67ο Πανελλήνιο Συνέδριο Ελληνικής εταιρείας Βιοχημείας και Μοριακής Βιολογίας

Hellenic Pasteur Institute

*Ld*PIBPnex, a secreted nexin-like protein from the protozoan parasite *Leishmania donovani*

Drosos Kourounis[1], Amalia Papadaki[1], Olympia Tziouvara[1], Haralabia Boleti[1,2]

1. Intracellular Parasitism group, Department of Microbiology, Hellenic Pasteur Institute, Athens, Greece

2. Light Microscopy unit, Hellenic Pasteur Institute, Athens, Greece

Introduction

Leishmania donovani is a protozoan parasite that causes visceral leishmaniasis, a disease of the hematopoietic organs, which can be fatal if left untreated.

The parasite has two developmental forms, the promastigote, which lives and proliferates inside the sandfly host and the intracellular amastigote living in the phagocytes of the mammal host. The pathogen is transmitted to mammals through the bite of phlebotomine sandflies (Fig. 1) [1].

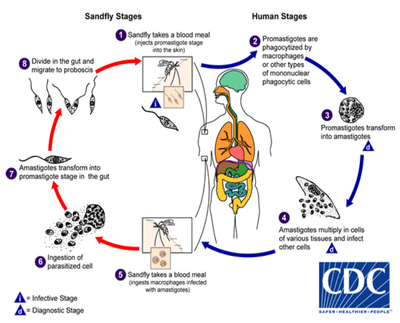


Fig. 1 : Life cycle of *L.donovani.*

Leishmania and other intracellular pathogens have evolved strategies to invade and persist within the host target cells. In some cases the underlying mechanisms involve the export of virulence factors into the host cell cytosol [2].

Proteomic analysis of the secretome of *Leishmania donovani* revealed a highly secreted protein encoded by the **LdBPK_352470.1** gene that is conserved in most *Leishmania* and *Trypanosoma* species. It's protein product is enriched in the extracellurar secreted fraction of a promastigote culture [3].

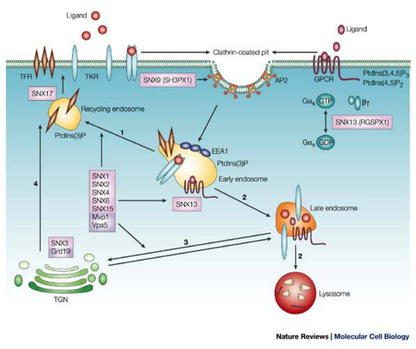


Fig. 3 : Molecular endocytic and sorting pathways regulated by the presence of different Nexins.

The LdBPK_352470.1 gene encodes for a **417 a.a protein** with **predicted MW 46,6 KDa,** a **PX phosphoinositide binding domain** and **structural features that classify it in the Sorting Nexin** (Fig. 2) [4] family of proteins. We named it ***Ld*PIBPnex**, from ***L**eishmania **d**onovani* **P**hosphoinositide **B**inding **P**rotein **nex**in.

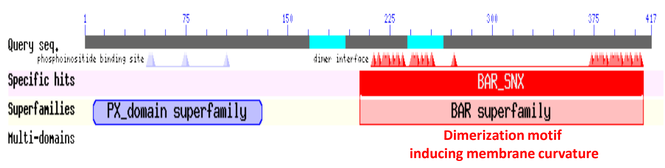
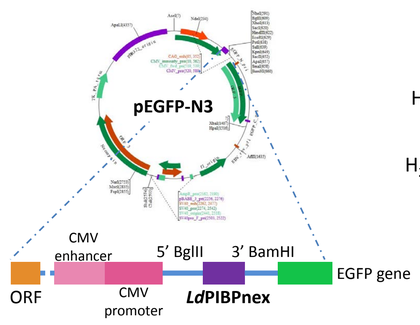


Fig. 2 : Predicted structural characteristics of *Ld*PIBPnex

Nexins are a large group of cytoplasmic proteins with the potential to **associate with membranes** either through their **lipid-binding PX domain** or **through protein–protein interactions with membrane-associated protein complexes**. Members of this family have been shown to participate in clathrin-dependent and –independent endocytosis, as well as in an increasing array of endosomal sorting events (Fig. 3) [5].

Cloning and Biochemical identification of *Ld*PIBPnex in *L. donovani* subcelllular protein fractions



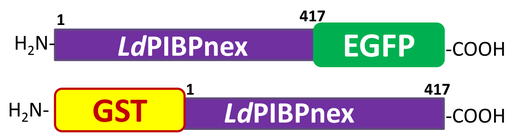
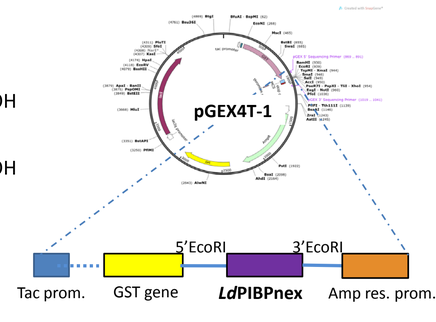


Fig 4. Schematic representation of plasmids and recombinant proteins generated in this study



Analysis of Secreted *L. donovani* proteins

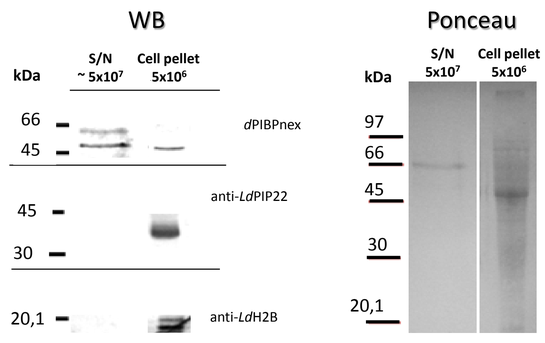


Fig. 5 : WB analysis of *L. donovani* proteins secreted at 25 ^{O}C for 6 hrs. The S/N containing secreted proteins was separated from the cells by a 2 step centrifugation (1000g, 10 min and 20.000 g, 20 min). Secreted proteins (S/N) were precipitated with acetone. Intracellular (cell pellet) and secreted proteins were detected by specific pAbs.

Kinetics of *Ld*PIBPnex expression in *L. donovani* promastigote cell cycle

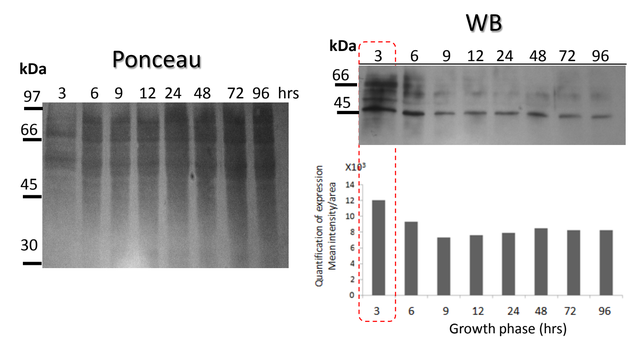
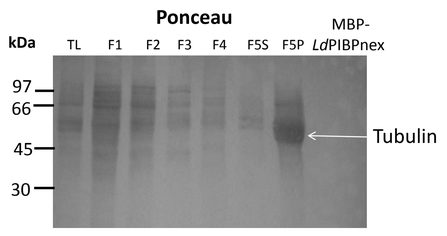


Fig. 6 : *L. donovani* lysates collected at specific time points during promastigote growth in cell culture were analyzed by SDS-PAGE (12% w/w) and WB, using specific rabbit a-*Ld*PIBPnex pAb, for the presence and abudance of *Ld*PIBPnex

Identification of *Ld*PIBPnex in *L. donovani* Subcellular fractions



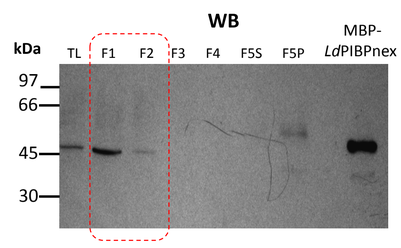


Fig. 7 : *L. donovani* promastigotes at the stationary phase of growth were fractionated by resuspension in buffers containing gradually increasing concentration of digitonin (20 μM, 200 μM, 1 mM and 10 mM). Protein fractions were analyzed by SDS-PAGE (12% w/v) and WB. Using a specific anti-*Ld*PIBPnex rabbit pAb generated against the C-terminal half of *Ld*PIBPnex produced in bacteria (i.e. MBP-*Ld*PIBPnex-C-term), used here as positive control. Fractions **F1** and **F2** are **enriched in soluble proteins**, fractions **F3-F5S** are **enriched in membrane proteins** and **F5P is enriched in cytoskeletal and nuclear proteins**.

BLAST analysis of *Ld*PIBPnex sequence

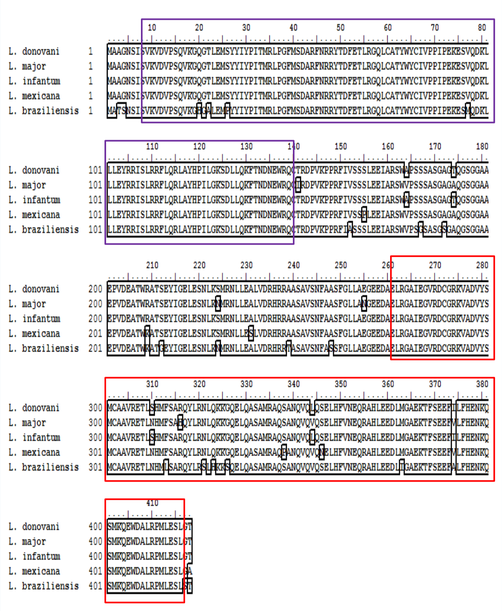


Fig. 8 : Comparison of *Ld*PIBPnex sequence with homologues from other *Leishmania* spp. indicates a high conservation. Purple framed area shows the PX domain, Red framed area the BAR domain.

Localization of *Ld*PIBPnex in *L. donovani* promastigotes

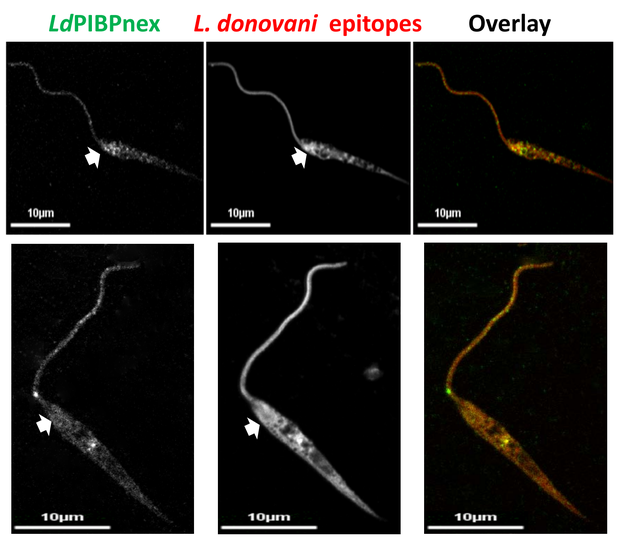


Fig. 9 : *L. donovani* promastigotes immunostained with a) anti-*Ld*PIBPnex (rabbit pAb) and anti-*L. donovani* (mouse pAb) specific Abs and second a-rabbit Alexa488 and a-mouse Alexa546 Abs respectively. Images are single optical sections of a series acquired by TCS-SP confocal Leica microscope with a 63X lense. *Ld*PIBPnex is localized as spots on the flagellum and in the cell body of the promastigote. Green and Red FL are presented as BW images. Overlay in color.

Localization of *Ld*PIBPnex epitopes in infected J774 macrophages with *L. donovani* promastigotes

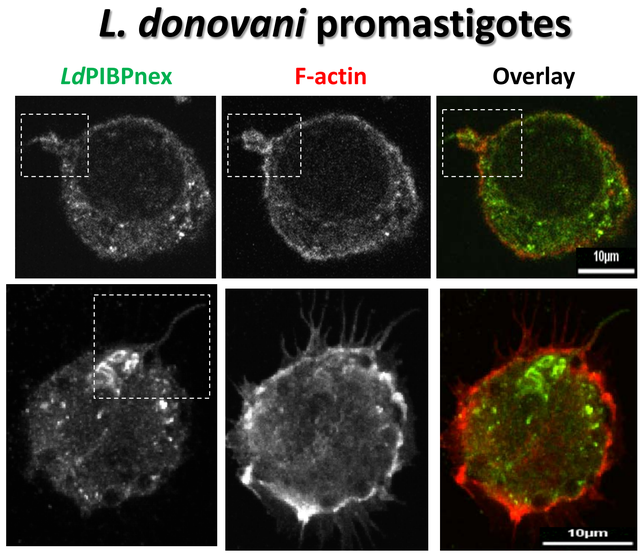


Fig. 10 : J744 Macrophages were infected with *L. donovani* promastigotes for 1 hr, fixed (4% PFA) and immuno-stained for *Ld*PIBPnex (a-*Ld*PIBPnex and a-mouse-**Alexa 488**) and F-actin (phaloidin-Alexa 546). Images are single optical sections of a series of images acquired by confocal TCS-SP Leica microscope with a 63X lense. *Ld*PIBPnex epitopes were detected on the macrophage on sites of early phagocytic events (framed areas)

Heterologous expression of *Ld*PIBPnex-GFP in HeLa cells

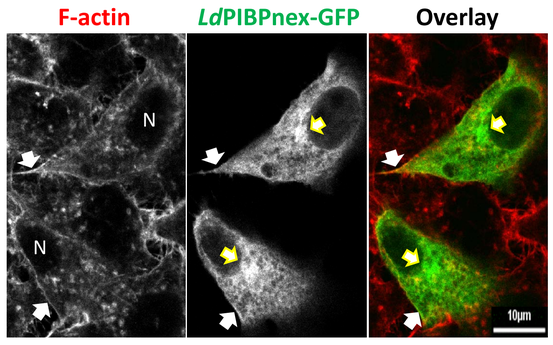


Fig. 11 : HeLa cells transfected with the pEGFP-*Ld*PIBPnex plasmid for 24 hrs, were fixed (PFA 4%) and stained for F-actin with phaloidin-Alexa 546. *Ld*PIBPnex-GFP shows a cytoplasmic membrane-like staining and partially colocalizes with F-actin at the cell cortex (white arrows). Yellow arrows indicate Golgi-like localization of *Ld*PIBPnex-GFP. Separate green and red FL are presented as BW images. Overlay in color.

Conclusions-Future work

1. LdPIBPnex is a nexin-like protein highly conserved amongst Leishmania spp (**Fig. 8**)
2. LdPIBPnex is expressed as a soluble protein (**Fig. 7**) at the logarithmic and stationary phases of the promastigote growth in culture (**Fig. 6**) with a higher expression at the lag phase indicating a **possible role at the parasite's S phase of growth**.
3. LdPIBPnex secretion was confirmed in L. donovani promastigotes cultured at 25^{o}C (temperature of arthropod host) (**Fig. 5**). The protein's secretion at 37^{o}C (temperature of mammalian host), is under investigation.
4. LdPIBPnex is localized in vesicle-like structures in L. donovani promastigote cell-body and flagellum and near the flagellar pocket (the main endo/exocytosis area) (**Fig. 9** arrows).
5. In mouse J774 macrophages infected with L. donovani promastigotes LdPIBPnex epitopes were mainly localized on macrophage surface membrane at site's of phagocytic events (**Fig. 10**, frames)
6. The GST-LdPIBPnex protein (**Fig. 4**) will be used to analyze binding to Phosphoinositides and in pull down assays to identify LdPIBPnex partners in Leishmania and macrophages.
7. The **function** of LdPIBPnex will be **studied** by generating LdPIBPnex KO L. donovani mutants or by overexpressing dominant negative mutants of the protein in L. donovani promastigotes.

Literature

1. *Alfred A.Pan, et al, 1993. Developmental Life Cycle of Leishmania—Cultivation and Characterization of Cultured Extracellular Amastigotes.*
2. Torrecilhas, A.C. et al, 2012. Vesicles as carriers of virulence factors in parasitic protozoan diseases.
3. *J Maxwell Silverman, et al, 2008. Proteomic analysis of the secretome of Leishmania donovani.*
4. *Jez Carlton, et al, 2005. Sorting Nexins – Unifying Trends and New Perspectives.*
5. *Peter J. Cullen 2008. Endosomal sorting and signalling: an emerging role for sorting nexins*

Funding : This work was partially funded by the Greek GSRT in the action **«ΑΝΑΠΤΥΞΙΑΚΕΣ ΠΡΟΤΑΣΕΙΣ ΕΡΕΥΝΗΤΙΚΩΝ ΦΟΡΕΩΝ -ΚΡΗΠΙΣ»**