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Identification, characterization, and expression of a unique secretory lipase from the human pathogen *Leishmania donovani*

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Abstract

Lipases have been implicated to be of importance in the life cycle development, virulence, and transmission of a variety of parasitic organisms. Potential functions include the acquisition of host

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resources for energy metabolism and as simple building blocks for the synthesis of complex parasite lipids important for membrane remodeling and structural purposes. Using a molecular approach, we identified and characterized the structure of an *LdLip3*-lipase gene from the primitive trypanosomatid pathogen of humans, *Leishmania donovani*. The *LdLip3* encodes a ~ 33 kDa protein, with a well-conserved sub-strate-binding and catalytic domains characteristic of members of the serine lipase-protein family. Further, we showed that *LdLip3* mRNA is constitutively expressed by both the insect vector (i.e., promastigote) and mammalian (i.e., amastigote) life cycle developmental forms of this protozoan parasite. Moreover, a homologous episomal expression system was used to express an HA epitope-tagged *LdLip3* chimeric construct (*LdLip3::HA*) in these parasites. Expression of the *LdLip3* chimera was verified in these transfectants by Western blots and indirect immunofluorescence analyses. Results of coupled immuno-affinity purification and enzyme activity experiments demonstrated that the *LdLip3::HA* chimeric protein was secreted/released by transfected *L. donovani* parasites and that it possessed functional lipase enzyme activity. Taken together these observations suggest that this novel secretory lipase might play essential role(s) in the survival, growth, and development of this important group of human pathogens.

Keywords

Leishmania; Human parasite; Gene structure; Trypanosomatid; Kinetoplastid protozoan; Lipase

Introduction

Leishmania spp. are a group of trypanosomatid protozoan pathogens which are transmitted to their mammalian hosts via the bite of infected sand fly vectors. In humans, these parasites cause a broad spectrum of diseases ranging from mild cutaneous ulcerations to severe and typically fatal visceral disease [1]. All *Leishmania* have a digenetic life cycle that includes two major parasite developmental stages: (1) extracellular flagellated promastigote forms that reside and multiply within the alimentary tract of their sand fly vector and (2) obligate intracellular nonflagellated amastigote forms which reside and multiply within the phagolysosomal system of infected mammalian macrophages [2]. It is important to point out that *Leishmania* are typically opportunistic facultative lipid scavengers. As obligate parasites they must salvage these macromolecules from their hosts for their own physiologic needs. In that regard, for example, during their life cycle these organisms undergo changes in physiology and architectural membrane remodeling/restructuring [3–5]. In addition, previous studies have shown that amastigotes have elevated fatty acid metabolism compared to promastigotes [6–12]. While precedence exists for such biological activities (i.e., beta oxidation and membrane remodeling) to date, no reports exist concerning the involvement of parasite derived lipases in these processes. Moreover, despite its apparent relevance, little direct evidence exists concerning the role(s) of such a lipase in the developmental biology of *Leishmania* parasites. Further, parasite proteins, in particular secretory enzymes which mediate the survival, growth and development of *Leishmania* generally remain to be elucidated. To that end, in the current report, we identified a secretory lipase enzymatic activity and used a molecular approach to characterize the gene that encodes a unique

secretory lipase. Further, we characterized its structure, expression, and localization in both developmental forms of this primitive human pathogen.

Experimental procedures

Reagents

All chemicals used, unless specified, were of analytical grade and purchased from Sigma-Aldrich Chemical Co. Enzymes were obtained from Roche Molecular Biochemicals; DNA molecular mass standards were from Invitrogen, Inc. and protein molecular mass standards were purchased from Amersham Biosciences.

Parasites and culture conditions

A cloned line of *L. donovani* (WHO designation: MHOM/SD/62/1S/1S-CL2D) [13, 14] was used in all experiments. Promastigotes were routinely grown as previously described [15]. Axenic amastigotes were routinely maintained as previously described [16].

Parasite cultures used for isolation of nucleic acids and proteins were harvested at ~ mid-log phase ($\sim 2 \times 10^7$ cells/ml) by centrifugation at $2,100 \times g$ for 15 min at 4°C [14]. The resulting cell pellets were washed twice in ice-cold PBS by centrifugation as above and finally resuspended in the appropriate buffers.

To assess whether *L. donovani* parasites released/secreted any measurable lipolytic activity during their growth in vitro, promastigotes were grown to $\sim 2 \times 10^7$ cells/ml as above in serum-free chemically defined medium according to Joshi [15]. Cells grown under these conditions were harvested by centrifugation and their cell-free culture supernatants were processed as previously described [14]. Aliquots of such culture supernatants were used for both enzyme assays and immunoprecipitation experiments.

Enzyme assays

Cell-free culture supernatants from wild-type *L. donovani* promastigotes were analyzed for their lipase activity. For these assays, the fluorogenic substrates 4-methylumbelliferyl-stearate, -butyrate, or -acetate were used to measure lipase activities with McIlvain buffer [17] (pH 6.0) at 37 and 42°C. The optimal pH for such assays was determined experimentally to be pH 6.0 in preliminary studies. The amount of the fluorescent 4-methylumbelliferone (4MU) product generated from these substrates was determined using 4MU standards. Fluorescence was measured with a Molecular Devices Fluorometer (SPECTRA Max Model M2) using a 360-nm excitation filter and a 450-nm emission filter. In these experiments, fresh parasite culture medium was used as a background control. Values obtained from such controls were subtracted from those generated with various test samples. For assays, a 100 μ l reaction volume containing 5 μ l sample or control media, 150 μ M substrate and McIlvain buffer, pH 6.0 were incubated in a 96-well microtiter plate for 30 min at the appropriate temperature. Reactions were terminated by the addition of stop buffer (1 M NaOH, 1 M glycine). The results of these enzyme assays are expressed as pmoles of 4MU product generated per min/mg of parasite culture supernatant protein. All samples

were assayed in quadruplicate, and these assays were repeated on multiple independently generated samples.

Nomenclature

The designations used in this report for genes, proteins, and plasmids follow the nomenclature for *Trypanosoma* and *Leishmania* as outlined by Clayton et al. [18].

Oligonucleotide primers, PCR, and probe preparation

Oligonucleotide primers were designed to amplify the *L. donovani* homolog of a putative serine lipase ORF (LmjF31.0830) identified in the *Leishmania major* (Freidlin) genome database, GeneDB [19]. This primer pair (pp1) consisting of (PCR-Fwd: 5'-ATGTTGCCCTCATCTTG CAGC and PCR-Rev: 5'-TTACAGGTACAGCATGGCG TC) were synthesized using an ExpediteTM nucleic acid synthesis system (PE Applied Biosystems) and used in PCR amplifications with *L. donovani* genomic (g) DNA as a template. It was possible to use gDNA as template since trypanosomatid protozoans generally do not possess introns within the coding region of their open reading frames (ORF) [20]. After an initial "hot start" at 94°C for 2 min, the conditions used for amplification were: 94°C for 15 s, 55°C for 30 s, 72°C for 30 s (35 cycles), and a final step at: 72°C for 5 min. The resulting 927-bp amplified product was cloned into the *pCR[®] 2.1-TOPO* vector (Invitrogen), and the resulting plasmid (*Ldon*-PCR927) was subjected to nucleotide sequencing. Analyses of the sequence data obtained from the *Ldon*-PCR927 clone showed that it had high sequence identity with the *L. major* ORF (LmjF31.0830) [19]. Based on this observation, the *L. donovani* ORF was designated as "*LdLip3*" to indicate its homology with this annotated putative serine lipase. Subsequently this cloned PCR fragment was labeled with digoxigenin-dUTP using the PCR Dig Labeling Kit according to manufacturer's instructions (Roche). The resulting digoxigenin-labeled probe (*Ldon*-DIG927) was used to screen an *L. donovani* cosmid library.

Cosmid library screening

A cosmid library of *L. donovani* gDNA (kindly provided by Dr. B. Ullman, Oregon Health Sciences University) was screened by hybridization using the *Ldon*-DIG927 probe under high stringency hybridization and washing conditions (0.1× SSC; 0.1% SDS at 65°C). Among the several positive clones identified from such screening one was chosen, *LdCos1*, for further analyses. DNA isolated from this cosmid clone was subjected to nt sequencing.

Nucleotide sequencing and analyses

DNA was sequenced using the fluorescent di-deoxy chain terminator cycle sequencing method [21] at the Johns Hopkins University DNA Analysis Facility (Baltimore, MD) or at the Genomics Sequencing Center at the University of Rhode Island (Kingston, RI). Sequence data obtained from both strands were analyzed using the Genetic Computer Group (GCG) software package [22] running on an N.I.H. Unix System and Sequencer 4.9 software (Gene Codes Corp.). Further, such sequences were also subjected to BLAST-N and BLAST-P analyses using the NCBI BLAST-link (<http://www.ncbi.nlm.nih.gov/BLAST/>). Signal sequence and protease cleavage sites were predicted using the SignalP link available

on the World Wide ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) available at: <http://www.expasy.ch/tools>. Protein domain analysis was done using the Simple Modular Architecture Research Tools (<http://smart.embl-heidelberg.de>). Protein multiple sequence alignments were done using the ClustalW program [23] using a MacVector 7.0 software package (GCG).

Isolation of gDNA and southern blot analysis

L. donovani gDNA was subjected to Southern blot analyses as previously described [13]. Subsequently, blots were hybridized under high stringency using a digoxigenin-labeled *Ldon*-DIG927 which corresponded to the *LdLip3* ORF. After washing at high stringency ($0.1\times$ SSC, 0.1% SDS at 65°C), the hybridized fragments were visualized according to manufacturer's instructions (Roche) and images were captured using BIOMAX™-MR X-ray film (Kodak). DNA was isolated from the *LdonCos1* cosmid clone and similarly subjected to Southern analysis as above.

Isolation of RNA and RT-PCR

Total RNA was isolated from promastigotes and axenic amastigotes using TRIzol® (Invitrogen) and subsequently treated with DNase I (Stratagene). Reverse transcription was carried out on such RNA using Superscript II (Invitrogen) and oligo dT to generate cDNA. PCR amplification reactions contained the oligo primers described above (PCR-Fwd and PCR-Rev) to amplify the 927 bp *L. donovani* ORF from 2 µl of the cDNA. The conditions for amplification were 95 °C for 1 min, 42°C for 1 min, 72°C for 1 min (40 cycles), and 72°C for 6 min. PCR products were analyzed by 1% agarose gel electrophoresis, and purified PCR products (Sephaglass™ Bandprep kit, Amersham Biosciences) were subjected to nucleotide sequence analysis.

Mapping of the 5' spliced-leader acceptor site

As indicated above, trypanosomatids generally do not possess introns within their ORFs. Interestingly, the pre-mRNAs in these organisms are joined to a 39 nt conserved spliced-leader at their 5'-end by trans-splicing to generate mature mRNAs capable of translation [24, 25]. To identify the 5'-splice acceptor site in the *LdLip3* ORF, RT-PCR analysis was performed as described previously [26]. For these reactions, cDNA was generated from total RNA isolated from both *L. donovani* promastigotes and axenic amastigotes. Such cDNAs were used as template in PCR with primer pair 2 (pp2) consisting of a forward primer (SpliceFwd) based on the *L. donovani* SL sequence [i.e., nt 5–31 of the 39 nt SL] [27]: 5'-AACGCTATATAAGT ATCAGTTTCTGTA and a reverse primer (*LdLip3*-RT/Rev) based on a portion of the 5'-end (i.e., nt 138–157) of the *LdLip3* ORF: 5'-ATGGCATCGATGTCAGGTAC. The resulting PCR-amplified products were cloned into the *pCR*® 2.1-*TOPO* plasmid vector (Invitrogen), sequenced, and analyzed. Sequence data obtained from these two parasite developmental forms were compared.

Generation of an epitope-tagged expression construct

The *pKSNEO* leishmanial vector [28] was used previously to express a variety of genes in several different species of *Leishmania* parasites [29–33]. In this report, this vector was used

to express a construct encoding an LdLip3-hemagglutinin-tagged (HA) chimeric protein in *L. donovani* parasites. This construct was generated by PCR using the *LdCos1* cosmid DNA as template with the following primers: (SpeFwd) 5' -

GGACTAGTATGTTGCCCTCATCTTGCA GC (*SpeI* restriction endonuclease site shown in bold); and (SpeHARev) 5' - **GGACTAGTTTAAGCGTAATCTGGAA**
CATCGTATGGGTACAGGTACAGCATGC (*SpeI* restriction endonuclease site in bold; stop codon in bold italics; and the -HA epitope tag is the underlined sequence). The conditions used for amplification were 94°C for 15 s, 55°C for 30 s, 72°C for 30 s (35 cycles), and a final step at 72°C for 5 min. The resulting amplified product was gel purified and cloned into the *pCR2.1-TOPO* vector (Invitrogen) to generate a *pCR2.1::LdLip3::HA* plasmid. The insert was excised from the latter plasmid using *SpeI* restriction endonuclease. Subsequently, the excised fragment was ligated into the *pKSNEO* (*SpeI*-linearized) plasmid to generate the *pKSNEO::LdLip3::HA* plasmid construct. The orientation of *LdLip3::HA* in *pKSNEO* was verified using restriction endonuclease analysis, and sequence of the *pKSNEO::LdLip3::HA* construct was verified by nt sequencing.

Transfection of plasmids into *Leishmania donovani* promastigotes

Log phase *L. donovani* promastigotes were transfected with either the *pKSNEO* control plasmid or the *pKSNEO::LdLip3::HA* construct by electroporation using previously described methods [31]. Subsequently transfectants were selected for in increasing concentrations (up to 200 µg/ml) of G418 over a period of several weeks. Promastigote cultures of such transfectants were routinely maintained and grown at 26°C in medium containing 50 µg/ml G418. For some experiments such transfected promastigotes were allowed to transform and grow under axenic amastigote growth conditions [16] in the presence of G418. Growth kinetics of both *pKSNEO* control and *pKSNEO::LdLip3::HA* transfected parasites were monitored at regular intervals during the course of their growth in vitro using methods previously described [31].

Western blot detection of the LdLip3::HA protein in transfected cells

Both *pKSNEO* control and *pKSNEO::LdLip3::HA* transfected *L. donovani* promastigotes and axenically grown amastigotes were prepared for SDS-PAGE [34] and Western blot analysis as previously described [31]. Such membranes were probed with an anti-HA monoclonal antibody (Covance Research Products) or an appropriately matched purified mouse IgG1, *k* control immunoglobulin (Sigma-Aldrich) followed by a goat anti-mouse horse radish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences). Immuno-detection was carried out using the ECL Western Blot Kit reagents (Amersham Biosciences), and images were captured from such blots using BIOMAX™-MR X-ray film (Kodak).

Immuno-fluorescence microscopy of transfected parasites

Mid-log phase cultures of both *pKSNEO* control and *pKSNEO::LdLip3::HA* transfected *L. donovani* promastigotes and axenically grown amastigotes were prepared for immuno-fluorescence microscopy as previously described [15]. Subsequently, cells were reacted with either an anti-HA monoclonal antibody (Covance Research Products), or an isotype-matched

control immunoglobulin (Sigma-Aldrich) followed by reaction with a fluorescein isothiocyanate (FITC)-conjugated, goat anti-mouse IgG secondary antibody (Sigma-Aldrich) and processed for microscopy [15]. Images were captured using a Zeiss Axioplan Microscope (Carl Zeiss, Inc.) equipped with epi-fluorescence, a cooled CCD camera (Photometrics), and appropriate FITC excitation/barrier filters. All captured images were processed using Adobe Photoshop 5.5 (Adobe Systems Inc.).

Western blot detection of secreted LdLip3::HA in culture supernatants of transfected parasites

Culture supernatant from both control *pKSNEO* and *pKSNEO::LdLip3::HA* transfectants were separated in SDS-PAGE (4–20% Tris–Glycine polyacrylamide gels, Invitrogen), trans-blotted onto PVDF membranes, and subjected to Western blot analysis as previously described [15]. Such membranes were probed with an anti-HA monoclonal antibody (Covance Research Products) or an appropriately matched purified mouse IgG1, *k* control immunoglobulin (Sigma-Aldrich) followed by a goat anti-mouse horse radish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences). Immuno-detection was carried out using the ECL Western Blot Kit reagents (Amersham Biosciences), and images were captured from such blots using BIOMAX™-MR X-ray film (Kodak).

Measurement of secreted/released LdLip3::HA lipase activity in parasite culture supernatants

Cell-free culture supernatants from both *pKSNEO* control and *pKSNEO::LdLip3::HA* transfected *L. donovani* promastigotes were analyzed for their lipase activity with 4MU-stearate as substrate in McIlvaine Buffer (pH 6.0) at 37 and 42°C as described above. All samples were assayed in quadruplicate and were repeated on multiple independently generated samples.

Immuno-affinity purification of secreted/released LdLip3::HA from parasite culture supernatants

To specifically determine whether the LdLip3::HA chimeric protein possessed lipase activity, cell-free culture supernatants from both *pKSNEO* control and *pKSNEO::LdLip3::HA* transfected promastigotes were subjected to purification with an anti-HA protein G-based affinity matrix (Roche) according to manufacturer's instructions. In initial experiments, we observed that the anti-HA antibody cross-reacted with a number of serum components present in the complete, FBS containing parasite culture medium. Therefore, to obviate any potential contamination issues, only promastigotes grown in serum-free chemically defined medium M199 were used in these studies. To that end, the affinity matrix columns were equilibrated with buffer (20 mM Tris pH 7.5, 0.1 M NaCl, 0.1 mM EDTA). Subsequently, culture supernatants from *pKSNEO* control and *pKSNEO::LdLip3::HA* transfected promastigotes were loaded onto the columns and the matrix washed 3 x with 20 bed volumes of wash buffer (20 mM Tris pH 7.5, 0.1 M NaCl, 0.1 mM EDTA, 0.05% Tween 20). Columns were eluted with 1 bed volume of elution buffer containing 1 mg/ml HA peptide in equilibration buffer. Eluted fractions were analyzed for protein concentration by Micro BCA (Pierce) and were subjected to Western

blot analysis with anti-HA antibody as described above. In parallel, eluted fractions were also analyzed for lipase activity using 4MU-stearate as outlined above. All samples were assayed in quadruplicate, and such assays were repeated using at least two independently generated samples. Results of these enzyme assays are expressed as specific activity in pmoles of 4MU-product generated per min/mg of protein. Fresh, unused culture media and buffers were used as controls in these assays.

Results

Measurement of lipase activity secreted/released by *L. donovani* promastigotes

To determine whether wild-type *L. donovani* promastigotes secreted/released any measurable lipase activity during their growth in vitro, aliquots of cell-free culture media supernatants from promastigotes were assayed for lipase activity using three different substrates, 4MU-stearate (C-18), 4MU-butyrate (C-4) and 4MU-acetate (C-2) to assay a broad range of lipase activities. Promastigotes cultures grown in chemically defined media were used in this study making it possible to identify specific secretory proteins produced by these organisms. Amastigotes can only be grown in complex serum containing media which complicates or renders impossible the identification and characterization of secretory enzymes in this developmental form. Fresh culture media were used as controls in these assays. Results of these enzyme assays showed that wild-type *L. donovani* promastigotes secreted/released lipase activity using 4MU-stearate as substrate (10.0 and 7.4 pmol/min/ml at 42 and 37°C, respectively). A putative nonspecific esterase activity was detected in parasite culture supernatants with the 4MU-acetate (135 pmol/min/ml at 42°C). Similarly using 4MU-butyrate as substrate, 60 pmol/min/ml of activity at 42°C were detected. Interestingly no activity was detected in parasite culture supernatants assayed at 37°C with 4MU-acetate and 4MU-butyrate substrates. These data indicated that these parasites produced and released/secreted both lipase and esterase activities during their growth in vitro. It is important to note that while one could detect protein present in culture supernatant samples by micro BCA, no one specific protein was present in quantities sufficient enough for isolation or purification. For example, in preliminary experiments we used a variety of different affinity-based bead matrices (e.g., binding to concanavalin A) in attempts to isolate sufficient quantities of this lipase for functional characterization including direct amino acid sequencing (data not shown). In that regard, despite repeated attempts, we were unable to obtain sufficient quantities of this protein for such purposes. Therefore, we adopted a molecular approach described below to identify the gene encoding this released/secreted lipase activity and to express it using a leishmanial expression vector (*pKSNEO*) to examine some of the biochemical/functional properties of this parasite enzyme.

Analysis of the *L. donovani* lipase gene

Genes encoding lipase enzymes have been reported from a wide variety of plant, animal and microbial sources as well as in the *L. major* (Freidlin) genome [19]. Despite their diverse origins, many lipases possess regions of amino acid sequence homology within their functional domains. Based on these observations, we used a PCR-based approach to identify a secretory lipase gene from the human pathogen *Leishmania donovani*. To that end, an annotated genome database of a closely related leishmanial species (*Leishmania major*

[Freidlin] GeneDB database) was searched for potential secretory lipase genes. Our search criteria included deduced proteins with a conserved lipase enzyme active site, a putative signal peptide and no apparent membrane anchor motifs or organelle targeting signals (e.g., KDEL). Among the 11 putative lipases in the *L. major* (Freidlin) database, only LmjF31.0830, annotated as a putative triacylglycerol lipase (i.e., no demonstrated experimental functional activities), met all of the above criteria and was pursued as a possible secretory lipase. Based on these observations, oligo primers were designed corresponding to the entire LmjF31.0830 ORF from the *L. major* (Freidlin) GeneDB database [19]. These primers were used in PCR amplifications with *L. donovani* gDNA as a template. The resulting 927 bp product obtained from such amplification reactions was gel purified, cloned (*Ldon-PCR927*) and subsequently sequenced. Such sequences were subjected to BLAST-N and BLAST-P analyses. Results of these analyses showed that the *Ldon-PCR927* clone contained an open reading frame (ORF), which showed both high nt and deduced aa sequence identity (94 and 89%, respectively) to LmjF31.0830 of *Leishmania major* [19] and to LinJ31.0860 of *Leishmania infantum* (99 and 99%, respectively). These results suggested that we had amplified a portion of a gene encoding a *L. donovani* lipase homolog. The *Ldon-PCR927* fragment was labeled with digoxigenin-dUTP. The resulting probe (*Ldon-DIG927*) was used to screen an *L. donovani* gDNA cosmid library by hybridization. Following several rounds of screening with this probe, a positive cosmid clone (*Ldon-Cos1*) was selected for further analysis. Results of nt sequence analyses revealed that the *Ldon-Cos1* clone contained a complete ORF (*LdLip3*) of 927 bp. The composition of this ORF is GC-rich (~62%) and the third base position of the codons used show a strong bias (~70%) toward G or C residues. These observations are consistent with the overall GC content of the *Leishmania* genome [35]. Further, the *LdLip3* ORF encodes a polypeptide of 308 amino acids with a calculated molecular mass of 33.2 kDa and a predicted pI of 8.52 (Fig. 1).

Comparison of the deduced amino acid sequence encoded by the *LdLip3* ORF to all available, non-redundant data bases using BLAST-P, showed that it has homology to a variety of lipases from diverse sources. Among those, the *LdLip3* deduced protein showed the highest level of aa sequence identity (i.e., 99 and 89%) with the LinJ31.0860 sequences from *L. infantum* and the LmjF31.0830 sequences from the *L. major* (Freidlin) genome data bases (Fig. 1). Moreover, such analyses showed that the *LdLip3* deduced protein also contained a signature sequence characteristic of the serine lipase family (i.e., spanning aa residues V₁₆₂-A₁₇₁) (Fig. 1). Taken together, these observations indicated that the *LdLip3* ORF encoded an *L. donovani* lipase.

Based on the von Heijne algorithm [36, 37] the hydrophobic, N-terminal, 24 amino acids of the *LdLip3* deduced protein constitute a putative signal peptide (Fig. 1). Cleavage at this site, presumably in the endoplasmic reticulum of this parasite, would result in a mature protein with V₂₅ as its N-terminal amino acid residue. Such cleavage would result in a mature protein consisting of 284 amino acids with a calculated molecular mass of 30.625 kDa and a predicted pI of 8.15. The conserved putative functional domains of the *LdLip3* protein are shown in Fig. 1. This includes the putative catalytic/active site of the enzyme which spans residues V₁₆₂-A₁₇₁. A comparison of the *LdLip3* functional domain with those

of its closest homolog, the LmjF31.0830 lipase [19], showed that within the putative catalytic site they share 100% aa sequence identity. The LdLip3 deduced protein was analyzed using: NetNGlyc, NetOGlyc and NetPhos web-based tools (available at <http://www.cbs.dtu.dk/services>) to identify aa residues that are potential sites for post-translational modifications of this protein. Such analyses showed that the LdLip3 possesses one potential site for N-linked glycosylation at N₄₄ (Fig. 1) and several potential N-myristoylation sites. In addition the LdLip3 deduced protein also contained multiple potential sites (S₃₂, T₄₆, S₆₇, T₁₀₄, Y₁₁₉, T₁₄₅, T₂₂₈, S₂₂₉) for phosphorylation by several different mechanisms (e.g., casein kinase II, protein kinase-C, etc.) (Fig. 1). As predicted by the Kyte-Doolittle algorithm [38] the LdLip3 deduced protein is hydrophilic in nature. Further, no apparent hydrophobic transmembrane domains or predicted glycosyl-inositol phosphate (GPI-) anchor signature sequences [39] were present in the C-terminus of this protein. Similarly, no KDEL- or analogous endoplasmic reticulum (ER) retention sequences [39, 40] or other intracellular organelle specific-targeting sequences were identified in the LdLip3 deduced protein. Based on its overall hydrophilicity, the presence of an N-terminal signal peptide and the absence of both membrane anchor and ER-retention motifs, suggest that the LdLip3 is a soluble/released protein.

Southern blot analysis of *LdLip3*

To examine the genomic organization and copy number of the *LdLip3* gene, *L. donovani* gDNA and *Ldon Cos1* DNA were subjected to Southern hybridization using the digoxigenin-labeled *Ldon-DIG927* probe (Fig. 2a) under high stringency conditions. Enzymes that did not cut within the *LdLip3* ORF (e.g., *XhoI*, *NotI* and *NcoI*) gave a single band of hybridization with the *Ldon-DIG927* probe (Fig. 2b). In contrast, gDNA digested with enzymes which cut within the *LdLip3* ORF (e.g., *HindIII*, and *SalI*) gave two or more bands of hybridization with this DIG-labeled probe (Fig. 2b). Taken together, these results summarized in the restriction map of the *LdLip3* locus (Fig. 2b), suggest that the *LdLip3* gene is present in a single copy within the diploid genome of this organism [19].

Expression of *LdLip3* mRNA in various *L. donovani* developmental stages

RT-PCR was carried out to examine the expression of *LdLip3* mRNA in various life cycle developmental stages of *L. donovani*. To that end, total RNA was isolated from in vitro grown promastigotes, and axenic amastigotes. Transcription of the *LdLip3* gene was assayed in a two-step process involving RT and PCR amplification. First, cDNA of promastigote and axenic amastigote mRNA was generated with oligo dT and reverse transcriptase. Second, an aliquot of each of these cDNAs was used as template in PCR with gene-specific primers (Fig. 3a). The results of these experiments showed that only a single amplified product of the predicted 927 bp was obtained using aliquots of promastigote or axenic amastigote cDNA as template (Fig. 3b PRO and AM lanes, respectively). These RT-PCR results were confirmed using multiple RNA-cDNA preparations (data not shown). Moreover, in control reactions in which cDNAs were not generated prior to PCR or in which forward or reverse primers were omitted from the reaction mix, no amplified products were obtained (data not shown). The specificity of the RT-PCR amplified products was verified by sequence analysis of gel purified PCR amplification products. Sequence analysis revealed that the primer pair specifically amplified a single product which corresponded to the *LdLip3* gene

sequence. Taken together these results indicated that *LdLip3* mRNA is actively transcribed by *L. donovani* promastigotes and axenic amastigotes. Thus, *LdLip3* appears to be constitutively transcribed throughout the developmental life cycle of *L. donovani*.

Mapping of 5' SL splice site

A unique feature of trypanosomatid parasites is that all of their mature, translatable mRNAs are capped at the 5'-end with a conserved 39 nt, SL [24, 25]. Such capping involves the addition (i.e., via trans-splicing) of a 39 nt SL to a specific splice acceptor site in the 5'-non-coding region of the parasite's mRNAs (Fig. 3a). To identify the 5'-splice acceptor site in the *LdLip3* transcript, RT-PCR analyses were performed using total RNA isolated from both *L. donovani* promastigotes and axenic amastigotes. cDNAs generated from such RNAs were used as template in PCR amplifications with a 5'-forward primer (SpliceFwd) corresponding to a portion of the *L. donovani* SL sequence and a reverse primer (*LdLip3*-RT/Rev) corresponding to an internal sequence in the *LdLip3* ORF (Fig. 3a). The nt sequence of the 245 bp product obtained in these reactions using cDNA from *L. donovani* promastigotes is shown in Fig. 3c. Analyses of these results indicated that the ATG start codon of the *LdLip3* ORF, was preceded by 54 nt of 5'-UTR (Fig. 3c). Further, these results showed that the SL acceptor site mapped to -55 nt from the ATG start codon of the *LdLip3* ORF. In addition to defining the 5'-UTR and SL acceptor site, these results also verified that both parasite developmental forms synthesized mature, translatable mRNAs for the *LdLip3* gene.

Homologous episomal expression of the *LdLip3* gene in *L. donovani* promastigotes and axenic amastigotes

A homologous episomal expression system was used to establish that the *LdLip3* gene encodes a protein with lipase activity and to further examine the role of the *LdLip3* protein in the developmental cycle of *L. donovani*. To that end, a chimeric construct was generated containing the complete ORF of *LdLip3* fused in frame at its 3'-end with a sequence encoding a hemagglutinin (HA) epitope. Following ligation into the *pKSNEO* leishmanial expression vector, this construct (*pKSNEO::LdLip3::HA*) was used to transfect *L. donovani* promastigotes (Fig. 4a). In parallel, promastigotes were transfected with the *pKSNEO* vector alone and these served as controls in all transfection experiments. Following electroporation, both the *pKSNEO::LdLip3::HA* and *pKSNEO* control transfectants were selected for growth in increasing concentrations of G418 over a period of several weeks. Subsequent to such drug selection, the growth kinetics of these transfectants were compared. Results of those studies showed that promastigotes transfected with either the *pKSNEO::LdLip3::HA* chimeric construct or the control plasmid (*pKSNEO*) had virtually identical growth kinetics over the time course of these experiments to non-transfected "wild-type" *L. donovani* promastigotes grown in complete medium lacking G418. Taken together, these observations indicate that these episomal transfections did not overtly alter the characteristic growth kinetics of the parental *L. donovani* promastigote cell line. *L. donovani* promastigotes transfected with either the *LdLip3::HA* or the *pKSNEO* control plasmid were placed under conditions (37°C, pH 5.5) to allow them to transform into, and grow as axenic amastigotes in vitro. Both promastigotes and axenic amastigotes of these *L. donovani* transfectants were

subsequently analyzed for their expression of the *LdLip3::HA* gene product using Western blots and immuno-fluorescence microscopy.

Expression of the *LdLip3::HA* chimeric protein in *L. donovani* transfectants

To determine if the *LdLip3::HA* transfectants of these two parasite developmental stages expressed the *LdLip3::HA*-tagged protein, lysates from promastigotes and axenic amastigotes of *pKSNEO* (control) and *pKSNEO::LdLip3::HA* transfectants were subjected to SDS-PAGE and reacted in Western blots with a mouse anti-HA monoclonal antibody or with an appropriately matched purified mouse control immunoglobulin. In such blots, the anti-HA antibody reacted with a single ~ 33 kDa *LdLip3::HA* chimeric protein present in lysates from both promastigotes and amastigotes of the *LdLip3::HA* transfected parasites (Fig. 4b). The anti-HA antibody appeared to react even more strongly with the *LdLip3::HA* chimeric protein present in lysates from axenic amastigotes transfectants (Fig. 4b). The anti-HA antibody showed no reactivity with lysates of control *pKSNEO* transfected promastigotes or axenic amastigotes (data not shown). Similarly, the isotype-matched purified control mouse immunoglobulin showed no reactivity with any of the samples tested in these assays (data not shown). Taken together, the results of these Western blot experiments demonstrated that the *LdLip3::HA* chimeric gene construct was readily transcribed and translated into an ~33 kDa chimeric protein by both promastigotes and axenic amastigotes of these transfectants. Further, our observations indicated that such expression appear significantly increased in the axenic amastigote form of these transfected parasites. This pattern of differential up-expression may reflect an inherent property of amastigotes per se but, might also be facilitated by the A2 gene-regulatory sequences [29, 30, 33] present in the backbone of the *pKSNEO* expression vector.

Immuno-fluorescence microscopy

The *LdLip3::HA* and *pKSNEO* transfectants of *L. donovani* were examined by indirect immuno-fluorescence microscopy to visualize the cellular distribution of the expressed *LdLip3::HA* chimeric protein. For these experiments, both transfected promastigotes and axenic amastigotes were fixed, permeabilized, reacted with anti-HA mouse monoclonal antibody, followed by a goat anti-mouse FITC-conjugated secondary antibody and examined using an epi-fluorescence microscope. Results of such observations revealed that the *LdLip3::HA* transfected promastigotes displayed only very low levels of intracellular immunofluorescence with the anti-HA antibody (Fig. 4c, panel Pro). In contrast, *LdLip3::HA* transfected axenic amastigotes showed very bright, punctuate intracellular fluorescence (Fig. 4c, panel Ax Am). Such staining is consistent with the processing of *LdLip3::HA* through the endoplasmic reticulum of these transfectants. Very similar IFA staining patterns have been reported for a variety of secretory proteins in these parasites [15]. No fluorescent signal was detected in either promastigotes or axenic amastigotes of *pKSNEO* (control) transfectants treated with the anti-HA monoclonal antibody (data not shown). Similarly, none of the cell samples tested showed any reactivity with the control isotype-matched mouse immunoglobulin used in these assays (data not shown). Results of these immuno-fluorescence assays demonstrated that both *LdLip3::HA* transfected promastigotes and axenic amastigotes synthesized and expressed the *LdLip3::HA* chimeric protein. Further, our immuno-fluorescence observations are in agreement with the Western blot data described

above, suggesting that the LdLip3::HA chimeric protein appeared to be up-expressed in the axenic amastigote form of these transfectants.

Detection of the secreted/released LdLip3::HA chimeric protein in culture supernatants of transfected parasites

To demonstrate that the LdLip3::HA chimeric protein was released/secreted from transfected parasites during their growth in vitro, culture supernatants of both *pKSNEO* control and *pKSNEO::LdLip3::HA* transfected *L. donovani* promastigotes were reacted in Western blots with a mouse anti-HA monoclonal antibody or with an appropriately matched purified mouse control immunoglobulin. Results of such blots demonstrated that the anti-HA antibody reacted only with a single ~ 33 kDa protein present in the supernatant of the *pKSNEO::LdLip3::HA* transfectant but not with the *pKSNEO* controls parasites (Fig. 5 a). These results demonstrated the *LdLip3::HA* chimeric gene was in fact transcribed and translated into released/secreted protein in transfected parasites.

Lipase activity of the secreted/released LdLip3::HA chimeric protein

Based on the above observations, experiments were designed determine whether the secreted/released *LdLip3::HA* expressed protein possessed functional lipase activity. For these experiments, cell-free supernatants from transfected cultures were analyzed directly for their lipase activity using 4MU-stearate as substrate. Results of such enzyme assays showed that *LdLip3::HA* transfected promastigotes released higher levels of lipase activity into their culture supernatants (16.0 pmol/min/mg at 37°C and 13.6 pmol/min/mg at 42°C) than similarly transfected *pKSNEO* control parasites (6.0 pmol/min/mg at 37°C and 4.0 pmol/min/mg at 42°C) (Fig. 5b). These results indicated that the *LdLip3::HA* transfected parasites had a 3.4 fold higher lipase activity at 37°C than *pKSNEO* control parasites (Fig. 5c). Similarly *LdLip3::HA* transfected parasites had a 2.5 fold higher lipase activity at 42°C a than *pKSNEO* control parasites (Fig. 5c). These results indicated that culture supernatant from the *LdLip3::HA* transfectants possessed higher lipase activity than the control transfectants.

Isolation of the LdLip3::HA chimeric protein by immuno-affinity binding

To demonstrate that the above increase in lipase activity was due to the LdLip3::HA expressed protein, culture supernatants from these transfectants were subjected to anti-HA affinity binding studies. For these experiments, culture supernatants of both *pKSNEO* control and *pKSNEO::LdLip3::HA* transfected *L. donovani* promastigotes were reacted with anti-HA antibody in a Protein G bead-based column matrix. The resulting immuno-affinity purified proteins were examined in Western blots with a mouse anti-HA monoclonal antibody or with an appropriately matched purified mouse control immunoglobulin. In such blots, the anti-HA antibody reacted with only a single ~ 33 kDa protein in culture supernatant from *pKSNEO::LdLip3::HA* transfected parasites (Fig. 6a). In contrast, the anti-HA antibody showed no reactivity in Western blots with similarly immuno-affinity processed culture supernatants from *pKSNEO* control transfected promastigotes (Fig. 6a). Similarly, the isotype-matched control mouse immunoglobulin showed no reactivity with any of the samples tested in these assays (data not shown). Results of these combined

immuno-affinity purification and Western blot experiments demonstrated that the full-length LdLip3::HA-tagged chimeric protein was constitutively synthesized and secreted/released by transfected *L. donovani* promastigotes during their growth in vitro.

Lipase activity of the anti-HA immuno-affinity purified secreted/released LdLip3::HA chimeric protein

To determine that the LdLip3::HA chimeric protein possessed lipase activity it was subjected to immuno-affinity absorption as above. The anti-HA immuno-affinity purified LdLip3::HA chimeric protein and *pKSNEO* controls were subsequently analyzed for lipase activity using 4MU-stearate as substrate. Results of these lipase activity assays showed that the affinity purified LdLip3::HA had 221 pmol/min/mg specific activity at 37°C and even higher specific activity at 42°C (332 pmol/min/mg) (Fig. 6b). No activity was detected in similarly processed *pKSNEO* control samples (Fig. 6b). Further, the immuno-affinity purified LdLip3::HA samples showed a 14 fold increase in specific lipase activity at 37°C and a 23 fold increase at 42°C when compared to the activity present in the 1× culture supernatants obtained from *LdLip3::HA* transfected parasites (Fig. 6c). These coupled immuno-affinity enzyme activity assays demonstrated that the LdLip3::HA chimeric protein did in fact possess lipase activity under the assay conditions used in this study. The cumulative results of these experiments demonstrated that the LdLip3::HA chimeric protein was secreted/released by the transfected parasites and that it possessed functional lipase activity. Taken together results of these studies demonstrated that the *LdLip3* gene encodes a unique functional secretory lipase in this important group of human pathogens.

Discussion

Leishmania donovani is an important protozoan pathogen of humans producing a fatal visceral disease [41]. Studies of this organism have been greatly facilitated because culture systems exist for generating its various life cycle developmental forms in vitro [16, 42–44]. All *Leishmania* spp. are opportunistic facultative lipid scavengers and as obligate parasites they must salvage these macromolecules from their hosts for their own physiologic requirements. We hypothesize that a secretory lipase would afford these organisms the ability to salvage fatty acids from both their insect vector and mammalian hosts. Such fatty acids could subsequently be used to synthesize complex lipids required for the growth and development of the parasite or used as potential carbon sources as fatty acids have been shown to be important substrates in energy metabolism via beta oxidation [5–12]. Previous studies have shown that amastigotes actually have elevated metabolism of fatty acids compared to promastigotes which typically prefer to metabolize proline and glucose [5, 9, 11]. In parallel, it has been shown that these organisms remodel their membrane lipid constituents throughout their life cycle as they transition between promastigote and amastigotes developmental forms. A variety of secretory enzymes have been shown to play critical functions in the growth, development, and survival of these organisms [15, 26, 45]. Taken together, we hypothesize that secretory lipases would also play important role(s) in the survival, growth, and development of these parasites. Considering the speculated importance of lipases to parasite growth, survival, and development, to date, no one had identified any secretory lipases in these organisms. In preliminary studies we showed that *L.*

donovani promastigotes released/secreted lipase activity into their culture supernatant during their growth in vitro. Thus, in light of the foregoing, this study was carried out to identify, characterize, and express the gene encoding a functional secretory lipase activity from this important human pathogen.

Using a PCR-based approach we identified a single copy, 927 bp ORF (*LdLip3*) in *L. donovani* parasites. The *LdLip3* encodes a deduced protein of 308 aa with a calculated molecular mass of 33.2 kDa which has homology to known lipases. Structural analysis of the *LdLip3* deduced protein showed that it possessed conserved functional domains characteristic of serine lipases in general [46–50]. Further, the active site of the *LdLip3* deduced protein contained a 9 aa consensus sequence characteristic of the catalytic domain of serine lipase enzymes [46–50]. This includes, a conserved terminal serine residue that has been shown to be critical for the catalytic function of this family of lipases [46–50]. Some members of this family include lipases from prokaryotes (e.g., *Helicobacter pylori* [46], *Pseudomonas aeruginosa* [47] and *Pseudomonas cepacia* [48]) and fungi (e.g., *Fusarium graminearum* [49] and *Candida albicans* [50]). Virtually all of these serine lipase family lipases are soluble-released/secretory proteins which have been shown to function extracellularly in these organisms. In that regard, results of our structural analyses suggested that the *LdLip3* deduced protein: 1) had an overall hydrophilic composition, 2) possessed a putative N-terminal signal peptide and 3) lacked both GPI- and transmembrane-anchor motifs. These predictions suggest that the *LdLip3* has properties typical of a soluble-released/secretory protein. In support of those predictions, results of our enzyme analyses showed that *L. donovani* parasites secrete/release lipase activity into their culture supernatants during their growth in vitro.

In trypanosomatid parasites, all mature, translatable mRNAs are capped at their 5'-end with a conserved 39-nt SL sequence [24, 25]. Results of our RT-PCR analysis using SL and *LdLip3* gene-specific primers demonstrated that both promastigotes and axenic amastigotes of *L. donovani* synthesized mature transcripts of the *LdLip3* gene. Taken together, these results indicate that this gene is actively transcribed throughout the developmental life cycle of the *L. donovani* parasite.

To determine the functional activity of the *LdLip3* protein in *L. donovani* parasites, an episomal expression system was devised using the *pKSNEO* leishmanial expression vector [28]. In these experiments, parasites were transfected with either a *pKSNEO* chimeric construct containing the *LdLip3* ORF joined at its 3'-end with a hemagglutinin (HA) epitope sequence (*pKSNEO::LdLip3::HA*) or the *pKSNEO* vector alone. Western blot results using whole cell lysates showed that the chimeric gene construct was translated into a ~ 33 kDa *LdLip3::HA* protein. Further, results of immunofluorescence assays were consistent with this chimeric protein being trafficked through the endoplasmic reticulum of both parasite developmental forms. In addition, Western blot analysis with culture supernatants from these transfectants demonstrated that the full-length chimeric protein was in fact secreted/released by both *LdLip3::HA* transfected promastigotes and axenic amastigotes during their growth in vitro. Moreover, results of our coupled anti-HA immuno-affinity purification/enzyme activity assays demonstrated that the *LdLip3::HA* chimeric protein which they secreted/released possessed functional lipase activity.

The cumulative results of this study have demonstrated for the first time that the *LdLip3* gene of *L. donovani* codes for a secretory protein with lipolytic activity. We assume this enzyme plays a role in acquiring host lipid constituents toward satisfying the parasite's need for energy metabolism (i.e., beta oxidation). Further, we hypothesize that it has important biological roles in lipid biosynthesis and/or in the structural remodeling of membrane lipids during parasite development. Based on the functional activities of this parasite enzyme we hypothesize that it could play a significant role in the pathophysiology associated with this human disease. For example, in the amastigote stage of this parasite the secretory lipase could alter the lipid constituents and structure of the host cell phagolysosome membrane. Alterations of this typically acidic and hydrolytic compartment could result in favorable conditions for parasite growth, survival, and development and have implications with regard to host immuno-responsiveness. In addition, release of this parasite secretory lipase into host extracellular spaces could account in part for the extensive tissue damage associated with the various forms of leishmaniasis.

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Abbreviations

Aa	Amino acid
Ab	Antibody
Bp	Base pair
DIG	Digoxigenin
FBS	Fetal bovine serum
gDNA	Genomic DNA
HA	Hemagglutinin
<i>LdLip3</i>	Gene encoding the secretory lipase of <i>Leishmania donovani</i>
4MU	4-Methylumbelliferone
Nt	Nucleotide
Oligo	Oligodeoxy-ribonucleotide
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RT	Reverse transcription

SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SP	Signal peptide
SL	Spliced leader

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<i>L. donovani</i>	MPHSSCSRIVVVAALLLLCGSARA VVLSREYSQTDARRSLQYANVTYADI	*	* 50
<i>L. infantum</i>	MPHSSCSRIVVVAALLLLCGSARA VVLSREYSQTDARRSLQYANVTYADI		50
<i>L. major</i>	MLPSSCSRIVVVAALLLLCGGARA VVLSGEYSQTDAIRSLQYANATYADS		50
<i>L. donovani</i>	DAIESWKCGGSCNANPSFKVTSIVKGDDAHSLLAYVGVD DGSAQVVVALR	*	100
<i>L. infantum</i>	DAIESWKCGGSCNANPSFKVTSIVKGDDAHSLLAYVGVD DGSAQVVVALR		100
<i>L. major</i>	DAVASWNCGGSCNANPSFKVTSIVKGDDAHS LHAYVG VDEGSAQVVVALR		100
<i>L. donovani</i>	GSATQQEKL MRRPAEPVL YDITSGC GLECRVHTGFQRSYLAV RRTVR AAV	* * *	150
<i>L. infantum</i>	GSATQQEKL MRRPAEPVL YDITSGC GLECRVHTGFQRSYLAV RRTVR AAV		150
<i>L. major</i>	GSATQQEQ LMRQLVEPVL YDITSGC GLECRV HAGFQRSYLAV RRTIRA AV		150
<i>L. donovani</i>	VRDLTMHPGY N <u>VLVTGH SVGA A</u> VALLAAVDVQA HVNRMFFVS RP I VS LYT		200
<i>L. infantum</i>	VRDLTMHPGY N <u>VLVTGH SVGA A</u> VALLAAVDVQA HVNRMFFVS RP I VS LYT		200
<i>L. major</i>	VRDLMMHPDY N <u>VLVTGH SVGA A</u> VALLAAIDVQA HVNRMFFVS RP I VS LYT		200
<i>L. donovani</i>	FGMPRVGNRAFAVWAAGMLSRGSHFRITSRHDPVP R MP SS GS AG FQH VPY	**	250
<i>L. infantum</i>	FGMPRVGNRAFAVWAAGMLSRGSHFRITSRHDPVP R MP SS GS AD FQH VPY		250
<i>L. major</i>	FGMPHVGNRAFAVWAAGMLSRGSHFRITSRHDPVP RL S SG SA DF QH VPY		250
<i>L. donovani</i>	EVC AAAAGTN CR VCED SADGD DPT C IVQASKVDMRDHF FY FG ER IS GG A		300
<i>L. infantum</i>	EVC AAAAGTN CR VCED SADGD DPT C IVQASKVDMRDHF FY FG ER IS GG A		300
<i>L. major</i>	EVC SAADGTN CR VCES VDSD DPT CIA HTSNVN MRDH FFY FG ER IS GG A		300
<i>L. donovani</i>	AGDAMLYL		308
<i>L. infantum</i>	AGDAMLYL		308
<i>L. major</i>	AGDAMLYL		308

Fig. 1.

The deduced aa sequence and structure of the *L. donovani* LdLip3. The deduced aa sequence of the *L. donovani* lipase (*LdLip3*) gene in Clustal W alignment with the homologs from the *L. major* (Freidlin) GeneDB database (LmjF31.0803) and the *L. infantum* database (LinJ31.0860). The **bold** sequence delineates a putative 24 aa signal peptide (M₁-A₂₄). The signal peptide cleavage site is indicated by the *arrow*. The **bold** and *underlined* aa (V₁₆₂-A₁₇₁) denote the conserved consensus sequence of the catalytic site of the serine class 3 lipase family. The single potential *N*-linked glycosylation site (N₄₄) is italicized, and eight potential phosphorylation sites are indicated by the asterisks (S₃₂, T₄₆, S₆₇, T₁₀₄, Y₁₁₉, T₁₄₅, T₂₂₈, and S₂₂₉)

Fig. 2.

Restriction map of the *L. donovani Lip 3* gene locus. a Schematic map showing the position of primers used to generate the *LdLip3* ORF probe. The *stippled box* indicates the 927 bp *LdLip3* ORF. The *arrows* represent the position of the primers used in these studies, and the *dashed line* indicates the predicted 927-bp amplified product used as a full-length gene probe (*Ldon-DIG927*). b Restriction map of *L. donovani* gDNA generated with the *Ldon-DIG927* probe. *L. donovani* gDNA was digested with various individual restriction endonucleases and combinations of two different enzymes (*Hind*III, *Xho*I, *Nco*I, and *Sal*I). Restriction fragments were separated by 1% agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with the *Ldon-DIG927* probe. This diagram summarizes all of the results generated in these Southern hybridization analyses. The *stippled box* represents the relative position of the *LdLip3* ORF within this genomic locus

Fig. 3.

RT-PCR and mapping of the SL acceptor site. **a** Schematic map representing the mature *LdLip3* mRNA. The *light gray box* indicates the 39-nt SL present on all mature mRNAs in these organisms. The *open box* indicates the 5' UTR. The *stippled box* represents the 927 nt encoded by the *LdLip3* ORF. The *dark gray box* indicates the 3' UTR/poly A tail. The *arrows* represent the positions of the specific primer pairs used in these studies, and the *dashed lines* indicate the predicted amplified products. Primer pair 1 (pp1) were used to amplify the entire *LdLip3* ORF to generate the predicted 927-bp amplified product. Primer pair 2 (pp2) were used to generate a 245-bp product to identify the 5' SL acceptor site and the 5' UTR of the *LdLip3* mRNA. **b** Ethidium bromide stained agarose gel showing the RT-PCR products from the *LdLip3* mRNA. *L. donovani* total RNA from promastigotes (Pro) and amastigotes (Am) was subjected to RT using oligo (dT). Aliquots of the resulting cDNA were subjected to PCR with pp1. The resulting 927-bp amplified products from each parasite developmental form are designated by the arrow at the right. *Lane M*, molecular mass standards, **c** Mapping of the *LdLip3* 5'-SL acceptor site. The nucleotide sequence of the RT-PCR product obtained with *LdLip3* mRNA amplified with pp2 (SL primer and an internal *LdLip3* primer). The conserved SL sequence is underlined, and the *arrow* marks the SL acceptor site at position minus-54 from the start codon of the gene. The 5'-untranslated region of the *LdLip3* gene is shown in *bold*. The ATG start codon of the *LdLip3* ORF is *italicized* and *underlined* and is marked with +1. The downstream sequence shown in *unbolded* font reflects the 5'-end of the *LdLip3* ORF

Fig. 4.

Episomal expression of the LdLip3::HA chimera in *L. donovani* promastigotes and axenic amastigotes. **a** Map of the LdLip3::HA chimeric construct. The *pKSNEO* plasmid: the gray box represents the nt sequence encoding neomycin phosphotransferase (Neo), the black line denotes the *pKSNEO* plasmid vector backbone, and *SpeI* indicates the restriction site used for insert cloning. A schematic representation showing the complete open reading frame (i.e., 1–924 bp, minus the terminal stop codon) of the *LdLip3* gene (stippled box) fused at its 3′-end with a 27-nt sequence encoding the hemagglutinin (HA) epitope (open box). The black box at the 5′-end represents nt-1 to nt-24 encoding the putative 24 aa signal peptide (SP) of the LdLip3 protein. The thick black line represents the *pKSNEO* plasmid expression vector, and the *SpeI* restriction endonuclease sites used for cloning are shown. The dark gray box and black lines represent Neo and the vector backbone as above. **b** Western blot. Whole cell lysates from *L. donovani* promastigotes (Pro) and axenic amastigotes (Am) transfected with the LdLip3::HA construct probed with a mouse anti-HA monoclonal antibody. Arrow denotes the ~33 kDa LdLip3::HA chimeric protein. Molecular mass standards in kDa are indicated on the right. **c** Indirect immunofluorescence images. LdLip3::HA transfected promastigotes (Pro) and axenic amastigotes (Am) were visualized by Differential Interference Contrast (DIC), stained with DAPI and probed with a primary mouse anti-HA monoclonal antibody and a FITC-labeled goat anti-mouse secondary antibody. DAPI and anti-HA samples were merged to orient the position of the nucleus and kinetoplast to the staining of the expressed protein

Fig. 5.

Episomal expression of the *LdLip3::HA* chimeric protein in 1 × culture supernatant from *L. donovani* transfectants. **a** Western blot of culture supernatant from *L. donovani* promastigotes transfected with the *pKSNEO* control plasmid (lane 1) or the *pKSNEO::LdLip3::HA* construct (lane 2) were probed with a mouse anti-HA monoclonal antibody. Arrow denotes the ~33 kDa *LdLip3::HA* chimeric protein. **b** Lipase activity in 1 × culture supernatants of *L. donovani* transfectants. Lipase activity was measured at pH 6.0 at 37 and 42°C with 1 × culture supernatants from both *pKSNEO* control and *pKSNEO::LdLip3::HA* transfectants using 4MU-stearate as substrate. Specific activity is reported as pmol/min/mg of protein. The stippled gray box and stippled black box represent the lipase activity of *pKSNEO* at 37 and 42°C, respectively. The solid gray and black boxes represent the lipase activity of *pKSNEO::LdLip3::HA* at 37 and 42°C, respectively. Error bars reflect standard error of the mean over several experiments done in quadruplicate. **c** The fold increase in lipase specific activity in 1 × culture supernatants from *pKSNEO::LdLip3::HA* versus *pKSNEO* transfectants. The solid gray and black boxes represent the fold increase in lipase specific activity of *pKSNEO::LdLip3::HA* versus *pKSNEO* at 37 and 42°C, respectively.

Fig. 6.

Western blot and activity analyses of immuno-affinity purified *LdLip3::HA* **a** Western blot of anti-HA immuno-affinity purified *LdLip3::HA* from culture supernatants of *L. donovani* transfectants. Culture supernatants of *L. donovani* promastigotes transfected with either the *pKSNEO* control plasmid (lane 1) or the *LdLip3::HA* construct (lane 2) were anti-HA affinity purified and reacted in Western blots with an anti-HA monoclonal antibody. Arrow denotes the ~33 kDa *LdLip3::HA* chimeric protein. **b** Anti-HA affinity purified samples from *pKSNEO* control and *pKSNEO::LdLip3::HA* transfectants were assayed for their lipase activities. Assays were carried out at 37 and 42°C with using 4MU-stearate as substrate. Specific activity is reported as pmol/min/mg of protein. No lipase activity was detected in the *pKSNEO* samples at 37 or 42°C. The solid gray and black boxes represent the lipase activity present in the immuno-affinity purified samples of *pKSNEO::LdLip3::HA* at 37 and 42°C, respectively. Error bars reflect standard error of the mean over several experiments done in quadruplicate. **c** The fold increase in lipase specific activity present in anti-HA affinity purified *pKSNEO::LdLip3::HA* versus 1× culture supernatants from these transfectants. The solid gray and black boxes represent the fold increase in lipase specific activity of anti-HA affinity purified *pKSNEO::LdLip3::HA* versus 1× culture supernatant form. *pKSNEO::LdLip3::HA* transfectants at 37 and 42°C, respectively