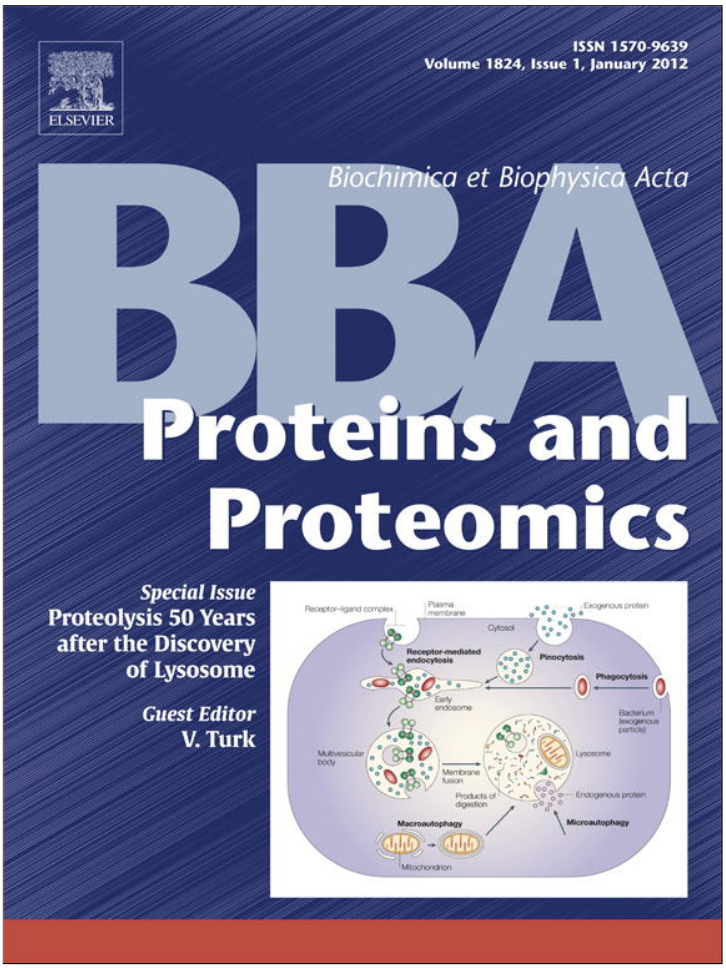

Biochimica et Biophysica Acta 1824 (2012) 195–206

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Review

The peptidases of *Trypanosoma cruzi*: Digestive enzymes, virulence factors, and mediators of autophagy and programmed cell death ☆

Vanina E. Alvarez, Gabriela T. Niemirowicz, Juan J. Cazzulo *

Instituto de Investigaciones Biotecnológicas (IIB-INTECH, Universidad Nacional de San Martín-CONICET). Avenida General Paz 5445, 1650 San Martín, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 10 March 2011
Received in revised form 11 May 2011
Accepted 12 May 2011
Available online 19 May 2011

Keywords:
Trypanosoma cruzi
Chagas disease
Peptidases
Cruzipain
Metacaspases
Autophagins

ABSTRACT

Trypanosoma cruzi, the agent of the American Trypanosomiasis, Chagas disease, contains cysteine, serine, threonine, aspartyl and metallo peptidases. The most abundant among these enzymes is cruzipain, a cysteine proteinase expressed as a mixture of isoforms, some of them membrane-bound. The enzyme is an immunodominant antigen in human chronic Chagas disease and seems to be important in the host/parasite relationship. Inhibitors of cruzipain kill the parasite and cure infected mice, thus validating the enzyme as a very promising target for the development of new drugs against the disease. In addition, a 30 kDa cathepsin B-like enzyme, two metacaspases and two autophagins have been described. Serine peptidases described in the parasite include oligopeptidase B, a member of the prolyl oligopeptidase family involved in Ca^{2+}-signaling during mammalian cell invasion; a prolyl endopeptidase (Tc80), against which inhibitors are being developed, and a lysosomal serine carboxypeptidase. Metallopeptidases homologous to the gp63 of *Leishmania* spp. are present, as well as two metallocarboxypeptidases belonging to the M32 family, previously found only in prokaryotes. The proteasome has properties similar to those of other eukaryotes, and its inhibition by lactacystin blocks some differentiation steps in the life cycle of the parasite. This article is part of a Special Issue entitled: Proteolysis 50 years after the discovery of lysosome.

1. Introduction

The American Trypanosomiasis, Chagas disease, is an endemic disease prevalent in all Latin American countries; the infected population is estimated as about 11–18 million, with many more people being at risk [1]. Only two drugs are available for treatment, Nifurtimox and Benznidazole, but they have a number of side effects and are not effective in all cases. This makes urgently necessary the development of new drugs, more efficient, less toxic and affordable to the poor people, who are most of the infected population. The disease is caused by a flagellated Protozoan parasite, *Trypanosoma cruzi*; the natural form of transmission is by a triatomine insect vector [1]. The life cycle of *T. cruzi* involves four major developmental stages (Fig. 1). The parasite enters the mammalian host when the insect defecates in the vicinity of the bite and the natural infective stage, the metacyclic trypomastigote is carried into the wound by scratching, and then penetrates and infects nearby cells. Once inside the cell, metacyclic trypomastigotes differentiate into amastigotes. These replicative forms multiply in the cytoplasm and, after several rounds of replication, differentiate into trypomastigotes which gain access into the bloodstream and eventually invade new cells, thus perpetuating the infection. When the insect bites an infected mammal, the trypomastigotes carried over with the blood meal differentiate into epimastigotes, which are a replicative form living in the insect gut. In the rectum, where the insect's urine is discharged, the epimastigotes differentiate to metacyclic trypomastigotes, which are able to start a new round of infection.

2. The peptidases of *Trypanosoma cruzi*

Since the identification of a number of proteolytic activities in cell-free extracts of epimastigotes in the late 1970s [2,3], several enzymes have been purified from the parasite and characterized. In addition, the completion of the *T. cruzi* Genome Project in 2005 [4] has identified a number of putative peptidases, most of which have not been biochemically characterized yet. They include cysteine peptidases (CPs), serine peptidases (SPs), metallo peptidases (MPs), aspartyl peptidases (APs) and the proteasome. Table 1 summarizes

Abbreviations: CPs, cysteine proteinases; SPs, serine proteinases; MPs, metalloproteinases; APs, aspartyl proteinases; 20S and 26S proteasome: proteasome oligomers with a sedimentation coefficient of 20S and 26S, respectively; Z, *N*-benzyloxycarbonyl; NHMec, amidomethyl coumarine; E-64, *trans*-epoxy succinyl amido (4-guanidino) butane; TLCK, *N*- α-tosyl-lysyl-chloromethylketone; MHC, major histocompatibility complex; C-T, C-terminal domain of cruzipain; Boc, *N*-*t*-butyloxycarbonyl; pNA, *p*-nitroanilide; PCR, polymerase chain reaction; gp63, *Leishmania* surface proteinase (leishmanolysin); POP Tc80, prolylendopeptidase Tc80 (collagenase); *Tc*SCP, *T. cruzi* serine carboxypeptidase; *Tc*MCP-1 and *Tc*MCP-2, *T. cruzi* metallocarboxypeptidase; BbCI, *Bauhinia bauhinioides* cysteine protease inhibitor; PCD, programmed cell death; PE, phosphatidylethanolamine

☆ This article is part of a Special Issue entitled: Proteolysis 50 years after the discovery of lysosome.

* Corresponding author. Tel.: +54 11 4580 7255; fax: +54 11 4752 9639.
E-mail address: jcazzulo@iibintech.com.ar (J.J. Cazzulo).

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doi:10.1016/j.bbapap.2011.05.011

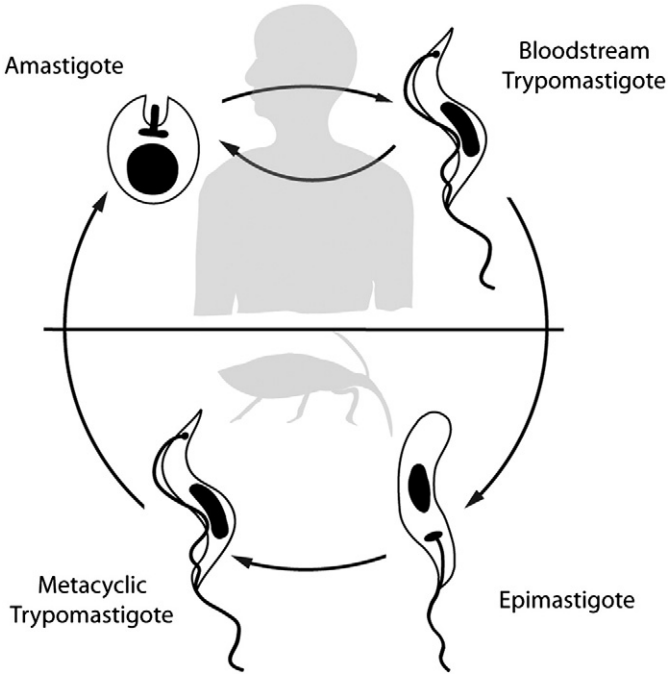

Fig. 1. The life cycle of *T. cruzi*. The parasite has a complex life cycle, with four major stages: an obligate intracellular replicative form, the amastigote, and a non-replicative one, invasive for host cells, the bloodstream trypomastigote, in the mammalian host; a replicative stage, the epimastigote, and a non-replicative one, the infective metacyclic trypomastigote, are present in the insect vector. These forms differ in size, in subcellular organization, and in antigenic and some metabolic properties.

the present knowledge of peptidases according to the data obtained from the MEROPS data base [5] and El-Sayed et al.[4]. Those biochemically characterized in the parasite are mentioned in the right column, and the numbers in brackets represent the estimated number of peptidases in each family.

The *T. cruzi* Genome Project [4] has annotated several clans and families of CPs, most of them belonging to either clan CA or CD. We will discuss in detail cruzipain (1 and 2) and the cathepsin B-like peptidases (family C1); the metacaspases (family C14) and the autophagins (family C54), which have been characterized biochemically; reference to most of the other families can be found in Ref. [6]. A remarkable fact is that 9 out of the 24 sequences related to calpains lack the peptidase domain and only 2 of the remaining 15 posses an intact catalytic triad; moreover, all of them lack the Ca^{2+} binding residues [7]. To date, one calpain-related protein has been studied. The protein has been shown to be present in the major parasite stages and although the recombinant protein has been expressed, its proteolytic activity has not been demonstrated [8]. It is also remarkable that the fully sequenced genomes of *Trypanosoma brucei*and *Leishmania major* Friedlin also present a high number of genes with some homology to calpains. To the best of our knowledge, none of the products of these genes has been shown to have proteolytic activity; however, their number and conservation in the three Trypanosomatids suggest that they must have some relevant, but still unknown, function.

Among SPs, the absence of members of the family S1 (trypsin and chymotrypsin-like enzymes) is noteworthy. Only three members of the clan SC have been detected; two of them, belonging to the S9 family, have been implicated in cell invasion (see below).

MPs represent the largest group of proteases in the *T. cruzi* genome covering nine different clans and 18 families including endopeptidases as well as oligopeptidases, amino and carboxypeptidases. In particular, the genes encoding the leishmanolysin-like metalloprotease or gp63 (clan MA, family M8) have been extensively amplified in the T. cruzi genome (more than 420 genes and pseudogenes) when compared to T. brucei and Leishmania spp. (13 and 6 respectively).

The genomic data [4] report only two APs, which have homology with those encoding presenilin and a signal peptide peptidase, both belonging to the family A22; genes predicting enzymes belonging to the A1 family (pepsin) have not been found. However, two enzymes, named "cruzipsins" have been recently described and are inhibited by inhibitors of the A1 family peptidases [9]. Since no amino acid sequences were reported [9] it is not possible to link these enzymes to any of the genes detected in *T. cruzi*.

The peptidases from *Leishmania* spp. have been recently reviewed [10]; cross-reference to some of the peptidases from *T. brucei* will be made in the following sections.

3. Cysteine peptidases

3.1. Cruzipain

The best characterized CP in *T. cruzi* is cruzipain [11], also known as cruzain [12] or GP57/51 [13]; the enzyme is expressed as a mixture of isoforms (see [14], for a recent review). The least homologous isoform, which has the most divergent properties, has been named "cruzipain 2" [15]. Cruzipain was first reported in cell-free extracts of epimastigotes by Itow and Camargo [2], and purified to homogeneity by Bontempi et al. [16]. The enzyme is expressed in the four main stages of the parasite, and is present in lysosome-related organelles; the highest concentration is found in an epimastigote-specific prelysosomal organelle called "reservosome" (Fig. 2) [17,18]. In addition there are minor isoforms associated to the plasma membrane, presumably through a GPI anchor [19]; the membrane localization is particularly significant in the amastigotes [20]. Some isoforms are secreted into the medium by the trypomastigotes, and this is highly relevant for the role of cruzipain as a virulence factor in Chagas disease [21,22]. Cruzipain 2 is mainly expressed in trypomastigotes and amastigotes [23].

Cruzipain is an endoproteinase able to digest proteins at acidic pH values (optimal pH 3–5), and blocked chromogenic and fluorogenic substrates with optimal pH values of 7 to 9. In the latter case, it prefers Arg or Lys at the P1 position, and a hydrophobic or a positively charged residue at P2 [11]. When acting on the oxidized A and B chains of insulin, however, it acted better on peptide bonds having bulky hydrophobic residues at P1 (with the exception of a major cleavage site at Glu in the B chain), and also at P2 and P3; the peptide bonds involving Arg and Lys in the B chain were not cleaved [24]. Several studies with synthetic substrates [25–27] indicated that specificity toward cruzipain was highest with Pro at P2', and confirmed a requirement for a hydrophobic residue at P2 (although a positively charged residue can also be accepted in this position) and a clear preference for Arg (or benzyl-Cys) at P1; on the other hand, the enzyme was able to accept a broad range of amino acid residues at P1'. The substrate specificity of cruzipain, which seems intermediate between those of cathepsins L and B, since the enzyme is able to accommodate either a hydrophobic or a positively charged residue at P2, is consistent with the presence of Glu at position 205 [28]. Recombinant cruzain has been shown to have carboxydipeptidase activity, very similar to that of cathepsin B [29].

The specificity of cruzipain 2 has been less studied, and differs from that of cruzipain, both when using kininogen as substrate and measuring bradykinin release [27], and for the hydrolysis of several synthetic substrates [23]. Recombinant cruzain and cruzipain 2 differ substantially in the specificity for the S2, S'1 and S'2 pockets [30].

Cruzipain is inhibited by a number of small synthetic molecules, E-64 (*trans*-epoxy succinyl amido (4-guanidino) butane) being the best inhibitor [31]. Protein inhibitors of cruzipain include cystatins, stefins, and kininogens [26,32,33], two members of the family of thyropins, namely a fragment of the Major Histocompatibility Complex (MHC)

class II-associated p41 invariable chain [34]; equistatin from the sea anemone *Actinia equina*[35]; an 18 kDa protein purified from seeds of *Bauhinia bauhinioides* (BbCI) [36] and a *T. cruzi* 12 kDa protein, chagasin [37]. BbCI is a Kunitz-type inhibitor which lacks disulfide bridges [38]; its structure has been determined by X-Ray crystallography [39]. The structure of chagasin has also been determined, both by NMR [40] and X-Ray crystallography, in the latter case free [41] and in complex with human cathepsin L [42]. The pro-domain of cruzipain has also been shown to be a potent selective inhibitor of the enzyme [43].

Table 1
Summary of peptidases detected in *T. cruzi* according to the Genome Project and the MEROPS database.

Clan	Family	Type peptidase	Experimentally characterized
Cysteine (C) peptidases			
CA			
	C1	Papain-like* (130)[a]	*Cruzipain, cathepsin B*
	C2	Calpain-like (24)	
	C12,19,65	Deubiquitinating enzymes (2 + 17 + 1)	
	C54	Autophagins (2)	*Atg4*
	C78	UfSP-1 peptidase (1)	
	C88	OTU1 peptidase (1)	
CD			
	C13	GPI transamidase (1)	
	C14	Metacaspase* (16)[b]	*TcMCA3, TcMCA5*
	C50	Separase (1)	
CE			
	C48	deSUMOylating enzyme (1)	
CF			
	C15	pyroglutamyl-peptidase I (1)	
PC			
	C26	γ-glutamyl hydrolase (1)	
Metallo (M) Peptidases			
MA			
	M1	Aminopeptidase N (3)	
	M3	Thimet oligopeptidase (2)	
	M8	Leishmanolysin-*like* (174)*	*GP63*
	M32	Carboxypeptidase *Taq* (2)	*TcMCP-1, TcMCP-2*
	M41	FtsH peptidase (5)	
	M48	Prenyl peptidase-1 (1)	
	M80	wss1 peptidase (1)	
MC			
	M14	Carboxypeptidase A (3)	
ME			
	M16	Pitrilysin-signal peptidase (3)	
MF			
	M17	Leucyl aminopeptidase (3)	
MH			
	M18	Aminopeptidase I (1)	
	M20	Glu carboxypeptidase* (9)	
MK			
	M22	O-sialoglycoprotein peptidase (2)	
MG			
	M24	Methionyl aminopeptidase 1 (4)	
MJ			
	M38	Isoaspartyl dipeptidase (1)	
MP			
	M67	AMSH deubiquitinating peptidase (3)	
M-			
	M76	Atp23 peptidase (1)	
	M79	prenyl peptidase 2 (1)	
Serine (S) Peptidases			
SB			
	S8	Subtilisin (2)	
SC			
	S9	Prolyl oligopeptidase (8)	*OPB, Tc80*
	S10	Carboxypeptidase Y* (22)[c]	*TcSCP*
	S15	Xaa-Pro dipeptidyl-peptidase (1)	
	S28	Lysosomal Pro-Xaa carboxypeptidase (1)	
	S33	Prolyl aminopeptidase (2)	
SF			
	S26	Signal peptidase I (2)	
Threonine (T) Peptidases			
PB			
	T1	Proteasome (15)	Proteasome
Aspartic (A) Peptidases			
AD			
	A22	Presenilin 1 and signal peptidase 2 (2)	

* The existence of sequences displaying high degree of identity makes unfeasible the exact discrimination into individual genes.
[a] Estimated by Campetella et al.. The figure given was estimated for the Tul2 strain; other strains have lower number of cruzipain genes [44].
[b] Estimated by Kosec et al.[91].
[c] Estimated by S. F. Parussini and J. J. Cazzulo (unpublished results).

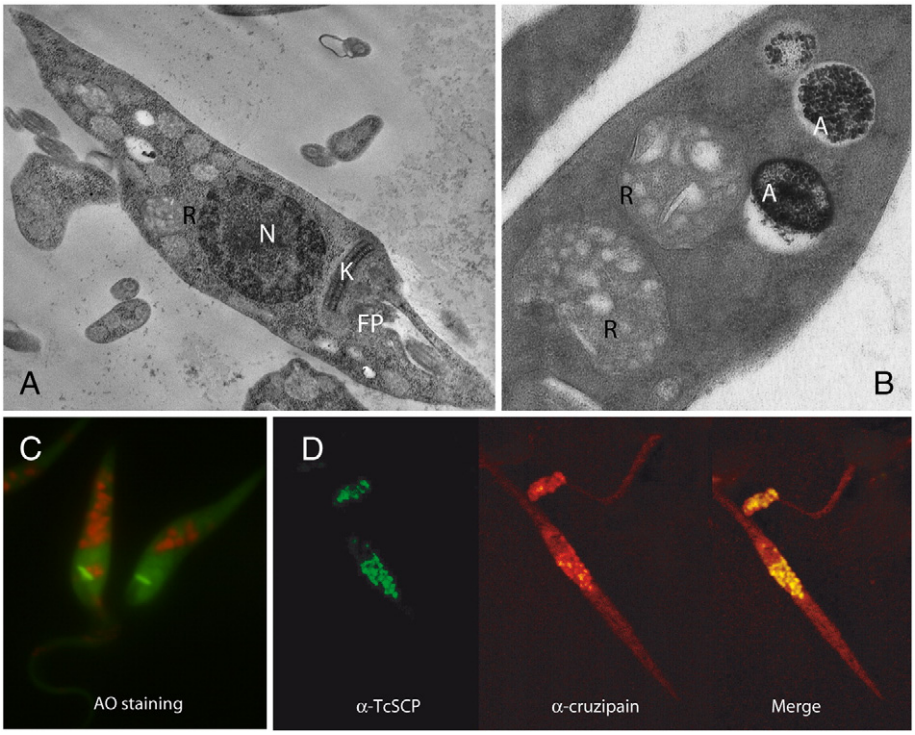

Fig. 2. Reservosomes represent the endpoint of the endocytic pathway in *T. cruzi* epimastigotes [109]. This organelle stores ingested proteins and lipids**.** The intracellular protein digestion is mainly carried out by cruzipain inside the reservosomes and seems to be modulated by its endogenous inhibitor chagasin. They are considered to represent a pre-lysosomal compartment due to the absence of *bona fide* lysosomal molecular markers and to pH evaluation at 6.0. (A and B) Transmission electron microscopy analysis of *T. cruzi* epimastigotes. N nucleus, K kinetoplast, FP flagellar pocket, A acidocalcisome and R reservosome. Reservosomes are round organelles (average diameter of 400–600 nm) delimited by a membrane and localized at the posterior region of epimastigotes. The organelle is composed of an electrondense protein matrix and electronlucent lipid inclusions. (C) Acridine orange (AO) staining of reservosomes. AO is a weak base that concentrates in acidic compartments (i.e. reservosomes and acidolcacisomes in *T. cruzi*). (D) Reservosomes are labeled by indirect immunofluorescence using anti-TcSCP (green) and anti-cruzipain (red) polyclonal antibodies. Merge image is shown at the right.

Cruzipain is encoded by a large number of genes arranged in head-to-tail tandems, located on 2 to 4 different chromosomes in different *T. cruzi* strains and clones. The maximum number of cruzipain repeat units has been estimated in about 60 for the CA-I/72 clone, and in about 130 for the Tul2 strain [44]. The genes, which, as most genes from Trypanosomatids contain no introns, encode a signal peptide, a propeptide, and the mature enzyme. Mature cruzipain consists of a catalytic moiety, with high sequence identity with some cathepsins, particularly cathepsin S, and a 130 amino acid long C-terminal extension (C-T), which seems so far restricted to Type I CPs from Trypanosomatids [45]. The C-T consists of a "core" of 76 amino acids, tightened by four disulfide bridges; an N-terminal segment of 27 amino acid residues containing 7 modified Thr residues and 7 Pro residues, which probably acts as a "hinge" linking the "core" to the catalytic moiety; and a highly hydrophilic C-terminal "tail" of 27 residues, containing 7 Arg, 2 His, 2 Asp and 5 Ser residues. Natural cruzipain is a complex of isoforms, as judged from Mono Q chromatography, reversed-phase HPLC and isoelectrofocusing [46], probably due to the simultaneous expression of several genes encoding amino acid substitutions, and to the presence of different cruzipain molecules bearing different post-translational modifications. Cruzipain is a glycoprotein; the sequence predicts three potential N-glycosylation sites. The first potential site in the catalytic moiety (Asn 33) is glycosylated *in vivo*, and bears only high mannose-type oligosaccharides [47]. The single N-glycosylation site in the C-T (Asn 255) bears either high mannose-type, hybrid monoantennary-type or complex biantennary-type oligosaccharide chains in different molecules [48]; moreover, this site also contains sulfated high-mannose type oligosaccharides; biantennary lactosaminic chains, some of them bearing sialic acid or fucose, have also been identified [49]. In addition, the C-T contains single units of O-linked N-acetylglucosamine, and sialic acid is also present, exclusively in N-linked oligosaccharide chains [50]. The seven modified Thr residues, however, seem not to be O-glycosylated, since the modifications were resistant to β-elimination [51]. Both the presence of sialic acid, which must be incorporated at the cell surface through the action of trans-sialidase, and the presence of polylactosaminic units, support the possibility that cruzipain reaches the lysosomal compartment via en endocytic recycling pathway, as proposed for *T. brucei*[52,53].

The gene encoding cruzipain 2, an isoform with 86% identity to cruzipain, has been cloned and sequenced [15]. The amino acid changes reported include substitutions at the S2, S1′ and S2′ subsites, in good agreement with the differences in substrate specificity mentioned above. The first potential N-glycosylation site at the catalytic domain (Asn 33), as well as the last Cys residue in the C-T, are absent in cruzipain 2.

Several X-ray crystallographic structures of a recombinant truncated form of the enzyme (cruzainΔc) have been determined, as a complex with several irreversible synthetic inhibitors [54–56]; the best resolution attained has been 1.1–1.2 Å [57]. All the crystallographic studies performed so far have used the recombinant truncated enzyme. There is no information, therefore, about the 3D-structure of the C-terminal domain, nor about its interactions with the catalytic moiety. Intact mature recombinant cruzipain expressed in the baculovirus/insect cell system [58] opens up the possibility of determining the complete structure in a near future.

Cruzipain is an immunodominant antigen, recognized by most sera from chronic patients of Chagas disease [13,59,60]. Most antibodies in natural infections and in immunized animals are directed against the C-T, and enzyme molecules with antibodies bound to this domain are still active, at least against small peptidic substrates [61]. Immunoprotection experiments performed in mice

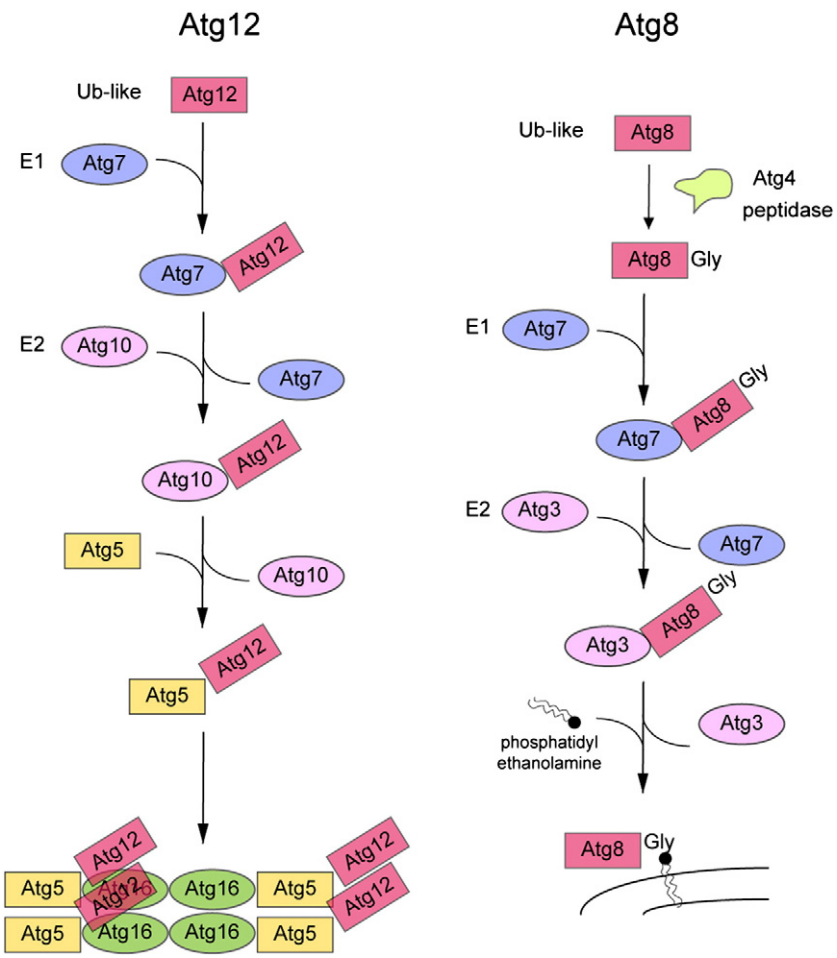

Fig. 3. Schematic representation of the two ubiquitin-like conjugation systems involved in autophagosome biogenesis [97]. The first one is the Atg12 system, where the ubiquitin-like Atg12 protein is conjugated to Atg5 protein in a process mediated by Atg7, an E1-like enzyme (ubiquitin activating enzyme), and Atg10, an E2-like enzyme (ubiquitin conjugating enzyme). The Atg12–Atg5 conjugate forms a complex with Atg16 protein, which is thought to form a transient coat that drives the deformation of the sequestering membrane during vesicle formation. The second ubiquitin-like protein that acts on vesicle expansion and completion is Atg8. This protein is proteolytically processed by the Atg4 protease (autophagin), thereby exposing a Gly residue which is then covalently attached to a phosphatidylethanolamine (PE) moiety by the concerted action of Atg7 and Atg3 proteins, the latter being a specific E2-like conjugating enzyme. This enables the previously cytosolic Atg8 protein to tightly associate with the membranes making Atg8-PE a suitable autophagosomal membrane marker. Before fusion with the vacuole, Atg8 is deconjugated from PE in the outer membrane by Atg4 and released to the cytosol in order to be reused for new vesicle formation.

with recombinant cruzipain have led to the suggestion that the enzyme is an important candidate for the development of a vaccine against Chagas disease [62,63].

As the major lysosomal proteinase of *T. cruzi*, cruzipain can be expected to play a prominent role in nutrition of the parasite, at least in the gut of the hematophagous insect vector. In addition, other roles, probably equally or more important, have been proposed for the enzyme. The enzyme participates in the process of invasion of mammalian cells by the parasite, which is essential for the development of Chagas disease. Cruzipain is able to induce the production of the proinflammatory peptide, Lys-bradykinin, directly by proteolysis of kininogen, or by activation of plasmatic pre-kallikrein [27]; kinin release was increased by 35-fold in the presence of heparan sulfate [23]. Since the invasion of host cells by the parasite correlates with the ability of the trypomastigotes to liberate kinins which, through interaction with B(2) receptors elicit an intracellular free calcium transient, cruzipain has been proposed as a virulence factor in Chagas disease [64]. In addition, a second, bradikinin-independent increase in parasite invasion elicited by the secreted cruzipain, presumably by activating an unidentified protein in the parasite cell membrane, has been detected [21].

Cruzipain may also provide a mechanism by which the parasite can escape the immune response of the host, by digestion at the “hinge” of all human IgG subclasses. At neutral pH, the fragments produced from IgG_1 and IgG_3 were Fab and Fc, whereas IgG_2 and IgG_4 yielded Fab_2 and Fc [65]. This pattern is compatible with the mechanism known as “fabulation”, by which the Fab_2 fragment is left protecting the antigen on the surface of the parasite, instead of opsonizing it for phagocytosis, or activating the complement cascade [66].

The proteinase has also been proposed to play a part in the differentiation steps of the parasite's life cycle, which are blocked by permeant irreversible inhibitors of the enzyme [67–69]. The possible participation of cruzipain in metacyclogenesis, first proposed by Bonaldo and co-workers [70] and supported by the results of Franke de Cazzulo and co-workers [67], has received further confirmation from the work of Tomas and co-workers [20], who showed that over-expression of cruzipain enhanced the differentiation of epimastigotes to metacyclics.

Some of the cruzipain inhibitors efficiently kill the parasite. Morpholinourea-Phe-homoPhe-vinyl sulphone phenyl was able to arrest epimastigote growth at 10 μM after incubation for 48 h, and all the parasites were dead after 5 days [71]. This effect was proposed to be due to inhibition of the auto-catalytic processing of the enzyme, leading to its accumulation in the Golgi and the disruption of the normal trafficking and processing of glycoproteins in the parasite [71]. Parasite stocks able to resist concentrations of two irreversible

inhibitors between 13- and 20-fold greater than the concentrations lethal for the parental strain have been obtained [72,73]. Resistance of *T. cruzi* to these CP inhibitors seems to have arisen by different mechanisms, in both cases preventing the damaging accumulation of cruzipain precursors in the secretory pathway of the parasite cells.

Treatment of *T. cruzi*- infected mice with N-Methyl-piperazine-Phe-homoPhe-vinyl sulphone phenyl (K777) resulted in their effective rescue from lethal infection, and parasitological cure of most of them [74]; this effect was observed even in an immunodeficient mouse model [75]. These results are very promising, since they clearly indicate that cruzipain can be considered a valid target for chemotherapy of Chagas disease; K777 is in the last stages of preclinical studies, and the beginning of clinical trials is envisaged for a near future [76].

3.2. Cathepsin B-like peptidase

A 30 kDa CP has been characterized in epimastigotes, amastigotes and trypomastigotes of *T. cruzi*[77]. The purified enzyme was able to hydrolyze the fluorogenic substrate N-succinyl-Leu-Leu-Val-Tyr-NHMec optimally at pH 6.5, and proteins such as bovine serum albumin and human type I collagen, optimally at pH 4. The sequence of the N-terminus suggested the highest identity with cathepsins B; this was confirmed by the cloning and sequencing of a gene (*tccb*) encoding a CP with 56% identity with human cathepsin B [78], although the two sequences did not match perfectly at the N-terminus. The Genome Project, however, has detected only one gene encoding this enzyme. The sequence predicted three potential N-glycosylation sites in the mature enzyme, in good agreement with the previous experimental finding [77] that the 30 kDa CP is a glycoprotein. A 30 kDa CP, located in the reservosomes and not immunologically cross-reactive with cruzipain, presented 44% identity at the N-terminus with the cathepsin B from *Caenorhabditis elegans*, but only 22% identity with the protein product of *tccb*[72]. Although this enzyme was proposed to be a cathepsin B, it seems more likely that it may be encoded by the gene annotated as "Cathepsin S" in the Genome data base.

3.3. Metacaspases

Metacaspases were originally identified as distant caspase relatives present in the genome of plants, fungi and protozoa [79]. Based on the conservation of the His–Cys catalytic dyad and a predicted common caspase-hemoglobinase fold, they have been assigned to the clan CD of cysteine peptidases [80,81]. Biochemical studies have shown that, as most clan CD enzymes, metacaspases display a very stringent specificity at P1 position; however, at variance with animal caspases which cleave after aspartic acid residues, metacaspases cleave proteins or peptides only after basic amino acids (Lys or Arg). Notably, all metacaspases studied so far are absolutely dependent on millimolar concentrations of calcium for their endopeptidase activity ([82–85] and our unpublished data). A combinatorial tetrapeptide library screening identified VRPR as the optimal substrate for *Arabidopsis thaliana* metacaspase 9 [86].

The best studied enzymes from this group are plant metacaspases (*Arabidopsis thaliana* and *Picea abies* metacaspases) and trypanosomatid metacaspases (*T. cruzi*, *T. brucei* and *L. major* metacaspases). Plant metacaspases can be divided into two subgroups based on their sequence similarity and domain architecture: type I metacaspases contain an N-terminal extension consisting of a Zinc-finger motif followed by a Pro-rich region, whereas type II metacaspases lack this N-terminal extension but have an insertion (from 90 up to 150 and approximately 80 amino acids residues for *A. thaliana* and *P. abies* type II metacaspases, respectively) between the regions corresponding to caspase p20 and p10 subunits [79,82,87]. Type II metacaspases were reported to be autocatalytically activated *in vitro* by a cleavage event at the p20–p10 boundary [82,87,88]. However, no proteolytic processing was observed when type I metacaspases are overexpressed *in vitro* or *in vivo*[82,89]. Trypanosomatid metacaspases can also be distinguished by their overall domain composition and gene copy number: a single copy gene (termed *LmMCA* in *L. major*, *TbMCA5* in *T. brucei* and *TcMCA5* in *T. cruzi*) is present in all three trypanosomatids and encodes a protein that bears a Pro, Gln and Tyr-rich C-terminal extension in addition to the catalytic domain; while multiple copy genes (4 genes in *T. brucei* named *TbMCA1-4* and about 16 genes in *T. cruzi* called *TcMCA3*) lacking the C-terminal region are present in *T. brucei* and *T. cruzi*[90,91]. The only metacaspase from *L. major* has been reported to undergo self-proteolytic processing that would enhance its peptidase activity *in vitro*[84]. In contrast, TbMCA2-3 as well as TcMCA3 and TcMCA5, do not require any processing in order to display maximal peptidase activity ([85] and our unpublished data).

Accumulating evidence indicates that metacaspases modulate programmed cell death (PCD) during plant embryogenesis, oxidative stress and as part of the hypersensitive response [87,89]. In addition, it has been shown that the Tudor staphylococcal nuclease is cleaved by *P. abies* metacaspase during both developmental and stress-induced PCD. This is the first described natural substrate of metacaspases; and it is also the first evolutionarily conserved substrate between metacaspases and caspases, since the human nuclease is cleaved by caspase-3 during apoptosis as well [92]. The role of trypanosomatid metacaspases is still elusive. It has been demonstrated that over-expression of metacaspase-5 in *T. cruzi* renders epimastigotes more susceptible to fresh human serum-induced PCD [91] and that *L. major* metacaspase can replace yeast caspase in its pro-cell death action during ageing [84]. Conversely, triple metacaspase null mutants (Δ*mca2/3* Δ*mca5*) did not impair the course of cell death induced by prostaglandin D_2 in *T. brucei* bloodstream forms when compared to wild type parasites. In fact, actual evidence points towards a potential role for trypanosomatid metacaspases in the progression of the cell cycle ([93,94] and our unpublished data).

Western blot analysis and proteomic data suggest that *T. cruzi* metacaspase-3 is differentially expressed in the mammalian forms (bloodstream trypomastigotes and amastigotes), although in a lesser extent, metacaspase-3 is expressed in epimastigote forms [6,91,95]. Metacaspase-5, on the other hand, seems to be exclusively present in epimastigotes. When subcellular localization of *T. cruzi* metacaspases-3 and -5 was analyzed by indirect immunofluorescence using polyclonal antibodies raised against the recombinant enzymes, they showed a cytosolic distribution with small spots distributed through the cytoplasm [91,95]. This finding was further confirmed with commercial monoclonal antibodies on transgenic parasites expressing tagged versions of the enzymes (our unpublished data). *L. major* metacaspase also presented a similar punctuate distribution in interphase cells, but becomes concentrated in the kinetoplast (mitochondrial DNA) at the time of the organelle's segregation and translocates to the nucleus during mitosis, where it associates with the mitotic spindle [94]. In contrast, all *T. brucei* metacaspases are located in Rab11 positive endosomes [93]. In *T. brucei* MCA2 and MCA3 are bloodstream form specific, whereas MCA5 is constitutive and expressed in both the mammalian bloodstream form and in the insect procyclic form [93]. *L. major* metacaspase has been shown to be expressed in promastigotes and amastigotes of the parasite [94].

Metacaspases are clearly different from classical caspases in substrate specificity and thus represent attractive drug targets for antiparasitic chemotherapy. Recently, a first series of inhibitors have been developed for TbMCA2 that could constitute the basis for a rational drug design [96].

3.4. Autophagins

Autophagy is the major mechanism used by eukaryotic cells to degrade and recycle proteins and organelles. During autophagy, portions of the cytoplasm are enclosed in double membrane vesicles called autophagosomes that deliver their content to the lysosomes

thereby enabling the degradation of the engulfed material by lysosomal hydrolases [97]. Genetic screens on yeast have identified more than 20 autophagy related genes (*ATG*s) required for autophagosome formation [98]. Among them, *ATG8* encodes a ubiquitin-like protein that acts on vesicle expansion and completion (Fig. 3) [99]. Atg8 protein is synthesized as a cytoplasmic precursor that is proteolytically processed after a conserved glycine residue by the CP Atg4 (autophagin). The exposed Gly on Atg8 is then conjugated to the amino group of phosphatidylethanolamine (PE) through a standard ubiquitin-conjugating enzyme cascade, which allows Atg8 to tightly associate with the membranes [100,101]. Before fusion with the lysosomes, Atg8 is deconjugated from PE in the outer membrane by the same protease Atg4 and released to the cytosol in order to be reused for new vesicle formation. A recent review [102] covers the field of autophagy in protists, including the Trypanosomatids.

Bioinformatic analysis of the genome of *T. cruzi* revealed the presence of all components of the Atg8 conjugation system, including two orthologs of the yeast Atg4 as well as two orthologs of yeast Atg8 [103]. Based on the ability to complement yeast *ATG4* deletion strains both *T. cruzi ATG4* gene products showed conserved functions. Furthermore, *in vitro* cleavage reactions performed with the purified recombinant proteins demonstrated that both autophagins are able to correctly process the two *T. cruzi* Atg8 after the conserved Gly residue. However, autophagin-2 displayed considerably lower proteolytic efficiency, suggesting that TcAtg4.1 is indeed the ortholog of the yeast Atg4, whereas the function of TcAtg4.2 is, similarly to the function of several autophagins in human, more elusive. Only one of the Atg8 variants, TcAtg8.1, partially reconstituted autophagy in the ATG8 deletion mutant. Moreover, TcAtg8.1 but not TcAtg8.2, was found by immunofluorescence to be located in autophagosome-like vesicles in HA-tagged TcATG8.1 transfected epimastigotes exposed to starvation conditions. The mutation of the conserved Gly residue to Ala abolished this effect, suggesting that the observed pattern is a consequence of Atg8.1-PE insertion into the lipid membranes and not of unspecific protein aggregation. Polyclonal anti-TcAtg8.1 antibodies confirmed this finding in wild-type nontransfected epimastigotes exposed to starvation [103].

Physiologically, autophagy is often triggered as a stress response, with starvation being the most common type of stress used in the experiments. In *T. cruzi* starvation of epimastigotes also occurs naturally in the gut of the insect vector, which is known to suffer long periods of lack of food (up to 12 months) [104]. Autophagy is also triggered during *T. cruzi* epimastigote to metacyclic trypomastigote spontaneous differentiation. A massive concentration of TcAtg8.1 in autophagosomes was only observed in morphologically identifiable intermediate stages and not in normal epimastigotes nor in fully developed metacyclic trypomastigotes, a clear indication of a very dynamic process, which is only activated when needed [103]. A critical role of autophagy in differentiation of protozoan parasites and other early-diverging eukaryotes is clearly emerging [105,106].

Autophagins were found to be constitutively expressed in all life-cycle stages of *T. cruzi*, which is in agreement with the earlier findings on human autophagins [107]. Despite its constitutive expression and apparent lack of existing endogenous inhibitors, the very strict specificity of autophagins may be sufficient to prevent unspecific proteolysis in the cytosol. This can be explained by the recently determined crystal structure of HsAtg4B [108]. The active site cleft of this family of enzymes was found to be masked by a loop of four amino acid residues and could be exposed only after Atg8 binding, which might also explain the apparent lack of activity of both *T. cruzi* autophagins on the short peptidic substrate Abz-TFGQ-EDDnp, designed on the basis of the cleavage sequence in TcAtg8.1.

Cruzipain is clearly involved in the last phase of autophagy, the degradation of autophagosomal contents after fusion with the reservosomes. Although this process happens inside the vector, it is noteworthy that cruzipain inhibition also inhibits the development and differentiation of the parasite inside the mammalian cell [67], and that the amastigote and trypomastigote stages present in the mammal also have a lysosomal compartment, in which cruzipain is located [109]. It is quite possible; therefore, that autophagy and cruzipain are also involved, together with the proteasome, in the differentiation events taking place inside the mammal. Cruzipain has already been characterized as a virulence factor in *T. cruzi*, whose inhibition is able to kill the parasite, and some inhibitors have recently been shown to be active in animal models of infection, and have been reported to be in late-stage preclinical development [75,110]. Since there are human pathological conditions, including cancer and neurodegeneration, where autophagy is believed to be overly active, targeting of the autophagin Atg4 by small synthetic inhibitors has been recently suggested to be an attractive new strategy for treatment. Considering the evolutionary conservation of the substrate specificity of this proteinase, it is possible that these inhibitors, once approved for human use, could also be tested, perhaps as a combined therapy with cruzipain inhibitors, for the treatment of Chagas disease.

4. Serine peptidases

4.1. Oligopeptidase B

An alkaline peptidase with a molecular mass of 120 kDa, oligopeptidase B (see [111], for a review), was purified to homogeneity by Santana and co-workers [112] and later shown to be indirectly involved in the penetration of the trypomastigote into the mammalian cell, by triggering a Ca^{2+}-signaling mechanism [113]. Cloning and sequencing of the gene encoding the enzyme showed that it belongs to the prolyl oligopeptidase family of SPs (family S9A), which are known in higher organisms to participate in the maturation of biologically active peptides [113]. It is possible, therefore, that oligopeptidase B processes a *T. cruzi* protein to a factor responsible for the Ca^{2+}-signaling activity for mammalian cells, necessary for parasite penetration. In terms of substrate specificity, the enzyme is essentially an arginyl hydrolase, since all peptides cleaved have Arg at P1. In addition, the enzyme presented a carboxypeptidase activity highly specific for basic amino acids, which required the presence of at least two basic residues at the C-terminus of the substrate, suggesting that the oligopeptidase may represent a new type of processing enzyme [114]. Since the knock-out of the gene encoding the enzyme strongly decreases the ability of *T. cruzi* trypomastigotes to invade mammalian cells, and to infect mice [115], and the *T. brucei* ortholog has been identified as a target for several drugs used in the treatment of sleeping sickness [116], the oligopeptidase B may become a target for the development of new drugs against Chagas disease.

4.2. Prolyl endopeptidase Tc80 ("POPTc80, collagenase")

A secreted 80 kDa proteinase with collagenase activity against human collagen types I and IV, as well as fibronectin, at neutral pH, was purified and partially characterized from *T. cruzi*[117]. Cloning and sequencing of the gene [118], which is present in a single copy per haploid genome, showed that the enzyme is a prolyl endopeptidase (EC 3.4.21.16), also belonging to the S9A SP family, but distinct from the oligopeptidase B. Structural modeling of POP Tc80, based on the crystallized porcine enzyme, suggest that POP Tc80 is composed of an α/β-hydrolase domain containing the catalytic triad Ser548–Asp631–His667 and a seven-bladed β-propeller non-catalytic domain. The authors suggested that binding of the triple-helical collagen to the catalytic site of POP Tc80 may occur in the vicinity of the interface between the two domains [118]. The enzyme, which is located in a vesicular compartment close to the flagellar pocket, the major place for exo- and endocytosis in trypanosomes, may be important for degrading the extracellular matrix and thus allowing the parasite to penetrate the tissues of the host, as well as for cell invasion. Selective

inhibitors of POP Tc80 have been synthesized [119–122], with K_i values in the low nanomolar range, and were able to prevent invasion of the host cells [123]; this inhibition happened when the parasites, but not the mammalian cells, were preincubated with the irreversible inhibitors, and correlated with POP Tc80 inhibition. This SP looks, therefore, as a new very promising target for the development of new drugs against Chagas disease [124].

4.3. Serine carboxypeptidase

A serine carboxypeptidase (*Tc*SCP) belonging to the S10 family has been purified and characterized from *T. cruzi* epimastigotes [125]. The enzyme is encoded by a number of genes per haploid genome, arranged in head-to-tail tandems, which predict a protein of 466 aminoacid residues (including a signal peptide and a short pro-domain). Sequence comparisons indicate that the enzyme belongs to the C group of serine carboxypeptidases, within the S10 family, and shows the higher similarity to plant and yeast enzymes. The enzyme is a lysosomal high-mannose type glycoprotein with a molecular mass of about 54 kDa. It prefers hydrophobic (Furyl-acryloyl-Phe-Phe) over charged substrates (Furyl-acryloyl-Ala-Lys), with an optimal pH at 4.5, and is strongly inhibited by 3,4-dichloroisocoumarin, with an IC_{50} value of 0.3 μM. Comparing with the yeast enzyme, all the residues involved in catalysis, and most of those involved in substrate binding, are conserved in the *T. cruzi* enzyme, as well as 8 out of 10 Cys residues known to be involved in disulfide bridges. *Tc*SCP was expressed in a baculovirus-insect cells system; the recombinant enzyme had a considerably lower specific activity as compared with the native one, and N-terminal sequencing suggested that part of the pro-domain had not been processed by the insect cells. Incubation of the recombinant protein with cruzipain, which is located in the same subcellular compartment [109] led to a three-fold increase in activity. This fact suggests that the natural processing proteinase for the SCP may be cruzipain (F Parussini, A. Chidichimo and J.J. Cazzulo, unpublished results). The functions of the enzyme are still unknown, but homologous genes have been found in *T. brucei* and *Leishmania major*[125].

5. Metallopeptidases

5.1. Leishmanolysin (gp63)-like metalloproteinases

Since the discovery of the surface metalloproteinase (gp63, leishmanolysin) of *Leishmania* spp. [126], genes homologous to those encoding the Leishmanial enzyme have been described in other Trypanosomatids. *T. cruzi* has 425 genes, 251 of which are pseudogenes [4]; from the 174 remaining true genes, there is proteomic evidence for the expression of 29 [95]. According to the Tritryp DataBase, mRNAs corresponding to 31 genes have been identified, but there is very little overlapping with the proteins identified by mass spectrometry. The MEROPS Database lists 95 leishmanolysin-like proteins, of which 74 are classified as leishmanolysins, and the others as non-peptide homologs. Although over-represented at the genomic level, the actual amount of metalloproteinase activity present is fairly low. In contrast *L. major* Friedlin has only 6 genes for leishmanolysin annotated in the TriTryps database, despite the fact that this protein is very abundant and covers about half the surface of the promastigote stage of the Leishmania parasites.

The first report of metalloproteinases in *T. cruzi* at a biochemical level was the work of Lowndes and co-workers [127] who detected the expression of a complex array of metalloproteinases in *T. cruzi*, apparently all of them membrane-bound, and showing considerable qualitative and quantitative variation in different strains and developmental stages of the parasite. Since no further characterization was made, it is not possible to identify the genes encoding them. Afterwards, Grandgenett and co-workers [128] cloned and sequenced four different cDNAs from the parasite, with high homology to each other, and showed that their expression was developmentally regulated in the different parasite stages. However, these authors did not identify the proteins encoded, and therefore a direct comparison with the activity data previously reported [127] is not possible.

Cuevas et al. demonstrated that *T. cruzi* has a family of gp63 genes composed of multiple groups [129]. Two of these groups, *Tcgp63-I* and *Tcgp63-II*, are present as high-copy number genes. The *Tcgp63-I* group, which encodes proteins of 543 amino acid residues containing a C-terminal region compatible with the addition of a GPI anchor, presents high homology with the sequences named gp63 1 to 4 by Grandgenett and co-workers [128]. *Tcgp63-II* encodes a protein with 566 amino acid residues, 42% identical to *Tcgp63-I,* but lacking the C-terminal hydrophobic sequence. Although the *Tcgp63-II* genes are far more represented in the genome that *Tcgp63-I*, the latter is preferentially expressed at the mRNA level, and the encoded protein (75–78 kDa, apparent mol. wt.) is the only one detectable at the protein level in Western blots, and has a low metalloproteinase activity detectable in gelatin-containing SDS-PAGE gels. This protein is present in the plasma membrane, bound by a GPI anchor. This MP, expressed in the four major parasite stages, may be involved in mammalian cell infection, since specific antibodies against a peptide derived from its sequence partially blocked (by about 50%) cell invasion [129].

Recently a different gp63-like MP has been detected in *T. cruzi* [130]; the protein, detected with a specific antiserum, is also expressed in the four major stages of the parasite; however, it has an apparent mol. wt. of 61 kDa in epimastigotes, trypomastigotes and amastigotes, and of 55 kDa in metacyclic trypomastigotes. Besides, whereas the 61 kDa protein is N-glycosylated and located at the plasma membrane in epimastigotes, the 55 kDa protein is not glycosylated and is intracellular in metacyclics. The antiserum against the 61 kDa MP was able, as the one recognizing the 78 kDa MP [129], to inhibit to a similar extent the infection of cells by the trypomastigotes; the effects of both antisera were partially additive, suggesting that both MPs are involved in infection [130].

5.2. CAAX prenyl protease (farnesylated-protein converting enzyme)

A gene encoding a protein highly homologous to the CAAX-prenyl protease, the enzyme which removes the last three amino acid residues at the C terminus after Cys prenylation, has been detected in *T. cruzi*, and is expressed, at least as mRNA, in the epimastigote stage of the parasite [131]. The enzyme, now known as CAAX prenyl protease 1, belongs to the M48 family; and is expressed at least in the epimastigote stage, as shown by proteomic studies (Tritryp Database). Although this enzyme has not been biochemically characterized in *T. cruzi*, its ortholog from *T. brucei* has been shown to be proteolytically active when expressed in insect cells, and its knock down by RNAi reduced significantly the growth of this parasite [132].

Another gene, also present in the *T. cruzi* genome, encodes an enzyme (CAAX prenyl protease 2) belonging to the M79 family, which has not been biochemically characterized so far, There is evidence for expression of one of two genes found in the parasite at the mRNA level, but not at the protein level.

5.3. Metallocarboxypeptidases (M32 family)

Two homologous genes to the *Thermus aquaticus* carboxypeptidase (*Taq*CP), a thermophilic enzyme reported to release a broad spectrum of amino acids from the C-terminus [133–135], were detected in *T. cruzi*[136]. These enzymes, named *T. cruzi* metallocarboxypeptidase-1 (*Tc*MCP-1) and *Tc*MCP-2, belong to the M32 family of peptidases, a group of hydrolases characterized by being restricted to a very limited phylogenetic range that includes bacteria, archaea, green algae and the trypanosomatidae family, but no metazoan

organisms. This fact makes these enzymes an attractive potential target for drug development [136].

Both *Tc*MCPs are 503 amino acids long and exist as homodimers. Although they are highly related in sequence (64% identity), their substrate preference and pattern of expression differs markedly. Thus, *Tc*MCP-1 is a cytosolic enzyme expressed in the four main stages of the parasite. This enzyme prefers basic amino acids (Lys or Arg) at P1′ position acting best at pH 6.2–7.3. Orthologous protein sequences (displaying 59–72% identity *Tc*MCP-1) were found in the genomes of *T. brucei* and *Leishmania* spp. *Tc*MCP-2, on the other hand, is species-specific. This cytosolic enzyme is present solely in the stages that occur in the insect vector, suggesting that its expression is developmentally regulated [136]. Recombinant *Tc*MCP-2 had a preference for aliphatic (including Gly), neutral and aromatic amino acids. Basic or acid residues at the P1′ position are not cleaved. Both enzymes are inhibited by metal chelators such as EDTA and *o*-phenanthroline [136].

No biological function has yet been assigned to peptidases of the M32 family. But, on the bases of its biochemical properties, and stage-specific expression, it has been suggested that protozoan M32 carboxypeptidases might play a role in peptide catabolism [137]. Other possible functions have been suggested based on the crystal structure of *Tc*MCP-1. This enzyme shows a strong topological similarity with archaeal, bacterial and mammalian metallopeptidases including angiotensin-converting enzyme, neurolysin and thimet oligopeptidase [138]. This fact, plus the restricted substrate preference of *Tc*MCP-1, does not rule out a possible regulatory role in the metabolism of small peptides. *Tc*MCP-1 can produce des-Arg bradykinin by hydrolysis of bradykinin. This peptide is known to promote the process of mammalian cell invasion by the trypomastigotes through B(1) receptors [139]. Recently, two reports have suggested that M32 peptidases are secreted by Trypanosomatids [140,141], a fact that might make feasible the latter hypothesis.

6. Threonine proteinases (the proteasome)

The Tritryps Database lists 63 genes encoding proteasome subunits (α-subunits 1, 2, 3, 5 and 7; β-subunits 1, 2, 3, 5, 6, and 7; 1, 2, 3 and 5 ATPase regulatory subunits; 2, 3, 5, 6, 7, 8, 9, and 11 ATPase non-regulatory subunits; and 3 genes for the proteasome activator protein pa26). Out of these genes, there is proteomic evidence of their expression, except for β7; ATPase 1 and 2, and non-ATPase 9 and 11. The MEROPS Database lists the β-subunits 1, 2 and 3, and the α-subunits 1, 2, 5 and 7, in addition to 7 non-peptidase homologs of the T1 family.

The presence of the proteasome was first reported in *T. cruzi* in 1996 [142], simultaneously with its demonstration in *T. brucei*[143]. The 20S proteasome was purified from epimastigotes of *T. cruzi* as a 670 kDa oligomer consisting of subunits of 25-35 kDa, with pI values ranging from 4.5 to 8.5 [142], and having the characteristic barrel-shaped electron microscopy image. The proteasome has also been biochemically characterized in *T. brucei*[144]. The proteasome activity was inhibited by lactacystin, and this inhibitor was able to prevent the differentiation of trypomastigotes to amastigotes, and the intracellular differentiation of amastigotes to trypomastigotes [142], thus showing that the proteasome is involved in the cellular remodeling essential for differentiation. Later the 26S proteasome was identified as a 1400 kDa complex, made up of about 30 different protein subunits with apparent mol.wt values of 25-110 kDa, with an ATP-dependent chymotrypsin-like activity against Suc-Leu-Leu-Val-Tyr-NHMec [145]. Several proteasome subunits have been cloned and sequenced from the parasite, and show homology to the corresponding subunits from other eukaryotic proteasomes [146,147].

7. Conclusions

The completion of the *T. cruzi* Genome Project in 2005, and the further genomic sequencing performed since in several laboratories, has allowed us to get a fairly complete idea of the peptidase repertoire of this human parasite. However, only a fraction of the peptidases predicted by genomic studies has been biochemically characterized. Since peptidases are becoming increasingly important as targets for the development of new drugs, efforts should be made to detect new potential targets for chemotherapy, in addition to the already validated cruzipain and the very promising oligopeptidase B and prolyl oligopeptidase. In addition, a better knowledge of the parasite's biology will require to get further insight in the functions of rather mysterious proteins, like the calpain homologs highly conserved in Trypanosomatids, or the huge number of leishmanolysin-like genes and proteins. The two S8 subtilisin-like proteins present in the *T. cruzi* genome are also very interesting, since recently similar enzymes have been shown to play a role in apoptosis in the plant *Avena sativa*[148], and as stated above, the mechanism of apoptosis in these parasites is still unknown, despite the studies on the metacaspases in the three most important Trypanosomatid parasites.

Acknowledgments

JJC and VEA are members of the Carrera del Investigador Científico from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET). GTN has a post-doctoral fellowship from CONICET. The work of the authors' laboratory reviewed here was supported by grants from CONICET, ANPCyT, Fundación Antorchas (Argentina), the World Bank/UNDP/WHO Special Programme for Research and Training in Tropical Diseases (TDR) and the Swedish Agency for Research Cooperation with Developing Countries (Sida/SAREC). We gratefully acknowledge Drs. Roberto Docampo and Celso Sant'Anna for Fig. 2A and B, and Dr. Fabiola Parussini for Fig. 2D.

References

[1] M.P. Barrett, R.J. Burchmore, A. Stich, J.O. Lazzari, A.C. Frasch, J.J. Cazzulo, S. Krishna, The trypanosomiases, Lancet 362 (2003) 1469–1480.
[2] S. Itow, E.P. Camargo, Proteolytic activites in cell extracts of *Trypanosoma cruzi*, J. Protozool. 24 (1977) 591–595.
[3] J.L. Avila, M.A. Casanova, A. Avila, A. Bretana, Acid and neutral hydrolases in *Trypanosoma cruzi*, characterization and assay, J. Protozool. 26 (1979) 304–311.
[4] N.M. El-Sayed, P.J. Myler, D.C. Bartholomeu, D. Nilsson, G. Aggarwal, A.N. Tran, E. Ghedin, E.A. Worthey, A.L. Delcher, G. Blandin, S.J. Westenberger, E. Caler, G.C. Cerqueira, C. Branche, B. Haas, A. Anupama, E. Arner, L. Aslund, P. Attipoe, E. Bontempi, F. Bringaud, P. Burton, E. Cadag, D.A. Campbell, M. Carrington, J. Crabtree, H. Darban, J.F. da Silveira, P. de Jong, K. Edwards, P.T. Englund, G. Fazelina, T. Feldblyum, M. Ferella, A.C. Frasch, K. Gull, D. Horn, L. Hou, Y. Huang, E. Kindlund, M. Klingbeil, S. Kluge, H. Koo, D. Lacerda, M.J. Levin, H. Lorenzi, T. Louie, C.R. Machado, R. McCulloch, A. McKenna, Y. Mizuno, J.C. Mottram, S. Nelson, S. Ochaya, K. Osoegawa, G. Pai, M. Parsons, M. Pentony, U. Pettersson, M. Pop, J.L. Ramirez, J. Rinta, L. Robertson, S.L. Salzberg, D.O. Sanchez, A. Seyler, R. Sharma, J. Shetty, A.J. Simpson, E. Sisk, M.T. Tammi, R. Tarleton, S. Teixeira, S. Van Aken, C. Vogt, P.N. Ward, B. Wickstead, J. Wortman, O. White, C.M. Fraser, K.D. Stuart, B. Andersson, The genome sequence of Trypanosoma cruzi, etiologic agent of Chagas disease, Science 309 (2005) 409–415.
[5] N.D. Rawlings, A.J. Barrett, A. Bateman, MEROPS: the peptidase database, Nucleic Acids Res. 38 (2010) D227–233.
[6] G. Kosec, V. Alvarez, J.J. Cazzulo, Cysteine proteinases of *Trypanosoma cruzi*: from digestive enzymes to programmed cell death mediators, Biocell 30 (2006) 479–490.
[7] K. Ersfeld, H. Barraclough, K. Gull, Evolutionary relationships and protein domain architecture in an expanded calpain superfamily in kinetoplastid parasites, J. Mol. Evol. 61 (2005) 742–757.
[8] V. Giese, B. Dallagiovanna, F.K. Marchini, D.P. Pavoni, M.A. Krieger, S. Goldenberg, *Trypanosoma cruzi*: a stage-specific calpain-like protein is induced after various kinds of stress, Mem. Inst. Oswaldo Cruz 103 (2008) 598–601.
[9] R.T. Pinho, L.M. Beltramini, C.R. Alves, S.G. De-Simone, *Trypanosoma cruzi*: isolation and characterization of aspartyl proteases, Exp. Parasitol. 122 (2009) 128–133.
[10] S. Besteiro, R.A. Williams, G.H. Coombs, J.C. Mottram, Protein turnover and differentiation in Leishmania, Int. J. Parasitol. 37 (2007) 1063–1075.
[11] J.J. Cazzulo, M.C. Cazzulo Franke, J. Martinez, B.M. Franke de Cazzulo, Some kinetic properties of a cysteine proteinase (cruzipain) from *Trypanosoma cruzi*, Biochim. Biophys. Acta 1037 (1990) 186–191.
[12] A.E. Eakin, A.A. Mills, G. Harth, J.H. McKerrow, C.S. Craik, The sequence, organization, and expression of the major cysteine protease (cruzain) from *Trypanosoma cruzi*, J. Biol. Chem. 267 (1992) 7411–7420.

[13] A.C. Murta, P.M. Persechini, S. Padron Tde, W. de Souza, J.A. Guimaraes, J. Scharfstein, Structural and functional identification of GP57/51 antigen of *Trypanosoma cruzi* as a cysteine proteinase, Mol. Biochem. Parasitol. 43 (1990) 27–38.

[14] V.G. Duschak, A.S. Couto, Cruzipain, the major cysteine protease of *Trypanosoma cruzi*: a sulfated glycoprotein antigen as relevant candidate for vaccine development and drug target. A review, Curr. Med. Chem. 16 (2009) 3174–3202.

[15] A.P. Lima, D.C. Tessier, D.Y. Thomas, J. Scharfstein, A.C. Storer, T. Vernet, Identification of new cysteine protease gene isoforms in *Trypanosoma cruzi*, Mol. Biochem. Parasitol. 67 (1994) 333–338.

[16] E. Bontempi, B.M. Franke de Cazzulo, A.M. Ruiz, J.J. Cazzulo, Purification and some properties of an acidic protease from epimastigotes of *Trypanosoma cruzi*, Comp. Biochem. Physiol. B 77 (1984) 599–604.

[17] M.J. Soares, T. Souto-Padron, W. De Souza, Identification of a large pre-lysosomal compartment in the pathogenic protozoon *Trypanosoma cruzi*, J. Cell Sci. 102 (1992) 157–167.

[18] T. Souto-Padron, O.E. Campetella, J.J. Cazzulo, W. de Souza, Cysteine proteinase in *Trypanosoma cruzi*: immunocytochemical localization and involvement in parasite-host cell interaction, J. Cell Sci. 96 (1990) 485–490.

[19] F. Parussini, V.G. Duschak, J.J. Cazzulo, Membrane-bound cysteine proteinase isoforms in different developmental stages of *Trypanosoma cruzi*, Cell. Mol. Biol. (Noisy-le-grand) 44 (1998) 513–519.

[20] A.M. Tomas, M.A. Miles, J.M. Kelly, Overexpression of cruzipain, the major cysteine proteinase of *Trypanosoma cruzi*, is associated with enhanced metacyclogenesis, Eur. J. Biochem. 244 (1997) 596–603.

[21] I.M. Aparicio, J. Scharfstein, A.P. Lima, A new cruzipain-mediated pathway of human cell invasion by *Trypanosoma cruzi* requires trypomastigote membranes, Infect. Immun. 72 (2004) 5892–5902.

[22] J.K. Yokoyama-Yasunaka, E.M. Pral, O.C. Oliveira Junior, S.C. Alfieri, A.M. Stolf, *Trypanosoma cruzi*: identification of proteinases in shed components of trypomastigote forms, Acta Trop. 57 (1994) 307–315.

[23] A.P. Lima, F.C. dos Reis, C. Serveau, G. Lalmanach, L. Juliano, R. Menard, T. Vernet, D.Y. Thomas, A.C. Storer, J. Scharfstein, Cysteine protease isoforms from *Trypanosoma cruzi*, cruzipain 2 and cruzain, present different substrate preference and susceptibility to inhibitors, Mol. Biochem. Parasitol. 114 (2001) 41–52.

[24] A. Raimondi, C. Wernstedt, U. Hellman, J.J. Cazzulo, Degradation of oxidised insulin A and B chains by the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* epimastigotes, Mol. Biochem. Parasitol. 49 (1991) 341–344.

[25] J.J. Cazzulo, M. Bravo, A. Raimondi, U. Engstrom, G. Lindeberg, U. Hellman, Hydrolysis of synthetic peptides by cruzipain, the major cysteine proteinase from *Trypanosoma cruzi*, provides evidence for self-processing and the possibility of more specific substrates for the enzyme, Cell Mol Biol (Noisy-le-grand) 42 (1996) 691–696.

[26] C. Serveau, G. Lalmanach, M.A. Juliano, J. Scharfstein, L. Juliano, F. Gauthier, Investigation of the substrate specificity of cruzipain, the major cysteine proteinase of *Trypanosoma cruzi*, through the use of cystatin-derived substrates and inhibitors, Biochem. J. 313 (1996) 951–956.

[27] E. Del Nery, M.A. Juliano, A.P. Lima, J. Scharfstein, L. Juliano, Kininogenase activity by the major cysteinyl proteinase (cruzipain) from *Trypanosoma cruzi*, J. Biol. Chem. 272 (1997) 25713–25718.

[28] A.P. Lima, J. Scharfstein, A.C. Storer, R. Menard, Temperature-dependent substrate inhibition of the cysteine proteinase (GP57/51) from *Trypanosoma cruzi*, Mol. Biochem. Parasitol. 56 (1992) 335–338.

[29] W.A. Judice, L. Puzer, S.S. Cotrin, A.K. Carmona, G.H. Coombs, L. Juliano, M.A. Juliano, Carboxydipeptidase activities of recombinant cysteine peptidases. Cruzain of *Trypanosoma cruzi* and CPB of *Leishmania mexicana*, Eur J Biochem 271 (2004) 1046–1053.

[30] F.C. dos Reis, W.A. Judice, M.A. Juliano, L. Juliano, J. Scharfstein, A.P. Lima, The substrate specificity of cruzipain 2, a cysteine protease isoform from *Trypanosoma cruzi*, FEMS Microbiol. Lett. 259 (2006) 215–220.

[31] J.J. Cazzulo, V. Stoka, V. Turk, The major cysteine proteinase of *Trypanosoma cruzi*: a valid target for chemotherapy of Chagas disease, Curr. Pharm. Design 7 (2001) 1143–1156.

[32] V. Stoka, M. Nycander, B. Lenarcic, C. Labriola, J.J. Cazzulo, I. Bjork, V. Turk, Inhibition of cruzipain, the major cysteine proteinase of the protozoan parasite, *Trypanosoma cruzi*, by proteinase inhibitors of the cystatin superfamily, FEBS Lett. 370 (1995) 101–104.

[33] B. Turk, V. Stoka, V. Turk, G. Johansson, J.J. Cazzulo, I. Bjork, High-molecular-weight kininogen binds two molecules of cysteine proteinases with different rate constants, FEBS Lett. 391 (1996) 109–112.

[34] T. Bevec, V. Stoka, G. Pungercic, J.J. Cazzulo, V. Turk, A fragment of the major histocompatibility complex class II-associated p41 invariant chain inhibits cruzipain, the major cysteine proteinase from *Trypanosoma cruzi*, FEBS Lett. 401 (1997) 259–261.

[35] V. Stoka, B. Lenarcic, J.J. Cazzulo, V. Turk, Cathepsin S and cruzipain are inhibited by equistatin from *Actinia equina*, Biol. Chem. 380 (1999) 589–592.

[36] C. de Oliveira, L.A. Santana, A.K. Carmona, M.H. Cezari, M.U. Sampaio, C.A. Sampaio, M.L. Oliva, Structure of cruzipain/cruzain inhibitors isolated from *Bauhinia bauhinioides* seeds, Biol. Chem. 382 (2001) 847–852.

[37] A.C. Monteiro, M. Abrahamson, A.P. Lima, M.A. Vannier-Santos, J. Scharfstein, Identification, characterization and localization of chagasin, a tight-binding cysteine protease inhibitor in *Trypanosoma cruzi*, J. Cell Sci. 114 (2001) 3933–3942.

[38] A.P. Araujo, D. Hansen, D.F. Vieira, C. Oliveira, L.A. Santana, L.M. Beltramini, C.A. Sampaio, M.U. Sampaio, M.L. Oliva, Kunitz-type *Bauhinia bauhinioides* inhibitors devoid of disulfide bridges: isolation of the cDNAs, heterologous expression and structural studies, Biol. Chem. 386 (2005) 561–568.

[39] D. Hansen, S. Macedo-Ribeiro, P. Verissimo, S. Yoo Im, M.U. Sampaio, M.L. Oliva, Crystal structure of a novel cysteinless plant Kunitz-type protease inhibitor, Biochem. Biophys. Res. Commun. 360 (2007) 735–740.

[40] D. Salmon, R. do Aido-Machado, A. Diehl, M. Leiderrt, O. Schmetzer, A.P. Lima, J. Scharfstein, H. Oschkinat, J.R. Pires, Solution structure and backbone dynamics of the *Trypanosoma cruzi* cysteine protease inhibitor chagasin, J. Mol. Biol. 357 (2006) 1511–1521.

[41] A.A. Figueiredo da Silva, L. de Carvalho Vieira, M.A. Krieger, S. Goldenberg, N.I. Zanchin, B.G. Guimaraes, Crystal structure of chagasin, the endogenous cysteine-protease inhibitor from *Trypanosoma cruzi*, J. Struct. Biol. 157 (2007) 416–423.

[42] A. Ljunggren, I. Redzynia, M. Alvarez-Fernandez, M. Abrahamson, J.S. Mort, J.C. Krupa, M. Jaskolski, G. Bujacz, Crystal structure of the parasite protease inhibitor chagasin in complex with a host target cysteine protease, J. Mol. Biol. 371 (2007) 137–153.

[43] F.C. Reis, T.F. Costa, T. Sulea, A. Mezzetti, J. Scharfstein, D. Bromme, R. Menard, A. P. Lima, The propeptide of cruzipain—a potent selective inhibitor of the trypanosomal enzymes cruzipain and brucipain, and of the human enzyme cathepsin F, FEBS J. 274 (2007) 1224–1234.

[44] O. Campetella, J. Henriksson, L. Aslund, A.C. Frasch, U. Pettersson, J.J. Cazzulo, The major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* is encoded by multiple polymorphic tandemly organized genes located on different chromosomes, Mol. Biochem. Parasitol. 50 (1992) 225–234.

[45] G.H. Coombs, J.C. Mottram, Proteinases of Trypanosomes and Leishmania, in: G. Hide, P.H. Holmes, G.H. Coombs, J.C. Mottram (Eds.), Trypanosomiasis and Leishmaniasis, CAB International, Oxford, 1997, pp. 177–197.

[46] J.J. Cazzulo, C. Labriola, F. Parussini, V.G. Duschak, J. Martinez, B.M. Franke de Cazzulo, Cysteine proteinases in *Trypanosoma cruzi* and other Trypanosomatid parasites, Acta Chim. Slovenica 42 (1995) 409–418.

[47] S.I. Metzner, M.C. Sousa, U. Hellman, J.J. Cazzulo, A.J. Parodi, The use of UDP-Glc: glycoprotein glucosyltransferase for radiolabeling protein-linked high mannose-type oligosaccharides, Cell. Mol. Biol. (Noisy-le-grand) 42 (1996) 631–635.

[48] A.J. Parodi, C. Labriola, J.J. Cazzulo, The presence of complex-type oligosaccharides at the C-terminal domain glycosylation site of some molecules of cruzipain, Mol. Biochem. Parasitol. 69 (1995) 247–255.

[49] M. Barboza, V.G. Duschak, Y. Fukuyama, H. Nonami, R. Erra-Balsells, J.J. Cazzulo, A.S. Couto, Structural analysis of the N-glycans of the major cysteine proteinase of *Trypanosoma cruzi*. Identification of sulfated high-mannose type oligosaccharides, FEBS J. 272 (2005) 3803–3815.

[50] M. Barboza, V.G. Duschak, J.J. Cazzulo, R.M. de Lederkremer, A.S. Couto, Presence of sialic acid in N-linked oligosaccharide chains and O-linked N-acetylglucosamine in cruzipain, the major cysteine proteinase of *Trypanosoma cruzi*, Mol. Biochem. Parasitol. 127 (2003) 69–72.

[51] J.J. Cazzulo, J. Martinez, A.J. Parodi, C. Wernstedt, U. Hellman, On the post-translational modifications at the C-terminal domain of the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi*, FEMS Microbiol. Lett. 79 (1992) 411–416.

[52] D.P. Nolan, M. Geuskens, E. Pays, N-linked glycans containing linear poly-N-acetyllactosamine as sorting signals in endocytosis in *Trypanosoma brucei*, Curr. Biol. 9 (1999) 1169–1172.

[53] M.J. McConville, K.A. Mullin, S.C. Ilgoutz, R.D. Teasdale, Secretory pathway of trypanosomatid parasites, Microbiol. Mol. Biol. Rev. 66 (2002) 122–154.

[54] M.E. McGrath, A.E. Eakin, J.C. Engel, J.H. McKerrow, C.S. Craik, R.J. Fletterick, The crystal structure of cruzain: a therapeutic target for Chagas' disease, J. Mol. Biol. 247 (1995) 251–259.

[55] S.A. Gillmor, C.S. Craik, R.J. Fletterick, Structural determinants of specificity in the cysteine protease cruzain, Protein Sci. 6 (1997) 1603–1611.

[56] L.S. Brinen, E. Hansell, J. Cheng, W.R. Roush, J.H. McKerrow, R.J. Fletterick, A target within the target: probing cruzain's P1' site to define structural determinants for the Chagas' disease protease, Structure 8 (2000) 831–840.

[57] L. Huang, L.S. Brinen, J.A. Ellman, Crystal structures of reversible ketone-Based inhibitors of the cysteine protease cruzain, Bioorg. Med. Chem. 11 (2003) 21–29.

[58] V. Alvarez, F. Parussini, L. Aslund, J.J. Cazzulo, Expression in insect cells of active mature cruzipain from *Trypanosoma cruzi*, containing its C-terminal domain, Protein Expr. Purif. 26 (2002) 467–475.

[59] J. Scharfstein, M. Schechter, M. Senna, J.M. Peralta, L. Mendonca-Previato, M.A. Miles, *Trypanosoma cruzi*: characterization and isolation of a 57/51,000 m.w. surface glycoprotein (GP57/51) expressed by epimastigotes and bloodstream trypomastigotes, J. Immunol. 137 (1986) 1336–1341.

[60] J. Martinez, O. Campetella, A.C. Frasch, J.J. Cazzulo, The major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* is antigenic in human infections, Infect. Immun. 59 (1991) 4275–4277.

[61] J. Martinez, O. Campetella, A.C. Frasch, J.J. Cazzulo, The reactivity of sera from chagasic patients against different fragments of cruzipain, the major cysteine proteinase from *Trypanosoma cruzi*, suggests the presence of defined antigenic and catalytic domains, Immunol. Lett. 35 (1993) 191–196.

[62] A.R. Schnapp, C.S. Eickhoff, D. Sizemore, R. Curtiss III, D.F. Hoft, Cruzipain induces both mucosal and systemic protection against *Trypanosoma cruzi* in mice, Infect. Immun. 70 (2002) 5065–5074.

[63] S.I. Cazorla, F.M. Frank, P.D. Becker, R.S. Corral, C.A. Guzman, E.L. Malchiodi, Prime-boost immunization with cruzipain co-administered with MALP-2 triggers a protective immune response able to decrease parasite burden and tissue injury in an experimental *Trypanosoma cruzi* infection model, Vaccine 26 (2008) 1999–2009.

[64] J. Scharfstein, V. Schmitz, V. Morandi, M.M. Capella, A.P. Lima, A. Morrot, L. Juliano, W. Muller-Esterl, Host cell invasion by *Trypanosoma cruzi* is potentiated by activation of bradykinin B(2) receptors, J. Exp. Med. 192 (2000) 1289–1300.

[65] P. Berasain, C. Carmona, B. Frangione, J.J. Cazzulo, F. Goni, Specific cleavage sites on human IgG subclasses by cruzipain, the major cysteine proteinase from *Trypanosoma cruzi*, Mol. Biochem. Parasitol. 130 (2003) 23–29.

[66] A. Krettli, N. Thomas, H. Eisen, Escape mechanisms of *Trypanosoma cruzi* from the host immune system, in: L. Israel, P. Lagrange, J.C. Salomon (Eds.), Les Colloques de l'INSERM. Cancer Immunology and Parasite Immunology, INSERM, Paris, 1980, pp. 553–558.

[67] B.M. Franke de Cazzulo, J. Martinez, M.J. North, G.H. Coombs, J.J. Cazzulo, Effects of proteinase inhibitors on the growth and differentiation of *Trypanosoma cruzi*, FEMS Microbiol. Lett. 124 (1994) 81–86.

[68] M.N. Meirelles, L. Juliano, E. Carmona, S.G. Silva, E.M. Costa, A.C. Murta, J. Scharfstein, Inhibitors of the major cysteinyl proteinase (GP57/51) impair host cell invasion and arrest the intracellular development of *Trypanosoma cruzi* in vitro, Mol. Biochem. Parasitol. 52 (1992) 175–184.

[69] G. Harth, N. Andrews, A.A. Mills, J.C. Engel, R. Smith, J.H. McKerrow, Peptide-fluoromethyl ketones arrest intracellular replication and intercellular transmission of *Trypanosoma cruzi*, Mol. Biochem. Parasitol. 58 (1993) 17–24.

[70] M.C. Bonaldo, L.N. d'Escoffier, J.M. Salles, S. Goldenberg, Characterization and expression of proteases during *Trypanosoma cruzi* metacyclogenesis, Exp. Parasitol. 73 (1991) 44–51.

[71] J.C. Engel, P.S. Doyle, J. Palmer, I. Hsieh, D.F. Bainton, J.H. McKerrow, Cysteine protease inhibitors alter Golgi complex ultrastructure and function in *Trypanosoma cruzi*, J. Cell Sci. 111 (1998) 597–606.

[72] V. Yong, V. Schmitz, M.A. Vannier-Santos, A.P. de Lima, G. Lalmanach, L. Juliano, F. Gauthier, J. Scharfstein, Altered expression of cruzipain and a cathepsin B-like target in a *Trypanosoma cruzi* cell line displaying resistance to synthetic inhibitors of cysteine-proteinases, Mol. Biochem. Parasitol. 109 (2000) 47–59.

[73] J.C. Engel, C. Torres, I. Hsieh, P.S. Doyle, J.H. McKerrow, Upregulation of the secretory pathway in cysteine protease inhibitor-resistant *Trypanosoma cruzi*, J. Cell Sci. 113 (2000) 1345–1354.

[74] J.C. Engel, P.S. Doyle, I. Hsieh, J.H. McKerrow, Cysteine protease inhibitors cure an experimental *Trypanosoma cruzi* infection, J. Exp. Med. 188 (1998) 725–734.

[75] P.S. Doyle, Y.M. Zhou, J.C. Engel, J.H. McKerrow, A cysteine protease inhibitor cures Chagas' disease in an immunodeficient-mouse model of infection, Antimicrob. Agents Chemother. 51 (2007) 3932–3939.

[76] J.H. McKerrow, P.S. Doyle, J.C. Engel, L.M. Podust, S.A. Robertson, R. Ferreira, T. Saxton, M. Arkin, I.D. Kerr, L.S. Brinen, C.S. Craik, Two approaches to discovering and developing new drugs for Chagas disease, Mem. Inst. Oswaldo Cruz 104 (Suppl. 1) (2009) 263–269.

[77] M.P. Garcia, O.T. Nobrega, A.R. Teixeira, M.V. Sousa, J.M. Santana, Characterization of a *Trypanosoma cruzi* acidic 30 kDa cysteine protease, Mol. Biochem. Parasitol. 91 (1998) 263–272.

[78] O.T. Nobrega, M.A. Santos Silva, A.R. Teixeira, J.M. Santana, Cloning and sequencing of tccb, a gene encoding a *Trypanosoma cruzi* cathepsin B-like protease, Mol. Biochem. Parasitol. 97 (1998) 235–240.

[79] A.G. Uren, K. O'Rourke, L.A. Aravind, M.T. Pisabarro, S. Seshagiri, E.V. Koonin, V.M. Dixit, Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma, Mol. Cell 6 (2000) 961–967.

[80] N.D. Rawlings, A.J. Barrett, Evolutionary families of peptidases, Biochem. J. 290 (1993) 205–218.

[81] L. Aravind, E.V. Koonin, Classification of the caspase-hemoglobinase fold: detection of new families and implications for the origin of the eukaryotic separins, Proteins 46 (2002) 355–367.

[82] D. Vercammen, B. van de Cotte, G. De Jaeger, D. Eeckhout, P. Casteels, K. Vandepoele, I. Vandenberghe, J. Van Beeumen, D. Inze, F. Van Breusegem, Type II metacaspases Atmc4 and Atmc9 of *Arabidopsis thaliana* cleave substrates after arginine and lysine, J. Biol. Chem. 279 (2004) 45329–45336.

[83] N. Watanabe, E. Lam, Two Arabidopsis metacaspases AtMCP1b and AtMCP2b are arginine/lysine-specific cysteine proteases and activate apoptosis-like cell death in yeast, J. Biol. Chem. 280 (2005) 14691–14699.

[84] I.J. Gonzalez, C. Desponds, C. Schaff, J.C. Mottram, N. Fasel, *Leishmania major* metacaspase can replace yeast metacaspase in programmed cell death and has arginine-specific cysteine peptidase activity, Int. J. Parasitol. 37 (2007) 161–172.

[85] C.X. Moss, G.D. Westrop, L. Juliano, G.H. Coombs, J.C. Mottram, Metacaspase 2 of *Trypanosoma brucei* is a calcium-dependent cysteine peptidase active without processing, FEBS Lett. 581 (2007) 5635–5639.

[86] D. Vercammen, B. Belenghi, B. van de Cotte, T. Beunens, J.A. Gavigan, R. De Rycke, A. Brackenier, D. Inze, J.L. Harris, F. Van Breusegem, Serpin1 of *Arabidopsis thaliana* is a suicide inhibitor for metacaspase 9, J. Mol. Biol. 364 (2006) 625–636.

[87] P.V. Bozhkov, M.F. Suarez, L.H. Filonova, G. Daniel, A.A. Zamyatnin Jr., S. Rodriguez-Nieto, B. Zhivotovsky, A. Smertenko, Cysteine protease mcII-Pa executes programmed cell death during plant embryogenesis, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 14463–14468.

[88] N. Watanabe, E. Lam, Calcium-dependent activation and autolysis of Arabidopsis metacaspase 2d, J. Biol. Chem. 286 (2011) 10027–10040.

[89] N.S. Coll, D. Vercammen, A. Smidler, C. Clover, F. Van Breusegem, J.L. Dangl, P. Epple, Arabidopsis type I metacaspases control cell death, Science 330 (2010) 1393–1397.

[90] J.C. Mottram, M.J. Helms, G.H. Coombs, M. Sajid, Clan CD cysteine peptidases of parasitic protozoa, Trends Parasitol. 19 (2003) 182–187.

[91] G. Kosec, V.E. Alvarez, F. Aguero, D. Sanchez, M. Dolinar, B. Turk, V. Turk, J.J. Cazzulo, Metacaspases of *Trypanosoma cruzi*: possible candidates for programmed cell death mediators, Mol. Biochem. Parasitol. 145 (2006) 18–28.

[92] J.F. Sundstrom, A. Vaculova, A.P. Smertenko, E.I. Savenkov, A. Golovko, E. Minina, B.S. Tiwari, S. Rodriguez-Nieto, A.A. Zamyatnin Jr., T. Valineva, J. Saarikettu, M.J. Frilander, M.F. Suarez, A. Zavialov, U. Stahl, P.J. Hussey, O. Silvennoinen, E. Sundberg, B. Zhivotovsky, P.V. Bozhkov, Tudor staphylococcal nuclease is an evolutionarily conserved component of the programmed cell death degradome, Nat. Cell Biol. 11 (2009) 1347–1354.

[93] M.J. Helms, A. Ambit, P. Appleton, L. Tetley, G.H. Coombs, J.C. Mottram, Bloodstream form *Trypanosoma brucei* depend upon multiple metacaspases associated with RAB11-positive endosomes, J. Cell Sci. 119 (2006) 1105–1117.

[94] A. Ambit, N. Fasel, G.H. Coombs, J.C. Mottram, An essential role for the *Leishmania major* metacaspase in cell cycle progression, Cell Death Differ. 15 (2008) 113–122.

[95] J.A. Atwood III, D.B. Weatherly, T.A. Minning, B. Bundy, C. Cavola, F.R. Opperdoes, R. Orlando, R.L. Tarleton, The *Trypanosoma cruzi* proteome, Science 309 (2005) 473–476.

[96] M. Berg, P. Van der Veken, J. Joossens, V. Muthusamy, M. Breugelmans, C.X. Moss, J. Rudolf, P. Cos, G.H. Coombs, L. Maes, A. Haemers, J.C. Mottram, K. Augustyns, Design and evaluation of *Trypanosoma brucei* metacaspase inhibitors, Bioorg. Med. Chem. Lett. 20 (2010) 2001–2006.

[97] D.J. Klionsky, Autophagy, Curr. Biol. 15 (2005) R282–283.

[98] D.J. Klionsky, J.M. Cregg, W.A. Dunn Jr., S.D. Emr, Y. Sakai, I.V. Sandoval, A. Sibirny, S. Subramani, M. Thumm, M. Veenhuis, Y. Ohsumi, A unified nomenclature for yeast autophagy-related genes, Dev. Cell 5 (2003) 539–545.

[99] H. Nakatogawa, Y. Ichimura, Y. Ohsumi, Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion, Cell 130 (2007) 165–178.

[100] Y. Ichimura, T. Kirisako, T. Takao, Y. Satomi, Y. Shimonishi, N. Ishihara, N. Mizushima, I. Tanida, E. Kominami, M. Ohsumi, T. Noda, Y. Ohsumi, A ubiquitin-like system mediates protein lipidation, Nature 408 (2000) 488–492.

[101] T. Kirisako, Y. Ichimura, H. Okada, Y. Kabeya, N. Mizushima, T. Yoshimori, M. Ohsumi, T. Takao, T. Noda, Y. Ohsumi, The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway, J. Cell Biol. 151 (2000) 263–276.

[102] M. Duszenko, M.L. Ginger, A. Brennand, M. Gualdron-Lopez, M.I. Colombo, G.H. Coombs, I. Coppens, B. Jayabalasingham, G. Langsley, S.L. de Castro, R. Menna-Barreto, J.C. Mottram, M. Navarro, D.J. Rigden, P.S. Romano, V. Stoka, B. Turk, P.A. Michels, Autophagy in protists, Autophagy, 7, 127-158.

[103] V.E. Alvarez, G. Kosec, C. Sant'Anna, V. Turk, J.J. Cazzulo, B. Turk, Autophagy is involved in nutritional stress response and differentiation in, J. Biol. Chem. 283 (2008) 3454–3464.

[104] A.H. Kollien, G.A. Schaub, The development of *Trypanosoma cruzi* in triatominae, Parasitol. Today 16 (2000) 381–387.

[105] S. Besteiro, R.A. Williams, L.S. Morrison, G.H. Coombs, J.C. Mottram, Endosome sorting and autophagy are essential for differentiation and virulence of *Leishmania major*, J. Biol. Chem. 281 (2006) 11384–11396.

[106] R.A. Williams, L. Tetley, J.C. Mottram, G.H. Coombs, Cysteine peptidases CPA and CPB are vital for autophagy and differentiation in *Leishmania mexicana*, Mol. Microbiol. 61 (2006) 655–674.

[107] J. Hemelaar, V.S. Lelyveld, B.M. Kessler, H.L. Ploegh, A single protease, Apg4B, is specific for the autophagy-related ubiquitin-like proteins GATE-16, MAP1-LC3, GABARAP, and Apg8L, J. Biol. Chem. 278 (2003) 51841–51850.

[108] K. Sugawara, N.N. Suzuki, Y. Fujioka, N. Mizushima, Y. Ohsumi, F. Inagaki, Structural basis for the specificity and catalysis of human Atg4B responsible for mammalian autophagy, J. Biol. Chem. 280 (2005) 40058–40065.

[109] C. Sant'Anna, F. Parussini, D. Lourenco, W. de Souza, J.J. Cazzulo, N.L. Cunha-e-Silva, All *Trypanosoma cruzi* developmental forms present lysosome-related organelles, Histochem. Cell. Biol. 130 (2008) 1187–1198.

[110] S.C. Barr, K.L. Warner, B.G. Kornreic, J. Piscitelli, A. Wolfe, L. Benet, J.H. McKerrow, A cysteine protease inhibitor protects dogs from cardiac damage during infection by *Trypanosoma cruzi*, Antimicrob. Agents Chemother. 49 (2005) 5160–5161.

[111] T.H. Coetzer, J.P. Goldring, L.E. Huson, Oligopeptidase B: a processing peptidase involved in pathogenesis, Biochimie 90 (2008) 336–344.

[112] J.M. Santana, P. Grellier, M.H. Rodier, J. Schrevel, A. Teixeira, Purification and characterization of a new 120 kDa alkaline proteinase of *Trypanosoma cruzi*, Biochem. Biophys. Res. Commun. 187 (1992) 1466–1473.

[113] B.A. Burleigh, E.V. Caler, P. Webster, N.W. Andrews, A cytosolic serine endopeptidase from *Trypanosoma cruzi* is required for the generation of Ca2+ signaling in mammalian cells, J. Cell Biol. 136 (1997) 609–620.

[114] J.P. Hemerly, V. Oliveira, E. Del Nery, R.E. Morty, N.W. Andrews, M.A. Juliano, L. Juliano, Subsite specificity (S3, S2, S1', S2' and S3') of oligopeptidase B from *Trypanosoma cruzi* and *Trypanosoma brucei* using fluorescent quenched peptides: comparative study and identification of specific carboxypeptidase activity, Biochem. J. 373 (2003) 933–939.

[115] E.V. Caler, S. Vaena de Avalos, P.A. Haynes, N.W. Andrews, B.A. Burleigh, Oligopeptidase B-dependent signaling mediates host cell invasion by *Trypanosoma cruzi*, EMBO J. 17 (1998) 4975–4986.

[116] R.E. Morty, L. Troeberg, R.N. Pike, R. Jones, P. Nickel, J.D. Lonsdale-Eccles, T.H. Coetzer, A trypanosome oligopeptidase as a target for the trypanocidal agents pentamidine, diminazene and suramin, FEBS Lett. 433 (1998) 251–256.

[117] J.M. Santana, P. Grellier, J. Schrevel, A.R. Teixeira, A Trypanosoma cruzi-secreted 80 kDa proteinase with specificity for human collagen types I and IV, Biochem. J. 325 (1997) 129–137.

[118] I.M. Bastos, P. Grellier, N.F. Martins, G. Cadavid-Restrepo, M.R. de Souza-Ault, K. Augustyns, A.R. Teixeira, J. Schrevel, B. Maigret, J.F. da Silveira, J.M. Santana, Molecular, functional and structural properties of the prolyl oligopeptidase of *Trypanosoma cruzi* (POP Tc80), which is required for parasite entry into mammalian cells, Biochem. J. 388 (2005) 29–38.
[119] S. Vendeville, L. Bourel, E. Davioud-Charvet, P. Grellier, B. Deprez, C. Sergheraert, Automated parallel synthesis of a tetrahydroisoquinolin-based library: potential prolyl endopeptidase inhibitors, Bioorg. Med. Chem. Lett. 9 (1999) 437–442.
[120] S. Vendeville, E. Buisine, X. Williard, J. Schrevel, P. Grellier, J. Santana, C. Sergheraert, Identification of inhibitors of an 80 kDa protease from *Trypanosoma cruzi* through the screening of a combinatorial peptide library, Chem. Pharm. Bull. (Tokyo) 47 (1999) 194–198.
[121] R. Joyeau, C. Maoulida, C. Guillet, F. Frappier, A.R. Teixeira, J. Schrevel, J. Santana, P. Grellier, Synthesis and activity of pyrrolidinyl- and thiazolidinyl-dipeptide derivatives as inhibitors of the Tc80 prolyl oligopeptidase from *Trypanosoma cruzi*, Eur. J. Med. Chem. 35 (2000) 257–266.
[122] S. Vendeville, F. Goossens, M.A. Debreu-Fontaine, V. Landry, E. Davioud-Charvet, P. Grellier, S. Scharpe, C. Sergheraert, Comparison of the inhibition of human and *Trypanosoma cruzi* prolyl endopeptidases, Bioorg. Med. Chem. 10 (2002) 1719–1729.
[123] P. Grellier, S. Vendeville, R. Joyeau, I.M. Bastos, H. Drobecq, F. Frappier, A.R. Teixeira, J. Schrevel, E. Davioud-Charvet, C. Sergheraert, J.M. Santana, *Trypanosoma cruzi* prolyl oligopeptidase Tc80 is involved in nonphagocytic mammalian cell invasion by trypomastigotes, J. Biol. Chem. 276 (2001) 47078–47086.
[124] G. Bal, P. Van der Veken, D. Antonov, A.M. Lambeir, P. Grellier, S.L. Croft, K. Augustyns, A. Haemers, Prolylisoxazoles: potent inhibitors of prolyloligopeptidase with antitrypanosomal activity, Bioorg. Med. Chem. Lett. 13 (2003) 2875–2878.
[125] F. Parussini, M. Garcia, J. Mucci, F. Aguero, D. Sanchez, U. Hellman, L. Aslund, J.J. Cazzulo, Characterization of a lysosomal serine carboxypeptidase from *Trypanosoma cruzi*, Mol. Biochem. Parasitol. 131 (2003) 11–23.
[126] C. Yao, Major surface protease of trypanosomatids: one size fits all? Infect. Immun. 78 (2010) 22–31.
[127] C.M. Lowndes, M.C. Bonaldo, N. Thomaz, S. Goldenberg, Heterogeneity of metalloprotease expression in *Trypanosoma cruzi*, Parasitology 112 (1996) 393–399.
[128] P.M. Grandgenett, B.C. Coughlin, L.V. Kirchhoff, J.E. Donelson, Differential expression of GP63 genes in *Trypanosoma cruzi*, Mol. Biochem. Parasitol. 110 (2000) 409–415.
[129] I.C. Cuevas, J.J. Cazzulo, D.O. Sanchez, gp63 homologues in *Trypanosoma cruzi*: surface antigens with metalloprotease activity and a possible role in host cell infection, Infect. Immun. 71 (2003) 5739–5749.
[130] M.M. Kulkarni, C.L. Olson, D.M. Engman, B.S. McGwire, *Trypanosoma cruzi* GP63 proteins undergo stage-specific differential posttranslational modification and are important for host cell infection, Infect. Immun. 77 (2009) 2193–2200.
[131] B.M. Porcel, L. Aslund, U. Pettersson, B. Andersson, *Trypanosoma cruzi*: a putative vacuolar ATP synthase subunit and a CAAX prenyl protease-encoding gene, as examples of gene identification in genome projects, Exp. Parasitol. 95 (2000) 176–186.
[132] J.R. Gillespie, K. Yokoyama, K. Lu, R.T. Eastman, J.G. Bollinger, W.C. Van Voorhis, M.H. Gelb, F.S. Buckner, C-terminal proteolysis of prenylated proteins in trypanosomatids and RNA interference of enzymes required for the post-translational processing pathway of farnesylated proteins, Mol. Biochem. Parasitol. 153 (2007) 115–124.
[133] S.H. Lee, E. Minagawa, H. Taguchi, H. Matsuzawa, T. Ohta, S. Kaminogawa, K. Yamauchi, Purification and characterization of a thermostable carboxypeptidase (carboxypeptidase Taq) from *Thermus aquaticus* YT-1, Biosci. Biotechnol. Biochem. 56 (1992) 1839–1844.
[134] S.H. Lee, H. Taguchi, E. Yoshimura, E. Minagawa, S. Kaminogawa, T. Ohta, H. Matsuzawa, Carboxypeptidase Taq, a thermostable zinc enzyme, from *Thermus aquaticus* YT-1: molecular cloning, sequencing, and expression of the encoding gene in Escherichia coli, Biosci. Biotechnol. Biochem. 58 (1994) 1490–1495.
[135] S.H. Lee, H. Taguchi, E. Yoshimura, E. Minagawa, S. Kaminogawa, T. Ohta, H. Matsuzawa, The active site of carboxypeptidase Taq possesses the active-site motif His-Glu-X-X-His of zinc-dependent endopeptidases and aminopeptidases, Protein Eng. 9 (1996) 467–469.
[136] G. Niemirowicz, F. Parussini, F. Aguero, J.J. Cazzulo, Two metallocarboxypeptidases from the protozoan *Trypanosoma cruzi* belong to the M32 family, found so far only in prokaryotes, Biochem. J. 401 (2007) 399–410.
[137] C.E. Isaza, X. Zhong, L.E. Rosas, J.D. White, R.P. Chen, G.F. Liang, S.I. Chan, A.R. Satoskar, M.K. Chan, A proposed role for *Leishmania major* carboxypeptidase in peptide catabolism, Biochem. Biophys. Res. Commun. 373 (2008) 25–29.
[138] G. Niemirowicz, D. Fernandez, M. Sola, J.J. Cazzulo, F.X. Aviles, F.X. Gomis-Ruth, The molecular analysis of *Trypanosoma cruzi* metallocarboxypeptidase 1 provides insight into fold and substrate specificity, Mol. Microbiol. 70 (2008) 853–866.
[139] A.G. Todorov, D. Andrade, J.B. Pesquero, C. Araujo Rde, M. Bader, J. Stewart, L. Gera, W. Muller-Esterl, V. Morandi, R.C. Goldenberg, H.C. Neto, J. Scharfstein, *Trypanosoma cruzi* induces edematogenic responses in mice and invades cardiomyocytes and endothelial cells in vitro by activating distinct kinin receptor (B1/B2) subtypes, FASEB J. 17 (2003) 73–75.
[140] A. Geiger, C. Hirtz, T. Becue, E. Bellard, D. Centeno, D. Gargani, M. Rossignol, G. Cuny, J.B. Peltier, Exocytosis and protein secretion in Trypanosoma, BMC Microbiol. 10 (2010) 20.
[141] J.M. Silverman, J. Clos, C.C. de'Oliveira, O. Shirvani, Y. Fang, C. Wang, L.J. Foster, N. E. Reiner, An exosome-based secretion pathway is responsible for protein export from Leishmania and communication with macrophages, J. Cell Sci. 123 (2010) 842–852.
[142] J. Gonzalez, F.J. Ramalho-Pinto, U. Frevert, J. Ghiso, S. Tomlinson, J. Scharfstein, E. J. Corey, V. Nussenzweig, Proteasome activity is required for the stage-specific transformation of a protozoan parasite, J. Exp. Med. 184 (1996) 1909–1918.
[143] S. Hua, W.Y. To, T.T. Nguyen, M.L. Wong, C.C. Wang, Purification and characterization of proteasomes from *Trypanosoma brucei*, Mol. Biochem. Parasitol. 78 (1996) 33–46.
[144] C.C. Wang, Z. Bozdech, C.L. Liu, A. Shipway, B.J. Backes, J.L. Harris, M. Bogyo, Biochemical analysis of the 20 S proteasome of *Trypanosoma brucei*, J. Biol. Chem. 278 (2003) 15800–15808.
[145] J.L. de Diego, J.M. Katz, P. Marshall, B. Gutierrez, J.E. Manning, V. Nussenzweig, J. Gonzalez, The ubiquitin-proteasome pathway plays an essential role in proteolysis during *Trypanosoma cruzi* remodeling, Biochemistry 40 (2001) 1053–1062.
[146] J.L. Bouzat, L.K. McNeil, H.M. Robertson, L.F. Solter, J.E. Nixon, J.E. Beever, H.R. Gaskins, G. Olsen, S. Subramaniam, M.L. Sogin, H.A. Lewin, Phylogenomic analysis of the alpha proteasome gene family from early-diverging eukaryotes, J. Mol. Evol. 51 (2000) 532–543.
[147] D.C. Bartholomeu, J.A. Batista, M.H. Vainstein, B.D. Lima, M.C. de Sa, Molecular cloning and characterization of a gene encoding the 29-kDa proteasome subunit from *Trypanosoma cruzi*, Mol. Genet. Genomics 265 (2001) 986–992.
[148] W.C. Coffeen, T.J. Wolpert, Purification and characterization of serine proteases that exhibit caspase-like activity and are associated with programmed cell death in *Avena sativa*, Plant Cell 16 (2004) 857–873.