVAA ^u MQEEQES--KPNENPVQSVIVFDPWHKPL	314
LmjF.35.3020	KEPRIHLLGHSFGGATCLAAALADTQAASERGGVSSIASTVVYDPWMIPL	305
Human PAF-AH2	DMSRVAVMGHSFGGATAILALAKETQFR-----CAVALDAWMFPL	265
	. : : : * : : * : * : *	
Tbg972.9.7760	QNEHFCNPLSDGRK---SYTVPTVTVHSDDWVKDSSEWEFFKRMKALVL	372
Tc00.1047053510743.60	QNELFLKPIEEGRN---RYTPTLMVHSDAWVQDRSIWNFFQRIITLLL	360
LmjF.35.3020	QKTMFYDKLTDRKQP--VHFTPTLQIFSEEWVRSKEQHAF ^u FEVKAIVD	353
Human PAF-AH2	ERDFYPKARGPVFFINTEKFQTMESVNL ^u MKKICAQHEQSRITIVLGSVHR	315
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Tbg972.9.7760	EQS-----AYASLNEVEKQALFGIVVTKNTNHL ^u SLVDVSVLSPVM	412
Tc00.1047053510743.60	SQP-----SISSLSEREKRMRFVTEKTGSTNHYSVSDVVLSPVI	400
LmjF.35.3020	AQPR ^u TTEESALVSAVD ^u AKLKVMKTSWYTIKDYRGTHLCTDVSLSF ^u PVL	403
Human PAF-AH2	SQT-----DFAFVTGNLIGKFFSTETRGSLDPYEGQEV ^u MVR--AM	353
	* . : : . : * : :	
Tbg972.9.7760	HGNIWATVSPRVQIMEWCNALLRFAKQNT ^u EV ^u STC-----	447
Tc00.1047053510743.60	HGCKNSILSPRVQIMEWSNTCLRFI ^u KEHAMYTSRTSAR-----	438
LmjF.35.3020	YRAAYMTASPRGCIVAF ^u AAETMR ^u FI ^u EVSGPLPLDTKLLNDSALAAALRG	453
Human PAF-AH2	LAFLQKHLDLKEDYNQWNNLIEGIGPSLTPGAPHHLSSL-----	392
	. : : : . : .	

Figure 1. Sequence alignment of PLA2/PAF-AH

Predicted amino acid sequences of putative PLA2/PAF-AH from *T. brucei gambiense* (Tbg972.9.7760), *T. cruzi* (Tc00.1047053510743.60), *L. major* (LmjF.35.3020), and human PAF-AH2 (NP_000428.2) were aligned using the Clustal Omega (1.0.3) algorithm. Shade area in LmjF.35.3020 represents predicted signal peptide and transmembrane helix. The lipase consensus motif (GX^uSXG) is underlined. Asterisks (*): fully conserved residues; colons (:): highly similar residues; periods (.): weakly similar residues.

Figure 2. *L. major* *pla2*⁻ mutants grow normally as promastigotes in culture

(A–B) Southern blot showing the replacement of *PLA2/PAF-AH* alleles with marker genes (*PAC/BSD*) in *pla2*⁻. Genomic DNAs from WT, *PLA2*^{+/-} (heterologous parasites in which one of the two *PLA2/PAF-AH* alleles has been deleted), and *pla2*⁻ promastigotes were digested with *Bgl*II and *Hind*III and resolved on an agarose gel. Following transfer to a PVDF membrane, a radiolabeled DNA probe was used to detect the *PLA2/PAF-AH* allele (~3.3 Kb) and the *PAC/BSD* replacement alleles (10.5–10.7 Kb). (A) Result from autoradiography. The asterisk represents a non-specific band recognized by the probe. For *pla2*⁻, *BSD* and *PAC* replacements appear as one band due to the similarity in size. (B)

Schematic representation of the Southern blot. (C) Promastigotes were inoculated in M199 medium and culture density was determined daily using a hemacytometer. Error bars represent standard deviations from 2 experiments.

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Figure 3. *L. major* parasites possess PLA2 activity against glycerophospholipids

Whole cell lysates of WT, *pla2*⁻, and *pla2*⁻/+PLA2 parasites were incubated with NBD-PE (A), NBD-PC (B), NBD-PS (C), or NBD-PG (D). Lipid products were separated from the substrates by TLC under the condition described in Materials and Methods (2.7). In A–D, four replicates are shown for each *Leishmania* sample. Negative controls: buffer only (A–C) or boiled WT lysate (D). Positive control: 1.0 unit of bovine PLA2 (B). Arrows indicate the direction of migration on TLC. FA: fatty acid. O: origin. F: solvent front. (E) Apparent PLA2 activity was quantified based on the amount of NBD-labeled fatty acid produced from

each reaction and the amount of protein in each sample. Error bars represent standard deviations from 2 experiments with 4 samples each time.

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Figure 4. PLA2/PAF-AH is responsible for the PAF-AH activity in *L. major*

Promastigote lysates were incubated with radiolabeled PAF and the release of acetate was measured as described in Materials and Methods (2.7). Results were calculated after subtracting the value from boiled WT lysate (raw data were summarized in Table S1). Error bars represent standard deviations from 4 repeats. ***: $p < 0.001$ (compared to *pla2*⁻).

Figure 5. *Pla2*⁻ parasites exhibit attenuated virulence

BALB/c mice were infected with purified metacyclic parasites (10^5 /mouse) in the left footpads. (A) Lesion sizes were measured weekly to monitor the progression of pathology. (B) Limiting dilution assay was conducted at the indicated times to determine parasite numbers in infected footpads (lesion sizes are indicated). Error bars represent standard deviations.

Figure 6. Localization of PLA2/PAF-AH in *L. major*

(A–E) Log phase promastigotes of *pla2*^{-/+}*PLA2-GFP* were subjected to immunofluorescence microscopy as described in Materials and Methods (2.4). (A) DIC image. (B) DNA staining with Hoechst 33342. (C) PLA2-GFP epifluorescence. (D) Immuno-staining with anti-BIP antibody to label ER. (E) Merge of C and D. (F–J) BM-MΦs were infected with *pla2*^{-/+}*PLA2-GFP* parasites for 48 hours and subjected to immunofluorescence microscopy as described in Materials and Methods (2.4). (F) DIC image of an infected MΦ. (G) DNA staining with Hoescht 33342. (H) Immuno-staining

with rabbit anti- GFP serum followed by goat anti-rabbit IgG conjugated with Texas Red. **(I)** Merge of **F**, **G**, and **H**. **(J)** Merged and magnified image of the boxed areas in **G** and **H**.

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Table 1

Predicted phospholipase genes in *L. major*

Annotation	Trityp DB ID	References
PLA1, putative	LmjF.31.2460	[34]; [25]; [26]
PLA2/PAF-AH	LmjF.35.3020	This study
Phospholipase C (PLC)-like protein	LmjF.30.2950	None
Phosphatidylinositol-specific PLC-like protein	LmjF.35.0040	None
Phosphoinositide-specific PLC, putative	LmjF.22.1680	None
Lysophospholipase, putative	LmjF.24.1840	None

Syntenic orthologues for these putative phospholipase genes are present in *L. braziliensis*, *L. infantum*, *T. brucei*, and *T. cruzi*.