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Trypanosoma rangeli: Differential expression of ecto-phosphatase activities in response to inorganic phosphate starvation

Claudia Fernanda Dick [a,b,c], André Luiz Araújo dos-Santos [b,c], André L. Fonseca-de-Souza [b,c], Juliana Rocha-Ferreira [b,c], José Roberto Meyer-Fernandes [b,c,*]

[a] *Instituto de Microbiologia Professor Paulo de Góes, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, UFRJ, Cidade Universitária, Ilha do Fundão, 21941-590 Rio de Janeiro, RJ, Brazil*
[b] *Instituto de Bioquímica Medica, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, UFRJ, Cidade Universitária, Ilha do Fundão, 21941-590 Rio de Janeiro, RJ, Brazil*
[c] *Instituto Nacional de Ciência e Tecnologia em Biologia Estrutural e Bioimagens, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, UFRJ, Cidade Universitária, Ilha do Fundão, 21941-590 Rio de Janeiro, RJ, Brazil*

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ABSTRACT

In this work, we showed that living cells of *Trypanosoma rangeli* express different ecto-phosphatase activities in response to different inorganic phosphate (Pi) concentrations in the culture medium. The ecto-phosphatase activity from *T. rangeli* grown at low-Pi concentration was inhibited by the increase of the pH, while the ecto-phosphatase of the cells grown at high Pi concentration was not modulated by the change of the pH of the medium. Okadaic acid inhibited only the ecto-phosphatase activity from cells grown at low-Pi concentration but not the ecto-phosphatase activity from cells grown at high-Pi concentration. Accordingly, phosphatase activity from *T. rangeli* grown at low Pi concentration was able to hydrolyze P-serine and P-threonine at high rate but not P-tyrosine. The phosphatase activity from *T. rangeli* grown at high-Pi concentration was able to hydrolyze P-serine, P-threonine and P-tyrosine with the same rate. The addition of anterior midgut homogenate of *Rhodnius prolixus* on the epimastigotes suspension inhibited the enzyme activity of *T. rangeli* grown at low-Pi concentration. On the other hand, anterior midgut homogenate had no effect in the ecto-phosphatase of *T. rangeli* maintained at high-Pi concentration. Altogether, the results described here indicate that ecto-phosphatase activities hydrolyzing phosphorylated compounds present in the extracellular medium of *T. rangeli* are regulated by the external Pi concentration.

1. Introduction

Trypanosomatids are a cosmopolitan group of protozoa that parasitize a large number of eukaryotic organisms (Vickerman, 1994). In the Trypanosomatidae family, the genus *Trypanosoma* comprises digenetic flagellates that usually have insects as vectors and infect humans beings and others animals as hosts (Hoare, 1972; Vickerman, 1994). *Trypanosoma rangeli* is a parasite of a several wild and domestic animals, with a wide geographical distribution in many Latin American countries, but apparently unable to elicit pathology (Hoare, 1972). This parasite has high immunological cross-reactivity with *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, which may lead to misdiagnosis (D'Alessandro-Bacigalupo and Saravia, 1992). Although *T. rangeli* and *T. cruzi* are biologically distinct, symptomatic transmission, often involving the same triatomine vector, has resulted in concomitant infections of humans with both flagellates (D'Alessandro-Bacigalupo and Saravia, 1992). Despite being harmless to mammals, *T. rangeli* is considered pathogenic to its insect vectors, being mainly transmitted by triatomine bugs of the genus *Rhodnius* (D'Alessandro-Bacigalupo and Saravia, 1992).

T. rangeli has a complex life cycle, involving distinct morphological and functional forms in the vector. *T. rangeli* after being ingested as blood trypomastigotes by its vectors multiplies as epimastigotes in the midgut and invade the hemocoel. In a few days after infection, short epimastigotes appear in the hemocoel of the insect and soon, they disappear to be replaced by a massive colonization by long epimastigotes (Mello et al., 1995; Gomes et al., 1999). The long epimastigotes survive in the hemolymph and/or inside the hemocytes and migrate to complete their development in the salivary glands (Takle, 1988).

The plasma membrane of cells may contain enzymes whose active sites face the external medium rather than the cytoplasm. The activities of these enzymes, referred to as ecto-enzymes, can be measured using intact cells (Meyer-Fernandes et al., 1997;

* Corresponding author. Address: Instituto de Bioquímica Medica, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, UFRJ, Cidade Universitária, Ilha do Fundão, 21941-590 Rio de Janeiro, RJ, Brazil. Fax: +55 21 2270 8647.
E-mail address: meyer@bioqmed.ufrj.br (J.R. Meyer-Fernandes).

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Meyer-Fernandes, 2002; Jesus et al., 2002; Gomes et al., 2006; Pinheiro et al., 2007; Peres-Sampaio et al., 2008). Knowledge about interactions between components of the external surface of the cells and the cellular elements of the host is of obvious importance for the understanding of the complex life cycle of *T. rangeli*. Ecto-phosphatases and ecto-kinases have been detected in several microorganisms, including protozoa (Fernandes et al., 1997; Sacerdoti-Sierra and Jaffe, 1997; Meyer-Fernandes et al., 1999; Dos Passos Lemos et al., 2002; De Jesus et al., 2002; Pinheiro et al., 2007; Amazonas et al., 2009), bacteria (Bliska et al., 1991, 1993; Braibant and Content, 2001; Madec et al., 2002) and fungi (Arnold et al., 1986; Bernard et al., 2002; Kneipp et al., 2003, 2004; Collopy-Junior et al., 2006; Kiffer-Moreira et al., 2007). Several biological roles for ecto-phosphatases have been proposed. In fungi these enzymes are involved with the infection of the epithelial cells (Kneipp et al., 2004; Collopy-Junior et al., 2006; Kiffer-Moreira et al., 2007). In addition, the regulation of the complex interactions required for differentiation, proliferation (Bakalara et al., 1995, 2000; De Almeida-Amaral et al., 2006; Fonseca-de-Souza et al., 2009) and infection of host cells (Furuya et al., 1998; Zhong et al., 1998; Martiny et al., 1999) is mediated in part by protein phosphorylation/dephosphorylation in higher eukaryotes as well as in trypanosomes.

Ecto-phosphatases regulated by exogenous phosphate content has been described in plant cells (Bozzo et al., 2004, 2006; Hur et al., 2007; Wang et al., 2008), fungi (Kneipp et al., 2004; Braibant and Content, 2001; Bernard et al., 2002) and more recently in protozoa parasites (Fonseca-de-Souza et al., 2008, 2009). A little of information is available about the mechanisms that regulate the complex cellular differentiation event in trypanosomatids. Nevertheless, it is known that an essential basis for the differentiation studies has been the identification and characterization of biochemical markers, which often correspond to genes that are expressed at defined steps during the process or in the fully differentiated phenotype (Derynck and Wagner, 1995). In this work, we have found differences on ecto-phosphatase activities present on the cell surface of *T. rangeli* in response to exogenous phosphate content in the proliferation medium.

2. Materials and methods

2.1. Parasites and growth conditions

Macias strain of *T. rangeli* (supplied by Dra. Maria Auxiliadora Sousa, from Fiocruz, Rio de Janeiro, Brazil) was maintained at liver infusion tryptose medium (LIT) supplemented with 20% fetal calf serum (Gibco) at 28 ± 2 °C and subcultivated at 5-day intervals in which the parasites achieve the stationary phase of growth (Fonseca-de-Souza et al., 2009). The high inorganic phosphate (Pi) culture medium consists of: sodium chloride 4.0 g/l; potassium chloride 0.4 g/l; disodium hydrogen phosphate 7.1 g/l; glucose 2 g/l; liver infusion broth 5.0 g/l; triptose 5.0 g/l; hemin 200 mg/l and folic acid 30 mg/l. The pH was adjusted to 7.2 with HCl. In the low-inorganic phosphate (Pi) culture medium, the disodium hydrogen phosphate was replaced by sodium bicarbonate 8.4 g/l. The pH was also adjusted to 7.2 with HCl. The measurements of concentrations of Pi in the high-Pi culture medium (50 mM) and in low-Pi culture medium (2 mM) were determinate according to Fiske and Subbarow (1925). For the experiments, the parasites were harvested from the culture medium by centrifugation at 1500 *g* at 4 °C for 10 min and washed twice in a cold buffer solution containing 100 mM sucrose, 20 mM KCl and 50 mM Tris (pH 7.2). Cellular viability was assessed, before and after incubations, by motility and Trypan blue dye exclusion (Leite et al., 2007). For Trypan staining the cells were incubated in the presence of 0.01% Trypan blue for 10 min in the buffer used in each experiment. The viability was not affected under the conditions employed here.

2.2. Lactate dehydrogenase (LDH) activity measurements

Cellular viability was also assessed measuring LDH activity in the cell-free supernatant fluids according to the method of Keiding et al. (1974). Two separate aliquots of *T. rangeli* intact cells and *T. rangeli* cells lysed by freeze thawing in liquid nitrogen were used to measured total LDH activity. Cell-free supernatant fluids from these samples were transferred to the corresponding wells of a flat-bottomed microtiter plate. These samples were incubated in a buffer solution containing 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1.2 mM sodium pyruvate and 0.15 mM of reduced form of nicotinamide adenine dinucleotide (NADH). The absorbance at 340 nm was recorded for 1 h at 5-min intervals. The blanks were prepared by replacement of cell-free supernatant fluids by the sample buffer.

2.3. Ecto-phosphatase activity measurements

The phosphatase activity was determined using *p*-nitrophenylphosphate (*p*-NPP) as substrate measuring the rate of *p*-nitrophenol (*p*-NP) production. Epimastigotes of *T. rangeli* (1.0×10^7 cells/ml) were incubated at 25 °C for 60 min in 0.5 ml of a reaction mixture containing 100 mM sucrose, 20 mM KCl, 50 mM Tris–HCl (pH 7.2) and 5 mM *p*-nitrophenylphosphate (*p*-NPP) as substrate. Reactions were started by the addition of intact cells and stopped by the addition of 1.0 ml of 1 N NaOH. The phosphatase activity was calculated by subtracting the nonspecific *p*-NPP hydrolysis measured in the absence of parasite cells. For determining the concentration of released *p*-NP, a product of *p*-NPP hydrolysis, the tubes were centrifuged at 1500 *g* for 10 min at 4 °C and the supernatant was measured spectrophotometrically at 425 nm, using a *p*-NP curve as a standard (Fernandes et al., 1997). We also tested phospho-aminoacids as phosphatase substrate. In these cases, the hydrolytic activities were spectrophotometrically analyzed by measuring the released Pi from these substrates, under the same conditions employed above (Fiske and Subbarow, 1925). The values obtained for *p*-nitrophenylphosphatase activity measured using both methods are exactly the same. In the experiments where high concentrations of $MgCl_2$, $CaCl_2$, $MnCl_2$, $ZnCl_2$, $CoCl_2$, $FeCl_2$, $CuCl_2$ and $NiCl_2$ were tested, possible precipitates formed were checked as previously described (Meyer-Fernandes and Vieyra, 1988). Under the conditions employed here, in the reaction medium containing 100 mM sucrose, 20 mM KCl, 50 mM Tris–HCl (pH 7.2) and 5 mM p-NPP, no phosphate precipitates were observed in the presence of these cations.

2.4. Insects and infection with T. rangeli

Insects were obtained from a colony of *Rhodnius prolixus* maintained at 28 °C and 70–80% relative humidity. For feeding, about 4 weeks after molting randomly chosen 5th-instar nymph were exposed to citrated human blood through a special feeding apparatus. The blood was decomplemented before addition of 5×10^7 epimastigotes/ml of *T. rangeli* previously grown at high- or low-phosphate culture medium. Seven days after feeding, anterior midguts of these insects were dissected and the number of parasites was counted in a Neubauer hemocytometer under a light microscope (Gomes et al., 2003).

2.5. Determination of protein content in anterior midgut

Anterior midguts of insects starved were dissected, washed in phosphate-buffered saline (PBS) (0.15 NaCl in 0.01 M sodium