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Biological Roles of Peptidases in Trypanosomatids

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Abstract: In this review, we report the recent developments in the characterization of peptidases and their possible biological functions in the Trypanosomatidae family. The focus will be on peptidases from *Trypanosoma cruzi*, *Leishmania* spp., African trypanosomes and plant and insect trypanosomatids. There are numerous events in parasite development where the involvement of peptidases has been established, and they will be approached in the present review. Also in this review we will discuss the central roles have been proposed for peptidases in diverse processes such as virulence, host cell interaction and invasion, catabolism of host proteins, differentiation, cell cycle progression and both stimulation and evasion of host immune responses.

Keywords: Peptidases, Trypanosomatids, Cruzipain, Cysteine peptidases, Serina peptidases, Metallopeptidases.

INTRODUCTION

Trypanosomatids are flagellate protozoa of the Kinetoplastida order, which is characterized by the presence of kinetoplast DNA (kDNA), an extracellular DNA network of circular molecules composed by maxi- and minicircles that corresponds to the parasite mitochondrial genome, localized near the basal body [1]. The Kinetoplastida order includes the Trypanosomatidae family, an exclusively parasitic taxon that infects a wide range of animals and plants and the Bodonidae family that includes parasitic and exclusively free-living organisms [1, 2].

The Trypanosomatidae family comprises four genera of digenetic organisms that parasitize vertebrate (*Trypanosoma*, *Leishmania* and *Endotrypanum*) or plant (*Phytomonas*) hosts, with insects or leeches serving as vectors and the monogenetic genera (*Leptomonas*, *Crithidia*, *Blastocrithidia*, *Herpetomonas* and *Rhynchoidomonas*) that are found largely in hemipteran and dipteran insect vectors with a limited distribution in seven other orders of insects, as well as in ciliates [2, 3]. Although the Trypanosomatidae family infects a diverse range of hosts, only two genera, *Leishmania* and *Trypanosoma*, are found in humans and are etiologic agents of important diseases such as leishmaniasis, Chagas' disease and African trypanosomiasis [3]. These trypanosomiases are neglected tropical diseases, since they are largely ignored by medical science, first-world public opinion and pharmaceutical companies [4]. The monoxenous trypanosomatids have been used as comparative models of study in order to understand the physiology, biochemistry, ultrastructure and the molecular biology of the pathogenic species [5-30]. In addition, trypanosomatids that are not normally infectious to humans have been isolated from immunosuppressed patients, mainly in HIV-positive individuals, where these parasites caused either visceral or cutaneous lesions [31].

Trypanosoma cruzi is the causative protozoan parasite of Chagas' disease, a debilitating and incurable chronic inflammatory condition characterized by cardiomyopathy and/or digestive disorders [32]. Its life cycle involves the obligatory passage through vertebrate mammals and invertebrate hematophagous triatomine bug hosts. The trypomastigotes ingested by the insect vector differentiate into proliferative epimastigotes that, on reaching the posterior intestine, evolve into metacyclic trypomastigotes. These forms invade vertebrate host cells and differentiate into non-flagellated amastigotes, which in turn transform into trypomastigotes that are responsible for the dissemination of the infection [33, 34]. Chagas' disease is endemic in Latin America and it affects an estimated 16-18 million people mainly in Central and South America, causing up to 45,000 deaths each year [35]. Recent surveys indicate that about 200,000 new cases occur yearly in areas where the disease is endemic, representing the third most common parasitic infection worldwide after malaria and schistosomiasis [36]. Most human infections occur through contact with infected bloodsucking triatomine species. The urbanization process in Latin America and immigration trends, however, have led to the disease being diagnosed in non-endemic areas where, even in the absence of the vector the infection can still be transmitted congenitally, by blood transfusion and by organ transplantation. In addition, oral transmission has aroused the attention of researchers, due to outbreaks in some particular areas such as the Brazilian Amazon, mainly associated with the consumption of Amazonian palm berry or açaí (*Euterpe oleracea* Mart.) juice [37]. Recently, in December 2007, an

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outbreak of Chagas' disease occurred in Caracas, Venezuela, related to ingestion of contaminated fruit juices [38]. Despite vector-control programs, this disease is an important public health concern because no vaccines have been developed and there are no prophylactic drugs to prevent infection by *T. cruzi*. Currently available medication, such as nitrofurans (nifurtimox) and nitroimidazoles (benznidazole) are effective during the acute phase of the infection but have little effect in the chronic stage of the disease [39]. Moreover, in addition to serious side effects, resistance against both compounds leads to increasing treatment failures [40]. Within this framework, an intense research program has been directed to develop strategies to produce a vaccine or efficient non-toxic drugs, through the investigation of specific targets such as molecules, organelles and metabolic pathways in *T. cruzi* [41].

African trypanosomes are devastating human and animal pathogens. *Trypanosoma brucei rhodesiense* and *T. b. gambiense* subspecies cause the fatal human disease known as African sleeping sickness and it continues to pose a major threat to 60 million people in 36 countries in sub-Saharan Africa [42]. The disease is caused by protozoan parasites of the genus *Trypanosoma* and comes in two types: East African human trypanosomiasis caused by *T. b. rhodesiense* and the West African form caused by *T. b. gambiense*. *T. brucei* is transmitted by the tsetse fly and alternates between the bloodstream and insect life cycle stages that are adapted to survive in the mammalian host and the insect vector, respectively [43]. The four main drugs used for human African trypanosomiasis are all toxic, and melarsoprol, the only drug that is effective for both types of central nervous system disease, is so toxic that it kills 5% of patients who receive it. Eflornithine, alone or combined with nifurtimox, is being used increasingly as first-line therapy for gambiense disease [4].

Leishmaniasis has a wide spectrum of diseased manifestations; hence, it is considered as a group of diseases rather than a single disease itself. The three main clinical forms are visceral, mucocutaneous and cutaneous. According to global estimates there are about two million new cases a year [44] and results in 2.4 million disability-adjusted life years annually [45]. *Leishmania* spp., the causative organism of leishmaniasis, is a protozoan parasite that lives as a promastigote in the digestive tract of sandflies and as an amastigote in the phagolysosomes of mammalian macrophages. At least 20 species of *Leishmania* are known to infect humans and they are the cause of a wide spectrum of clinical manifestations resulting in substantial morbidity and mortality with 10% of the world's population at risk of infection [46]. The increase in the incidence of the disease, associated with higher morbidity rates, the spread of some forms of leishmaniasis to new geographical areas and *Leishmania*-HIV co-infection, has become an important public health problem in the world [47].

The designation 'lower trypanosomatids' referrers to the parasites that possess a monoxenous life cycle style and are usually found in insect hosts but are not considered capable of causing parasitic diseases in vertebrates. 'Lower trypanosomatids' also refers to the species of trypanosomatids belonging to the *Phytomonas* genus, which circulate between a phytophagous vector and a plant [48]. The genus *Phytomonas* comprises plant flagellate trypanosomatids that are etiological agents of diseases affecting various fruits and plants, some of them causing great economical losses in agriculture. For instance, phloem-dwelling phytomonads cause acute and chronic yellowing of leaves in coffee plants, 'hartrot' in coconut palms and 'marchitez wilt' in oil palms, while the latex-associated *P. françai* is found associated with a disease known as ''chochamento das raízes'', which means ''empty roots'', in the latex of cassava (Manihot esculenta Crantz); and the disease is characterized by poor root system development and general chlorosis of the aerial part of the plant [15, 49]. However, phytomonads also parasitize lactiferous plants without any apparent pathogenicity [50]. These parasites are transmitted by phytophagous hemipteran insects of the families Coreidae, Lygaeidae, Pyrrhocoridae and Pentatomidae [49]. In comparison to the pathogenic trypanosomatids belonging to the *Leishmania* and *Trypanosoma* genera, very modest attention has been given to their lower counterparts. Nevertheless, lower trypanosomatids have been used as organism models to studies concerning cell biology, biochemistry, nutrition, ultrastructure, symbiosis and genetic approaches in the Trypanosomatidae family [48, 51]. Surprisingly, some genera of monoxenous lineage have been isolated from other hosts such as plants and mammals [31, 49].

Recent advances in our understanding of the biochemistry and molecular biology of the protozoa have focused attention on specific parasite molecules that are essential to the parasite life cycle or the pathogenesis of the diseases they produce. One group of enzymes that plays myriad roles in these processes is the parasite-derived peptidases [52, 53]. This review focuses on the role of peptidases from trypanosomatids, objecting the understanding of the biochemistry and cell biology of these molecules during the parasite life cycle or in the interaction with host cells.

PEPTIDASES

Peptidases are a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins. Nowadays the MEROPS database is a source of information on peptidases and contains all properties and characteristics of these enzymes including origin, nomenclature and classification, inhibitors, substrates, amino acid sequences and the structure if known [54]. The name peptidase is preferred to proteases, but they are also called proteolytic enzymes or proteinases. There are two sets of subclasses of peptidases: the exopeptidases [EC 3.4.11-19], enzymes that cleave peptide bonds at the amino- or carboxy-terminus, and the endopeptidases [EC 3.4.21-24 and EC 3.4.99], which cleave peptide bonds internally in a polypeptide. Peptidases have recently been classified using three different methods: [i] by the chemical mechanism of catalysis represented by serine, cysteine, threonine, aspartic, glutamic or the metallo catalytic type and only a few remain of unknown catalytic type, [ii] by the details of the catalytic reaction, as they are invariably selective of the bonds they hydrolyze and [iii] by molecular structure and homology. The last classification is the most recent of the three methods; it depends on the data availability for amino acid sequences and three-dimensional structures [53].

The study of peptidase activities in protozoan parasites, trypanosomatids in particular, has attracted considerable attention over the last decade, since some of these enzymes play important roles in host/parasite interactions [52, 53], which include modulation of the host immune system, invasion, destruction of host tissues, parasite migration, growth and development and/or acquisition of essential nutrients for survival and proliferation required for continued infection [52, 53]. Furthermore, these enzymes are considered virulence factors [52, 53]. Therefore, they are promising targets for the design of antimicrobials against trypanosomatid diseases [39-41, 55]. In addition, the ability of lower trypanosomatids to express homologues of *Leishmania* and *Trypanosoma* virulence factors seems to play essential roles in the nutrition as well as in the interaction with the insect epithelial cells. Table **1** summarizes the major peptidases found in trypanosomatids.

PEPTIDASES IN *TRYPANOSOMA CRUZI*

Cysteine Peptidase Families

Cysteine peptidases of *T. cruzi* have been implicated in a variety of biological events including nutrition, invasion of host cells, immune evasion, pathogenesis and virulence and some have also been validated as promising drug targets [52-55, 56]. The major cysteine peptidase of this parasite, the

Table 1. Major Peptidases of Trypanosomatids

Trypanosomatids	Peptidase Class	Peptidase Name
Trypanosoma cruzi	Cysteine peptidases	Cruzipain Cathepsin B-like Calpains Metacaspases
	Metallopeptidases	Gp63 Metallocarboxypeptidases (TcMCP-1 and TcMCP-2) Matrix Metallopeptidases (MMP-9-like)
	Serine peptidases	Oligopeptidases B Prolyl and endopeptidase Tc 80
	Treonine peptidases	Proteasome
	Aspartic peptidases	Cruzipsin-I (CZP-I) and cruzipsin-II (CZP-II).
Leishamania spp.	Cysteine peptidases	Cathepsin L-like CPA and CPB Cathepsin B-like CPC Calpains
	Metallopeptidases	gp63
	Serine peptidases	56 kDa 68 kDa 110 kDa
	Aspartic peptidases	Cathepsin D -like
	Treonine peptidases	Proteasome
African group	Cysteine peptidases	CP1 and CP2 (congopain or trypanopain-Tc) Trypanopain-Tb (or brucipain) Rhodesain
	Metallopeptidases	TbMSP-A, TbMSP -B and TbMSP –C
	Serine peptidases	Oligopeptidase B (OpdB),
	Treonina peptidases	Proteasome
Lower trypanosomatids	Cysteine peptidases	40-120 Kda Calpains- like
	Metallopeptidases	gp63-like

cruzipain has been characterized and is located in clan CA, family C1 (Fig. **1**). Recently, the calpains of clan CA and family C2 have been studied in this parasite (Fig. **1**).

Another cysteine peptidase found in *T.cruzi* is the metacaspase, which is also located in clan CD and belongs to the family C14, (Fig. **1**). Clan CD enzymes differ fundamentally from those in clan CA, which allows them to be readily distinguished. The catalytic cysteine–histidine (CH) dyad is crucial to all cysteine peptidases for activity. For instance, the order of catalytic CH residues in protein differs in clan CD (HC) and CA (CH). The clan CD is not inhibited by E-64, an archetypal and potent cysteine peptidase inhibitor. Whereas the catalytic dyad CH is sufficient for clan CD; however, other cysteine peptidases possess an additional highly conserved amino acid that is involved in the catalytic mechanism. For instance, either an asparagine or aspartic acid residue is present in clan CA, serving to orientate the active site histidine [57].

Family C1 (Cruzipain and Others)

Cruzipain also called gp57/51 and cruzain (the recombinant catalytic domain of cruzipain) is the major cysteine peptidase in *T. cruzi* and it is expressed as a complex mixture of isoforms. The enzyme is found in all developmental forms of different *T. cruzi* isolates, with levels 10-fold higher in epimastigotes and is active in the pH range of 5.0 to 7.5 [58-60]. Cruzipain has an N-terminal catalytic domain linked to an antigenic C-terminal extension, the gp25 [61, 62]. Structural characterization of the oligosaccharide chains revealed the presence of sulfated residues that are essential for cruzipain recognition by IgG antibodies from the serum of chagasic patients [63, 64].

This peptidase is localized in different cell compartments, depending on the life cycle stage. The enzyme in the late endosome compartments of *T. cruzi* epimastigotes is called reservosome [65]. Meanwhile, in the intracellular amastigote, the principal stage for drug targeting, much of

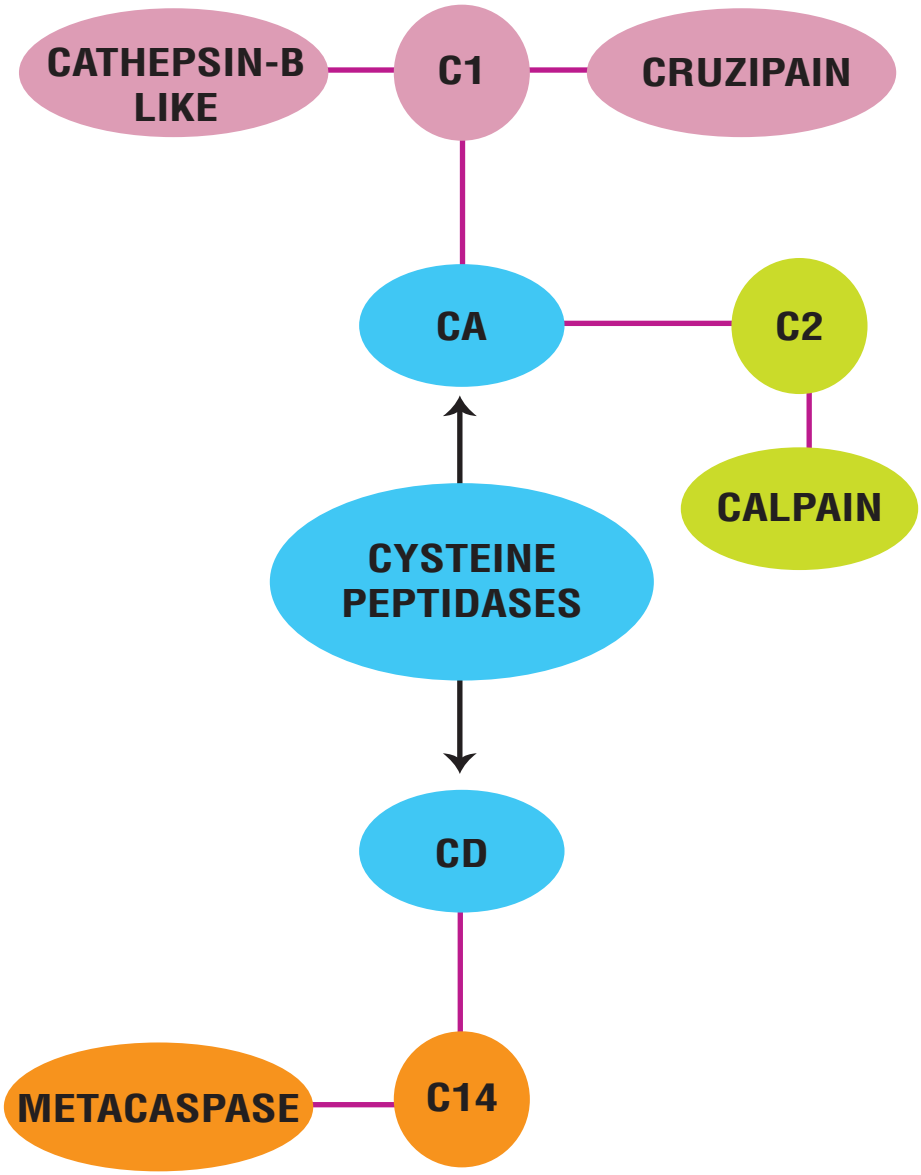

Fig. (1). Overview of representative enzymes in *Trypanosoma cruzi* that belong to cysteine peptidase families.

cruzain is found on the surface of the parasite, directly in contact with host cell cytoplasm [65, 41].

Beyond its function in parasite nutrition as a lysosomal peptidase, the enzyme has been implicated in other cellular processes including cell invasion, proliferation, parasite differentiation and metacyclogenesis [60, 66]. Selective inhibitors of this peptidase block the proliferation of both extracellular epimastigotes and intracellular amastigotes and arrest metacyclogenesis *in vitro*, indicating that the enzyme performs essential functions for parasite survival such as differentiation and growth [67-69].

Host peptidase inhibitors such as α-2-macroglobulin (α-2M) and kininogens interact with *T. cruzi* cruzipain in extravascular infection sites, linking inflammation to innate immunity by different mechanisms [70]. Cruzipain has the ability to generate kinin released from kininogens due to its activity of 'kininogenase' [71, 72]. Although soluble kininogens are not efficiently processed by cruzipain, the interactions of kininogens with sulfated glycosaminoglycans displayed at cell surface render the kinin-precursor molecules sensitive to proteolytic processing by cruzipain [73]. Once liberated, kinins bind to the bradykinin B2 receptor B_2R, which is expressed by a wide range of cell types, including cardiomyocytes, or promote the signaling of the inducible receptor B_1R [74-76]. Chagasin, the endogenous cruzipain inhibitor, is a *T. cruzi* protein that also plays an important role in parasite differentiation and infectivity [77].

In recent years cruzipain had been studied as a drug target and several inhibitors are in development at experimental levels as well as by use of computer-assisted molecular design such as the classical two-dimensional quantitative structure-activity relationships (2D QSAR) and hologram QSAR (HQSAR) studies [52]. Currently, a cruzipain inhibitor, the vinyl sulfone K777 has entered in preclinical drug development investigations [41].

In addition to the cruzipain, all the *T. cruzi* life cycle forms express a cathepsin B-like cysteine peptidase with a molecular mass of 30 kDa, which may also be considered an immunogenic molecule, since antibodies against this peptidase were detected in chagasic patients as well as in patients with mucocutaneous leishmaniasis and kala-azar patients [78, 79].

A novel cysteine peptidase released to the culture medium during *T. cruzi* metacyclogenesis was described by Duschak *et al*. [80]. This proteolytic activity, named *Tc*CPmet, has a molecular mass between 97 and 116 kDa, being active at pH 6.0 in SDS-PAGE. The enzyme was not recognized by anti-cruzipain antibody, suggesting a different nature of both cysteine peptidases. This new cysteine peptidase activity seems to be a feature of metacyclic trypomastigote and makes this finding more attractive by suggesting that it is developmentally regulated. This fact reinforces a possible role for this cysteine peptidase during metacyclogenesis. In addition, the authors also suggest the involvement of this enzyme in the protein degradation processes necessary for this stage-specific transformation [80].

Family C2 (Calpains)

Calpains (calcium-activated non-lysosomal peptidases, CAPN) are neutral peptidases that respond to Ca^{2+} signals by cleaving specific protein substrates, thereby irreversibly modifying their function [81]. Members of this peptidase family are present in a variety of organisms including trypanosomatids and are involved in diverse cellular processes including apoptosis, signal transduction, differentiation and cytoskeleton remodeling [82, 83]. They contain several calcium-binding sites, which affect allosterically the enzyme activity [84, 85].

Genome sequences of the Tritryps (*T. cruzi, T. brucei and Leishmania major*) have recently become available: the cysteine peptidases in these species are a large family of peptidases that include calpain-like genes [86]. A gene encoding the *T.cruzi* calpain (CALP), *TcCALPx*11 (GeneDBID Tc00.1047053506563.210), was identified by microarray analysis as being differentially expressed by *T. cruzi* during metacyclogenesis. Its expression during nutritional stress preceding *T. cruzi* metacyclogenesis was 2.53 times higher than that observed in epimastigotes [83]. Antiserum against a recombinant *TcCALPx*11-GST-fusion protein reacted with a protein band at 80 kDa in *T. cruzi* epimastigote protein extracts by western blot analysis, which corresponds to the estimated molecular mass of *TcCALPx*11 without post-translational acylation, thereby confirming the prediction from in silico analysis [83, 86]. Western blot with parasite extracts during metacyclogenesis showed an increase in the abundance of *Tc-CALPx*11 in parasites under nutritional stress. Three types of stress (nutritional, temperature and acidic pH) were tested and the *TcCALPx*11 expression increased irrespective of the type of stress applied to the parasite. *TcCALPx*11 was not detected during *T. cruzi* amastigogenesis [83]. *T. cruzi* calpain also presented a strong cross-reactivity with anti-*Drosophila melanogaster* calpain and anti-cytoskeleton-associated protein from *T. brucei* antibodies, and labelling was found mainly intracellularly. No significant cross-reactivity was found with anti-human brain calpain antibody. The expression of calpain was decreased in cells kept for long periods in axenic cultures in comparison to a strain recently isolated from mice. Different levels of calpain expression were also detected in distinct phylogenetic lineages, like Y strain (lineage TCII), Dm28c (TCI) and INPA6147 strain (Z3 zymodeme). Interestingly, the calpain inhibitor III (MDL28170) at 70 μM promoted a strong reduction on the growth rate of *T. cruzi* Dm28c strain after 48 h [87].

Family C14 (Metacaspases)

Caspases are a conserved family of central effector cysteine peptidases involved in inflammatory disease, neurodegenerative disorders and apoptosis [88]. Recent works report the presence and functionality of metacaspases in *L. major, T. brucei, and T. cruzi* [89-93]. Five metacaspase genes (*TbMCA*1–*TbMCA*5) have been identified to date in *T. brucei* [89]. The genome of *T. cruzi*, contains two genes, *TcMCA*3 and *TcMCA*5, with homology to those encoding metacaspases, distantly related to the caspases involved in programmed cell death (PCD) in higher eukaryotes. *TcMCA*3 is present in the CL Brener clone at 16 copies per haploid genome, arrayed in two tandems located in chromosomes of 0.54 and 0.98 Mbp. *TcMCA*5, on the other hand, is present as a single copy gene [91, 94]. The proteins encoded were expressed in *Escherichia coli* BL21 (DE3) cells, to

generate antibodies, which showed that *TcMCA*3 is expressed in the four major stages of the parasite, whereas *TcMCA*5 is expressed only in the epimastigote form. Moreover, recombinant *TcMCA*3, but not *TcMCA*5, was recognized in most sera from chronic chagasic patients, showing that the protein is expressed during natural infections [91]. Using synthetic caspase substrates, Jimenez *et al.* [95] detected an increase in caspase-like activity in epimastigotes at the stationary phase of growth, indicating that these enzymes may be involved in some of the apoptotic features.

gp63 and other Metallopeptidases

In *T. cruzi* several types of metallopeptidases have been described, some of them specifically expressed during metacyclogenesis in various strains and clones of *T. cruzi* [96, 97]. In addition, multiple isoforms of the endopeptidase gp63 (clan MA, family M8) family have been found as well as matrix metallopeptidases-like peptidases [98, 99]. Recently, exopeptidases such as metallocarboxypeptidases were found in the cytoplasm of the parasite [100, 101].

gp63

T. cruzi expresses multiple isoforms of the gp63 family of metallopeptidases at all life stages [98]. A polyclonal antiserum against recombinant gp63 of *T. cruzi* (*Tcgp*63) was used as a tool in order to study *Tcgp*63 expression and localization in this parasite. By Western blot analysis, biotinylation, endoglycosidase digestion and immunofluorescence, *Tcgp*63 was detected as a surface glycoprotein with a molecular mass of 61 kDa in epimastigotes and as a non N-glycosylated and intracellular protein with a molecular mass of 55 kDa in metacyclic trypomastigotes. Amastigote presented a molecular mass of 61 kDa [102]. Moreover, preincubation of trypomastigotes with either *Tcgp*63 antiserum or a purified *Tcgp*63 C-terminal subfragment reduced infection of host myoblasts suggesting that isoforms play a role in host cell infection

Metallocarboxypeptidases

Two cytosolic metallocarboxypeptidases of the M32 family, isolated from *T. cruzi* CL Brener clone, have recently been characterized: *TcMCP*-1 and *TcMCP*-2 [100]. Both of them showed to be closely related in sequence (67%) by displaying temporary expression patterns and substrate preferences. These peptidases differ in their pattern of expression: while *Tc*MCP-1 is present in all life stages of *T. cruzi*, *Tc*MCP-2 is mainly restricted to the stages that occur in the invertebrate host. *Tc*MCP-1 removes basic C-terminal residues, whereas TcMCP-2 prefers hydrophobic/aromatic residues. The structure of *Tc*MCP-1 shows strong topological similarity with archaeal, bacterial and mammalian metallopeptidases including the angiotensin-converting enzyme, neurolysin and thimet oligopeptidase. These enzymes, like other *T. cruzi* peptidases, could be a potential target in Chagas' disease at the molecular level and provide a template for the design of novel therapeutic approaches [100, 101].

Matrix Metallopeptidases

Matrix metallopeptidases (MMPs) are a family of structurally related zinc-dependent peptidases that are able to hydrolyze the major components of the extracellular matrix (ECM), such as collagen, elastin, fibronectin and laminin [103]. Thereby, these enzymes play a central role in processes that involve ECM remodeling, including embryonic development, bone remodeling, invasive processes such as angiogenesis, tumor metastasis and tissue repair [104, 105]. Moreover, MMPs help mediate inflammatory cell migration because they can degrade all components of the ECM, clearing the path for migrating cells of the immune and repair responses [106]. MMPs have distinct but overlapping substrate specificities, and apart from the extracellular matrix, MMPs may cleave a wide range of non-matrix substrates, such as pro-forms of other MMPs, cell-adhesion molecules, chemokines, cytokines, growth factors, their receptors, and junctional proteins, either to augment or to inhibit the functional activity of these substrates [107].

Increased levels of various MMPs (collagenases, stromelysins, and gelatinases) have been associated with inflammatory diseases of connective tissues. The actions of the collagenases MMP-2 and MMP-9 are involved in regulation of the inflammatory response in several circumstances, including the direct cleavage of immune system proteins. In the acute phase of *T. cruzi* infection, myocardial inflammatory infiltrate produces a significant tissue injury, which may cause acute morbidity and mortality. This inflammation is modulated by cytokines and chemokines produced by leukocytes and cardiomyocyte during *T. cruzi* infection [108, 109]. Gutierrez *et al.* [110] investigated the role of MMP-2 and MMP-9 in the myocarditis induced by *T. cruzi*, using immunohistochemical, zymography with gelatin as substrate and enzyme-linked immunosorbent assays as well as real-time polymerase chain reaction as methodologies. Mice treated with the MMP inhibitor doxycycline, showed a decrease of heart inflammation, delayed peak in parasitemia and improved survival rates when compared with the control group. Reduced levels of cardiac tumor necrosis factor–α, interferon-γ, and serum nitrate were also observed in the treated group. These results show that MMPs play a role in Chagas myocarditis. An involvement of MMPs were also observed for viral myocarditis with high levels of MMP-9, MMP-2 and MMP-12 [106]. In addition, peptidase-dependent extracellular matrix remodeling is one of the events that it is emerging as a key regulator of *T. cruzi* infection and pathogenesis of Chagas' disease. In this sense, earlier studies from our group showed MMP-9-like activity in the cytoplasm of *T. cruzi* during *in vitro* infection of embryonic hepatocyte cells [99].

Serine Peptidase

Serine peptidases described in *T. cruzi* include two peptidases of the prolyl oligopeptidase family (clan SC, family S9): (i) Oligopeptidase B, involved in Ca^{2+}-signalling during mammalian cell invasion and (ii) a prolyl endopeptidase Tc80 (POP Tc80).

Oligopeptidases B (Tc OP or T. cruzi OpdB)

Oligopeptidases B is a cytosolic enzyme with a molecular mass of 120 kDa, and belongs to the prolyl oligopeptidase family of serine peptidases. Oligopeptidases only hydrolyze peptides smaller than 30 amino acid residues and as a result have no naturally occurring inhibitors. It was proposed that the Ca^{2+} agonist generated by oligopeptidase B is exported from the parasite and binds to a receptor on the

surface of cells, activating phospholipase C and generating inositol phosphate, which binds to its receptor on the membrane of endoplasmic reticulum and promotes Ca^{2+} release [111-113]. Oligopeptidase B null mutant trypomastigotes are defective in mobilizing Ca^{2+} from thapsigargin-sensitive stores in mammalian cells and in establishing infection *in vitro* and *in vivo* [113]. The enzyme is directly involved with the parasite ability to invade a wide variety of mammalian cells and consequently in the establishment of the infection [52, 111-113]. This enzymatic activity could also be associated with Chagas' disease pathogenesis by hydrolyzing host proteins and inducing host immune responses [114].

Prolyl Endopeptidase Tc 80 (POP Tc80)

This enzyme has been identified in cell-free extracts of *T. cruzi* trypomastigotes, amastigotes and epimastigotes. This peptidase mediates the specific degradation of purified human types I and IV collagens and fibronectin. Thus, the enzyme may facilitate *T. cruzi* migration through the extracellular matrix, gaining access to cells in virtually any part of the host body, or may be involved in the host cell invasion, cleaving collagen and interacting with integrin receptors [115-117].

Proteasome

Protein modification by ubiquitin and ubiquitin-like proteins is one of the most complex and intensely studied mechanisms of post-translational protein regulation in eukaryotes. Conjugation of the 76-amino-acid protein ubiquitin is first and foremost a signal for targeting proteins to the proteasome for degradation, but there is evidence that ubiquitin also plays diverse roles in the regulation of numerous biological pathways [118]. Two research groups first identified ubiquitin genes in *T. cruzi* nearly two decades ago [119, 120]. Later, the presence of the 26S proteasome and the ubiquitin pathway in *T. cruzi* were reported for the first time by de Diego *et al*. [121]. The 26S proteasome of *T. cruzi* epimastigotes was identified as a high molecular mass complex (1400 kDa) with an ATP-dependent chymotrypsin-like activity against the substrate Suc-LLVY-Amc (Succinyl Leu-Leu-Val-Tyr-7-amino-4- methylcoumarin).

Experiments with specific inhibitors of proteasome activity such as peptide aldehydes (e.g. N-Acetyl-Leu-Leu-Leucinal, MG132), lactacystin and gliotoxin have helped to define the role of the proteasome in various parasitic protozoa processes, including replication and differentiation [122]. Lactacystin treatment in *T. cruzi* inhibits both the transformation of trypomastigotes to amastigotes and the development of amastigotes into trypomastigotes, thus implicating proteasomes in parasite remodeling [123]. Moreover, lactacystin treatment promotes the conjugation of ubiquitin to flagellar proteins and the accumulation of ubiquitinated products during transformation [121]. Recently, Cardoso *et al.* [124] found that epimastigotes treated with lactacystin were still able to adhere to the substrate, but did not differentiate into metacyclic trypomastigotes. In addition, lactacystin treatment blocked epimastigote multiplication, since flow cytometry data demonstrated that *T. cruzi* epimastigotes cultured in lactacystin-containing LIT medium were arrested at the G2 phase of the cell cycle. These findings suggest that proteasomes are involved in *T. cruzi* cell growth and metacyclogenesis *in vitro*.

Antigenic peptides derived from intracellular proteins are continuously presented to the immune system by MHC class I molecules on the surface of immune and non-immune cells and the major proteolytic system generating peptide ligands are dependent on proteasomes [125]. The 20S proteasome is the key peptidase generating peptides for the MHC class I antigen presentation pathway. In this sense, some studies have been done in order to ask whether *in vitro T. cruzi* infection might influence the gene expression or protein profile of the 20S proteasome. This could be the explanation for the chronic phase of the Chagas' disease in the same way that certain viruses have developed strategies to evade class I-restricted antigen processing and presentation [126]. When Faria *et al.* [127] compared *T. cruzi*-infected HeLa cells with non-infected cells, no differences between the composition of 20S proteasome and expression of its subunits were observed. This included the interferon-γ inducible subunits and failed to induce the formation of immunoproteasome. However, the proteasome trypsin- and chymotrypsin-like activities were higher in infected cells than in non-infected cells.

Recently, three monoclonal antibodies were produced against *T. cruzi* proteasomes (7E5, 25A10 and 18F7) and all of them reacted with a single band of 27 kDa on immunoblots of purified proteasomes. Using the 7E5 antibody which recognizes the α-subunit of protozoan peptidase, *T. cruzi* 20S proteasomes were found not only in the cytoplasm and nucleus but also in the kinetoplast [128]. The proteasome 20S was present in all *T. cruzi* stages in similar amounts. These results point to a possible role of the ubiquitin proteasome system in the kinetoplast biochemistry. This multicatalytic system could be involved in different biological functions such as the cell cycle in the replicative stages of *T.cruzi* or remodeling in trypomastigotes that do not divide. However, further studies need to be carried out to establish the complete function of this structure on *T. cruzi* biology [128].

Aspartic Peptidases

Recently, two aspartic peptidases were isolated from *T. cruzi* epimastigotes, named cruzipsin-I (CZP-I) and cruzipsin-II (CZP-II). Cruzipsin-II was isolated from a soluble fraction and the other was solubilized with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) [129]. Both peptidase activities were inhibited by the classic inhibitor pepstatin A and the aspartic active site labeling agent 1,2-epoxy-3-[phenyl-nitrophenoxy] propane (EPNP). Although the genome of *T. cruzi* has been already decoded, no pepsin-like peptidase has been detected [94]. However, the authors explain that probably several sequences could not be correctly identified because of the great difficulty to correlate homologous genes by using the current computer techniques.

PEPTIDASES IN *LEISHMANIA* SPP.

Cysteine Peptidases

In parasitic protozoa, cysteine peptidases are particularly important for *Leishmania* survival, replication, development, metabolism, host cell infection and evasion of the host immune response. Thus, they have attracted considerable inter-

est as targets for the design of new chemotherapeutic agents and vaccines [57]. *Leishmania* spp. contain three types of clan CA cysteine peptidases the cathepsin L-like CPA and CPB and the cathepsin B-like CPC [57]. These CPs are frequently referred to as the only CPs in *Leishmania*. However, a detailed analysis on the database of the *L. (L.) major* genome reveals a total of 56 genes that codify for hypothetical CPs, which are divided into 4 clans and 13 families. It is possible that these CPs play crucial roles for the parasite, although until now the role of most of these CPs can only be predicted, based on the current knowledge of homologous enzymes [57].

So far, functionally well characterized cysteine peptidases are from the New World species of the *Leishmania mexicana* group. In this complex, the *cpb* genes are multicopy and are located in a single locus of 19 copies arranged in a tandem repeat [130, 131]. The first two copies of CPB, CPB1 and CPB2, are expressed in the infective metacyclic promastigote stage of the parasite, while the others are expressed predominantly in the intracellular amastigote stage [130-132]. Information about the roles and importance of the enzymes in host-parasite interactions was obtained by the generation of a *L. (L.) mexicana* CPB-deficient (*Δcpb)* mutant. It was shown that *Δcpb* promastigotes are less infective to macrophages than wild-type parasites *in vitro* and that *Δcpb* promastigotes and amastigotes are able to form only small, slow-growing lesions in BALB/c mice [130, 133, 134]. Curiously, only marginal recovery of lesion growth, at best, could be restored to *Δcpb* mutants by reinserting individual CPB genes on episomes [130, 134]. However, the reinsertion of multiple CPB genes in these *Δcpb* mutants was able to effectively restore virulence [135]. Therefore, the level of CPB expression or the diversity in CPB isoenzymes or both are important for parasite virulence, which provides an explanation for the presence in *L. mexicana* of a multicopy tandem array of CPB genes [135]. Also shown was that the absence of the *cpb* genes resulted in a shift in the immune response from predominantly Th2 immune response (wild-type) to the Th1 response, normally observed when the CPB isoenzymes are present [133]. It has also been suggested that in *L. mexicana* these enzymes are vital for autophagy and differentiation of the parasite [136]. Recently, our group described that drug-induced *Leishmania* autophagy is accompanied by enhanced cpb expression [137].

The members of the *L. (L.) donovani* complex also possess multiple classes of cysteine peptidases, which are developmentally regulated [138, 139] and are involved in intracellular parasite survival [140]. Furthermore, it was proposed that *L. donovani* and *L. chagasi* cysteine peptidases activate the kinin system, a group of potent vasoactive peptides proteolytically liberated from kininogens, which have been recognized as signals alerting the innate immune system [141].

In despite of the substantial advance in knowledge of the cysteine peptidases from the *L. mexicana* complex and to a lesser degree in the *L. donovani* complex, less is known about the *L. braziliensis*. The genome annotation is in progress and the major CPs gene groups have already been identified. However, there are limited reports on the occurrence of CP activities in this parasite. So far, only cysteine peptidases ranging from 20 kDa to 65 kDa have been reported in *L. braziliensis* [142].

A polyclonal antibody to *L. amazonensis* CPB was able to recognize polypeptides in *L. braziliensis* [143, 144] and its expression seems to be correlated to parasite virulence [144]. A *cpb* gene from *L. (V.) braziliensis* was cloned and expressed, revealing differences in substrate utilization between *L. mexicana* and *L. braziliensis* CPs [145]. Curiously, although several reports have related parasite virulence to the expression of key virulence factors, a recent report indicated that the lizard non-pathogenic to humans *L. tarentoale* expresses an amastin-like, CPB, lipophosphoglycan (LPG) and gp63 molecules at comparable levels to those in *L. major* and *L. infantum*, both at the mRNA and protein levels [146]. Indeed, the "non-pathogenic to humans" concept needs to be revisited in the light that reports of leishmaniasis-like diseases caused by presumed monoxenous trypanosomatids have been increasing drastically in recent years [31, 147].

Calpains, as cited in the topic *T. cruzi* peptidases, were detected in *Leishamnia* spp.. In *L. major*, a total of 27 calpain-like proteins have been described based on a genome search for conserved domains [148]. As yet there are no data on the specific functions for any of the calpain-like proteins in *Leishmania*. In *L. major*, it was demonstrated that a calpain related protein (LmCALP20.2) is up regulated in the promastigote insect stage and LmCALP20.1, coded by the adjacent gene, is up regulated in the subsequent metacyclic insect stage [149]. Our group demonstrated that MDL28170, a potent calpain inhibitor, is capable of reducing promastigote growth in culture and induces cell death [150], probable through apoptosis (unpublished data). A proteomics screen implicated a calpain-related protein in drug resistance in *L. donovani* clinical field isolates, probably by modulating drug-induced apoptosis [151]. Highly sensitive gene expression microarray analysis revealed that calpains, among other genes, are differentially expressed in *Leishmania* parasites isolated from post kala-azar dermal leishmaniasis (PKDL) patients in comparison with those from visceral leishmaniasis (VL) [152]. A calcium-activated peptidase in the cytosolic fraction of *L. donovani* has been found to digest different endogenous proteins when subjected to SDS-PAGE. Gelatin-embedded gel electrophoresis confirms the presence of calcium-dependent peptidase [153]. However, there is no protein sequence linking this activity to a calpain and the absence of amino acid residues essential for catalytic activity and the moderate overall degree of sequence identity of calpains genes suggest that most calpain-like proteins do not act as cysteine peptidases [148].

Metallopeptidases

Leishmanolysin, promastigote surface protease (PSP), major surface protease (MSP), EC 3.4.24.36 or gp63 are the designations for the most abundant surface glycoprotein of *Leishmania* spp. [154], which is distributed over the entire surface of the promastigote forms, including the flagellum [155-157]. Each *L. major* promastigote in the stationary phase is estimated to have 500,000 copies of gp63, constituting about 1% of the organism's total protein content [158]. Experiments using surface biotinylation, cytofluorimetry and immunoelectron microscopy showed that three-quarters of *L. mexicana* gp63 occur on the cell surface, whereas the re-

mainder is located intracellularly [158]. Moreover, gp63 is also released by promastigotes to the extracellular medium in both membrane-associated and free forms [155, 159]. Although the GPI anchor can be cleaved *in vitro* with phospholipase C (PLC) revealing the cross-reacting determinant (CRD) epitope, evidence using antibody against CRD indicates the GPI anchor is not enzymatically cleaved *in vivo* during the release from the *Leishmania* cell [160, 161]. The release of *L. amazonensis* gp63 into the extracellular environment has been shown to be powerfully reduced either in the presence of a metal chelator 1,10-phenanthroline, or in the case of gp63 mutation at the active site. This suggests that gp63 release is dependent on autoproteolysis [160].

All gp63 studied to date in the different *Leishmania* species share high nucleotide sequence identity and the enzyme has been shown to be encoded by a family of tandemly linked genes, all of which map to a single chromosome [162, 163]. Additionally, the crystal structure of gp63 purified from *L. major* promastigotes reveals that this peptidase is a member of the metzincin family of zinc metallopeptidases, with an active site sequence motif of HE*XX*H*XX*G*X*H [164]. Structural studies indicate that the gp63 likely exists as homodimers and both amphiphilic and hydrophilic forms have been found in the same cell [165]. As described above, several *Leishmania* spp. contain distinct classes of *gp63* genes that are developmentally regulated in different parasite life cycle stages, including the amastigote, logarithmic promastigote and metacyclic promastigote forms [166]. Consequently, gp63 plays vital roles in the different stages of the *Leishmania* life cycle.

Also gp63 presents wide substrate utilization, including extracellular matrix components such as type IV collagen and fibronectin, which could facilitate the migration through the sub-endothelial basement membrane, facilitating the dissemination of the parasite in the tissues [167]. On the other hand, the *in vivo* substrates of gp63 are unknown. Several possible additional roles were suggested for the gp63 molecule when the promastigote is inside the mammalian host: (i) it helps promastigote evasion of complement-mediated lysis, by cleaving C3 to its breakdown products [168] as well as converting C3b to the inactive form iC3b [169], (ii) it enhances phagocytosis of promastigotes through macrophage receptors such as CR3 [169-171], (iii) it contains the sequence SRYD that is antigenically related to the RGDS sequence of fibronectin, suggesting a potential interaction of gp63 with macrophage fibronectin receptors [172], (iv) it promotes degradation of host cytosolic MARCKS-related protein [173], (v) it is capable of cleaving surface CD4 and could diminish T cell responses [174] and (vi) it also cleaves intracellular peptides presented by MHC class I molecules [175].

The relevance of gp63 molecules on the interaction with the invertebrate host has been the subject of recent publications. Gp63 is predominantly present on the surface of promastigotes, which reside in the midgut of the phlebotomine sandfly vector [176, 177]. However, *L. major* gp63 knockout presented a similar number of metacyclic promastigotes in the anterior gut of *Phlebotomus dubosqui* [178]. On the other hand, the down-regulation of gp63 in *L. amazonensis* affected considerably the parasite development in *Lutzomyia longipalpis* [179]. Our group has demonstrated that leishmanolysin-like proteins from insect and phytoflagellate trypanosomatids can function as adhesive molecules, promoting the connection of live parasites to the invertebrate host epithelial cells [15]. Indeed, an insect receptor able to bind purified gp63 has been identified [29].

Although there are no records about MMPs in *Leishmania* spp., Costa and co-workers *et al.* [180] observed the presence of MMP-9-like activity in *L. chagasi*-hepatocyte-macrophage co-culture supernatants, where the higher MMP-9 activity coincided with an increase in TGF-β production which took place when macrophage leishmanicidal activity was the highest. This fact suggests that this cytokine could be participating in metallopeptidase activation/conversion and probably in matrix deposition by hepatocytes in culture.

Serine Peptidases

The first experimental evidence of serine peptidases in *Leishmania* spp. was demonstrated in 2000 by Almeida-Campos and Horta [181]. The authors correlated the lytic effects of crude extracts of *L. amazonensis* to macrophages due to a serine peptidase, based on the inactivation of this effect by specific inhibitors. A serine peptidase-like triad was later identified in *L. major*, the enzyme hydrolyzed benzoyl-Arg-p-nitroanilide, an activity sensitive to specific serine peptidase inhibitors [182]. In *L. amazonensis*, subcellular localization studies by immunoelectron microscopy suggested that a promastigote serine peptidase of approximately 56 kDa is predominantly located in the flagellar pocket and in vesicular structures that are morphologically similar to the compartments of mammalian endocytic as well as exocytic pathways. In amastigotes, the enzyme was found to be located in electron dense structures corresponding to megasomes in addition to subcellular structures like flagellar pocket and cytoplasmic vesicles, as seen in promastigotes [183]. An additional 68 kDa [184] enzyme was identified and seems to be present at the cell surface, as well as in cytoplasmic membranous compartments of the parasite [185]. In addition, a 110 kDa serine peptidase that occurs in the detergent-soluble extract of this parasite seems to be a dimer of 60 and 45 kDa [186]. A released enzyme of 110 kDa was also identified and seems to occur as a homodimer [187]. Similar enzymes were also identified *in L. braziliensis* [188] and *L. donovani* [189]. A common feature of these enzymes seems to be their ability to digest a wide range of proteinaceous substrates and several enzymes are homodimeric or heterodimeric proteins. As yet, no specific functions have been addressed to these enzymes. The toxic effects of classical serine peptidase inhibitors on *L. amazonensis* parasites were correlated to enzymatic inhibition [190].

Aspartic Peptidases

The activity of aspartic peptidases was first demonstrated in 2005 [191]. Alves and co-workers showed that metallo, serine and aspartic peptidase activities are down-regulated during the shock-induced transformation of promastigotes into amastigotes, by the hydrolysis of specific chromogenic substrates. Later, Valdivieso *et al.* [192] reported the presence of an enzyme capable of degrading the synthetic substrate benzoyl-Arg-Gly-Phe-Phe-Leu-4-methoxy-β-naphthyl-

amide, which is selective for cathepsin D like aspartic peptidases. Diazo-acetyl-norleucinemethylester (DAN) not only inhibited the enzyme activity but also interfered in parasite growth in culture. The dramatically increasing numbers of patients co-infected with *Leishmania* and HIV [193], together with data from fungal infections showing that the inhibitors of HIV aspartic peptidases (HIV-PIs) have a direct effect on opportunistic pathogens [194] instigated researchers to seek for direct effects of HIV-PIs on *Leishmania*. In this context, Savoia and co-workers in 2005 [195] first demonstrated the dramatic effects of indinavir and saquinavir on the growth of *L. major* and *L. infantum*. Later, it was demonstrated that HIV-PIs powerfully reduce *L. infantum* infection in macrophages, either co-infected or not with HIV [196]. Our research group demonstrated that the hydrolysis of a HIV peptidase substrate by *L. amazonensis* extract was inhibited by pepstatin A and several HIV-PIs, suggesting that an aspartic peptidase may be the intracellular target of the inhibitors [137]. In addition, HIV-PIs also impaired *L. amazonensis* growth and interaction with macrophages, indicating that the HIV-PIs are active against a wide range of *Leishmania* species. Also, the HIV-PIs induced several critical ultrastructural alterations in *L. amazonensis* promastigotes, which culminated with parasite death, probably through an imbalance between apoptosis and autophagy [137]. Despite all these beneficial effects, the HIV-PIs induced an increase in the expression of cpb and gp63, two well-known virulence factors expressed by *Leishmania* spp. [137]. The anti-proliferative effect of aspartic peptidase inhibitors makes this enzymatic class a putative new target for the development of leishmanicidal drugs. In addition, in the face of the leishmaniasis/HIV overlap, it is critical to further comprehend the sophisticated interplays among *Leishmania*, HIV and macrophages. In addition, there are many unresolved questions related to the management of *Leishmania*-HIV-coinfected patients. For instance, the efficacy of therapy aimed at controlling each pathogen in coinfected individuals remains largely undefined

Proteasome

Earlier studies have demonstrated the presence of proteasome in several species of the *Leishmania* genus [52]. The *L. mexicana* 20S proteasome has a molecular mass of around 670 kDa, which is consistent with the size of 20S proteasomes from other species [197], and it is composed of at least 10 distinct subunits in the 22 to 32 kDa size range. However, the molecular mass of the *L. mexicana* proteasome increases to 1200 kDa in the presence of ATP, consistent with there being a 26S proteasome in the parasite, extending, thus, the range of eukaryotic species known to have the 26S form of the proteasome. However, this proteasome has a substrate preference profile more similar to proteasomes from higher eukaryotes than from the related protozoan *T. brucei*, which has an unusually high chymotryptic peptidase activity [198]. On the other hand, the purified *L. chagasi* 20S proteasome possesses proteins with molecular masses of 22-35 kDa and exhibits a higher trypsin-like activity compared to chymotrypsin-like activity, as previously reported by Hua and colleagues for *T. brucei* [198].

Similar to what has been observed in *T. cruzi*, studies with specific inhibitors of proteasome activity have also helped to define the role of the proteasome in the *Leishmania* genus. For instance, lactacystin and its active form clasto-lactacystin b-lactone blocked the *in vitro* growth and intracellular survival of *L. chagasi* promastigotes and arrested the intracellular development of the lactacystin-treated parasites during the infectivity of *L. chagasi* promastigotes in mouse peritoneal macrophages [199]. The involvement of the proteasome in the regulation of *L. mexicana* cell cycle progression was demonstrated by the addition of a peptide aldehydes inhibitor (MG132), since this drug caused an accumulation of promastigotes with 4n DNA content. This fact suggests that proteasome is required after DNA synthesis and before completion of mitosis, such that in conditions in which the proteasome is inactivated, parasite growth is arrested at this particular point of the cell cycle [200].

PEPTIDASES IN AFRICAN TRYPANOSOMES

Cysteine Peptidases

The major proteolytic activities in African trypanosomes result from cysteine peptidases, whose orthologs are found in all the species analyzed and collectively named trypanopains [201-203]. These enzymes share structural and functional similarities with *T. cruzi* cruzipain [203-206].

T. congolense possesses at least two families of CPs, named CP1 and CP2, the latter also known as congopain or trypanopain-Tc [207], while the enzymes in *T. b. brucei* and *T. b. rhodesiense* are termed trypanopain-Tb (or brucipain) and rhodesain, respectively [204, 205].

Congopain is a lysosomal enzyme that was purified from bloodstream forms [208], but it differs from mammalian cathepsins B and L by its broad pH activity profile and its high stability at neutral pH, together with the presence of a C-terminal extension [209]. Congopain is a dominant antigen of trypanosomes, eliciting both humoral and cellular responses in cattle [206] and antibody-mediated inhibition of trypanosomal CPs may contribute to mechanisms of trypanotolerance [210]. Detection of procongopain and trypanosomal enzymatic activity in the plasma of *T. congolense*-infected cattle suggests that the enzyme may interact with host proteins during infection, such as to reduce the proliferation of bovine peripheral blood mononuclear cells in response to mitogens and antigens, as well as to interact with bovine kininogens to trigger the release of kinins [206]. It is also known that α-2-macroglobulin effectively inhibits cysteine peptidase activity in African trypanosomes [204]. These data, together with the capacity of procongopain to be activated at a weakly basic pH, suggest that procongopain may be extracellularly processed in the presence of blood vessel glycosaminoglycans, supporting the hypothesis that the enzyme acts as a pathogenic factor in the interaction of the parasite with its host [211].

Brucipain has also been shown to form active complexes with kininogen, and these circulating complexes may confer some advantage to the parasite by modulating host physiology or the immune response [212]. Brucipain and rhodesain are also located in the lysosome, mainly in *T. b. rhodesiense* in long slender forms and in *T. b. brucei* in short stumpy forms [205]. These activities are considered targets for chemotherapy and inhibitors of these peptidases were shown to kill the parasites both in culture and in experimentally

infected animals, the killing being correlated with inhibition of trypanopains [205, 213]. The investigation into the roles of the endogenous inhibitor of cysteine peptidase (ICP) revealed that ICP acts as a regulator of brucipain activity, playing an important role in the modulation of surface coat exchange during differentiation, intracellular proteolysis and parasite infectivity to mice [214]. It was also demonstrated that brucipain plays an important role in the bloodstream forms of *T. b. gambiense* transendothelial migration of the human blood-brain barrier, which is correlated to the ability to evoke calcium fluxes in brain microvascular endothelial cells [215].

T. brucei also possesses a cathepsin B-like peptidase that is mainly located inside the endosomal vesicles, although it is actively found in the form of a circulating antigen as well [201, 216].

Metallopeptidases

Besides cysteine peptidases, another class of proteolytic enzymes found in African trypanosomes corresponds to homologues of *Leishmania* spp. major surface protease (MSP) [217]. *T. brucei* has three classes of *MSP* genes, called *TbMSP*-A, -B and –C that are differentially expressed during the parasite life cycle. In addition, TbMSP-B protein is a surface-located metallopeptidase that plays a role in the release of the variant surface glycoprotein (VSG) during differentiation from bloodstream forms to procyclic trypanosomes, being constitutively expressed in the latter [218].

Serine Peptidases

African trypanosomes also contain a serine oligopeptidase, oligopeptidase B (OpdB), which is implicated in the pathogenesis of these parasites, since it is released into the bloodstream of infected mammalian hosts [219-221], where it retains its catalytic activity and may participate in the anomalous degradation of host peptide hormones [222]. In this sense, oligopeptidases B display similar properties to several hormone-processing enzymes, thus host-peptide hormones are candidates for cleavage by the parasite oligopeptidases [222]. In fact, the enzyme has been identified as a target of several trypanocidal drugs, such as suramin [223] and its proper characterization may lead to the design of highly selective inhibitors to be employed as trypanocidal drugs [222].

Proteasome

The first proteasome to be purified and characterized from a parasite was *T. brucei* proteasome and several differences between *T. brucei* and mammalian proteasomes have been found, such as their molecular mass (the 20S proteasome has a molecular mass of 630 kDa in trypanosomes compared with 700 kDa in mammals), and substrate specificity (*T. brucei* proteasome exhibits high trypsin-like but lower chymotrypsin-like activities compared with the mammalian counterparts) [52]. Nevertheless, the evidence of 26S proteasome from *T. brucei* demonstrated that the absence of degradation of mammalian ornithine decarboxylase-antienzyme complex, which catalyzes the first step in the polyamine biosynthetic pathway, is the real difference between mammalian and trypanosomal 26S proteasome [224].

Preceding studies reported that lactacystin inhibited the proliferation of both the mammalian and the insect forms of *T. brucei* and arrest them in the G2/M and G1/S phases, respectively [200]. Two of five peptide trileucine methyl vinyl sulphones tested, containing different substituents at the N-terminus (or P4 position) showed trypanocidal activities *in vitro* using culture-adapted bloodstream forms of *T. brucei*, since they inhibited trypsin-like activity [122, 225]. This may be attributed to the high and the low chymotrypsin-like activity of the trypanosomal proteasome, while the opposite is the case with the mammalian proteasome [198]. Therefore, proteasome inhibitors specifically targeting the proteasomal trypsin-like activity may be the rational choice for future anti-sleeping sickness drug development.

PEPTIDASES IN LOWER TRYPANOSOMATIDS

Up to now, the proteolytic activities extensively described in lower trypanosomatids belong to the metallo and cysteine peptidase classes [5, 14, 18, 226]. In this context, peptidases with similar biochemical and/or immunological properties to the gp63 [165] and cruzipain [227] were identified in different species of *Herpetomonas* (*H. samuelpessoai*, *H. roitmani*, *H. anglusteri* and *H. megaseliae*), *Leptomonas* (*L. samueli*, *L. seymouri*, *L. collosoma* and *L. wallacei*) *Crithidia* (*C. fasciculata*, *C. guilhermei* and *C. deanei*), *Blastocrithidia* (*B. culicis*) and *Phytomonas* (*P. serpens* and *P. françai*) [6-18, 20-26, 28-30, 154, 228-232].

Cysteine Peptidases

Lower trypanosomatids also produce cysteine peptidase activities, whose expression is dependent of growth conditions, like culture medium and time of incubation. However, all the cysteine peptidases are active at acidic pH and completely restrained by E-64, a powerful cysteine peptidase inhibitor. In addition, the cysteine peptidases produced by these flagellates were exclusively detected in the parasite cellular extracts, except for *C. desouzai*, *C. deanei* [23] and *P. serpens* [16, 20], whose cysteine peptidase activities were also characterized in the extracellular medium. At least in *P. serpens*, both cellular and extracellular cysteine peptidases were able to degrade several proteinaceous substrates, suggesting a possible participation of these enzymes in parasite nutrition [16, 20]. Moreover, a 40 kDa cysteine peptidase synthesized by *P. serpens* cells cleaved a 115 kDa surface polypeptide located at the surface of a salivary gland from *Oncopeltus fasciatus*, a phytophagous insect, implicating a possible participation of this cysteine peptidase in interaction processes [20]. Corroborating the preceding statement, cysteine peptidase inhibitors were capable of blocking the adhesion process between *P. serpens* promastigote cells and salivary glands of *O. fasciatus* [16]. In *H. samuelpessoai*, a 45 kDa cysteine-type peptidase was down-modulated during the cellular differentiation triggered by both temperature switch and dimethylsulfoxide treatment [13, 232]. Cysteine peptidase production was up-modulated by the presence of endosymbiont in *B. culicis* [26].

In lower trypanosomatids two distinct cysteine-type peptidases were reported with biochemical/immunological similarities to corresponding enzymes produced by pathogenic flagellates designated as cruzipain-like (detected in *B. culicis* and *P. serpens*) and cpb-like (identified in *H. samuelpessoai*)

[16, 26, 232]. Cruzipain-like molecules expressed by *P. serpens* were immunocytochemically evidenced along the whole extension of the membrane, coating the cell body and the flagellum, as well as in cytoplasmic compartments [16]. The 40 kDa cruzipain-like molecule located at the *P. serpens* cell surface is attached to membrane domains *via* a GPI and fluorescence microscopy showed that cruzipain-like molecules were preferentially located on the posterior end of *P. serpens* cells, resembling *T. cruzi* cruzipain distribution [232].

Furthermore, calpain molecules containing some degrees of similarity with calpain produced by *Drosophila melanogaster* were also reported in two insect flagellates, *C. deanei* [25] and *H. samuelpessoai* [232]. Interestingly, the 80 kDa calpain-like molecules, produced by *C. deanei* cells, were purified from the culture filtrate, showing distinct properties from the well-characterized mammalian calpains, but some degree of similarity was displayed to invertebrate calpain-related enzymes [25]. In *H. samuelpessoai*, a 80 kDa calpain-like molecule was identified and its expression was enhanced after DMSO-triggered differentiation, suggesting a participation during the process of promastigote into paramastigote transformation [232].

Metallopeptidases: gp63-Like

Gp63-like molecules expressed in lower trypanosomatids were detected on both cell-associated and secreted forms. Typically, the cellular gp63-like molecules were mainly attached to the cell surface by a GPI anchor; however, a small amount of this enzyme can be detected in cytoplasmic compartments, which may indicate the occurrence of hydrophilic isoforms, or the detection of enzymatic activity from precursor molecules that are further transported to the plasma membrane [13]. Curiously, two distinct populations with different affinities for the anti-gp63 antibody were identified in *H. samuelpessoai*, *L. wallacei*, *C. deanei* and *C. guilhermei* by means of flow cytometry analyses, indicating that gp63-related molecules are not equally expressed on the surface of trypanosomatid cells [19, 28, 231]. The lack of equal expression may be correlated to the parasites growth and/or cell cycle phases, since flagellate cultures were not synchronized. Furthermore, the occurrence of distinct subpopulations could alternatively denote a different expression of surface gp63-like molecules in the different developmental stages or even a diminished accessibility to external ligands in cell subsets, as previously reported for other trypanosomatid cell surface molecules [12, 27]. Conversely, the extracellular isoform can be released from parasite cells by two mechanisms that follow the classic secretory pathway with routing of gp63 from the endoplasmic reticulum to the Golgi network and then into the flagellar pocket. Vesicles containing both membrane-bound and free gp63 fuse with the flagellar pocket membrane and thereafter the free gp63 is released extracellularly [30], while the membrane-bound form is located throughout the external cell membrane and a proportion of this is released directly *via* autoproteolysis [19], in a similar mechanism to that observed in *Leishmania* [160, 161]. Both cellular and released gp63-like molecules had similar biochemical features, including optimum pH around 5.0–6.5 (the prevailing pH range of the insect gut and the pH of phagolysosome), susceptibility to metallo-type peptidase inhibitors mainly 1,10-phenanthroline, molecular mass around 50–70 kDa, immunological reactivity against anti-gp63 antibodies, best temperature at 37ºC (mammalian temperature) instead of 26ºC (invertebrate temperature) and broad hydrolytic capability (degrading gelatin, hemoglobin, casein, albumin, mucin, immunoglobulin and insect proteinaceous components), corroborating the hypothesis of conservation of an ancient and functionally relevant molecule shared by both heteroxenous and monoxenous trypanosomatids [15].

The gp63-like molecules contribute to adhesive processes in lower trypanosomatids. In this respect, binding assays with explanted guts or salivary glands of insects incubated with purified gp63 (in an active form or inactivated by heating or removal of essential ions from the active site) and the pre-treatment of trypanosomatids with anti-gp63 antibodies, metallopeptidase inhibitors or phospholipase C showed a reduced adhesion of these trypanosomatids to the insect gut wall or salivary glands, respectively (Fig. **2**). At least to *Aedes aegypti*, a suitable experimental model to study the interaction of monoxenous trypanosomatids and insects, a receptor of 50 kDa for the gp63 purified molecule was identified in the protein insect gut extract [28].

Another interesting remark regarding the monoxenous trypanosomatid is that some of them present a bacterium symbiont in their cytoplasm, conferring a suitable model for the symbiosis studies. It is well known that the symbiont interferes with several aspects of the host trypanosomatid physiology [233], including the production of proteolytic enzymes [10, 23, 26]. In this sense, previous studies showed that endosymbiont-bearing strains are more efficient in binding to insect cell lines and midguts, as compared to symbiotic free strains, which is probably related to differential expression of surface molecules [27, 234]. For example, the endosymbiont of *C. deanei* influences the expression of surface gp63 molecules. Additionally, the number of wild *C. deanei* bound to *A. aegypti* explanted guts was twice as many as those of aposymbiotic parasites. Flow cytometry assays revealed that the reactivity of the wild strain with anti- p63 antibodies was approximately double that of the aposymbiotic strain, suggesting that the higher expression of surface gp63 by the wild strain of *C. deanei* may positively influence this interaction, posing a prominent advantage for the endosymbiont-containing trypanosomatids [30]. In a similar way, the endosymbiont positively influenced the interaction of *C. deanei* with mammalian fibroblasts and the surface gp63-like molecules of this parasite were also implicated in this adhesive process [230].

PERSPECTIVES

Trypanosomatids elaborate a large array of peptidases, which are intracellular and/or extracellular, with different specificities and many of them have been purified and characterized and their genes cloned and sequenced. In this sense, much progress has been made in understanding peptidase function in protozoan parasites. We are gaining a fascinating picture of the often unique and the highly specialized roles of peptidases in these organisms, playing central functions in diverse processes such as nutrition, host cell invasion, catabolism of host proteins, parasite differentiation, cell cycle progression and both stimulation and evasion of host

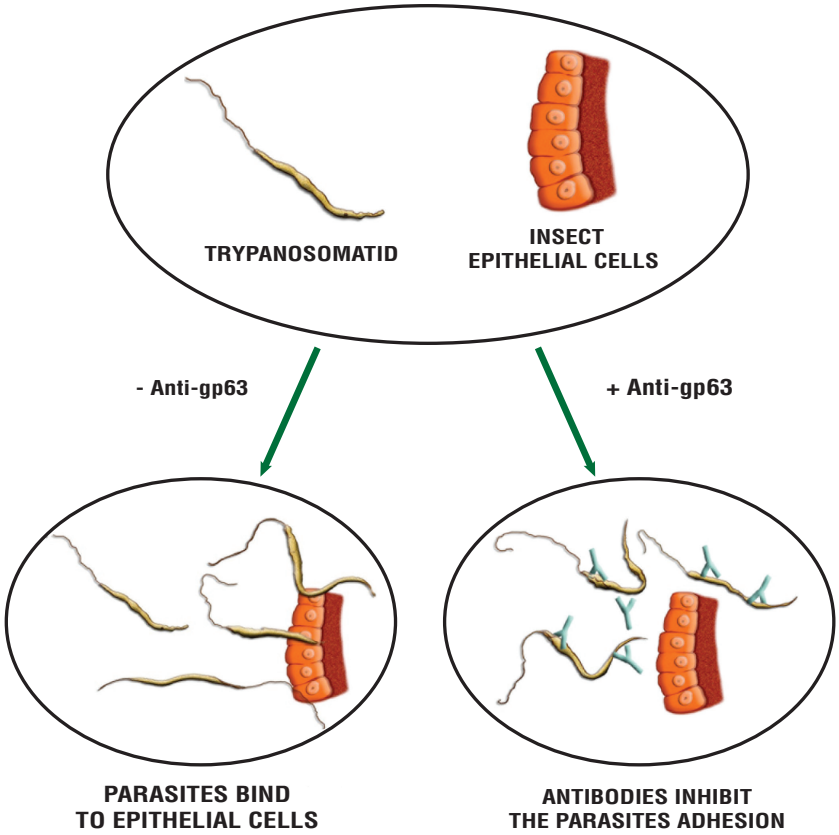

Fig. (2). The schematic representation shows a trypanosomatid cell expressing at the surface gp63-like molecules. The adhesion rate to the insect epithelium *in vitro* is evaluated in untreated (– anti-gp63) or parasites previously treated with anti-gp63 antibodies (+ anti-gp63). This treatment led to reduced levels of adhesion.

immune responses. In addition, because of their unusual structural features, developing a new generation of chemotherapeutic agents for parasitic diseases, such as Chagas' disease and leishmaniasis, is also a critical research priority. Furthermore, the lower trypanosomatids share similar molecules from classical trypanosomatid pathogens, including virulence factors like peptidases [15, 17]. These observations indicate that the life cycle of lower trypanosomatids has been underestimated and that homologous to virulence attributes from pathogens should fulfill a more diverse role for these trypanosomatids than previously thought and confirm their importance as a model for biochemical and molecular studies among the trypanosomatids.

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ABBREVIATIONS

CPs = cysteine peptidases

gp63 = glycoprotein with 63kDa

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