MSc 2017

«Cloning and Molecular characterization of the secreted nexin *Ld*PIBPnex with phosphoinositide binding domain from the protist *Leishmania donovani*»

Drosos KOYROUNIS
National Kapodistrian University of Athens and Hellenic Pasteur Institute

ABSTRACT

Leishmania are dimorphic protozoan parasites from the *Trypanosomatidae* family *(kinetoplastid class*) living as extracellular flagellated forms (promastigotes) in their sandfly vector gut and as intracellular aflagellated forms (amastigotes) inside modified phagolysomes of the mammalian host phagocytes. As other intracellular pathogens, *Leishmania* species have evolved strategies supporting invasion and persistence within the mammalian phagocytes. In some cases the underlying mechanisms involve export of virulence factors into the host cell cytosol. Proteomic analysis of the secretome of *Leishmania donovani/L. donovani* (causative agent of the fatal disease visceral Leishmaniasis/VL) revealed several proteins secreted in the extracellular medium of cultured promastigotes, possibly through an exosome-like route (Silverman, Chan et al. 2008). **One of these was the product of the *Ld*BPK_352470.1 gene**, highly conserved in *Leishmania* species, which **encodes for a secreted protein** of 417 a.a. (predicted MW 46,6 kDa) with a PX phosphoinositide binding domain and structural features classifying it in the **Sorting Nexin protein family**, a large group of cytoplasmic proteins that bind to membranes either through their lipid-binding PX domain or through protein–protein interactions with membrane-associated protein complexes. Members of the Nexin family are shown to participate in an increasing array of endosomal sorting and intracellular membrane traffic events (Cullen 2008) (van Weering, Verkade et al. 2010) (Carlton, Bujny et al. 2005). We named the ***Ld*BPK_352470.1 gene** product ***Ld*PIBPnex**, for ***L**eishmania* ***d**onovani* **P**hosphoinositide **B**inding **P**rotein **nex**in. Herein we present results from a) cloning of the LDBPK_352470.1 gene in different expression vectors b) expression of the recombinant *Ld*PIBPnex protein in bacteria and mammalian cells, c) localization of the endogenous *Ld*PIBPnex in *Leishmania* cells and infected macrophages using an anti-*Ld*PIBPnex specific pAb that we generated, d) enrichment of *Ld*PIBPnex in soluble protein fractions of *L. donovani* axenic (cultured) promastigotes and e) secretion in the promastigote culture extracellular medium. Cloning of the *ldpibp$_{nex}$* gene into the pEGFP-N3, pGEX4T-1 and pLexsy-mCherry

plasmids, was successful only in the first two cases. The recombinant pEGFP-*ldpibp*$_{nex}$ plasmid, was used in order to track the sub-cellular localization of *Ld*PIBPnex-GFP in the mammallian epithelial HeLa cells, with fluorescence microscopy, by observing the green fluorescence of the EGFP protein. We localized *Ld*PIBPnex-EGFP in the cytosol, and on the plasma membrane of the transfected HeLa cell where it colocalized with the cortical F-actin. It also showed a polarized perinuclear localization similar to the Golgi apparatus localization. Regarding the recombinant pGEX-*ldpibp*$_{nex}$ plasmid, it will be used in future studies to express the GST-*Ld*PIBPnex recombinant protein in order to analyze biochemichaly its binding to Phosphoinositides and to identify *Ld*PIBPnex partners in *Leishmania* and macrophage cell extracts by pull down assays.
In parallel we localized *Ld*PIBPnex epitopes in macrophages infected with *L. donovani* promastigotes at macrophage surface membrane site's of phagocytic events. Regarding the localization of the protein in *L. donovani* promastigote cells, we detected *Ld*PIBPnex in vesicle-like structures in the cell-body and the flagellum as well as near the flagellar pocket.
7
The expression of *Ld*PIBPnex in promastigote parasites was also confirmed biochemically using Western blot, while analysis of the expression levels of *Ld*PIBPnex in axenic promastigote cultures, during the parasite's growth stages showed that the protein is expressed as soluble both at the logarithmic and stationary phases of the promastigote growth in culture, with a higher expression detected at the lag phase. Next, we confirmed the secretion of *Ld*PIBPnex in *L. donovani* promastigote cultures at 25$_{o}$C while the experiments for analysis of secretion at 37$_{o}$C will have to be repeated. Lastly, *L. donovani* promastigotes at the stationary phase of growth were fractionated by resuspension in buffers with containing gradually increasing concentration of the detergent digitonin and further analysis with SDS-PAGE and Western Blot. Using the anti-LdPIBPnex specific polyclonal Ab, we detected *Ld*PIBPnex in the fractions, enriched in soluble parasitic proteins.