MSc Thesis

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Cloning, molecular and functional characterization of the putative PI phosphatase, *Ld*PIP_22, of the parasitic protozoan *Leishmania donovani*

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ABSTRACT

Leishmaniasis is a group of anthropozoonotic diseases with 1.6 million new cases per year in humans. The causative agent is the infection by parasitic protozoa of the genus *Leishmania*, which are transmitted by about 30 species of sandflies (vector) of the genus *Phlebotomus* and *Lutzomyia*. This group of diseases affects the poorest people on the planet and is associated with malnutrition, population displacement, poor housing, weak immune system and lack of resources. The treatment of the disease causes serious side effects, it is expensive, while the lack of vaccines and the resistance to some of the currently available drugs are causing the need for developing new and non-toxic drugs and vaccines.

Specific phosphatases of the parasites could be potential drug targets (inhibitors). Phosphatases, in general, are molecular targets in the treatment of different diseases, infectious or not, but in the *Leishmania* parasite their function is not yet clarified and their regulation has not been studied.

The kinetoplastids' (family to which the *Leishmania* species belong) phosphatome contains a large number of hypothetical dual specificity phosphatases (serine/threonine and tyrosine) with considerable divergence from classic dual-specificity phosphatases in higher eucaryotes (aDSPs). A subgroup of the aDPSs from *Leishmania* species with a similar catalytic P-loop to the *M. tuberculosis* virulence factor MptpB phosphoinositide (PI) phosphatase designated as atypical lipid phosphatases (ALPs) was recently described. These proteins are detected in bacteria and lower eukaryote genomes but not in higher eukaryote genomes.

This work studies one member of the ALP enzyme family from the unicellular protozoan parasite *Leishmania donovani*, the causative agent of visceral leishmaniasis (Kala-Azar). The orthologue protein of *M. tuberculosis* is a virulence factor. This and the fact that the interference in the regulation of the phosphoinositide metabolism of the host is an important pathogenicity mechanism of many human bacterial pathogens was the motivation of studying the putative phosphoinositide phosphatase *Ld*PIPh22 (***L***. ***d****onovani* **P**hospho**I**nositide **Ph**osphatase located in chromosome **22**). The bacterial phosphoinositide phosphatases known to interfere with host phosphoinositide metabolism and act as virulence factors are attractive antimicrobial drug targets and this is something that may also apply to the *Ld*PIPh22 protein. A Blast analysis of the *Ld*piph22 gene sequence revealed orthologues in all *Leishmania* species with high sequence identity, which means that this protein is vital for the parasite. Additionally, it has orthologues in pathogenic bacteria but not in higher eukaryotes, including the human species.

The main aim of this study was the generation of molecular (plasmids, antibodies) and cellular tools (transgenic parasites) in order to examine the biochemical and functional properties of the *Ld*PIPh22 protein.The *Ld*piph22 gene was amplified by PCR from the genomic DNA of *Leishmania donovani* parasites (LG13 strain) and cloned into bacterial, eukaryotic and *leishmanial* expression vectors for overexpression in bacterial, leishmanial (*L. donovani* and *L. tarentolae*) and several eukaryotic cell lines. Detection and subcellular localization of the protein in *L. donovani* cells were conducted by indirect immunofluorescence microscopy and biochemically by Western blotting after fractionation of total parasite proteins. The *Ld*PIPh22 protein was detected by

specific antibodies, produced in the context of this study, for both methods, and localized on the parasite body, perinuclearly, on the flagellar pocket, around membrane vesicles and on structures resembling the parasite actin cytoskeleton. Biochemically, the *Ld*PIPh22 protein was found in protein fractions enriched in membrane and/or cytoskeletal proteins. The recombinant rN15-*Ld*PIPh22-8His protein (with an additional 15-peptide in N-terminus and 8 histidines in the C-terminus), which was isolated from bacteria soluble extracts by high affinity chromatography, has phosphatase activity *in vitro*, with the ability to dephosphorylate the generic phosphatase substrate pNPP, with comparable activity values reported in the literature for a protein homologue. When the *Ld*PIPh22 was ectopically expressed as a GFP or an 8His tagged protein in mammalian tissue culture cells, it was localized on sites of dynamic actin polymerization, where phosphoinositides play important role.

This work is continued towards the exploration of the possibility that *Ld*PIPh22 is secreted by *L. donovani* parasites and of the biochemical properties of the *Ld*PIPh22 as a phosphatase, while the natural peptide and lipid (phosphoinositides) substrates and the substrate specificity are under investigation.