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Immunocytochemistry of Proteases in the Study of *Leishmania* Physiology and Host-Parasite Interaction

Raquel Elisa da Silva-López
Department of Natural Products, Farmaguinhos
Oswaldo Cruz Foundation, Rio de Janeiro,
Brazil

1. Introduction

Leishmaniasis is a chronic disease caused by parasites from *Leishmania* genus and still represents a severe public health problem in the world and the incidence is increasing (Desjeux, 2004). There is no effective vaccine for prevention of any form of leishmaniasis and programs of prevention and drug therapy are the main mechanisms for disease control. On the other hand, current chemotherapy is the only way to treat cases of leishmaniasis. Since the 1940s, the pentavalent antimony compounds (e.g., Glucantime, Pentostam, or branded pentavalent formulations) have been the mainstays of antileishmanial therapy (Aït-Oudhia et al., 2011). Although these drugs are usually effective, they produce serious side effects, present difficulties of administration and high cost, the parasite persists in the scars of clinically cured patients (Schubach et al., 1998), and drug resistance has been observed (Castillo et al., 2010). Second-line drugs are used in areas with high rates of unresponsiveness to antimonial treatment or when it was not possible to administrate it. However, these drugs are even more toxic than antimony compounds and expensive, and these include pentamidine, amphotericin B, anti-fungal, allopurinol, and more recently, miltefosine, paramomicine and sitamaquine. Furthermore, they have low therapeutic index when compared to antimonial compounds (Almeida and Santos, 2011). Instead of determining treatment based on rational therapeutic indications, treatment of choice is frequently dictated by economic considerations and in a large majority of countries, chemotherapeutic approaches for all forms of leishmaniasis rely on the use of pentavalent antimonial compounds (Aït-Oudhia et al., 2011). The mechanism of pentavalent antimony compounds action is the inhibition of glycolytic pathway and β-oxidation enzymes of the parasites (Baiocco et al., 2009), but being a heavy metal it is non-selective and it is believed to interfere with other metabolic pathways of parasites and hosts. Furthermore, these drugs can interact with the zinc finger domain of proteins, and many proteins have this motif in their tridimensional structures (Demicheli et al., 2008).

Attempts to develop vaccines against *Leishmania* and drugs to treat cases of leishmaniasis are a continuing effort in search for novel parasite antigens. Various candidate molecules have also been tested and some degrees of protection against different species of *Leishmania* infection were observed (Chawla and Madhubala, 2010). In order to develop a rational drug

for leishmaniasis chemotherapy, the biochemistry of *Leishmania* parasites needs to be better understood for the identification of these strategic targets. Immunocytochemistry strategies have localized these targets in *Leishmania*, providing valuable information about the roles of these molecules in the parasite life cycle and in the pathogenesis of leishmaniasis. So, the purpose of this chapter is to focus on the employment of immunolabeling and electron microscopy in order to localize proteases which are critical for the survival of the *Leishmania* parasites. Furthermore, this chapter will cover aspects of leishmaniasis epidemiology, *Leishmania* morphology, potential drug targets in *Leishmania* and proteases as targets in *Leishmania*, highlighting the function and subcellular localization of cysteine proteases, gp 63 metalloprotease and serine proteases.

2. Leishmaniasis and *Leishmania*

Leishmaniasis is one of the most significant neglected diseases and occurs in the tropical and subtropical regions of America, Asia, Africa and Europe. This disease is considered to be endemic in 88 countries, 72 of which are developing countries (Kaye and Scott, 2011). About 350 million people are at risk of *Leishmania* infection and as many as 12 million people in the world are believed to be currently infected. Approximately 1–2 million estimated new cases every year with the annual mortality rate of about 60,000 (Okwor and Uzonna, 2009). Leishmaniasis is a disease associated with the poverty, environmental changes, such as deforestation, building of dams, urbanization, and the accompanying migration of non-immune people to endemic areas. However, due to underreporting - notification of leishmaniasis is compulsory in only 32 of the 88 affected countries - and misdiagnosis, actual case numbers are expected to be higher. Furthermore, most affected people are hidden because the social stigma associated with deformities and disfigurement scars and due to they live in remote areas. Leishmaniasis-related disabilities impose a great social burden, and reduce economic productivity (WHO, accessed in August 15th, 2011). Over the past 20 years, leishmaniases have increasingly been recognized as an opportunistic infection in HIV-infected patients, with *Leishmania*–HIV co-infection common in areas where both diseases are endemic. The highest prevalence of co-infection cases occurs mostly in Spain and southwestern Europe, among injectable drug users. The presence of both pathogens concomitantly in the same host cell (macrophage) influences the expression and multiplication of both pathogens. HIV-1 infection increases the risk of developing visceral leishmaniasis by 100 to 2,300 times in endemic areas, reduces the likelihood of a therapeutic response and greatly increases the probability of relapse. Moreover, *Leishmania* promotes an increment in viral load and a more rapid progression to AIDS, which reduces life expectancy of infected patients (Ezra et al., 2010).

Clinical manifestations of leishmaniasis range from self-healing cutaneous, mucocutaneous skin ulcers and a long-lasting diffuse cutaneous in cellular-mediated immune response deficient hosts to a lethal visceral form (i.e., visceral leishmaniasis or kala-azar) and post-kala-azar dermal leishmaniasis. The clinical spectrum of this disease is associated with the species of *Leishmania* involved (Desjeux, 2004). Today, about 30 species of protozoan of the *Leishmania* genus (Order Kinetoplastida and Family Tripanosomatidae) are known and approximately 20 are pathogenic for humans and are the causative agents of the "Old" and "New Worlds" leishmaniasis. All members of the genus *Leishmania* Ross, 1903 are parasites of mammals. The two subgenera, *Leishmania* and *Viannia*, are separated on the basis of their location in the vector's intestine and isoenzyme analysis was used to define species

complexes within the subgenera. These species generally present different epidemiological and clinical characteristics related to different genetic and phenotypic profiles. All species of the subgenus *Viannia* were isolated in the 'New World', while those of the subgenus *Leishmania* were isolated from the "Old World", except for species of the *L. (L.) mexicana* complex, *L. (L.) hertigi*, *L. (L.) deanei*—which are found in the 'New World' only—and *L. (L.) infantum/chagasi* and *L. (L.) major*, which are found in both the "New" and "Old Worlds" (Bañuls et al., 2007).

These parasites can be transmitted by female sandflies via anthroponotic or zoonotic cycles, transplacental, blood transfusion and through contaminated needles by injecting drug users. Vector transmission is the commonest way of parasite dissemination (Molina et al., 2003). *Leishmania* parasites have a dimorphic life-cycle: The flagellated, motile forms of *Leishmania* spp. are called promastigotes (Figure 1 A). They are found into the very alkaline digestive tract of the sandfly and progress through various morphologically distinct stages of differentiation to ultimately become the non-dividing, infectious 'metacyclic' promastigotes that are transmitted during a sandfly bite. These 'metacyclic' promastigotes are phagocyted by professional phagocytes such as macrophages and, inside these cells parasites survive and multiply as amastigotes (Figure 1B), a smaller form of *Leishmania* with non-exteriorized flagellum and very metabolic active (Seifert, 2011).

All members of the genus *Leishmania* are obligated intracellular parasites of several mammalian cells and survive under very acid, oxidant and hostile conditions into parasitophorous vacuoles environment, and they have evolved several mechanisms to avoid their degradation (Mougneu et al., 2011). These mechanisms include specific organelles and molecules, such as proteases, that are secreted or are intracellular expressed (Silva-López et al., 2005; Yao, 2003). Special organelles found in trypanosomatid *Leishmania* include mitochondrion and kinetoplast (De Souza et al., 2009a), megasomes (De Souza et al. 2009b) and glycosomes (Michels et al., 2006). Some of these organelles and the evolutive forms of *Leishmania* are schematically represented in the figure 1.

3. Potential drug targets in *Leishmania*

One of the features in the process of drug development is target identification in a biological pathway. In theory, during this identification in a pathogen, it is important that the putative target should be either absent in the host or substantially different from the host homolog so that it can be exploited as a drug target. Trypanosomatids, phylogenetically, branch out quite early from the higher eukaryotes. In fact, their cell organization is significantly different from the mammalian cells and thus, it is possible to find targets that are unique to these pathogens. Secondly, the target selected should be absolutely necessary for the survival of the pathogen. It is also important to consider the stage of the life-cycle of the pathogen in which the target protein is expressed. So, the most important targets are enzymes, since they regulate a specific biochemical pathway and their active sites can bind specific inhibitors that can be designed or found in the nature. A good enzyme target means that its inhibition should lead to loss in cell viability. Furthermore, it is important that the target selected should be assayable (Shukla et al., 2010). Many enzymes have been investigated in their capacity to control or regulate essential *Leishmania* biochemical pathways or some mechanisms that guarantee the parasite survival and proliferation for infection maintenance, such as the enzymes that regulate or participate in sterol biosynthesis,

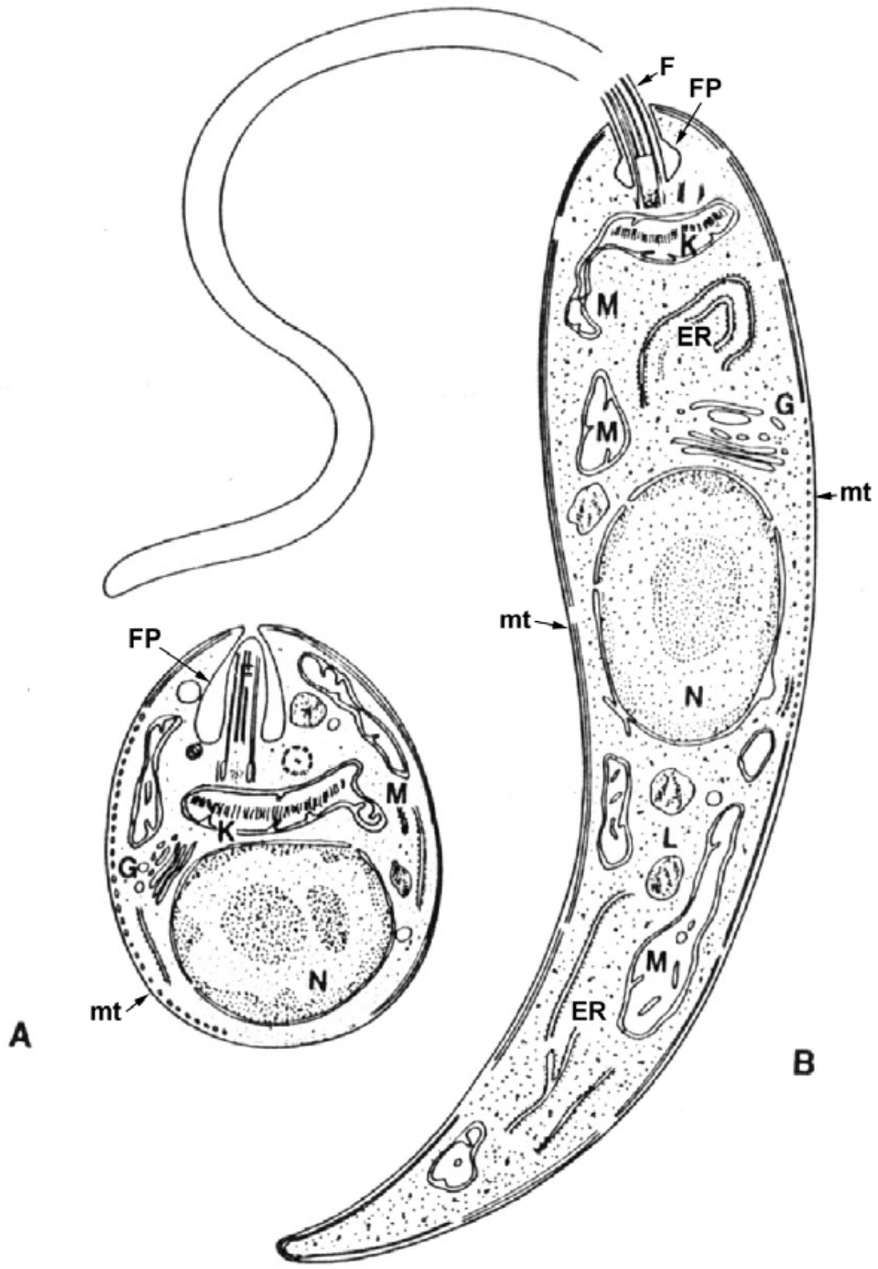

Fig. 1. Schematic representation of *Leishmania* sp forms and their organelles. A) Amastigotes. B) Promastigotes. F, flagellum; K, kinetoplast; N, nucleus; FP, flagellar pocket; ER, endoplasmic reticulum; M, mitochondrion; mt, microtubules; G, Golgi; L, lysosome (adapted by the author from Rey, 1991).

hypusine biosynthesis, glycolysis, purine salvage, glycosylphosphatidylinositol biosynthesis, folate biosynthesis and glyoxalase and trypanothione system or special enzymes such as protein kinases, topoisomerases and proteases (Chandra et al., 2010; Chawla and Madhubala, 2010).

Before discussing about the proteases, which is the purpose of this chapter, it is worth to mention about some enzymatic systems that are considered good targets in *Leishmania* (Table 1). Sterols are important components of the cell membrane and are essential to the cellular function. The sterol biosynthesis of trypanosomatids is very different from humans, because these parasites synthesize ergosterol and 24-methyl sterols, instead of cholesterol. The extensively studied squalene synthase and D24,25-sterol methyltransferase enzymes, only present in trypanosomatids, perform crucial roles in regulation of sterol metabolism and their specific inhibitors have showed anti-proliferative and growth inhibition effects of *Leishmania in vitro* (Goto et al., 2009).

Leishmania compartmentalize several important metabolic systems in special peroxisomes which are designated glycosomes. These organelles contain seven of the ten enzymes of glycolytic pathway, the pentose-phosphate pathway, β-oxidation of fatty acids, purine salvage, biosynthetic pathways for pyrimidines, ether-lipids and squalenes (Moyersoen et al., 2004). The glycolytic enzymes of *Leishmania* exhibited large phylogenetic distance with the mammalian hosts and, for this reasons, specific inhibitors have been designed for the most important regulator enzyme, the glyceraldehyde-3-phosphate dehydrogenase. These compounds inhibited growth of *L. (L.) mexicana* by blocking the energy production, since glycolysis is the most important source of energy for these parasites (Saunders et al., 2010). Furthermore, the biogenesis of these organelles occurs via peroxins self-interactions and the reduction of peroxin expression or their self-interaction inhibition induced the *Leishmania* death (Michels et al., 2006).

Protozoans of *Leishmania* genus lack the metabolic machinery to the synthesis of purine nucleotides and the parasites have to depend upon the purine salvage system to utilize purine from their hosts. Three phosphoribosyltransferases were identified in *Leishmania*, and the hypoxanthine-guanine phosphoribosyl transferase is the most important enzyme involved in purine salvage (Carter et al., 2008). Various inhibitors have been designed to target this enzyme due to its difference in substrate specificity with the host enzyme. Allopurinol is the most common inhibitor that is phosphorylated by the enzyme and incorporated into nucleic acid, leading to selective death of the parasite (Loiseau and Bories, 2006). Allopurinol has been shown to be effective against cutaneous and visceral leishmaniasis, but when used with other anti-leishmanial drugs was found to be even more effective (Castillo et al., 2011; Almeida and Santos, 2011). Besides, purines are transported through the parasite cell surface by nucleoside transporters and these transporters also uptake toxic nucleoside analogs which inhibits the parasite growth (Al-Salabi and Koning, 2005). So, these transporters represent an alternative strategy for interfering in *Leishmania* purine metabolism and develop novel drugs to leishmaniasis treatment. Some of these synthetic and natural products that inhibited specifically crucial steps in a metabolic pathway of *Leishmania* inducing the parasite death and reducing leishmaniatic lesion progression in susceptible animal models have been tested in controlled clinical trials. These compounds have showed different degrees of efficacy, therapeutic index and in general caused less adverse effects than that observed in patients treated with antimonials or with the second-line drugs that is currently being used for leishmaniasis treatment (Fernandes Rodrigues et al., 2008; Valdivieso et al., 2010; Almeida and Santos, 2011; Pereira et al., 2011).

Target enzymes	**Methods of location**	**Subcellular location**	**Biological functions**
Squalene synthase and *D24,25-sterol methyl-transferase.*	Subcellular fractionation	Membrane, glycosomes and mitochondrial/ microsomal vesicles.	Sterol biosynthesis, which are components of the cell membrane and *Leishmania* signaling (Goto et al., 2009).
Deoxyhypusine synthase and *deoxyhypusine hydroxylase*	not applied	not determined	Hypusine biosynthesis that are involved in *Leishmania* proliferation, differentiation, and biosynthesis of macromolecules (Chawla et al., 2010).
Glyceraldehyde-3-phosphate dehydrogenase	Subcellular fractionation	Glycosomes	Regulates the glycolysis that is the most important metabolic pathway in *Leishmania* ATP supply (Saunders et al., 2010).
Hypoxanthine-guanine phosphoribosyl transferase	Subcellular fractionation	Glycosomes	Purine salvage for nucleotides and nucleosides (Carter et al., 2008).
Glycosylphosphatidylinositol biosynthetic enzymes	Subcellular fractionation immunofluorescence	Tubular subdomain of the endoplasmic reticulum	Glycosylphosphatidyl-inositol acts as a membrane anchor for many cell-surface proteins of eukaryotes (Ilgoutz et al., 1999).
methylene-tetrahydrofolate dehydrogenase/cyclohydrolase and formyl-tetrahydrofolate ligase.	Subcellular fractionation western blotting immunofluorescence	Cytosol	Formyl-tetrahydrofolate biosynthesis. Folates are used in purine biosynthesis and mitochondrial initiator methionyl-$tRNA^{Met}$formylation (Vickers et al., 2009)
Glyoxalase system	immunofluorescence	kinetoplast	catalyzes the formation of d-lactate from methylglyoxal, a toxic product of glycolysis, using trypanothione as substrate (Chauhan and Madhubala, 2009)

Target enzymes	Methods of location	Subcellular location	Biological functions
Trypanothione system	Subcellular fractionation immunofluorescence	mitochondria and cytosol	H_2O_2-detoxification (Krauth-Siegel et al., 2007)
Protein kinases	immunofluorescence immunocytochemistry	cytosol	Enzymatic activity regulation by addition of phosphate (Baqui et al., 2000).
Topoisomerases	immunofluorescence	Kinetoplast mitochondria	Topoisomerases I catalyze the cleavage of one strand of DNA, whereas topoisomerases II catalyze the cleavage of a double-stranded DNA, requiring ATP as cofactor (Banerjee et al, 2011)
Proteases	Subcellular fractionation immunofluorescence immunocytochemistry	Membrane, flagellar pocket, megasomes and endocytic/ exocytic vesicles	Hydrolysis of peptide bonds in proteins and peptides. They are crucial in *Leishmania* life cycle, in host-parasite relationship, and in leishmaniasis pathogenesis (Silva-López et al., 2010a, b).

Table 1. Main protein targets in *Leishmania* sp.

4. Proteases as targets in *Leishmania*

Proteases, also known as peptidases, are hydrolytic enzymes that cleave peptide bounds in proteins and peptides, releasing peptides with variable sizes and free amino acids. Unlike most enzymes, proteases lack specificity toward a substrate, i.e., a specific protein. Instead, they are very specific for a peptide containing the scissile peptide bond and the amino acids involved in the neighborhood of the peptide bonds instead of the whole molecule (Garcia-Carreno and Del Toro, 1997). They are ubiquitously found in all living beings from unicellular to higher organisms and are the most studied class of enzyme (Shinoda and Miyoshi, 2011). They participate in various physiological processes through the modification of proteins, such as digestion of food proteins, tissue remodeling, neuropeptides, hormones, and proenzyme processing, cellular metabolism by proteasomes, blood clotting, complement activation cascade reactions, metabolism regulation and a vast number of other biological phenomena as well as they are encoded by 2% of all genes in an organism (López-Otín and Bond, 2008). These enzymes are grossly classified as: exopeptidases which act on the ends of protein substrates and are designated as amino-or carboxypeptidases, and

endopeptidases acting on the interior of protein substrates. Endopeptidases classification rests on the type of residue at the active site: the hydroxyl group of serine proteases (EC 3.4.21) and the sulfidryl group of cysteine proteases (EC 3.4.22) are the nucleophile during catalysis, while activated water is the nucleophile for aspartic (EC 3.4.23), and metalloproteases (EC. 3.4.24) (Barret, 1994). Proteases are further classified according to their structure in families and Clans. A family is a set of homologous proteolytic enzymes that show significant similarity in amino acid sequence, and homologous families that have arisen from a single evolutionary origin of peptidases are grouped together in a Clan (Rawlings et al., 2010). The different types of proteases have particular characteristics that allow their proteolysis to function in a huge diversity of environments. They are very well adapted to surrounding conditions and can act in acid or basic pH, hypo and hyper osmolarity, higher or lower temperatures, and for these reasons they can be found in all cellular compartments and in all organs of the higher organisms.

Besides their physiological necessity, proteases are potentially hazardous to their proteinaceous content and their activity must be precisely controlled by the respective cell or organism. However if this activity is unregulated it can destroy cells, tissues and organs and can kill organisms. So, the control has to be very efficient and is normally achieved by regulated expression/secretion, zymogen production, enzyme activation, degradation of the mature enzymes and most important by protease inhibitors, that can react with the active site or other domain of the enzyme, impeding its capacity to bond and hydrolyze the substrate. In general, there are two types of inhibitors: (a) small non-proteinaceous compounds, secreted by microorganisms that irreversibly modify the amino acid residue of the enzyme active site, and (b) the huge number of natural inhibitors, which are pseudo substrates isolated from various cells, tissues and organisms often that accumulate in high quantities in plant seeds, and various body fluids. Inhibitors of different types occur commonly among living organisms and viruses, which stresses their ultimate role in physiological processes (Otlewski et al., 1999; Krowarsch et al., 2003; Silva-López, 2009). These inhibitors are valuable tools for investigation of the biochemical properties and the biological functions of proteases, besides they are employed in the treatment of many diseases and are under investigation as chemotheraphic in the treatment of leishmaniasis (Silva-López et al., 2007; Valdivieso et al., 2010; Olivier and Hassani, 2010; Pereira et al., 2011).

Many studies have focused their attention on the crucial roles of proteases in the *Leishmania* life cycle, in the host-parasite relationship, and in the pathogenesis of leishmaniasis. These enzymes are important virulence factors and they have been implicated in a wide variety of adaptation mechanisms for in-host parasite survival, which include modulation of the host immune system, invasion and destruction of host connective tissues, enabling parasites to migrate to specific sites for growth and development and/or acquire essential nutrients that guarantee survival and proliferation for infection maintenance (Mottran et al., 2004; McKerrow et al., 2006; Matos Guedes et al., 2010; Gómez and Olivier, 2010; Silva-López, 2010 b; Yao, 2010; Swenerton et al, 2010; Swenerton et al, 2011) and their importance has been confirmed through findings that specific protease inhibitors kills *Leishmania* parasites (Silva-López et al., 2007).

It is important to point out that the subcellular location of *Leishmania* proteases provides knowledge about the function of these enzymes in the parasite physiology, and consequently if they are potential targets to develop new chemotherapy for leishmaniasis

treatment. There are some strategies to determine the proteases localization and immunocytochemistry techniques are the most capable for specifically detecting and localizing macromolecules within thin sections of any cell type, preserving the structure of most antigenic sites and retaining the antigenicity (Herman, 1988). Using these strategies, many proteases were successfully localized in both forms of *Leishmania* and their importance in the parasite physiology and infection was elucidated. Cysteine proteases, gp63 metalloprotease and, more recently serine proteases are the most investigated proteolytic enzymes of the genus *Leishmania* and some considerations about their functions and subcellular localization using immunocytochemistry studies will be focused in this chapter.

4.1 Cysteine proteases

North and Coombs in 1981 reported for the first time the proteolytic activity in both promastigote and amastigote forms of *Leishmania*. They demonstrated that the highest activity was found in amastigotes and belonged to the cysteine protease class (North and Coombs, 1981), and since then these enzymes were extensively studied. So, at this time proteases were also considered virulence factors in *Leishmania*, because as in other parasites these enzymes have a recognized role in the mechanisms of invasion, survival and migration in host tissues (Kozar, 1961). The immunocytochemistry constitute an universal assay capable of detecting and localizing macromolecules in any cell type using specific unlabeled primary antibody directed at the antigen of interest and then indirectly localizing the primary antibody with a second label consisting of antibodies conjugated to an electron-dense material (Lunedo et al., 2011) and considerations about this valuable technique will be done at the end of this chapter. These cysteine proteases were localized for the first time in amastigotes of *L. (L.) mexicana*, a member of *mexicana* complex, by the post-embedding immunocytochemistry assays using as the primary antibody IgG fraction purified from a rabbit antiserum against *L. (L.) mexicana* amastigote cysteine protease and the anti-rabbit IgG immunospecificity complexed with gold colloidal (5-10 nm diameter) as the secondary antibody. The acid ester hydrolase arylsulfatase (EC 3.1.6.1) was also employed as a marker of lysosomes using 4-nitrocatechol sulphate as substrate and barium chloride as capture agent, forming electron dense barium deposits at reaction sites (the enzyme location), that is visualized by electron microscopy. These enzymes, cysteine protease and arylsulfatase, were found into larges organelles that contain putative lysosomal enzymes that was previously named "megasomes" (Pupkins et al., 1986). Megasomes are large lysosome-like structures, previously described in amastigote forms of *Leishmania* belonging to the mexicana complex, whose major constituents are the cysteine proteinases (Ueda-Nakamura et al., 2001). It is important to point out is that *L. (L.) mexicana* has the ability to cause both a cutaneous and a diffused cutaneous leishmaniasis in South and Central America and, is a member of the mexicana complex that is formed by *L. (L.) amazonensis*, *L. (L.) pifanoi*, *L. (L.) garnhami*, *L. (L.) venezuelensis* and *L. (L.) forattinni*. Besides, *L. (L.) amazonensis* is one of the most important etiologic agent that causes cutaneous and diffuse cutaneous leishmaniasis in Latin America, *L. (L.) donovani* causes cutaneous and mainly visceral diseases in the "Old World" and *L. (L.) major* is associated with cutaneous and mucocutaneous leishmaniasis in the "Old World" (Bañuls et al., 2007). Comparative studies with amastigotes of *L. (L.) amazonensis*, *L. (L.)*

donovani, revealed that *L. (L.) amazonensis* was similar to *L. (L.) mexicana* in possessing both high content of cysteine protease activity in amastigotes and a large numbers of megasomes, whereas the other two species lacked both of these features (González et al., 2008). The presence of numerous megasomes in the amastigote is a characteristic of the *Leishmania* subgenus, particularly of mexicana complex (Pupkis et al., 1986).

Further studies also employing light microscopy and post-embedding immunocytochemistry and the weak base 3-(2,4 dinitroanilino)-3'-amino-N-methyldipropylamine (DAMP) as a probe, localized acidic compartments of *L. (L.) amazonensis* amastigotes since it is known that DAMP concentrates in acidic compartments of cultured cells. This probe was mainly accumulated within megasomes and in dense inclusion vacuoles, proving that megasomes have a low pH maintained by an active process, besides suggesting that these organelles may be targets for amino acid derivatives (Antoine, 1988). It is important to consider some immunocytochemical aspects to localize the DAMP probe in *Leishmania*. In this assay the anti-DNP (2,4-Dinitrophenol) immune serum was prepared in rabbit by injecting human IgG-DNP mixed with a complete Freund´s adjuvant. After fixation and reaction with an antibody against-DNP it can be visualized by anti-rabbit IgG conjugated to gold (7 nm) particles as secondary antibody (Antoine, 1988).

The immunocytochemistry is a powerful technique for investigating the location of specific proteins in a cell. The biosynthesis, enzymatic processing, and immunocytochemical localization of a major cysteine protease of *L. (L.) pifanoi*, were investigated employing *L. (L.) pifanoi* axenic cultured amastigotes and *L. (L.) amazonensis* lesion-derived and, both polyclonal antisera and monoclonal antibodies specifically recognized either the mature cysteine protease or the carboxyl-terminal extension domain (Duboise et al., 1994) using post-embedding protocols. *L. (L.) pifanoi* is another member of the mexicana complex that causes cutaneous leishmaniasis in humans (Colemares et al., 2002). It is important to emphasize that all proteases are synthetized as a high molecular weight zymogen without proteolytic activity and the formation of mature protease involves the processing of the zymogen by cleavage of specific peptides bounds and removal of C or N-terminal domains (Neurath, 1984). Electron microscopic immunolocalization of both catalytic and C-terminal domains showed intense labeling of megasomes, indicating that this cleavage occurs in this organelle. Furthermore, specific cysteine proteinase inhibitors blocked the processing of cysteine protease *in vivo* and also inhibited parasite cell division. Moreover, a low level of the mature protease was also associated with the flagellar pocket and plasma membrane. Consistent with this observation, a low level secretion of this cysteine protease into the culture medium was detected (Duboise et al., 1994). It is known that the flagellar pocket is a secluded extracellular compartment in the anterior portion of trypanosomatids, formed by an invagination of the plasma membrane at the base of the flagellum and is the only part of the cell surface that supports exocytosis and endocytic traffic in *Leishmania* and other trypanosomatids because of its lack of attached microtubules (De Souza, 2006; Bonhivers et al., 2008). The flagellar pocket membrane is an obligatory intermediary station for membrane-bound molecules trafficking between intracellular membranes and the cell surface and vice-versa (Overath et al., 1997).

The processing and trafficking of cysteine proteases were also studied in *L. mexicana* (L.) using axenic amastigotes and anti-cysteine protease B antiserum and the study showed that

the proteolytic processing of zymogen and maturation of the enzyme is redundant and required other types of cysteine proteases and is not easy to paralyze it with inhibitors. However, cysteine proteases are addressed to megasomes via the flagellar pocket and therefore differs from trafficking in mammalian cells (Brooks et al., 2000), making this pathway an important target to block the *Leishmania* growth.

Post-embedding immunocytochemistry assays, using antibody against *Leishmania* cysteine protease, was also employed to prove the importance of these proteases in the pathogenesis of leishmaniasis. This *Leishmania* protease was detected in the lesion sections from infected mice by *L. (L.) mexicana* amastigotes, possibly due to amastigotes lysis and releasing of megasomes content into the parasitophorous vacuole of infected macrophages. These proteases were also found extracellularly in the host mice tissue presumably as a result of macrophage rupture and appear to persist within the lesion, where they may damage host cells and the extracellular matrix proteins (Ilg et al., 1994). Additionally, it was demonstrated that metacyclic promastigotes of *L. (L.) mexicana,* the infective form of parasite, exhibited higher proteolytic activity than multiplicative promastigotes and amastigotes, expressing quantitatively more and with a distinct pattern composed of multiple proteases (Bates et al., 1994). Suggesting that the expression of proteases varies according to evolutive forms of parasite and are important both to survival within the host, and infection of the mammalian cells.

The information about the cellular location and distribution of cysteine proteases in *Leishmania* parasites draws attention to the importance of these enzymes in the parasite physiology. Further studies have clarified the biochemical properties and functions of these enzymes, as well as their gene expression (Ramos et al., 2004; Hide and Bañuls, 2008). Cysteine proteases are the major proteolytic activity in *Leishmania* and they are required for survival and growth of protozoan into fagolisosomes and leishmaniasis pathogenesis (Marín-Villa et al., 2008), because of this, they are considered the most important virulence factors of *Leishmania* since they influence the interaction between the parasite and mammalian host (Mottram, et al., 2004). Genome analysis has revealed the great diversity of cysteine proteases of *Leishmania* (Hide and Bañuls, 2008) and they are distributed in eight families within clan CA. Family C1 contains cysteine protease A and cysteine protease B, which are both cathepsin L-like, and cysteine protease C, which is cathepsin B-like. Cysteine protease B is unusual as it has a 100-amino acid C-terminal extension in comparison with most cysteine proteases of the group, and exists as multiple isoenzymes, which are encoded by a tandem array of similar cysteine protease B genes located in a single locus (the arrays comprise eight genes in *L. major*) (Saffari and Mohabatkar, 2009).

Although the exact roles of cysteine proteases in *Leishmania* pathogenesis are unclear, it has been demonstrated that *Leishmania* cannot grow within macrophages in the presence of specific protease inhibitors (Duboise et al., 1994). Besides, it was demonstrated that *L. (L.) chagasi* cathepsin L-like cysteine protease (Ldccys2) are specifically expressed in amastigote and is necessary for macrophage infection and for survival of the parasite within macrophage cells (Mundondi et al., 2005). The role of the same enzyme, Ldccys2, was investigated in *L. (L.) pifanoi* and *L. (L.) amazonensis* employing post-embedding immunocytochemistry using antibodies against recombinant C-terminal extension of Ldccys2 and anti-Ldccys2 catalytic domain both of *L. (L.) pifanoi* and 10nm-gold labeled goat

anti-rabbit as a secondary antibody (Marín-Villa et al, 2008). The polyclonal antibody specific to Ldccys2 C-terminal extension recognized cysteine proteases from both parasites and also detected a predominant location of this peptide in the lysosome and flagellar pocket of cultured axenic amastigotes of both parasite species. However, its location was shifted towards the surface of the parasites during macrophage infection. This same antibody significantly reduced macrophage infection in both *L. (L.) pifanoi* and *L. (L.) amazonensis*, confirming that Ldccys2 C-terminal domain is essential for macrophage infection. Importantly, the entrance into macrophages is mediated by the endocytosis of opsonized parasites, which are recognized by membrane receptors present on the macrophage surface (Rittig and Bogdan, 2000). Besides, confirming previous reports that C-terminal extensions of proteases are highly immunogenic in *T. cruzi*, antibodies against this peptide in sera of leishmaniasis patients was detected. This study suggests an essential role for *Leishmania* cysteine proteinases C-terminal extensions at early stages of infection (Marín-Villa et al, 2008). Other studies demonstrated that when *Leishmania* parasites are exposed to various stress conditions, such as heat shock and oxidant agents, they release cysteine protease C, a cathepsin B-like enzyme, which is involved in the cell death cascade of the parasite (El-Fadili et al., 2010).

Beside the roles described above, *Leishmania* cysteine proteases, specifically of type B, can modulate the immune response of mammalian hosts to favor parasite survival and proliferation. They are themselves immunogenic, since *L. (L.) mexicana* cysteine proteases are T cell immunogen, resulting in the development of potentially protective Thl cell lines. This finding suggests that the cysteine proteases could also be a vaccine candidate and that homologous enzymes in other parasites species may also be so (Khoshgoo et al., 2008; Saffari and Mohabatkar, 2009; Fedelli et al., 2010, Doroud et al, 2011).

Many evidences indicate that *Leishmania* cysteine proteases could be targets to develop rational drugs to treat leishmaniasis, so specific inhibitors were produced by combinatorial synthetic chemistry optimization using models of both *L. (L.) major* cathepsin B and L, through a structure based drug design screen (Scheidt et al, 1998). These compounds were tested against *Leishmania* cysteine proteases and both amastigotes and promastigotes (Selzer et al., 1999; Schurigt et al., 2010). The electron microscopy and post-embedding immuno-cytochemical localization strategies were also used to study the effect of hydrazine derivatives in parasite and to confirm target protease localization at the site of inhibitor-induced abnormalities (Selzer et al., 1999). After 24 h of treatment, ultrastructural alterations included autophagic vacuoles, undigested cell debris, and multivesicular bodies into dilated megasomes and flagellar pocket were observed. These abnormalities resemble alterations seen in lysosomal storage diseases caused by lysosomal hydrolases deficiency. Using a polyclonal antiserum raised against the native *L. (L.) major* cysteine protease B and a secondary goat antibody to rabbit IgG conjugated with 10-nm gold particles it was possible to observe, only in treated promastigotes, heavily labeled in dilated megasomes and in flagellar pocket, confirming the specific effect of inhibitors in the site of cysteine proteases location (Selzer et al., 1999). Other cysteine protease inhibitors from natural resources, such as plant cystatins, or chemically synthetized, such as pseudopeptide substrate analogues, derivatives of aziridine, triazoles, α-ketoheterocycles and NO-donors, were assayed against *Leishmania*. These compounds provided different degrees of inhibition in promastigote growth and viability, amastigote survival and reduction in the macrophages infection rate (Duboise et al., 1994; Pral et al., 1996; Alves et al., 2001; Tornøe et al., 2004; Ascenzi et al.,

2004; Ordóñez-Gutiérrez et al., 2009, Schurigt et al., 2010; Steert et al., 2010). Although cysteine proteases inhibitors look promising, the activity of the three cysteine proteases families would need to be blocked to completely prevent parasite invasion or replication in the host cells and lesion development, and non-selective inhibitors can also affect the host cysteine proteases. An alternative to this problem would be to develop inhibitors which prevent the cysteine proteases precursor processing resulting in a retrograde accumulation of unprocessed proteases and proteins in organelles of endocytic/exocytic pathway which lead to the parasite´s death.

4.2 The major surface protein or gp 63 metalloprotease

After the discovery of cysteine proteases in both amastigote and promastigote forms of *Leishmania*, a protease of 63 kDa was purified and characterized as the major surface protein of promastigotes also called glycoprotein 63 (gp63) (Etges et al., 1986). This protease was identified in different species of *Leishmania,* including *L. (L.) major*, *L. (L.) donovani*, *L. (L.) infantum, L. (L.) tropica, L. (L.) mexicana*, *L. (L.) amazonensis*, *L. (V.) braziliensis*, and *L. (L.) enriettii*, and was proved to be structurally and functionally conserved in Old and New World *Leishmania* species (González et al., 2008). It is important to note that *L. (L.) major*, *L. (L.) donovani*, *L. (L.) infantum* and *L. (L.) tropica* cause cutaneous and mucocutaneous leishmaniasis in the "Old World" while *L. (L.) enriettii* is non-pathogenic for humans (Bouvier et al., 1987). In amastigote forms of *L. (L.) mexicana* the surface glycoprotein gp63 was localized by post-embedding immunocytochemistry strategies using a monoclonal antibody against promastigote gp63 of *L. (L.) mexicana* and goat anti-mouse IgG conjugated with 5-nm gold particles. This protease was located in amastigote surface, however the label was more intense within the flagellar pocket of the parasite, which is also involved with endocytosis and secretion of molecules (Overath et al., 1997), and is primarily associated with dense material in the lumen of this pocket (Medina-Acosta et al., 1989). The isolation and analysis of surface proteins from lesion amastigotes indicated that gp63 is also the most abundant protein on the amastigote surface (Medina-Acosta et al., 1989).

Gp63 (EC 3.4.24.36) is a zinc metalloprotease that accounts for about 1% of the total protein in promastigotes of *Leishmania* and is also termed as major surface protease, surface acid protease, promastigote surface protease, and leishmanolysin. This metalloproteases belongs to the M8 family of endopeptidases, sharing several characteristics with mammalian matrix metalloproteases (Yao et al., 2003). This enzyme hydrolyzes only proteins, but not peptides, at various pH values (acid, neutral or basic) depending on the protein substrate (Tzinia and Soteriadou, 1991). These observations suggest that gp63 can catalysis in different environment conditions, such as in the sandfly midgut and macrophage parasitophorous vacuoles, because this enzyme is present in both promastigotes and amastigotes *Leishmania* surfaces. Since then, the functions of this strategic protease were extensively investigated. Gp63 plays several important roles in the pathogenesis of leishmaniasis, including (i) evasion of complement-mediated lysis, (ii) facilitation of macrophage infection by promastigotes, (iii) interaction with the extracellular matrix, (iv) inhibition of natural killer cellular functions, (v) resistance to antimicrobial peptide killing, (vi) degradation of macrophage and fibroblast cytosolic proteins, and (vii) promotion of survival of intracellular amastigotes (Yao, 2010). The gp63 overexpression caused increased host infection and intracellular parasite survival, on the other hand, gp63-deficient parasites infecting

macrophages mice, showed a diminished infection and survival (Yao, 2003). Thus, gp63 contributes to parasite virulence by exerting a novel type of control over complement fixation. Organisms expressing gp63 can exploit the opsonic properties of complement while avoiding its lytic effects (Brittingham et al., 1995).

The major surface protease is also important in the *Leishmania*-sandfly interaction. The development and forward migration of *Leishmania* parasites in the sandfly gut was accompanied by morphological transformation to highly mobile, non-dividing 'metacyclic' forms. Metacyclogenesis is associated with developmentally regulated changes in expression of gp63 and its expression in *L. (L.) major* promastigotes surface was studied by post-embedding immunocytochemical analysis using the GP63-specific monoclonal antibodies which demonstrates a clear expression from 2 to 7 days post-blood feeding (Davies et al., 1990). This protease proved to be essential in the development and survival of parasite into sandfly gut, because it degrades hemoglobin and other proteins in the blood meals, thereby providing nutrients needed for the growth of promastigotes and protects promastigotes from degradation by the midgut digestive enzymes (Hajmová et al., 2004). Besides all the functions of Gp63 in *Leishmania*-hosts interactions, the native protein, recombinant or specific peptides from this cleavage was able to elicit a protective immunity to many species of *Leishmania* infection in a variety of animal models (Handman, et al., 1990; Abdelhak et al., 1995; Bhowmick et al., 2008; Mazumder et al., 2011). The immunogenicity and antigenicity of gp63 is very well known. Additionally, it was immunolocalized in the lumen of flagellar pocket indicating that this protease is secreted by *Leishmania* parasites and, and it explains why can be found antibodies against gp63 in patient sera with leishmaniasis (Sayal et al., 1994). For these reasons, gp63 is one of the major candidate molecules for vaccine development against leishmaniasis (Chawla and Madhubala, 2010). Unlike the *Leishmania* cysteine proteases inhibitors that are extensively investigated for drug development to leishmaniasis treatment, studies about gp63 inhibitors did not identify any compound that block the biological functions of this protease and, as the immunogenicity and antigenicity of gp63 has always been recognized many studies were conducted to develop vaccines against *Leishmania* using gp63 or its derivatives as immunogen.

4.3 Serine proteases

Although serine proteases are the most studied enzymes in all living organisms, the first studies about proteases of *Leishmania* identified important proteolytic activity belonged to cysteine protease class, as discussed before (North and Coombs, 1981). Almost two decades later the activity of a serine peptidase was purified and characterized from soluble extracts of *L. (L.) amazonensis* promastigotes (Andrade et al., 1998). Unlike other proteases described in *Leishmania*, it does not hydrolyze proteins or large peptides, but cleaves only small peptides substrates, at the carboxyl side of basic residues and aromatic residues preceding basic residues, which characterizes the enzyme as an oligopeptidase. This was the first study that reports the presence of serine peptidase activity in *Leishmania* and even more an oligopeptidase (Andrade et al., 1998). It is important to consider that *Trypanosoma* species do not express enzymes showing serine protease activities, but only serine oligopeptidases with specific functions in many steps of mammalian cell invasion (Silva-López et al., 2008; Alvarez et al., 2011).

Besides the oligopeptidase activity, *L. (L.) amazonensis* showed expressive activity of serine proteases (Silva-López et al., 2004; Silva- López and De Simone, 2004 a, b; Silva-López et al., 2005). This type of proteolytic enzymes were first obtained from a cell-free supernatant of axenic *L. (L.) amazonensis* promastigotes and was proven to be originated from the parasite despite having been purified from culture supernatant. The post-embedding immunocytochemistry strategy was critical to demonstrate the relationship between the extracellular serine protease and promastigotes, using a rabbit antiserum raised against a heat-inactivated 56-kDa serine protease obtained from culture supernatant and purified using aprotinin-agarose affinity chromatography, and anti-rabbit antibody labeled with 10-nm gold particles (Silva-López et al., 2004). In this study amastigotes from lesions of infected mice were also used in order to investigate the subcellular location of this serine protease and infer possible functions for this enzyme. It was possible to observe that the antibody reacted poorly with the parasite surface and moderately with internal structures in most samples (about 95%) of both forms of the parasite (Figure 2).

In promastigotes, gold particle labeling showed the serine protease to be predominantly located in the flagellar pocket and in vesicular structures which are morphologically similar to the compartments found in mammalian endocytic/exocytic pathways (Figure 2 A and B). It is worth noting, as mentioned previously, that the flagellar pocket is a secluded extracellular compartment in the anterior portion of *Leishmania* formed by an invagination of the plasma membrane at the base of the flagellum and is the only part of the cell surface that supports exocytosis and endocytic traffic of molecules (De Souza, 2006; Bonhivers et al., 2008). This pocket is an obligatory intermediary station for membrane-bound molecules trafficking between intracellular membranes and the cell surface and vice-versa (Overath et al., 1997). Both membrane-bound and secreted proteins appear on the cell surface, underscoring the role of this membrane in delivery of proteins to the cell surface and exterior (Bonhivers et al., 2008). In amastigotes, the enzyme was detected not only in subcellular structures similar to those of promastigotes, such as the flagellar pocket and cytoplasmic vesicles (Figure 2), but also in electron-dense structures corresponding to megasomes (Figure 2 C and D). As commented before, megasomes are large lysosome-like structures and are the main sites of proteolytic activity in *Leishmania* belonging to the mexicana complex, whose major constituents are the cysteine proteases, which results in differentiation process participation and in parasite intracellular survival (Ueda-Nakamura et al. 2002).

The processing and trafficking of cysteine proteases, the best studied lysosomal *Leishmania* proteases, has been reported in *L. pifanoi* and *L. (L.) mexicana* and is targeted to megasomes via the flagellar pocket and has been previously discussed (Duboise et al., 1994; Brooks et al. 2000). These results demonstrated that *L. (L.) amazonensis* secretes a 56-kDa serine protease into the culture supernatant through the flagellar pocket with the participation of different components that resemble mammalian endocytic/exocytic organelles. Furthermore, the fact that this enzyme is located in megasomes, where cysteine proteases are also found, indicate that the serine protease can contribute, in association with the cysteine proteases, to maintain the parasite life cycle and leishmaniasis pathogenesis and so, also represents a novel target in *Leishmania* parasite. This secreted serine protease was further purified and their biochemical characteristics and kinetics parameters were investigated. This enzyme is a dimeric protein of about 115 kDa, with subunits of 56kDa, very well adapted to the environment conditions and certainly contributes to survival and growth of the parasite

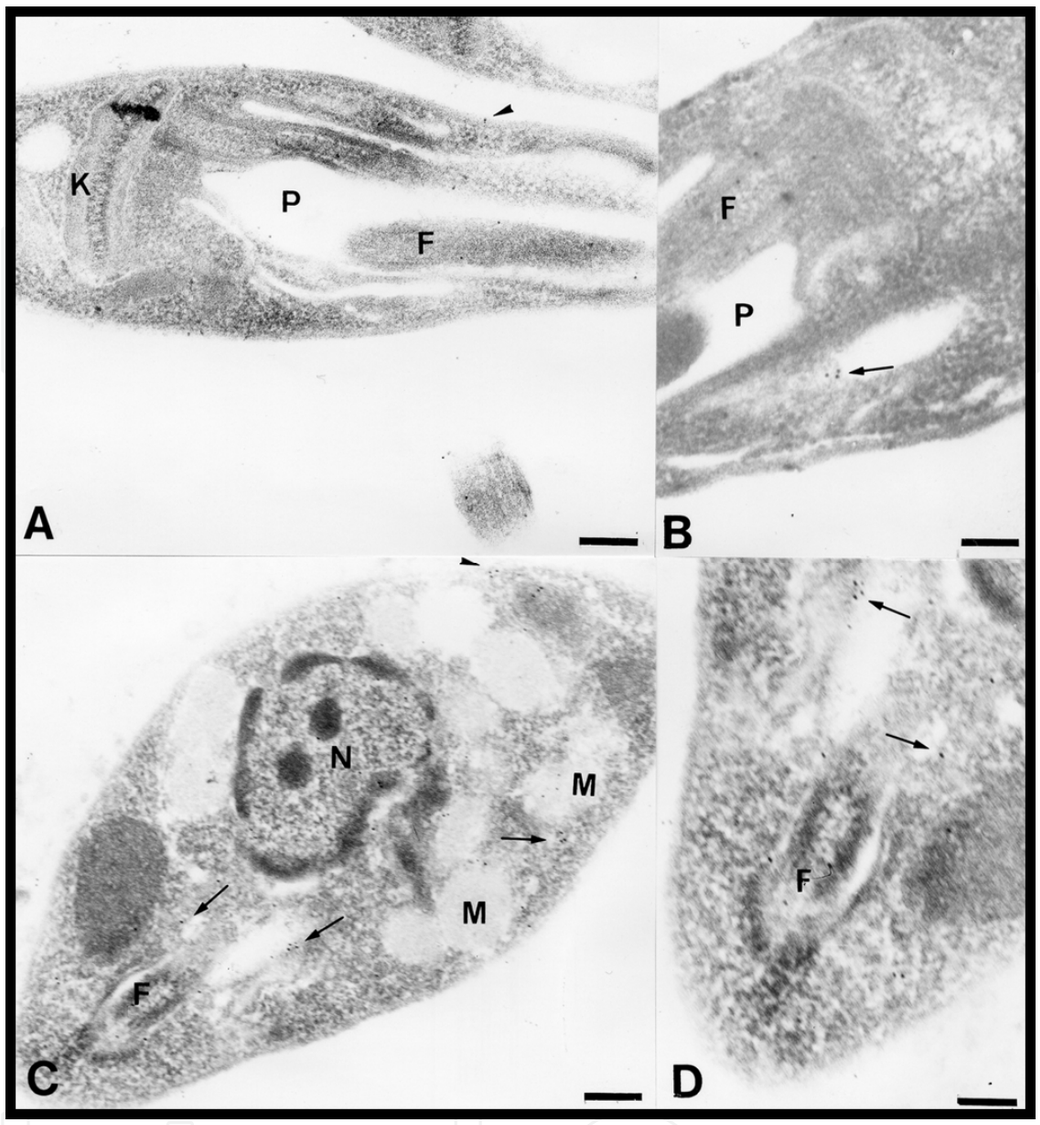

Fig. 2. Subcellular location of serine protease in *L. (L.) amazonensis* promastigotes. (A) Promastigote forms showing immunolabeling at flagellar pocket. In both forms, the cell surface was poorly labeled (Arrowhead). (C) Amastigotes displayed moderate labeling in flagellar pocket, cytoplasmic vesicles (Arrows) and in megasomes. Scale bars; A and C: 2.0 µm. High magnification images of the anterior region of (B) promastigote and (D) and amastigote showing immunolabeling at cytoplasmic vesicles (Arrows) that subtending the flagellar pocket in both forms of *L. (L.) amazonensis*. Scale bars; B and D: 3.2 µm. Flagellar pocket (P), flagellum (F), kinetoplast (K), megasome (M) and nucleus (N) (Silva-López et al., 2004).

inside their hosts, since it is found in promastigotes and amastigotes forms of *Leishmania* (Silva-López et al., 2005).

Two other serine proteases were also purified from water and detergent soluble intracellular extracts of *L. (L.) amazonensis* promastigotes and exhibited different properties and must

perform distinct functions in the *Leishmania* metabolism and physiology (Silva- López and De Simone, 2004 a and b). The subcellular location of the 68-kDa serine protease, an enzyme purified from the water soluble intracellular extracts of promastigote parasites, was performed by electron microscopy post-embedding immunocytochemistry, using the antiserum raised in rabbit by injecting the 68-kDa serine protease purified as previously described (Silva-López and Giovanni De Simone 2004 a). As observed in the figure 3, the antibody reacted against the parasite cytoplasmic membrane and internal structures in the analyzed cells. Cytoplasmic gold particles are seen bound to the external surface and to the flagellar pocket membrane (Figure 3 A), and were localized predominantly in cytoplasmic vesicular structures morphologically similar to that of the endocytic/ exocytic pathways and tubulovesicular structures close to the flagellar pocket region (Figure 3 B and C) (Morgado-Díaz, et al., 2005).

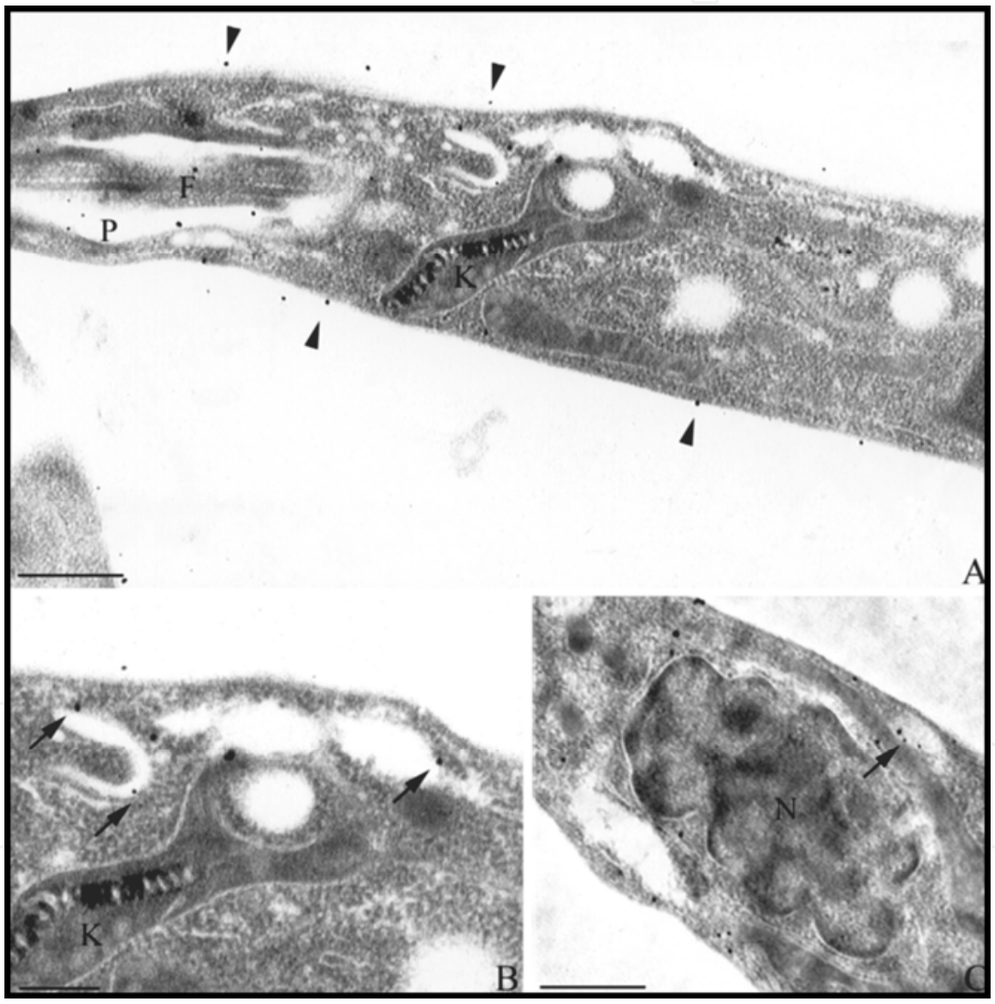

Fig. 3. Subcellular localization of 68 kDa serine protease in *L. (L.) amazonensis* promastigotes. A: gold particles are seen bound to the external surface (arrowheads) and to the flagellar pocket membrane, B: high magnification showing labeling in cytoplasmic vesicles and tubulovesicular structures close to the flagellar pocket (arrows). C: high magnification showing labeling (arrow) in cytoplasmic vesicles. P: flagellar pocket; F: flagellum; K: Kinetoplast; N: nucleus. Bar = 0.25 μm (Morgado-Díaz, et al., 2005).

It is important to compare the localization of both 68 kDa intracellular and 56 kDa secreted serine proteases: While the 68 kDa enzyme is mainly located in membranes of intracellular compartments and plasma membrane, the 56 kDa protease, previously described, reacted poorly with the parasite surface and moderately with internal structures. However, it was predominantly located in the flagellar pocket, megasomes and structures that are morphologically similar to the compartments that are found in mammalian endocytic/exocytic pathways (Silva-López et al. 2004), which justified the released into the extracellular environment.

The activity of serine proteases were also isolated from aqueous, detergent and extracellular extracts of *L. (V.) braziliensis* promastigotes employing aprotinin-agarose affinity chromatography (Matos Guedes at al., 2007). *L. (V.) braziliensis* is the major species of *Leishmania* associated with cutaneous and mucosal forms of leishmaniasis in Brazil and Latin America (Bañuls et al., 2007). These proteases display some biochemical similarities with *L. (L.) amazonensis* serine proteases, demonstrating a conservation of this class of proteolytic activity in the *Leishmania* genus and suggesting similar subcellular location and functions. This was the first study to report the purification of a serine protease from *L. (V.) braziliensis* (Matos Guedes at al., 2007).

The first evidences of the possible functions of these serine proteases in *Leishmania* were obtained using specific serine protease inhibitors. These compounds induced parasite death, with regard to time and doses dependence and, significant morphological alterations. These structural changes were observed in the region of the flagellar pocket and included the appearance of vesicles, which were accompanied by bleb formations of the membrane that covers this pocket which was importantly altered (Silva-López et al., 2007). These effects in the flagellar pocket (a structure of intense exocytic/endocytic activities) indicated that these compounds are endocyted through this structure, and inhibited the serine proteases which are located in this pocket, as previously described by immunocytochemical studies (Silva-López et al., 2004). In the cytoplasm the presence of vesicles that resemble autophagic vacuoles was also noted. The autophagy is a catabolic process involving the degradation of a cell's own components through the lysosomal machinery and is required for normal turnover, starvation, stress responses differentiation, development and in a certain type of cell death (Kiel, 2010). Serine proteases inhibitors caused cell death of *L. (L.) amazonensis* promastigotes inducing the formation of autophagic vacuoles, since the features associated with *Leishmania* apoptosis were not observed (Paris et al., 2004). No modification was found in any of the other cellular structures of the parasites treated with these inhibitors. Furthermore, all parasites exhibited shape alterations (Silva-López et al., 2007). Although the described results indicate that these enzymes are essential for parasite survival, their functions in *Leishmania* physiology are unclear. If these enzymes participate in the exocytosis/endocytosis pathway through the processing of intracellular proteins or even in the maintenance of morphological organization of *Leishmania* remains to be elucidated. However, these findings suggest that *Leishmania* serine proteases appear to be promising targets for the development of specific inhibitors for leishmaniasis chemotherapy.

Serine protease activities were also described for *L. (L.) chagasi*, the causative agent of visceral leishmaniasis in Latin America (Bañuls et al., 2007). These enzymes were isolated from aqueous, detergent soluble and culture supernatant of *L. (L.) chagasi* promastigote extracts and respectively named as LCSII, LCSI and LCSIII. The characterization of these

enzymes employed similar strategies used for *L. (L.) amazonensis* serine proteases (Silva-López et al., 2010a). The same rabbit antiserum against the 56-kDa extracellular protease and anti-rabbit antibody labeled with 10-nm gold particles were used to determine the subcellular localization of the serine proteases in *L. (L.) chagasi* promastigotes employing post-embedding immunocytochemistry strategies. The reactivity of this antiserum was first assayed with all three serine proteases by immunoblotting proteases and indicated that serine proteases of both parasites are related proteins. The antibody did not react with the parasite surface but strongly with internal structures in most samples. The gold particles labeling confirmed that serine proteases are located in the flagellar pocket region and intracellular vesicles (Figure 4), demonstrating that LCSIII which was obtained from culture supernatant, follow the same route of secretion to the extracellular environment utilizing the flagellar pocket. *L. (L.) chagasi* serine proteases were also located in contractile vacuoles and in vesicles located at the posterior region of the parasite body, next to the nucleus. The contractile vacuoles are intracellular vesicles immediately adjacent to the plasma membrane of the flagellar pocket and are involved in fluid secretion via this pocket (Linder and Staehelin, 1979). Other serine peptidases, such as the extracellular serine peptidases from *L. (L.) amazonensis* (Silva-López et al., 2004) (Figure 2) and *T. cruzi* (Silva-López et al., 2008) employed the same route of secretion, since they were also evidenced in the flagellar pocket and contractile vacuoles.

Notably, the endocytic pathway of the *Leishmania* promastigotes comprises a network of tubular endosomes, multivesicular bodies and un unusual multivesicular tubule (MVT)–lysosome, originally observed in *L. (L.) mexicana* (Alberio et al., 2004), and are the main sites of proteolytic activity in *Leishmania*, as well as being crucial for the differentiation process and parasite intracellular survival (Ueda-Nakamura et al., 2007).

Recent studies demonstrated that *L. (L.) donovani* express a very similar secreted serine protease like *L. (L.) amazonensis* which was also located in the flagellar pocket of promastigotes by post-embedding immunogold labeling using anti-115-kDa serine protease antibody and a gold 10 nm particles conjugated secondary antibody. Besides, this enzyme is particularly expressed in virulent strains and is also associated with the metacyclic stage of *L. (L.) donovani* promastigotes. It is postulated that 115 kDa serine protease could be a potential vaccine candidate since it plays important roles in the macrophage infection and is secreted to extracellular environments (Choudhury et al., 2010).

Besides the expression of secreted 115-kDa serine protease, two other serine proteases were identified and characterized in *L. (L.) donovani* promastigotes, using biochemical and molecular strategies: subtilisin (Swenerton et al., 2010) and oligopeptidase B (Swenerton et al., 2011) serine proteases. The functions of *Leishmania* subtilisin (Clan SB, family S8) was studied in parasites with gene knock-out for this enzyme, which resulted in reduced ability to undergo promastigote to amastigote differentiation *in vitro* and amastigotes revealed abnormal membrane structures, retained flagella, and increased binucleation. These "knock-out" parasites displayed reduced virulence in both hamster and murine infection models. Furthermore, proteomic analysis indicated that *Leishmania* subtilisin is the maturase for tryparedoxin peroxidases that detoxifies reactive oxygen intermediates and maintain redox homeostasis that is essential for *Leishmania* virulence (Swenerton et al., 2010). Using similar proteomic strategies was demonstrated that *L. (L.) donovani* oligopeptidase B (Clan SC, family S9A) regulate the function of enolase, since parasites "knock-out" of this peptidase

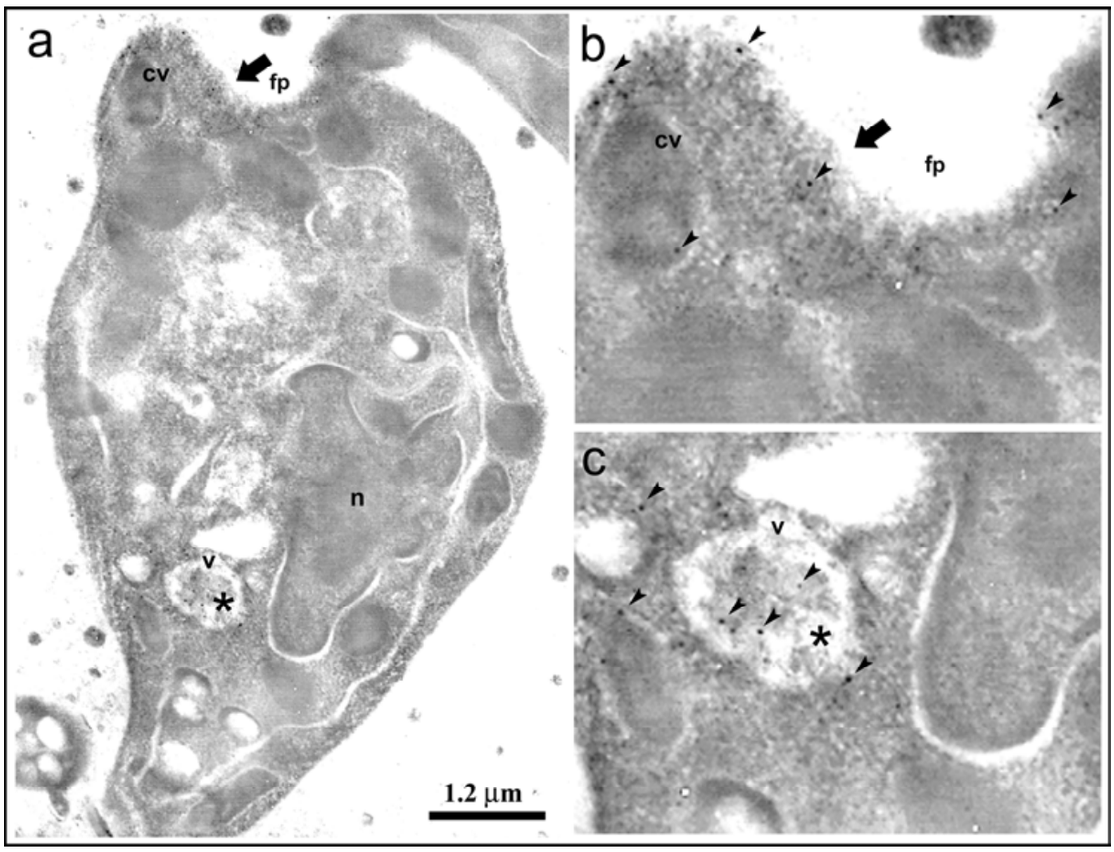

Fig. 4. Immunolocalization of serine proteases in promastigotes of *L. (L) chagasi*. Representative ultrathin section of promastigotes labeled with polyclonal antiserum to 56-kDa *L. amazonensis* serine protease. (a) Gold particles are seen bound to the flagellar pocket membrane (arrow) and into intracellular vesicles (asterisk) of *L. chagasi* promastigote. (b-c) High magnification images showing labeling in the anterior region of promastigote, in flagellar pocket (arrow head), contractile vacuoles and into intracellular vesicles (arrow head and asterisk), respectively. Flagellar pocket (fp), contractile vacuole (cv), vesicle (v) and nucleus (n) (Silva-López et al., 2010a).

showed enolase abnormally increased but enzymatically inactive. Aside from its classic role in carbohydrate metabolism, enolase was found to localize in cytoplasmic membranes, where it binds host plasminogen and functions as a virulence factor for several pathogens. As expected, there was a striking alteration in macrophage responses to *Leishmania* when oligopeptidase B was deleted, so the enzyme interfered in parasite enolase activity and immune evasion. Besides, these "knock-out" parasites displayed decreased virulence in the murine footpad infection model (Swenerton et al., 2011).

It is also important to emphasize the roles of serine proteases in the host immune system modulation. Mice vaccination with soluble proteases isolated from *L. (L.) amazonensis* promastigote antigens directly activated IL-4, IL-10 and TGF-beta production by immune cells and primed mice to respond to parasite challenge with a strong Jones-Mote cutaneous hypersensitivity reaction, and increased susceptibility to infection. So, serine proteases are key components of *L. (L.) amazonensis* promastigote antigens responsible for disease-

promoting immunity (Matos Guedes et al., 2010) and besides being important targets to drug development against *Leishmania*, they are also vaccine candidates for leishmaniasis.

In addition to the proteases already discussed, aspartic protease activity was identified and characterized in *L. (L.) mexicana* promastigotes (Valdivieso et al., 2007). This activity was target of anti proliferative effect on *Leishmania* sp. promastigotes and axenic amastigotes by HIV aspartyl-protease inhibitors, Ac-Leu-Val-Phenylalaninal, Saquinavir mesylate and Nelfinavir. The latter two compounds are currently used as part of antiretroviral therapy. This effect appears to be the result of cell division blockage. In addition, these drugs induced in culture a decrease in the percentage of co-infected HIV/*Leishmania* monocytes and amastigotes of *Leishmania* per macrophage. The finding of a dose-dependent inhibition of *Leishmania* aspartyl-protease activity by these drugs allows us to propose this activity as the drug parasite target. A direct action of these HIV aspartyl-protease inhibitors on *Leishmania* parasites would be correlated with the effect that highly active antiretroviral therapy has had in the decrease of HIV/*Leishmania* co-infection, opening an interesting perspective for new drugs research development based on this novel parasite protease family (Valdivieso et al., 2010).

It is very clear that the employment of specific protease inhibitors could block *Leishmania* proteolytic activity and interfere in the progression of leishmaniasis. A recent study demonstrated that host uncontrolled matrix metalloprotease activity in the cutaneous lesions caused by *L. (V.) braziliensis* may result in intense tissue degradation and, consequently, poor healing wounds, which were associated with unsatisfactory response to antimonials treatment (Maretti-Mira et al., 2011). Thus a pharmaceutical formulation containing protease inhibitors can inhibit both host and parasite proteases and helps heal leishmaniac lesions.

5. Technical considerations

All immunocytochemical experiments begin with tissue fixation, which serves the dual purpose of preserving the cellular structure and the *in vivo* distribution of antigens. However, antigens are chemically modified by fixation and further denatured by dehydration and embedment. Formaldehyde fixation preserves most antigenic sites but it is reversible and it does not maintain good ultrastructure. Osmium post fixation is essential to preserve membrane structure and ultrastructural detail; unfortunately, osmium often irreversibly destroys antigenic sites. In immunocytochemical protocols to localize serine proteases in *L. (L.) amazonensis* and *L. (L.) chagasi*, the parasites were fixed in 4% paraformaldehyde/1% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.3 (Silva-López et al., 2004; Morgado-Díaz et al. 2005; Silva-López et al., 2010a). The samples were dehydrated in methanol and embedded at progressively lowered temperature in Lowicryl K4M resin. Lowicryl is a hydrophilic acrylic resin that tolerates partial dehydration and is processed and photopolymerized at subfreezing temperatures. The hydrophilic properties of Lowicryl result in excellent antigen retention and consequently in high label density, specificity, and low background (Herman, 1988). After embedment, thin sections were collected on 400 mesh uncoated nickel grids, incubated for 30 min at room temperature in phosphate buffered saline (PBS) containing 1.5% bovine serum albumin and 0.01% Tween 20, pH 8.0 (blocking buffer) in order to block unspecific bounds. The grids were incubated for 60 min in the presence of the primary antibodies: anti-56 kDa extracellular serine

protease or anti-68 kDa intracellular serine protease diluted in blocking buffer. Both antibodies were produced using similar protocols. Serine proteases were purified using affinity chromatography on aprotinin-agarose columns (Silva-López and De Simone, 2004b; Silva-López et al., 2005). The antiserum was raised in rabbit by injecting the homogeneous heat inactivated purified serine proteases emulsified in complete (first booster) and incomplete (subsequent boosters) Freud's adjuvant. After the fourth injection the antibody reactivity was checked by immunoblotting. So, grids containing embedded parasites were finally incubated for 60 min with goat anti-rabbit antibody labeled with 10-nm gold particles. The grids were subsequently washed with PBS and distilled water, stained with uranyl acetate and lead citrate and observed in a Zeiss EM10C transmission electron microscope (Silva-López et al., 2004; Morgado-Díaz et al. 2005; Silva-López et al., 2010a). The quality of antibodies is essential to obtain reliable results in immunocytochemistry assays, since they must be specific and sensitive enough to bind the antigen of interest in the cellular structure without labeling other intracellular sites that do not contain the antigen. Furthermore, to localize antigens by electron microscopy, it is necessary to impart electron density to the bound antibodies. Colloidal gold probes have been extensively adopted for use in post-embedding and pre-embedding immunocytochemistry assays (Bendayan et al. 1987).

Immunocytochemical experiments may be accomplished with various procedures for pre- or post-embedding labeling. Each method offers distinct advantages and disadvantages regarding to specificity, density of antigen labeling, and structural preservation. Pre-embedding immunocytochemistry assays requires cryoprotected tissue and labeled with primary and indirect electron-dense labels which enter the tissue by diffusion. The labeled tissue is then embedded, sectioned, and examined. The primary advantage is the excellent antigen retention as the consequence of few pre-labeling and processing steps. The disadvantages result from the poor penetration of both primary antibodies and secondary labels into the tissue, limiting the label to a gradient in the superficial few micrometers, and require costly instrumentation. Post-embedding immunocytochemistry is the most employed technique in most electron microscopy subcellular location studies and was discussed above. In these procedures tissues are fixed, dehydrated, and embedded in plastic using protocols similar to those of conventional EM. Thin plastic sections are labeled by immersion in solutions of primary antibodies followed by electron-dense second label. The main advantages are that the skills and methods are similar to those employed in conventional EM, and no specialized equipment beyond that found in any EM laboratory is required (Herman, 1988).

6. Concluding remarks

The immunocytochemistry is a technique of choice that permits routine and reproducible localization of most moderately abundant antigens using specific antibodies against certain antigens. The subcellular location of enzymes suggests their function in the metabolism of specific organelle, cell or organism. The immunocytochemical localization of *Leishmania* proteases in megasomes, flagellar pocket, cytoplasmic membrane, contractile vacuoles, cytoplasmic vesicles, tubulovesicular structures and as secreted enzymes into the extracellular environment indicates the versatility of these proteases. They participate in exocytic/endocytic pathways, in processing of endogenous proteins or enzymes, in the

digestion of exogenous proteins for parasite nutrition or signaling. The localization of *Leishmania* proteases in membrane or in the extracellular medium suggests that these enzymes could be important mediators with their hosts, and thus modulate a host immune response. Since *Leishmania* proteases perform crucial roles in parasite physiology and in the host-parasite interaction, they are absolutely necessary for the survival of the pathogen and the leishmaniasis progression and, in addition they are substantially different from the host homolog. So, they are considered important targets in *Leishmania*. Furthermore, specific protease inhibitors induced important alterations in parasite morphology, reduced the viability and growth of *Leishmania* and killed axenic promastigotes and amastigotes and intracellular amastigotes thus becoming a promising candidate for leishmaniasis treatment (Silva-López et al., 2007; Valdivieso et al., 2010; Olivier & Hassani, 2010; Pereira et al., 2011). In conclusion, immunocytochemical strategies contributed and continue to contribute for the specific identification of targets in *Leishmania* which is a rational approach for drug development in the leishmaniasis treatment.

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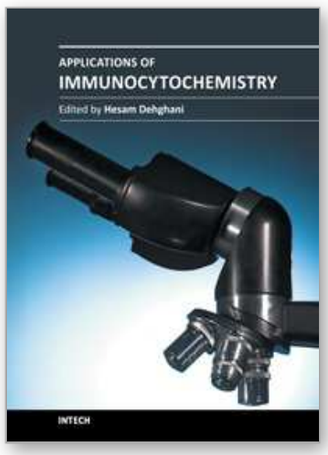

Applications of Immunocytochemistry
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Immunocytochemistry is classically defined as a procedure to detect antigens in cellular contexts using antibodies. However, over the years many aspects of this procedure have evolved within a plethora of experimental setups. There are different ways to prepare a given specimen, different kinds of antibodies to apply, different techniques for imaging, and different methods of analyzing the data. In this book, various ways of performing each individual step of immunocytochemistry in different cellular contexts are exemplified and discussed. Applications of Immunocytochemistry offers technical and background information on different steps of immunocytochemistry and presents the application of this technique and its adaptations in cell lines, neural tissue, pancreatic tissue, sputum cells, sperm cells, preimplantation embryo, arabidopsis, fish gonads, and Leishmania.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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