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Disruption of the *Plasmodium falciparum* 2-Cys peroxiredoxin gene renders parasites hypersensitive to reactive oxygen and nitrogen species

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Abstract In parasitism, *Plasmodium falciparum* is exposed to toxic reactive oxygen species and reactive nitrogen species (RNS). Peroxiredoxins (Prx) are ubiquitously distributed antioxidant enzymes. In bacteria and yeast, Prx have also been implicated in detoxifying RNS. Here, we used a gene targeting strategy to investigate the physiological role of 2-Cys Prx of *P. falciparum*, PfTPx-1, in living parasite cells. The PfTPx-1-null parasite line was more sensitive to paraquat (a superoxide donor) and sodium nitroprusside (a nitric oxide donor), than wildtype. These findings suggest that PfTPx-1 protects the parasite cells from oxidative and nitrosative stresses.

Key words: Peroxiredoxin; Reactive nitrogen species; Reactive oxygen species; Thioredoxin peroxidase; *Plasmodium falciparum*

1. Introduction

Plasmodium falciparum is the parasitic pathogen that causes falciparum malaria, one of the most debilitating and life-threatening diseases in tropical regions of the world. Despite years of intensive research, an effective vaccine is still not available, and the parasite displays increasing resistance towards the commonly used plasmodicidal drugs. To combat falciparum malaria, a better understanding of the basic biology of *P. falciparum*, especially the mechanism of adaptation to environmental conditions, is needed.

P. falciparum lives in an oxygen- and nitrogen-rich environment in humans and is exposed to toxic reactive oxygen species (ROS) and reactive nitrogen species (RNS). When the parasite infects erythrocytes, it is exposed to superoxide (O_2^-) and nitric oxide (NO) from the host immune system [1]. Because hemoglobin has a high affinity for NO, the parasite would likely be exposed to NO derivatives in erythrocytes [2]. In addition, O_2^- and pro-oxidative heme iron are produced by the parasite when it digests hemoglobin for nutrition [3]. The significant decrease in glutathione (GSH) in parasite-infected erythrocytes suggests that the parasites need efficient antioxidant systems [4,5]. *P. falciparum* has been shown recently to possess two major functional antioxidant systems: a thioredoxin system [5–7] and a glutathione system [5,8]. To date, an enzymatic defense against RNS has not been identified in *P. falciparum*.

Peroxiredoxins (Prx) are a family of antioxidants that are found in all organisms from bacteria to humans [9,10]. Alignment of the amino acid sequences among this family of proteins revealed the existence of two types of Prx, which differ in the number of conserved cysteine residues. The 2-Cys Prx contains both conserved residues (Cys47 and Cys170), whereas the 1-Cys group lacks the Cys170 residue [9,11,12]. Several Prx proteins act as terminal peroxidases which reduce hydrogen peroxide and organic hydroperoxides using electrons donated by the thioredoxin system [9] and other physiological reductants [11,12]. 2-Cys Prx have also been implicated in detoxifying RNS in bacteria [13] and yeast [14].

In *P. falciparum*, we and other groups have reported three Prx subfamilies (reviewed in [7]). It was suggested that these Prx subfamilies in *P. falciparum* be named Pf1-Cys-Prx, PfTPx-1, and PfTPx-2 [7]. PfTPx-1 is the 2-Cys Prx, which contains two VCP motifs [15–17]. The PfTPx-1 protein is expressed during the trophozoite stage [15]. Recombinant PfTPx-1 protein showed peroxidase activities when H_2O_2, *t*-butylhydroperoxide, and cumene hydroperoxide are available as substrates [15–17] and was suggested to be the terminal peroxidase in the thioredoxin (Trx)/Trx reductase (TrxR) system during the intraerythrocytic stage [15–18].

In the present study, we investigated if PfTPx-1 acts as an antioxidant in parasite cells and detoxifies ROS and RNS with a targeted gene disruption strategy.

2. Materials and methods

2.1. Parasite culture

The FCR-3 strain of *P. falciparum* was cultured with a modification of the method of Trager and Jensen [19] and synchronized with 5% D-sorbitol [20].

2.2. PfTPx-1 gene disruption construct

For disruption of the PfTPx-1 gene, a 545-bp fragment corresponding to nucleotides 3–527 of the coding region was amplified by polymerase chain reaction (PCR). The primers used were 5′-GCG GCG GCC GCG GCA TCA TAT GTA GG-3′ and 5′-GCG GGA TCC TTT TTC CAG TTT GCT GGG CA-3′. The primers contained a *Not*I site and *Bam*HI site (underlined), respectively, for cloning of the PCR product into pDT.Tg23 [21] (MRA-97, Malaria Research and Reference Reagent Resource (MR4) Center, Manassas, VA, USA), a kind gift of Y. Wu and T.E. Wellems (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA). pDT.Tg23 contains the dihydrofolate reduc-

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Abbreviations: GSH, glutathione; Prx, peroxiredoxin; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNP, sodium nitroprusside; TPx, thioredoxin peroxidase; Trx, thioredoxin

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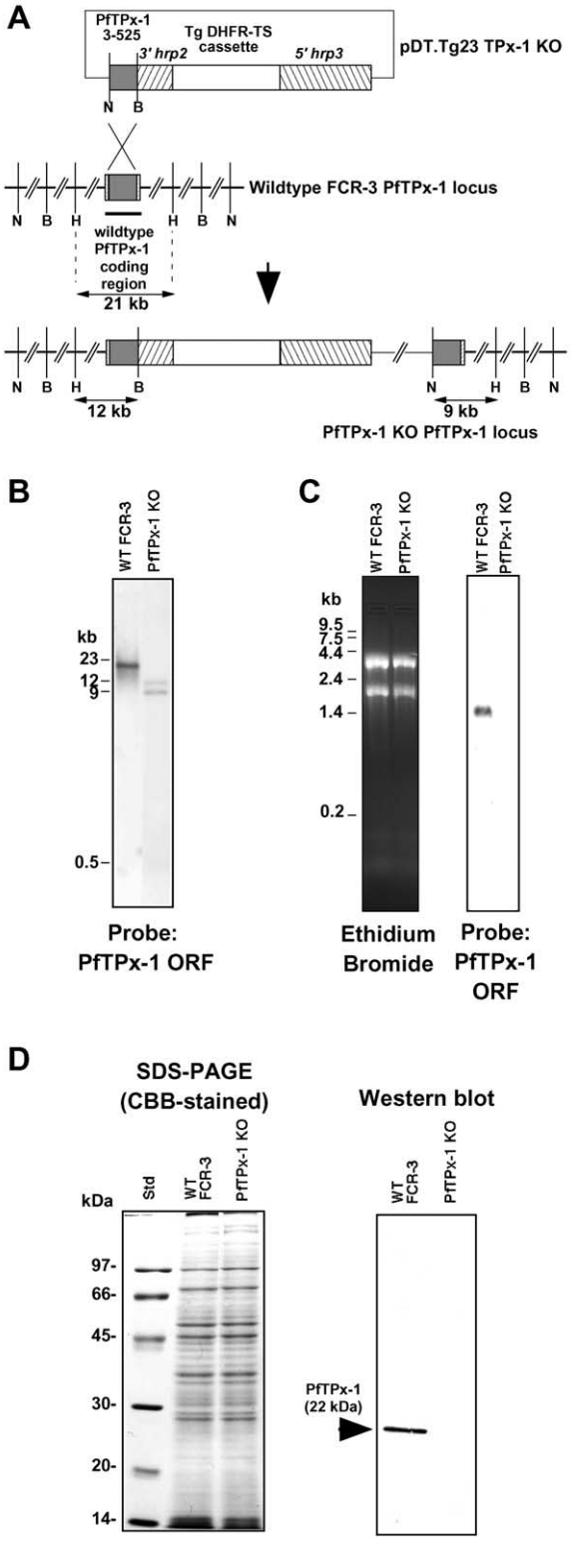
tase-thymidylate synthase (DHFR-TS) gene of *Toxoplasma gondii*, which confers resistance to pyrimethamine to transfectants. The plasmid construct made in this study was named pDT.Tg23 TPx-1 KO.

2.3. Transformation and selection of parasite lines

Parasites were transfected with 100 μg of pDT.Tg23 TPx-1 KO DNA by electroporation as described previously [22]. In brief, 10^9 erythrocytes with 5% parasitemia of ring-stage parasites were transfected with vector DNA in incomplete Cytomix solution [22]. The Gene Pulsar II system (Bio-Rad) was used at 0.31 kV and 975 μF. Drug selection with 1 μM pyrimethamine was initiated 48 h after electroporation, and this concentration was used thereafter. Transformed parasites grew slowly immediately after the start of drug selection. Continuous culture resulted in an increased growth rate, suggesting that episomally transformed parasites had been replaced by parasites in which the plasmid construct was integrated into the genome [23]. Ten weeks after the transformation, the integration of the plasmid construct into the targeted locus was detected by Southern hybridization analysis (described below). Single-cell clones were obtained by limiting dilution after integration was verified. As a control for transfection and selection, empty pDT.Tg23 plasmid was electroporated into parasites under the same conditions used for pDT.Tg23 TPx-1 KO. Drug selection with 1 μM pyrimethamine was initiated 48 h after electroporation, and this concentration was used thereafter. Twelve weeks after transfection, the growth rate of pDT.Tg23-transformants had increased as much as that of pDT.Tg23 TPx-1 KO. This transfectant was used as a control in the assay of parasite sensitivities to ROS and RNS.



2.4. Southern blot analysis

Genomic DNA used for Southern blot analysis was extracted from parasite-infected erythrocytes by the standard phenol–chloroform method [24]. For Southern blot analysis, genomic DNA was triply restricted with *Not*I, *Bam*HI, and *Hin*dIII. Digested DNAs were separated by agarose gel electrophoresis and transferred onto HiBond N^+ membranes (Amersham Pharmacia Biotech). The membrane was probed with the entire coding sequence of PfTPx-1. Labeling of the DNA probe, hybridization, and chemiluminescent signal detection were performed with the AlkPhos direct system (Amersham Pharmacia Biotech).

2.5. Northern blot analysis

For Northern blot analysis, the parasite-infected erythrocytes were harvested from the synchronized culture when the parasite reached the late trophozoite stage. The parasite-infected erythrocytes were lysed with phosphate-buffered saline (PBS) containing 0.05% saponin. The parasite pellet was washed several times with PBS and treated for total RNA extraction with TRIzol reagent (Gibco Invitrogen). Five microgram of total RNA was subjected to electrophoresis in a 1.5% agarose–formaldehyde gel and transferred to HiBond N^+ membranes (Amersham Pharmacia Biotech) by the standard capillary method [24]. The membrane was probed with the entire coding sequence of PfTPx-1. Labeling of the DNA probe, hybridization, and chemilumi-

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Fig. 1. Design and experimental verification of PfTPx-1 gene disruption in *P. falciparum*. A: Design of disruption construct pDT.Tg23 TPx-1 KO, schematic of the PfTPx-1 locus, and organization following the single crossover homologous recombination event. The partial open reading frame (ORF) between nt 3 and 525 of PfTPx-1 (darkly shaded region) was PCR-amplified and cloned into the *Not*I/*Bam*HI sites of the original plasmid (pDT.Tg23). The vector contains the *T. gondii* DHFR-TS gene as a selectable marker (open box). *hrp*, histidine-rich protein. The restriction sites used to verify the integration event are indicated on the illustration (B, *Bam*HI; H, *Hin*dIII; N, *Not*I). B: Southern blot analysis of genomic DNA samples triply digested with *Bam*HI, *Hin*dIII, and *Not*I. DNA samples from wildtype (FCR-3) and the PfTPx-1 KO line were digested and loaded onto 0.7% agarose gel and run for 4 h at 50 V in TAE buffer. After electrophoresis, DNAs were transferred onto nylon membrane and probed with the coding sequence of PfTPx-1. DNA size markers in kb are indicated on the left. C: Northern blot analysis of wildtype and the PfTPx-1 KO line. Total RNAs (5 μg) extracted from trophozoite/schizont cultures were separated by electrophoresis in a 1.5% agarose–formaldehyde gel (left panel) and probed with the coding sequence of PfTPx-1 (right panel). Ethidium bromide-stained gel shows equal loading. Sizes in kb are indicated on the left. D: Western blot analysis of wildtype and the PfTPx-1 KO line. Cell lysates (10 μg) extracted from trophozoite/schizont cultures were separated by 12.5% SDS–PAGE (left panel) and probed with anti-PfTPx-1 rabbit serum (right panel). Coomassie brilliant blue (CBB)-stained gel shows equal loading. Molecular weight markers in kDa are indicated on the left.

nescent signal detection were performed with the AlkPhos direct system (Amersham Pharmacia Biotech).

2.6. Western blot analysis

For Western blot analysis, late trophozoite parasite pellets were prepared by the same method as used for the Northern blot analysis and solubilized in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer containing 5% 2-mercaptoethanol [25]. After separation by SDS–PAGE (12.5%), parasite proteins were transferred electrophoretically to polyvinylidene difluoride sheets (Immobilon; Millipore) and reacted with anti-recombinant PfTPx-1 rabbit serum [15]. Immune complexes were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Cappel). Protein concentrations were determined with a BCA protein assay kit (Pierce).

2.7. Assay of parasite sensitivities to ROS and RNS

To evaluate parasite sensitivity to ROS and RNS, cultures were maintained in the presence or absence of 40 μM paraquat (Sigma) as an O_2^- donor [26] and 20 μM sodium nitroprusside (SNP; Sigma) as an NO donor [27]. Growth inhibitions by the reagents were assayed with parasite cultures in the ring stage with initial parasitemia of 0.1%. The culture medium was replaced every 24 h, and at the same time parasite growth was evaluated. A drop of the cultured erythrocytes was smeared on a slide glass and Giemsa stained. The number of parasites in 10 000 erythrocytes was counted and defined as the level of parasitemia.

2.8. Statistical analysis

Differences were evaluated using Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

To investigate the function of PfTPx-1 in *P. falciparum*, the PfTPx-1 gene was disrupted. pDT.Tg23 TPx-1 KO was transfected into parasite cells, and cells that were transformed with the plasmid construct were selected by pyrimethamine pressure. After 10 weeks of selection, integration of the construct into the PfTPx-1 locus was detected by genomic Southern hybridization (data not shown). A clonal line with the desired integration was established by limiting dilution. Two clonal lines with disruptions of the PfTPx-1 locus were established. Representative results of Southern hybridization for one cloned line (PfTPx-1 KO) are shown in Fig. 1B. A single 21-kb signal was detected in the genome of the wildtype (Fig. 1B). In the PfTPx-1 KO line, 12-kb and 9-kb bands were detected on Southern blots (Fig. 1B), indicating a single targeted integration and disruption of the PfTPx-1 locus had occurred (Fig. 1A). No 0.5-kb band (partial open reading frame between nt 3 and 525 of PfTPx-1; Fig. 1A) was found to result from episomes, indicating this was lost in the clone. Northern blotting revealed a single RNA species of about 1.4 kb, corresponding to the PfTPx-1 mRNA, in FCR-3 that was not detectable in the PfTPx-1 KO line (Fig. 1C).

To characterize the phenotype of the PfTPx-1 KO line, Western blot analysis with the PfTPx-1 protein-specific antiserum was performed (Fig. 1D). Lysates of trophozoite-rich cultures of wildtype and the PfTPx-1 KO cell line were probed with anti-PfTPx-1 serum. A band with a molecular mass of 22 kDa, which matches the mass of PfTPx-1 [15], was detected in wildtype lysates, whereas no band was observed in lysates of PfTPx-1 KO cells. This result verified the PfTPx-1-null phenotype of the PfTPx-1 KO line.

The PfTPx-1 KO line did not differ from wildtype cells in either growth rate or morphology under normal culture conditions (Fig. 2A). To confirm if PfTPx-1 acts as an antioxidant in living cells, we tested the sensitivity of PfTPx-1 KO cells to paraquat and SNP, which produce intracellular O_2^- and NO respectively. The concentrations used were determined by preliminary experiments with a variety of concen-

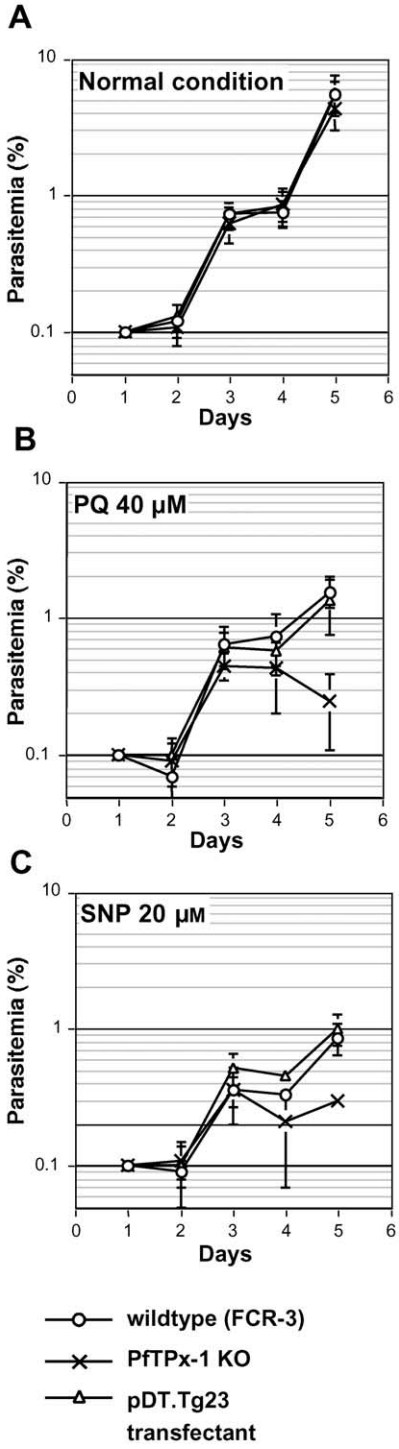

Fig. 2. Sensitivities of the PfTPx-1 KO line to paraquat and SNP. A: Growth curves for wildtype (FCR-3 strain, open circles), PfTPx-1 Prx KO line (cross symbols), and pDT.Tg23 transfectant (control, open triangles) under normal culture conditions. B: Sensitivities of parasite lines to paraquat. Parasite lines were cultured in complete medium containing 40 μM paraquat. C: Sensitivities of parasite lines to SNP. Parasite lines were cultured in complete medium with 20 μM SNP. All data are mean ± S.D. of three independent experiments.

trations ranging from 10 to 1000 μM (data not shown). The PfTPx-1 KO line showed a reduced growth rate in culture medium with 40 μM paraquat in comparison with wildtype (day 5, $P < 0.05$; Fig. 2B). The growth rate of the control transfectant showed no significant difference to wildtype under this condition. In parasite cells, superoxide anions released because of paraquat can be reduced by parasite superoxide dismutase [28], thereby generating H_2O_2 in the cytoplasm. It is likely that the parasite uses PfTPx-1 to reduce this H_2O_2 [15–17] in connection with the Trx/TrxR system [5–7]. In the presence of 20 μM SNP, the growth rate of the PfTPx-1 KO line was also decreased in comparison with that of wildtype (day 5, $P < 0.05$; Fig. 2C). The growth rate of the control transfectant showed no significant difference to wildtype under this condition. These results suggest that PfTPx-1 is used for detoxifying both intracellular ROS and RNS. Because the host hemoglobin mediates delivery and capture of NO as a vasodilator [2], the parasite is perhaps exposed to RNS in erythrocytes. In addition, NO and its derivatives function in the host immune system to combat microbial pathogens [1]. Although there have been no reports of anti-RNS activity by PfTPx-1, the peroxynitrite reductase activity, which was recently found in the bacterial [13] and yeast [14] 2-Cys Prx subfamilies, may underlie the NO hypersensitivity of the PfTPx-1 KO line. The parasites in erythrocytes are most likely fighting ROS and RNS, which are present in the O_2^-- and NO-rich environment. Independent of these external stresses, the parasite produces pro-oxidative heme iron in the cell during the late trophozoite stage when it digests host hemoglobin for nutrition [3]. Taken together these findings suggest that the parasite must have an efficient antioxidant system to ensure intraerythrocytic development under stressful conditions. Results from our gene targeting study suggest that the parasite uses PfTPx-1 as a part of its antioxidant system to detoxify intracellular ROS and RNS.

The hypersensitivity of the PfTPx-1 KO line to paraquat and SNP compared to wildtype was not detected immediately after the addition of each reagent into the culture medium (Fig. 2B,C). Reduced parasite growth appeared on the third day of the assay (48 h of culture), indicating that the toxic effects of ROS and RNS were cumulative and could only be detected after one complete round of the parasite cell cycle. An additional delay of culture until day 9 with 40 μM paraquat or 20 μM SNP resulted in a further decrease of the growth rate of FCR-3, the control transfectant, and PfTPx-1 KO and in an increase of the difference in parasitemia between FCR-3 and PfTPx-1 KO (data not shown). ROS and RNS damage biological macromolecules via lipid peroxidation and oxidation of proteins. In addition, ROS and RNS damage DNA and are known to be powerful mutagens [29,30]. PfTPx-1 may prevent critical damage to DNA by ROS and RNS and thus protect the parasite from death caused by lethal accumulation of mutations [31].

Successful disruption of the PfTPx-1 gene in the parasite genome indicates that the PfTPx-1 gene is not essential for parasite survival under in vitro culture conditions and that there is redundancy in parasite antioxidant defenses. Systems such as the Trx and GSH systems [5–8] may cooperate with each other to compensate for the absence of PfTPx-1. Most likely, other peroxiredoxins, such as PfTPx-2, Pf1-Cys-Prx or TPx_{GI}, a glutathione peroxidase-like thioredoxin peroxidase [7], directly compensate for loss of PfTPx-1 function. The question of how much TPx-1 contributes to malarial parasite survival in vivo may be interesting to answer. Studies of rodent malaria parasites with TPx-1 gene disruption in mice may provide some insights.

The results of the present study suggest that 2-Cys Prx of *P. falciparum*, PfTPx-1, protects parasite cells from ROS and RNS. Further studies of the in vivo functions of Prx would clarify the mechanism underlying parasite adaptation and may facilitate development of an alternative malaria chemotherapy.

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