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Expression of profilin in *Trypanosoma cruzi* and identification of some of its ligands

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ABSTRACT

The role and regulation of actin in *Trypanosoma cruzi* and other related parasites is largely unknown. Based on early genome analysis, it was proposed that there was a reduced dependency on the acto-myosin system in the trypanosomatid parasites. However, more recent studies have extended the set of potential actin regulatory proteins, particularly for *T. cruzi*. One of the identified actin-binding proteins in trypanosomatids is profilin. In other systems, it is capable of simultaneously binding both monomeric actin and several actin-regulatory factors. Hence, the study of profilin and its ligands may help to identify novel pathways in which actin is involved. In *T. cruzi*, profilin is encoded by a single copy gene. In this work, we demonstrated that this gene is constitutively expressed in both insect and mammalian stages of the parasite, and that the protein is diffusely distributed. Furthermore, we identified some of its potential ligands by LC-MS using GST-profilin pull-down assays of parasite's protein extracts. Many of them were trypanosomatid specific proteins with unknown functions, although proteins from the carbohydrate metabolism, and two metallopeptidases were also detected. As expected, known ligands of profilin in other organisms were identified, including actin, the microtubule components, and the elongation factor 1-alpha. Our work suggests that profilin and the actin system may be regulated by unknown factors and participate in novel biological processes.

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1. Introduction

Microfilaments and microtubules are the most conserved elements of the eukaryotic cytoskeleton. These filaments are linear polymers whose components are a globular protein named actin and α/β-tubulin dimers. In cells, actin polymerization into microfilaments is tightly regulated to rapidly start filament formation or degradation at the right place and time. This is performed by a diverse set of proteins that bind monomeric or polymeric actin, named the actin binding proteins (ABPs) [1]. Some ABPs control the initiation of polymerization by stabilizing actin oligomers that serve as seeds for the formation of new filaments. Others regulate the elongation of filaments and the turnover of actin subunits into them. There are ABPs that sever or bind to the ends of microfilaments to restrict its length or promote polymerization, whereas additional proteins can stabilize bundles or networks of microfilaments.

We are interested in the function and regulation of the actin cytoskeleton in the protist parasite *Trypanosoma cruzi*. This species is the etiological agent of Chagas' disease, and is classified within the trypanosomatids. It has been suggested that these parasites have a characteristic cytoskeleton mainly constituted by microtubules [2]. In support of this view, the genome sequencing projects of the model trypanosomatids *T. cruzi*, *T. brucei*, and *Leishmania major*, led to the recognition of several unique microtubule-organizing factors and kinesins [3]. In contrast, a reduced set of homologous proteins to canonical ABPs were identified. Additionally, it was not possible to detect proteins involved in the cross-talk between the actin and microtubule cytoskeletons, and there was a limited set of myosins. It was then concluded that in trypanosomatids there is a reduced dependency on the acto-myosin system that is compensated by a more complex microtubule cytoskeleton. However, further scrutiny of these genomes revealed the presence of a few additional ABPs. These included coronin and the end-binding protein 1, which are proteins with microfilament binding

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domains known to be involved in the cross-talk between the actin and tubulin cytoskeletons [4]. It has also been shown that *T. cruzi* has an expanded set of actins, as well as actin regulatory proteins like formins and the Cap-Z complex, and of myosins [5–7]. This growing repertoire of actins and ABPs could be increased by lineage specific proteins that may have either equivalent functions to those characterized in other organisms, or novel functions.

To get more insight into the regulation and function of actin in *T. cruzi*, we began the study of profilin. Profilin is a key regulatory factor of the dynamics of actin polymerization in most cells [8]. Although the amino acid sequences of different profilins have less than 25% identity across different kingdoms, they have a remarkable conservation in their three dimensional structure and in their described ligands [9]. Distinct domains for the interaction with actin and proteins bearing proline-rich stretches are typical structural features of profilins. They allow profilin to interact with a wide range of proteins besides actin. Most of them are related with the actin regulation, but some are involved in other functions such as transcription, RNA splicing, or endocytosis [8]. Regarding actin regulation, profilin binds to monomeric actin and prevents the spontaneous formation of microfilaments. When polymerization is activated, profilin:actin complexes bind to nucleation and elongation factors (e.g. VASP, WASP, formins) by the poly-proline binding domain of profilin [10,11]. This promotes the dissociation of the profilin:actin complex, and the subsequent delivery of the actin molecule to the barded end of a microfilament. In trypanosomatids, the only study of profilin demonstrated its expression at the mRNA level in *Trypanosoma brucei*, and the ability of its encoding gene to complement a yeast negative mutant for profilin [12]. This work suggested that trypanosomatid's profilin is able to interact with both actin and ABPs. Therefore, we considered this protein as an attractive bait for the identification of novel actin regulatory and functional elements in *T. cruzi*. In this work, we demonstrated the expression of profilin in *T. cruzi* and identified some of its potential ligands.

2. Material and methods

2.1. Bioinformatic analysis

Multiple sequence alignment was performed with Clustal Ω (www.ebi.ac.uk/Tools/msa/clustalo/), and analyzed in the Jalview program [13]. 3D structural modeling and analysis was performed in Phyre2 [14] and Chimera [15].

2.2. Parasites

Mid-log phase *T. cruzi* CL-Brener epimastigotes were obtained by culture at 28 °C in liver infusion tryptose (LIT) medium [16] supplemented with 10% fetal bovine serum, 60 μg/mL penicillin, 100 μg/mL streptomycin, and 25 mg/L haemin. Amastigotes and trypomastigotes were obtained as previously described [5].

2.3. Expression of recombinant profilin and production of polyclonal sera

Full-length CDS of *T. cruzi* profilin (TcCLB.510911.10) was cloned into the pGEX4T1 (GE Healthcare) and pMALc2C (New England Biolabs) expression vectors to express profilin as a *N*-terminal fusion with either GST (GST-profilin) or MBP (MBP-profilin). Recombinant protein expression was conducted in *E. coli* BL-21 (DE3) using standard procedures. GST-profilin was purified by affinity using Glutathion-Sepharose High Performance (GE Healthcare) following the manufacturer's instructions. For MBP-profilin experiments, total bacterial lysates were used.

Affinity purified GST-profilin (10 μg per dose) was used to immunize Balb/c mice intraperitoneally. For the primary immunization the protein was emulsified in Freund's complete adjuvant. Two boost doses were administrated within 7 weeks intervals, but using incomplete Freund's as adjuvant. Blood samples were collected two weeks before the first immunization to be used as pre-immune controls. Immune sera were obtained fifteen days after the last boost. Positive sera obtained from two immunized mice were pooled at a ratio of 1:1 to be used in this study. The pooled pre-immune sera from the same mice was used as negative control. Animal handling and immunization protocol were approved by local Bioethics Committee for Animal Research.

2.4. Western blot

To produce total protein extracts, the parasites were harvested by centrifugation (1000×*g*, 10 min), washed with PBS, resuspended in 2X Laemmli's buffer, and boiled at 94 °C for 5 min. Parasite lysates were separated in 12% SDS-PAGE and transferred to PVDF membranes. Protein from 5×10^6 parasites was loaded per lane. PVDF membranes were blocked for 2 h with 5% non-fatty milk prepared in 0.05% Triton X-100 in PBS (PBS-T). Then, mice immune sera or its pre-immune controls (dilution 1:2000) were incubated for 2 h, followed by three washes (5 min each) with PBS-T. Monoclonal anti-tubulin-α antibody (Sigma, T6199) was used as a loading control (dilution 1:100000). As a secondary antibody, anti-mouse IgG-HRP (Thermo Scientific) (dilution 1:60000) was incubated for 1 h, followed by washes as described. Finally, the chemiluminescent signal was revealed by exposure to film. The antibodies were diluted in 2% non-fatty milk in PBS-T, and the incubations were performed at room temperature (RT) with gentle shaking.

2.5. Immunofluorescence microscopy

Cells were fixed with 3.7% paraformaldehyde for 20 min at 4 °C, attached on silanized coverslips, permeabilized with 0.5% Triton X-100 for 5 min, and blocked with 2% BSA for 2 h. Then, anti-profilin immune sera or its pre-immune control (dilution 1:400) were added and incubated for 1 h. After three washes with 1% BSA, the secondary anti-mouse antibodies conjugated with Alexa-Fluor® dyes (Molecular Probes) were added, incubated for 1 h and then washed as described above. Finally, the DNA was stained with TOTO®-3 (Molecular Probes), and the samples were mounted for fluorescence. The images were obtained using a Zeiss LSM 5 Pa confocal microscope and the images analyzed with ImageJ v. 1.49p.

2.6. GST pull-down assays

To produce epimastigote protein extracts, 150 mL of parasite's culture were harvested by centrifugation (1000×*g*, 10 min, RT), washed in PBS, resuspended in lysis buffer (1% Triton X-100 in PBS with cOmplete (Roche) protease inhibitors), and incubated for 15 min on ice. Protein extracts were cleared by centrifugation (13000×*g*, 20 min, 4 °C), and the soluble proteins were quantified with the DC kit (Bio-Rad) using BSA standard curves. For the pull-down assays, protein extracts from bacteria expressing GST-profilin or GST alone were incubated with 100–200 μL of affinity beads for 1 h, and washed three times with the lysis buffer. Then, the recombinant proteins associated to the beads were incubated with 2–3 mg of parasite's protein extract overnight at 4 °C, and then washed five times with PBS. Finally, the bound proteins were

eluted by boiling the beads at 94 °C for 5 min in 1 vol of 2X Laemmli's buffer.

2.7. Mass spectrometry

Laemmli's buffer containing the eluted samples from pull-down assays were loaded and concentrated in SDS-PAGE. Samples were run for short periods only to allow the concentration of the sample into a single protein band. Gels were stained with Coomassie blue. The single band contains all proteins in the sample, including the recombinant protein and the associated *T. cruzi* proteins. This band was excised, unstained with 50% methanol and 5% acetic acid for 12 h, washed, and incubated twice with 100 mM ammonium bicarbonate for 15 min. Then, the samples were reduced with 50 mM DTT for 45 min, alkylated with 20 mM iodoacetamide for 2 h, dehydrated with 100% acetonitrile, and in-gel digested with 30 μL of 20 ng/μL porcine trypsin for 18 h at 37 °C. The peptides were extracted by sonication in 50% acetonitrile and 5% formic acid, resuspended in 1% formic acid, and desalted and concentrated in Ziptip C18 columns using 3% acetonitrile and 0.1% formic acid as the mobile phase. Finally, the samples were analyzed by LC-MS in a Synapt G2S (Waters) instrument at the proteomic facility services of UNAM (Biousai, UNAM). The obtained data was used for searching in a database of 19661 predicted protein sequences from the *T. cruzi* CL-Brener genome (retrieved from www.tritrypdb.org on September 2015) using the ProteinLynx Global Server software (Waters). Only protein hits with >95% of probability were considered. The annotation of each protein hit was corroborated by domain searches using the Pfam (pfam.xfam.org/) and CCD (www.ncbi.nlm.nih.gov/Structure/cdd/docs/cdd_search.html) tools.

3. Results

3.1. Sequence comparison and 3D structural model of T. cruzi profilin

The gene encoding for a homologous amino acid sequence to profilin (Tc-profilin) is located at chromosome 35 of *T. cruzi* CL-Brener. Orthologous genes for this sequence are identified at the other 24 sequenced trypanosomatid species available at the TriTrypDB. In trypanosomatids profilin is encoded by single copy genes, with the exception of *Trypansoma vivax* that has two copies arranged in tandem. All of them have the same length (150 amino acids; 16 kDa), and show amino acid sequence identities > 70%. In a homology-based 3D model, Tc-profilin adopts the typical folding of profilins (Fig. 1A). Further analysis of the sequence showed that the amino acids involved in actin binding are poorly conserved (Fig. 1B). In contrast, most of the residues of the poly-proline binding site are identical (Fig. 1B). The previously reported specific insertion present in the profilin of *T. brucei* [12] is also present in Tc-profilin and in the orthologous protein sequence of *Leishmania major*. In our 3D model, it is partially folded as an α-helix and it is located before the C-terminal α-helix of the protein (Fig. 1A). The function of this insertion is unknown.

3.2. Tc-profilin is expressed along the life cycle

Mice were immunized with affinity purified GST-profilin to obtained polyclonal sera against the protein. In western blot analysis, immune sera detected a recombinant Tc-profilin fused to a different tag (MBP-profilin) but not the MBP tag alone, supporting

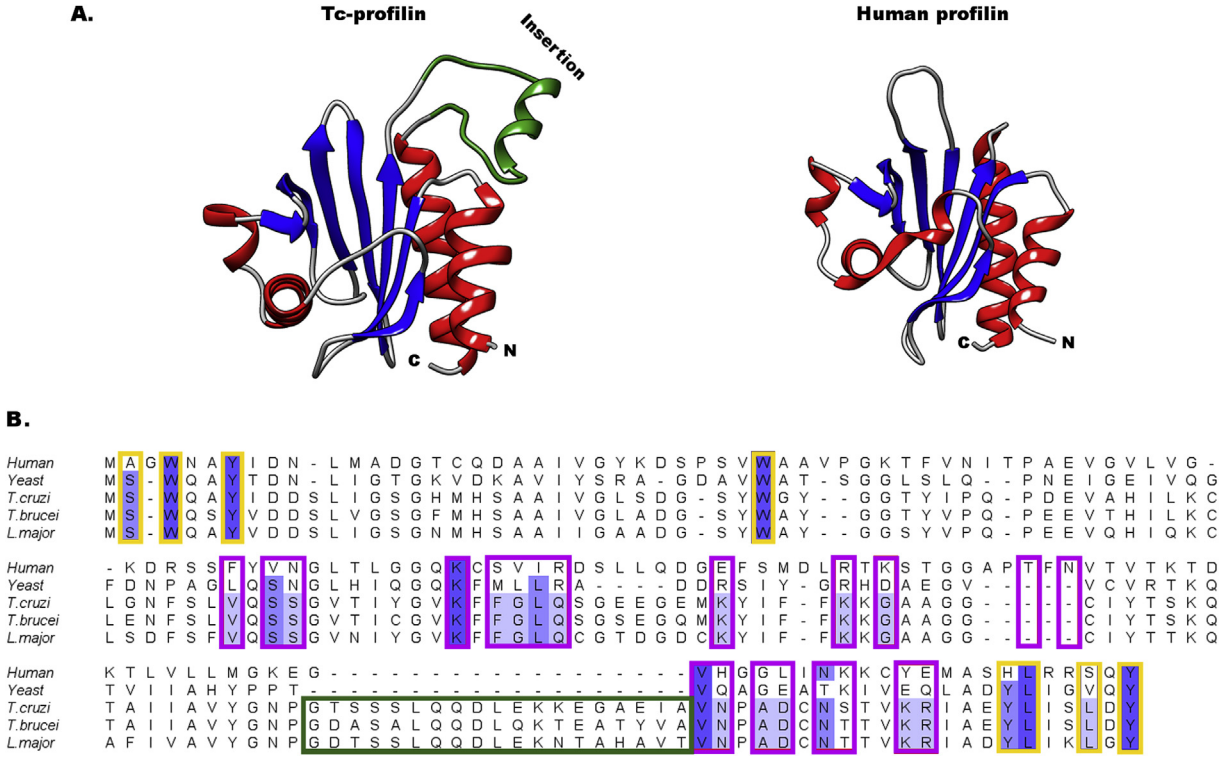

Fig. 1. Sequence analysis of trypanosomatid's profilin **A**. 3D structural model of Tc-profilin compared with that from human profilin (PDB: 2PBD). The characteristic 7 anti-parallel β-sheets (in blue) surrounded by 4 α-helices (in red) of profilins can be identified. The position of the described insertion is shown in green **B**. Sequence alignment of profilin amino acid sequences from model trypanosomatid species (TriTrypDB: TcCLB.510911.10, Tb427tmp.01.5350, LmjF.32.0520) with that from human and yeast (UniProt: P07737, P07274). Residues involved in actin-binding [26], and poly-proline stretches [11,27] are delimited with magenta and yellow border boxes, respectively. These amino acids are shaded according to its conservation (blue more conserved, white less conserved). The position of the insertion is delimited with a green border box (residues 110–128). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

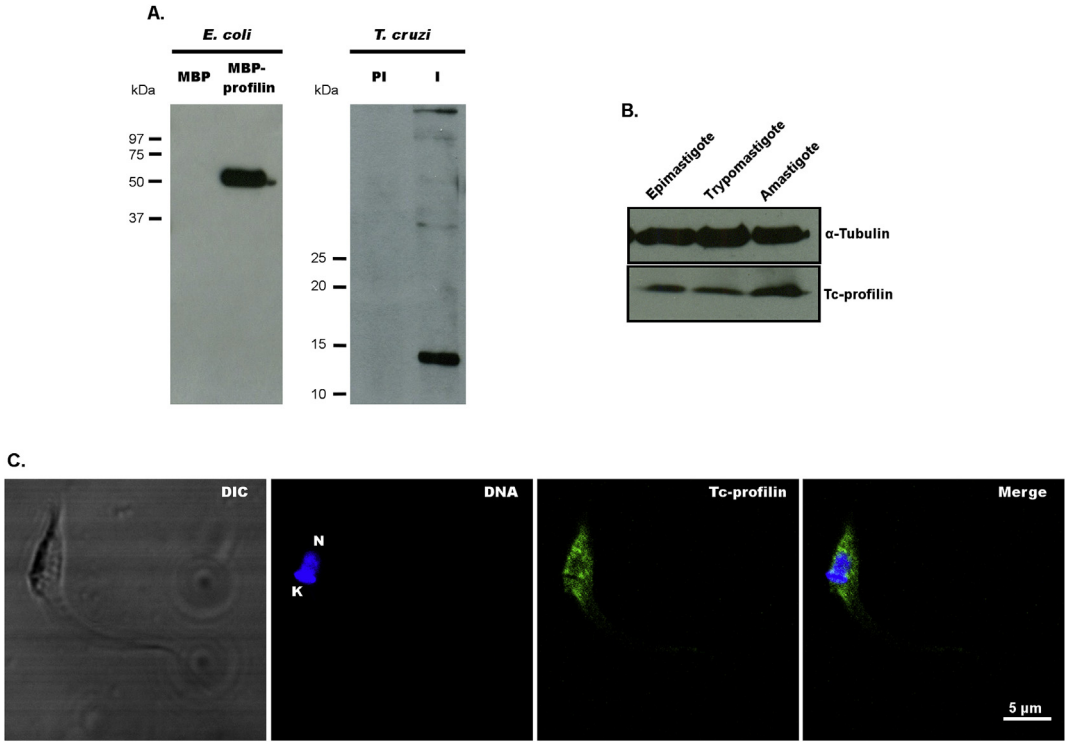

Fig. 2. Expression of Tc-profilin in different life cycle stages **A**. Western blot analysis of total *E. coli* protein extracts expressing recombinant MBP-profilin (59 kDa) and MBP (50 kDa) using polyclonal immune sera. The same analysis was conducted with total epimastigote's protein extracts, but using immune (I) and preimmune (PI) sera **B**. Constitutive expression of Tc-profilin in three developmental stages of the parasite. An anti-α-tubulin antibody was used as loading control **C**. Subcellular localization of Tc-profilin in epimastigotes revealed by confocal microscopy. The location of the nucleus (N) and kinetoplast (K) are indicated. A confocal section of 0.4 μm is shown.

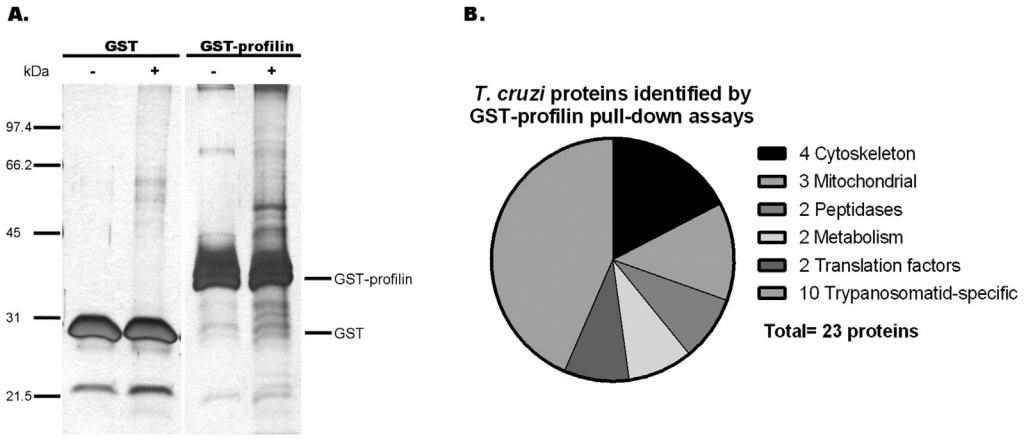

Fig. 3. *T. cruzi* proteins identified in GST-profilin pull-down assays by LC-MS **(A.)** SDS-PAGE analysis of a representative GST-profilin pull-down assay with epimastigote's protein extracts, stained with $AgNO_3$. Samples from experiments using GST-profilin (42 kDa) and GST (26 kDa) that were eluted from the affinity beads before (−) and after (+) the incubation with the *T. cruzi* protein extracts are shown **(B.)** Pie chart showing the number of detected GST-profilin ligands according to its deduced function or cell location. The number of detected proteins is indicated for each group.

the specific recognition of epitopes of the target protein. To study the expression of the native protein, we conducted the same analysis but with total parasite's proteins. In extracts obtained from epimastigotes, immune sera identified a band of the expected size (16 kDa) that was not observed with the preimmune control (Fig. 2A, left panel). A band of the same size is also detected in protein extracts from cell-derived trypomastigotes and amastigotes (Fig. S1), confirming the expression of the protein in different developmental stages of the parasite. The expression of Tc-profilin seems to be constitutive during the parasite's life cycle (Fig. 2B). We then analyzed the localization of the protein by confocal microscopy using our polyclonal sera. The specificity of the observed signal was evaluated with the corresponding preimmune control, which showed only background fluorescence (Fig. S2). As described for other profilins (e.g. Refs. [17,18]), our immune sera detected a diffuse distribution of the protein throughout the cell body of epimastigotes (Fig. 2C). A similar localization was observed in intracellular amastigotes (Fig. S3).

3.3. Profilin binds several proteins in vitro

To identify Tc-profilin ligands, we conducted GST-profilin pull-

Table 1
T. cruzi proteins identified in two of pull-down assays with GST-profilin using LC-MS. Proteins identified in the two GST-profilin pull-down assays are show in bold. Only proteins that were not detected in the GST pull-down controls were considered. In bold and with an asterisk those proteins identified in the two pull-down assays.

#	TriTrypDB ID	Protein description	Deduced function/location
Cytoskeleton			
1	**TcCLB.458759.10***	Profilin (recombinant protein)	Actin polymerization
2	**TcCLB.510571.30***	Actin	Microfilament
3	**TcCLB.411235.9***	Alpha-tubulin	Microtubule
4	**TcCLB.506563.40***	Beta-tubulin	
Mitochondrial			
5	**TcCLB.506211.160***	ADP ATP carrier protein 1	Transport, inner mitochondrial membrane
6	TcCLB.508741.229	Mitochondrial outer membrane protein porin	Transport, outer mitochondrial membrane
7	TcCLB.507029.30	Heat shock 70 kDa protein	Protein folding
Metabolism			
8	TcCLB.506297.190	Pyruvate phosphate dikinase	Carbohydrate metabolism
9	TcCLB.510105.100	UDP glucose dehydrogenase	
Peptidases			
10–11	**TcCLB.510837.9***, TcCLB.509213.120	Peptidase M20/M25/M40 superfamily	Metallopeptidase, TcCLB.510837.9 corresponds to the acetylornithine deacetylase
Translation factors			
12	**TcCLB.511369.30***	Elongation factor 1-alpha	Protein synthesis, actin binding
13	TcCLB.511585.190	Elongation initiation factor 4-alpha	Protein synthesis
Trypanosomatid specific			
14	TcCLB.507009.10	Gim5A	Glycosome surface in *T. brucei* [28]
15–18	TcCLB.507555.80, TcCLB.508285.10, TcCLB.509815.10, TcCLB.506845.60	Retrotransposon hot spot protein family	Associated with subtelomeric regions in *T. brucei* [29]
19	TcCLB.507771.30	Hypothetical	HIT zinc finger motif (Pfam: PF04438)
20	TcCLB.504019.3	Hypothetical	Chromosome segregation ATPase domain (CDD: COG1196)
21	TcCLB.504137.50	Hypothetical (fragment)	Unknown
22	TcCLB.508221.220	Hypothetical	Unknown
23	TcCLB.506507.20	Hypothetical	Unknown

down assays using 1% Triton X-100 soluble protein extracts obtained from epimastigotes. For this aim, recombinant GST-profilin expressed in *E. coli* was first immobilized on affinity beads. A control with immobilized GST was performed to discard *T. cruzi* proteins that bound to the tag and not to the profilin portion. A few bacterial proteins besides the recombinant proteins remained bound to the beads before incubation with *T. cruzi* proteins, as showed by SDS-PAGE analysis (Fig. 3A). Several unique bands were observed with GST-profilin compared with the GST control after the incubation with the parasite's protein extracts. These bands are expected to be Tc-profilin ligands in the parasite. To identify them, samples from two independent pull-down experiments were loaded in SDS-PAGE and briefly run to obtain a single band that was excised and subjected to trypsin digestion and LC-MS analysis. In total, 23 *T. cruzi* proteins were identified in the GST-profilin assays and not in the controls with GST (Table 1). Six of them were detected in the two experiments. Four of these are known ligands of profilin in other organisms: actin, α and β tubulins [17,19], and the elongation factor 1-alpha [20]. Another ten were trypanosomatid specific proteins most of them with unknown functions. Proteins from the carbohydrate metabolism, and two metallopeptidases were also detected. In GST controls only one *T. cruzi* protein was identified. The confidence values for each protein hit are shown in Table S1.

4. Discussion

We are interested in characterizing the actin system of *T. cruzi* and its regulation. As an initial approach, we selected profilin due to its ability to interact with actin and other ABPs. We demonstrated that the protein is expressed in various parasite stages. Then, using GST-profilin pull-down assays, we identified several of its potential ligands. One of them was the conventional actin, despite the low conservation of the amino acids potentially involved in the interaction with this protein. The α and β tubulins have also been reported as ligands of profilin. The evidence for the interaction of profilin with the tubulin cytoskeleton was first provided by the identification of tubulin in pull-down assays of protein extracts from mouse brain using immobilized recombinant profilin as a bait [19]. Later, specific antibodies against both monomeric actin and profilin showed partial co-localization with tubulin in human fibroblasts [21]. Recently, stronger co-distribution of profilin with microtubules was uncovered in mouse melanoma cells treated with a non-ionic detergent before cell fixation [17]. Additionally, the authors demonstrated the physical interaction between these proteins by co-immunoprecipitation assays. These works indicate that profilin could be an additional link for the cross-talk of the actin and tubulin cytoskeletons. It is possible that this is also true for *T. cruzi*, suggesting a stronger communication between actin and tubulin in this organism than previously suggested [3].

In addition to the cytoskeletal ligands, proteins with other reported functions were identified in our pull-down assays. Most of them were trypanosomatid specific proteins that have not been characterized, so our work may shed light on its role. The other hits consisted of proteins with other reported functions. Based on the available literature, no plausible explanation for their interaction with profilin can be proposed. A possibility would be protein moonlighting, which refers to the co-option of proteins for multiple functions. This has been proposed as a phenomenon that may explain the discordance between the apparently reduced number of proteins revealed by genome sequences and the complexity of cells [22]. In fact, one example of protein moonlighting is the participation of the elongation factor 1-alpha (EF1-α) in the actin cytoskeleton regulation. This association was first reported in the slime mold, where the direct binding of EF1-α with actin promoted the formation of microfilament bundles *in vitro* [23,24]. In yeast this

association is conserved, and specific phenotypes in the actin cytoskeleton organization triggered by the overexpression of point mutants of EF1-α were described [25]. In this organism, a complex containing both EF1-α and profilin was also described [20]. Therefore, interactions with no apparent biological importance such as those reported in here are feasible *in vivo*. However, unspecific interactions produced by the protein solubilization procedure, and the high abundance of the identified proteins cannot be discarded.

We have verified that profilin is expressed in different developmental stages of *T. cruzi*. Using *in vitro* experiments with *T. cruzi* protein extracts, we detected several potential ligands of profilin in this parasite. This list surely represents an incomplete picture of the profilin binding proteins in *T. cruzi*. In fact, expected profilin ligands such as proteins with proline-rich regions (e.g. formins), or other actins isoforms were not detected. However, our data suggests that novel pathways of profilin and actin regulation and function should be operating in this parasite. Future studies will address the biological significance of reported interactions, and search for additional actin regulatory elements and functions in this parasite.

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