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Short communication

Catalytic properties, localization, and *in vivo* role of Px IV, a novel tryparedoxin peroxidase of *Trypanosoma brucei*

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ABSTRACT

Px IV is a distant relative of the known glutathione peroxidase-type enzymes of African trypanosomes. Immunofluorescence microscopy of bloodstream cells expressing C-terminally Myc_6-tagged Px IV revealed a mitochondrial localization. Recombinant Px IV possesses very low activity as glutathione peroxidase but catalyzes the trypanothione/tryparedoxin-dependent reduction of hydrogen peroxide and, even more efficiently, of arachidonic acid hydroperoxide. Neither overexpression in bloodstream cells nor the deletion of both alleles in bloodstream or procyclic parasites affected the *in vitro* proliferation. *Trypanosoma brucei* Px IV shares 58% of all residues with TcGPXII. The orthologous enzymes have in common their substrate preference for fatty acid hydroperoxides. However, the *T. cruzi* protein has been reported to be localized in the endoplasmic reticulum and to be specific for glutathione as reducing agent. Taken together, our data show that Px IV is a low abundant tryparedoxin peroxidase of *T. brucei* that is not essential, at least under culture conditions.

Catalase and classical glutathione peroxidases (GPx) do not occur in African trypanosomes. Hydroperoxides are detoxified by 2-Cys-peroxiredoxins (Prxs) and non-selenium glutathione peroxidase-type (Px) enzymes which both act as tryparedoxin peroxidases (for reviews see [1–4]). Whereas the Prxs use hydrogen peroxide and peroxynitrite as main substrates [5], the Px-type enzymes preferably detoxify lipid-derived hydroperoxides [6]. Both types of peroxidases occur in the cytosol and mitochondrion [7,8]. In African trypanosomes, three virtually identical Px-type enzymes (Px I, II, and III) [9] are encoded on chromosome 7. RNA interference against the Px-type enzymes causes a severe growth defect in both bloodstream (BS) and procyclic (PC) *Trypanosoma brucei* [8,10]. Proliferation of the Px-depleted BS parasites can, however, be fully restored by supplementing the medium with the vitamin E-analogue Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] [6]. The specific knockout (KO) of the mitochondrial Px III results in BS *T. brucei* that are fully viable and infectious in the mouse model. In contrast, parasites that lack the cytosolic Px I and II undergo a disintegration of their lysosome followed by cell lysis [6,11]. The lethal phenotype is prevented by Trolox and attenuated by the iron chelator deferoxamine in accordance with its close link to the endocytic uptake of iron-loaded transferrin by BS *T. brucei* [11]. In the insect stage of *T. brucei*, both Px I–II KO and Px III KO cells are fully viable. However, deletion of the complete peroxidase locus is lethal. Either the mitochondrial or the cytosolic form of the peroxidases is required and sufficient to prevent mitochondrial damage and cell lysis [12].

Here we report on the catalytic properties, subcellular localization and *in vivo* role of Px IV (TriTryp, Tb927.11.15920), another Px-type protein in *T. brucei* which is encoded by a single copy gene on chromosome 11. The detailed experimental procedures and list of primers (Table S1) are provided in the supplementary data. Px IV has an overall sequence identity of 57% and 46% with its *T. cruzi* (TcGPXII [13]) and *L. major* orthologues but only of 30% when compared with *T. brucei* Px I–III. Clearly, all trypanosomatid Px IV type proteins display the structural characteristics of glutathione peroxidases, such as three conserved regions which harbor the residues of the catalytic tetrad composed of Secys/Cys, Gln, Trp, and Asn [14].

The *pxIV* coding region was cloned into several pET vectors and expressed in various *E. coli* strains at different temperatures. In all cases, the fusion proteins were nearly completely present in insoluble form. Finally, the recombinant protein was expressed from the pETGB-1a-*pxIV* plasmid and purified on Talon columns (for details see supplementary data). From a five liter bacterial culture, three mg of pure, soluble and tag-free *T. brucei* Px IV was obtained. The recombinant Px IV was subjected to tryparedoxin peroxidase and glutathione peroxidase assays. The specificity towards different hydroperoxides was measured in the standard tryparedoxin perox-

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Table 1
The tryparedoxin peroxidase activities were measured in a total volume of 150 μl of 100 mM Tris, 5 mM EDTA, pH 7.6, containing 240 μM NADPH, 100 μM TS_2, 150 mU TR, 10 μM Tpx and 1–5 μM Px IV [6]. The assays for comparing H_2O_2 and HpETE contained 0.1% Triton X-100, 17% ethanol, and 50 μM hydroperoxide substrate. The glutathione peroxidase assay was essentially that published for the *T. cruzi* orthologue [13]. In a total volume of 150 μl of 100 mM Tris, 5 mM EDTA, pH 8.0, the reaction mixture contained 200 μM NADPH, 3 mM GSH, 1.4 U human glutathione reductase and 7–30 μM Px IV. The reaction mixtures were pre-incubated until a stable baseline was obtained, the hydroperoxide substrate was added and the absorption decrease at 340 nm was followed at 25 °C. The rate of the spontaneous reduction of the hydroperoxide by the respective thiol system, in the absence of Px IV, was subtracted before the specific activity was calculated. 15(S)-HpETE, 15(*S*)-hydroperoxide of arachidonic acid. The data represent the values of at least two independent measurements, each conducted at least as double determinations. n. d., not determined.

Substrate	Concentration [μM]	$T(SH)_2$/Tpx	GSH
		Activity [U/mg]	
H_2O_2	100	0.2–0.3	n. d.
t-Butyl hydroperoxide	100	<0.02	n. d.
Cumene hydroperoxide	100	<0.04	n. d.
H_2O_2	50	0.2	0.02–0.05
15(S)-HpETE	50	0.4–0.7	n. d.

idase system [6,9]. With H_2O_2, Px IV had an activity of 0.2-0.3 U/mg (Table 1). Control assays containing all components except for Tpx, revealed only the rate for the spontaneous reduction of H_2O_2 by $T(SH)_2$. Thus, Px IV is not reduced by $T(SH)_2$ but requires Tpx for its peroxidase activity. Virtually no activity was detectable with t-butyl hydroperoxide or cumene hydroperoxide whereas with HpETE, Px IV showed an activity that was two to three times higher compared to H_2O_2. In the glutathione peroxidase system, the activity of Px IV towards H_2O_2 dropped by a factor of 4–10 compared to the tryparedoxin peroxidase activity. Although less pronounced, this specificity towards both its reducing and oxidizing substrates corresponds that of the Px I–III enzymes [6]. The deficiency of *T. brucei* Px IV to reduce t-butyl hydroperoxide and cumene hydroperoxide and its preference for fatty acid hydroperoxides agrees with the properties of TcGPXII [13]. However, the *T. cruzi* orthologue was shown to lack any activity with H_2O_2 and, most remarkably, to act specifically as a glutathione peroxidase. In addition, compared to the activity of TcGPXII with linoleic acid hydroperoxide in the presence of 3 mM GSH [13], the tryparedoxin peroxidase activity of *T. brucei* Px IV with HpETE was more than an order of magnitude higher. Taking into account that the concentration of free GSH in trypanosomatids is usually below 1 mM [2] and that the K_m-value of TcGPXII for GSH is 5 mM [13], the physiological significance of the glutathione peroxidase activity of the *T. cruzi* enzyme remains to be elucidated. The finding that TcGPXII did not display any activity with the tryparedoxin system may, at least partially, be explained by the low concentrations of only 20 μM $T(SH)_2$ and 2.5 μM Tpx used in these assays. Taken together, our data revealed that *T. brucei* Px IV acts as tryparedoxin peroxidase that catalyzes the reduction of both H_2O_2 and fatty acid hydroperoxides.

For none of the trypanosomatid Px IV-type proteins, a clear-cut targeting sequence or common subcellular localization could be identified. To determine the subcellular localization, BS *T. brucei* 2T1 cell lines were generated that allowed the tet-inducible expression of either N- or C-terminally Myc_6-tagged Px IV. Western blot analyses confirmed the inducible expression of the proteins (Fig. S1A). When cultured in the presence or absence of tet, the mutant cells grew like the WT controls (Fig. S1B). The mutant parasites were subjected to immunofluorescence microscopy using antibodies against BiP and Prx as well as MitoTracker Red (MT) to visualize the ER, cytosol and mitochondrion, respectively. Cells that expressed Px IV-C-Myc_6 showed a perfect overlap of the Myc signal with the MT staining (Fig. 1A), but none with the BiP signal (Fig. 1B). Visual inspection of 204 induced cells revealed for 80% of the cells a clear co-localization of the Myc and MT signals, but no colocalisation between Myc and BiP. The Pearson's correlation coefficients were calculated using the program ImageJ. Between the Myc and MT signals the value was 0.80 ± 0.05 whereas for the Myc and BiP pair the value was 0.46 ± 0.09, even lower than that for the background overlay of the ER and mitochondrial markers (0.67 ± 0.07) (not shown). This clearly revealed that the C-terminally Myc_6-tagged Px IV was targeted to the mitochondrion. In contrast, cells that expressed the N-terminally tagged Px IV displayed an overlap of the Myc and Prx signals (Fig. 1C). Visual inspection of 298 parasites revealed for 90% of the cells a co-localization of Px IV with the cytosolic marker protein. Again no overlap with the ER protein BiP was observed (Fig. 1D). N-Myc_6-Px IV was retained in the cytosol, probably because an N-terminal targeting sequence was blocked. In Western blot analyses of total lysates from cells expressing either the N- or the C-terminally Myc-tagged protein, both Px IV species revealed an identical running behavior, indicating that the putative targeting sequence is either very short or not processed after translocation of the protein into the mitochondrion. Although none of the trypanosomatid Px IV-type proteins has a classical C-terminal retention signal, *T. cruzi* GPXII appears to be located in the ER of the parasite [13]. There are examples for ER-resident proteins that lack an ER retrieval signal. In a protein disulfide isomerase of *Dictyostelium*, the C-terminal domain has been shown to be necessary and sufficient for its ER localization [15]. Thus, one cannot rule out that introduction of the C-terminal tag may have interfered with a putative ER targeting. Alternatively, Px IV could have a dual cellular localization or even be secreted as it is the case for glutathione peroxidase in *Brugia malayi* [16] or mammalian GPx3 [14]. Both ER and mitochondrial targeting rely on N-terminal signal peptides. Remarkably, in several secretory mammalian proteins, the ER signal peptides can mediate alternative targeting to the mitochondrion [17]. A common feature of the recombinant *T. cruzi* and *T. brucei* enzymes is their preference for lipid hydroperoxides as substrates, a property that should be required in the ER as site of cellular lipid biosynthesis as well as the mitochondrion as a main site of endogenous lipid peroxidation. Phospholipid transport from the ER into the mitochondria appears to proceed *via* close membrane contacts, and components of these tethering complexes are functionally connected to phospholipid biosynthesis [18].

A genome-wide RNA-interference approach indicated that Px IV is essential in BS *T. brucei* [19] whereas our specific RNA-interference studies towards Px IV in BS cells did not reveal any altered proliferation phenotype (not shown). Therefore, we decided to generate Px IV KO cell lines, replacing the Px IV alleles by puromycin and neomycin resistance genes (for details see supplementary data and Fig. S2A). PCR analyses verified the successful removal of both *px IV* alleles and the insertion of the resistance cassettes in the *px IV* locus (Fig. S2B). Both BS and PC Px IV KO cell lines proliferated like the respective WT parasites (Fig. S2C and D) indicating that Px IV is dispensable.

Since the subcellular localization studies revealed a mitochondrial localization of Px IV in *T. brucei* whereas the *T. cruzi* orthologue has been reported to be located in the ER [13], we subjected the Px IV KO cell lines to organelle-specific stressors. To induce an ER stress, BS and PC Px IV KO cells were treated with DTT. Under these conditions, WT parasites immediately stopped moving and died within 24 h, essentially as reported previously [20]. The Px IV KO cells displayed an identical behavior (Fig. 2A). In the next step, the parasites were exposed to menadione or paraquat. The redox cycling agents generate superoxide anions which are converted into H_2O_2 and the mitochondrion represents a main subcellular compartment for their mode of action [20]. Exposure to menadione (Fig. 2B) and paraquat (Fig. 2C) affected the proliferation of the BS and PC Px IV KO cells to the same degree as the WT controls.