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Study of specific Leishmania Atypical Lipid Phosphatases (ALPs) as putative virulence factors for parasite survival in host phagocytes and as potential drug targets

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Introduction

The kinetoplastids' phosphatome contains a large number of atypical protein phosphatases most of which belong to the dual-specificity phosphatase (DSP) family and show considerable divergence from classic DSPs in higher eukaryotes in both the domain organization and sequence features in their catalytic domain. These are referred as atypical DSPs (aDPs)(Brenchley R. et al., BMC Genomics, 2007, 26;8:434) (Fig1). The distinct traits of the aDPs from the Leishmania sps can be exploited in the selection of suitable new targets for drug development taking advantage of the already extensive knowledge on protein phosphatase inhibitors (Sobhia ME. et al.2012, Expert Opin. Ther Pat. 22(2):125-5); Heneberg P. (2009). Curr Med Chem. 16(6):706-33)

A subgroup of the aDSPs from Leishmania sps with similar catalytic P-loop with the *M.tuberculosis* virulence factor MptpB phosphoinositide (PI) phosphatase belong to a family of PI phosphatases designated as atypical lipid phosphatases (ALPs) recently described in bacteria and lower eukaryotes (Beresford et al., 2010, BMC Genomics, 11:457). Using the P-loop signature (HCXXGKDR) as a "diagnostic motif", 200 uncharacterized putative phosphatase sequences with conserved signature were identified in bacteria, fungi and protozoa (Beresford et al. ,2010). One of these proteins is the MptpB, a virulence factor from Mycobacterium tuberculosis, with distinct active site **P-loop signature**, that shares characteristics with eukaryotic lipid phosphatases and protein tyrosine phosphatases (Fig 2). Sequences with the characteristic P-loop signature HCXXGKDR were identified in the Leishmania major chromosomes 22 and 33. Preliminary data from biochemical analysis of the Leishmania major members of this family indicated that like the MptpB and the Listeria monocytogenes homologues they have phosphatase activity towards phosphoinositides. No biological role has been yet assigned to any of these proteins.



Heterologous expression of the recombinant rLdPIPh22 in bacteria (E.coli BL21) as a 8His-tagged protein & purification from bacteria lysates with the Ni2+ agarose beads

Transformation of *E.coli* (BL21 stain) with the recombinant pTriex 1.1-*La*PIPh22 plasmid Expression of the LaPIPh22-8His-tagged protein in E.coli BL21 cells Batch purification of the 8His-tagged protein from bacteria using the Ni2+agarose system



The **ALP** enzyme family in microorganisms, with a conserved P-loop motif and distinct sequence and biochemical characteristics to classic eukaryotic lipid phosphatases and no human homologues, are attractive potential pharmaceutical targets against infectious diseases.

The parasites of the genus Leishmania are protozoan organisms of the Trypanosomatidae family. When transmitted to the mammal host by the bite of the insect host (sandflies of the genus Phlebotomus or Lutzomyia) (Fig. 3) cause a wide spectrum of diseases called Leishmaniases, the most serious of which is visceral Leishmaniasis (kala azar) which if left untreated is fatal. The Leishmania parasites exist in two forms, the promastigote which lives in the vertebrate host and the amastigote that propagates in the macrophages of the vertebrate host (Fig 4).

Although several anti-Leishmanial drugs are available, the need to develop new specific drugs is urgent due to severe side effects of the existing ones, high cost and development of drug resistant parasite strains.



(Brenchley R. et al., 2007)

 Eukaryotic lipid phosphatases	PTP domain PTP active site PH-GRAM Domain
 Bacterial, plant and Leishmania lipid phosphatases	<pre>PDZ-domain FYVE domain C2 domain Transmembrane segments Prokaryotic membrane lipoprotein lipid</pre>

Fig.2 Domain organization of Atypical Lipid Phosphatases. ALP and

PFA lipid phosphatases lack the classic lipid binding domains. Only

Lmo1800 (from Listeria monocytogenes) contains a predicted lipid attachments sequence (Beresford et al., 2010)







Fig. 7 A) Analysis of bacteria lysates (total lysate) from *E.coli* expressing the *Ld*PIPh22-8His w/o (NI) or with (IN) induction (0,1mM IPTG, 3hrs) [SDS-PAGE (left panel); Western Blot using an anti-His mAb (right panel)]; B) SDS-PAGE analysis of all the LaPIPh22-8His purification steps. The protein was eluted from the Ni2+ beads with 250 mM imidazole. C) Western Blot of total lysate, soluble fraction (S/N) and insoluble fraction (pellet) from the purification procedure. More than 75% of the protein was recovered in the soluble fraction. In this case the protein was produced by growinng the bacteria w/o IPTG O/N at 25oC;

Phosphatase activity of the recombinant rLdPIPh22-8His produced in *E.coli*

AIM

We study two members of the ALP enzyme family [i.e. LDBPK_220120 (LdPIPh22) and LDBPK_332990 (LdPIPh33) (Fig.1 blue frame) putative PI dual specificity phosphatases] from the unicellular protozoan parasite L.donovani, the causative agent of visceral Leishmaniasis, as potential molecular targets for the development of new anti-leishmanial drugs.

Methodology-Results

Cloning of LdPIPh22 and LdPIP33 from L.donovani LG13 strain

The LaPIPh22 (LDBPK_220120) and LaPIPh33 (LDBPK_332990) genes were amplified by PCR using primers specific for the beginning and end of ORFs and L.donovani LG13 Ethiopian strain genomic DNA as template. Subsequently the PIPh22 & PIPh33 PCR products were inserted in the vector pTriex 1.1 (Fig. 5A) to produce 8His tagged fusion protein, in pLEXSY-satmRFP (Fig. 5B), specific for expression in protozoan parasites of the Trypanosomatidae family and in pEGFP-N3 (Fig. 5C) for expression in mammalian cells as a GFP fusion protein. Insertion was confirmed after digestion of the PCR product (Fig. 5) and the vectors with the suitable restriction enzymes and ligation of the digested DNA fragments. The PIPh22 and PIPh33 sequences were verified by sequencing of the pTriex1.1plasmids.





Fig. 5. Plasmid maps and agarose gels showing the analysis of PCR products of LdPIPh22 cloned into the pTriex1.1, pLEXSY-sat and pEGFP-N3 expression vectors.

Bioinformatics analysis



LdPIPh33 from LG13 strain

Comparison with homologues from other Leishmania sps

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LdLG13_PIPh33	MTSVIQRLRI	RVEAEGLENLRNLGG	YHTNNSTKTKF	RRGVVYRSDQL	FRIPACMAQE	RGLVDQLRIRH	VYDLCDSTEV	SEKRY
LdBPK_332990	MTSVIQRLRI	RVEVEGLENLRNLGG	YHTNNSTKTK	RRGVVYRSDQL	FRIPACMAQE	RGLVDQLRIRH	VYDLCDSTEV	SEKRY
LinJ33_V3.2990	MTSVIQRLRI	RVEVEGLENLRNLGG	YHTNNSTKTTF	RRGVVYRSDQL	FRIPACMAQE	RGLVDQLRIRH	VYDLCDSTEV	SEKRY
LmjF33.2840	MTSVIQRLRI	REVEGLENLRNLGG	YHTNNSTKTTF	RWGVVYRSDQL	FRVPADVAQ	VLVDQLHIRH	VYDLRANTEV	SAKRY
LMXM_32_2840	MTSVIQRLRI	RVEVEGLENLRSLGG	YHTNNSTKTTF	RWGVVYRSEQL	CRVPADVAQ	R VLVDQLHIHH	VYDLRDNTEV	SAKRY
LtaP33.3090	MAAVIQRLRI	RVEVEGLENLRNLGG	<u>үнт</u> к <mark>и</mark> бтктти	WGVVYRSDQI	SRVPTDVVQ	<u>ζνιΓεριηι</u> ήσ	VYDLRDNNEV	LARPY

LdLG13_PIPh33 SLLHMQHTSLPIDMSNANRFLKEGENLKQVATAHRFMQEIDREFVRSYALTVGLIIKGIIGSKASCDKAFLIHCTAGKD
LdBPK_332990 SLLHMQHTSLPIDMSNANRFLKEGENLKQVATAHRFMQEIDREFVRSYALTVGLIIKGIIGSKASCDKAFLIHCTAGKD
LINJ33_V3.2990 SLLHMQHTSLPIDMSNANRFLKEGENLKQVATAHRFMQEIDREFVRSYALTVGFIIKGIIGSKASCDKAFLIHCTAGKD
LmjF33.2840 SLPHMQYTSLPIEMSNANRFLKEGENLKQVATAHRFMQEIYREFVRSYAPTMGLIIKGIIDSKASCDNAFLIHCTAGKD
LMXM_32_2840 SLTHMQRTSLPIDMSNANQFLKEGEDLKQVATAHRFMQEIYRDFVRSHEPTVGLIIKGIIDNKASCDNAFLIHCTAGKD
Ltap33.3090 SLPHMQRTSLPIDMSNTHQFLKKGDDLKHVATAHVFMQEIYREFVRSYAPTVGLVIKGIIHSKASCDNASLIHCTAGKD

LdLG13_PIPh33 TGWCCYVLLTLLDVTEKEKRADYLLTNTFVGIPADAWDYNGAEGMSEEAMAALWAAFNEYLDAALDELSKMGGI...... Linj33_V3.2990 TGWCCYVLLTLLDMTEKEKRADYLLTNTFVGIPADAWDYNGAEGMSEEAMAALWAAFNEYLDAALDELSKMGGIYKYAKS Linj33.2840 TGWCCYVLLTLLDVMEEKKRSDYLLTNTFVGIPADAWDYNGAEGMSEEAMAALWAAFNEYLDAALDELSKMGGIYKYAKS LMXM_32_2840 TGWCCYVLLTLLDVMEEKKRADYLLTNTFVGIPADAWDYDGAEGMSEEAMTALWTVFHEYLDAALDELNKMGGIYKYAKS LMXM_32_000 TGWCCYVLLTLLDVTEEKRADYLLTNTFVGIPADAWDYDGAEGMSEEAMTALWTVFHEYLDAALDELNKMGGIYKYAKS LMXM_32_000 TGWCCYVLLTLLDVTEEKRADYLLTNTFVGIPADAWDYDGAEGMSEEAMTALWTVFHEYLDAALDELNKMGGIYKYAKS

Comparison with Mycobacterial ptpB (MptpB) related phosphatases from pathogenic bacteria

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Fig. 8 Purified rLdPIPh22-8His (A: SDS-PAGE with purified fractions) was used to measure phosphatase activity (B) using the synthetic substrate p-nitrophenyl-phosphate. Phosphatase activity was measured at 5 different pH values and at 25oC and 37oC. Activity is presented as the OD405 values of the generated product p-nitrophenolate (C). Values represent averages from 2-4 measurements obtained with protein from 2 different purification batches. Activity at 25oC is 21% higher than at 37oC.

Heterologous expression of *Ld*PIPh22 -GFP in AGS gastric epithelial cells, in NIH3T3 fibroblasts and in HeLa cells



Fig.9 Heterologous expression of *La*PIPh22-GFP or *La*PIPh-8His was performed in AGS human adenocarsinoma gastric epithelial cells, in NIH3T3 mouse fibroblasts and in HeLa cervical carcinoma cells by transfection with the pEGFP-LdPIPh22, pTriex1.1-LdPIPh22 plasmids (Fig.5 A&C).

AGS cells, 12 hrs post-transfection were infected for 16 hrs with Helicobacter pylori.

All cells were analysed 30 hrs post-transfection by confocal microscopy after staining for filamentous actin with Phaloidin-Alexa-546 (B) or for DNA with Propidium lodide (A) or with the anti-His mAb in the case or LdPIPh22-8His expression (D).

LdPIPh22-GFP localizes more intensely in sites of dynamic actin polymerization (arrows) i.e in lamelipodia, filopodia (C, inset), cortical actin leading edges (D), cell adhesion and focal adhesion points (**B**, **D**).

In the AGS cells infected with H.pylori (A, red FL), LaPIPh22-GFP localized on protrusions and elongations (A, arrows) induced by the bacterium through the intracellular CagA protein action on actin cytoskeleton dynamics (Wessler S. et al. Cell Comunication and signalling 2011, 9:27).

All localizations observed for rLdPIPh22 are sites rich in phosphoinositides and PI phosphatases that modulate signaling related to actin cytoskeleton reorganization directing growth, invasion, migration, and focal adhesions.

The nuclear localization in the pEGFP-LdPIPh22 transfected cells is due to GFP protein produced by additonal translation initiation from the GFP ATG codon in the pEGFP plasmid.

Expression and localization of endogenous *Ld*PIPh22 in *L.donovani* LG13 strain



Fig. 10. The endogenous *Ld*PIPh22 was localized in *L.donovani* LG13 strain by

MAVRELPGAWNFRDVADTATA-AIVSERILE LESVENERDMEGYASKNERKI-KMERKLYRSSSILVNIN-EINDAALLAKSENIVNITVOLRSSSEVKAOPTPAIE TLKPGSQIKLEGAVNVRDLGGYKTTDGLT-IKPHKLIRSAELANUSDSDKKKUVNTYDLSHIVDERTSSEVATKPDPKLT TESHPSLIPLDGGINERDLGGNLAADGRR-IKPGULERSGSLDRUS-TNDCDFUSHSNVTQIIDYRDADEVQAKPDVLWP VTVAERRLPLQGAFNERDMEGYETTEGRK-VKMEKLYRSEELAGUT-EMDIDYLQKSGUKLUCDYRTDFEVKHKPNPEIT MptpB Imo1935 Imo1935 Imo1800 Imo1800 ypes bant ypes bant Ld_LG13_PIPh22G MptpB DG Imo1935 G LdLG13_PIPh33 LHMQHTSLPIDMSNANRFLKEGE----NLKQVATAHRFMQEIDREFVRSYALTVGLI-IKGIIGSKASCD------lmo1800 MDMTHDSVMKDNGTSTSTQDLTASLAKMDNPETFLINANKSFITDETSIQAYKDFFDILLANQ-------ĂĦMĦŊŢPĂŊPŢS--SĒVŊAŊLĒKLTŊETLATEDV<u>RA</u>EMEELYHRLPENSLĀMQŪLVNLLQNCASTOPVASSVVHS ypes bant ·ARQVCLEVMQDL - AKDLNINEFFQVGDLSMLGKPGEYLVKMNQDFVSGNEAFVNFLNLAQNPE - - - - - - - - 1 200 270 200 200 500 510 5 1 Ld_LG13_PIPh22 MptpB Imo1935 lmo1800 ypes bant

Ld_LG13_PIPh22

- 2 I LE RADAFFLE L CEGEV CKRYGSVNAYMEKELGISVEQLEKLRSYVVRPTSSSS GVLGVRAEYLAAARQTIDE TYGSLGGYLR-DAGI SQATVNRMRGVLLS-----PMAEARPEYLEJAFDEMKKQYGSVANYLEKGIGUTATEKAAFQKEMLE-----MptpB Imo1935
- TNAAFDETNAKYGSMONFILKEKLGITDAKKEQLKKAYLY-----
- lmo1800 ypes bant
- FVLSAREEFIQTTLRSIHERYGSRERWLKHEFGLGSIEREKLQSYFLE-----AMFEARAEYLQAAIDEVKKQYGSVEAYAEKALGFTKESLEEMKVLLLEDK----

TL	LOLG13_PIPh33	CHMQHISLPIDM <u>S</u> NANRFI	LKEGENLK	QVATAHREMQETUR	EFVRSYALIVGLI	- IKGIIGSKASC	D
	MptpB	PDGIDVHLLPFPDLADDD	ADDSAPHETAFK	RLLTNGGSNGESGE	SSQSINDAATRYM	TDEYRQFPTRNG	AQRALHRVVTL
	Imo1935	EGILNKHIPIGTAKNEET	КЦР	VTNDTTIYEPLMGE	SYRVFVQSVEGFR	EIFTEVLEDAKA	G
SG	Imo1800	TOVDYTHOSVMKDNGTSTS	STQDLTAS LA	KMDNPETFLINANK	SFITDETSIQAYK	DFFDILLANQDG	
	ypes	PGAHYHNIPANPLSSEVNA	ANLEKLTNETLA	TFDVR-AFMFELYH	RLPFNSLAYQQLV	NLLQNCASTDPV	ASSVVHSG
	bant	TGARQVCLPVMQDLAKDLI	NINEFFQVGDLS	MLGKPGEYLVKMNQ	DFVSGNEAFV	NFLNLAQNPENL	
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SD	LdLG13_PIPh33	KAFLIHCTAGKDRTC	SWCCYVLLTLLD	VTEKEKRADYLLTN	TFVGIPA	DAWDYNGAEGMS	EEAMA
	MptpB	LAAGREVLTHCEAGKDRTC	SFVVALVLEAVG	LDRDVIVADYLRSN	DSVPQLRARISEM	IQREDTELAPE	VVTFTKARLSD
	Imo1935	LPFVFHCTAGKDRTC	σνισαιιιτιτο	VPEKTIFDDYAITN	RYQDDILQEMGGI	VSLFSSGTEKID	LETFR
	lmo1800	SVLWHCTAGKDRAK	SFGTALVLSALG	ν <u>σκητνι</u> σργμες η	KYRADENKKALEA	VAAKTD NKKV	I DGMT
	ypes	DVVHRGIVOHCAVGKDRT	SVGAALVLFALG	ADESTVLEDYLLTE	TTLKPFREHMLAE	LALKLN DQA	LAQFT
	bant	PLVNHCTAGKDRTC	3 F G S A L L L L L L G	VPEKTVMEDYLLSN	GFRERLNEKMMAF	LGAKLQ NDES	RAILG
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		330	340 350	360	370		
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	LdLG13_PIPh33	ALWAAFNEYLDAALDELS	(MGG)				
	MptpB	GVLGVRAEYLAAARQTIDE	ETYGSLGGYLR-	DAGISQATVNRMRG	VLLG		
	Imo1935	PMAEARPEYLETAFDEMK	ROYGSVANYLEK	GIGITATEKAAFQK	EMLE		
	lmo1800	AVMENRE SYLINAAFDE INA	AKYGSMDNFLKE	KLGLTDAKKEQLKK	AYLY		

LRSIHERYGSRERWLKHEFGLGSIEREKLQSYFLE-

EARAEYLQAAIDEVKKQYGSVEAYAEKALGFTKESLEEMKVLLLEDK

Fig. 6 (A,C) ClustalW multiple alignment of LaPIPh22 and LaPIP33 protein sequences from LG13 strain with corresponding genes from L.donovani strain BPK282A1 (BPK_220120), L.infantum (LinJ_22_0120), L.major Friedlin (LmjF_22_0250), L.mexicana strain MHOM (LmxM_22_0250) and L.tarentolae Parrot-Tarll (LtaP22.0250). PIPh22 and PIP33 genes are highly conserved amongst Leishmania sps (seq.identity>90%) apart from L.braziliensis (~6% identity) while they are not found in Trypanosoma. (B,D) Sequence alignment of LaPIPh22 or LaPIPh33 genes from LG13 strain with homologues from Mycobacterium tuberculosis (MptpB, GenBank: CCC62750.1, 15,1% identity), Listeria monocytogenes [Imo1800, NP_465325, 29% identity; Imo1935, NP_465459) 24% identity], Yersinia pestis KIM10+ [NP_667783.1), 19% identity] and Bacillus anthracis strain Ames [(NP_845680), 25,2% identity]. The red frame indicates the P-loop motif sequence.

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CONCLUSIONS-FUTURE PERSPECTIVES

- * The LdPIPh22 and LdPIPh33 ALPs (Mycobacterial ptpB (MptpB) related phosphatases) from L.donovani have orthologues in all Leishmania sps, except Leishmania braziliensis, with high (>90%) sequence identity.
- The LdPIPh22 has high sequence identity with homologues fro pathogenic bacteria as Listeria monocytogenes and the Bacillus anthracis homologues (24-29%identity).
- Recombinant LdPIPh22-8His isolated from bacteria dephosphorylates the pNPP substrate with optimum pH=6.0. The substrate specificity of LdPIPh22-8His is under investigation.

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- Endogenous LdPIPh22 in parasites localizes in structures resembling leishmanial actin while it is absent from the flagellum. This localization is going to be confirmed by colocalization experiments with anti-LdACT antibodies.
- + Heterologous Expression of LdPIPh22-GFP and LdPIPh22-8His in mammalian tissue culture cells localizes on sites of dynamic actin polymerization (lamelipodia, filopodia, cell adhesion an dfocal adhesion points, where PI metabolism plays important role.
- Parasites overexpressing LdPIPh22-8His and LdPIPh33-8His and knock out strains are being generated and will be analyzed for growth, morphological and virulence characteristics in order to deduce a functional role for the LdPIPh22 protein in the parasites.

immunofluorescence and confocal microscopy.

Cells were stained with an anti-*Ld*PIPh22 rabbit polyclonal antibody generated by us using the rLdPIP22-8His as an antigen. Leishmania cells were fixed with 4%w/v PFA and subsequently stained with the first antibody and a second anti rabbit Alexa-488. DNA was stained with Propidium lodide.

The staining of LdPIPh22 resembles the staining pattern observed for the Leishmanial actin published by *Kapoor et al. in 2008*. (Fig 11)



Fig. 11 In vivo analysis of LdACT (actin) filaments labelled with anti-LdACT antibodies (red). Kapoor P et al. J. Biol. Chem. 2008;283:22760-22773)