Leishmania Subtilisin Is a Maturase for the Trypanothione Reductase System and Contributes to Disease Pathology

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Proteases are a ubiquitous group of enzymes that play key roles in the life cycle of parasites, in the host-parasite relationship, and in the pathogenesis of parasitic diseases. Furthermore, proteases are druggable targets for the development of new anti-parasitic therapy. The subtilisin protease (SUB; Clan SB, family S8) of Leishmania donovani was cloned and found to possess a unique catalytic triad. This gene was then deleted by gene knock-out, which resulted in reduced ability by the parasite to undergo promastigote to amastigote differentiation in vitro. Electron microscopy of SUB knock-out amastigotes revealed abnormal membrane structures, retained flagella, and increased binucleation. SUB-deficient Leishmania displayed reduced virulence in both hamster and murine infection models. Histology of spleens from SUB knock-out-infected hamsters revealed the absence of psammoma body calcifications indicative of the granulomatous lesions that occur during Leishmania infection. To delineate the specific role of SUB in parasite physiology, two-dimensional gel electrophoresis was carried out on SUB−/− versus wild-type parasites. SUB knock-out parasites showed altered regulation of the terminal peroxidases of the trypanothione reductase system. Leishmania and other trypanosomatids lack glutathione reductase, and therefore rely on the novel trypanothione reductase system to detoxify reactive oxygen intermediates and to maintain redox homeostasis. The predominant tryparedoxin peroxidases were decreased in SUB−/− parasites, and higher molecular weight isoforms were present, indicating altered processing. In addition, knock-out parasites showed increased sensitivity to hydroperoxide. These data suggest that subtilisin is the maturase for tryparedoxin peroxidases and is necessary for full virulence.

Protozoan parasites of the genus Leishmania cause a variety of vector-borne diseases in vertebrates, including cutaneous, mucocutaneous, and visceral leishmaniasis in humans. Due to the lack of safe and effective treatments for this disease, leishmaniasis is classified by the World Health Organization as a Tropical Disease Research Category I disease, an emerging or uncontrolled disease (1). This kinetoplastid parasite has a relatively simple dimorphic life cycle consisting of promastigote and amastigote stages. Leishmania promastigotes multiply extracellularly as spindle-shaped flagellates in the midgut of the phlebotomine sandfly vector. The parasites are then transmitted to a mammalian host when an infected sandfly bites to take in a blood meal. In the naïve host the parasites infect macrophages and differentiate into amastigotes. This form of the parasite is an ovoid intracellular aflagellate. Throughout its life cycle, Leishmania is exposed to a variety of reactive oxygen species that it must detoxify to survive. Antioxidant defense is particularly important for amastigotes, because they must also survive the oxidative burst generated by the host macrophages (2).

Recent advances in parasite molecular biology and bioinformatics have enabled us to strategically identify and study Leishmania proteins as therapeutic targets. Parasite proteases are viable drug targets, because many of them are required for the pathogenic life cycle of the parasite (3, 4). We have identified an unusual Clan SB, family S8 subtilisin-like serine protease in Leishmania as one of these therapeutic targets. This family of endopeptidases is conserved across all biological kingdoms (5). Subtilisins are protein-processing enzymes and known virulence factors for both Plasmodium and Toxoplasma parasites (6). In Plasmodium falciparum a subtilisin-like serine protease is required for erythrocyte egress by infectious merozoites (7, 8) and is believed to be the convertase for the maturation of merozoite surface protein 1 and SERA proteins (9, 10). In Toxoplasma gondii a subtilisin is involved in rhoptry organelle protein processing (11, 12).

In this study we describe the identification and phenotypic characterization of Leishmania subtilisin. This protease was found to process the terminal peroxidases of the trypanothione reductase system. This system plays an important role in Leishmania survival within host macrophages and is being intensely studied as a target for antiparasitic drug development (13). This study has found that subtilisin is an important regulator of this system and is key for parasite infectivity and virulence.

EXPERIMENTAL PROCEDURES

Animals and Parasite Strains—Commercially bred, 6- to 8-week-old, female BALB/c mice (Mus musculus) were used for the murine footpad infection model (Charles River Laboratories International, Inc., Davis, CA). Commercially bred, 4- to 5-week-old, male Golden Syrian hamsters (Mesocricetus auratus) were used for the visceral infection model (Simonsen Lab-

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oratories, Inc., Gilroy, CA). *Leishmania donovani* donovani MHOM/ET/67/HU3 cloned stock and *Leishmania major* LV39 MRHO/SU/59/P were used for knock-out studies and for animal infections. *Leishmania* promastigotes were cultured at 27 °C in M199 (Sigma) liquid medium as previously described (14). Axenic amastigotes of *L. donovani* were cultured at 37 °C in 100% fetal bovine serum (Omega Scientific Inc., Tarzana, CA) as previously described (15).

**Subtilisin Cloning and Sequencing**—Genomic DNA from *L. donovani* was isolated as previously described (16). The *subtilisin* gene was then amplified from this genomic DNA by PCR using the Expand High Fidelity PCR System (Roche Applied Science). The forward primer (5′-CAC GCA TCA GCC GGT AC-3′) and reverse primer (5′-ACT AGT TCA ACA CGA GAG AGT CGA TTC TGA CG-3′) were used for PCR amplification of *L. donovani* SUB. Three clones were independently evaluated for protease activity. Supernatant from induced cultures was harvested by centrifugation at 3,000 × g for 10 min, followed by 0.2-μm filtration (Nalg Nunc). Expressed SUB protein was then buffer-exchanged with 50 mM Tris-Cl, pH 7.5, and concentrated on an Amicon Ultra-4 10,000 MWCO filter device (Millipore, Billerica, MA). This concentrate was then fractionated by hydrophobic interaction. The sample was diluted 1:1 to a final buffer concentration 30 mM Tris-Cl, pH 8.0, with 1 mM ammonium sulfate and loaded onto a HiTrap Octyl-Sepharose 4FF hydrophobic interaction column (Amersham Biosciences). A 10 mM Tris-Cl, pH 8.0, buffer with 1 mM ammonium sulfate was used for column equilibration, sample loading, and washing at a 1 ml/min flow rate. The ammonium sulfate concentration was decreased from 1–0 mM over 40 column volumes to elute the SUB protein. The eluate was desalted by buffer exchanging with 50 mM Tris-Cl, pH 7.5, and concentrated by Amicon. SUB protein concentration was measured by inhibition titration with PPACK (H-D-Phe-Pro-Arg-CMK) and NanoDrop 1000.

Protease activity was measured using peptide substrates containing C-terminal 7-amino-4-carbamoylmethylcoumarin (AMC) as the fluorogenic leaving group. The synthetic substrates Z-VFRSLK-AMC, Z-RVRR-AMC, and Z-RR-AMC were used. Initial test reactions contained 20 μM substrate. For *Km* determination VFRSLK was serially diluted from 100–0.05 μM, and RFRV was serially diluted from 20 to 0.025 μM. Enzyme samples were mixed with substrate in 50 mM Tris-Cl, pH 7.5, with 0.2% DMSO in 150 μl of total volume. Hydrolysis of the substrates was measured at 25 °C using a FlexStation microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA). Excitation/emission for AMC and 7-amino-4-carbamoylmethylcoumarin were 350/460 nm and 380/460 nm, respectively. *Vmax* values were calculated using the accompanying SoftMax Pro v4.8 software.

**Southern and Northern Blot Analyses**—For Southern blot analysis, genomic DNA was digested with indicated restriction endonucleases (New England Biolabs, Ipswich, MA and Roche Diagnostics, Indianapolis, IN), and fragments were separated by electrophoresis on a 0.6% agarose gel (17). These were then transferred to Hybond-N+ (Amersham Biosciences) nylon filters by the manufacturer's instructions for alkali transfer. Southern blot probes were 32P-labeled using a Rediprime II Random Prime Labeling System (Amersham Biosciences) as per the manufacturer's instructions. Hybridization and wash-thione reductase; TS, and T(H2)2, oxidized and reduced trypanothione; TXN, tryptaredoxin; TXNPx, tryptaredoxin peroxidase; Prx, peroxidoxin; Z, benzoylxy-carbonyl.
ing conditions were performed as previously described (18). RNA was isolated from *L. donovani* promastigotes and axenic amastigotes (19). RNA (5 μg/lane) was size-fractionated, and Northern blot hybridization was performed as previously described.

**Constructs for Targeted Gene Deletion of SUB**—Two knock-out cassettes (one for each allele) were created to delete the *L. donovani* and *L. major* SUB genes. These cassettes each contained an antibiotic resistance gene, *hyg* (conferring hygromycin B resistance) (20), *pac* (conferring puromycin resistance, used for *L. donovani* only) (21), or *sat* (conferring nourseothricin resistance, *L. major* only) (22), followed by 1.5 kb of the 3'-untranslated region of the *L. major dhfr-ts* gene (23). This untranslated region ensures high level expression during the life cycle of *Leishmania*. To target these knock-out cassettes to the *SLB* locus, 5' and 3' targeting flanks were created and ligated into their respective sides of the cassettes. These targeting flanks were generated by PCR amplification of the untranslated regions directly 5' of the *SLB* ORF and 3' of the *SLB* catalytic core (*L. donovani*) or 3' of the ORF (*L. major*). PCR primers were designed based on the published *L. infantum* and *L. major* sequences (GenDB): *L. donovani* 5' flank forward (5'-CTC ACT AGT CGC CTC TCT GTC GCA CTC-3') and reverse (5'-CTC TCT AGA CAC CAC TAC CTC AAT CGG AGC G-3') (1.3-kb fragment), 3' flank forward (5'-CTC ACT AGT TGG TCA TCT ACG GCC GTG GTA GC-3') and reverse (5'-CTC TCT AGA CGT GCC CTG ATC TGC GGC AGC-3') (0.7-kb fragment); *L. major* 5' flank forward (5'-CTC ACT AGT TGC GCA ACC ACA GCG GTC ATC-3') and reverse (5'-CTC TCT AGA TAC CTC AAT GGG AGC GTG CCT G-3') (1.5-kb fragment), 3' flank forward (5'-CTC ACT AGT TCG TTT GAG AGG CCA ACG GCC CG-3') and reverse (5'-CTC TCT AGA CGA GTG GCC GTG GCC CGT C-3') (0.8-kb fragment) primers (Spel sites, in bold, and XbaI sites, underlined, were included for cloning). These constructs were maintained and amplified in the pGEM-9Zf+(Promega). For targeted gene deletion, 50 μg of the targeting constructs was excised from their vectors using the flanking restriction endonucleases Spel and XbaI (New England Biolabs) and purified by electrophoresis on 0.8% agarose gels then purified using the QIAEX II Gel Extraction Kit (Qiagen Inc., Valencia, CA).

**Leishmania Transfections and Clone Isolation**—Purified transfection constructs described above were used to transfected *L. infantum* promastigotes by electroporation (2.25 kV/cm, 500 microfarads) as previously described (23). After electroporation, the cells were grown and selected using hygromycin B, puromycin, or nourseothricin on both plates and in liquid media and clones were isolated as previously described (24).

**Replication Rates and in Vitro Differentiation**—Day 4 SUB knock-out and wild-type parasites were split in triplicate into new M199 (for promastigote replication rates) and into 37°C fetal bovine serum (for axenic amastigote replication). Parasite culture densities were determined on days 1–4 post-split by cell counting on a Multisizer 3 Coulter Counter (Beckman Coulter, Inc., Fullerton, CA). Axenic amastigote differentiation was observed by microscopy.

**Transmission Electron Microscopy**—Approximately 10⁸ day 4 *L. donovani* axenic amastigotes from wild-type or SUB−/− cultures were pelleted and washed 3× in PBS. The parasites were processed for conventional EM by freeze-substitution in 1% OsO₄/0.1% uranyl acetate in acetone and embedded in Epon resin. Sections were cut with a Leica Ultracut UCT Ultramicrotome (Leica Microsystems, Bannockburn, IL) and viewed on a Tecnai T20 electron microscope (FEI Co., Hillsboro, OR) with a 4000 × 4000 UltraScan charge-coupled device camera (Gatan Inc., Pleasanton, CA).

**Hamster and Mouse Infections**—Hamsters were infected intraperitoneally with 10⁸ day 4 SUB knock-out or wild-type *L. donovani* promastigotes (groups of three) (25). Animals were weighed weekly over the length of the experiment. Hamsters were culled 200 days post-infection by CO₂ inhalation followed by thoracotomy. Pieces of each spleen and liver were fixed in 10% formalin in PBS and then embedded in parafin for histology. Sections were cut at 5 μm and stained with either Wright-Giemsa or hematoxylin and eosin by the University of California at San Francisco (UCSF) Morphology Core using standard protocols. Psammoda bodies were identified by microscopy and counted. Mice were infected with metacyclic *L. major* promastigotes purified from day 4 SUB−/− and wild-type cultures using negative selection by binding to peanut agglutinin as has been previously described (26). BALB/c mice (groups of 5) were anesthetized by isoflurane inhalation and infected subcutaneously with 5 × 10⁶ metacyclic promastigotes in 50 μl of Hanks’ balanced salt solution. Footpad swelling was measured weekly after inoculation using a Mitutoyo caliper. Parasites were recovered from infected mice by resection of the left popliteal lymph node.

**Two-dimensional Gel Electrophoresis**—Three experimental replicates were prepared from separately cultured samples of both wild-type and SUB knock-out *L. donovani*. Approximately 10⁶ cells were pelleted, washed 3× with PBS, and stored at −80°C. Lysates were prepared by resuspending the cell pellets in 2 ml of native lysis buffer containing 20 mM HEPES, pH 7.5, 250 mM sucrose, 3 mM MgCl₂, 0.5% Nonidet P-40, 1 mM DTT, and 1× Halt EDTA-free protease inhibitor mixture (Pierce). Cells were then broken by mechanical lysis using 70 strokes of a Dounce homogenizer. The lysates were centrifuged at 12,000 × g for 20 min at 4°C, and the clarified supernatants were dialyzed overnight against 50 mM Tris-HCl, pH 7.5, 100 mM NaCl using 8-kDa molecular weight cut-off dialysis membranes. The following day the protein samples were concentrated and washed by precipitation using the ReadyPrep 2-D Cleanup Kit (Bio-Rad Laboratories, Inc., Hercules, CA). Approximately 300 μg of protein per gel was brought up to 300 μl in Bio-Rad Rehydration/Sample buffer and was passively loaded onto 17-cm, 3–10 pH immobilized pH gradient isoelectric focusing strips. Isoelectric focusing was performed using slow increases in voltage over multiple steps up to 10 kV for a total of 60–65 kVh focusing time. Next, the strips were reduced and alkylated using sequential 10-min incubations in 2% DTT then 2.5% iodoacetamide and dissolved in sample equilibration buffer. The isoelectric focusing strips were run in the second dimension on 17 × 17 cm, 12.5% acrylamide Tris-glycine, SDS-PAGE gels. Gels were stained with SYPRO Ruby and imaged using a
Typhoon Trio Variable Mode Imager (Amersham Biosciences). These images were utilized for spot intensity analysis using Bio-Rad PDQuest software (v. 7.4). For proteomic analysis, gels were silver-stained (27), and selected protein spots were excised and in-gel-digested with trypsin (28, 29). The resulting peptides were extracted and analyzed by on-line liquid chromatography/mass spectrometry using an Eksigent nanoflow pump (Dublin, CA) coupled to a QStar Pulsar quadrupole orthogonal acceleration, time-of-flight hybrid instrument (Applied Biosystems, Foster City, CA). The reversed-phase chromatographic column was controlled with a Famos autosampler (Sunnyvale, CA) and Eksigent software to run at a 5–50% acetonitrile gradient and the bacterial subtilisins. This distinguishes L. donovani SUB from the more closely related L. infantum (a subspecies from within the L. donovani complex) has a C-terminal truncation after amino acid 1192 (LinJ13_V3.0940). The Pfam predicted Subtilase family core for L. donovani SUB is between amino acids 86–414. Comparisons of L. donovani SUB to other trypanosomatid SUBs are summarized in Table 1. Interestingly, L. donovani SUB has a non-canonical catalytic triad with the catalytic Glu in place of the standard Asp due to a single C to G base pair change. L. infantum SUB also has Glu in place of the Asp, indicating that this adaptation may be specific to parasites in the L. donovani complex. The SUB catalytic core amino acid (aa) sequences are relatively conserved within the Leishmania species; however, these sequences have diverged considerably from those of the trypanosomes, with only a 40% identity between the genera. L. donovani has a truncated C terminus. All the trypanosomatid SUB proteins fall between 126 and 191 kDa.

### Results

**L. donovani SUB Uses a Non-canonical Catalytic Triad**—The gene encoding L. donovani SUB was cloned as described above. Sequencing yielded a 5,235-bp gene. This sequence was submitted to GenBank™ with accession number ADA81891. The resultant 1,744-amino acid protein has an estimated molecular mass of 184.7 kDa. This protein has a predicted signal peptide (SignalP V2.0 HMM probability of 0.995) with a cleavage site between amino acids 38–39 (0.421 probability), and a probable C-terminal transmembrane helix between amino acids 1709–1731 (TMHMM v. 2.0). The published L. major SUB (GeneDB: LmjF13.1040) shares this general layout; however, SUB from the more closely related L. infantum (a subspecies from within the L. donovani complex) has a C-terminal truncation after amino acid 1192 (LinJ13_V3.0940). The Pfam predicted Subtilase family core for L. donovani SUB is between amino acids 86–414. Comparisons of L. donovani SUB to other trypanosomatid SUBs are summarized in Table 1. Interestingly, L. donovani SUB has a non-canonical catalytic triad with the catalytic Glu in place of the standard Asp due to a single C to G base pair change. L. infantum SUB also has Glu in place of the Asp, indicating that this adaptation may be specific to parasites in the L. donovani complex. The SUB catalytic core amino acid sequences are relatively conserved within the Leishmania species; however, these sequences have diverged considerably from those of the trypanosomes, with only a 40% identity between the genera.

To determine the subfamily of Leishmania subtilisin, the catalytic core sequence was compared with the cores of other Clan SB, family S8 family members. Core sequences were aligned using ClustalW2 (EMBL-EBI), and a phylogenetic tree was generated (Fig. 1). The Leishmania SUBs group with the subfamily S8A proteases, which include the eukaryotic Site-1 peptidases and the bacterial subtilisins. This distinguishes Leishmania SUB from the Toxoplasma and Plasmodium SUBs and from the subfamily S8B kexins and furins. Site-1 peptidases are restricted to metazoan organisms and are known to process sterol regulatory element binding proteins, which are not found in trypanosomatids (32).

**L. donovani and L. major SUBs Were Recombinantly Expressed in P. pastoris**—The catalytic cores of the subtilisin proteins from L. donovani and L. major were successfully recombinantly expressed. Site-1 proteases, to which Leishmania SUB is most similar, have a requirement for a lysine or arginine in the P4 position. For this reason SUB activity was evaluated using synthetic substrates with and without P4 Arg. Cleavage of the synthetic substrates RVRR and VFRSLK was detected in all of the SUB-expressing Pichia supernatants compared with the X-33 background strain. Slight activity against the RR substrate was only detected in one L. major clone. In this clone the RRV Vmax was over six times that of RR. Poor cleavage of RR and the lack of detected protease activity in the X-33 control strain indicate that the cleavage is not due to endoge-

### Table 1

Comparison of predicted SUB proteins from trypanosomatids

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene Sub</th>
<th>aa identity %</th>
<th>Core identity %</th>
<th>aa identity</th>
<th>Core identity</th>
<th>Length</th>
<th>Molecular mass kDa</th>
<th>Catalytic triad</th>
</tr>
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<tbody>
<tr>
<td>L. donovani</td>
<td>SUB1</td>
<td>100.0</td>
<td>100.0</td>
<td>1744</td>
<td>184.7</td>
<td>Glu-97, His-130, Ser-395</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. infantum</td>
<td>SUB1</td>
<td>99.6</td>
<td>99.7</td>
<td>1192</td>
<td>126.2</td>
<td>Glu-97, His-130, Ser-395</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. major</td>
<td>SUB1</td>
<td>89.0</td>
<td>94.5</td>
<td>1722</td>
<td>182.7</td>
<td>D99, His-132, Ser-397</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. braziliensis</td>
<td>SUB1</td>
<td>70.0</td>
<td>83.0</td>
<td>1785</td>
<td>190.7</td>
<td>D97, His-130, Ser-405</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. major</td>
<td>SUB1</td>
<td>70.0</td>
<td>83.0</td>
<td>1430</td>
<td>160.5</td>
<td>D219, His-269, Ser-502</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. cruzi</td>
<td>SUB1</td>
<td>23.1</td>
<td>40.3</td>
<td>1408</td>
<td>158.0</td>
<td>D196, His-246, Ser-480</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. brucei</td>
<td>SUB1</td>
<td>24.4</td>
<td>39.5</td>
<td>1487</td>
<td>161.5</td>
<td>D191, His-238, Ser-476</td>
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*Note: The catalytic cores of the subtilisin were determined by ClustalW2 (EMBL-EBI), and a phylogenetic tree was generated (Fig. 1). The Leishmania SUBs group with the subfamily S8A proteases, which include the eukaryotic Site-1 peptidases and the bacterial subtilisins. This distinguishes Leishmania SUB from the Toxoplasma and Plasmodium SUBs and from the subfamily S8B kexins and furins. Site-1 peptidases are restricted to metazoan organisms and are known to process sterol regulatory element binding proteins, which are not found in trypanosomatids (32).*
Leishmania Subtilisin Regulates Tryparedoxin Peroxidase

Major KEX2. These results show that, like the Site-1 proteases, Leishmania SUB prefers a basic P4 residue. L. donovani and L. major SUB was isolated from the Pichia supernatants, and $k_{cat}$ and $K_m$ values were determined for both RVRR and VFRSLK substrates (Table 2). Both enzymes catalyzed the RVRR substrate at about a 10-fold faster rate than VFRSLK. The L. donovani SUB had a similar affinity for both substrates, whereas L. major SUB had a 10-fold higher affinity for RVRR. Interestingly, L. donovani SUB had a much lower rate of catalysis for each substrate (126- and 62-fold lower for RVRR and VFRSLK, respectively) when compared with L. major SUB. This difference could potentially be due to the non-canonical catalytic triad of L. donovani.

Subtilisin (SUB) −/− Parasites Have Defects in Promastigote to Amastigote Differentiation—The published L. major and L. infantum genomes (GeneDB) indicate that Leishmania sp. contain a single copy of SUB per haploid genome. This was confirmed to be true in L. donovani and L. major by Southern blot analysis of genomic DNA (data not shown). Deletion of both alleles of the single copy gene was carried out by two rounds of targeted gene replacement in L. donovani and confirmed by Southern blot (Fig. 2). Only one allele could be deleted from L. major despite multiple attempts at targeting the second allele.

Wild-type, SUB+/−, and −/− L. donovani promastigotes were cultured at 27 °C in M199. These parasites replicated at a rate comparable to wild-type parasites. To test for the ability to differentiate into axenic amastigotes, stationary phase (day 5 post-split) promastigotes were split 1:10 in FBS at 37 °C. Wild-type and SUB+/− parasites differentiated readily, however SUB −/− did not (Fig. 3). The SUB −/− parasites remained as elongated, flagellated spindles. These cells did not form aggregates of cells typically seen in axenic amastigote cultures. To test for differentiation under lower dilution conditions, stationary phase SUB −/− promastigotes were diluted 1:2 in FBS at 37 °C. After 4 days, some of the cells appeared to differentiate; however, cell aggregation did not occur.

Electron Microscopy of SUB −/− Amastigotes Revealed Abnormal Ultrastructures—Wild-type and SUB −/− axenic amastigotes from the low dilution differentiation were processed for transmission electron microscopy. Wild-type axenic amastigotes had typical amastigote morphology: a rounded cell body measuring ~3 μm in diameter, no external flagellum, and a condensed electron-dense kinetoplast (Fig. 4A). SUB −/− axenic amastigotes exhibited many abnormalities (Fig. 4, B–D). Although most of these cells had rounded cell bodies, many were still elongated and spindle-shaped. Those that were rounded often had invaginations in the plasma membrane. Additionally, many of these cells were binucleated. Unlike the wild-type amastigotes, the SUB −/− cells also had multiple flagellar cross sections, including flagella appearing in the cytoplasm, outside of their expected location within the flagellar pocket. These images indicate that the SUB −/− cells were not successfully differentiating into amastigotes.

SUB Regulates Levels of Peroxidases from the Trypanothione Reductase System—Site-1 peptidases and the subtilisins from apicoplast parasites such as Toxoplasma and Plasmodium are maturases that process proteins within vesicles (9, 11, 33). The presence of a signal peptide and C-terminal transmembrane domain on Leishmania SUB indicates that it may perform a similar function. We employed two-dimensional gel electrophoresis to study the main differences in protein expression and processing between wild-type and SUB −/− parasites. Gels were run in triplicate (Fig. 5). On average, gels had ~325 well defined spots, a value typical for two-dimensional gels of this size. These protein species repre...

**TABLE 2**

Kinetic analysis of L. donovani and L. major SUB

<table>
<thead>
<tr>
<th>AMC Substrate</th>
<th>L. donovani SUB</th>
<th>L. major SUB</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$ S$^{-1}$</td>
<td>$K_m \mu M$</td>
<td>$k_{cat}/K_m$ S$^{-1}$ μM$^{-1}$</td>
</tr>
<tr>
<td>RVRR</td>
<td>2.19 × 10$^{-2}$</td>
<td>1.20</td>
</tr>
<tr>
<td>VFRSLK</td>
<td>1.88 × 10$^{-2}$</td>
<td>0.91</td>
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</table>
sent up to 4% of the predicted 8,195 protein-coding genes from the
L. donovani complex (34). Spots that differed in intensity between
the wild-type and SUB\(+/\)H11002 parasites were selected for peptide
sequencing by mass spectrometry. The identities of these proteins
are presented in Table 3. Interestingly, nine distinct spots were
found to be tryparedoxin peroxidases, the terminal peroxidases of
the trypanothione reductase system. In the L. donovani complex,
this family of peroxidases is comprised of three cytoplasmic try-
paredoxin peroxidases encoded in a multigene array, TXNPx1–3
(TRYP1–3), and a mitochondrial peroxidoxin, Prx (35). Interest-
ingly, L. major encodes seven cytoplasmic tryparedoxin peroxi-

<table>
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<tr>
<th>Promastigotes</th>
<th>Axenic Amastigotes</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>SUB(+/-)</td>
</tr>
<tr>
<td>KO</td>
<td>SUB(-/-)</td>
</tr>
</tbody>
</table>

FIGURE 3. Wild-type, SUB\(+/-\), and \(-/-\) L. donovani promastigotes were
cultured successfully at 27 °C in M199. To test for the ability to differenti-
ate into axenic amastigotes, stationary phase (day 5 post-split) promastigotes of
each culture were split 1:10 in FBS at 37 °C. Wild-type and SUB\(+/-\) parasites
differentiated readily, however SUB\(-/-\) did not. The SUB\(-/-\) parasites
remained as elongated, flagellated spindles. These cells did not form aggre-
gates of cells typically seen in axenic amastigote cultures.

FIGURE 4. Wild-type axenic amastigotes (A) had normal rounded cell bod-
ies measuring \(\sim 3 \mu m\) in diameter (scale bar = 1 \mu m) with no external
flagellum and a condensed electron-dense kinetoplast. SUB\(-/-\) axenic
amastigotes (B–D) exhibited many abnormalities. Although most of these
cells had rounded cell bodies, many were still elongated and spindle-shaped
(D). Those that were rounded often had invaginations in the plasma mem-
brane (B). Additionally, many of these cells were binucleated (C). Unlike the
wild-type amastigotes, the SUB\(+/-\) cells also had multiple flagellar cross-se-
tions, including flagella appearing in the cytoplasm (B), outside of their
expected location within the flagellar pocket \(\{N = \text{nucleus}, K = \text{kinetoplast},
FP = \text{flagellar pocket}, F = \text{flagellum}\}\).

TABLE 3

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein</th>
<th>Relative to WT</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>No Prx detected</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Prx Elevated</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>TXNPx2 Same</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>TXNPx1/3 Decreased</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>TXNPx1/3 Decreased</td>
<td>Elevation</td>
</tr>
<tr>
<td>6</td>
<td>TXNPx1/3 Elevated</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>No TXNPx detected</td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 5. Wild-type L. donovani had high levels of TXNPx1 and -3 forming
a single doublet of spots (WT spots 4 and 5, left). The level of TXNPx2 was
low (spot 3) and Prx was not detectable (spot 1). In the SUB\(+/-\) parasites (right),
the wild-type TXNPx1/3 doublet decreased (SUB\(+/-\) spots 4 and 5) and two
new TXNPx1/3 doublets were present at a higher molecular weight (spots 6 and
7) and at a higher molecular weight with a lower pi (spots 8 and 9). Prx
levels (spots 1 and 2) were elevated in these parasites.
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dases, TXNPx1–7, in addition to the single Prx. These enzymes are all 2-Cys peroxiredoxins and have complementary roles in parasite protection against oxidative stress (31). *L. donovani* TXNPx1 and -3 share 99% amino acid identity and have 94% identity to TXNPx2 (GeneDB).

Spot densitometry was performed on the peroxidase spots in triplicate. Wild-type *L. donovani* had high levels of TXNPx1 and -3 forming a single “wild-type doublet” of spots. The level of TXNPx2 was low and Prx was not detectable. In the SUB−/− parasites, the TXNPx1/3 wild-type doublet decreased (to ~35% of the wild-type spot density), and two new TXNPx1/3 “mutant doublets” were present at a higher molecular weight and at a higher molecular weight with a lower pI. In addition, Prx levels were elevated in these parasites, which could be a compensation for the decreased level of wild-type TXNPx1/3. TXNPx2 spot density was not significantly different between the wild-type and SUB−/−. The range of mass spectrometry amino acid coverage was nearly complete for all TXNPx spots (supplemental Fig. S1), with the notable exception of the wild-type doublet spots 4 and 5 for both WT and SUB−/−. For these spots the C-terminal 20 amino acids were never detected, despite high protein abundance and the fact that these amino acids were detected in the higher molecular weight doublets. These data suggest that the lower molecular weight wild-type doublet is C-terminally cleaved. The molecular weight shift of the mutant doublets is ~2 kDa, which is consistent with the retention of the 20-amino acid C terminus.

**SUB Knock-out Parasites Have Increased Sensitivity to Oxidative Damage—Proteomic analysis of SUB-deficient Leishmania indicated that SUB is required for normal regulation of the trypanothione reductase system. Alteration of this system would hinder the parasite’s ability to detoxify hydroperoxides and thus render it more sensitive to oxidative damage (13). To evaluate sensitivity of wild-type and SUB-deficient Leishmania to oxidative damage, a hydroperoxide sensitivity assay was performed (Fig. 6). As expected, SUB knock-out Leishmania was significantly more sensitive to hydroperoxide compared with wild type. In 100 μM tert-butylhydroperoxide, wild-type parasites were over 60% viable while SUB knock-out parasites cultures had less than 10% viability.

**Loss of SUB Results in Delayed Lesion Formation in Mice and the Absence of Psammoma Body Lesions in Hamsters—**Within the host, Leishmania is exposed to a variety of oxidative stresses particularly within host macrophages. It was predicted that SUB-deficient parasites would have reduced virulence in animal infection models due to the altered regulation of the trypanothione reductase system. Indeed, SUB-deficient Leishmania were found to be less virulent in both the mouse and hamster systems.

BALB/c mice were infected subcutaneously into their left hind footpads with either wild-type or SUB-deficient parasites. Footpad swelling was measured weekly (Fig. 7A). Swelling was evident in the wild-type-infected mice after 7 weeks; however, significant swelling (compared with the contralateral footpad) was not observed in mice infected with SUB-deficient parasites until after 14 weeks. The SUB-deficient infections were not self-limiting and continued to increase footpad swelling, however the lesion size was consistently 7–8 weeks delayed compared with the wild-type infections.

For the visceral leishmaniasis infection model, male Golden Syrian hamsters were infected intraperitoneally with wild-type or SUB-deficient parasites. At 200 days post-infection, the hamsters were sacrificed, and their spleens were sectioned and histologically examined. Spleens were enlarged in both wild-type- and knock-out-infected animals. All wild-type-infected hamsters’ spleens contained psammoma body calcifications (Fig. 7, B and C), indicative of granulomatous lesions that occur in visceral leishmaniasis (36, 37). Strikingly, no psammoma bodies were observed in the spleens of hamsters infected with SUB-deficient Leishmania (Fig. 7, D and E).

**DISCUSSION**

*Leishmania* is a dimorphic parasite that must survive and replicate in two vastly different environments: the gut of a phlebotomine sandfly and within the parasitophorous vacuole in phagocytic cells of vertebrates. Throughout this life cycle, the parasite is exposed to a variety of oxidative insults, including the reactive oxygen species produced by host macrophages. Antioxidant defense is therefore extremely important for parasite survival. *Leishmania*, along with the other kinetoplastids, uses an unusual hydroperoxide metabolic pathway, the trypanothione reductase system, which employs trypanothione as the main transporter of electrons (38). This system has been identified as an important target for antiparasitic drug development. Our research has shown that *Leishmania* parasites contain an unusual subtilisin-like enzyme that governs the levels of key peroxides in the trypanothione reductase system. This serine protease, therefore, represents a potential target for rational drug design (39, 40).

We have identified and cloned a novel subtilisin-like protease from the parasite *L. donovani*. Phylogenetic sorting of known subtilisin catalytic cores showed that the *Leishmania* SUBs fall within the subfamily S8A and are most closely related to site-1 proteases. Interestingly, *L. donovani* (and the related *L. infantum*) SUB has a non-canonical catalytic triad. Clan SB serine
proteases use an Asp-His-Ser triad, whereas *L. donovani* complex SUBs use a Glu in place of the Asp. Glu and Asp are identical save for one additional carbon in the side chain of Glu. Subtilisins are known to be pliable enzymes; however, a search of the MEROPS database has shown that there are currently no other known cases of Glu in the catalytic triad of a Clan SB protease (33). To verify that *L. donovani* subtilisin is an active enzyme, the catalytic cores of both *L. donovani* and *L. major* were recombinantly expressed in *Pichia pastoris*. Activity was recovered for both SUB cores indicating that the Glu-His-Ser catalytic triad is functional; however, $k_{\text{cat}}$ values for this non-canonical triad were around 100-fold lower than for the canonical triad. Proteolytic cleavage preferentially occurred when substrates had a basic residue in the P4 position, much like the site-1 proteases (41, 42). This strengthens the placement of *Leishmania* subtilisin in subfamily S8A.

To phenotypically characterize the function of subtilisin in *Leishmania*, the genes were disrupted in *L. donovani* and *L. major* by homologous recombination. Both alleles of the *L. donovani* gene were knocked out; however, only one allele could be deleted in *L. major* despite multiple attempts at gene targeting. This could be due to either a greater requirement for subtilisin in *L. major* or a compensatory change in *L. donovani* that allowed for the full knock-out to be generated. Knock-out parasites of both species grew well *in vitro* as promastigotes; however, attempts to grow the *L. donovani* SUB$^{-/-}$ parasites as amastigotes revealed a defect in their ability to differentiate. This indicates that subtilisin may be beneficial for survival in the amastigote stage. Electron microscopy of amastigote-like cells from the SUB$^{-/-}$ differentiation experiments revealed that these cells were either extremely abnormal or had not fully differentiated. Commonly seen ultrastructural abnormalities included elongated cell bodies, severe membrane invaginations, binucleation, and multiple flagellar cross sections. These abnormalities are likely due to parasite distress in response to the lack of subtilisin enzyme. This research suggested that the biological role of subtilisin within *Leishmania* may be as a maturase for a protein or pathway that promotes amastigote survival.

To test this hypothesis and to uncover the pathway catalyzed by subtilisin, proteomic analysis was performed on *Leishmania* wild-type and SUB knockouts. We uncovered five sets of protein spots that differed considerably between wild-type and SUB$^{-/-}$ parasites. All five of these sets were identified as members of the tryparedoxin peroxidase family, the terminal peroxidases of the trypanothione reductase system. Both the cytoplasmic tryparedoxin peroxidases (TXNPx1, -2, and -3) and the mitochondrial peroxidoxin (Prx) were identified. Wild-type
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Leishmania had high amounts of TXNPx1 and -3 forming a single doublet of spots. Knocking out SUB resulted in a reduction of this wild-type doublet and the appearance of higher molecular weight mutant doublets. TXNPx2 was unchanged following SUB knock-out. Peptide analysis of these spots revealed that all the wild-type TXNPx1 and -3 spots did not contain the C terminus of the proteins, whereas the mutant spots retained these termini (supplemental Fig. S1). Subtilisin is therefore putatively responsible for C-terminal processing of the wild-type tryparedoxin peroxidases 1 and 3. This conclusion is further supported by the fact that the mutant doublet had a mass shift of 2 kDa, which is the calculated mass of the removed C termini. Interestingly, TXNPx2, which was not found to be processed, already has an abbreviated C terminus.

Although the tryparedoxin peroxidases are primarily cytoplasmic, along with other members of the trypanothione reductase system, can be targeted to the trypanosomatid peroxisomes, known as glycosomes (43). The targeting of these enzymes to the glycosomes requires a canonical type-1 peroxisome/glycosome-targeting signal, PTS1 (44). The PST1 is comprised of a short C-terminal extension with a terminal tripeptide SKL, or a conserved variant of SKL (45, 46). Both TXNPx1 and -3 C termini encode a PTS1 (SKL and SKQ, supplemental Fig. S1); however, TXNPx2 lacks this tripeptide targeting sequence. In addition, TXNPx1 and -3, but not TXNPx2, contain a potential subtilisin cleavage motif at the site where processing is believed to occur. This Lys-Lys-Gly-Ala motif is nearly identical to a potential autocatalytic cleavage site of Leishmania subtilisin, Lys-Tyr-Gly-Ala. C-terminal proteolytic processing of TXNPx1 and -3 by subtilisin can therefore have a role in balancing levels of the peroxidases in the glycosomes and in the cytoplasm. Trypanothione reductase, which itself can be targeted to the glycosome using a PTS1, also contains a potential subtilisin cleavage motif (Lys-Met-Gly-Ala) (GeneDB), indicating that subtilisin may control targeting of multiple enzymes in the tryparedoxin peroxidase pathway.

Proteomic analysis of SUB−/− parasites also showed increased levels of Prx compared with wild-type. The function of mitochondrial Prx is believed to be complementary to that of cytosolic TXNPxs (31), thus the increase in Prx may be a compensatory change due to the reduction of functional TXNPxs. This hypothesis is supported by the fact that both alleles of the SUB gene could not be deleted in L. major. Although L. donovani complex parasites encode three cytosolic tryparedoxin peroxidase genes in the TXNPx gene array, L. major encodes seven cytosolic tryparedoxin peroxidases (GeneDB). L. major therefore relies more heavily on these cytosolic enzymes, thereby making a sufficient compensatory increase in Prx more difficult.

SUB-deficient Leishmania was found to have increased sensitivity to hydroperoxides compared with wild-type parasites in vitro. Reduced viability of SUB-deficient L. major and L. donovani amastigotes was also exhibited in vivo using the murine and hamster infection models, respectively. In both systems, the SUB knock-out parasites had clearly reduced virulence. Our research has shown that subtilisin promotes survival of Leishmania amastigotes by serving as a maturase for the trypano-thione reductase system, thus aiding in redox homeostasis and protecting the parasite from oxidative stresses in the host macrophage. Because parasite proteases are known to be viable chemotherapeutic targets (3, 4), Leishmania subtilisin represents a new potential target for rational drug design.

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