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ORIGINAL ARTICLE

Trypanosoma cruzi Proline Transport Presents a Cell Density-dependent Regulation

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ABSTRACT

Trypanosoma cruzi, the etiological agent of Chagas disease, uses proline as its main carbon source, essential for parasite growth and stage differentiation in epimastigotes and amastigotes. Since proline is mainly obtained from extracellular medium by transport proteins, in this work we studied the regulation of the *T. cruzi* proline transporter *Tc*AAAP069. Proline uptake and intracellular concentration presented oscillations during epimastigote growth phases, increasing during the early exponential phase (322 pmol/min) and decreasing to undetectable levels during the late exponential phase. Transporter expression rate correlated with proline uptake, and its subcellular localization alternated from both, the plasma membrane and close to the flagellar pocket, when the transport is higher, to only the flagellar pocket region, when the transport decreased until proline uptake and *Tc*AAAP069 protein became undetectable at the end of the growth curve. Interestingly, when parasites were treated with conditioned medium or were concentrated to artificially increase the culture density, the proline transport was completely abolished resembling the effects observed in late exponential phase. These data highlight for the first time the existence of a density-associated regulation of relevant physiological processes such as proline metabolism.

CHAGAS disease or American trypanosomiasis is the result of human infection by the protozoan parasite *Trypanosoma cruzi* (Chagas 1909). Along the life cycle, this parasite is exposed to different environments in the gut of the insect vectors, the bloodstream of the mammalian hosts, and also within different cell types. The availability of nutrients in these dissimilar environments determines the need for complex metabolic adaptations. Energy production in several stages of this parasite is based mainly on the consumption of carbohydrates and amino acids, essentially proline, which is abundant in the gut of the insect vector and can be metabolized through the Krebs cycle (Bringaud et al. 2012; Cazzulo 1994; Sylvester and Krassner 1976). In addition to its relevant role in metacyclogenesis (Contreras et al. 1985; Homsy et al. 1989), proline is also important during life cycle, for host cells infection, and intracellular differentiation (Martins et al. 2009; Silber et al. 2009; Tonelli et al. 2004).

Transporters are in contact with extracellular molecules and work, not only as permeases carrying the solutes into the cell but also as environmental sensors. The transport of metabolites represents the first step of many metabolic routes and may also regulate such processes (Silber et al. 2005).

The only amino acid transporter family identified in kinetoplastids is the AAAP (Amino Acid/Auxin Permease; TC 2.A.18), one of the major transporter families of the APC (amino acid/polyamine/organocation) superfamily (Bouvier et al. 2004). Some permeases of the AAAP family transport specific amino acids, while others have a very broad specificity for all twenty amino acids, including D-isomers (Young et al. 1999). This gene family has been characterized in *Trypanosoma cruzi* by our group (Bouvier et al. 2004), and the function of many of these transporters was studied (Carrillo et al. 2010; Inbar et al. 2012; Miranda et al. 2012; Saye et al. 2014). Previous works showed that L-proline is transported from the extracellular media through two different active transport systems (Silber et al. 2002), and recently, a D-L-proline transporter (TcAAAP069) was identified in *T. cruzi* (Saye et al. 2014).

A higher intracellular proline concentration, produced by the overexpression of TcAAAP069, causes an improved resistance to trypanocidal drugs (benznidazol and nifurtimox) and also to reactive oxygen species including hydrogen peroxide and nitric oxide, emulating some natural physiological situations. In addition, an increased proline concentration also generates a higher amount of ATP in the transgenic parasites (Saye et al. 2014). On the other side, *T. cruzi* parasites with reduced intracellular proline levels are more sensitive to oxidative imbalance (Paes et al. 2013), and cell viability and nutrient stress resistance are negatively affected in the presence of proline analog T4C (L-thiazolidine-4-carboxylic acid) (Magdaleno et al. 2009). Many essential processes involving proline have been reported in *T. cruzi*. For example, the metabolic pathway in which L-proline is oxidized to pyrroline-5-carboxylate by a mitochondrial proline dehydrogenase regulating the redox state of the cell and the respiratory metabolism (Mantilla et al. 2015; Paes et al. 2013). Finally, proline transport and associated processes have been thoroughly studied in other trypanosomatids such as *T. brucei*, *Leishmania* spp., *Crithidia*, and *Phytomonas* (Canepa et al. 2007; Galvez Rojas et al. 2008; L'Hostis et al. 1993; Law and Mukkada 1979). In these organisms, proline has been shown to play critical roles in osmoregulation, drug resistance, and amino acid homeostasis (Bringaud et al. 2012).

Considering the importance of proline in many critical processes as is the case of drug resistance, energy metabolism, and progression of parasite life cycle; in the present study, we explored the regulation mechanisms of proline transport in *Trypanosoma cruzi*.

MATERIALS AND METHODS

Cell cultures

Epimastigotes of the Y strain were cultured at 28 °C in plastic flasks (25 cm^2), containing 5 ml of BHT medium (started with 5×10^6 parasites per milliliter) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Camargo 1964). Cells were counted using a hemocytometer. Viability assays were performed using "Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (MTS)" (Promega, Madison, WI) according to the manufacturer instructions.

Conditioned medium was obtained from day 15 parasite culture. Briefly, culture was centrifuged at 3,000 *g* for 10 min and the supernatant was carefully separated and used as conditioned medium.

Proline transport assays

Aliquots of epimastigote cultures (10^7 parasites) were centrifuged at 8,000 *g* for 30 s, and washed once with phosphate-buffered saline (PBS). Cells were resuspended in 0.1 ml PBS and then 0.1 ml of the transport mixture was added containing 1 mM L-(^{3}H) proline (0.4 μCi, PerkinElmer's NEN®; Radiochemicals, Waltham, MA) followed by incubation at 28 °C. Reaction was stopped by adding 1 ml of ice-cold PBS. Cells were centrifuged as indicated above, and washed twice with ice-cold PBS. Cell pellets were resuspended in 0.2 ml of water and counted for radioactivity in UltimaGold XR liquid scintillation cocktail (Packard Instrument Co., Meriden, CO) (Pereira et al. 1999). Assays were run at least by triplicate. Cell viability was assessed by direct microscopic examination. Non-specific uptake and carry-over were measured in transport mixture at T_0, or incubated at 4 °C. Transport assays were performed when uptake activity starts to be detectable, after day 4 of parasite culture.

Plasmid constructions and parasite transfection

TcAAAP069 (GeneDB: *Tc*CLB.504069.120) was amplified using genomic *T. cruzi* DNA as template. Amplification product was subcloned into a modified pTREX (Vazquez and Levin 1999) expression plasmid called pTREXL (Bouvier et al. 2013) and fused with the tri-FLAG epitope (tF-TcAAAP069). Constructions were transfected into *T. cruzi* epimastigotes as follows. 10^8 parasites grown at 28 °C in BHT medium were harvested by centrifugation, washed with PBS, and resuspended in 0.35 ml of electroporation buffer (PBS containing 0.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$). This cell suspension was mixed with 50 μg of plasmid DNA in 0.2 cm gap cuvettes (Bio-Rad Laboratories, Hercules, CA). The parasites were electroporated using a single pulse of 400 V, 500 μF with a time constant of about 5 ms.

Proline determinations

Measurements of proline concentration were adapted from the ninhydrin colorimetric reaction (Bates 1973). Briefly, 10^7 parasites were washed with PBS, resuspended in Tris-HCl 100 mM pH 7.0 and then ruptured by sonication. The supernatant was treated with 5% TCA for an hour at 4 °C. The new supernatant was mixed with ninhydrin acidic solution (0.25 g ninhydrin dissolved in 6 ml glacial acetic acid and 4 ml phosphoric acid 6 M) and glacial acetic acid and then incubated for an hour at 100 °C. The reaction was stopped in ice, followed by toluene addition and thorough mix. The organic phase was recovered in a new tube and measured at 520 nm.

Fluorescence microscopy

Epimastigote samples were washed twice with PBS. After letting the cells settle for 30 min at room temperature onto poly-L-lysine coated coverslips, parasites were fixed at room temperature for 20 min with 4% formaldehyde in PBS, followed by a cold methanol treatment for 5 min. Slides were incubated for 45 min with anti-tri-FLAG monoclonal antibody produced in mouse (Catalog Number F1804; Sigma-Aldrich, St. Louis, MO) diluted 1:200, or in combination with the anti-TCLP1 antibody diluted 1:50. Slides were washed and incubated with anti-mouse IgG with fluorophore Alexa Fluor® 488 polyclonal antibody or in combination with anti-rabbit IgG with fluorophore Alexa

Fluor® 594 made in goat (catalog numbers ab150113 and ab150080; Abcam, Cambridge, UK), both diluted 1:500. Slides were mounted using Vectashield (Vector Laboratories, Burlingame, CA). Cells were observed in an Olympus BX60 fluorescence microscope. Images were recorded with an Olympus XM10 camera.

Western blot analysis

Western blots were performed using total parasite extracts fractioned by electrophoresis in polyacrylamide denaturing gels and transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were treated for 1 h with 5% nonfat dry milk in PBS and incubated overnight with the anti-tri-FLAG monoclonal antibody produced in mouse (Catalog Number F1804; Sigma-Aldrich) diluted 1:4,000. Membranes were washed and incubated with the secondary antibody for 2 h (anti-mouse HRP made in horse diluted 1:2,500, catalog number PI-2000; Vector Laboratories). Detection was done by chemiluminescence (SuperSignal™ West Pico Chemiluminescent Substrate, product number 34080; Pierce Biotechnology, Rockford, IL). Total protein Ponceau S-staining was used for sample normalization.

Flow cytometry analysis

A total of 1×10^7 parasites were washed in PBS and fixed with 500 µl of 70% ethanol in PBS at 4 °C overnight. Then, fixed parasites were washed again with PBS and incubated for 30 min at 37 °C with 500 µl of PI staining solution (2 mM EDTA, 10 mg/ml DNAse-free RNAseA, and 20 mg/ml Propidium Iodide en PBS). Samples were processed in a flow cytometer (FACSAria II; BD Biosciences, Franklin Lakes, NJ) and results were analyzed using the FlowJo software. Fluorescence intensity, which is proportional to the DNA content present in parasite populations, was plotted against cell count for each point to monitor the progression of cell cycle. Experiments were carried out in triplicate.

RESULTS

Variations of proline transport along the parasites' growth curve

Preliminary observations suggested that proline transport is tightly regulated during growth of epimastigote culture. When transport was evaluated using stationary phase parasites proline uptake was undetectable (Sayé et al. unpublished data). These data were the starting point to begin studying the proline uptake at different stages of the growth curve using transgenic parasites overexpressing the proline transporter *Tc*AAAP069 fused with the tri-FLAG epitope (tF-*Tc*AAAP069). Under standard culture conditions, epimastigotes had a first phase of growth up to day 6 (d6) and a second phase from d6 to d10, increasing the parasite densities from 0.5×10^7 parasites/ml per day ($R^2 = 0.97$) to 2.15×10^7 cells/ml per day ($R^2 = 0.99$, Fig. S1). Proline transport rates were determined at different parasite densities during the growth curve. After a culture passage, during the first 5 d of culture, the proline transport dramatically increased from undetectable levels up to 264 pmol/min on d4 and reaching the maximum value of 322 pmol/min at d5. From d6 to d10 proline transport constantly decreased until it was completely abolished (Fig. 1, black line). This seems to represent an oscillation of transport rates during the culture passages, between 0 and 322 pmol/min.

Since compensatory mechanisms could exist, variations in proline transport activity do not necessarily imply differences in this amino acid intracellular level. To establish whether or not the amount of intracellular proline correlates with transport rates along the culture progression, proline determinations were made simultaneously with transport assays. Interestingly, the maximum proline concentration was found at slightly higher parasite densities than those found for maximum transport rates. In addition, when transport rates decreased to undetectable levels, proline concentration was diminished from 6.8 mM on d6 to 4.2 mM on d10 and remained constant up to the next culture passage (Fig. 1, gray line). To evaluate how proline availability varies during the growth curve of epimastigotes, extracellular proline concentration was quantified in samples of fresh culture medium and medium obtained from parasites of d15 culture. No significant differences were observed between both groups, and corresponded to 5 mM (±0.5) and 4.7 mM (±0.4), respectively.

Expression analysis by immunofluorescence revealed that *Tc*AAAP069 is expressed from d4 to d8, and after that it was undetectable (Fig. 2A). Throughout this period of time, the transporter was located close to the flagellar pocket, as previously described (Inbar et al. 2012; Miranda et al. 2012; Saye et al. 2014), and it was also detected in the plasma membrane on d4, d5 and d6. Results obtained

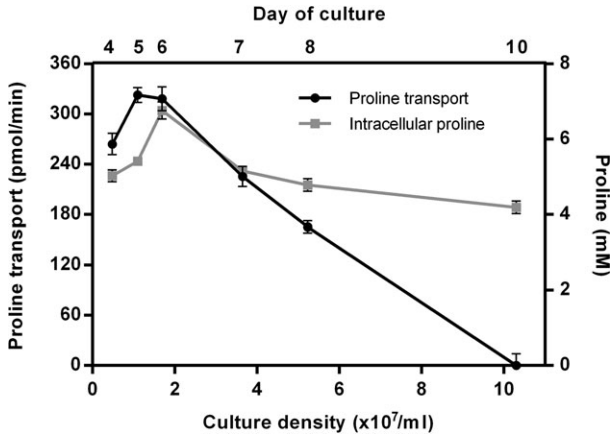

Figure 1 Proline flow dynamics in *Trypanosoma cruzi* epimastigotes. Proline uptake (circle, black line) and intracellular proline concentration (square, gray line) were measured from epimastigote cultures from d4 to d10 (upper *X*-axis) as stated under "Materials and Methods". The corresponding parasites densities are also indicated in the graphic (lower *X*-axis). All uptake assays were performed using 10^7 parasites.

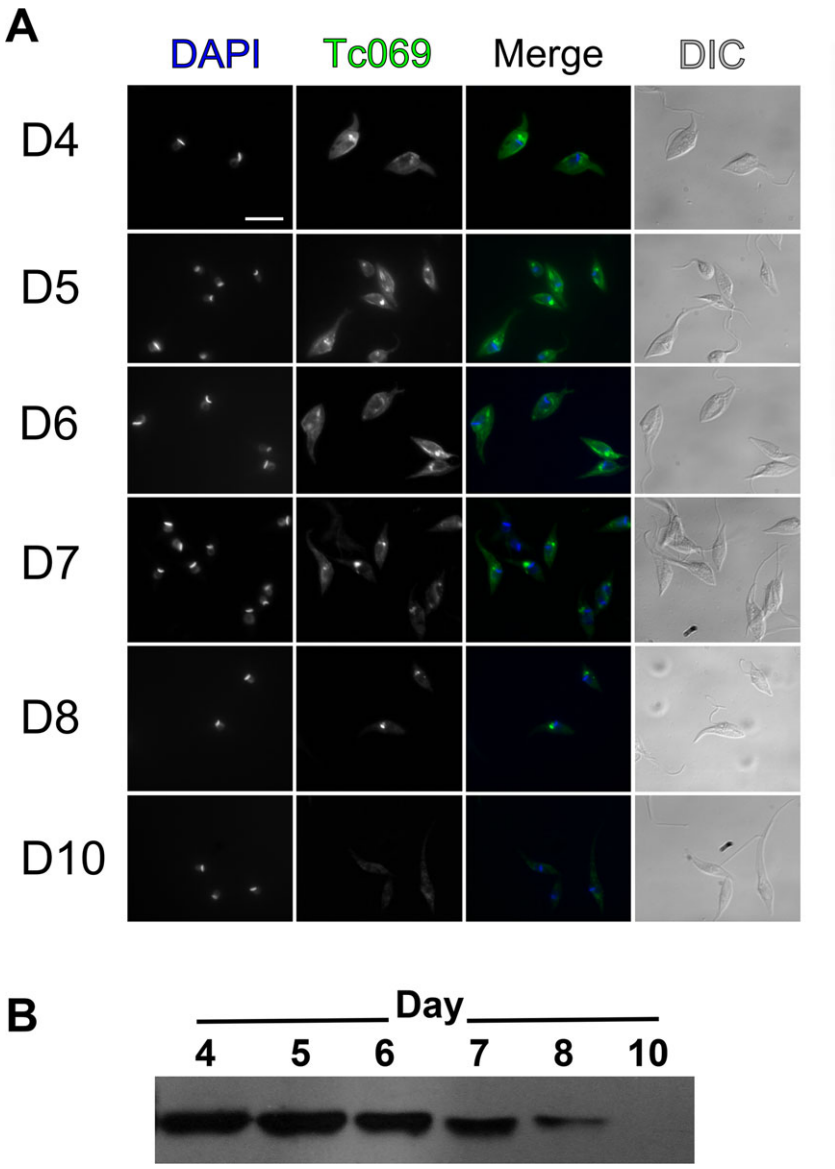

Figure 2 Regulation of expression of the proline permease TcAAAP069. (**A**) Parasites transfected with the plasmid pTREXL containing the full length TcAAAP069 gene fused to the tri-FLAG epitope (tF-*Tc*AAAP069) were analyzed by indirect immunofluorescence using anti-tri-FLAG antibodies (green) and DAPI (blue). Each row represents 1 d of culture (e.g. D4 = day 4). Scale bar = 10 μm. (**B**) Transgenic epimastigote samples from different days were analyzed by Western Blot using anti tri-FLAG antibodies. Loading control was performed by total protein staining using Ponceau S and further quantitation using the ImageJ program (National Institute of Health, Bethesda, MD).

by Western Blot analysis using anti-tri-FLAG antibodies correlated with proline transport activity and immunofluorescence data. The transporter *Tc*AAAP069 expression decreased along the growth curve and was undetectable since d8 (Fig. 2B). We also evaluated colocalization with TCLP1 (Trypanosomatid CesT-like Protein 1) which is a *T. cruzi* protein that localizes predominantly to the intracellular portion of the flagellar pocket area, and it might be associated with endocytic processes (Durante et al. 2015). No colocalization was detected throughout the growth curve (Fig. S2).

To test if proline uptake or *Tc*AAAP069 expression were undetectable after d8 due to an unrelated phenomenon, parasites viability was evaluated using a vital stain; d10 Tc069 parasites and wild type controls did not present significant differences (73% ± 7 and 68% ± 10). As an additional control, flow cytometry analysis revealed that there was no cell cycle arrest of Tc069 parasites along the exponential phase of growth, nor differences in cells size (Fig. S3). These results suggest that the observed lack of proline transport is not a consequence of a decreased cell metabolism or a cell cycle arrest.

To validate the transgenic model, the regulation of proline transport activity and intracellular proline concentration were studied in wild type parasites. The obtained curves for both parameters presented shapes similar to those obtained for transgenic parasites.

Regulation of proline transport

To identify the factors involved in the modulation of proline uptake the first step was to evaluate the possible regulation by substrate availability. Proline transport was measured in parasites incubated 24 h in PBS only, PBS supplemented with 10 mM proline, and as control PBS supplemented with 10 mM lysine, an unrelated amino acid. Cell viability was evaluated using a vital stain; parasites starved 24 h in PBS and controls did not present significant differences (97% ± 0.4 and 100% ± 0.9, respectively). Whereas proline is an important energy source and its transport is energy-dependent (Silber et al. 2002), uptake was also compared with parasites incubated with PBS supplemented with 10 mM glucose. After incubation in PBS-proline or PBS-glucose, proline uptake velocity presented a significant increase of 2.9-fold (± 0.28) compared with parasites incubated in PBS-lysine or PBS-alone ($p < 0.0001$) (Fig. S4). No significant differences were found between PBS-proline vs. PBS-glucose and PBS-lysine vs. PBS only. These results support the hypothesis that either proline or glucose provides the energy needed for proline uptake without specifically regulating the transport.

To check whether a medium component might influence somehow proline uptake, epimastigote cultures from d5 (10^7 parasites/ml) were switched to the following culture conditions: (a) fresh medium, (b) conditioned medium obtained from d15 parasites, and (c) control group without treatment. Then, proline uptake was evaluated periodically during 6 d. After 24 h, proline transport rates of groups A and C showed a slight increase while in the group B transport decreased three times (Fig. 3A). These results demonstrated the effect of conditioned medium components over the proline transport. Group B reached the minimum transport rate at d3 after treatment while in groups A and C transport rates decreased continuously until d6. As stated above, no significant differences in extracellular proline concentration were observed between the mentioned culture media.

Considering the obtained results, we hypothesize that regulation of proline uptake by medium components could be mediated by an unknown parasite density-derived factor. To preliminary test this hypothesis, parasites from d5 were assayed for proline uptake in two cell densities, 10^7 parasites/ml (density reached at d5) for the control, and 7×10^7 parasites/ml (high-density, equivalent to a d8 culture). It is important to note that both groups, control and high-density, were resuspended in fresh medium. In concordance with the prior experiment, after 24 h the proline transport rate of control group remained constant, while in the concentrated parasites it decreased three times (Fig. 3B). High-density culture reached the minimum transport rate at d2 after treatment while in control group transport rates decreased continuously until d6. Interestingly, in control parasites the proline transport rate at d6 after treatment was slightly lower than that presented at d1 for concentrated parasites (65.3 ± 6.6 and 87.5 ± 7.7 pmol/min, respectively) and the culture densities at those days after treatment were similar (1.3×10^8 and 1.82×10^8 parasites/ml). According to previous data, the depletion of medium components in only 24 h is improbable (Alonso et al. 2001), being the accumulation of some factor dependent on the parasites density a more feasible hypothesis.

DISCUSSION

L-proline is the most abundant amino acid in the midgut and haemolymph of trypanosomatids insect vectors (Bringaud et al. 2006), and is oxidized to glutamate in the parasite mitochondria (Mantilla et al. 2015; Paes et al. 2013; Sylvester and Krassner 1976). *Trypanosoma cruzi* is capable to metabolize D-proline since it also contains proline racemases (Chamond et al. 2003). After its conversion into glutamate, part of the proline is fully oxidized through the TCA cycle (Sylvester and Krassner 1976). Our results suggest that intracellular proline is accumulated, as a result of a finely regulated process, prior to increasing the cell replication velocity. The correlative increase of TcAAAP069 protein, transport rate, intracellular proline concentration and finally the cell replication velocity, suggests that parasites accumulate proline prior to the initiation of the exponential growth phase.

Accumulation of free proline is the result of a balance between uptake from the extracellular medium, biosynthesis and degradation. So, it cannot be excluded that the observed changes in the intracellular pool of proline are due not only to an increased transport but also to variations in the metabolism of proline. We can speculate that proline transport regulation occurs to maintain the cell energy or redox homeostasis, both of them, or even the osmotic equilibrium (Magdaleno et al. 2009; Paes et al. 2013; Rohloff et al. 2004; Saye et al. 2014; Sylvester and Krassner 1976).

Other interesting finding is the transient localization of the transporter in the plasma membrane, in addition to the previously reported focus close to the flagellar pocket (Inbar et al. 2012; Miranda et al. 2012; Saye et al. 2014). This localization correlates with maximum transport rates, suggesting the possibility that the proline uptake is more active when *Tc*AAAP069 transporter is located both in the plasma membrane and close to the flagellar pocket. All *T. cruzi* amino acid transporters described up to day were detected in the same focus close to the flagellar pocket, but the presence in the plasma membrane was not reported yet.

Formerly, it was described that proline transport decreases during the passage from intracellular epimastigotes to trypomastigotes (Silber et al. 2009; Tonelli et al. 2004). However, and despite the high proline transport activity, intracellular epimastigotes do not have high levels of intracellular free proline, and they actually have the low-

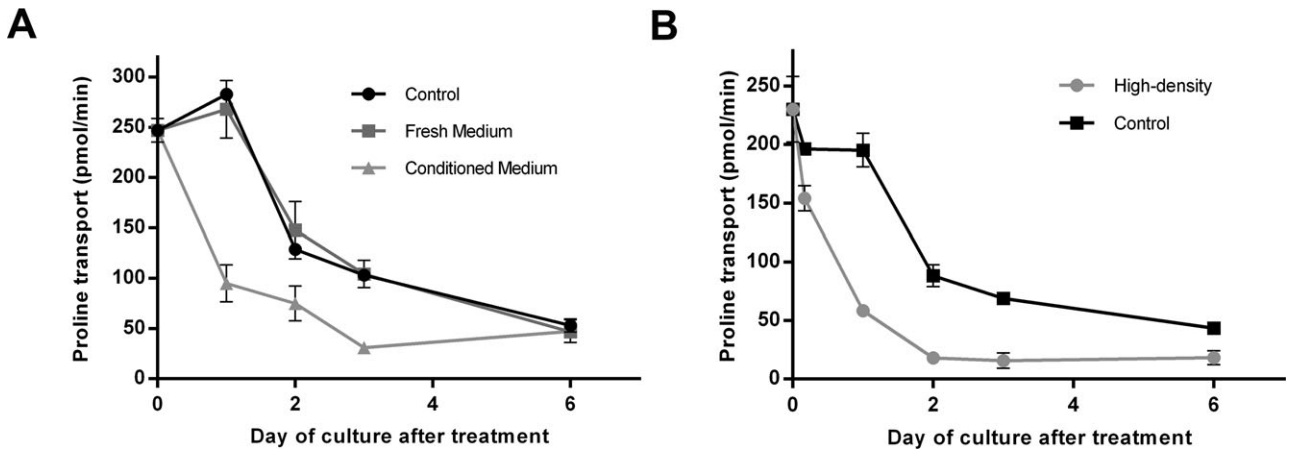

Figure 3 Modulation of proline transport by parasites density related factor. (**A**) Epimastigote cultures from d5 (10^7 parasites/ml) were switched to the following conditions: (1) fresh medium (square), (2) conditioned medium obtained from d15 parasites (triangle), and (3) control group without treatment (circle). Then, proline uptake was evaluated periodically for 6 d. (**B**) Epimastigotes from d5 (10^7 parasites/ml) were assayed for proline transport periodically after dividing the culture into two groups: (1) control group without treatment (square) and (2) parasites concentrated seven times respect to the control, equivalent to a d8 culture density (~7 × 10^7 parasites/ml, circle). Both groups, control and concentrated, were resuspended in fresh medium.

est levels among the mammalian stages of *T. cruzi* (Silber et al. 2009). Our results showed that epimastigotes, like intracellular epimastigotes, also present high proline transport activity, and this activity decreases during the growth curve until it completely disappears in the late exponential phase, when the epimastigotes are in transition to metacyclic trypomastigotes. Related to the putative regulation mechanisms, post-translational modifications of the *Tc*AAAP069 could produce down-regulation of the transporter, indeed the *T. cruzi* phosphoproteome reported the presence of five phosphorylation positions and also SUMOylation sites (Marchini et al. 2011).

There are many reports about the effects of the conditioned medium over cellular processes in *T. cruzi* epimastigotes, i.e. cell division, metacyclogenesis, arginine kinase expression, arginine transport, aspartate transport, cysteine transport, lysine transport, soluble adenylate kinase activity, and nuclear adenylate kinase localization (Alonso et al. 2001; Bouvier et al. 2006; Camara Mde et al. 2013; Canepa et al. 2005, 2009; de Camara Mde et al. 2013; Inbar et al. 2012; Pereira et al. 2002). In the case of *T. brucei,* the slender to stumpy differentiation is a density-dependent response that resembles quorum sensing in microbial systems (Mony et al. 2014). In this sense, our results about the down-regulation of proline uptake in parasites, growing at high densities or in conditioned medium, suggest the presence of a parasite density-derived factor that can regulate such process. The release of parasite proteins was studied extensively. Parasite-associated extracellular vesicles have been reported and also their contribution to host–pathogen interactions (Twu and Johnson 2014). In the specific case of *T. cruzi*, the presence of different secretion pathways to excrete or secrete proteins has been demonstrated. Moreover, some functions of such vesicles were reported, for example, metacyclic forms may use extracellular vesicles to deliver cargo into host cells (Bayer-Santos et al. 2013). One or more of these secreted proteins could be responsible for the regulation of proline transport, however, further investigation is needed.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Epimastigote growth curve. In vitro growth curve of *Trypanosoma cruzi* epimastigotes overexpressing the proline transporter TcAAAP069. To determine the growth rate, 10^6 cells/ml were seeded in BHT medium and maintained at 28 °C for 10 d. Parasites were counted using a hemocytometer chamber.

Figure S2. Colocalization with TCLP1 protein. *Trypanosoma cruzi* TcAAAP069 parasites of day 5 (D5) and day 8 (D8) were analyzed by indirect immunofluorescence using anti-tri-FLAG (green), anti-TCLP1 (red) antibodies and DAPI (blue). Scale bar = 10 μm.

Figure S3. Cell cycle and cell size analysis. *Trypanosoma cruzi* TcAAAP069 parasites of day 5 (D5) and day 7 (D7) were analyzed by flow cytometry after iodide propidium staining. (**A**) Cell cycle analysis. The percent of cells in each phase was calculated using FlowJo software. (**B**) Cell size analysis of sample parasites. Dot plot of forward scatter vs. side scatter was used to compare cell size between D5 and D7 parasites. The corresponding histograms are also shown (horizontal for FSC-A and vertical for SSC-A).

Figure S4. Proline transport after PBS starvation with or without proline, lysine, or glucose. *Trypanosoma cruzi* TcAAAP069 parasites of day 5 (1×10^7 parasites/ml) were incubated for 24 h in PBS alone (PBS), PBS supplemented with 10 mM proline (PRO), PBS supplemented with 10 mM lysine (LYS) or PBS supplemented with 10 mM glucose (GLUC). Uptake assays were performed using 10^7 parasites.