

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

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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 pro-oxidants. 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 lo-

calized 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* (LimTXNPrx), as well as for the cytoplasmic peroxiredoxins from *L. infantum* (LicTXNPrx), *L. chagasi* (LcPxn1, LcPxn2) and *L. donovani* (LdH6TXNPrx), 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 LcPxn1 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 nonselective 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

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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 H₂O₂ and *t*-BOOH (44). There are increasing numbers of reports that describe apoptosis- or PCD-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 H₂O₂, *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 H₂O₂, 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 H₂O₂-mediated PCD, pointing to a vital role for this peroxiredoxin in the survival of the parasite.

(This report includes part of the doctoral thesis of M. Bente.)

MATERIALS AND METHODS

Cultivation of cells. *L. donovani* strain Lo8, 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 × 10⁶ 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 mildly acidic medium (pH 5.5, 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% thioglycolate 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 sterile PBS. PECs were washed once and seeded at a density of 10⁶ cells per 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% CO₂ at 37°C for 24 h, PECs were incubated with *L. donovani* parasites at a parasite-to-PEC ratio of 10:1 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* logarithmic phase promastigotes was prepared with the Puregene DNA purification system (Gentra Systems) 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 from *L. major* (accession no. 6066432): sense primer Prx-S26(NdeI) (5'-GAGACATATGCTCCGCGTCTT TCCA-3') and antisense primer Prx-AS27(XhoI) (5'-GAGACTCGAGTCACA TGTCTTCTCGA-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 pJC45, a derivative of pJC40 (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-nitrilotriacetic 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 semidry blotting technique with electrophoresis buffer (0.25 M Tris, 0.5 M glycine, 1% SDS) as blotting buffer. Polyclonal 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-indolylphosphate (BCIP)-Nitro Blue Tetrazolium color developmental substrate (Promega).

Immunoelectron microscopy. Cells were harvested by centrifugation (10 min, 690 × 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 Ultracut E (Reichert) and placed on 200-mesh 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, following 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), 1:2,000 in PBS, cells were mounted in mounting medium (Dako Cytomation) and examined with a Zeiss Axioskop 2 plus immunofluorescence microscope and the improvisation software.

Intracellular amastigotes on coverslips were incubated with 1 nM MitoTracker 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 H₂O₂ and *t*-BOOH was evaluated with 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 H₂O₂ 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 H₂O₂ and *t*-BOOH were determined spectrometrically by using known amounts of H₂O₂ 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 µM FeCl₃, 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

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 Prx-S26(NdeI) and Prx-AS27(XhoI) were used to PCR amplify the coding region of LdmPrx. The product was digested with NdeI and XhoI, and the 5' overlapping ends were filled in by using Klenow polymerase to create blunt ends. The vector was digested with EcoRV and ligated with the prepared insert. The correct orientation and sequence were reconfirmed by nucleotide sequencing.

Transfection of *L. donovani* promastigotes. Plasmid DNA was purified with a Nucleobond 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. Electrotransfection 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 × 10⁶) harvested at early log phase and transfected either with LdmPrx/pX63pol or pX63pol in 100 µl of Hanks balanced salt solution were exposed in triplicate to various concentrations of different oxidative stress inducers in 96-well plates at 25°C. H₂O₂ and *t*-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-1,4-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 × 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, 10⁷ promastigotes harvested at early log phase and transfected with either LdmPrx/pX63pol or pX63pol or untransfected cells from the early (2 × 10⁶) and late (6 × 10⁷) logarithmic phase in 1 ml of M199 medium were exposed to 1 to 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 indicated 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 *M_r* 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 second 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 *M_r* 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 deoxyri-

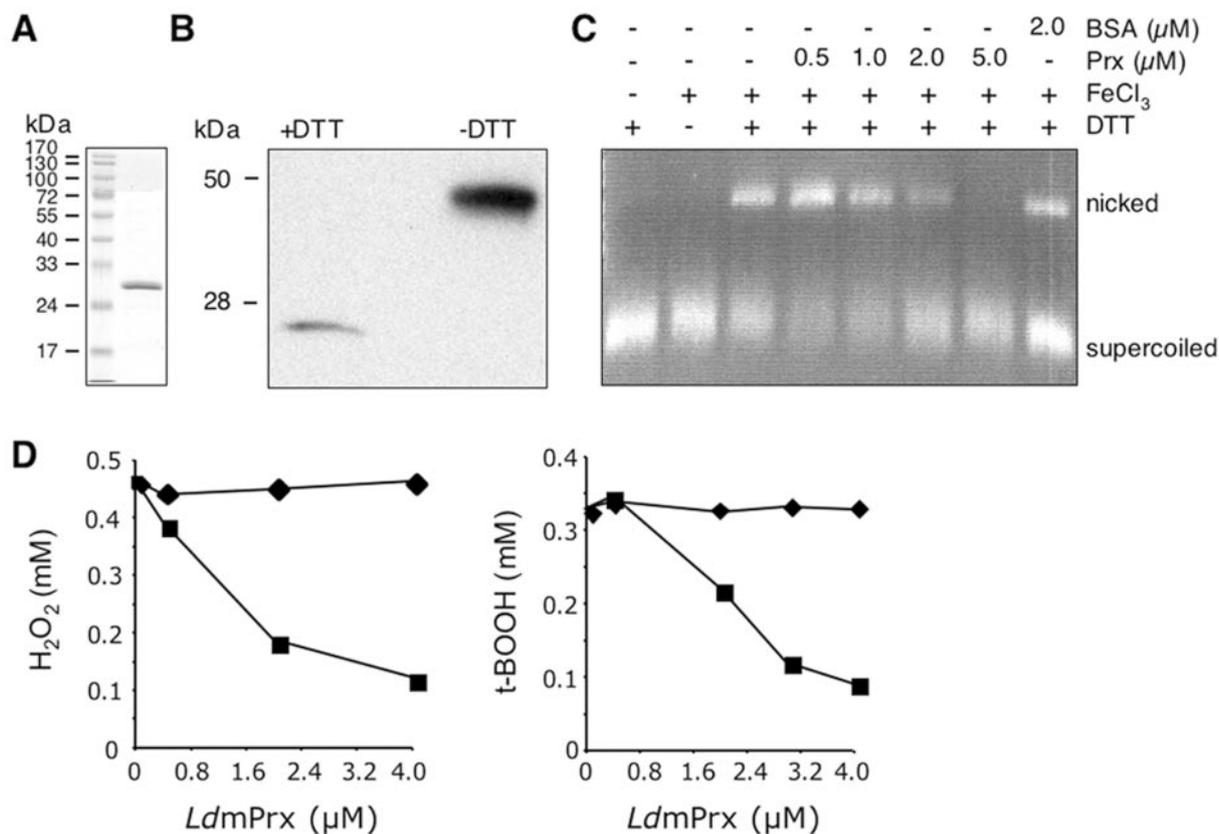


FIG. 1. Recombinant LdmPrx shows peroxiredoxin enzyme activities in vitro. (A) Twelve percent SDS-PAGE of purified rLdmPrx under reducing conditions. Molecular mass standards are indicated on the left. (B) Western blot assay of *L. donovani* promastigote lysates. Cells (5×10^6) from the stationary growth phase ($6 \times 10^7/\text{ml}$) were lysed directly in hot SDS sample buffer under nonreducing conditions (without DTT) and reducing conditions (in the presence of 20 mM DTT). Blots were developed with anti-LdmPrx polyclonal antibodies. Molecular mass standards are indicated on the left. (C) Nicking assay with recombinant LdmPrx was performed as described in Materials and Methods. Samples were then loaded onto a 1% agarose gel. Lane 1, only DTT; lane 2, only FeCl₃; lane 3, DTT plus FeCl₃; lane 4, DTT plus FeCl₃ plus rLdmPrx (0.5 μM); lane 5, DTT plus FeCl₃ plus rLdmPrx (1 μM); lane 6, DTT plus FeCl₃ plus rLdmPrx (2 μM); lane 7, DTT plus FeCl₃ plus rLdmPrx (5 μM); lane 8, DTT plus FeCl₃ plus bovine serum albumin (BSA; 2 μM). (D) Peroxide assay with rLdmPrx performed as described in Materials and Methods. Symbols: squares, untreated rLdmPrx; diamonds, rLdmPrx incubated at 70°C for 5 min.

boses (17). In the presence of DTT and Fe³⁺, part of plasmid pUC18 was converted into the nicked form after 3 h 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 μM recombinant protein is necessary for complete DNA protection. It was shown that 5 μM 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 H₂O₂, 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 H₂O₂,

as well as *t*-BOOH (10). The enzymatic kinetics observed for the removal of H₂O₂ 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). This result was consistent with the result obtained from the proteomic approach to *L. donovani* stage differentiation (7). 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

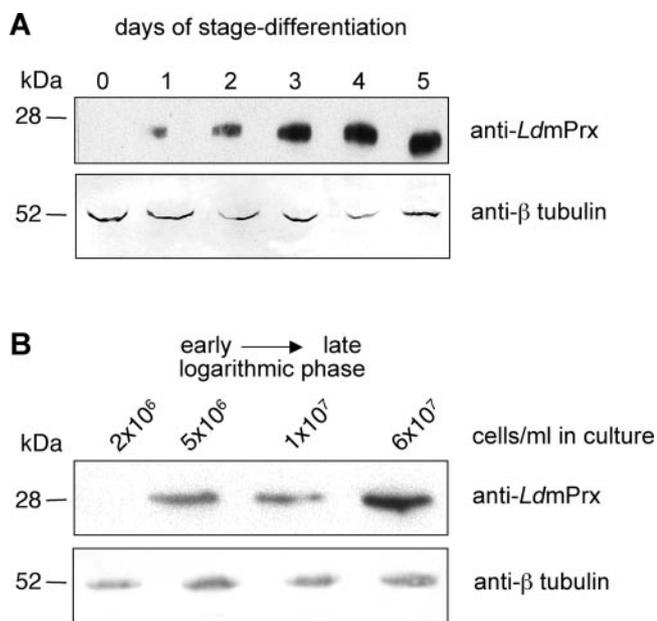


FIG. 2. Expression pattern of LdmPrx in *L. donovani*. Western blot assays of *L. donovani* lysates from cells of each day of stage differentiation (as mentioned in Materials and Methods, the transformation starts with parasites grown to the early logarithmic phase [2×10^6 cells/ml]) (A) or cells from different time points of promastigote cell culture (B) are shown. Lane 1, 2×10^6 cells/ml; lane 2, 5×10^6 cells/ml; lane 3, 1×10^7 cells/ml; lane 4, 6×10^7 cells/ml. From each time point, 5×10^6 cells 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.

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 (2×10^6 cells/ml), no detectable amount of LdmPrx could be observed by Western blot analysis. At a cell density of 5×10^6 /ml, a peroxiredoxin signal could be detected which showed an increase in intensity when cells were cultivated to the stationary growth phase (6×10^7 /ml). Exposing the promastigote cells to sublethal doses of H_2O_2 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).

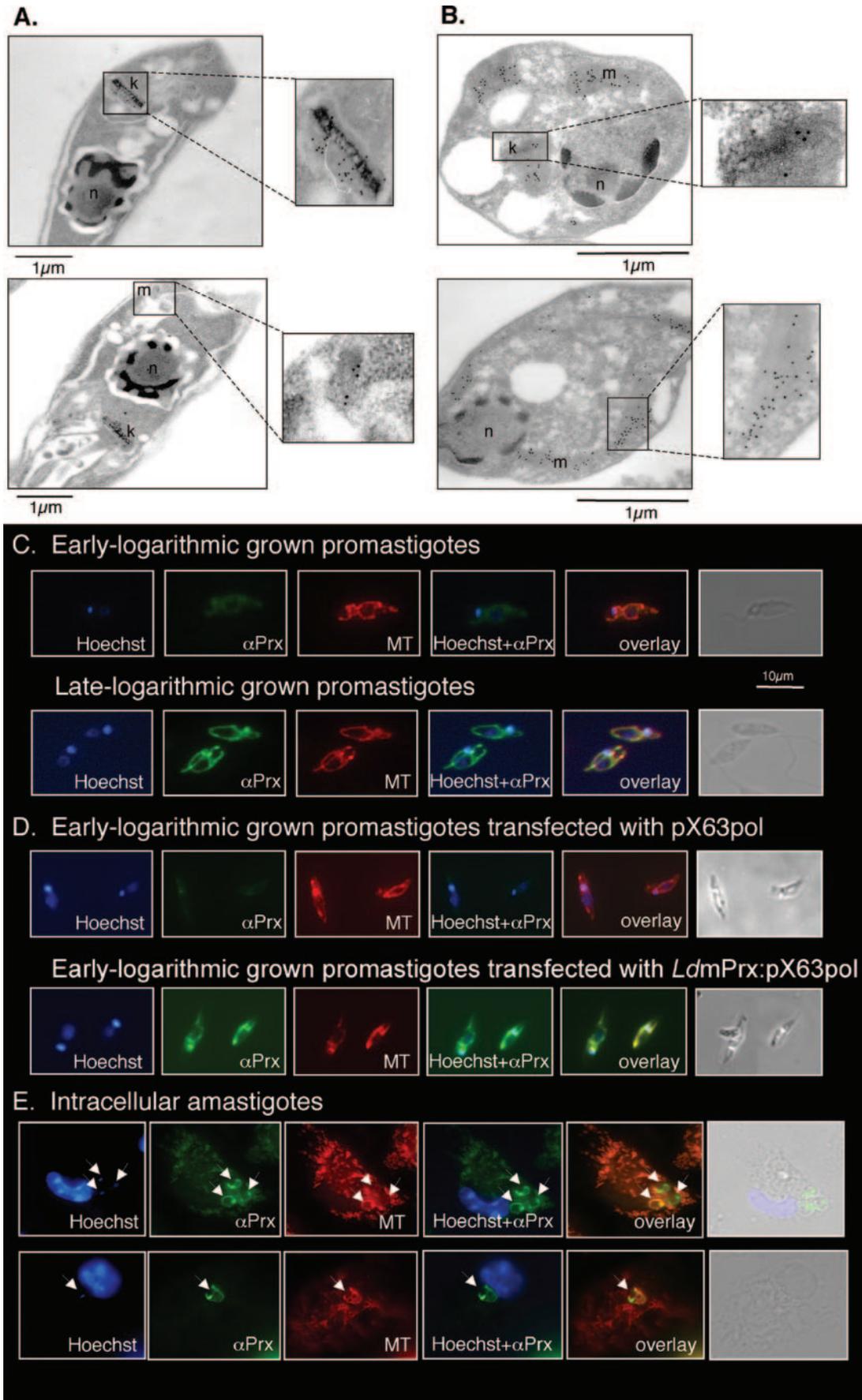
As suggested by the presence of a mitochondrial targeting sequence and indicated by the electron microscopic studies,

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). Overexpressing 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-transformed 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 (H_2O_2 , *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 DMNQ 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 LimTXNPx, significant resistance to *t*-BOOH, but not to H_2O_2 also, was observed (11).

Overexpression of LdmPrx prevents DNA breakdown in *L. donovani* promastigotes after exposure to H_2O_2 . Exposure to H_2O_2 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 H_2O_2 (18). They demonstrated that



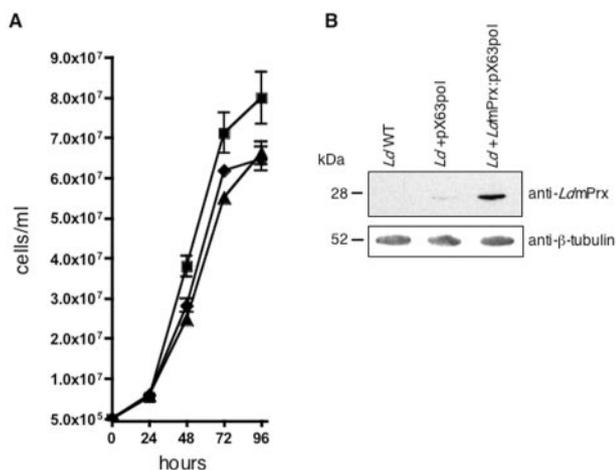


FIG. 4. Overexpression of LdmPrx in *L. donovani* promastigote cells. (A) Wild-type (squares), pX63pol-transformed (diamonds), and LdmPrx/pX63pol-transformed (triangles) parasites were cultured for 4 days, and cells were counted every 24 h with a Casy Cell Counter (Schärfe System). The experiment was 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.

parasites undergo necrosis-like death with doses greater than 4 mM H_2O_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 with pX63pol (Fig. 5A and B) after 3 h of treatment with 1 to 3 mM H_2O_2 . About 60% of the cells counted became TUNEL positive. In contrast to that, only approximately 8% of the LdmPrx-overexpressing parasites became TUNEL positive after the appropriate treatment (Fig. 5A and B), even though no difference in the survival rate between peroxiredoxin-overexpressing *Leishmania* and the respective controls was observed (Table 1).

The same resistance to PCD was observed when cells grown to the early and late logarithmic phase were compared. For cells grown to the late logarithmic phase, which also showed an increase in the amount of peroxiredoxin, approximately 5% TUNEL-positive cells were counted. Cells from the early logarithmic growth phase were 30 to 90% TUNEL positive (Fig. 5B).

It is known that H_2O_2 is toxic for *Leishmania*. At the concentrations used for the TUNEL assay, all cells die after 3 h of

TABLE 1. Relationships among percent viabilities of parasites transfected with either LdmPrx/pX63pol or pX63pol (control) under different stress conditions

Oxidative stress inducer	Concn (nM)	Relationship ^a
H_2O_2	1.0	1.04
<i>t</i> -BOOH	2	0.97
MAHMA NONOate	2	1.07
PAPA NONOate	0.5	1.00
Spermine NONOate	0.3	1.07
DMNQ	2	1.04
GSNO	2	1.52
Sin-1	0.3	0.54
NOR-3	0.3	1.02
NOC-7	0.3	0.71
NOC-9	0.8	0.71
NOC-12	1	1.12

^a The same amount of viable cells in both populations was defined as 1.

exposure to H_2O_2 but only those cells having an increased amount of mitochondrial peroxiredoxin are protected from PCD. Nevertheless, H_2O_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 ($O_2^{\cdot-}$), H_2O_2 , peroxynitrite, and lipoygenase 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_2O_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 $\times 630$. Phase-contrast images of the preparations are also included. Intracellular amastigotes in panel E are indicated by arrowheads.

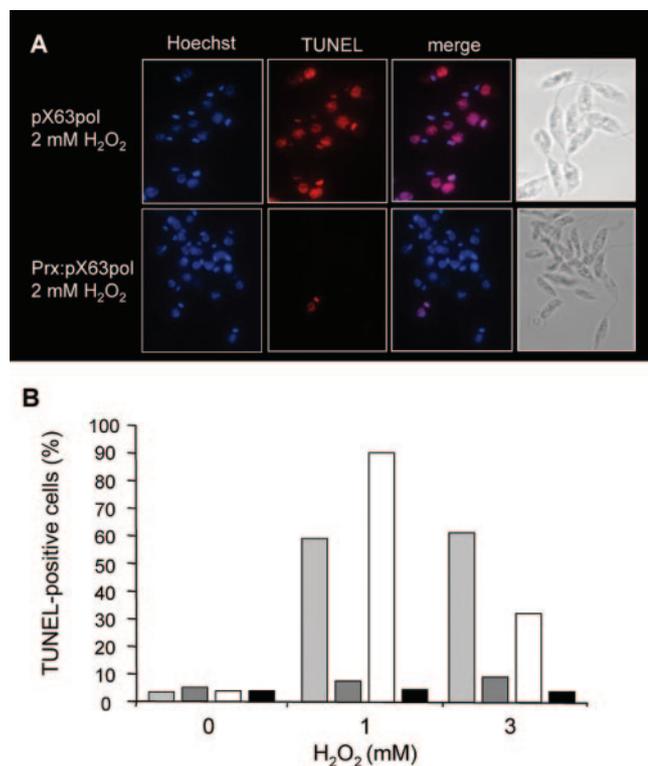


FIG. 5. DNA fragmentation in *L. donovani* promastigotes overexpressing LdmPrx. (A) Microscopic images of TUNEL-stained, pX63pol- and LdmPrx/pX63pol-transfected promastigotes (early logarithmic phase, 2×10^6 /ml) after 3 h of exposure to 2 mM H₂O₂. DNA was stained with Hoechst. Parasites were photographed at a magnification of $\times 630$. Phase-contrast images of the preparations are also included. (B) pX63pol (light gray bars)- and LdmPrx/pX63pol (dark gray bars)-transfected parasites (early logarithmic phase), as well as parasites grown to the early (white bars) and late (black bars) logarithmic phase, were exposed to various concentrations of H₂O₂ for 3 h, and DNA fragmentation was detected by TUNEL staining.

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 *T. cruzi*. This protein was also found to be concentrated around the kinetoplast (56). Relevant information regarding the localization of other *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 *L. chagasi* peroxiredoxin 1 (LcPxn1), for example, is predominantly expressed in the amastigote stage, whereas LcPxn2 and LcPxn3 are expressed mainly in the promas-

tigote stage, with LcPxn3 being far less abundant than LcPxn2 (6). Nevertheless, diverse expression of *Leishmania* mitochondrion-localized peroxiredoxins was not known until now. Regulation of mitochondrial peroxiredoxins was reported from yeast and humans. Prx1p from *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 *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 H₂O₂. When expression of the mitochondrial peroxiredoxin TbMPX was lowered, no change in growth rate or sensitivity to exogenous H₂O₂ 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 *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 H₂O₂-mediated signal transduction (58) and involvement in the regulation of apoptosis (13, 44). We showed that overexpression of LdmPrx in *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 *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 *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 H₂O₂, *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 *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 H₂O₂ 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 H₂O₂ (13). Depletion of PrdxIII results in increased mitochondrial accumulation of H₂O₂, 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 H₂O₂ and *t*-BOOH (44). In summary, the authors suggested that the mitochondrial protein regulates the physiological level of H₂O₂ and protects cells from apoptosis induced by high levels of H₂O₂ (44). This emerging picture fits ideally with our results. LdmPrx seems to detoxify the H₂O₂ 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 H₂O₂. But only those cells having an increased amount of mitochondrial peroxiredoxin are protected from PCD. H₂O₂ 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 H₂O₂-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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