

Actin expression in trypanosomatids (Euglenozoa: Kinetoplastea)

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Heteroxenic and monoxenic trypanosomatids were screened for the presence of actin using a mouse polyclonal antibody produced against the entire sequence of the Trypanosoma cruzi actin gene, encoding a 41.9 kDa protein. Western blot analysis showed that this antibody reacted with a polypeptide of approximately 42 kDa in the whole-cell lysates of parasites targeting mammals (T. cruzi, Trypanosoma brucei and Leishmania major), insects (Angomonas deanei, Crithidia fasciculata, Herpetomonas samuelpessoai and Strigomonas culicis) and plants (Phytomonas serpens). A single polypeptide of approximately 42 kDa was detected in the whole-cell lysates of T. cruzi cultured epimastigotes, metacyclic trypomastigotes and amastigotes at similar protein expression levels. Confocal microscopy showed that actin was expressed throughout the cytoplasm of all the tested trypanosomatids. These data demonstrate that actin expression is widespread in trypanosomatids.

Key words: actin - confocal microscopy - trypanosomatids - *Trypanosoma cruzi*

Actin is a conserved filamentous protein that plays a major role in diverse eukaryotic cell processes, including cytokinesis and motility. These processes depend on the interactions of actin monomers and filaments with numerous other proteins and are driven by a dynamic remodeling of the actin cytoskeleton, including the assembly of G-actin (a monomeric form) into F-actin (a filamentous form) and the reverse disassembly (Pollard & Cooper 2009, Lee & Dominguez 2010). Furthermore, there is increasing evidence, from studies involving a number of cell types, demonstrating the importance of actin during endocytosis (Robertson et al. 2009, Galleta et al. 2010).

Although actin is abundant and well characterised in higher eukaryotic cells, until recently, very little information existed on its expression and distribution in protozoa of the Trypanosomatidae family (Kohl & Gull 1998). Actin filaments have been only poorly visualised in trypanosomatids through transmission electron microscopy (Sahasrabudhe et al. 2004). However, their presence has been demonstrated by immunofluorescence in the cytoplasm of the pathogenic species *Trypanosoma brucei* (García-Salcedo et al. 2004), *Trypanosoma cruzi* (de Souza et al. 1983, Mortara 1989, Melo et al. 2008, Cevallos et al. 2010) and *Leishmania major* (Sahasrabudhe et al. 2004). Interestingly, the immunogenic and filament-forming properties of *Leishmania* actin differ significantly from those of actin in other eukaryotes, particularly plasmodia, yeast and mammalian actins (Sahasrabudhe et al. 2004).

The first studies describing the sub-cellular localisation of actin in trypanosomatids were performed using heterologous antibodies against actin from other organisms. Antibodies raised against rabbit actin detected a 43 kDa protein in *T. cruzi* and *Leishmania mexicana* (Mortara 1989) and antibodies raised against *Entamoeba histolytica* actin showed a positive reaction in the flagellum of *T. cruzi* (de Souza et al. 1983). More recently, the sub-cellular localisation of *T. cruzi* actin was evaluated using a homologous antibody and an immunofluorescence analysis of the different developmental forms of this parasite (epimastigotes, bloodstream trypomastigotes and amastigotes). This assay revealed many rounded and punctuated structures, resembling patches, distributed throughout the cytoplasm, which had no association with subpellicular microtubules or with the nuclear or kinetoplast DNA (Melo et al. 2008). Actin-like filaments were also demonstrated in *L. major* using a homologous antibody and actin was present in the promastigote form as granules, as short filaments or bundles and as patches (Sahasrabudhe et al. 2004).

Here, we have screened pathogenic and non-pathogenic species of trypanosomatids for the presence of actin by western blotting using a homologous antibody coded with the entire sequence of the *T. cruzi* actin gene (TcActin). Furthermore, we have analysed the expression of this protein in different developmental forms of *T. cruzi* (cultured epimastigotes, in vitro-derived trypomastigotes and cell culture-derived amastigotes) and studied its subcellular localisation by confocal microscopy.

MATERIALS AND METHODS

Reagents - Bovine serum albumin (BSA) was purchased from Sigma (Sigma Chemical Co, St. Louis, MO, USA). Alexa Fluor 488-conjugated rabbit anti-mouse immunoglobulin G (IgG) was purchased from Molecular Probes (Carlsbad, CA, USA). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

doi: 10.1590/0074-0276108052013015

Financial support: CNPq, FIOCRUZ

MJS and SPF receive research fellowships from CNPq.

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Received 12 December 2012

Accepted 26 February 2013

Molecular weight markers (Benchmark Protein Ladder, 10-220 kDa) and the fluorescent dye Hoechst 33342 trihydrochloride were purchased from Invitrogen (Eugene, OR, USA). A polyclonal mouse antibody against *T. cruzi* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was kindly provided by F Morini [Carlos Chagas Institute, Oswaldo Cruz Foundation (Fiocruz), state of Paraná, Brazil].

Parasites - *T. cruzi* clone Dm28c (Contreras et al. 1988) epimastigote forms were grown at 28°C in liver infusion tryptose (LIT) medium (Camargo 1964) supplemented with 10% foetal bovine serum (FBS), with weekly passages. Epimastigotes were differentiated in vitro into metacyclic trypomastigotes in triatomine artificial urine (TAU)3AAG medium (190 mM NaCl, 17 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂ and 8 mM phosphate buffered saline (PBS) at pH 6.0 supplemented with 10 mM L-proline, 50 mM L-sodium glutamate, 2 mM L-sodium aspartate and 10 mM D-glucose) (Contreras et al. 1985). Briefly, five-day-old cultured epimastigotes were harvested by centrifugation for 10 min at 8,500 g. The parasites were then incubated at a density of 5 x 10⁸ cells/mL for 2 h at 28°C in TAU medium. Cells (at a 1:100 dilution) were further incubated for 96 h at 28°C in culture flasks that contained no more than 1 cm deep of TAU3AAG medium. The number of living epimastigotes and metacyclic trypomastigotes was then determined by cell counting via a Neubauer chamber after 24 h, 48 h and 72 h. The metacyclic trypomastigotes were purified from the 72 h-old cultures by DEAE-51 cellulose chromatography (Contreras et al. 1985).

To obtain amastigote forms, metacyclic trypomastigotes derived in vitro were used to infect Vero cells (ATCC CRL-1586), which were then cultivated in Dulbecco's Modified Eagle's medium (Sigma) supplemented with 5% FBS and incubated in a humidified atmosphere with 5% CO₂ at 37°C. After 10 days of infection, the amastigotes released into the supernatant were harvested by centrifugation at 1,000 g for 5 min.

L. major promastigotes were grown at 28°C in Schneider's medium (Sigma) supplemented with 10% FBS and 10 mg/L haemin. Procyclic trypomastigote forms of *T. brucei* were cultivated at 28°C in SDM79 medium (Invitrogen) supplemented with 10% FBS and 10 mg/L haemin (Brun & Schonenberger 1979). *Strigomonas culicis* (formerly *Blastocrithidia culicis*) (Teixeira et al. 2011), *Angomonas deanei* (formerly *Crithidia deanei*) (Teixeira et al. 2011) with its bacterial endosymbiont, *Crithidia fasciculata* and *Herpetomonas samuelpessoai* were grown at 28°C in LIT medium supplemented with 10% FBS with daily passages. *Phytomonas serpens* was grown at 28°C in GYPMY medium (Itow-Jankevicius et al. 1993) with passages every three days.

Cloning and expression of TcActin - The complete coding sequence of TcActin (Dm28c clone) was amplified using synthetic primers that were based on the *T. cruzi* CL Brener sequence (accession Tc00.1047053510571.30) available from the *T. cruzi* genome database (genedb.org). The putative open reading frame of 1,131 bp, encoding a protein of 41.9 kDa, was amplified by poly-

merase chain reaction (PCR) using forward primer 5'CGGAATTCATGTCTGACGAAGAACAGTCCGC-TATT3', reverse primer 5'ATTCGTCGACTCATTGT TGTGCACAATGCTTGGGCCTGCCTCGTC3' and the genomic DNA as a template. The PCR products, flanked by EcoRI and Sall sites, were inserted into a vector (pGEX-4T₁, Invitrogen) containing glutathione-S-transferase (GST). Positive clones were purified with a QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA, USA) and sequencing was performed at Macrogen Inc (Seoul, Korea). The correct sequence was confirmed by the BLAST algorithm. The amplified fragments inserted into the pGEX-4T₁ vector (Invitrogen) were used to transform *Escherichia coli* strain Top 10. Production of the recombinant protein was induced by adding 0.3 mM isopropyl-β-D-1-thiogalactopyranoside. Inclusion bodies containing the recombinant protein were isolated and the recombinant protein was purified by electro-elution.

Polyclonal antiserum - A polyclonal antiserum against the expressed recombinant TcActin was produced in albino Swiss mice. Approximately 50 µg of purified, GST-tagged TcActin was mixed 1:1 with complete Freund's adjuvant and inoculated intraperitoneally in mice. After 15 days, the animals received three consecutive boosts at two-week intervals, each containing an additional 20 µg of the antigen and 77 µL of Alu-Gel (Serva, Heidelberg, Germany) as adjuvant. The antiserum was harvested five days after the last booster injection. This study was carried out in strict accordance with the recommendations in the guide for animal use of the Fiocruz and the protocol was approved by the Committee on Animal Experimentation (P-0434/07).

Western blot analysis - For immunoblotting analysis, all of the parasites' total protein extracts were prepared by resuspending the PBS-washed parasites (10⁶ cells/µL) in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer to lyse the cells (10⁷ cells/lane). The proteins were fractionated in 13% polyacrylamide gels by SDS-PAGE and transferred onto nitrocellulose membranes (Hybond C, Amersham Biosciences, England), according to standard protocols (Sambrook et al. 1989). Following the transfer of the proteins to nitrocellulose, the membranes were blocked with 5% non-fat milk and 0.05% Tween-20 in PBS. The membranes were then incubated for 2 h with a blocking buffer containing the polyclonal antiserum raised against recombinant protein TcActin (1:200 dilution). After three washings with 0.05% Tween-20 in PBS, the nitrocellulose was incubated for 1 h with HRP-conjugated rabbit anti-mouse IgG (1:10,000 dilution). The membrane was again washed three times with 0.05% Tween-20 in PBS and the reactive bands were visualised using the BCIP-NBT solution, as described by the manufacturer.

Furthermore, 15 µg of the total proteins obtained from the *T. cruzi* cultured epimastigotes, metacyclic trypomastigotes and cell culture-derived amastigotes, as well as those from the Vero cells, were resolved on SDS-PAGE (13%) gels. Following the transfer of the proteins to nitrocellulose, the membrane was incubated for 2 h with a blocking buffer containing both the polyclonal

antiserum raised against recombinant protein TcActin (1:200 dilution) and a polyclonal antiserum against *T. cruzi* GAPDH. After three washings with 0.05% Tween-20 in PBS, the membranes were incubated as described above. The relative amount of actin and GAPDH (1:250 dilution) expressed by each developmental stage was estimated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence assays - All of the parasites (including *T. cruzi* epimastigotes, amastigotes and metacyclic trypomastigotes) were washed and resuspended at a density of 10^7 cells/mL in PBS. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT), washed twice in PBS and then adhered to 0.1% poly-L-lysine-coated coverslips, which were themselves incubated for 20 min at RT. The cells were made permeable by 2 min of incubation with 0.1% Triton X-100 in PBS; next, they were washed with PBS, blocked by incubation overnight with 1% BSA in PBS and incubated for 1 h with the polyclonal antiserum against TcActin (1:150 dilution). After another washing, the samples were incubated with Alexa Fluor 488-conjugated rabbit anti-mouse IgG at a 1:800 dilution. The nuclear and kinetoplast DNA were stained by incubation with Hoechst 33342. After extensive washing, the coverslips were mounted onto glass microscope slides using N-propyl-gallate for anti-fading. The samples were examined using a Leica SP5 confocal laser microscope (Leica Microsystems, Mannheim, Germany) and the images were processed for better contrast using Adobe Photoshop CS3 software.

RESULTS

A polypeptide of approximately 41.9 kDa, encoded by the entire sequence of the *TcActin*, was used to obtain a polyclonal TcActin antibody prepared in mice. When used in western blotting to probe for the presence of actin in trypanosomatids, this antibody reacted with a peptide of approximately 42 kDa in the whole-cell lysates of multiple species (Fig. 1A), including parasites targeting mammals (*T. cruzi* epimastigotes, *T. brucei* procyclic forms and *L. major* promastigotes), insects (*A. deanei*, *C. fasciculata* and *S. culicis*) and plants (*P. serpens*). Interestingly, two close weak bands near that weight were recognised in the *P. serpens* blot.

The expression of actin was also analysed by western blotting in different life stages of *T. cruzi*, as well as in Vero cells, using the TcActin antibody (Fig. 1B). This procedure identified a single peptide of approximately 42 kDa (Fig. 1B, upper bands) in the whole-cell lysates of all tested *T. cruzi* stages (culture epimastigotes, metacyclic trypomastigotes and cell-derived amastigotes). A similar actin expression level was observed in all of the developmental forms, as confirmed by band densitometry analysis conducted using ImageJ software (Fig. 1C). The GAPDH banding pattern (Fig. 1B, lower bands) was used as a loading control.

Confocal microscopy and the TcActin antibody were used to visualise the cellular distribution of actin in different trypanosomatids, both monoxenic (*A. deanei* with its bacterial endosymbiont, *C. fasciculata*, *H.*

samuelpessoai and *S. culicis*) and heteroxenic (*P. serpens*). In the tested parasites, this antibody recognised the protein in all of the cells in the population (data not shown). The positive reaction was homogeneously dispersed throughout the cytoplasm of all the cells (Fig. 2). No reaction was observed in the flagellum. In *A. deanei*, the labelling was more intense at the bacterial endosymbionts, which are located at the posterior cell region.

In *T. cruzi*, punctate labelling for TcActin was found throughout the cytoplasm of epimastigotes, metacyclic trypomastigotes and amastigotes. No reaction was observed in microtubule-containing structures such as the flagellum and the plasma membrane (Fig. 3). In the trypomastigotes, the positive granules were arranged in linear arrays (Fig. 3H). In the epimastigotes and amastigotes, positive reactions were occasionally found in elongated structures that may represent the cytostome/cytopharynx (Fig. 3B).

DISCUSSION

In the present study, a mouse TcActin polyclonal antibody was cross-reacted with different trypanosomatids, including some monoxenic species found in insects (*A. deanei*, *C. fasciculata*, *H. samuelpessoai* and *S. culicis*) as well as a plant species (*P. serpens*). Both the presence of actin genes and the expression of the encoded protein have previously been demonstrated in some pathogenic heteroxenic trypanosomatids, such as *T. brucei* (García-Salcedo et al. 2004), *T. evansi* (Li et al. 2009), *T. cruzi* (de Souza et al. 1983, Mortara 1989, Melo et al. 2008), *T. rangeli* (Toaldo et al. 2001) and *L. major* (Sahasrabudde et al. 2004). The cross-reaction of our TcActin polyclonal antibody with *T. brucei* procyclic forms and *L. major* promastigotes, both of which are parasites known to ex-

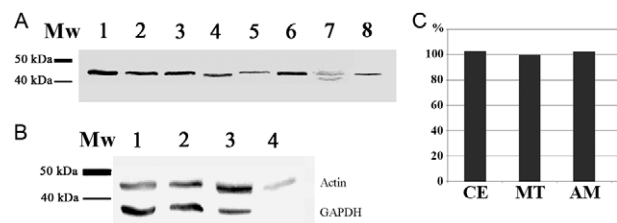


Fig. 1: western blot using the *Trypanosoma cruzi* actin gene (TcActin) antibody. A: different trypanosomatids were probed with detection of a single band of about 42 kDa, except for *Phytomonas serpens* (Lane 7) where two close faint bands could be observed. Lanes 1-8 contained 10^7 cells (1: *T. cruzi*; 2: *Trypanosoma brucei*; 3: *Leishmania major*; 4: *Strigomonas culicis*; 5: *Crithidia fasciculata*; 6: *Angomonas deanei* with symbiont; 7: *Phytomonas serpens*; 8: *Herpetomonas samuelpessoai*); B: different *T. cruzi* stages probed against the anti-TcActin antibody (upper bands) and an anti-glyceraldehyde 3-phosphate dehydrogenase antibody (lower bands) [1: culture epimastigote (CE) in liver infusion tryptose (LIT) medium; 2: metacyclic trypomastigote (MT); 3: amastigote (AM); 4: Vero cells (actin control); 1-4 contained 15 μ g protein/lane]; C: actin expression level as evaluated by integrated density of protein bands (using GAPDH band as normaliser) analysed by the ImageJ software (n = 3). To evaluate the actin expression in AM, the Vero cell band signal was subtracted from the AM band signal; MW: molecular weight markers.

press actin (García-Salcedo et al. 2004, Sahasrabudde et al. 2004), validated our polyclonal antiserum. Taken together, these data indicate that actin is present and conserved throughout the Trypanosomatidae family.

In a previous work, an antiserum raised against the entire actin from *T. cruzi* recognised a single band in one-dimensional electrophoresis. However, five actin isoforms were identified by this antiserum in two-dimensional electrophoresis (Cevallos et al. 2010). Therefore, it seems plausible that our polyclonal antibody (produced against the entire actin sequence) also recognised multiple actin isoforms within a single band generated by one-dimensional electrophoresis. The presence of two reacting bands in the *P. serpens* lysate suggests that this parasite possesses actin isoforms of different sizes.

In a previous study, an anti-*Leishmania* actin antibody was cross-reacted with cell lysates of various *Leishmania* species as well as of *T. cruzi*. However, that antibody failed to recognise the actin bands in the whole-cell lysates of *Plasmodium falciparum*, *Saccharomyces cerevisiae*, BHK21 cells and human erythrocytes, in-

dicating that the actin of trypanosomatids is divergent from that of other eukaryotic cells (Sahasrabudde et al. 2004). Accordingly, a rabbit anti-human α -actin polyclonal antibody readily recognised the actin band in whole-cell lysates of *P. falciparum*, *S. cerevisiae*, BHK21 cells and human erythrocytes, but did not cross-react with *Leishmania* or *Trypanosoma* actin (Sahasrabudde et al. 2004). A polyclonal antibody against a conserved C-terminal peptide of rabbit actin only poorly recognised *T. cruzi* actin. However, an antibody against *T. cruzi* actin was not species-specific, as proteins of a similar size were also detected in lysates from *L. mexicana* procyclics, *Toxoplasma gondii* sporozoites and *Trichomonas vaginalis* trophozoites (Cevallos et al. 2010).

The homology levels of *T. cruzi* actin compared with the eukaryotic actins of *L. major*, *T. brucei*, *P. falciparum*, *Dictyostelium discoideum* and *Homo sapiens* are 95%, 99%, 71%, 69% and 86%, respectively (Melo et al. 2008). Thus, the properties of trypanosomatid actin differ from those of the yeast, *Plasmodium* and human actins. The major differences were confined to the amino acids located on the surfaces of the yeast and mammalian actins, including amino acid residues 42-46, which constitute the major self-association sites for actin filament formation (Sahasrabudde et al. 2004). F-actin has not yet been clearly identified in trypanosomatids, which might be due to filament instability, deficient assembly or accentuated severing, events that are not yet understood (Melo et al. 2008).

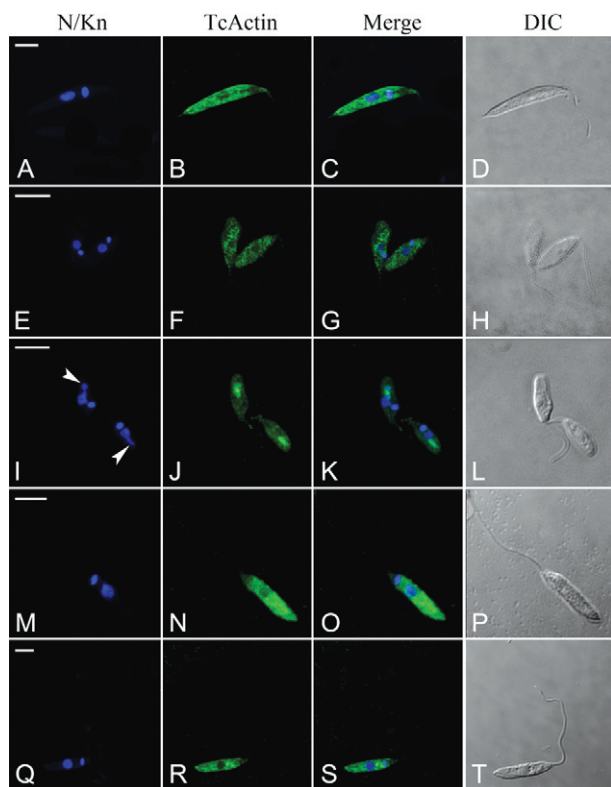


Fig. 2: actin localisation in different trypanosomatids by confocal microscopy by using the *Trypanosoma cruzi* actin gene (TcActin) antibody (actin detection with TcActin mouse polyclonal antibody plus Alexa Fluor 488-conjugated rabbit anti-mouse immunoglobulin G; note the staining throughout the cell body). A-D: *Strigomonas culicis*; DIC: differential interference contrast images of the parasite body; E-H: *Crithidia fasciculata*; I-L: *Angomonas deanei* with symbiont; Merge: merged Hoechst and actin images; M-P: *Phytomonas serpens*; N/Kn: staining of nuclear (N) and kinetoplast (Kn) DNA with Hoechst 33342; Q-T: *Herpetomonas samuelpessoai*. Arrowheads in I indicate staining of the bacterial endosymbiont. Bar = 5 μ m.

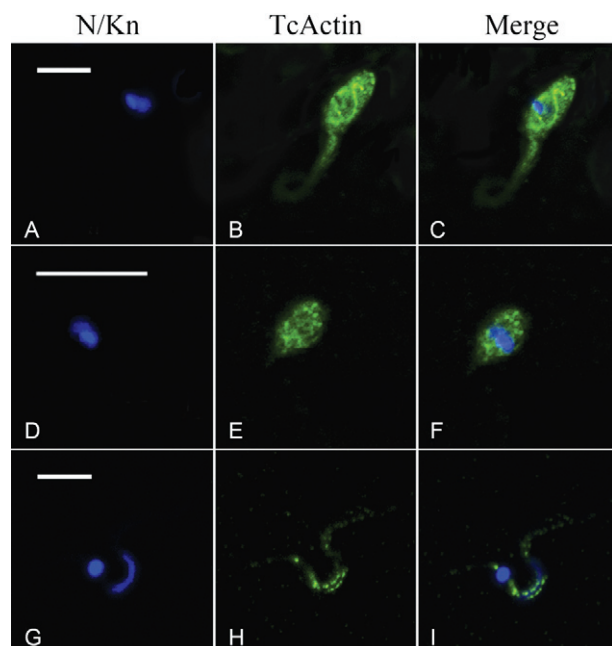


Fig. 3: actin localisation in *Trypanosoma cruzi* by using the *T. cruzi* actin gene (TcActin) antibody (actin detection with TcActin mouse polyclonal antibody plus Alexa Fluor 488-conjugated rabbit anti-mouse immunoglobulin G). A-C: epimastigote form (note the positive labelling at an elongated structure that may represent the cytopharynx); D-F: amastigote form; G-I: trypomastigote form; N/Kn: staining of nuclear (N) and kinetoplast (Kn) DNA with Hoechst 33342; Merge: merged Hoechst and actin images. Bar = 5 μ m.

With confocal microscopy, we found positive reactions for actin dispersed throughout the cytoplasm of all the tested trypanosomatids. This cytoplasmic pattern has also been observed in *Leishmania* sp. (Sahasrabudhe et al. 2004), *T. cruzi* (de Souza et al. 1983, Mortara 1989, Melo et al. 2008, Cevallos et al. 2010) and *T. brucei* (García-Salcedo et al. 2004). A previous study described the subcellular localisation of actin in *T. cruzi* by using an antibody produced against an actin fragment containing the conserved N-terminal region (SKKLFVG-DEAQAKR), which is present in all three *T. cruzi* actin alleles available from GenBank (Melo et al. 2008). This experiment resulted in actin labelling throughout the parasite cell body. In the present work, we have confirmed the cellular localisation of actin in *T. cruzi* epimastigotes, trypomastigotes and amastigotes by using an antibody produced against the entire actin coding region. Furthermore, our data indicate that actin is expressed at similar levels in these developmental stages. It has previously been shown that actin is equally expressed in different evolutive forms of *T. brucei* (García-Salcedo et al. 2004). Thus, actin can act as an optional normaliser for protein expression studies investigating the *T. cruzi* differentiation process.

This is the first study to describe actin localisation in *T. cruzi* metacyclic trypomastigotes, as previous works have analysed actin distribution only in cell-derived trypomastigotes (Cevallos et al. 2010) or bloodstream trypomastigotes (Melo et al. 2008). Although similar in their distribution of cellular organelles, these forms constitute different stages of the parasite. Thus, the linear array of actin granules observed in metacyclic trypomastigotes is interesting and may represent a distinct actin arrangement.

An increased signal was detected near the bacterial endosymbionts located at the posterior cell end of *A. deanei*, suggesting that some type of actin-based cytoskeleton might be related to the symbionts' positioning in this region. It has been previously speculated that such an actin-based cytoskeleton might also be related to anchoring RNA granule components in *T. cruzi*, as granules that remain motionless were associated with actin filaments, whereas those that moved in the cytoplasm remained associated with microtubules (Holetz et al. 2010).

Some studies of trypanosomes showed that actin participates in endocytosis and intracellular transport in these organisms (García-Salcedo et al. 2004). Thus, a repression of actin expression by iRNA in the bloodstream forms of the African trypanosome *T. brucei* resulted in the cessation of growth, loss of the endocytic activity and termination of vesicular traffic from the flagellar pocket membrane (García-Salcedo et al. 2004). Whereas in procyclic forms, actin was uniformly distributed in the cytoplasm, in bloodstream forms, actin was primarily located at the posterior end of the cell, clearly concentrated between the nucleus and the kinetoplast, a site of highly active endocytic activity (García-Salcedo et al. 2004). A proteomic analysis of the American trypanosome *T. cruzi* indicated the presence of actin in the reservosome, an end organelle of the endocytic pathway (Sant'Anna et al. 2009). Accordingly, thus far, the only demonstrated func-

tional role of actin in *T. cruzi* has been related to endocytosis (Bogitsh et al. 1995, García-Salcedo et al. 2004). These data reinforce evidence from other eukaryotic cells indicating the importance of an actin cytoskeleton during endocytosis (Robertson et al. 2009, Galletta et al. 2010). The possible localisation of actin at the cytostome/cytopharynx of *T. cruzi* epimastigotes would imply the presence of a contractile cytoskeleton at this region. Further experiments are needed to confirm this hypothesis.

ACKNOWLEDGEMENTS

To Nilson Fidêncio, Vanessa Stella and Crisciele Kuligowski, for technical assistance.

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