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On the occurrence of thioredoxin in *Trypanosoma cruzi*☆

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Abstract

The full coding sequence for thioredoxin from *Trypanosoma cruzi* (TcTRX) strain Tulahuen O has been cloned into the pRSETA vector. The protein was expressed in *Escherichia coli* with an N-terminal extension of six histidine residues for purification through metal ion chromatography. The biological activity of recombinant TcTRX was proved utilizing the insulin reduction assay. Amino acid sequence alignment indicates a high identity of TcTRX with thioredoxins from different sources. Immunocytochemistry assays showed that TcTRX is present in epimastigote forms of *T. cruzi*, thus, indicating that the gene is expressed in vivo, rather than being a pseudogene. The in vivo occurrence of TcTRX points out the necessity of considering this protein as a molecular component of the redox metabolism in trypanosomatids.

Keywords: *Trypanosoma cruzi*; Trypanosomatids; Redox metabolism; Thioredoxin

1. Introduction

Trypanosoma cruzi is the causative agent of Chagas' disease. As aerobic parasites, all trypanosomatids, are inevitably exposed to several reactive oxygen species (ROS), namely superoxide anions, hydrogen peroxide, and myeloperoxidase products. These chemical species are generated during the host defense reaction and also as byproducts of the aerobic metabolism. The ability of trypanosomatids to cope with such oxidative stress conditions appears oddly weak. Trypanosomatids possess an iron-containing superoxide dismutase (Le Trant et al., 1983) to scavenge phagocyte-derived superoxide anions, whereas they lack catalase and their glutathione peroxidase-like system exhibits low efficiency (Hillebrand et al., 2003; Schlecker et al., 2005). Catalase- and selenocysteine-containing glutathione peroxidases are the major hydroperoxide metabolizing enzymes in host organisms (Chance et al., 1979; Mezzetti et al., 1990).

In members of the family Trypanosomatidae, peroxide metabolism mainly involves a glutathionyl derivative of spermidine, trypanothione [N^1,N^8-bis (glutathionyl)-

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spermidine, $T(SH)_2$]. A system involving three distinctive oxidoreductases is able to catalyze the $T(SH)_2$-dependent hydroperoxide removal. These enzymes are trypanothione reductase (TR), homologous to glutathione reductase (Fairlamb and Cerami, 1992; Krauth-Siegel et al., 1987); a thioredoxin-related protein called tryparedoxin (TXN); tryparedoxin peroxidases (TXNPx), peroxiredoxin-type, or glutathione peroxidase-type proteins (Hillebrand et al., 2003; Nogoceke et al., 1997; Reckenfelderbaumer et al., 2000; Wilkinson et al., 2000a,b). It has been proposed that in trypanosomatids these redox components operate instead of the specific thioredoxin (TRX)-thioredoxin reductase (TRXR) system, found in different organisms (Fairlamb and Cerami, 1992). In fact, TRXR seems to be absent in these microorganisms (Hirt et al., 2002; Muller et al., 2003).

Classical TRXs have been characterized in several parasites: *Plasmodium falciparum* (Kanzok et al., 2002); *Fasciola hepatica* (Salazar-Calderon et al., 2001); *Schistosoma mansoni* (Alger et al., 2002). Also, TRX was identified and fully characterized in *Trypanosoma brucei brucei* (Reckenfelderbaumer et al., 2000; Schmidt et al., 2002; Schmidt and Krauth-Siegel, 2003). The presence of a gene encoding a putative TRX has been revealed by the genome sequencing project of *Leishmania major* (Myler et al., 1999). No evidence for the occurrence of TRX in *T. cruzi* is available, an issue being of relevance to fully understand the redox metabolism of this parasite. The *T. cruzi* CL Brener database (http://tcruzidb.org, http://www.genedb.org) show a putative *trx* gene. Here, we present results that add value to this database, reporting the cloning of a full gene (*trx*) coding for TRX from *T. cruzi* (Tulahuen O), its expression in *Escherichia coli*, and the chromatographic purification of the recombinant protein. Furthermore, the presence of TRX in epimastigote forms is evidenced immunologically.

2. Materials and methods

2.1. Parasites

Epimastigote forms of *T. cruzi* Tulahuen O strain were grown in CIEN medium (Barrios et al., 1980) at 28 °C. Collected parasites were washed three times with phosphate buffered saline (PBS), pH 7.4. Total RNA of *T. cruzi* Tulahuen O epimastigotes was isolated by using a commercial kit (SV total RNA isolation system, from Promega), whereas genomic DNA was prepared by standard methods (Maniatis et al., 1982).

2.2. Bacteria and plasmids

E. coli Top 10 F′ cells (Invitrogen) were used for cloning. The strain *E. coli* BL21 (DE3) was utilized in routine plasmid construction and expression experiments. The vector pGEM-T Easy (Promega) was selected for cloning and sequencing purposes. The expression vector was pRSET A (Invitrogen). DNA manipulations, *E. coli* cultures and transformations were performed according to standard protocols (Maniatis et al., 1982).

2.3. Amplification of the TcTRX gene

Specific oligonucleotide primers were designed from the *T. cruzi* CL Brener database (http://tcruzidb.org, http://www.genedb.org). The forward primer contained a *Bam*HI site and overlapped the 5′-end of the coding sequence (5′-GGATCCATGCCAGTGGTGGATGTGTACAGC-3′), and the reverse primer overlapped the 3′-end of the coding sequence and contained a *Hind*III site (5′-AAGCTTTTAACTATTGCTAATGATTTCCC-3′). A gene putatively encoding TRX in *T. cruzi* (*tctrx*) was amplified by PCR using genomic DNA of *T. cruzi* Tulahuen O (94 °C, 10 min; 94 °C, 1 min; 65 °C, 1 min; 72 °C, 2 min; 35 cycles; 72 °C, 10 min). The amplified DNA was cloned into the pGEM-T Easy vector and used to transform competent *E. coli* TOP 10 F′, as well as for sequencing. Total RNA (1.5 μg) from *T. cruzi* was reverse transcribed using a SuperScript™ II reverse transcriptase (Invitrogen) and amplified using a Taq DNA polymerase (Invitrogen). The reverse gene-specific primer was used for first strand cDNA synthesis and the forward gene-specific primer for the following amplification. The PCR products were electrophoretically separated on 2% agarose gel.

2.4. Southern blot

Genomic DNA from *T. cruzi* (10 μg) was digested with each of the following restriction enzymes (Promega): *Bam*HI, *Hind*III or *Eco*RI. The resulting restriction fragments were separated by 2% submerged agarose gel electrophoresis (SAGE) for 80 min at 90 V in standard TBE buffer, and subsequently blotted onto Hybond™-N+ membrane (Amersham Pharmacia Biotech) by capillary transfer. The DNA was cross-linked to the membrane by UV irradiation. Southern hybridization was performed according to "The DIG System User's Guide for Filter Hybridization" (Boehringer Mannhein). DIG-labeled *tctrx* gene was used as probe for hybridization assay.

2.5. Cloning, expression and purification of TcTRX

Standard preparations of the plasmid [pGEM-T Easy/TcTRX] were digested with *Bam*HI and *Hind*III enzymes. The TcTRX encoding fragment was cloned into a pRSET A expression vector between the same restriction sites. *E. coli* BL21 (DE3) transformed with the recombinant plasmid [pRSET A/TcTRX] was grown at 30 °C and 160 rpm in LB medium with 25 μg/ml of ampicillin, to an OD_{600} of 0.6–0.8. At this time, the overexpression of the recombinant TcTRX was induced with 1 mM IPTG and incubated for 6 h. The optimal expression time was revealed by analysing samples taken at different times after induction. The cell culture was centrifuged at 5000 × *g* for 10 min, the pellet was resuspended in binding buffer (20 mM Tris–HCl buffer, pH 8.0, and 100 mM NaCl). Cells were then disrupted by sonication and centrifuged at 5000 × *g* for 20 min. Soluble and insoluble fractions were subjected to SDS-PAGE. The soluble fraction was loaded (flow rate of 2 ml/min) onto a metal affinity resin column charged with Co^{2+} (Clontech) previously equilibrated with binding buffer. The column was washed twice with 10 bed volumes of binding buffer containing 10 mM imidazole. The His-tagged TRX was eluted with 20 mM Tris–HCl, pH 8.0, containing 100 mM NaCl, and 100 mM imidazole. Active fractions were pooled and dialyzed against 100 mM potassium phosphate buffer, pH 8.0.

2.6. Insulin disulfide reduction assay

The TRX activity was assayed using the standard chemical protocol previously described (Arner et al., 1999). The assay medium contained 100 mM potassium phosphate (pH 7.0), 1 mM EDTA, 130 μM human insulin (Invitrogen), 500 μM DTT, and varying concentrations (1, 3, and 6 μM) of TcTRX in a total volume of 900 μl.

2.7. Antibody production and Western blotting

To have an immunological tool to detect TRX in *T. cruzi*, we raised antibodies against the previously characterized protein from *T. brucei brucei* (TbbTRX). We amplified the gene *tbbtrx* (GenBank accession number AJ239128) from genomic DNA (kindly provided by Dr. Antonio Uttaro, Universidad Nacional de Rosario, Argentina). The gene was sequenced to confirm its identity. The amplified gene was cloned in pMALC2 vector (New England Biolabs) for expression as a fusion protein to maltose binging protein (MBP-TbbTRX). The recombinant protein was purified in an amylose column (New England Biolabs), and then utilized for rabbit immunization according to Vaitukaitis et al. (1971). This serum was used as primary antibody to identify TcTRX in immunological tests.

Detection of specific proteins by Western blotting was performed after standard techniques (Maniatis et al., 1982). Proteins in SDS-PAGE gels were blotted onto PVDF membranes using a Mini-PROTEAN II (Bio-Rad) apparatus. The membrane was blocked overnight at 4 °C, subsequently incubated with primary antibody (dilution 1:100) at room temperature for 1 h, and then incubated with a biotinilated anti-rabbit IgG secondary antibody for 1 h. After washing, the membrane was incubated with alkaline phosphatase-conjugated streptavidin. Detection was carried out with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Inmuno Select™, Gibco) in 100 mM NaCl, 100 mM Tris–HCl, pH 9.5, and 5 mM $MgCl_2$. The non-specific recognition of TcTRX by anti-MBP antibodies (produced in our laboratory using the protocol described above but using MBP) was tested by using a 1:100 dilution of a primary antibody corresponding to a whole rabbit serum raised against *E. coli* MBP.

2.8. Immunoprecipitation

2.8.1. Step I: cell lysate preparation

Approximately, 5 g of *T. cruzi* Tulahuen O (5×10^{10} cells) were washed three times with 20 ml of PBS in a conical tube and spun at 3000 × *g* for 10 min. After the last wash, the supernatant was removed completely and the cell pellet resuspended in 8 ml of cold Lysis buffer (50 M Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40) containing 50 μg/ml of PMSF. Cell suspension was vortex mixed and incubated on ice for 30 min, with occasional swirling. The cell lysate was centrifuged at 10,000 × *g* for 15 min at 4 °C, and the supernatant carefully collected (without disturbing the pellet) in a clean tube.

2.8.2. Step II: cell lysate preclearing

Protein A Sepharose beads (50 μl, Amersham Pharmacia Biotech) were washed twice with 450 μl cold Lysis buffer and microcentrifuged at full speed for 5 min. The beads were resuspended in 50 μl of fresh Lysis buffer, added to the cell lysate, incubated on ice for 60 min at 4 °C, and then centrifuged for 10 min as before. The clear supernatant (containing no bead) was transferred to a clean tube.

2.8.3. Step III: immunoprecipitation

The cold precleared lysate was incubated overnight with an 1/100 dilution of anti-MBP-TbbTRX serum at

4 °C. Fifty microlitres of washed Protein A beads (prepared as described in Section 2.8.2) were added. The suspension was incubated for 1 h on a rocking platform, and microcentrifuged at full speed for 2 min at 4 °C. The supernatant was completely removed and the beads were washed five times with 500 μl of Lysis buffer. The supernatant was carefully removed and the beads were resuspended in 50 μl of 1× Laemmli sample buffer (Maniatis et al., 1982), followed by incubation in a boiling water bath for 10 min and centrifugation during 5 min. The resulting supernatant was divided in 25 μl aliquots and used for SDS-PAGE and Western blot analysis.

2.9. Immunohistochemistry

Epimastigote forms of *T. cruzi* Tulahuen O strain were fixed on a slide in 10% (v/v) formaldehyde in 0.05 M PBS, pH 7.5 for 10 min at 4 °C. The cells were incubated with 30 volumes of methanol:H_2O_2 (10:1) to inhibit endogenous peroxidase present in trypanosomatids. The fixed parasites were incubated at 4 °C with a 1:100 dilution of anti-MBP-TbbTRX serum for 14–16 h. After washing with 0.05 M PBS, pH 7.5 for 10 min, parasites were incubated for 30 min at 37 °C with a 1:400 dilution of the secondary antibody: biotinylated anti-rabbit IgG. Then, the sample was incubated with a 1:1200 dilution of peroxidase-conjugated streptavidin (HRP) for additional 30 min. Detection was performed in 3.3 ml of a medium containing 50 mM Tris–HCl, pH 7.4, 2.3 mg diaminobencidine (DAB) (Invitrogen) and 50 μl H_2O_2 (30 volumes). Four different negative controls were carried out in parallel: (i) without primary antibody; (ii) with an 1:100 dilution of rabbit preimmune serum; (iii) with an 1:100 dilution of a rabbit anti-MBP-TbbTRX serum preincubated for 96 h with 20 μg of MBP-TbbTRX; (iv) with an 1:100 dilution of anti-MBP primary antibody. Controls were stained at 25 °C with Mayer's hematoxylin prepared as described in: http://www.ihcworld.com/_protocols/counterstain_solutions/mayer_hematoxylin.htm.

3. Results

3.1. Cloning and sequencing of a thioredoxin gene from T. cruzi

The gene encoding thioredoxin (*tctrx*) has been amplified from genomic DNA of *T. cruzi* (Tulahuen O) epimastigotes and cloned into the pGEM-T Easy vector for sequencing. The *tctrx* gene encodes a protein of 107 amino acid residues with a molecular weight of 12,120 and a calculated p*I* of 7.9. The in vivo transcription

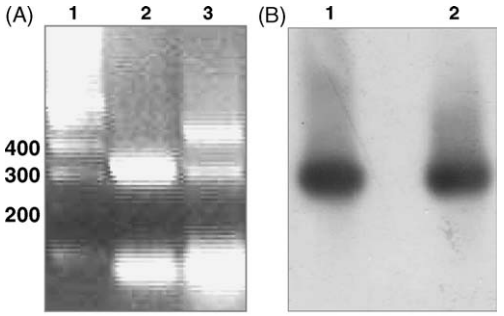

Fig. 1. PCR and RT-PCR products amplified from *T. cruzi*. (A) Lane 1, SAGE of DNA molecular marker (Invitrogen); lane 2, *tctrx* amplified from genomic *T. cruzi* DNA; lane 3, *tctrx* amplified from cDNA reverse transcribed from total *T. cruzi* RNA. (B) *Southern blot*: PCR and RT-PCR products were separated electrophoretically in 2% agarose gel and transferred to nylon membranes. For Southern hybridization, 2 μg of the DIG-labeled *tctrx* was used. Lane 1, amplification of *tctrx* from genomic *T. cruzi* DNA and lane 2, amplification of *tctrx* from cDNA reverse transcribed from total *T. cruzi* RNA. Size standards (bp) are indicated on the left side.

of *tctrx* was revealed by RT-PCR/Southern hybridization experiments (Fig. 1). The *tctrx* was amplified from genomic DNA of epimastigote *T. cruzi* (Fig. 1A, lane 2) and also from *T. cruzi* total RNA (Fig. 1A, lane 3). The identity of fragments amplified from both genomic DNA and total RNA was confirmed by hybridization assay with the DIG-labeled *tctrx* (Fig. 1B, lanes 1 and 2, respectively).

Genomic DNA from *T. cruzi* was digested with different restriction enzymes, which do not cut within the *tctrx* but do cut DNA into predicted fragments. In the Southern blot analysis, the DIG-labeled PCR probe hybridized with a 1.5 kb *Eco*RI fragment, and recognized 4.9 and 8.6 kb fragments in *Bam*HI and *Hin*dIII digests, respectively (Fig. 2). This result suggests that *trx* is present as a single copy gene in *T. cruzi*, which fully agrees with a search in the *T. cruzi* genome project database.

3.2. Expression, purification and reductive activity of recombinant TcTRX

The full open reading frame of *tctrx* was cloned into *Eco*RI/*Hin*dIII digested pRSETA vector. Competent *E. coli* BL21 (DE3) cells were transformed with the resulting expression construct (pRSETA/*tctrx*), rendering a system useful for the expression and purification of the protein (Fig. 3). Transformed cells overexpressed TcTRX with a tag of six histidine residues in its N-terminus (Fig. 3A). The protein was purified on a Co^{2+} column (Fig. 3B) and probed with anti-MBP-TbbTRX or anti-MBP rabbit polyclonal antibodies.

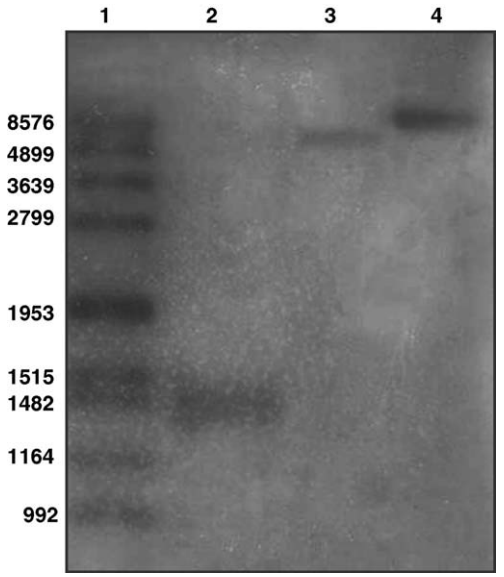

Fig. 2. Southern blot of *T. cruzi* genomic DNA digested with different restriction enzymes. The *T. cruzi* genomic DNA restriction fragments were separated by SAGE and transferred to nylon membranes. For Southern hybridization 2 μg of the DIG-labeled PCR product (*tctrx*) were used. Lane 1, DNA molecular markers Dig-labeled VII (Boehringer Mannheim); lane 2, *T. cruzi* genomic DNA digested with *Eco*RI; lane 3, *T. cruzi* genomic DNA digested with *Bam*HI; lane 4, *T. cruzi* genomic DNA digested with *Hin*dIII. Size standards (bp) are indicated on the left side.

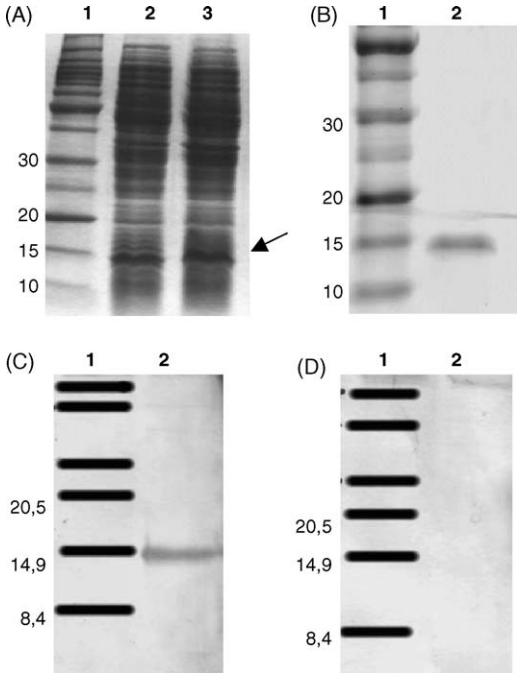

Fig. 3. Electrophoretic and immunological identification of recombinant TcTRX. (A) SDS-PAGE stained with Coomassie blue corresponding to protein molecular markers (Invitrogen) (lane 1); soluble (lane 2) and insoluble (lane 3) fractions of extract from [pRSET A/TcTRX]-transformed cells. (B) SDS-PAGE stained with Coomasie blue of protein molecular markers (lane 1), and purified H6TcTRX (lane 2). (C) Western blot using a rabbit antiserum raised against TbbTRX. Lane 1, prestained protein molecular markers; lane 2, purified H6TcTRX. (D) Western blot using a rabbit antiserum raised against MBP. Lane 1, prestained protein molecular marker; lane 2, purified H6TcTRX. Size standards are given on the left side in kDa. The arrow show the recombinant TcTRX.

The anti-MBP-TbbTRX serum recognized a protein of 14.9 kDa (Fig. 3C). Western blots using anti-MBP antibodies gave no recognition signal, which is critical to show that the anti-MBP-TbbTRX specifically reacted with TRX (Fig. 3D). The recombinant protein is about 3 kDa larger than the authentic protein, in agreement with the enlargement caused by the histidine-tag.

Fig. 4 shows an alignment of amino acid sequences between TcTRX and homologous proteins from different sources. The *T. cruzi* protein exhibited different degrees of identity with other TRXs: *T. brucei* (74%), *L. major* (65%), *F. hepatica* (35%), *P. falciparum* (33%), *Homo sapiens* (31%), and *E. coli* (23%). The amino acid sequence identity was absolute at the WCGPCK motif representing the redox active site of TRXs (Holmgren et al., 1975).

The redox activity of the recombinant TcTRX was determined in the insulin assay. The method is based on the ability of DTT-reduced TRX to catalyze the reduction of insulin resulting in the precipitation of the insoluble β-chain. In the absence of TcTRX, no increase in turbidity was observed during the first 45 min of incubation (Fig. 5). In contrast, the presence of varying concentrations of purified recombinant TcTRX (1, 3, and 6 μM) gave increasing rates of insulin reduction (Fig. 5), which was detectable after approximately 2 min of incubation (6 μM TcTRX). We also determine that TcTRX could be reduced by TRXR from *Entamoeba histolytica* (data not shown). In fact, when in the medium described for insulin assay, DTT was replaced by TRXR (150 nM) from *E. histolytica* and NADPH (200 μM), the reduction of TcTRX could be followed by measuring the extinction of absorbance at 340 nm, thus, indicating the functionality of the system (Arias et al., unpublished research). This latter activity was found proportional to the amount of TcTRX (between 1 and 20 μM) in the medium.

3.3. Cellular localization of TcTRX

Attempts to evidence the presence of TRX in *T. cruzi* crude extracts by Western assays gave negative results. Therefore, our strategy was to concentrate the protein by immunoprecipitation. Using the anti-MBP-TbbTRX

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          Trypanosoma cruzi TRX   --MPVVDVYSVEQFREIVNEDLLTVAWFTAVWCGPCKTIERAMEKITFEF
Trypanosoma brucei brucei TRX     --MSVVDVYSVEQFRNIMSEDILTVAWFTAVWCGPCKTIERPMEKIAYEF
          Leishmania major TRX    --MPFTEVYGVEQFRDIANDPTLTVVCFSAVWCGPCKTIEKDLDRLTYEF
          Fasciola hepatica TRX   --MRLLRTAADLEKLINENKGRLIVVDFFAQWCGPCRNIAPKVEALAKEI
      Plasmodium falciparum TRX   ---MVKIVTSQSEFDSIISQNELVIVDFFAEWCGPCKRIAPFYEECSKTY
          Escherichia coli TRX    MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEY
              Homo sapiens TRX    -MVKQIESKTAFQEALDAAGDKLVVVDFSATWCGPCKMINPFFHSLSEKY

          Trypanosoma cruzi TRX   P-TVRFAKVDADNNSEIVSKCKVLQLPTFMLVKNG--KLIGYVIGANLDT
Trypanosoma brucei brucei TRX     P-TVKFAKVDADNNSEIVSKCRVLQLPTFIIARSG--KMLGHVIGANPGM
          Leishmania major TRX    A-SVRFAKVDADNNTDIVSKCRVMQLPTFMLVRAG--QMLGYVIGADLAQ
          Fasciola hepatica TRX   P-EVEFAKVDVDQNEEAAAKYSVTAMPTFVFIKDG--KEVDRFSGANETK
      Plasmodium falciparum TRX   T-KMVFIKVDVDEVSEVTEKENITSMPTFKVYKNG--SSVDTLLGANDSA
          Escherichia coli TRX    QGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVASATKVGALSKGQ
              Homo sapiens TRX    S-NVIFLEVDVDDCQDVASECEVKCTPTFQFFKKG--QKVGEFSGANKEK

          Trypanosoma cruzi TRX   LKRKIREIISNS-
Trypanosoma brucei brucei TRX     LRQKLRDIIKDN-
          Leishmania major TRX    LKAKIREEANKS-
          Fasciola hepatica TRX   LRETITRHK----
      Plasmodium falciparum TRX   LKQLIEIYAA---
          Escherichia coli TRX    LKEFLDANLA---
              Homo sapiens TRX    LEATINELV----
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Fig. 4. Alignment of TRXs amino acid sequences. The amino acid sequence deduced for TcTRX was aligned with TRXs from *Leishmania major* (GenBank accession number AE001274), *Trypanosoma brucei brucei* (GenBank accession number AJ239128), *Fasciola hepatica* (GenBank accession number AJ250097), *Plasmodium falciparum* (GenBank accession number NP702434), *Escherichia coli* (GenBank accession number M54881), *Homo sapiens* (GenBank accession number J04026). Conserved residues are in white, the WCGPC motif and the highly conserved residues D58, G81, and P73 are in bold white.

immune serum prepared in our laboratory and Protein A Sepharose, we enriched TRX present in *T. cruzi* Tul 0 (Fig. 6). Fig. 6A shows an SDS-PAGE of the proteins immunoprecipitated from the crude extract obtained by cellular lysis of *T. cruzi*. It is evident that many proteins co-precipitated which may – at least partially – be due to their interaction with TcTRX. From results shown in Fig. 6A, considering the Coomassie brilliant blue detection limit and comparing staining intensity given by known proteins, we could estimate an amount of ca. 0.2 μg TcTRX present in the extract from 5 g of the parasite (5×10^{10} cells). Proteins in the gel were transferred to a PVDF membrane for Western analysis using the anti-MBP-TbbTRX serum. As shown in Fig. 6B, of the many proteins co-immunoprecipitated, only TcTRX was revealed in the Western analysis, which indicates a high specificity of the polyclonal antibody utilized.

Based on the results described above, the cellular occurrence of TRX in *T. cruzi* was determined using

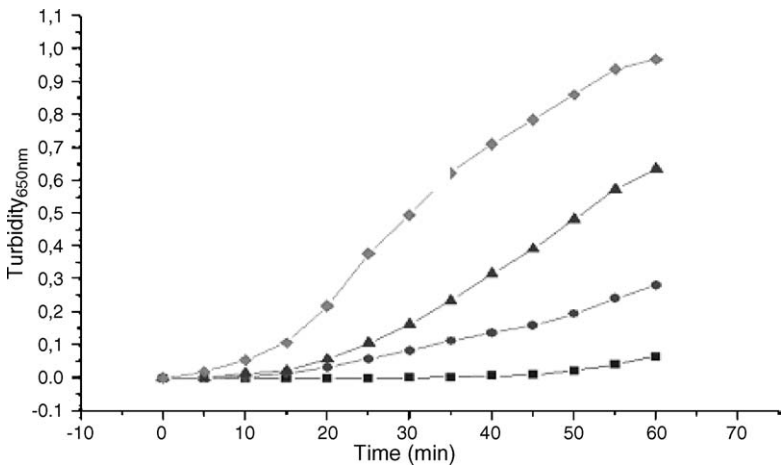

Fig. 5. Insulin reduction by recombinant TcTRX. Reactions were performed in triplicate containing 500 μM DTT and different concentrations of TcTRX: 6 μM (diamonds), 3 μM (triangles), 1 μM (circles), or in the absence of recombinant TcTRX (squares).

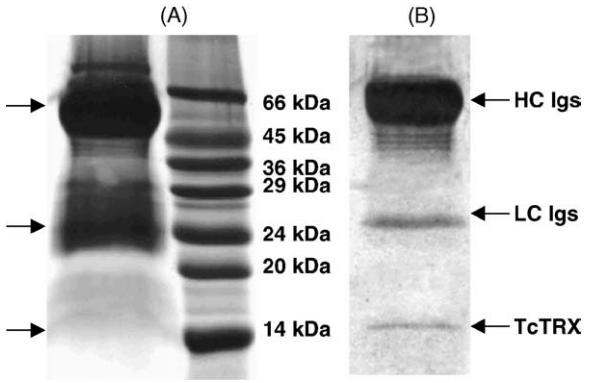

Fig. 6. Immunoprecipitation of TRX in *T. cruzi* Tulahuen O extracts. Immunoprecipitation from cell lysates using anti-MBP-TbbTRX serum and Protein A Sepharose was performed as described under Section 2. (A) SDS-PAGE of the samples stained with Coomassie blue. Lane 1, proteins obtained after immunoprecipitation (25 μl of the final supernatant); lane 2, protein molecular markers. (B) Western blot using a rabbit-antiserum raised against MBP-TbbTRX. Lane 1, protein molecular markers; lane 2, proteins obtained after immunoprecipitation (25 μl of the final supernatant): TcTRX, thioredoxin from *T. cruzi*; HC Igs, heavy chain of immunoglobulins; LC Igs, light chain of Igs, are indicated with arrows.

the immunoperoxidase system (HRP/DAB) and optical microscopy to visualize the reaction (Fig. 7). As illustrated in Fig. 7A, treatment of the parasites with the TcTRX-specific antibody followed by HRP/DAB resulted in a diffuse cytoplasmic staining with continuity to the flagellum. As expected, no immunological reaction was observed for negative controls, which required Mayer's hematoxylin staining for microscopic observation (Fig. 7B–E). This result, together with that of RT-PCR (see Fig. 1) support that the *tctrx* gene is transcribed and translated in *T. cruzi*. This fact is strongly reinforced by immunoprecipitation experiments showing the presence of TRX protein in the parasite extracts (Fig. 6).

4. Discussion

T. cruzi is the causal agent of Chagas' diseases, a severe sickness affecting more than 20 million persons from South of the USA to the South of Argentina. The knowledge of the metabolic pathways present in *T. cruzi* is a main prerequisite for the rational design of chemicals specifically affecting key enzymes in the parasite. TRX

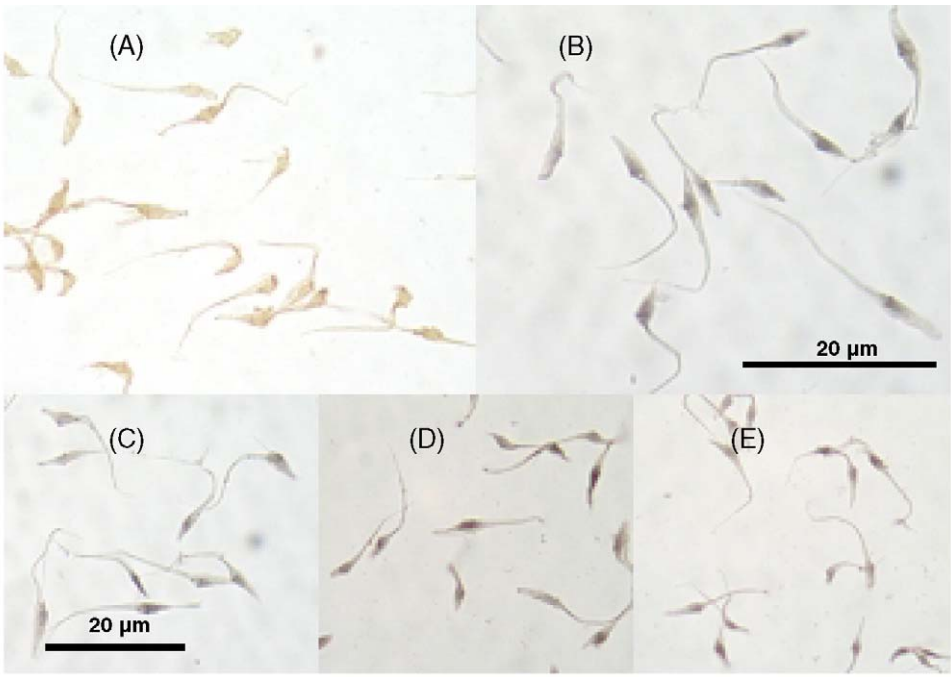

Fig. 7. Cellular localization of TRX in *T. cruzi* Tulahuen O. Immunoperoxidase analysis of Tulahuen O strain epimastigotes of *T. cruzi* recognized by antibodies raised against TRX from *T. brucei brucei*. (A) Positive reaction, sequentially incubated with rabbit anti-MBP-TbbTRX serum and HRP-labeled anti-rabbit IgG. (B) Negative control, sequentially incubated with rabbit preimmune serum and HRP-labeled anti-rabbit IgG. (C) Negative control, only incubated with HRP-labeled anti-rabbit IgG. (D) Negative control, sequentially incubated with rabbit anti-MBP serum and HRP-labeled anti-rabbit IgG. (E) Negative control, sequentially incubated with rabbit anti-MBP-TbbTRX (pretreated at 4 °C for 96 h with 20 μg of MBP-TbbTRX) and HRP-labeled anti-rabbit IgG. All negative controls were stained with Mayer's hematoxyline.

is a redox protein that plays a number of relevant roles in metabolism. As it has been demonstrated in different organisms, TRX is involved in the synthesis of deoxyribonucleotides, in the detoxification of ROS, as well as in the regulation of transcription factors (Holmgren, 1979a; Williams, 2000). The many metabolic functions of TRX make of relevance the identification of this protein in this pathogenic trypanosomatid.

In the present work, we show that TRX, previously found in *L. major* and *T. brucei brucei*, is also present in *T. cruzi* strain Tulahuen O. A putative nucleotide sequence encoding TRX (*tctrx*) was obtained from the *T. cruzi* CL Brener database (http://tcruzidb.org, http://www.genedb.org). The identity of the amplified *tctrx* (324 bp) gene was confirmed by DNA sequencing (GenBank accession no. AY688958). Southern blot analysis suggests the presence of *tctrx* as a single copy gene (Fig. 2). The amino acid sequence deduced from *tctrx* (107 amino acid residues) showed a high degree of identity when compared with trypanosomatid TRXs. When the analysis is extended to TRXs from different organisms the sequence identity is largely restricted to the active site (WCGPC) region. TcTRX conserves amino acid residues D58, G81, and a P73 also present in the protein from other sources. In fact, D58 and G81 are considered to contribute to the right folding of the protein, whereas the cisP73 was reported to interact with the active site in the 3-D structure of TRX (Holmgren et al., 1975). When compared with the fully characterized TRX from *T. brucei brucei* (Schmidt and Krauth-Siegel, 2003), it is found a great similitude with TcTRX. Mainly, a D26 residue, highly conserved in several TRXs and being critical for catalytic activity (Dyson et al., 1991), is replaced by a W residue in TcTRX and TbbTRX (Friemann et al., 2003). The presence of a W26 residue was shown to produce a change in the environment of the protein active site that modifies the pK_a of two critical cystein residues (Friemann et al., 2003).

The *tctrx* gene was expressed in the *E. coli* BL21 (DE3)/pRSET A system, and the recombinant TcTRX protein was recovered in the soluble fraction and purified to homogeneity by metal ion affinity chromatography. The biological activity of the recombinant molecule was probed by the insulin reduction assay, where precipitation of the B chain of insulin was dependent on increasing TcTRX (which is chemically reduced by DTT) in the assay medium (Holmgren, 1979b). The identity of recombinant TcTRX was further confirmed in Western blot experiments by using specific antibodies, which also were useful for the immunolocalization of TcTRX in epimastigote forms of *T. cruzi* immobilized on glass. Diffuse cytoplasmic staining with continuity to the flagellum was observed by optical microscopy. These results agree with previous reports about the cellular localization of TRX in other organisms (Cha et al., 2003; Li et al., 2004; Maggioli et al., 2004; Powis and Montfort, 2001).

In our hands, Western blot detection of TcTRX in crude extracts from *T. cruzi* required a previous concentration by immunoprecipitation. Despite this, the protein was clearly visualized in the epimastigote forms of the parasite by immunocytochemistry. Many reasons could explain this difference in the ability of detection. For instance, the immunocytochemical approach could be detecting the antigen that is locally concentrated within the cell. Also, the efficiency of TRX recovery in the crude extract preparation and/or in the process of transfer to the PVDFmembrane could markedly reduce the concentration of the protein in Western blots. Similar experimental observations have been reported by others, for example, in the characterization of acid glycohydrolases in rat spermatocytes (Abou-Haila and Tulsiani, 2001; Skudlarek et al., 2000).

Previous studies have pointed out that trypanosomatids specifically utilize TXN to detoxify peroxides, and the absence of the TRX/TRXR system in these organisms has been proposed (Flohe et al., 2002; Hirt et al., 2002; Muller et al., 2003; Reckenfelderbaumer et al., 2000). However, it was demonstrated that *L. major* and *T. brucei brucei* possess TRX (Myler et al., 1999; Reckenfelderbaumer et al., 2000), and that TbbTRX can transfer reduction equivalents to TXNPx and other peroxidases for subsequent reduction of hydroperoxides (Hillebrand et al., 2003; Schmidt et al., 2002). In addition, in *T. brucei brucei*, $T(SH)_2$ was shown to be able in reducing TXN, and TRX as well (Schmidt and Krauth-Siegel, 2003). This latter report raises the intriguing questions of how TRX is ultimately reduced, and which specific function it plays in trypanosomatids. Is $T(SH)_2$ the only way for reducing TRX in the absence of TRXR? Is TRX involved, in some way, in peroxide detoxifying mechanisms? Although it has been hypothesized that in *T. brucei brucei* TRX is not essential for survival (Muller et al., 2003; Schmidt and Krauth-Siegel, 2003), further studies are necessary to characterize the actual function (thought non-essential, but relevant) of the protein in the scenario of the parasite metabolism. Results presented herein on the characterization of TRX in *T. cruzi* contribute to establish the occurrence of this protein in this trypanosomatid. From this, the involvement of TRX in the (redox) metabolism of these parasites remains an open question.

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