RNA Interference Identifies Two Hydroperoxide Metabolizing Enzymes That Are Essential to the Bloodstream Form of the African Trypanosome

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Detoxification of hydroperoxides in trypanosomines is mediated by a series of linked redox pathways that are dependent on the parasite-specific thiol trypanothione for reducing equivalents. These pathways are characterized by differences in subcellular location, electron transport molecules, and substrate specificity. To determine the functional significance of the enzymes involved, we have used a tetracycline-inducible RNA interference system to down-regulate expression of each of the corresponding transcripts in bloodstream form Trypanosoma brucei. We have identified two peroxidases, a cytosolic peroxiredoxin (TbCPX) and a member of the non-selenium glutathione-dependent peroxidase family (TbGPXI), that appear to be essential for the viability of this clinically relevant stage of the parasite life cycle. The addition of tetracycline to the cultures resulted in a major reduction in mRNA levels and enzyme activity, a dramatic fall in growth rate, and significant cell death. Furthermore, within 20 h of adding tetracycline, cells in which the cytosolic peroxiredoxin transcript was targeted were found to be 16-fold more susceptible to killing by exogenous hydrogen peroxide. We also observed that knockdown of the tryparedoxin TbT-PNI, a thioredoxin-like protein that facilitates electron transport to both TbCPX and TbGPXI, resulted in a reduction in growth rate. These experiments therefore identify redox pathways that are essential for oxidative defense in T. brucei and validate the corresponding peroxidases as targets for drug design.

Despite trypanosomatids contain significant levels of glutathione, their predominant low molecular weight thiol is a glutathione-spermidine conjugate called trypanothione (N<sup>4</sup>,N<sup>8</sup>-bis-glutathionylspermidine) (8). In conjunction with trypanothione reductase, an NADPH-dependent flavoprotein that maintains the thiol in its reduced form (9) (Fig. 1), trypanothione participates in a number of cellular processes that are carried out by glutathione in other organisms. Of these, its role in protecting the parasite from oxidative damage is perhaps the most important.

Several trypanothione-dependent hydroperoxide-metabolizing pathways have now been characterized in trypanosomatids (10–21) (Fig. 1). In all cases, transfer of reducing equivalents from trypanothione to the hydroperoxide occurs by a two-step redox cascade. Initially, electrons are transferred from trypanothione to an intermediary molecule such as tryparedoxin (a thioredoxin-like protein), glutathione, or ascorbate. These then facilitate the redox flux to the appropriate peroxidase. Five distinct trypanosomal peroxidases have been reported, each exhibiting differences in their subcellular location, substrate specificity, and electron donor.

Two members of the peroxiredoxin family of antioxidant enzymes have been identified in each of the trypanosomatids that cause disease in humans (14, 15, 20). They are located in either the cytosol or mitochondrion, use tryparedoxin as an electron donor, and can reduce substrates including H<sub>2</sub>O<sub>2</sub> and small chain organic hydroperoxides (Fig. 1, A and B). A second type of protein, related to heme peroxidases found in plants has been identified in the endoplasmic reticulum of T. cruzi (17). This enzyme has a substrate specificity restricted to H<sub>2</sub>O<sub>2</sub> and uses ascorbate as source of reducing equivalents (Fig. 1D). The remaining two proteins share extensive similarity to the non-selenium glutathione-dependent peroxidase family. One (GPXI) has been found in both the cytosol and glycosomes of T. cruzi (16) and additionally in the mitochondrion of T. brucei (21). Glycosomes are parasite-specific organelles that contain enzymes involved in a number of biochemical pathways including glycolysis and fatty acid biosynthesis (22–25). The second (GPXII), is present in the endoplasmic reticulum of T. cruzi (18). These enzymes can use glutathione as an electron donor (Fig. 1C), albeit with a low efficiency, although tryparedoxin can also act as an alternative source of electrons for GPXI from both T. cruzi and T. brucei (16, 21) (Fig. 1A). This ability to scavenge reducing equivalents from different sources may reflect the absence of one intermediary (either glutathione or tryparedoxin) from one of the subcellular locations where GPXI is found. The two T. cruzi glutathione-dependent peroxidases have a narrow substrate range, preferentially detoxifying hydroperoxides found in fatty acids and phospholipids, and...
have no activity toward H₂O₂. In contrast, the T. brucei GPXI can metabolize H₂O₂ (21). Phylogenetic analysis suggests that trypanosomal GPXIs are of plant origin and may have been acquired following an endosymbiotic event early in the evolution of the trypanosomatid lineage (18). Together, these trypanothione-dependent hydroperoxide-metabolizing pathways (Fig. 1) allow the parasite to mount an effective response to a number of oxidative insults that may arise within the cell.

Overexpression studies in T. cruzi have shown that the various peroxidases play an important role in protecting the parasite from hydroperoxide-mediated damage (14, 16, 17). In most cases, elevated levels of the peroxidase correlate with an increase in resistance toward exogenous hydroperoxides. To examine the reciprocal situation, we have employed an inducible RNA interference (RNAi) system to generate a series of T. brucei cell lines where many of the genes implicated in the above pathways can be down-regulated. These experiments have identified two peroxidases that appear to be essential for the viability of the bloodstream form of the parasite.

EXPERIMENTAL PROCEDURES

Parasites—T. brucei single marker cell line (SMB) bloodstream forms that constitutively express T7 polymerase and the tetracycline repressor protein (26) were grown at 37 °C under a 5% CO₂ atmosphere in modified Iscove’s medium (27) containing 2 μg ml⁻¹ G418. Tetracycline-free fetal calf serum (Autogen Bioclear) was used in the growth medium. DNA and total RNA were extracted from parasites using the DNeasy Tissue and RNeasy mini kits (Qiagen), respectively.

Genes—Five T. brucei genes were targeted in this study. These have been designated TbCPX (accession numbers AF283104 and AF326293) (15), TbMPX (AA828496) (15), TbGPXI (AJ298281) (21), TbTPNI (AF199570 and AJ006403) (15, 28), and TbTPNII (AC093543) and were identified from either the T. brucei genome project (available on the World Wide Web at www.sanger.ac.uk/Projects/T_brucei/) or EMBL/GenBank™ data bases. The properties of the corresponding enzymes are outlined in the Introduction.

RNAi Constructs— Fragments (between 237 and 423 bp) corresponding to the 5'-coding sequence of each gene were amplified from genomic DNA (see Table I for details) and ligated into XcmI-digested p2TT7TA-2, a TA cloning vector based on the construct p2TT7 (7) that confers hygromycin resistance (29). In these vectors, the inserted DNA is flanked by two opposing T7 promoters with each promoter under the control of a tetracycline operator. The amplified products were sequenced using a dye terminator cycle sequencing kit (Applied Biosystems) and fractionated using an ABI Prism 377 DNA sequencer. Constructs were linearized with NotI and electroporated into bloodstream form parasites, which were cloned as described (30). Transformed parasites were selected in modified Iscove’s medium containing 2 μg ml⁻¹ G418 and 2.5 μg ml⁻¹ hygromycin B. RNAi induction was initiated by adding 1 μg ml⁻¹ tetracycline to the culture. Three isoforms of TbGPXI have recently been described, each with differing amino and carboxyl termini (21). The RNAi construct generated here was designed to target the transcripts expressed by all three genes.

Detection of the Growth Inhibition Phenotype—Bloodstream form trypanosomes, transformed with each of the RNAi constructs, were seeded at 1 × 10⁶ cells ml⁻¹ and incubated at 37 °C in the presence of tetracycline. Every 24 h, parasite growth was monitored microscopically, and the culture was diluted to 1 × 10⁷ cells ml⁻¹. Control cultures incubated in the absence of tetracycline were grown in parallel. Under these conditions, untreated cell lines grew with a doubling time of 7–8 h.

Peroxide Sensitivity Experiments—Bloodstream form parasites grown in either the absence or presence of tetracycline were seeded at 2.5 × 10⁶ ml⁻¹ in a 96-well plate in 200 μl of growth medium containing 18 different concentrations of H₂O₂ over the range 1.25–1000 μM. After incubation at 37 °C for 90 min, 20 μl of the vital stain Alamar blue (BIOSOURCE UK Ltd.) was added to each well and the plates incubated for a further 6 h. The fluorescence of each culture was determined using a Gemini Fluorescent Plate reader (Molecular Devices) at an
excitation wavelength of 530 nm, an emission wavelength of 585 nm, and a filter cut-off at 550 nm. The color change resulting from the reduction of Alamar blue is proportional to the number of live cells, which was established following production of a standard curve.

Enzyme Assays—Fractionation studies were carried out on tetra-cycline-induced and -uninduced T. brucei cultures as described (31). 1-Liter cultures of bloodstream form parasites were pelleted; washed once in 25 mM Tris-Cl, pH 7.6, 1 mM EDTA, 0.32 mM sucrose (buffer A); and then resuspended in buffer A (5 × 10⁷ cells ml⁻¹) containing protease inhibitors (Roche Applied Science). Silicon carbide was added to the cell paste, and the cells were lysed in a Dounce homogenizer. Differential centrifugation was performed to remove abrasive (100 × g; 3 min) and nuclei/cell debris (1000 × g; 10 min). A final centrifugation (14,500 × g; 10 min) produced a “large granule” pellet. Linear density gradients from 0.4 to 2 M sucrose in 25 mM Tris-Cl, pH 7.6, 1 mM EDTA were layered upon a 2.5 M sucrose cushion. The large granule fraction was resuspended in buffer A and applied to the top of the gradient. Isopycnic centrifugation was then carried out using a SW40 rotor in a Beckman L8–80 ultracentrifuge at 200,000 g for 150 min at 4 °C. 1.0-mL fractions were collected and assayed for hexokinase activity (32). Glutathione-dependent and trypanothione-dependent peroxidase activities were measured by monitoring NADPH oxidation (13, 16). Fractions were extensively dialyzed against 25 mM Tris-Cl, pH 7.6, 1 mM EDTA prior to analysis. For glutathione-dependent peroxidase (TbGPXI) activity, a standard reaction mixture (1 mL) containing 100 mM Tris-Cl, pH 7.6, 5 mM EDTA, 0.2 mM β-NADPH, 1 mM Na₂SO₃, 10 mM GSH, 0.1% (v/v) Triton X-100, 1.4 units of glutathione reductase, and the dialyzed fraction 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). For trypanothione-dependent peroxidase (TbCPX) activity, a standard reaction mixture (1 mL) containing 50 mM HEPEs, pH 7.6, 0.5 mM EDTA, 200 μM NADPH, 0.5 μM trypanothione, 50 μM trypanothione reductase, and the dialyzed fraction 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 H₂O₂ (Sigma). In both cases, enzyme activity was calculated using an ε value of 2220 M⁻¹ cm⁻¹. Protein concentrations were determined by the BCA protein assay system (Pierce).

RESULTS

Generation of the T. brucei RNAi Cell Lines—Several enzymes involved in hydroperoxide metabolism in T. brucei have now been identified and characterized at the biochemical level. Using an inducible RNAi system we set out to determine their importance for parasite viability. The full-length nucleotide sequences of four of the corresponding genes were identified from the EMBL/GenBank™ data base. These were the cytosolic (TbCPX) and mitochondrial (TbMPX) peroxiredoxins (15), a non-selenium glutathione-dependent peroxidase (TbGPXI) (21), and a trypanorxin (TbTPNII) (28). In addition to the above, we also identified a previously uncharacterized tryparedoxin in the T. brucei genome project data base (available on the World Wide Web www.sanger.ac.uk/Projects/T_brucel) after BLAST analysis with the T. cruzi TeTPNII sequence (16) (accession number AAF04973). The deduced T. brucei protein possesses two distinctive signatures that identify it as a member of the tryparedoxin family of oxidoreductase proteins (33–35) (Fig. 2). First, it contains a WCPC motif that in other tryparedoxins functions as the redox active center of the molecule, and second, it has several conserved amino acids that are proposed to be involved in binding trypanothione (Fig. 2). TbTPNII differs from most other tryparedoxins in that it possesses an insertion sequence and a hydrophobic carboxyl-terminal extension (Fig. 2). Both of these features are also present in the T. cruzi counterpart. The precise roles of both of these proteins and the redox pathways in which they participate have yet to be elucidated. The presence of a home-containing ascorbate-dependent peroxidase homologous to that reported in T. cruzi (17) (Fig. 1D) has yet to be confirmed in T. brucei.

DNA fragments from each of the above genes were cloned into the trypanosomal RNAi vector pGTTTA (see “Experimental Procedures”). Constructs were transformed into bloodstream form T. brucei SMB (26), and integration into the genome was confirmed by Southern hybridization (data not shown).

Knockdown of TbCPX and TbGPXI Severely Curtails Cell Growth—To examine whether RNAi-mediated down-regulation of any of the targeted genes affected the growth rate of bloodstream form T. brucei, the cumulative cell density of tetracycline-treated parasites was followed and compared against untreated cultures (Fig. 3). In the absence of tetracycline, all five recombinant cell lines were found to grow at approximately the same rate as the parental cells. When tetracycline was added, there was no major alteration in the growth rate of the parasites that had been transformed with the TbMPX and TbTPNII constructs (Fig. 3A). In contrast, induction of double-stranded RNA corresponding to TbCPX had a dramatic effect (Fig. 3B). Within 24 h, a significant reduction in the growth rate of these cells could be observed. In the next 24 h, most of the cells in the population died, and those that remained alive exhibited greatly decreased motility. The experiment was repeated with a second clone. There was a similar rapid decline in growth rate, such that 2 days after exposure to tetracycline, the cumulative cell density of treated parasites was less than 1% that of the noninduced cultures. Interestingly, in the third and subsequent days after induction, we observed an outgrowth of viable parasites in the case of this clone (Fig. 3B). This type of reversion has been observed previously following induction of RNAi against essential genes in T. brucei (36, 37).

In cells where TbGPXI was down-regulated, there was also a major effect on growth rate. Induction of RNAi resulted in a >99% reduction in the cumulative cell density obtained over a period of 3–6 days, depending on the clone examined (Fig. 3C). Three isoforms of TbGPXI have been identified (21). The 268-bp fragment inserted into the RNAi vector is 95% identical across the three genes, including a stretch of 134 nucleotides where the sequence is completely conserved. It can be inferred that each of the corresponding transcripts should be equally susceptible to RNAi-mediated knockdown (see Fig. 4). The cell line in which the transcript encoding the tryparedoxin TbTPNII had been targeted also displayed a significant reduction in growth rate following tetracycline treatment (Fig. 3D). However, the decline in the cumulative cell density was less than that exhibited by the TbCPX and TbGPXI cell lines. In addition, we did not observe any obvious signs of cell death or see an effect on motility.

To confirm that the effects induced by tetracycline were associated with down-regulation of the target transcript, RNA from each of the cell lines was examined by Northern blotting. RNA was isolated from cells that had been maintained in the presence of tetracycline for 4 days. In the case of the TbCPX cell line, RNA had to be isolated after only 20 h of treatment due to the induction of cell death at later time points. For all of the
genes examined, there was a significant reduction in the level of the targeted transcript (Fig. 4). In two cases, TbMPX and TbGPXI, in addition to the disappearance of the endogenous transcript, we observed the appearance of a smaller band on the autoradiographs (Fig. 4). Previous T. brucei RNAi studies have also noted the appearance of smaller hybridizing RNAs in tetracycline-treated cultures (37, 38). These molecules have been reported to correspond either to the RNAi transcript itself or to fragments produced by the degradation of the endogenous targeted mRNA.

For parasite cell lines where the TbCPX and TbGPXI mRNA transcripts were down-regulated, we also investigated whether the corresponding enzyme activity was reduced (Table II). With the TbCPX cells, homogenized extracts from tetracycline-induced cultures were used for enzyme assays. The results showed a significant decrease in the activity of the targeted enzymes, consistent with the reduction in mRNA levels. The same trend was observed for the TbGPXI cells.

Fig. 2. Sequence analysis of TbTPNII. The sequence of TbTPNII was aligned with other tryparedoxins. The residues that are common with the TbTPNII sequence are represented by dots; dashes represent gaps in the sequence made to optimize the alignments. The conserved WCPPC motif is highlighted (33, 34), and the amino acids implicated in trypanothione-binding are marked (+) (35). The hydrophobic carboxyl-terminal extension is indicated by a bar, and the boxed region identifies the insertion present in T. brucei and T. cruzi TPNIIs. TbTPNII, T. brucei tryparedoxin II, AC093543; TcTPNII, T. cruzi tryparedoxin II, AAF04973; TcTPNI, T. cruzi tryparedoxin I, CAC85916; CfTPNI, C. fasciculata tryparedoxin I, AAD20445 and AAC72299; TbTPNI, T. brucei tryparedoxin I CAA07003.

Fig. 3. Growth of RNAi cell lines. The growth of induced and noninduced T. brucei was monitored daily over a 6-day period (see “Experimental Procedures”). For all of the recombinant lines, cell counts were performed on three independent cultures from which the mean cell density was determined. The data are expressed as a percentage growth difference between the tetracycline-treated and control cultures. A, comparison of parental (P), RNAi-MPX (M), and RNAi-TPNII (TII) cell lines. B, comparison of parental cell line (P) and two RNAi-CPX (C1 and C2) clones. C, comparison of parental cell line (P) and two RNAi-GPXI (G1 and G2) clones. D, comparison of parental (P) and an RNAi-TPNI cell line (TI).

Fig. 4. Down-regulation of gene expression by RNAi. Blots containing 5 μg of T. brucei total RNA from tetracycline-induced (+) and noninduced (−) cell lines were hybridized with radiolabeled probes as indicated. RNA loading was judged by hybridization using T. brucei tubulin.
duced and uninduced parasites (generated as for RNA isolation) were first fractionated on a continuous sucrose gradient, and fractions were assayed for hexokinase and thiol-dependent peroxidase activities (see “Experimental Procedures”). The highest trypanothione-dependent peroxidase activity in untreated controls, using H$_2$O$_2$ as substrate, was observed in fractions corresponding to the cytotoxic part of the gradient. This is in agreement with the previously determined location of TbCPX (15). Induction of RNAi against the TbCPX transcript resulted in a 7.3-fold reduction in this peroxidase activity within 20 h. For TbGPXI, the precise location of this enzyme has yet to be determined, although it has recently been suggested that it may be present at multiple sites within the parasite (21). In the TbGPXI experiments, the total glutathione-dependent peroxidase activity was found to be 3.8-fold lower in dialyzed extracts derived from tetracycline-treated cells compared with untreated controls. Although T. brucei does express a second glutathione-dependent peroxidase, designated TbGPXII, this enzyme does not have any activity toward cumene hydroperoxide, the substrate used in this study. 3 In all cases, the hexokinase activities of RNAi-induced cultures were comparable with untreated controls (Table II). These experiments therefore confirm a link between tetracycline-induced down-regulation of TbCPX and TbGPXI mRNA and a reduction in the corresponding enzyme activities.

TbCPX Has a Major Role in Protecting Bloodstream Form T. brucei from H$_2$O$_2$—The overexpression of hydroperoxide-metabolizing enzymes has shown that they play an important role in protecting T. cruzi from exogenous oxidants (14, 16, 17).

Here we investigated whether down-regulation of the activity of these enzymes in T. brucei altered sensitivity to H$_2$O$_2$. The parasite cell lines were treated with tetracycline under the same conditions that were used when examining mRNA levels and then exposed to H$_2$O$_2$ for 90 min (see “Experimental Procedures”). This allowed the concentration of oxidant that killed 50% of the cells (LD$_{50}$) to be determined (Fig. 5). In all but one case, there was no significant difference between induced and noninduced cells (the LD$_{50}$ values ranged from 140 to 160 μM). However, with the TbCPX cell line, treatment with tetracycline for 20 h led to a 16-fold increase in susceptibility of bloodstream forms to killing by H$_2$O$_2$ (LD$_{50}$ of untreated parasites, 147 ± 3 μM; LD$_{50}$ of treated parasites, 9 ± 1 μM).

### DISCUSSION

In T. brucei, RNAi techniques have proved to be a rapid and flexible method for addressing biological function, particularly in cases where “double-knockout” approaches could result in a lethal phenotype (36, 37, 39–42). Here we have used an inducible RNAi system to assess the degree of functional redundancy within the redox pathways involved in hydroperoxide metabolism (Fig. 1) as a step toward the identification and validation of new drug targets. Our results indicate that the peroxidases TbCPX and TbGPXI are both essential for survival of bloodstream forms of the parasite. We were able to correlate the observed phenotype to a reduction of the corresponding mRNAs and enzyme activity (Figs. 3 and 4 and Table II). The effects observed when the TbCPX transcript was targeted were particularly striking and suggest that the corresponding redox pathway is the major mechanism for protecting T. brucei from H$_2$O$_2$. RNAi-mediated knockdown of the TbCPX transcript resulted in considerable cell death within 48 h, presumably because the parasite is rendered unable to deal with endogenous production of this oxidant. Induction of RNAi was also associated with a 16-fold increase in susceptibility to exogenous H$_2$O$_2$. In both sets of circumstances, it can be inferred that TbGPXI and other hydroperoxide-metabolizing systems are unable to compensate for the reduction in TbCPX levels.

When TbGPXI was down-regulated by RNAi, this did not result in an increased susceptibility to killing by H$_2$O$_2$. When considered together with the results obtained with the TbCPX cell line, this suggests that within bloodstream form trypanosomes, TbGPXI does not have a direct or critical role in detoxifying this peroxide. Both TbCPX and TbGPXI are capable of metabolizing H$_2$O$_2$ in vitro, and both enzymes are present in the cytosol (15, 16, 21). However, TbGPXI has also been detected in mitochondrial fractions, and the T. cruzi homologue is known to be glycosomal as well as cytosolic. On the basis of sequence, TbGPXI is a member of the phospholipid peroxidase

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**Table II**

| Parasite cell line | Assay | Tetracycline | Activity | Ratio (−TET/+/TET) |
|-------------------|-------|-------------|----------|-------------------|
| RNAi-TbGPXI       | GPX   | 34          | 3.8      |                   |
|                   |       | +           | 9        |                   |
|                   |       | Hexokinase  | 846      | 1.0               |
| RNAi-TbCPX        | CPX   | 377         | 7.3      |                   |
|                   |       | +           | 52       |                   |
|                   |       | Hexokinase  | 928      | 1.0               |
Down-regulation of Hydroperoxide Metabolism in Trypanosomes

subgroup of glutathione-dependent peroxidases. In T. cruzi, the analogous enzyme (TcGPXI) has a substrate specificity that is restricted to fatty acid, phospholipid and short chain organic hydroperoxides, and the major biological role of this enzyme appears to involve minimizing the effects of secondary oxidation (e.g. by preventing damage caused by lipid peroxidation) (13). Therefore, the significant growth inhibition that is observed 3–6 days following knockdown of TbGPXI may result primarily from cumulative membrane damage arising from the reduced capacity of the cell to reverse lipid peroxidation.

When expression of the mitochondrial peroxidase TbMPX was lowered, no changes in growth rate or sensitivity to exogenous H$_2$O$_2$ were observed. (Figs. 3 and 5). The apparent non-essential role of TbMPX probably reflects a reduced oxidative burden in the mitochondrion of the bloodstream form of the parasite and correlates with a cessation in many mitochondrial associated functions, including the respiratory cycle (43–46). Therefore, either the level of hydroperoxide generation within the mitochondrion in the bloodstream form of the parasite is insufficient to result in lethal damage, or the loss of TbMPX mitochondrial activity can be complemented by other mechanisms such as nonenzymatic interactions with thiols or a possible TbGPXI mitochondrial redox pathway. It will be of interest to investigate whether this peroxidase is essential for viability in the trypanosomatida Leishmania and T. cruzi, parasites in which the mitochondrial is active during the mammalian stages of the life cycle.

The tryparedoxin TbTPNI mediates transfer of reducing equivalents from trypanothione to TbCPX and TbGPX (15, 21) (Fig. 1). It can also participate in other pathways including acting as a ribonucleotide reductase (47, 48). When TbTPNI gene expression was reduced after RNAi induction, growth inhibition was observed (Fig. 3), although the extent was less significant than that seen when TbCPX or TbGPX were down-regulated. There was also no change in the sensitivity of these cells to exogenous H$_2$O$_2$ (Fig. 5). The less pronounced phenotype changes observed in this experiment may reflect the high levels of TbTPNI in the cell and an inability of RNAi to reduce this to a critical level. In other trypanosomatids, TPNI can account for 3–5% of the total soluble protein (10, 16). A second possibility is that other classes of tryparedoxin molecules or alternative sources of reducing equivalents could partially compensate for the reduced levels of TbTPNI (49, 50). For example, glutathione is able to act as a source of electrons to TbGPX (Fig. 1), although less efficiently than TbTPNI (16, 21). We also tested a second tryparedoxin, TbTPNII. This protein, which contains a number of novel features (Fig. 2), appears to be nonessential to bloodstream form functions. The function of TbTPNII and the redox pathways in which it participates are unknown. In T. cruzi, however, we have shown that in vitro the analogous protein TcTPNII is able to act as a source of electrons to the mitochondrial peroxidase TcMPX but not to TcGPX (16).

In other eukaryotes, multiple enzyme-mediated mechanisms operate to protect subcellular compartments from hydroperoxide-mediated damage. For example, in mammalian cells, both glutathione and thioredoxin-dependent peroxidases function to remove H$_2$O$_2$ from the mitochondria, whereas in the peroxisome of plants, catalases and ascorbate-dependent peroxidases perform the same role (51–55). The presence of these dual pathways may reflect biochemical differences, such that one pathway is effective when peroxide levels are low, whereas the other system functions optimally at higher concentrations (16, 56). The overlapping and complementary nature of these pathways has been demonstrated. It is possible to generate viable cell lines/organisms completely lacking or having reduced levels of a peroxidase, a process that is often accompanied by an increase in the activity of the alternative pathway (56–60). In these situations, the phenotypic effect of deleting one pathway is often not evident until the cell line/organism is exposed to oxidative stress. Based on the lethal effects observed when TbCPX and TbGPX are down-regulated, T. brucei apparently lacks such functional redundancy in some areas of its hydroperoxide metabolizing capacity. This has obvious implications for drug development.

The enzyme trypanothione reductase is central to hydroperoxide metabolism (Fig. 1) and has been found to be essential in all the trypanosomatida so far examined (61–63). However, in T. brucei, trypanothione reductase activity has to be reduced by >90% to observe a major effect on growth rate. Therefore, any drug designed to target this enzyme would have to be extremely effective to be pharmaceutically successful (63). Based on our data, additional components of the parasite oxidative defense system can now also be considered as valid targets for chemotherapy. The cysteine peroxidase TcCPX would appear to have most promise, particularly since the availability of a three-dimensional structure for this enzyme (64) should aid drug design.

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