Inhibition of *Trypanosoma cruzi* proline racemase affects host-parasite interactions and the outcome of in vitro infection

Leticia Coutinho[1,2], Marcelo Alves Ferreira[1], Alain Cosson[3], Marcos Meuser Batista[2], Denise da Gama Jaén Batista[2], Paola Minoprio[3], Wim M Degrave[1], Armand Berneman[3], Maria de Nazaré Correia Soeiro[2]/+

[1]Laboratório de Genômica Funcional e Bioinformática [2]Laboratório de Biologia Celular, Instituto Oswaldo Cruz-Fiocruz, Av. Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brasil [3]Institut Pasteur, Laboratoire d'Immunobiologie des infections à *Trypanosoma*, Département d'Immunologie, Paris, France

Proline racemase is an important enzyme of Trypanosoma cruzi *and has been shown to be an effective mitogen for B cells, thus contributing to the parasite's immune evasion and persistence in the human host. Recombinant epimastigote parasites overexpressing* TcPRAC *genes coding for proline racemase present an augmented ability to differentiate into metacyclic infective forms and subsequently penetrate host-cells in vitro. Here we demonstrate that both anti* T. cruzi *proline racemase antibodies and the specific proline racemase inhibitor pyrrole-2-carboxylic acid significantly affect parasite infection of Vero cells in vitro. This inhibitor also hampers* T. cruzi *intracellular differentiation.*

Key words: *Trypanosoma cruzi* - proline racemase - host-parasite interaction - enzyme inhibition

The kinetoplastid protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas disease, a serious public health problem in most of the Latin American countries, and affecting about 12 million people (Dias 2007). In addition, due to migration, Chagas disease becomes a threat in several additional countries (Schmunis 2007). The parasitic illness has two main phases: the acute, which appears shortly after infection, and the chronic phase, which develops in about one-third of the infected individuals, after a silent period of years or decades. Due to this long asymptomatic state, Chagas disease is often considered a "silent killer", impairing early specific diagnosis and treatment (Tarleton 2007, Bilate et al. 2008). The main clinical manifestations of Chagas disease are cardiac and digestive alterations, and the pathogenesis has been attributed to host immune system disturbances associated with a low-grade parasite presence (Rocha et al. 2007, Marin-Neto et al. 2008). Up to now, there is no immediate prospect for the development of a vaccine and current clinical therapy based on heterocyclic nitro compounds (nifurtimox and benznidazol) is quite unsatisfactory for chronic patients, thus calling attention to the search for new therapeutic approaches (Coura et al. 2002, Dias 2006).

The life cycle of *T. cruzi* comprises distinct developmental stages such as amastigotes and epimastigotes that are multiplicative forms, found respectively in the cytoplasm of mammalian cells and within the midgut of the insect vectors and trypomastigotes, which are non-dividing infective forms present in both vertebrate and invertebrate hosts (De Souza 2008).

Proline racemases are enzymes that catalyze the interconversion of L- and D-proline enantiomers and have been identified in *T. cruzi* (Reina-San-Martin et al. 2000). This enzyme was the first eukaryotic amino acid racemase described and is encoded in *T. cruzi* by two paralogous genes per parasite haploid genome, *TcPRACA* and *TcPRACB*, that are thought to give rise, respectively, to secreted and intracellular protein isoforms (Chamond et al. 2003). The *Tc*PRACA is an effective mitogen for host B cells thus contributing to the *T. cruzi* immune evasion and persistence, triggering non-specific polyclonal lymphocyte activation (Reina-San-Martin et al. 2000, Chamond et al. 2003).

In view of the fact that (i) the establishment of a *T. cruzi* infection depends on a series of consecutive biological events, involving interactions of diverse parasite molecules with host components (Yoshida 2006, Covarrubias et al. 2007, Fernandes et al. 2007), and (ii) the overexpression of *Tc*PRAC isoforms leads to an increase of parasite differentiation into infective forms and of the efficiency of host cell penetration (Chamond et al. 2005), we decided to further address the impact of proline racemase during the intracellular cycle of *T. cruzi* besides exploring the implication of these enzymes in parasite interactions with mammalian cells in vitro.

MATERIALS AND METHODS

Mammalian cell cultures - For obtaining trypomastigotes or for the interaction assays, Vero cells (from African green monkey kidney) were seeded at a density of 1,5 x 10^6 cells in 25 cm^2 culture flasks or at 5 x 10^4 cells/well into 24-well culture plates containing round glass slides. The cultures were maintained in RPMI 1640 medium

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+ Corresponding author: soeiro@ioc.fiocruz.br
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(Roswell Park Memorial Institute - Sigma Aldrich - USA) supplemented with 5% fetal bovine serum and 1 mM L-glutamine and kept at 37°C in an atmosphere of 5% CO_2.

Parasites - Cell culture-derived trypomastigotes from *T. cruzi* CL Brener (clone F11-F5) were isolated from the supernatant of Vero cells, which had been previously infected with bloodstream trypomastigotes (De Souza et al. 2003).

Infection and treatment of the Vero cell cultures - Vero cell cultures were infected for 24 h at 37ºC with cultured trypomastigotes at a 10:1 parasite/cell ratio in the presence or absence of increasing doses of freshly made dilutions of 10 µM, 100 µM or 1.000 µM of the specific proline racemase inhibitor pyrrole-2-carboxylic acid (PYC), which was previously dissolved in dimethyl sulfoxide (DMSO). Alternatively, Vero cell lines were infected for 24 h at 37°C with trypomastigote forms in the presence or absence of 42, 84 or 168 µg/mL of affinity purified rabbit anti-*Tc*PRAC polyclonal IgG antibodies. The latter were produced by hyperimmunization with *Tc*PRACA recombinant protein lacking the first 30 amino acids, corresponding to the presumed signal peptide. Mature *TcP*RACA and *Tc*PRACB proteins possess 96% identity (7 amino acid differences). Anti-*Tc*PRAC polyclonal antibodies recognize both isoforms of the enzyme and were used throughout the experiments. To further investigate the effect of the racemase inhibitor upon the parasite or host cells, trypomastigotes or Vero cell cultures were pre-treated separately for 2 h at 37°C with PYC (0, 10, 100 or 1.000 µM), rinsed three times with phosphate buffered saline (PBS) and then used in a 24 h infection assay (10:1 parasite: host cell ratio). To verify if the proline racemase inhibitor could also exert an effect on the parasite intracellular cycle after 24 h of untreated parasite-host cell interaction, infected cultures were rinsed three times and incubated for up to 96 h with 10-1.000 µM PYC. After the different treatments, the cultures were washed with PBS, fixed with Bouin's solution and stained with Giemsa, according to Araujo-Jorge et al. (1989). The number of infected host cells and of parasites per infected cell was then scored in 400 host cells in three independent experiments, each run in duplicate. Results were expressed as the percentage of infected cells, the mean number of parasites per infected cell or by the endocytic index (EI) which represents the percentage of infected cells v.s. mean number of parasites per infected cell (Da Silva et al. 2008). In some assays, after the interaction for 24 h with PYC-treated parasites, the infected cultures were rinsed and followed up to eight days post infection. The number of parasites released into the cell culture supernatant was then counted daily. As controls, parasites were incubated only in culture medium, in medium with comparable DMSO concentrations as the samples with diluted PYC, or in the presence of non-immunized rabbit antiserum when indicated. Statistical analysis was carried out using the Student *t*-test, with the level of significance set at $p < 0.05$. In previous control experiments, the absence of toxicity of PYC towards Vero cells was verified incubating the inhibitor PYC at concentrations of 1-1.000 uM for 24 and 48 h with Vero cells and testing cell viability by Trypan Blue exclusion (0,4% in PBS). Cells remained viable and did not incorporate the dye. No toxicity was observed. As positive controls, Vero cells were treated with 50 mM of $NiCl_2$ in PBS. In the latter case, dying Vero cells presented apoptotic vesicles, as examined by optical microscopy and dead Vero cells detached from the support.

Immunofluorescence - In order to analyze the expression of proline racemase during the intracellular cell cycle, Vero cells were infected with *T. cruzi* and after 2-72 h of infection were rinsed twice with PBS, fixed for 20 min at 4°C with 4% paraformaldehyde (PFA) and washed again with PBS. The cells were permeabilized for 10 min with 0.25% Triton X-100 in PBS. Next, the samples were incubated for 30 min in 3% bovine serum albumin (BSA) to prevent nonspecific labeling. The cultures were then incubated for 60 min at 37ºC with affinity purified rabbit IgG anti-*Tc*PRAC diluted in PBS containing 0.25% Triton X-100 and 3% BSA. After two washes with PBS, the samples were incubated for 30 min at 37°C with goat anti-rabbit rhodamine-IgG or fluorescein isothiocyanate-IgG (FITC) (1:100, Sigma) diluted in 3% BSA. Samples were further incubated with 10 µg/mL 4,6-diamidino-2-phenylindole (DAPI) to enable visualization of parasite nuclei and kinetoplasts and mounted with 2.5% 1.4-diazabicyclo-(2.2.2)-octane. As negative controls, primary antibodies were omitted or non-immune rabbit serum was used as indicated. Cultures were examined using a Zeiss Axioplan 2 microscope equipped with epifluorescence (Zeiss INC, Thornwood, NY) or with a confocal laser scanning microscope (BX51 Olympus). Epimastigote and metacyclic trypomastigote forms were treated in a similar fashion and the presence of proline racemase revealed with goat anti-rabbit tetramethyl rhodamine isothiocyanate (TRITC) or FITC and DAPI.

Ultrastructural imunocytochemistry - *T. cruzi* epimastigote forms, or Vero cells after 48 h of infection, were rinsed three times with PBS and fixed for 60 min at 4ºC with 4% PFA plus 0.1% glutaraldehyde and 0.2% picric acid in 0.1 M sodium cacodylate buffer, pH 7.2. After rinsing, the cells were dehydrated throughout an ascending methanol series and then embedded in Lowicryl resin (Hespanhol et al. 2005). The ultrastructural immunocytochemistry was performed in the unstained ultrathin sections that were blocked with 50 mM NH_4Cl and incubated for 20 min at RT in a blocking buffer containing 4% BSA plus 1% Tween 20. The samples were then incubated for 60 min at RT with affinity purified rabbit anti-*Tc*PRAC IgG. After washing, the samples were incubated for 30 min at 37°C with the goat anti-rabbit IgG coupled to 15-nm colloidal gold particles (Sigma Chemical Co, St. Louis, MO, USA). Uranyl-stained grids were analyzed using an EM10C Zeiss transmission electron microscopy. The primary antibodies were omitted or the samples were incubated with rabbit non-immunized sera. We did not observed non-specific binding of the primary or the secondary antibody, as indicated in the figures when appropriate.

RESULTS

Localization of TcPRAC during the T. cruzi life cycle - Previous studies indicated that *T. cruzi* differentially expresses both intracellular and secreted forms of the *Tc*PRAC, possibly in a life stage specific manner (Reina-San-Martin et al. 2000, Chamond et al. 2005). In order to analyze in more detail the sub-cellular localization of *Tc*PRAC during the life cycle, and particularly in the intracellular form of the parasite in epithelial cells of the African green monkey, we used affinity purified rabbit polyclonal IgG raised against *Tc*PRAC that recognizes both isoforms of the enzyme in immunofluorescence experiments. The analysis by confocal laser scanning microscopy associated with differential contrast interference confirmed that similarly to epimastigote (Fig. 1A-C) and metacyclic trypomastigote (Fig. 1D) insect stages, *Tc*PRAC localizes in the cytoplasm of intracellular amastigote forms of the parasites (Fig. 2). The intensity of anti-*Tc*PRAC labeling varied according to the time post infection reaching the highest signal of *Tc*PRAC expression after 48 h, while the number of intracellular parasites increased by amastigote multiplication (Fig. 2D). The analysis by fluorescent microscopy of released parasites (trypomastigotes and amastigotes) after 96 h of infection showed a positive, but weaker diffuse labeling (not shown). The negative control where the primary antibody was omitted did not show any labeling (Fig. 2A). Non infected Vero cells are not labeled by anti-*Tc*PRAC antibodies or by the goat anti-rabbit IgG-TRITC, as shown in Fig. 2B. In ultrastructural analysis using rabbit anti-PRAC IgG developed with coloidal gold-goat-anti-rabbit antibodies, the immunolabeling (Fig. 3 A-D) confirmed the cytoplasmatic enzyme localization: colloidal gold particles were found dispersed in the cytoplasm and within vesicles present nearby the flagellar pocket in epimastigotes or in recently released trypomastigotes (not shown). When non-immune serum was used, no labeling was found (Fig. 3E).

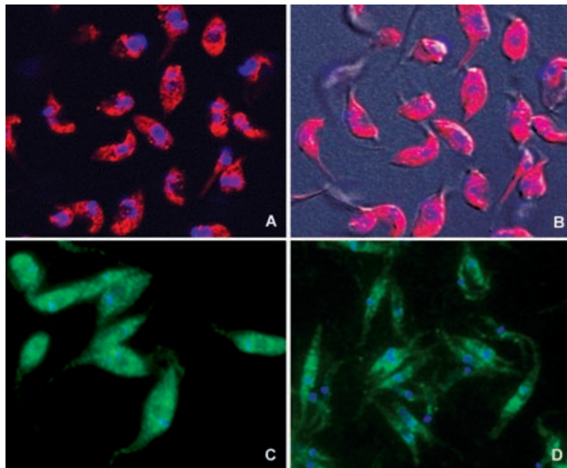

Fig. 1: analysis of *Tc*PRAC expression in the epimastigote (A-C) and metacyclic trypomastigote (D) forms of *Trypanosoma cruzi*. Confocal laser scanning microscopy and differential contrast interference merge analysis. A-B: epimastigote forms were incubated with tetramethyl rhodamine isothiocyanate-labeled goat anti-rabbit IgG followed by 4,6-diamidino-2-phenylindole (DAPI) staining (blue, revealing parasite nuclei and the stronger labeled kinetoplast). Lower panel: epimastigote (C) and metacyclic trypomastigote forms (D) were incubated with fluorescein isothiocyanate-IgG-labeled goat anti-rabbit IgG followed by DAPI staining.

Anti-TcPRAC specific antibodies reduce parasite infection of Vero cells - Since the overexpression of the *Tc*PRAC enzyme in metacyclic forms potentiates their infectivity (Chamond et al. 2005), we next investigated the possible participation of *Tc*PRAC during the invasion of tissue culture derived trypomastigotes into the host cells. These assays were performed by adding anti-proline racemase antibodies during the interaction between trypomastigotes and host cells. When increasing doses of the specific antibody (42, 84 and 168 µg/mL) were added to the culture medium, an important dose-dependent decrease in both the percentage of infected Vero cells as well as in the mean number of parasites per infected host cell could be noticed (Fig. 4 A-B) as measured 24 h after infection. A light microscopy

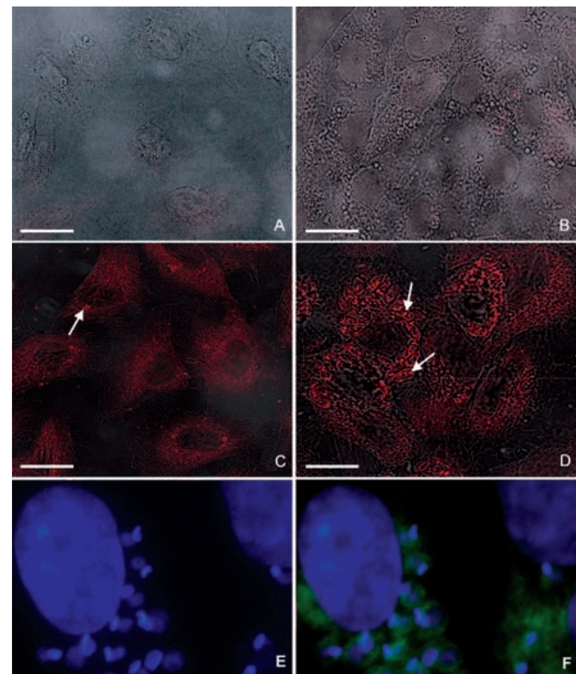

Fig. 2: analysis of *Tc*PRAC expression during the intracellular cycle of *Trypanosoma cruzi*. Confocal laser scanning microscopy and differential contrast interference merge analysis (A-E). A: uninfected Vero cell cultures were incubated with rhodamin labeled goat anti-rabbit IgG; B: uninfected Vero cell cultures were incubated with rabbit anti-*Tc*PRAC IgG antibodies followed by rhodamin labeled goat anti-rabbit IgG; C-F: infected Vero cell cultures were incubated with rabbit anti *Tc*PRAC antibodies that recognize both *Tc*PRACA and *Tc*PRACB isoforms of the protein and further developed by rhodamin-labeled (C-D) or fluorescein isothiocyanate-IgG-labeled (E, F) goat anti-rabbit IgG and 4,6-diamidino-2-phenylindole staining (E, F) - *T. cruzi*-infected Vero cell cultures after 24 h (C), 48 h (D) or 96 h (E, F) of parasite interaction. Often strong labeling can be observed for some amastigotes (arrows). Note that Vero cells do not possess receptor for Fc of immunoglobulins and that the whole affinity purified IgG was used in the experiments. Bars (panels A-D) = 10 µm.

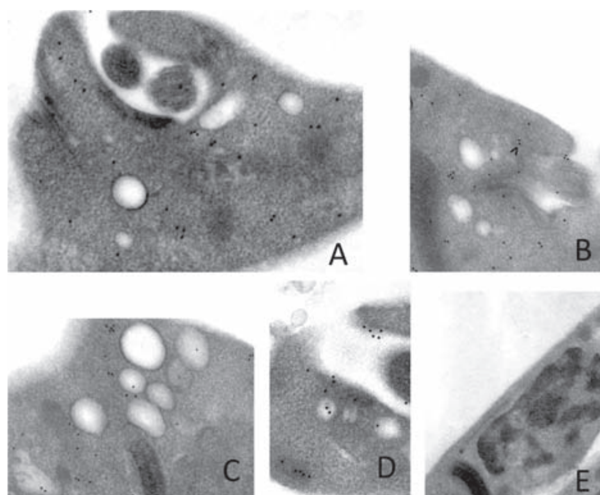

Fig. 3: ultrastructural imunolabeling of epimastigote parasite forms confirming the cytoplasmatic enzyme localization. Colloidal gold particles were found dispersed at the cytoplasm and within vesicles present nearby the flagellar pocket. Samples were incubated with affinity purified rabbit anti-TcPRAC IgG, followed by revelation with goat anti-rabbit IgG coupled to 15-nm colloidal gold particles (A-D). Cells incubated with non-immune rabbit serum did not show any labeling (E).

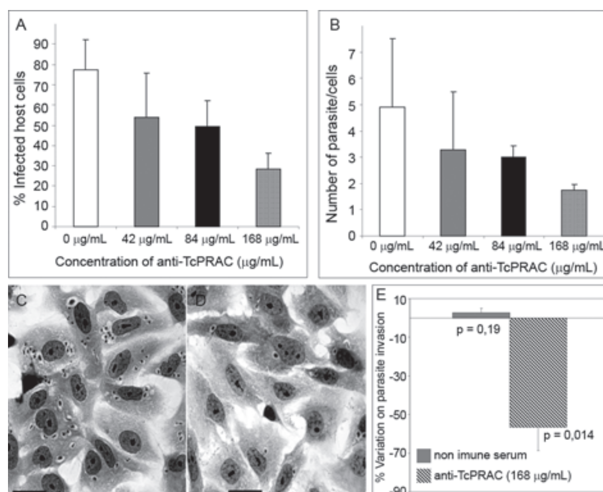

Fig. 4: anti-*Tc*PRAC antibodies hamper the *T. cruzi*-host cell interaction. A and B show the frequencies observed of host cell infection in cultures where the parasite invasion was realized in the presence of affinity purified rabbit anti-*Tc*PRAC antibodies (A) and the number of parasites per infected cell (B) after 24 h of interaction performed in the present of increasing doses (42 µg/mL, 84 µg/mL and 168 µg/mL) of purified rabbit anti-*Tc*PRAC polyclonal antibodies. The data show a dose-dependent inhibition on both the mean number of infected host cells and on the number of parasites per host cell, reaching 59% (A) and 64% (B) of decrease, respectively. Light microscopy of untreated (C) and purified rabbit anti-*Tc*PRAC polyclonal antibodies (168 µg/mL) treated cell cultures (D) after 24 h of infection show a decrease in parasite invasion due to treatment with anti-*Tc*PRAC. E: the negative control performed by the addition of non-immune serum, which did not induce alteration on the parasite invasion. Bars (panels C and D) = 10 µm.

analysis shows the untreated cells (Fig. 4C) and with the addition of anti-*Tc*PRAC at 168 µg/mL (Fig. 4D). The latter condition lowered by nearly 60% the number of infected cells ($p \leq 0.000029$) and by 64% the number of parasites per host cell, while addition of rabbit non immune serum did not induce any alteration on the parasites invasion profile (Fig. 4E).

A specific inhibitor of TcPRAC reduces host cell invasion by T. cruzi - Previous crystallographic data have shown that the incubation of *Tc*PRAC with its competitive inhibitor promotes considerable alteration of the enzyme structure, thus interfering with its binding to B-cell expressed ligands (Buschiazzo at al. 2006). It is worth noting that *Tc*PRAC enzymatic activity only accepts free L and D-proline, thus excluding the interconversion by this enzyme of proline residues bound to peptide chains expressed by the host cells. To investigate the possible effect of *Tc*PRAC on parasite invasion, we added increasing concentrations of PYC in supernatants of parasite invasive assays using adherent Vero cell cultures. Our results showed that when increasing PYC concentrations (10, 100, 1.000 µM) were added concomitantly with the interaction of trypomastigotes and Vero host cells at a 10:1 cell ratio in vitro, a clear dose-dependent effect was noticed with less infected host cells and lower mean numbers of parasites per infected cell, as measured 24 h after the onset of interaction. Thus, a significant decrease in the IE (Fig. 5) ($p \leq 0.0001$) was observed when the culture cells were treated with higher doses, reaching 86% of inhibition (Fig. 5C). Control samples treated with comparable amounts of DMSO did not show alterations in the analyses (Fig. 5C). A light microscopy analysis illustrates the dose dependent effect of PYC treatment, in 10 µM (Fig. 5E), 100 µM (Fig. 5E, F) and 1.000 µM (Fig. 5E, G), demonstrating a significant decrease in both the mean number of parasites per host cell and in the number of infected host cells (arrows) when they are compared with the untreated sample (Fig. 5D).

Inhibition effect of PYC in pre-treated parasites or host cells before their interaction - In order to clarify whether the interference of proline racemase inhibitor with parasite invasion could be related to an effect of PYC on the host cell or on the *Tc*PRAC enzyme, we performed invasion assays after pre-treatment of the parasites or of the Vero cells for 2 h before their interaction, using different doses of PYC (10, 100 and 1.000 µM). Our data revealed that the pre-treatment of the parasites with 1.000 µM of PYC did not lead to changes in their morphology and motility, but resulted in an up to 54% reduction in the percentage of parasitized cells (Fig. 6A) and about 30% less parasites per cell (Fig. 6B) when cultures were counted at day 4 after infection. This resulted in an up to 96% reduction in the number of released trypomastigotes in the supernatant at this time-point (Fig. 6C). When the Vero cells were pre-treated with the inhibitor, no alteration of parasite invasion was observed (Fig. 7A, B), which also demonstrated the lack of toxicity of PYC on the cells under these experimental conditions. Negative controls adding DMSO to the medium in equivalent concentrations after PYC dilution did not alter any of the infectivity parameters (Figs 6, 7).

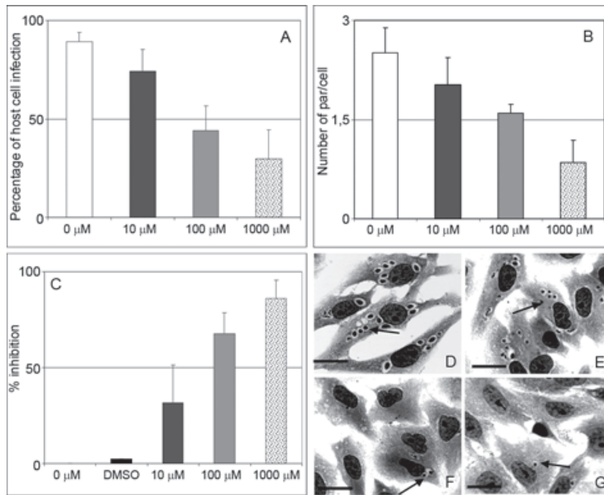

Fig. 5: effect of the pyrrole-2-carboxylic acid (PYC) inhibitor on *Trypanosoma cruzi*-host cell interaction. A-G: Vero cell cultures were previously infected for 24 h with trypomastigote culture forms of *T. cruzi* in the presence or not of different concentrations of PYC (0-1.000 μM). A dose-dependent effect of PYC was noticed in the percentage of infected host cells (A), in the number of the parasites per infected cells (B) and thus in the endocytic index. The control samples treated only with dimethyl sulfoxide (DMSO) did not show alterations in the analyses (A-C). Light microscopy of infected Vero cell cultures left untreated (D) or submitted to treatments with 10 (E), 100 (F) and 1.000 μM PYC (G). The data shows a decrease in both host cell infection and in the number of parasites (arrow) per cell already in cultures treated with low concentrations of the inhibitor (i.e. 10 μM final concentrations), as compared to untreated samples (D). Bars (panels D-G) = 10 μm.

Effect of PYC on T. cruzi intracellular differentiation - Our results indicated that TcPRAC localizes in the cytoplasm of intracellular amastigotes. Thus, we also investigated whether inhibition of the intracellular enzyme would influence parasite differentiation, more precisely from amastigote to trypomastigote forms. For this purpose, Vero cells were infected for 24 h and washed to eliminate extracellular parasites. Different PYC concentrations (10, 100 and 1.000 μM) were added to the medium and the infection was followed for 96 h. In untreated cultures, a significant decrease in the number of amastigote parasites was seen with time, as expected, due to their natural differentiation into trypomastigotes (Fig. 8A, B). However, in the cultures treated with PYC, there was a dose-dependent increase in the percentage of amastigotes thus suggesting that proline racemase activity might be involved in the process of differentiation of intracellular parasites to trypomastigotes (Fig. 8A, C).

DISCUSSION

Several mechanisms have been reported regarding the invasion of host cells by *T. cruzi*, involving diverse receptor-ligand systems, triggering signaling cascades and leading to actin-dependent and independent internalization processes (Rosestolato et al. 2002, Woolsey et al. 2003, De Souza et al. 2005, Villalta et al. 2008, Yoshida & Cortez 2008). In the present study, we analyzed the involvement of proline racemase during the invasion and differentiation steps of the parasite and verified the localization of proline racemase during the cell cycle of *T. cruzi*. *Tc*PRAC is a homodimeric enzyme, whose monomers are folded in two symmetric subunits separated by a deep crevice (Buschiazzo et al. 2006). The enzyme is believed to be expressed as two isoforms: an intracellular and a secreted form (Reina-San-Martin et al. 2000). Previous studies demonstrated that *Tc*PRAC is a potent host B cell polyclonal activator, which contributes to the prevention of specific humoral immune responses in early infection and is thus crucial for parasite evasion and fate (Minoprio 2001). Recent studies have also suggested that the enzyme, through the availability of D-proline in the cytoplasm, allows for further addition of D-proline to parasite peptide chains (Coatnoan et al. 2009). Thus, the presence of D-proline in expressed *T. cruzi* proteins could bring additional advantage to the parasite by contributing to the initial lack of specificity of host B-cell responses and to the improved parasite resistance to host cellular or extracellular proteases.

Both fluorescent and ultrastructural analysis showed the localization of proline racemase in the cytoplasm but also near the flagellar pocket of the parasites. The latter is a specialized region of the plasma membrane involved in endocytosis and exocytosis of kinetoplastid protozoans. Expression and possible secretion of proline racemase by intracellular amastigotes might possibly contribute to proline sequestration by the parasite. We have previously shown that *Tc*PRAC secreted by metacyclic parasites is involved as a mitogen for host B. It is also conceivable that the release of *Tc*PRAC by *T. cruzi* amastigotes liberated by disrupted host cells may also contribute to the maintenance of B cell polyclonal lymphocyte activation and thus ensure parasite escape, persistence and pathology associated with the chronic form of Chagas disease (Minoprio 2001, Chamond et al. 2003).

In the present study we show that the intensity of the fluorescence in the intracellular amastigotes was stronger after 48 h of parasite interaction when, in this experimental model, the parasites are in the process of active multiplication. The expression of *Tc*PRAC decreases later on, with the onset of amastigote to trypomastigote differentiation (not shown). Interestingly, we found that the addition of the specific proline racemase inhibitor PYC in *T. cruzi*-infected cultures resulted in a dose-dependent decrease of the conversion of amastigotes to trypomastigotes (Fig. 8), suggesting that this enzyme, which is up-regulated in actively multiplying parasites, may be related to parasite differentiation triggering steps. This corroborates our previous results demonstrating that the over-expression of *Tc*PRAC results in an increase in epimastigote differentiation into infective metacyclic forms and that a critical impairment of parasite viability could also be noticed in functional knock-down parasites (Chamond et al. 2005).

The addition of specific anti-proline racemase antibodies (Fig. 4) or the PYC inhibitor (Fig. 5) during the initial steps of parasite-host cell interaction led to a significant initial reduction of *T. cruzi* invasion in vitro. Proline racemase does not act on proline incorporated

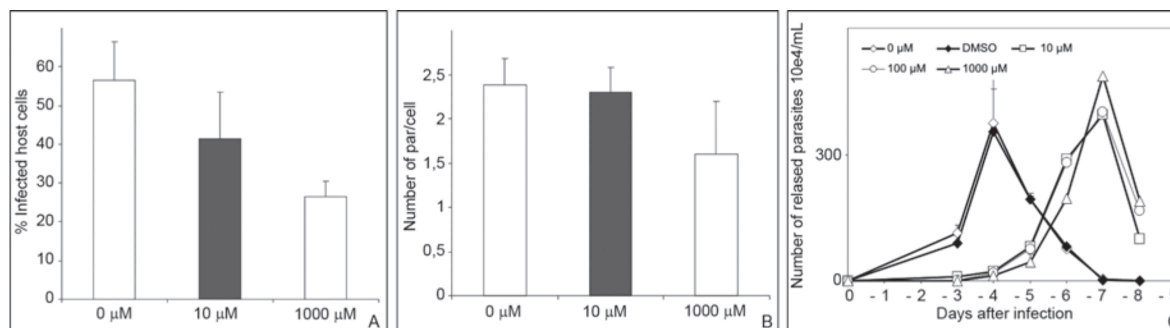

Fig. 6: effects of the pre-treatment of *Trypanosoma cruzi* with pyrrole-2-carboxylic acid (PYC) on parasite invasion. Cellular infection was scored after 24 h of Vero cell infection (A-B) and regularly after and up to eight days post infection (C). Trypomastigote forms of the parasite were pre-treated for 2 h with different concentrations of PYC (10 μM, 100 μM or 1.000 μM) before their interaction with the host cells. The results show a dose-dependent effect on both the percentage of infected host cells (A) and the mean number of parasites per cell (B). After four days, cultures infected with pre-treated parasites present a mean number of released parasites in the supernatants that is significantly reduced (C) when compared to cultures incubated with dimethyl sulfoxide (DMSO) alone that depicted similar levels of released parasites as compared to untreated samples.

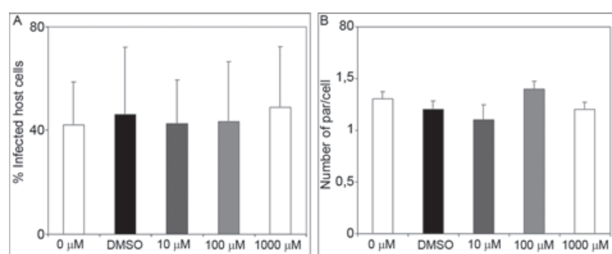

Fig. 7: effects of the pre-treatment of Vero cells with pyrrole-2-carboxylic acid (PYC) on *Trypanosoma cruzi* invasion. The host cells were pre-treated for 2 h with different concentrations of PYC (0 μM, 10 μM, 100 μM, 1.000 μM) before their interaction with the trypomastigote parasites (A-B). The results show no significant differences on both the percentage of infected host cells (A) and the mean number of parasites per cell (B) between treated and untreated cultures. Note that mammalian cells do not possess proline racemase genes, corroborating the absence of effect of PYC on a putative host enzyme. The results also show an absence of toxicity of PYC toward Vero cells. The treatment of host cells with dimethyl sulfoxide (DMSO) also did not show any difference in both analyses (A and B), as compared to untreated samples.

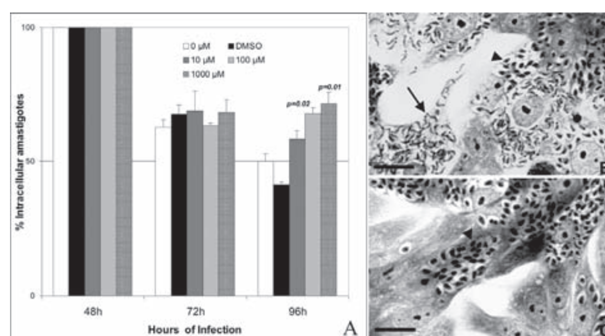

Fig. 8: effect of the pyrrole-2-carboxylic acid (PYC) inhibitor on *Trypanosoma cruzi* intracellular differentiation. Vero cell cultures were infected for 24 h with trypomastigote forms, washed to remove free parasites and then maintained in the continuous presence or in the absence of different concentrations of PYC (10 μM, 100 μM and 1.000 μM) (A). As expected, after 96 h of infection a decrease in the percentage of amastigotes, due to their natural conversion to trypomastigote forms, can be noticed in untreated cultures, while a dose-dependent increase of amastigote numbers is observed in the PYC-treated cultures (A). Light microscopy of cells after 96 h of infection of untreated (B) as compared to the cultures developed in the presence of 1.000 μM PYC (C) shows that untreated cells display higher levels of trypomastigotes (arrows) than inhibitor-treated cells that present higher numbers of amastigote forms. DMSO: dimethyl sulfoxide.

into polypeptides, but the inhibition of the enzyme has a direct or indirect influence on the parasite metabolism, differentiation and virulence. The previous incubation of host cells with PYC did not alter the infectious process. However, treatment of trypomastigotes with PYC showed a significant influence of the *Tc*PRAC inhibition in the reduction of parasite invasion. These results indicate that PYC may additionally affect intracellular *Tc*PRAC and consequently interfere with metabolic pathways, differentiation or with the expression of parasite epitopes implicated with invasion. Furthermore, it is worth recalling that *T. cruzi* host cell invasion is dependent on components of the extracellular matrix (Ortega-Barria & Pereira 1991, Herrera et al. 1994). It is also possible that *Tc*PRAC allows for the modification of cellular matrix components facilitating parasite adhesion. In similar experiments reported by Moro et al. (1995), the involvement of a *T. cruzi* peptidyl-prolyl cis-trans isomerase (*Tc*MIP) during the infection of host cells by trypomastigotes was demonstrated. Similar to our assays, these authors demonstrated that antibodies raised against *Tc*MIP significantly inhibited parasite infection possibly impairing the ability of the secreted form of the enzyme to interact with host cell surface components (Moro et al. 1995).

Corroborating the data on the implication of *Tc*PRAC, the specific inhibitor PYC considerably reduced the ability of *T. cruzi* to invade host cells supporting the idea that proline racemase activity is important for the recognition process of the parasite by host cell ligands. These results extend our previous observations, which showed that

metacyclic trypomastigotes overexpressing *Tc*PRAC are more infective as compared to the wild-type parasites (Chamond et al. 2005). Although previous work of the our group has shown that the *Tc*PRAC-PYC interaction has an influence on the overall structure of the protein precluding its interaction with B-cell expressed ligands (Buschiazzo et al. 2006), the exact mechanism regarding *Tc*PRAC involvement in *T. cruzi* invasion of host cells still remains unknown. Additionally, our present data indicate that the PYC inhibitor hampers intracellular amastigote to trypomastigote differentiation (Fig. 8), which no doubt would contribute to diminished parasite development and fate. We found further evidences that the enzyme localizes nearby and in the flagellar pocket of the parasite forms, which can give rise to important repercussions for the immune system responses during the parasite infection in both acute and late phases of the disease. As a result, reduced levels of parasite specific responses throughout infection by mitogenic stimulation at the tissue environment may certainly contribute to parasite persistence and consequently to progressive pathology and tissue damage.

Our present data substantiate earlier findings showing that proline racemase plays a role in both infectivity and differentiation of *T. cruzi* (Chamond et al. 2005) and support its use as a critical lead for the development of chemotherapy against Chagas disease. PYC is a known inhibitor of proline racemases with very poor solubility in water and our recent efforts using medicinal chemistry to synthesize more soluble PYC variants have not yielded better inhibitors of *Tc*PRAc with a higher affinity for the enzyme (manuscript in preparation). However, molecular dynamics studies based on PYC were used to design pharmacophoric models to screen libraries of chemical compounds. The results of those studies will certainly be important to allow further and better appreciation of *Tc*PRAC impact in vivo.

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