



The Journal of Young Investigators

The premier undergraduate research journal

**Southern Hybridization and Restriction Map Analyses of the LdLIP3 locus in the human pathogen
*Leishmania donovani***

Mary Stromberg and Alison Shakarian
Salve Regina University

Volume 19, Issue 15
September 2009

Southern Hybridization and Restriction Map Analyses of the *LdLIP3* locus in the human pathogen *Leishmania donovani*

Mary Stromberg and Alison Shakarian
Salve Regina University

Abstract

In the present study Southern hybridization analysis of *Leishmania donovani* gDNA was performed to generate a restriction map of the *LdLIP3* gene loci and to determine its copy number in the genome. The gDNA was digested with restriction endonucleases and subjected to agarose gel electrophoresis. Restriction fragments were visualized with ethidium bromide staining and subjected to Southern hybridization with a digoxigenin labeled probe of the full length *LdLIP3* gene. Results indicated that the gene is present as single copy. Moreover, our data supports the hypothesis that *L. donovani* possess a gene that encodes a secreted lipase. To date, there are no published studies concerning the characterization of any lipase genes in *Leishmania* thus this research provides some of the tools necessary to better understand the role of lipases to the biology and malignance of this important human pathogen.

Introduction

Several species of *Leishmania* are pathogenic parasites of human hosts and are endemic to tropical and subtropical areas throughout the world. In the life cycle of *Leishmania donovani* the parasite is injected into a healthy human host by an infected *Phlebotomus* sandfly. The parasites are phagocytized by and multiply within the host's macrophages causing the disease leishmaniasis. There are over 20 million people infected with various species of *Leishmania* and 1.5 million new cases are reported each year. (WHO 1999)

The infection of leishmaniasis may be either cutaneous (infection of the the skin or mucus membranes) or visceral (infection of the internal organs) and is dependent upon the species that infects the human host. Cutaneous leishmaniasis caused by species such as *Leishmania major* and *Leishmania mexicana* is characterized by the presence of ulcerative skin lesions that appear where the sandfly took a blood meal. Visceral leishmaniasis or kala-azar is caused by *L. donovani* and is characterized by fever, weight loss, and hepatosplenomegaly (enlargement of the liver and spleen). Visceral leishmaniasis is fatal if left untreated and is becoming more common with the spread of HIV and other causes of immuno-suppression. (WHO 1999)

Parasite secreted proteins which mediate the survival, growth and development of *Leishmania* generally remain to be elucidated (Shakarian et al. 1997; Ellis, Shakarian and Dwyer 1998; Shakarian and Dwyer 1998; Shakarian and Dwyer 2000). One secretory molecule thought to be involved in these processes is a parasite derived secreted lipase. Lipases are enzymes that break down triglycerides into fatty acids and

glycerol. They are highly functional both in aqueous and non-aqueous environments giving them a broad range of activity. (Subramanian and Wasan 2003; Stinchcombe et al. 2006) Lipases can have a broad range of functions from antibacterial and antiviral activities (Stavrinides, McCann, and Guttman 2008) to roles in pathogen metabolism, growth and development (Stehr et al. 2004; Schaller 2005; Yordanov et al. 2008; Brunke and Hube 2006; Pignède et al. 2000). It is assumed therefore that lipases might make suitable targets for the development of novel drug treatments (Belardinelli et al. 2007; Singh et al. 2007; Briken 2008).

As a blood parasite that can cause extensive tissue damage, it is hypothesized that lipases of *Leishmania* may be involved in such pathology and at minimum that it is essential for the survival and development of the parasite throughout its life cycle. Despite its apparent relevance, to date little direct evidence exists concerning the role(s) of such a lipase in the developmental biology of *Leishmania* parasites in either their sandfly vector or human host. Mapping the locus and determining the copy number of the gene that encodes the secreted lipase enzyme of *Leishmania* should benefit our understanding of the underlying roles that secreted lipases play in the virulence of this parasite. These studies should provide insight into the structure of the DNA surrounding the lipase gene and examine a mechanism of regulating protein expression by simple duplication of the gene copy number. Such expression strategies have been reported in other parasitic organisms such as *Plasmodium* (Anderson, Patel and Ferdig 2009). To date however, no such experimental evidence has been reported in *Leishmania*. Thus this report

constitutes the first characterization of the structure of the gene encoding this novel *L. donovani* enzyme.

Materials and Methods

Reagents

All chemicals used, unless specified, were of analytical grade and purchased from Sigma-Aldrich Chemical Co. Enzymes used for molecular studies were obtained from New England Biolabs; DNA molecular mass standards were from Invitrogen, Inc. or from Roche.

Parasites and culture conditions

Parasites used in this study were *L. donovani* strain 1S-CL2D from Sudan, World Health Organization (WHO) designation (MHOM/SD/62/1S-CL2D). Parasite used for isolation of genomic DNA (gDNA) were grown and maintained at 26°C in medium M199 (Invitrogen) supplemented according to Debrabant et al. (2004).

Isolation of gDNA

L. donovani parasite cultures were harvested at mid-log phase ($\sim 2 \times 10^7$ cells ml⁻¹) by centrifugation at 2100 x g for 15 min at 4°C (Shakarian et al. 1997). The resulting cell pellets were washed twice in ice-cold phosphate buffered saline (PBS, 10 mM sodium phosphate, 145 mM NaCl, pH 7.4) by centrifugation as above and resuspended in the appropriate buffers for gDNA isolation. gDNA was prepared using the GNome DNA isolation kit (BIO 101) according to the manufacture's instructions.

Restriction digests of *L. donovani* gDNA

For Southern blot analyses gDNA was digested with several restriction endonucleases. Restriction endonucleases were chosen based upon the length of the enzyme recognition site and the nucleotide sequence of the recognition site. The parameters for length of recognition site were 6-8 nucleotides to minimize the frequency of cut sites. The sequences of the sites were chosen to contain at least one GC base pair as these organisms are known to be ~60% GC rich in their genome content (Chanda et al. 2007). In addition, previous sequence analysis of the *LdLIP3* 927 bp open reading frame (Shakarian unpublished) showed that *Hind* III cuts once within the gene at position 321 bp from the +1 start and that *Sal* I cuts within the gene twice at positions 261 bp and 685 bp. Taking all these factors into consideration, the following restriction

endonucleases were chosen for this study: *Sal* I, *Hind* III, *Xho* I and *Nco* I.

Single and double restriction endonuclease digests were set up with 5µg gDNA and the buffer appropriate for each restriction endonuclease and were incubated at 37°C overnight. Sample and control DNAs were separated by 0.8% agarose gel electrophoresis in 1 X TBE at 15 V overnight. Gels were stained with ethidium bromide and visualized on a Gel Logic 440 Imaging System (Kodak).

Oligonucleotide primers, PCR and probe preparation

Oligonucleotide primers: PCR-Fwd and PCR-Rev (Fig 1A) were designed to amplify the entire ORF of the secreted-lipase gene (*LdLIP3*) previously cloned from *L. donovani* (Shakarian unpublished). These primers (PCR-Fwd: 5'-ATGTTGCCCTCATCTTGCAGC and PCR-Rev: 5'-TTACAGGTACAGCATGGCGTC) were synthesized by β cyanoethylphosphoramidite chemistry using an Expedite™ nucleic acid

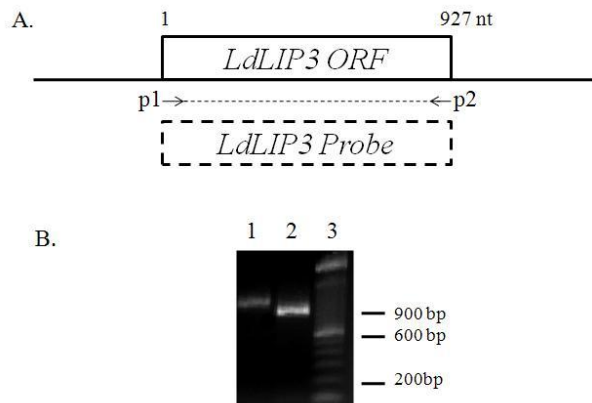


Figure 1. *LdLIP3* open reading frame and gene probe A. Schematic map showing the position of primers used in these studies. The open box indicates the 927 bp *LdLip3* ORF. The arrows represent the specific primer pairs used in these studies to generate a full length gene probe and the dashes indicate the expected 927 bp amplified product. B. Generation of *LdLIP3* gene probe. The *LdLIP3* gene was labeled by PCR using the digoxigenin-labeling kit (Roche). Reaction products were visualized by ethidium bromide stained 0.8% agarose gel electrophoresis. Lane 1, digoxigenin labeled *LdLIP3* probe, Lane 2, unlabeled control PCR reaction (expected 927 bp amplicon), Lane 3, 100 bp ladder DNA molecular mass standards, sizes are given to the right of the gel.

synthesis system (IDT). PCR amplifications with the *LdLIP3* plasmid as template and primers were

carried out using the PCR Digoxigenin Labeling Kit according to manufacturer's instructions (Roche). An unlabeled control reaction using Sigma Ready Mix, *LdLIP3* plasmid and primers was run under identical amplification reaction conditions. After an initial "hot start" at 94°C for 2 min, the conditions used for amplification were: [95°C for 30 sec, 60°C for 1 min, 72°C for 1 min] for 30 cycles, 72°C for 2 min, 4° hold. A 0.8% agarose gel in 1x TBE was run at 50 V for 1 hr to ensure that the digoxigenin-labeled probe reaction was successful (Fig 1B). The resulting digoxigenin-labeled probe (*LdLIP3* probe, Fig 1A and Fig 1B, lane 1) was used to in Southern hybridization analysis of *L. donovani* gDNA.

Southern blot analysis

The gels containing the restriction fragments of *L. donovani* gDNA were processed for Southern blotting according to Sambrook and Russell (2001). The separated DNA restriction fragments were transferred from the agarose gels to positively charged nylon membranes (Roche) by capillary transfer overnight in 10 X SSC and subsequently cross-linked to the membranes by UV irradiation using a Stratalinker® 2400 (Stratagene).

Nylon membranes were hybridized and washed under high stringency conditions using the digoxigenin-labeled DNA probe (i.e. *LdLIP3* probe) according to manufacturer's recommendations (Roche). The hybridized fragments were visualized using an anti-digoxigenin-alkaline phosphatase conjugated antibody in conjunction with a chemiluminescent reagent (CSPD) also according to manufacturer's instructions (Roche). Images were captured from such blots and analyzed using Gel Logic 440 Imaging System (Kodak).

Data analysis

DNA fragments that hybridized to the *LdLIP3* probe were compared to the known sized DNA fragments of the digoxigenin-labeled DNA molecular mass marker to determine the sizes of those hybridized fragments. In addition, the nucleotide sequence of the *LdLIP3* gene was entered into Sequencer™ 4.9 (Gene Codes) to determine which of the restriction endonucleases used in this study would cleave the DNA within the gene open reading frame itself. Using this information, together with determined sizes of the hybridized fragment, a relative restriction endonuclease map was constructed for the *LdLIP3* gene locus.

Results

To characterize the genomic structure and to approximate the copy number of the *L. donovani* *LdLIP3* secretory gene locus, gDNA isolated from *L. donovani* promastigotes was digested with both single and double restriction endonucleases combinations and subjected to agarose gel electrophoresis (Fig 2). Results revealed that the

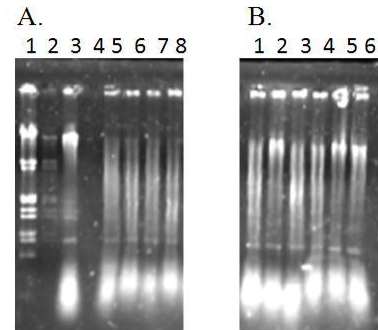


Figure 2. Ethidium bromide stained gel of the *L. donovani* gDNA digested with various individual restriction endonucleases A. Lane 1, unlabeled molecular mass marker III (Roche), Lane 2, digoxigenin-labeled molecular mass marker III (Roche), Lane 3, uncut *L. donovani* gDNA, Lane 4, blank, Lane 5, *Sal I*, Lane 6, *Hind III*, Lane 7 *Xho I*, Lane 8, *Nco I* or with double digestions containing 2 restriction endonucleases as shown in B. Lane 1, *Sal I* and *Hind III* Lane 2, *Xho I* and *Sal I*, Lane 3, *Xho I* and *Hind III*, Lane 4, *Nco I* and *Sal I*, Lane 5, *Nco I* and *Hind III*, Lane 6, *Nco I* and *Xho I*. Restriction fragments from both single and double restriction endonuclease digestion reactions were separated in a 0.8% agarose gel and visualized by ethidium bromide staining.

gDNA was completely digested when a restriction endonuclease was present in the reaction as shown by a smear in the ethidium stained gel (Fig 2 A lanes 5-8, for single digests and Fig 2 B lanes 1-6 for double digests). Uncut gDNA controls, as expected, showed minimal migration into the gel matrix (Fig 2A lane 3).

The agarose gels above were subjected to Southern hybridization with a full length *LdLIP3* gene probe (Fig 1). The gDNA restriction fragments that bound to the *LdLIP3* gene probe by nucleotide complementation were visualized using Gel Logic 440 Imaging System (Kodak). Hybridized fragments were compared to the

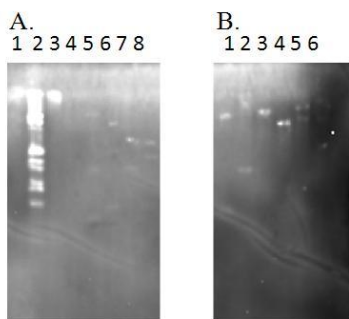


Figure 3. Southern hybridization. DNA from the agarose gel in Fig 2 was transferred to a nylon membrane by capillary action, cross-linked to the membrane by UV irradiation and hybridized with the digoxigenin-labeled *LdLip3* probe. Sizes of hybridizing fragments were determined by comparison to the DNA molecular mass standards (in bp) shown on the right. **A. Single restriction endonuclease digests.** Lane 1, unlabeled molecular mass marker III (Roche), Lane 2, digoxigenin-labeled molecular mass marker III (Roche), Lane 3, uncut *L. donovani* gDNA, lane 4, blank, Lane 5, *Sal* I (4142 bp and 424 bp fragments), Lane 6, *Hind* III (1354 bp and 18391 bp fragments), lane, 7 *Xho* I (6658 bp fragment), Lane 8, *Nco* I (4487 bp fragment). **B. Double restriction endonuclease digests.** Lane 1, *Sal* I and *Hind* III (4142 bp fragment), Lane 2, *Xho* I and *Sal* I (4659 bp and 575 bp fragments), Lane 3, *Xho* I and *Hind* III (939 bp and 702 bp fragments), Lane 4, *Nco* I and *Sal* I (3042 bp 1075 bp fragments), Lane 5, *Nco* I and *Hind* III (3102 bp and 702 bp fragments), Lane 6, *Nco* I and *Xho* I (4041 bp fragment).

digoxigenin-labeled molecular mass standards (Roche) to determine fragment sizes (Fig 3).

The lengths of the hybridizing restriction fragments and the information from the previous sequence analysis of the *LdLIP3* gene were used to construct a relative restriction endonuclease site map of the *LdLIP3* locus. These data are summarized in the restriction map of the *LdLIP3* locus (Fig 4) and are consistent with *LdLIP3* being present as a single copy gene in the parasite genome.

Discussion

Leishmania donovani is an important protozoan pathogen of humans throughout India and the Sudan and is the major causative agent of human visceral leishmaniasis in the Old World (Grimaldi and Tesh 1993). Considering the speculated importance of parasite secreted proteins to pathogen survival and transmission (Shakarian et

al. 1997; Ellis, Shakarian and Dwyer 1998; Shakarian and Dwyer 1998; Shakarian and Dwyer 2000), to date little evidence exists per se concerning their roles in the biology of *Leishmania*. The *LdLIP3* gene which encodes a secretory lipase is a previously unstudied open reading frame which could hold a key to a better understanding of host-pathogen interaction and may help to determine the role lipases play to the development and survival of these important human pathogens.

The current study was carried out to characterize the *L. donovani* lipase gene, *LdLIP3*. A restriction map of the *LdLIP3* gene locus was constructed using Southern hybridization analysis of *L. donovani* gDNA digested by various restriction endonucleases. The restriction enzymes were used in both single and double digestion reactions and restriction fragments were separated by gel electrophoresis and transferred to nylon membranes. Hybridization of these blotted membranes with an *LdLIP3* full length gene probe under stringent conditions resulted in specific banding patterns upon detection. This data was used to construct a relative restriction map of the *LdLIP3* gene locus in *L. donovani* gDNA (Fig 4) and indicates that *LdLIP3* is present as a single copy within the genome of these

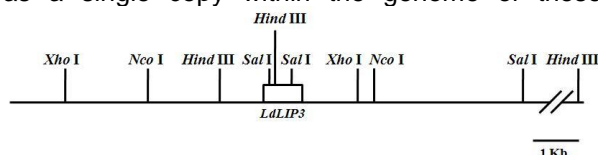


Figure 4. Restriction map and structure of the *LdLIP3* gene locus. The ORF of the *LdLIP3* gene is 927bp in length and is indicated by the open box. Analysis of the previously cloned and sequenced *LdLIP3* gene revealed the ORF is cut once by *Hind* III at 321 bp, and is cut twice by *Sal* I at 261 bp and 685 bp. *Hind* III cuts outside the gene at -1023 bp and 18712 bp. *Nco* I cuts outside the gene at -2781bp and 1706 bp. *Xho* I cuts outside the gene at -4398 bp and 1260bp. *Sal* I cuts outside the gene at 4827 bp. Based upon this data the restriction map of the *LdLIP3* locus was generated.

important human parasites. This is interesting as gene copy number has been shown to provide a simple way of changing protein expression levels in some parasites (Anderson, Patel and Ferdig 2009). This expression strategy ultimately alters a parasite's phenotype without requiring a change in the DNA sequence.

This relative restriction map is the first report of the structure of a secreted lipase gene locus from any species of *Leishmania*. In future studies this data will be used to evaluate what happens if the *LdLIP3* gene locus is knocked-out from the *L. donovani* genome and what happens if it is over-expressed. Such functional studies are facilitated by the knowledge gained from the current study that this gene is present in single copy within the diploid genome of these organisms and should give a better understanding of the roles this enzyme has in the survival and virulence of these pathogens.

Acknowledgments

This project was supported in part by Salve Regina University faculty small grants program and a program project grant funded by RI-INBRE Grant # P20RR016457 from the NCRR/NIH. The contents of this document are solely the responsibility of the authors and do not necessarily represent the official views of Salve Regina University, NCRR or NIH.

References

Anderson T.J., Patel J. and Ferdig M.T. (2009) Gene copy number and malaria biology. *Trends Parasitol.* 25, 336-43.

Belardinelli, M., L. Guerra, G. Gambellini, M. Maroli, M. Mazzini and A. M. Fausto (2007) Immunolocalization of a lipase-like protein in the reproductive apparatus of female *Phlebotomus papatasi* at various stages of gonotrophic cycle. *Annals of Tropical Medicine and Parasitology* 101, 611–619.

Briken, V. (2008), Molecular mechanisms of host-pathogen interactions & their potential for the discovery of new drug targets. *Current Drug Targets*, 9, 150-157.

Brunke S, and Hube B. (2006) MfLIP1, a gene encoding an extracellular lipase of the lipid-dependent fungus *Malassezia furfur*. *Microbiology.* 152, 547-54.

Chanda I., Pan A., Saha S.K., Dutta C. (2007) Comparative codon and amino acid composition analysis of Trityps-conspicuous features of *Leishmania major*. *FEBS Lett.* 581(30), 5751-8.

Debrabant A, Joshi MB, Pimenta PF, Dwyer DM (2004) Generation of *Leishmania donovani* axenic amastigotes: their growth and biological characteristics. *Int J Parasitol.* 34, 205-17.

Ellis S.L, Shakarian A.M, and Dwyer D.M. (1998) *Leishmania*: amastigotes synthesize conserved secretory acid phosphatases during human infection. *Exp Parasitol.* 89, 161-8.

Grimaldi, G., Jr., and Tesh, R. B. (1993) Leishmaniases of the New World: current concepts and implications for future research. *Clin Microbiol Rev* 6, 230-250.

Pignède G, Wang H, Fudalej F, Gaillardin C, Seman M, Nicaud JM. (2000) Characterization of an extracellular lipase encoded by LIP2 in *Yarrowia lipolytica*. *J Bacteriol.* 182(10), 2802-10.

Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A laboratory manual*. 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schaller M, Borelli C, Korting HC, Hube B. (2005) Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses.* 48(6), 365-77.

Shakarian AM, Ellis SL, Mallinson DJ, Olafson RW, Dwyer DM. (1997) Two tandemly arrayed genes encode the (histidine) secretory acid phosphatases of *Leishmania donovani* Gene. 196:127-37. Erratum in: *Gene* 203(2), 251.

Shakarian, A.M., and Dwyer, D.M. (1998) The *Ld Cht1* gene codes the secretory chitinase of the human pathogen *Leishmania donovani*. *Gene* 208, 315-322.

Shakarian AM, Dwyer DM. (2000) Structurally conserved soluble acid phosphatases are synthesized and released by *Leishmania major* promastigotes. *Exp Parasitol.* 95, 79-84.

Singh, N., Rishi K. Somvanshi, S., Sharma, S., Dey, P. K. and Tej P.S. (2007) Structural elements of ligand recognition site in secretory phospholipase A2 & structure-based design of specific inhibitors *Current Topics in Medicinal Chemistry*, 7, 757-764.

Stavrínides, J., McCann, H.C. and Guttman, D.S. (2008) Host-pathogen interplay & the evolution of bacterial effectors; *Cellular Microbiology* 10, 285–292.

Stehr F, Felk A, Gácsér A, Kretschmar M, Mähns B, Neuber K, Hube B, Schäfer W. (2004) Expression analysis of the *Candida albicans* lipase gene family during experimental infections and in patient samples. *FEMS Yeast Res.* 4(4-5), 401-8.

Stinchcombe, J.C., Majorovits, E., Bossi, G., Fuller, S. and Griffiths, S.M. (2006) Centrosome polarization delivers secretory granules to the immunological synapse. *Nature*, 443,28.

Subramanian, R. and Wasan, K. M. (2003) Effect of lipid excipients on *in vitro* pancreatic lipase activity. *Drug Development and Industrial Pharmacy*, 29, 885–890.

UNDP/WorldBank/World Health Organization. (1999) Tropical Disease Research: Progress 1997-1998: Fourteenth Programme Report of UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. World Health Organization, Geneva, Switzerland.

Yordanov, M., Dimitrova, P., Patkar, S., Saso, L. and Ivanovska, N. (2008) Inhibition of *Candida albicans* extracellular enzyme activity by selected natural substances and their application in *Candida* infection; *Can. J. Microbiol.* 54, 435–440.