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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.

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.

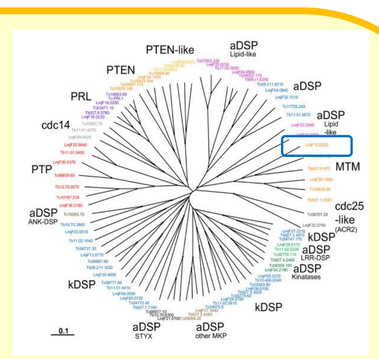


Fig.1 Radial phylogenetic tree of TriTryp DSPs and PTPs. (Brenchley R. et al., 2007)

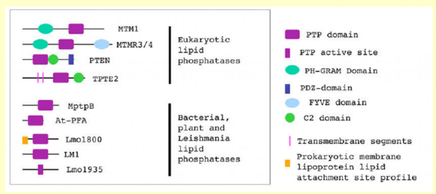


Fig.2 *Domain organization of Atypical Lipid Phosphatases.* ALP and PFA lipid phosphatases lack the classic lipid binding domains. Only Lmo1800 (from *Listeria monocytogenes)* contains a predicted lipid attachments sequence *(Beresford et al. , 2010*)

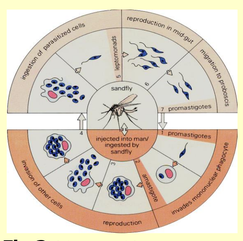


Fig.3: Life cycle of *Leishmania sp*.

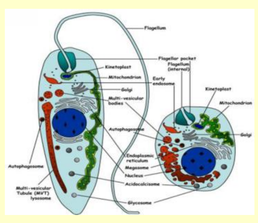
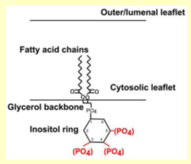


Fig. 4 Promastigoes (left) and amastigotes (right) forms of Leishmania



Structure of phosphoinositide (PI)

AIM

We study two members of the ALP enzyme family [i.e. LDBPK_220120 (*Ld*PIPh22) and LDBPK_332990 (*Ld*PIPh33) (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 *Ld*PIPh22 and *Ld*PIP33 from *L.donovani* LG13 strain

The *Ld*PIPh22 (LDBPK_220120*)* and *Ld*PIPh33 (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-sat-mRFP (***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.

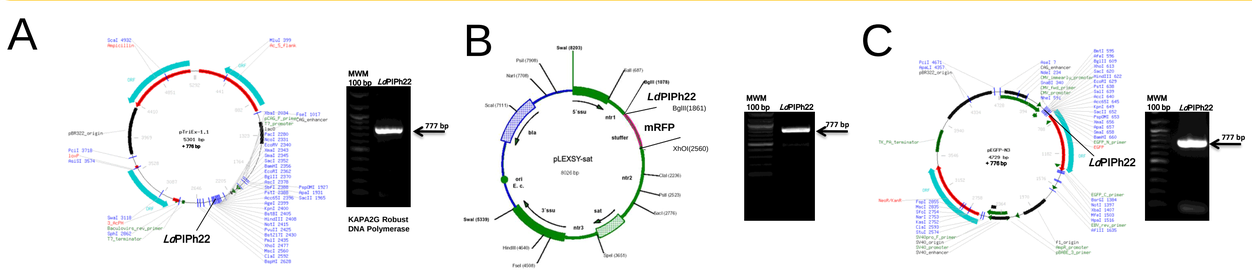


Fig. 5. Plasmid maps and agarose gels showing the analysis of PCR products of *Ld*PIPh22 cloned into the pTriex1.1, pLEXSY-sat and pEGFP-N3 expression vectors.

Bioinformatics analysis

***Ld*PIPh22 from LG13 strain**

***Ld*PIPh33 from LG13 strain**

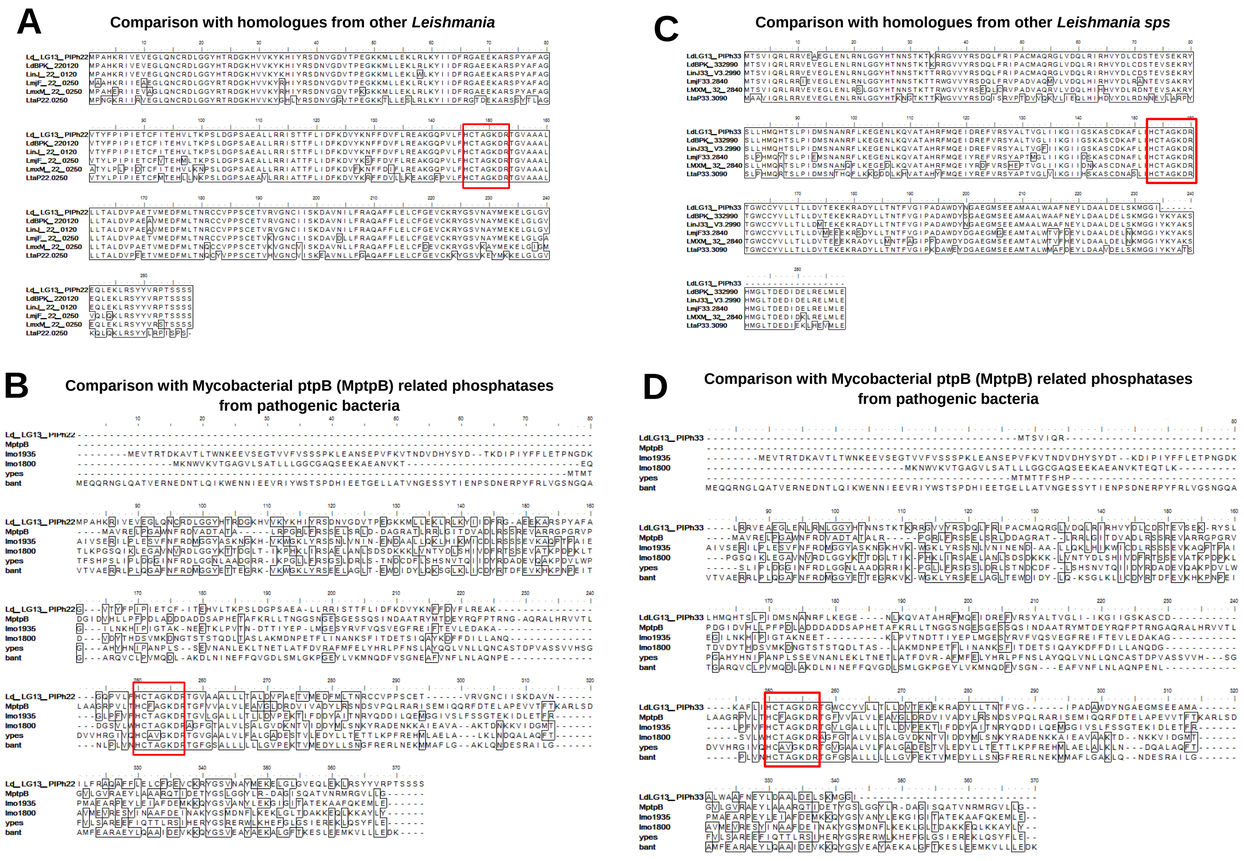


Fig. 6 (A,C) ClustalW multiple alignment of *Ld*PIPh22 and *Ld*PIP33 protein sequences from LG13 strain with corresponding genes from *L.donovani* strain BPK282A1 (*BPK_220120), L.infantum (LinJ_22_0120), L.major Friedlin (LmjF_22_0250), L.mexicana strain* MHOM *(LmxM_22_0250) and L.tarentolae Parrot-TarII* (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 *Ld*PIPh22 or *Ld*PIP33 genes from LG13 strain with homologues from *Mycobacterium tuberculosis* (MptpB, GenBank: CCC62750.1, **15,1% identity**), *Listeria monocytogenes* [lmo1800, NP_465325, **29% identity**; lmo1935, 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.

Heterologous expression of the recombinant r*Ld*PIPh22 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-*Ld*PIPh22 plasmid
- Expression of the *Ld*PIPh22-8His-tagged protein in *E.coli* BL21 cells
- Batch purification of the 8His-tagged protein from bacteria using the Ni2+agarose system

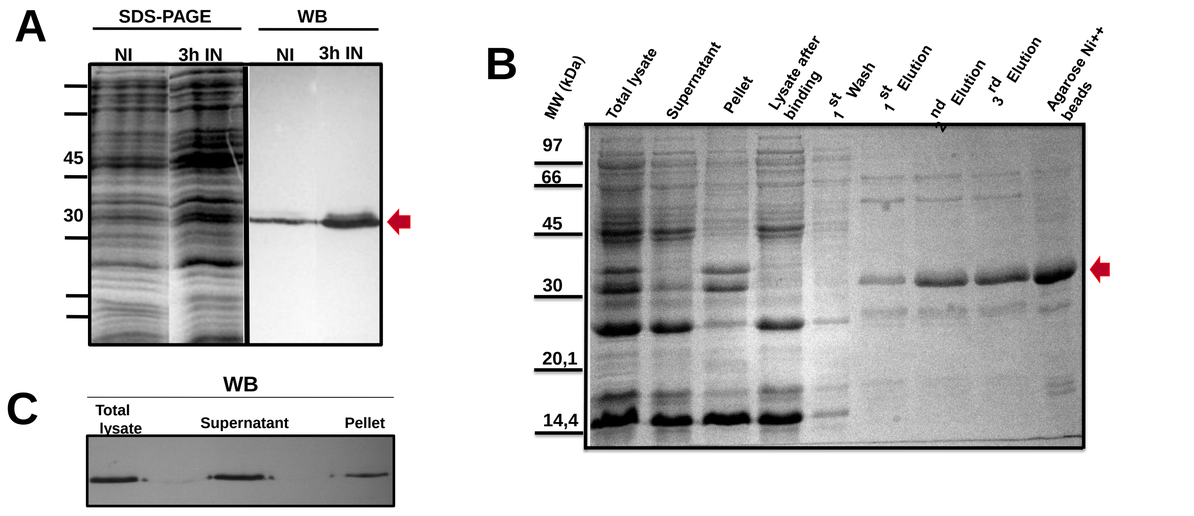


Fig. 7 **A**) Analysis of bacteria lysates (total lysate) from *E.coli* expressing the *Ld*PIPh22-8His w/o (NI) or with (IN) induction (0,1mM IPTG, 3hrs) [SDS-PAGE (left panel); Western Blot using an anti-His mAb (right panel)]; **B**) SDS-PAGE analysis of all the *Ld*PIPh22-8His purification steps. 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 r*Ld*PIPh22-8His produced in *E.coli*

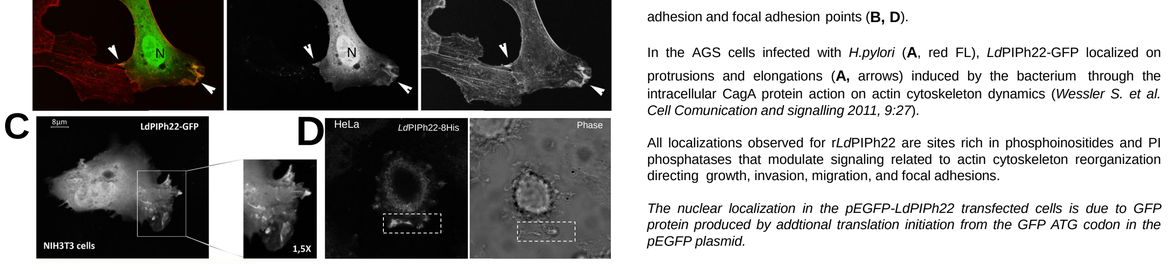


Fig. 8 Purified r*Ld*PIPh22-8His (**A**: SDS-PAGE with purified fractions) was used to measure phosphatase activity (**B**) using the synthetic substrate p-nitrophenyl-phosphate. Phosphatase activity was measured at 5 different pH values and at 25oC and 37oC. Activity is presented as the OD405 values 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 *Ld*PIPh22-GFP or *Ld*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-*Ld*PIPh22, pTriex1.1-*Ld*PIPh22 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 Iodide (**A**) or with the anti-His mAb in the case or *Ld*PIPh22-8His expression (**D**).

*Ld*PIPh22-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), *Ld*PIPh22-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 r*Ld*PIPh22 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 addtional translation initiation from the GFP ATG codon in the pEGFP plasmid.

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

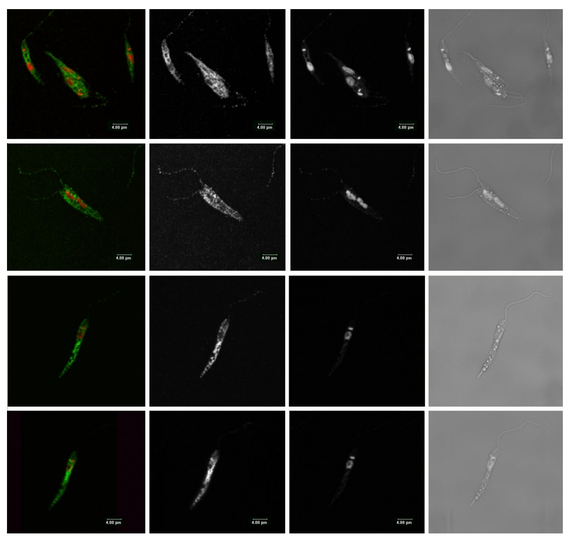


Fig. 10. The endogenous *Ld*PIPh22 was localized in *L.donovani* LG13 strain by immunofluorescence and confocal microscopy.

Cells were stained with an anti-*Ld*PIPh22 rabbit polyclonal antibody generated by us using the r*Ld*PIP22-8His 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 Iodide.

The staining of *Ld*PIPh22 resembles the staining pattern observed for the Leishmanial actin published by *Kapoor et al. in 2008*. (**Fig 11)**

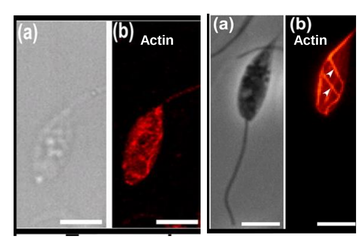


Fig. 11 In vivo analysis of *Ld*ACT (actin) filaments labelled with anti-*Ld*ACT antibodies (red). Kapoor P et al. *J. Biol. Chem*. 2008;283:22760-22773)

CONCLUSIONS-FUTURE PERSPECTIVES

- The *Ld*PIPh22 and *Ld*PIPh33 ALPs (Mycobacterial ptpB (MptpB) related phosphatases) from *L.donovani* have orthologues in all *Leishmania sps*, except *Leishmania braziliensis,* with high (>90%) sequence identity.
- The *Ld*PIPh22 has high sequence identity with homologues fro pathogenic bacteria as *Listeria monocytogenes* and the *Bacillus anthracis* homologues (24-29%identity).
- Recombinant *Ld*PIPh22-8His isolated from bacteria dephosphorylates the pNPP substrate with optimum pH=6.0. The substrate specificity of *Ld*PIPh22-8His is under investigation.
- Endogenous *Ld*PIPh22 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-*Ld*ACT antibodies.
- Heterologous Expression of *Ld*PIPh22-GFP and *Ld*PIPh22-8His 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 *Ld*PIPh22-8His and *Ld*PIPh33-8His 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 *Ld*PIPh22 protein in the parasites.

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