Study of specific *Leishmania* histidine acid phosphatases as putative virulence factors for parasite survival in host phagocytes and as potential drug targets

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Abstract

Leishmaniases are (re)emerging neglected Tropical diseases caused by *Leishmania*. Chemotherapy treatment is difficult to administer, expensive and progressively ineffective due to emergence of drug resistance. The need for new and effective leishmanicidal agents is urgent and identification of novel drug targets is a strong challenge for Biomedical Research. One class of molecular targets are **phosphatases**.

We study a putative extracellular membrane bound (***Ld*MAcP**) and a secreted (***Ld*SAcP1**) histidine acid phosphatase from *L.donovani,* molecules highly conserved within the *Trypanosomatidae* family of protozoan parasites (*Sacharian et al. 2003*). Our overall objective is the identification of distinct molecular and functional characteristics of these enzymes that will be valuable in designing selective inhibitors with potential therapeutic value.

Introduction

***Leishmania sp.* are protozoan parasites**. The life cycle of the parasite includes a non-intracellular stage (promastigote phase) in the insect host and an **obligatory intracellular stage (amastigote phase) in phagosomes of mammalian host phagocytes** where they proliferate and establish a replicative niche (**Fig 1**). *Leishmania donovani* is the causal agent of the potentially fatal disease, visceral leishmaniasis (VS-Kala azar), in humans.

The **histidine phosphatase superfamily** is a large functionally diverse group of proteins. They share a conserved catalytic core centered (**Fig. 2**) on a histidine residue which becomes phosphorylated during the course of catalysis. The superfamily contains two branches sharing very limited sequence similarity. Human representatives of both branches are of considerable medical interest, and various parasites contain superfamily members whose inhibition might have therapeutic value (*Rigden D. J. 2008*).

Genes encoding distinct members of the histidine-acid phosphatase enzyme family from *Leishmania donovani* (***Ld* SAcP-1 , *Ld*SAcP-2 & *Ld*MAcP**), *have been* identified and partly characterized since the early 90's *(Shakarian A. Dwyer DM, et al., 2003)* **(Fig. 3).** In these studies, it was suggested that these enzymes may play essential roles in the growth, development and survival of this organism, but their specific biological roles have not been explored.

Aim of this work is to characterize further the structural properties of these enzymes and develop strategies to study their function in the life cycle of the *L.donovani* parasite and their potential virulence activity.

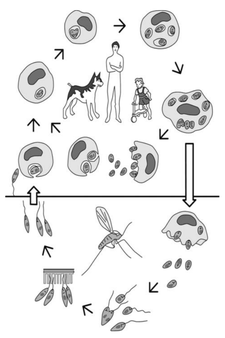

Fig. 1: Life cycle of *Leishmania sp.*

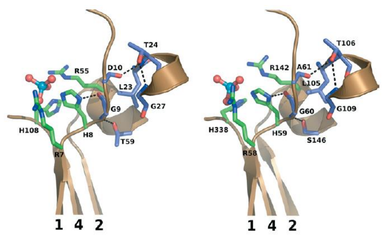

Fig. 2: The near-superimposable conserved catalytic cores of E. coli SixA (left) & A. fumigatus phytase (right) (*Rigden D. J. 2008*).

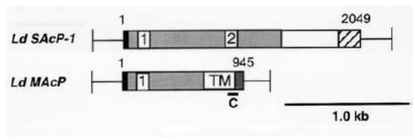

Fig. 3: Diagrammatic representations of the *L.donovani* Histine acid phosphatase gene family *(Shakarian A. Dwyer DM, et al., 2002)*

Methodology-Results

Sequence comparison of *Ld* MAcP & *Ld*SAcP-1 and predictions for structural motifs and post-translational modifications

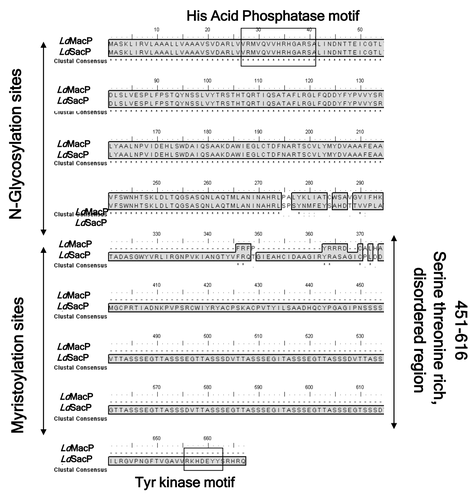

Fig 4: Alignment of *Ld*SAcP1 (***LinJ36_V3.6770***) & *Ld*MAcP (***LinJ36_V3.6740***) protein sequences *(ClustalW2)*. His-acid phosphatase and tyr kinase motifs are indicated. Areas with predicted post-translational modifications are shown on the sides.

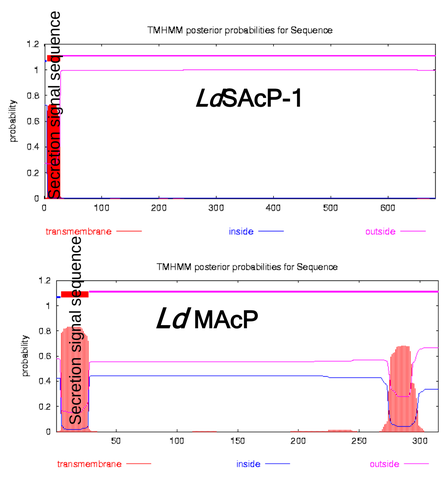

Fig 5: Prediction profiles for transmembrane helices in *Ld*SAcP & *Ld*MAcP protein sequences (***TMHMM Server v. 2.0***)

Blast analysis for *Ld* MAcP & *Ld*SAcP-1

Construction of *Ld*MAcP plasmids

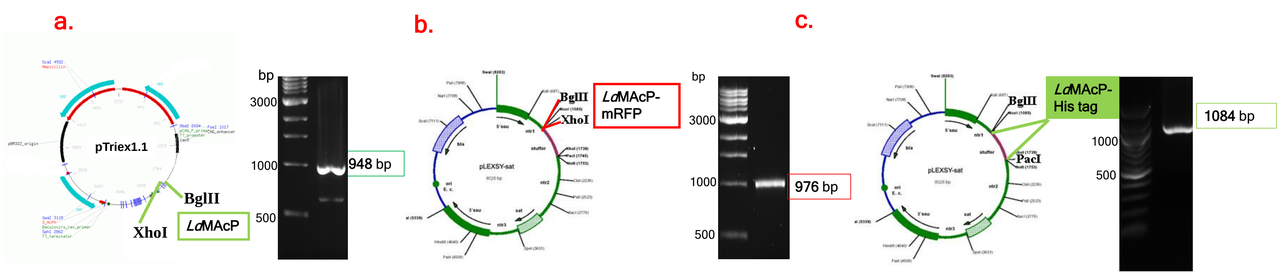

Fig 5: The *Ld*MAcP gene was amplified by PCR using primers specific for the beginning and end of the ORF and genomic *L. donovani* DNA as template. The MAcP PCR products with appropriate restriction sites added were inserted after RE digestion into the pTriex 1.1 vector (***Fig. 5a***) in front of the sequence of a 8His tag or in the pLexsy-sat-mRFP (*Kotini M. et al. and Boleti H.2009*) plasmid (***Fig. 5b***) in front of the mRFP gene. The *Ld*MAcP-8His tag gene was inserted into the pLexsy-sat vector. (***Fig. 5c***). The pTriex1.1 vector allows expression in *E.coli* and mammalian cells, The pLexsy-Sat vector is specific for expression in protozoan parasites of the *Trypanosomatidae* family.

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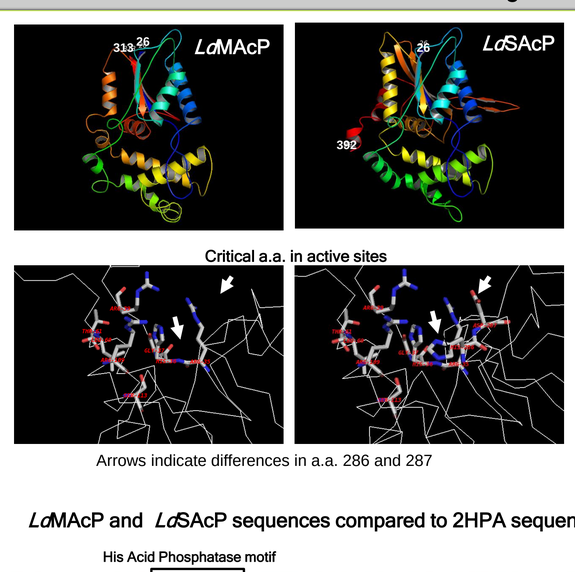
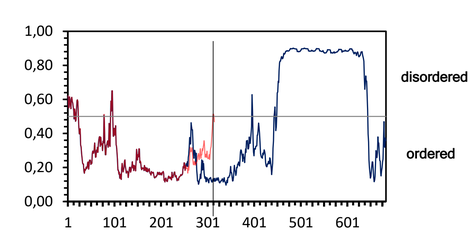
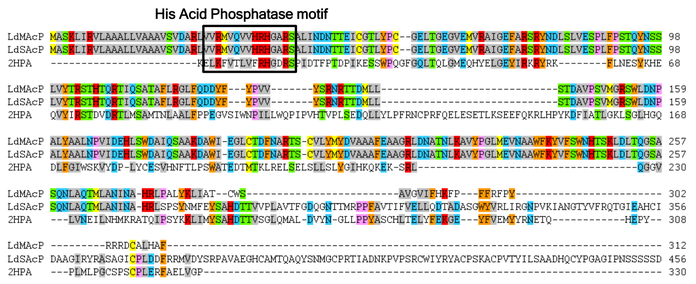
3D -modeling of *Ld*MAcP & *Ld*SAcP-1



Probability for *Ld*SAcP and *Ld*MAcP for disordered structure



*Ld*MAcP and *Ld*SAcP sequences compared to 2HPA sequence



*Ld*MAcP superimposed on 2HPA and *Ld*SAcP

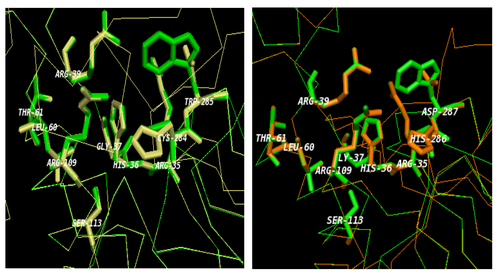


Fig 6 : 3D Molecular Models of ***Ld*MAcP** (residues 26 - 313) and Ld**SAcP** (26-392) were generated by comparative modeling, based on the structure of the human prostatic acid phosphatase (PDB code: 2HPA, residues 1001-1343). Residues conserved throughout the histidine acid phosphatase superfamily are also conserved in *Ld*MAcP and *Ld*SAcP1, with the exception of **His-286** and **Asp-287**, which are missing in *Ld*MAcP (*Swiss Model Workspace* [Arnold K., et al. (2006)], *Procheck* [Laskowski et al. (1993)] & *Verify3D* [Luthy R., Nature (1992)]. Structures were visualized and molecular images were generated using *Pymol* [DeLano Scientific, USA, http://www.pymol.org/])

Expression and purification of the *Ld*MAcP-8His-tagged protein in *E.coli* / Generation of anti-*Ld*MacP pAb

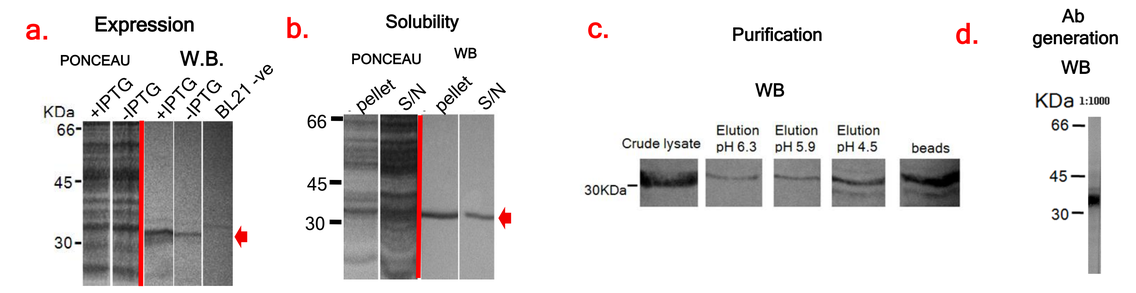


Fig 7: Expression of the *Ld*MAcP-8His-tagged protein (31,7 kDa) in *E.coli* (BL21 stain) bearing the pTriex1.1-*Ld*MacP plasmid (***Fig. 7a***) after induction with IPTG (0,1 mM). *Ld*MAcP-8His was recovered mainly in the insoluble fraction (***Fig. 7b***) and was purified under denaturing conditions (***Fig. 7c***). Detection of the 8His-tagged was performed with the anti-6His mAb (*SM1693PS, Acris Abs*). *E.coli* produced *Ld*MacP-8His (a.a. 1-274) was used to generate pAbs in mice. In (***Fig. 7d***) the mouse pAb was tested against *E.coli Ld*MAcP-8His lysates.

Localization and acid phosphatase activity of *Ld*MAcP-mRFP & *Ld*MacP-8His in *L.donovani* parasites

Phosphatase on parasites

Fig 8: *L.donovani*-MAcP-mRFP transgenic parasites (***Fig. 8a***). *L.donovani* wt parasites labeled by IF with the mouse anti-*Ld*MAcP antibody (***Fig. 8b***).Typical ER and plasma membrane staining was observed (arrows). The star indicates the nucleus.The flagelum of the parasites was not stained.

Heterologous expression of *Ld*MAcP- 8His in HeLa human epithelial cells and assay of extracellular phosphatase activity of the surface expressed *Ld*MAcP-8His

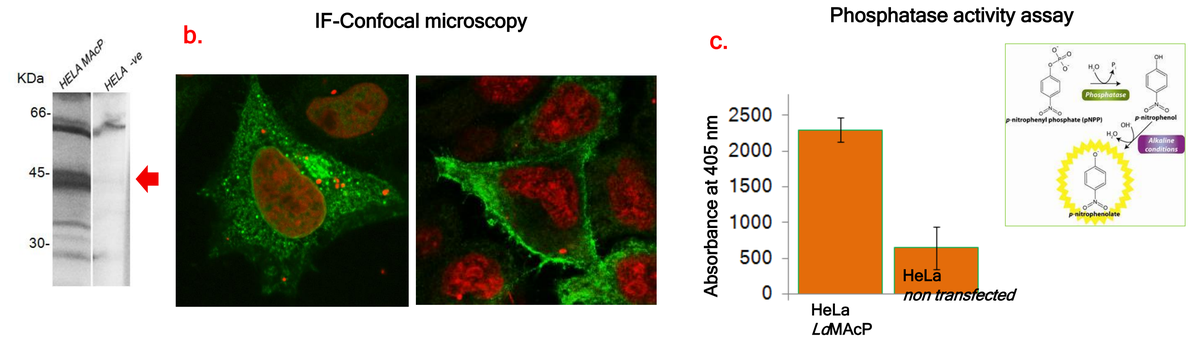


Fig 9: HeLa cells transfected with pTriex1.1-*Ld*MacP plasmid for 24 hs were subjected to WB (*9a*) or IF (*9b*) analysis using the anti-His mAb. *Ld*MacP-8His was detected as a ~45kDa protein indicating post-translational modifications (i.e. glycosylation). It showed ER, Golgi and plasma membrane localization. Transfected cells had **~ 5X higher extracellular** acid phosphatase activity from non-transfected cells.

Conclusions

- ***Ld*MacP localises at the ER and plasma membrane** (as predicted from its secretion signal sequence) of *L.donovani* parasites.
- ***Ld*MacP** expressed heterologously **in HeLa cells localizes at the cell surface**, is post-translationally modified (MW ~45 KDa vs 31,7 KDa) and **exhibites extracellular acid phosphatase activity** (according to predicted topology).
- The **3D structures** of ***Ld*MacP** *and* ***Ld*SacP1** due to the high sequence identity with human prostatic acid phosphatase were **reliably modeled**. In *Ld*MacP the His 286 and Asp287 (highly conserved in the His phosphatase superfamily) are Cys and Trp (more hydrophobic). This results in a more "open" and less charged active site, that could accommodate much bulkier substrates, such as phosphorylated proteins and facilitate interactions with more hydrophobic parts of the substrate.
- ***Ld*SacP** is **predicted to have an intrinsically disordered region.**

Future studies → Is *Ld*MacP a *Leishmania* virulence factor ? Assesement as potential drug target !

- Transgenic parasites overexpressing *Ld*MacP-mRFP and *Ld*MacP-8His have been generated and will be assesed for improved