The *Trypanosoma cruzi* Enzyme TcGPXI Is a Glycosomal Peroxidase and Can Be Linked to Trypanothione Reduction by Glutathione or Tryparedoxin*

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Trypanosoma cruzi glutathione-dependent peroxidase I (TcGPX) can reduce fatty acid, phospholipid, and short chain organic hydroperoxides utilizing a novel redox cycle in which enzyme activity is linked to the reduction of trypanothione, a parasite-specific thiol, by glutathione. Here we show that TcGPXI activity can also be linked to trypanothione reduction by an alternative pathway involving the thioredoxin-like protein tryparedoxin. The presence of this new pathway was first detected using diazylated soluble fractions of parasite extract. Tryparedoxin was identified as the intermediate molecule following purification, sequence analysis, antibody studies, and reconstitution of the redox cycle in vitro. The system can be readily saturated by trypanothione, the rate-limiting step being the interaction of trypanothione with the tryparedoxin. Both tryparedoxin and TcGPXI operate by a ping-pong mechanism. Overexpression of TcGPXI in transfected parasites confers increased resistance to exogenous hydroperoxides. TcGPXI contains a carboxyl-terminal tripeptide (ARI) that could act as a targeting signal for the glycosome, a kinetoplastid-specific organelle. Using immunofluorescence, tagged fluorescent proteins, and biochemical fractionation, we have demonstrated that TcGPXI is localized to both the glycosome and the cytosol. The ability of TcGPXI to use alternative electron donors may reflect their availability at the corresponding subcellular sites.

Members of the family Kinetoplastida cause a variety of diseases that afflict humans including African sleeping sickness (*Trypanosoma brucei*), Chagas’ disease (*Trypanosoma cruzi*), and visceral/cutaneous leishmaniasis (*Leishmania* spp.). The search for new drug targets is a priority because existing chemotherapeutic regimes are unsatisfactory. One potential Achilles heel is the apparent limited ability of these parasites to deal with reactive oxygen species such as the superoxide anion and various hydroperoxides (1, 2). A number of drugs in current use are believed to mediate part of their activity by inducing oxidative stress either directly by increasing the levels of reactive oxygen species (e.g. nifurtimox) (3, 4) or indirectly by affecting the components that mediate the detoxification of reactive oxygen species (e.g. difluoromethylornithine, pentamidine, and trivalent arsenicals) (5–8). Therefore, the functional analysis of the oxidative defense pathways in these parasites may be of importance in terms of improved chemotherapy.

In most eukaryotes glutathione plays a central role in protecting cells from oxidative damage. Reducing equivalents are transferred from NADPH to the hydroperoxide by the concerted action of glutathione reductase, glutathione, and glutathione-dependent peroxidases. Trypanosomatids lack glutathione reductase, and this pathway is modified (Fig. 1). They possess an analogous redox system centered upon the trypanosomatid-specific thiol trypanothione. Here trypanothione, a conjugate of glutathione and spermidine (N7,N8-bisglutathionylspermidine), is maintained as dihydrotrypanothione by the activity of the NADPH-dependent flavoprotein trypanothione reductase. Dihydrotrypanothione has been shown to drive distinct pathways. In these pathways, reducing equivalents are transferred from dihydrotrypanothione to the hydroperoxide via a two-step oxidoreductase cascade involving either tryparedoxin and peroxiredoxin enzymes (Fig. 1A) or glutathione and glutathione-dependent peroxidases (Fig. 1B) (9–14). In *T. cruzi* two distinct peroxiredoxins have been identified. These enzymes can metabolize hydrogen peroxide and short chain organic hydroperoxides generated within different compartments of the cell; one peroxiredoxin has a cytosolic location (*T. cruzi* cytoplasmic peroxiredoxin), and the other is found in the mitochondrial (*T. cruzi* mitochondrial peroxiredoxin) (11). For the glutathione-dependent peroxidase, we have identified two *T. cruzi* glutathione-dependent peroxidases, TcGPXII and TcGPXII (12, 14), enzymes that had previously been reported to be absent from trypanosomatids (1, 2, 15–17). Although both enzymes are members of the phospholipid hydroperoxide glutathione-dependent peroxidase subgroup of glutathione-dependent peroxidases, they can be distinguished from each other on the basis of sequence and their substrate specificity; both enzymes can metabolize fatty acid and phospholipid hydroperoxides, but only TcGPXI can metabolize short chain hydroperoxides. Neither TcGPXI nor TcGPXII can metabolize hydrogen peroxide (12, 14). The identification of these distinct pathways demonstrates that *T. cruzi* does possess an effective enzymatic hy-

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1 The abbreviations used are: TcGPXI, *T. cruzi* glutathione-dependent peroxidase I; TcGPXII, *T. cruzi* glutathione-dependent peroxidase II; gGAPDH, glycosomal glyeraldehyde-3-phosphate dehydrogenase; RFP, red fluorescent protein; eGFP enhanced green fluorescent protein; RACE, rapid amplification of cDNA ends; PBS, phosphate-buffered saline.
TcGPXI has dual localization in between trypanothione and TcGPXI. We also demonstrate that domain (12). This led us to speculate that some other pathway may act the glycosome and the cytosol. This distribution may account the redox pathways linked to this reaction. A, the tryparedoxin mediated pathway. Peroxiredoxins (TPx) reduce hydroperoxide (ROOH) to the corresponding alcohol (ROH) at the expense of dihydrotrypanothione, tryparedoxin (TPN) (11, 14). B, the glutathione-dependent pathway. Dihydrotrypanothione can also interact with oxidized glutathione (GSSG) to generate reduced glutathione (GSH). The glutathione-dependent peroxidases TcGPXI and TcGPXII reduce hydroperoxides at the expense of reduced glutathione (12, 14). Here we show that TcGPXI can also reduce hydroperoxides via a tryparedoxin-mediated pathway.

droperoxide metabolizing capability, a question that until recently had been open to debate (18).

Kinetic analysis of the glutathione-dependent nature of TcGPXI revealed that the enzyme has a high $K_m$ for this thiol (>5 mM) and a low activity toward the hydroperoxide substrate (12). This led us to speculate that some other pathway may act as an additional source of reducing equivalents for this enzyme. Here we describe the purification and identification of a tryparedoxin molecule that acts as an alternative redox shuttle between trypanothione and TcGPXI. We also demonstrate that TcGPXI has dual localization in T. cruzi and is present in both the glycosome and the cytosol. This distribution may account for the ability of TcGPXI to scavenge reducing equivalents from different sources.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions—**T. cruzi (MHOM/BR/78/Silvio - X10/6 or CL-Brener) epimastigotes were grown at 28 °C in RPMI 1640 medium (Sigma) containing the supplements previously described (19). Recombinant T. cruzi were maintained in the same medium containing either 200 μg of G418 ml−1 alone or 100 μg of G418 ml−1 with 20 μg of hygromycin ml−1 where appropriate.

**DNA and RNA Extractions—**T. cruzi genomic DNA was isolated from exponentially growing cells using the proteinase K/SDS method (20). Intact T. cruzi chromosomes for contour clamped homogenous electric field analysis were extracted using an agarose-embedding technique (21). T. cruzi total RNA was prepared using the guanidium thiocyanate lysis method (20). DNA was sequenced using a dye terminator cycle sequencing kit (Applied Biosystems) and fractionated on an ABI Prism 377 DNA sequencer.

**Enzyme Purification—**Recombinant His-tagged TcGPXI was purified from Escherichia coli BL-21 on a nickel-nitrilotriacetic acid column as described (12). Tryparedoxin (designated TcTPNI) was purified as follows. 15 liters of epimastigotes in the late logarithmic phase of growth were harvested, washed, and resuspended in argon-saturated, ice-cold buffer 1 (20 mM bis-Tris propane, pH 7.2, 1 mM EDTA, 1 mM dithiothreitol) containing a mixture of protease inhibitors (100 μg ml−1 phenylmethylsulfonyl fluoride, 1 μg ml−1 E-64, 0.5 μg ml−1 leupeptin, 1.7 μg ml−1 pepstatin A). The cells were lysed by repeated (three times) rounds of freeze thawing, the debris was removed by centrifugation at 13,000 × g for 20 min at 4 °C, and the clarified lysate was concentrated using Centricon YM-3 columns (Amicon). The retene was then dialyzed extensively (twice for 2 h, once overnight) against argon-saturated buffer 2 (20 mM bis-Tris propane, pH 7.2) at 4 °C. The extract was loaded onto a DEAE-Sepharose column (Amersham Biosciences) and eluted with a step gradient of KCl (0, 35, 70, and 300 mM in buffer 2). The redox active fractions were pooled and dialyzed extensively against argon-saturated buffer 3 (20 mM potassium phosphate, pH 7.2) at 4 °C. The sample was loaded onto a 2.5′ ADP-Sepharose 4B column (Amersham Biosciences), and the active, flow through fraction was collected. This was concentrated on Centricon YM-3 columns (Amicon) and then dialyzed extensively against buffer 4 (25 mM Tris-Cl, pH 7.8, 10% glycerol) at 4 °C. The sample was loaded onto a Mono Q column (Amersham Biosciences) and eluted with a linear gradient of NaCl (0–300 mM) in buffer 4.

**Identification of Tryparedoxin by Protein Sequencing—**Amino acid sequencing was carried out by Severn Biotech. Because tryparedoxin was shown to be amino-termially blocked, the protein was first treated with 2% dithiothreitol and then cleaved with CNBr in 70% formic acid. Peptides were sequenced using the Applied Biosystems 477A protein sequencer linked to a 120A phenylthiohydantoin-derivative analyzer.

**Cloning the Tryparedoxin Gene—**A DNA fragment containing TcTPNI was identified by a 3′-RACE approach using T. cruzi cDNA prepared from epimastigote mRNA with the primer ANCGTATACGTTAATC (Stratagene). A derivative of T. cruzi genomic DNA using the primers TPNI-3 (agatctTGGTTTGGCGAAGTACCTC) and TPNI-4 (aagcttTTA-

**Enzyme Assays—**Trypanothione-dependent peroxidase activity was measured by monitoring NADPH oxidation (18). A standard reaction mixture (1 ml) containing 50 mM HEPES, pH 8.0, 0.5 mM EDTA, 200 μM NADPH, 0.5 μM trypanothione reductase, 20 μM trypanothione, 2 μM recombinant TcGPXI, and 1 μM TcTPNI (recombinant or trypanosome-derived) was incubated at 30 °C for 5 min. The background rate of NADPH oxidation was determined, and the reaction was initiated by the addition of cumene hydroperoxide (Sigma). The enzyme activity was calculated using a ε value of 6220 M−1 cm−1. The peroxidase activity caused by auto-oxidation of trypanothione and any co-purified E. coli proteins was negligible. Glutathione-dependent TcGPXI peroxidase activity was carried out as described (12).
Construction of T. cruzi Vectors and Parasite Transformation—A DNA fragment encoding a TcGPXI (12) (GenBank accession number AJ313314) was amplified from T. cruzi CL-Brener genomic DNA using the primers GPXI-1 (actagtagTTGGAATCCATTTTGCCTGTGGTCTAC- TTG) and GPXI-2 (atgattCTAGACCATTCCACTTCAACACCAA). Restriction sites were incorporated into the primers to facilitate cloning of the amplified product into the T. cruzi expression vector pRIBOTEX (22).

The open reading frame encoding the red fluorescent protein (RFP) was amplified from the plasmid pDsRED-C1 (CLONTECH) using the primers RED-1 (gatattcATGGTGCGCTCCTCCAAGAA) and RED-2 (gatattcTTATCTAGATCCGGT) and cloned into the T. cruzi expression vector pTEX (25). A DNA fragment encoding the carboxyl-terminal region (last 31 amino acids) of TcGPXI was amplified from the expression vector pTrcHis-GPXI (12) and then cloned in-frame with the RFP. The entire gene fusion was then transferred across into the vector pTEX-HYb(2).

The open reading frame encoding the enhanced green fluorescent protein (eGFP) minus its stop codon was amplified from the plasmid pEFGFP (CLONTECH) using the primers GFP1 (ctgcagGTCGACTTCTCTAG) and GFP2 (TAATGCCGACTTCTAGATGATGATGATCCCTAGAG) and cloned into the T. cruzi expression vector pTEX (23). In this context, the sequence immediately 3' to eGFP encodes the carboxyl terminus of T. cruzi glyceraldehyde-3-phosphate dehydrogenase (gGADPDP). A ClaI site between these two sequences was cleaved and end-filled using the DNA polymerase I (Klenow) fragment, and the vector was religated. This produced an in-frame fusion of eGFP gene with the 3' gGADPDP sequence. The open plasmids were introduced into T. cruzi by electroporation and selected using the conditions previously described (24).

Peroxide Sensitivity Experiments—Epimastigotes in the logarithmic phase of growth were seeded at 5 x 10^6 ml^-1 into Nuncen multiwell plates (24 wells) in 2 ml of growth medium supplemented with the agent under investigation. After 5 days of growth at 27°C, the cell density of each culture was determined, and the concentration of hydroperoxide that inhibited parasite growth by 50% (IC50) was established (25). Each experiment was performed in triplicate.

Generation of Antiserum Against TcGPXI and TcTPXI—The Histagged TcGPXI or T. cruzi TcTPXI proteins were excised from Coomassie-stained SDS-PAGE gel and macerated in the presence of liquid nitrogen. The resultant material was suspended in Freund's complete adjuvant, sonicated (six times for 10 s), and then inoculated into mice (BALB/c). At 2-week intervals further inoculations were carried out using gel-purified protein suspended in Freund's incomplete adjuvant. After a total of five inoculations, the mice were bled, and the specificity of the antiserum was tested.

Localization Studies—For indirect immunofluorescence microscopy, T. cruzi in the exponential phase of growth were harvested, washed in 137 mM NaCl, 4 mM NaHPO4.1, 1.7 mM KH2PO4, 2.7 mM KCl (PBS) and fixed with paraformaldehyde (2% (w/v) in PBS). Parasites (2.5 x 10^7/ml well) were air-dried onto a microscope slide and then permeabilized with 0.1% Triton X-100 (w/v) in PBS. The parasites were then blocked with 5% fetal bovine serum (Sigma), diluted in PBS, and probed with either preimmune or antiserum against TcGPXI diluted 1:200 in blocking solution. The parasite DNA was then stained with fluorescein isothiocyanate conjugate (ImmunoResearch Laboratories) and probed with either preimmune or antiserum against TcGPXI diluted 1:200 (w/v). The specificity of the antiserum was tested.

RESULTS

Reconstitution of a Trypanothione-dependent, TcGPXI-mediated Peroxidase Activity—TcGPXI can metabolize a wide range of hydroperoxides using glutathione but not trypanothione as the direct electron donor (12). To determine whether an alternative factor could also act as an electron donor to TcGPXI, we measured the activity of a recombinant form of the enzyme in the presence of T. cruzi cell extract dialyzed extensively against 100 mM HEPES, 0.5 mM EDTA, pH 8.2 using cumene hydroperoxide as substrate. NADPH oxidation was observed when trypanothione and excess trypanothione reductase were added to the assay. This reconstitution of TcGPXI activity showed that a factor(s) within the extract could act as a redox shuttle between trypanothione and TcGPXI. To identify this molecule, 15 liters of T. cruzi epimastigotes in the late logarithmic phase of growth (Fig. 2) were harvested and lysed by three rounds of freeze thawing, and the extract was clarified. Separation of the lysate on a DEAE-Sepharose column resulted in the binding of this factor to the matrix as a fraction that could be readily eluted using 35 mM KCl (“Experimental Procedures”). The active fractions were purified further on an ADP-Sepharose column to remove any NAPDH/NADH binding enzymes and then fractionated on a Mono Q column. Elution of the proteins bound to the Mono Q matrix using a linear NaCl gradient enabled the isolation of a 16-kDa protein (Fig. 2) that was able to link TcGPXI activity to the reduction of trypanothione. The overall yields of the final purification are given in Table I. Based on the purification it would appear that the 16-kDa protein is relatively abundant constituting ~3% of the total soluble protein within T. cruzi

The Shuttle Molecule That Mediates Electron Transfer from Trypanothione to TcGPXI Is a Trypoxidin—Preliminary attempts at amino acid sequencing of the 16-kDa protein demon-
stratized that the factor was blocked at its amino terminus ("Experimental Procedures"). After cleavage with CNBr, a peptide was sequenced and shown to be identical to part of a tryaredoxin molecule that we had cloned previously and designated as TcTPNI. Sequence analysis revealed that TcTPNI has extensive identity (51–63%) to tryaredoxins from other trypanosomatids and lower identity (33%) to a second tryaredoxin from T. cruzi (designated TcTPNII) (Fig. 3). TcTPNII is also distinct from other tryaredoxins in that it contains an insertion in the sequence, the role of which has yet to be determined. One feature that all tryaredoxins share is the motif WCPPPC located near their amino terminus (Fig. 3, region A). This motif has been shown to play a crucial role in oxidoreductase redox activity (29).

Analysis of TcTPNI using PSORT did not identify any potential targeting signals, consistent with previous observations that these molecules are cytosolic in Crithidia fasciculata and T. brucei (13, 30).

The genomic organization of TcTPNI within T. cruzi was investigated by Southern hybridization and by contour clamped homogenous electric field analysis (data not shown). This indicated that TcTPNI is single copy and is located on a 1.4 megabase pair chromosome.

To confirm that TcTPNI could function as a redox shuttle, the full-length TcTPNI gene was ligated into the vector pTrcHis-C (Invitrogen) and expressed in E. coli XL-1Blue after isopropyl-β-D-thiogalactopyranoside induction. In this system, the recombinant TcTPNI is tagged at its amino terminus with a histidine-rich sequence and an epitope detectable with the anti-Xpress monoclonal antibody (Invitrogen) and can be identified as an 18-kDa protein within the soluble fraction of E. coli extracts (data not shown). The recombinant fusion protein could be readily purified by one round of affinity chromatography on a nickel-nitrilotriacetic acid column. Using recombinant TcTPNI, a series of assays were carried out aimed at reconstituting the peroxidase pathway (Fig. 4). The components of the tryaredoxin-mediated hydroperoxide metabolizing pathway were added sequentially to the reaction mixture, and TcGPXI activity was measured by following the change in NADPH oxidation. When any of the constituents of the pathway were missing, no activity was detected. In situations where all the components of this pathway (trypanothione reductase, trypanothione, TcTPNI, and TcGPXI) were present, TcGPXI activity was restored. The pathway could not be reconstituted when recombinant His-tagged TcTPNII was used in place of TcTPNI. These experiments clearly demonstrate that TcTPNI is part of the flux of reducing equivalents from trypanothione to TcGPXI.

Western blot analysis was also carried out to confirm that TcTPNI was the same factor as the 16-kDa protein that was purified from the parasite. Antisera generated against the 16-kDa T. cruzi-derived molecule ("Experimental Procedures") was used to probe blots containing recombinant TcTPNI and TcTPNII, parasite cell extracts, and the purified 16-kDa molecule itself (Fig. 5). The antisera cross-reacted with TcTPNI, and no signal was observed in lanes containing TcTPNII. A single band of 16 kDa, the same size as the purified parasite shuttle molecule, was observed in T. cruzi extracts. No other bands were detected within the cell extract, indicating that the antisera was specific to the molecule purified from the trypanosome. Thus, based upon sequence, the ability to reconstitute a TcGPXI peroxidase activity and cross-reactivity against antisera, the T. cruzi factor that links trypanothione reduction to TcGPXI activity is the tryaredoxin TcTPNI.

**Investigating the Biochemical Properties of the Tryaredoxin-mediated Pathway—**Each step within the tryaredoxin-mediated peroxidase pathway was examined in detail. Assays were carried out to investigate the role of trypanothione using a range of thiol concentrations (12.5–250 μM) with fixed levels of the other components in the system, with cumene hydroperoxide as substrate (Fig. 6, reaction I). The data indicated that trypanothione could readily saturate the pathway. When the results were analyzed using a Hanes’ plot, there was a linear relationship that allowed the apparent $K_{m}$ for trypanothione to be calculated as 40.9 ± 4.9 μM. Further analysis showed that the apparent $V_{max}$ for this reaction was 1174 ± 41 nmol NADPH oxidized min⁻¹ mg⁻¹ and that the catalytic specificity ($K_{cat}/K_{m}$) was 7.6 × 10⁵ M⁻¹ s⁻¹. These assays were extended to investigate the mechanism by which TcTPNI interacts with TcGPXI. The reactions were carried out using three concentrations of trypanothione (25, 50, and 100 μM), at various [TcGPXI] with the levels of TcTPNI and cumene hydroperoxide fixed. Double reciprocal plots were linear at all thiol concentrations (Fig. 6, reaction II). The plots did not converge but remained parallel, a pattern characteristic of a ping-pong mechanism. Secondary plot analysis allowed the $K_{m}$ for TcGPXI to be determined at 2.2 ± 0.3 μM. This also generated a $V_{max}$ value of 1989 ± 119 nmol NADPH oxidized min⁻¹ mg⁻¹ and a $K_{cat}/K_{m}$ value of 2.4 × 10⁵ M⁻¹ s⁻¹. To better understand the mechanism by which TcGPXI interacts with the hydroperoxide, assays were carried out using four concentrations of TcTPNI (0.0625, 0.125, 0.5, and 1 μM), at various concentrations of cumene hydroperoxide with the levels of trypanothione and TcGPXI fixed. As with reaction II, double reciprocal plots were linear at all TcTPNI concentrations (Fig. 6, reaction III). Again, the plots were parallel and indicated that the interaction between the peroxidase and the hydroperoxide occurred via a ping-pong mechanism. Secondary plot analysis allowed the $K_{m}$ for cumene hydroperoxide to be determined as 16.1 ± 1.0 μM. The kinetic data generated from the secondary plots yielded a $V_{max}$ of 1899 ± 155 nmol NADPH oxidized min⁻¹ mg⁻¹ and gave a $K_{cat}/K_{m}$ value of 3.5 × 10⁴ M⁻¹ s⁻¹. Analysis of the kinetic constants calculated for each reaction indicates that the rate-limiting step within the tryaredoxin-mediated pathway is the interaction between trypanothione and TcTPNI. Because the rate of hydroperoxide reduction is between 8–15-fold higher using the tryaredoxin-mediated pathway than that determined previously for the glutathione-dependent pathway (12), it would appear that tryaredoxin is more efficient at transferring reducing equivalents to TcGPXI than glutathione.

**Subcellular Localization of TcGPXI in T. cruzi**—A distinctive feature of TcGPXI is the presence at its carboxyl terminus of a SKL-type glycosomal targeting tripeptide (ARI) (31). In
trypanosomatids, the glycosome is the location of a number of biochemical pathways, including glycolysis and fatty acid β-oxidation (32–35). As an initial step to determine the subcellular localization of TcGPXI, we used antisera raised against the recombinant peroxidase and indirect immunofluorescence (Fig. 7). The specificity of the antisera was tested on blots containing recombinant TcGPXI and wild-type parasite extracts. It recognized the 18-kDa recombinant TcGPXI and a 16-kDa band corresponding to endogenous TcGPXI in lanes containing T. cruzi extracts. No other bands were detected in the cell extract. The antisera was used to stain parasite cells. These were then analyzed by confocal microscopy and showed a punctate pattern suggestive of a microsomal location (Fig. 7B, panel 2). TcGPXI was not found in the nucleus or mitochondrion or flagellum (Fig. 7A, panel 4).

To further examine the localization, a homogenized extract from a T. cruzi cell line overexpressing TcGPXI (see below) was fractionated on a continuous sucrose gradient (0.4–2 M). The fractions were collected and assayed for hexokinase, NADPH-dependent isocitrate dehydrogenase and trypanothione reductase activities. These were used as markers for the glycosome, mitochondrion, and cytosol, respectively. Hexokinase activity was associated with fractions found toward the bottom of the gradient, whereas the major trypanothione reductase activity was found toward the top (Fig. 8). NADPH-dependent isocitrate dehydrogenase activity was found between the hexokinase and trypanothione reductase active fractions. All fractions were then extensively dialyzed and assayed for TcGPXI activity using glutathione as the electron donor and cumene hydroperoxide as substrate. The predominant glutathione-dependent peroxidase activity in the TcGPXI overexpressing line (see below) was associated with fractions toward the top of the gradient (i.e. within the cytosol), although a significant proportion (26%) was found in fractions toward the bottom (i.e. the glycosome). The fractionation studies were repeated using wild type T. cruzi cultures. Here the glutathione-dependent peroxidase activity associated with the glycosomal fractions was 7-fold lower and that associated with the cytosolic fractions was 3-fold lower than that of the TcGPXI overexpressing cell line. Therefore, it can be concluded that the higher level of glutathione-dependent peroxidase activity observed with the transformed cells in the glycosomal fraction is attributable to the increased expression of the transfected TcGPXI. In three independent experiments we also noted that a small portion of trypanothione reductase activity coincided with the glycosomal fractions of the gradient (Fig. 8). It has been pointed out that trypanothione reductases possess a carboxyl-terminal extension with a weak SKL-type glycosomal targeting tripeptide (SSL for T. brucei; ASL for T. cruzi), and it has been postulated that this enzyme could have dual localization (31).

Fluorescent proteins in conjunction with confocal microscopy are a powerful tool in determining whether amino acid motifs can function as localization signals. To investigate whether this approach could be applied to T. cruzi, the genes encoding the eGFP and RFP were cloned into the T. cruzi expression vector pTEX, and the resultant constructs were used to transform epimastigotes ("Experimental Procedures"). Analysis of fixed parasites showed that expression of both proteins resulted in fluorescence throughout the cell including within the nucleus, mitochondrion, and the flagellum (Fig. 9A, panels 1 and 2). To determine whether the carboxyl-terminal region of TcGPXI, including the ARI peptide, could function as a glycosomal targeting signal, a DNA sequence encoding the last 31 amino acids of the peroxidase was cloned in-frame with the 3′-end of the RFP gene generating a RFP-GPXI fusion. As control, we used a DNA sequence encoding the last 51 amino acids from the T.
cruzi gGADPH including the carboxyl-terminal ARL tripeptide that has been previously shown to target marker proteins to the glycosome (36). This was fused to the 3′ end of eGFP producing an eGFP-gGAPDH chimera. Both recombinant genes were cloned into expression vectors and used to transform T. cruzi to generate co-expressing cell lines (“Experimental Procedures”). Analysis of cells co-expressing eGFP-gGAPDH and RFP-GPX demonstrated that both proteins gave a punctate pattern of fluorescence, although for the RFP-GPX fusion a diffuse cytosolic signal was also observed (Fig. 9 B, panels 2 and 3). With both recombinant proteins, no fluorescence was detected in the nucleus, mitochondrion, or flagellum (Fig. 9 A). When the eGFP-gGAPDH and RFP-GPX images were superimposed, the pattern suggested that both fusion proteins were co-localized in the cell (indicated by yellow staining) (Fig. 9 B, panel 4). Therefore, based on possession of a putative glycosomal targeting tripeptide at its carboxyl terminus, immunofluorescence, biochemical fractionation, and fluorescence protein-tagging studies, our data suggest that TcGPXI is found within both the glycosomal and cytosolic compartments of T. cruzi.

Overexpression of TcGPXI in T. cruzi Confers Resistance toward Exogenous Peroxides—To examine the function of TcGPXI within the parasite, the entire TcGPXI ORF was cloned into the expression vector pRIBOTEX (22), and the resultant construct was used to transform T. cruzi (“Experimental Procedures”). The presence of multiple copies of the episome within the parasite was demonstrated by Southern hybridization (data not shown), and elevated expression of the gene was confirmed by RNA hybridization (Fig. 10 A). The higher level of TcGPXI expression was also detected as an increased glutathione-dependent peroxidase activity in glycosomal and cytosolic fractions during biochemical localization studies (see above). The recombinant and wild type cell lines were grown in the presence of H₂O₂ or t-butyl hydroperoxide, and the concentration of each oxidant that inhibited parasite growth by 50% (IC₅₀) was determined (Fig. 10, B and C). For the TcGPXI overexpressing cell lines a small but significant increase in resistance toward both hydroperoxides was observed. These studies were extended to investigate the effects of the trypanocidal agents nifurtimox and gentian violet. There was no significant difference in growth inhibition between the overexpressing and control cell lines.

DISCUSSION

It is increasingly apparent that a number of peroxidases from other organisms, previously classified on the basis of activity with one electron donor, can also scavenge reducing equivalents from other sources (37–39). In common with other members of the phospholipid hydroperoxide glutathione-dependent peroxidase group of enzymes, TcGPXI lacks several residues required for efficient glutathione binding. Additionally, the kinetic constants determined for TcGPXI revealed that it has a high Kₓ for glutathione (millimolar) and a low Vₘₐₓ (12). This compared unfavorably with the cytosolic tryparedoxin-dependent peroxiredoxins from C. fasiculata and T. brucei that have reaction rates ~12-fold higher in the case of t-butyl hydroperoxide (9, 13). This led us to suggest that TcGPXI might also use alternative molecules as electron donors.
Fig. 6. Investigating the kinetic properties of the trypanoxin-dependent peroxidase pathway. Postulated scheme for the metabolism of hydroperoxides (ROOH) via a trypanoxin-mediated peroxidase pathway. Trypanoxin (TcTPNI) acts as a redox shuttle between trypanothione and TcGPXI. Trypanothione disulfide (T[SH]₂) is converted to dihydrotrypanothione (T[SH]₂⁻) at the expense of NADPH by the activity of trypanothione reductase (TR). Reaction I, interaction between trypanothione and TcTPNI. TcTPNI activity was assayed by following the oxidation of NADPH in the presence of T[SH]₂⁻ (12.5–250 μM), TcTPNI (1 μM), TcGPXI (3 μM), and cumene hydroperoxide (COOH) (20 μM). Reaction II, interaction between TcTPNI and TcGPXI. TcTPNI activity was assays in the presence of T[SH]₂⁻ (25 μM, ●, 50 μM, ○, or 100 μM, ▼), TcTPNI (1 μM), TcGPXI (0.125–10 μM), and cumene hydroperoxide (50 μM). Reaction III, interaction between TcGPXI and cumene hydroperoxide. TcGPXI activity was assayed by following the oxidation of NADPH in the presence of T[SH]₂⁻ (50 μM), TcTPNI (0.0625 μM, △, 0.125 μM ▼, 0.5 μM, ○, or 1.0 μM, ●), TcGPXI (2.5 μM), and cumene hydroperoxide (2–20 μM). All assays were initiated by the addition of the hydroperoxide. TcTPNI and TcGPXI activities are expressed as nmol NADPH oxidized min⁻¹ mg⁻¹, whereas [T[SH]₂⁻], [TcTPNI], [TcGPXI], and [cumene hydroperoxide] are expressed in μM.

(12) We therefore investigated whether other molecules present in T. cruzi extracts could activate TcGPXI.

Using dialyzed, soluble T. cruzi extracts in the presence of NADPH and cumene hydroperoxide, we were unable to reconstitute TcGPXI activity unless trypanothione and excess trypanothione reductase were added. Because trypanothione cannot act as direct electron donor to TcGPXI, it was implicit that a factor within the extract must be mediating the transfer of reducing equivalents from trypanothione to TcGPXI. Following purification and sequencing, this factor was identified as a 16-kDa factor (29). Confirmation that the 16-kDa factor and TcTPNI could readily saturate the reactions downstream. Analysis of the TcTPNI/TcGPXI and TcGPXI/hydroperoxide reactions revealed that both of these interactions occur via a ping-pong mechanism with limiting \( K_m \) values, kinetics typical of oxidoreductases. Based upon the Michaelis constant, reaction rates, and catalytic specificity, the rate-limiting step in this pathway appears to be the interaction between trypanothione and trypanoxin. This interaction has also been identified as the rate-limiting step in other trypanosomal hydroperoxide metabolizing pathways (9, 10). The mechanism of interaction between TcGPXI and hydroperoxide observed for the trypanoxin-mediated pathway is in contrast to that reported previously using glutathione as electron donor (12). These differences may be attributable to the rate that reducing equivalents are transferred along the redox chain. For the trypanoxin pathway, this flux occurs rapidly such that the overall turnover of hydroperoxide is 8–15-fold higher than that of the glutathione-mediated pathway. This suggests that in this instance the forward rate of hydroperoxide metabolism is the interaction between glutathione and TcGPXI, confirming the observations reported previously (12). Therefore, any alterations in glutathione levels would have a greater effect on the overall rate of hydroperoxide reduction than changes in the levels of trypanoxin or trypanothione. This would account for the apparent ordered kinetics observed using glutathione as opposed to the ping-pong mechanism noted here.

In most organisms, glycolysis occurs within the cell cytosol.
However, in trypanosomatids this pathway, along with pathways involved in \( \text{H}_2\text{O}_2 \)-oxidation of fatty acids, ether lipid biosynthesis, and purine salvage, is compartmentalized to the glycosome (32–35, 42, 43). Glycosomes are single membrane-bound organelles related to peroxisomes (35). Peroxisomes have been shown to be a major source of reactive oxygen species (44, 45), and most organisms have evolved a peroxisomal oxidant defense system that includes copper/zinc superoxide dismutase, glutathione peroxidase, and catalase (39, 46–48). 

*Trypanosoma cruzi* microsomal fractions have been demonstrated to generate reactive oxygen species (49), although the detoxification pathways employed have yet to be determined. Catalase has been reported to be absent from trypanosomatids (1, 2, 15–17).

The carboxyl-terminal tripeptide of TcGPXI (ARI) was identified as conforming to the SKL-type motif that has been shown to mediate protein import into glycosomes and peroxisomes (35). Peroxisomes have been shown to be a major source of reactive oxygen species (44, 45), and most organisms have evolved a peroxisomal oxidative defense system that includes copper/zinc superoxide dismutase, glutathione peroxidase, and catalase (39, 46–48). *T. cruzi* microsomal fractions have been demonstrated to generate reactive oxygen species (49), although the detoxification pathways employed have yet to be determined. Catalase has been reported to be absent from trypanosomatids (1, 2, 15–17).

The carboxyl-terminal tripeptide of TcGPXI (ARI) was identified as conforming to the SKL-type motif that has been shown to mediate protein import into glycosomes and peroxisomes. In general, the terminal tripeptide sequences required for transport into the glycosome appear to be less stringent than for peroxisomes, and it has been shown that some SKL-type tripeptides function more efficiently as targeting signals than others (31, 50). Using antiserum raised against TcGPXI in conjunction with indirect immunofluorescence, cell fractionation studies, and the tagging of fluorescent proteins (Figs. 7–9), we confirmed that TcGPXI was localized to the glycosome, although a cytosolic fraction was also observed. This dual localization may have functional significance and be related to the observation that TcGPXI can use both tryparedoxin and glutathione as electron donors. Studies on yeast and mammalian peroxiredoxin V have shown that these enzymes are distributed in the peroxisome and cytosol (39, 51–54) and can also use both thioredoxin and glutathione as an electron donor (39, 55). Because peroxisomes lack thioredoxin but do contain glutathione (41), it has been proposed that peroxiredoxin V functions as a thioredoxin-dependent enzyme within the cytosol, whereas within the peroxisome the glutathione-dependent activity predominates. It will be of interest to determine whether analogous redox pathways involving TcGPXI occur within the glycosomal and cytosolic compartments of *T. cruzi*.

To examine the role of TcGPXI, the protein was overexpressed in *T. cruzi*. We found that transformed cells had an increased resistance to exogenous 1,2-butyl hydroperoxide and \( \text{H}_2\text{O}_2 \), even though \( \text{H}_2\text{O}_2 \) is not a substrate for this enzyme (Fig. 10) (12). As an uncharged molecule, \( \text{H}_2\text{O}_2 \) can readily pass through biological membranes, and our results suggest that it...
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been shown that the parasite has evolved a number of complementary strategies for hydroperoxide metabolism (11, 12, 14, 56). Within these pathways trypanothione plays a central role acting as the source of reducing equivalents to both tryparedoxin and glutathione. The identification and characterization of the tryparedoxin-mediated TcGPXI pathway further strengthens the case for regarding trypanothione metabolism as a target for chemotherapy. However, parasites can survive even when trypanothione reductase activity is reduced by 90%, at least in the case of T. brucei (57). Other components of hydroperoxide metabolism should therefore also be considered as additional targets.

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