Expression of a Mitochondrial Peroxiredoxin Prevents Programmed Cell Death in Leishmania donovani†

Simone Harder,1 Meike Bente,1 Kerstin Isermann,2 and Iris Bruchhaus1*

Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany,1 and Zoological Institute of the University of Kiel, Kiel, Germany2

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Leishmania promastigote cells transmitted by the insect vector get phagocytosed by macrophages and convert into the amastigote form. During development and transformation, the parasites are exposed to various concentrations of reactive oxygen species, which can induce programmed cell death (PCD). We show that a mitochondrial peroxiredoxin (LdmPrx) protects Leishmania donovani from PCD. Whereas this peroxiredoxin is restricted to the kinetoplast area in promastigotes, it covers the entire mitochondrion in amastigotes, accompanied by dramatically increased expression. A similar change in the expression pattern was observed during the growth of Leishmania from the early to the late logarithmic phase. Recombinant LdmPrx shows typical peroxiredoxin-like enzyme activity. It is able to detoxify organic and inorganic peroxides and prevents DNA from hydroxyl radical-induced damage. Most notably, Leishmania parasites overexpressing this peroxiredoxin are protected from hydrogen peroxide-induced PCD. This protection is also seen in promastigotes grown to the late logarithmic phase, also characterized by high expression of this peroxiredoxin. Apparently, the physiological role of this peroxiredoxin is stabilization of the mitochondrial membrane potential and, as a consequence, inhibition of PCD through removal of peroxides.

Leishmania parasites affect more than 12 million people worldwide, with an estimated 2 million new cases each year (WHO World Health Report, 2004, http://www.who.int/whr/en). Depending on the species involved, symptoms range from the self-healing cutaneous form (Leishmania major) to the fatal visceral form (L. donovani). The parasite is transmitted as the infective promastigote form from the gut of its insect vector, female phlebotomine flies of the genera Phlebotomus and Lutzomyia, to mammalian hosts. Promastigotes get phagocytosed by macrophages and convert into the amastigote form, which is able to survive and replicate within phagolysosomes. During phagocytosis of Leishmania promastigotes, the macrophages produce different reactive oxygen species (ROS) to kill the parasites. ROS readily react with proteins, DNA, and lipids and have been implicated in a wide variety of cell functions, such as signal transduction, redox homeostasis, apoptosis, aging, tumor progression, and pathogen infection (9, 19, 42, 58).

Numerous reports have shown that Leishmania parasites are susceptible to ROS- and RNS (reactive nitrogen species)-mediated toxicity (41, 57). In order to survive and establish an infection, they have to cope with these prooxidants. In Trypanosomatidae, it was shown that peroxiredoxins are the major antioxidant enzymes that can use different ROS and RNS like H2O2, hydroperoxides, and ONOO− as substrates (56). Peroxiredoxins are found in a great variety of organisms, where they fulfill distinct functions, such as detoxification, signaling, or differentiation (25). In different members of the family Trypanosomatidae, cytosolic, as well as mitochondrial, peroxiredoxins were found (6, 10, 11, 21). Peroxiredoxins localized in the cytosol appear to be ideal to protect the parasite against oxidative attack from the outside. Peroxiredoxins localized in the mitochondrion seem to be of particular interest, since kinetoplasts are known to generate H2O2 as a by-product of their own mitochondrial energy metabolism (51).

For the mitochondrial peroxiredoxin of L. infantum (LimTXNPx), as well as for the cytoplasmic peroxiredoxins from L. infantum (LicTXNPx), L. chagasi (LcPx1, LcPx2) and L. donovani (LdH6TXNPx), it was shown that they all can detoxify ROS, with a preference for H2O2 and tert-butyl hydroperoxide (t-BOOH) (6, 10, 11, 21). It was further shown that overexpression of LcPx1 in L. chagasi enhanced survival when exposed to different ROS and RNS and also enhanced survival within U937 macrophage cells (6). L. infantum overexpressing the cytoplasmic peroxiredoxin showed increased resistance to H2O2, as well as t-BOOH, whereas parasites overexpressing the mitochondrial peroxiredoxin showed only resistance to t-BOOH and no resistance to H2O2 (11).

Mitochondria are important checkpoints for the control of programmed cell death (PCD). In addition, they are an important site for the production of ROS. During the process of oxidative phosphorylation, part of the consumed oxygen is released in the mitochondria as ROS like H2O2, superoxide radical anions, singlet oxygen, and hydroxyl radicals. Mitochondrial H2O2 can induce apoptosis by inducing the release of proapoptotic factors from the mitochondria. Release of these factors like cytochrome c or the apoptosis-inducing factor into the cytosol occurs through opening of a nonsselective mitochondrial permeability transition pore and results in activation of caspases (31, 43). Apparently, mammalian mitochondrial PrxIII is an important regulator of H2O2 in the mitochondria. Depletion of PrxIII results in increased mitochondrial accumulation of H2O2 and leads to an increase in the rate of apoptosis induced by staurosporine or by tumor necrosis factor alpha and cycloheximide. This further leads to an increase in...
membrane permeability, formation of protein-permeable channels, and release of proapoptotic proteins (13). Furthermore, it was shown that overexpression of PrxIII in a mammalian cell line protects the cells from apoptosis caused by H$_2$O$_2$ and t-BOOH (44). There are increasing numbers of reports that describe apoptosis- or PCDu-like processes also in unicellular organisms such as trypanosomatids (2–4, 18, 33, 39, 59), bacteria (47), yeast (37), and Plasmodium (1). However, very little is known about the molecular mechanisms by which PCD occurs in unicellular organisms. Das and colleagues showed that, upon exposure to suitable doses of H$_2$O$_2$, L. donovani promastigotes express several markers common to metazoan apoptosis, including nuclear condensation, accumulation of intracellular calcium, activation of caspase-like proteases, a decrease in intracellular trypanothione content, fragmentation of cellular DNA, formation of DNA ladders, cleavage of a poly-(ADP)ribose polymerase-like protein, and loss of cell volume (18). Furthermore, it was shown that during activation of PCD by H$_2$O$_2$, loss of the mitochondrial membrane potential takes place (39).

In the present study, we characterized a mitochondrial peroxiredoxin of L. donovani (LdmPrx). Its expression prevents PCD. During development of the parasite, it changes expression from the kinetoplastid area in promastigotes to the entire mitochondrion in amastigotes, accompanied by dramatically increased expression. This expression level correlates with protection against H$_2$O$_2$-mediated PCD, pointing to a vital role for this peroxiredoxin in the survival of the parasite.

MATERIALS AND METHODS

Cultivation of cells. L. donovani strain Lo$_9$, a gift from D. Zilberstein (Department of Biology, Technion, Israel Institute of Technology, Haifa, Israel), was used for all experiments. Promastigotes (day 0) frozen directly after passage through BALB/c mice were thawed and cultivated at 25°C in M199 medium supplemented with 25% fetal calf serum and 20 μg/ml gentamicin. In vitro differentiation to amastigotes was achieved as described previously (30). Briefly, promastigotes (day 0, early logarithmic stage, 2 × 10$^6$ cells/ml) were heat shocked at 37°C for 24 h (day 1) and then cultivated for up to 5 days at 37°C in mid- to late-logarithmic phase (days 2 to 5).

PEC infection assay (intracellular amastigotes). Peritoneal exudate cells (PECs) from 4- to 6-week-old female C57black/6 mice were used for infection assays. Mice were treated with 5% thiglycolate in phosphate-buffered saline (PBS) given intraperitoneally 4 days prior to experiment. On day 4, mice were sacrificed and PECs were prepared by rinsing the peritoneum with 10 ml of PBS. PECs were washed once and seeded at a density of 10$^6$ cells/well in a 12-well plate on coverslips in RPMI medium supplemented with 10% fetal calf serum, 5 mM glutamine, and 50 μg/ml gentamicin. After incubation under 5% CO$_2$ at 37°C for 24 h, PECs were incubated with L. donovani parasites at a parasite-to-PEC ratio of 1:10 for 48 h. Nonengulfed parasites were washed away three times with warm RPMI medium, and cells on coverslips were used for immunofluorescence studies.

Genomic DNA isolation. Genomic DNA from L. donovani was isolated according to the manufacturer's recommendations.

Cloning and sequencing of the LdmPrx gene. Two primers were designed on the basis of the sequence of peroxiredoxin L. major (accession no. 6066432): sense primer Prx-S26(NdeI) (5′-GAGACATATGCTCCGGCTCTT TCCA-3′) and antisense primer Prx-AS27(XhoI) (5′-GAGACGTAGCTCACA TGTTTCCTCCGTA-3′). Prx-S26(NdeI) and Prx-AS(XhoI) were used to PCR amplify L. donovani genomic DNA (95°C for 1 min, 50°C for 1 min, 72°C for 2 min; 30 cycles with a Perkin-Elmer DNA Thermal Cycler 480). The amplified product (679 bp) was gel purified and cloned into the pCR2.1-TOPO vector. The gene was sequenced with the Big Dye Terminator PCR cycle sequencing kit in accordance with the manufacturer's (Applied Biosystems) instructions.

Expression and purification of recombinant protein. The PCR-amplified DNA fragment coding for LdmPrx was cloned into prokaryotic expression plasmid pC45, a derivative of pC40 (16), with restriction enzymes NdeI and XhoI. Following transformation into Escherichia coli BL21(DE3)(pAPlacIQ), the protein was expressed according to standard procedures. Recombinant protein was isolated with Ni-nitri triacetic acid resin according to the manufacturer's (QIAGEN, Hilden, Germany) recommendations.

Generation of polyclonal antibodies. Two hundred micrograms of recombinant LdmPrx was injected subcutaneously into a chicken. The first injection was done in combination with complete Freund's adjuvant, and the following two booster injections were done in combination with incomplete Freund's adjuvant after 2 weeks. Antibodies were purified from eggs with increasing concentrations of polyethylene glycol 6000.

Western blot assays. Twelve percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions. Samples from promastigotes and in vitro-derived amastigotes were obtained by lysing the cells directly in hot SDS sample buffer (95°C, 125 mM Tris-HCl [pH 6.8], 20% glycerol, 20% SDS, 20 mM dithiothreitol (DTT), 0.001% bromophenol blue). Western blot analyses were carried out by the semidyblotting technique with electrophoresis buffer (0.25 M Tris, 0.5 M glycine, 1% SDS) as blotting buffer. Polycional chicken antisera (LdmPrx, 1:10,000) or monoclonal mouse antibodies (anti-β tubulin clone Tub2.1 [Sigma] and alkaline phosphatase-conjugated anti-chicken immunoglobulin M (IgM) or anti-mouse IgG (Sigma) as the secondary antibody were used to detect the protein with the 5-bromo-4-chloro-3-indolyphosphate (BCIP)–Nitro Blue Tetrazolium color developmental substrate (Promega).

Immunoelectron microscopy. Cells were harvested by centrifugation (10 min, 6000 × g, 4°C), washed twice with PBS, and fixed for 24 h at 37°C in 200 mM sodium cacodylate buffer with 4% paraformaldehyde. Fixed cells were dehydrated in ethanol and embedded in LR-White. Ultrathin sections were prepared on an Ultratoc E (Reichert) and placed on 200-nm Ni grids. Anti-LdmPrx antibodies (1:500) or preimmune antibodies were incubated with the grids for 1 h at 37°C and then overnight at 4°C. The sections were then treated with rabbit anti-chicken antibody (1:300; Jackson Immunolab) and with protein A-gold (10 nm, 1:100; Biocell). Electron micrographs were taken on a Philips CM-10 transmission electron microscope.

Immunofluorescence assay. L. donovani promastigotes were incubated with 1 nM MitoTracker red CMXROS (Molecular Probes) diluted in M199 medium for 30 min, washed once with medium alone, added to poly-l-lysine-covered glass slides, and air dried. Cells were fixed with 3.7% formaldehyde in M199 medium for 15 min, washed three times in PBS, and permeabilized in PBS-0.2% Triton X-100, followed by three washes in PBS. Subsequently, cells were incubated for 30 min in PBS containing 10% fetal calf serum (FCS). After blocking, cells were incubated with anti-LdmPrx, diluted 1:1,000 in PBS–10% FCS, followed by three washes in PBS. Slides were incubated with Cy2-conjugated donkey anti-chicken IgG antibody (Dianova), diluted 1:1,000 in PBS–10% FCS, and washed another three times in PBS. After incubation with Hoechst 33258 (Molecular Probes), 1% agarose was added, and the PECs were mounted in mounting medium (Dako Cytomation) and examined with a Zeiss Axioskop 2 plus immunofluorescence microscope at the improvement software.

Intracellular amastigotes on coverslips were incubated with 1 nM MitoTracker red CMXROS diluted in RPMI medium, washed once with medium alone, and fixed with 4% paraformaldehyde in PBS. After three washes in PBS, cells were permeabilized in absolute methanol at −20°C. Further staining steps were performed as described above.

Peroxide assay (ferrous ammonium sulfate-potassium thiocyanate). The ability of LdmPrx to remove H$_2$O$_2$ and t-BOOH was evaluated using the ferrithiocyanate system (50). Reaction mixtures (1-ml reaction volume) containing 25 mM HEPES (pH 7.0) and LdmPrx protein (10 to 100 μg) were preincubated with 3 mM DTT for 10 min at 37°C. After preincubation, 480 μM H$_2$O$_2$ or 350 μM t-BOOH (final concentration), respectively, was added and the reaction was allowed to proceed for another 30 min. The reaction was stopped by addition of 8% (vol/vol) trichloroacetic acid, and protein was removed by centrifugation (5 min, 12,000 × g). Subsequently, 0.2 volume of 10 mM ferrous ammonium sulfate and 0.1 volume of 2.5 M potassium thiocyanate were added and the absorbance was measured at 480 nm. The amounts of H$_2$O$_2$ and t-BOOH were determined spectrometrically by using known amounts of H$_2$O$_2$ and t-BOOH as standards.

Nicking assay. The ability of LdmPrx to protect DNA from hydroxyl radical-induced nicking was determined as previously described (34, 48). Reaction mixtures containing 0.1 mM HEPES (pH 7.2), 3.3 mM FeCl$_3$, 10 mM DTT, 5 mM EDTA (pH 8), and various concentrations of LdmPrx were incubated in a total volume of 50 μl at 37°C for 3 h. After incubation, 1 μg of supercoiled pUC18 plasmid DNA (Invitrogen) was added to the reaction mixture and the mixture

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was incubated at 37°C for an additional 3 h. The DNA was separated on a 1% agarose gel at a 100-V constant voltage. All solutions were made fresh immediately before use.

**Construction of expression vectors.** Leishmania-specific expression vector pX63pol (kindly provided by Martin Wiese, Bernhard Nocht Institute, Hamburg, Germany) was used to express LdmPrx in *L. donovani* promastigotes. Primers Pro-S26(NdeI) and Pro-AS27(XhoI) were used to PCR amplify the coding region of LdmPrx. The product was digested with NdeI and XhoI, and the 5' orientation and sequence were reconfirmed by nucleotide sequencing.

**Transfection of *L. donovani* promastigotes.** Plasmid DNA was purified with a Nucleoscale AX PC2000 Maxiprep Kit (Macherey & Nagel), and 100 μg of DNA was used per transfection. Parasites were transfected by electroporation. Cells were harvested during the late log phase of growth, washed twice in ice-cold PBS and once in prechilled electroporation buffer (21 mM HEPES [pH 7.5], 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM glucose), and suspended at a density of 10⁸/ml in electroporation buffer. Chilled DNA was mixed with 0.4 ml of the cell suspension, which was immediately used for electroporation with a Bio-Rad Gene Pulser apparatus. Electrottransfection was carried out with a 4-mm electroporation cuvette at 3,750 V/cm and 25 μF.

After electroporation, cells were kept on ice for 10 min before being transferred into 10 ml of drug-free medium. After 24 h, transfectants were selected with 7.5 μg/ml bleomycin (Calbiochem).

**Viability assays.** Promastigotes (5 x 10⁵) harvested at early log phase and transfected either with LdmPrx/pX63pol or pX63pol in 100 μl of Hanks balanced salt solution were resuspended in various concentrations of antiperoxidative stress inducers in 96-well plates at 25°C. H₂O₂ and r-BOOH (Merck) were used as peroxide donors. MAHMA NONOate, PAPA NONOate, spermine NONOate, NOC-7, NOC-9, NOC-12 (Calbiochem), and GSNO (S-nitroso-L-glutathione; Alexis Biochemicals) were used as nitric oxide donors. Sin-1 (Calbiochem) yields NO and superoxide anion radicals. NOR-3 (Calbiochem) is cell permeating and releases NO intra- and extracellularly. DMNQ (2,3-dimethoxy-1-naphthoquinone; Alexis Biochemicals) induces intracellular superoxide anion formation. The investigated concentrations ranged from 0 to 5 mM. After incubation (1 h), the stress inducers were removed and parasite viability was measured by monitoring incubation in Cell Proliferation Reagent WST-1 (Roche) for 3 h. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium salt to formazan. Formazan was detected on a microplate reader at 440 nm. Percent viability was calculated from the ratio of optical density readings in wells with stress inducers to those in control wells x 100. In order to compare the ratio of control viabilities to viabilities of overexpressing parasites more easily, control values were equated to 1.

**In situ labeling of DNA fragments.** Cells undergoing apoptosis generate abundant DNA fragments in their nuclei. In situ detection of DNA fragments by terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) was performed with an in situ cell death detection kit, TMR red (Roche), according to the manufacturer’s instructions. Briefly, 10⁶ promastigotes harvested at early log phase and transfected with either LdmPrx/pX63pol or pX63pol or untransfected cells from the early (2 x 10⁶) and late (6 x 10⁶) logarithmic phase in 1 ml of M199 medium were exposed to 1 or 3 mM H₂O₂ for 3 h. Control cells were incubated in PBS. Cells were used to coat poly-l-lysine-covered slides and fixed with 4% paraformaldehyde. Permeabilization was done with 0.1% Triton X-100; 0.1% sodium citrate for 2 min on ice, followed by incubation in a TUNEL reaction mixture containing TdT and TMR red-labeled nucleotides for 1 h. The samples were counterstained with Hoechst 33258 (Molecular Probes), diluted 1:2,000 in PBS, mounted in mounting medium (Dako Cytomation), and visualized with a Zeiss Axioskop 2 plus immunofluorescence microscope. At least 500 cells in two independent experiments were counted. All counts were done with coded samples to prevent bias.

**RESULTS**

**Sequencing of a mitochondrial peroxiredoxin gene of *L. donovani* and analysis of its amino acid sequence.** In the course of a proteome analysis of in vitro stage differentiation of *L. donovani*, a mitochondrial peroxiredoxin was found to be expressed in a stage-specific manner (7). Primers deduced from the coding region of a homologous *L. major* peroxiredoxin gene (accession no. CAJ03825) were used to amplify the corresponding DNA by PCR with *L. donovani* genomic DNA as the template. A product of 679 bp was obtained; it was 98% identical to its *L. major* homologue. Southern blot analysis demonstrated that the LdmPrx gene is a single-copy gene (data not shown).

The gene for the *L. donovani* peroxiredoxin encodes a protein of 226 amino acid residues with a calculated Mr of 25,000 and a pI value of 6.4. LdmPrx contains a 27-amino-acid N-terminal mitochondrial targeting sequence, as predicted by the program MitoProtII (http://ihg.gsf.de/ihg/mitoprot.html). The amino acid sequence displays 90% identity to the mitochondrial targeting sequences of peroxiredoxins of *L. infantum* and *L. major* and 47% identity to that of *Trypanosoma brucei*. The overall similarities of the whole sequence range from 55 to 60% (higher eukaryotic organisms like *Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster,* and *Homo sapiens*) to more than 97% identity to the other *Leishmania* peroxiredoxins. LdmPrx is a 2-Cys peroxiredoxin (for alignment, see the supplemental material). Classical 2-Cys peroxiredoxins are characterized by two highly conserved cysteine residues (25). The N-terminal cysteine is embedded in a VCP motif as is typical for peroxiredoxins. It appears to be the one that is attacked by the hydroperoxide and has been shown to be essential for the activity in several peroxiredoxins. The redox-active cysteine, the one that interacts with the reductant, is located near the C terminus and is also part of a VCP motif in most of the 2-Cys peroxiredoxins (25). The C-terminal conserved cysteine residue of LdmPrx is different from the VCP motif found in other peroxiredoxins but identical to the IPC motif found in the mitochondrial peroxiredoxins of *T. brucei, T. cruzi, L. major,* and *L. infantum.* This indicates that LdmPrx belongs to a peroxiredoxin subfamily, together with the mitochondrial homologues of *T. cruzi,* *T. brucei,* *L. major,* and *L. infantum,* distinct from the cytosolic enzymes from these organisms, which also display a VCP motif within the C-terminal cysteine.

**Biochemical characterization of recombinant LdmPrx.** The full-length LdmPrx protein was expressed in *E. coli* as an N-terminally His-tagged protein. The purified protein (rLdmPrx) was about 28 kDa in size, which corresponds to the predicted Mr of mature LdmPrx of 25,000, taking the His tag with its molecular mass of approximately 3 kDa into account (Fig. 1A). Matrix-assisted laser desorption ionization–time of flight mass spectrometric analysis of the product after digestion with trypsin confirmed the identity of rLdmPrx (data not shown). With a specific antibody generated against rLdmPrx, a protein with a molecular mass of 26 kDa was detected by Western blot analysis of promastigote cells grown to stationary phase. Under nonreducing conditions, the molecular mass of the native protein shifted to about 50 kDa, suggesting dimerization of the protein (Fig. 1B). The capacity of peroxiredoxins to form dimers is mediated by the cysteines within the two conserved domains (12, 20).

The antioxidant activity of rLdmPrx was characterized in a thiol mixed-function oxidation system (Fig. 1C). Peroxiredoxins are known to prevent DNA damage by ROS. ROS can be produced by incubating DTT with Fe³⁺, which catalyzes the reduction of O₂ to H₂O₂. The latter is further converted by the Fenton reaction to hydroxyl radicals (34, 35). It has been demonstrated that these hydroxyl radicals can induce strand breaks in DNA, as well as chemical changes in the bases and deoxyr-
In the presence of DTT and Fe\(^{3+}\)/H\(_{1100}\), part of plasmid pUC18 was converted into the nicked form after 3h of incubation (Fig. 1C, lane 3). Addition of rLdmPrx completely abolished the conversion of the DNA into the nicked form (Fig. 1C, lane 7), while bovine serum albumin had no effect (Fig. 1C, lane 8). The degree of protection correlated with the amount of rLdmPrx added to the assay (Fig. 1C, lanes 4 to 7). Several recombinant peroxiredoxins from different organisms like *Plasmodium falciparum*, *Chlamydomonas reinhardtii*, *Brugia malayi*, *Onchocerca volvulus*, and *Entamoeba histolytica* were also tested for their DNA-protective effects. For most of them, 1 to 16 \(\mu\)M recombinant protein is necessary for complete DNA protection. It was shown that 5 \(\mu\)M rLdmPrx inhibits nicking of DNA completely, which is in good correlation with the properties of the other peroxiredoxins (14, 23, 24, 27, 36).

Purified rLdmPrx was also able to detoxify \(\mathrm{H}_2\mathrm{O}_2\) as well as \(t\)-BOOH, in vitro in a concentration-dependent manner. Removal of the two peroxides required the presence of DTT (3 mM), indicating that LdmPrx possesses thiol peroxidase activity (Fig. 1D). It was also shown for the recombinant *L. infantum* mitochondrial peroxiredoxin that it reduces \(\mathrm{H}_2\mathrm{O}_2\), as well as \(t\)-BOOH (10). The enzymatic kinetics observed for the removal of \(\mathrm{H}_2\mathrm{O}_2\) is comparable to that of peroxiredoxins from *P. falciparum*, *C. elegans*, human neutrophils, and *Pisum sativum*, where similar peroxidase assays were used (Fig. 1D) (8, 26, 27, 32).

**Expression and localization of LdmPrx in *L. donovani* promastigotes and amastigotes.** A polyclonal antiserum against rLdmPrx was used to detect the protein in Western blot assays of *L. donovani* cellular extracts of all days of stage differentiation from the promastigote to the amastigote form under reducing conditions (Fig. 2A). No protein could be detected in the promastigote stage (day 0 of stage differentiation). A single and specific polypeptide band of about 26 kDa could be detected directly after the heat shock (day 1 of stage differentiation), and the highest intensity was observed from day 3 of the stage differentiation process (Fig. 2A). To study if the differential expression of LdmPrx is specific for the differentiation process or can also be induced in the presence of other culture conditions, Western blot assays of promastigote parasites harvested...
at different time points from the early logarithmic to the late logarithmic phase of culture were performed (Fig. 2B). As shown also in Fig. 2A for promastigotes grown to the early logarithmic phase (2 × 10⁷ cells/ml), no detectable amount of LdmPrx could be observed by Western blot analysis. At a cell density of 5 × 10⁶/ml, a peroxiredoxin signal could be detected which showed an increase in intensity when cells were cultured to the stationary growth phase (6 × 10⁶/ml). Exposing the promastigote cells to sublethal doses of H₂O₂ and t-BOOH, on the other hand, did not have any effect (data not shown). Therefore, the increase in expression may occur in response to differentiation-induced signals.

Immunoelectron microscopy and immunofluorescence assays were used to determine the subcellular localization of LdmPrx. Promastigote cells showed labeling only in the kinetoplast area (Fig. 3A, magnifications). In amastigotes, labeling of the kinetoplast area was also observed. In addition, the amastigotes exhibited extensive labeling of the whole mitochondrion (Fig. 3B, magnifications), with a strong increase in the amount compared to that of the promastigotes. These results indicate correct targeting of LdmPrx into the mitochondrion and confirm the differences in protein amounts between the two stages detected by Western blot assay and the proteomic approach (7).

As suggested by the presence of a mitochondrial targeting sequence and indicated by the electron microscopic studies, immunofluorescence analysis also corroborated that this protein localizes to the single mitochondrion of the parasite. The anti-LdmPrx antibody staining perfectly colocalized with the MitoTracker dye, a marker for mitochondria (Fig. 3C and D). Late logarithmic phase promastigotes and in vivo-derived amastigotes (Fig. 3C and E) revealed anti-LdmPrx staining all over the mitochondrion. As shown in Fig. 3E, Mitotracker dye stains the Leishmania mitochondrion, as well as the mitochondria of the macrophages. As supposed from the electron microscopy studies and Western blot analyses, early logarithmic phase promastigotes showed only weak anti-LdmPrx staining (Fig. 3C). The immunofluorescence studies also indicate that promastigotes transformed with the expression vector LdmPrx/pX63pol, which led to overexpression of the peroxiredoxin in early logarithmic phase promastigotes, showed distinct staining only in the mitochondrial area. Clear colocalization with the Mitotracker dye was observed (Fig. 3D). Therefore, overexpression of LdmPrx leads to correct targeting of the molecule. No mislocalization could be detected.

Overexpression of LdmPrx did not decrease the sensitivity of L. donovani promastigotes to exogenously produced oxidative stress. To investigate the role of LdmPrx in protection against oxidative and nitrosative stress, we overexpressed the protein within the parasites by using Leishmania-specific expression vector pX63pol. LdmPrx/pX63pol-transformed parasites and control cells transfected with plasmid pX63pol showed no substantial growth rate alterations compared to wild-type parasites (Fig. 4A). Overexpression of LdmPrx in early logarithmic phase promastigotes revealed a significant increase in expression of the protein compared to that in control cells (Fig. 4B).

To see if overexpression of the protein in L. donovani promastigotes protects parasites against oxidative stress, we measured the viability of pX63pol- and LdmPrx/pX63pol-transfected parasites after incubation with different oxidative stress inducers. Table 1 shows the relationship of the viability of overexpressing cells to that of control cells after exposure to oxidative stress. The same amount of viable cells in both populations was defined as 1. Treatment with neither peroxides (H₂O₂, t-BOOH, Sin-1) nor nitric oxides (MAHMA NONOate, PAPA NONOate, spermine NONOate, GSNO, NOC-7, NOC-9, NOC-12, Sin-1) had any effect on the viability of LdmPrx-overexpressing cells compared to that of control cells. Overexpression of the protein did not even show a protective effect after exposure to cell-permeating ROS producers like DMNO and NOR-3, which generate superoxide anion radicals and nitric oxides intracellularly. These results are consistent with the observation that, after exposure to oxidative stress, no upregulation of LdmPrx occurred. In contrast to the missing resistance to ROS of L. donovani overexpressing LdmPrx, for L. infantum overexpressing LimTXNPrx, significant resistance to t-BOOH, but not to H₂O₂, also was observed (11).

Overexpression of LdmPrx prevents DNA breakdown in L. donovani promastigotes after exposure to H₂O₂. Exposure to H₂O₂ triggers apoptosis in numerous mammalian cells and yeast (15), leading to membrane blebbing, cytoplasmic and nuclear condensation, and chromatin aggregation with accompanying DNA breakage (52). Das and colleagues studied the death-associated phenotype in L. donovani promastigotes that occurs after exposure to H₂O₂ (18). They demonstrated that
parasites undergo necrosis-like death with doses greater than 4 mM H$_2$O$_2$. Lesser doses, on the other hand, precipitate apoptosis-like death, resulting in breakdown of DNA material detected by TUNEL staining. We also were able to generate detectable DNA fragments in our control parasites transfected by TUNEL staining. We also were able to generate detectable DNA fragments in our control parasites transfected by TUNEL staining. 

It is known that H$_2$O$_2$ is toxic for Leishmania. At the concentrations used for the TUNEL assay, all cells die after 3 h of exposure to H$_2$O$_2$ but only those cells having an increased amount of mitochondrial peroxiredoxin are protected from PCD. Nevertheless, H$_2$O$_2$ is stable enough to diffuse through the whole cell and is not only involved in induction of PCD. It also has dramatic cytoplasmic effects leading, for example, to disruption of biochemical pathways and therefore to necrosis-like death. Since LdmPrx is localized only in the mitochondria, the necrosis-like death is not affected.

**DISCUSSION**

During its life cycle, *Leishmania* faces oxidants from different sources, either during the oxidative burst after phagocytosis of the parasites, in the course of which superoxide radicals (O$_2^-$), H$_2$O$_2$, peroxynitrite, and lipoxygenase products are produced (40) or as a by-product of the parasite’s aerobic metabolism, whose most important source is the mitochondrial electron transport chain (51). Therefore, *Leishmania* survival depends on strategically localized antioxidant enzymes, which are able to quickly eliminate the oxidants in the respective cell compartments.

The peroxiredoxin gene characterized in the present study encodes a mitochondrial 2-Cys peroxiredoxin protein that is a homologue of previously identified tryparedoxin peroxidases. However, it forms a novel group within the peroxiredoxins together with the homologue molecules of *L. infantum* (11), *T. brucei* (49), and *T. cruzi* (56), all of them displaying mitochondrial localization.

All *Leishmania* peroxiredoxins, including the one analyzed in the present study, have been shown to display peroxidase activity in vitro, with H$_2$O$_2$ and t-BOOH as favored substrates (6, 10, 11, 21). Furthermore, LdmPrx reduces hydroxyl radical-

![FIG. 3. Intracellular localization of LdmPrx in *Leishmania* parasites. The peroxiredoxin enzyme was localized by immunoelectron microscopy, L. donovani promastigote cells grown to the early logarithmic phase (A) and in vitro-derived amastigote cells (B) were analyzed with anti-LdmPrx polyclonal antibodies. n, nucleus; k, kinetoplast; m, mitochondrion. L. donovani promastigotes grown to the early and late logarithmic phase (C), promastigotes grown to the early logarithmic phase and transfected with pX63pol or LdmPrx/pX63pol (D), and intracellular amastigote cells (E) were stained in vivo with the MitoTracker dye (MT) and then processed for immunofluorescence assay with anti-LdmPrx polyclonal antibodies. DNA was stained with Hoechst. Parasites were photographed at a magnification of ×630. Phase-contrast images of the preparations are also included. Intracellular amastigotes in panel E are indicated by arrowheads.](image-url)

![FIG. 4. Overexpression of LdmPrx in *L. donovani* promastigote cells. (A) Wild-type (squares), pX63pol-transformed (diamonds), and LdmPrx/pX63pol-transformed (triangles) parasites were cultured for 4 days, and cells were counted every 24 h with a Casy Cell Counter (Scha¨rfe System). The experiment was done three times in duplicate. (B) Western blot assays of *L. donovani* wild-type (lane 1), pX63pol-transformed (lane 2), and LdmPrx/pX63pol-transformed (lane 3) promastigote cells from the early logarithmic phase. Cells (5 × 10$^6$) were lysed directly in hot SDS sample buffer under reducing conditions, fractionated by 12% SDS-PAGE, and transferred to nitrocellulose membrane. Blots were developed with either anti-LdmPrx polyclonal antibodies or anti-β-tubulin monoclonal antibodies (as a loading control). Molecular mass standards are indicated on the left.](image-url)

![FIG. 5. Intracellular amastigote cells from the early logarithmic phase. Cells (5 × 10$^6$) were cultured for 4 days, and cells were counted every 24 h with a Casy Cell Counter (Scha¨rfe System). The experiment was done three times in duplicate.](image-url)

![TABLE 1. Relationships among percent viabilities of parasites transfected with either LdmPrx/pX63pol or pX63pol (control) under different stress conditions](table-url)
induced nicking of DNA. This observation, together with the localization of LdmPrx in the vicinity of the kinetoplast, suggests that one function of this enzyme is protection of the mitochondrial genome from direct or indirect peroxide-mediated damage. A similar localization was observed for a mitochondrial peroxiredoxin of \textit{T. cruzi}. This protein was also found to be concentrated around the kinetoplast (56). Relevant information regarding the localization of other \textit{Leishmania} peroxiredoxins is not available.

During stage differentiation from the promastigote to the amastigote form but also during the transformation of promastigotes grown from the early logarithmic phase to the late logarithmic phase, the amount of peroxiredoxin within the cells increases dramatically. These observations from Western blot analyses were confirmed by immunofluorescence and electron microscopic studies, which showed a uniform distribution through the whole tube-shaped mitochondrion in amastigotes and promastigotes grown to the late logarithmic phase. Stage-specific expression has been described for other leishmania peroxiredoxins. Cytoplasmic \textit{L. chagasi} peroxiredoxin 1 (LcPxn1), for example, is predominantly expressed in the amastigote stage, whereas LcPxn2 and LcPxn3 are expressed mainly in the promastigote stage, with LcPxn3 being far less abundant than LcPxn2 (6). Nevertheless, diverse expression of \textit{Leishmania} mitochondrion-localized peroxiredoxins was not known until now. Regulation of mitochondrial peroxiredoxins was reported from yeast and humans. Prx1p from \textit{S. cerevisiae} is upregulated when cells use the respiratory pathway, as well as in response to oxidative stress (45). There is also evidence for differential expression of the mammalian mitochondrial peroxiredoxin PrdxIII. This protein shows a change in expression pattern in neurodegenerative disorders like Down syndrome and Pick’s disease (29).

Peroxiredoxins seem to be essential for parasite survival as there is no reported knockout of a peroxiredoxin gene in kinetoplastids so far. Wilkinson and colleagues therefore chose an RNA interference (RNAi) approach to investigate the biological function of peroxiredoxins. RNAi with TbCPX in \textit{T. brucei} bloodstream forms had a dramatic effect. Within 24 h, a significant reduction in the growth rate could be observed and in the following 24 h most of the cells died and those that remained alive exhibited greatly decreased mortality (55). Induction of RNAi was also associated with a 16-fold increase in susceptibility to exogenous H$_2$O$_2$. When expression of the mitochondrial peroxiredoxin TbMPX was lowered, no change in growth rate or sensitivity to exogenous H$_2$O$_2$ was observed, which correlates with the cessation of many mitochondrion-associated functions, including the respiratory cycle, in the bloodstream form of the parasite (55). In \textit{Leishmania}, the mitochondrion is active during the whole life cycle of the parasite and the homologous protein is apparently essential for the parasite as we were not able to generate viable cells after targeted gene deletion.

Displaying peroxidase activity in vitro does not imply that the only in vivo function of peroxiredoxin proteins is defense against oxidative stress. Other biological roles for peroxiredoxins have been described, including regulation of H$_2$O$_2$-mediated signal transduction (58) and involvement in the regulation of apoptosis (13, 44). We showed that overexpression of LdmPrx in \textit{L. donovani} does not decrease the sensitivity of promastigotes to exogenously produced oxidative stress. Previous reports have shown that both ROS and RNS contribute to the early control of \textit{Leishmania} infection (22, 41). Clearly, they need an antioxidant defense system against ROS to provide a selective advantage for survival. Overexpression of cytoplasmic peroxiredoxins in \textit{Leishmania} is sufficient to show resistance to exogenous peroxides (5, 11, 56). Therefore, LdmPrx is not well positioned to protect the parasite against host-derived oxidative stress and the data derived from viability assays also indicate another biological role for this enzyme.

A cell suicide pathway analogous to the process described as PCD in metazoa has evolved in some parasitic protozoa. Especially for trypanosomatids, there are numerous reports describing PCD-like processes with the goal to elucidate the molecular mechanisms underlying this procedure (2–4, 18, 33, 39, 59). Upon exposure to suitable doses of H$_2$O$_2$, \textit{L. donovani} promastigotes display several features comparable to apoptotic metazoan cells like nuclear condensation, accumulation of intracellular calcium, activation of caspase-like proteases, decreased intracellular glutathione-like (trypanothione) content, fragmentation of cellular DNA, formation of DNA ladders, cleavage of a poly(ADP)ribose polymerase-like protein, and loss of cell volume (18). In \textit{T. brucei} procyclic forms, similar
morphological features of PCD could be induced in vitro by concanavalin A treatment or oxidative stress (46, 54). Additionally, *L. amazonensis* promastigotes display DNA laddering and chromatin condensation in dense clusters upon heat shock, corresponding to classical features of PCD (38). Recently, it was shown that the death response of *L. donovani* to H$_2$O$_2$ results in loss of mitochondrial membrane potential, suggesting the involvement of mitochondria in cell death (39). Mitochondria are the prime checkpoints for the control of apoptosis (43, 53). It is postulated that the existence of PCD in *Leishmania* may maximize the biological fitness of the parasites. In this context, it has been suggested that limiting nutritional resources in the gut of the insect vector leads to death of the excess of promastigotes by PCD. Furthermore, it is postulated that cells that did not differentiate from the promastigote into the infectious metacyclic form die, so that they may not compete with the differentiated cells for available nutrients. In addition, it is speculated that the ability of intracellular amastigotes to undergo PCD leads to a reduction of the host immune response and facilitates the survival of residual parasites (28).

Apparently, LdmPrx protects from PCD. This assumption correlates well with the known function of the mammalian mitochondrial peroxiredoxin PrdxIII. For PrdxIII, it was shown that it is an important regulator of the abundance of mitochondrial H$_2$O$_2$ (13). Depletion of PrdxIII results in increased mitochondrial accumulation of H$_2$O$_2$, which led to an increase in the rate of apoptosis (13). Furthermore, it was shown that overexpression of PrdxIII in a mammalian cell line protects the cells from apoptosis caused by H$_2$O$_2$ and t-BOOH (44). In summary, the authors suggested that the mitochondrial protein regulates the physiological level of H$_2$O$_2$ and protects cells from apoptosis induced by high levels of H$_2$O$_2$ (44). This emerging picture fits ideally with our results. LdmPrx seems to detoxify the H$_2$O$_2$ within the mitochondria and therefore protects from PCD. Nevertheless, at the concentrations used for the TUNEL assay in the present study, all cells die after 3 h of exposure to H$_2$O$_2$. But only those cells having an increased amount of mitochondrial peroxiredoxin are protected from PCD. H$_2$O$_2$ is stable enough to diffuse through the whole cell and is not only involved in induction of PCD. It also has dramatic cytoplasmic effects leading, for example, to the disruption of biochemical pathways and therefore to necrosis-like death. Since LdmPrx is localized only in the mitochondria, necrosis-like death is not affected.

In summary, we can conclude that LdmPrx is, as PrdxIII has been described, an important H$_2$O$_2$-eliminating enzyme in mitochondria.

PCD in trypanosomatids could be a remnant process of eukaryotic cell evolution that does not possess a particular function and is induced in response to diverse stimuli. Signals that might indicate PCD include drugs, oxidative stress, inhibitors of signaling molecules, exposure to human serum, nutritional deprivation, heat shock, and nitric oxide (59). Upregulation of a protein like the mitochondrial peroxiredoxin that protects against this kind of cell death could therefore be inevitable under stress conditions.

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