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Research paper

Redox metabolism in *Trypanosoma cruzi*. Biochemical characterization of dithiol glutaredoxin dependent cellular pathways

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ABSTRACT

In *Trypanosoma cruzi*, the modification of thiols by glutathionylation–deglutathionylation and its potential relation to protective, regulatory or signaling functions have been scarcely explored. Herein we characterize a dithiolic glutaredoxin (*Tcr*Grx), a redox protein with deglutathionylating activity, having potential functionality to control intracellular homeostasis of protein and non-protein thiols. The catalytic mechanism followed by *Tcr*Grx was found dependent on thiol concentration. Results suggest that *Tcr*Grx operates as a dithiolic or a monothiolic Grx, depending on GSH concentration. *Tcr*Grx functionality to mediate reduction of protein and non-protein disulfides was studied. *Tcr*Grx showed a preference for glutathionylated substrates respect to protein disulfides. From *in vivo* assays involving *Tcr*Grx overexpressing parasites, we observed the contribution of the protein to increase the general resistance against oxidative damage and intracellular replication of the amastigote stage. Also, studies performed with epimastigotes overexpressing *Tcr*Grx strongly suggest the involvement of the protein in a cellular pathway connecting an apoptotic stimulus and apoptotic-like cell death. Novel information is presented about the participation of this glutaredoxin not only in redox metabolism but also in redox signaling pathways in *T. cruzi*. The influence of *Tcr*Grx in several parasite physiological processes suggests novel insights about the protein involvement in redox signaling.

1. Introduction

During the life cycle, *Trypanosoma cruzi* is exposed to both reactive oxygen (ROS) and nitrogen (RNS) species. These dangerous species are generated endogenously as by-products of the parasite aerobic metabolism, a phenomena reported for many other organisms, or exogenously generated from the host immune system [1]. Both ROS and RNS can affect the cellular redox balance, consequently altering other major cell functions. In biological systems, thiols have an essential function in scavenging ROS and RNS either spontaneously or catalytically. Thiol redox state is a mediator in metabolic, transcriptional and signaling cell processes, supporting cellular functions under basal physiological and/or oxidative (or nitrosative) stress conditions [2]. Glutathionylation is one of the most common thiol modification mechanisms inside the cell, which mainly operates during oxidative stress but also physiologically by mediating redox signals exerted by ROS [2,3]. Glutaredoxin (Grx), an oxidoreductase with deglutathionylating activity, has a relevant role in intracellular homeostasis of protein thiols. Grx has a strong preference for glutathione mixed disulfides [4] and its cellular functionality has been mainly related to regulation of enzyme activity by glutathionylation–deglutathionylation [2,4,5].

The cellular redox status can influence cell viability through regulation of cellular proliferation, growth arrest or programmed cell death (PCD) [6]. Depending on diverse factors (type of organism, tissue or apoptotic stimulus), Grxs can act linked to pro-apoptotic or anti-apoptotic pathways [5]. PCD was previously described in protists [7], including *T. cruzi* [8,9]. Although the biological significance of PCD in protozoan is still unclear, it was postulated that apoptosis-like cell death can function as a way for

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improving infectivity [8,10]. In trypanosomatids, most of the molecular effectors that would be involved in the sequence of apoptosis-like events were not elucidated. In these microorganisms, different cellular actions were found related to the apoptosis-like cell death induced by human serum complement. Such cell processes include: i) decrease of $T(SH)_2$ and GSH levels [8], ii) ROS production [8], iii) release of cytochrome *c* from mitochondria to cytosol [8] and iv) accumulation of calcium into mitochondria [8,11].

In trypanosomatids, trypanothione [$T(SH)_2$] dependent mechanism involved in redox homeostasis and ROS/RNS detoxification has been relatively well characterized [12,13]. Conversely, even when these parasites contain free glutathione, the modification of thiols by glutathionylation–deglutathionylation and its potential relation to protective, regulatory or signaling functions have been poorly explored. In fact, glutathionylation was exclusively studied as a mechanism for protecting several enzymes of the antioxidant network in *Trypanosoma brucei* [14]. A dithiolic glutaredoxin in *T. cruzi* (*Tcr*Grx) was previously characterized by our group [15], we reported on the identification of two genes encoding dithiolic Grx in the *T. cruzi* genome (Tc00.1047053506475.116 and Tc00.1047053511431.40) having a high identity between them and coding for two identical proteins (*Tcr*Grx). At the moment, this is the unique dithiol Grx reported in *T. cruzi*. Afterward, Ceylan and co-workers [16] advanced in the study of two glutaredoxins in African trypanosomes, one of them (namely Grx2) sharing a high identity percentage (higher than 80%) with the dithiolic Grx previously reported in *T. cruzi* [15]. However, the characterization of Grx in trypanosomatids, concerning biochemistry, kinetics and cellular functionality is far from complete. In this work we report on the many faceted biochemical properties of pure recombinant *Tcr*Grx, showing its relevance in maintaining intracellular physiological concentrations of GSH, and other low molecular mass thiols. Additionally, we present information about the relevance of *Tcr*Grx activity (glutathionylation–deglutathionylation) on the parasite vital processes.

2. Experimental procedures

2.1. Reagents

Trypanothione disulfide and glutathionyl-spermidine disulfide were acquired from Bachem (Torrance, CA, USA). DIEGSSG was from Cayman Chemical (Michigan, USA). All other reagents and chemicals were of the highest quality commercially available from Sigma–Aldrich (St. Louis, MO, USA) or similar.

2.2. Expression and purification of TcrGrx and mutants TcrGrxC31S and TcrGrxC34S

*Tcr*Grx was obtained as previously described [15]. Mutants of *Tcr*Grx in active site cysteine residues were obtained by site-directed mutagenesis on *tcrgrx* gene cloned in pRSET-A by PCR-driven using specifically designed primers, following QuickChange™ mutagenesis protocol. Expression and purification of *Tcr*GrxC31S and *Tcr*GrxC34S was performed in the same way as *Tcr*Grx [15]. Purity of the recombinant protein was analyzed by SDS-PAGE [17]. Protein content was determined by the method of Bradford [18].

2.3. Gel filtration chromatography

The estimation of the molecular size of native *Tcr*Grx was performed in a Superdex 200 (GE-Healthcare) column equilibrated with 25 mM Tris–HCl pH 7.5, 1 mM EDTA and 100 mM NaCl. The purified protein was analyzed chromatographically together with different molecular mass standards: bovine serum albumin (66 kDa), ovoalbumin (43 kDa), carbonic anhydrase (29 kDa) and lysozyme (14 kDa).

2.4. Activity assays

Thioltransferase activities were measured spectrophotometrically by following consumption of NADPH at 340 nm and 30 °C, by means of coupled assays that guarantee the regeneration of reduced *Tcr*Grx, using a Multiskan Ascent one-channel vertical light path filter photometer (Thermo Electron Co.). The standard reaction medium (50 μL final volume) contained 100 mM Tris–HCl pH 7.5, 2 mM EDTA and 300 μM NADPH, and the different coupled reactions were analyzed after the specific additions. $T(SH)_2$-dependent reduction of GSSG or CySS or *S*-nitrosothiols by *Tcr*Grx was performed in a reaction mixture containing 100 μM TS_2, 1 μM *Tcr*TR, 15–1000 μM GSSG or CySS or GSNO or CySNO and different concentrations (0.5–5 μM) of *Tcr*Grx. GSH-dependent reduction of TS_2, $(Gsp)_2$ or HEDS by *Tcr*Grx was performed at different concentrations (0.25–5 μM) of *Tcr*Grx and 3 mM GSH, as previously reported [15,19]. The ability of *Tcr*Grx for transferring electrons to *Tcr*cTXNPx, *Tcr*mTXNPx and *Tcr*GPxI, was determined by measuring the reduction of *t*-bOOH [20]. The capacity of *Tcr*Grx for transferring electrons to *Tcr*MSRAs was determined by measuring the L-Met(*S*) SO reduction [21]. GSH-dependent reduction of dehydroascorbate (DHA) by *Tcr*Grx was performed as previously reported [22].

Reduction of the two interchain disulfide bonds of insulin catalyzed by recombinant *Tcr*Grx was analyzed in an assay adapted from the previously described by Holmgren [23]. The ability of *Tcr*Grx to participate in redox regulation of enzymatic activities was assayed using *Tcr*UDP-GlcPPase as a target, assay for UDP-GlcPPase activity was performed according to Fusari et al. [24]. Briefly, the formation of PPi from UTP and Glc-1P was monitored at 37 °C in a reaction mixture containing 100 mM MOPS pH 8.0, 1 mM $MgCl_2$, 1 mM UTP, 1 mM Glc-1P and 1U pyrophosphatase. Pure recombinant *Tcr*UDP-GlcPPase was reduced in presence of 1 mM DTT for 30 min at 30 °C, the excess of reducer agent was removed by desalting in an ultrafiltration device (Microcon, Millipore). Pre-reduced *Tcr*UDP-GlcPPase was inactivated by oxidative treatment with 2 mM diamide or 10 mM GSSG in standard reaction medium. The excess was removed as mentioned previously. Oxidized *Tcr*UDP-GlcPPase was then incubated under the same conditions with 10 mM GSH and variable concentrations of *Tcr*Grx. At different times, aliquots were withdrawn and assayed for UDP-GlcPPase activity.

All kinetic parameters are the mean of at least three independent sets of data, which were reproduced within ±10%.

2.5. Glutathionylation of TcrUDP-GlcPPase with dieosinediglutathione (DIEGSSG)

Glutathionylation of *Tcr*UDP-GlcPPase was evaluated using DIEGSSG, following a protocol adapted from previously reported [25]. *Tcr*UDP-GlcPPase (11 μM) was incubated with DIEGSSG (33 μM) in 0.1 M phosphate buffer pH 7 for 30 min at 37 °C. The excess was removed by ultrafiltration in spin tubes, and diafiltered against 0.1 M phosphate buffer pH 7. Deglutathionylation was performed in a reaction containing 0.5 mM DTT, or 0.1 mM GSH, 4 U mL^{-1} glutathione reductase, 1 mM NADPH, with or without 10 μM *Tcr*Grx depending on the experiment. Proteins in the reaction mixtures were subjected to non-reducing SDS-PAGE. Before staining with Coomassie blue the gel was scanned in Typhoon 9400 (GE Healthcare). The DIEGSSG has low fluorescence, having the eosin-GSH molecules higher fluorescence intensity. Thus,

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glutathionylated proteins are labeled with the fluorescent signal corresponding to eosin isothiocyanate (λ_{exc} = 518 nm; λ_{em} = 545 nm).

2.6. Glutathione-Grx redox equilibrium

Redox equilibrium assays were carried out incubating the oxidized protein (10–40 μM) for 4 h at 30 °C in a reaction mixture containing 100 mM Tris–HCl pH 7.5, 2 mM EDTA and 1 mM GSH. After incubation, 5% (w/v) TCA was added to separate proteins from the reaction mixture. In an aliquot of the reaction mixture the remaining GSH concentration was estimated using DTNB reagent [26]. The GSSG generated was determined by a kinetic method as previously described [22].

2.7. Measurement of thiol pKa by UV absorption

The pH-dependent nucleophilic cysteine ionization was followed by the absorption of the thiolate anion at 240 nm [27,28]. The proteins were analyzed at 25 °C in a pH range from 2 to 11. Spectra of 1–5 μM *Tcr*Grx or *Tcr*GrxC34S oxidized or reduced (with 10 mM DTT for 10 min at room temperature), were recorded between 200 and 340 nm. The spectra were measured against buffer solution in a stoppered quartz cuvette in a Boeco S-22 UV–Vis spectrophotometer and the absorbance was converted into molar extinction coefficient. For a single thiolate group a value between 4 and 6 mM^{-1} cm^{-1} was used, according to previous reports [22,29].

2.8. Determination of thiol pKa of nucleophilic cysteine by DTNB reduction assay

To determine the p*Ka* value of cysteinyl thiol groups the reaction rate as a function of pH was measured. We used the mutant in the Cys 34, according to a previous reported strategy [30]. The rate of DTNB reduction by *Tcr*GrxC34S was determined by incubation of reduced protein (20 μM) with 500 μM DTNB in 50 μl of the reaction media at different pH values, at 25 °C. The rates of DTNB reduction were measured by monitoring the absorbance at 405 nm. The apparent second order rate constants were calculated and plotted against pH.

2.9. T. cruzi cultures, pTEX constructs and transfection procedures

T. cruzi Dm28c strain was cultured in LIT medium supplemented with 10% foetal bovine serum at 28 °C. Plasmid constructs for *T. cruzi* transfections were based on pTEX vector [31], using restriction sites in the multicloning site (EcoRI or BamHI and XhoI). *T cruzi* epimastigotes were transformed with the constructs pTEX (empty vector), pTEX/GFP, pTEX/*Tcr*Grx, pTEX/RFP-*Tcr*Grx and pTEX/*Tcr*Grx-RFP. Transfection procedures were performed as described by Piñeyro and co-workers [32].

2.10. Peroxide sensitivity and induction of apoptotic − like cell death

Suspensions of logarithmic-phase *T. cruzi* (transfected stably with pTEX, pTEX/GFP or pTEX/*Tcr*Grx) epimastigotes at 2×10^6 mL^{-1} were prepared in fresh LIT (containing 2 g L^{-1} glucose) + 10% SFB medium and transferred in aliquots of 1 mL to 48 well plates. Glucose oxidase was added to different wells to obtain 0–0.1 mM final concentrations. Samples were taken at different times of incubation (15, 45, 180 and 240 min) under oxidative condition to evaluate epimastigote viability [33]. Experimental data refer to the analysis of three independent experiments, which were reproduced within ±10%.

For apoptotic-like cell death induction assay, logarithmic-phase epimastigotes were collected by centrifugation and resuspended in PBS (control condition) or PBS 10–15% human fresh serum, at a density of 1×10^7 parasites mL^{-1}. Aliquots of 1 mL were dispensed in 48 wells plate. Samples were separated at different times for control and apoptotic-like phenotype quantification and TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay using ApopTag® Plus Fluorescein *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA, USA), following manufacturer instructions.

For caspase-3-like activity quantification, control or HFS treated epimastigotes suspensions (10 mL) were prepared in the same way, incubated in 25 cm^2 T culture flask, for a period of 6 h. Caspase-like activity in extracts containing 50 μg protein were measured using Caspase 3 assay kit, colorimetric (Sigma, St. Louis, MO, USA), following the instructions of the manufacturer.

2.11. Cell infection and invasion assay

Infection of HeLa cells [34] was performed following a protocol adapted from Ref. [35]. Briefly, HeLa cells (2.5×10^5 cells/well) were cultured in 12 well plates, on DMEM 10% SFB in 5% CO_2 atmosphere chamber at 37 °C. Semiconfluent cells were infected with *T. cruzi* trypomastigotes (pTEX or pTEX/*Tcr*Grx) at a ratio 5:1 (trypomastigote:cell). Non internalized trypomastigotes were removed 2 h later by washes with PBS, and fresh DMEM was added. The plates were incubated in 5% CO_2 atmosphere chamber at 37 °C. At 0 and 48 h post-infection, cultures were sampled and subjected to nuclear staining with DAPI. Cells and intracellular parasite counts were performed from epifluorecence microphotographs of several microscopy fields with CellNote application (http://cellnote.up.pt/). Experimental data are the mean of three independent experiments.

2.12. Western blotting

Protein extracts for western blotting were prepared resuspending 1×10^7 washed parasites directly in SDS-PAGE sample buffer, boiling 10 min. Protein in SDS-PAGE gels were blotted onto nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS, subsequently incubated with primary antibody at 4 °C overnight, and then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody for 1 h. Detection was carried out using Supersignal detection kit (Pierce, Rockford, IL, USA).

2.13. Localization studies

Digitonin differential membrane permeabilization protocol was performed as described in Ref. [36]. A pellet containing 10^8 parasites was treated successively with increasing concentrations of digitonin (0–4 mg mL^{-1}) in extraction buffer (20 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA and 300 mM saccharose pH 7.5). Protein fractions in the supernatants were subjected to SDS-PAGE and western blot analysis. Polyclonal sera raised against *Tcr*cTXNPx, *Tcr*mTXNPx, *Tcr*APX, *Tcr*GlcK, *Tb*cytC were used as cytoplasmic, mitochondrial matrix, endoplasmic reticulum, glycosomal and mitochondrial intermembrane space markers, respectively.

Indirect inmunofluorescence localization approach was adapted from Wilkinson and co-workers (2008) [37]. Epimastigotes stably transformed with pTEX/RFP-*Tcr*Grx and pTEX/*Tcr*Grx-RFP were settled in silanized slides and fixed with 4% (w/v) formaldehyde, and then permeabilized 10 min with 0.2% (v/v) Triton-X100 at room temperature. Permeabilised parasites were blocked with 2% (w/v) BSA and washed with PBS 1X. Afterward, slides were incubated for 1 h at room temperature with the specific localization markers:

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anti-*Tcr*mTXNPx for mitochondria, anti-*Tcrc*TXNPx for cytoplasm, anti-*Tbr*cytC for inner-mitochondrial membrane space or anti-*Tbr*Bip for endoplasmic reticulum. After washes, the slides were incubated with goat anti-rabbit DyLight 488 (Pierce, Rockford, IL, USA) secondary antibodies. Finally, after washes slides were mounted with antifade mounting solution plus DAPI, and visualized under a confocal microscope.

3. Results

3.1. Monomeric TcrGrx disulfide is reduced by glutathione, glutathionylspermidine and trypanothione

We have reported recently [15] the identification of two genes encoding two identical proteins, having identical amino acid sequence (*Tcr*Grx). We have described a procedure to recombinantly produce *Tcr*Grx at high purity degree, based on one of the mentioned sequences. The functional protein was found to have a monomeric structure, as it eluted in a single peak corresponding to a molecular mass of ~16 kDa from a Superdex 200 column, which agrees with the expected size for the recombinant protein expressed as a fusion to a histidine-tag. On the other hand, the electrophoretic profiles of *Tcr*Grx treated with different oxidants or reducing agents in non-reducing SDS-PAGE showed a similar mobility, thus indicating that *Tcr*Grx would not form covalent oligomers after redox modification. Our findings agree with previous reports on Grxs from other sources [4,38].

T. cruzi has two TXNs (TXNI and TXNII) actively involved in redox metabolism that can use various low molecular mass-thiols [22]. To explore in detail the functionality of *Tcr*Grx, we sought for similar properties in using the thiol compounds $T(SH)_2$, GSH, Gsp-SH, and dihydrolipoamide. An increasing oxidation rate of NADPH directly proportional to *Tcr*Grx concentration was observed in kinetic studies performed with these thiols, except for dihydrolipoamide. The reduction of *Tcr*Grx by $T(SH)_2$ followed second-order kinetics. The second order rate constant (k') thus calculated was about 2-fold lower than that value determined in the case of $T(SH)_2$-dependent reduction of *Tcr*TXNI (Table 1). Differently, third-order kinetics were observed for *Tcr*Grx reduction by monothiols (GSH or Gsp-SH), in agreement with the behavior observed for other members of the TRX-family [22,39,40]. When initial velocities were determined at various GSH or Gsp-SH concentrations two kinetic phases were observed for the reduction of *Tcr*Grx depending on monothiol concentration (more details in Supporting information, Fig. S1). Data were fitted to the kinetic model corresponding to the overall range of GSH concentrations [39], to obtain the parameters corresponding to each kinetic phase (Table 1). These parameters support that GSH and Gsp-SH are oxidized by *Tcr*Grx with similar catalytic performance.

On the other hand, no activity was detected when $T(SH)_2$, GSH or Gsp-SH were replaced by dihydrolipoamide, indicating a putative glutathionyl-moiety dependence for using reducing substrates.

Table 1
Kinetic constants for *Tcr*Grx reduction by low molecular mass thiols, calculated in presence of 1 mM GSSG [for $T(SH)_2$ or GSP-SH oxidation] or 100 μM TS_2 (for GSH oxidation) at pH 7.5 and 30 °C.

Redoxin	*Low molecular weight thiols*				
	$T(SH)_2$	GSH		Gsp-SH	
	k' (M^{-1} s^{-1})	k' (M^{-1} s^{-1})	k'' (M^{-2} s^{-1})	k' (M^{-1} s^{-1})	k'' (M^{-2} s^{-1})
*Tcr*TXNI	7.3×10^4	62	2.7×10^5	2.6×10^2	2.3×10^7
*Tcr*Grx	4.5×10^4	2.0×10^2	4.2×10^6	4.3×10^2	1.3×10^7

3.2. Reduction of non-protein substrates by TcrGrx, TcrGrxC31S and TcrGrxC34S

The active site of *Tcr*Grx is formed by the motif CEYC in the positions 31 to 34 of the protein. To obtain further experimental support about the participation of cysteine residues in the catalytic mechanism, two *Tcr*Grx mutants were generated. The resulting *Tcr*Grxs having the respective active sites CEYS (*Tcr*GrxC34S) and SEYC (*Tcr*GrxC31S) were kinetically studied. *Tcr*Grx-dependent reduction of non-protein disulfides, such as GSSG and CySS, were kinetically analyzed using enzymatic coupled systems. Second-order rate constants (k') thus obtained (Table 2) indicated a better catalytic performance for *Tcr*Grx in reducing GSSG than CySS, with a difference of two orders of magnitude. Moreover, second order rate constants for GSSG-reduction catalyzed by *Tcr*Grx resulted about 2000-fold higher than that corresponding to the spontaneous GSSG reduction by $T(SH)_2$ (10 M^{-1} s^{-1}) [22] at pH 7.5 and 30 °C. As shown in Table 2, the kinetic behavior of the mutant *Tcr*GrxC34S for this reaction was similar to the wild type protein, which suggests that Cys 34 is not critical for the ability of *Tcr*Grx to reduce GSSG. These results agree with previous reports determined for other dithiol Grxs [30,41]. *Tcr*GrxC31S showed no reductase activity, indicating the essential role of the Cys 31 residue for this protein functionality.

Table 2 also provides additional information about *Tcr*Grx. DHA reductase activity reported in Grxs having an active site motif CPYC [4,42], was found for *Tcr*Grx. $T(SH)_2$ dependent reduction of DHA was characterized by a second order rate constant of 22 M^{-1} s^{-1} (at pH 6.5) [43]. *Tcr*Grx-catalyzed reduction of DHA by GSH at pH 7.5, showed a rate constant two orders of magnitude higher than the spontaneous reaction (Table 2). Concerning the second-order rate constants values detailed in Table 2 for GSSG, CySS and DHA reduction by *Tcr*Grx, they are in good agreement with those rates reported for TXNs from *T. cruzi* (Table 2), *T. brucei* [28] and *Crithidia fasciculata* [44]. We also analyzed the ability of *Tcr*Grx and *Tcr*GrxC34S to reduce *S*-nitrosyl derivatives such as GSNO and CySNO (see Table 2), determining that both proteins increased by three orders of magnitude the rate constant of the direct reaction between GSNO and $T(SH)_2$ (k'_{GSNO} = 2 M^{-1} s^{-1} at pH 7.5 and 30 °C, Ref. [22]). Conversely reduction of CySNO was not catalyzed by *Tcr*Grx.

3.3. TcrGrx is a more effective reductant of glutathionylated substrates than protein disulfides

In trypanosomatids, reduction of hydroperoxides can be catalyzed by TXNPxs [45] and GPxs [46]. After hydroperoxides

Table 2
Kinetic constants for the reduction of different substrates by *Tcr*Grx, calculated in presence of 100 μM $T(SH)_2$ or 3 mM GSH (for DHA, TS_2, $(Gsp)_2$ or HEDS reduction) at pH 7.5 and 30 °C. Control assays, in absence of the respective assayed redoxin, were included (and subtracted to evaluated kinetics for Grx). N.A.: no activity was detected. (–): not determined.

Oxidant		k' (M^{-1} s^{-1})		
		*Tcr*TXNI	*Tcr*Grx	*Tcr*GrxC34S
Low molecular weight thiols	GSSG	1.5×10^4	2.5×10^4	1.6×10^4
	TS_2	–	7.0×10^3	–
	$(Gsp)_2$	–	4.0×10^4	–
	GSNO	8.7×10^2	2.3×10^3	1.3×10^3
	CySNO	1.4×10^2	N.A.	–
	CySS	1.7×10^3	2.4×10^2	–
Non thiol	DHA	1.0×10^3	1.8×10^3	–
Protein thiols	*Tcrc*TXNPx	5.7×10^5	N.A.	–
	*Tcrm*TXNPx	1.1×10^5	N.A.	–
	*Tcr*GPxI	6.1×10^5	N.A.	–
	*Tcr*MSRA10	1.2×10^4	N.A.	–
	*Tcr*MSRA180	3.2×10^4	N.A.	–

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reduction, TXN is the main reducer of the disulfide bonds formed in *Tcr*TXNPxs and *Tcr*GPxs [20,47]. We evaluated *Tcr*Grx capacity to perform the reduction of the disulfide in *Tcr*Pxs in standard coupled reactions. *Tcr*Grx neither catalyzed the reduction of *Tcr*TXNPxs (cytosolic or mitochondrial) nor of *Tcr*GPxI (Table 2), which is in agreement with the kinetic properties reported for Grx2 from *T. brucei* [16]. In a similar way, in assays of disulfide reduction in *Tcr*MSRAs, we evidenced that *Tcr*Grx could not replace *Tcr*TXN (Table 2), whose functionality has been previously probed [21]. On the other hand, *Tcr*Grx was not effective to reduce insulin in standard protein disulfide assays performed in the presence of either GSH or $T(SH)_2$, as previously reported for other Grxs, such as human Grx1 and Grx2 [42] .

On the other hand, we recently characterized, the redox regulation of a UDP-glucose pyrophosphorylase from *Entamoeba histolytica* (*Ehi*UDP-GlcPPase) [48], revealing the presence of several reactive cysteines relevant for the enzyme activity. We identified in the *T. cruzi* genome project (www.tritrypdb.org) a nucleotide sequence (Tc00.1047053506359.60) coding for a *Tcr*UDP-GlcPPase with high identity to *Ehi*UDP-GlcPPase. The recombinant *Tcr*UDP-GlcPPase was generated for enzymatic analysis (data not shown), and it exhibited kinetics and redox regulation behavior similar to that reported for *Ehi*UDP-GlcPPase [48]. Thus, *Tcr*UDP-GlcPPase was inactivated by more than 95% by 30 min incubation with 2 mM diamide, after which the activity was recovered to 82% of the initial value by 5 min treatment with 25 μM *Tcr*TXNI. Noteworthy, *Tcr*Grx was not effective for recovering activity even after 30 min incubation. When the oxidation of *Tcr*UDP-GlcPPase was performed with GSSG, a significant loss of activity was observed, suggesting that *Tcr*UDP-GlcPPase was glutathionylated.

To further explore glutathionylation of *Tcr*UDP-GlcPPase we performed the oxidation with DIEGSSG (dieosinediglutathione). This glutathione derivative has low fluorescence due to quenching phenomena, having the monomeric form high fluorescence signal. It is used to fluorescently label cysteine residues when they are glutathionylated. As shown in Fig. 1A, after treatment with DIEGSSG the band corresponding to *Tcr*UDP-GlcPPase becomes labeled with the fluorescent glutathione. No fluorescence was detected in the lane corresponding to *Tcr*UDP-GlcPPase alone, the reaction mixture without the protein or DIEGSSG reagent. On the other hand, in Fig. 1B it is shown that the signal corresponding to *Tcr*UDP-GlcPPase labeled disappear after treatment with DTT, and the level of the signal diminishes with GSH, or even more with GSH plus *Tcr*Grx treatments. In terms of enzyme activity, as shown in Fig. 1C, the loss of activity after GSSG oxidation was effectively reverted by GSH in a *Tcr*Grx mediated (and concentration-dependent) reaction. Hence, *Tcr*Grx showed a restriction towards mixed disulfide between *Tcr*UDP-GlcPPase and GSH.

3.4. Physicochemical properties determinations: redox potential of *TcrGrx* and pKa for Cys 31

Redox potential of *Tcr*Grx was determined using a method based in the equilibrium with GSH. Different ratios of oxidized *Tcr*Grx and GSH were incubated for 6 h, after which the remaining GSH concentration was estimated using DTNB reagent [26] and the GSSG generated was determined separately by a kinetic method based on NADPH-dependent reduction of GSSG by GR, using a GSSG calibration curve. The reduced and oxidized fractions of *Tcr*Grx were estimated analyzing ratios of GSH:GSSG and, with these data, the equilibrium constants for *Tcr*Grx reduction by GSH was determined [49]. Afterward, the redox potential of *Tcr*Grx was calculated from the Nernst equation: $E^{o}_{7.5\ \mathrm{TcrGRX}} = E^{o}_{7.5\ \mathrm{GSSG/GSH}} + (R \cdot T/z \cdot F) \cdot \ln([\mathrm{TcrGRX}] \cdot [\mathrm{GSSG}]/[\mathrm{TcrGRX} - \mathrm{S_2}] \cdot [\mathrm{GSH}]^2)$. Analysis of different reaction mixtures resulted in a standard redox potential at pH 7.5 of -203 ± 11 mV for *Tcr*Grx.

The obtained data were confirmed by means of Haldane relations as described by Arias et al. [22]. The apparent equilibrium constant ($K_{eq\ app}$) for the reaction: 2 GSH + $TcrGrx_{Ox}$ ↔ GSSG + $TcrGrx_{Red}$, $K_{eq\ app}$ is calculated as the ratio between the third-order kinetic constant (direct reaction) and the second-order kinetic constant (inverse reaction). Accordingly, using the Nernst equation and the $K_{eq\ app}$, a global standard redox potential at pH 7.5 for *Tcr*Grx of -180 ± 10 mV was obtained. Consequently, considering the experimental error, values obtained by both methods differ in less than 10%. For the couple GSSG/GSH a value of −270 mV at pH 7.5 was used for all calculations [6].

For several thiol disulfide oxidoreductases, the first cysteine thiol found in the active site (CXXC) is the nucleophile responsible for the reactivity [50]. In order to obtain a better knowledge about the molecular basis of *Tcr*Grx properties, the p*Ka* value of Cys 31 was determined (Fig. 2) using two independent methods: *i*) measurement at 240 nm of the thiol ionization states of Cys 31 (p*Ka* = 6.8 ± 0.2, Fig. 2A) and *ii*) pH dependence highlighted of the reduction rate of DTNB by *Tcr*GrxC34S (p*Ka* = 6.6 ± 0.1, Fig. 2B). In both cases, the p*Ka* value was consistent with the inflection point observed in the pH dependent *Tcr*Grx activity profile (Supporting

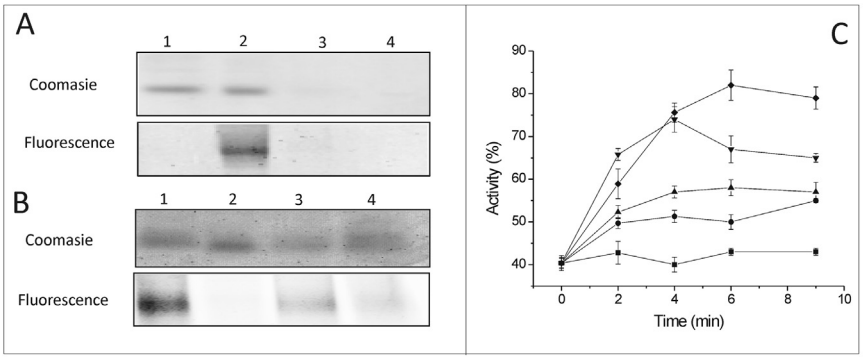

Fig. 1. *Tcr*UDP-GlcPPase glutathionylation and deglutathionylation by *Tcr*Grx. A) *Tcr*UDP-GlcPPase glutathionylation is detected by reaction with DIEGSSG. Lane 1: Control of *Tcr*UDP-GlcPPase without reagents. Lane 2: *Tcr*UDP-GlcPPase reaction with DIEGSSG (the glutathionylated form called *Tcr*UDP-GlcPPase-S-SGE). Lane 3: *Tcr*UDP-GlcPPase reaction with DIEGSSG after precipitation of proteins. Lane 4: DIEGSSG. B) *Tcr*UDP-GlcPPase deglutathionylation. Lane 1: *Tcr*UDP-GlcPPase reaction with DIEGSSG (*Tcr*UDP-GlcPPase-S-SGE). Lane 2: *Tcr*UDP-GlcPPase-S-SGE treated with DTT (0.5 mM). Lane 3: *Tcr*UDP-GlcPPase-S-SGE treated with 0.1 mM GSH, 4 U mL^{-1} glutathione reductase, 1 mM NADPH. Lane 4: *Tcr*UDP-GlcPPase-S-SGE treated 0.1 mM GSH, 4 U mL^{-1} glutathione reductase, 1 mM NADPH, plus 10 μM *Tcr*Grx. C) Recovery of *Tcr*UDP-GlcPPase activity catalyzed by *Tcr*Grx, evolution of percentage of activity recovery for pre-oxidized *Tcr*UDP-GlcPPase with 10 mM GSSG, then incubated with: 10 mM GSH (-□-), 10 mM GSH plus 2 μM *Tcr*Grx (-■-),10 mM GSH plus 5 μM *Tcr*Grx (-●-),10 mM GSH plus 20 μM *Tcr*Grx (-▲-),10 mM GSH plus 50 μM *Tcr*Grx (-◆-).

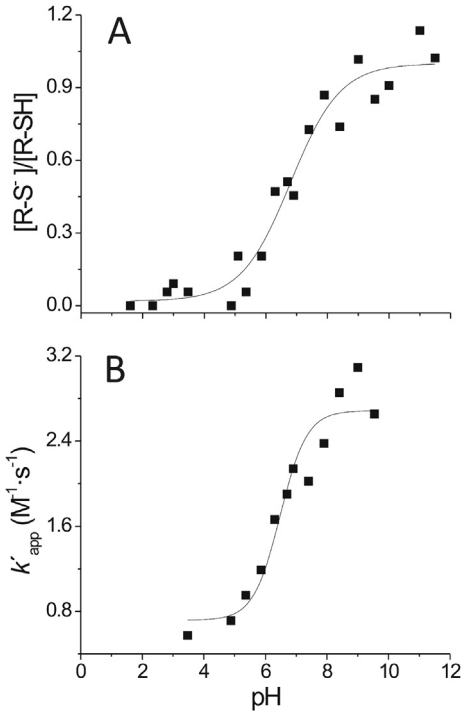

Fig. 2. Evaluation of nucleophilic cysteine thiol p*Ka* A) Nucleophilic cysteine ionization approach. Titration curve of absorption of the thiolate anion at 240 nm was obtained by subtracting ε_{240} values for oxidized *Tcr*Grx to ε_{240} values for *Tcr*GrxC34S, in a pH range from 2 to 11. B) Reaction rate for DTNB reduction by *Tcr*GrxC34S as a function of pH. The rate of DTNB reduction by *Tcr*GrxC34S was determined by incubation of reduced protein and DTNB in the reaction media at different pH values by monitoring the absorbance at 405 nm. The apparent second order rate constants were calculated and plotted against pH.

information Fig. S2), suggesting a dependence of the disulfide reductase activity with the ionization state of redox active cysteines. It is worth noting that the p*Ka* value for the nucleophilic Cys of *Tcr*Grx is higher than the p*Ka* values (average 3.0–4.0) reported for classic Grxs [30].

3.5. *TcrGrx is expressed in the different developmental stages of T. cruzi. Subcellular localization*

To further explore on the functional role of *Tcr*Grx we performed studies evaluating the relevance of the protein for the physiology of the parasite. We sought to establish the intracellular localization of *Tcr*Grx and also analyze if levels of the active protein are related with the parasite resistance to oxidative stress as well as with its virulence. It is known that the complex life cycle of *T. cruzi* includes several developmental stages in vertebrates and invertebrates host. These stages have different morphology, expression patterns and biochemical properties [51,52]. We investigated the expression of *Tcr*Grx in different morphologic stages of *T. cruzi* by using immunologic and fluorescent techniques. In all morphological stages analyzed (epimastigote, cell-derived trypomastigote and amastigote), a protein band of the expected size (ca. 15 kDa) was recognized by polyclonal antiserum produced in rabbit against pure recombinant *Tcr*Grx (Supporting information Fig. S3).

We obtained epimastigotes overexpressing *Tcr*Grx fused to red fluorescent protein (RFP) at either the N-terminus (RFP-*Tcr*Grx) or the C-terminus (*Tcr*Grx-RFP), which were subjected to co-immunolocalization and confocal microscopy (Fig. 3). Specific antibodies against proteins of known localizations were applied as compartment markers: anti-*Tcr*mTXNPx for mitochondria [45], anti-*Tcrc*TXNPx for cytoplasm [45], anti-*Tbr*cytC for inner-mitochondrial membrane space [53] or anti-*Tbr*Bip for endoplasmic reticulum [54]. The same pattern of fluorescence was observed for RFP-*Tcr*Grx and *Tcr*Grx-RFP epimastigotes (Supporting information Fig. S4). A control assay was performed using anti-*Tcr*Grx to confirm the same localization for *Tcr*Grx, RFP-*Tcr*Grx and/or *Tcr*Grx-RFP. We found fluorescence signal co-localization only when antibodies against *Tcrc*TXNPx were used, which suggests a cytosolic localization for *Tcr*Grx. Furthermore, RFP fusion (either at the N- or the C-terminus) also gave fluorescence in the nuclear compartment (Fig. 3). It is worth mentioning that the nucleotide sequences coding for *Tcr*Grx (GeneIDs: Tc00.1047053506475.116 and Tc00.1047053511431.40) did not include any sequence coding for an import signal to nucleus (or any other compartment signal). It could be presumed that *Tcr*Grx would be translocated to the nucleus following a cellular mechanism (interaction with other proteins) already described for mammalian Grx1 [55]. On the other hand, to confirm the cytosolic localization, epimastigotes of *T. cruzi* were successively subjected to lysis with increasing concentrations of digitonin [36]. Analysis of soluble fractions by western blot revealed that *Tcr*Grx was present in the protein fraction released from epimastigotes at a digitonin concentration of 0.05 mg mL^{-1}, the fraction were *Tcrc*TXNPx was also detected (Supporting information Fig. S5).

3.6. *TcrGrx is involved in the resistance to oxidative stress and in-host replication in cell cultures*

We evaluated if *Tcr*Grx overexpression in *T. cruzi* could help to cope with an oxidative environment. Thus, *T. cruzi* cells stably transfected with pTEX (empty vector), pTEX/GFP or pTEX/*Tcr*Grx were challenged by means of glucose oxidase. The addition of glucose oxidase directly to the culture medium, which contains glucose, resulted in a continuous generation of H_2O_2. Under basal condition, all three *T. cruzi* cell lines showed similar proliferation kinetics (Supporting information Fig. S6), which indicates that overexpression of GFP and *Tcr*Grx has no detrimental effect on epimastigote proliferation. It came up also from kinetics under basal condition (Supporting information Fig. S6) that the overexpression of Grx did not affect the method of measure of proliferation and viability (based in the reduction of rezasurin [33]). The overexpression of *Tcr*Grx was tested by western blot assay using specific antibody anti *Tcr*Grx (Supporting information Fig. S6-inset). Under oxidative stress conditions, epimastigote forms overexpressing *Tcr*Grx showed significantly higher viability percentages ($p < 0.05$, Fig. 4C), when compared with cultures of epimastigotes overexpressing GFP or parasites transfected only with pTEX vector (Fig. 4). This result supports the contribution of *Tcr*Grx to the general resistance against oxidative damage, even when *in vitro* kinetic study (see Section 3.2 & Table 2) indicates that it was unable to couple to *Tcr*TXNPxs or *Tcr*GPxI for detoxifying peroxides.

The involvement of *Tcr*Grx in the infectivity of *T. cruzi* was also studied. The infection assay of non-phagocytic HeLa cells was performed with *Tcr*Grx overexpressing cell-derived *T. cruzi* trypomastigotes, or control parasites transformed only with the empty vector (pTEX). After 2 h of interaction between HeLa cells and cell-derived trypomastigotes, a counting of internalized amastigotes was completed. The analysis in terms of infected cell percentages did not show differences in the infectivity of both *Tcr*Grx overexpressing trypomastigotes and the pTEX-transfected trypomastigotes (Fig. 5). We considered relevant to evaluate if *Tcr*Grx had an effect on intracellular amastigotes replication. Thus, amastigotes overexpressing *Tcr*Grx and the corresponding control were counted inside the cells at 48 h post-infection. We observed a slightly higher number of amastigotes per cell in cultures infected with the *Tcr*Grx overexpressing

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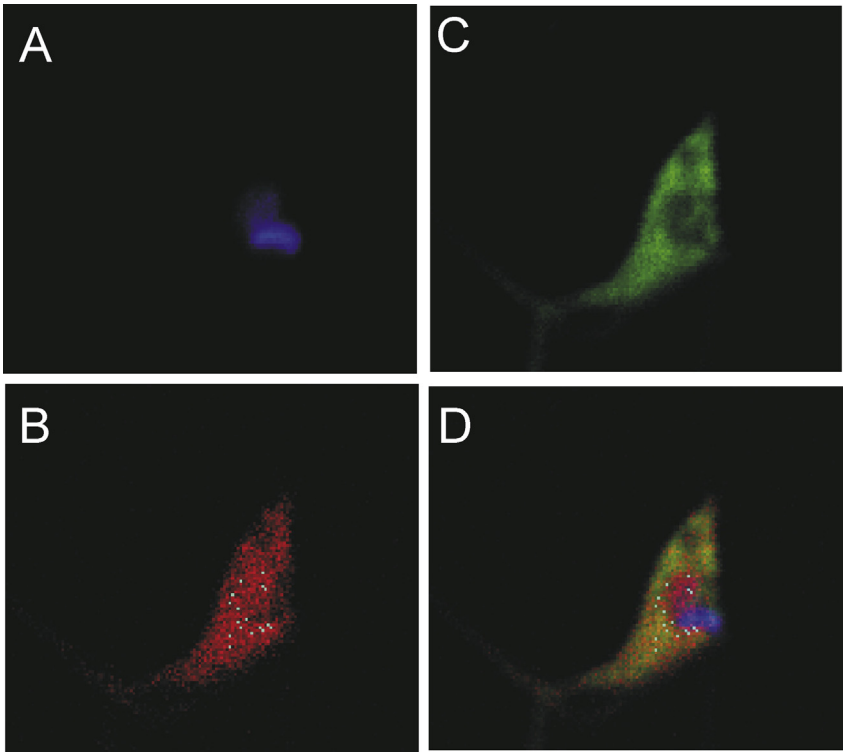

Fig. 3. Confocal microphotographs showing co-immunolocalization of cytosolic marker (*Tcrc*TXNPx) and RFP-*Tcr*Grx. A) DAPI stain. B) Localization of red fluorescence from RFP-*Tcr*Grx. C) *Tcrc*TXNPx cytosolic localization revealed with anti-*Tcrc*TXNPx primary antibodies and goat anti-rabbit DyLight 488, as a green fluorescence. D) Merged images showing co-localization of RFP-*Tcr*Grx and *Tcrc*TXNPx.

parasites (Fig. 5), the observed difference is significant at the level of 95% (*t*-test statistics). Our results suggest that *Tcr*Grx would be not essential in the process of internalization of trypomastigotes into the host cell, but point a clue about the relevance of *Tcr*Grx in the survival of parasites once they are inside the host cell.

3.7. *TcrGrx is linked to apoptosis-like cell death in T. cruzi*

We searched for other putative physiological functions for *Tcr*Grx by quantifying the magnitude of apoptotic-like cell death events generated after stimulus. Cultures of *T. cruzi* epimastigotes (stably transfected with pTEX, pTEX/GFP or pTEX/*Tcr*Grx) were stimulated to apoptosis by addition of human fresh serum (HFS) as a complement source. Different parameters (previously established as phenotypes characteristic for this kind of programmed cell death) were analyzed: *i*) morphological changes in epimastigote forms of the parasite (immediate response after stimulus) [9], *ii*) increased caspase-like activity (medium time elapsed from stimulus) [8], or *iii*) TUNEL labeling of apoptotic nuclei (long time response after stimulus) [8]. In our hands, 15 min after addition of 10% HFS into axenic cultures of *Tcr*Grx overexpressing epimastigotes, the percentage of cells with an altered morphology (spheroid shaped cells that have lost motility) was increased significantly. In all time intervals studied, it was observed a higher percentage of apoptotic epimastigotes in cultures overexpressing *Tcr*Grx than in the case of cultures of epimastigotes transfected with both pTEX/GFP and pTEX alone (Fig. 6A). This phenomenon was increased even more in cultures supplemented with 15% HFS (Fig. 6A). The differences observed were significant at a level of 95% (ANOVA-test). HFS-treated epimastigotes displayed TUNEL-labeled positive nuclei, being the levels of TUNEL-positive cells higher in cultures of parasites overexpressing *Tcr*Grx than in cultures of the microorganism transfected with pTEX empty vector. These differences (significant at a level of 90%) persisting on time (Fig. 6B), suggest that apoptotic-like cell death occurrence in *T. cruzi* could be regulated in a reaction catalyzed by *Tcr*Grx. Furthermore, we analyzed, in the above mentioned parasites lines, the levels of caspase-like activity after 3 h from apoptosis induction (Fig. 6C). The highest activity was found in epimastigotes overexpressing *Tcr*Grx. As shown in Fig. 6C, significant differences (90% of confidence) were found between epimastigotes overexpressing *Tcr*Grx before and after apoptosis induction. Such behavior was not observed in epimastigote cells transfected with pTEX empty vector were tested.

4. Discussion

The ability of *T. cruzi* to cope with oxidative stress is essential for survival during its infection to mammalian tissues [13,56,57]. Currently, there is a general understanding about the mechanisms responsible for this antioxidant resistance, but the biochemical and kinetic characterization of several actors of the redox metabolism is still incomplete. Similar to Grxs in *T. brucei* [16], *Tcr*Grx was unable to transfer reducing equivalents to 2-Cys peroxiredoxins (both cytoplasmatic and mitochondrial) or glycosomal GPxI. Recombinant *Tcr*Grx exhibited the ability to accept reducing equivalents from GSH, Gsp-SH or $T(SH)_2$; being, in its reduced status, able to reduce low molecular mass disulfides, such as GSSG or CySS. Our results suggest that GSH-dependent reduction of DHA can be catalyzed by *Tcr*Grx, which could be an alternative pathway for keeping ascorbate under its reduced form in the parasite.

Moreover, the redox regulation of enzymes activities or cellular signaling has been scarcely elucidated in trypanosomatids. We present evidence suggesting that *Tcr*Grx is functional for *in vitro* modifying the activity of UDP-GlcPPase (we have previously demonstrated this activity on a glyceraldehyde-3-Pdehydrogenase

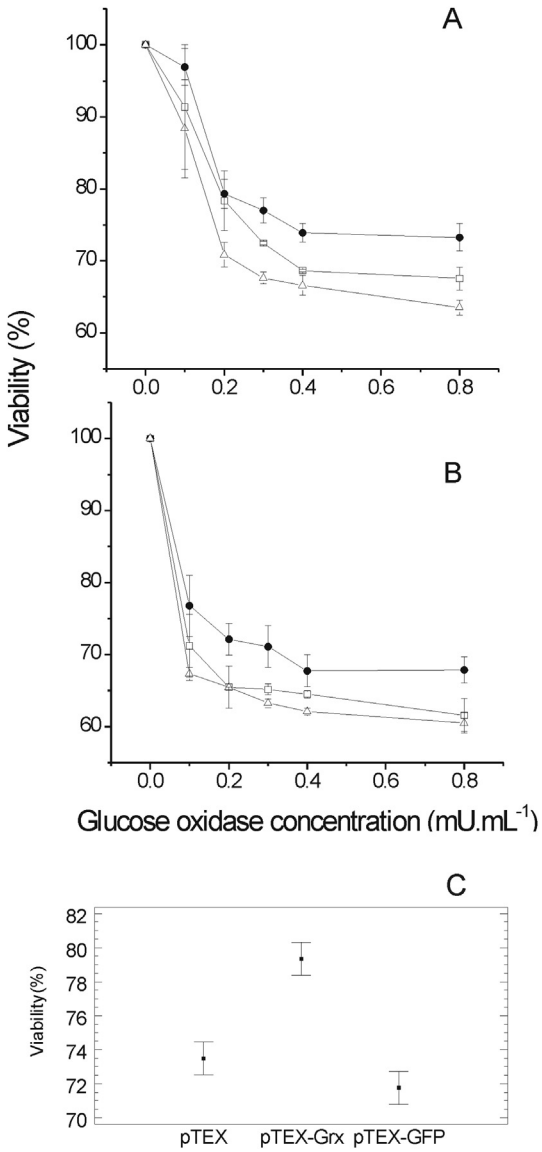

Fig. 4. *T. cruzi* epimastigotes cultures under oxidative stress. Viability percentages of cultures of *T. cruzi* cells challenged with different concentrations of glucose oxidase (0.1–0.8 mg mL^{-1}), after different times of exposition: A) 15 min B) 240 min. Cultures of *T. cruzi* were stably transfected with: pTEX (-□-), pTEX-GFP (-△-) and pTEX*Tcr*Grx (-●-) C) Results of analysis of variance for viability in cultures of cultures of *T. cruzi* stably transfected with: pTEX, pTEX-GFP and pTEX-*Tcr*Grx challenged with different concentrations of glucose oxidase (0.1–0.8 mg mL^{-1}), after different times of exposition (15, 45, 180 and 240 min). Representation of media value of viability for each parasite transformed line and LSD intervals (95% confidence), taking as variable factors time of exposition and concentrations of glucose oxidase.

from *Triticum aestivun* [15]) through glutathionylation/deglutathionylation reactions, which could be highly relevant for protecting key sulfhydryl groups in this and other proteins of the parasite. *Tcr*Grx showed a strong specificity for GSH-mixed disulfides, a property already reported for Grxs from other organisms, such as *Escherichia coli*, *Saccharomyces cerevisiae*, or mammalian cells [38,58–60]. Additionally, it exhibited differential capacity for catalyzing $T(SH)_2$ dependent reduction of *S*-nitrosothiols, with specificity for GSNO. The capacity of *Tcr*Grx for reducing GSNO could work as a part of an antioxidant system against stress generated by RNS. Possibly, GSH would react with exogenous NO, producing GSNO, which would be then regenerated by $T(SH)_2$/*Tcr*Grx or GSH/*Tcr*Grx systems to yield GSH and HNO (it reacts with

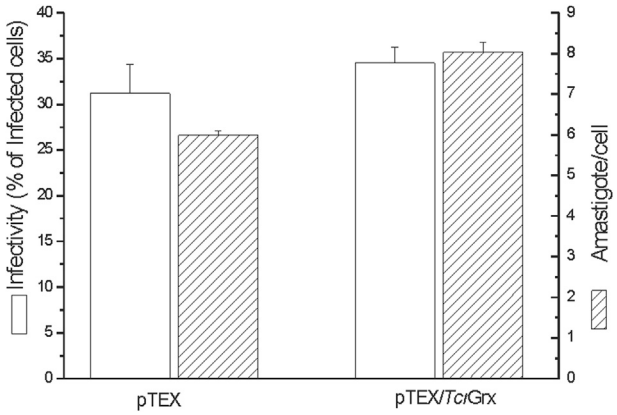

Fig. 5. Parasites infectivity and in-host proliferation. Semiconfluent monolayer of HeLa cells were infected with *T. cruzi* trypomastigotes transfected with pTEX or pTEX/*Tcr*Gr. Invasion capacity of parasites (white bar) was evaluated by counting HeLa cells containing internalized parasites immediately after infection, and calculated as the percentage of total cells that were infected. Proliferation inside host cell (striped pattern bar) was evaluated by counting intracellular amastigotes 48 h post-infection, and expressed as amastigote number per infected cell. Values represent mean of three biological replicates.

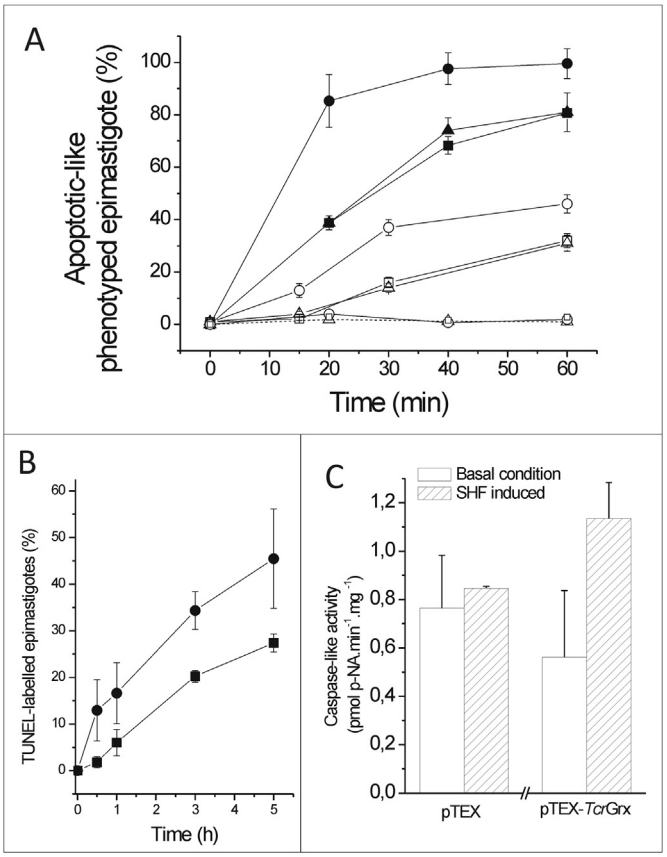

Fig. 6. Apoptosis-like programmed cell death in cultures of epimastigotes of *T. cruzi*. A) Evolution of apoptotic-like phenotyped counting, epimastigotes transfected with pTEX (-□-),pTEX-GFP (-△-) and pTEX-*Tcr*Grx (-○-) after stimulus with 10% HFS, and pTEX (-■-), pTEX-GFP (-▲-) and pTEX-GRX (-●-) after addition of 15% HFS. Evolution in control cultures with heat inactive serum is showed for pTEX (–□–), pTEX-GFP (–△–) and pTEX-*Tcr*Grx (–○–). B) Evolution of percentage of TUNEL labeled epimastigotes of *T. cruzi* transfected with pTEX (-■-) or pTEX-GRX (-●-), after induction of apoptotic-like programmed cell death with 10% human fresh serum. C) Caspase-like activity in extracts of *T. cruzi* transfected with pTEX or pTEX-GRX incubated in control condition (empty bars) or HFS treatment (striped pattern bars), performed as indicated in Experimental procedures section. Values represent mean of three biological replicates.

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O_2 to generate NO_2^-). Similar mechanisms were proposed for GSNO detoxification in *Plasmodium falciparum* and mammalian cells [61,62]. The present work provides results to support the hypothesis of an active participation of $T(SH)_2$/*Tcr*Grx or GSH/*Tcr*Grx redox pairs in the regulation of intracellular levels of GSNO from *T. cruzi* living under oxidative stress condition. Fig 7 summarizes the reaction proposed for *Tcr*Grx as a component of the redox network operating in *T. cruzi*.

The standard redox potential value of *Tcr*Grx (average value of −190 mV at pH 7.5) thermodynamically justifies the capacity of this redox protein to use $T(SH)_2$ or Gsp-SH ($E^{\circ\prime}$ of −242 mV [28]), and GSH ($E^{\circ\prime}$ of −240 mV [26]) as reducing substrates. It is coherent with a flow of reducing equivalents transported through a trypanothione-dependent system. The estimated p*Ka* value for nucleophilic cysteine of *Tcr*Grx (p*Ka* = 6.6 at 25 °C) is atypical regarding "classical" Grxs. It was previously demonstrated that residues of the dipeptide between cysteine residues in the active site play an important role in several physicochemical properties of proteins in the thioredoxin family [28,63]. The presence of a proline residue in this dipeptide is a feature of "classical" Grx [4] that is determinant for the tertiary structure of the protein and the consequent reactivity of residues around cysteines in the active site, influencing their electrostatic interactions with thiols [63]. The absence of this proline residue between Cys 31 and Cys 34 in *Tcr*Grx, could be a reason for the atypical p*Ka* value for the nucleophilic cysteine. Nevertheless, at the intracellular pH (about 7.0), more than 50% of the Cys 31 is in the thiolate state, as required for reactivity in thiol-disulfide oxidoreductases [64]. Comparing with the nucleophilicity of cysteines from other relevant thiol-disulfide oxidoreductases in the parasite a lower p*Ka* value than *Tcr*TXNI (p*Ka* = 7.0) or *Tcr*TXNII (p*Ka* = 7.3) [22], was observed for *Tcr*Grx.

By means of specific antibodies designed against *Tcr*Grx, we evidenced the occurrence of Grx in cellular extracts from *T. cruzi* epimastigote, trypomastigote and amastigote stages. The protein showed cellular localization in cytoplasm and probably occurs also in the nucleus. Probably, it performs deglutathionylation in different molecular targets in both cell compartments, a hypothesis that would be matter of future studies. We analyzed, through *in vivo* assays, the role of *Tcr*Grx in the protection of the parasite living under oxidative conditions. This task was previously described for Grxs from others organisms, for example mammalian Grx2 [65] and yeast Grxs [66]. RNAi-based experiences are usually exploited for exploring gene function in several cell types. Unfortunately RNAi machinery is not present in *T. cruzi* [67], being knockout experiments in this parasite extremely laborious and practically unreliable in several cases as a consequence of the physiologic characteristic of *T. cruzi* [68]. Overexpression of the gene of interest is frequently the viable alternative for studying gene function in *T. cruzi*. In our experiments, the overexpression of *Tcr*Grx did not affect the normal proliferation parameters of the cultures. However, under conditions of oxidative stress, parasites overexpressing *Tcr*Grx showed an improved resistance, which suggests that the protein could play a role in the response of *T. cruzi* to hostile (oxidative) environments. It is tempting to speculate that *Tcr*Grx could help to maintain both DHA and GSH intracellular (or nuclear) levels. Moreover, one of the possible mechanism for glutathionylation is catalyzed by Grxs [69], for example, human Grx2 which can catalyze thiol oxidation and glutathionylation of proteins in the mitochondrial membrane [70]. It is possible that glutathionylation catalyzed by *Tcr*Grx collaborates with sulfhydryl homeostasis, preventing irreversible oxidation of proteins [71].

We have also verified that the overexpression of *Tcr*Grx slightly improves *T. cruzi* survival inside the cell host. The evaluation of *T. cruzi* multiplication level inside infected cells has been previously used as indicator of the relevance of antioxidant molecules, like *Tcr*Grx, for the parasite viability in an oxidative environment [32,57]. Based in our experimental data, *Tcr*Grx could be also involved in a cellular pathway that connects the apoptotic-like stimulus, and the final phenotype of apoptotic-like death. As observed in other studies [5], several components of apoptosis

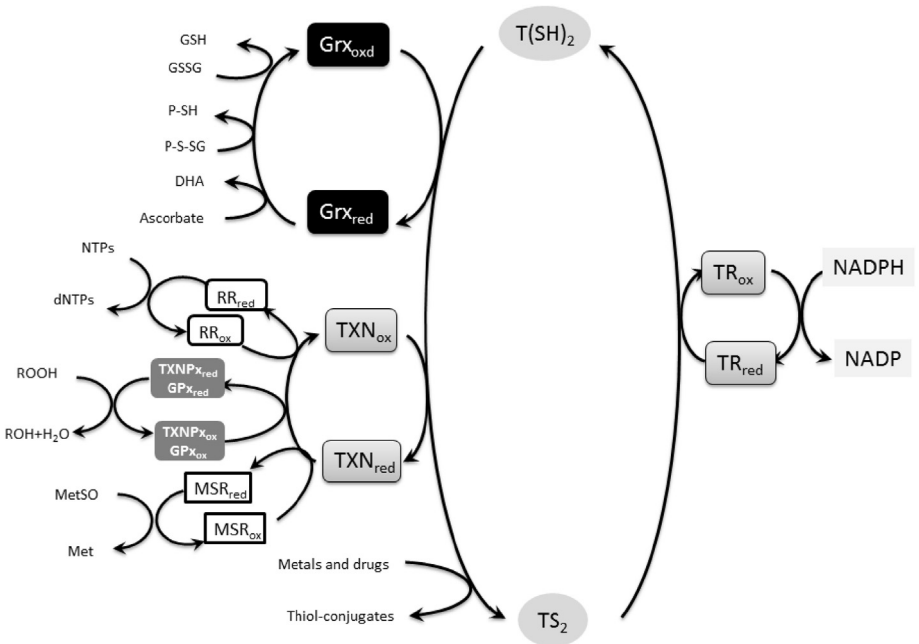

Fig. 7. Proposed reactions catalyzed by *Tcr*Grx in the redox metabolism of *T cruzi*. In this system, reduced trypanothione can transfers reduction equivalents to compounds for detoxification [12], or to oxidoreductases such as *Tcr*Grx or *Tcr*TXN [13]. *Tcr*Grx can reduce ascorbate, GSSG or glutathionylated proteins (P-S-SG), while *Tcr*TXN can reduce protein disulfides ribonucleotide reductase (RR), effector proteins against oxidative stresses for example *Tcr*TXNPx, *Tcr*GPx or reparative enzymes like *Tcr*MSR. Trypanothione is reduced by TR (trypanothione reductase) at expenses of NADPH [73].

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signaling pathways could be subject of regulation by Grxs, resulting in augmented or decreased levels of apoptosis, depending mainly on apoptotic stimulus and cells type. There is a considerable variability in the responses observed in different systems, it is clear that Grx became important for regulating cell signaling even when components subjected to regulation can vary in different contexts (such as the cell type, tissue or cellular injury). In our experiments we observed responses that suggest different roles for *Tcr*Grx as a result of the varied challenges evaluated. In trypanosomatids, the understanding of the apoptotic signaling pathways is still not completed, but different pathways are being outlined depending on the inducer (summarized in Ref. [72]). In view of these antecedents for PCD in trypanosmomatids, it will be necessary to identify *Tcr*Grx targets for establishing the actual function played by *Tcr*Grx in cell death in *T. cruzi*.

5. Conclusions

Antioxidants defences are essential for *T. cruzi* to face the oxidative environment they will find after host cells infection. Our work provides information about the biochemical and enzymatic properties of *Tcr*Grx as well as on its contribution to the parasite antioxidant defence. In *T. cruzi*, *Tcr*Grx is an oxidoreductase probably involved in reduction of glutathione, dehydroascorbic acid and mixed disulfides, such as glutathionylated proteins. Novel information reported herein proposes the participation of this Grx in redox signaling pathways in *T. cruzi*, probably through glutathionylation-deglutathionylation mechanisms. Our results support a functional relationship between *Tcr*Grx and programmed cell death in *T. cruzi*. The involvement of *Tcr*Grx in several parasite physiological processes suggests novel insights about the protein involvement in redox signaling.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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List of abbreviations

Cys	cysteine
CySNO	*s*-nitrosocysteine
CySS	cystine
DHA	dehydroascorbic acid
DIEGSSG	dieosinediglutathione
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
GFP	green fluorescent protein
Grx	glutaredoxin
Grx-S_2	glutaredoxin, disulfide form
Grx-SSR	glutaredoxin mixed disulfide
GSH	glutathione, reduced form
GSNO	*S*-nitrosoglutathione
Gsp-SH	glutathionylspermidine, reduced form
$(Gsp)_2$	glutathionylspermidine, oxidized form
GSSG	glutathione, oxidized form
HEDS	2-hydroxyethyl disulfide
HFS	human fresh serum
IMS	intermembrane mitochondrial space
PCD	programmed cell death
PDI	protein disulfide isomerase
RFP	red fluorescent protein
RNS	reactive nitrogen species
ROS	reactive oxygen species
RSH	monothiol
$T(SH)_2$	trypanothione, reduced form
*Tcr*APx	ascorbate peroxidase from *T. cruzi*
*Tcr*GPxI	glutathione peroxidase from *T. cruzi*
*Tcr*Grx	glutaredoxin from *T. cruzi*
*Tcr*GrxC31S	glutaredoxin from *T. cruzi*, mutant in Cys 31
*Tcr*GrxC34S	glutaredoxin from *T. cruzi*, mutant in Cys 34
*Tcr*MSRAs	methionine sulfoxide reductases A from *T. cruzi*
*Tcr*TXNI	tryparedoxin I from *T. cruzi*
*Tcr*TR	trypanothione reductase from *T. cruzi*
*Tcr*UDP-GlcPPase	UDP-glucose pyrophosphorylase from *T. cruzi*
TR	trypanothione reductase
Trx	thioredoxin
TS_2	trypanothione, oxidized form
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
TXN	tryparedoxin
TXNPx	tryparedoxin peroxidase

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2014.07.027.

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