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Molecular characterization and interactome analysis of *Trypanosoma cruzi* Tryparedoxin 1

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ABSTRACT

Trypanosoma cruzi tryparedoxin 1 (*Tc*TXN1) is an oxidoreductase belonging to the thioredoxin superfamily, which mediates electron transfer between trypanothione and peroxiredoxins. In trypanosomes TXNs, and not thioredoxins, constitute the oxido-reductases of peroxiredoxins. Since, to date, there is no information concerning *Tc*TXN1 substrates in *T. cruzi*, the aim of this work was to characterize *Tc*TXN1 in two aspects: expression throughout *T. cruzi* life cycle and subcellular localization; and the study of *Tc*TXN1 interacting-proteins. We demonstrate that *Tc*TXN1 is a cytosolic and constitutively expressed protein in *T. cruzi*. In order to start to unravel the redox interactome of *T. cruzi* we designed an active site mutant protein lacking the resolving cysteine, and validated the complex formation *in vitro* between the mutated *Tc*TXN1 and a known partner, the cytosolic peroxiredoxin. Through the expression of this mutant protein in parasites with an additional 6xHis-tag, heterodisulfide complexes were isolated by affinity chromatography and identified by 2-DE/MS. This allowed us to identify fifteen *Tc*TXN1 proteins which are involved in two main processes: oxidative metabolism and protein synthesis and degradation. Our approach led us to the discovery of several putatively *Tc*TXN1-interacting proteins thereby contributing to our understanding of the redox interactome of *T. cruzi*.

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1. Introduction

Trypanosoma cruzi, the causative agent of Chagas disease, constitutes a major sanitary problem in Latin America. This illness mainly affects the poor rural areas of Latin America, where the number of estimated infected persons is around 10 million, 28 million people are at risk of infection and ~20,000 deaths occur per year in endemic regions [1]. Remote location of infected people makes Chagas disease neglected and unattractive for search of new drugs. No vaccines are available at present, and drugs currently in use, nifurtimox

Abbreviations: TXN tryparedoxin, *Tc*TXN *T. cruzi* tryparedoxin; c-*Tc*TXNPx, *T. cruzi* cytosolic tryparedoxin peroxidase; H_2O_2, hydrogen peroxide; DAPI, 4′,6-diamidino-2-phenylindole.

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and benznidazol, were developed decades ago and are effective only during the symptomatic stage of Chagas disease and have undesirable side effects. Programs have focused on disease prevention by eliminating the vector, and prevention of other means of transmission, such as infection by blood donors or maternal transmission [2]. However, new means of transmission have been described, such as oral infection by contaminated food and accidental contamination during laboratory work and via organ transplantation [3]. Due to human migration, there are estimations that 100,000 chronically infected people from endemic countries are now living in non-endemic countries which do not screen donors serologically for Chagas disease [4]. There has been reported more number of cases in urban locations, novel means of transmission, vector resistance, new carriers and disease reactivation in AIDS individuals. Chagas disease can become an international threat. New effective treatments are a priority so novel specific targets for drug development against *T. cruzi* need to be discovered.

The protozoan parasite *T. cruzi* has a complex life cycle which alternates between two intermediate hosts (the first, a triatomine insect and the second, a mammalian vertebrate which could be a human), where it invades different cell types [5]. During macrophage invasion, parasites are temporarily located in phagocytic vacuoles, where reactive oxygen and nitrogen species are synthesized [6]. Oxidizing species such as peroxynitrite and hydrogen peroxide (H_2O_2) can be toxic to parasites and therefore, their ability to circumvent such an oxidative environment will determine the success and persistence of the infectious process. Therefore, *T. cruzi* antioxidant mechanisms constitute an active field of investigation, since they could provide the basis for a rational drug development.

As revealed by its genome sequencing [7], *T. cruzi* lacks genes for glutathione reductase, thioredoxin reductase, catalase and selenium-dependent glutathione peroxidases. Instead, the trypanosomes redox metabolism is based on the low molecular mass dithiol trypanothione (N(1), N(8)-bis (glutathionyl) spermidine). Thiol redox homeostasis in trypanosomes is efficiently maintained through the participation of different peroxidases which are part of a complex system of enzymes that includes trypanothione synthetase trypanothione reductase and tryparedoxins (for a review see [8]). Several of these enzymes are found exclusively in trypanosomes. This is the case of tryparedoxins (TXNs), oxidoreductases belonging to the thioredoxin superfamily which transfer reducing equivalents from trypanothione to peroxidases such as peroxiredoxins and glutathione peroxidases [9–11].

The regulation of a number of phenomena in the cell has been linked to the reversible conversion of disulfides to dithiols thereby modulating the activities of the respective proteins [12]. This conversion activity is mainly carried out by thioredoxins, another type of oxidoreductase. Several recent articles have described the identification of thioredoxin-interacting proteins in eukaryotes [13–17]. The putative target proteins are involved in many processes, including oxidative stress response (e.g. peroxiredoxins), nucleotide metabolism (e.g. ribonucleotide reductase) and protein synthesis (e.g. several elongation factors), among others.

However this does not seem to be the case for trypanosomes since there is no thioredoxin reductase, and thioredoxins are expressed at very low concentrations [18]. The predominant low molecular mass dithiol proteins in trypanosomes are tryparedoxins (TXNs), which are uniquely expressed in these parasites, and despite belonging to the thioredoxin superfamily they are quite different to thioredoxins. TXNs have a different active site motif (WCPPCR instead of WCG(A)PK in most thioredoxins), and are also considerably larger than thioredoxins due to several insertions summing up to about 5 kDa [19]. As in thioredoxins, the reduction of protein disulfides by TXNs is based on a dithiol exchange mechanism [19] in which the N-terminal cysteine residue of the CXXC motif (Cys40 in *T. cruzi*) initiates a nucleophilic attack on the disulfide target resulting in the formation of a mixed disulfide. The intermolecular disulfide bond is subsequently cleaved by the C-terminal resolving cysteine residue of the active site motif (Cys43 in *T. cruzi*), yielding the reduced substrate and the oxidized TXN. In trypanosomes TXNs, and not thioredoxins, constitute the oxidoreductases of peroxiredoxins (known as tryparedoxin peroxidases) and ribonucleotide reductase, while thioredoxins seem to be less relevant, as can be inferred from gene knock out and dsRNAi experiments, showing no phenotypic changes [20].

In *Trypanosoma brucei* and *Leishmania infantum* there are two TXN isoforms, one cytosolic (TXN1) and one mitochondrial (TXN2) [21,22]. The importance of cytosolic TXNs in trypanosomatids was evidenced by experiments demonstrating that they are essential for parasite survival. In *T. brucei* the depletion of *Tb*TXN1 showed that it plays a pivotal role in the hydroperoxide metabolism, and parasites become significantly more sensitive to oxidative damage [23]. In *L. infantum* the replacement of both chromosomal *Li*TXN1 alleles was only possible upon parasite complementation with an episomal copy of the gene. Furthermore, *ex vivo* infection assays suggest that wild-type levels of *Li*TXN1 are required for optimal *L. infantum* virulence [24]. The role of TXN1 in immunopathological processes has also been described in *L. infantum* by targeting B-cell effector functions, leading to IL-10 secretion and production of specific antibodies [25]. These results must not only be considered in the context of oxidative metabolism; since in trypanosomes TXNs may substitute thioredoxins in some functions, we can expect the presence of protein targets from different cellular processes.

In *T. cruzi* two genes that code for TXN have been described: *Tc*TXN1 and *Tc*TXN2. It has been shown that *Tc*TXN1 is capable of reducing *T. cruzi* glutathione peroxidase [26]. However, up to date, no molecular studies have been performed in *T. cruzi*'s TXN1 (*Tc*TXN1). In the first part of this work we present our findings upon cloning the gene and conducting studies to determine its subcellular localization and expression profile. In order to begin the unraveling of the TXN1 interactome, we developed an *in vivo* approach, by expressing a mutated form of *Tc*TXN1 in *T. cruzi*. This mutated *Tc*TXN1 contains a substitution in its resolving cysteine as well as an additional 6xHis tag (*Tc*TXN1C43S). Complexes between mutant TXN1 and targets were affinity purified and analyzed by two dimensional electrophoresis and mass spectrometry (MALDI-TOF-TOF). Our approach led us to the identification of some potential TXN1 binding partners. The advantage of this *in vivo*

approach is that it was carried out maintaining intracellular conditions, and thus allowing a better appreciation of physiological roles of disulfide oxidoreductases.

2. Materials and methods

2.1. Parasites

T. cruzi parasites of the Dm28c strain were used throughout this work [27]. Epimastigotes were grown in liver infusion tryptose (LIT) medium supplemented with 10% heat inactivated fetal bovine serum (FBS) at 28 °C. Vero cell derived trypomastigotes, amastigotes and metacyclic trypomastigotes were obtained as described in [28].

2.2. Production of polyclonal antiserum against *Tc*TXN1 and western blot

Rabbits were immunized with 200 μg of *Tc*TXN1 protein (purified as described below) in Freund's Complete Adjuvant (Sigma), for the first immunization. They were boosted three times at 2 weeks intervals with the same amount of protein using Freund's Incomplete Adjuvant (Sigma). Pre-immune serum was taken prior to the first immunization and immune serum was taken 2 weeks after the last immunization. For immunoblots, *T. cruzi* extracts were prepared as previously described [28]. Blots were incubated with anti-*Tc*TXN1 serum used at a dilution of 1:1000; bound antibody was detected with Horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Sigma) and then revealed with the Supersignal Detection Kit (Pierce).

2.3. Immunofluorescence microscopy

Parasites were harvested and washed twice with PBS, resuspended at a density of 10^7 cells/ml and fixed in PBS 4% paraformaldehyde for 20 min at room temperature. They were then washed with PBS, incubated in PBS 0.1 M glycine for 10 min at room temperature and permeabilized in PBS 0.2% Triton X-100. Cells were attached to poly-L-Lysine coated coverslips, rinsed with PBS and incubated for 1 h with antiserum against *Tc*TXN1 (dilution 1:300) in PBS 5% BSA. After three washes with PBS-0.1% Tween 20 they were incubated with goat anti-rabbit IgG coupled to FITC (Invitrogen) 1:200 in PBS 5% BSA, washed three times with PBS-0.1% Tween 20, once with PBS alone and mounted on slides with Prolong with DAPI (Invitrogen). Slides were observed with an Olympus IX 81 microscope coupled to a Hamamatsu Orca-ER camera (Diagnostic Instruments).

2.4. Generation of *Tc*TXN1C43S by site-directed mutagenesis

*Tc*TXN1C43S was generated following the instructions of the commercial protocol for QuikChange® Site-Directed Mutagenesis kit (Stratagene) with some technical adaptations. Briefly, we designed two fully complementary primers containing the nucleotide to be mutated in the center of these oligonucleotides: *Tc*TXN1C43Sforw: 5′-GGTGCCCGCCC**A**GCG CGGCTTC-3′ and *Tc*TXN1C43Srev: 5′-GAAGCCGCGGC**T**GGGCG GGCACC-3′. PCR reaction (50 μl) was performed using as a template 10 ng of pET28c/*Tc*TXN1 [29] plasmid purified from bacteria with DNA methylase activity, 1 μM primers, 0.2 mM dNTPs, and 2.5 U *Pfu* DNA polymerase (Fermentas) under the following conditions: 94 °C for 5 min; 12 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 11 min. After PCR, the amplified product was treated for 3 h at 37 °C with 10 U of *Dpn*I restriction enzyme (Fermentas), which has the ability to degrade the parental methylated DNA plasmid. Then, *E. coli* TOP10F′ competent cells (Invitrogen) were transformed with the *Dpn*I digested plasmid and the recombinant clones were selected to verify the mutation by sequencing.

2.5. Expression and purification of recombinant proteins

The *Tc*TXN1 and *Tc*TXN1C43S genes were cloned in the pET-28c plasmid (Novagen).The recombinant proteins were obtained as hexahistidine-tag proteins from the BL21 (DE3) *E. coli* strain and purified in a Ni^{2+}-NTA resin column. *Tc*TXN1 and *Tc*TXN1C43S were expressed for 4–6 h at 25 °C in LB medium with 25 μg/ml kanamicin and 1% glucose after adding 0.5 mM IPTG. Cells were resuspended in 50 mM phosphate buffer pH 8, 300 mM NaCl, 20 mM imidazole, and then treated with 80 μg/ml lysozyme for 30 min in ice, disrupted by sonication, and centrifuged at 15,000 × *g* for 15 min at 4 °C. Soluble recombinant proteins were purified via Ni^{2+}-HiTrap affinity column (GE Healthcare) and eluted in the same buffer plus 150 mM imidazole. N-terminal His tag was cleaved overnight at 4 °C with 3 U thrombin (Sigma) per mg of protein. The histidine-tag was removed by passage through the Ni-HiTrap affinity column. Fractions were pooled, desalted and concentrated using Vivaspin ultrafiltration spin columns (Vivascience) against 50 mM HEPES-HCl pH 7.6. Purified proteins were analyzed by SDS-PAGE. *Tc*TXNPx protein was obtained as previously described [30].

2.6. Purification of *T. cruzi* TXNPx/TXN1C43S complex in vitro

Recombinant proteins *Tc*TXNPx and *Tc*TXN1C43S were reduced by incubation with dithiothreitol (DTT) in >10 fold molar excess in ice. Reductant was removed by gel filtration through a desalting Hitrap column (Amersham Bioscience). 250 μM *Tc*TXNPx, 400 μM *Tc*TXN1C43S and 320 μM hydrogen peroxide were incubated for 30 min at room temperature. *Tc*TXNPx/*Tc*TXN1C43S complex was purified by gel filtration in Superdex 200 10/30 column (GE Healthcare) and analyzed by SDS-PAGE according to [31].

2.7. Generation of *Tc*TXN1C43S expressing parasites

The entire *Tc*TXN1C43S gene plus a sequence coding for an N-terminal six histidine tag was amplified from pET-28c. Amplification product was cloned in pGEM-T (Promega) and subcloned as a 500 pb *Bam*HI/*Hind*III fragment in the trypanosomal expression vector pTEX [32]. Epimastigotes were transformed by electroporation using the same conditions as in Piñeyro et al. [28]. Transfected parasites were grown in the presence of continually increasing G418 (GIBCO)

until a final concentration of 0.5 mg/ml. Overexpression was confirmed by western Blot with anti-*Tc*TXN1 antibody.

2.8. *Purification of TcTXN1/target complexes in vivo*

A 5 ml culture of epimastigotes transfected either with pTEX or pTEX-*Tc*TXN1C43S were grown at 28 °C, and then diluted in 50 ml of LIT medium. Parasites were collected after 3 days, washed with PBS and resuspended in 6.5 ml of phosphate buffer pH 8.0, 300 mM NaCl, 1% triton X-100, 1 mM PMSF. They were subjected to 3 cycles of thawing/frosting and then treated with *DNase*I for 30 min at 4 °C. Soluble proteins were recovered by centrifugation for 1 h at 6000 x*g*. Supernatants containing proteins were applied to Dynabeads® His-Tag Isolation and Pulldown (Invitrogen), and His-tagged proteins were purified as recommended by the manufacturer. Two sequential elutions were conducted, the first with phosphate buffer (phosphate buffer pH 8.0, NaCl 300 mM, 0,01% Tween 20, 1 mM PMSF) with 10 mM β-mercaptoethanol, and the second with the same phosphate buffer with 300 mM imidazole.

2.9. *SDS-PAGE and 2-D electrophoresis*

For 2-DE proteins were precipitated with 10% trichloroacetic acid in acetone with 20 mM DTT and washed twice with cold 100% acetone with 20 mM DTT. The protein pellets were air dried and resuspended in buffer (7 M urea, 2 M thiourea, 40 mM Tris base, 4% CHAPS, 1 mM PMSF). IEF was carried out on an IPGPhor System (GE Healthcare) with the following conditions: 7 cm non linear pH 3–10 gradient IPG strips (GE Healthcare) were passively rehydrated with the sample for 12 h at 20 °C and isoelectric focusing was gradually increased to 5000 V and run for 30,000 Vh at 20 °C and a maximum current of 50 μA/strip. After IEF each strip was equilibrated in 6 M urea, 75 mM Tris–HCl pH8.8, 29.3%(v/v) glycerol, 2%SDS, 0.002% bromophenol blue containing 1% DTT for 15 min, followed by a further 15 min equilibration in the same solution containing 25 mg per ml of iodoacetamide instead of DTT. Electrophoresis was performed in Tris/Glycine/SDS buffer according to the standard conditions and gels were stained with $AgNO_3$ as previously reported [33].

2.10. *In-gel digestion and MALDI-TOF-TOF MS*

Peptide mass fingerprinting of selected protein spots was carried out by in-gel trypsin treatment (Sequencing-grade Promega) overnight at 37 °C. Peptides were extracted from the gels using 60% acetonitrile in 0.2% trifluoroacetic acid, concentrated by vacuum drying and desalted using C18 reverse phase micro-columns (OMIX Pippete tips, Varian). Peptide elution from micro-column was performed directly into the mass spectrometer sample plate with 3 μl of matrix solution (a-cyano-4-hydroxycinnamic acid in 60% aqueous acetonitrile containing 0.2% TFA). Mass spectra of digestion mixtures were acquired in a 4800 MALDI-TOF/TOF instrument (Applied Biosystems) in reflector mode and were externally calibrated using a mixture of peptide standards (Applied Biosystems). Collision-induced dissociation MS/MS experiments of selected peptides was performed. Proteins were identified by NCBI database searching with peptide m/z values using the MASCOT software and using the following search parameters: monoisotopic mass tolerance, 0.05 Da; fragment mass tolerance, 0.2 Da; methionine oxidation and cysteine carbamidomethylation were set as possible modifications and one missed tryptic cleavage was allowed.

3. Results and discussion

3.1. *Tryparedoxin 1 is a cytosolic and constitutively expressed enzyme in T. cruzi*

Based on the GenBank sequence (Accession number AJ313314.1), we designed specific oligonucleotides to PCR amplify the gene coding for *Tc*TXN1 from *T. cruzi* Dm28c strain genomic DNA. The amplified product was cloned and analyzed by sequencing, showing to be identical to the reference gene (not shown). The predicted gene product is a 144 amino acid protein with a theoretical molecular mass of 16 kDa and a calculated isoelectric point of 5.27. The gene was cloned and expressed in *E. coli* as described, with an additional six-histidine tag at the N-terminus. The protein was affinity purified and used for rabbit polyclonal antiserum production.

Since trypanosomes display different morphological forms throughout their life cycle, each with a specific pattern of protein expression, we studied the expression of *Tc*TXN1 along the life cycle by western blot analysis (Fig. 1A). The experiments showed that the antiserum recognized a unique band of 15 kDa, detected with similar intensity in all stages of *T. cruzi*'s life cycle; differences between lanes represent disparities in the amount of protein loaded, as deduced from the Ponceau S dye and normalization with antiserum against c-*Tc*TXNPx [28] (Fig. 1A).

Subcellular localization of *Tc*TXN1 was studied by indirect immunofluorescence microscopy. Fig. 1B shows that the protein is homogeneously distributed in the cell with a typical cytosolic pattern, as its homologous TXN1 from *Crithidia fasciculata* and *T. brucei* [34,35].

From these results we conclude that *Tc*TXN1 is a constitutive cytosolic protein, in accordance to our previous description of its substrate localization c-*Tc*TXNPx which is also a cytosolic protein expressed throughout the parasites's life cycle [28]. As both enzymes act co-ordinately, these results confirm that this cytosolic metabolic pathway is necessary both in the insect and mammal stages. The presence of *Tc*TXN1 in all stages indicates its relevance and constitutes an important element for considering it a potential target for drug design.

3.2. *Mutated tryparedoxin 1 is able to form a complex with tryparedoxin peroxidase*

The major goal of this work was to isolate *Tc*TXN1 partners being expressed *in vivo* within *T. cruzi* parasites. To attain this objective we based our strategy on the reaction mechanism of TXNs, which is based on their two conserved cysteines. Cys40 residue and the oxidized target form a transient disulfide bridge, which is later resolved by Cys43 of *Tc*TXN1, releasing the oxidized TXN and the reduced protein target. Based on this, we generated a mutation at the active site of *Tc*TXN1, by replacing the resolving cysteine residue (Cys43) with a serine

residue. This mutant protein (*Tc*TXN1C43S) can form disulfide bridges with its targets, but these cannot be resolved due to the lack of the resolving cysteine residue, generating heterodimers between targets and *Tc*TXN1.

Validation of our *in vivo* approach was carried out through the demonstration that *Tc*TXN1C43S was able to form a complex with a known partner. For that purpose we used c-*Tc*TXNPx, the most widely demonstrated tryparedoxin substrate in trypanosomes. Both proteins, c-*Tc*TXNPx and *Tc*TXN1C43S were purified (Fig. 2 A and B) and co-oxizidized with H_2O_2. The resulting fractions were separated by gel permeation, obtaining two peaks: one major peak with a Mr of approximately 350 kDa which is in consonance with the expected mass of the complex, according to a previous report in *T. brucei* [31]; and another one corresponding to a molecular mass of around 30 kDa. Both peaks were collected and analyzed by SDS-PAGE. Fig. 2C shows that peak 1 contains bands with sizes matching both c-*Tc*TXNPx and *Tc*TXN1C43S, and these bands are only present in reducing conditions, indicating the reduction of the heterodisulfide bridge. During our analyses, we found the formation of a dimer of tryparedoxin that is sensitive to DTT, suggesting the formation of a disulfide bond between *Tc*TXN1 homodimers. Although this can be considered a consequence of the generation of the mutant, we also evidenced the presence of these dimers in the wild type tryparedoxins (see below). All the bands were excised from the gels, trypsin digested and analyzed by MALDI-TOF-MS, confirming their identity (data not shown).

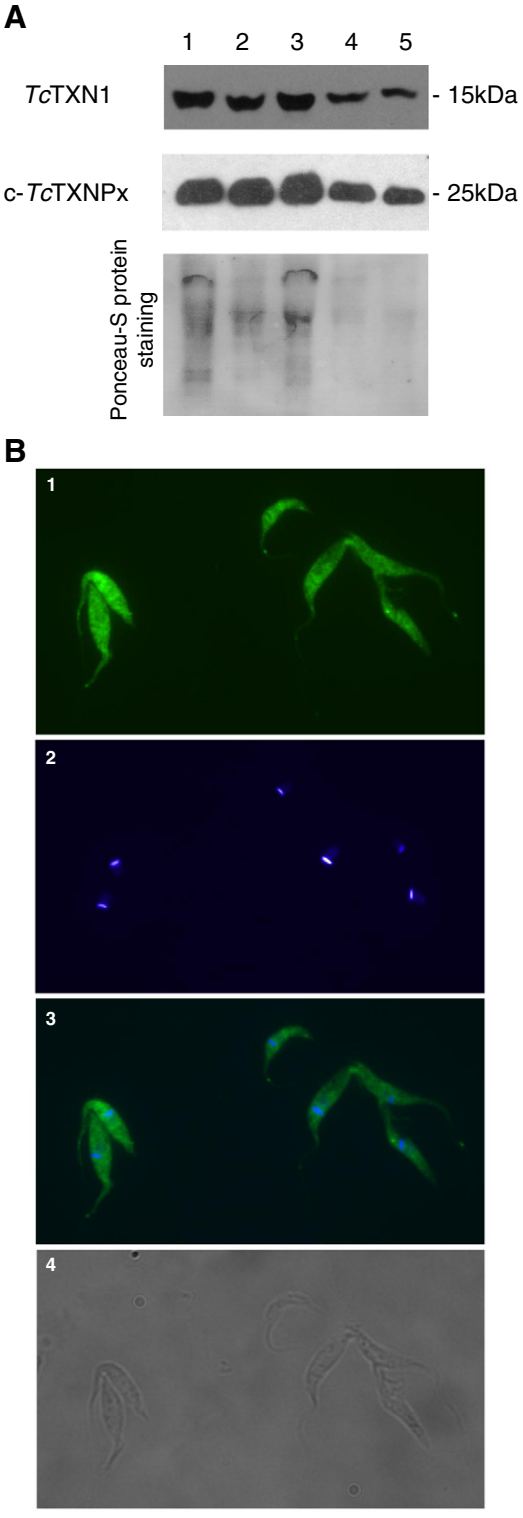

Fig. 1 – Tryparedoxin 1 is a cytosolic protein expressed along the life cycle of *T cruzi*. A—Western Blot with 15 μg of total protein extracts of each sample. Lanes: 1—epimastigotes; 2—nutritionally stressed epimastigotes; 3—metacyclic trypomastigotes; 4—bloodstream trypomastigotes; 5—amastigotes. As loading control immunostaining with antisera against c-*Tc*TXNPx, and Ponceau-S-protein staining of same membrane were performed. B—Analysis of *Tc*TXN1 localization in epimastigotes parasites by immunofluorescence. Parasites were fixed, permeabilized and incubated with: 1—antisera against *Tc*TXN1 (green labeling); 2—DAPI (blue labeling); 3—Merge of 1 and 2; 4—contrast image.

3.3. *Transfection of Trypanosoma cruzi with TcTXN1C43S*

The expression of His-tagged *Tc*TXN1C43S in *T. cruzi* was achieved by transfecting parasite lines with the pTEX-*Tc*TXN1C43S vector [32], and was confirmed by western blot. As shown in Fig. 3, extracts from the transfected cell line present a double band, one corresponding to the genome coded tryparedoxin (15 KDa) and a 15.5 KDa band, corresponding to the mutant protein, the mass difference being due to the additional histidine tag. As an experiment control we used pTEX transfected parasites. We did not find significant differences in growth rates and morphology of transfected parasites when compared with wild types, but the fact that the native enzyme is also being expressed in transfectants must be taken into account. Although phenotypic differences were not the scope of this work, it is a matter that should be studied more in depth in order to analyze changes in thiol content as well as alterations in the different pathways in which *Tc*TXN1 takes part.

3.4. *Identification of candidate tryparedoxin 1 interacting proteins*

In order to isolate the disulfide intermediates and based on the dithiol exchange mechanism catalyzed by tryparedoxins, protein cellular extracts of *T. cruzi* transfected with pTEX and pTEX-*Tc*TXN1C43S were applied on Dynabeads magnetic beads

for isolating histidine-tagged proteins, under nondenaturating conditions. Eluted proteins were separated by 2-DE, and differential spots were selected for mass spectrometry identification using MASCOT software and the parameters described in Materials and methods. This allowed us to successfully identify twenty-two protein spots. As can be seen in Fig. 4 and Table 1, in some cases the same protein was found in spots with the same molecular mass but different isoelectric point, probably as a consequence of post-translational modifications. Interestingly, most of them belonged to pathways related to oxidative metabolism, cysteine and methionine related pathways, and protein translation and degradation.

Previously published research in *Trypanosoma cruzi* allowed us to demonstrate that c*Tc*TXNPx is a substrate for tryparedoxin [36,37], and in this work we showed that these two proteins can form a complex *in vitro*. The presence of c*Tc*TXNPx in the eluate of proteins interacting with *Tc*TXN1 constitutes a positive control of our experimental approach. Only the cytosolic isoform was found as a partner, which is in concordance with the *Tc*TXN1 cytosolic localization determined in this work. We also detected *Tc*TXN1 in the eluate, indicating the generation of a dimer of tryparedoxins, and this observation coincides with the dimer detected in the in vitro experiments (Fig. 2). MS analysis of *Tc*TXN1 spots revealed that dimers were formed between mutated *Tc*TXN1 monomers. Taking into account that Cys40 and Cys43 are the only cysteines present on its primary structure, this result indicates that an intermolecular disulfide bridge is formed between Cys40 residues from each monomer. This makes sense if we consider that Cys40 is exposed to the solvent according to the structure of TXN1 from *C. fasciculata* [38], but *Cf*TXN1 forms an intramolecular disulfide. It should be noted that *Cf*TXN1 contains a third cysteine in position 68, with unknown function, and this is not the case for *Tc*TXN1. Western blot experiments showed the same homodimerization result: under nonreducing conditions a specific band of 30 kDa appeared in the control parasites (Fig. 3, lane 1), and this band disappeared under reducing conditions, suggesting that homodimerization of *Tc*TXN1 through a disulfide bond is not only a consequence of the mutation of Cys43. Despite the fact that thioredoxin dimerization from other organisms is emerging as a relevant event, this constitutes a non described

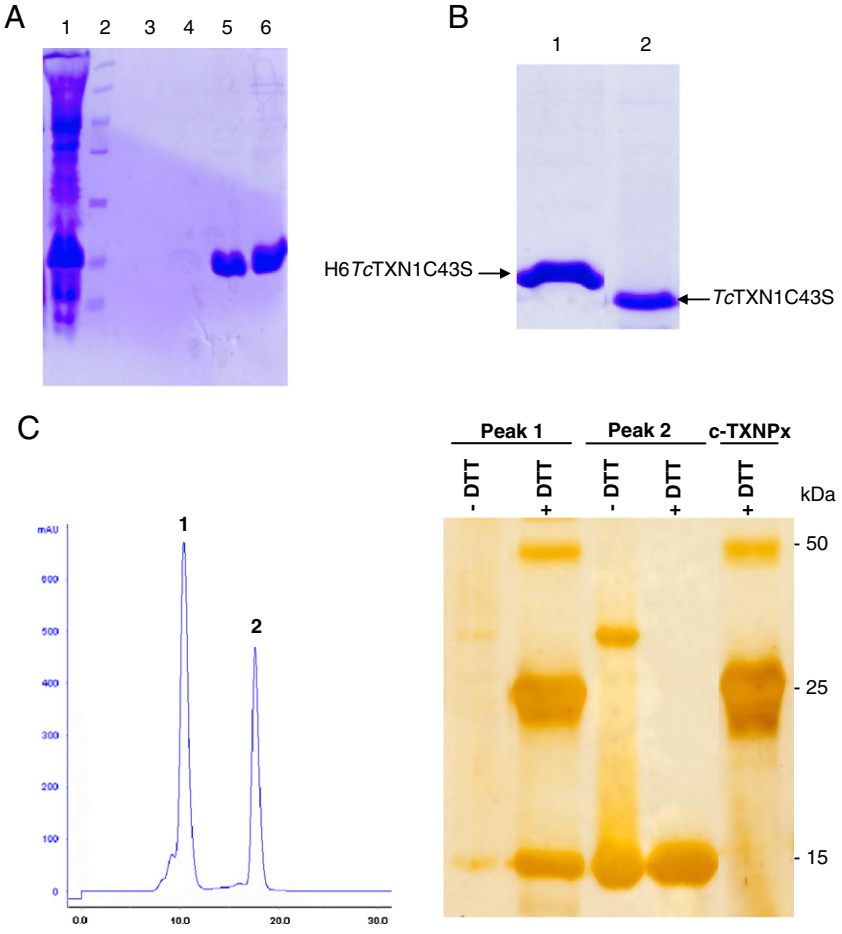

Fig. 2 – *In vitro* validation of the *Tc*TXNPx/*Tc*TXN1C43S complex. A—Purification of *Tc*TXN1C43S after affinity chromatography on His-bind columns. Lanes: 1—Total soluble fraction before purification; 2—Molecular mass markers; 3 to 6—purified elution fractions. B—Thrombin cleavage. Lanes: 1—Hexa hystidine *Tc*TXN1C43S; 2—*Tc*TXN1C43S after thrombin cleavage. C—Purification of *T. cruzi* c-TXNPx/TXN1C43S complex *in vitro* by gel filtration. Elution profile in terms of protein (E280) of covalent complex with an apparent Mr of 350,000 and excess of *Tc*TXN1C43S is completely separated. SDS-Page analysis of gel filtration purified peaks and purified c-TXNPx.

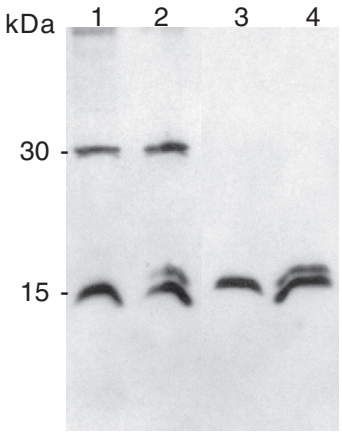

Fig. 3 – Expression of *Tc*TXN1 in transfected parasites. Western Blot analysis of *Tc*TXN1 in total protein extracts. Lanes: 1 and 3—pTEX transfected epimastigotes; 2 and 4—pTEX-HisTXN1C43S transfected epimastigotes. 1 and 2—without DTT, 3 and 4—with DTT.

phenomenon in tryparedoxins. For human thioredoxins it has been suggested that dimerization may provide a mechanism for protein regulation, or a means of sensing oxidative stress [39]. Recently, by mutating the resolving cysteine from the *B. subtilis* thioredoxin A, the formation of a stable homodimer was observed. This homodimer preserves the thioredoxin domain of each unit [40] and, interestingly, is stabilized by a disulfide bond between the N-terminal cysteines of each monomer, as occurs with *Tc*TXN1. Although our findings constitute preliminary results, we cannot discard a possible biological relevance related with tryparedoxin dimerization in trypanosomes, as is the case for some thioredoxins.

Through our *in vivo* approach we were able to pinpoint several proteins as new candidates to interact with *Tc*TXN1, among which a group of them should be emphasized, due to their relationship with polyamine precursors: cystathionine γ-lyase, methylthioadenosine phosphorylase (MTPA) and amidino transferase. Cystathionine γ-lyase is an enzyme that participates in reverse transsulfuration. This pathway has been previously described in fungi and mammals, and includes the complete process leading to cysteine from methionine [41]. In literature *T. cruzi* appears to be the first protist proven to have two independent pathways for cysteine production: sulfur-assimilation from sulfate and transsulfuration from methionine via cystathionine. Extracellular L-cysteine, but not L-cystine, has been shown to be essential for growth of the *Trypanosoma brucei* bloodstream form [42]. Since trypanosomes require cysteine not only for protein biosynthesis, but also for formation of trypanothione and glutathione, trypanosomes may require multiple cysteine-acquiring pathways to ensure this amino acid is available to fulfill the needs of their redox metabolism. Methylthioadenosine phosphorylase (MTPA) catalyzes the reversible phosphorolytic cleavage of methylthioadenosine (a by-product of the polyamine synthesis), producing methylthioribose-1-phosphate (MTRP) and adenine; MTRP is recycled to methionine and adenine is redirected to nucleoside synthesis. Since trypanosomes lack *de novo* purine biosynthetic pathways, and have high demands for S-adenosylmethionine (AdoMet), a metabolite precursor for polyamines [43], MTPA should enable the salvage of adenine and methionines. Interestingly, in mammals MTPA is redox regulated [44], as it can be inactivated by specific oxidation of two conserved cysteines to sulfenic acid. In addition, a disulfide bridge is formed between two other conserved cysteines. In *T. cruzi* MTPA these conserved cysteines are present in the sequence, constituting good candidates to be attacked by *Tc*TXN1. Finally, we want to point out that spot 17 (Table 1), although annotated as a hypothetical protein, is an amidino transferase, an enzyme that can use arginine as substrate, and may be involved in polyamine biosynthesis in trypanosomes. Again, the alignment of this protein shows a conserved cysteine (residue 187) greatly increasing the chance that this residue is the target of *Tc*TXN1. It should be pointed out that in other eukaryotic organisms thioredoxins have been described to participate in the regulation of different enzymes of the same metabolic pathways [13–17], allowing us to speculate that in trypanosomes, tryparedoxins can fulfill this role, so the relationships between *Tc*TXN1 activity and polyamines metabolism should be analyzed more in depth.

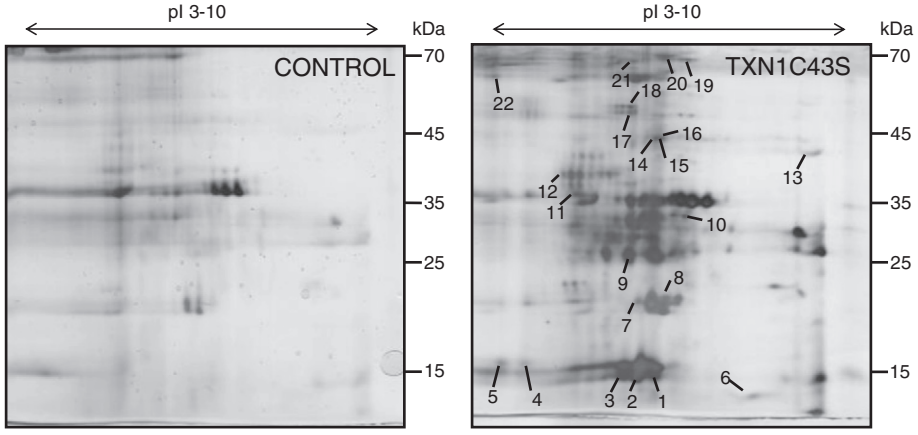

Fig. 4 – Identified proteins on a representative pH 3–10 2-D gel of TXN-complexed proteins of *T. cruzi*. Identified spots are marked. Numbered spots correspond to identified proteins listed in Table 1.

Table 1

Spot	Protein name	Accession number	Theoretical MW (kDa)	Theoretical *pI*	Sequence coverage (%)	Protein Score	Peptide Score
1	Tryparedoxin	71411917	16,027	5.27	82	127(>84)	70(>51)
2	Tryparedoxin	19171158	16,057	5.27	84	177(>84)	147(>52)
3	Tryparedoxin	71411917	16,027	5.27	82	218(>84)	147(>51)
4	Hypothetical protein	71408281	16,325	4.36	50	115(>84)	80(>51)
5	Hypothetical protein	71408281	16,325	4.36	50	115(>84)	80(>51)
6	Hypothetical protein	71407758	12,951	7.85	57	133(>84)	52(>51)
7	Eukaryotic initiation factor 4a	71666845	49,771	5.98	23	105(>84)	67(>50)
8	Eukaryotic initiation factor 4a	71666325	49,771	6.29	28	207(>84)	106(>51)
9	Tryparedoxin peroxidase	71413203	22,246	5.96	24	86(>84)	
10	Methylthioadenosine phosphorylase	71665924	33,156	6.89	28	114(>84)	62(>49)
11	Methylthioadenosine phosphorylase	71665924	33,156	6.89	35	181(>84)	141(>49)
12	Methylthioadenosine phosphorylase	71665924	33,156	6.89	20	90(>84)	62(>49)
13	Cystathione gamma lyase	71662043	44,461	7.57	24	170(>84)	123(>53)
14	Nli-interacting factor	71408266	39,739	6.61	27	156(>84)	94(>47)
15	Dtdp-glucose 4,6-dehydratase	71407377	36,624	6.27	17	90(>84)	
16	Pyruvate dehydrogenase e1 component alpha subunit	322818032	42,641	7.97	20	91(>84)	54(>47)
17	Hypothetical protein	322822648	43,143	5.82	20	85(>84)	
18	Imidazolonepropionase	71407562	46,704	7.75	3		51(>47)
19	Hypothetical protein	71656512	72,257	7.08	29	164(>84)	87(>52)
20	Hypothetical protein	71656512	72,257	7.08	44	196(>84)	
21	Ubiquitin-activating enzyme e1	71411317	114,332	5.62	1		42(>40)
22	Aminopeptidase p	71649060	57,680	5.93	11	86(>84)	

Identification of TXN-complexed proteins by MALDI-TOF/TOF MS and NCBInr database searching using Mascot. Spots were identified using PMF data in combination with MS/MS data. Scores greater than indicated in parentheses were considered significant ($p<0.05$) according to the Mascot algorithm. Spot numbers correspond to that shown in the 2D gel of Fig. 4.

A second group of partners is related to protein synthesis and degradation. The eukaryotic translation initiation factor eIF4AI is a DEAD-box RNA helicase that is a subunit of the translation initiation factor complex eIF4F, that is well characterized in *T. brucei* [45]. Here we identified the cytosolic eIF4AI but not its homologous eIF4AIII (a nuclear protein); this is to be expected due to the cytosolic location of *Tc*TXN1. The interaction of eIF4A with thioredoxin has been previously reported in *A. thaliana*, and the presence of highly conserved cysteines in this family has been pointed out [46]. In concordance with this finding, the presence of two conserved cysteines in the eIF4AI primary structure of kinetoplastids is noteworthy (residues 129 and 297 in *T. cruzi*), particularly residue 129, a highly conserved cysteine present in all homologous proteins from human to bacteria. E1 ubiquitin activating enzyme (E1), is a highly conserved small protein present in all eukaryotic cells, and it is a part of the cascade that covalently attaches ubiquitin to substrate proteins. E1 activates ubiquitin by C-terminal adenylation, and subsequently forms a highly reactive thioester bond between its catalytic cysteine and ubiquitin's C-terminus. Interestingly, the highly conserved catalytic domain presents two cysteines that are present in all members of the family, residues 453 and 599 in *T. cruzi*. Finally we highlight the finding of aminopeptidase P, a conserved and non-well characterised protease, whose conserved cysteines participate in intramolecular disulfide bridges, probably related with its function [47]. Interestingly the related protease aminopeptidase M was found as a thioredoxin interacting protein in *A. thaliana* [17]. It should be noted that the capture of partners involved in protein biosynthesis and degradation have been also widely reported in experiments with mutated thioredoxins and glutaredoxins [13,17,48] and this also seems to be the rule for tryparedoxins.

It must be pointed out that, as all the methods described for capture of partners through the generation of heterodisulfide dimers, false positives cannot be discarded. Some proteins that are in transit from cytosol to organelles could be captured by *Tc*TXN1, even if they were not natural substrates. Such seems to be the case for the mitochondrial pyruvate dehydrogenase E1 subunit. However, these results stimulate us to further investigate its possible interaction with *Tc*TXN2, the proposed mitochondrial tryparedoxin in *T. cruzi*. Possibly imidazolonepropionase represents another example of a false positive since it has been demonstrated that this protein coordinates a metal ion with its own hystidines. Moreover, since the substrate of the enzyme contains a diazol-like ring [49], it is a candidate protein to be immobilized by Co^{2+} and eluted with imidazole.

4. Conclusions

This proteomic approach reports the identification of putative partners of tryparedoxin 1 from *T. cruzi*. We demonstrate that *Tc*TXN1 is a protein expressed along the life cycle of *T. cruzi*, without significant changes on its expression, and it is located in the cytosol. In order to start unravelling the redox interactome of *T. cruzi*, and based on the mechanism of action of tryparedoxins, we propose an improved approach for capture of partners. The pipeline for this method is outlined in Fig. 5. After cloning and expression of the mutated gene, the protein is purified, and the *in vitro* formation of a complex with

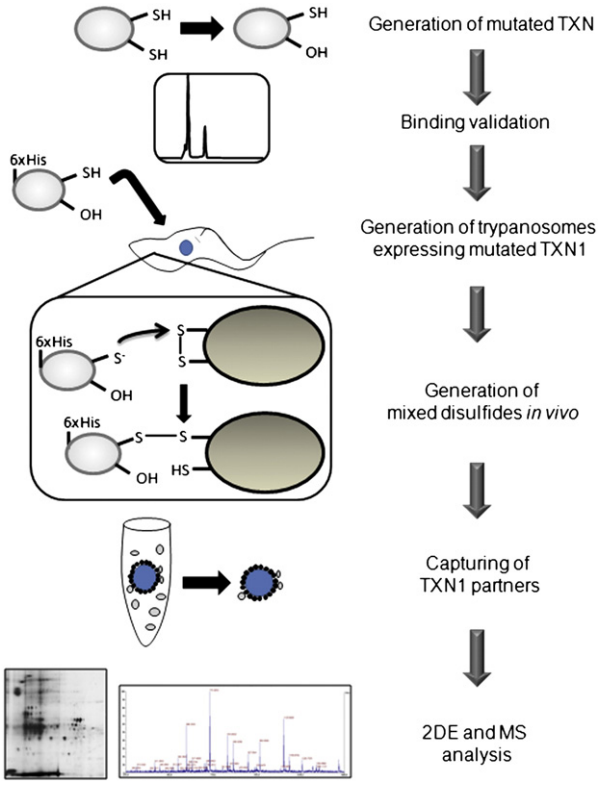

Fig. 5 – Diagram showing experimental approach undertaken in this work.

a known partner is used as a "quality control" of the experimental approach. Peroxiredoxins are good candidate partners at this point. Afterwards the gene with an additional hexahystidine tag is expressed in the cell, which implies that the hetero disulfide will be formed in a physiological environment. Finally, proteins are extracted, affinity chromatography purified and identified by 2DE separation and MS identification. By using this approach with *Tc*TXN1, twenty-two spots were found, corresponding to fifteen proteins, belonging to two main processes: oxidative metabolism, and protein metabolism (synthesis, ubiquitination, degradation). Most of the proteins are localized (or predicted to be) in the cytosol and, interestingly most of them have either homologues that have been described to interact with thioredoxin in other organisms, or highly conserved cysteines. Our approach led us to the discovery of several putatively *Tc*TXN1-interacting proteins thereby contributing to our understanding of the redox interactome in *T. cruzi*. These results give clues on the relevance of *Tc*TXN1 and lead us to consider it as a target for drug development. Further studies with immobilized protein are necessary in order to complement this *in vivo* approach.

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