Involvement of tyrosine phosphatases in *Leishmania* differentiation and virulence

by

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ABSTRACT

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Leishmania protozoan parasites, the causative agent of leishmaniasis, a disease endemic in more than 80 countries, undergo two main developmental stages during its life cycle: the extracellular flagellated promastigote residing in the midgut of the sandfly vector and the obligate intracellular amastigotes which multiply in the phagolysosome of infected macrophages within the mammalian host. The differentiation process from promastigote to amastigote allows *Leishmania* parasites to adapt to different environments and is essential for parasite proliferation and survival. However, the molecular events that regulate this process are not well understood.

In higher eukaryotes, cellular proliferation, differentiation and function are governed largely by protein phosphorylation, which is controlled by protein kinases and phosphatases. The research described in this thesis has investigated the role of protein tyrosine phosphatase in controlling the differentiation and proliferation of the *Leishmania* pathogen in different life cycle stages, by analogy to what happens in higher eukaryotes. The focus was on protein phosphatases because in general, there are fewer phosphatases than kinases in the eukaryotic cells and therefore there is less likelihood of redundancy under conditions where it is possible to genetically develop mutants in phosphatase genes.

By undertaking a predominantly genetic approach, we show the protein tyrosine phosphatase may play a role in *L. donovani* differentiation and is clearly required for parasite virulence as defined by survival in the mammalian host.

The results from this study suggest that *Leishmania* PTP1 represents a potential drug target. However it is also revealed that the overall three dimensional structure of the active site of *Leishmania* PTP1 is very similar to the human PTP1B arguing that it may be difficult to develop parasite specific inhibitors. Taken together this study represents the first genetic analysis of a key regulatory gene in *Leishmania, which* establishes the foundation for future more biochemical approaches to study protein phosphorylation in *Leishmania.*

Résumé

Ph.D **Mirna Nascimento** **Microbiologie et Immunologie**

La *Leishmaniose* est une maladie endémique dans plus de 80 pays et met environ 350 millions de personnes en danger autour du monde. La maladie est définie par le parasite protozoaire *Leishmania* dont le cycle de vie présente deux grandes étapes. Les promastigotes extracellulaires, qui sont flagellées et qui résident dans la région digestive du vecteur la mouche des sables, et les amastigotes intracellulaires qui se multiplient dans les phagolysosomes des macrophages infectés de l'hôte mammifère. Le procédé de différentiation du promastigote à l'amastigote permet au parasite *Leishmania* de s'adapter aux différents environnements, un mécanisme essentiel à sa survie et prolifération. Cependant, les événements moléculaires qui règlent ce processus ne sont pas bien compris.

Chez les eucaryotes de niveau plus élevé, la prolifération et différentiation sont en grande partie déterminées par la phosphorylation de protéines, ce qui à son tour est commandée par les kinases et phosphatases de protéines. La recherche décrite dans cette thèse étudie le rôle de la Tyrosine phosphatase qui commande la différentiation et prolifération du pathogène *Leishmania* pendant les différentes étapes de son cycle de vie. Cette étude a été réalisée en comparant ces événements avec ceux se produisant chez les eucaryotes supérieurs. L'emphase a été mise sur les phosphatases des protéines car, en général, il y a moins de phosphatases que de kinases dans les cellules eucaryotiques. Etant donné leur faible nombre, il y a moins de risques de redondance de fonction, ce qui facilite la création de différentes mutants de gènes de phosphatases.

En entreprenant une approche principalement génétique, nous démontrons que la tyrosine phosphatase a un rôle important dans la différentiation de *L. donovani.* De plus, ce processus de différentiation est nécessaire pour la virulence du parasite et par le fait même pour sa survie dans l'hôte mammifère.

Les résultats de cette étude suggèrent que *Leishmania* PTP1 pourrait représenter une cible potentielle pour le développement d'un médicament. Par contre, nous indiquons également que la structure tridimensionnelle globale du site actif de *Leishmania* PTP1 est très semblable au PTP1B humain, ce qui pourrait rendre difficile le développement d'inhibiteurs parasite-spécifiques. De façon générale, cette étude représente la première analyse génétique d'une enzyme de régulation clé de *Leishmania*, ce qui établit une base important pour les futures études biochimiques de la phosphorylation des protéines chez *Leishmania.*

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I would like to express my most sincere gratitude to my academic supervisor, Dr. Greg Matlashewski, for his advices and support throughout my PhD, granting his expertise to help me conduct and complete this work. I would also like to thank the members of my advisory committee, Dr. Armando Jardim and Dr. Brian Ward, for their advice and assistance. I am very glad and grateful for the collaboration of Drs. Michel Tremblay, Martin Olivier and Albert Berghuis during this work and for their priceless advice, suggestions and technical support.

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CONTRIBUTION OF AUTHORS

In accordance with the guidelines concerning thesis preparation of the faculty of Graduate Studies and Research from McGill University, the experimental portions of this thesis is presented in manuscript format. Manuscripts are presented in chapters 2 and 3 as they appear in the literature or as prepared for journal submission. Prefaces provided within, prior to each of those chapters, have the purpose to connect the texts.

Contribution of authors for chapters 2 and 3 is specified and listed as follows:

Chapter 2

Mirna Nascimento, Nay Abourjeily, Anirban Ghosh, Wen-Wei Zhang and Greg Matlashewski (2003). Heterologous expression of a mammalian protein tyrosine phosphatase gene in *Leishmania*: effect on differentiation. *Molecular Microbiology* **50:** 1517-1526.

In this manuscript, I was responsible for transfecting and selecting *L. donovani* promastigotes with the plasmid expressing human PTP1B and determining the involvement of the ectopic phosphatase in parasite differentiation and virulence. Consequently, I performed the p-NPP phosphatase activity assay, Western blots analysis of A2 and hPTP1B expression and the morphological studies on transfected *L. donovani*. The kinase inhibition studies (using AG1433) were performed jointly between myself and Ms. Nay Abourjeily. Dr. Wen-Wei Zhang constructed the plasmid pALTneoPTP1B for expression of the hPTP1B in *Leishmania* parasites. Dr. Anirban Ghosh provided experimental advice.

Chapter 3

Mirna Nascimento, Wen-Wei Zhang, Anirban Ghosh, Douglas R. Houston, Albert M. Berghuis, Martin Olivier and Greg Matlashewski (2006). Identification and characterization of a protein tyrosine phosphatase in *Leishmania;* involvement in virulence. Under revision for the *Journal of Biological Chemistry*

I carried out the expression and purification of the LmPTP1 by performing PCR amplification of the *L. major* and *L. donovani* PTP1 gene and the hPTP1B gene, construction of the A2-tag pcDNA3-LmPTP1 and pcDNA3-hPTP1B and transfection of mammalian cells. I also performed the activity assays on the cloned phosphatases and evaluated the effect of phosphatase inhibitors on the His-tag purified enzymes. Southern blot evaluation of specific LdPTP1 targeted deletion was performed by myself , as well as culture proliferation and morphological studies of *L. donovani* PTP1 null mutants. I also carried out PCR amplification and generation of the add-back constructs pSPYneoLdPTP1, transfection into *L. donovani* null mutants, Southern blot analysis of the added-back strain and BALB/c infections (performed with the help of Dr. Wen-Wei Zhang). Dr. Anirban Ghosh provided experimental advice.

Dr. Wen-Wei Zhang carried out the following : (i) subcloning of the *L. donovani* PTP1 gene and generation of the pBSLdPTP1 plasmid, (ii) construction of the targeting plasmid pBSLdPTP Hyg, (iii) transfection of pBSLdPTP Hyg into *L. donovani* to give rise to the double knockout homozygous null mutant parasites (LdPTP1 -/-), with Southern blot confirmation of the enzyme catalytic domain deletion.

Dr. Douglas Houston from Dr. Albert Berghuis' laboratory performed the three-dimensional *in silico* structural analysis of the *Leishmania* LPTP1 enzyme compared to the human hPTP1B enzyme structure based on the sequence information derived from my research.

CONTRIBUTIONS TO ORIGINAL SCIENTIFIC KNOWLEDGE

1. This research established that expression of the Heterologous protein tyrosine phosphatase 1B in *L. donovani* promastigotes mediates partial differentiation of these parasites towards the amastigote stage and increases infection levels *in vivo* and *in vitro*.

2. This research demonstrated that inhibition of protein tyrosine kinases in promastigotes results in partial differentiation towards amastigotes and increased virulence.

3. This research identified the *Leishmania major* homologue to human PTP1B, the *Leishmania* PTP1 enzyme (LPTP1), and sequenced the *L. donovani* PTP1 gene.

4. This research confirmed the activity of the *L. major* PTP1 gene product.

5. This research demonstrates the role of the *Leishmania* PTP1 enzyme in the parasite life cycle, by showing mutant parasites are able to proliferate as promastigotes but were poorly infective as amastigotes in BALB/c mice. This confirmed the importance of this enzyme for the ability of the parasite to survive in the mammalian host.

6. This research revealed that although *Leishmania* PTP1 represents a potential drug target because of its importance for survival in the mammalian host, it's active site is structurally very similar to the human PTP1B enzyme suggesting it may be difficult to identify parasite specific PTP1 inhibitors.

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CHAPTER 1: Introduction

A. *LEISHMANIA*

Leishmaniasis represents a collection of diseases with clinical diversity that mostly afflicts economically less privileged individuals throughout the world (approximately 80% of the two million new cases/year) (Davies *et al.*, 2003). According to the World Health Organization (WHO), leishmaniasis is an emergent and uncontrolled disease (Murray *et al.*, 2005) and is therefore considered one of the most relevant parasitic infectious diseases. *Leishmania* spp., the causative agent of leishmaniasis, is a unicellular eukaryotic organism with a dimorphic life cycle that alternates between its insect vector and the mammalian host, where it causes a spectrum of clinical manifestations. Considered a neglected disease, recent discoveries and advances in the research studies of leishmaniasis bring hope that more attention and funding will be given to facilitate prevention, diagnosis, treatment and vaccine development against this destructive disease.

Infection and Pathogenicity

Infection with the protozoan flagellated parasites belonging to the Genus *Leishmania*, Family *Trypanosomatidae* and Order *Kinetoplastida*, results in the spectral disease leishmaniasis, which is characterized by a multitude of clinical forms in humans that are mainly related to the species of parasites. Leishmaniasis is caused by 20 different species of human pathogenic *Leishmania* spp. transmitted through the bite of infected female phlebotomine sandfly vectors (reviewed in Desjeux, 2004a). The *Trypanosomatidae* family diverged early in eukaryotic evolution and

trypanosomatids encompass numerous protozoan genera. Members of this order are eukaryotes and exhibit usual features such as the presence of a nucleus delimited by a nuclear membrane, and organelles such as the endoplasmic reticulum, the golgi apparatus, the mitochondrion, among others. However, some organelles have unique characteristics: for example, the mitochondria is present as a single unit per cell, is large and elongated, and its whole DNA content is condensed into a sub-structure called the kinetoplast, hence the name of this order (reviewed in Bastin *et al*, 2000). The kinetoplast is a specialized region of the mitochondrion (Fig. 1) rich in small circular DNA molecules (kDNA) containing tens of maxicircles and thousands of interlocked minicircles into a single network in each cell and is the site of RNA editing (reviewed in Lukes et al., 2002).

Different species of *Leishmania* cause distinct forms of the disease, which depends on properties of the parasite like temperature sensitivity, tissue tropism, ability to evade the immune system and chronic persistence in the host. Development of leishmaniasis is also dependant on the immunological status of the patient (reviewed in Murray, 2005). Clinical forms appear as visceral, mucocutaneous and cutaneous leishmaniasis. Visceral leishmaniasis (VL), which is fatal if not treated, involves the entire reticuloendothelial system and is caused by species of the *L. donovani* complex (*L. donovani, L. infantum* and *L. chagasi*). Mucocutaneous leishmaniasis (MCL), a mutilating and disfiguring disease caused mainly by *L. braziliensis,* can lead to a great psychological trauma in inflicted patients and creates social and aesthetic stigma. Cutaneous leishmaniasis (CL), caused by *L. major*, *L. tropica* and *L. mexicana*, among others, may appear as multiple skin lesions (diffuse

type) in individuals with defective cell-mediated immune response or as single (localized) lesions that can heal spontaneously (reviewed in Desjeux, 2004b).

CL usually appears two to three weeks after the bite of the infected sand fly as a small papule on the skin. This lesion usually grows slowly, often becomes ulcerated and can lead to the development of secondary infections (reviewed in Matlashewski, 2001). Moreover, diffuse lesions, characterized by disseminated nodules similar to lepromatous leprosy, may develop and are caused by *L. mexicana*, *L. amazonensis* and *L. aethiopica* infections. Lesions may also appear in the oral and nasopharyngeal mucosa if the species involved have tropism to mucocutaneous membranes, like *L. braziliensis* (reviewed in Desjeux 2004b). Similar to CL, VL begins with a nodule at the site of the insect bite. This lesion rarely ulcerates and often disappears, but signs of systemic disease like fever, weight loss, weakness, hepatosplenomegaly, lymphadenopathy, anemia, and leucopenia appear. In this form of the disease, parasites invade internal organs (liver, spleen, bone marrow, and lymph nodes) and attack the reticuloendothelial cells, wherein they multiply and eventually burst those cell types; and this infection is fatal if not treated (reviewed in Schwartz *et al.*, 2006, Baron *et al.*, fourth edition, 1996).

Classification

Systematic classification of *Leishmania* species has divided them into Old World species, whose main examples for causing CL are *Leishmania tropica*, *L. major*, and *L. aethiopica* (causing oriental sore) and for VL, *L. donovani* and *L*

infantum. These species cause disease in the Middle East, Africa, southern Europe and Asia. New World species, found throughout Central and South America and Southern USA, which cause CL are *L. mexicana* (chiclero ulcer), *L. amazonensis*, *L. peruviana* (cutaneous disease locally known as Uta), *L. braziliensis* (causing MCL also known as espundia), *L. panamensis*, *L. guyanensis* (causing dermal leishmaniasis), while *L. chagasi* and *L. infantum*, which are considered identical, are known to cause VL (Guerin *et al.*, 2002, Baron *et al.*, fourth edition, 1996) (Table 1). *Leishmania* species are still further divided into two sub-genera, *Leishmania* (present both in the Old World and the New World) and *Viannia* (New World only), according to their development in the phlebotomine sandfly digestive tract (Table 1). Members of the *Leishmania* subgenus such as *Leishmania* (*Leishmania*) *major* and *L.* (*Leishmania*) *donovani* grow at the pyloric (foregut) and midgut regions, while *Viannia* species grow at the hindgut region, where they attach by flagellar hemidesmosomes to prevent their loss during excretion of blood meal, and later migrate to the midgut and foregut. Examples of the latter are *L.* (*Viannia*) *braziliensis* and *L.* (*Viannia*) *panamensis*. Despite this division, biochemical and molecular criteria are also used to differentiate *Leishmania* parasites according to the presence of specific markers. Clinical and morphological criteria are used alongside immunoreaction with monoclonal antibodies, and biochemical analyses (zymodeme, restriction enzymes, DNA hybridization, isoenzymatic profiles and PCR-based techniques) for an accurate positioning of species (reviewed in Correa *et al*, 2005).

Life cycle

Leishmania parasites have a digenetic life cycle (Fig. 2), alternating between two different morphological and developmental forms (Fig. 1): the intracellular and apparently aflagellated round amastigotes, which actually present a very short flagellum limited to the flagellar pocket, which is an invagination of the plasma membrane where the flagellum emerges from the cell body (Overath *et al.*, 1997). Amastigotes have a diameter of 2-6 μm and are found within the phagolysosome compartment (or the parasitophorous vacuole) of infected macrophages of the mammalian host (Fig. 1). The motile flagellated procyclic promastigote form is present in the alimentary tract of the invertebrate dipteran vector, the sandfly. They have a long and slender body of approximately 15-30μm by 2-3μm (Fig. 1). The procyclic forms undergo intense replication and become attached to the epithelial midgut of the insect vector (reviewed in Sacks and Kamhawi, 2001). The flagellum is considered a functional organelle since it is involved in the parasite's motility and attachment to insect-specific tissues and it may also be involved in signaling via its action as an environmental sensor (reviewed in Bastin *et al.* 2000). Following replication, procyclics undergo another developmental transition, transforming into metacyclic promastigotes (Fig. 2), which represent the infective stage for the mammalian host. These *Leishmania* developmental stages express different molecules and markers and their surface is coated by stage-specific surface glycocalyxes. The cell surface of promastigotes contains a thick and dense glycocalyx, whose main components are the surface glycoconjugate termed lipophosphoglycan (LPG), GPI-anchored proteins and free

glycoinositolphospholipids (GIPLs), which are not linked to proteins; LPG re-structure and re-organization occur during metacyclogenesis and seem to be very important for the parasite infectivity (reviewed in Naderer *et al.*, 2004). Amastigotes dramatically down-regulate LPG expression and hence lack a surface glycocalyx, but nonetheless they seem to be coated with GIPLs and host glycosphingolipids. GPI-anchored proteins, like the surface zinc metalloprotease gp63, also follow this pattern of expression and are downregulated once promastigotes differentiate into the amastigote stage in the mammalian host (reviewed in Naderer *et al.*, 2004).

Transmission of leishmaniasis occurs through the bite of an infected sandfly of the genera *Phlebotomus* (for Old World species) or *Lutzomya* (New World species) (Fig. 2). Female sandflies (*Phlebotomus* and *Lutzomyia* spp) become infected when they take up blood from infected human hosts (anthroponoses) or terrestrial mammals (zoonoses) during its bloodmeal (Desjeux, 2004b). Regarding zoonoses, a number of reservoir hosts are involved in the maintenance of *Leishmania* in nature, varying from domestic to wild mammals. The reservoirs for wild zoonotic CL can be from different orders, which include rodents, hyraxes, marsurpials and edentates, while dogs are considered important reservoirs for species that have a peri-domestic or domestic transmission (reviewed in Farell *et al.*, 2002- vol. 4). When a female sandfly takes a blood meal from an infected mammalian host, amastigotes are ingested and once in the insect digestive tract they transform and replicate as promastigotes. During the next bloodmeal, infective metacyclic promastigotes present in the proboscis (mouthpart) are regurgitated and injected into the skin of the mammalian host. At this point they are phagocytosed by cells of the mononuclear

phagocytic system, mostly macrophages, and complete their life cycle by transforming back into amastigote and multiplying by mitosis (reviewed in Farell *et al.*, 2002- vol. 4) (Fig. 2). Vector competence in most species seems to be controlled by the parasite's ability to resist proteolytic enzymes during the bloodmeal digestion and avoid excretion by binding to the midgut. Binding to the midgut epithelium occurs through lectin-like interactions and is mediated by the promastigote polymorphic LPG, whose oligosaccharide side chains differ between species (reviewed in Naderer *et al.*, 2004). From the insect's point of view, the protein from the family of galectins PpGalec expressed in the midgut was shown to be critical for this sandfly-*Leishmania* association, survival of parasite in the digestive tract and therefore for *Leishmania* transmission (Beverley and Dobson, 2004). Overall, risk of acquiring infection in endemic areas is determined by the occurrence of local sandfly populations and by the presence of infected animals or a human reservoir (reviewed in Murray *et al.*, 2005).

Incidence and Geographic Distribution

The global prevalence of leishmaniasis is estimated at approximately 12 million or more infected people and 2 million new cases affecting over 88 countries in every continent, except Oceania and Antarctica (reviewed in Desjeux, 2004b) (Fig. 3). It is estimated that 1-1.5 million new cases of CL and 500.000 cases of VL occur every year, and that a population of 350 million people around the world are at risk of infection, although these numbers likely underestimate the reality due to a number of

limiting factors including undiagnosed/misdiagnosed or unreported cases (Murray *et al.*, 2005, Desjeux, 2004a). Ever-increasing incidence rates have drawn more attention to this disease and are raising great concern among the medical community, since leishmaniasis poses as a major public health problem in many countries. This amplification in incidence figures in recent years is largely due to population movement either with a significant increase in international traveling to affected areas and migration from rural to urban regions. In addition, factors including environmental changes (e.g. deforestation and global warming), lack of efficient vector control and an effective vaccine, *Leishmania* resistance to treatment, or individual risk factors such as HIV infection and malnutrition contribute to a higher incidence (Desjeux, 2004a,b). Another great concern is that the increasing numbers of patients co-infected with HIV are a potential source for drug resistance emergence. These patients have a high parasite burden associated with a weak immune response and respond to treatment slowly, which could lead to drug-resistance. Furthermore, identification of transmission via needle sharing in these patients is another route for the spread of resistant parasites (reviewed in Croft *et al.*, 2006). Ninety per cent of those affected by VL live in India, Bangladesh, Nepal, Brazil and Sudan while 90% of CL cases occur in Afghanistan, Peru, Brazil, Syria, Algeria, Iran and Saudi Arabia (Fig. 3). Even though a great number of countries that have been affected by this disease, including the United States (canine leishmaniasis) and Southern European countries from the Mediterranean basin, 72 of those are listed as underdeveloped or developing countries (reviewed in Desjeux, 2004b). Additionally, those more likely to be infected are the poor population living in remote areas far from health care

centers. Therefore, prospects for financial return following the development of an antileishmanial drug are unlikely, which is not appealing to major pharmaceutical companies. As a result, despite all these striking figures of disease incidence and mortality, leishmaniasis still remains a neglected disease (WHO, 2005 www.who.int/leishmaniasis/burden/magnitude, reviewed in Murray *et al.* 2005, Hailu *et al.*, 2005, Desjeux, 2004a).

Measures to control and prevent leishmaniasis vary according to areas affected, but they traditionally represent disease treatment, vector control with the use of insecticide-treated bed nets or insecticide house spraying, and animal reservoir control (in cases of zoonosis) with use of impregnated dog collars or vaccination of dogs (reviewed in Murray *et al.*, 2005, Desjeux, 2004b). However, these methods are expensive and difficult to sustain for prolonged periods.

Immunology of Leishmania infections

The immune response in *Leishmania* infections is one of the main components involved in the disease outcome and pathogenesis, since it dictates the resolution of the infection or susceptibility to the parasite. Therefore the role of cytokines and the type of immune response they generate have been extensively investigated. Experimental infections with *L. major* have created the well documented and established Th1/Th2 paradigm associated with resistance and susceptibility to the disease. The murine model has been of great importance regarding the understanding of *in vivo* mechanisms of T cell protection and it has been widely used to characterize

the immune response against *Leishmania*. There are two functionally and phenotypically distinct subsets of CD4+ T cells, distinguishable by their cytokine pattern, namely the T helper cell type 1 (Th1) and type 2 (Th2). Classical experiments have showed that C57BL/6 mice infected with *L. major* develop a Th1 type response with production of interleukin (IL)-12 that initially activates natural killer (NK) cells to produce interferon-gamma (IFN-γ), and a polarization of naïve-T cells into Th1 cells. This Th1 bias is characteristic of cell-mediated immunity and is important against intracellular pathogens. IFN-γ activated macrophages, in turn, produce tumor necrosis factor (TNF) and reactive nitrogen intermediates (RNIs), in particular nitric oxide (NO), by upregulation of inducible nitric oxide synthase (iNOs), enabling these mice to kill intracellular *Leishmania*, control the parasite infection and therefore become resistant to *L. major* by activation of CD4+ and CD8+ T cells (reviewed in Murray *et al.*, 2005, Sacks and Noben-Trauth, 2002). Conversely, BALB/c mice mount a Th2 response with expression of IL-4, among other cytokines, that counter-regulate the Th1 response. This has a powerful deactivating effect on infected cells and favours the development of a humoral response by inhibiting IFN-γ secretion. Consequently, these mice fail to control the infection and develop progressive lesions and systemic disease and are considered susceptible to *L. major*. Studies in IL-13 deficient and transgenic mice have shown that this cytokine also has a role in *Leishmania* susceptibility, as it seems to have similar properties to IL-4 (Matthews *et al.*, 2000), and IL-10-deficient BALB/c mice are markedly more resistant to *L. major* infection than wild-type mice, indicating a role for IL-10 in susceptibility (reviewed in Campos-Neto, 2005, Gumy *et al.*, 2004,

Sacks and Noben-Trauth, 2002). All these studies show how important and relevant is the balance of the Th1/Th2 immune response in order to regulate the outcome of leishmaniasis. But is also important to keep in mind there are evolutionary divergences in distinct species of *Leishmania* causing significant differences in host-parasite mechanisms that ultimately lead to different patterns of disease and different immune mechanisms that mediate curing or non-curing responses (reviewed in McMahon-Pratt and Alexander, 2004).

Macrophage Entry

In order to establish a successful infection, the obligate intracellular pathogen *Leishmania* needs to silently invade macrophage cells and circumvent the host's immune response by keeping macrophage functions deactivated. Despite the fact macrophages have microbicidal properties, it is remarkable that *Leishmania* parasites can still invade and survive within these cells. Molecules present on the parasite's surface or secreted by *Leishmania* have been linked to such invasion and evasion mechanisms (reviewed in Sacks and Kamhawi, 2001). Entry into macrophages is basically a passive process to the parasite, with uptake of both amastigote and promastigote forms facilitated by receptor-mediated endocytosis of the opsonized parasite (reviewed in Denkers and Butcher, 2005) and binding may involve serum factors, complement and sandfly saliva (reviewed in Titus *et al.*, 2006 and in Farell *et al.*, 2002- vol.4, Zer *et al.*, 2001). Different species of *Leishmania* bind to complement receptors CR1/CR3, mannose-fucose receptor (MFR), fibronectin receptor (FnR) and a receptor for a non-enzymatic glycosylation end product (Chang

et al., 1990), due to the differences in surface molecule composition of each species (reviewed in Olivier *et al.*, 2005). Amastigotes may also bind macrophages via a similar mechanism or through the Fc receptor and C-reactive protein receptor (Alexander *et al.*, 1999).

The most abundant component of the *Leishmania* promastigote cell surface is LPG (lipophosphoglycan), whose structure varies between *Leishmania* species and has been implicated in promastigote uptake and in protection from killing by macrophages (reviewed in Matlashewski, 2001). Another important surface molecule is the glycoprotein gp63, a zinc-dependent metalloprotease that is proposed to have an important role in parasite uptake, inhibition of complement-mediated lysis and parasite protection in the phagolysosome compartment (reviewed in Yao *et al.*, 2003). Once inside the host cell, promastigotes prevent the induction of NO and of many cytokines and therefore inhibit macrophage functions that are important for an effective immune response to protect against leishmaniasis. This, in turn, allows the parasite to survive and multiply within macrophage phagolysosome compartment.

Diagnosis and Treatments

The severe pathologies that arise from leishmaniasis, which may ultimately lead to death of the patient, emphasize the need for an early diagnosis of *Leishmania* infections. Diagnostic methods for CL are based on direct microscopic visualization of the parasite from smears, scrapings or biopsies of lesions or indirectly by culturing material obtained from biopsies (reviewed in Schwartz *et al.*, 2006). Direct

visualization of amastigotes after Giemsa staining is useful and fast. However, this process is a painful process to patients, identification of infected cells is difficult and sensitivity varies from 19 - 77 %. Indirect methods, on the other hand, require experience and technical skills to be properly performed and have 58 - 62 % sensitivity (reviewed in Schwartz *et al.*, 2006, Desjeux, 2004b). However, a combination of both microscopy and culture methods increases the sensitivity to detect infection to over 85 % (reviewed in Murray *et al.*, 2005). Another test that is positive in almost all patients with CL is the leishmanin skin or Montenegro test that measures Delayed type hypersensitivity (DTH) reactions to an intradermal injection of a suspension of killed promastigotes. The DTH test is also an important instrument for epidemiological and immunological studies, though it may yield a negative result in the beginning of an infection (reviewed in Schwartz *et al.*, 2006, Manzur and ul Bari, 2006). For VL, serological tests such as the enzyme-linked immunoabsorbant assay (ELISA), direct agglutination test (DAT), the more recent fast agglutination-screen test (FAST) (Schoone *et al.*, 2001) and indirect Immunofluorescence test (IFAT) are commonly used to detect antileishmanial antibodies. The use of the recombinant *Leishmania* antigen k39, a conserved amastigote epitope, in a dipstick system and urine antigen detection tests (latex agglutination) are currently under evaluation as a diagnostic method for VL (reviewed in Hailu *et al.*, 2005 and Murray *et al.*, 2005). Parasitological diagnostics rely on the demonstration of parasites in the spleen, bone marrow or lymph node aspirates. Despite the fact that these methods have an average of 95 % sensitivity,

they are quite invasive and potentially dangerous to patients (reviewed in Hailu *et al.*, 2005, Silva *et al.*, 2005, Desjeux, 2004b, Guerin *et al.*, 2002).

Unfortunately, none of the diagnostic methods above are species-specific and the differentiation of species is a crucial step for appropriate treatment and prognosis determination. Detection of parasite DNA material by a molecular method such as PCR represents a more sensitive and powerful diagnostic approach (with the sensitivity to detect one single parasite or less). Diagnostic PCR can also be specific for certain *Leishmania* species when primers for the conserved regions of the minicircle kinetoplast DNA (kDNA), which also posses a variable region, are used in amplifications (reviewed in Sundar and Rai, 2002a) or when species-specific primers based on single nucleotide polymorphism (SNP) are chosen (Zhang *et al.*, 2006). However, diagnostic PCR is still an uncommon practice in remote places of developing countries where most of leishmaniasis cases occur. Therefore, diagnostic methods that are simpler, quicker and more specific are in great demand.

Treatment

Treatment of VL is limited and pentavalent antimonial drugs (Sb^V), such as sodium stibogluconate (Pentostam) or meglumine antimoniate (Glucantime), have been the mainstay of therapy for about 60 years in most parts of the world. These agents are very toxic and can cause several side effects such as cardiotoxicity, nausea, abdominal pain and pancreatitis. The exact mechanism of action of Sb^V still remains to be completely understood and reports show controversial results, although it is widely accepted that these pentavalent antimonials are "prodrugs" and need to be

reduced to their trivalent form Sb^{III}, by the parasite itself, to be effective (reviewed in Croft *et al.*, 2006). The location and mechanisms of reduction, however, are not completely clear, but stage-specific reduction leading to amastigote susceptibility has been shown, whereas promastigotes do not seem to be affected (reviewed in Croft *et al.*, 2006). Treatment with antimonial drugs has also been threatened by the emergence of parasite resistance. Pentamidine, the second line of treatment against VL, was considered an inadequate substitute for antimony because of great irreversible toxicity, such as renal failure, and the appearance of resistance cases, causing its use to be abandoned (Guerin *et al.*, 2002). Amphotericin B (Fungizone) is an alternative choice and is currently considered a second-line drug for treatment. It is a polyene antibiotic used as an antifungal that is selectively targeted to the parasite due to its higher affinity for ergosterol in trypanosomatids compared to cholesterol in mammalian cells (reviewed in Croft *et al.*, 2006). Amphotericin B then causes the formation of pores and leads to membrane permeability and killing of *Leishmania* (reviewed in Sundar and Rai, 2002b). Nevertheless, serious adverse reactions have also been reported in patients and other drawbacks include its high cost and need for hospitalization due to required infusions (reviewed in Murray *et al.*, 2005, Hailu *et al.*, 2005). The development of lipid-associated (liposome) formulations of amphotericin B (AmBisome), more efficient to target macrophages (possibly related to parasite load), allows it to be administered in a single dose and is better tolerated by patients, producing fewerside-effects (reviewed in Croft *et al.*, 2006, Murray *et al.*, 2005, Guerin *et al.*, 2002). The high costs of this effective drug still restrict its use in underdeveloped countries. Miltefosine is an alkylphosphocholine originally

developed as an anti-tumor agent and is the first and only oral drug effective against VL and is associated with moderate gastrointestinal side effects (reviewed in Berman *et al.*, 2006, Desjeux, 2004a, Sundar and Rai, 2002b). Studies suggest that miltefosine triggers changes in the parasite associated with apoptosis such as cell shrinkage, DNA fragmentation and phosphatidylserine exposure (Paris *et al.*, 2004). But it is an expensive and teratogenic drug and therefore cannot be used for women of childbearing age. In addition, there is a theoretical risk of developing resistance if not used in combination with other antileishmanial drugs due to its long half-life and consequently prolonged subtherapeutic levels in the blood (Berman *et al.*, 2006). Paramomycin (also known as aminosidine) is an antibiotic of the aminoglycoside family that was shown to be highly effective against VL as a single agent or when used in combination with sodium stibogluconate (reviewed in Hailu *et al.*, 2005), showing minimum toxicity and lower costs (Murray *et al.*, 2005). Sitamaquine is a novel drug in the testing phase against leishmaniasis. It is an orally active 8-aminoquinoline analog (lepidine) that has a broad-spectrum antiprotozoan activity, but its specific mode of action is yet to be known (reviewed in Croft *et al.*, 2006). This drug is in phase II trials and it has shown a cure rate in patients with VL of about 80%. Overall, Sitamaquine was efficacious and generally well tolerated with few side effects; however, further tests are still needed in order to confirm drug toxicity in different organs (Wasanna *et al.*, 2005).

While lesions from CL can spontaneously self-cure, depending on different factors, treatment is recommended to accelerate cure, avoid scarring and prevent mucosal invasion. Treatment is also likely to be given for persistant, multiple or

larger cutaneous lesions (reviewed in Murray *et al.*, 2005). The same drugs used for VL, such as amphotericin B and miltefosine, are used for cutaneous disease. Sb^V is administered systemically for the treatment of CL but a prolonged treatment schedule is necessary for cases of mucocutaneous disease (MCL) (Schwartz *et al.*, 2006). Local treatment is chosen instead of systemic modalities when lesions are smaller and there is no risk of MCL. For Old world CL species, intralesional antimony treatment is often used, although infiltrations are painful; topical formulations are also available but they need to penetrate into the dermis to be effective (reviewed in Schwartz *et al.*, 2006, Murray *et al.*, 2005).

Patients generally start to respond to treatment within 6 weeks for CL with noticible lesion healing and conditions often improve within 2 weeks for VL. But treatment failure (unresponsiveness) and relapse (reappearing of lesion) can occur in 5-10% of treated case (Murray *et al.*, 2005). When this happens, a second or third course of Sb^V must be administered and often a combination therapy or a different treatment regimen is given to patients. HIV patients also present special problems since they respond slowly to treatment and relapse often occurs.

Some immunointerventions have been used alone or in combination with traditional Sb^V therapy in an attempt to activate/stimulate the immune system to produce a protective response against *Leishmania* parasites. The basis for immunotherapy comes from the old practice of leishmanization, which is the inoculation of material from lesions to produce a smaller lesion and long-term immunity against subsequent infections. Different approaches have been tested experimentally with the use of killed promastigotes and adjuvants like BCG, use of

cytokines, such as IFN-γ or immunomodulators (reviewed in Davies *et al.*, 2003, Lee and Hasbun, 2003, Sundar and Rai, 2002). In one study, Imiquimod, which is an immune response modifier that can activate macrophages to release NO, was given locally as a cream in combination with standard meglutamine antimoniate in CL patients with prior unresponsiveness to Sb^V alone and produced a 90% cure rate (Arevalo *et al.*, 2001). A second study also demonstrated that topical Imiquimod 5% cream is a superior therapy to Sb^V alone in the treatment of CL by reducing the time required to cure the disease and by improving scar quality (Miranda-Verastegui *et al.*, 2005).

Drug resistance

A major problem that has come up from the use of different drugs against leishmaniasis in the past 15 years is acquired parasite resistance, which has become a clinical threat. Some reasons for the emergence of drug resistance are misuse of the drugs, especially Sb^V. In the case for the region of Bihar, India, drug misuse practices exposed the parasite to drug pressure and progressive tolerance was reached (Croft *et al.*, 2006). The mechanisms by which *Leishmania* acquire resistance against antimonial drugs have been the focus of many research studies, even though results seem to be quite contradictory. I*n vitro* studies show a decrease in the reduction of Sb^V to Sb^{III} in *L. donovani* amastigotes resistant to sodium stibogluconate (Shaked-Mishan *et al.*, 2003). A different study showed aquaglyceroporins mediate the uptake of Sb^{III} and it has been suggested that a decrease in the expression of these molecules could be responsible for resistance. In support of this hypothesis, transfection of

vectors expressing aquaglyceroporyns into resistant *Leishmania* renders them sensitive to that antimonial and a disruption of one of its alleles causes the cell to become resistant to Sb^{III} (Gourbal *et al.*, 2004). There have been no reports so far on the identification of a pump that may be responsible for drug efflux or its role in antimonial resistance (Croft *et al.*, 2006). A couple of amphotericin B resistance cases in *Leishmania*/HIV co-infected patients have been reported. However studies on the mechanism of resistance were inconclusive and only *in vitro* studies showed that resistant promastigotes change their plasma membrane sterol to an ergosterol precursor (reviewed in Croft *et al.*, 2006). In relation to paramomycin, no clinical cases of resistance have been reported and only one report of resistance in cultured promastigotes has been identified thus far. This case was related to decreased drug uptake with no enzymatic modification or mutations in *Leishmania* rRNA, which is the target in bacteria to prevent protein synthesis. However, in *Leishmania*, paramomycin treatment appears to affect mitochondrial ribosomes and induces respiratory dysfunction and mitochondrial membrane depolarization (reviewed in Croft *et al*, 2006).

Overall, measures to prevent the potential spread of resistance are imperative. The development of new drugs is essential and must be complemented by an understanding of the mechanism of drug action, including both the identification of their intracellular targets and parasite mechanisms of defense.

Drug targets

Currently, the main tool against leishmaniasis is pharmacological treatment, since an effective vaccine has yet to leave the arena of clinical trials. The occurrence of parasite drug resistance makes crucial the development of new drugs and identification of their targets, as well as the minimization of treatment duration, costs and toxicity. The best approach for the development of novel drugs involves knowledge of parasite biology and rational design, such as targeting well-characterized unique gene products essential for *Leishmania* survival in the mammalian host or gene products implicated in metabolic pathways specific to *Leishmania*. These essential pathogen proteins should be more resistant to changes through mutations due to their importance in function and therefore become attractive molecular targets. Additionally, data coming from the completion of the *L. major* and *L. infantum* genome projects should facilitate this selection process. A recent analysis of the sequencing results from the Tritryp species (*L. major, T. cruzi* and *T. brucei*) indicates the apparent acquisition of bacterial enzymes, which gives advantages to parasites, are potential drug targets (Kissinger, 2006). Overall, a number of genes have been the focus of different studies and some will be discussed herein.

Leishmania cathepsin L-like cysteine proteases (CP) have attracted some attention for the development of specific protease inhibitors because they play a key role in parasite infection. These enzymes exhibit stage-specificity with some being expressed in the infective metacyclic stage (CPB in *L. chagasi* and CPB1 and 2 in *L.mexicana*) and others in the amastigote stage (CPBs in *L. mexicana* and CPA in *L. chagasi* and *L. donovani*) (Denise *et al.*, 2003) and they have been shown to be

involved in nutrition, host cell invasion and evasion and modulation of the host immune system (reviewed in Matlashewski, 2001, Frame *et al.* 2000). Macrophage and BALB/c murine models infected with CPB mutant *L. mexicana* showed that promastigotes have reduced survival compared to wildtype, suggesting these enzymes are potential targets for antileishmanial drugs (Frame *et al.*, 2000).

Another molecule involved in *Leishmania* virulence is the elongation factor EF-1α, required for protein synthesis and other important cell functions. Nandan *et al.* (2002) demonstrated that *Leishmania* EF-1α is involved in the pathogenesis of *L. donovani* infections by binding and activating host SHP-1 phosphatase and consequently impairing macrophage functions important to pathogen elimination, such as iNOs expression. Even though the degree of homology between the *Leishmania* and human EF-1α is very high (82%), sequence differences have been found, which account for structural differences that may be involved in the host-pathogen interaction, establishing a proof-of-principle for a specific drug target (Cherkasov *et al.*, 2005).

It has been recently suggested that destabilizing the structure of oligomerized proteins by changing their quaternary structure and preventing protein-protein interactions of important biological regulators might be a key mechanism to inactivate enzymes. GDP-mannose pyrophosphorylase (GDP-MP) is an enzyme responsible for mannose metabolism in *Leishmania* by catalyzing the conversion of mannose 1-phosphate to GDP-mannose. This molecule, in turn, is used as a building block for glycoconjugates, critically important for virulence and found in the outer glycocalyx of the parasite (Davies *et al.*, 2004). The self-association and consequent

activation of GDP-mannose pyrophosphorylase has been shown in a recent study (Davies *et al.*, 2004) and disruption of the GDP-MP oligomeric state might inhibit the activity of this important enzyme in *Leishmania*, providing insight into the design of novel drugs (Perugini *et al.*, 2005).

DNA topoisomerases are ubiquitous enzymes that play pivotal roles in cellular processes mainly related to DNA topology, organization and metabolism, namely replication, transcription, recombination and repair are targets for antitumor and antimicrobial drugs (reviewed in Das *et al.*, 2006). Following the same rationale, these enzymes could also be targeted in *Leishmania* parasites. Studies have shown these *Leishmania* enzymes are sufficiently distinct from their human counterparts, to allow differential chemical targeting (Das *et al.*, 2006). There are three different categories of DNA topoisomerases: type IA, IB and type II. Despite homology between trypanosomatid DNA topoisomerase and other eukaryotic type 1B enzymes, *Leishmania* and *Trypanosoma* type I DNA topI differ structurally from all other eukaryotes: they are heterodimers, with each subunit (core and catalytic domain) being encoded by different genes located in different chromosomes, but once synthesized they associate with each other through protein-protein interaction and form an active heterodimeric enzyme (reviewed in Reguera *et al.*, 2006, Das *et al.*, 2006).

Genome content

Leishmania parasites have a compact genome of approximately 35 Mb with 60% of its content being composed of G+C rich sequences. Genome size was based

on *Leishmania major*-Friedlin (MHOM/IL/81/Friedlin) genome, the reference strain used in the *Leishmania* Genome Sequencing Project. *Leishmania* are diploid organisms, although some chromosomes are aneuploid, with 36 chromosome pairs, in general, ranging in size from 0.28 to 2.8 Mb. Species of the new world have 34 or 35 chromosomes, with chromosomes 8+29 and 20+36 fused in the *L. mexicana* group and 20+34 in the *L. braziliensis* group. But gene order and sequences are highly conserved among 30 different species (Ivens *et al.* 2005): the *Leishmania* karyotype seems to be conserved among *Leishmania* strains and species overall, although observations of a small percentage of distinct patterns due to modest chromosome size polymorphisms among strains and greater size polymorphisms among species have been demonstrated (reviewed in Sunkin *et al*, 2000).

Not much is known about transcription initiation in trypanosomatids, and only a few promoters have been examined in regards to their function (Ivens *et al*, 2005). Promoters for protein-encoding genes (RNA polymerase II promoters) have not been identified yet (Clayton, 2002), with the only exception being the SL RNA gene promoter dependent on RNA pol II (reviewed in Palenchar and Bellofatto, 2006). Genes in *Leishmania* chromosomes are organized into large polycistronic units and are characterized by a unique arrangement of directional gene clusters (DGC), oriented in such a way that their mRNAs are transcribed into long polycistronic mRNAs either convergently from or divergently toward the telomeres (Ivens *et al*, 2005, Laurentino *et al*, 2004). The *L. major* genome is organized into 133 gene clusters of tens to hundreds of protein-coding genes, with unrelated predicted function, on the same DNA strand (Ivens *et al*, 2005). Furthermore, *Leishmania*

genes are known to be mostly free of introns and constitute a high-density genome, with 45% of which estimated to be protein coding regions (Laurentino *et al*, 2004).

Trypanosomatids have three polymerases with typical functions: RNA polymerase (RNA pol) I transcribes rRNA; RNA pol II transcribes protein-enconding genes from mRNAs and RNA pol III transcribes small RNAs (tRNAs and snRNAs); RNA pols I and II are also involved in polycistronic transcription (reviewed in Palenchar and Bellofatto, 2006 and Beverley, 2003). Some studies suggest that polycistronic transcription by RNA pol II initiates bidirectionally within the divergent strand-switch (STS) region, and terminates within the convergent STS region, which usually contains tRNA, rRNA and/or snRNA genes (reviewed in Palenchar and Bellofatto, 2006, Ivens *et al.*, 2005). Some long DGCs hold intervening tRNA and snRNA, which is suggestive of more than one polycistron (Ivens *et al.*, 2005). As mentioned above, transcription proceeds towards the telomeres in most cases. Findings indicate that trypansomatid genomes, *Leishmania* included, is disproportionately high in the number of proteins with a zinc-finger domain. This fact, along with the presence of polycistronic gene organization and paucity of both RNA pol II and transcriptional regulation are consistent with posttranscriptional control mechanisms responsible for the control of gene expression (Ivens *et al.*, 2005). mRNA processing is also carried out in a distinctive manner to generate mature mRNAs with trans-splicing of a spliced-leader (mini-exon) RNA to the 5' of almost all mRNA and with polyadenylation, whose site is determined by trans-splicing of the downstream mRNA (reviewed in Beverley, 2003 and Shapira *et al.*, 2001). Cis-splicing is a rare event in trypanosomatids, but both cis- and trans-

splicing seem to be catalysed by the spliceosome (Ivens *et al.*, 2005). At the postranslational level, protein modification seems to engage typical eukaryotic processes, namely, phosphorylation, glycosylation and lipidation (Ivens *et al.*, 2005). Nevertheless, several essential modifications that facilitate membrane attachment and/or protein-protein interactions have also been characterized in trypanosomatids like glycosylphosphatidylinositol (GPI)-anchor addition, acylation and prenylations (Ivens *et al*, 2005).

Trypanosomatids possess a two-unit genome: besides their genomic DNA, trypanosomatids also have the mitochondrial DNA in the kinetoplast, namely the kDNA. These two genomes are replicated periodically in the cell cycle and data from timing of the cell cycle suggests DNA synthesis is triggered in the mitochondrion at the same time as in the nucleus, although segregation of the kDNA occurs before separation of the replicated nuclear genome at mitosis (reviewed in Gull, 2001). The kDNA is made up of two types of circular DNA: the network of several thousands of concatenated minicircles, each 0.5-2.5 kb in size, depending on the species, and 20-30 maxicircles which contain genetic information equivalent to a normal mitochondrial genome, with sequences that hybridize with mitochondrial gene sequences from other organisms (genes for mitochondrial proteins), and mitochondrial ribosomal RNAs. The minicircles in this organelle are responsible for encoding guide RNAs that have complementary regions to the edited mRNA segments involved in mitochondrial RNA editing, which results in the insertion or deletion of uridine residues in the maxicircle transcripts to create functional open reading frames (ORFs) (reviewed in Costa-Pinto *et al.*, 2001, Morris *et al*, 2001, Weissmann *et al*, 1990).

Post-transcriptional Control

In trypanosomatids, mature mRNAs are formed by processing of polycistronic pre-mRNA units transcribed by RNA polymerase II, as discussed above. No evidence for the transcriptional activation of developmentally regulated genes has been found to date, and mRNA levels are exclusively determined by post-transcriptional mechanisms involving different processing of the polycystronic transcript mRNA and mRNA stability (reviewed in Shapira *et al.*, 2001). Maturation of mRNAs involves two processing reactions, namely *trans*-splicing of a capped 39-nucleotide (nt) miniexon near the 5' end of the coding sequence and specific polyadenylation. Although *trans*-splicing precedes polyadenylation, the two mechanisms are coupled (Ullu *et al.*, 1993) and polyadenylation occurs at a fixed distance of ~250 nt upstream of the trans-splicing site, with polypyrimidine tracks and AG splice accepting sites serving as signal for these two mechanisms (reviewed in Clayton, 2002, Shapira *et al.*, 2001, LeBowitz *et al.*, 1993). Not much is known about control elements in *Leishmania* but the regulatory needs during their life cycle must be accomplished through downstream mechanisms that involve RNA and protein regulatory processes (reviewed in Beverley, 2002). That, in turn, could affect gene expression, mRNA stability or translation. Different studies indicate that elements in the 3' unstranslated region (UTR) and intergenic regions of mRNAs are involved in both altered mRNA stability and mRNA translation (McNicoll *et al.*, 2005, Larreta *et al.*, 2004, Zilka *et al.*, 2001, Charest *et al.*, 1996).

Vaccines

Despite the fact that leishmaniasis is one of the most significant parasitic diseases and is considered a category 1 disease (emerging and uncontrolled) by the WHO (Murray et al., 2005), there are currently no available effective vaccines for human use. Leishmaniasis may be a preventable disease by vaccination as it is known that individuals affected with primary cutaneous disease are protected from re-infection and are afforded life-long immunity. Experimentally, the development of CD4 Th1 and cell-mediated responses are the main players in mice resistance. However, in vaccines tested so far (animal models or clinical trials), a polarized Th1 response showed protection but rarely gave long-term immunity or complete protection against *Leishmania* (reviewed in Scott *et al.*, 2004, Davies *et al.*, 2003). It is possible that the lasting immunity observed in previously infected individuals is due to the presence of a persistent low number of parasites and inability to reach sterile immunity, even after "cure" (reviewed Sacks and Noben-Trauth, 2005). Therefore, the solution for this impasse may come from a better understanding on how memory T cells develop and function to help comprehend this issue (reviewed in Scott *et al.*, 2004). A second matter to be taken into consideration is the fact that the immune response differs according to the species of *Leishmania* involved in the infection, suggesting that the requirements for vaccines intended to protect from CL may be different from those for visceral disease (Selvapandiyan *et al.*, 2006). Nonetheless, studies continue in an effort to find an effective vaccine against *Leishmania* infection.

Vaccines against leishmaniasis developed so far are comprised of three categories: 1) live vaccines, including genetically altered organisms, 2) first generation vaccines, consisting of fractions or whole killed parasites and 3) second generation vaccines, where all defined vaccines, with recombinant proteins, DNA, etc, are included (Khamesipour *et al*, 2006).

The inoculation of material from *Leishmania* lesions in hidden areas of the body of uninfected children is an ancient practice in parts of the Middle East and is the first documented use of live parasites to attempt immunization. The major problem with the use of live virulent parasites, though, is the dangers of uncontrolled pathogenesis, including large skin lesions, which caused this practice to be discontinued in the 1990's (reviewed in Handman, 2001). Recent advances in molecular biology techniques have allowed the manipulation of the *Leishmania* genome to generate live attenuated vaccines. This may involve the introduction of or, more commonly, the elimination of genes to produce avirulent promastigotes and amastigotes that are unable to produce pathology or to revert to a virulent form. Protection from avirulent challenges in mice was achieved after deletion of genes, such as the enzyme dihydrofolate reductase-thymidylate synthetase (DHFR-TS) in *L. major*, the glucose transporter gene family or the cysteine protease in *L. mexicana* (reviewed in Selvapandiyan *et al.*, 2006, Handman, 2001). Very recently, a non-pathogenic species of *Leishmania* to humans, *L. tarentolae*, was used as a live vaccine in BALB/c mice. This vaccine was able to elicit a protective Th1 immune response against subsequent inoculation of the infectious *L. donovani* by inducing T cell proliferation and production of IFN-γ (Breton *et al.*, 2006). However,

speculations that a uniform large scale production of avirulent strains with well defined conditions is not feasible and could cause a halt in the development process of a live-attenuated parasite vaccine.

The use of killed parasites started in the 1940's in Brazil, but it was in the late 1970's that Mayrink *et al.* obtained promising results with the development of a killed vaccine composed of five isolates of *Leishmania* containing four different species, and later on with the use of a single strain of *L. amazonensis* in the Leishvaccin ® (Biobras) (Mayrink *et al.*, 1979, Marzochi *et al.*, 1998). These vaccines were both used prophylactically and therapeutically with antimonial drugs, and when adjuvants were introduced, such as Bacillus Calmette Guerin (BCG), there was an increase in cure rates (reviewed in Handman, 2001). During the past 20 years clinical trials have been conducted in Brazil to evaluate the response to these vaccines. Recently De Luca *et al.* (2001) confirmed the presence of acquired T lymphocyte-mediated immune responses against various dermotropic *Leishmania* species (but not against VL) characterized by the production of IFN-γ and a predominance of the CD8+ T cell phenotype among the *Leishmania*-reactive cells in 50-70% of individuals. Their *in vitro* results also showed a significant increase in *Leishmania* antigen-induced IFN-γ and IL-2 production and the absence of increase in IL-4 production by T cells. Old World species, such as killed *L. major*, has also been tested but no significant protection was achieved unless alum (aluminum hydroxide) was used in combination with killed parasites + BCG. This vaccine also showed increased cure rates when used therapeutically with antimonial drugs (reviewed in Khamesipour *et al*, 2006). Various crude *Leishmania* fractions have been demonstrated to be immunogenic and

protective in mice. An example of one of these, the fucose-manose ligand (FML) antigen has been used in trials for a dog vaccine and has induced around 90% of protection in naturally exposed vaccinated dogs (reviewed in Khamesipour *et al*, 2006). Another fraction from lysates of *L. major* or *L. infantum* promastigotes, comprised of proteins with molecular weights between 67-94 kDa and termed F-2, induced resistance to promastigote challenges in mice (Frommel *et al.*, 1988). The disadvantage of the use of both killed parasite and partial fraction methods is the lack of reproducibility and of knowledge about the involvement of molecules and epitopes that could promote a protective immune response.

Refinement of vaccine strategies led to the use of better studied and defined candidates. Finalization of the *Leishmania* genome project has also allowed identification of a number of genes present in *Leishmania*, especially in the amastigote stage which is responsible for all clinical manifestations in humans. One of the first recombinant molecules used was the membrane glycoprotein gp63. Both recombinant and native proteins seem to be protective, even though results in humans and animal models have been variable. The glycolipid LPG also provided a good level of protection, depending on the adjuvant used in combination with this molecule (reviewed in Handman, 2006). The *Leishmania* homologue of a receptor activated for C kinase (LACK) expressed both in promastigotes and amastigotes was able to protect mice from subsequent infections when combined with IL-12 (Mougneau *et al.*, 1995). Cysteine proteases, which are primarily expressed and active in the amastigote stage, are also recognized by the immune system of *Leishmania* infected patients. Immunization studies with cysteine proteases conducted in mice

demonstrated that, in combination with adjuvants, they can induce immunity against *L. major* infection (Zadeh-Vakili *et al.*, 2004, Rafati *et al.*, 2002, 2001, 2000). Candidate molecules have been tested against VL; the amastigote-specific A2 recombinant proteins provided significant protection (89% reduction in liver parasite burden with high IFN- γ production) when used to immunize mice followed by challenge with *L. donovani* (Ghosh *et al.*, 2001). *L. chagasi* LCR1, which shares homology with *Trypanosoma* flagellar protein, is another protein that induced IFN-γ production and partially protected BALB/c mice against *L. infantum* (Wilson *et al.*, 1995, Streit *et al.*, 2000).

A sandfly (*Phlebotomus* spp.) salivary protein of 15 kDa termed SP15 was also used as a vaccine, along with the salivary gland homogenate (SGH), and was able to provide protection in mice challenged with *L. major* plus SGH (Valenzuela *et al.*, 2001). This is consistent with reports that individuals from endemic areas naturally develop antisandfly saliva antibodies and when they acquire leishmaniasis, through the bite of the insect vector, they develop a cell mediated Th1 immune response (reviewed in Titus *et al.*, 2006).

Access to the genome database has allowed the use of diverse *Leishmania* DNA sequences in the development of DNA vaccines in an effort to express *Leishmania* proteins *in vivo* in the host (in a mammalian expression vector) with these antigens being appropriately presented to the immune system as occurs in natural infections. DNA vaccination can elicit both humoral and cellular responses and immunostimulatory molecules like IFN-γ or IL-12 have been used to increase vaccine effectiveness; DNA unmethylated CpG motifs have also been used in an effort to

mediate a Th1 response in the host (reviewed in Gurunathan *et al.*, 2000). Many of the molecules mentioned in the section above have also been tested in DNA vaccines against leishmaniasis. The LACK antigen has given varying degrees of protection against *L. major*, but not against other species, in immunized mice (reviewed in Kubar and Fragoki, 2005). The A2 genes showed great potential in DNA vaccination in combination with the HPV E6 gene (used to inhibit the tumour suppressor p53 and therefore inhibit cellular effects like apoptosis or DNA repair mechanisms), as the combined vaccination conferred a good level of protection in mice against subsequent challenges with *L. donovani* (Ghosh *et al.*, 2001). A DNA vaccine containing the SP15 gene of the sandfly salivary protein was also able to control *Leishmania* infection in mice. In this study, vaccinated mice deficient in B cells were infected with *L. major* plus SGH, they presented humoral and DTH responses against the parasites. These results argued that a DTH response against saliva provides protective effects and that salivary gland proteins or the DNA that encodes them could be used as vaccine alone or in combination with an anti-*Leishmania* vaccine, which may allow the host exposed to the bite of infected sand flies to develop minor or no pathology and a strong anti-*Leishmania* immunity (Valenzuela *et al.*, 2001).

Thus far, results with DNA vaccination show great potential and much has been learned from these trials, especially about how the nature of the antigen or the adjuvant, dose and route of administration can influence the outcome of the responses to DNA vaccination. But achievement of a more effective vaccine may only be reached when a cocktail of several potentially immunogenic molecules is used to

maximize the host response to elicit a better protection against infections with different species of *Leishmania*.

Differentiation

Leishmania parasites cycle between vector and host (Fig. 2) and are therefore exposed to extremely different environments, undergoing a developmental program with differentiation into highly adapted forms that enable their survival. This transformation is linked to remarkable biochemical and morphological changes, including flagellum restructuring and synthesis of new surface molecules correlated with differential gene expression (Bente *et al.*, 2003), or most likely to differential protein expression, in response to environmental changes. However, the molecular and biochemical mechanisms involved in this differentiation remains to be understood and thus far knowledge is still limited.

In the mammalian host, *Leishmania* grows and survives as intracellular aflagellated amastigotes in pH 5.0 and temperatures of 37 °C, and in the cold-blooded sandfly vector midgut as extracellular flagellated promastigotes in temperatures of around 27 °C and alkaline pH. Procyclic promastigotes intensely replicate in the digestive tract of the insect vector and become attached by their flagella to the sandfly epithelial midgut cells. Upon proliferation, non-infective and immature procyclic forms lose their epithelial cell attachment property and differentiate into the virulent, non-dividing metacyclic, that migrates to the sandfly mouth parts prior to transmission (Sacks and Kamhawi, 2001). Metacyclogenesis is accompanied by

morphological changes, as well as differences in gene expression and structural modification of surface molecules like LPG, which mediates release of metacyclics from the insect midgut, followed by migration towards the prosbocis and injection into the skin of the mammalian host during the sandfly bloodmeal (Sacks, 2001). Morphologically, metacyclics are shorter and slender than procyclics with their flagella measuring twice the length of their cell body. Metacyclogenesis seems to be a pre-requisite for complement-mediated lysis resistance and therefore important for host invasion (McConville *et al.*, 1992). This process can be replicated *in vitro* in axenic cultures, where a fraction of non-infective logarithmic (stationary) phase procyclics differentiate into infective metacyclics (Sacks and Perkins, 1984). Few metacyclic-specific proteins have been identified to date (Knuepfer *et al.*, 2001) but the main marker of this process is still the developmental differences in LPG structure, which is the basis for current methods of metacyclic purification (Spath and Beverley, 2001).

A number of genes preferentially expressed in the promastigote stage of the *Leishmania* life cycle have been identified to date. One example is the 3' nucleotidase/nuclease, involved in purine salvaging in *Leishmania*, which is capable of breaking down and dephosphorylating exogenous purine sources to nucleosides and making them suitable for transport across the membrane. This enzyme is expressed in procyclic and metacyclic promastigotes, but is absent from the amastigotes (Sopwith *et al.*, 2002). The paraflagellar rod (PFR), restricted to the flagella of kinetoplastids, is a cytoskeletal structure that runs along the length of the flagellum next to the axoneme once it emerges from the flagellar pocket and is

essential for flagellar motility in *Leishmania* promastigotes; however, it is absent from the attenuated flagellum of amastigotes (Bastin *et al.*, 1998). Its main components, the PFR1 and PFR2 proteins, have promastigote-specific expression with abundance of mRNAs only observed in promastigotes, correlating well with the presence or absence of the PFR structure during the *Leishmania* life cycle (Moore *et al.*, 1996). LPG and the metalloprotease gp63 are genes with higher expression in the promastigote stage that have been well studied due to their roles in facilitating *Leishmania* survival in the mammalian host.

Transformation from promastigotes to amastigotes occurs when the former are phagocytosed by macrophages, multiply and reside in the phagolysosome compartment and are exposed to an environment with lower pH (4.5 - 6.0). *Leishmania* parasites are able to quickly adapt to this acidic pH, and once transformed into amastigotes, many of their enzymes have optimal activity conditions in the new environment, although intracellular pH of both promastigotes and amastigotes remain constant (Zilberstein and Shapira, 1994). It has long been known that exposure to reduced pH and elevated temperatures initially trigger differentiation and influence protein synthesis. Therefore, the heat shock system has been the focus of many studies regarding differentiation, since the drastic changes *Leishmania* encounters induce expression of Heat shock proteins (HSPs), which are involved in cellular response to stress. In *Leishmania*, increased accumulation of mainly HSP70, HSP83 (also known as HSP90) mRNAs, due to posttranscriptional upregulation, has been detected after heat shock conditions *in vitro* and *in vivo* (Brandau *et al.*, 1995), although these genes are transcribed constitutively and their transcription is not

induced by heat shock. Abundance of another protein, HSP 100, is only detected in amastigotes but not in the promastigotes (Krobitsch *et al.*, 1998). These results argue HSPs could be important players in the differentiation process.

Recently, Barak *et al.*, (2005) showed the course of events that ultimately lead to differentiation in *Leishmania* parasites: five hours after the initial differentiation signals happen (shift in temperature and pH), morphological changes start to be noticed and, induced by heat, cells arrest at G1 within the first three hours, although they returned to the cell cycle and resumed growth at hour six after signal. These authors also showed that acidic pH and higher temperatures have very specific roles in the differentiation process; while an increase in temperature induces growth arrest, low pH releases growth arrest and induces heat adapted promastigotes to transform into amastigotes. Investigation of genes involved in this process still remains to be established.

Stage-specific gene expression in *Leishmania* can be mimicked *in vitro* by applying changes in temperature and pH similar to those occurring during the parasite life cycle. Several species have been used to produce axenic amastigotes with each one requiring special conditions (related to temperature, pH and nutrients) for this differentiation (Gupta *et al.*, 2001). However, generation of *L. major* axenic amastigotes seems to be difficult (Gupta *et al.*, 2001) and trials with different strains haven't been successful thus far (Debrabant *et al.*, 2004). To date, a limited number of genes have been shown to be differentially expressed in either promastigote or amastigote stages. Axenic amastigotes are more infective than promastigotes and express stage-specific proteins like A2, amastin, specific proteases (CPA cysteine

proteases in *L. chagasi and L. donovani* and CPB in *L. mexicana*), and HSP 100 (Charest and Matlashewski, 1994;Wu *et al.*, 2000;Denise *et al.*, 2003). Exposure to higher temperatures typically up-regulates expression of HSPs, as discussed above, and down-regulates the expression of β- tubulin or surface molecules like LPG, similarly to *bona fide* amastigotes (Gupta *et al.*, 2001). Genes that are preferentially expressed in response to various extracellular stimuli probably play an important role in *Leishmania* survival and pathogenicity within macrophages. This is extremely important since amastigotes are responsible for all clinical manifestation in the mammalian host. The identification and characterization of such genes and their control mechanisms will help better understand stage-specific gene expression in amastigotes and may provide enough insight for the development of novel therapeutic strategies.

A2 proteins

Among the amastigote-specific proteins, the A2 family (seven proteins that differ according to the number and length of repeated motifs) range from 45 to 100 kDa, are solely expressed in the amastigote stage of *L. donovani* and *L. mexicana* complexes but are not present in the *L. major* or *L. tropica* complexes (Ghedin *et al.*, 1997;Zhang *et al.*, 1996). A2 is one of the best markers for amastigote differentiation to date and its accumulation requires both an increase in temperature and acidic pH. However, pH shift is considered the major trigger for developmental expression of the A2 gene and mRNA slowly accumulates after transfer of cells, with A2 protein accumulation following similar kinetics (Charest *et al.*, 1996). As early as 1 hour

after transfer to higher temperature and lower pH, promastigotes start to express low molecular weight A2 and after 5 hours they express all A2 proteins at similar levels to amastigotes (Barak *et al.*, 2005). Accumulation of transcripts is regulated by post-transcriptional mechanisms at the level of mRNA stability and involves regulatory elements within the 3' untranslated region (UTR) (Ghedin *et al.*, 1997; Zhang *et al.*, 1996). There are at least 7 copies of the A2 gene per haploid genome clustered in arrays and tandemly associated with copies of related sequences termed with A2rel, although no homology has been found between these 2 genes. However unlike A2, A2rel is not developmentally expressed but is constitutively expressed throughout the *L. donovani* life cycle. Its sequence is conserved in different *Leishmania* species (*L. donovani, L. mexicana, L. tropica* and *L. braziliensis* complexes) (Ghedin *et al.*, 1998). The N-terminal of the A2 protein contains a leader sequence, but the protein has no hydrophobic or anchor region that could indicate it is a membrane protein and it has been suggested it might be secreted, even though it was not found in the supernatant of amastigote-like cells and was only be observed in the cytoplasm of A2 transfected *L. donovani* (Zhang *et al.*, 2006). Zhang and Matlashewski (1997) investigated the biological role of the A2 proteins by using antisense RNA to study their involvement in amastigote survival within macrophages. Amastigotes with inhibited or impaired A2 expression (94% less A2 compared to wild-type amastigotes) could proliferate in culture in a manner indistinguishable from wild-type cells, and showed no difference in their ability to invade macrophages but had a reduced ability to proliferate and multiply within macrophages *in vitro* and could barely survive in BALB/c mice. These results indicated A2 is a virulence factor

required for leishmanial survival in the mammalian host (mouse model) but it is not required for *L. donovani* proliferation or differentiation in axenic culture. Other studies with A2 (Zhang and Matlashewski, 2001) and its expression in *L. major* showed these proteins might be involved in visceralization of leishmaniasis since it could increase the ability of that species, which is not typically viscerotropic, to survive in the spleen of infected mice, resulting in splenomegaly.

B. SIGNAL TRANSDUCTION

Cells are highly responsive to their environment and their physiological state is determined by exogenous and endogenous signals that ultimately lead to gene transcription and protein expression. Signal transduction cascades mediate this process in order to detect, amplify and integrate various external signals to regulate many biochemical processes, generating responses such as changes in enzyme activity, gene expression or ion-channel activity (Rosenfeld *et al.*, 2006).

Some external stimuli can be sensed by cells through surface molecules, while others can be directly internalized by surface receptor interactions. As a result, an extracellular signal is transduced into the cells and a sequence of reactions leads to a specific cellular response. There are different types of cell-surface receptors that interact with soluble ligands and they are classified as: G protein-coupled receptors, ion-channel receptors, tyrosine kinase-linked receptors and receptors with intrinsic enzymatic activity (reviewed in Lodish et al., 2000). Binding of the ligand to these

receptors is specific and may or may not induce formation of a second-messenger, which, in turn, is responsible for the transmission of the signal.

Discussion of the following sections will focus mainly on signaling through receptors with intrinsic activity, where activation by binding of ligands leads to dimerization of the receptor and activation of its kinase activity; these receptors, also known as receptor serine/threonine kinases or receptor tyrosine kinases, are able to autophosphorylate residues on their cytosolic domains and can also phosphorylate their target substrates.

Phosphorylation

Proteins are able to perform numerous functions relying on the versatility of the 20 existing amino acids. However, they can be covalently modified through the addition of groups other than amino acids. Covalent modification, and in particular phosphorylation, are reversible and act to alter - activate or inhibit - a wide variety of cellular proteins in response to environmental signals. Many proteins in a typical eukaryotic cell are modified by phosphorylation, which is the most prevalent reversible covalent modification and one of the most common methods utilized to regulate protein activity, stability, protein-protein interactions and function (Zhang *et al.*, 2002). Phosphorylation is a highly effective mechanism for controlling the activity of proteins for structural, thermodynamic, kinetic, and regulatory reasons since the phosphoryl group adds two negative charges to a modified protein, leading to structural changes that can alter substrate binding and activity. The phosphate

group can also form hydrogen bonds, allowing specific interactions with hydrogen-bond donors. Phosphorylation is an incredibly fast mechanism and it induces great cellular effects because a single enzyme (kinase) can phosphorylate a number of target substrates in a short interval, and further amplification can arise from this first action because target proteins may be different enzymes that could affect other substrate molecules (reviewed in Berg *et al.*, 2002).

As briefly mentioned above, protein kinases are the enzymes responsible for the catalysis of the phosphorylation process through the addition of a phosphate group onto proteins. The terminal phosphoryl group of ATP, the most commonly used source of phosphate group, is transferred to specific serine and threonine residues by serine/threonine kinases and to specific tyrosine residues by tyrosine kinases with the release of a large amount of free energy when the phosphate bond in ATP is broken to produce ADP (Fig. 4). Kinases have been extensively studied and constitute one of the largest protein families known, and each is responsible for phosphorylating a different protein or set of proteins. The diversity of these enzymes allows regulation and fine-tuning according to tissue specificity, time or substrate (Berg *et al.*, 2002). This reaction is reversed by another group of enzymes, the phosphatases, which in turn remove the phosphate group by catalyzing its hydrolytic removal from proteins (Fig. 4). Therefore, cellular phosphorylation is controlled by activities of both kinases and phosphatases. There are many different phosphatases in the cell, some of them are highly specific and remove phosphate groups from only one or a few proteins, whereas others act on a broad range of proteins and some are targeted to specific substrates by regulatory subunits (Zhang *et al.*, 2002). Kinases that

phosphorylate proteins in eukaryotic cells belong to a very large family of enzymes, which share a catalytic sequence of 250 amino acids. Various family members contain different amino acid sequences on either side of the kinase sequence, and often have short amino acid sequences inserted into loops within it. Some of these additional amino acid sequences enable each kinase to recognize the specific set of proteins it phosphorylates; other sequences allow the activity of each enzyme to be tightly regulated, so it can be turned on and off in response to different signals.

So, as a result of the combined actions of kinases and phosphatases, the phosphate groups on proteins are constantly turning over by additions and removals, leading to different status of protein activation in the cell. In higher eukaryotes, protein phosphorylation and dephosphorylation is a key mechanism involved in a number of physiological processes such as cell cycle control, cellular proliferation, growth, transformation and differentiation.

Kinases

AGC group

The AGC group includes the cAMP-dependent protein kinase A (PKA), cGMP-dependent protein kinase G (PKG), phospholipid-dependent protein kinase C (PKC) and Phosphoinositide-dependent protein kinase 1 (PDK1). Members of this family have received considerable attention because they are downstream effectors of intracellular second messengers (cAMP, cGMP or phospholipids and Ca^{2+}) in animals and yeast. The structurally related AGC kinases phosphorylate their substrates at

serine and threonine residues and participate in a variety of crucial and well-known processes such as protein synthesis, gene transcription, cell growth, apoptosis and cytoskeletal remodeling (Bogre *et al.*, 2003). The members of this family also play critical roles in receptor tyrosine kinase (RTK) signal transduction, essential for regulating many important biological events just mentioned above. Kinases from this group display a high degree of primary sequence conservation within their kinase domains and activation loops; however, outside the catalytic domain the AGC kinases usually have little similarity. Their activity depends upon phosphorylation of the activation loops and each member also has a highly conserved phosphorylation site (Parker and Parkinson, 2001;Peterson and Schreiber, 1999). The representative member of this group is PKA, composed of the relatively small catalytic (C) and regulatory (R) subunits. The catalytic subunit is considered a prototype because of its constitutive activity and apparent simplicity, containing short flanking regions at the N- and C-termini in addition to the conserved core (Taylor *et al.*, 2005;Breitenlechner *et al.*, 2004). The active protein is released only upon binding of cAMP, when the catalytic subunit is assembled as a fully phosphorylated enzyme (Taylor *et al.*, 2005). In its inactive state the catalytic domain is sequestered by its association with a regulatory subunit. The regulatory domain, on the other hand, also displays key functions such as targeting the kinases to the appropriate cellular location and regulation of the kinase activity by serving as an autoinhibitory module (Newton, 2003).

PKG shares similar structural and biochemical properties with PKA, however, unlike protein kinase A, PKG does not dissociate into catalytic and regulatory

subunits upon activation. PKG is a dimer, each containing a regulatory domain (R) and a catalytic domain (C) on a single polypeptide chain (reviewed in Francis and Corbin, 1999). There are two homologous forms of PKG: type I, with an acetylated amino (N)-terminal, usually associated with the cytoplasm and type II, with a myristylated N-terminal, associated with the membrane (reviewed in Francis and Corbin, 1999). PKG is an important regulator of diverse cellular processes and many reports have indicated that PKG might regulate cell function by activating members of the MAPK family of signaling proteins whose function is to regulate the contractile activity of the smooth muscle cell (Komalavilas *et al.*, 1999).

PKC comprises a superfamily of isoenzymes activated in response to various stimuli, inducing activation of different isoforms. These isoenzymes are grouped into subclasses according to the domain composition of the regulatory moiety and its activity depends on Ca^{2+} and on the phospholipid diacylglycerol (DAG). The two basic modules are the C1 and C2 domains: the diacylglycerol and the Ca^{2+} sensors, respectively. Some PKCs contain both C1 and C2 domains while others contain only the C1 domain; however the C1 domain can also be found in a number of non-kinase molecules (reviewed in Newton, 2003). Generation of DAG and Ca^{2+} recruits PKC to the membrane by engaging the C1 and C2 domains. This interaction at the cell surface allows substrate binding and phosphorylation (Newton, 2003). This enzyme is believed to be involved in signaling pathways that control cellular metabolism, mitogenesis, apoptosis and differentiation.

Phosphorylation of the AGC family kinases in the activation loop has been found to be mediated by PDK1. This serine/threonine kinase is able to phosphorylate

the conserved threonine residue in the activation loop of AGC family kinases. However, it is not yet established whether all these kinases are direct physiological substrates of PDK1 or whether there are additional substrates. This kinase contains a catalytic domain near its N-terminal and a pleckstrin homology (PH) domain (allowing interactions with lipids) at its C-terminal, which is responsible for targeting to the plasma membrane and hence for substrate interactions; it also contains several autophosphorylation sites (Mora *et al.*, 2004).

CaMK group

Calcium signaling plays an important role in many aspects of cellular growth and development and it's recognized as one of the most important intracellular signals in eukaryotes. Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) play pivotal roles in Ca^{2+} signaling pathways. An increase in intracellular Ca^{2+} on cellular stimulation results in binding of Ca^{2+} to calmodulin (CaM). This Ca^{2+}/CaM complex activates several Ca^{2+}/CaM-dependent enzymes and elicits a variety of Ca^{2+}-dependent cellular responses. Among these enzymes, a group of Ser/Thr protein kinases activated by Ca^{2+}/CaM, called CaMKs, plays a pivotal role in the Ca^{2+} signaling pathways. They are classified into two different categories: the CaMKs with strict substrate specificity (responsible for the phosphorylation of a specific substrate in response to Ca^{2+}) and the multifunctional CaMKs that can phosphorylate multiple protein substrates and are involved in a greater range of physiological responses through phosphorylation of various substrates; it can be exemplified by CaMK-II, the best characterized CaM kinase (Ishida *et al.*, 2003). When

Ca^{2+}/calmodulin binds a subunit of CaMK II, calmodulin displaces the autoregulatory domain of the subunit, enabling it to become phosphorylated by the catalytic domain of a neighboring subunit. Once phosphorylated, the subunit can remain active for a prolonged period, even in the absence of a sustained elevation in Ca^{2+} (Curtis and Finkbeiner, 1999). CaMKII is present in abundance in the brain and is known to play important roles in the central nervous system through the regulation of the synthesis, release, and signaling of neurotransmitters, although it is also involved in carbohydrate metabolism, transcription, cytoskeletal organization and cardiac functions, among others (Ishida *et al.*, 2003).

CMGG and STE groups

The Mitogen-activated protein kinases (MAPKs) and the Cyclin-dependent kinases (CDKs) are included in this group; the STE group is involved in the MAPK pathways and this family refers to the three classes of protein kinases that lie sequentially upstream of the MAPKs (MEK or MAPKK, MEKK or MAPKKK and MEKKK) (reviewed in Plowman *et al.*, 1999). The MAPK family of kinases connects external stimuli through cascades, transmitting these extracellular signals to their intracellular targets, leading to varied cellular responses ranging from activation or suppression of gene expression to the regulation of cell mortality, growth, and differentiation (reviewed in Rubinfeld and Seger, 2005). Each cascade consists of several protein kinases that sequentially activate each other by phosphorylation (Fig. 5A). The core cascade is usually composed of MAPK kinase kinase (MAP3K or MEKK), MAPKK, and MAPK, all of which having highly conserved molecular

architecture. The MAPK is activated by phosphorylation on a conserved tyrosine and threonine in the activation loop by a dual-specificity MAPK kinase (MAPKK or MEK), which, in turn, is phosphorylated on conserved serine and threonine by a MAPKK kinase (MAPKKK or MEKK). The four distinct mammalian MAPK cascades are organized according to the subgroup of their MAPK components: the extracellular signal regulated kinases 1 and 2 (ERKs), the c-Jun N-terminal kinase (JNK), p38MAPK and ERK5 (Fig. 5B). The most extensively studied is ERK1/2, activated by a variety of extracellular agents, which include growth factors, hormones, and neurotransmitters. Extracellular factors, which can act through G protein-coupled receptors, tyrosine kinase receptors, ion channels and other mechanisms, can initiate a variety of intracellular signaling responses that result in the activation of the ERK (MAPK) cascade, which often requires adaptor proteins (reviewed in Rubinfeld and Seger, 2005). After activation, ERK1/2 is able to phosphorylate downstream substrates related to a multitude of cellular functions such as cell survival, motility, proliferation and differentiation (reviewed in Kohno and Pouyssegur, 2006).

Cyclin-dependent kinases (CDKs) are heterodimeric serine/threonine kinases with crucial roles in the regulation and control of eukaryotic cell division. Their enzymatic activity is modulated by protein-protein interactions as well as by both inhibitory and activating phosphorylations. Association with regulatory subunits (cyclins), synthesized and degraded in a cell-cycle-dependent manner, activates CDKs. Cyclins are regulatory partners of CDKs and comprise a diverse family of proteins found in all organisms that share a conserved sequence of 100 amino acids,

the cyclin box, which is necessary for CDK binding and activation (reviewed in Nebreda, 2006). Cyclin binding provides the CDK with targeting domains important for substrate selection and subcellular localization, which in turn determine the biological specificity. Although cyclins play an important role in cell cycle progression, they are not always required for CDK activation (reviewed Nebreda, 2006). Historically, the first member of the CDK family (Cdk1) was identified in genetic screens for *Schizosaccharomyces spp.* mutants with defects in the cell division cycle. This protein, then designated Cdc2 in *S. pombe* and Cdc28 in *Saccharomyces cerevisiae*, was shown to be essential for cell-cycle progression. Subsequently, many members of the Cdk family were cloned and a unifying nomenclature was adopted (the term 'cyclin-dependent kinase') (reviewed in Malumbres and Barbacid, 2005). Cell cycle progression requires a different cyclin-dependent kinase through each stage (Fig. 6): progression through the G0/G1 transition was found to be primarily under the control of cyclin D/Cdk4,6 and the restriction point in the exit from the G1/S transition is controlled by complexes of Cdk2 and E-type cyclin. Two other cyclins (A and E), respectively paired with Cdk2 and Cdc2, were found to be required for progression through S phase (Cdk2/cycA) or driving of the G2/M transition (Cdc2/cycB) (reviewed in Sanchez and Dynlacht, 2005, Cooper, 2000).

Protein Tyrosine kinase (TK) group

Tyrosine phosphorylation is a fundamental mechanism for numerous important aspects of eukaryote physiology (reviewed in Schlessinger, 2000). This

group of enzymes is divided into two families: the transmembrane receptor tyrosine kinases (RTK) and the non-receptor or cytosolic family. Their tyrosine kinase domain has approximately 300 residues with a two-domain architecture shared by serine/threonine kinases; the ATP binding site is located in the cleft between the N and C-terminal lobes and the tyrosine-containing residue of the substrate interacts with residues in the C-terminal lobe (reviewed in Chiarugi, 2005). Tyrosine kinases are the main mediators in the transmission of extracellular signals into the cell and therefore are essential enzymes regulating cellular signaling processes such as growth, differentiation and metabolism.

Signaling by RTKs, which are single-pass membrane proteins with an extracellular ligand-binding domain and an intracellular kinase domain, is perhaps the most extensively studied pathway to date. One of the RTK categories includes the receptors for most polypeptide hormones and growth factors, the latter organized in different subfamilies, such as the epidermal growth factor receptors (EGFRs or ErbBs), fibroblast growth factor receptors (FGFRs), insulin and the insulin-like growth factor receptors (IR and IGFR), platelet-derived growth factor receptors (PDGFRs) and the vascular endothelial growth factor receptors (VEGFRs) (reviewed in Li and Hristova, 2006). RTKs conduct biochemical signals via lateral dimerization in the plasma membrane and ligand binding results in receptor autophosphorylation with subsequent activation of downstream signaling cascades that include mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), protein kinase C and small GTPases, among others. Although these events occur mostly in the cytosol, transcription and cell-cycle regulation are affected by receptor

signaling, which requires the activation and nuclear translocation of transcription factors (reviewed in Massie and Mills, 2006). Studies have shown that nuclear events, once thought to be mediated only by signaling cascades, can also be affected directly by some RTKs and their adaptor molecules (i.e. peripheral membrane proteins that associate with membranes through phosphoinositide and protein interactions with the plasma membrane and endosomes) since they can be directly translocated to the nucleus to affect gene expression, transcription and DNA repair (reviewed in Massie and Mills, 2006). RTK signaling has an important role in the regulation of a diverse set of cellular processes, such as control of cell growth, differentiation, and metabolism and has been implicated in diseases such as cancer and immune deficiencies (reviewed in Alonso *et al.*, 2004).

About one third of protein tyrosine kinases (PTKs) are grouped as non-receptor tyrosine kinases. They are found in the cytoplasm, do not contain a transmembrane domain, and they usually function downstream of the RTKs. Typical cytoplasmic PTKs (cPTK) contain domains that mediate protein-protein interactions (SH2 and SH3 domains), protein-lipid interactions (pleckstrin homology [PH] domain) or can be lipid modified (reviewed in Chiarugi, 2005). SH2, or Src homology region 2, is a domain that binds polypeptides that contain pTyr with significant specificity; SH3 is a small beta-barrel module that presents a non-polar groove complementary to the target short peptides in a polyproline-II conformation, i.e, it binds to proline rich peptides and hydrophobic residues. Cytoplasmic PTKs include Src and Janus kinases, among others. The mechanisms for their activation involve heterologous protein-protein interactions and are therefore more complex.

The extensively studied Src family is the prototype in this group: inhibition of kinase activity is achieved by orchestrated interactions between the SH2 domain and the phosphorylated Tyr in the C-terminal and between the SH3 domain and the SH2 kinase linker. Binding of ligand to the SH2 or SH3 domain and/or dephosphorylation of the C-terminal phospho-Tyr (pTyr) by tyrosine phosphatases (PTPs) releases inhibition, leading to autophosphorylation of the Tyr residue in the activation loop (reviewed in Chiarugi, 2005). The Src family is capable of communicating with a large number of different receptors. It was first identified as the transforming protein (v-Src) of the oncogenic retrovirus Rous sarcoma virus (RSV). Later studies identified Src-related proteins that regulate cellular events in addition to cell proliferation and carcinogenesis, such as cytoskeletal alterations, differentiation, survival, cell adhesion, migration, immune cell function and even learning and memory (reviewed in Thomas and Brugge, 1997).

Phosphatases

About one-third of all eukaryotic proteins are controlled by phosphorylation of specific serine, threonine, and/or tyrosine residues (reviewed in Barford *et al.*, 1998). Most phosphorylation mechanisms are reversible, reflecting the balanced activity between protein kinases and phosphatases. Changes in the phosphorylation state can result from changes in the activities of either of these enzymes. Protein phosphatases are defined by three distinct families: the PPP and PPM families, both encoding protein Ser/Thr phosphatases, whereas the protein tyrosine phosphatase (PTP) family

includes both tyrosine-specific and dual-specificity phosphatases. Within each, structural diversity is generated by the attachment of regulatory and targeting domains and/or subunits to the protein catalytic domain. Regulatory subunits and domains serve to direct the protein to a particular subcellular localization and modulate protein specificity, functions that are regulated by allosteric modification using second messengers and reversible protein phosphorylation (reviewed in Barford *et al.*, 1998).

In higher eukaryotes, four types of serine/threonine specific protein phosphatase catalytic subunits have been identified in a variety of species and are differentiated by their substrate specificity, regulation by peptide inhibitors and bivalent cation requirements. Based on amino acid sequence comparisons, three enzymes, protein phosphatases (PP) 1, 2A and 2B, are members of the same gene family (PPP) possessing a conserved catalytic core and are regulated by Ca^{2+}/calmodulin. Their common catalytic domain has 280 residues and most divergences are found within their noncatalytic N- and C-termini. They are also distinguished by their associated regulatory subunits and form a diverse variety of holoenzymes (reviewed in Ceulemans and Bollen, 2004, Barford *et al.*, 1998). PP1 is involved in controlling multiple cellular functions including glycogen metabolism, muscle contraction, cell cycle progression, neuronal activities, and the splicing of RNA (reviewed in Barford *et al.*, 1998). Protein phosphatase 2A (PP2A) is a highly abundant and ubiquitously expressed Ser/Thr phosphatase whose activity is found in numerous cellular processes. It has long been implicated in cell cycle regulation in many different organisms (reviewed in Ceulemans and Bollen, 2004). PP2B is

characterized by its dependence on Ca^{2+} for activity. It consists of an A-subunit with an N-terminal catalytic domain and a C-terminal regulatory region containing binding sites for the B-subunit and calmodulin as well as an auto-inhibitory sequence at the extreme C-terminal. The fourth enzyme, PP2C, appears to be in a distinct family (PPM) due to different mechanics and structure and Mg^{2+} requirement. Many other phosphatases have been discovered in the PPP family, namely PP4, PP5 and PP6. The former and the latter are similar to the PP2A subfamily on the basis of catalytic domain structure; PP5 is grouped into a separate subfamily not only on the basis of their catalytic domain, but also because of a fused amino-terminal domain that contains three to four tetratricopeptide repeat (TPR) motifs involved in the regulation its phosphatase activity (reviewed in Barford, 1996).

Tyrosine phosphatases

Protein tyrosine phosphatases (PTPs), are highly regulated enzymes and together with PTKs play an important role in regulating cellular growth, metabolism, cell cycle, cell-cell communication (including within the immune system), differentiation, gene transcription and survival; they exert both positive and negative effects on a signaling pathway (reviewed in Chiarugi, 2005, Mustelin *et al.*, 2005, Zhang *et al.*, 2002). Members of PTP family are very diverse and have been identified from prokaryotes to eukaryotes (reviewed in Elchebly *et al.*, 2000). PTPs comprise a superfamily of enzymes with more than 100 members and its hallmark is the presence of the signature motif or active site - the H/V**C**X_5**R**(S/T) within the catalytic domain - containing the invariant cysteine (Cys) and arginine (Arg) residues

required for phosphatase activity (reviewed in Alonso *et al.*, 2004, Zhang *et al.*, 2002).

PTPs are divided according to their sequence homology and substrate specificity into "classical" tyrosine-specific phosphatases, low molecular weight (LMW) phosphatases or dual-specific phosphatases, which can cleave phosphoester bonds in proteins that contain phosphotyrosine (pTyr), as well as phosphoserine (pSer) and phosphothreonine (pThr), the latter exemplified by MAPK phosphatases and Cdc25 phosphatases (Fig. 7) (reviewed in Wang *et al.*, 2003). Many of the PTP substrates have the related SH2 domain sequence that binds pTyr in a sequence-specific manner (reviewed in Hunter, 1989). Tyrosine-specific PTPs are further divided according to their localization into receptor PTPs and cytosolic PTPs (cPTPs): the first can be exemplified by CD45 and generally have an extracellular putative ligand-binding domain composed of a variety of structural domains, a transmembrane region and one or two cytoplasmic PTP domains within the C-terminus (reviewed in Chiarugi, 2005, Elchebly *et al.*, 2000). cPTPs, which include PTP1B and LMW phosphatases, contain a single catalytic domain, N- or C-terminal extensions such as SH2 domains which may have targeting or regulatory functions (reviewed in Chiarugi, 2005, Zhang *et al.*, 2002, Elchebly, *et al* 2000). All PTPs are characterized according to their sensitivity to vanadate, a phosphatase inhibitor, ability to hydrolyze *p*-Nitrophenyl phosphate (pNPP), insensitivity to okadaic acid, a serine/threonine phosphatase inhibitor, and lack of metal requirements for catalysis (reviewed in Denu and Dixon, 1998). The signature motif, which forms the phosphate recognition site, is located at the base of the PTP loop in the active site,

also know as the cleft. The active site pockets of different PTPs vary in size and shape, which defines the depth of the cleft and gives specificity for substrates: the active site is located in a deep pocket in tyrosine-specific PTPs to select exclusively pTyr-containing substrates, while the active site cleft is more superficial in dual-specificity phosphatases and may recognize pTyr or pSer/Thr (reviewed in Chiarugi, 2005, Tonks, 2003,Wang *et al.*, 2003). The active site is also surrounded by different superficial loops that are important for catalysis and substrate recognition (reviewed in Wang *et al.*, 2003).

Catalysis of the phosphate group involves a two-step mechanism and starts with the sulfur atom of the thiol group of the invariant and active cysteine (Cys) residue. The thiolate side chain of Cys serves as a nucleophile to accept the phosphoryl group of the substrate, forming a cysteinyl-phosphate catalytic intermediate. The arginine (Arg) residue makes a hydrogen bond with the substrate phosphoryl group and has an important role in both substrate binding and transition state stabilization during catalysis (reviewed in Wang *et al.*, 2003). PTPs also make use of an aspartic acid (Asp) residue in an accessory loop, which acts as general acid by protonating the ester oxygen of the substrate tyrosyl-leaving group and enhancing the rate of phosphoenzyme formation. In the second step, mediated by a glutamine (Gln) residue from a different accessory loop, the dephosphorylation and hydrolysis of the phosphoenzyme intermediate occurs by attack of a nucleophilic water molecule assisted by the same Asp residue functioning now as a base, with subsequent release of enzyme and inorganic phosphate (reviewed in Tonks, 2003, Wang *et al.*, 2003).

RPTPs are mainly regulated through dimerization, like most transmembrane receptor proteins. However, RPTPs are often inhibited by dimerization through symmetrical interactions between the catalytic site and the inhibitory structural wedge (reviewed in Chiarugi, 2005). By catalyzing the removal of a phosphate group from a tyrosine residue, PTP can act either as an "on" or "off" switch for signal transduction. Although PTPs share a common catalytic mechanism, they have distinct and often unique biological functions, which may also be determined by the identity of the substrates upon which they act in the cell (Zhang *et al.*, 2002)

PTP1B

In higher eukaryotes, protein tyrosine phosphatase 1B (PTP1B) is the prototype of the PTP superfamily, first isolated from human placenta. This PTP was the first one shown to exist as a phosphoprotein *in vivo* and is widely expressed in cells (Tonks *et al.*, 1988). It has been implicated in numerous signaling pathways, possessing a dynamic role as a regulator of multiple receptor tyrosine kinases, such as the insulin receptor (IR), platelet derived growth factor receptor (PDGR) and epidermal growth factor receptor (EGFR) (reviewed in Dube and Tremblay, 2004, Sarmiento *et al.*, 2000). Because of this great versatility, PTPs are considered to have promiscuous activity *in vitro*. Their structure consists of an N-terminal catalytic domain (PTP domain) with the signature motif (I/VH**C**XX**R**S/T), where the essential cysteine residue is at position 215 and, together with the arginine residue, is important for catalysis; accessory loops, namely WPD and Q loops, are also involved in catalysis (reviewed in Tonks, 2003). At the C-terminus the presence of a cleavable

proline-rich motif allows interaction with SH3-domain-containing proteins and a small hydrophobic stretch of 35 amino acid residues is necessary and sufficient to localize the enzyme to the endoplasmic reticulum (ER) facing the cytoplasm (reviewed in Tonks, 2003). Subcellular localization of PTP1B allowing access to substrates in a spatial and temporal manner and is thought to influence the broad range of substrate specificity this enzyme has, since it is proposed it could regulate nascent RTKs during their biosynthesis and transport to the membrane by preventing their premature activation (reviewed in Dube and Tremblay, 2004, Sarmiento *et al.*, 2000). The C-terminus of PTP1B is not only involved with localization and activation of this enzyme, but also exerts an effect on catalysis: a truncated form of the enzyme lacking 75 amino acid residues from the C-terminus has increased PTP activity, which suggest a role for the C-terminus as an activity suppressor (reviewed in Tonks, 2003).

PTP1B has been implicated in the regulation of metabolic signaling (insulin and leptin) (reviewed in Elchebly *et al.*, 2000) and perhaps this is the area where it has been most studied since it is the negative regulator of insulin (i.e. it impairs insulin signaling) through dephosphorylation of key residues within the insulin receptor (IR) and therefore it decreases tyrosine kinase activity. This inhibition leads to insulin resistance through decreased secretion of insulin from the pancreas, raising plasma glucose. Deregulation of this signaling pathway leads to the development of deseases and PTP1B is known to be involved in the etiology of type II diabetes (diabetes mellitus) (reviewed in Elchebly *et al.,* 2000), although its role goes beyond metabolic function and it has recently been shown to be involved in Ras signaling,

behaving as a positive or negative regulator of oncogenesis (reviewed in Dube & Tremblay, 2004, Dube *et al.*, 2004).

PTP1B is a major target for drug development for the treatment of diabetes and obesity (reviewed in Dube and Tremblay, 2004) and with the discovery of its potential involvement in oncogenic signaling, it may also be pursued as a target against cancer.

Phosphorylation in Trypanosomatids

In trypanosomatids, as in higher eukaryotes, signal transduction is an important mechanism to control intracellular events. The role of various components of signaling pathways and the molecular basis of this process have been extensively studied in eukaryotic cells; in trypanosomatids, the identification and characterization of several receptor proteins, kinases, phosphatases and second messengers have been reported, but their function and signaling pathways in which they are involved in have not been elucidated.

Kinases

Trypanosomatid kinome (Serine/threonine protein kinases)

Trypanosomatids consist of parasites of the *Trypanosoma* and *Leishmania* genera, which are two major pathogens of human and domestic animals. *Trypanosoma cruzi* causes Chagas disease, a debilitating disease in South America

affecting multiple organ systems; *T. brucei gambiense* and *T.b. rhodesiense* are responsible for African sleeping sickness in humans, a tsetse fly (*Glossina* spp.) transmitted disease. Another subspecies, *T.b. brucei,* does not infect humans, but it causes a disease called nagana in native antelopes and other African ruminants (reviewed in Vickerman *et al.*, 1988). In the bloodstream and tissue fluids of the mammalian host, *T. brucei* proliferates as a long slender form and differentiates into a growth-arrested short stumpy form pre-adapted for survival in the fly. When bloodstream forms are taken up during a blood meal by the insect vector, the stumpy form differentiates rapidly to the dividing procyclic (insect) form in the lumen of the fly midgut. The parasite continues its life cycle by progressing through a series of further developmental stages (epimastigotes) culminating, in the salivary glands of the fly, in differentiation to the metacyclic form, which is infective for the mammalian host (reviewed in Vickerman *et al.*, 1988). Therefore, *Trypanosoma sp.*, just like *Leishmania*, responds to external and intracellular signals with changes in metabolism and protein profile as they adapt to different environments in their complex life cycle.

Mechanisms of signal transduction have been reported in these parasites, and although there are similarities with mammalian molecules, little is known about the intracellular processes involved in their life cycle. Completion of the genome project for three trypanosomatid species pathogenic in humans (*Trypanosoma brucei, T. cruzi and L. major*) has enabled studies of their "kinomes", that is, the protein kinase genomes (Parsons *et al.*, 2005). The trypanosomatid kinome is 33% larger than that of *S. cerevisae* and twice the size of *P. falciparum*, although compared to humans it is

only one third of the size and has significant differences from the mammalian host (reviewed in Naula *et al.*, 2005). There are approximately 179, 156 and 171 eukaryotic protein kinases (ePKS) and 17, 20 and 19 atypical protein kinases (aPKs) in *L. major*, *T. brucei* and *T. cruzi* respectively. Some of the aPKs lack sequences involved in peptide binding or characteristic domains in ePKs (Parsons *et al.*, 2005). The three kinomes (Tritryp kinomes) analyzed are closely related and only a small number of genes seems to be unique to each species (20 in *L. major*, 11 in *T. cruzi* and 3 in *T. brucei*). Most of their ePKs had highly significant BLAST scores against at least one member of the human, worm, fly and yeast database used (Parsons *et al.*, 2005). About 8% of ePKS from each species are, however, supposedly catalyticly inactive due to the presence of mutations within essential residues (Parsons *et al.*, 2005).

ePKs are grouped by the amino acid sequences of the catalytic domain and comprise a diverse set of proteins broadly divided into two categories: serine/threonine kinases and tyrosine kinases. These kinases are key elements in the regulation of different physiological processes such as transcriptional control and cell cycle progression in eukaryotic cells and, therefore, are ubiquitous and important regulators of signal transduction. But in trypanosomatids, the mechanisms for gene regulation occur at the post-transcriptional level and the results of signaling pathways are unlikely to be at the level of transcription. Therefore, the control mechanisms still remain unknown (reviewed in Parsons and Ruben, 2000, Parsons *et al.*, 2005). A few receptor proteins have been identified in the tritryp kinome with previous reports on the identification of receptor-like proteins, such as adenylate cyclase in *T. brucei*

linked to regulation of transition processes during differentiation (Seebeck *et al.*, 2004, Alexandre *et al.*, 1996), or of a surface receptor for the insulin-like growth factor (IGF-I) on *Leishmania* promastigotes, speculated to be used for survival and proliferation within the host (Gomes *et al.*, 2001). However, trypanosomatids lack typical signaling receptors, as well as SH2 domains, transcriptional factors and heterotrimeric G proteins (Parsons *et al.*, 2005, reviewed in Seebeck *et al.*, 2004). Molecules that could potentially bind to second messengers have also been identified, although no intermediate steps of signal transduction have been defined to date (Parsons *et al.*, 2005).

Analysis of the tritryp kinomes classified kinases into seven groups related to ePKs, based on similarities in the active site. These organisms have representatives of the AGC, CAMK, CMGC and STE groups, but members of both the TK and TKL (tyrosine kinase and tyrosine kinase-like) groups are missing. Most mammalian receptor kinases are from the TK group. However, in plants most receptor proteins are from the serine/threonine group (reviewed in Parsons *et al.*, 2005). Although kinases bearing a transmembrane domain were found in the kinome studies, especially within the STE group, not much is know about the surface display of such enzymes, despite the fact there are reports of ectokinases in *Leishmania* (Lester *et al.*, 1991). Kinases that did not fit into any of those groups above were classified as "other". Within each of these groups, a number of families were also identified. The most important groups will be discussed in the following sections.

AGC group

The trypanosomatid AGC group is structurally related to kinases that respond to second messengers and encompasses: protein kinases A (PKA), responsive to cAMP, protein kinase G (PKG), responsive to cGMP and protein kinase C (PKC), responsive to the phospholipid DAG (Parsons *et al.*, 2005). Trypanosomatids have approximately half the number of human AGC kinases (Parsons *et al.*, 2005), although no orthologs of PKG have been identified in *Leishmania* and *Trypanosoma* (Allocco *et al.*, 2006). cAMP is an important mediator of cell differentiation, and that is also true for trypanosomatids: in *T. brucei*, the slender form secretes a factor termed SIF (for stumpy-induction factor) that stimulates parasites to transform into the non-dividing stumpy forms and which acts through cAMP signaling. Most cAMP-induced effects are mediated through protein kinase A (reviewed in Naula and Seebeck, 2000;Parsons and Ruben, 2000).

PKAs have been extensively studied in eukaryotes and are tetramers made up of two catalytic subunits. Binding of cAMP, generated by adenylate cyclases, results in dissociation and activation of monomeric C (catalytic) subunits, which in turn phosphorylate a wide range of substrates in the nucleus and cytosol (reviewed in Skalhegg and Tasken, 2000). Therefore PKAs play an important role in signal exchange between the external environment, cytoplasm and nucleus via adenylate cyclase-cAMP signal transduction pathways (reviewed in Seebeck *et al.*, 2004). However, the *T. cruzi* enzyme seems to be activated by cGMP rather than by cAMP. The biological relevance of this finding is still not known (reviewed in Seebeck *et al.*,

2004). PKAs have also been identified and cloned in *L. donovani* and *L. amazonensis* (Genestra *et al.*, 2001; Banerjee and Sarkar, 1992).

In eukaryotes, PKC uses ATP as a phosphate donor and consists of a family of ten isoenzymes. These isotypes contain a catalytic domain, including an ATP binding site, and a regulatory domain with Ca^{2+}, DAG and phospholipid binding sites (reviewed in Epand and Lester, 1990). In trypanosomatids, little is known about the functional role of PKCs.

Leishmania parasites

In *Leishmania*, as in *Trypanosoma*, cAMP, the activator of PKAs, is related to differentiation from metacyclic promastigotes to amastigotes *in vitro*, although little is known about the downstream effectors of cAMP signaling (reviewed in Seebeck *et al.*, 2004). *L. donovani* PKA appears to have some of the properties of mammalian PKA (Banerjee and Sarkar, 1992). However, in contrast to general rules, the enzyme in this species does not seem to be regulated by cAMP (reviewed in Seebeck *et al.*, 2004) and may be regulated in a similar fashion as *T. cruzi* PKA. In *L. major*, the catalytic subunit of a PKA termed LmPKAC1 is primarily expressed in promastigotes, but not amastigotes, suggests it is regulated during the differentiation process (Siman-Tov *et al.*, 1996). Two genes encoding PKA catalytic subunit isoforms (LmPKAC2a and C2b) have been recently characterized, but their biological role remains to be determined (reviewed in Seebeck *et al.*, 2004, Siman-Tov *et al.*, 2002). In *L. amazonensis*, studies showed the activity of a PKA was higher in metacyclic promastigotes compared to non-infective procyclic promastigotes with low activity in amastigotes, suggesting a role in the metacyclogenesis process

(Genestra *et al.*, 2004). *Leishmania* PKAs have mostly been studied in the context of differentiation, but other functions have been suggested for these proteins need to be experimentally confirmed.

PKC has been implicated in different cellular processes in *Leishmania* (Becker and Jaffe, 1997). *L. amazonensis* PKC seems to be involved in parasite uptake, as the enzyme activity may modulate *L. amazonensis* interactions with macrophages (Vannier-Santos *et al.*, 1995). In 2002, Alvarez *et al.*, suggested *L. infantum* PKC also play a critical role in the attachment and internalization steps of parasitic invasion, since inhibition of this enzyme inhibits the parasite-host cell invasion process. However, information about second messengers or the actual role of these enzymes in *Leishmania* signal transduction pathways is still scarce.

Little has been described about the phosphorylation activity associated with cyclic-3'-5' guanosine monophosphate (cGMP) in trypanosomatids. A cGMP-dependent activity of a protein kinase was detected in *L. amazonensis* metacyclics but the biological significance of this data remains to be defined (Geigel and Leon, 2003), although it is possible this kinase is part of metabolic pathways with a potential role in the life cycle or infectivity of the parasite based on its pattern of expression.

CAMK group

This is a relatively poorly represented group in the three sequenced trypanosomatid genomes that includes Ca^{2+}/calmodulin regulated kinases and AMP-dependent kinases. It includes 13 CaMKs predicted to be active in *T. cruzi*, 14 in *T. brucei* and 16 in *L. major* (Parsons *et al.*, 2005).

Ca^{2+} has a critical role as a second messenger in a variety of eukaryotic cell functions, such as cell division, secretion and motility, and is considered a universal signaling molecule. Trypanosomatids posses all the machinery to transduce Ca^{2+} signals, including acidocalcisomes, which are Ca^{2+} storage organelles with special significance for the propagation of signals in intracellular trypanosomatids (amastigote stage) (reviewed in Parsons and Ruben, 2000). Exposure to Ca^{2+} chelators in trypanosomatids leads to a decrease in host cell invasion (reviewed in Moreno and Docampo, 2003). In general, the Ca^{2+} system in trypanosomatids share great similarities with that of the mammalian host, but regulation differs from the processes that occur in higher eukaryotes (reviewed in Moreno and Docampo, 2003, Parsons and Ruben, 2000). These organisms also contain a variety of Ca^{2+} binding proteins (CaBPs) and, possibly, the regulatory calmodulin (CaM). However, only a small number of target enzymes seem to depend on CaM (reviewed in Parsons and Ruben, 2000).

A Ca^{2+}/calmodulin dependent kinase in *T. cruzi* epimastigotes (TcCaMK) with autophosphorylating activity was found to be associated with the parasite cytoskeleton and to play an important role in regulating Ca^{2+}-dependent processes in the parasite (Ogueta *et al.*, 1996; 1994). This protein also appears to be involved in the phosphorylation of a different set of proteins throughout the parasite life cycle (Ogueta *et al.*, 1998). Some isoforms of adenylate cyclase are also Ca^{2+} binding proteins and a variety of others have been cloned, such as the EF-hand CaBP that can sense or buffer Ca^{2+}: this protein is located in the flagellum of trypanosomatids and, due to its localization, may be involved in motility and environmental sensing

(reviewed in Parsons and Ruben, 2000; Godsel and Engman, 1999; Maldonado *et al.*, 1997).

Leishmania

Intracellular Ca^{2+} concentration in *Leishmania* and other typanosomatids has been demonstrated to change during their interaction with host cells and upon heat shock, suggesting a role in the differentiation process. However, Ca^{2+} physiological role as second messenger is yet to be established (reviewed in Moreno and DoCampo, 2003, Sarkar and Bhaduri, 1995). In *Leishmania*, a handful of proteins have been shown to bind Ca^{2+}, including Ca^{2+}-dependent ATPases in *L. donovani* (Mazumder *et al.*, 1992) and in *L. mexicana* (Benaim *et al.*, 1993), although hardly any CaM kinases have been characterized in these parasites.

CMGG

This is a significantly expanded family of kinases in trypanosomatids that include cyclin-dependant kinases (CDKs), whose family members were named CRK for cdc2-related kinase, mitogen-activated protein kinases (MAPKs) and extracellular-signal-regulated kinase (ERK), a subdivision of MAPKs. The increased number of such kinases in trypanosomatids may reflect the requirement to control their life cycle and cell cycle, along with the need to carry out correct replication and segregation of organelles (reviewed in Naula *et al.*, 2005).

Cyclin-dependent kinases

Trypanosomatids have a family of cdc2-related kinases that share approximately 45-55% identity with the human cdc2. Due to the importance of the

division process to differentiation during their life cycle, a number of investigators have tried to identify the molecular mechanisms that control the cell cycle in trypanosomatids. In *L. major* and *T. brucei*, this family has 11 members, while *T. cruzi* has 10 (reviewed in Naula *et al.*, 2005). A few reports on the characterization and potential roles of these CDKs have been published and in *T. brucei* RNAi studies indicated CRK1, 2,3, 4 and 6 have potential role in cell cycle, since donwregulation of CRK1 in the procyclic and bloodstream forms leads to reduced growth and increase duration of the G1 phase. Concurrent downregulation of CRK1 and either CRK2, 4 or 6 further enhanced that phenotype, although neither CRK2 nor 6 alone are essential for cell cycle progression. CRK3 is the functional homologue of CDK1 controlling entry into mitosis and its downregulation in *T. brucei* both procyclic (insect stage) and bloodstream forms led to mitotic block and growth arrest (Tu and Wang, 2004).

CDKs require cyclins as regulatory subunits and activating partners and analysis of the tritryp genome identified 10 cyclins in each parasite. It has been demonstrated that CRK3 interacts with CYC2 and CYC6 and downregulation of these two cyclins correlate with results of CRK3 downregulation. Collective data suggest the CRK3:CYC2 complex is required for cell cycle progression through G1 (reviewed in Naula *et al.*, 2005).

In *T. cruzi*, a couple of CRKs functioning as cyclin-dependent kinases (Gomez *et al.*, 2001) have been identified and characterized. One of them, the TzCRK3, is most likely a homologue of CDK1 whose activity is maximal at the G2/M phase boundary. The second CRK studied, TzCRK1, is active throughout the

G1 and S phases (reviewed in Naula *et al.*, 2005). They were both shown to phosphorylate histones H1, but the relevance of this information is not yet known (da Cunha *et al.*, 2005).

Leishmania

In *L. mexicana*, two cdc2-related genes have been isolated: CRK1, expressed in all life-cycle stages but active and able to phosphorylate histone H1 only in the promastigote stage and CRK3, the functional homologue of CDK1 controlling entry into mitosis and active both in promastigotes and amastigotes (Grant *et al.*, 2004;Grant *et al.*, 1998;Mottram *et al.*, 1996). LmexCRK1 is essential in promastigotes, but its specific function in the cell cycle control is yet to be established: it is proposed to have a similar function to *T. brucei* CRK1 in cell cycle progression, since the latter can complement LmexCRK1 mutants (reviewed in Naula *et al.*, 2005). Inhibition of LmexCRK3 activity with flavopiridol, a chemical inhibitor of CDKs and a growth inhibitor with a structure related to natural alkaloids, showed cells arrested in G_2/M phase and this kinase is essential to control cell cycle progression at the entry into mitosis (Grant *et al.*, 2004, Hassan *et al.*, 2001). *L. major* CRK3, which is 99% identical to LmexCRK3, is able to complement *S. pombe* cdc2 mutant and was shown to be essential for parasite growth (Hassan *et al.*, 2001, (Wang *et al.*, 1998).

MAP kinases

A large number of MAPK genes (14 in *T. brucei*, 13 in *T. cruzi* and 15 in *L. major*) have been identified in the tritryp kinome, possibly reflecting the importance of the MAPK cascade in coordinating environmental sensing (Parsons *et al.*, 2005).

Four MAP kinases have been cloned and characterized in *T. brucei*, all of which can also be classified as ERKs. One is highly expressed in the bloodstream stage of the parasite and appears to be induced by IFN-γ; it also appears to be essential for parasite survival and proliferation (Hua and Wang, 1997). This MAPK was termed KFR1. TbMAPK2, the second member, is not required for *T. brucei* bloodstream form but it was shown to be important for cell cycle progression, growth and differentiation to procyclics (Muller *et al.*, 2002). The third MAPK, the TbECK1, is an ERK-like kinase that shares characteristics of CDKs and is constitutively expressed throughout the trypanosome life cycle. This kinase has an unusual C-terminal region that acts as negative regulator of procyclic proliferation, suggesting an important role in cell cycle (Ellis *et al.*, 2004), making this molecule an attractive drug target. The recently characterized TbMAPK5 is involved in growth and differentiation of bloodstream forms. Deletion of this gene results in reduced mice infection due to premature differentiation to stumpy forms: mutant procyclics were able to proliferate normally in culture, could be transmitted to tsetse fly and were able to infect mice, however the parasitemia level was three times lower (Pfister *et al.*, 2006).

Leishmania

Nine MAPKs (LmxMAPK-LmxMPK9) genes with developmentally regulated mRNA expression (Parsons *et al.*, 2005) have been cloned and partially characterized in *L. mexicana* (reviewed in Naula *et al.*, 2005). LmxMPK, the first MAPK identified, is essential for infection since it is required for amastigote survival and proliferation inside the host macrophage (Wiese, 1998). Subsequently, eight more

genes, present in one copy per haploid genome, were cloned from *L. mexicana*, as well as one homologue from *L. major*, *L. infantum*, *L. amazonensis* and *L. braziliensis* (Wiese and Gorcke, 2001) and *L. donovani* and *L. panamensis* (Wiese *et al.*, 2003a;Wiese *et al.*, 2003b). In *L. tropica*, *L. aethiopica* and *L. donovani* this gene is present in at least two copies per haploid genome (Wiese and Gorcke, 2001). Of these *L. mexicana* MAPKs, only a couple more, the LmxMPK4 and LmxMPK9, apart from LmxMPK, have been fully characterized: LmxMPK4 is essential for promastigotes and amastigotes (Wang *et al.*, 2005) and LmxMPK9, exclusively found in the promastigote stage, is involved in the regulation of flagellar length by inducing flagellar shortening (Bengs *et al.*, 2005).

STE group

This is another expanded group identified in the trypanosomatid kinomes, with many STEs having been studied in *Leishmania*. In the MAP kinase signaling pathway cascade they are the upstream regulators and involve activators of MAPK, such as MAP kinase kinases (MAP2Ks), that mediate activation of the former through phosphorylation on tyrosine or threonine in the activation loop (Parsons *et al.*, 2005). Although several reports of members of the group exist, it has been impossible to predict the signaling pathways with which the members of this group are involved based only in sequence analysis.

Leishmania

In *L. mexicana* two MAP kinase kinases (LmxMKK and LmxPK4) and one MAPKKK (LmxMPK3) have been identified. The LmxMKK is only expressed in the

promastigote stage and is required for regulation of flagellar assembly and maintenance of a full-length flagellum, promastigote shape and size and for cell motility (Wiese *et al.*, 2003a). LmxPK4, also found in other kinetoplastids, is expressed throughout the promastigote stage and during differentiation to amastigotes, but is not required for growth *in vitro* since mutants show no defect in proliferation (Kuhn and Wiese, 2005). However mutants lacking the enzyme have delayed proliferation within infected cells and have difficulty to establish infection, showing LmxPK4 may have an important role in the differentiation process towards the amastigote stage *in vivo* (Kuhn and Wiese, 2005). The MPK3 is exclusively expressed in promastigotes and its mRNA is downregulated in the amastigote stage. Mutation of the LmxMK3 gene leads to the appearance of a reduced flagellum that resembles LmxMKK mutants, indicating these two kinases are components of a common signal transdcution pathway. And indeed, LmxMKK is able to phosphorylate and activate LmxMK3 (Erdmann *et al.*, 2006). It is likely there are two MAP kinase signal transduction cascades influencing the control of flagellar length: one with LmxMKK and its potential activators and substrates kinases regulating elongation of the flagellum and a second with LmxMPK9 and its activating kinases affecting shrinkage of flagellum (Bengs *et al.*, 2005).

TK and TKL groups

This group involves receptor-linked or cytosolic tyrosine kinases and tyrosine kinase-like enzymes. According to analysis of the tryp kinomes, these two groups are absent in trypanosomatids, despite the fact there are numerous reports on tyrosine

phosphorylation in these organisms. It has been suggested that tyrosine phosphorylation is due to the action of atypical tyrosine kinases, such as dual-specificity kinases, that could phosphorylate serine, threonine and tyrosine residues (Sadigursky and Santos-Buch, 1997). For this matter, sequence data alone could not accurately predict specific signaling pathways in trypanosomatids and additional biochemical studies will be required for that purpose (reviewed in Parsons *et al.*, 2005, Naula *et al.*, 2005).

Nonetheless, tyrosine phosphorylation has been well documented in trypanosomatids (Wheeler-Alm and Shapiro, 1992) and it seems to be an important mechanism for differentiation, where tyrosine phosphorylation is stage-regulated rather than cell cycle-regulated (Parsons *et al.*, 1995; Parsons *et al.*, 1991), and for parasite invasion (Favoreto S Jr *et al.*, 1998), where tyrosine phosphorylation is absent in slender blood forms of *T. brucei*, but stumpy forms show close profile to procyclic forms with a higher phosphotyrosine containing proteins (Parsons *et al.*, 1990). However no TKs have been purified from trypanosomatids to date and the enzymes that carry out this process have not yet been characterized (Doerig *et al.*, 2002;Gonzalez, 2000).

Leishmania

Tyrosine phosphorylation activity and its effects have also been broadly reported in *Leishmania* parasites, but to date no characterization of TKs has been described. The effects of differential tyrosine phosphorylation are generally related to environmental changes, adaptation to acidic conditions, transformation from the promastigote to the amastigote stage (Salotra *et al.*, 2000;Rivero-Lezcano *et al.*,

1997;Dell and Engel, 1994) or associated with mediation of promastigote entry into macrophages (Ghosh and Chakraborty, 2002).

"Other" group

This group involves all kinases that don't have any homology to groups from the ePKs used for analysis. The NEK family has a significant number of members in the tritryp kinome (20-22) when compared to the human genome (15). Not much is known about the role of NEK members, but in some model systems they seem to be involved in cell cycle and skeletal function and appear to work in cascades. One NEK in *T. brucei,* termed Nrk, posseses higher expression levels in procyclics than in bloodstream forms (Reviewed in Naula *et al.*, 2005, Parsons *et al.*, 2005).

Some identified members from this group include proteins involved in cell division in other organisms, DNA replication/repair and stress response (Parsons et al., 2005). Activators of CAMKs are also included here, as well as the casein kinase I (CK1) family. CK1 was identified in *L. mexicana* and *T. cruzi* and an interesting fact about this kinase is that it can bind a group of CDK inhibitors (flavopiridol), unlike the mammalian CK1 (Knockaert *et al.*, 2000), making it an attractive target for drug development.

Additionally Identified and Characterized Kinases

Ectokinases

A few kinases in *Leishmania* have been identified on the external membrane of the parasite and were termed ectokinases (Vieira *et al.*, 2002, Mukhopadhyay *et al.*, 1988; Lester *et al.*, 1991). These enzymes either reside on or are shed from the cell surface and have the ability to phosphorylate exogenous substrates. This external orientation may have an important role in regulating host-parasite interactions, parasite survival and signal transduction (Lester *et al.*, 1991). Cell surface histone-specific kinase activity has been detected in Old and New world *Leishmania* species, such as *L. donovani*, *L. major*, *L. tropica*, *L. panamensis* and *L. amazonensis* and is present in higher levels in metacyclics than in procyclics (Mukhopadhyay *et al.*, 1988). In 1991 (Hermoso *et al.*, 1991) demonstrated that *L. major* promastigote ectokinase LPK-1 is able to phosphorylate components of the complement cascade indicating this enzyme may play a role in the interaction with the host, reinforcing the idea that kinases released by this parasite may be involved in host-parasite interactions and host immune system modulation. It has also been shown that *L. major* is capable of releasing/shedding its ectokinases with different activities either in the presence or even in the absence of substrates, depending on the ectokinase, and that the constitutively shed leishmanial ectokinase LCK is a casein kinase (CK1)-like enzyme (Sacerdoti-Sierra and Jaffe, 1997). Constitutive shedding of the casein kinases 1 and 2, active in promastigotes of *L. donovani,* is synergistically inhibited by low pH and high temperatures, conditions found in the transfer from insect vector to macrophages, indicating optimal conditions for the release of CK1 from

promastigotes are consistent with the local environment in the insect vector. Nonetheless, activity is still detected in low pH, suggesting that casein kinases could potentially have a role in parasite survival within macrophages perhaps by phosphorylating host proteins to facilitate the invasion process (Vieira *et al.*, 2002). Recently, *L. major* CK1 isoform 2 has been identified as the primary high-affinity binding protein of a PKG inhibitor, which leads to inhibition of promastigote growth in culture, which is consistent with an essential biochemical function for CK1 in the insect stage of *Leishmania* promastigotes (Allocco *et al.*, 2006).

Phosphatases

Reversible protein phosphorylation is recognized as a fundamental mechanism to regulate protein function. As previously reviewed, the dephosphorylation of phosphoproteins is catalyzed by protein phosphatases, which are classified into the tyrosine phosphatases (PTPs) and serine/threonine phosphatases (PPs) groups. The genome-wide analysis of the tritryp kinomes revealed protein kinases comprised approximately 2 % of each genome (Parsons *et al.*, 2005). According to the genome database, there are approximately 57 phosphatases in *L. major*, which correspond to less than 1% of its genome. Yet, even less information about these phosphatases is available.

Tyrosine phosphatases

Although there was no direct evidence, reports of PTP activity in trypanosomatids started in the 1990's (Parsons *et al.*, 1991;Aboagye-Kwarteng *et al.*, 1991;Cool and Blum, 1993). Bakalara *et al.* (1995b) showed *T. brucei* and *T. cruzi* lysates were able to dephosphorylate peptides at their tyrosine residues and the cytosolic enzyme activity was inhibited with a PTP inhibitor. Those authors also showed PTP activity was located both in the soluble and particulate fractions and that a 55-kDa protein was found in the bloodstream and procyclic forms, suggesting tyrosine phosphatase is stage-regulated. A membrane-associated PTP was also identified in *T. brucei*, which is present in the bloodstream stage, but not in the procyclic stage. Presented data suggested TbPTPase is stage-regulated and has an optimal pH of 4.0-5.0, possibly a reflection of its external location, and that it might play a role in trypanosome growth and differentiation (Bakalara *et al.*, 1995a). A PTP closest to the class of phosphatases of regenerating liver (PRL) was isolated and characterized in *T. cruzi* (Cuevas *et al.*, 2005). TcPRL-1 has several residues characteristic of PRLs and the conserved catalytic residues common to all PTPs and, like other PRLs, this protein is farnesylated at the C-terminal, a modification that is essential for its membrane localization and biological function. In epimastigotes, TcPRL-1 is located in endocytic membranes, co-localizing with the cysteine proteinase and its functional role in cellular processes has not been determined (Cuevas *et al.*, 2005). *T. cruzi* also has an ecto-protein tyrosine phophatase in the trypomastigote and amastigote stages with optimal activity on acidic pH that could indicate physiological relevance since trypomastigotes enter the acidic compartment

of mammalian cells; amastigotes present a higher activity of the ecto-PTP, which may suggest a role during infections, maybe as an antiphagocytosis mechanism (Furuya *et al.*, 1998).

Leishmania

In *Leishmania*, little is known about PTPs, although there are about nine identified tyrosine phosphatases in the *L. major* database. To date, most studies suggest the involvement of host PTPs in the pathogenesis of *Leishmania* infections. In 1993, Cool and Blum first demonstrated PTP activity in *L. donovani* and the possible presence of multiple enzymes that change the balance of tyrosine phosphorylation and dephosphorylation at different stages of the culture. A subsequent study on the involvement of phosphatases in the differentiation process showed the tyrosine phosphorylation profile of a virulent strain of *L. donovani* is reduced after heat shock, mimicking amastigote transformation, most likely due to increase in protein phosphatase activity (Salotra *et al.*, 2000). However, one investigation on the involvement of tyrosine phosphatases in the differentiation process of *L. donovani* showed that overexpression of the human PTP1B in promastigotes induces partial amastigote differentiatiation and expression of the amastigote-specific A2 proteins; similar results are also obtained when the tyrosine kinase inhibitor tyrphostin AG1433 (an inhibitor of RTKs with more affinity to PDGF receptor) is used in promastigote cultures (Nascimento *et al.*, 2003). Moreover, the hPTP1B expressing promastigotes display increased virulence, when compared to wild-type *L. donovani*, both *in vivo* and *in vitro*. These data strongly suggested tyrosine phosphorylation plays a role in *L. donovani* differentiation and

virulence (Nascimento *et al.*, 2003). More recently, a report on membrane PTP activity in *L. major* has been published (Aguirre-Garcia *et al.*, 2006) with the identification of a 55-60 kDa protein band. The Western blot band identified when antibodies against the catalytic domain of hPTP1B or a *T. cruzi* PTP was used, indicate metacyclics have higher levels of the enzyme than procyclics. Immunolocalization studies also showed that in metacyclics this PTP shifts its localization from cytoplasmic to the membrane vicinity, on the tip of the parasite that usually establishes contact with the host, suggesting a possible role in adaptation to confront host mechanisms, with external membrane expression or even release of the enzyme (Aguirre-Garcia *et al.*, 2006). However further studies will be necessary to determine the precise role of this PTP. Also very recently, Almeida-Amaral *et al.* (2006) have identified and characterized the activity of another ecto-phosphatase in *L. amazonensis*. This phosphatase seems to be inhibited by both a classical inhibitor of acid phosphatase as well as phosphotyrosine phosphatase inhibitors such as sodium orthovanadate and bpV(phen), suggesting it may be a phosphohydrolase ectophosphatase with PTP activity. However, the biological relevance of this finding remains to be determined. An unusual PTP with dual function has also been characterized in *L. major* and was termed LmACR2 (Zhou *et al.*, 2006). This enzyme was first identified as a metalloid reductase that exhibits Sb(V) reductase activity and functions as a physiological drug activator in *Leishmania (*Zhou *et al., 2004).* However, its closest homologues are members of the dual phosphatase Cdc25 PTP family, sharing the same active site. LmACR2 is capable of dephosphorylating a phosphotyrosine-containing peptide and its function is inhibited when a PTP inhibitor

is used, indicating it also has PTP activity (Zhou *et al.*, 2006). The role of a conserved cysteine residue (Cys75) in the active site motif was also investigated and results with mutant enzymes show this residue, along with an Arg81 residue, is important for the enzyme phosphatase activity, which is proposed to be the physiological function of *Leishmania* ACR2 (Zhou *et al.*, 2006).

Serine/threonine phosphatases

Regarding serine/threonine phosphatases, the first identification of PPs in trypanosomes goes back to 1991 (Erondu and Donelson, 1991) with the characterization of catalytic subunits of protein phosphatase 1 and 2A from *T. brucei* with high degree of homology with their mammalian homologues. Very few phosphatases have been isolated and characterized since then and their function is not completely understood, although numerous reports on the use of PPs inhibitors suggest their involvement in nutrition, cellular differentiation and virulence. The biological roles of PP1 and PP2A have been inferred after pre-treatment of *T. cruzi* trypomastigotes with a PP1 inhibitor, which induces amastigote differentiation, suggesting involvement in the differentiation process (Grellier *et al.*, 1999). When a specific inhibitor of PP2A is used, but not inhibitors of PP1 or PP2B, cells do not completely transform from trypomastigotes to amastigotes *in vitro*, suggesting a role for these enzymes in *T. cruzi* remodeling (Gonzalez *et al.*, 2003). Another report of two PPs in *T. cruzi*, termed TcPP1α and TcPP1β and expressed in both epimastigotes and metacyclic trypomastigotes, indicate they may be involved in cell division and maintenance of parasite shape since inhibition with PP1 inhibitor affects epimastigote

growth and morphology (Orr *et al.*, 2000). The identification of a *T. brucei* PP5 (TbPP5) and analysis of its expression has been reported (Chaudhuri, 2001). This type 5 PP has 45-48% overall identity and 60-65% similarity with PP5 from different species and is expressed as a 52 kDa protein in the bloodstream and procyclic proliferative forms. TbPP5 is a predominantly cytoplasmic phosphatase with a conserved region at the C-terminal that appears to be essential for localization and seems to be expressed at higher levels in mid-log phase than in stationary phase promastigotes. The role of TbPP5 is yet to be determined and future studies will be required to analyze its function in cellular proliferation (Chaudhuri, 2001).

Ectophosphatases

Protein phosphatases have been detected at the cell surface of several members of the *Trypanosomatidae* family and in other intracellular pathogens such as *Yersinia*. Biological and physiological roles for these enzymes have been proposed and are supposed to involve nutrition, through hydrolysis of metabolites in order to provide the parasite with inorganic phosphate, virulence and differentiation. Acid phosphatases (AcPs) are known to exist in the plasma membrane of trypanosomatids, although these enzymes are typically found in the lysosome compartment since they're able to hydrolyse a number of substrates at acidic pH. An acidic protein phosphatase, most likely from a different family of acidic ectophosphatases, has been characterized and cloned from *T. brucei*. This enzyme, named TryAcP115, is modified by N-linked glycans and is expressed in patchwork patterns on the cell surface. This unequal distribution is related to its C-terminal intracellular domain. It has been proposed TryAcP115 may be implicated in cell growth and host-parasite

interaction (Bakalara *et al.*, 2000). The demonstration of ecto-phosphatase activity of a tartrate-sensitive membrane-bound acid phosphatase in intact and live bloodstream forms of *T. brucei*, but not in procyclics, also suggests a role in cell-cell interactions (Cedro *et al.*, 2003). In *T. rangeli*, a parasite of wild and domestic animal unable to elicit pathology, an external acidic phosphatase present in the epimastigote (insect) stage with optimal pH in the acidic range is also predicted to have a role in (invertebrate) host-parasite interaction and/or transduction of external stimuli (Gomes *et al.*, 2006). A *T. brucei* type I membrane bound histidine acid phosphatase termed TbMBAP1 is a tartrate-sensitive acid phosphatase downregulated during differentiation from the bloodstream to the procyclic insect stage and is essential for the maintenance of endo- and exocytosis in the mammalian stage of *T. brucei* (Engstler *et al.*, 2005).

Leishmania

Identification of a type 2C serine/threonine protein phosphatase (PP2C) in *L. chagasi* and *L. amazonensis* through amino acid sequence comparison demonstrates this enzyme is present in both the promastigote and amastigote stage; the authors also demonstrate the presence of PP1-like activity in *Leishmania* (Burns, Jr. *et al.*, 1993). Further characterization studies identified the *L. donovani* PP2C as a cytosolic enzyme with great similarities to the mammalian PP2C and a Mg^{2+} requirement for activity (Nandi and Sarkar, 1995), but the PP2C function in *Leishmania* is still unknown. A cytosolic Ca^{2+}/calmodulin dependent PP2B, which is ubiquitously expressed in higher eukaryotes, has also been described in *L donovani* promastigotes. It has close similarity with other well-characterized PP2B from mammalian sources,

however its biochemical role also remains to be elucidated (Banerjee *et al.*, 1999). A study by Becker and Jaffe (1997) evaluated the effect of two different inhibitors of serine/threonine kinases on parasite growth and morphology with the use of a kinase inhibitor approach, which would mimic phosphatase activity: by inhibiting serine/threonine kinases, promastigote growth was inhibited, while amastigote differentiation and proliferation were not affected, which may indicate a role for serine/threonine phosphatases in promastigote proliferation.

Ectophosphatases

Many *Leishmania* surface membrane molecules have been studied and one of the best characterized is the family of histidine acid phosphatases. They include the unique tartrate-resistant externally oriented cell surface membrane-bound acid phosphatase (MAcP) and the heavily glycosylated/phosphorylated, tartrate-sensitive secretory acid phophatase (SAcPs) present in a several *Leishmania* species (Shakarian *et al.*, 2003). According to studies of two main acid phosphatases in *L. mexicana* promastigotes, the membrane bound and the secreted enzymes have homologous but not identical N-terminal sequences and are encoded by different genes (Menz *et al.*, 1991).

One of the first studies to identify the distribution of acid phosphatases over external surface membranes of *L. donovani* promastigotes, including the flagellar membrane was carried out by Gottlieb and Dwyer (1981b; 1981a). These authors showed activity and broad substrate specificity exhibited asymmetrical distribution in the exterior surface. These membrane bound acid phosphatases were also detected in the outer face of the plasma membrane of infective and non-infective *L. amazonensis*

promastigotes and amastigotes (Pimenta and de, 1986). However, studies of *L. donovani* acid phosphatase indicate virulent clones have higher levels of enzyme activity than avirulent ones and that there are quantitative and qualitative differences between virulent and avirulent *L. donovani* regarding the amount of produced enzyme and the pattern of electrophoretic mobility (Katakura and Kobayashi, 1988) that this could be used as a marker to differentiate virulent from avirulent clones (Singla *et al.*, 1992). The *L. donovani* cell-surface acid phosphatase ACP1, which dephosphorylates phosphopeptides at serine residues, was shown to be able to inhibit the generation of superoxide anions by phagocytes (macrophage oxidative burst) (Das *et al.*, 1986) and similar results are obtained with a purified preparation of *L. donovani* acid phosphatases that are able to suppress host phagocytic cells by reducing their ability to produce oxygen metabolites (Remaley *et al.*, 1985). In 2002, Shakarian *et al.* identified and characterized a novel *L. donovani* tartrate-resistant membrane-bound acid phosphatase (MacP). The gene is present in a single copy in the L. *donovani* genome and the RNA and protein expression indicate it is constitutively present both in membranes of promastigotes and amastigotes. This enzyme contains an N-terminal peptide sequence that is also conserved in SacPs (which includes the histidine AcPs catalytic domain) that is required, along with the C-terminal, for surface membrane targeting (Shakarian *et al.*, 2002). Activity of the *L. mexicana* membrane bound acid phosphatase (LmxMBAP) is detected in both promastigote and amastigote stages (Menz *et al.*, 1991) and cloning of LmxMBP indicate it is a type I membrane protein with hydrophilic N-terminus responsible for enzyme activity (Weise *et al.*, 2005) and that its COOH-terminus is required for

endosomal targeting, where the enzyme is located in the endosomal/lysosomal compartment between the flagellar pocket and golgi apparatus (Wiese et al., 1996). The role of LmxMBP remains to be elucidated, but the enzyme doesn't seem to be required for promastigote growth in cultures, for infection or survival of amastigotes within macrophages (Benzel *et al.*, 2000).

The extracellular secreted acid phosphatase (SAcP), with an optimal pH of 4.5 - 5.0 and N-linked (glycosylated) side chains, was first detected in promastigotes of *L. donovani* and was capable of dephosphorylating a wide variety of substrates; comparative studies showed it was distinct from the previously described surface membrane-bound acid phosphatase (Gottlieb and Dwyer, 1982). Analysis of different *Leishmania* species demonstrated the presence of secreted acid phosphatase activity in all cultured promastigotes examined, except for *L. major* and *L. tarentolae.* However, activity has been detected in *L. major* promastigotes lysates when a sensitive method is used (Shakarian and Dwyer, 2000). Differences among species regarding the level of enzyme activity and their inhibition by tartrate were detected. This is due to either differences in the amount of released enzyme or to different enzymatic activity, as well as different pattern of electrophoretic mobility (Lovelace and Gottlieb, 1986; Doyle and Dwyer, 1993). The *L. donovani* SAcPs are composed of tandemly arrayed single-copy genes (sacp1 and sacp2) constitutively secreted by promastigotes (Shakarian *et al.*, 1997). In *L. mexicana*, two tandemly arranged genes (lmsap1 and lmsap2), highly conserved to LdSAcPs, encoding a tartrate-sensitive acid phosphatase located in the flagella pocket have also been describe and characterized from promastigotes (Wiese *et al.*, 1996; Wiese *et al.*, 1995). Biosynthesis and

secretion of *L. donovani* acid phosphatase was investigated and results showed this enzyme is the major glycoprotein secreted in large amounts by promastigotes *in vitro*. It is composed of two related heterodisperse subunits, synthesized as two intracellular precursors, and its secretory pathway includes N-glycosylation in the endoplasmic reticulum with further glycosylation and modification involving the Golgi apparatus prior to secretion (Bates and Dwyer, 1987). It was demonstrated *L. donovani* amastigotes are also able to produce this enzyme *in vivo* and *in vitro*, which suggests its importance for survival of both developmental stages survival (Bates *et al.*, 1989; Doyle *et al.*, 1991; Ellis *et al.*, 1998). *L. mexicana* promastigotes release a single type of SAP filament that is actually a copolymer of both SAP1 and SAP2, while amastigotes do not seem to have SAP (Wiese *et al.*, 1995). LdSAcP1 and LdSAcP2 are actively transcribed in promastigotes and have a high degree of sequence conservation to each other, with the presence of a signal peptide, a catalytic domain with N-linked glycosylation sites and the consensus sequence of histidine acid phosphatases, a Ser/Thr rich repeat region and a common C-terminus. LdSAcPs are heterogeneous glycoproteins that possess similarities to the LPG structure due to the sharing of carbohydrate epitopes in their abundant C-terminal phosphoglycans within the ser/thr rich region (Lippert *et al.*, 1999). The filamentous complexes of LmSAPs are composed of a central chain of globular particles with a surrounding glycocalyx that are assembled in the flagellar pocket. Similar to LdSAcP, these complexes are modified by N-linked glycans and phosphoserine mannosyl residues and by capped phosphosaccharide repeats, all attached to the serine residues within the Ser/Thr-rich repeat domain (Stierhof *et al.*, 1998). The role of the extensively studied LdSAcPs is

speculated to be related to hydrolysis of substrates to provide nutrients or to modify the host environment to the parasite advantage, enabling them to live in hydrolytic conditions within the sandlfy. Some of these conclusions were drawn after investigation of LdSAcP activity in the presence of proteases, which showed overall enhancement of LdSAcP activity most likely due to involvement of the phosphoglycan modifications in those enzymes (Joshi *et al.*, 2004). However, studies of the involvement of *L. mexicana* SAP in the survival of metacyclic promastigotes within the insect vector showed this phosphatase is not present in the gel-like plug produced by the parasites in the sandfly midgut (Stierhof *et al.*, 1999).

Leishmania Infection and Altered Host Signaling

Leishmania parasites are able to infect cells of the monocytic/macrophage lineage and evade the host immune response by impairing macrophage and dentritic cell function. *Leishmania* impairs host cell function in part by manipulation of intracellular kinases and phosphatases and, therefore, suppresses the activation of signaling pathways, transcription factors and gene expression, leading to compromised macrophage functions (reviewed in Murray *et al.*, 2005). The presence of surface molecules in *Leishmania* have been the main focus of studies regarding interaction and manipulation of macrophage functions: LPG, gp63 and GIPL have been extensively linked to entry into the host cell (reviewed in Olivier *et al.*, 2005 and Sacks and Kamhawi, 2001). The secretion of molecules, such as secreted acid phosphatases and the presence of ecto-kinases/phosphatases, have also been linked to

parasite survival and pathogenesis in the mammalian host as well as to the *Leishmania*-induced suppressive effects through manipulation of host signaling cascades (reviewed in Denkers and Butcher, 2005).

Parasite-driven impairment of macrophage PKC, or its major substrate MARCKS, is also an important event during *Leishmania* infection, since it's linked to a decrease in oxidative mechanisms due to interference by LPG (reviewed in Denkers and Butcher, 2005;Nandan and Reiner, 2005;Brandonisio *et al.*, 2000). LPG has also been associated with the inactivation of macrophage MAPK, important for the activation of NF-κβ-dependent signaling pathways that regulates proinflammatory cytokines release (Prive and Descoteaux, 2000). Several studies indicate Ca^{2+} concentration is increased in *Leishmania* infected cells since there is increased Ca^{2+} uptake linked to the fact LPG seems to chelate intracellular Ca^{2+} (reviewed in Olivier *et al.*, 2005). It's well established that *Leishmania* infections are able to downregulate the IFN-γ response. As a consequence, the pathway downstream of the IFN-γ receptor, namely Janus Kinases (JAKs)/STAT1 signaling, is also inhibited and shows defective phosphorylation of its components with reduced PTK activity, resulting in inhibition of transcription of IFN-γ induced genes, such as iNOs (reviewed in Olivier *et al.*, 2005). Results from different studies also suggest a role for the Src homology domain 2 containing tyrosine phosphatase 1 (SHP-1) in the pathogenesis of leishmaniasis, since infection of macrophages with *Leishmania* was shown to induce an increase in the specific activity of host SHP-1 (reviewed in Olivier *et al.*, 2005 and Nandan and Reiner, 2005). This phosphatase is predominantly expressed in hematopoietic cells and is typically involved in signal termination (it is a negative

regulator involved in the immune response). It appears that SHP1 is the main component of the macrophage PTP repertoire and interferes with JAK/STAT pathways following cytokine stimulation and therefore has a direct role in negative signaling induced by *Leishmania* infection (Blanchette *et al.*, 1999). It has also been showed that *L. major* infection in SHP1 deficient mice, which shows a normal expression level of iNOs and signaling molecules (e.g. STAT1, NFκβ), does not produce footpad swelling (Forget *et al.*, 2001), further establishing the involvement of this phosphatase in *Leishmania* progression (reviewed in Olivier *et al.*, 2005).

Leishmania Kinases and Phosphatases as Drug Targets

Phosphorylation of serine, threonine and tyrosine residues play an essential role in many molecular aspects of cellular processes and cell cycle in eukaryotes. Protein kinases, and the well-studied cyclin-dependant kinases (CDKs), have been extensively characterized in many organisms, including some in trypanosomatids as discussed above. Therefore, there is a great potential for using kinases as a target for novel drugs to interfere with key signaling pathways (reviewed in Sebolt-Leopold and English, 2006).

A number of specific kinase inhibitors have been recently developed and are under clinical evaluation (reviewed in Sebolt-Leopold and English, 2006 and in Naula *et al.*, 2005), and understanding the function and structure of human kinases and their interactions with inhibitors may also be useful to identify *Leishmania* homologues and drug targets specific to the parasite enzymes. Hence, developing a rational

strategy to identify new targets is a critical step and justified much of the work described in this thesis.

In *Leishmania*, orthologues of MAPK, MAPKK, CDK and PKA have been identified and demonstrated to be involved in a number of essential functions, especially in the regulation of cell cycle, differentiation and response to stress during the complex parasite life cycle (reviewed in Doerig *et al.*, 2002, Parsons and Rubens, 2000). The potential of MAPKs and CDKs from the CMGC kinase family to be used as targets is therefore vast, since they are abundant (Parsons *et al.*, 2005) and most likely required to insure proper growth and cell division. Moreover, their sequences diverge significantly from the mammalian homologues (reviewed in Knockaert *et al.*, 2002) and there are unique features in trypanosomatid cell cycle biology, such as the absence of key cell cycle checkpoints, that could be used as specific targets (reviewed in Naula *et al*, 2005). A number of CDK inhibitors, none of which selective for a single CDK (reviewed in Knockaert *et al.*, 2002), have been tested in *Leishmania* and have been shown to affect kinase functions (Knockaert *et al.*, 2002, Knockaert *et al.*, 2000). Recently, a library screen of CRK3 kinase inhibitors was used against the *L. mexicana* CRK3 and four classes of inhibitors were shown to inhibit parasite CRK3 causing disruption of cell cycle and a change in DNA content, growth arrest and aberrant cell morphology (Grant *et al.*, 2004). Diverse protein kinase inhibitors have also been known to affect *Leishmania* growth, morphology and infectivity (Alloco *et al.*, 2006; Di Giorgio C. *et al.*, 2004; Nascimento *et al.*, 2003 ; Alvarez et al., 2002 ; Becker *et al.*, 1997).

Alternatively, blocking essential phosphatases may also result in either deactivation of vital pathogenic mechanisms or activation of specific phosphorylation events that can be deleterious to *Leishmania* parasites and could therefore be used as potential drug targets. In 1998, Olivier *et al.* showed inhibition of phosphatases in *L. donovani* promastigotes by bpV(phen), a potent PTP inhibitor, inhibits parasite growth in culture and in macrophages, when these cells are treated with the inhibitor prior to infection, due to an increase in the production of NO. Moreover, when mice were treated with bpV(phen), they were able to control infection and the development of cutaneous lesions. A different study by Pathak and Yi (2001) showed sodium stibogluconate, an established pentavalent antimonial used as a common treatment for leishmaniasis, is able to strongly inhibit the *in vitro* activity of PTP1B and SHP1, among other PTPs, but not of dual phosphatases. Both bpV(phen) and the pentavalent sodium stibogluconate are able to inhibit PTPs and these studies reinforce the idea that blocking vital phosphatase activity in *Leishmania*-infected cells may be an important mechanism to control infections. The recent identification of the *L. donovani* PTP1 enzyme and its involvement in parasite survival in mice (Nascimento *et al*, 2006) also support the connection of PTP as necessary in the course of *Leishmania* infection. Consequently, identification of highly selective and potent phosphatases inhibitors that modulate essential parasite functions may be an effective strategy to inhibit specific pathways involved in *Leishmania* infectivity or survival within the host and could be used against this disease.

According to a recent study, there is a high probability specific inhibitors can be designed to target kinases with less than 60% homology to the mammalian

enzymes, which is the case for most of the parasite kinomes (reviewed in Naula *et al.*, 2005), and most likely the same idea can be applied to phosphatases. The use of multi-targeted inhibitors that affect other related signaling molecules, or a rational drug combination, should also be taken into consideration in order to avoid mechanism of acquired resistance (reviewed in Sebolt-Leopold and English, 2006 and Naula *et al.*, 2005). In the past few years advances in the field of cancer and diabetes has resulted in the development of kinase and phosphatase inhibitors. *Leishmania* research can also benefit from these studies, especially through screening of inhibitor libraries to facilitate the identification of novel potential targets to combat leishmaniasis.

Leishmania Phosphorylation and differentiation

Over the years, the crucial involvement of the complex network of protein phosphorylation and dephosphorylation in the regulation of several cellular processes in higher eukaryotes, such as signal transduction, cellular growth and transformation has become well established. Studies in yeast have also established a central role of protein phosphorylation in cell cycle control. The final steps of this mechanism involve the phosphorylation/dephosphorylation of transcription factors that ultimately regulate gene expression. Similarly, in *Leishmania* the counterbalanced activity of protein kinases and phosphatases has been detected and showed to be regulated during their development, suggesting a critical role during parasite differentiation to amastigotes or during metacyclogenesis (Espiau *et al.*, 2006;Genestra *et al.*, 2004).

As previously discussed, tyrosine phosphorylation may play a role in the differentiation of *Leishmania* parasites and other trypanosomatids, since the phosphorylation pattern is known to change during these parasite development (Parsons *et al.*, 1991). Through the action of tyrosine kinases and phosphatases, during the log growth phase there is a general increase in tyrosine phosphorylation, suggesting it is due to tyrosine kinases, and in the late log/stationary phase there is an overall decrease in tyrosine phosphorylation, possibly indicating the activity of tyrosine phosphatases (Salotra *et al.*, 2000, Cool and Blum, 1993); however no specific enzymes have been identified in any of these studies. The changes in the phosphorylation status of the cells are most likely due to the fact these parasites go through a great variation in their environment during their life cycle, alternating between the sandfly environment (26 ^{0}C pH 7.0) and that of the mammalian host (37 ^{0}C, pH 5.0), and they need to adapt to those changes. It has also been shown that the level of tyrosine phosphorylation in virulent *L. donovani* promastigotes decreases after heat shock, while avirulent promastigotes didn't present changes in their phosphorylation pattern (Salotra *et al.*, 2000), which may indicate effective differentiation towards the amastigote stage in the former strain. However, the significance of these studies and the specific role of tyrosine phosphatases in amastigote differentiation and virulence are far from clear. In higher eukaryotic cells, where adaptation to changes in the environment is initiated by extracellular signals, phosphorylation at tyrosine residues is an important regulatory mechanism involved in cellular division, growth and differentiation and by analogy it should also be important for cellular function in trypanosomatids (Salotra *et al.*, 2000).

RATIONALE AND THESIS OBJECTIVES

Leishmania parasites are dimorphic protozoans that are exposed to markedly different environments during their life cycle. It is well established that environmental factors such as pH and temperature trigger the differentiation of *Leishmania*. Transformation is linked to biochemical and morphological changes, flagellum restructuring and synthesis of new surface molecules correlated with altered protein function in response to environmental changes. However, the molecular basis of amastigote transformation and virulence remains poorly understood.

Signal transduction, through phosphorylation of tyrosine residues, plays a key role in differentiation, among other cellular events, in higher eukaryotes. This process is one of the most important regulatory mechanisms for biochemical changes in the cell. By analogy to higher eukaryotes, protein phosphorylation is likely to be important for *Leishmania* intracellular events and may play an important role in parasite survival and differentiation.

As discussed herein, several studies in *Leishmania* parasites suggest phosphorylation levels change as the parasite goes through its life cycle with different patterns of tyrosine phosphorylation being reported. It is therefore possible that tyrosine phosphorylation is life cycle stage-regulated, since higher phosphorylation levels are detected in promastigotes compared to amastigotes (Dell and Engel, 1994), where, upon heat shock, differentiation and dephosphorylation occur, possibly indicating the activity of tyrosine phosphatases (Solotra *et al*, 2000, Cool and Blum, 1993). However, such studies were based on analysis of general *Leishmania* phosphorylation patterns and none of them provided in-depth investigation on the

potential enzymes involved in the process, including the tyrosine kinases and phosphatases linked to this regulatory mechanism.

Therefore, we have chosen to investigate the involvement of tyrosine phosphatases in the *Leishmania* differentiation process and virulence focusing the research on a genetic approach. The work presented in Chapter 2 aimed to determine whether a heterologous tyrosine phosphatase would affect the control of *L. donovani* differentiation and virulence levels. Our results indicate participation of a tyrosine phosphatase in the processes mentioned above.

Once it was established that tyrosine phosphatases are linked to differentiation and virulence, we extended our investigations to identify and characterize the *Leishmania* endogenous tyrosine phosphatase responsible for the effects reported in Chapter 2 and to determine the role of this enzyme on *L. donovani* life cycle, proliferation and survival in culture and in mice, as reported in Chapter 3. This Chapter also examined the potential of the endogenous *Leishmania* tyrosine phosphatase as a drug target.

Overall, the aim of this research was to determine the contribution of tyrosine phosphatases to *Leishmania* differentiation and proliferation, as well as in the virulence and survival of these parasites within mammalian cells.

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FIGURE LEGENDS

Table I. Pathologies linked to *Leishmania* species and geographic distributions.

Figure 1. *Leishmania* developmental stages. **A)** Amastigote form cartoon and an amastigotes-infected macrophage and **B)** promastigote cartoon and culture. Notice the different morphology of each stage and parasite intracellular components. Adapted from www.leishmania.org/pagine/leishmaniosi_canina/eziologia-promastigote.asp, www.dpd.cdc.gov/dpdx/HTML/ImageLibrary and www.bact.wisc.edu/foodsafety/parasite/gallery.html.

Figure 2. *Leishmania* life cycle in the sandfly and the human host. Developmental stages and transition forms of *Leishmania* are seen as the parasite goes though its biological cycle that depends on whether they are founding in the insect vector or within the mammalian host.

Figure 3. Worldwide distribution of leishmaniasis. A map showing regions of the world affected by leishmaniasis, as seen in dark and hatched area. The majority of countries affected are composed of developing or under-developed nations. Adapted from Davies *et al.*, *BMJ.* **326**:377-82 (2003).

Figure 4. Phosphorylation process. Counterbalanced action of protein kinases and phosphatases carry out the phosphorylation process through addition of a phosphate group from ATP and production of ADP by protein kinases. To reverse this action, protein phosphatases remove the phosphate from the side chain of target proteins with the release of inorganic phosphate (Pi).

Figure 5. Simplified representation of the MAP kinase cascade. MAP Kinases are activated in response to a variety of stimuli such as growth factors and other signaling

molecules that eventually leads to the activation of transcription factors and gene expression. **A)** The cascade is composed of a succession of events that start with the phosphorylation of MAPKKK, leading to its activation and ultimately results in activation of MAPK to control cellular events. **B)** Activation of the four different MAP kinase cascades (ERK1/2, ERK5, JNK and p38): extracellular factors start the intracellular signaling responses that can result in activation of either gene expression or CDKs. Each MAPK cascade is activated either by a small GTP-binding protein (e.g Ras family) or by an adaptor protein, which transmits the signal either directly or indirectly through a mediator MAP4K to the MAP3K level. Signal is transmitted down the cascade by MAPKK and MAPK. Adapted from Rubinfeld and Seger. *Mol Biotechnol.* **31**:151-74 (2005).

Figure 6. Diagram of CDKs and cyclin participation in the cell cycle progression. CDK activity is modulated by binding of cyclins. This complex, in turn, regulates a number of checkpoints during the cell cycle. In animal cells, progression through the G_1 restriction point is regulated by complexes of Cdk4 and 6 with cyclin D. Cdk2/cyc E complexes function later in G_1 and are crucial for the G_1/S transition. Cdk2/cyc A complexes are required for progression through the S phase and Cdc2/cyc B complexes drive the G_2 to M transition.

Figure 7. Structure of Protein tyrosine phosphatases (PTPs). The PTP family comprises cytosolic PTPs - that includes low-molecular-weight PTPs (LMW-PTPs) - receptor PTPs (RPTPs) and dual-specificity PTPs (DUSPs). Cytosolic (or soluble) PTPs and RPTPs have different structures however they share a conserved catalytic domain. The PTP catalytic domain is highly conserved with the presence of a single cysteine used in a cysteinyl-phosphate enzyme intermediate during

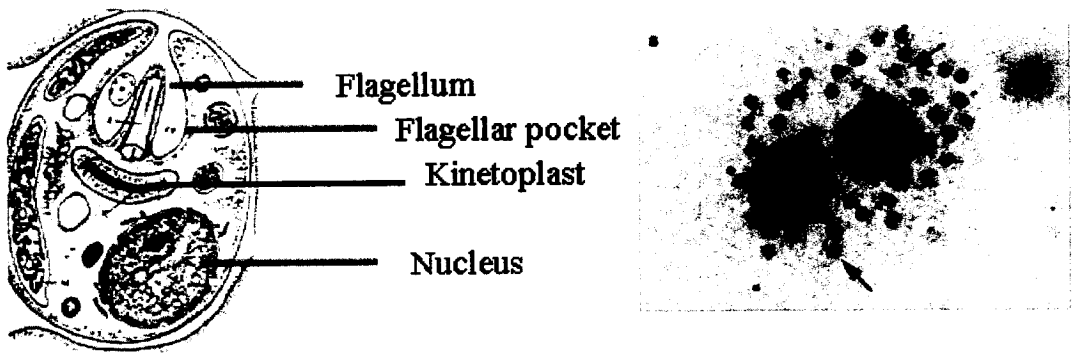
dephosphorylation. On the other hand, DUSPs can dephosphorylate phosphorylated tyrosine, threonine and serine residues. Adapted from Larsen *et al.*, *Nature* **4**:700-711 (2003).

Table I. Pathologies linked to *Leishmania* spp. and geographic distributions

Species	Pathology	Location
Old World		
L. (L.) donovani	VL, PKDL	Middle East, China, India, East Asia, Pakistan.
L. (L.) infantum	VL, PKDL	Mediterranean, Middle East, Asia, Africa, China
L. (L.) major	CL	Middle East, India, Africa Pakistan, northwest China.
L. (L.) tropica	CL	Middle East, Mediterranean, West Asia, India.
L. (L.) aethipica	CL, DCL	Ethiopia, Kenya, Yemen.
New World		
L. (L.) chagasi	VL	South and Central America
L. (L.) mexicana	CL , DCL	Mexico, Central America, Texas
L. (L.) amazonensis	CL, DCL	Amazon basin, Brasil, Texas
L. (V.) braziliensis	MCL, CL	Brasil, Peru, Bolivia, Ecuador Venezuela
L. (V.) peruviana	CL	Andes region, Peru
L.(V.) guyanensis	CL	Guyana, Surinam, Amazon basin

Figure 1.

A) *Leishmania* Amastigote



B) *Leishmania* promastigotes

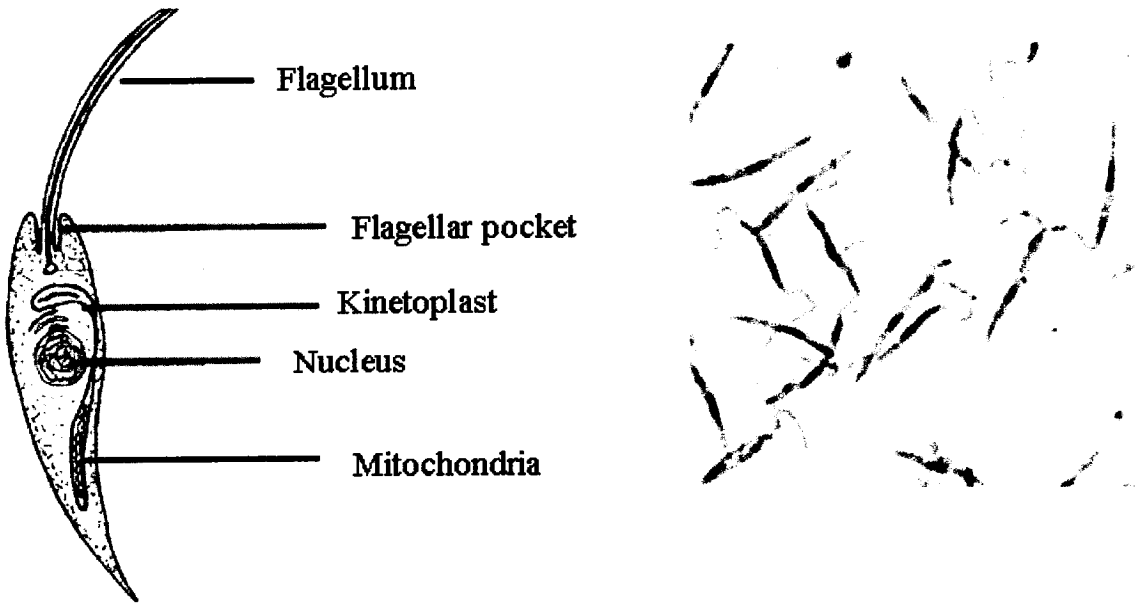


Figure 2

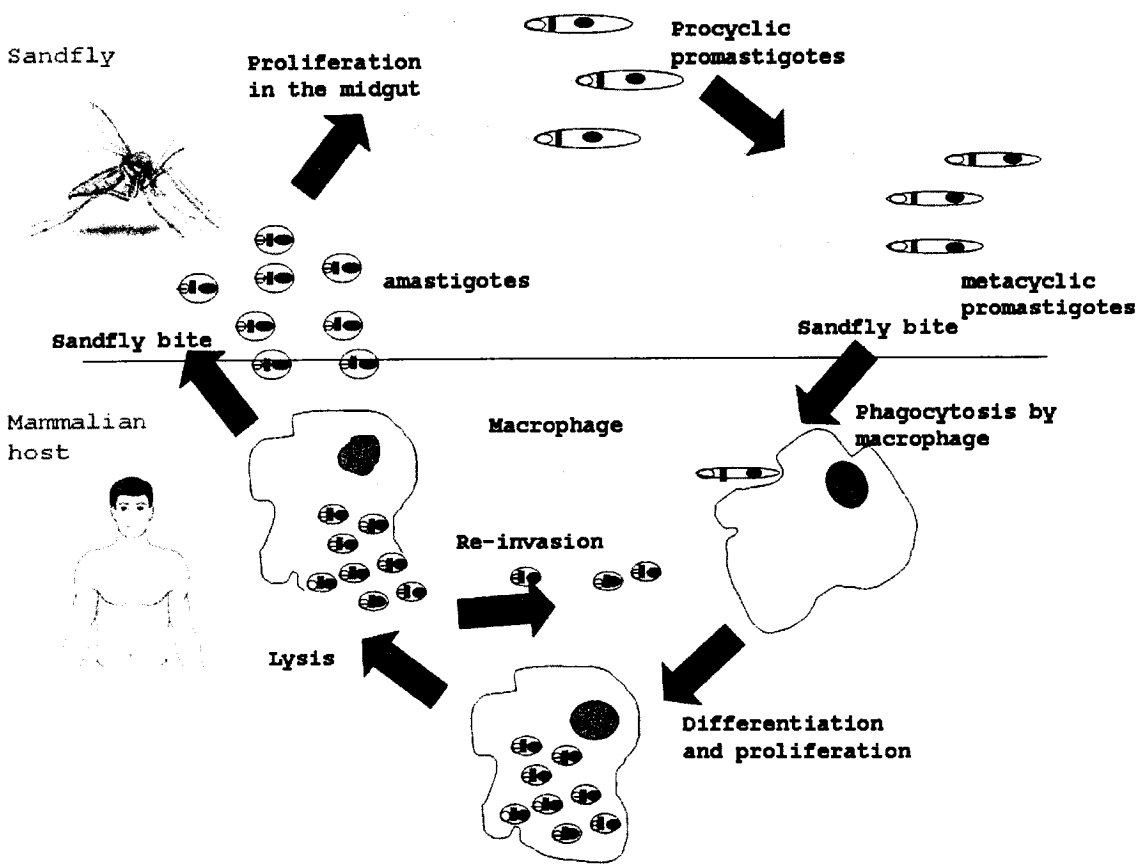

Sandfly
Proliferation in the midgut
Procyclic promastigotes
amastigotes
metacyclic promastigotes
Sandfly bite
Sandfly bite
Mammalian host
Macrophage
Phagocytosis by macrophage
Re-invasion
Lysis
Differentiation and proliferation

Figure 3

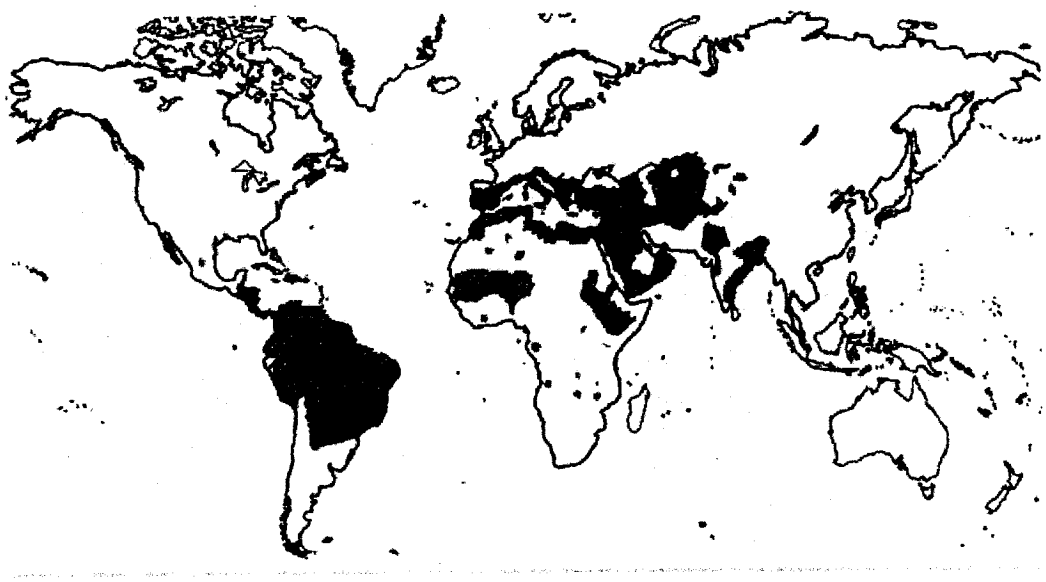


Figure 4

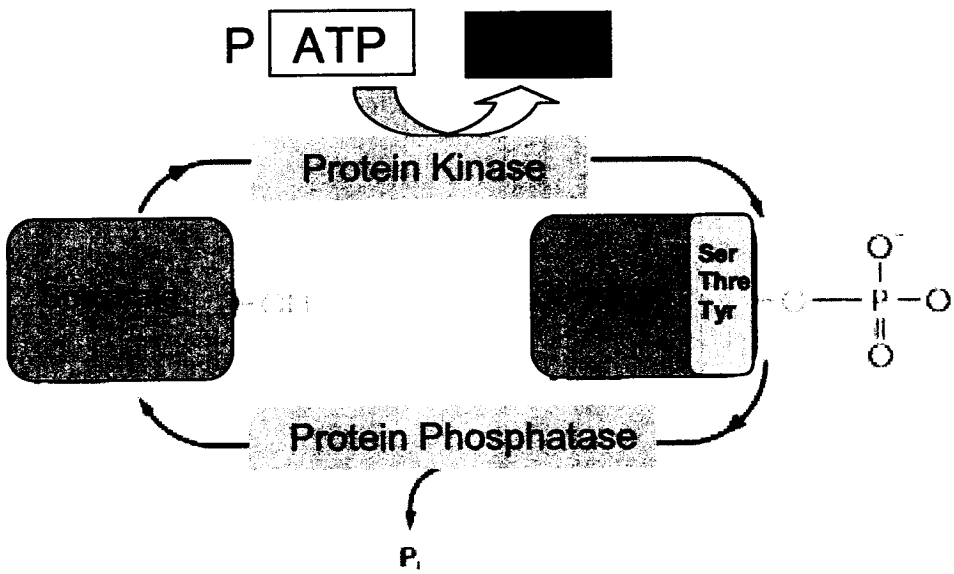
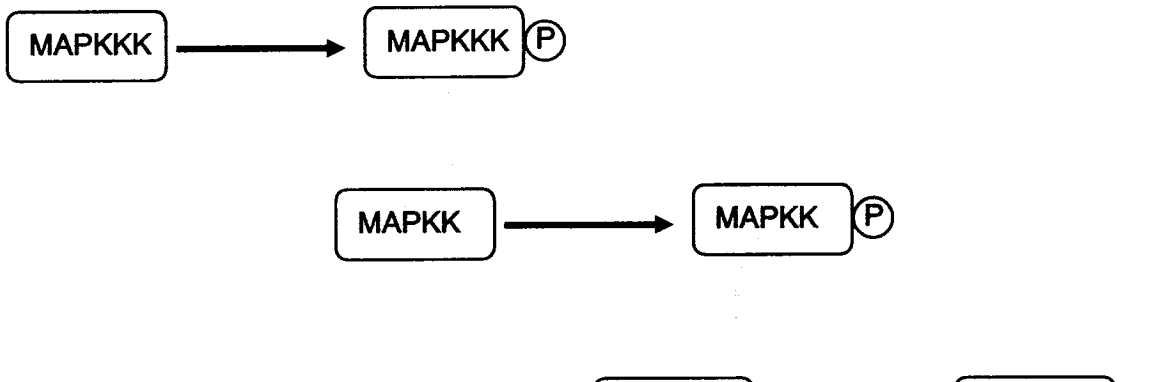


Figure 5

A)


MAPKKK
MAPKKK
P
MAPKK
MAPKK
P
MAPK
MAPK
P

B)

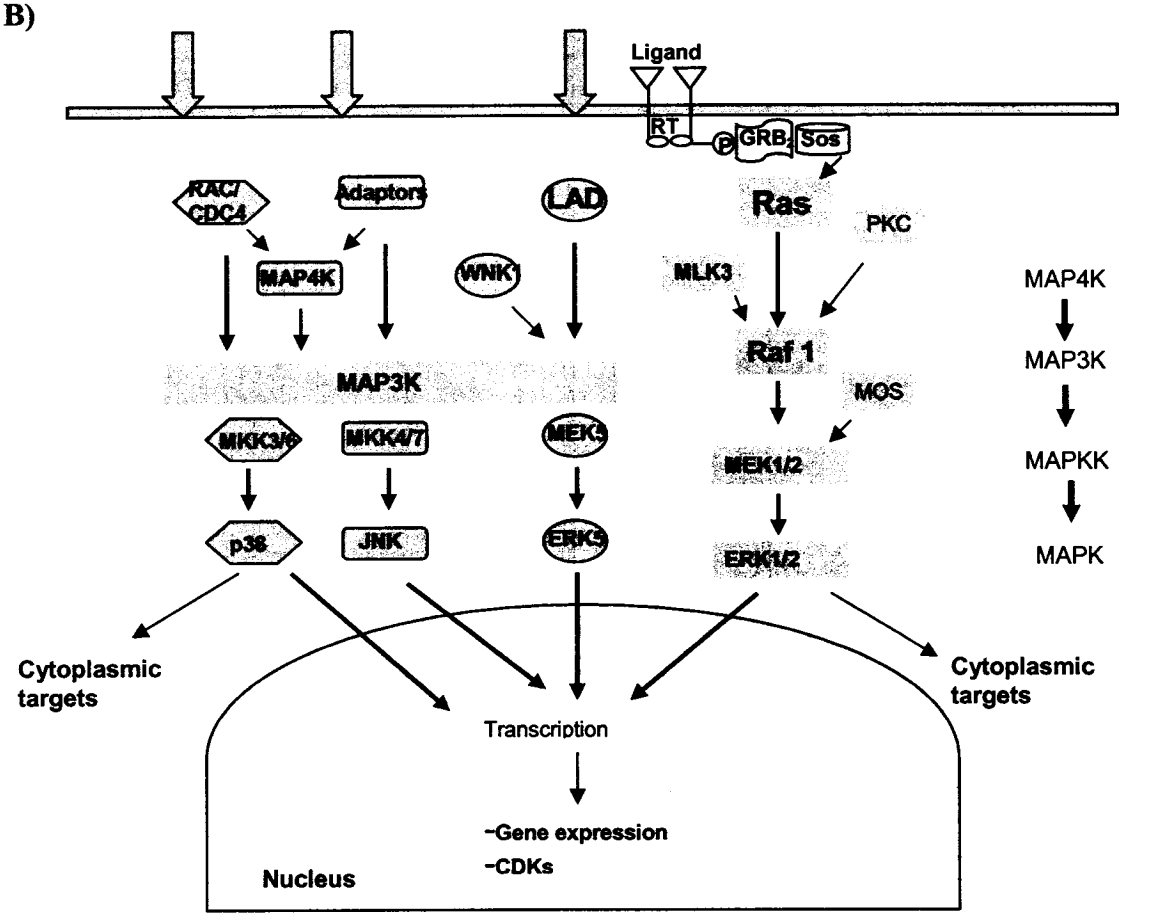

Ligand
RT
P
GRB
Sos
RAC/ CDC4
Adaptors
LAD
Ras
PKC
MAP4K
WNK1
MLK3
Raf 1
MAP3K
MOS
MKK3/6
MKK4/7
MEK5
MEK1/2
p38
JNK
ERK5
ERK1/2
MAP4K
MAP3K
MAPKK
MAPK
Cytoplasmic targets
Transcription
-Gene expression
-CDKs
Nucleus
Cytoplasmic targets

Figure 6

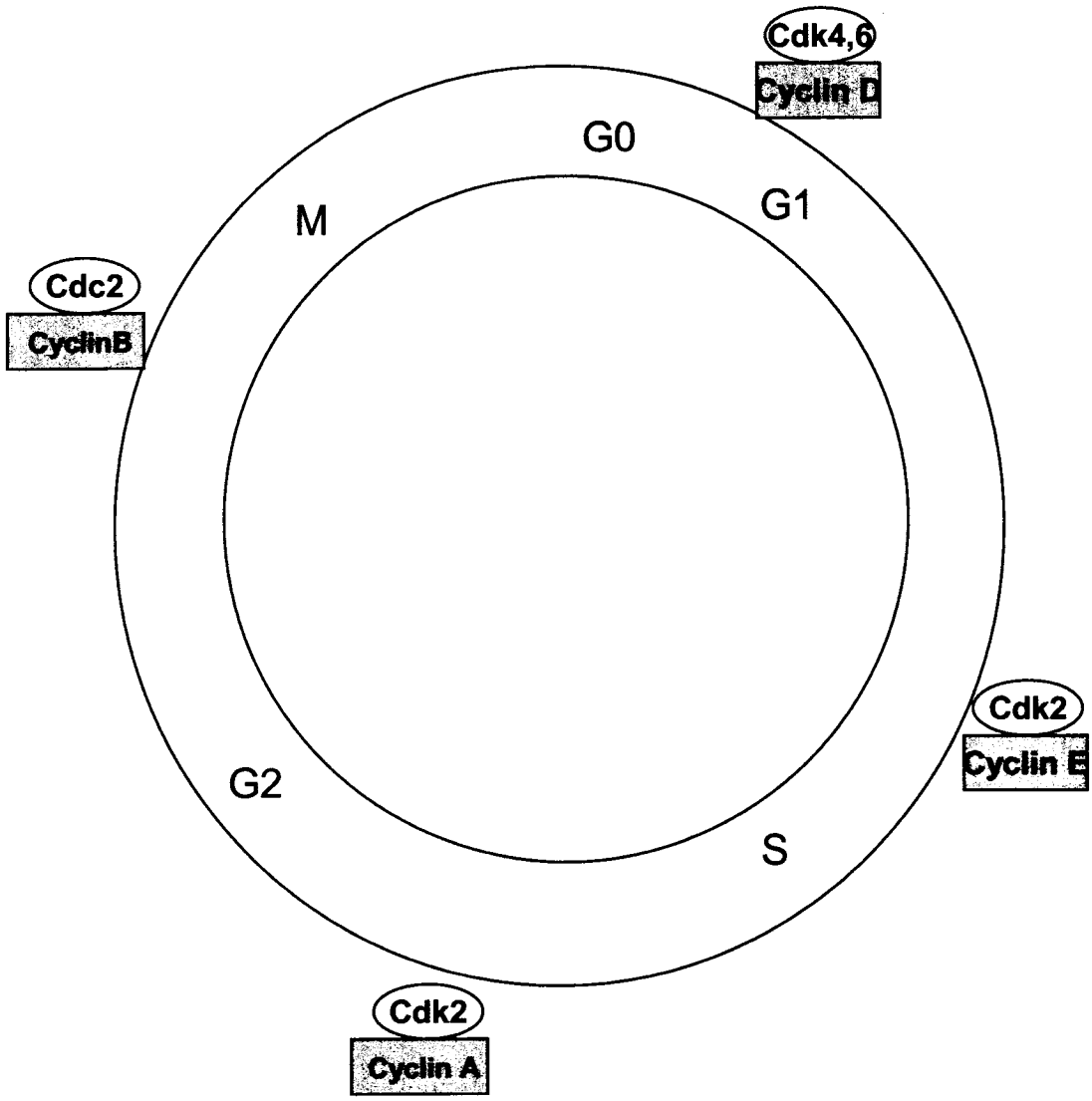
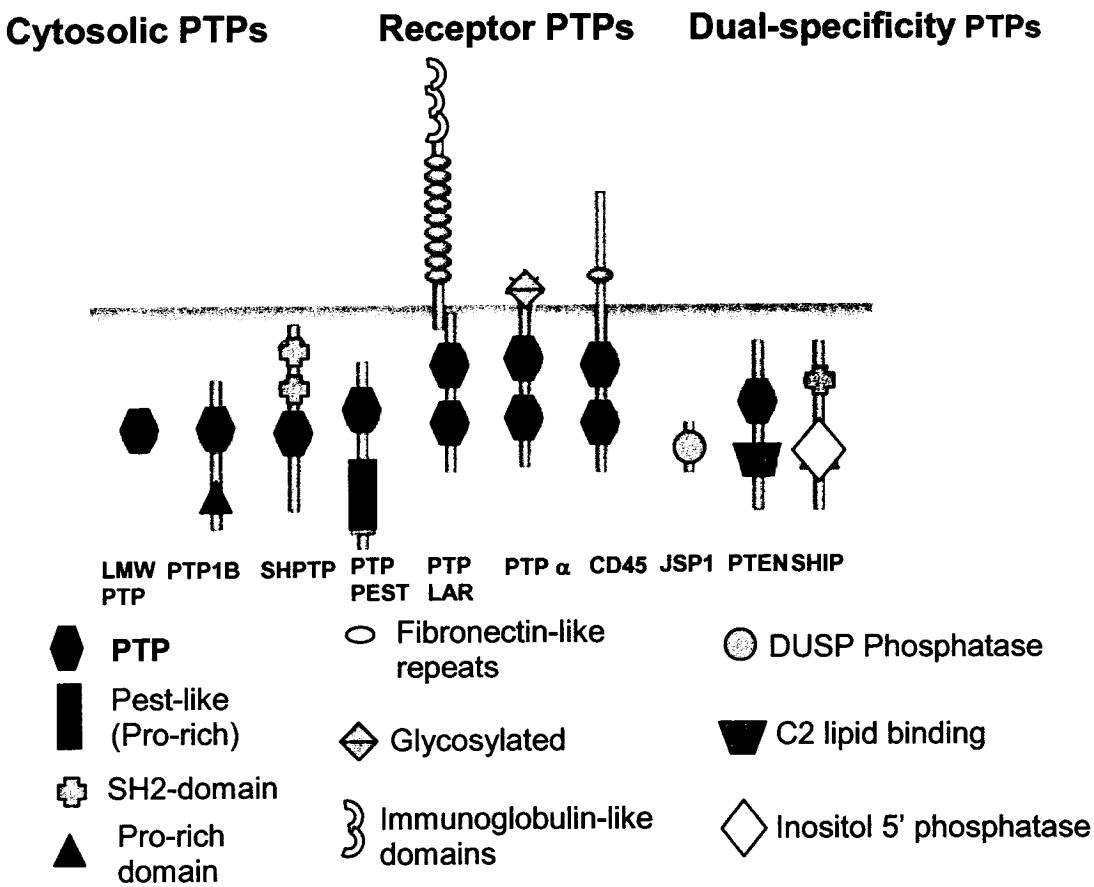

Cdk4,6
Cyclin D
G0
G1
M
Cdc2
CyclinB
Cdk2
Cyclin E
G2
S
Cdk2
Cyclin A

Figure 7


Cytosolic PTPs
Receptor PTPs
Dual-specificity PTPs
LMW PTP
PTP1B
SHPTP
PTP PEST
PTP LAR
PTP α
CD45
JSP1
PTEN
SHIP
PTP
Pest-like (Pro-rich)
SH2-domain
Pro-rich domain
Fibronectin-like repeats
Glycosylated
Immunoglobulin-like domains
DUSP Phosphatase
C2 lipid binding
Inositol 5' phosphatase

PREFACE TO CHAPTER 2

Signaling pathways involved in adaptation to environmental changes and regulation of growth, differentiation and proliferation are not well understood in *Leishmania*. However, it is known these parasites use phosphorylation and dephosphorylation of proteins as a regulatory mechanism during their life cycle. In higher eukaryotes, tyrosine phosphorylation is responsible for cellular differentiation and proliferation. Regulation of protein tyrosine kinases (PTKs) and phosphatases (PTPs) is likely to be important for *Leishmania* intracellular events, including differentiation, since these parasites encounter a wide range of environmental conditions. With this study, we aimed to identify the involvement of tyrosine phosphatase in *Leishmania donovani* differentiation either through overexpression of the human PTP1B or through inhibition of PTKs; we also report herein the influence of tyrosine phosphatase on *L. donovani* virulence *in vitro* and *in vivo*.

CHAPTER 2: Heterologous expression of a mammalian protein tyrosine phosphatase gene in *Leishmania*: effect on differentiation.

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Summary

Leishmania is a protozoan pathogen, which is transmitted to humans through the bite of an infected sandfly. This infection results in a spectrum of diseases throughout the developing world, collectively known as leishmaniasis. During its life cycle, *Leishmania* differentiates from the promastigote stage in the sandfly vector into the amastigote stage in the mammalian host where it multiplies exclusively in macrophage phagolysosomes. Although differentiation of *Leishmania* is essential for its survival and pathogenesis in the mammalian host, this process is poorly understood. In higher eukaryotic cells, protein tyrosine phosphorylation plays a central role in cell proliferation, differentiation and overall function. We have therefore investigated the role of protein tyrosine phosphorylation in *Leishmania* differentiation by undertaking complementary approaches to mediate protein tyrosine dephosphorylation *in vivo*. In the present study, *L. donovani* were engineered to express a mammalian protein tyrosine phosphatase, or were treated with inhibitors of protein tyrosine kinases, and the resulting phenotype was examined. Both approaches resulted in a partial differentiation from promastigotes to amastigotes including the expression of the amastigote specific A2 protein, morphological change and increased virulence. These data provide support for the involvement of tyrosine phosphorylation in the differentiation of *Leishmania*.

Introduction

Leishmaniasis is an infectious disease with an overall prevalence of 12 million cases and some 350 million people at risk of infection (Desjeux, 2001). The aetiologic agent of this disease is the *Leishmania* protozoan parasite, which is transmitted through the bite of an infected sandfly. This infection is firmly established in over 88 countries in the developing world, and it is now emerging in previous unaffected areas such as in Southern Europe and expanding its presence in South America and East Asia (Desjeux, 2001). Leishmaniasis is typically associated with three types of pathology: visceral leishmaniasis, cutaneous leishmaniasis and mucocutaneous leishmaniasis (reviewed in Herwaldt, 1999). Visceral disease is responsible for widespread mortality and morbidity whereas the cutaneous and mucocutaneous diseases have a severe impact on the quality of life but are non-fatal (Herwaldt, 1999). Treatment options are limited and there is no available vaccine. It is therefore important to define the molecular basis for this infection in order to identify potential new targets for drug and vaccine development. In order to complete its life cycle, *Leishmania* promastigotes, which are transmitted to a mammalian host by the bite of an infected sandfly, must differentiate into amastigotes in order to survive inside the macrophage phagolysosomal vacuole (reviewed in Matlashewski, 2001; Matlashewski, 2002). A number of biochemical and biological properties have been established which delineate the differences between promastigotes and amastigotes (Gupta *et al.*, 2001), however, the molecular basis for regulating differentiation from promastigotes to amastigotes is not understood. In both higher and lower eukaryotes, protein kinases regulate major biological processes including

cell differentiation, proliferation, signal transduction and cell morphology (Hunter, 1996; Cohen, 2000). Although protein phosphorylation has been described in *Leishmania* (Lester *et al.*, 1990; Saltora *et al.*, 2000), it remains unclear what role this plays in differentiation. Some protein kinase inhibitors have been shown to be toxic to *Leishmania* and mediate changes in growth and morphology suggesting that protein kinases are important for proliferation and viability (Becker and Jaffe, 1997). Protein tyrosine phosphorylation is regulated in eukaryotic cells through the balance of protein tyrosine kinases and protein tyrosine phosphatases (Hunter, 1996; Cohen, 2000; Ibarra-Sanchez *et al.*, 2000). The transfection of mammalian genes into lower eukaryotic cells, including yeast, has been used successfully to identify and characterize key regulatory pathways involved in cell proliferation and differentiation (Nurse *et al.*, 1998). We have therefore undertaken this approach to study the role of tyrosine phosphorylation in *Leishmania* differentiation. The human tyrosine phosphatase1B (PTP-1B) has been characterized in detail and has been reported to be promiscuous with respect to its target protein specificity (Cheng *et al.*, 2001). As the human PTP-1B gene and relevant PTP-1B specific antibodies were available, we determined whether it was possible to express PTP-1B in *L. donovani* and whether this affected the state of differentiation using previously established biological, morphological and phenotypic characteristics for *Leishmania* amastigotes. It is demonstrated within that ectopic expression of human PTP-1B in *L. donovani* resulted in several changes associated with differentiation towards amastigotes including increased virulence. Consistent with this observation, it is further revealed that treatment of promastigotes with protein tyrosine kinase inhibitors is also

associated with differentiation towards amastigotes and that this was reversible upon the removal of the inhibitor. These results reveal the feasibility of studying *Leishmania* differentiation through ectopic expression of key regulatory enzymes from higher eukaryotes, and provides evidence that tyrosine phosphorylation plays a role in the differentiation and life cycle of *Leishmania*.

Results

Expression of mammalian protein tyrosine phosphatase-1B in L. donovani

We initially determined whether it was possible to stably express the human protein tyrosine phosphatase-1B (PTP-1B) gene in *L. donovani*. PTP-1B was chosen in this study because it had been shown to be promiscuous with respect to target protein tyrosine dephosphorylation, the corresponding gene has been previously cloned and antibodies specific to the protein were available (Muise *et al.*, 1996; Cheng *et al.*, 2001). Promastigotes were transfected with a PTP-1B gene expression plasmid (PALTneo-PTP1B) and following selection, the expression of PTP-1B was determined by Western blot analysis. As confirmed in Fig. 1A, it was possible to transfect and select for promastigotes expressing the PTP-1B protein. Consistent with the expression of the human PTP-1B protein in *L. donovani* promastigotes, there was also a higher level of protein tyrosine phosphatase activity in the promastigotes expressing PTP-1B than in the control plasmid transfected and selected or wild-type non-transfected promastigotes (Fig. 1B). One of the most widely used protein markers specific for amastigotes is the expression of the A2 family of proteins, which ranges in molecular weight from 45 kDa to 110 kDa, and is expressed at higher levels

in amastigotes than promastigotes (Zhang *et al.*, 1996; Gupta *et al.*, 2001). To determine whether PTP-1B expression mediated an induction of A2 expression, Western blot analysis was carried out on stationary phase cultures of control transfected and the PTP-1B expressing *L. donovani* promastigotes. As shown in Fig. 2A, there was a clear increase in the level of A2 protein expression in the PTP-1B transfectants in comparison to the control vector transfected cells. It is also evident that PTP-1B expressing cells did not express as much A2 protein as the control axenic amastigotes arguing that ectopic expression of PTP-1B resulted in a partial differentiation towards amastigotes. Re-transfection and selection of PTP-1B in *L. donovani* revealed that this pattern of induction of A2 expression in PTP-1B expressing cells was reproducible. Stationary phase axenic cultures of *L. donovani* promastigotes (cultured at 27 ^{0}C, pH 7.2) are long and cylindrical and contain flagella. By comparison, axenic cultures of amastigotes (cultured at 37 ^{0}C, pH 5.5) are smaller, lack flagella and display a more rounded morphology, similar to macrophage derived amastigotes which reside in the low pH environment of the macrophage phagolysosome (Doyle *et al.*, 1991; Gupta *et al.*, 2001). We therefore examined the morphology of the PTP-1B/A2 expressing promastigote culture to determine whether they had assumed an amastigote like morphology. As shown in Fig. 2B, many of the individual stationary phase *L. donovani* expressing PTP-1B (cultured at 27 ^{0}C, pH 7.2) had assumed a more rounded morphology and had lost or retained shorter flagella, properties associated with axenic amastigotes. In comparison, the control transfected and selected promastigotes were uniformly long and cylindrical which is typical of cultured promastigotes. These data demonstrate that ectopic expression of

human tyrosine phosphatase PTP-1B gene in stationary phase cultured *L. donovani* was associated with an increase in A2 protein expression and an altered morphology consistent with a partial differentiation towards amastigotes. It has been previously established that amastigotes are more infective to macrophages than are promastigotes (Pan, 1984; Bates, 1994; Gupta *et al.*, 2001). Because the PTP-1B expressing *L. donovani* expressed A2 and displayed a more amastigote-like morphology, it was important to determine whether the PTP-1B transfectants were more virulent *in vitro* and *in vivo*. Given that only the control pALTNeo plasmid and pALTNeoPTP1B plasmid transfectants underwent transfection and G418 selection, only these were directly compared to each other for the infection assays to ensure that any phenotypic differences would be due to PTP-1B expression and not a result of phenotypic changes caused by the transfection and selection procedure. As shown in Fig. 3A and B, the PTP-1B expressing promastigotes were more virulent *in vitro* than the control transfected promastigotes with respect to both the percentage of macrophage infected and the number of amastigotes per macrophage. Moreover, PTP-1B expression also increased virulence in mice as determined by measuring visceral infection levels 4 weeks following infection via the tail vein (Fig. 3C). Taken together, these observations demonstrate that ectopic expression of human PTP-1B in *L. donovani* increased the virulence of this pathogen both *in vitro* and *in vivo*. These observations supported the argument that increased tyrosine phosphatase activity in *L. donovani* mediated at least a partial differentiation toward the amastigote phenotype. To confirm that the phenotypic change was associated with the PTP-1B gene transfer, we attempted to reverse the phenotype by inhibiting PTP-1B with a protein tyrosine

phosphatase inhibitor, vanadate. However, vanadate proved to be toxic to the *L. donovani* cultures and therefore it was not possible to specifically inhibit PTP-1B activity in this manner. It was therefore necessary to consider other pharmacological approaches to substantiate the above observations.

L. donovani treatment with protein tyrosine kinase inhibitors

The above experiments argued that increased protein tyrosine phosphatase activity, which reduces tyrosine phosphorylation, was associated with differentiation from promastigotes to amastigotes. This suggested that inhibition of tyrosine kinase to diminish tyrosine phosphorylation could likewise mediate a differentiation from promastigotes to amastigotes. To test this hypothesis, we examined *L. donovani* promastigotes following treatment with tyrphostin (AG1433), a generic inhibitor of protein tyrosine kinases (Levitzki, 1990; Gazit *et al.*, 1996) at concentrations which did not impair viability in culture. As shown in Fig. 4A, AG1433 was able to induce the expression of the A2 protein in a dose-dependent manner. Similar results were obtained with a second protein tyrosine kinase inhibitor, butein (data not shown). Moreover, AG1433 treatment induced morphological changes associated with the amastigote phenotype (Fig. 4B). Consistent with the induction of A2 expression and the amastigote morphology, AG1433 also increased the infection of *L. donovani* in macrophages *in vitro* (Fig. 4C). An advantage of undertaking the pharmacological approach, compared to the gene transfer approach, is that it is possible to confirm whether removal of the tyrosine kinase inhibitor results in phenotypic reversion. The AG1433 treated *L. donovani* promastigotes were therefore washed and placed back into culture in the absence of AG1433 and their proliferation and morphology was

compared to the same number of control promastigotes which were not previously exposed to AG1433. As shown in Fig. 5A, following the removal of AG1433, the proliferation rate of the AG1433 treated promastigotes was the same as the control non-treated promastigotes. Moreover, following the removal of AG1433, the morphology of the AG1433-treated promastigotes reverted back to the slender flagella containing form, which was indistinguishable from the control non-treated promastigotes (morphology data not shown). As shown in Fig. 5B, following the removal of AG1433 from the culture, the expression of the amastigote specific A2 proteins was also lost, consistent with differentiation back into promastigotes. These data confirmed the viability of the AG1433-treated cells and their ability to differentiate back into promastigotes following the removal of AG1433. Taken together, these data argue that the generic tyrosine kinase inhibitor AG1433 mediated phenotypic changes associated with differentiation from promastigotes to amastigotes in a manner similar to ectopic expression of protein tyrosine phosphatasePTB-1B.

Discussion

Leishmania survives as amastigotes for the vast majority of its life cycle – from months to years – within the mammalian host. In contrast, promastigote survive for only a few days in the sandfly vector until it is transmitted to another mammalian host. The mechanism of differentiation from promastigotes to amastigotes is therefore essential for the survival and pathogenesis of this parasite in the human host. Protein tyrosine phosphorylation is among the most important regulatory mechanisms in

eukaryotic cells. We therefore examined differentiation of *Leishmania* following ectopic expression of a human protein tyrosine phosphatase gene, and in a complementary approach, by treatment with protein tyrosine kinase inhibitors. Several significant observations are reported within. First, it was possible to stably express the human PTP-1B enzyme in *L. donovani* and this was associated with higher protein tyrosine phosphatase activity in the transfected cells. Second, the PTP-1B expressing promastigotes displayed several features consistent with their differentiation towards amastigotes including the expression of A2, morphological changes and infection in macrophages and mice. Third, similar phenotypic changes were induced using a complementary pharmacological approach to inhibit protein tyrosine kinase and this was reversible upon the removal of the inhibitor. Taken together, targeting tyrosine phosphorylation either enzymatically or pharmacologically in promastigotes had similar effects on *Leishmania*, stimulating differentiation towards amastigotes. Although the PTP-1B transfected and AG1433 treated *L. donovani* displayed several features associated with amastigotes, we consider these not to be fully differentiated amastigotes as many of the individual parasites shown in Figs. 2 and 4 had not completely lost their flagella and were not completely round as the authentic axenic *L. donovani* amastigotes. Nevertheless, it was clear that the expression of the human PTP-1B enzyme in *Leishmania* did have a measurable and relevant phenotypic influence, most notably the induction of A2 expression and increased virulence *in vitro and in vivo*, properties associated with amastigotes. It is of interest to compare the present study to a previous study in which we expressed high levels of the biologically active human p53 tumour suppressor

gene in *L. donovani* in which no phenotypic change was evident (Zhang *et al.*, 1995). As p53 is a transcriptional regulator, it was perhaps not surprising that it was inert in *Leishmania.* This argues that the phenotypic changes in *Leishmania* mediated by ectopic expression of PTP-1B was dependent on its activity and not due to a non-specific effect caused by the expression of a foreign higher eukaryotic gene. The fact that different independent approaches which target tyrosine phosphorylation in eukaryotic cells had very similar phenotypic outcomes on *Leishmania* provides support for the notion that tyrosine phosphorylation plays a significant role in *Leishmania* differentiation. However, it is noteworthy, that we did not detect specific changes in the tyrosine phosphoproteins in the PTP-1B transfectants compared to the control promastigotes using one dimensional gel electrophoresis and Western blot analysis with monoclonal antibodies specific to phosphotyrosine containing proteins. This may be because PTP-1B expressing cells with more global changes in phosphotyrosine proteins levels may have been selected against as a result of a negative impact on parasite survival in culture. The PTP-1B mediated changes in phosphotyrosine proteins are therefore likely to be more subtle and as a result may only be detectable following more sensitive and extensive two dimensional gel analysis and Western blotting with several different phosphotyrosine protein specific monoclonal antibodies. These studies are currently under consideration with the objective of obtaining sequence information on the target proteins using emerging proteomic approaches. The present study therefore sets a firm biological foundation for such future proteomic studies.

Consistent with the observations reported within, reduced protein tyrosine phosphorylation has also been observed in *L. donovani* promastigotes upon switching them to amastigote culture conditions (Saltora *et al.*, 2000). Reduced levels of protein phosphorylation have also been reported in *L. major* amastigotes compared to promastigotes, and this was associated with increased protein phosphatase activity in amastigotes (Dell and Engel, 1994). Although these studies did not examine the effects of ectopic expression of a protein tyrosine phosphatase gene in *Leishmania* or of tyrosine kinase inhibitors, they are consistent with the observations reported within in arguing that protein tyrosine phosphorylation plays a role in *Leishmania* differentiation. The expression of A2 is dependent on both an increase in temperature and a decrease in pH resulting in an increased stability of A2 mRNA in amastigotes (Charest *et al.*, 1994; 1996). In the present study, it was possible to induce the expression of A2 by ectopic expression of a protein tyrosine phosphatase or protein tyrosine kinase inhibitors in the absence of temperature and pH induction. These observations demonstrate that A2 protein expression is regulated directly or indirectly through signal transduction pathways involving protein tyrosine phosphorylation and that reduced pH or increased temperature can be bypassed to achieve A2 expression under promastigote culture conditions. This argues that environmental changes encountered during the life cycle of *Leishmania* act to influence tyrosine phosphorylation. As A2 proteins are amastigote specific, the characterization of the signal transduction pathway regulating their expression could facilitate identifying additional amastigote specific proteins. Future studies will also require the identification and characterization of endogenous protein tyrosine phosphatase genes

using the data emerging from the *Leishmania* genome project currently nearing completion (Myler *et al.*, 2000; Almeida *et al.*, 2002). The genetic and biological experimental approaches described within using heterologous genes will be useful to confirm the functions of the endogenous protein tyrosine modifying enzymes.

Experimental procedures

Plasmids, antibodies, biochemicals and transfections

The anti-A2 monoclonal antibody C9 was developed in our laboratory (Zhang *et al.,* 1996). The human PTP-1B gene and antibodies have been previously described (Muise *et al.*, 1996) and the gene was cloned into the *Leishmania* vector pALTNeo and transfected into *Leishmania* as detailed below. Sheep anti-mouse IgG and donkey anti-rabbit IgG horseradish peroxidase conjugated (Amersham Pharmacia Biotech, QC) were used as secondary antibodies and the enhanced chemiluminescence (ECL) (AmershamPharmacia Biotech, QC) was used for signal visualization. The protein tyrosine kinase inhibitor; tyrphostin AG1433 [2-(3,4-Dihydroxyphenyl)-6,7 dimethylquinoxaline, HCl; SU 1433] (Levitzki, 1990; Gazit *et al.*, 1996) was purchased from CalBiochem. AG1433 was dissolved in methanol, aliquoted, speed vacuumed and maintained at -20 ^{0}C according to the manufacturer recommendation until needed. Dried aliquots were resuspended in DMSO (Fisher Scientific, NJ) at a concentration of 10 mM. The final DMSO concentrations did not exceed 0.1% (v/v) in the culture medium and had no affect on the *Leishmania* cultures.

Expression of human PTP1B in Leishmania

To make the construct pALTneoPTP1B, a 1.5 kb human PTP1B cDNA containing the entire coding sequence was removed from plasmid pZeohPTP1B (Muise *et al.*, 1996) with restriction enzymes *BamH* I and *Xba* I and inserted into the corresponding sites downstream of the *Leishmania* tubulin gene intergenic sequence in the *Leishmania* expression vector pALTneo which has been previously described (Laban *et al.*, 1990; Zhang *et al.*, 1995). The pALTneo vector does not contain a promoter sequence as promoters are nonfunctional in *Leishmania.* pALTneo however, does contain *L. major* tubulin intergenic regions which contains the processing sites required to express mature *trans*-spliced mRNA in *Leishmania* as previously detailed (Laban *et al.*, 1990). pALTneoPTP1B and the control vector pALTneo were electroporated into *Leishmania donovani* promastigotes as previously described (Zhang *et al.*, 1995). Transfectants were selected and maintained in culture with 50 mg ml^{-1} of G418.

Western blot analysis of A2 and PTP1B proteins

For Western blot analysis, promastigotes, amastigotes and AG1433 treated cultures were washed three times with chilled PBS, resuspended to 5.0 x 10^6cells/10 ml, and immediately lysed with boiling 2X SDS-PAGE sample buffer. Ten microlitres of the lysate was then resolved on 10% SDSPAGE gels, and transferred onto nitrocellulose membrane (Bio-Rad Laboratories). TBST 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% (v/v Tween-20) was used for all subsequent washing steps at room temperature. Membranes were first washed twice and then blocked with 10% non-fat milk in TBST for two hours. Following three washes, the detection of A2

proteins was preformed as described previously by Zhang *et al.* (1996) with the anti-A2 monoclonal antibody C9.

For the detection of PTP-1B, anti-PTP-1B (BD Biosciences 1, 2000) was used as the primary antibody and incubated for one hour at room temperature. Membranes were washed three times and incubated for one hour with sheep antimouse IgG (1 : 3000). Following five washes, proteins were detected using ECL (Amersham). To insure equal loading of protein in each lane, membranes were stripped and reblotted with polyclonal anti-tubulin antibody (Oncogene 1, 2000), the secondary antibody in this case was donkey anti-rabbit IgG (1 : 3000).

Leishmania culture, differentiation, and macrophage infections

Leishmania donovani 1S2D promastigotes kindly provided by Dr Denis Dwyer were cultured in M199 medium (Gibco) supplemented with 10% fetal bovine serum (FBS), at pH 7.2 and 27 ^{0}C. Every four days, late-log phase cultures were split 1 : 100 (v/v) into fresh promastigote medium. Promastigotes were induced to differentiate into amastigotes by inoculating 1 : 50 (v/v) late-log phase promastigotes into M199 medium supplemented with 20% FBS, pH 5.5 and 37 ^{0}C, conditions which mimic the transition into the macrophage phagolysosomal vacuole. Amastigotes were differentiated back into promastigotes by placing them back into promastigote culture conditions. The macrophage cell line Raw 264.7 (ATCC TIB-71) was used to carry out *in vitro* infection experiments with promastigotes and amastigotes. Cells were split 1 : 10 once a week in RPMI-1640 medium (Gibco) supplemented with 10% FBS, 1 M Hepes (pH 7.3), 100 U ml^{-1} of penicillin and streptomycin. Macrophage infections were carried out as previously detailed (Buates and Matlashewski, 2001).

Briefly, infections were carried out using stationary phase parasite cultures in RPMI 1640 medium supplemented with 10% FBS at a 1 : 1 ratio of parasite to macrophage cell (10^6 macrophages ml^{-1}) in non-adherent polystyrene tubes. It was not feasible to first isolate metacyclic parasites as it was not possible to identify typical metacyclics within the PTP-1B expressing lines or following treatment with AG1433. Parasites were washed free of AG1433 prior or just prior to carrying out the infections. Following 12 h contact at 37 ^{0}C, free parasites were washed away from the infected cells by washing four times with PBS and an aliquot was cytocentrifuged. Cytocentrifuged infected cells on polylysine-coated slides were fixed with methanol and stained with Giemsa and the number of internalized amastigotes per 1000 macrophage nuclei and the percent of infected cells was counted in triplicate. The experiment was repeated three times and the results reported in bar graphs are the mean ± SE. Statistical methods: Microsoft Excel was used to calculate the Student's *t*-test and results were considered to be statistically significant if $P < 0.05$.

Protein tyrosine phosphates activity determination and inhibition of protein tyrosine kinase

Protein tyrosine phosphatase activity was determined as previously described (Kozlowski *et al.*, 1993; Blanchette *et al.*, 1999). Briefly, 107 *L donovani* cells were washed in PBS and disrupted in ice-cold lysis buffer containing 50 mM Tris-HCl pH 7.0, 0.1 mM EGTA, 0.1% 2-beta-mercaptoetanol (v/v), 1% NP40 and protease inhibitors. Total protein tyrosine phosphatase activity was determined on 20 mg of protein in a reaction mixture containing 50 mM Hepes pH 7.5, 0.1% 2-beta-mercaptoethanol and 10 mM of the substrate 4-Nitrophenyl phosphate (pNPP).

Enzymatic hydrolysis of pNPP was determined by measuring the absorbance at 405 nm at 15 and 30 min of incubation. Experiments involving the inhibition of *L. donovani* tyrosine kinase were carried out using concentrations of tyrphostin AG1433 which were non-toxic as determined by carrying out growth curves in the presence of different concentrations of AG1433. The proliferation of promastigotes cultured in 20 mM AG1433 and below was not impaired.

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Figure Legends

Figure 1. A. Western blot analysis of human PTP1B expression in *Leishmania donovani* transfected with plasmids pALTNeoPTP1B expressing PTP1B or the control plasmid, pALTNeo as indicated. **B**. Total cellular tyrosine phosphatase activity determined in the PTP1B transfectants (PTP1B), in the control transfected (pALTNeo), and non-transfected wild-type *L. donovani* promastigotes.

Figure 2. A. Western blot analysis of A2 expression in stationary cultures of *Leishmania donovani* transfected with plasmids pALTNeoPTP1B expressing PTP1B, the control plasmid, pALTNeo, or cultured wild-type amastigotes as indicated. Equal loading of protein was verified by reprobing the blot with an anti-alpha-tubulin antibody. **B**. Morphology of wild-type promastigotes (cultured at 27 ^{0}C, pH 7.2); wild-type amastigotes (37 ^{0}C, pH 5.5); PTP1B transfected promastigotes (27 ^{0}C, pH 7.2); and control pALTNeo plasmid transfected promastigotes (27 ^{0}C, pH 7.2) as indicated. Note that the arrows demonstrate that ectopic PTP1B expression induced a

more rounded morphology resembling the cultured amastigotes. In comparison, promastigotes are long and spindle shaped whereas amastigotes are rounder.

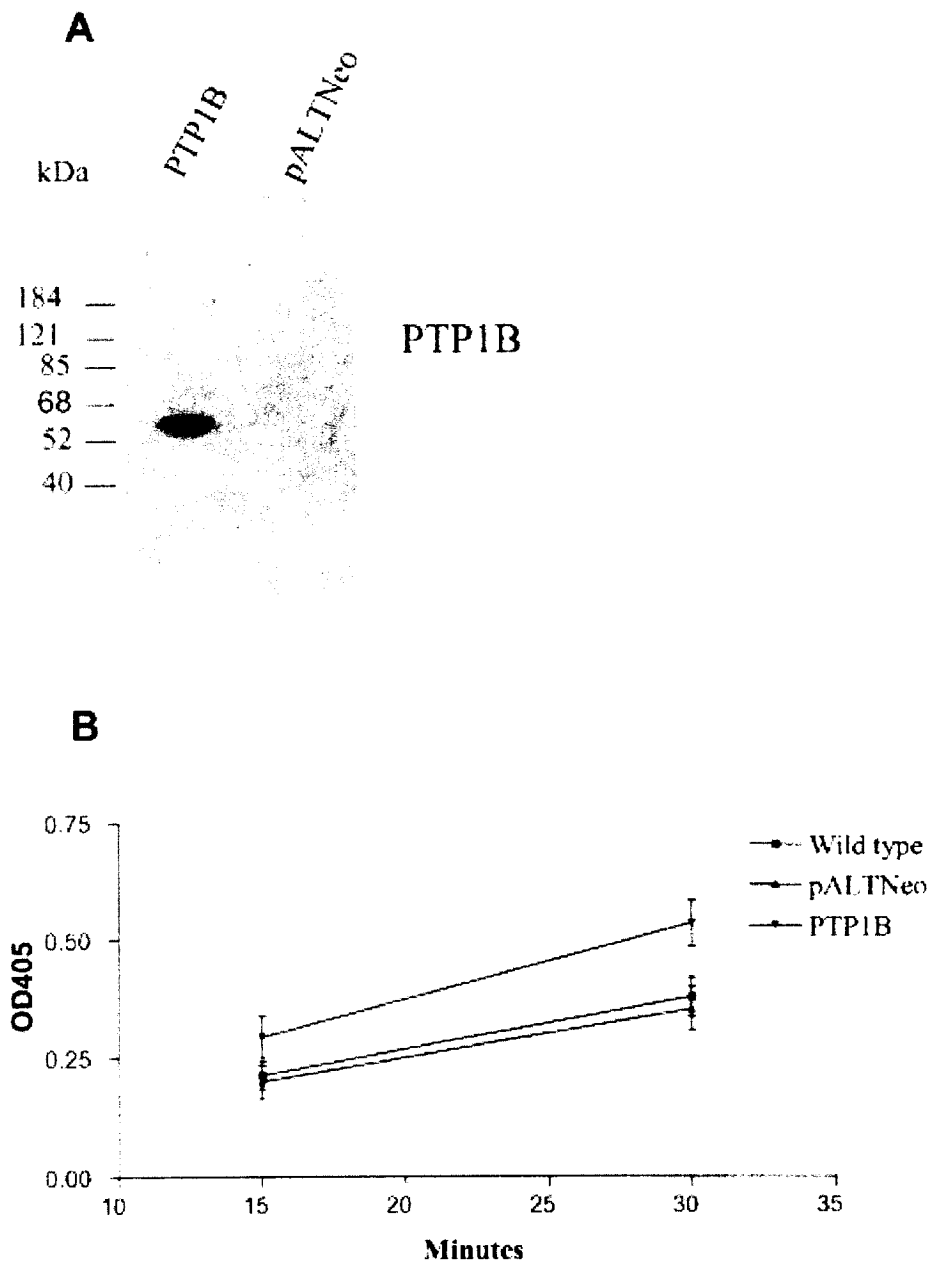
Figure 3. Infection of macrophages and mice with the PTP1B and control plasmid pALTNeo transfected promastigotes. **A.** Percentage of macrophages infected after12 h of infection at a 1 : 1 promastigote/macrophage ratio. **B.** The number of amastigotes per macrophage after 12 h of infection at a 1 : 1 promastigote/macrophage ratio. **C.** Infection in mice 28 days following tail vein infection with 2 x 10^8 promastigotes showing the number of amastigotes per 1000 cells and the Leishman-Donovan Units (LDU): number of amastigotes per 1000 cells x liver weight (g).

Figure 4. Phenotypic characterization of AG1433 treated promastigotes. **A.** Expression of the A2 protein in Promastigotes (Lane P), Amastigotes (Lane A), and promastigotes cultured in various concentrations of AG1433 as indicated. Below the levels of tubulin in each lane was determined to ensure equal levels of protein in each lane. **B.** Morphology of wild-type (WT) cultured Promastigotes, wild-type (WT) Amastigotes, and AG1433 15 mM treated Promastigotes. **C.** Macrophage infection levels determined as the number of amastigotes per 1000 macrophages following infection with promastigotes, amastigotes and promastigotes cultured inAG1433 as indicated. Note that the amastigotes and the AG1433-differentiated promastigotes were more infective than the control untreated promastigotes.

Figure 5. Removal of AG1433 resulted in the reversion back into wild-type promastigotes. **A.** Promastigotes cultured in 10 mM and 15 mM AG1433 were washed to remove the AG1433, and then placed into promastigote culture media in

the absence of AG1433 and their proliferation rate was compared with an equal number of non-treated promastigotes. **B**. Western blot analysis of A2 protein in promastigotes (Lanes 1 and 4); promastigotes cultured in the presence of 10 mM and 15 mM AG1433 (lanes 2 and 3); and promastigotes previously cultured in the presence of AG1433 following the removal of in AG1433 (lanes 5 and 6). Note that removal of AG1433 resulted in the loss of A2 protein expression.

Figure 1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pALTNeo
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PTP1B
B
OD405
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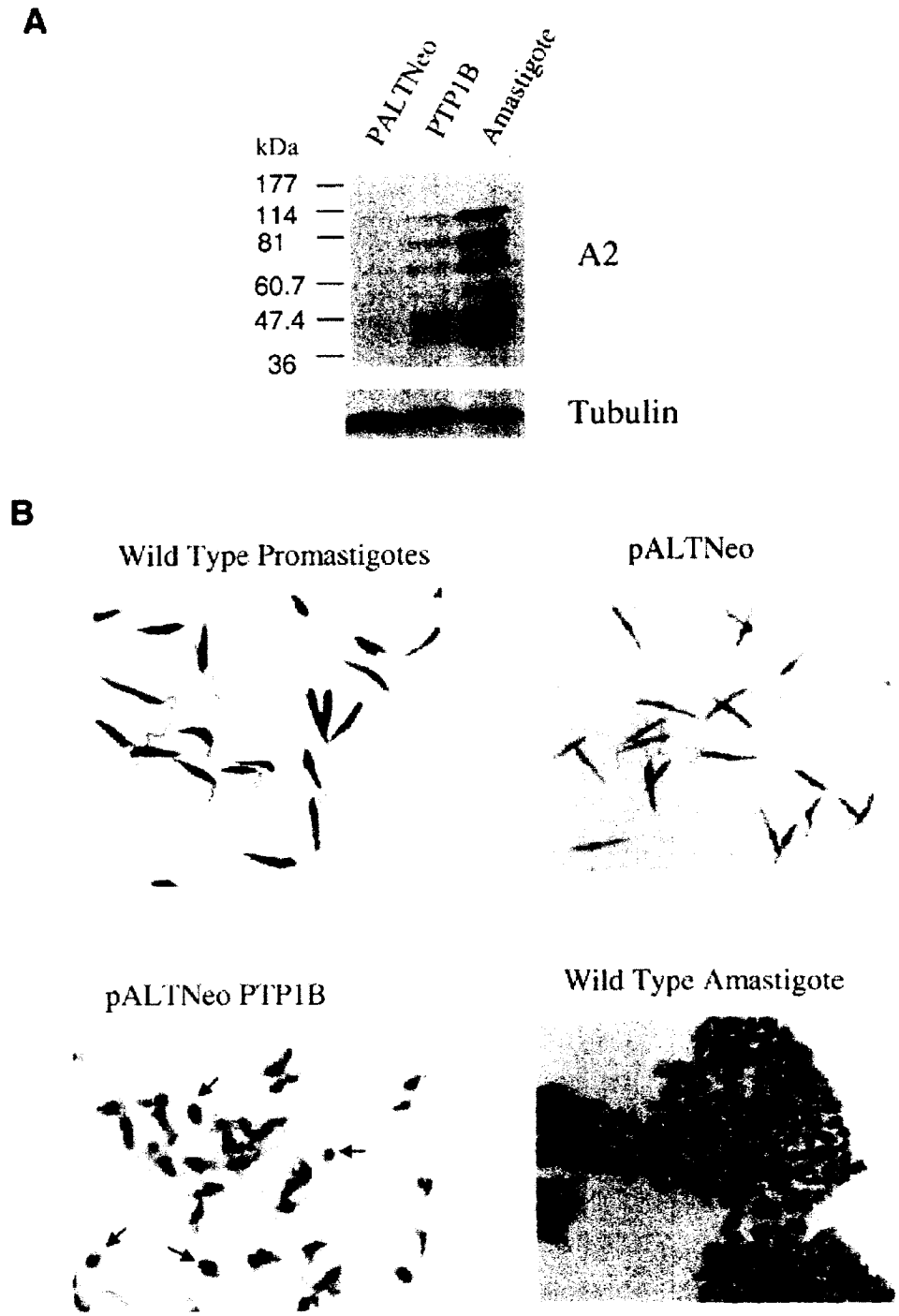
30
35
Minutes
Wild type
pALTNeo
PTP1B

Figure 2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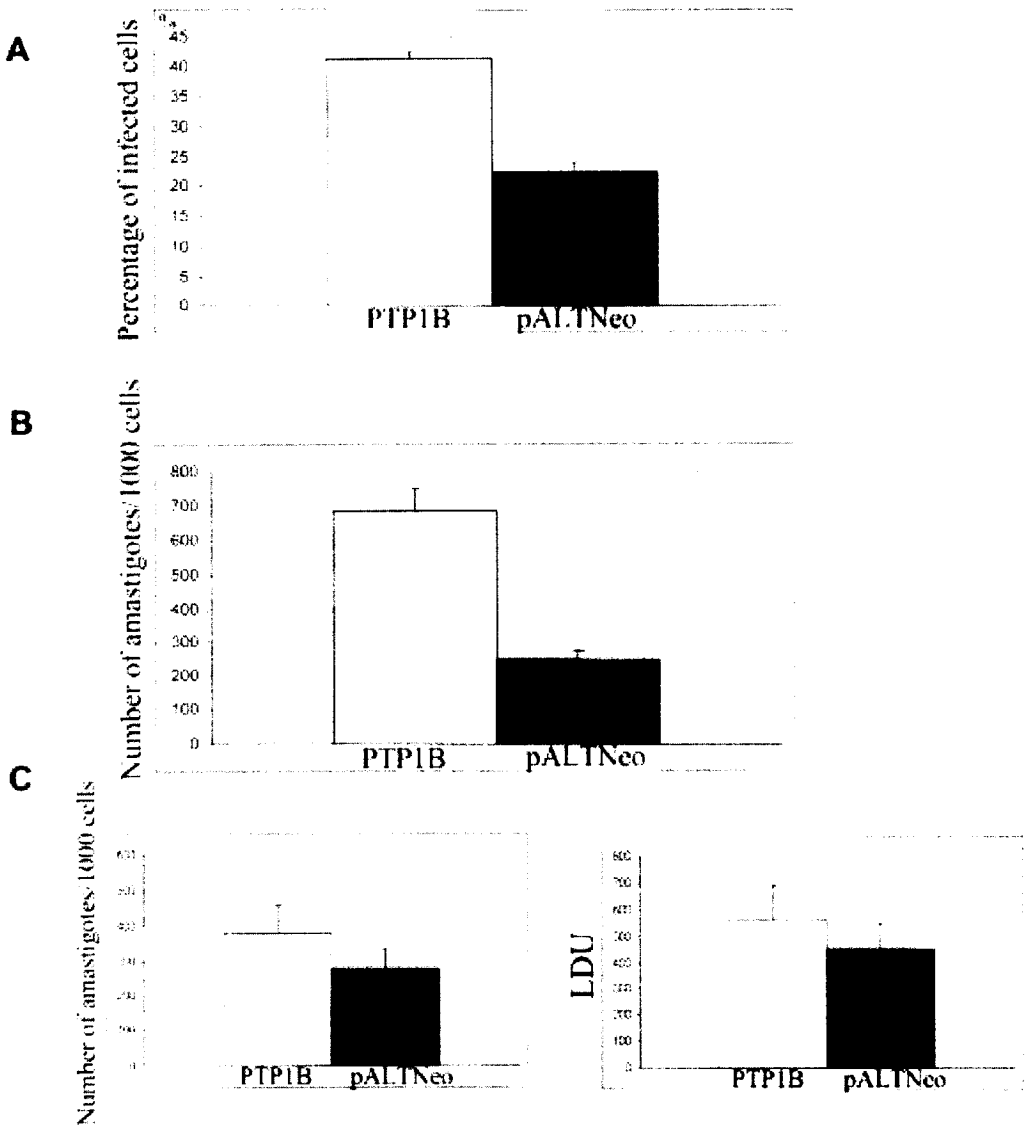
Wild Type Amastigote

Figure 3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Percentage of infected cells
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25
20
15
10
5
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PTP1B
pALTNeo
B
Number of amastigotes/1000 cells
800
700
600
500
400
300
200
100
0
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C
Number of amastigotes/1000 cells
PTP1B
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PTP1B
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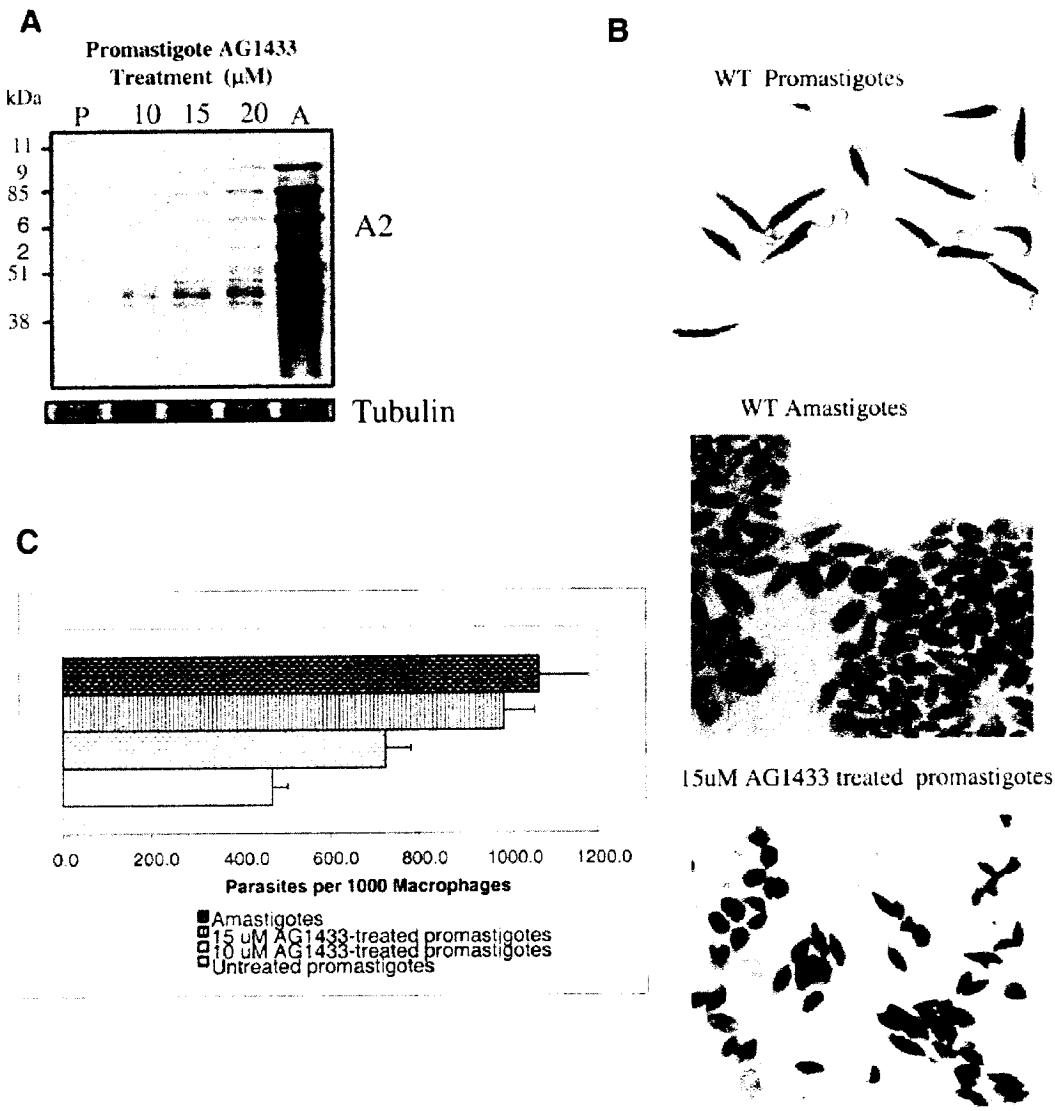
Figure 4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Promastigote AG1433
Treatment (μM)
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11
9
85
6
2
51
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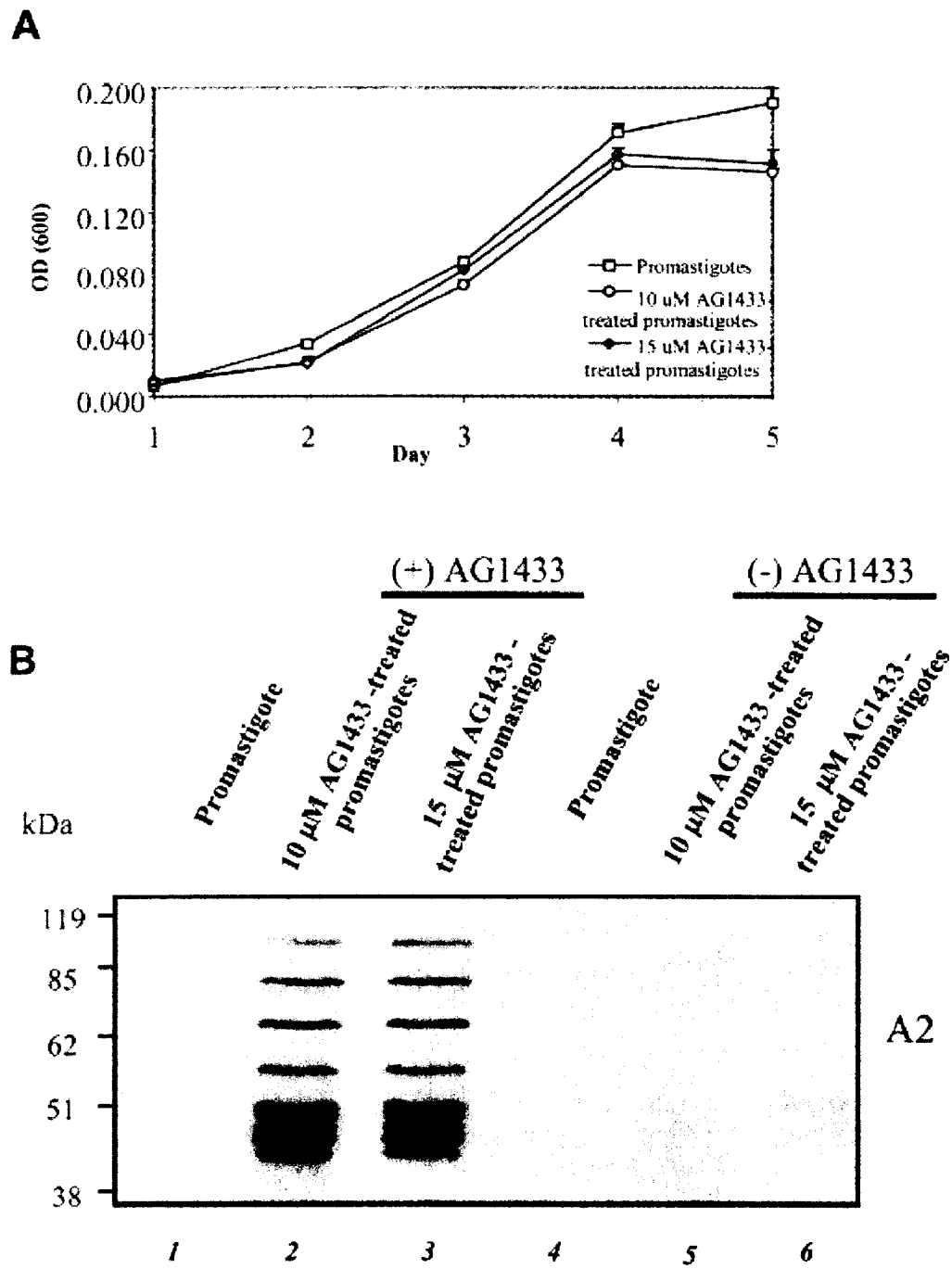
Tubulin
B
WT Promastigotes
WT Amastigotes
15uM AG1433 treated promastigotes
C
0.0
200.0
400.0
600.0
800.0
1000.0
1200.0
Parasites per 1000 Macrophages
Amastigotes
15 uM AG1433-treated promastigotes
10 uM AG1433-treated promastigotes
Untreated promastigotes

Figure 5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119
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PREFACE TO CHAPTER 3

In the previous chapter, the results showed tyrosine phosphatases are involved in the amastigote differentiation process of *L. donovani*, since higher expression of a heterologous and promiscuous PTP and the use of a PTK inhibitor lead to partial amastigote differentiation. It was also demonstrated that higher tyrosine phosphatase activity is linked to an increase in *L. donovani* virulence levels. This study extends the investigation on the contribution of tyrosine phosphatases in pathways related to *L. donovani* differentiation and infectivity. The next Chapter describes the identification of the *Leishmania* homologue to hPTP1B through sequence analysis and the confirmation the LPTP1 is an active enzyme. In addition, the development of *L. donovani* mutant clones lacking this phosphatase provides further evidence of its involvement in mechanisms of *Leishmania* survival inside the host; *in silico* studies also investigate the prospect of finding specific inhibitors to target the *Leishmania* PTP1 enzyme.

CHAPTER 3: Identification and Characterization of a Protein Tyrosine Phosphatase in *Leishmania;* Involvement in Virulence.

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Running title: Protein tyrosine phosphatase and *Leishmania* virulence

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Abstract

Leishmania parasites are eukaryotic protozoans responsible for a variety of human diseases known as leishmaniasis, which ranges from skin lesions to fatal visceral infections. *Leishmania* is transmitted by the bite of an infected sandfly where it exists as promastigotes and, upon entry into a mammalian host, differentiates into amastigotes, which replicate exclusively in macrophages. The biochemical pathways enabling *Leishmania* to differentiate and survive in the mammalian host are poorly defined. We have therefore examined the role of protein tyrosine phosphorylation, which is essential in regulating cell function in higher eukaryotes. Using the recently completed *Leishmania* genome, we have identified and cloned a *Leishmania* protein tyrosine phosphatase gene (LPTP1) by virtue of its homology with the human protein tyrosine phosphatase 1B gene (hPTP1B). The enzyme activity of recombinant LPTP1 was confirmed using a combination of PTP specific substrates and inhibitors. We further demonstrate, by creating LPTP1 null mutants through gene targeting, that LPTP1 is necessary for survival as amastigotes in mice but it is dispensable for survival as promastigotes in culture. Human PTPs, including the PTP1B enzyme, are actively pursued drug targets for a variety of diseases. The observations with the LPTP1 mutants in mice suggest it may also represent a drug target against the mammalian amastigote stage. However, *in silico* structure analysis of LPTP1 revealed a striking similarity with hPTP1B in the active site suggesting that, although this is an attractive drug target, it may be difficult to develop an inhibitor specific for the *Leishmania* LPTP1.

INTRODUCTION

Leishmaniasis is a disease caused by infection with *Leishmania* protozoan parasites, which results in a spectrum of clinical manifestations ranging from self-healing cutaneous lesions to fatal visceral disease (reviewed in 1). There are over two million new cases of leishmaniasis each year and over 12 million people currently suffering from this infection in 88 tropic and subtropic countries (2, 3). During its lifecycle, *Leishmania* alternates between promastigotes in the sandfly vector and amastigotes in the mammalian host. Once transmitted to the mammalian host through the bite of an infected sandfly, the promastigotes differentiate into non-flagellated intracellular amastigotes whereupon they multiply exclusively in the phagolysosome organelle of infected macrophages. Amastigotes are responsible for the diverse pathologies associated with leishmaniasis, which depends to a large extent on the *Leishmania* species (reviewed in 1). The biochemical changes associated with differentiation from promastigotes to amastigotes and with the long-term survival of amastigotes in the mammalian host are poorly understood, and consequently the biological role of protein phosphorylation remains largely unknown in *Leishmania.*

Protein phosphorylation is among the most important regulatory biochemical changes in higher eukaryotic cells. Phosphorylation of tyrosine residues is controlled by protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP). Particularly, protein tyrosine phosphorylation and dephosphorylation regulate multiple central processes including cellular phenotypic functions, differentiation, proliferation, and cell death (reviewed in 4). The overall protein phosphorylation pattern in *Leishmania* parasites and related trypanosomatids has been shown to change during differentiation associated with different lifecycle stages, suggesting

that protein kinases and phosphatases play a role in these processes (5). More recently, it has been established that *L. major* promastigote extracts contain protein tyrosine phosphatase activity, although the corresponding gene(s) has not yet been identified (6).

Due to their central role in higher eukaryotic cell function, we have begun to investigate the potential contribution of protein tyrosine phosphorylation to the *Leishmania* life cycle and virulence. We had previously observed that heterologous expression of a prototype human protein tyrosine phosphatase 1B (hPTP1B) in *L. donovani* mediates partial differentiation towards the amastigote stage, induces expression of amastigote-specific proteins, and increases virulence in BALB/c mice (7). These observations argue that protein tyrosine phosphatases (PTPs) play a role in amastigote survival in the mammalian host and provide strong justification for the characterization of endogenous *Leishmania* PTP genes as detailed within.

In the present study, we describe the identification of a PTP gene from *L. major, L. infantum,* and *L. donovani* which has extensive sequence and corresponding structural homology with the human PTP1B gene product. These *Leishmania* genes have been designated LmPTP1, LiPTP1 and LdPTP1, respectively. The enzyme activity of LmPTP1 has been confirmed with relevant substrates and inhibitors. We have also developed LdPTP1 heterozygous and homozygous null mutant knock-out clones which proliferated in a similar fashion to wildtype promastigotes in culture but were severely impaired with respect to survival as amastigotes in BALB/c mice. Through *in silico* structural analysis, we also show that the *L. infantum* PTP1 (LiPTP1) and human PTP1B (hPTP1B) shared remarkable structural conservation in

the active site; however, notable differences outside this region are also present. This observation suggests that from a phenotypic perspective, the LPTP1 represents an attractive drug target. However, from a structural perspective, it may be difficult to develop a small molecule specific to the active site of LPTP1.

Experimental Procedures

Parasite cultures - The *L. donovani* 1S/Cl2D and *L. major* Friedlin V9 promastigotes were routinely cultured at pH 7.2 and 27 °C in M199 medium (Gibco) supplemented with 10 % fetal bovine serum (FBS). *L. donovani* differentiation into amastigotes was performed by shifting to amastigote culture media (37 °C, pH 5.5 in RPMI 1640 plus 10 % FBS) overnight, which mimics the temperature and pH of the host macrophage phagolysosome.

Cloning, sequencing and tagging of L. major PTP1 - The 1.5 Kb *L. major* DNA fragment homologous to the human PTP1B was identified in the *Leishmania major* database by BLAST search (Entry GeneDB **LmjF36.5370**, www.genedb.org). Based on the sequence obtained from the database, two primers were designed to PCR amplify the LmPTP1 gene and to incorporate a sequence encoding a His-tag at the N-terminal and the amplified product was then ligated into the mammalian expression vector pcDNA3. The primers used were HisF 5'-aagcttATGGGCCATCATCATCATCATCATATGTGTGAAAAGCAACTCAAGGAG-3', which contained a *Hind* III site and reverse R 5'-ggatccTTACACAAACGAAGGCGAGAAGCGC-3' contained a *Bam* HI site. The resulting His-tagged LmPTP1 containing plasmid was used as template in a second

PCR reaction, where a new primer incorporated a 10 amino acid epitope tag from the *L. donovani* specific A2 protein which is recognized by an anti-A2 monoclonal antibody (8) A2-HisF 5'-aagcttATGCAGTCCGTTGGCCCGCTCTCCGTTGGCCCGCATCATCATCATCATCATCAT-3', with a *Hind* III site and the same R 5'-ggatccTTACACAAACGAAGGCGAGAAGCGC-3' with a *Bam* HI site was used. The final amplified product was then cloned into the pcDNA3 vector and called pcDNA3-LmPTP1 as shown in Fig. 2A. As a positive control for subsequent experiments, the human PTP1B sequence was also PCR amplified with the A2/His-tag, using the oligonucleotide primers A2HisF (hPTP) 5'-ggatccATGCAGTCCGTTGGCCCGCTCTCCGTTGGCCCGCATCATCATCATCATCATCAT-3', with an *Eco* RI site and the A2HisR (hPTP) 5'-gaattcCTATGTGTTGCTGTTGAACAGGAAC-3' with a *Bam* HI site.

The *L. donovani* LdPTP1 gene was PCR amplified from *L. donovani* 1S/Cl2D genomic DNA with the same primers used above for *L. major* and sequenced (GenBank bankit827438 DQ862810). In brief, the amplified fragment was cloned into the TOPO TA cloning vector (Invitrogen), termed pLdPTP1-TOPO, and M13 Reverse and M13 Forward primers were used in sequencing reactions with the MegaBace500 (Molecular Dynamics of GE Healthcare). Sequencing reactions were performed by DYEnamic ET TerminatorCycle Sequencing Kit with Thermo Sequenase II DNA polymerase, and post-reaction cleanup was achieved by extensive ethanol precipitation before adding formamide loading solution.

Transfection of Cos7 cells and purification of A2His-tagged LmPTP1 and hPTP1B - The Cos7 cell line was transiently transfected using lipofectamine reagent according to the manufacturer's protocol (Invitrogen). Briefly, a 100mm dish of Cos7 cells (approximately $8x10^5$ cells) was transfected with a total of 8 μg DNA (6 μg of pcDNA3-LmPTP1 and 2 μg of a β-galactosidase expression plasmid) in 20 μl of lipofectamine. The following day (~20 h after transfection), cells were washed with cold media and lysed with NP-40 lysis buffer (150 mM NaCl, 1 % NP-40, 20 mM Tris pH 8.0) + protease inhibitors (Roche® complete cocktail tablets) on ice for 30 minutes. Cell lysates were centrifuged and β-galactosidase assays performed to determine the levels of transfection efficiency in each dish and to normalize the amount of protein used in each assay. His-bind® resin (Novagen) was used for purification of the A2-His tagged LmPTP1 and hPTP1B according to the manufacturer's protocol. Briefly, His-bind® resin was activated in 1X Binding buffer (8X= 4 mM NaCl, 160 mM Tris-HCl, 40 mM Imidazole, pH 7.9) + 0.1 % NP-40. 60 μl of activated His-bind® resin was added to 200 μl cell lysates and incubated with agitation for 3 h at 4 ^{0}C. His-bind® resin was thoroughly washed 5x in 1.0 ml of 150 mM NaCl, 1%NP-40, 20 mM Tris pH 8.0, buffer + protease inhibitors (Roche® complete cocktail tablets) and half of the His-bind® resin with the purified A2-His tagged LmPTP1 or hPTP1B was used for activity assays, and the other half used for Western blot analysis with anti-A2 tag monoclonal antibodies.

Protein tyrosine phosphatase activity and inhibition assays - The 4 p-Nitrophenylphosphate (pNPP) assay was used for detection of total phosphatase activity as described previously (7). Briefly, 30 μl of His-bind® resin containing

purified PTP proteins was washed once in 1.0 ml 150 mM NaCl, 1 % NP-40, 20 mM Tris pH 8.0, Roche® complete protease inhibitor buffer and placed in a 96 well plate. 180 µl reaction buffer (50 mM Hepes pH 7.5, 0.1 % β-Mercaptoethanol containing 10 mM fresh pNPP) was added to each well and the plate was incubated at 37 ^{0}C overnight. Plates were read at 405 nm.

The Malachite green phosphatase activity assay with the insulin receptor (IR) phosphopeptide is a more specific assay for hPTP1B, since the IR is a major substrate of this enzyme (9, 10). The assay was performed according to manufacturer's instructions (Sigma, PTP101, non-radioactive phosphotyrosine phosphatase assay).

Two inhibitors, 1 mM of Sodium orthovanadate (Na_3VO_4), and 10 µM of potassium bisperoxo(1,10-phenanthroline)oxovanadate (V) [bpV (phen)] were used to further confirm the activity of LmPTP1 and the hPTP1B as the control. bpV (phen) is a member of a class of potent and specific PTP inhibitors (11, 12). For the inhibition assays, His-bind® resin containing LmPTP1 and hPTP1B was washed 5 times with 1.0 ml 150 mM NaCl, 1 % NP-40, 20 mM Tris pH 8.0, Roche® complete protease inhibitor buffer and then incubated with inhibitors for 1 h at 4 ^{0}C. His-bind® resin containing the PTPs was washed 4x with 1.0 ml 150 mM NaCl, 1 % NP-40, 20 mM Tris pH 8.0, Roche® complete protease inhibitor buffer and then assayed for phosphatase activity using pNPP as substrate, as detailed above.

Disruption of the LdPTP1 genes from L. donovani- The *L. donovani* PTP1 gene disrupted strains were generated by homologous gene targeting as outlined in Fig. 4A. A *Hind* III and *Xba* I fragment containing the *L. donovani* PTP1 gene from the pLdPTP1-TOPO plasmid described above was subcloned into a pBluescript vector.

The resulting pBSLdPTP1 plasmid was digested with *Bcl I* to remove the 357 bp catalytic region of the LdPTP1 gene. The fragment containing the hygromycin resistance gene was removed from the pSPY hygromycin vector (13) with *Bam HI* and *Bgl II* and inserted into the *Bcl* I site within the LdPTP1 sequence, generating the plasmid pBSLdPTP Hyg. The linear fragment containing the hygromycin gene and the LdPTP flanking sequences was then electroporated into *L. donovani.* Transfectants were initially selected, in the first round targeting, with 50 μg/ml hygromycin to obtain the heterozygous single LdPTP1 knockout mutant. The double knockout homozygous null mutant for the LdPTP1 gene was achieved by increasing the hygromycin concentration to 200 μg/ml in selection culture medium.

Complementation of the double knockout null mutant with a plasmid containing the LdPTP1 gene was carried out as follows. The following oligonucleotide primers LdPTPF1 5'cccaagcttTCACTTTTTGTTGCCCTTGGT with a *Hind* III site and the reverse LdPTPR1 5'cgagatctCAGAGGTGCAGCCAGTCATA with a *Bgl* II site were used to amplify a 3140 bp fragment from *L. donovani* genomic DNA which contained the LdPTP1 gene open reading frame including 655 bp upstream and 1000 bp downstream flanking sequences. The 3140 bp fragment was then inserted into *Hind* III and *Bam* HI sites of plasmid pSPY-Neo (13) to generate the complementing plasmid pSPYNeoLdPTP1 as shown in Figure 7A.

Southern Blotting - For Southern blot analysis, 10 μg of *Leishmania* genomic DNA was digested with restriction enzymes *Pst* I and *Sst* I and separated in a 0.7 % agarose gel. Hybridization and washing were performed as previously described (14). The LdPTP1 active domain encoding DNA (357 bp *Bcl* I fragment from nucleotides 677-

1034) was used as a probe for DNA from the single (+/-) and double knock-out (-/-) clones described above to demonstrate the disruption of the LdPTP1 gene (Fig. 4B). Southern blot using the hygromycin gene demonstrated specific targeting into the LdPTP1 gene (Fig. 4C). The *L. major* 1.5 kb LmPTP1 gene containing fragment was used in the southern blot to confirm the presence of the episomal LdPTP1 added back to the double knockout null clone Ld1PTP1 -/- (Fig. 7B).

Infection of Balb/c mice and recovery of amastigotes - Female BALB/c mice (Charles River Breeding laboratories) weighing 20-25 g (n= four mice per group) were injected via tail vein with $1.5x10^8$ late log phase promastigotes in 100 µl PBS, as described previously (15). After 4 weeks of infection, mice were examined for *L. donovani* parasite burden by counting the number of amastigotes in the Giemsa-stained liver imprints. Liver parasite burden, expressed as Leishman-Donovan Units (LDU) was calculated by multiplying the number of amastigotes per 1000 cell nuclei x liver weight (g). Spleen parasite burden were determined by limiting dilution in 96 well plates as previously detailed (16).

Proliferation in culture - Parasite growth was evaluated by determination of the optical density (O.D.) at 600 nm of diluted cultures (starting from 10^6 cell/ml) grown in 96 well plates from days 0 to 8 (promastigotes) or 0 to 5 (amastigotes).

LiPTP1 catalytic domain modeling - The alignment between the *L. infantum* PTP1 amino acid sequence and human PTP1B obtained with ClustalW (Fig. 1) in conjunction with the PTP1B crystal structure (PDB code 1SUG) were used in Modeller 8 (version 2, default configuration, 17) to create the *L. infantum* PTP1 homology model. A 20-residue stretch of amino acids present in the *Leishmania*

PTP1 sequence that was outside of the enzyme active site was not present in the human enzyme sequence and, as a result, was not modeled (residues 32-51). In addition, the construct used to elucidate the human enzyme structure did not contain any residues beyond position 319. Therefore, these two regions were removed from the *Leishmania* homology model.

RESULTS

We began this study by performing a BLAST search of the *L. major* database (18, www.genedb.org) for sequences which could represent PTP genes by virtue of their homology with the human PTP1B gene. In total, 9 potential PTP genes were identified in the *Leishmania* genome. The one with the greatest identity with human PTP1B was LmjF36.5370 located on chromosome 36, which we have designated LmPTP1. The *L. infantum* PTP1 (LiPTP1) sequence was also identified in this manner from the *L. infantum* data base (LinJ36.5860, www.genedb.org). Based on these sequences, we designed PCR primers to amplify, clone and sequence the *L. donovani* LdPTP1 homolog (GenBank bankit827438 DQ862810) as detailed in the Experimental Procedures. Alignment comparison of the various *Leishmania* PTP1 and hPTP1B proteins revealed they share approximately 40% sequence identity, including a number of important conserved amino acid residues within the hPTP1B signature catalytic domain [(I/V)HCXXGXXR(S/T/G)] which contains the essential cysteine and arginine residues required for enzyme activity (boxed region in Fig.1) (reviewed in 4). In addition to the conserved catalytic domain, LPTP1s share relevant

accessory motifs with hPTP1B including the adjacent signature WPD and the Q residues (boxed region highlighted in bold in Fig. 1), which play a role in maintaining the conformation of the active site. The LPTP1s and hPTP1B also share a proline rich region from amino acids 325-340 which are responsible for SH3 domain protein-protein type interactions and cellular localization in hPTP1B (4). Southern blot analysis of genomic DNA from *L. major* demonstrated that LmPTP1 was a single copy gene in the haploid genome (our unpublished data). Included in Figure 1 is the closest PTP sequence homolog from *S. cerevisae*, which is more divergent from hPTP1B than is the LPTP1s.

Although the *Leishmania* PTP1 sequences shown in Figure 1 suggest that they encode for a hPTP1B homolog, it was necessary to validate this experimentally using relevant enzyme substrates and inhibitors. We designed primers to amplify, clone and insert the LmPTP1 gene into a eukaryotic expression vector (pcDNA3) for expression in transfected simian Cos7 cells. In order to detect and partially purify the LmPTP1 from Cos7 cells, a 10 amino acid epitope tag derived from the *L. donovani* A2 protein, followed by a 7-Histidine tag (His_7 tag) encoding sequence, were inserted at the 5' end of the LmPTP1 gene (Figure 2A). In this manner, the Cos7 cell expressing A2-His_7 tagged LmPTP1 could be detected with anti-A2 monoclonal antibodies (Mabs) (8) and partially purified from cell lysates by affinity chromatography with His-tag bind® resin. We also generated the same construct using the human hPTP1B gene as a positive control for subsequent comparison.

Expression of the A2-His_7 tagged LmPTP1 and hPTP1B genes in Cos7 cells was analyzed by Western blot analysis with anti-A2 Mabs 24hr after transfection. As

shown in Fig 2B, the hPTP1B and LmPTP1 proteins were detectable at the predicted molecular weights of 50 kDa and 55 kDa respectively (Lanes 2 and 3). The control empty pcDNA3 vector did not produce bands (Lane 1) confirming the specificity of the Western blot for A2-tagged PTPs. The A2-His_7 tagged proteins were subsequently partially purified from the transfected Cos7 cell lysate using His-tag bind® resin and washed extensively, followed by Western blot analysis with anti-A2 Mabs. As shown in Fig. 2B, lanes 5 and 6, the washed His-tag binding resin contained approximately equal amounts of hPTP1B and LmPTP1. These data confirmed that it was possible to express, detect, and partially purify similar levels of hPTP1B and LmPTP1 from transfected Cos7 cells.

We next determined whether we could detect PTP enzyme activity in the washed His-tag bind® resin containing the extracted hPTP1B and LmPTP1 from the transfected Cos7 cells. Two protein phosphatase substrates were used for these assays including 4-*p*-Nitrophenylphosphate (pNPP) and a specific tyrosine phosphatase substrate, insulin receptor (IR) phosphopeptide (Fig. 3A and B). For this assay, the hPTP1B and LmPTP1 expression constructs were transfected into Cos7 cells, purified on His-Tag resin, assayed for activity and further subjected to parallel Western blot analysis to confirm similar levels of hPTP1B and LmPTP in each assay. As shown in Figure 3A and B, both LmPTP1 and hPTP1B enzyme activities were detected on the His-tag bind® resin with the pNPP and IR-phosphopeptide substrates when compared to the control (His-tag binding resin from control pcDNA3-transfected cells).

To confirm that the activity detected in the samples shown was due to PTP activity, we determined whether it was possible to specifically inhibit this activity using the protein phosphatase inhibitor sodium orthovanadate (Na_3VO_4), and a more specific protein tyrosine phosphatase inhibitor potassium bisperoxo(1,10-phenanthroline)oxovanadate (V) [bpV (phen)] (12, 19). As shown in Figure 3C and D, both the LmPTP1 and hPTP1B were inhibited with Na_3VO_4 and bpV (phen) (white bars). Accompanying Western blots confirmed there were similar levels of LmPTP1 and hPTP1B assayed in the presence (+, white bars) and absence (-, black bars) of these inhibitors. Importantly, the inhibitors did not affect the background activity observed on His-tag binding resin from the control vector transfected cells (pcDNA3 control). Taken together, these data confirm the bioinformatic prediction that the LmPTP1 gene encodes for a protein tyrosine phosphatase enzyme which, when assayed under these conditions, had a similar level of activity as hPTP1B.

Once the *Leishmania* PTP1 gene had been identified and characterized as detailed above, it was necessary to determine its role in the parasite's life cycle by developing null PTP1 mutants. We performed single and double knockouts of the catalytic domain of the PTP1 gene from *L. donovani* and characterized the resulting mutant parasite phenotype. *L. donovani* was chosen for this analysis because it can be cultured *in vitro* as both promastigotes and amastigotes whereas *L. major* can only be cultured as promastigotes. Additionally, it has been previously shown that overexpression of a transfected hPTP1B gene in *L. donovani* resulted in increased virulence in the amastigote stage (7).

Since *Leishmania* are diploid organisms, the two alleles of the catalytic domain of the LdPTP1 gene were targeted for deletion as summarized in Figure 4A. The *Bcl* I restriction enzyme fragment containing the LdPTP1 catalytic domain (nucleotides 677-1034, Fig. 1) was replaced with a *Bam* HI – *Bgl* II fragment containing the selectable marker gene conferring hygromycin (Hyg) resistance. The resulting plasmid, termed pBsLdPTP1hyg, was linearized and targeted into the LdPTP1 site of the *L. donovani* genome by transfection, and transformants were selected for hygromycin resistance. The first round of gene targeting (heterozygous deletion) was carried out using 50 μg/μl of hygromycin selection and the second round (homozygous deletion) was carried out using 200 μg/μl of hygromycin. In this manner, both LdPTP1 alleles could be targeted with one selectable marker. Cultures were then subjected to serial dilution to isolate individual LdPTP1 knock-out clones.

Southern blot analyses were performed to confirm the heterozygous and homozygous targeted deletion of the catalytic domain of the LdPTP1 gene in the cloned *L. donovani* mutant cultures. For this analysis, the 357 bp *Bcl* I restriction enzyme fragment, which was deleted by gene targeting (see Fig. 4A), was used as the hybridization probe. As shown in Figure 4B, the *Bcl* I LdPTP1 gene fragment encoding the catalytic domain was eliminated from the *L. donovani* clones as indicated by the absence of the band containing this sequence in the null mutant double knock out (-/-) cultures and further by a 50% reduction in the single knock out clone (+/-), as compared to wild type *L. donovani* (+/+) culture. PCR analysis of the LdPTP1 gene in these mutant clones confirmed the Southern blot data showing deletion of the catalytic domain (data not shown). We further confirmed the accurate

replacement of the Hygromycin resistance gene specifically into the LdPTP1 locus on the same clones by performing Southern blot analysis with a probe specific for the Hygromycin resistance gene. As shown in Figure 4C, the hygromycin resistance gene was only present in the specific site for the LdPTP1 gene. Taken together, these Southern blot analyses demonstrated that the active site region of the LdPTP1 gene was specifically and completely disrupted in the null mutant clones, thereby confirming their suitability for subsequent phenotypic analysis.

Initially, the phenotype of the LdPTP1 null mutants was compared with that of the wildtype *L. donovani* using well established *in vitro* axenic culture protocols for promastigotes and amastigotes. As observed in Figure 5A, the two individual homozygous LdPTP1 null mutant clones (Ld1PTP1-/-, Ld2PTP1-/-), cultured under promastigote conditions (26 ^{0}C, pH 7.2), proliferated at a slightly slower rate compared to wild type *L. donovani* (L.dWT). The heterozygous single knockout clone (LdPTP+/-) proliferated at a similar rate to the null mutant clones. Under amastigote culture conditions (37 ^{0}C, pH 5.5), the homozygous null mutants (Ld1PTP1-/-, Ld2PTP1-/-) also demonstrated a slight reduction in proliferation compared to the single knockout clone (LdPTP+/-) and the wildtype culture (LdWT) (Fig. 5B). With respect to promastigote morphology, there did not appear to be any difference between the null mutant clones and the wildtype cultures as shown in Figure 5C.

The most stringent assay for *L. donovani* virulence is its ability to survive in the visceral organs in a mammalian host. We therefore compared the ability of the LdPTP1 null mutant clones and the parental wildtype *L. donovani* to survive in the

liver 4 weeks following injection in the tail vein of BALB/c mice. As shown in Figure 6, the two LdPTP1 null mutant clones (Ld1PTP1-/-, Ld2PTP1-/-) displayed significantly reduced virulence compared to the wildtype parasites as indicated by both the number of amastigotes per nuclei in liver imprints (Upper panel) and by calculating the Leishman-Donovan Units (LDU) determined by multiplying the level of infection by liver weight (Lower panel). Both LdPTP1 null mutant clones displayed the same phenotype with approximately an 80-90 % reduction in virulence as determined by their ability to survive in the liver. The single knock-out clone LdPTP1+/- showed a clear intermediary reduction in virulence, consistent with reduced expression of LdPTP1. These results argue that the LdPTP1 gene plays a significant role in parasite survival in the mammalian host.

We next determined whether it was possible to restore the virulence of the null mutant clone (Ld1PTP1-/-) by complementation with the wildtype LdPTP1 gene. This was performed by introducing the LdPTP1 gene including its flanking regulatory sequences into a plasmid (Fig. 7A). The LdPTP1 containing plasmid (pSPYneoLdPTP1) was transfected into the null mutant clone (Ld1PTP1-/-) and transformants were selected in G418 and cloned by limiting dilution. For subsequent infections in mice, the wildtype *L. donovani* promastigotes which had been in culture for several months and used to generate the null mutants characterized in Figure 6 were no longer available. Nevertheless, the heterozygous single knockout clone LdPTP1-/+ also represented a relevant control because it had retained one wildtype PTP1 allele (Fig. 4B), was more virulent than the null mutant clones (Fig. 6) and had undergone the same selection procedure as the null mutant clone, Ld1PTP1-/-.

Southern blot analysis with the entire LdPTP1 gene probe confirmed that the pSPYneoLdPTP1 plasmid had been successfully introduced into the null mutant clone (Fig 7B, Lanes 5 and 6).

We compared the virulence of the mutant Ld1PTP1-/- containing the complementary pSPYneoLdPTP1 plasmid to the parental null mutant Ld1PTP1-/- and the heterozygous mutant (LdPTP1+/-), which retained one intact endogenous LdPTP1 allele. In addition, a newly established *L. donovani* culture was used for comparison. As shown in Figure 7C and D, adding back the plasmid-derived LdPTP1 gene to the Ld1PTP1-/- null mutant increased virulence in the liver (Lanes 4) in comparison to the parental Ld1PTP1-/- null mutant (Lanes 3). The Ld1PTP1-/- parasites containing the add-back LdPTP1 was similar in virulence to the heterozygous LdPTP1+/- clone (Fig. 7C and D, Lanes 2) which retained one functional endogenous allele for the LdPTP1 gene. It is also noteworthy that the infection levels shown in Figure 7C, D, for both the heterozygous LdPTP1+/- (Lanes 2) and null mutant Ld1PTP1-/- (Lanes 3) were almost identical to the previous experiment shown in Figure 6. As expected, the newly thawed culture of wildtype *L. donovani* (Fig. 7C, D, Lanes 1) was considerably more virulent than the previous *L. donovani* culture used to generate the original LdPTP1 null mutants and which had been maintained in culture for several months (Fig. 6A and B, Lanes 1).

To further examine the ability of the added back LdPTP1 gene to restore virulence to the null mutant, we compared infection levels in the spleen. As shown in Figure 7 E, adding back the plasmid derived LdPTP1 gene to the Ld1PTP1-/- null mutant resulted in increased spleen parasite levels (Lane 4) compared to the parental

Ld1PTP1-/- null mutant (Lane 3) and was similar in virulence to the heterozygous single allele knockout clone (+/-, Lane 2). Taken together, these infection results in the mouse liver and spleen demonstrate that adding back the LdPTP1 gene on a plasmid partially complemented the virulent phenotype in the null mutant Ld1PTP1-/- clone, thus confirming the importance for LdPTP1 in amastigote survival in the mammalian host.

The preceding observations provide an argument that inhibition of *Leishmania* PTP1 in amastigotes may have therapeutic potential and therefore may represent a drug target. With this in mind, *in silico* homology modeling was performed to compare the three-dimensional structures of the *Leishmania infantum* PTP with the hPTP1B (Fig 8A). This was performed to determine whether there were significant differences in the active sites between these enzymes which could be exploited to develop specific small molecule inhibitors. The structure of the complex between a tyrosine-phosphorylated peptide substrate (sequence etdy(Ptr)rkggkgll) and human PTP (HEPTP, PDB code 1G1G), was used to model the position of the peptide ligand into the *L. infantum* homology via superposition. Enzyme residues within eight angstroms of the ligand were used to perform the superposition. The hydrogen-bonding pattern seen in the substrate peptide-human HEPTP complex between the phosphate group and the backbone of the protein appears to be very well conserved in the *L. infantum* PTP1 homology model, and a high level of structural conservation between the human and *Leishmania* enzymes is concentrated in and around the active site (Figs. 8A and B). Thus, it seems likely that the active sites of the two proteins are similar, although there are significant differences in residues further away from

the active site, including residues 32-51 (Fig. 8A and B). Two differences close to the active site are worth mentioning: while the *L. infantum* enzyme has a proline residue at position 67, the human enzyme has an arginine. Second, residue 205 in the *L. infantum* sequence is a glutamine, while in all human tyrosine phosphatases there is a phenylalanine residue at this position. These differences affect the hydrophobicity of the substrate binding pocket and thus, potentially, the preferred substrate. This altered specificity may provide scope for designing an inhibitor that binds more tightly to the *L.infantum* enzyme than to the human homologues.

DISCUSSION

Among the most important signaling mechanisms in eukaryotic cells are those that involve protein tyrosine phosphorylation and dephosphorylation (4). It was therefore important to identify a prototype PTP related gene in the *Leishmania* genome, determine its role in the parasite life cycle, and its potential as a drug target. We were particularly interested in identifying a *Leishmania* PTP1B-type gene because we had previously observed that overexpression of the human PTP1B increased the virulence of *L. donovani* and appeared to mediate certain aspects of differentiation towards the amastigote stage (7). The present study confirmed that *Leishmania* parasites do have a functional PTP1B-like gene which we termed LPTP1. Deletion of the *L. donovani* PTP1 gene resulted in attenuated amastigotes in BALB/c mice but did not significantly impair promastigote survival in culture. These results argue that *Leishmania* PTP1 plays a significant role in biochemical pathways

associated with amastigote survival in the mammalian host. On the other hand, we did not obtain evidence that *Leishmania* PTP1 was directly involved in differentiation into amastigotes as previously suggested (7) since the LdPTP1-/- knockout mutant promastigotes were capable of morphologically differentiating into and proliferating as amastigotes in culture. In addition, we did not observe any changes in the expression of the amastigote-specific protein A2 in the LdPTP-/- null mutant clones (data not shown).

This study further supports the recent conclusion that *L. major* has PTP enzyme activity (6) and provides insight into the primary and three-dimensional structural features of the *Leishmania* PTP1 gene. Comparison of the primary structure of the human PTP1B and the *Leishmania* PTP1 proteins revealed striking similarities. Firstly, the 10 amino acid active site containing the central cysteine residue is highly conserved with only a two amino acid difference in this region. As revealed in Figures 1 and 8, human PTP1B and the *Leishmania* PTP1 enzymes share additional important motifs, including the invariant Asp residue in the WPD loop and the Gln residue in the Q loop which are also involved in the catalysis of the cysteinyl–phosphate catalytic intermediate (4). The *in silico* three dimensional structural analysis in the presence of a substrate peptide further confirms the close similarities between the *Leishmania* and human PTPs. This is further supported by the observations in Figure 3, which showed that when assayed under identical conditions in the presence of the same substrates and inhibitors, the *Leishmania* PTP1 and human PTP1B had similar levels of activity. Outside of the active site, the proline rich region at the C-terminal section of human PTP1B is involved in its association

with substrates containing SH3-domains (4, 20); this proline enrichment of the C-terminal region was also present in the *Leishmania* PTP1 sequence. Subcellular localization of the human PTP1B enzyme is largely mediated through the highly hydrophobic C-terminal region which anchors this enzyme to the endoplasmic reticulum (21 - 23). Likewise, the last 35 amino acids of *Leishmania* PTP1 are also highly hydrophobic, suggesting a potential association with cell membranes similar to the human PTP1B protein. This is consistent with the recent report that PTP activity is associated with *L. major* membrane fractions (6).

To define the role of the *Leishmania* PTP1 gene in the parasite life cycle stages and virulence, the active site of the enzyme was deleted by gene targeting. It is noteworthy that although LdPTP1 is a single copy gene, it was relatively easy to develop both heterozygous and homozygous PTP1 gene knockouts in promastigotes indicating that this enzyme does not play a major role in the survival of promastigotes in culture. Morphologically, the LdPTP1 mutant promastigotes were similar to wildtype cultures. However, the LdPTP1 mutants were severely attenuated in comparison to the wildtype *L. donovani* with respect to survival in the liver and spleen of BALB/c mice. Future studies will be necessary to identify the endogenous targets (parasite or host) of *Leishmania* PTP1 to define the biochemical pathways involved in mediating parasite survival in the mammalian host. Although there is no secretion leader sequence on the *Leishmania* PTP1 enzyme, its strong homology with human PTP1B makes it tempting to speculate that it might also target host cell substrates. This would be similar to other obligate macrophage pathogens including *Yersinia* spp., *Salmonella typhimurium,* and *Mycobaterium tuberculosis*, which all

secrete PTP enzymes into the host cell that play significant roles in the virulence of these pathogens (reviewed in 24, 25).

This study represents an example of how recent tools including the *Leishmania* genome and *in silico* protein structure analysis can be combined with more traditional molecular biological approaches including gene targeting and virulence analysis in mouse models to define potential drug targets. We have focused on PTPs because of the central role of tyrosine phosphorylation in the biology of higher eukaryotic cells making this among the most intensely studied areas of cell biology and drug development. This study revealed a strikingly high conservation between the *Leishmania* and human PTPs. Because the active sites of these enzymes are highly conserved, it is difficult to conclude that *Leishmania* PTP1 represents a viable target for drug development. Nevertheless, there may be subtle differences in charge within the active site suggesting that screening a library of PTP inhibitors for *Leishmania* PTP1 specific inhibitors may be justified. The combination of the technologies used in this study should however prove beneficial in defining additional drug targets against this important human parasite.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Amino acid sequence alignment of PTPs from *Leishmania* spp., *S. cerevisae,* and human PTP1B. *Dashes* (-) indicate conserved residue among the sequences. *Dots* (.) were included in the sequence comparison for alignment purposes only. *Stars* (*) highlight conserved residues in *Leishmania* and human PTP1B sequences. The *L. major* 13 residues making up the conserved catalytic domain is shown in the box (231-243) with the active cysteine (236) residue in bold and highlighted. The WPD (202-204) and Q (281) loops conserved in *Leishmania* and human PTPs are in bold and highlighted with boxes. Proline residues in the C-terminal region are also in bold and indicated by arrows.

Figure 2. Expression, detection and purification of *L. major* PTP1 and human PTP1B from transfected Cos 7 cells. *Panel A*, relevant regions of the recombinant expression plasmids (pcDNA3-LmPTP1, pcDNA3-hPTP1B) containing the CMV promoter (CMV), a 10 amino acid epitope-tag derived from the A2 protein (A2), a His7-tag (His7) followed by either the *L. major* LmPTP1 or human hPTP1B genes. *Panel B*, Western blot analysis of LmPTP1 and hPTP1B using the anti-A2 Mab following transfection of Cos7 cells with pcDNA3-LmPTP1 (*Lanes 3, 6*) or pcDNA3-hPTP1B (*Lanes 2, 5*) or the control empty plasmid pCDNA3 (*Lanes 1 and 4*) in total cell lysates (*Lanes 1-3*), or following purification on His-bind® resin (*Lanes 4-6*). These are representative data from three independent experiments showing the same results, including performing the Western blot, each time to confirm similar levels of PTP in each assay.

Figure 3. LmPTP enzymatic activity and inhibition following expression in Cos7 cells. LmPTP1 and hPTP1B purified on the His-bind® resin were assayed on PTP substrates; pNPP (*Panel A*) and insulin receptor (IR) phosphopeptide (*Panel B*) as indicated. Also shown is a Western blot confirming equal levels of PTP protein used for each substrate enzyme assay. For the enzyme inhibition assays, LmPTP1 and hPTP1B purified on the His7-tag resin were assayed in the *absence* (-) or *presence* (+) of the PTP inhibitors; Na_3VO_4 (*Panel C*) and bpV(phen) (*Panel D*) as indicated. Also shown is a Western blot confirming equal levels of PTP protein used in the *presence* (+) or the *absence* (-) of the inhibitors in each assay. Note than both inhibitors impaired the enzyme activity to levels similar to that derived from the

control transfected cells (control pcDNA3). Values reported are the average mean of three independent experiments and results in bar graphs are the mean ±SE (standard error). Microsoft Excel was used to calculate the Student's test. *P≤ 0.05 and ** P≤0.01 demonstrates statistical difference from control.

Figure 4. Removal of PTP1 gene active site from *L. donovani* by gene targeting. *Panel A*, Gene targeting strategy. A hygromycin selectable marker gene was inserted into a *Bcl* I site of the pBsLdPTP1 construct containing the LdPTP1 gene, resulting in the construct pBsLdPTP1-Hyg which was then linearized and transfected into *L. donovani* promastigotes. For the single gene targeting (heterozygous LdPTP1 knockout), recombinant parasites were selected in 50 μg/ml hygromycin and for the double gene targeting (homozygous LdPTP1 knockout) recombinant parasites were selected in 200 ug/ml hygromycin. *Panel B*, Southern blot analysis of genomic DNA derived from the wildtype (+/+), the heterozygous (+/-), and homozygous (-/-) gene targeted clones following digestion with *Pst* I or *Sst* I. The probe consisted of the 357 bp fragment (see *Panel A*) derived from the LdPTP1 active site encoding region. *Panel C*, Southern blot analysis as in *B* above with a probe consisting of the Hyg^R gene. Note that these Southern blots confirm the removal of the 357bp fragment containing the active site of the LdPTP1 gene in the homozygous (-/-) gene targeted clones and the Hyg^R gene targeted specifically into the LdPTP1 gene in all clones tested.

Figure 5. Comparison of wildtype *L. donovani* and LdPTP1 gene-targeted mutant clone proliferation and morphology in culture. *Panel A*, Proliferation of promastigote cultures (27°C pH 7.2): wildtype (LdWT), single knockout (LdPTP1+/-) and two different double knockout null mutant (LdPTP1-/-) clones. *Panel B*, Proliferation of amastigote cultures (37°C pH 5.5): wildtype (LdWT), single knockout (LdPTP1+/-), and two different double knockout null mutant (LdPTP1-/-) clones. *Panel C*, Morphology of log phase promastigote cultures (100 X): wildtype (LdWT), double knockout (LdPTP1 -/-). *Leishmania* proliferation curves are representative of two independent experiments showing the same results.

Figure 6. Comparison of the virulence between the single (+/-) and double (-/-) knockout LdPTP1 mutants. Liver parasite burden was determined 4 weeks following infection by quantifying the number of amastigotes/1000 nuclei in liver imprints (*Upper Panel*) and by Leishman-Donovan Units (LDU) (*Lower panel*) calculated by multiplying the number of amastigotes per 1000 cell nuclei x liver weight. Note that two independent clones (Ld1PTP1-/-, Ld2PTP1-/-) were severely attenuated compared to the single knockout clone retaining one wildtype PTP1 allele (LdPTP+/-) or the wildtype *L. donovani* (L.d.WT). Values reported in bar graphs are the mean ±SE of the average mean of four mice per group. Microsoft Excel was used to calculate the Student's test. *$P \leq 0.05$ and ** $P \leq 0.01$ demonstrate statistical difference from the wildtype control.

Figure 7. Complementation of the LdPTP1 null mutant. *Panel A*, Representation of the pSPYNeoLdPTP1 complementing construct which was transfected into the Ld1PTP1 -/- null mutant. *Panel B*, Southern blot analysis of Genomic DNA cut with *Pst* I (*Lanes 1, 3, 5*) and *Sst* I (*Lanes 2, 4, 6*) probed with the full length LdPTP1 sequence. *L. donovani* DNA was derived from; wildtype (*Lanes 1, 2*); Ld1PTP1-/- null mutant (*Lanes 3, 4*); Ld1PTP1-/- null mutant with added back pSPNeoLdPTP1 (*Lanes 5, 6*); *Markers*, M. Parasite levels in the liver 4 weeks after infection as determined by: *Panel C*, number of amastigotes per nuclei in liver imprints or *Panel D*, Leishman donovani Units for; wildtype (*Lane 1*), heterozygous knockout clone retaining one wildtype LdPTP1 allele (*Lane 2*), homozygous knockout null mutant Ld1PTP1-/- (*Lane 3*). Homozygous knockout null mutant Ld1PTP1-/- plus the add-back pSPNeoLdPTP1 (*Lane 4*). *Panel E*, Infection levels in the spleen 6 weeks after infection for: wildtype (*Lane 1*), heterozygous knockout clone retaining one wildtype LdPTP1 allele (*Lane 2*), homozygous knockout null mutant Ld1PTP1-/- (*Lane 3*). Homozygous knockout null mutant, Ld1PTP1-/-, plus the add-back pSPNeoLdPTP1 (*Lane 4*). For figures 7 C and D, results reported in bar graphs are the mean ±SE of the average mean of four mice per group. Microsoft Excel was used to calculate the Student's test. *$P \leq 0.05$ demonstrates statistical difference from the heterozygous knockout clone (+/-) and the homozygous knockout null mutant Ld1PTP1-/- containing the add-back pSPNeoLdPTP1 (-/- + LdPTP1). For figure 7 E, the spleens from the 4 mice in each group were pooled before determining parasite numbers.

Figure 8. Three-dimensional homology model for the structure of *Leishmania infantum* PTP1. *Panel A*, The homology model is shown as a cartoon representation, highlighting alpha-helices, beta-strands and loops. The color scheme used is according to the level of conservation at each residue position between the *L. infantum* sequence and the human PTP1B sequence with red representing identical residues; orange, conserved substitutions; green, semi-conserved substitutions; turquoise, positional conservation; blue, no conservation. Those stretches of sequence outside the active site (32-51 and 319-493) that could not be modeled, and therefore were not included, are represented schematically by ovals, and their size is indicative of the number of residues missing from the model. The oval representing the residues 32-51 that are present in the *Leishmania* sequence but not the human sequence is colored blue, the oval that represents residues 319-493 that are present in both enzyme sequences but were not present in the structure of the human enzyme is colored mauve. *Panel B*, Using the identical orientation as in (A), a molecular surface representation is shown, which is colored using the same scheme as in (A). The active site is shown occupied by the mono-phosphorylated peptide substrate from the human PTP1B-peptide complex (PDB code 1G1G), which is represented by a stick model colored by atom type as follows: white, carbon; blue, nitrogen; red, oxygen; yellow, phosphorous. Also indicated are residues proline 67 and glutamine 205, which may affect substrate preference.

Figure 1

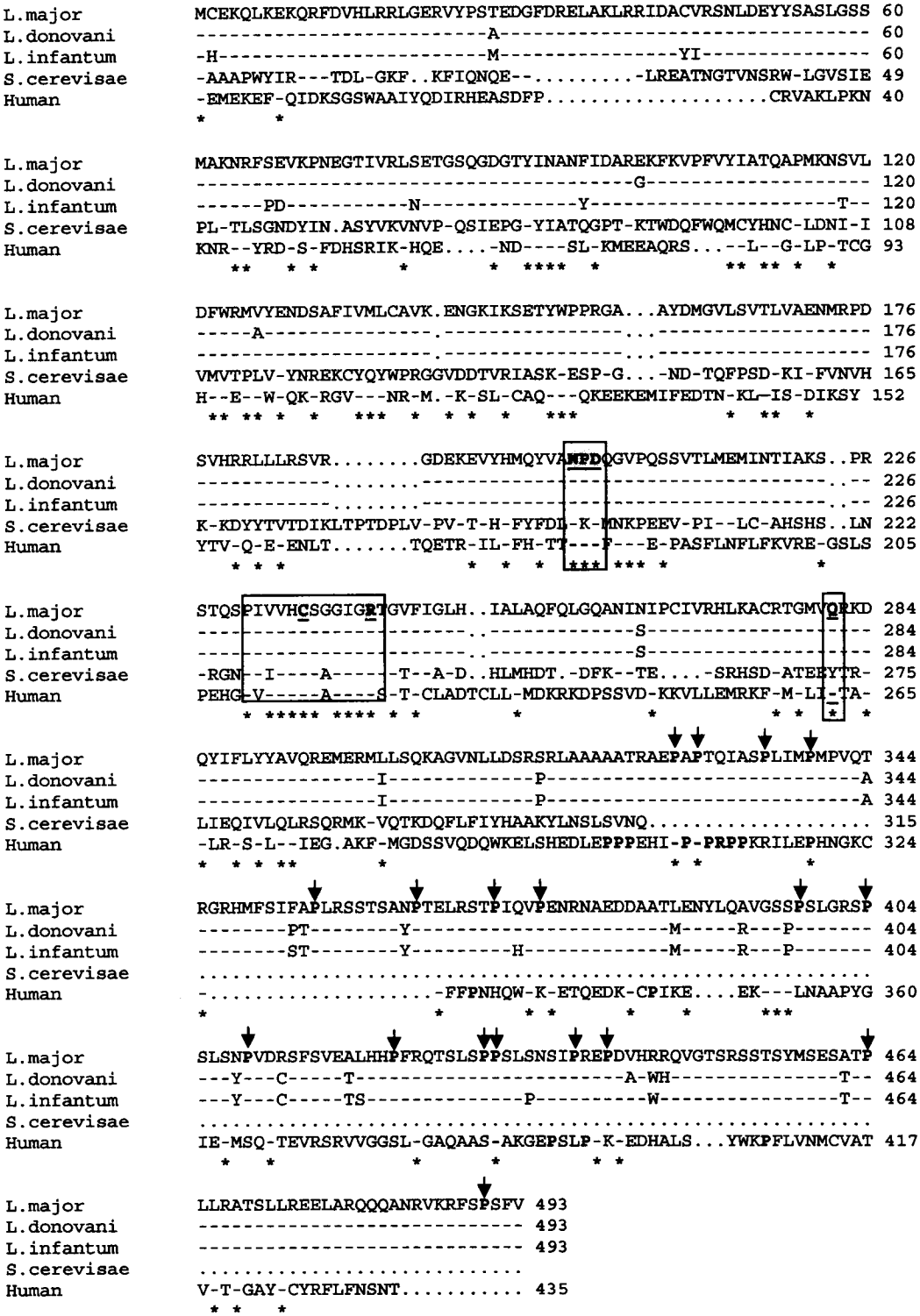


Figure 2

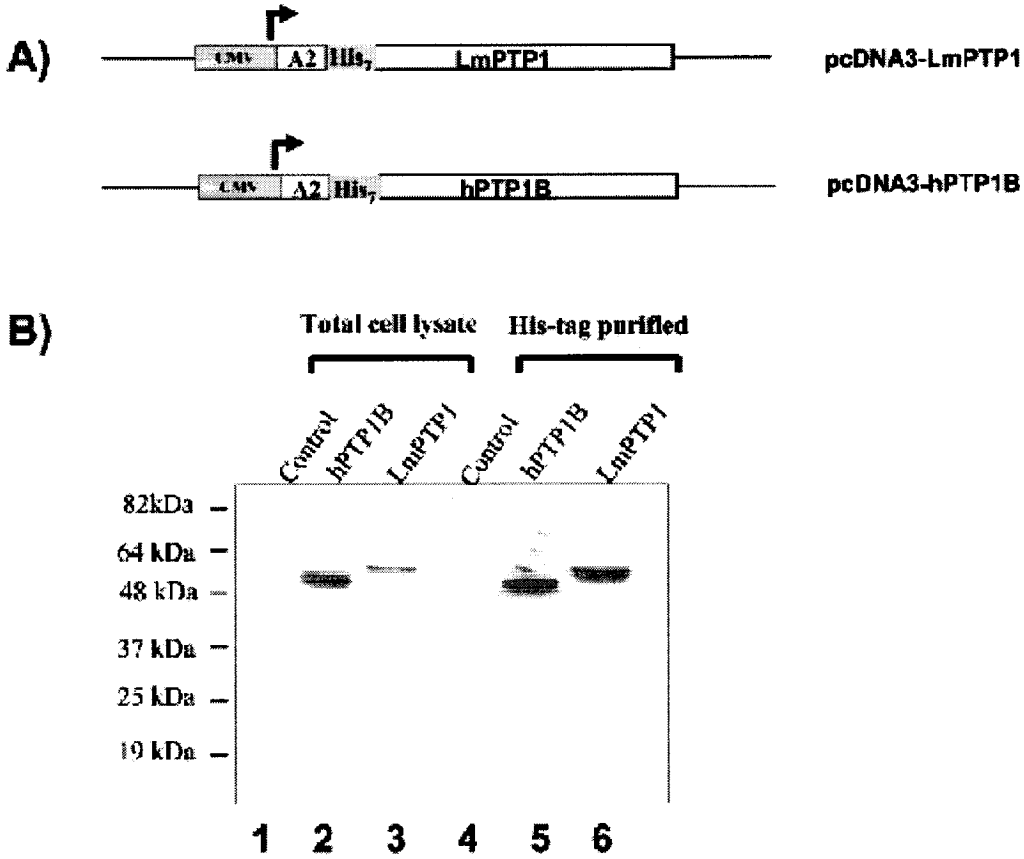

A)
CMV
A2
His6
LmPTP1
pcDNA3-LmPTP1
CMV
A2
His6
hPTP1B
pcDNA3-hPTP1B
B)
Total cell lysate
His-tag purified
Control
hPTP1B
LmPTP1
Control
hPTP1B
LmPTP1
82kDa
64 kDa
48 kDa
37 kDa
25 kDa
19 kDa
1 2 3 4 5 6

Figure 3

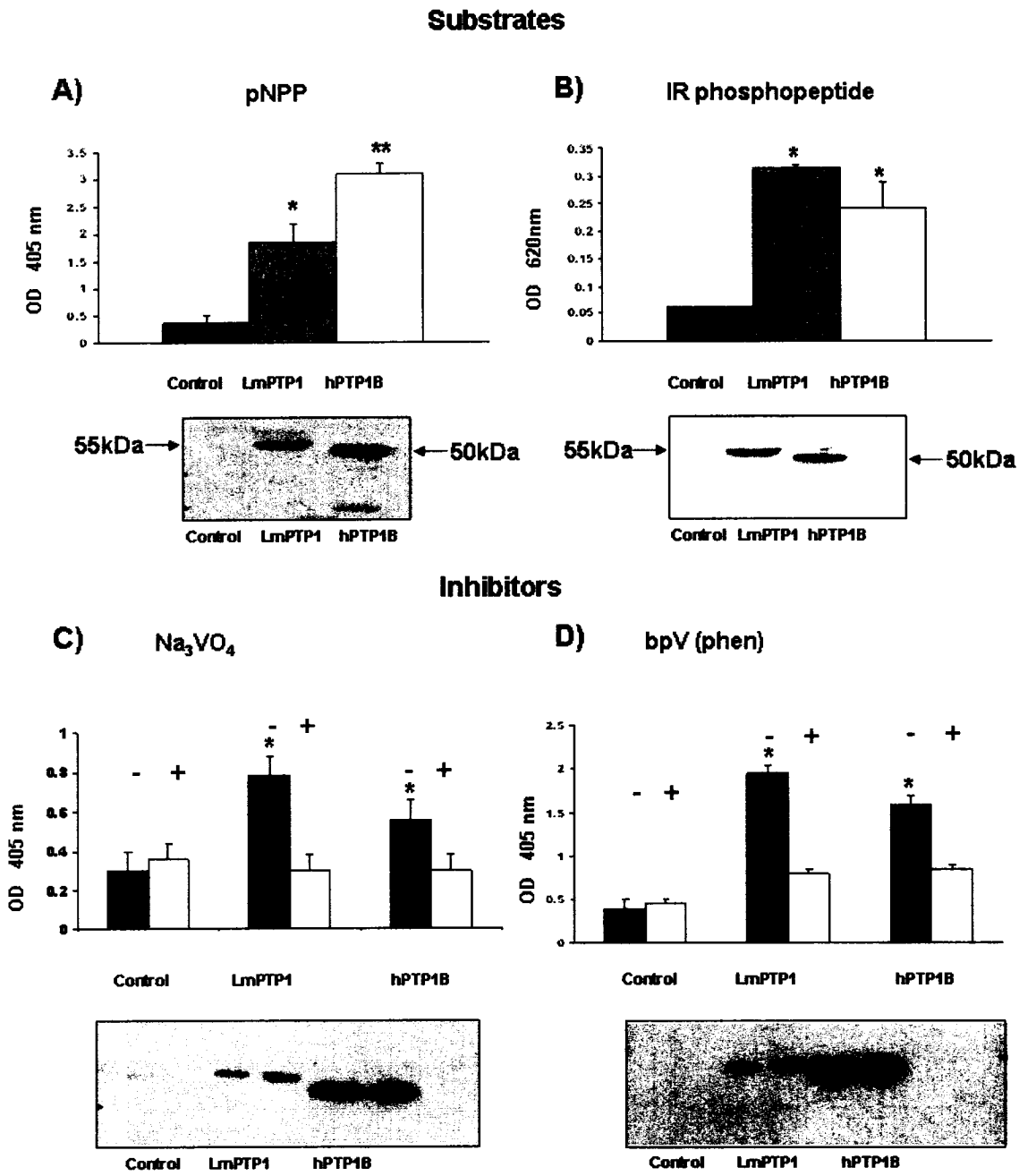

Substrates
A)
pNPP
OD 405 nm
Control
LmPTP1
hPTP1B
55kDa
50kDa
B)
IR phosphopeptide
OD 620nm
Inhibitors
C)
Na_3VO_4
D)
bpV (phen)

Figure 4

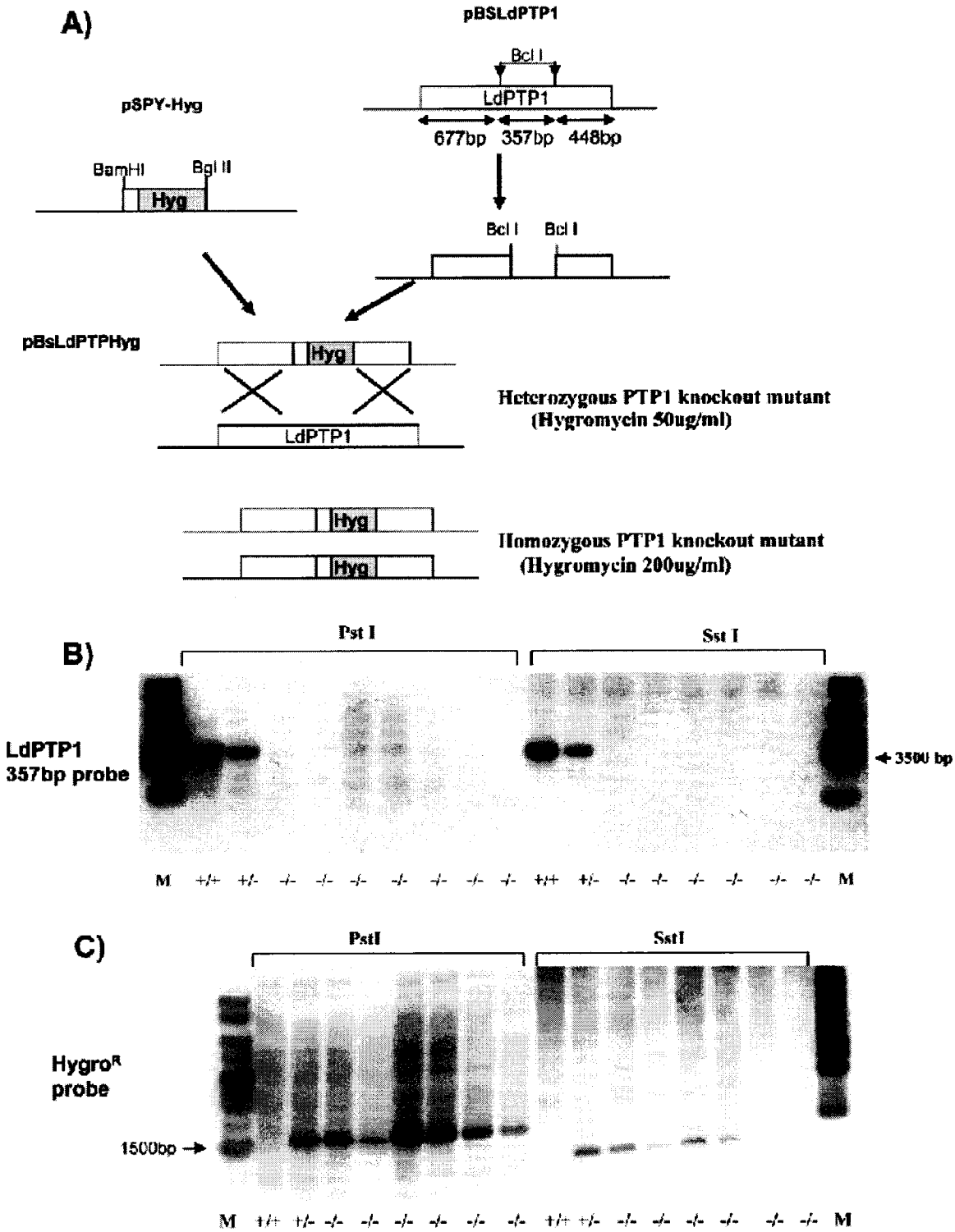
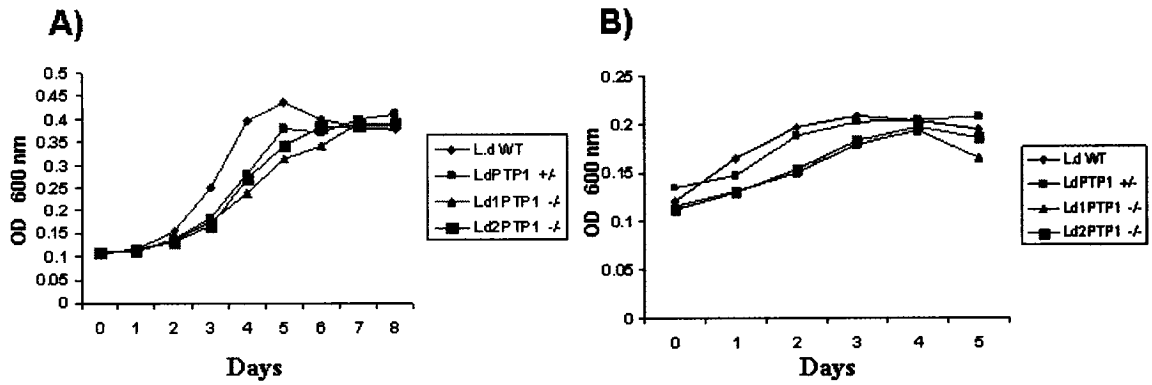
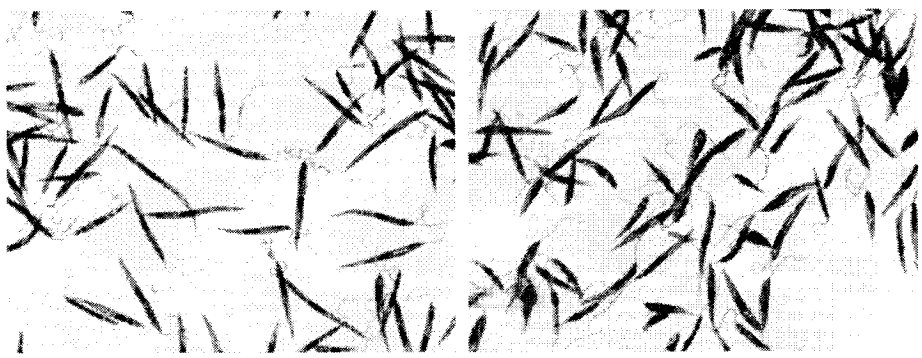

A)
pBSLdPTP1
Bcl I
LdPTP1
677bp 357bp 448bp
pSPY-Hyg
BamHI
Bgl II
Hyg
Bcl I
Bcl I
pBsLdPTPHyg
Hyg
LdPTP1
Heterozygous PTP1 knockout mutant
(Hygromycin 50ug/ml)
Hyg
Hyg
Homozygous PTP1 knockout mutant
(Hygromycin 200ug/ml)
B)
Pst I
Sst I
LdPTP1
357bp probe
3500 bp
M +/+ +/- -/- -/- -/- -/- -/- -/- -/- +/+ +/- -/- -/- -/- -/- -/- -/- M
C)
PstI
SstI
Hygro^R
probe
1500bp
M +/+ +/- -/- -/- -/- -/- -/- -/- +/+ +/- -/- -/- -/- -/- -/- -/- M

Figure 5

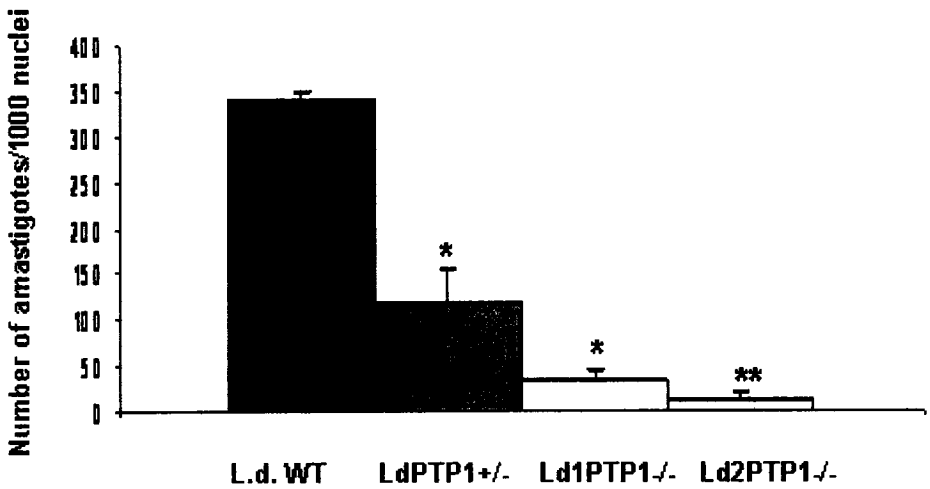


C)



L.d. WT LdPTP1-/-

Figure 6


Number of amastigotes/1000 nuclei
400
350
300
250
200
150
100
50
0
*
*
**
L.d. WT
LdPTP1+/-
Ld1PTP1-/-
Ld2PTP1-/-

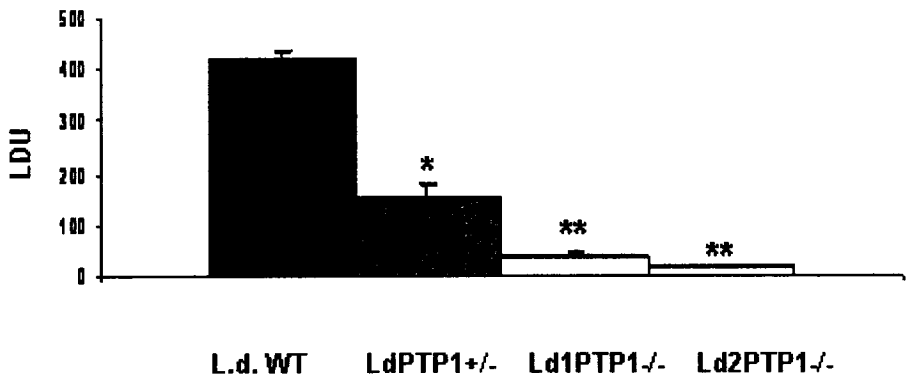

LDU
500
400
300
200
100
0
*
**
**
L.d. WT
LdPTP1+/-
Ld1PTP1-/-
Ld2PTP1-/-

Figure 7

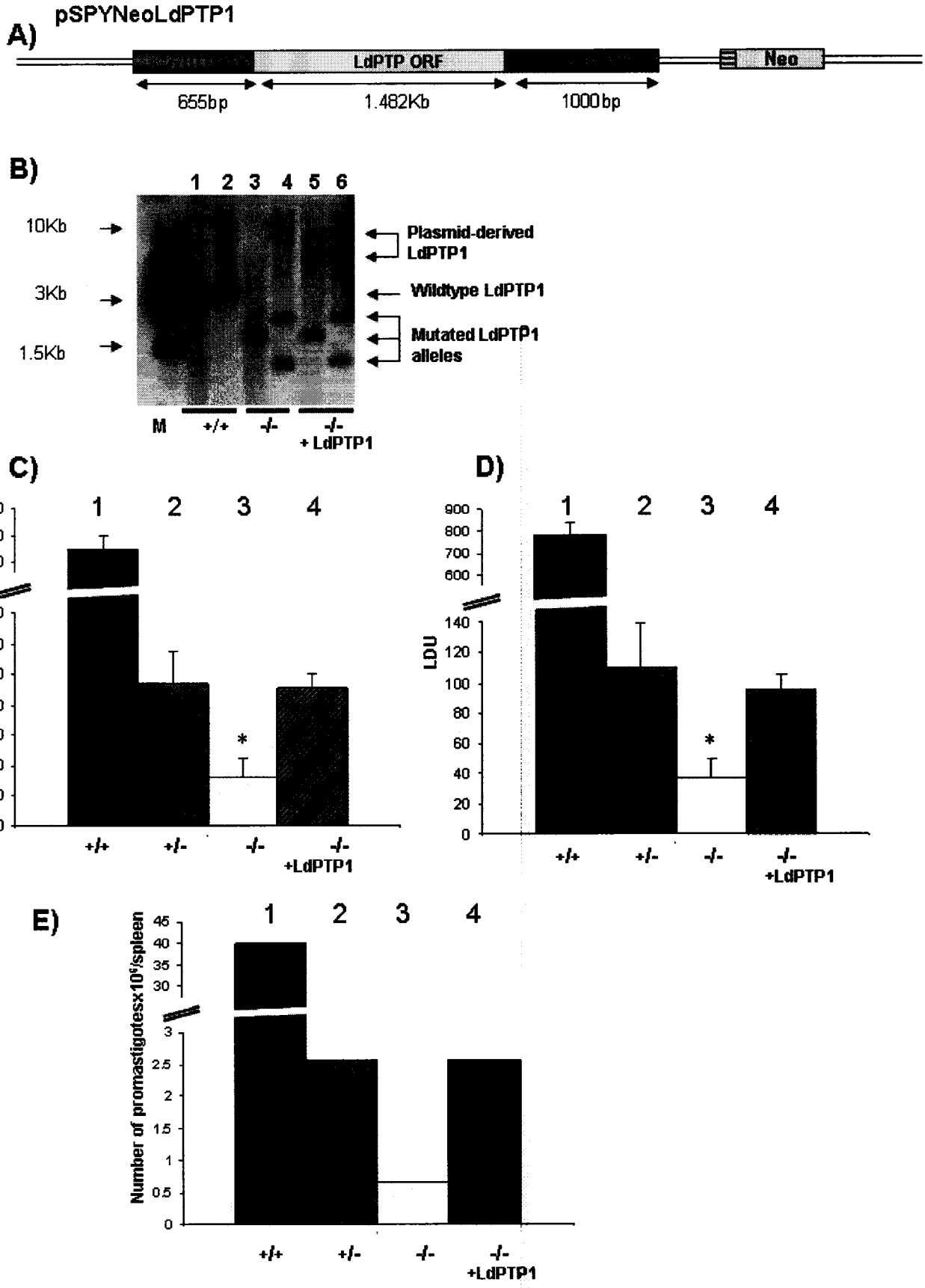
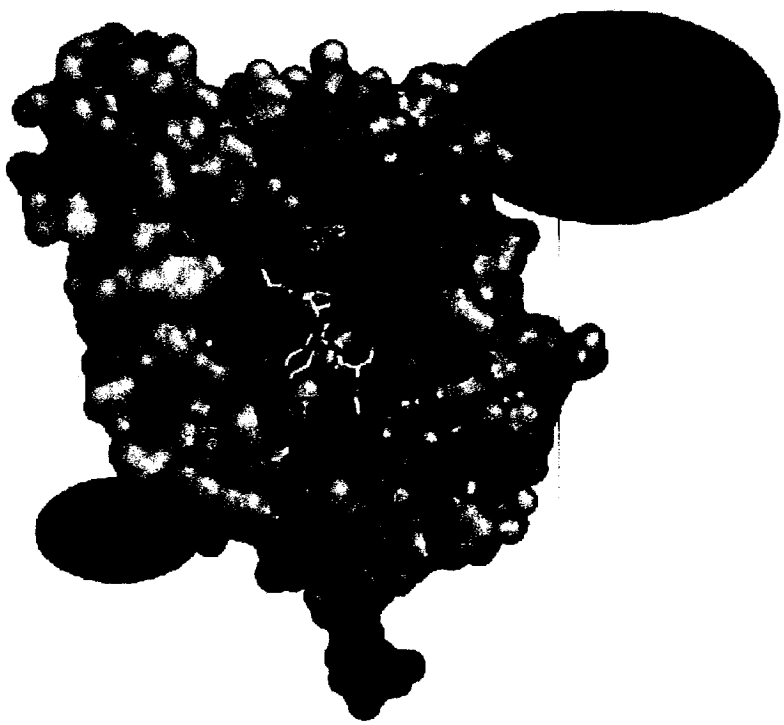

A)
pSPYNeoLdPTP1
LdPTP ORF
Neo
655bp
1.482Kb
1000bp
B)
1 2 3 4 5 6
10Kb
3Kb
1.5Kb
Plasmid-derived LdPTP1
Wildtype LdPTP1
Mutated LdPTP1 alleles
M
+/+
-/-
-/- + LdPTP1
C)
Number of amastigotes/1000 nuclei
1 2 3 4
+/+
+/-
-/-
-/- +LdPTP1
D)
LDU
1 2 3 4
+/+
+/-
-/-
-/- +LdPTP1
E)
Number of promastigotes x10⁶/spleen
1 2 3 4
+/+
+/-
-/-
-/- +LdPTP1

Figure 8

A)

B)



CHAPTER 4: General Discussion

The importance of networks of tyrosine phosphorylation in a number of cellular processes in higher eukaryotes, ranging from differentiation to transformation, is well established in different organisms. As demonstrated in this thesis, tyrosine phosphorylation also plays an important role for *Leishmania* parasites with respect to survival in the host. Enzymes involved in regulating tyrosine phosphorylation have not been studied in detail in these protozoans and the research described in this thesis has begun to address this issue. The identification of tyrosine phosphatases has been reported in *Leishmania* (Dell and Engel, 1994, Cool and Blum, 1993). However, their biological roles in physiological processes are not known and many of the studies on tyrosine phosphorylation are restricted to the demonstration of different patterns of phosphorylation, without identifying the corresponding enzymes involved in the process.

The most important process *Leishmania* parasites undergo during their life cycle is the passage from the invertebrate sandfly to the mammalian host and back to the sandfly, where they encounter a significant change in environmental conditions, especially regarding temperature and pH. Signal transduction is likely to be involved in pathways that control adaptations to such changes, leading to differential expression of proteins that ultimately results in altered metabolism and changes in *Leishmania* biochemistry and morphology. Therefore, protein kinases and phosphatases may act as signal transduction mediators and, as a result, may be implicated in *Leishmania* differentiation, which is an essential process for parasite replication and survival inside the host macrophage (reviewed in Matlashewski, 2001 and Gupta *et al.*, 2001).

In search of the role of tyrosine phosphorylation in the differentiation and virulence levels of *Leishmania* parasites, we initially investigated the effects of overexpression of the human PTP1B in *L. donovani* promastigote cultures (Nascimento *et al.*, 2003). We took this approach initially because the *Leishmania* genome had not been completed and therefore we did not have direct access to the *Leishmania* PTP gene (s). As demonstrated within, our first results argue tyrosine phosphatases may play a role in amastigote differentiation of *L. donovani* parasites, since overexpression of the heterologous human phosphatase PTP1B, known act on a broad range of targets (reviewed in Dube and Tremblay, 2004), induces partial amastigote differentiation of transfected *L. donovani* promastigotes including the induction of A2 gene expression, a marker for amastigote differentiation (Charest and Matlashewski, 1994; Zhang *et al.*, 1996). This was further supported by using a tyrosine kinase inhibitor, which mimics the action of tyrosine phosphatases. Both approaches resulted in a partial differentiation toward the amastigote stage. It was of particular interest that expression of human PTP1B in *Leishmania* also mediated an increase in virulence in mice. This would argue that dephosphorylation of target proteins plays a significant role in virulence as determined by increased infection levels. It will be important in future studies to define these target proteins.

Subsequent studies led to the identification of the *Leishmania* sequence homologue to hPTP1B in the *L. major* (LmPTP1) *and L. infantum* (LiPTP1) genome databases. *In vitro* activity assays confirmed that these sequences code for an active enzyme, a fact confirmed by the ability of the LmPTP1 enzyme to dephosphorylate phosphotyrosine peptides and by its inhibition with tyrosine phosphatase inhibitors.

This study also showed for the first time the utility of using an A2 epitope tag to identify gene products expressed in *L. major* which have lost the ability to express A2 (Ghedin *et al.*, 1997; Zhang *et al.*, 2003). However, disruption of LdPTP1 in *L. donovani* did not indicate any specific involvement of this phosphatase in amastigote differentiation since the LdPTP1 knockouts grew and differentiated into amastigotes *in vitro* similarly to wild-type cells. Tyrosine phosphorylation is a tightly regulated and controlled process within the cell. Higher levels of the ectopic hPTP1B expression in *L. donovani*, an enzyme considered promiscuous with a number of targets, possibly led to the dephosphorylation of target(s), which is/are involved in differentiation, and caused the observed change in phenotype. In 1993, Lammers *et al.* showed transient overexpression of hPTP1B in human kidney fibroblast 293 cells dephosphorylates receptor tyrosine kinase precursors in the endoplasmic reticulum, which is in accord with its cellular localization. Those authors also proposed that PTP specificity is defined by a combination of cellular localization and target recognition. However, high levels of expression may interfere with PTP1B specificity and activity regulation. The PTK inhibitor used in the first study, tyrphostin AG1433, also targets RTK, similarly to hPTP1B. The same observed phenotype in AG1433-treated promastigotes, i.e. partially amastigote differentiated cells, may be due to the fact that both act on similar substrates. An alternative explanation might be that ectopic expression of hPTP1B or treatment with AG1433 caused a non-specific stress on the promastigotes which served as a signal to differentiate toward the amastigote stage in culture. However, the effect on

differentiation was not observed in the control pALTNeo transfected *L. donovani* or with p53 expressing *Leishmania* (Zhang *et al.*, 1995).

The *Leishmania* PTP1 enzyme and its physiological target(s), on the other hand, are involved in the pathogenicity of *Leishmania* related to the ability of this parasite to survive in mice. This was demonstrated by a striking ~85% decrease in LdPTP1 -/- virulence in mice (Nascimento *et al.*, 2006). This was also consistent with the observation that higher ectopic phosphatase activity leads to increased virulence *in vitro* and *in vivo*, (Nascimento *et al.*, 2003). Therefore, it is clear that *Leishmania* PTP1 activity is important for parasite survival in the mammalian host, since its loss may prevent or delay proliferation inside macrophages.

The fact that a tyrosine phosphatase may be associated with *Leishmania* virulence and pathogenicity doesn't come as a surprise, since a number of other intracellular pathogens have their tyrosine phosphatases related to virulence. That is the case for *Yersinia* spp., *Mycobacteria tuberculosis* and *Salmonella* spp. However, *Yersinia*, and other prokaryotic pathogens, translocate phosphatases directly into the host cell cytosol through a contact- dependent type III secretion system (Hu *et al.*, 2004), thus affecting phosphorylation of host cells by causing a direct interference with the host signal transduction pathways. This in turn, leads to impairment of phagocytic functions and disruption of normal cytoskeletal regulation and structure (reviewed in Stebbins and Galan, 2001; DeVinney *et al.*, 2000). In *Leishmania*, molecules that directly interfere with the host are usually located in the external membranes, such as LPG, gp63 and acid phosphatases. The *Leishmania* PTP1 seems to be cytosolic, as indicated by sequence analysis and its homology to the amino acid

sequence of the cytosolic hPTP1B. However, PTP1B is able to anchor in membranes and it suggest that this may also be the localization of the LPTP1. A study by Aguirre-Garcia *et al.*, (2006) detected the presence of a PTP in *L. major* membrane fractions, though no sequence identification was presented. These authors also reported higher PTP expression in metacyclics and suggested a role for this PTP in *Leishmania* adaptation to host defense mechanisms. Further studies will be required to determine the localization of LPTP1 in *Leishmania* cells and elucidate its role and targets. It is, however, tempting to speculate this enzyme may be able to interfere with host signaling given its strong homology with the human enzyme in the active site.

Analysis of the LPTP1 sequence, both at the primary level of amino acid sequence and three-dimensional structure, showed considerable homology to hPTP1B in the active site. Additionally, two loops, namely the WPD and Q loops, involved in the mechanisms of ligand binding and phosphate group catalysis (reviewed in Tonks, 2003), are also present in the *Leishmania* PTP1. This suggests that it may be difficult to identify inhibitors specific for *Leishmania* PTP1. Nevertheless, two amino acid residues are different in the parasite active site sequence. These differences may be exploited as a target for small molecule specific inhibitors, since this may account for differences in the hydrophobicity of the pocket and may provide a unique structural opportunity for achieving selective inhibition. As discussed previously, intense research on PTP1B for treatments against type II diabetes and obesity, has led to an extensive study on structure-based drug design for human PTPs in the last decade (Hu *et al.*, 2004). The screen of inhibitor libraries would be a valuable resource for the

identification of specific LPTP1 inhibitors targeting binding sites present only in the parasite phosphatase enzyme.

Inhibition of LPTP1 could be used as a treatment for leishmaniasis, since, as demonstrated in this study, disruption of this phosphatase activity severely limits *L. donovani* survival in mice. In BALB/c mice, analysis of parasite ability to survive in visceral organs in a long-term infection with wildtype *L. donovani* shows an increase in liver LDU (first 4 weeks) and spleen LDU (first 6-8 weeks), marking the peak of parasitemia, followed by a reduction in parasite burden after 6-8 weeks; at week 10 the level of parasitemia is very low with a small number of parasite detected (our unpublished data). Although the effects of a long-term infection with PTP1-/- *L. donovani* are presently unknown, it is possible that the host could eliminate parasites deficient in phosphatase activity and control infection. The use of a specific phosphatase inhibitor could also affect *Leishmania* ability to survive intracellularly or abolish its interference with host signaling, potentially causing the immune response to be more effective against this pathogen. However, at this stage, we cannot say with certainty whether the inhibitor effects would modulate parasite survival or macrophage functions, since we have no further knowledge of LPTP1 target(s). Reports on the use of tyrosine phosphatase inhibitors, such as bpV(phen) in mice (Olivier *et al.*, 1998), or the pentavalent drug sodium stibogluconate (Pathak and Yi, 2001) against leishmaniasis strongly argue that *Leishmania* tyrosine phosphatases are involved in the parasite pathogenicity and that inhibiting this pathway could influence the course of *Leishmania* infections. The priority for future studies will therefore be to define the target proteins for LPTP1.

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APPENDIX A: Research Compliance Certificates