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Review

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

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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

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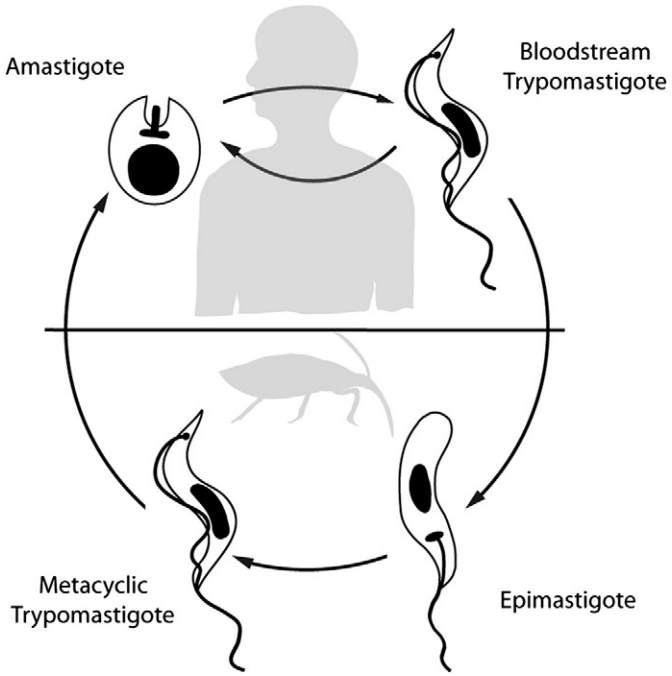

Fig. 1. The life cycle of *T. cruzi*. The parasite has a complex life cycle, with four major stages: an obligate intracellular replicative form, the amastigote, and a non-replicative one, invasive for host cells, the bloodstream trypomastigote, in the mammalian host; a replicative stage, the epimastigote, 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 pre-lysosomal 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)