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Peptidases and gp63-like proteins in *Herpetomonas megaseliae*: Possible involvement in the adhesion to the invertebrate host

Ana C. Nogueira de Melo [a], Claudia M. d'Avila-Levy [a], Felipe A. Dias [a], Jorge Luís A. Armada [b], Heriberto D. Silva [b], Angela H.C.S. Lopes [a], André L.S. Santos [a], Marta H. Branquinha [a], Alane B. Vermelho [a,*]

[a] *Departamento de Microbiologia Geral, Instituto de Microbiologia Prof. Paulo de Góes (IMPPG), Bloco I, Centro de Ciências da Saúde (CCS), Universidade Federal do Rio de Janeiro (UFRJ), Cidade Universitária, Ilha do Fundão, Rio de Janeiro, RJ 21941-590, Brazil*
[b] *Departamento de Genética, Instituto de Biologia, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Rodovia BR 465, Km 7, Seropédica, RJ 23890-000, Brazil*

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Abstract

The cell-associated and extracellular peptidases of *Herpetomonas megaseliae* grown in brain–heart infusion and in modified Roitman's complex media were analyzed by measuring peptidase activity on gelatin, casein and hemoglobin in zymograms. Casein was the best proteinaceous substrate for the peptidase detection on both growth conditions. However, no proteolytic activity was detected when hemoglobin was used. Our results showed that cellular cysteine peptidase (115–100, 40 and 35 kDa) and metallopeptidase (70 and 60 kDa) activities were detected on both media in casein and gelatin zymograms. Additionally, the use of casein in the gel revealed a distinct acidic metallopeptidase of 50 kDa when the parasite was cultured in the modified Roitman's complex medium. Irrespective of the culture medium composition, *H. megaseliae* released metallopeptidases exclusively in the extracellular environment. The presence of gp63-like molecules on the *H. megaseliae* surface was shown by flow cytometry using anti-gp63 antibody raised against recombinant gp63 from *Leishmania mexicana*. The pre-treatment of parasites with phospholipase C reduced the number of gp63-positive cells, suggesting that these molecules were glycosylphosphatidylinositol-anchored to the surface. Additionally, the supernatant obtained from phospholipase C-treated cells and probed with anti-cross-reacting determinant confirmed that at least a 52 kDa gp63-like molecule is glycosylphosphatidylinositol-anchored. Furthermore, we assessed a possible function for the gp63-like molecules in *H. megaseliae* on the interaction with explanted guts of its original host, *Megaselia scalaris*, and with an experimental model employing *Aedes aegypti*. Parasites pre-treated with either anti-gp63 antibody or phospholipase C showed a significant reduction in the adhesion to *M. scalaris* and *A. aegypti* guts. Similarly, the pre-treatment of the explanted guts with purified gp63 diminished the interaction process. Collectively, these results corroborate the ubiquitous existence of gp63 homologues in insect trypanosomatids and the potential adhesion of these molecules to invertebrate host tissues.

Keywords: *Herpetomonas megaselia*; *Megaselia scalaris*; *Aedes aegypti*; Insect trypanosomatid; Peptidase; gp63; Cellular interaction

1. Introduction

Although the Trypanosomatidae family includes parasites of plants, insects and vertebrates, only two genera, *Leishmania* and *Trypanosoma*, are usually found in humans and are etiologic agents of important illnesses such as leishmaniasis, Chagas' disease and African trypanosomiasis (McGhee and Cosgrove, 1980). In addition to these digenetic parasites, several genera, including *Herpetomonas*, *Crithidia*, *Blastocrithidia* and *Leptomonas* are composed of monogenetic parasites of the midgut and associated organs of a wide range of insects (McGhee and Cosgrove, 1980). The monogenetic trypanosomatids have been used as comparative models of study in order to understand the physiology, biochemistry, ultrastructure and the molecular biology of the pathogenic species (Schneider and Glaser, 1993; Nogueira de Melo et al., 2001; Santos et al., 2002, 2006; Vermelho et al., 2003; d'Avila-Levy et al., 2003a, 2006; Jaffe and Dwyer, 2003). In addition, trypanosomatids normally not infectious to humans were

* Corresponding author. Tel.: +55 21 2562 6743; fax: +55 21 2560 8344.
E-mail address: abvermelho@micro.ufrj.br (A.B. Vermelho).

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isolated from immunosuppressed patients, mainly in HIV-positive individuals, in whom the parasites caused either visceral or cutaneous lesions (reviewed by Chicarro and Alvar, 2003). Collectively, these studies emphasize the need for further investigation in the biochemical machinery of the insect trypanosomatids.

Peptidases have been extensively studied in order to elucidate their roles in parasite survival and pathogenicity, and their biochemical characterization is of interest not only for understanding these enzymes in general but also for comprehending their roles in the parasite (Rao et al., 1998). Of the four major catalytic types (cysteine, metallo, aspartic and serine peptidases), all but the aspartic peptidases have been described in trypanosomatids (Branquinha et al., 1996; Sajid and McKerrow, 2002; Yao et al., 2003; Silva-Lopez and Giovanni-de-Simone, 2004; Santos et al., 2005a). The major surface peptidase of *Leishmania* spp. is the best-characterized metallopeptidase group (named gp63 or leishmanolysin) in the Trypanosomatidae family and homologues of these enzymes have been described in a large number of monogenetic trypanosomatids belonging to the genera *Blastocrithidia*, *Crithidia*, *Herpetomonas* and *Leptomonas* (Inverso et al., 1993; Schneider and Glaser, 1993; Branquinha et al., 1996; Jaffe and Dwyer, 2003; Elias et al., 2006; d'Avila-Levy et al., 2003a, 2006). Recently, gp63 homologues have been described in trypanosomatids that also have a vertebrate host, such as *Trypanosoma cruzi* (Cuevas et al., 2003) and *Trypanosoma brucei* (Lacount et al., 2003). The gp63 is especially abundant in the vector stage (promastigote forms) of *Leishmania*, but it has been implicated in many aspects of the interaction with the vertebrate host (reviewed by Yao et al., 2003). In this context, the extracellular release of gp63 is especially relevant for lesion formation in animal models via the degradation of extracellular matrix (McGwire et al., 2003).

The current study was carried out in order to evaluate the cellular and extracellular peptidase profiles in *Herpetomonas megaseliae*, a monogenetic trypanosomatid isolated from the phorid fly *Megaselia scalaris* (Dagget et al., 1972), and the role of gp63-like molecules on the adhesion of this trypanosomatid to explanted guts from insect hosts.

2. Materials and methods

2.1. Chemicals

Media constituents, polyethyleneglycol 4000 (PEG 4000), reagents used in electrophoresis, buffer components, nitrocellulose membrane and reagents for chemiluminescence detection were purchased from Amersham Life Science (Little Chalfont, England). Low molecular mass standards were acquired from GIBCO BRL (Grand Island, NY, USA). The proteolytic inhibitors (*trans*-epoxysuccinyl L-leucylamido- (4-guanidino) butane [E-64] and 1,10-phenanthroline), the thiol stimulating agent (dithiothreitol [DTT]), *Bacillus thuringiensis* phospholipase C (PLC), the proteinaceous substrates (gelatin, casein and hemoglobin) and the horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody were purchased from Sigma Chemical Co (St Louis, MO, USA). All other reagents were analytical grade.

2.2. Microorganisms and cultivation

Herpetomonas megaseliae (ATCC 30029) was provided by Dr Erney Camargo (Universidade de São Paulo, SP, Brazil). The trypanosomatid was maintained by weekly transfers at 26 °C in two different media: brain–heart infusion (BHI) [3% BHI and 2 mg% hemin] and modified Roitman's complex medium (MRC medium) [0.5% yeast extract, 0.5% peptone, 2% KCl, 2% sucrose and 2 mg% hemin]. Both media were supplemented with 1% heat-inactivated FCS. *Leishmania* (*Leishmania*) *major* (MHOM/SU/1973/5-ASKH) was grown for 4 days at 26 °C to obtain promastigote forms, in the BHI medium supplemented with 10% FCS. Cellular growth was assessed by counting the parasites in a Neubauer chamber.

2.3. Parasite extracts

Herpetomonas megaseliae (1.0×10^8 cells) and the culture supernatant (equivalent to 1.0×10^9 cells) were obtained as previously described (d'Avila-Levy et al., 2001). Briefly, log phase cultures were harvested by centrifugation at $1500\times g$ for 10 min at 4 °C and cells were then lysed at 0 °C by the addition of 100 μl of SDS-PAGE sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol and 0.002% bromophenol blue). The spent culture media were passed over a 0.22 μm filtration unit and concentrated 20-fold by dialysis (cut off 9 kDa) against PEG 4000 overnight at 4 °C. The concentrated culture supernatant was mixed with SDS-PAGE sample buffer in a 7:3 (sample:buffer) ratio.

2.4. Effect of culture medium composition on peptidase production

The effect of each culture medium on the cellular and extracellular proteolytic activity was evaluated on 10% SDS-PAGE with 0.1% gelatin, casein or hemoglobin incorporated as substrate (Nogueira de Melo et al., 2002). Briefly, the gels were loaded with 10 μl of parasite extract (equivalent to 1.0×10^7 cells) and 20 μl of concentrated culture supernatant (equivalent to 3.0×10^7 cells) per slot, and following electrophoresis at a constant voltage of 170 V at 4 °C for 2 h, they were soaked for 1 h in 2.5% Triton X-100. The gels were then incubated for 24 h at 37 °C in 50 mM sodium phosphate buffer, pH 5.5, in the presence or in the absence of 2 mM DTT or 10 μM E-64 and in 50 mM glycine–NaOH buffer, pH 10.0, with or without 10 mM 1,10-phenanthroline. The gels were stained for 1 h with 0.2% Coomassie brilliant blue R-250 in methanol–acetic acid–water (50:10:40) and destained in the same solvent. The gels were dried, scanned and digitally processed. The molecular mass of the peptidases was calculated by comparison with the mobility of low molecular mass standards.