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Iron Superoxide Dismutases Targeted to the Glycosomes of *Leishmania chagasi* Are Important for Survival

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Kinetoplastid glycosomes contain a variety of metabolic activities, such as glycolysis, β-oxidation of fatty acids, lipid biosynthesis, and purine salvage. One advantage of sequestering metabolic activities is the avoidance of cellular oxidative damage by reactive oxygen species produced as a by-product of metabolism. Little is known about how glycosomes themselves withstand these toxic metabolites. We previously isolated an iron superoxide dismutase from *Leishmania chagasi* that is expressed at low levels in the early logarithmic promastigote stage and increases toward the stationary promastigote and amastigote stages. We have since identified a second highly homologous *Lcfesodb* gene that is expressed at high levels in the early logarithmic promastigote stage and decreases toward the stationary promastigote and amastigote stages. Localization studies using green fluorescent protein fusions have revealed that LcFeSODB1 and LcFeSODB2 are localized within the glycosomes by the last three amino acids of their carboxyl termini. To better understand the specific role that FeSODB plays in parasite growth and survival, a single-allele knockout of the *Lcfesodb1* gene was generated. The parasites with these genes exhibited a significant reduction in growth when endogenous superoxide levels were increased with paraquat in culture. Furthermore, the FeSODB1-deficient parasites exhibited a significant reduction in survival within human macrophages. Our results suggest that LcFeSODB plays an important role in parasite growth and survival by protecting glycosomes from superoxide toxicity.

Despite the evolution of a complex mammalian immune response against foreign pathogens, *Leishmania* continues to plague humans, causing death and disease worldwide. This places an emphasis on elucidating the molecular mechanisms employed by *Leishmania* to establish a successful infection. *Leishmania* is an intracellular protozoan parasite that infects mammalian macrophages. These parasites possess a digenic life cycle, consisting of an extracellular promastigote form that multiplies and develops within the alimentary tract of the sand fly vector and an intracellular amastigote form that resides and multiplies within the phagosome of the mammalian host macrophage.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are toxic metabolites that damage all living cells. The superoxide anion ($O_2^{\cdot -}$) is produced by the reduction of oxygen and is the fundamental oxidant among a battery of reactive intermediates, as it is involved in numerous reactions generating a plethora of increasingly toxic intermediates, including hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), and peroxynitrite ($ONOO^-$). Collectively, these prooxidants are toxic to DNA and can cause peroxidation of lipids and the inactivation of iron-sulfur enzymatic centers of dehydratases (15). Important reactions involving $O_2^{\cdot -}$ are demonstrated in the following reactions: $O_2^{\cdot -} + Fe^{3+} \rightarrow Fe^{2+} + O_2$; $2\,O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$; $O_2^{\cdot -} + H_2O_2 \rightarrow {}^{\cdot}OH + {}^{-}OH + O_2$; $O_2^{\cdot -} + HOCl \rightarrow {}^{\cdot}OH + O_2 + Cl^-$; and $O_2^{\cdot -} + {}^{\cdot}NO \rightarrow ONOO^-$.

In transforming from promastigotes to amastigotes, *Leishmania* parasites undergo numerous biochemical changes in order to adapt to their new environment. Numerous genes have been shown to be differentially expressed in the promastigote and amastigote stages, and many morphological and metabolic changes have been documented. A particularly interesting organelle that houses many metabolic activities, such as glycolysis, β-oxidation of fatty acids, ether lipid biosynthesis, and purine salvage, is the glycosome. Glycosomes are found in *Leishmania* and other kinetoplastid parasites. One of the benefits of compartmentation of metabolic activities is the avoidance of cellular oxidative damage caused by ROS that are produced as a result of metabolism. Little is known about the antioxidant defenses of *Leishmania* that protect these organelles and the important metabolic enzymes contained in them from oxidative damage.

Superoxide dismutases (SODs) comprise a family of metalloenzymes containing iron (FeSOD), manganese (MnSOD), nickel (NiSOD), or copper and zinc (Cu/ZnSOD) at the active site (1, 35). SODs dismutate $O_2^{\cdot -}$ into H_2O_2 and O_2 before it exacerbates prooxidant damage by initiating the formation of even more toxic species, such as $^{\cdot}OH$ and $ONOO^-$. SODs have been shown to contribute to the pathogenicity of many parasites by protecting them from $O_2^{\cdot -}$ toxicity (2, 3, 6, 16, 25, 28, 32, 34). We have previously isolated an FeSOD from *Leishmania chagasi*, *Lcfesodb1*, which is expressed at high levels in the stationary promastigote and amastigote stages of infection. We present here the isolation from *L. chagasi* of a second member of the FeSODB gene family (*Lcfesodb2*) that is expressed at high levels in the early logarithmic promastigote stage. We have shown that both LcFeSODB1 and LcFeSODB2 are localized to the glycosomes by the last three amino acids of their carboxyl termini, suggesting that these proteins may act to protect important glycosomal enzymes from $O_2^{\cdot -}$ toxicity. In order to further characterize the role that *Lcfesodb* plays in parasite survival, we generated a single-allele knockout of the

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Lcfesodb1 gene using homologous-recombination technology. A single-allele knockout of *Lcfesodb1* resulted in a decreased level of growth when the endogenous level of $O_2^{\cdot -}$ within the parasites was increased with paraquat and a decreased level of survival within macrophage cells, suggesting that *Lcfesodb1* is important for the growth and survival of *L. chagasi*. Such an antioxidant defense within *Leishmania* glycosomes had not been clearly defined previously.

MATERIALS AND METHODS

Materials. All enzymes, [α-^{32}P]dCTP, Hybond N$^+$, Rapid-Hyb buffer, and protein molecular weight markers, were purchased from Amersham Biosciences. DNA and RNA molecular weight markers, agarose and parasite medium, medium components, and hygromycin B and Geneticin drugs were supplied by Life Technologies, Inc. All other chemicals were purchased from Sigma unless stated otherwise. Nucleic acid quantifications were performed using the Beckman DU 640 spectrophotometer.

Parasites. *L. chagasi* (HMOM/BR/00/1669), *Leishmania donovani* (1S2D), and *Leishmania major* (Friedlin strain) parasites were used in this study. *L. chagasi* promastigote parasites were cultured at 26°C in hemoflagellate minimal essential medium consisting of minimal essential medium supplemented with sodium pyruvate (1.1×), essential (0.5×) and nonessential (1×) amino acids, glucose (0.15%), sodium bicarbonate (0.22% [wt/vol]), 10% fetal calf serum (inactivated at 56°C for 30 min), 10 μg of hemin/ml, and 35 mM HEPES sodium salt. The promastigotes were inoculated at 10^6/ml and harvested at log or stationary phase, defined by morphological and concentration criteria as previously described (36). Log-phase parasites were ovoid to cigar shaped and at a concentration of 4×10^6 to 8×10^6/ml. Stationary-phase parasites were needle shaped and at a concentration of 5×10^7 to 7×10^7/ml.

Infection of U937 cells. Differentiation of the human macrophage cell line U937 (American Type Culture Collection) into macrophages and infection with stationary-phase *Leishmania* promastigotes were performed using standard methods as previously described (31). U937 cells were seeded at a concentration of 2.5×10^5/cm^2 in eight-chamber slides and differentiated into adherent macrophages by treatment with 7.5 ng of phorbol myristate acetate (Sigma) per ml of RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, and 50 μg of Gentamicin (RPMI)/ml with 5% CO_2 at 37°C for 72 h. Adherent cells were washed three to five times with warm RPMI medium, followed by incubation with *L. chagasi* parasites at a parasite-to-U937-cell ratio of 10:1 for 6 h. Nonengulfed parasites were washed away three to five times with warm RPMI. Warm fresh RPMI medium was then added to the flask containing the infected macrophages, and the flask was incubated with 5% CO_2 at 37°C. The level of infection was determined 6, 18, and 42 h postinfection by optical microscopy following Diff Quick staining of cell preparations (22). Values are expressed either as the percentage of macrophages infected or as the total number of amastigotes per 100 macrophages.

Sequencing of iron-SOD genes. Two converging primers, Forward Primer 1 (5′-CTGCACCACTCGAAGCACCA-3′) and Reverse Primer 2 (5′-CAGGTA GTACGCGTGCTCCCA-3′), based on conserved amino acid sequence of FeSODs (24), were synthesized based on *Leishmania* codon usage.

The 426-bp product obtained by amplifying *L. chagasi* genomic DNA was subsequently labeled with [α-^{32}P]dCTP (Amersham Biosciences) and used as a probe to screen a λZAPII *L. chagasi* (MHOM/BR/82/BA-2, CI) sheared genomic library, kindly provided by S. Reed (Seattle Biomedical Research Institute, Seattle, Wash.). A total of ~10^5 PFU were plated and lifted in duplicate. Potential positive clones were treated by secondary screening to recover isolated positive clones. Isolated clones were amplified using standard protocols, and DNA was extracted using a Lambda Maxi kit (Qiagen). Sequencing of the purified recombinant λ DNA was performed at the University of Calgary DNA Sequencing Facility, Calgary, Alberta, Canada.

Southern and Northern blots. Genomic DNA was isolated from logarithmic promastigotes by lysing parasites in lysis buffer (10 mM Tris-Cl, pH 8.3, 50 mM EDTA, 1% sodium dodecyl sulfate [SDS]). RNase A (100 μg/ml) was added to the suspension and incubated overnight at 37°C, followed by the addition of 100 μg of proteinase K/ml and incubation at 42°C overnight. DNA was extracted by performing a phenol-chloroform treatment followed by ethanol precipitation. Genomic DNA was digested with various restriction enzymes and separated on a 1% agarose gel. The DNA was transferred to Hybond N+ membranes via capillary action and fixed with long-wave UV light for 5 min. The blots were blocked with Rapid-Hyb buffer and incubated at 68°C with [α-^{32}P]dCTP random-primed labeled DNA probes for 2 h, after which the blots were washed successively with decreasing salt concentrations. Total RNA was isolated from promastigotes using the acid guanidinium isothiocyanate method (4). Total RNAs were separated in a 1.2% formaldehyde-containing agarose gel, transferred onto Hybond N$^+$ membranes, and baked at 80°C for 2 h. The blots were blocked with Rapid-Hyb buffer and incubated at 62°C with [α-^{32}P]dCTP random-primed labeled DNA probes for 2 h and washed in decreasing salt concentrations. The RNA blots were hybridized with [α-^{32}P]dCTP-labeled α-tubulin as a loading control.

Generation of *Lcfesodb1* gene knockouts. The generation of knockout constructs was based on the pX63-HYG and pX-NEO vectors (kindly provided by S. M. Beverley), which consist of a pSP6-T3 (Life Technologies Inc.) backbone and a cassette comprising a gene encoding resistance to hygromycin B (*hyg*) or Geneticin (*neo*) and flanking dihydrofolate reductase-thymidylate synthase (DHFR) sequences that are required for proper gene expression (5). To disrupt the *Lcfesodb1* locus, the target *Lcfesodb1* DNA sequences were cloned into pX63-HYG and pX63-NEO such that the *hyg* expression cassette (2,837 bp) and *neo* expression cassette (2,687 bp) were flanked at both ends by *Lcfesodb1* DNA. This strategy permitted subsequent excision of the entire insert, excluding vector DNA, for transfection into the parasites to promote homologous recombination with chromosomal *Lcfesodb1* sequence. The 5′ *Lcfesodb1* homologous-region insert was PCR amplified, yielding a 466-bp fragment (nucleotides 80 to 538) that was cloned into the *Hin*dIII/*Xho*I sites in both vectors. The unique 3′ *Lcfesodb1* homologous-region insert was PCR amplified, yielding a 1,456-bp (nucleotides 888 to 2217) fragment that was cloned into the *Sma*I/*Bgl*II sites of both vectors, resulting in the pX63HYGFeSODB1KO and pX63NEOFeSODB1KO vectors. The nucleotide numbers refer to the *Lcfesodb1* sequence (GenBank accession no. AF003963). The vectors were digested with *Hin*dIII and *Bgl*II, and the knockout fragment containing the selectable gene marker was purified. The procedures used in the transfection of *L. chagasi* promastigotes have been described previously (14, 23). Wild-type parasites were transfected with 5 μg of the linearized knockout constructs by electroporation, and transformants were selected at 50 μg of hygromycin B/ml. For gene complementation studies, the entire coding region of the *Lcfesodb1* gene was cloned into the *Bam*HI sites of the pX-Neo expression vector to generate the pX-FeSODB1 vector. Plasmids were sequenced for the correct orientation. Single-allele knockout parasites were transfected with 40 μg of the pX-FeSODB1 plasmid by electroporation and selected at 1 mg of Geneticin/ml. It is known that continuous subpassage of parasites may result in a decrease in virulence; therefore, the Δ*sodb1hyg* and wild-type strains were electroporated and subpassaged in parallel the same number of times under the same culture conditions.

Western blot analysis. *L. chagasi* lysates were prepared by harvesting log- and stationary-phase parasites as described previously (20). Briefly, the parasites were washed three times with phosphate-buffered saline (PBS; 1×), resuspended in hypotonic buffer (5 mM Tris-Cl [pH 7.8], 0.1 mM EDTA [pH 8.0], 5 mM phenylmethylsufonyl fluoride, 100,000 IU of aprotinin, 20 μg of leupeptin/μl) and freeze-thawed in liquid nitrogen. The equivalent of 5×10^6 parasites/lane were boiled for 10 min and separated on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane by semidry blotting. Western blot analysis was performed using anti-LcFeSODB1 antiserum and an enhanced-chemiluminescence kit (Amersham Biosciences). Anti-LcFeSODB1 polyclonal antiserum was obtained from the University of Calgary Animal Research Facility. His-tagged LcFeSODB1 protein was excised from a Coomassie blue-stained SDS-polyacrylamide gel electrophoresis (PAGE) gel, macerated in the presence of liquid nitrogen, resuspended in Freund's complete adjuvant, and passed several times through a 21.5-gauge needle before inoculation into New Zealand White rabbits. Further inoculations were done at 4-week intervals using gel-purified protein resuspended in Freund's incomplete adjuvant. After four inoculations, the rabbits were bled and the specificity of the antiserum was tested. Monoclonal antibody (E7) to β-tubulin was purchased from the Developmental Studies Hybridoma Bank, University of Iowa.

SOD cytochrome *c* activity assay. The SOD activity in *L. chagasi* parasite lysates was determined by the reduction of ferricytochrome *c* according to standard methods (19). Briefly, reaction mixtures contained 0.05 M potassium phosphate buffer at pH 7.8, 0.02 mM EDTA, 0.01 mM ferricytochrome *c*, 0.05 mM xanthine, and 1.6×10^{-2} U of xanthine oxidase/ml. Absorbance at 550 nm was monitored at 25°C, and the rate of reduction of ferricytochrome *c* was calculated. Under these conditions, the amount of SOD required to inhibit the rate of reduction of ferricytochrome *c* by 50% is defined as 1 U of activity.

Subcellular localization of LcFeSODB. To determine the subcellular localization of LcFeSODB1 and LcFeSODB2 within *L. chagasi*, various LcFeSODB fusions were constructed with green fluorescent protein (GFP). The gene fusions were expressed in the parasites in the pXNEO expression vector. Fusion con-

structs were generated by PCR amplifying the coding region of GFP with Forward Primer 3 (5′ GTCGGATCCATGGTGAGCAAGGGCGAGG 3′) and Reverse Primer 4 (5′ CCGGAATTCGTACTTGTACAGCTCGTCC 3′) containing a mutated GFP stop codon (TAC) to allow fusion with a downstream gene. The entire coding regions of LcFeSODB1 and LcFeSODB2 that were to be fused to GFP were PCR amplified using Forward Primer 5 (5′ CCGGAATTCATGCC GTTCGCTGTTCAGCCG 3′) (for both LcFeSODB1 and LcFeSODB2) and Reverse Primer 6 (5′ CCGTCTAGATTAAAGCTGGCTAGAGGCGAAAT CCC 3′) (for LcFeSODB1) and Reverse Primer 7 (5′ CCGTCTAGATTACAG ATCACTGTTGACGTAGTGGG 3′) (for LcFeSODB2). Amplification of the LcFeSODB coding regions without their respective SQL and SDL amino acids was done using Forward Primer 5 and Reverse Primer 8 (5′ CCGTCTAGATT AAGAGGCGAAATCCCAGTC 3′) (for LcFeSODB1 without SQL) and Reverse Primer 9 (5′ CCGTCTAGATTAGTTGACGTAGTGGGAGCC 3′) (for LcFeSODB2 without SDL). Amplification of GFP with the amino acids SQL or SDL added to the carboxyl terminus was done using Forward Primer 3 and Reverse Primer 10 (5′CCGTCTAGATTAAAGCTGGCTGTACTTGTACAGC TCGTCCATGCCG 3′) (for GFP with SQL) and Reverse Primer 11 (5′ CCGT CTAGATTACAGATCACTGTACTTGTACAGCTCGTCCATGCCG 3′) (for GFP with SDL). The underlined sequences identify the restriction sites used and the start codon, stop codon, or matched stop codon. The GFP and LcFeSODB PCR products were digested with *Eco*RI and ligated together. The ligated products were PCR amplified using Forward Primer 3 and the corresponding reverse primers and cloned into the pXNEO *Bam*HI/*Xba*I restriction sites. The newly constructed vectors were transfected into *L. chagasi* parasites by electroporation and selected with Geneticin to a final concentration of 50 μg/ml.

Approximately 5×10^6 mid-log-phase parasites were washed with 1× PBS and applied to poly-L-lysine-coated coverslips for 15 min. The parasites were washed with PBS and fixed with 4% paraformaldehyde–0.01% electron microscopy-grade gluteraldehyde in PBS (pH 7.0) at 24°C for 30 min and washed twice with PBS. The parasites were then incubated in 50 mM glycine (in 1× PBS) for 15 min and then washed with PBS. The coverslips were blocked, and the cells were permeabilized with 2% goat serum–0.1% Triton X-100 for 30 min. Primary antibody was diluted (1:200) in 2% goat serum, centrifuged at 18,000 × *g* for 10 min, and applied to the coverslips for 1 h. The coverslips were washed six times with PBS (5 min per wash). Secondary antibody, Alexa Fluor 594 (Molecular Probes), was diluted (1:500) in 2% goat serum, centrifuged for 10 min, and applied to the coverslips for 1 h in the dark. The coverslips were then washed six times with PBS (5 min per wash). Twenty microliters of 50% glycerol was applied to a glass slide, and the coverslips (parasite side down) were placed on top and sealed with nail polish. The parasites were photographed with a fluorescent microscope.

In vitro drug assays. Treatment of parasites with paraquat involved seeding the stationary-phase parasites at a density of 10^6/ml in hemoflagellate minimal essential medium as previously described (23). The parasites were permitted to recover for 24 h prior to the addition of freshly prepared paraquat at a concentration that inhibited wild-type parasite growth by 50% under the conditions used (500 μM). After 6 days of exposure to paraquat, parasite viability was measured microscopically according to flagellar motility by assessing 200 promastigotes as motile (viable) or nonmotile (nonviable), as previously described (36). Experiments with <95% parasite viability before treatment with paraquat were discounted.

GenBank accession numbers. The coding regions of the *fesodb* genes were amplified from *L. donovani* (IS2D) and *L. major* (Friedlin strain) genomic DNAs using primers based on *L. chagasi Lcfesodb1* and *Lcfesodb2* sequences (GenBank accession numbers AF003963 and AF312581, respectively). The sequences of the genes isolated from *L. donovani* and *L. major* have been deposited in GenBank with the following accession numbers: *L. donovani* LddFeSODB1, AF312585, and LddFeSODB2, AF312582; *L. major* LmFeSODB1, AF312586, and LmFeSODB2, AF312583.

RESULTS

Isolation and characterization of the *Lcfesodb* gene cluster. One *Lcfesodb* gene (now called *Lcfesodb1*) was previously isolated (23). The isolation of the second *fesodb* gene (now called *Lcfesodb2*) is presented herein. A λZAPII *L. chagasi* genomic DNA library was screened, and a positive clone containing *Lcfesodb* sequence was digested with *Eco*RI, *Pst*I, and *Bam*HI (Fig. 1A). Extensive sequencing and Southern blot analysis of the insert revealed that the *Lcfesodb2* gene is situated in tandem upstream of the *Lcfesodb1* gene (Fig. 1B). The chromosomal localization of the *Lcfesodb1* and *Lcfesodb2* genes determined by pulsed-field gel electrophoresis revealed that they colocalize to the same high-molecular-weight chromosome, which supports their tandem genomic organization (data not shown). Similar results were obtained for the *fesodb1* and *fesodb2* genes of *L. donovani* (data not shown).

FeSODB2 consists of 209 amino acids with a predicted molecular mass of 23.2 kDa, whereas LcFeSODB1 consists of 196 amino acids with a predicted molecular mass of 21.7 kDa. The amino acid sequences of LcFeSODB1 and LcFeSODB2 are 88% identical and differ primarily by the presence of a 13-amino-acid extension found at the carboxyl terminus of LcFeSODB2 (Fig. 2). The last three amino acids of LcFeSODB1 (SQL) and LcFeSODB2 (SDL) resemble the typical glycosomal localization signal sequence, SKL, suggesting that these proteins may be localized to the glycosomes. We have also amplified *fesodb1* and *fesodb2* genes from *L. donovani* (LddFeSODB1 and LddFeSODB2) and *L. major* (LmFeSODB1 and LmFeSODB2) genomic DNAs using primers flanking the coding region sequences of *Lcfesodb1* and *Lcfesodb2.* Sequence comparisons of the *fesodb1* and *fesodb2* genes in the different species revealed >92% identity, suggesting a high degree of conservation of *fesodb* genes in *Leishmania* (Fig. 2).

***Lcfesodb1* and *Lcfesodb2* genes are differentially expressed.** To study the expression pattern of *Lcfesodb1* and *Lcfesodb2* as the parasites progress through their life cycle, total RNAs were isolated from parasites at early logarithmic phase, stationary phase, and an amastigote-like stage (derived from infected U937 cells). The amount of RNA loaded in each lane was normalized using a probe complementary to the constitutively expressed α-tubulin gene of *Leishmania* (Fig. 3C). Northern blot hybridization using the specific 3′ untranslated region (UTR) of *Lcfesodb1* revealed a single band of 3.8 kb that increased in intensity ~1.7-fold from the early logarithmic to the stationary phase and ~2.1-fold from the early logarithmic to the amastigote stage (Fig. 3A). In contrast, Northern blot hybridization using the specific 3′ UTR of *Lcfesodb2* revealed a single band of 2.4 kb that decreased in intensity ~2.4-fold from the early logarithmic to the stationary phase and ~3.1-fold from the early logarithmic to the amastigote stage (Fig. 3B). These results demonstrate that the *Lcfesodb* genes are differentially expressed: *Lcfesodb1* is expressed at low levels in the early logarithmic promastigote stage and at high levels in the stationary promastigote and amastigote stages, whereas *Lcfesodb2* is expressed at high levels in the early logarithmic promastigote stage and at low levels in the stationary promastigote and amastigote stages. Similar results were observed for *Ldfesodb1* and *Ldfesodb2* in *L. donovani* (data not shown).

Replacement of the *Lcfesodb1* gene by homologous recombination. The high expression of *Lcfesodb1* in the stationary promastigote- and amastigote-like stages suggests that the LcFeSODB1 protein may play an important role in intracellular survival. To help define the role of *Lcfesodb1* in parasite survival, we attempted to create a homozygous *Lcfesodb1* gene deletion in *L. chagasi* using sequential rounds of gene replacement to replace both alleles of the endogenous *Lcfesodb1* gene with HYG and NEO drug-resistant genes. In the first round of transfection, the knockout fragment containing a *HYG*/*DHFR* resistance cassette flanked by the 5′ and unique 3′ homologous

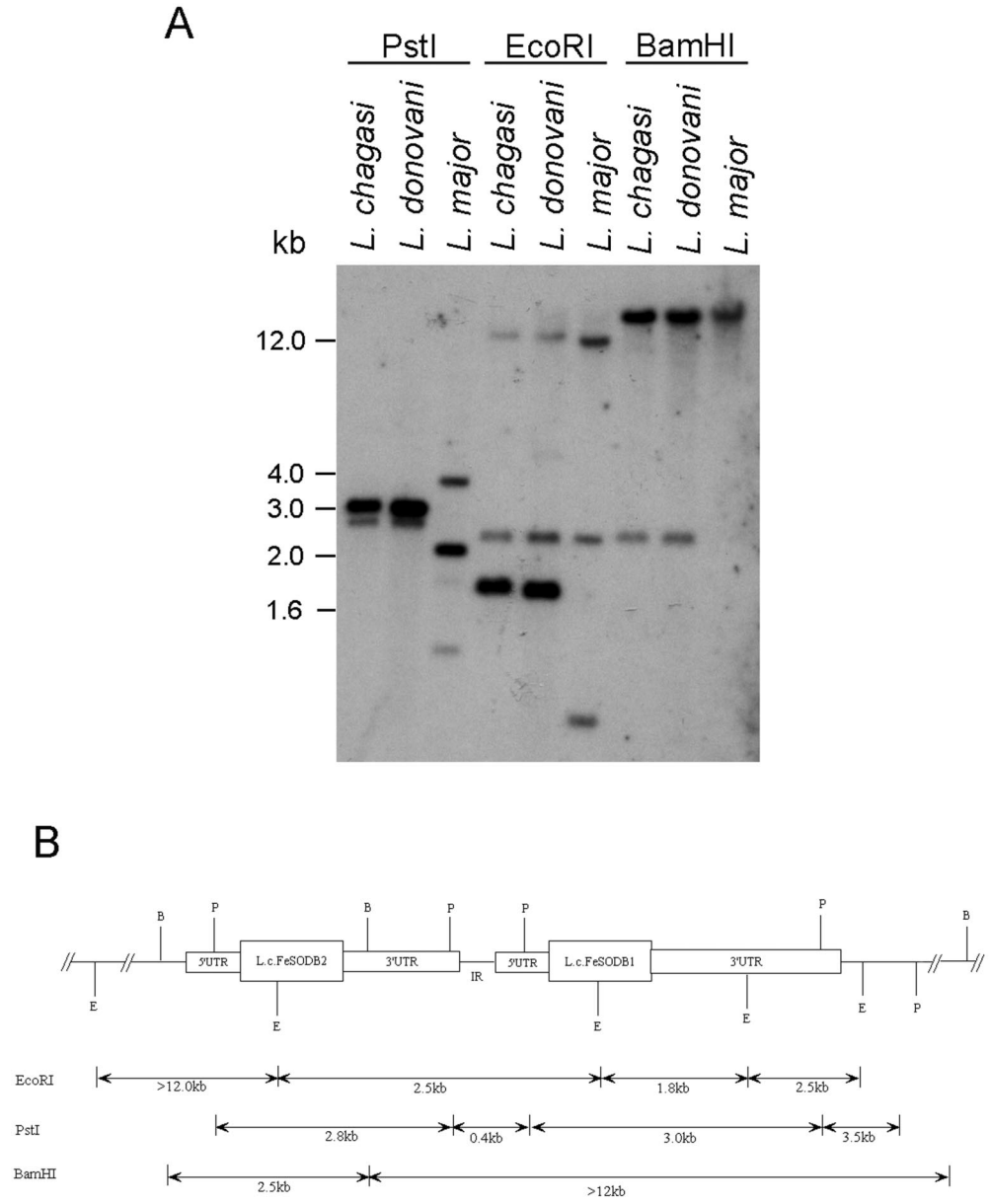

FIG. 1. Southern blot analysis and restriction map of the *Lcfesodb* cluster. (A) Total genomic DNAs from *L. chagasi*, *L. donovani*, and *L. major* (500 ng) were isolated; restriction digested with *Pst*I, *Eco*RI, and *Bam*HI; resolved on a 1% agarose gel; and transferred to a nitrocellulose membrane. The membrane was hybridized with a radiolabeled *Lcfesodb1* coding-region probe. (B) Restriction map of the *L. chagasi Lcfesodb* cluster determined by Southern blot hybridization, library screening, and sequence analysis of genomic DNA. The tentative positions of the 3′ UTR of *Lcfesodb1* and the intergenic region (IR) are based on the known 5′ UTR *Lcfesodb1* sequence. The sequence located upstream of the 5′ UTR of *Lcfesodb1* and downstream of the *Lcfesodb2* stop codon is representative of the 3′ UTR of *Lcfesodb2* and the IR. The restriction fragment lengths shown below the schematic correspond to the hybridizing bands observed with several probes used for Southern blot analysis. P, *Pst*I; E, *Eco*RI; B, *Bam*HI.

Lcfesodb1 regions (pX63HygFeSODB1) was transfected into wild-type *L. chagasi* promastigotes (Fig. 4A). The predicted homologous-recombination event was designed to knock out the aspartate-161 and histidine-165 amino acid residues near the carboxyl terminus of LcFeSODB1, which are essential in coordinating the iron atom in the active site of FeSOD enzymes for activity (Fig. 4A) (24). We were aware that this knockout strategy could replace one *Lcfesodb1* allele alone or both *Lcfesodb1* and *Lcfesodb2* alleles, because *Lcfesodb2* is situated upstream of *Lcfesodb1* and shows similarity to the 5′ UTR and the 5′ half of the coding region of *Lcfesodb1*.

Genomic DNAs from a hygromycin B-resistant mutant (Δ*sodb1hyg*) and wild-type parasites were isolated, digested with *Bam*HI and *Pst*I, and analyzed using Southern blotting to confirm proper integration of the knockout fragment into only one *Lcfesodb1* allele (Fig. 4C and D). Digestion of the wild-type genomic DNA with *Bam*HI resulted in two bands (>12 and 2.5 kb), whereas the knockout resulted in three bands

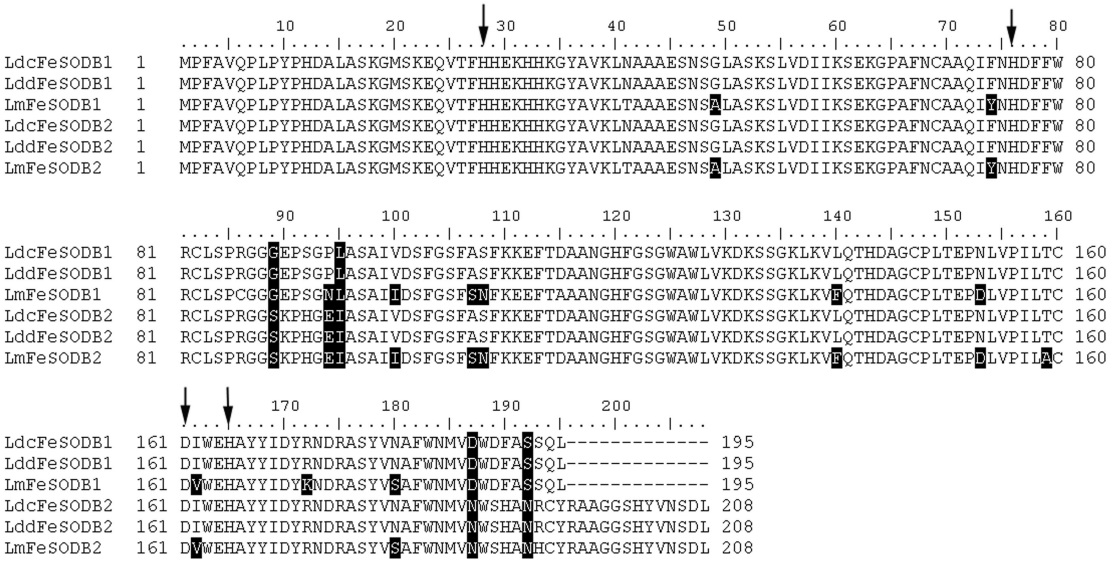

FIG. 2. Amino acid sequence comparison of *L. chagasi*, *L. donovani*, and *L. major* FeSODB coding regions. Sequence alignment of FeSODB1 and FeSODB2 reveals a higher degree of divergence in the carboxyl termini, where FeSODB2 contains a 13-amino-acid extension that is not present in FeSODB1. The solid boxes indicate amino acid differences. The arrows indicate residues involved in iron binding at the active site. To maintain alignment, dashes represent missing amino acids.

(>12, 3.8, and 2.5 kb) when hybridized with the *Lcfesodb1* coding region probe (Fig. 4C, lanes 1 and 3). The 2.5-kb band was present in both the wild-type and knockout parasites at the same intensity and was determined to contain the *Lcfesodb2* gene by using *Lcfesodb2*-specific probes (data not shown). The fact that the bands were of equal intensity supports the idea that *Lcfesodb2* was not disrupted by a homologous-recombination event. The >12-kb band was present in both the wild-type and knockout parasites; however, the band in the knockout parasites was approximately half the intensity of the wild-type band, as would be seen if one allele of *Lcfesodb1* was knocked out. The 3.8-kb hybridizing band in the Δ*sodb1hyg* digest, absent in the digested wild-type DNA, is a result of the predicted integration of the *HYG*/*DHFR* cassette into the *Lcfesodb1* gene, which introduces a *Bam*HI site that is not present in this location in the wild-type allele (Fig. 4B and C, lane 3). Hybridization of these digests with a *hyg*-specific probe resulted in a single band of 3.8 kb corresponding to a fragment containing *hyg* gene sequences, as expected (Fig. 4B and C, lane 3).

Hybridization of *Pst*I-digested Δ*sodb1hyg* genomic DNA with the *Lcfesodb1* coding region-specific probe also revealed the predicted pattern that would result from accurate integration into only the *Lcfesodb1* gene locus (Fig. 4B). The 2.8- and 3.0-kb bands appearing in both the wild-type and Δ*sodb1hyg* digests (Fig. 4C, lanes 2 and 4) represent fragments containing the uninterrupted *Lcfesodb2* and *Lcfesodb1* alleles, respectively (Fig. 4B). Hybridization with probes specific to each 3′ UTR verified their identities (data not shown). The additional 1.3-kb band appearing only in the Δ*sodb1hyg* digested DNA (Fig. 4C, lane 4) represents the disrupted *Lcfesodb1* allele fragment, because the *HYG*/*DHFR* cassette contains an internal *Pst*I site (Fig. 4B). Hybridization of this blot with a *hyg*-specific probe showed the expected absence of hybridizing bands in the wild-type DNA and the presence of 4.3- and 1.3-kb bands containing *hyg* gene sequences (Fig. 4B and C, lane 4).

In further support of the finding that one allele of *Lcfesodb1* had been disrupted, Northern blot analysis using a 3′ UTR-specific probe from *Lcfesodb1* revealed a significant decrease in the intensity of the *Lcfesodb1* transcript in the Δ*sodb1hyg* parasites compared to the wild-type parasites (data not shown). Northern blot analysis using a 3′ UTR-specific probe from *Lcfesodb2* did not reveal a difference in intensity of the *Lcfesodb2* transcript in the Δ*sodb1hyg* parasites compared to the wild type, suggesting that the *Lcfesodb2* allele was not disrupted (data not shown). Taken together, these results confirm that one *Lcfesodb1* allele was successfully disrupted by the hygromycin cassette and that the *Lcfesodb2* gene was not af-

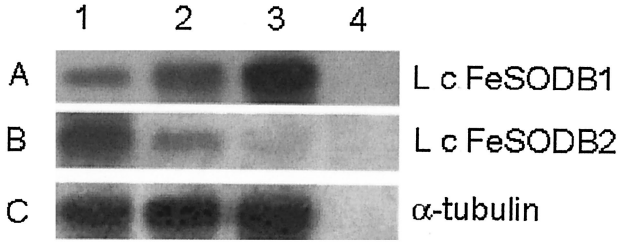

FIG. 3. Northern blot analysis. Ten micrograms of total RNAs from early log-phase *L. chagasi* parasites (lane 1), stationary-phase *L. chagasi* parasites (lane 2), U937 cells infected with *L. chagasi* parasites (lane 3), and uninfected U937 cells (lane 4) was extracted and resolved on a 1.2% agarose gel under denaturing conditions, blotted, and probed with the 3′ UTR of *Lcfesodb1* (SA, 7.5×10^8 cpm/μg) (A), the 3′ UTR of *Lcfesodb2* (specific activity, 5.3×10^8 cpm/μg) (B), or α-tubulin from *L. chagasi* (loading control) (specific activity, 3.5×10^8 cpm/μg) (C). No hybridizing bands of any size were observed with the α-tubulin probe and the RNA from the uninfected U937 cells (C, lane 4). Exposures: 26 h at −80°C (A and B) and 2 h at room temperature (C).

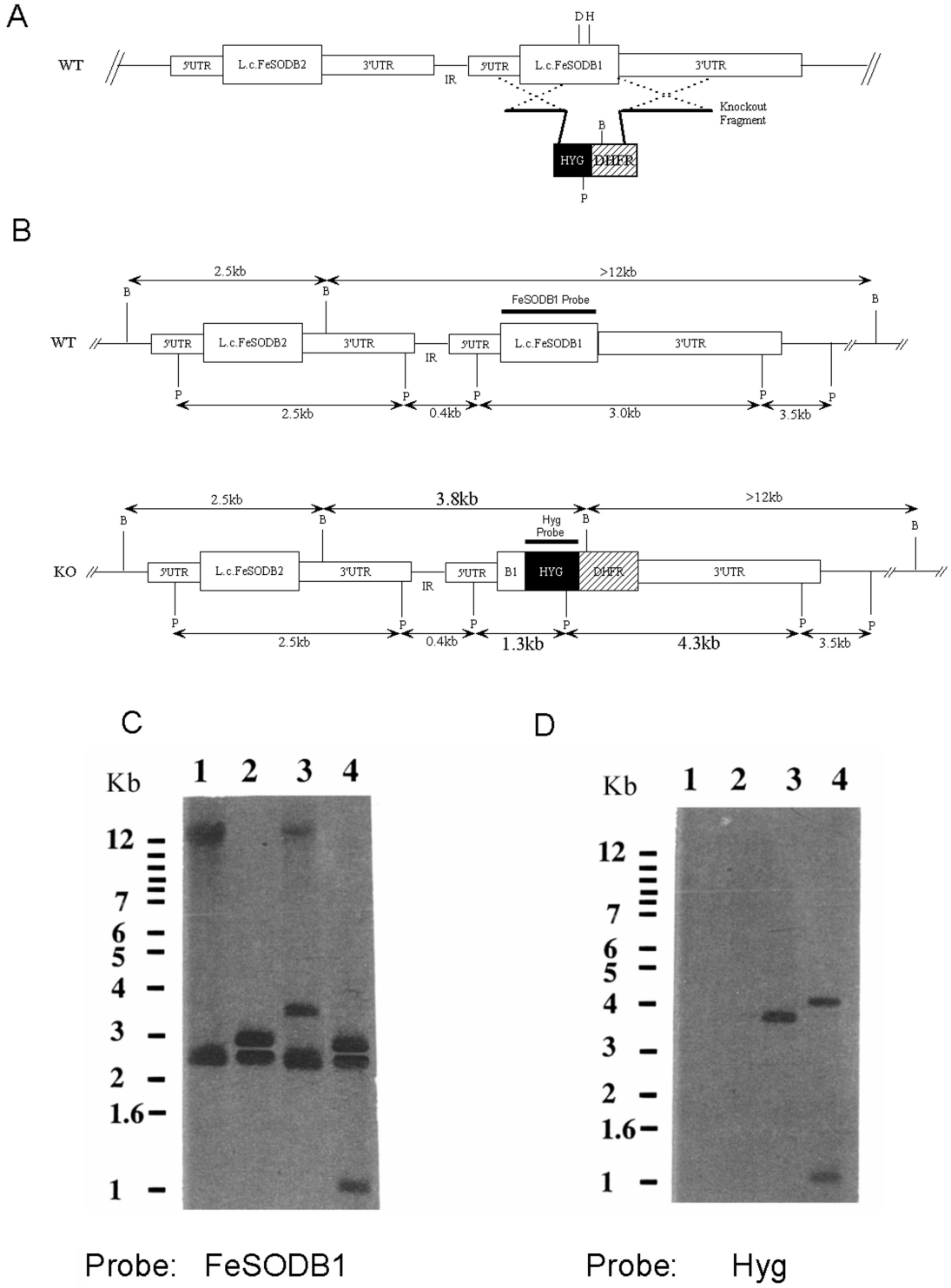

FIG. 4. Schematic representation and Southern blot analysis of *Lcfesodb1* knockout parasites. (A) Schematic of homologous-recombination strategy employed to knock out the 360-bp region of *Lcfesodb1* that contains the active-site aspartic acid-161 (D) and histidine-165 (H) residues, which act as ligands to coordinate the iron. The transfected knockout fragment consists of a 466-bp fragment, homologous to the 5′ UTR and coding region of *Lcfesodb1*, and a 1,465-bp fragment, homologous to the 3′ UTR of *Lcfesodb1*, that flank the HYG/DHFR cassette. The homologous-recombination event between the *Lcfesodb1* allele and the knockout fragment DNA is represented by two dashed crosses. IR, intergenic region. (B) Schematic depicting the endogenous wild-type (WT) allele of the *Lcfesodb* cluster and the disrupted allele following homologous recombination. The expected *Bam*HI (B) and *Pst*I (P) restriction fragment sizes are given. (C and D) Southern blot analyses of *L. chagasi* Δ*sodb1hyg* and wild-type genomic DNAs using *Lcfesodb1*-specific (C) and hygromycin (*hyg*)-specific (D) probes. Lanes 1, wild-type DNA digested with *Bam*HI; lanes 2, wild-type DNA digested with *Pst*I; lanes 3, Δ*sodb1hyg* DNA digested with *Bam*HI; lanes 4, Δ*sodb1hyg* DNA digested with *Pst*I. Molecular size marker positions are shown to the left of each blot.

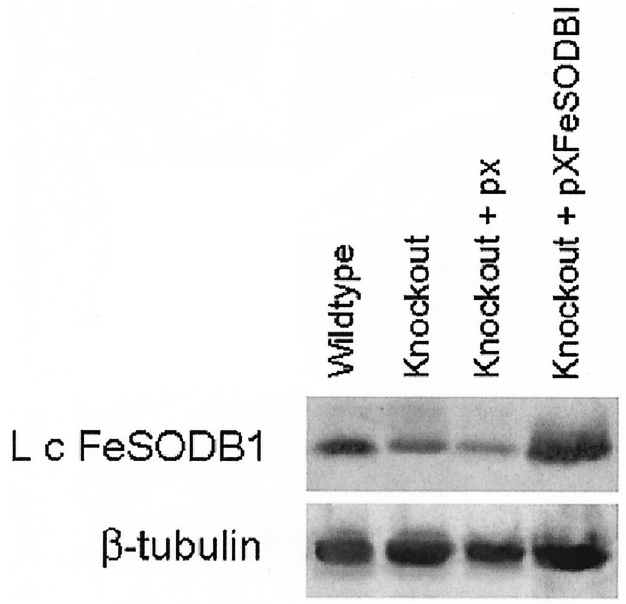

FIG. 5. Western blot analyses of wild-type and Δ*sodb1hyg* parasites. Lysates from stationary-phase wild-type, Δ*sodb1hyg* (Knockout), Δ*sodb1hyg*+pX (Knockout + px), and Δ*sodb1hyg*+pX-FeSODB1 (Knockout + pXFeSODB1) parasites were separated on an SDS–12% PAGE gel and subjected to Western blotting using LcFeSODB1 antiserum (top). The same blots were stripped and incubated with monoclonal β-tubulin antibody to serve as a loading control (bottom).

fected by the homologous-recombination event. Repeated attempts to knock out the second *Lcfesodb1* allele with the *neo*, *sat*, or GFP gene were unsuccessful. It is possible that both alleles of the *Lcfesodb1* gene are essential for parasite survival; however, further tests would need to be done to come to this conclusion.

Reduced SOD activity within Δ*sodb1hyg* parasites is due to the lack of one *Lcfesodb1* allele. Western blot and densitometry analyses of wild-type and Δ*sodb1hyg* promastigote lysate revealed an ~1.5-fold decrease in LcFeSODB1 protein in the Δ*sodb1hyg* parasites compared to the wild-type parasites (Fig. 5). To determine if there is a decrease in SOD activity in the Δ*sodb1hyg* parasites compared to the wild type, a cytochrome *c* assay was performed on parasite lysate. The results revealed a significant ($P < 0.025$) decrease in SOD activity in the Δ*sodb1hyg* parasites ($3.6 \times 10^{-6} \pm 3.6 \times 10^{-6}$ U/mg) compared to the wild type ($12.7 \times 10^{-6} \pm 1.4 \times 10^{-6}$ U/mg) (The activity is the average ± standard error of five independent trials.)

To determine whether the decrease in SOD activity observed in the Δ*sodb1hyg* parasites was in fact due to the absence of one *Lcfesodb1* allele, the Δ*sodb1hyg* parasites were rescued by transfection with a plasmid containing the *Lcfesodb1* gene (pX-FeSODB1). Western blot analysis of the Δ*sodb1hyg* parasites overexpressing LcFeSODB1 showed an ~1.6-fold increase in LcFeSODB1 protein compared to Δ*sodb1hyg* parasites transfected with the pX vector alone (Fig. 5). Cytochrome *c* analysis of lysates from Δ*sodb1hyg*+pX-FeSODB1 parasites showed that the SOD activity in the Δ*sodb1hyg*+pX-FeSODB1 parasites ($12.4 \times 10^{-6} \pm 2.7 \times 10^{-6}$ U/mg) increased to a level similar to that of the wild type ($12.7 \times 10^{-6} \pm 1.4 \times 10^{-6}$ U/mg) and considerably higher than that of lysates from Δ*sodb1hyg*+pX parasites ($5.9 \times 10^{-6} \pm 1.6 \times 10^{-6}$ U/mg). Taken together, these complementation results show that the decreased level of SOD activity in the Δ*sodb1hyg* parasites can be attributed to the absence of one *Lcfesodb1* allele.

Cellular localization of *LcFeSODB1* and *LcFeSODB2*. To gain a better understanding of the roles that LcFeSODB1 and LcFeSODB2 play in parasite survival, we studied the cellular localization of these proteins within *L. chagasi.* The last three amino acids of LcFeSODB1 (SQL) and LcFeSODB2 (SDL) conspicuously resemble the glycosomal targeting signal sequence, SKL. Previous mutational analysis of the SKL glycosomal targeting signal in *Trypanosoma brucei* showed that the signal is highly degenerate (30).

We created GFP-LcFeSODB1 and GFP-LcFeSODB2 fusion protein gene constructs and overexpressed them in *L. chagasi* parasites (selected at 50 μg of Geneticin/ml) to determine their localization. Fluorescence microscopy showed a distinct punctate pattern of fluorescence, suggesting compartmentation of the proteins in an organelle (Fig. 6A). Colocalization of the GFP fusion proteins with the glycosomal proteins HGPRT (29) (Fig. 6, columns 3 and 4) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (10, 11) (data not shown) confirmed that the GFP fusion proteins are localized to the glycosomes. To test whether the proposed targeting signal sequences SQL and SDL were capable of targeting the cytosolic GFP protein to the glycosome, we individually fused the SQL and SDL sequences to the carboxyl terminus of GFP. The fluorescence patterns resembled the punctate pattern seen with the LcFeSODB1 and LcFeSODB2 fusion proteins and were colocalized with the glycosomal marker proteins HGPRT (Fig. 6A) and GAPDH (data not shown). GFP-LcFeSODB1 and GFP-LcFeSODB2 fusion proteins lacking the SQL and SDL signal sequences exhibited GFP fluorescence similar to that in parasites expressing GFP alone (Fig. 6B). These parasites exhibited fluorescence throughout the organism, including the flagella, characteristic of a cytoplasmic localization pattern. We have thought about generating monoclonal antibodies to LcFeSODB1 and LcFeSODB2 proteins to further complement our localization studies; however, because the amino acid sequences of these proteins are virtually identical, it is highly likely that we would not be able to differentiate between the LcFeSODB1 and LcFeSODB2 proteins in immunolocalization or fractionation studies. Therefore, we have used specific GFP fusion protein constructs and immunolocalization with control glycosomal proteins to demonstrate that the LcFeSODB1 and LcFeSODB2 proteins are localized to the glycosomes, and we have used this technology to identify the specific amino acids involved in targeting these proteins to the glycosomes.

Δ*sodb1hyg* parasites exhibit decreased survival upon exposure to paraquat and within human macrophages. In order to determine whether the single-allele knockout of *Lcfesodb1* affects parasite viability compared to the wild type, Δ*sodb1hyg* parasite growth was monitored with and without hygromycin B. No significant differences were observed in the growth curves or in the gross morphology of the Δ*sodb1hyg* parasites compared to the wild type (data not shown). To determine the effect that knocking out one allele of *Lcfesodb1* has on the ability of *L. chagasi* to survive an increased level of endogenous $O_2^{\cdot -}$, the ability of the parasites to survive in culture in the

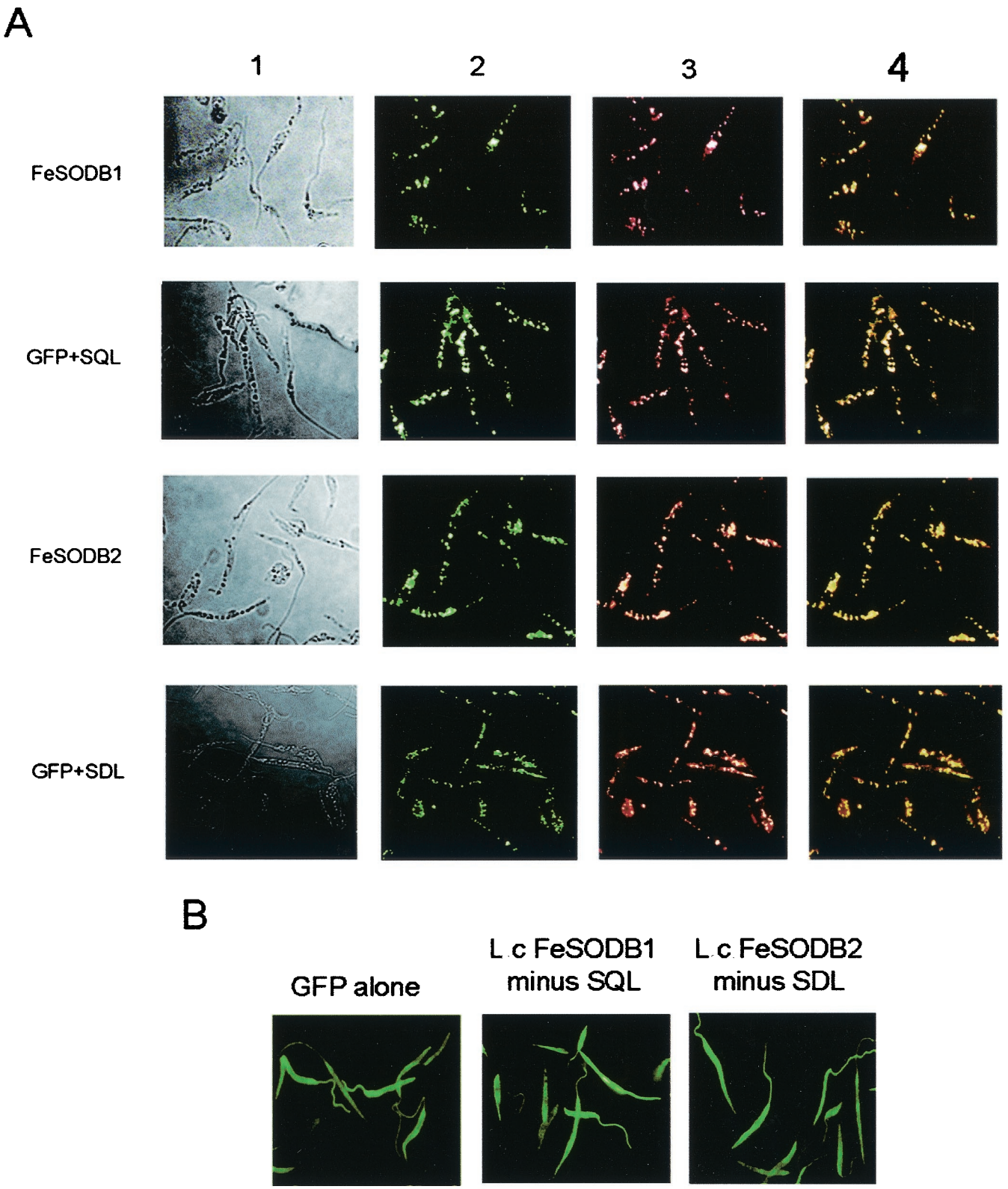

FIG. 6. Cellular localization of LcFeSODB1 and LcFeSODB2. (A) Fluorescence patterns of *L. chagasi* parasites expressing GFP fusion proteins with LcFeSODB1 (FeSODB1), SQL alone (GFP+SQL), LcFeSODB2 (FeSODB2), or SDL alone (GFP+SDL). Column 1, parasites observed under bright-field illumination; column 2, GFP fluorescence; column 3, Alexa Fluor 594 fluorescence; column 4, merged images of columns 2 and 3. (B) Fluorescence patterns of *L. chagasi* parasites expressing GFP alone and GFP fusion proteins with LcFeSODB1 lacking the last three amino acids (SQL) and LcFeSODB2 lacking the last three amino acids (SDL).

presence of paraquat was examined. Paraquat is a redox-cycling drug that penetrates the cytosol to significantly increase endogenous $O_2^{\cdot -}$ production. After 6 days of exposure to paraquat, the percent survival of the Δ*sodb1hyg* parasites (46.2% ± 9.7%) was significantly ($P < 0.02$) lower than that of the wild-type parasites (76.9% ± 6.3%). The deficiency in survival of the Δ*sodb1hyg* parasites was rescued to a level slightly higher than that of the wild type after transfection with pX-FeSODB1 (86.6% ± 1.6%) and considerably higher than that of Δ*sodb1hyg* transfected with pX (52.7% ± 2.3%). (The data represent the averages ± standard errors of four independent trials.)

To study the effect of knocking out one allele of the *Lcfesodb1* gene on the ability of the parasites to survive intracellularly, we performed an infection study using U937 cells. Differentiated U937 cells were infected with stationary-phase wild-type and Δ*sodb1hyg* parasites and incubated for various times. Remarkably, after 48 h of infection, the total number of Δ*sodb1hyg* amastigotes per 100 macrophages decreased to less than half that of the wild-type amastigotes (475 and 1,162, respectively) (Fig. 7A). The reduced infection level of the Δ*sodb1hyg* parasites was rescued by overexpressing LcFeSODB1 in these parasites, which exhibited a slightly increased infection level after 48 h compared to the control wild-type parasites transfected with the pX vector alone (1,110 and 900, respectively) (Fig. 7B). This could imply that the parasites overexpressing FeSODB1 exhibit enhanced survival and replication compared to the control wild-type parasites. These numbers were significantly ($P < 0.001$) higher than the infection level of Δ*sodb1hyg* amastigotes transfected with the pX vector alone (390). The percentages of infected macrophages in the wild-type and Δ*sodb1hyg* parasites and in the Δ*sodb1hyg* parasites transfected with pX and the Δ*sodb1hyg* parasites transfected with pX-FeSODB1 were similar at 6, 24, and 48 h postinfection (Fig. 7C). These results suggest that LcFeSODB1 is important for the survival of *L. chagasi* under conditions of increased endogenous $O_2^{\cdot -}$ and for survival within macrophages.

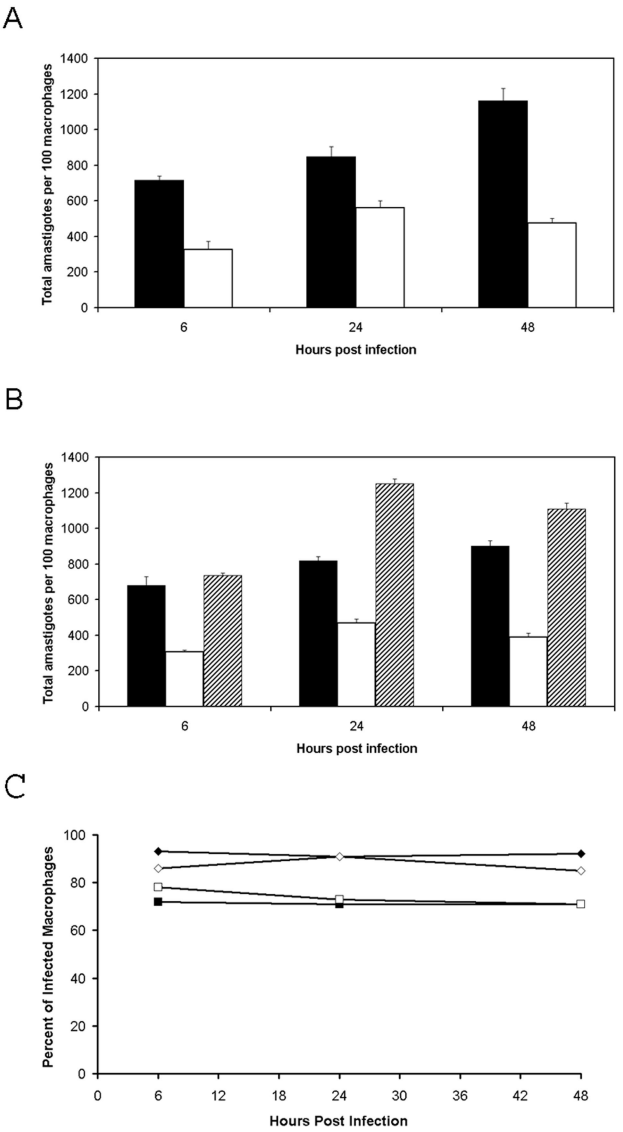

FIG. 7. Survival of *L.chagasi* wild-type and Δ*sodb1hyg* parasites within U937 cells. (A and B) Differentiated U937 cells were incubated with stationary-phase wild-type (solid bars) or Δ*sodb1hyg* (open bars) promastigotes (A) and with wild-type promastigotes transfected with the pX vector (solid bars), Δ*sodb1hyg* promastigotes transfected with the pX vector (open bars), or Δ*sodb1hyg* promastigotes transfected with the pX-FeSODB1 vector (shaded bars) (B) for 6 h as described in Materials and Methods. Nonengulfed parasites were washed away, and the infected U937 cells were incubated for 6, 24, and 48 h, after which the infected macrophages were stained with Diff Quick and examined by optical microscopy to determine the level of infection, which was expressed as the total number of amastigotes per 100 infected macrophages. (C) Percentages of infected macrophages for wild-type parasites (solid diamonds), Δ*sodb1hyg* parasites (open diamonds), Δ*sodb1hyg* parasites transfected with pX (solid squares), and Δ*sodb1hyg* parasites transfected with pX-FeSODB1 (open squares). The averages plus standard errors of four independent experiments are shown. *, $P < 0.001$.

DISCUSSION

With the emergence of multidrug-resistant *Leishmania* parasites, the toxicity of current drug therapy and the increased tendency for patient relapse have strengthened the drive for the development of better drugs and an effective vaccine. Understanding the mechanisms that allow *Leishmania* to survive intracellularly is important for designing such treatment strategies and for drug development. The production of $O_2^{\cdot -}$ has been recognized as an important mechanism involved in the killing of *Leishmania* and many other pathogens (8, 13, 21, 26, 33). The ability of SODs to dismutate $O_2^{\cdot -}$ and our previous findings that *L. chagasi* possesses FeSODs which are not found in humans attracted our interest to these proteins as potential targets for therapeutics. We have previously hypothesized that LcFeSODB may protect the parasites from macrophage-derived $O_2^{\cdot -}$ toxicity; however, the evidence presented here has caused us to reevaluate our previous hypothesis. Interestingly, we have isolated a second *fesodb* gene (*Lcfesodb2*) that is highly homologous to *Lcfesodb1* and whose expression is high in the early logarithmic promastigote stage and decreases toward the stationary promastigote and amastigote stages. This contrasts with the expression of *Lcfesodb1*, which is low in the early logarithmic stage and increases toward the stationary and amastigote stages. We demonstrate that LcFeSODB1 and LcFeSODB2 are both localized to the glycosomes by the final three amino acids located at the ends of their carboxyl termini.

Furthermore, we show that a single-allele knockout of the *Lcfesodb1* gene results in a significant decrease in survival of the parasites under conditions of increased endogenous $O_2^{\cdot -}$ levels and within macrophages.

In this study, we present a distinct member of the *L. chagasi fesodb* gene cluster, *Lcfesodb2*, which was determined to be located in tandem with and upstream of the *Lcfesodb1* gene. Southern blot hybridization patterns of *L. chagasi* and *L. donovani* genomic DNAs appear to be very similar, which suggests that the two organisms have maintained similar genomic arrangements of the *fesodb* genes throughout their evolution. Although the Southern blot hybridization pattern of the *fesodb* genes in *L. major* differs from those in *L. chagasi* and *L. donovani* (Fig. 1A), sequence analysis of isolated *fesodb1* and *fesodb2* genes from *L. chagasi*, *L. donovani*, and *L. major* demonstrates a high degree of conservation, with >90% identity in amino acid sequence (Fig. 2). This suggests a low rate of evolution for the individual *fesodb* genes, which in turn may reflect an important role of FeSODB in oxidative-stress defense. Although we have not performed biochemical or gene knockout studies of FeSODB in *L. donovani* or *L. major*, their high amino acid sequence identity and the presence of identical glycosomal targeting signal sequences leads us to hypothesize that the FeSODB genes may have similar functions in those species.

Northern blot analysis revealed differential expression of the *Lcfesodb1* and *Lcfesodb2* genes in *L. chagasi*, where the expression of *Lcfesodb1* increases from the promastigote to the amastigote stage and *Lcfesodb2* expression decreases from the promastigote to the amastigote stage. The high level of expression of *Lcfesodb2* in the early logarithmic promastigote stage suggests that the gene may be important for survival within the midgut of the sandfly, and the high level of expression of *Lcfesodb1* in the stationary promastigote and amastigote stages suggests that it may be important for survival within macrophages. The possession of SODs in both the promastigote and amastigote stages is clearly beneficial to the parasites. Research indicates that the midgut environment of the sandfly is potentially lethal for the developing parasite and that expression of stage- and species-specific molecules promotes parasite survival and growth during this stage (27). It is very likely that between blood meals, the parasites endure conditions of overcrowding and starvation within the sandfly. Such conditions have been shown to result in increased oxidative stress on other cells, leading to the induction of antioxidant enzymes, such as catalase (18). As the parasites enter the mammalian macrophage, they are exposed to an entirely different environment, which is much harsher than the environment in the gut of the sandfly. Among other changes, heat shock and an increased acidic and oxidative environment undoubtedly impose many metabolic stresses on the parasites that lead to increased intracellular oxidative pressures. The possession of SODs, such as LcFeSODB1 and LcFeSODB2, would therefore be beneficial for parasite survival in controlling endogenous superoxide levels. What is not clear at the moment is why *Leishmania* possesses two highly homologous FeSODs, both targeted to the glycosomes and with completely different expression profiles. Perhaps *Lcfesodb2* predated *Lcfesodb1* in evolution, and over time the parasites acquired an FeSODB that is up-regulated toward the amastigote stage and as a result acquired an enhanced ability to survive intracellularly.

To advance our understanding of the specific role that LcFeSODB1 plays in survival, we used GFP fusion proteins to determine the localization of LcFeSODB within the parasites. The results show that LcFeSODB1 and LcFeSODB2 are both localized to the glycosomes and hence are not likely to be available to react directly with external $O_2^{\cdot -}$ generated from macrophages, since $O_2^{\cdot -}$ is a charged molecule that does not diffuse through lipid membranes (12, 17). This suggests that the proteins may have evolved to protect the parasites against $O_2^{\cdot -}$ formed in the glycosomes as a result of changes in metabolism that may occur in the parasites in response to oxidative stress, starvation, and/or overcrowding within the sandfly gut or within the phagolysosomes of macrophages. Glycosomes are known to provide an enclosed environment separate from the cytoplasm for a variety of essential activities, such as glycolysis and β-oxidation of fatty acids, which generate $O_2^{\cdot -}$ as a by-product. When $O_2^{\cdot -}$ reaches toxic levels, it may inactivate important proteins essential for parasite survival or it may exacerbate oxidative damage through its conversion into and/or reaction with other ROS and RNS. A defense against glycosomal $O_2^{\cdot -}$ has not been clearly defined. There was a brief report of Cu/ZnSOD activity using SOD inhibitors within the glycosomes of *L. donovani* (7), but a Cu/ZnSOD gene or protein has never been isolated.

To further elucidate the functional role of LcFeSODB1 in the survival of *Leishmania*, we generated a single-allele knockout of the *Lcfesodb1* gene. Analysis of the *Lcfesodb1* single-allele knockout (Δ*sodb1hyg*) parasites revealed a significant decrease in the survival of these parasites within macrophages and when exposed to paraquat in culture. The infection data (Fig. 7) suggest that the defect in Δ*sodb1hyg* parasite growth could be due to the inability of the parasites to deal with their own endogenous superoxide. If the defect in Δ*sodb1hyg* parasite growth was due to intracellular killing by macrophage-derived superoxide produced by the respiratory burst which occurs in the early stage of infection, we would have observed the same initial infection level as for the wild-type control parasites, followed by a transient decrease in the infection level. Figure 7A and B show that the initial infection level of the Δ*sodb1hyg* parasites is low and remains low over a period of 48 h. Our findings that the Δ*sodb1hyg* parasites exhibit a decreased level of survival compared to wild-type control parasites upon exposure to paraquat, which increases superoxide levels in the cytosol, suggests that the knockout parasites have a defect in protection from endogenous superoxide toxicity, which further supports our infection studies suggesting that the Δ*sodb1hyg* parasites have an impaired ability to deal with their own endogenous superoxide levels.

The results show that LcFeSODB1 and LcFeSODB2 both act within the glycosomes, and we propose that their role is to protect glycosomal proteins involved in key metabolic processes from $O_2^{\cdot -}$ toxicity. These findings also highlight the importance of the glycosomes in parasite survival. It is noteworthy that macrophage-derived $O_2^{\cdot -}$ still has an important role in contributing to the intracellular killing of *Leishmania* (8, 13, 21, 26, 33), perhaps by its conversion into and/or reaction with other ROS and RNS. While this paper was in preparation, an antisense knockout against FeSOD from

Leishmania tropica (38% identical in amino acid sequence to LcFeSODB1) was published demonstrating that FeSOD is important for survival in mouse macrophages, apparently providing protection against macrophage-derived superoxide (9). Interestingly, our results suggest that LcFeSODB1 does not provide protection against macrophage-derived $O_2^{\cdot -}$. Although we were only able to create a single-allele knockout of the *Lcfesodb1* gene, we were able to demonstrate its importance for survival, and because of the absence of FeSODs in mammals, LcFeSODB is undoubtedly an attractive target for future drug design.

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