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Molecular characterization and interactome analysis of *Trypanosoma cruzi* tryparedoxin II

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ABSTRACT

Trypanosoma cruzi, the causative agent of Chagas disease, possesses two tryparedoxins (*Tc*TXNI and *Tc*TXNII), belonging to the thioredoxin superfamily. TXNs are oxidoreductases which mediate electron transfer between trypanothione and peroxiredoxins. This constitutes a difference with the host cells, in which these activities are mediated by thioredoxins. These differences make TXNs an attractive target for drug development. In a previous work we characterized *Tc*TXNI, including the redox interactome. In this work we extend the study to *Tc*TXNII. We demonstrate that *Tc*TXNII is a transmembrane protein anchored to the surface of the mitochondria and endoplasmic reticulum, with a cytoplasmatic orientation of the redox domain. It would be expressed during the metacyclogenesis process. In order to continue with the characterization of the redox interactome of *T. cruzi*, we designed an active site mutant *Tc*TXNII lacking the resolving cysteine, and through the expression of this mutant protein and incubation with *T. cruzi* proteins, heterodisulfide complexes were isolated by affinity chromatography and identified by mass spectrometry. This allowed us to identify sixteen *Tc*TXNII interacting proteins, which are involved in a wide range of cellular processes, indicating the relevance of *Tc*TXNII, and contributing to our understanding of the redox interactome of *T. cruzi*.

Biological significance
T. cruzi, the causative agent of Chagas disease, constitutes a major sanitary problem in Latin America. The number of estimated infected persons is ca. 8 million, 28 million people are at risk of infection and ~20,000 deaths occur per year in endemic regions. No vaccines are available at present, and most drugs currently in use were developed decades ago and show variable efficacy with undesirable side effects. The parasite is able to live and prolipherate inside macrophage phagosomes, where it is exposed to cytotoxic reactive oxygen and nitrogen species, derived from macrophage activation. Therefore, *T. cruzi* antioxidant mechanisms constitute an active field of investigation, since they could provide the basis for a rational drug development.

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Peroxide detoxification in this parasite is achieved by ascorbate peroxidase and different thiol-dependent peroxidases. Among them, both mitochondrial and cytosolic tryparedoxin peroxidases, typical two-cysteine peroxiredoxins, were found to be important for hydrogen peroxide and peroxynitrite detoxification and their expression levels correlated with parasite infectivity and virulence. In trypanosomes tryparedoxins and not thioredoxins act as peroxiredoxin reductases, suggesting that these enzymes substitute thioredoxins in these parasites. *T. cruzi* possesses two tryparedoxin genes, TcTXNI and TcTXN II.
Since thioredoxins are proteins with several targets actively participating of complex redox networks, we have previously investigated if this is the case also for TcTXNI, for which we described relevant partners (J Proteomics. 2011;74(9):1683–92). In this manuscript we investigated the interactions of TcTXNII. We have designed an active site mutant tryparedoxin II lacking the resolving cysteine and, through the expression of this mutant protein and its incubation with *T. cruzi* proteins, hetero disulfide complexes were isolated by affinity chromatography purification and identified by electrophoresis separation and MS identification. This allowed us to identify sixteen *Tc*TXNII interacting proteins which are involved in different and relevant cellular processes. Moreover, we demonstrate that TcTXNII is a transmembrane protein anchored to the surface of the mitochondria and endoplasmic reticulum.

1. Introduction

Trypanosomatids are unicellular organisms of the order Kinetoplastida that parasitize a wide variety of invertebrate and vertebrate hosts [1]. The most relevant specimens for human and animal health belong to the genera *Trypanosoma* and *Leishmania*, which annually affect half a million people around the world (WHO; http://www.who.int/en/). *Trypanosoma cruzi* is the etiologic agent of Chagas disease, an infection that affects several million people in Latin America [2,3]. No vaccines are available at present, and currently used drugs (nifurtimox and benznidazol) are effective firstly during acute phase of the disease, and present undesirable side effects. In the last years, efforts have been focused on the characterization of proteins and pathways that constitute potential drug targets due to their absence in humans. In that sense, oxidative metabolism differs significantly between trypanosomatids and humans: *T. cruzi* lacks genes for glutathione reductases, thioredoxin reductases, catalases, and selenium dependent glutathione peroxidases, as revealed by its genome. Instead, redox metabolism is based on a low molecular mass dithiol, the trypanothione. The thiol redox homeostasis is maintained through the participation of a complex system of enzymes, most of them found exclusively in trypanosomatids [1], as is the case of tryparedoxins (TXNs). TXNs are low molecular mass dithiol proteins in trypanosomatids [1,2]. Belonging to the thioredoxin (TRX) oxidoreductase superfamily, but they represent a distinctive molecular group within the superfamily, being characterized by a WCPPC motif at their catalytic center [4,5]. Although TXNs and TRXs have the same core structure [6,7] they have low sequence similarity, which is restricted to the active site region and a few other adjacent amino acid residues, making these enzymes suitable targets of drugs. The N-terminal cysteine (Cys^{42}) of the WCPPC active site motif is exposed to the solvent and its nucleophilicity is warranted by a fast proton shuttling that involves the second cysteine (C45) and a network of uncharged internal residues [8–10]. The thiolate anion reacts with disulfides from specific proteins leading to mixed disulfides between TXN and the respective target molecule. Attack of this mixed disulfide by the vicinal Cys of TXN releases the reduced target protein and oxidizes TXN [9–11]. In *T. cruzi*, two genes coding for TXN have been identified and functionally characterized [1,12,13]. 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. This conversion activity is mainly carried out by TXNs. Recently we have described the identification of TXNI interacting proteins in *T. cruzi* using an in vivo approach. The target proteins are involved in many processes, including oxidative stress response (e.g. peroxiredoxins) and protein synthesis (e.g. several elongation factors), among others.

We focused this work on TXN II: first we show new insights into the function and subcellular localization of the enzyme and, in order to extend our knowledge of its physiological significance in the parasitic cell, a proteomic approach is presented, in order to determine target proteins of *T. cruzi* TXNII (TXNII interactome). We performed an affinity chromatography method using an active site mutant of *Tc*TXNII as bait. This mutated *Tc*TXNII contains a substitution in its resolving cysteine. Complexes between mutant TXNII and targets were affinity purified and analyzed by one dimensional electrophoresis and mass spectrometry (MALDI TOF–TOF). Our approach led us to the identification of some potential TXNII binding partners.

2. Materials and methods

2.1. Materials

Bacteriological media were purchased from Britania Laboratories (Argentina), *Taq* DNA polymerase and restriction enzymes were purchased from Promega (Argentina), while CNBr-activated Sepharose 4B was acquired from GE-Healthcare (Argentina). All other reagents and chemicals were of the highest quality commercially available from Sigma-Aldrich (Argentina) or similar.

2.2. Bacteria and plasmids

Escherichia coli TOP10 F′ and *E. coli* BL21 (DE3) cells (Invitrogen, Argentina) were utilized in routine plasmid construction and

protein expression assays, respectively. The vector pET-28c (Novagen) was used as the expression vector. DNA manipulation, *E. coli* culture, and transformation procedures were performed according to standard protocols [14].

2.3. Generation of TcTXNIIC45S by site-directed mutagenesis, overexpression and purification of recombinant protein

*Tc*TXNIIC45SΔ22C were 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: TXNIIC45Sforw: 5′-GTGTCCTCCGTCTCGATT TTTC-3′ and TXNIIC45Srev: 5′-GAAAAATCGAGACGGAGGAC AC-3. PCR reaction (50 μl) was performed using as a template 10 ng of purified pET28c/*Tc*TXNIIΔ22C plasmid 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. Fidelity and correctness of the gen was confirmed on both strands by complete sequencing (Macrogen, South Korea). The recombinant protein was obtained as N-terminal hexahistidine-tag protein from *E. coli* BL21 (DE3) culture and purified in a Ni^{2+}–Hi-Trap resin column under identical conditions as described by Arias and co-workers [15]. Pure protein was stored at −80 °C in 20 mM Tris–HCl, pH 7.5; and 1 mM EDTA and 5% (v/v) glycerol.

2.4. Parasite strain, culture procedure and preparation of parasite cell extract

*T. cruzi Dm*28c epimastigote cells were cultivated axenically at 28 °C in LIT media supplemented with 10% (w/v) bovine fetal serum and 20 $\mu g \cdot ml^{-1}$ hemin, as previously reported [16]. *T. cruzi Dm*28c metacyclogenesis was performed by treating epimastigotes in TAU3AAG medium as previously described [17].

For preparing the parasite cell extract, the cellular pellet (~10^9 parasites) was diluted in 1.5 ml of buffer containing 100 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 2 mM EDTA. Parasites were disrupted by four cycles of freezing and thawing in a water bath at room temperature followed by sonication at 4 °C. After centrifugation at 18,000 *g* for 30 min at 4 °C, the obtained supernatant was used as cell lysate for affinity chromatography column.

2.5. Preparation of TcTXNIIC45SΔ22C-Sepharose 4B resin

About 1 mg of the pure mutant *Tc*TXNIIC45SΔ22C supernatant in coupling buffer (100 mM sodium carbonate, 500 mM NaCl, pH 8.3) was incubated for 1 h at room temperature under gentle agitation with 4 ml CNBr-activated Sepharose 4B resin, which had been swelled in 1 mM HCl according to the manufacturer's instructions. After termination of the coupling reaction by centrifugation and washing of the resin with coupling buffer, unmodified reactive groups of the resin were blocked by incubation with 1 M ethanolamine, pH 8.0 for 4 h at room temperature. Then, the resin was washed with 10 volume of acetate buffer (0.1 M sodium acetate, 0.5 M NaCl pH 4.0) and subsequently with 10 volume of coupling buffer.

2.6. Capturing and identification of the target proteins by immobilized TcTXNIIC45SΔ22C

T. cruzi cell lysate was prepared from 10^9 epimastigotes (suspended in 100 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA) by freeze–thaw (four cycles) and sonication. Then, the soluble fraction was obtained by centrifugation at 18,000 ×*g* for 30 min at 4 °C. The soluble extract (1 ml) containing 20 mg of total soluble proteins was incubated with 1 ml of the *Tc*TXNIIC45SΔ22C-Sepharose 4B resin at room temperature for at least 1 h under gentle stirring. The column was then extensively washed with 100 mM Tris–HCl, pH 7.5, 500 mM NaCl, and 2 mM EDTA to remove non-specifically bound proteins. Subsequently, the potential target proteins linked by the newly formed heterodisulfide were eluted by incubation with a buffer containing 50 mM DTT for 30 min at room temperature. The DTT-eluted fractions with protein were concentrated and separated by SDS-PAGE. The bands of interest were excised, and then subjected to tryptic digestion and MALDI–TOF analysis as described below. A negative control pull down experiment was performed with CNBr-activated Sepharose 4B resin without immobilized protein was used to study unspecific binding of proteins. This control experiment was carried out in analogy to the original pull down experiment. Peptide mass fingerprinting of selected protein bands was carried out by in–gel digestion and MALDI–TOF–TOF MS as previously described [18]. 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 was set as possible modifications and one missed tryptic cleavage was allowed.

2.7. Immunolocalization by confocal microscopy

Epimastigote cells of *T. cruzi*, obtained from axenic culture, were washed twice for 15 min at room temperature in a phosphate buffered saline solution (PBS; 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, 0.24 g/l KH_2PO_4, pH 7.4) and fixed in 4% (v/v) formaldehyde. After washing they were permeabilized and blocked during 30 min in a medium containing PBS plus the addition of 0.1% (v/v) Triton X-100 and 3% (w/v) BSA. Fixed samples were incubated first with specific polyclonal antibodies (1/100 dilution) and second with FITC-conjugated goat anti-rabbit antibody (Life Technologies) and TRITC-conjugated goat anti-mouse antibody (both 1/1000 dilution). Incubations with the primary and secondary antibodies were performed at 25 °C for 1 h. Finally, after washes slides were mounted with antifade mounting solution plus DAPI and visualized under a confocal microscope (DIC/Nomarski, Eclipse TE-2000-E2—Nikon).

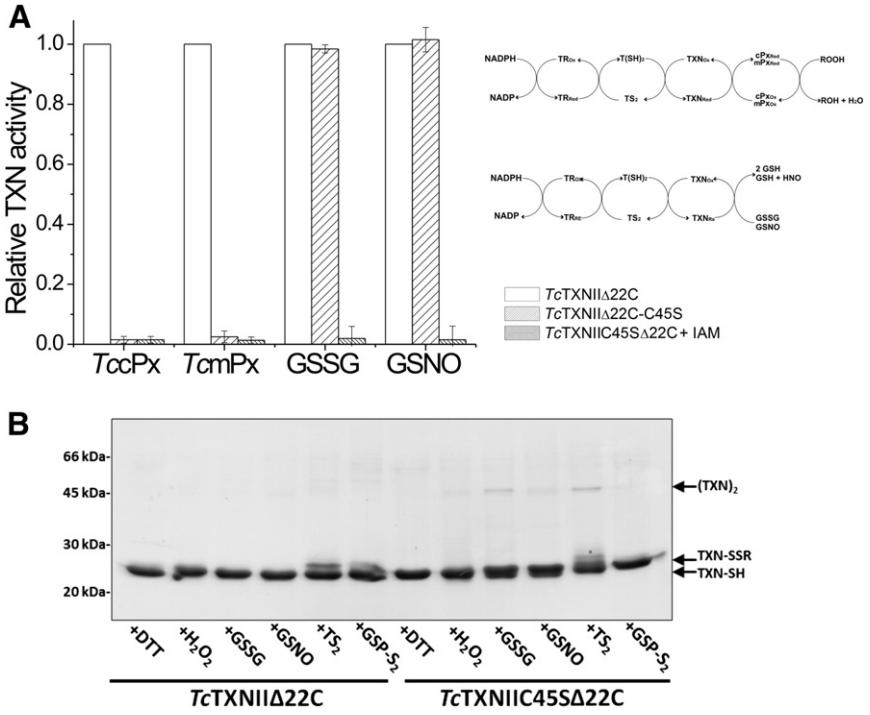

Fig. 1 – A) Trypanothione-dependent disulfide reductase activity of *Tc*TXNIIΔ22C and *Tc*TXNIIC45SΔ22C. The reactions were performed in 100 mM TRIS–HCl, pH 7.5, 2 mM EDTA, 300 μM NADPH, 1 μM *Tc*rTR, 100 μM TS_2, 1.5–10 μM *Tc*TXNIIΔ22C or *Tc*TXNIIC45SΔ22C and 0.26 μM *Tc*cTXNPx (plus 70 μM *t*-bOOH), 1 μM *Tc*mTXNPx (plus 70 μM *t*-bOOH), 1 mM GSSG or 1 mM GSNO, at 30 °C. As negative control, alkylated *Tc*TXNIIC45SΔ22C (with iodoacetamide, IAM) was used. B) Sensitivity of *Tc*TXNIIΔ22C and *Tc*TXNIIC45SΔ22C to oxidative treatments. Pre-reduced TXNs (5 μM) were subjected to 15 min oxidative treatments with different physiological reagents (1 mM final concentration) at 30 °C. After incubation, the proteins were resolved by non-reducing SDS-PAGE.

2.8. Subcellular fractionation of *T. cruzi* epimastigote

T. cruzi epimastigotes ($5{\cdot}10^8$ cells) were pelleted and suspended in 1 ml of 10 mM Tris–HCl, pH 7.5, and 1 mM EDTA (plus cocktail of protease inhibitors) and disrupted under hypotonic conditions by passage through a narrow hypodermic needle. The lysate was fractionated by serial differential centrifugation (from 500 ×*g* to 18,000 ×*g*) for 30 min at 4 °C. The last soluble fraction was treated with 8 mM $CaCl_2$ (for vesicles and microsomes precipitation [19]) and newly centrifuged for 30 min at 18,000 ×*g*. The obtained pellets (each centrifugation step) were mixed with SDS-PAGE sample buffer, boiled for 5 min and stored at −20 °C. Aliquots were analyzed by western blot using rabbit polyclonal antisera against *Tc*TXNII, *Tc*TXNI, *Tcm*TXNPx, *Tcc*TXNPx, *Tc*GlcK, *Tc*Cruzipain and *Tc*APx. HRP-conjugated anti-rabbit antibodies (Life Technologies) were employed as secondary antibody. Bands were visualized using the ECL Western blotting detections reagents (Thermo Scientific).

3. Results and discussion

3.1. The Cys^{45} of *Tc*TXNII is essential to protein disulfide reduction, but not for low molecular mass disulfide reduction

As previously described [9–11], the thiolate anion of N-terminal Cys reacts with disulfides from specific proteins. This event leads to mixed disulfides between TXN and the respective target molecule. Then, the attack of this mixed disulfide by the vicinal Cys (C-terminal, Cys^{45} of *Tc*TXNII) of TXN releases the reduced target protein and oxidizes TXN to disulfide. We tested the ability of *Tc*TXNIIC45SΔ22C to catalyze the reduction of *T. cruzi* peroxiredoxins (cTXNPx or mTXNPx) by $T(SH)_2$. This was evaluated by the coupled assay for trypanothione-dependent *t*-bOOH reduction via a tryparedoxin/peroxidase-mediated reaction [15]. As positive control, *Tc*TXNIIΔ22C was used in equivalent conditions. Fig. 1-A shows that *Tc*TXNIIΔ22CC45S was not active for transferring reducing equivalents to cytoplasmatic (*Tcc*TXNPx) or mitochondrial (*Tcm*TXNPx) 2-Cys peroxiredoxins. However, when 2-Cys peroxiredoxins were replaced by GSSG or GSNO as final acceptor in the coupled assay, *Tc*TXNIIC45SΔ22C exhibited identical catalytic efficiency to *Tc*TXNIIΔ22C. These results indicate that reduced *Tc*TXNIIC45SΔ22C [plus $T(SH)_2$] exhibited a capacity to reduce low molecular mass disulfides via monothiol mechanism (similar to monothiol glutaredoxins [15,20,21]). This result was consistent with the formation of adducts between low molecular mass disulfides with monothiolic TXNII version (*Tc*TXNIIC45SΔ22C, Fig. 1-B). In addition, the mutant TXNII showed a slight tendency to form covalent dimers in the presence of physiologic oxidant substrates, compared to the wild type version (Fig. 1-B). These results indicate the essential role of C-terminal cysteine (Cys^{45} in *Tc*TXNII) in the resolution of protein mixed disulfides, which correlates with previously published data on the catalytic mechanism of TXNs [15,18,22].

3.2. *TcTXNII is a transmembrane protein anchored to the surface of the mitochondria and endoplasmic reticulum*

As previously described [15], *Tc*TXNII is an integral membrane protein exhibiting its redox domain with cytoplasmatic orientation. Subcellular localization of *Tc*TXNII was studied by cellular fractionation of epimastigote cells followed by western blot. As show Fig. 2, *Tc*TXNII is co-detected together with *Tc*mTXNPx (mitochondria marker) and *Tc*APx (endoplasmatic reticulum marker) proteins. Conversely, *Tc*TXNII was not detected in the same fraction that *Tc*TXNI (soluble citoplasmatic marker), as previously reported [18]. Additionally, subcellular localization of *Tc*TXNII was further analyzed by confocal immunofluorescence microscopy of epimastigote cells (using the same antibodies, Fig. 3). The obtained images revealed a pattern of recognition signals compatible with a co-localization with *Tc*mTXNPx, *Tc*APx and *Tcc*TXNPx (cytoplasm marker). The latter is consistent with previously published data [15] where the protein has a membrane topology oriented toward the cytoplasm. Our results suggest that the cellular locations of *Tc*TXNII are endo-membrane systems such as mitochondrial outer membrane and endoplasmatic reticulum. Similar results were previously reported for *Leishmania infantum* TXNIII, which exhibited an external mitochondrial membrane localization [23].

3.3. *Tryparedoxins are present during the metacyclogenesis process*

As previously described, *Tc*TXNs were found to be expressed in epimastigotes, cell derived trypomastigotes and intracellular amastigotes from *T. cruzi* [15,18]. Since a number of changes occur in protein patterns during metacyclogenesis we analyzed this process more in detail. The expression of *Tc*TXNs during the metacyclogenesis of *T. cruzi* epimastigote cells (in TAU3AAG media) were analyzed by western blot using total extracts from the different stages of the process. As shown in Fig. 4, we observed that the levels of both TXNs decrease at the beginning of the metacyclogenesis process (24 h), reaching similar levels to the initial at 72 h. A similar profile was observed for *Tc*mTXNPx. Conversely, *Tcc*TXNPx levels were similar in almost the entire process. Toward the end of the process (168 h) the levels of the proteins evaluated decreased significantly. In addition, when analyzed subcellular localization of *Tc*TXNII in metacyclic trypomastigotes, a similar pattern as that of epimastigotes was observed, exhibiting a vesicular distribution (Fig. 4).

3.4. *Identification of protein substrates for TcTXNII*

In order to isolate the putative protein substrates for *Tc*TXNII (via disulfide intermediates of TXNs), protein cellular extract of *T. cruzi* epimastigotes was applied on *Tc*TXNIIC45S Δ22C-Sepharose 4B column for isolating *Tc*TXNII-interacting proteins, under non-denaturing conditions. Eluted proteins were separated by reducing SDS-PAGE, and differential bands (versus control column) were selected (Fig. 5) for mass spectrometry identification using MASCOT software and the parameters described in Materials and methods section. In addition, we performed other in vitro experimental control. In this case, the Cys residue from active site of *Tc*TXNIIC45SΔ22C (the only Cys in the protein) was blocked with iodoacetamide, generating inactive bait. This inactive protein was used as negative control for pulldown experiments. We observed a great difference in the number and abundance of captured proteins (using the active bait) with respect to this negative control (data not shown).

The pulldown experiments allowed us to successfully identify sixteen protein bands listed in Table 1. Interestingly,

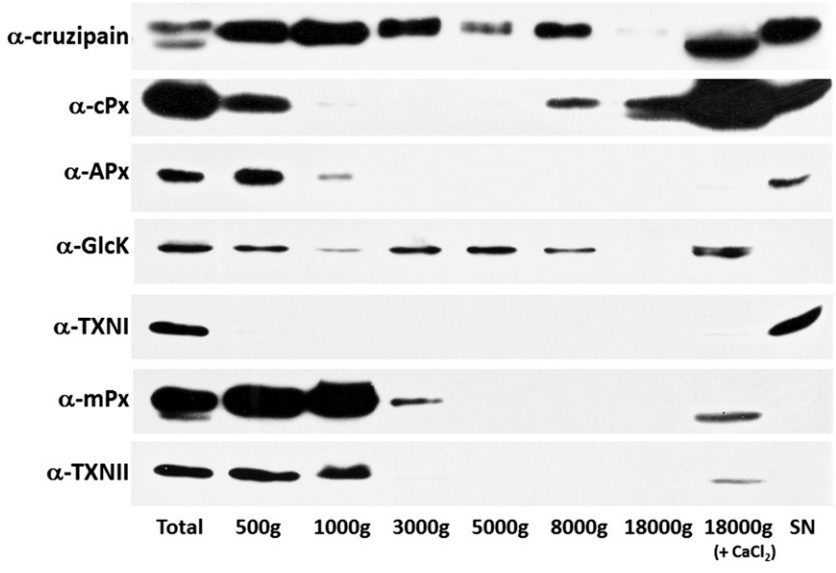

Fig. 2 – Subcellular fractionation of *T. cruzi* epimastigote cells. Epimastigote cells were treated as described in Materials and methods section. The supernatants were analyzed by western blot using specific polyclonal antibodies against different cellular targets: *Tc*TXNII (diluted 1/1000), *Tc*TXNI (diluted 1/5000), *Tc*mTXNPx (diluted 1/5000), *Tcc*TXNPx (diluted 1/10,000) *Tc*GlcK (diluted 1/1000), *Tc*Cruzipain (diluted 1/10,000) or *Tc*APx (diluted 1/10,000). HRP-conjugated anti-rabbit was employed as a secondary antibody. Bands were visualized using the ECL Western blotting detections reagents.

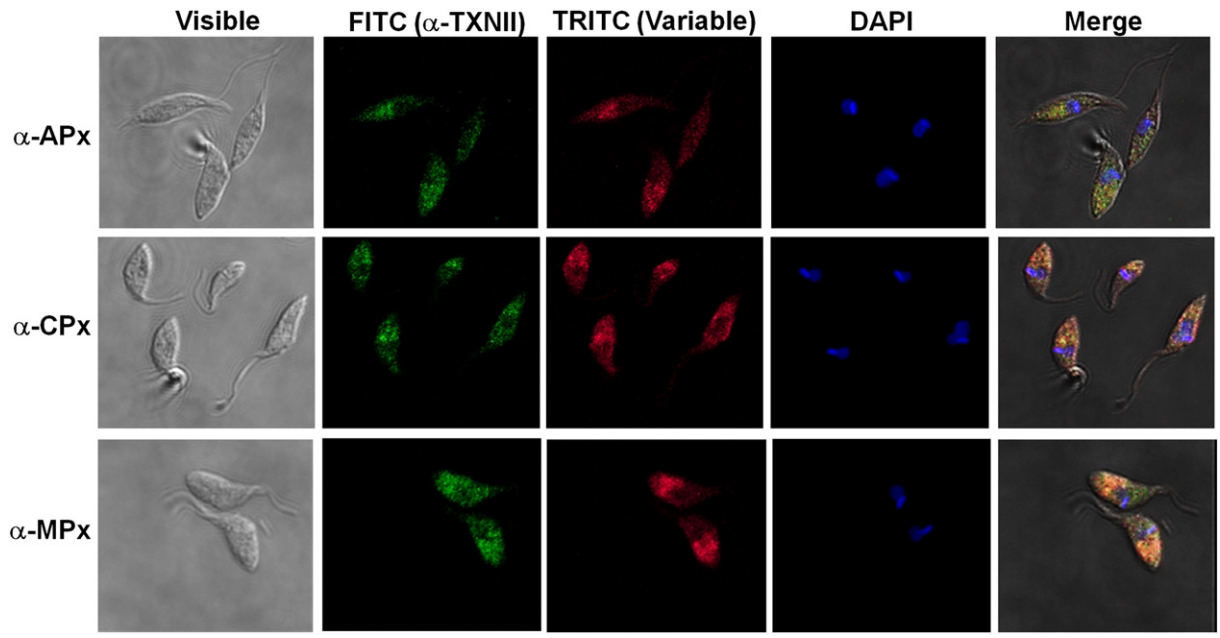

Fig. 3 – Confocal microscopy images of *T. cruzi* epimastigote cells. Parasites were stained with DAPI for visualization of nucleus and kinetoplast (blue) and labeled with rabbit anti-*Tc*TXNII antibody and FITC conjugated goat anti-IgG as a secondary antibody (green). Different known localization cellular targets were used as cellular markers: *Tcc*TXNPx (cytoplasm), *Tcm*TXNPx (mitochondria) or *Tc*APx (endoplasmatic reticulum), labeled with specific mousse antibody and TRIC conjugated goat anti-IgG as a secondary antibody (red).

most of them belonged to pathways related to antioxidant system, energy metabolism, cytoskeleton, and protein translation. Previously, we demonstrated that *Tcc*TXNPx and *Tcm*TXNPx (two isoforms of 2-Cys peroxiredoxin) are substrates for *Tc*TXNII [15], and in this work we show that these two proteins were present in the eluate of proteins interacting with *Tc*TXNII. Both peroxiredoxin isoforms were found as partners, which is in concordance with the *Tc*TXNII localization. This result constitutes a validation of our experimental approach. In addition, new candidates to interact with

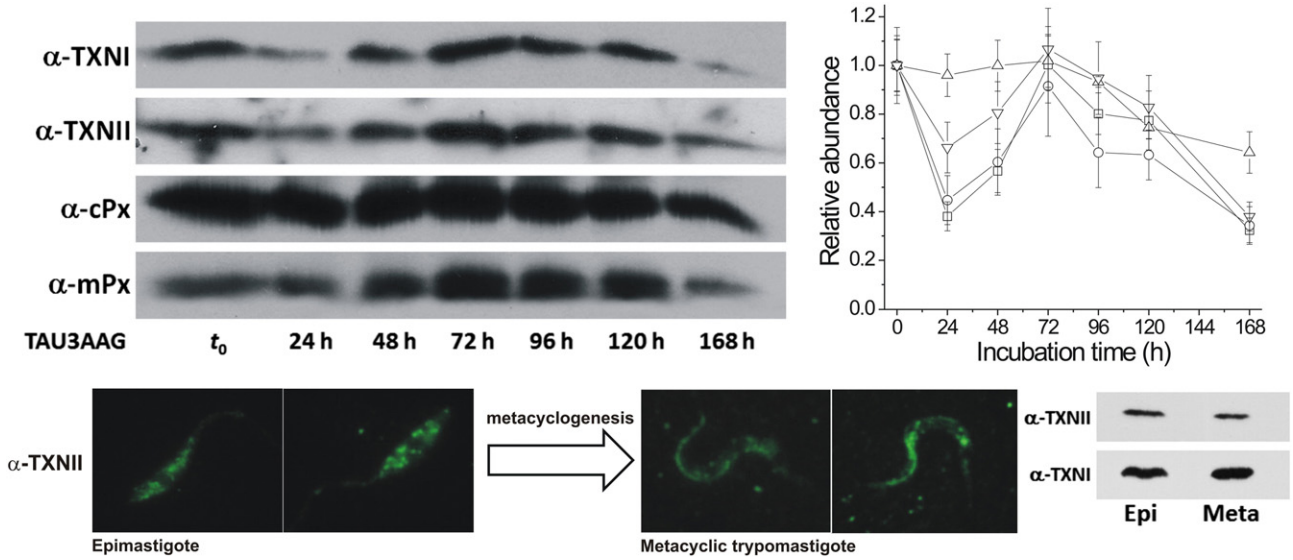

Fig. 4 – Tryparedoxins are present during the metacyclogenesis process. Relative expressions of *Tc*TXNs during metacyclogenesis were estimated by western blot and densitometry. Western blot experiments were performed using 30 μg of *T. cruzi* crude extract from each incubation time. The relative intensities (to initial time, t_0) of *Tc*TXNII, *Tc*TXNI, *Tcc*TXNPx and *Tcm*TXNPx in western blot were represented in the graphic. Indirect immunofluorescence of epimastigotes or metacyclic trypomastigotes, stained with DAPI and labeled with anti-*Tc*TXNII purified antibody. Comparative TXN western blots from 10^7 epimastigote (Epi) or metacyclic trypomastigote (Meta) total cell extracts were performed using rabbit anti-*Tc*TXNII or anti-*Tc*TXNI antibodies.

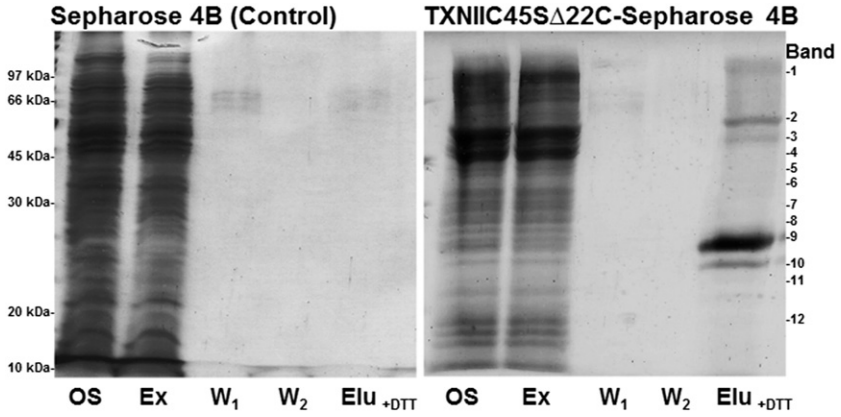

Fig. 5 – SDS-PAGE profile of the captured proteins by *Tc*TXNIIC45SΔ22C affinity chromatography. *Tc*TXNIIC45SΔ22C was immobilized on CNBr-activated Sepharose 4B resin before incubating the column with 20 mg of *T. cruzi* epimastigote soluble cell lysate and extensive washing steps with NaCl-containing buffer. Target proteins were eluted with 50 mM DTT. The obtained protein samples were separated on reducing SDS-PAGE, and protein bands were identified after tryptic digestion by MALDI–TOF analysis. The isolated proteins are indicated in the figure and enlisted in Table 1. As negative control, experiment was performed with CNBr-activated Sepharose 4B resin without immobilized *Tc*TXNIIC45SΔ22C.

*Tc*TXNII were found. The F0F1-type ATP synthase is a type of ATPase found in bacterial plasma membranes, in mitochondrial inner membranes, and in chloroplast thylakoid membranes. The activation of the ATP synthase due to the redoxin-dependent reduction was studied in depth in several organisms [24]. So far, there is little information about this type of regulation in *T. cruzi*. The amidinotransferase (or L-arginine:glycine amidinotransferase) is an enzyme that catalyzes the transfer of an amidino group from L-arginine to glycine, to produce L-ornithine and glycocyamine. The latter, is an immediate precursor of creatine [25] and the formation of glycocyamine is the rate-limiting step of creatine biosynthesis [25]. The amino acid sequence of this protein shows nine cysteine residues that increase the chance that this protein is a target of *Tc*TXNII. 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 [26–28]. Interestingly, this protein has been identified as interactor of TXNI [18], suggesting a possible redox regulation of amidinotransferase of *T. cruzi*. Other captured target was the S-adenosyl-L-homocysteine hydrolase (SAHH), an enzyme of the activated methyl cycle, responsible for the reversible hydration of S-adenosyl-L-homocysteine into adenosine and homocysteine [29]. Consequently, SAHH plays a critical role in maintaining normal levels of S-adenosyl-L-homocysteine in the cell. Inhibition of SAHH results in cellular accumulation of S-adenosyl-L-homocysteine and inhibition of adenosyl-methionine-dependent methyltransferases. Since methylation is important in a wide range of cellular processes, SAHH has been proposed as a drug target for parasitic diseases including malaria, leishmaniasis and trypanosomiasis [30]. In *Plasmodium falciparum*, this enzyme has been identified to interact with thioredoxin, glutaredoxin and plasmoredoxin [28,31]. In *T. cruzi*, the enzyme has conserved cysteine in its amino acid sequence,

Table 1 – Potential *Tc*TXNII target proteins identified in *T. cruzi* epimastigotes.

Protein name	Protein ID	Theoretical MW (kDa)	N° Cys	Sequence coverage (%)	Protein score	Band
Hypothetical protein (Radial spoke)	gi:70878892	37.7	5	32	159	5
Beta tubulin 1.9	gi:18568141	49.7	12	29	202	2
Porin 3	gi:70864370	29.6	9	3	63	6
Alpha-tubulin	gi:1314208	48.9	11	10	208	2
Hypothetical protein TCSYLVIO_6332 (F0F1-type ATP synthase, delta subunit)	gi:322821007	28.6	1	26	138	7
Cytoplasmic tryparedoxin peroxidase	gi:71396508	22.4	7	61	497	9
Mitochondrial tryparedoxin peroxidase	gi:70870571	25.5	4	6	110	9
Tryparedoxin II	gi:70872315	21.9	2	25	258	10
Histone H2A	gi:1781355	14.3	0	7	61	12
Elongation factor 2	gi:205278864	94.1	18	26	153	1
I/6 autoantigen putative	gi:407859521	23.0	4	21	101	11
Amidino transferase	gi:407851021	43.1	9	41	125	4
S-adenosylhomocysteine hydrolase	gi:70873492	48.4	11	3	70	3
D-isomer specific 2-hydroxyacid dehydrogenase-protein	gi:70876009	38.5	5	33	87	5
Epsilon-tubulin	gi:407398727	27.9	5	48	151	8
Hypothetical protein TCSYLVIO_000159	gi:407867802	34.2	5	14	129	5

constituting good candidates to be attacked by *Tc*TXNII. Another captured protein was the D-isomer specific 2-hydroxyacid dehydrogenase (or R-lactate dehydrogenase). This enzyme catalyzes the NAD-dependent oxidation of *R*-lactate to pyruvate and is part of the detoxification pathway of methylglyoxal (dependent of glyoxalase I, glyoxalase II and low molecular mass thiols) in trypanosomatids [32]. In a previous study the enzymes form *Trypanosoma conorhini* and *Crithidia fasciculata* have been characterized, and it was observed that the enzymatic activity of this protein was activated in the presence of cysteine [33]. These previous results and those obtained here strongly suggest a redox regulation of this enzyme in trypanosomatids.

A group of TXNII partners related to cytoskeleton were identified: α-, β-, ε-tubulin, I/6 autoantigen (an internally repetitive cytoskeletal protein [34]) and radial spoke protein. In the case of tubulin, it has been observed that the assembly and polymerization of this protein depend on redox changes [35–37]. For example, in mammalian microtubule [38], cysteine oxidation to disulfides altered the ability of the proteins to promote the assembly of microtubules from tubulin. The treatment with reducing molecule (such as thioredoxin) fully restores the ability to promote microtubule assembly [38]. So far, in trypanosomes is unclear the dependence of redox state and polymerization (and assembly) of tubulin. On the other hand, the radial spoke is a multi-unit protein structure found in the axonemes of eukaryotic cilia and flagella [39]. The flagellum of *Trypanosoma* is a multifunctional organelle with critical roles in motility and other aspects of the trypanosome life cycle [40]. In *Trypanosoma brucei*, RNA interference to ablate expression of radial spoke protein and central pair components presented the first evidence that flagellar beating is important for cell division (cytokinesis) and proposed the drive flagellar beat as drug targets for the treatment of African sleeping sickness [40]. So far, in trypanosomes is unclear the dependence of redox state and polymerization (and assembly) of tubulin on the function of flagellar proteins.

A component of the protein biosynthesis was identified as a potential target. Here we identified the eukaryotic elongation factor 2 (eEF2) as a target of *Tc*TXNII. The eEF2 catalyzes the translocation of tRNA and mRNA down the ribosome at the end of each round of polypeptide elongation [41]. Previous studies reported that oxidative stress inhibits protein synthesis at the elongation step in mammals [42]. Moreover the elongation factor in chloroplasts has been captured by the chloroplast Trx [43]. The interaction of eEF2 with thioredoxin has also been reported in *Arabidopsis thaliana* [44], *Dictyostelium discoideum* [45], *Entamoeba histolytica* [46], *E. coli* thioredoxin [47] and *P. falciparum* [31]. Finally, it was observed a regulation of translation by the redox state of the elongation factor in *Synechocystis* sp. [48]. Taken together these reports indicate a strong relationship between elongation of peptides and redox state, with a relevant participation of thioredoxins. In concordance with this finding, *T. cruzi* eEF2 present eighteen highly conserved cysteine residues in its primary structure; this would allow interaction via disulfide bridges TXNII. Interestingly, in a previous report, no interaction was observed between TXNI and eEF2. The redox interaction was only detected with the eukaryotic initiation factor 4a [18] which strongly suggests a possible specific interaction of TXNII with eEF2.

A protein target of *Tc*TXNII that was interesting due to the cellular localization which might occur is porin 3, a voltage-dependent anion channel of the outer mitochondrial membrane. The voltage-dependent anion channel regulates the flux of mostly anionic metabolites through the outer mitochondrial membrane [49]. The *T. cruzi* protein has high identity (98%) with the protein from *T. brucei*, which is the main metabolite transporter in the outer mitochondrial membrane in this organism [49]. The single *T. brucei* mitochondrial porin is essential only under growth conditions that depend on oxidative phosphorylation [49]. In plants, it has been shown that thioredoxin interacts with mitochondrial porin via thiol/disulfide exchange reaction [24]. In addition, in mammalian cells, the voltage sensor of the mitochondrial permeability transition pore is tuned by the oxidation-reduction state of vicinal thiols (increase of the gating potential by oxidants and its reversal by reducing agents) [50]. This result was extremely interesting given the cellular location of *Tc*TXNII on the outer mitochondrial membrane.

A hypothetical protein (TCSYLVIO_000159) was isolate by this methodology as TXNII-partner. Although we could not identify conservation in this protein, it has four Cys residues, of which the residue Cys^{202} is conserved in all trypanosomatids (data not shown). Future experiments will be needed to determine the function of this protein.

Other unrelated protein that was a target of *Tc*TXNII was the histone H2A. It must be pointed out that, despite having used a method based on capture of partners through the generation of heterodisulfide dimers, false positives cannot be discarded. That seems to be the case for the histone H2A, which has no cysteine residues, and so its capture by affinity chromatography could be due to co-purification with any of the other targets isolated. However, these results stimulate us to further investigations.

4. Conclusions

In trypanosomatids, TXNs transfer reducing equivalents from trypanothione to redox pathways involving thiol/disulfide exchange [4,9]. Herein, we analyze the expression and cellular localization of *Tc*TXNII. By means of specific antibodies designed against *Tc*TXNII, we evidenced the occurrence of this protein through of metacyclogenesis process of *T. cruzi* epimastigotes. The protein showed cellular co-localization with *Tc*mTXNPx (mitochondria marker) and *Tc*APx (endoplasmatic reticulum marker) proteins. In addition, the identity of *Tc*TXNII as a true integral membrane protein with a cytoplasmatic orientation of the redox domain was previously demonstrated [15]. With this previously data, together with the results here obtained, we may conclude that *Tc*TXNII is a transmembrane protein anchored to the surface of the mitochondria and endoplasmic reticulum.

In order to deepen the studies about the redox interactome of *T. cruzi* TXNs, an in vitro proteomic approach allowed us to identify putative partners of TXNII in this parasite. After cloning and expression the *Tc*TXNIIC45SΔ22C, the observed in vitro redox activity indicated the incapacity of mutant protein to catalyze the thiol/disulfide exchange with protein substrates. This result validates the use of this protein for the preparation of the affinity column for isolation of potential

interactors. After performing affinity chromatography, purified proteins were isolated and identified by SDS-PAGE and MS. By using this approach with *Tc*TXNII, sixteen putative partner proteins were identified, belonging to four main processes: antioxidant system, energy metabolism, cytoskeleton, and protein translation. 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 TRX in other organisms. This in vitro approach led us to the discovery of several putatively *Tc*TXNII-interacting proteins thereby contributing to our understanding of the redox interactome in *T. cruzi*. The involvement of TXNs in several parasite physiological processes suggests novel insights about the protein involvement in redox signaling.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.03.001.

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