TcGPXII, a glutathione-dependent *Trypanosoma cruzi* peroxidase with substrate specificity restricted to fatty acid and phospholipid hydroperoxides, is localized to the endoplasmic reticulum

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Until recently, it had been thought that trypanosomes lack glutathione peroxidase activity. Here we report the subcellular localization and biochemical properties of a second glutathione-dependent peroxidase from *Trypanosoma cruzi* (TcGPXII). *TcGPXII* is a single-copy gene which encodes a 16 kDa protein that appears to be specifically dependent on glutathione as the source of reducing equivalents. Recombinant TcGPXII was purified and shown to have peroxidase activity towards a narrow substrate range, restricted to hydroperoxides of fatty acids and phospholipids. Analysis of the pathway revealed that TcGPXII activity could be readily saturated by glutathione and that the peroxidase functioned by a Ping Pong mechanism. Enzyme reduction was shown to be the rate-limiting step in this pathway. Using immunofluorescence, TcGPXII was shown to co-localize with a homologue of immunoglobulin heavy-chain binding protein (BiP), a protein restricted to the endoplasmic reticulum and Golgi. As the smooth endoplasmic reticulum is the site of phospholipid and fatty acid biosynthesis, this suggests that TcGPXII may play a specific role in the *T. cruzi* oxidative defence system by protecting newly synthesized lipids from peroxidation.

Key words: hydroperoxide metabolism, reactive oxygen species, trypanothione.

INTRODUCTION

The protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas' disease. It has been estimated that 16–18 million people in Latin America are infected with the parasite, making this a major public heath problem [1]. Throughout its life cycle *T. cruzi* is exposed to a variety of reactive oxygen species (ROS). These can cause cellular damage directly by reacting with various biological macromolecules, or indirectly by generation of the highly reactive hydroxyl radical via the transition metal-mediated Haber–Weiss and Fenton reactions. In addition to ROS produced by various endogenous reactions, *T. cruzi* can also be exposed to oxidants produced as a result of external factors, including drug metabolism. The drugs currently used in the treatment of Chagas' disease, benznidazole and nifurtimox, are unsatisfactory as they often have toxic side effects and can fail to clear parasitaemia. The precise mode of action of these drugs is unknown, but nifurtimox has been shown to undergo redox cycling within the parasite [2–4]. This, coupled with the apparently limited ability of *T. cruzi* to metabolize peroxides [5], has led to the suggestion that functional dissection of the parasite's oxidative defence system may be of importance in terms of improved chemotherapy [6–8].

Many aspects of the oxidative defence system of *T. cruzi*, particularly those involved in hydroperoxide metabolism, are distinct from the pathways used by other organisms. For example, the parasite has been reported to lack catalase, an enzyme that plays a front-line role in detoxifying ROS in most other eukaryotes [6,7,9–11]. In addition, it displays unique properties in terms of thiol content and the mechanism of thiol reduction. *T. cruzi* does contain glutathione, the major thiol found in most organisms, but lacks glutathione reductase activity. Instead the parasite has evolved an analogous system in which the trypanosomatid-specific thiol trypanothione (N^1,N^8-bisglutathionylspermidine) is maintained as dihydrotrypanothione by the activity of the NADPH-dependent flavoprotein trypanothione reductase [12]. Dihydrotrypanothione acts as source of reducing equivalents to glutathione through a disulphide-exchange reaction that can occur spontaneously [13] and via a reaction catalysed by Tc52, a trypanothione-glutathione thioltransferase that shares motifs with glutathione S-transferases of other organisms [14].

A number of different hydroperoxide detoxification pathways have now been characterized in *T. cruzi*. In all cases trypanothione has been shown to play a pivotal role by facilitating the flux of reducing equivalents to either tryparedoxin (a thioredoxin-like molecules specific to trypanosomatids) or glutathione. These molecules then transfer electrons to the peroxidase. In *T. cruzi*, two members of the 2-Cys peroxiredoxin family have been identified and localized to the cytosol (*T. cruzi* cytosolic peroxiredoxin; TcCPX) and mitochondrion (*T. cruzi* mitochondrial peroxiredoxin; TcMPX) [15,16]. Enzymes of this class have been shown to be tryparedoxin-dependent and appear to function as general hydroperoxide scavengers. A second type of enzyme in *T. cruzi* is related to glutathione-dependent peroxidases (GPXs)

Abbreviations used: ROS, reactive oxygen species; GPX, glutathione-dependent peroxidase; TcGPX, *T. cruzi* GPX; cGPX, cytosolic GPX; PHGPX, phospholipid hydroperoxide GPX; Ni-NTA, Ni^{2+}-nitrilotriacetic acid; BiP, homologue of immunoglobulin heavy-chain binding protein; TcCPX, *T. cruzi* cytosolic peroxiredoxin; TcMPX, *T. cruzi* mitochondrial peroxiredoxin; ER, endoplasmic reticulum; CHEFE, contour-clamped homogeneous electric field electrophoresis.

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The nucleotide sequence for TcGPXII reported in this paper has been deposited in the EMBL/GenBank Nucleotide Sequence Database under accession number AJ313313.

found in other organisms. This was an unexpected finding as GPXs have been reported to be absent from *T. cruzi* [6,7,9–11]. The enzyme *T. cruzi* GPX I (TcGPXI) was shown to metabolize a wide range of hydroperoxides using either glutathione or tryparedoxin as immediate electron donors [17,18]. The ability to accept reducing equivalents from different donor molecules may have functional significance, as this enzyme has also been shown to have a dual localization and is found within the cytosol and glycosome.

Here we report the identification and functional analysis of a second *T. cruzi* GPX (TcGPXII). We demonstrate that TcGPXII is localized to the endoplasmic reticulum (ER) and that it is distinct from other trypanosomal peroxidases, since its activity can be linked to trypanothione reduction only by glutathione. Moreover, TcGPXII can metabolize fatty acid and phospholipid hydroperoxides, but not short-chain alkyl hydroperoxides, suggesting that it may have a specific role in protecting cellular lipids from ROS-mediated damage.

EXPERIMENTAL

Isolation of parasite DNA and RNA

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 described previously [19]. Genomic DNA was isolated using the proteinase K/SDS method [20], and chromosomes for contour-clamped homogeneous electric field electrophoresis (CHEFE) analysis were extracted using an agarose-embedding technique [21]. DNA was sequenced using a dye terminator cycle-sequencing kit (Applied Biosystems) and fractionated with an ABI Prism 377 DNA sequencer. Total RNA was prepared using the guanidinum thiocyanate lysis method [20].

Isolation of *TcGPXII*

A DNA fragment encoding a *T. cruzi* GPX (designated *TcGPXII*) was amplified from parasite epimastigote RNA using a sense primer corresponding to the *T. cruzi* spliced leader sequence (GGATCCACAGTTTCTGTACTATTG) and the antisense primer GPX2-1 (AAGCTTACTGCGGCCAAGGCGTCACATC). Restriction sites (underlined) were incorporated into the primers to facilitate cloning of the amplified product into pBluescript KS(−) (Stratagene). A derivative of *TcGPXII* was amplified from *T. cruzi* genomic DNA using the primers GPXII-5 (AGATCTGGGCAGCAGCACGCTGTTCG) and GPXII-1 (AAGCTTTCATGCACCCCGTTGCGGCCC). The amplified product was cloned into the *Bgl*II and *Hin*dIII sites of the *Escherichia coli* expression vector pTrcHis-C (Invitrogen).

Heterologous expression and purification of His-tagged TcGPXII

E. coli BL-21, transformed with the plasmid pTrcHis-GPXII, was grown in NZCYM broth (Sigma) and protein expression induced by isopropyl β-D-thiogalactoside [17]. Recombinant His-tagged TcGPXII was affinity purified on a Ni^{2+}-nitrilotriacetic acid (Ni-NTA) matrix column under native conditions as recommended by the manufacturer (Qiagen). The cell-lysis, column-wash and elution steps were all carried out in the presence of protease inhibitors (100 $\mu g \cdot ml^{-1}$ PMSF, 1 $\mu g \cdot ml^{-1}$ E-64, 0.5 $\mu g \cdot ml^{-1}$ leupeptin and 1.7 $\mu g \cdot ml^{-1}$ pepstatin A). Fractions were analysed by SDS/PAGE. Protein concentrations were determined by bicinchoninic acid protein assay system (Pierce). Cultures of non-transformed *E. coli* BL-21 were treated as above in control experiments.

Enzyme activity

GPX activity was measured by monitoring NADPH oxidation [17,22]. A standard reaction mixture (1 ml) containing 100 mM Tris/HCl, pH 8.0, 5 mM EDTA, 0.2 mM β-NADPH, 1 mM NaN_3, 3 mM glutathione, 0.1 % (v/v) Triton X-100, 1.4 units of glutathione reductase and 2.5 μM TcGPXII was incubated at 30 °C for 5 min. The background rate of NADPH oxidation was determined and the reaction initiated by the addition of the hydroperoxide substrate. The non-enzymic activity due to the auto-oxidation of glutathione and the activity of any potentially co-purified *E. coli* proteins were also examined. The enzyme activity was calculated using $\epsilon = 6220\ M^{-1} \cdot cm^{-1}$. Data were analysed by fitting to a rectangular hyperbola (Kinenort program, supplied by Dr A. G. Clark, University of Wellington, Wellington, New Zealand).

Trypanothione-dependent peroxidase activity was assayed in a similar fashion except that 20 μM dihydrotrypanothione and 0.5 μM trypanothione reductase were used in place of glutathione and glutathione reductase. Trypanothione-dependent peroxidase activity was also assayed in the presence of dialysed soluble *T. cruzi* extract (250 μg) or recombinant His-tagged tryparedoxin (2.5 μM) [15]. Positive controls for the activity of a trypanothione-dependent redox cycle were carried out as described in [15].

The hydroperoxides tested as substrate were linoleic acid hydroperoxide, L-α-phosphatidylcholine hydroperoxide, cumene hydroperoxide, t-butyl hydroperoxide and H_2O_2. Linoleic acid hydroperoxide and L-α-phosphatidylcholine hydroperoxide were prepared from linoleic acid and L-α-phosphatidylcholine (both from Sigma), respectively, using soya-bean lipoxidase (also from Sigma) [17,22].

Generation of TcGPXII antiserum

The His-tagged TcGPXII protein was excised from a Coomassie Brilliant Blue-stained SDS/PAGE gel and macerated in the presence of liquid nitrogen. The resultant material was suspended in Freund's complete adjuvant (Sigma), sonicated (6 × 10 s), then inoculated into BALB/c mice. Further inoculations were carried out at 2-week intervals using gel-purified TcGPXII suspended in Freund's incomplete adjuvant (Sigma). After a total of five inoculations the mice were bled and the specificity of the antiserum was tested.

Localization by immunofluorescence

Trypanosomes in the exponential phase of growth were harvested, washed twice in PBS and fixed with paraformaldehyde [2 % (w/v) in PBS]. Parasites (2.5×10^5/well) were air-dried on to a microscope slide and then permeabilized with methanol for 2 min at −20 °C followed by a 30 min incubation in PBS containing 1 % saponin and 10 $mg \cdot ml^{-1}$ heat-treated RNase. The cells were then blocked with 10 % fetal bovine serum (Sigma) diluted in PBS and co-labelled with anti-TcGPXII serum (1:200) and anti-TbBiP serum [raised against the *Trypanosoma brucei* homologue of immunoglobulin heavy-chain binding protein (BiP); 1:400] raised in rabbits (a gift from James D. Bangs, University of Wisconsin-Madison Medical School, Madison, WI, U.S.A.). Dilutions were carried out as indicated. After 90 min, the slides were washed extensively in PBS and then incubated for 60 min with Alexa-Fluor 488 goat anti-mouse and Alexa-Fluor 546 goat anti-rabbit sera (Molecular Probes), both diluted 1:400. The parasite DNA was then stained with 5 μM TOTO-3 (Molecular Probes) in PBS containing 1 % saponin

and 10 mg · ml^{-1} heat-treated RNase. Parasites were visualized using a Zeiss LSM 510 microscope.

RESULTS

Isolation of TcGPXII from *T. cruzi*

An expressed sequence tag sequence (accession number AI046216) from the *T. cruzi* genome project was identified as encoding the C-terminal region of a putative GPX. A primer was designed to the adjacent 3′ untranslated region and used with the *T. cruzi* spliced leader sequence in a reverse transcriptase-PCR with *T. cruzi* epimastigote RNA as a template (see the Experimental section for details). This generated a fragment of 550 bp. Sequence analysis identified an open reading frame of 480 bp with the potential to encode a 16 kDa protein (designated TcGPXII; Figure 1). Examination of the TcGPXII sequence showed that it contained three regions characteristic of the GPX family of enzymes (Figure 1, regions A, B and C). Studies have shown that in GPXs each of these regions contains an amino acid that contributes to the catalytic triad that mediates peroxidase activity. The amino acids contributed by regions B and C, glutamine and tryptophan, are conserved in all members of this family [23]. However, a number of GPXs, particularly those of mammalian origin, contain the amino acid selenocysteine in region A, whereas many other GPXs contain a cysteine. A substitution of this nature has been shown to affect both the peroxidase activity and sensitivity towards peroxide inactivation [24,25]. TcGPXII contains cysteine. Despite the similarity at the catalytic core, GPXs show considerable variation elsewhere in their sequences. This, together with their substrate specificity and subcellular localization, has allowed these enzymes to be divided into a number of groups. Amino acid alignments between members of two groups, the cGPXs (cytosolic GPXs) and PHGPXs (phospholipid hydroperoxide GPXs), indicate that many of the observed differences could be attributable to two insertions in the cGPXs that are not present in the PHGPXs (Figure 1, regions I and II). These insertion sequences may mediate oligomerization since cGPXs are dimeric/tetrameric whereas PHGPXs are monomeric [23]. In addition, they may also be the basis of the observed differences in substrate specificity; PHGPXs can metabolize phospholipid hydroperoxides whereas cGPXs cannot. Using this criterion, TcGPXII appears to belong to the PHGPX group. However, TcGPXII has no greater similarity toward either clade (sharing 31–37% identity with a range of PHGPXs and cGPXs, including another GPX from *T. cruzi*, TcGPXI [17]). A search of the *T. brucei* genome project databases (http://www.sanger.ac.uk/Projects/T_brucei and http://www.tigr.org/tdb/mdb/tbdb) identified two sequences (AL465487 and AQ944786) that, when assembled, correspond to a homologue of TcGPXII (designated TbGPXII). As with the *T. cruzi* peroxidase, TbGPXII contains a cysteine

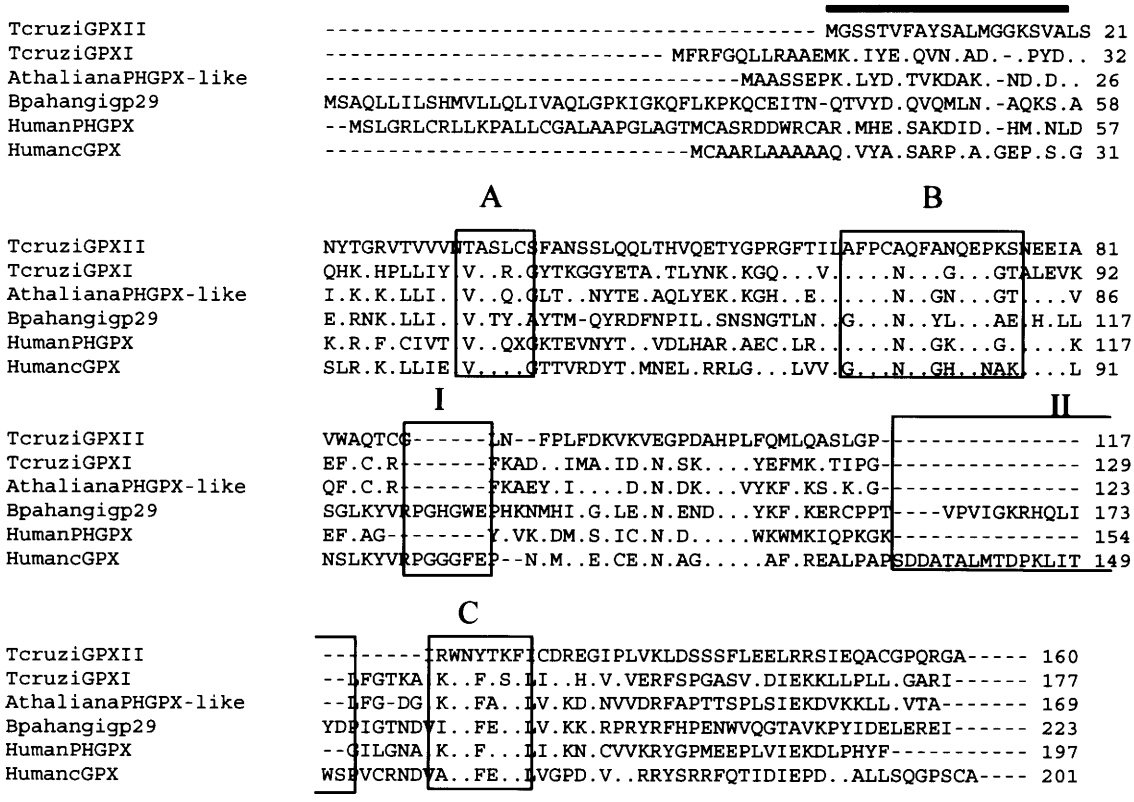

Figure 1 Sequence alignment of TcGPXII with other GPXs

TcruziGPXII, *T. cruzi* GPXII (accession no. AJ313313); TcruziGPXI, *T. cruzi* GPXI (AJ313312); AthalianaPHGPX-like, *Arabidopsis thaliana* PHGPX-like protein (BAA24226); Bpahangigp29, *Brugia pahangi* surface glycoprotein (S23062); humanPHGPX, human PHGPX (NP002076); humancGPX, human cGPX (P07203). The residues that are common with the TcGPXII sequence are represented by dots; dashes represent gaps in the sequence made to optimize the alignments. Differences between the sequences when compared with the TcGPXII are indicated. A, B and C represent the regions containing residues implicated in the redox activity of these enzymes [23]. The putative TcGPXII signal sequence is indicated by a bar. The X in region A represents a selenocysteine residue. I and II represent regions that are missing in members of the PHGPX clade.

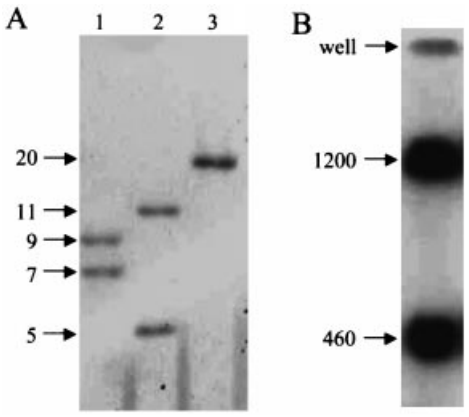

Figure 2 Genomic organization of *TcGPXII*

(**A**) *T. cruzi* CL-Brener genomic DNA digested with *Eco*RI (lane 1), *Sal*I (lane 2) and *Hin*dIII (lane 3). (**B**) CHEFE-separated chromosomes from *T. cruzi* CL-Brener were analysed by Southern hybridization using *TcGPXII* as a probe. Sizes given are in kb.

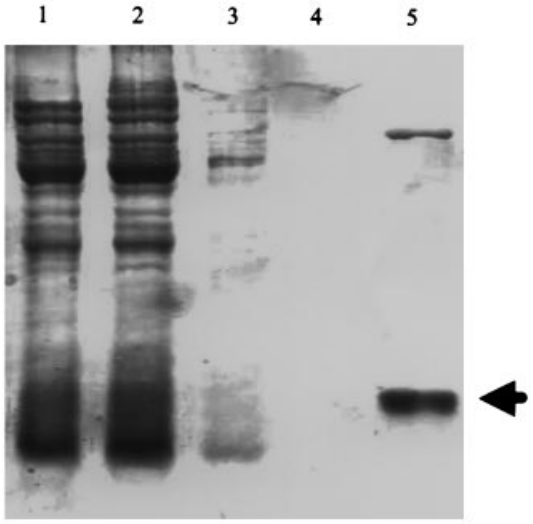

Figure 3 Purification of recombinant TcGPXII on a Ni-NTA affinity column

Fractions obtained at various stages of the purification of TcGPXII were resolved by SDS/PAGE (10% gel) and visualized by Coomassie Brilliant Blue staining. A clarified fraction of an *E. coli* BL-21 (pTrcHis-GPXII) cell lysate after 3 h of isopropyl β-D-thiogalactoside induction (lane 1) was loaded on to a Ni-NTA column and the flow-through collected (lane 2). The column was washed extensively with 20 mM imidazole (lanes 3 and 4). The recombinant protein was eluted at 200 mM imidazole (lane 5). The band of 18 kDa (indicated) corresponds to recombinant TcGPXII.

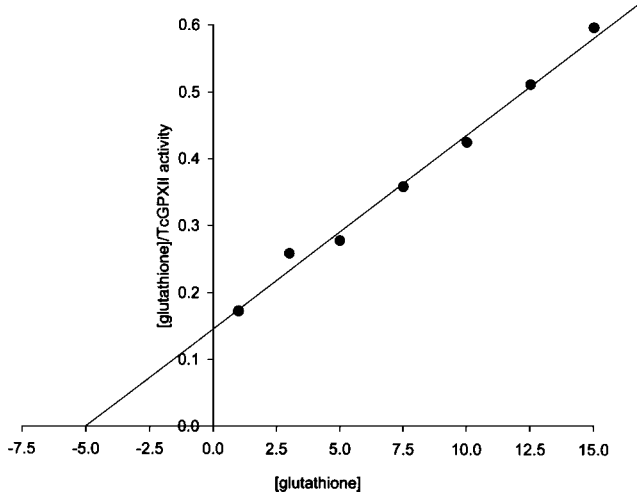

Figure 4 Saturation of TcGPXII activity with glutathione

TcGPXII activity was assayed by following NADPH oxidation [17,22] in the presence of various concentrations of glutathione (1–15 mM), 1.4 units of glutathione reductase and 2.5 μM TcGPXII. The reactions were initiated by the addition of a fixed concentration (20 μM) of linoleic acid hydroperoxide. TcGPXII activity is expressed as nmol of NADPH oxidized · min^{-1} · mg of protein^{-1} and [glutathione] is expressed in mM.

residue in region A (see Figure 1) and also lacks the two insertions present in the mammalian cGPX. Comparison between the two trypanosomal sequences indicate that they share 51% identity.

The genomic organization of *TcGPXII* was determined by Southern hybridization and by CHEFE analysis. This indicated that *TcGPXII* is a single-copy gene localized on two chromosome homologues of 1200 and 460 kb (Figure 2). With certain restriction enzymes, such as *Eco*RI and *Sal*I, two bands corresponding to the two allelic forms were detected (Figure 2A, lanes 1 and 2). Interestingly, the cytosolic iron superoxide dismutase gene has also been shown to be located on these two chromosome homologues [26].

Heterologous expression and enzyme activity

To investigate the biochemical properties of TcGPXII, the gene was cloned into the vector pTrcHis-C and expressed in *E. coli* BL-21 (see the Experimental section for details). In this system, recombinant TcGPXII is tagged at its N-terminus with a histidine-rich sequence and an epitope detectable with the anti-Xpress monoclonal antibody (Invitrogen). The 18 kDa recom-

Table 1 Activity of TcGPXII towards different substrates

Glutathione-dependent peroxidase assays were performed in triplicate using 10 different hydroperoxide concentrations with a fixed concentration of glutathione (3 mM). The data were analysed statistically using the Kinenort program. The values for the apparent K_m and V_{max} values ($\pm$S.E.M.) are given. An apparent V_{max} of $<$ 0.33 nmol of NADPH oxidized · min^{-1} · mg of protein^{-1} is at the limit of detection.

Substrate	Apparent K_m (μM)	Apparent V_{max} (nmol of NADPH oxidized · min^{-1} · mg of protein^{-1})
Linoleic acid hydroperoxide	0.66 ± 0.04	13.43 ± 0.15
L-α-Phosphatidylcholine hydroperoxide	2.6 ± 0.35	16.77 ± 0.79
t-Butyl hydroperoxide	–	< 0.33
Cumene hydroperoxide	–	< 0.33
Hydrogen peroxide	–	< 0.33

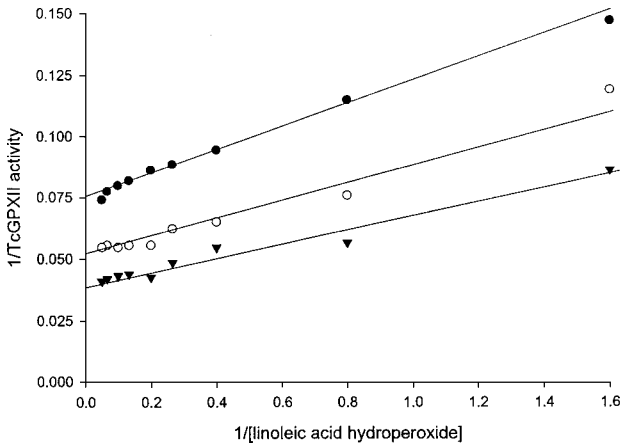

Figure 5 Kinetic properties of TcGPXII against linoleic acid hydroperoxide

TcGPXII activity was assayed by following NADPH oxidation [17,22] in the presence of 3 mM (●), 5 mM (○) or 10 mM (▼) glutathione. The reactions were initiated by the addition of linoleic acid hydroperoxide (0.625–20 μM). TcGPXII activity is expressed as nmol of NADPH oxidized · min^{-1} · mg of $protein^{-1}$ and [linoleic acid hydroperoxide] is expressed in μM.

binant protein could be readily purified by one round of affinity chromatography on a Ni-NTA column (Figure 3).

The substrate specificity of TcGPXII was investigated using a glutathione/glutathione reductase redox cycle. TcGPXII activity was measured by following the change in NADPH oxidation in response to various hydroperoxides. Assays were carried out using either purified recombinant TcGPXII or extracts of non-transformed *E. coli* BL-21 that had been affinity purified in parallel. The control assays accounted for any non-enzymic background due to auto-oxidation and also any *E. coli* peroxidase activity that had been co-purified along with TcGPXII. Where a TcGPXII-specific activity was detected, the apparent K_m and V_{max} values were determined by plotting 1/TcGPXII activity against 1/[hydroperoxide] at a fixed concentration of glutathione (3 mM; Table 1). Such plots were linear in each case. TcGPXII exhibited a high affinity for phospholipid and fatty acid hydroperoxides. No activity towards H_2O_2 or short-chain hydroperoxides, such as cumene hydroperoxide and t-butyl hydroperoxide, was detected, although we cannot exclude the possibility that these hydroperoxides may inactivate TcGPXII.

To determine whether TcGPXII activity could be saturated by glutathione, assays were carried out using various concentrations of glutathione (1–15 mM) with a fixed amount of linoleic acid hydroperoxide (20 μM). A Hanes plot yielded a linear relationship from which the K_m for glutathione was determined to be 5.0 ± 0.7 mM (Figure 4). Further analysis of the data gave a V_{max} of 34.4 ± 1.7 nmol of NADPH oxidized · min^{-1} · mg^{-1} and a catalytic specificity (k_{cat}/K_m) value of 2.0×10^3 $M^{-1} \cdot s^{-1}$. These assays were extended to investigate the type of kinetics that TcGPXII displays towards the hydroperoxide. Here peroxidase activity was measured at three different glutathione concentrations (3, 5 and 10 mM) over a wide range of linoleic acid hydroperoxide concentrations. Plots of 1/TcGPXII activity against 1/[linoleic acid hydroperoxide] generated a series of linear, almost parallel, plots indicative of the Ping-Pong-type kinetics exhibited by mammalian GPXs (Figure 5) [27,28]. Analysis of the data by secondary plot analysis gave a true K_m for linoleic acid hydroperoxide of 0.67 ± 0.07 μM. This also generated a true V_{max} of 38.9 ± 2.1 nmol of NADPH oxidized · min^{-1} · mg^{-1} and gave a k_{cat}/K_m of 1.8×10^4 $M^{-1} \cdot s^{-1}$. Examination of the kinetic values indicates that the rate-limiting step for this pathway is the interaction between glutathione and TcGPXII. The rate of hydroperoxide metabolism determined for this system is comparable with the glutathione-dependent activity shown by TcGPXI [17]. It has been demonstrated recently that TcGPXI

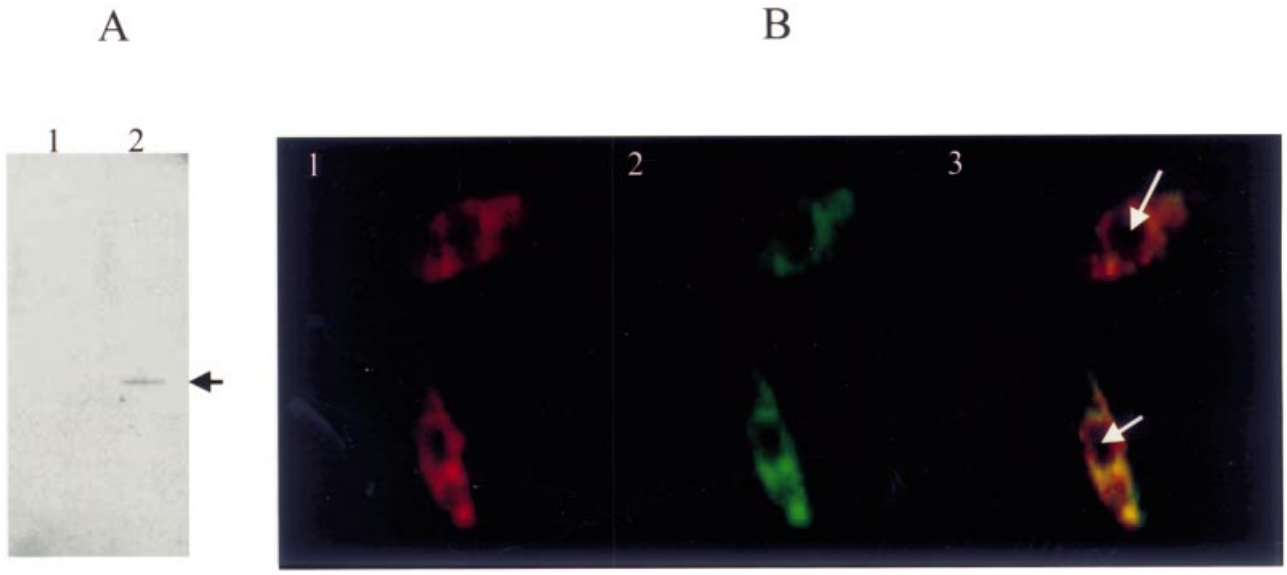

Figure 6 Immunolocalization of TcGPXII to the ER in *T. cruzi* epimastigotes

(**A**) Specificity of the TcGPXII antiserum was examined by probing blots containing 10 μg of soluble (lane 1) and 10 μg of pellet (lane 2) fractions of *T. cruzi* lysates. Protein loading was judged by Amido Black staining. The arrow indicates the single band identified in the pellet fraction (16 kDa). (**B**) *T. cruzi* epimastigote cells (2.5×10^5/well) were fixed and dried on to microscope slides. After permeabilization, parasites were stained with antiserum raised against *T. brucei* BiP (red; panel 1) and antiserum raised against recombinant TcGPXII (green; panel 2). Merged images show co-localization of TcGPXII and BiP (yellow; panel 3). The white arrows indicate the position of the nucleus, as judged by TOTO-3 staining (results not shown).

can utilize both glutathione and tryparedoxin to link hydroperoxide metabolism back to trypanothione reduction [17,18]. To determine whether TcGPXII had similar properties, enzyme activity was measured in the presence of other trypanosome-derived factors. Replacement of glutathione and glutathione reductase with trypanothione and trypanothione reductase did not reconstitute the peroxidase activity. When these experiments were extended to examine if TcGPXII-mediated hydroperoxide metabolism could be linked to trypanothione reduction by the addition of dialysed soluble parasite extracts or recombinant tryparedoxin [18], no activity was detected (results not shown). Therefore, TcGPXII activity could only be reconstituted in the presence of a glutathione redox cycle.

Localization of TcGPXII within *T. cruzi*

The subcellular location of TcGPXII was examined using antiserum raised against the recombinant protein. The specificity of the antiserum was tested on blots containing recombinant TcGPXII and *T. cruzi* extracts. It recognized the 18 kDa recombinant TcGPXII (results not shown) and a 16 kDa band corresponding to endogenous TcGPXII in lanes containing *T. cruzi* extracts (Figure 6A). No other bands were detected in the cell extract. Staining of fixed permeabilized parasites with anti-TcGPXII resulted in a punctate pattern throughout much of the cell, particularly around, but not within, the nucleus. There was no labelling in the flagellum. This type of pattern is similar to that reported for proteins localized to the ER [29,30]. To confirm this observation, *T. cruzi* cells were co-labelled with anti-TcGPXII and antiserum raised against the *T. brucei* chaperone BiP (anti-TbBiP) [29] (Figure 6B, panels 1 and 2). When the images were superimposed, the pattern of co-localization (Figure 6B, yellow staining) indicated that BiP was always present in TcGPXII-containing compartments, although BiP was also observed in other vesicular structures (Figure 6B, panel 3). Three-dimensional image analyses confirmed this co-localization (results not shown). TcGPXII contains a hydrophobic N-terminal that could act as a signal peptide (Figure 1) but lacks either the conventional (KDEL) or trypanosomal (MDDL) C-terminal ER retention tetrapeptide [29,31]. It will be of interest to determine the mechanism by which TcGPXII is retained within this organelle.

DISCUSSION

Investigation of the oxidative defence system of *T. cruzi* has revealed previously that this parasite expresses cytosolic, mitochondrial [15] and glycosomal peroxidases [18], whose activity can be linked to the reduction of the parasite-specific thiol trypanothione. Here we demonstrate that *T. cruzi* expresses a fourth peroxidase, TcGPXII, whose activity is distinct from the other trypanosomal peroxidases. It has a substrate range restricted to phospholipid and fatty acid hydroperoxides, is solely dependent upon glutathione as source of reducing equivalents and is located in the ER.

To identify the rate-limiting step within the TcGPXII pathway, we investigated the biochemical mechanisms by which the peroxidase interacts with glutathione and the hydroperoxide substrate (Figures 3 and 4). The amino acid substitution cysteine/selenocysteine (Figure 1, region A) has been shown to affect the kinetic properties exhibited by peroxidases towards glutathione. For many selenium-dependent GPXs, including mammalian cGPX and PHGPX, the interaction with glutathione occurs via pseudo-first-order kinetics, whereas the activity of non-selenium enzymes such as *Brugia* GPX and TcGPXI can be readily saturated by this thiol [17,27,28,32]. Here we found that glutathione can readily saturate TcGPXII activity but with a low affinity (K_m of 5.0 mM). Analysis of the TcGPXII/linoleic acid hydroperoxide interaction revealed that the enzyme has a high affinity for the substrate (as judged by its low true K_m) and functions via a Ping Pong mechanism, typical of oxidoreductases [27,28]. Comparison of the Michaelis constant and catalytic specificity suggest that the pathway is limited predominantly by the glutathione-mediated reduction of oxidized TcGPXII, analogous to that observed for TcGPXI [17]. The reaction rates for the TcGPXI and TcGPXII-mediated glutathione pathways were comparable.

The recent observations that GPXs from other organisms can use alternative molecules as electron donors [18,33–35] led us to investigate whether TcGPXII displayed such properties. Previously we have demonstrated that both glutathione and tryparedoxin can transfer reducing equivalents to TcGPXI with the result that the tryparedoxin-mediated pathway has a 10-fold greater activity than the glutathione-dependent system [18]. Attempts to use the trypanothione system as electron donor in place of the glutathione redox cycle gave no demonstrable activity even when dialysed soluble *T. cruzi* extracts or tryparedoxin were added to the assays. Thus the only electron donor so far identified for TcGPXII is glutathione. As the parasite lacks glutathione reductase activity, glutathione is maintained in its reduced form through an exchange reaction with dihydrotrypanothione [13,14]. Therefore, although TcGPXII does not interact directly with trypanothione, its activity is ultimately dependent upon this parasite-specific thiol. In *T. cruzi*, glutathione concentrations can reach millimolar levels [13,36,37], sufficient to drive significant TcGPXII activity. Some subcellular compartments may even experience a higher localized glutathione concentration.

In this paper, the evidence for localization of TcGPXII in the ER was inferred from experiments in which *T. cruzi* cells were co-stained with antibodies against *T. brucei* BiP and TcGPXII (Figure 6). In eukaryotes the ER is the site where secreted and membrane proteins are synthesized, modified and folded (rough ER) and where fatty acid and phospholipids are synthesized and metabolized (smooth ER). Both types of ER have been isolated from trypanosomatids [38]. For many of these processes an oxidized environment is required, and this is reflected in the relatively low glutathione/glutathione disulphide ratio found in the lumen of the ER (approx. 2:1 compared with approx. 50:1 in the cytosol) [39]. This redox state is maintained by the combined activity of glutathione and/or glutathione disulphide transporters and enzymes that produce oxidizing compounds [39–44]. One side effect of such reactions is the generation of ROS. Unlike mitochondria and peroxisomes, the biochemical pathways for the metabolism of hydroperoxides in the ER are unclear. Although a number of antioxidant enzymes are synthesized or trafficked through the ER, it is uncertain whether they exhibit peroxidase activity in this organelle [45–47]. It is believed that mammalian PHGPX is present in the ER, where in addition to its ROS-detoxifying role, it may modulate the activity of other enzymes such as those involved in prostaglandin biosynthesis [48,49], a process that has been identified recently in *T. brucei* [50]. In *T. cruzi* it appears that TcGPXII has the potential to play a crucial role in the oxidative defence system by minimizing and/or preventing damage resulting from lipid peroxidation reactions within the ER. Peroxidation of lipids makes them more hydrophobic, causing perturbation of normal membrane function including transporter and receptor processes.

Two distinct GPXs from *T. cruzi* have now been reported. The activity of both enzymes can be coupled to trypanothione reduction by glutathione, although in the case of TcGPXI an

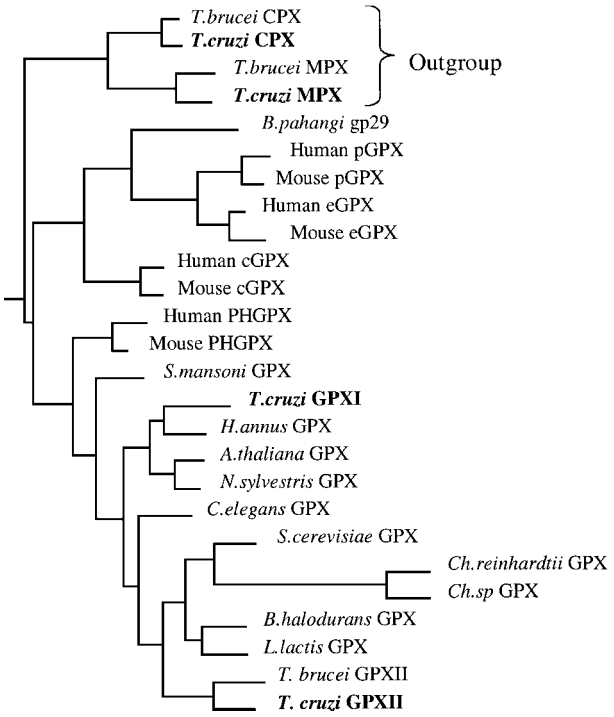

Figure 7 Phylogenetic analysis of GPXs

Maximum-parsimony tree (2480 steps) of GPXs from various organisms was constructed after ClustalW (http://www.ebi.ac.uk/clustalw) alignment using a gap penalty of 5. Further analysis was carried out with the programs Seqboot, Protpars, Consense and Drawtree from PHYLIP [55]. The accession numbers for TcGPXI, TcGPXII, *B. pahangi* gp29, human cGPX, human PHGPX and *A. thaliana* PHGPX-like protein are given in Figure 1. Other accession numbers are as follows: *T. brucei* CPX (AAG45225), TcCPX (AJ012101), *T. brucei* MPX (AAG28496), TcMPX (AJ006226), human plasma GPX (NP 002075), mouse plasma GPX (P46412), human epididymis GPX (eGPX; O75715), mouse epididymis GPX (P21765), *Schistosoma mansoni* GPX (AAA29885), *Helianthus annus* GPX (O23968), *Nicotiana sylvestris* GPX (CAA42780), *Caenorhabditis elegans* GPX (O62327), *Saccharomyces cerevisiae* GPX (P40581), *Chlamydomonas reinhardtii* GPX (T09638), *Chlamydomonas* sp. GPX (BAA83594), *Bacillus halodurans* GPX (NP 243696), *Lactococcus lactis* GPX (O32770), *T. brucei* GPXII (AL465487/AQ944786). The enzymes from *T. cruzi* are in bold. Outgroup refers to an unrelated family of enzymes.

alternative pathway involving tryparedoxin has also been demonstrated [17,18]. Although functionally related as members of the PHGPX clade, phylogenetic analysis of TcGPXI and TcGPXII suggests that these two enzymes may have different evolutionary origins (Figure 7). TcGPXI shares greatest similarity with GPXs found in plants (50% identity), whereas TcGPXII clusters with a range of GPXs sharing 31–37% identity. It has been noted previously that other trypanosomal enzymes have surprisingly close relationships with their plant counterparts [51–53]. Such proteins may have been acquired by an ancestral trypanosomatid during an engulfment event followed by transfer of genetic material into the nucleus of the host cell. This process is believed to account for the presence of a plastid in Euglenids, related photosynthetic organisms that share many of the peculiar trypanosomatid traits and which are traditionally grouped with the Kinetoplastida in the phylum Euglenozoa [54]. In this phylogenetic analysis (Figure 7) the trypanosomal peroxiredoxins TcCPX, TbCPX (*T. brucei* cytosolic peroxiredoxin), TcMPX and TbMPX (*T. brucei* mitochondrial peroxiredoxin) clustered as an outgrouping of the GPX family.

We have now identified five distinct pathways that have a role in hydroperoxide metabolism in *T. cruzi* [15,17,18]. This has altered our view of oxidative defence in this parasite. Rather than being deficient, as was the consensus view, it is now clear that the mechanisms and pathways involved are both sophisticated and flexible. Oxidative defence appears to be highly compartmentalized, to utilize a number of overlapping redox pathways and to involve enzymes with varying substrate specificity. This probably reflects their precise biological roles. For example, some enzymes seem to function as general hydroperoxide scavengers (TcCPX and TcMPX), whereas others may have more specific roles, such as the protection of newly synthesized lipids from hydroperoxide-mediated damage (TcGPXII). Further examination of these pathways is warranted to assess the potential of these antioxidant enzymes as chemotherapeutic targets.

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