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Detection of matrix metallopeptidase-9-like proteins in *Trypanosoma cruzi*

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ABSTRACT

In this study, the cell-associated and extracellular peptidases of *Trypanosoma cruzi* grown in modified Roitman's complex (MRC) medium were analyzed by measuring peptidase activity in gelatin-containing zymograms. Our results showed that the cell-associated peptidases as well as peptidases extracellularly released by *T. cruzi* displayed two distinct proteolytic classes: cysteine and metallopeptidase activities. The major cysteine peptidase, cruzipain, synthesized by *T. cruzi* cells was detected in cellular parasite content, as a 50 kDa reactive polypeptide, after probing with anti-cruzipain antibody. In addition, metallo-type peptidases belonging to the matrix metallopeptidase-9 (MMP-9) family were revealed, after Western blotting, as a 97 kDa protein band in cellular extract and an 85 kDa polypeptide in both cellular and secreted parasite extracts. The MMP-9-like activity present in cells and spent culture medium was immunoprecipitated by an anti-MMP-9 polyclonal antibody. The surface location of MMP-9-like proteins in *T. cruzi* was also evidenced by means of flow cytometry analysis. Furthermore, doxycycline that has direct MMP-9 inhibiting properties *in vitro,* inhibited MMP-9-like activities in gel zymography, immunoprecipitation and flow cytometry analyses. This is the first report of the presence of MMP-9-like molecules in *T. cruzi*. The presence of a matrix extracellular-degrading enzyme may play a role in the *T. cruzi*-host cell interaction, making this enzyme a potential target for future drug development against this pathogenic trypanosomatid.

1. Introduction

The flagellated protozoan *Trypanosoma cruzi*[1] is the causative agent of Chagas' disease, a pathology characterized by chronic inflammation associated with cardiomyopathy and/or digestive disorders (Engman and Leon, 2002). Chagas' disease is a neglected tropical disease that affects over 11 million people and causes an estimated 50,000 deaths annually in Latin America (WHO, 2006). Most human infections occur through contact with the infected bloodsucking triatomine species. However, the urbanization process in Latin America and immigration trends have led to the disease being diagnosed in non-endemic areas where, even in the absence of the vector; the infection can still be transmitted congenitally, by blood transfusion and by organ transplantation (Bern et al., 2007). In addition, oral transmission has aroused the attention of researchers, due to outbreak in some particular areas such as the Brazilian Amazon, mainly associated with the consumption of Amazonian palm berry or açaí (Euterpe oleracea Mart.) juice (Yoshida, 2009).

Peptidases produced by the pathogenic trypanosomatids play an important role in several steps of the host infection including: adsorption, penetration, intracellular survival, replication, differentiation, infectivity, immune evasion and nutrition (Vermelho et al., 2007). Trypanosomatids elaborate a large array of peptidases, which are intracellular and/or extracellular, with different specific-

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[1] *Index descriptions and abbreviations: Trypanosoma cruzi*; matrix metallopeptidase-9; gelatin–SDS–PAGE; immunoprecipitation; MMP-9, matrix metallopeptidase-9; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MRC, modified Roitman's complex medium; MMP, matrix metallopeptidase; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; FACS, fluorescence activated cell sorting; ECM, the extracellular matrix.

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ities and many of them have been purified and characterized and their genes cloned and sequenced. Because of their unusual structural features, parasite peptidases are considered to have a high potential as targets for novel antiparasitic agents (Brack et al., 2008). The immunodominant nature of many peptidases offers potential for serodiagnosis and vaccine development.

Trypanosoma cruzi has been shown to contain several proteolytic activities (Vermelho et al., 2007). Cruzipain, a cysteine peptidase of the papain family, is the most prominent proteolytic activity synthesized by all developmental stages of *T. cruzi*. Cruzipain is also a proposed therapeutic target for treatment of Chagas' disease (Cazzulo, 2002). Previous studies have demonstrated that *T. cruzi* infection can be cured in cell, mouse and dog models by treatment with irreversible inhibitors of cruzipain (Engel et al., 1998; Barr et al., 2005). In addition to cruzipain, other cysteine peptidases identified in the parasite include a 30-kDa cathepsin B-like peptidase which exhibited an important hydrolytic activity on human type I collagen (Garcia et al., 1998) and *Tc*CPmet secreted from metacyclic trypomastigotes during metacyclogenesis (Duschak et al., 2006). Two serine peptidases belonging to the prolyl oligopeptidase family have also been characterized in *T. cruzi*: the parasite calcium-signaling activity is linked to the 120-kDa cytosolic serine peptidase, named Tc-OP, which generates an active calcium agonist from a cytosolic precursor molecule (Burleigh and Woolsey, 2002) and the 80-kDa serine prolyl oligopeptidase (POP Tc80) facilitates *T. cruzi* dissemination throughout host tissues due to its ability to mediate the cleavage of some extracellular matrix components (Bastos et al., 2005). The expression of metallopeptidase activities have been previously described in various strains and clones of *T. cruzi*, some of them specifically observed during metacyclogenesis (Bonaldo et al., 1991; Lowndes et al., 1996). Moreover, Cuevas et al. (2003) described that *T. cruzi* possesses a family of gp63 genes composed of multiple groups. Two of the groups, *Tcgp63*-I and *Tcgp63*-II, are present as high-copy-number genes and antibodies against *Tcgp63*-I partially blocked the infection of Vero cells by trypomastigotes, which suggests a possible role for this metallopeptidase during the infection process *in vitro*. Recently, Pinho et al. (2009) described for the first time the isolation of two aspartic peptidases from *T. cruzi*, which have been named CPZ-I and CPZ-II.

Extracellular matrix (ECM)-eukaryotic cell interactions are regulated by a family of zinc-dependent peptidases known as matrix metallopeptidases (MMPs) that can be classified broadly by substrate specificity (Van den Steen et al., 2002). Through turnover of extracellular matrix proteins, MMPs are involved either in normal matrix remodeling or pathological tissue destruction (Gomis-Rüth, 2009). Among the members of the MMP family, the gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are known to be key players in many physiological and pathological processes. In this context, earlier studies from our group showed matrix metallopeptidase-9 (MMP-9)-like activity in the cytoplasm of *T. cruzi* during *in vitro* infection of embryonic hepatocyte cells (Nogueira de Melo et al., 2004). Peptidase-dependent extracellular matrix remodeling is one of the events that is emerging as a key regulator of *T. cruzi* infection and pathogenesis of Chagas' disease. Here in this study we look at the detection of MMP-9-like proteins in cells and spent culture medium of *T. cruzi* Y strain and discuss the possible implications of this enzyme in the pathology of Chagas' disease.

2. Materials and methods

2.1. Chemicals

Media constituents, reagents used in electrophoresis, buffer components and the nitrocellulose membrane were obtained from Amersham Life Science (Little Chalfont, England). The metallo (1,10-phenanthroline) and the cysteine (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane [E-64]) peptidase inhibitors, dithiothreitol (DTT) and secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit Fc) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rat IgG_{2b} κ isotype and anti-MMP-9 polyclonal antibody were purchased from BD Pharmigen and Santa Cruz (Biotechnology), respectively. All other reagents were analytical grade.

2.2. Parasite and growth conditions

T. cruzi Y strain is part of our trypanosomatid culture collection and it was cultivated in modified Roitman's complex (MRC) medium (0.5% yeast extract, 0.5% peptone, 2% KCl, 2% sucrose and 2 mg% hemin) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) for 6 days at 28 °C. Cellular viability was assessed by motility, trypan blue cell dye exclusion and the absence of lactate dehydrogenase, an intracellular enzyme, in the cell-free culture supernatants (d'Avila-Levy et al., 2003).

Bloodstream trypomastigote forms of *T. cruzi* (Y strain) were maintained cyclically in Swiss mice and they were obtained at the peak of parasitemia (7 days post-infection) as previously described (Meirelles et al., 1984). Briefly, immediately after euthanasia intra-cardiac function was made for blood collection. The blood samples were centrifuged at 1000 rpm for 20 min and the plasma containing trypomastigote forms of *T. cruzi* was removed. The parasites were seeded by centrifugation (3500 rpm for 15 min) and resuspended in Dulbecco's modified Eagle medium (DMEM). The total number of parasites/ml was determined by quantification in a Neubauer chamber. Intracellular amastigotes were obtained after the infection of Vero cell monolayers with trypomastigote forms of *T. cruzi*, Y strain. The highly infected cells ruptured releasing amastigote forms of *T. cruzi* after 5–7 days of infection. The parasites were harvested, centrifuged and resuspended in DMEM (Souto-Padrón et al., 1990).

2.3. Parasite extracts

Six-day-old cultured epimastigotes, at the log growth phase, were harvested by centrifugation (1500*g*, 15 min, 4 °C), washed three times with phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7.2), then 1×10^7 cells were lysed at 0 °C by the addition of 100 μL of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol and 0.002% bromophenol blue). Amastigote and trypomastigote cells (1×10^7 cells) were also washed three times with phosphate-buffered saline and lysed as described above. The spent culture medium was passed over a 0.22-μm filtration unit and concentrated 30-fold in a 10,000-molecular-weight cut-off Centricon micropartition system (AMICON, Beverly, MA). The concentrated culture supernatant was mixed with SDS–PAGE sample buffer in a sample:buffer ratio of 8:2. Protein concentration was determined by the method of Lowry et al. (1951), using BSA as a standard.

2.4. Analysis of peptidase activity

The cellular and extracellular proteolytic activities were evaluated on 10% SDS–PAGE with 0.1% gelatin incorporated as substrate (Heussen and Dowdle, 1980). Briefly, the gels were loaded with 10 μL of parasite extract (equivalent to 1×10^7 cells) and 40 μL of concentrated culture supernatant (equivalent to 100 μg of protein) per slot and after electrophoresis at a constant voltage of 170 V for 2 h at 4 °C, they were soaked for 1 h in 2.5% Triton X-100 under constant agitation. The gel strips containing the cellular and released peptidases were then incubated for 48 h at 37 °C in