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Trypanosoma cruzi nucleoside triphosphate diphosphohydrolase 1 (TcNTPDase-1) biochemical characterization, immunolocalization and possible role in host cell adhesion

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ABSTRACT

Previous work has suggested that *Trypanosoma cruzi* diphosphohydrolase 1 (TcNTPDase-1) may be involved in the infection of mammalian cells and serve as a potential target for rational drug design. In this work, we produced recombinant TcNTPDase-1 and evaluated its nucleotidase activity, cellular localization and role in parasite adhesion to mammalian host cells. TcNTPDase-1 was able to utilize a broad range of triphosphate and diphosphate nucleosides. The enzyme's K_m for ATP (0.096 mM) suggested a capability to influence the host's ATP-dependent purinergic signaling. The use of specific polyclonal antibodies allowed us to confirm the presence of TcNTPDase-1 at the surface of parasites by confocal and electron microscopy. In addition, electron microscopy revealed that TcNTPDase-1 was also found in the flagellum, flagellum insertion region, kinetoplast, nucleus and intracellular vesicles. The presence of this enzyme in the flagellum insertion region and vesicles suggests that it may have a role in nutrient acquisition, and the widespread distribution of TcNTPDase-1 within the parasite suggests that it may be involved in other biological process. Adhesion assays using anti-TcNTPDase-1 polyclonal antibodies as a blocker or purified recombinant TcNTPDase-1 as a competitor revealed that the enzyme has a role in parasite–host cell adhesion. These data open new frontiers to future studies on this specific parasite–host interaction and other unknown functions of TcNTPDase-1 related to its ubiquitous localization.

Abbreviations: NTPDas, nucleoside triphosphate diphosphohydrolase; TcNTPDase-1, *T. cruzi* NTPDase-1; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate.

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1. Introduction

Trypanosoma cruzi is a flagellate protozoan known to be the etiological agent of Chagas disease (Chagas, 1909). The World Health Organization estimates that 8 million people are infected with *T. cruzi* worldwide, predominantly in Latin America (WHO, 2010). The disease is expanding to non-Latin American countries and remains a serious health problem because it is difficult to diagnose and to treat the chronic form of the disease, and there is no vaccine.

Ecto-Nucleoside Triphosphate Diphosphohydrolases (NTPDases) are enzymes that hydrolyze ATP and other tri- and diphosphate nucleosides (Plesner, 1995; Zimmermann, 1999). Extracellular nucleotides act as signaling molecules in the immune response of mammalian hosts, and they may be hydrolyzed by parasite ectonucleotidases. This hydrolysis could interfere with several events, such as ADP-dependent platelet aggregation and the ATP-dependent inflammatory response (Bours et al., 2006; de Almeida Marques-da-Silva et al., 2008; de Souza et al., 2010; Maioli et al., 2004; Sansom et al., 2008).

T. cruzi has ectonucleotidase activity on its surface, and an NTPDase gene was identified and cloned (TcNTPDase-1); subsequently, the recombinant protein was expressed in a bacterial system (Fietto et al., 2004; Santos et al., 2009). In these previous studies, we demonstrated a positive correlation between extracellular ATP hydrolysis and the infectivity and virulence of *T. cruzi*, and we suggested that TcNTPDase-1 would be a good target for rational drug design for Chagas disease chemotherapy, mainly because anti-TcNTPDase-1 antibodies decreased the infection (Santos et al., 2009). Other authors believe that high ecto-ATPase activity in pathogens is an adaptive parasitic behavior, and it has made these organisms more virulent because it could interfere with extracellular purinergic signals (Bisaggio et al., 2003; Sansom et al., 2007; Silverman et al., 1998). Based on the typical function of this family of proteins, it has been proposed that these enzymes can modulate biological responses induced by extracellular nucleotides and metabolites (Sansom et al., 2008). Furthermore, trypanosomatids are unable to synthesize purine rings *de novo* (Cohn and Gottlieb, 1997), depending instead on the salvage pathway (Borst and Fairlamb, 1998), in which NTPDases are suggested to have a role in extracellular purine acquisition (Berredo-Pinho et al., 2001).

Previously, we demonstrated that polyclonal antiserum against TcNTPDase-1 significantly decreased rates of *T. cruzi in vitro* infection. It did not, however, inhibit the enzymatic activity (nucleotidase activity) of the recombinant TcNTPDase-1 protein, suggesting a possible non-activity-dependent role for this enzyme in *in vitro* infection (Santos et al., 2009), possibly at an initial infection step, such as adhesion. In the present work, to better understand the role of TcNTPDase-1 in *T. cruzi* infection and in parasite biology, we expressed, purified and characterized the recombinant enzyme by its substrate preference and used it to investigate its immunolocalization and role in host cell-adhesion.

2. Materials and methods

2.1. Bacterial heterologous expression and TcNTPDase-1 purification

The recombinant form of *T. cruzi* NTPDase-1 (Accession no. AY540630) was expressed in a bacterial heterologous system as described previously (Santos et al., 2009). The purification and protein refolding were performed following previously described protocols (Areas et al., 2002) with few modifications. The lysis buffer contained 50 mM Tris pH 8.0, 100 mM NaCl and a protease inhibitor cocktail (aprotinin [1 μg/mL], pepstatin [1 μg/mL] and leupeptin [1 μg/mL]). Lysozyme (1 mg/mL) was added to facilitate cell lysis, which was conducted using sonication (6 pulses of 10 s with 10 s intervals between each pulse of 20 Hz amplitude). The centrifugation steps were performed at 12,500 × *g*, and the pellet was solubilized and suspended in buffer (50 mM Tris pH 8.0, 500 mM NaCl) and stored at 4 °C for 24 h before use. A second purification step was performed using nickel affinity chromatography Ni-NTA-agarose (GE-Healthcare®). The equilibrium/wash buffer contained 50 mM Tris pH 8.0, 100 mM NaCl, 10 to 20 mM imidazole, 8 M urea and 10 mM β-mercaptoethanol. The elution buffer contained 250 mM imidazole and decreasing concentrations of urea, with fixed concentrations for the other constituents.

2.2. TcNTPDase-1 activity

The enzymatic activity was measured using the malachite green method (Ekman and Jager, 1993) with modifications. The assays were conducted in a total reaction volume of 80 μL, including the activity buffer (50 mM Tris, pH 8.0; 50 mM HEPES, pH 8; 2.5 mM $MgCl_2$; 116 mM NaCl; 5.4 mM KCl; and 2.5 mM nucleotide) and 0.5 μg of purified TcNTPDase-1 for 30 min at 37 °C. TcNTPDase-1 presents linear hydrolysis until 1 h (data not shown). After the addition of the colorimetric reagent, the reactions were read at 650 nm. To determine the K_m and V_{MAX} values, 0.5 μg of purified TcNTPDase-1 were incubated in the same reaction medium described above in the presence of varying concentrations of ATP. The ATPase activity was measured at different periods of time, and the ATP hydrolysis did not exceed 10%. In these experiments, the ATPase activity was determined by measuring the hydrolysis of [γ-^{32}P] ATP (specific activity of approximately 10^4 Bq/nmol ATP) (Lemos et al., 2000). To evaluate the stability of the refolded protein, each of three samples was divided into three different aliquots. From each sample, one aliquot was stored at −22 °C, another one at 4 °C and the last one at 22 °C. Then, enzymatic activity (UDPase) assays were performed for 20 consecutive days starting from time "zero" after purification. Before each test, all samples were kept on ice for 5 min.

2.3. Anti-TcNTPDase-1 polyclonal antiserum production and the purification of specific antibodies

The recombinant TcNTPDase-1 purified by nickel affinity chromatography was used to produce specific polyclonal antiserum as previously described (Santos et al., 2009). All of the procedures were performed according to the guidelines of the Brazilian College of Animal Experimentation (COBEA). The immune antiserum was used to purify specific anti-TcNTPDase-1 antibodies. To purify the specific IgGs against TcNTPDase-1 that were present in the polyclonal antiserum, the purified recombinant TcNTPDase-1 protein was coupled to CNBr-Sepharose Fast Flow 4B according to the manufacturer's instructions (GE®). Specific anti-TcNTPDase-1 IgGs were purified as described previously (Chandler, 2007).

2.4. Parasites

We used a *T. cruzi* Y strain isolated from an acute human case. This strain leads to low parasitemia and high mortality in mice (Silva and Nussenzweig, 1953). *T. cruzi* epimastigotes grown in LIT medium and frozen in liquid nitrogen were thawed and seeded in Grace's medium (Sigma) containing 5% fetal bovine serum (FBS) at 28 °C.

2.5. Western blot

To immunodetect the TcNTPDase-1 in *T. cruzi* protein extract, parasites grown in LIT medium were centrifuged at 786 × *g* for 5 min at 4 °C to remove the growth medium and resuspended in

PBS. A 10% SDS-PAGE gel was loaded with 20 μg of total protein extract from non-infective axenic epimastigotes. The proteins were separated by electrophoresis and blotted on a nitrocellulose membrane. We used anti-TcNTPDase-1 purified antibodies (1:1.000) as primary antibodies and anti-rabbit-IgG conjugated with FITC (Sigma®) as secondary antibodies (1:10,000).

2.6. Adhesion assay and blocking with anti-TcNTPDase-1 antibodies

A modification of the method previously described (Santos et al., 2009) was used to determine whether epimastigotes attached to VERO cell monolayers. Briefly, the epimastigote form of the parasite was grown in Grace's medium at 26 °C until the culture reached the mid-log phase of growth and was then resuspended in RPMI with 5% FBS. Epimastigotes (20:1 parasites per cell) were gently washed with PBS and placed in contact with VERO cells that had been previously cultured for 48 h in RPMI with 5% FBS, removed with trypsin and plated on sterile coverslips (13 mm) at 5×10^5 cells per coverslip. The parasites interacted with the cells for 30 min at 4 °C. The coverslips were gently washed with PBS at 4 °C, fixed with *Bouin* solution for 15 min, stained with Giemsa and analyzed by light microscopy (Santos et al., 2009). Anti-dog IgG (Santa Cruz Biotechnology®) was used as negative control, and VERO cells plus epimastigotes were used as positive control. The percentage of adhered parasites was determined by counting 300 cells, in triplicate, in the presence or absence of polyclonal anti-TcNTPDase-1 at a dilution ratio of 1:100. The adhesion assays were performed in three independent experiments.

2.7. Adhesion assays and inhibition by competition with recombinant TcNTPDase-1

The adhesion competition assays with TcNTPDase-1 were conducted at different protein concentrations (0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 4.0 μg/mL). The protein concentrations were determined by the Bradford method (Bradford, 1976) using 96-well microplates (Biorad®). VERO cells were cultured for 48 h on sterile, round, glass coverslips in a 24-well tissue culture plate at a density of 5×10^5 cells per coverslip in RPMI with FBS 5% at 37 °C with 5% CO_2. To study the inhibition of adhesion, VERO cells were incubated with the recombinant protein for 5 min. Epimastigotes were then centrifuged, counted and resuspended at the desired concentration in RPMI with 5% FBS and added to the cell monolayers as described above (Santos et al., 2009). Albumin (4 μg/mL), recombinant protein elution buffer and denatured TcNTPDase-1 (0.5 μg/mL, 5 min boiled at 95 °C) were used as negative controls, and VERO cells plus epimastigotes was used as a positive control. The percentage of adhered parasites was determined by counting 300 cells in triplicate in the presence or absence of TcNTPDase-1 at each recombinant protein concentration. The adhesion assays were performed as three independent experiments.

2.8. Immunolocalization of TcNTPDase-1 in epimastigotes by confocal laser scanning microscopy

The immunolocalization of TcNTPDase-1 in epimastigotes was performed with epimastigotes obtained as described above. The parasites were washed twice in PBS and settled onto glass slides containing 1% poly-lysine. After one wash with PBS, they were directly fixed for 10 min at room temperature with PBS containing 4% paraformaldehyde and then blocked in PBS plus 2% BSA. The samples were incubated with a purified polyclonal antibody against TcNTPDase-1 (dilution 1:50) in PBS plus 2% BSA for 1 h at room temperature. The slides were washed in blocking solution and subsequently incubated for 30 min at 37 °C with Alexa 488-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen Life Technologies) at a dilution of 1:400. The glass slides were mounted with Prolong Gold Antifade Reagent containing DAPI (Molecular Probes) and examined by confocal microscopy (Leica, SP5) at the Faculdade de Medicina de Ribeirao Preto-USP, Ribeirao Preto, SP (Baqui et al., 2000).

2.9. Ultrastructural immunocytochemistry

For transmission electron microscopy analysis, epimastigotes were fixed in 4% paraformaldehyde, 0.5% glutaraldehyde, 5 mM calcium chloride, and 3.7% sucrose in a 100 mM sodium cacodylate buffer (pH 7.2). The samples were gradually dehydrated in alcohol at low temperatures, infiltrated, and finally set in LR White resin at 60 °C. Ultrathin sections were collected on nickel grids of 300 mesh and incubated for 20 min at room temperature in 50 mM ammonium chloride in PBS at pH 7.2. Next, the sections were incubated in PBS (pH 8.0) containing 1.5% albumin and 0.01% Tween 20 for 20 min at room temperature and then overnight in the presence of purified anti-TcNTPDase1 (1:100 or 1:50 as indicated) except for control grids. The grids were washed in PBS and finally incubated (1:30) with a secondary anti-rabbit IgG produced in goat and conjugated with 10 nm gold particles for 60 min. The ultrathin sections were contrasted with solutions of 3% uranyl acetate and 0.2% lead citrate. All of the materials were observed and photographed in a transmission electron microscope (Zeiss EM 109) at the Núcleo de Microscopia e Microanálise at Universidade Federal de Viçosa, Minas Gerais, Brazil.

2.10. Statistical analysis

The data were statistically analyzed using the ANOVA Holm–Sidak method using SigmaPlot software, Version 11.0 2008, and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. TcNTPDase-1 heterologous expression, purification and biochemical characterization

The TcNTPDase-1 gene was previously described by our group (Fietto et al., 2004), and its protein was demonstrated to be a virulence factor and facilitator of infectivity (Santos et al., 2009). To expand on the TcNTPDase-1 studies and biochemical characterization, we expressed the recombinant TcNTPDase-1 in a bacterial system and used it in biochemical and biological studies. The purified recombinant protein presented only one protein band in a Coomassie blue-stained gel as previously shown (Santos et al., 2009). The recombinant protein was purified in its active form and showed a greater ability to hydrolyze diphosphate nucleosides over their respective triphosphate nucleosides (Fig. 1A and B). A substrate specificity characterization demonstrated that TcNTPDase-1 is a genuine apyrase enzyme. The hydrolysis intensity followed the order GDP = UDP > GTP = UTP > ADP = ATP (Fig. 1A), consistent with the results shown for ATP and ADP in a previous study from our group (Santos et al., 2009). The refolding and temperature stability tests using UDP as a substrate showed that the recombinant TcNTPDase-1 was more stable when stored at 4 °C than at room temperature (22 °C) or at a freezing temperature (−22 °C) (Fig. 1B). Furthermore, the activity increased until five days after purification, remained stable until approximately day 10, and then decreased after this time, descending to levels lower than 50% of the highest activity (obtained at day 5 after purification).

Previous studies have reported the expression of the human NTPDases 5 and 6 in bacterial systems, and we observed similarities between the profiles of TcNTPDase-1 and these enzymes with

A

Table 1: Substrate Specificity of *T.cruzi* NTPDase-1

Substrate	nmol Pi x mg^{-1} x min^{-1}
ATP	84.3 ± 13.9
ADP	142.3 ± 59.2
AMP	0
GTP	633.6 ± 175.1
GDP	1335.6 ± 321.4
UTP	533.0 ± 149.0
UDP	1224.1 ± 120.9

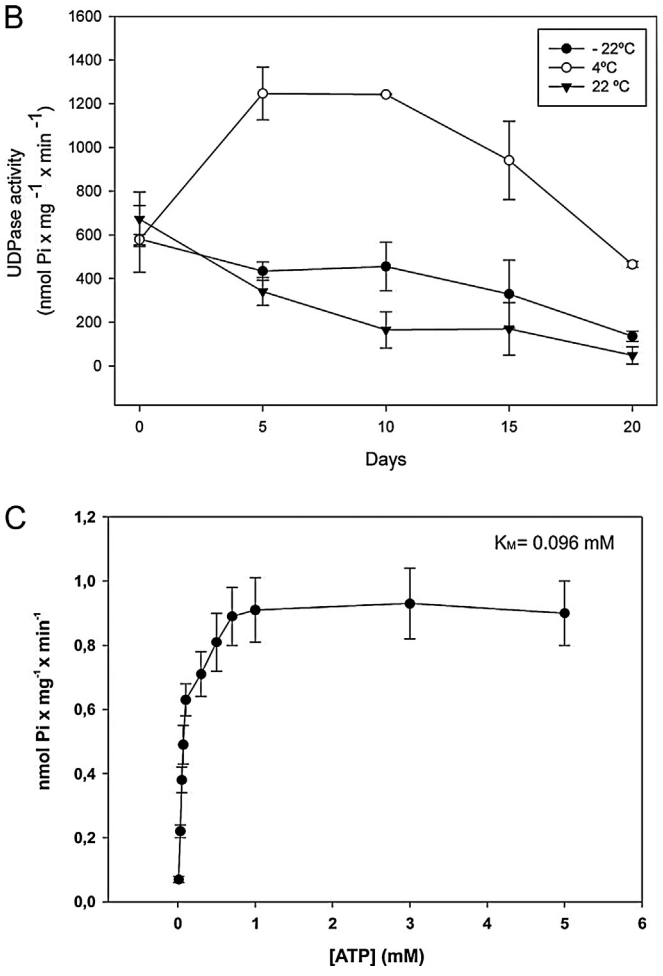

Fig. 1. Heterologous TcNTPDase-1 substrate specificity, stability and K_m. Enzyme activity was assayed using the malachite green method in a reaction buffer containing 50 mM HEPES, 50 mM Tris, 116 mM NaCl, 5.4 mM KCl, 2.5 mM $MgCl_2$ and 2.5 mM substrate, with the pH adjusted to 8.0. The results are the means ± SD from at least three independent experiments. After TcNTPDase-1 purification, each of the three samples was aliquoted and stored to verify the ideal storage temperature. Activity assays were performed for 20 days using UDP as a substrate (B). (C) The K_m for ATP. Activity was determined by measuring the hydrolysis of [γ-^{32}P] ATP. The results are the means ± SD from at least three independent experiments.

some distinctions. The human NTPDase 6 hydrolyzes GDP more effectively than UDP and GTP more effectively than UTP, whereas TcNTPDase-1 hydrolyzes GDP and UDP equally well; likewise, it hydrolyzes GTP and UTP equally well. Similar observations can be made from the study of human NTPDase 5 (Ivanenkov et al., 2003; Murphy-Piedmonte et al., 2005; Santos et al., 2009).

It is important to note that the hydrolytic capabilities assayed using an excess of substrate and a long time reaction (Fig. 1A) may not be directly related to the sensitivity to or affinity for the nucleotides, as demonstrated in the kinetic test with recombinant TcNTPDase-1, using ATP as the substrate. In this assay, we verified a K_m of 0.096 mM (Fig. 1C), whereas the K_m for UDP was almost 10 times higher (data not shown), suggesting that we cannot rule out the importance of ATP hydrolysis, even though it had the lowest hydrolysis intensity in the substrate specificity assay (Fig. 1A). These data strengthen an idea proposed in our previous works (Fietto et al., 2004; Santos et al., 2009), in which we suggested that the hydrolytic activity of this enzyme might have a role in modulating the host ATP-dependent purinergic signaling, such as that involved in the immune system. This supposition is based only on the K_m for ATP (*e.g.*, CD39 K_m for ATP is 0.01–0.2 mM) (Zimmermann et al., 2012) and P2 receptors EC_{50}, that would allow its desensitization by TcNTPDase activity (*e.g.*, P_2X_7 EC_{50} for ATP is 0.1 mM) (Khakh et al., 2001). Nevertheless, because we used a purified protein, the real role in physiological conditions needs to be better characterized in future studies. The importance of these receptors in triggering the immune response of the host is well known (Bours et al., 2006; Burnstock, 2007). Recent studies have demonstrated the importance of purine receptors in the elimination of intracellular pathogens such as *Leishmania*. It has been shown that, during infection, the host cell increases its expression of these receptors, but they are inactive or have their activation blocked by an unknown mechanism (Chaves et al., 2009; Marques-da-Silva et al., 2011).

3.2. Immunodetection and immunolocalization of TcNTPDase-1 using specific antibodies

We purified specific antibodies using affinity chromatography, and these antibodies were able to detect the recombinant TcNTPDase-1. Western blotting analysis revealed a single band of approximately 70 kDa in epimastigotes (non-infective form) (Fig. 2A). This molecular weight is compatible with the weight of TcNTPDase-1 previously predicted by our group (Fietto et al., 2004).

Assays of *in vitro* enzymatic activity using the anti-TcNTPDase-1 antibody had not previously shown inhibitory effect against the recombinant protein, suggesting that this antibody might recognize a protein homologous to TcNTPDase-1 or that it might bind to TcNTPDase-1 in a region inessential for enzymatic activity (Santos et al., 2009). The antiserum, however, was able to inhibit the *in vitro* infectivity by approximately 50%, suggesting that binding to these other regions of TcNTPDase-1 still affects the infective ability of the trypomastigote.

To better understand the cellular role and localization of TcNTPDase-1, we used the purified antibodies to localize TcNTPDase-1 in the *T. cruzi* epimastigotes by confocal microscopy. We observed a distribution of TcNTPDase-1 throughout the epimastigote cell bodies in both non-permeabilized paraformaldehyde-fixed and acetone-fixed cells (data not shown), indicating that the protein is located on the cell surface of the parasite (Fig. 2B). Additionally, strong staining was observed in the inner middle parasite's body (white arrow), near the flagellar pocket region. This result could suggest a role for TcNTPDase-1 in the acquisition of nucleosides and purines because the flagellar pocket is the major region involved in endocytic and exocytic

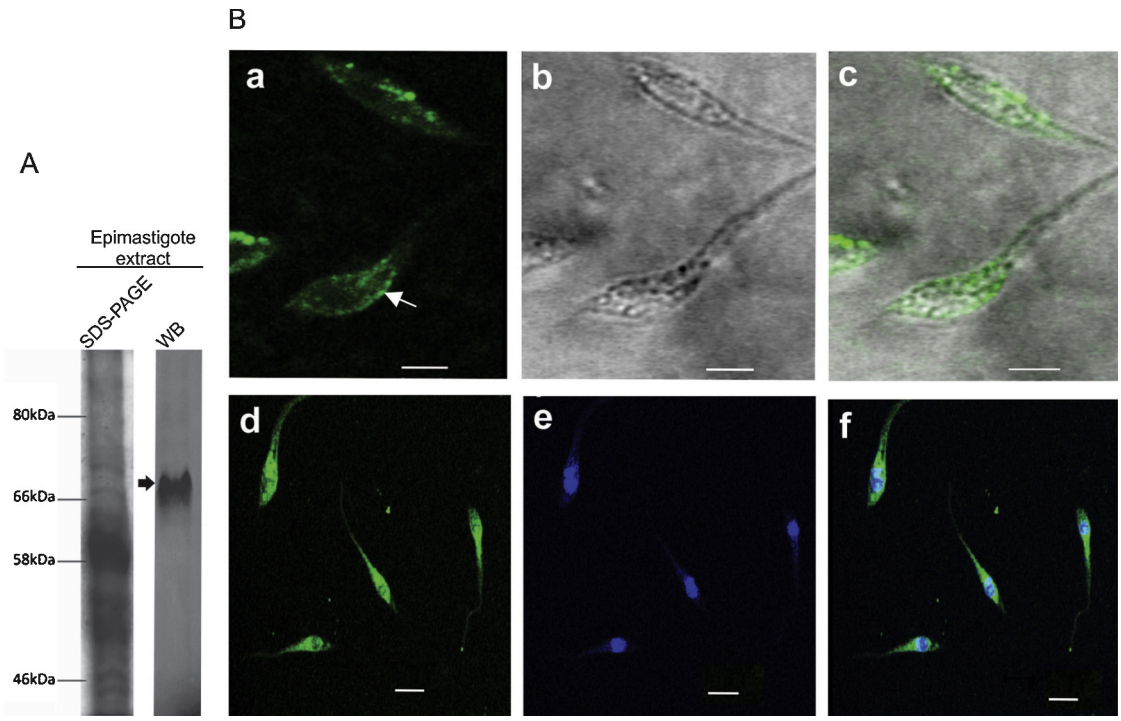

Fig. 2. Immunodetection of TcNTPDase-1 in *T. cruzi*. (A) Western blot (WB) analysis of TcNTPDase-1 expression in epimastigote extract. After incubation with rabbit antibodies anti-NTPDase-1 (1:1000) and anti-IgG conjugated with FITC (1:10,000), nitrocellulose membranes were analyzed in FLA 5100 (Fujifilm®). The SDS-PAGE gel was stained with Coomassie blue. (B) TcNTPDase-1 distribution in epimastigotes by confocal microscopy. Non-permeabilized parasites were fixed and stained with anti-TcNTPDase-1 (1:50) and visualized with Alexa 488-conjugated goat anti-rabbit IgG (a, d) (white arrow indicates the inner middle body), phase contrast (b) and merged (c, f). Nuclei and kinetoplasts were labeled with DAPI (e). Bar = 5 μm.

activities in epimastigotes (Field and Carrington, 2009; Landfear and Ignatushchenko, 2001).

The subcellular localization of the enzyme in the epimastigote form was determined by immunoelectron microscopy using purified polyclonal antibodies against TcNTPDase-1. In an ultrastructural analysis, gold particles were visualized predominantly in the cell body, nucleus, flagellum and flagellar pocket region (Fig. 3). The presence of TcNTPDase-1 in the flagellum insertion region recapitulates the data suggested by the confocal images (Fig. 2B) and reinforces the possible role of this protein in nutrient acquisition by epimastigotes because they are a replicative form of the parasite, which has a metabolism that requires high levels of purines and derivatives to replicate DNA, transcribe RNA and execute other purine-dependent pathways (Berens et al., 1981).

Staining was also observed in the kinetoplast, in cytoplasmic vesicles and, to a lesser extent, on the external cell surface (white arrowhead) and inner cell surface (black arrowhead) (Fig. 3). No staining was observed in the control assay (*e.g.*, Fig. 3H). The vesicular localization of this enzyme could be reservosomes. A recent and specific reservosome proteomic study revealed the presence of TcNTPDase-1 in these organelles (Sant'Anna et al., 2009). These data reinforce the possible involvement of TcNTPDase-1 in the metabolic nutrition of epimastigotes because reservosomes are described as multivesicular bodies that are the main site for the storage of ingested proteins and lipids, as well as for secretory proteins that are synthesized by protozoans. Nevertheless, TcNTPDase-1 vesicular localization requires further investigation. In addition, TcNTPDase-1 has a signal peptide in its amino-terminal domain (Fietto et al., 2004). The peptide signal suggests TcNTPDase-1 may also be secreted, but this cannot be currently verified experimentally.

The observed cell surface localization is in accord with previous data that described NTPDase activity on the cell surface of *T. cruzi* (Bernardes et al., 2000; Bisaggio et al., 2003; Fietto et al., 2004; Meyer-Fernandes et al., 2004; Santos et al., 2009). Furthermore, immunolocalization using non-homologous anti-*T. gondii* NTPDase1 antibodies revealed the presence of a NTPDase-homologous protein on the surface of *T. cruzi* (Fietto et al., 2004). Our data support this observation with ultrastructural immunolocalization using specific purified antibodies, and the lower level of surface detection is related to this post-inclusion technique.

The spread profile of TcNTPDase-1 subcellular localization is reinforced by recent findings showing similar ubiquitous localization of an NTPDase in *Leishmania braziliensis* and *Leishmania amazonensis* promastigotes, suggesting their involvement with various biological process within these parasites (Detoni et al., 2013; Porcino et al., 2012).

3.3. *TcNTPDase-1 may play a role in parasite adhesion to mammalian cells*

Ectonucleotidases are very important for many biological processes, and their importance in infections caused by pathogens, including protozoa such as *T. cruzi* (Sansom et al., 2008), has been studied. We demonstrated that a high ratio of extracellular ATP/ADP hydrolysis is important for the trypomastigote-stage parasite to maintain the ability to infect VERO cells (Santos et al., 2009). In that work, we found that the partial inhibition of ecto-ATPDase activity decreased *in vitro* infectivity and parasite virulence. Alternately, the antibodies produced against the recombinant TcNTPDase-1 did not inhibit the ATPDase activity of the recombinant protein or the ecto-ATPDase activity in live parasites. However, the same antibodies were able to inhibit an *in vitro* infection, suggesting a specific role for TcNTPDase-1 that would be partially independent of enzymatic activity.

In this work, we used recombinant TcNTPDase-1 and specific polyclonal antibodies to evaluate its biological role at the early

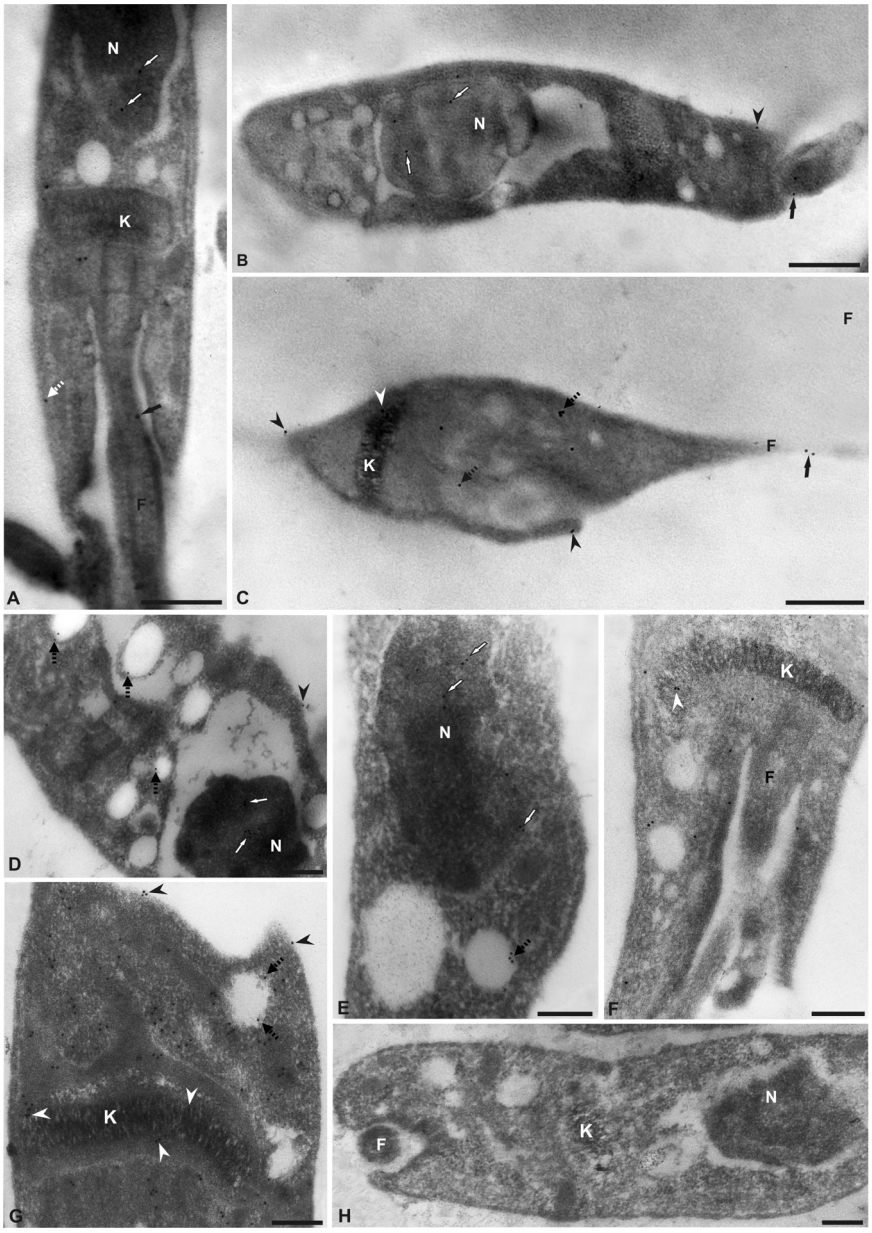

Fig. 3. Ultrastructural analysis of TcNTPDase-1 subcellular localization in epimastigotes. (A–G) Electromicrographs using polyclonal anti-TcNTPDase-1 (1:110 A–C, and 1:50 D–G) and anti-IgG conjugated to 10 nm colloidal gold. TcNTPDase-1 is shown in the nucleus (N) (white arrows), kinetoplast (K) (white arrowhead), internal vesicles (dashed black arrow), flagellum (F) (black arrow), flagellum insertion region (panel F surrounding the flagellum), and outer and inner cell surface (black arrowheads and dashed white arrow, respectively). No staining was observed in the control (H). Bars: A, C–H = 0.2 μm; B = 0.5 μm.

stage of infection in mammalian cells, specifically in the adhesion step.

The adhesion assays were performed using epimastigotes at the log phase of growth. Direct adhesion assays were performed using VERO cells as a model. To avoid internalization, the assays were conducted at 4 °C for 30 min as previously described (Bisaggio et al., 2003). The adhesion assays were conducted either with cells that had been previously incubated in the presence of purified recombinant protein in a competition assay (Fig. 4A) or with epimastigotes that had been previously incubated with purified anti-TcNTPDase-1 to directly block native TcNTPDase-1 (Fig. 4B). In the presence of increasing amounts of the recombinant protein (Fig. 4A), we observed a significant dose-dependent inhibition of epimastigote adhesion, suggesting the presence of binding sites on the host cell that would be partially blocked by recombinant TcNTPDase-1. Furthermore, the nucleotidase activity of the recombinant protein could also influence this event. There was no inhibitory effect when the assay was performed in the presence of an unrelated protein (albumin) or denatured TcNTPDase-1 (Fig. 4A). The parasites that had been previously incubated with purified polyclonal anti-TcNTPDase-1 antibodies (Fig. 4B) exhibited a significant decrease in motility (data not shown) and a significant decrease in adhesion (compared to the controls) in the absence of antibodies or in the presence of an unrelated antibody (anti-dog IgG). The effects of anti-TcNTPDase-1 are specific and independent of the conserved portion of IgGs, as shown by the fact that the control using unrelated anti-IgG did not differ from the control without any antibodies (Fig. 4B). Although the decrease in motility could contribute to the observed decrease in adhesion.

These results suggest that TcNTPDase-1 could participate in cell adhesion. We can envision three scenarios that would explain

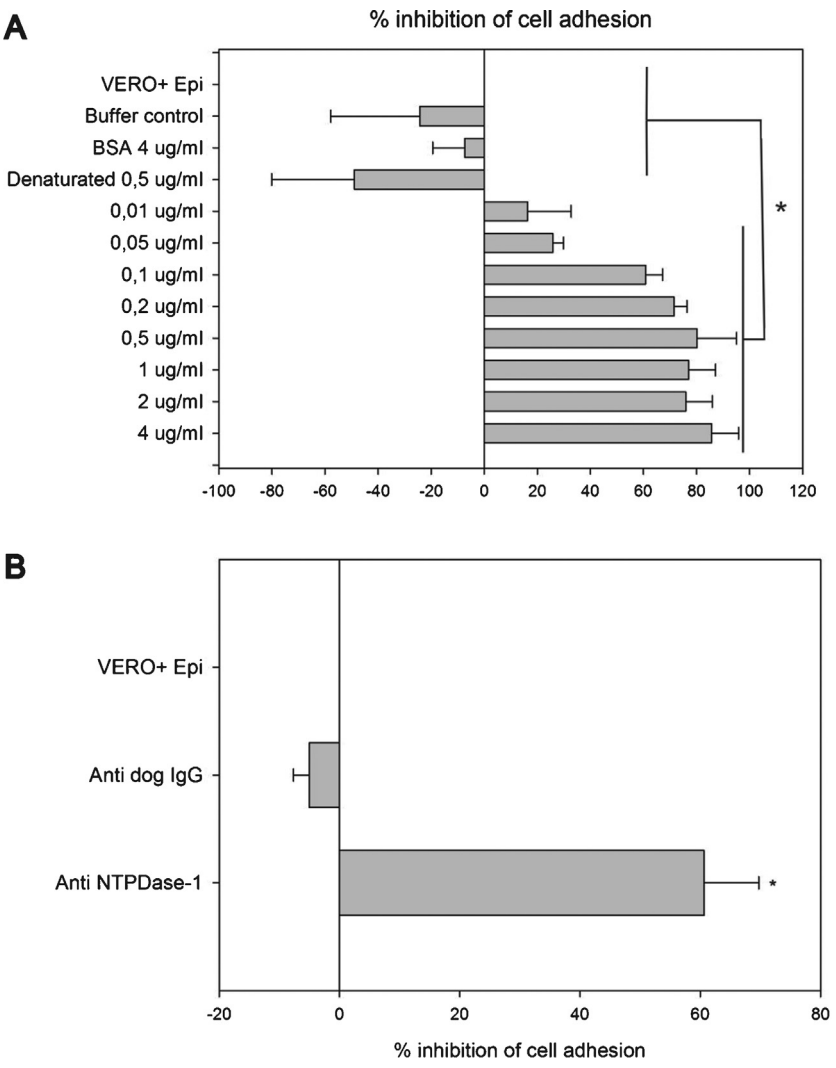

Fig. 4. Participation of TcNTPDase-1 in cell adhesion. (A) Adhesion assays by competition with TcNTPDase-1. VERO cells were previously placed in contact with various concentrations of recombinant protein for 5 min (0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 4.0 μg/mL). Epimastigotes were then added (20 parasites per cell). (B) Adhesion assays in the presence or absence of polyclonal anti-TcNTPDase-1. Epimastigotes were previously incubated with antibodies for 10 min and then placed in contact with the VERO cells (20:1). After incubation, coverslips were washed, fixed, stained and analyzed by light microscopy. Albumin (4 μg/mL BSA), the elution buffer of the recombinant protein (Buffer control), denatured TcNTPDase-1 (0.5 μg/mL), Anti-dog-IgG (Santa Cruz Biotechnology®) and VERO cells with epimastigotes only (VERO + Epi) were used as controls. The percentage of adhered parasites was determined by counting 300 cells in triplicate on each coverslip tested. The results are the means ± SD from at least three independent experiments.

this hypothesis: firstly, antibodies may bind to the modulatory regions of the enzyme, thus preventing it from responding to certain stimuli; alternately, the action of antibody binding could generate a steric hindrance on the surface of the parasite, impairing its adhesion to host cells; finally, there could be a synergic interaction between the enzyme and a host receptor, and the anti-TcNTPDase-1 would also be capable of inhibiting the adhesion to the host cell. All three hypotheses are currently under investigation.

4. Conclusions

Our results provide new information regarding TcNTPDase-1 nucleotidase activity, demonstrating its ability to use a broad range of triphosphate and diphosphate nucleosides. Additionally, we used specific antibodies to provide new data regarding several aspects of subcellular localization. The ubiquitous localization of TcNTPDase-1 in epimastigotes highlights the need to investigate the participation of this enzyme in other processes, in addition to its possible roles in both purine acquisition and virulence. Furthermore, we demonstrated that TcNTPDase-1could be involved in host cell adhesion. This specific parasite–host interaction and the possible unknown functions of TcNTPDase-1 (related to its ubiquitous localization in replicative parasite cells) merit further study.

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