LEISHMANIA SUBTILISIN IS A MATURASE FOR THE TRYPANOTHIONE REDUCTASE SYSTEM AND CONTRIBUTES TO DISEASE PATHOLOGY*
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Running head: Leishmania subtilisin regulates tryparedoxin peroxidase
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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 knockout, which resulted in reduced ability by the parasite to undergo promastigote to amastigote differentiation in vitro. Electron microscopy of SUB knockout 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 knockout-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 wildtype parasites. SUB knockout 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, knockout 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 leishmaniases 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 (ROS) that it must detoxify in order to survive. Antioxidant defense is particularly important for amastigotes, as 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, as 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 endo-peptidases is conserved across all biological kingdoms (5). Subtilisins are protein processing enzymes and known virulence factors for both Plasmodium and Toxoplasma parasites (6). In P. 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 infectious merozoites (7,8) and is believed to be protease is required for erythrocyte egress by (6). In order for both processing enzymes and known virulence factors all biological kingdoms (5). This family of endopeptidases is conserved across Leishmania subtilisin is involved in Toxoplasma gondii 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, 6-8 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, 4-5 week-old, male Golden Syrian hamsters (Mesocricetus auratus) were used for the visceral infection model (Simonsen Laboratories, Inc., Gilroy, CA). Leishmania donovani donovani MHOM/ET/67/HU3 cloned stock and Leishmania major LV39 MRHO/SU/59/P were used for knockout studies and for animal infections. Leishmania promastigotes were cultured at 27°C in M199 (Sigma, USA) 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 Diagnostics, Indianapolis, IN) in two overlapping pieces, termed the 5’ and 3’ halves. For each half, an external primer from the non-coding flanks of the gene and an internal primer were designed based on regions of identity between the known L. infantum and L. major sequences (Sanger Institute GeneDB: LinJ13_V3.0940 and LmjF13.1040). Both halves were cloned into the pGEM-7Zf(-) vector (Promega, Madison, WI) and then spliced together using an internal HindIII site. The open reading frame was sequenced using the following primers: 5’-CAT GCA TCA GCC GGT AC-3’, 5’-GCA TGG TCA TCT AC-3’, 5’-TAC TCA CAA TCT CTA CG-3’, 5’-CAC CAG TAA GAG TGC GG-3’, 5’-GAA GAG CCG CCA CCG TG-3’, 5’-CGT GCT GCC AGG ACA GC-3’, 5’-TCC TTG TTG AGG GTG CG-3’, 5’-TAG CCA TAG CCC ACC GC-3’, 5’-CTC CTC TTT GAG GTG GC-3’, 5’-CGC TCT GTC TCG AGG CG-3’, 5’-GTG TGG GGC AGC GGC AG-3’, 5’-ACC GTT GTC TCG AGG CG-3’, 5’-CGT TAG GAG ACG CCG CAC CA-3’, 5’-CGT CTG CTA GAG AGG AG-3’, 5’-ACC CAC CTT CCG CTC CG-3’, 5’-CGT GTG CCA GCC GAC CAC GG-3’, 5’-TAG GCA GCG GTG CCG AC-3’, 5’-AAC GGC AGC AGG CTC TC-3’, 5’-GTC GGC ACC GCT GCC TA-3’, 5’-ATC GGC TAT AGG ATT CC-3’, 5’-CAG TAG CCC GCA GTG GC-3’, 5’-CGT TGT CTG TCG CGA CC-3’, 5’-ACT GAT CAG CCA AGG CG-3.’ The amino acid sequence of L. donovani subtilisin catalytic core was identified using Pfam (Accession number PF00082, Subtilase family) and was aligned with homologous sequences from L. infantum, L. major, L. braziliensis, T. cruzi (1 & 2), T. brucei (1 & 2), P. falciparum (1, 2 & 3), T. gondii (1a, 1b & 2), B. licheniformis, B. amyloliquefaciens, B. subtilis, H. sapiens (furin & Site-1), M. musculus, S. cerevisiae, S. pombe, C. intestinalis, A. mellifera, X. laevis, and D. rerio (respectively: LinJ13_V3.0940, LmjF13.1040, LbrM13_V2.0860, Tc00.104705351104540, Tu00.104705351185960, Tb11.02.1280, Tb927.3.4230, CAD51440, XP_001348051, CAD51437, XP_002368971, XP_002364650, P00780, P00782, P04189, P09958, EAW95506, P23188, P13134, Q09175, XP_002122807, XP_395754, NP_001087381, and CAK04389) using the ClustalW algorithm from MegAlign (DNASTAR, Madison, WI).

**Expression and Purification of Recombinant SUB from Pichia pastoris—**
Transformation constructs were generated by PCR amplification of *L. donovani* and *L. major* SUB cores, adding a 5' Sall site (bold) followed by a Kex2 cleavage site (underlined) and a 3' SpeI site (underlined bold) using forward (*L.d.*: 5'-CTC GTC GAC AAA AGA GCA CAC CGT TCC ACA GAT GCG-3'; *L.m.*: CTC GTC GAC AAA AGA GCA CGC CGT TCC ACC GAT GCG) and reverse (*L.d.*: 5'-CTC ACT AGT TCA ACA CGG GCA AGT CGA TTC TGA C-3'; *L.m.*: 5'-CTC ACT AGT TCA ACA CGA GAG AGT CGA TTC TGA CG-3') primers, and then cloned into pPICZα-SUB constructs were electroporated into *X.33* and RVRR were used. Substrates were generated by PCR (underlined bold) using forward (*L.d.*: 5'-CTC Kex2 cleavage site (underlined) and a 3' SpeI site cores, adding a 5' SalI site (bold) followed by a ACT AGT GCA AGT CGA TTC TGA C -3'; *L.m.*: ACT AGT GCA CGC CGT TCC -3') primers, and then cloned into pPICZα-SUB constructs were electroporated into *X.33* *P. pastoris* and expression clones were isolated and induced for 96 hr as per the manufacturer’s protocol. For each *L. donovani* and *L. major* 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 (Nalge Nunc). Expressed SUB protein was then buffer exchanged with 50 mM Tris-HCl, pH 7.5 and concentrated on an Amicon Ultra-4 10,000 NMWL 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-HCl, pH 8.0 with 1 M ammonium sulfate (AS) and loaded onto a HiTrap Octyl Sepharose 4FF hydrophobic interaction column (GE Healthcare). A 10 mM Tris-HCl, pH 8.0 buffer with 1 M AS was used for column equilibration, sample loading, and washing at a 1 mL/min flow rate. The AS concentration was decreased from 1-0 M over 40 column volumes to elute the SUB protein. The eluate was desalted by buffer exchanging with 50 mM Tris-HCl, pH 7.5 and concentrated by Amicon. SUB protein concentration was measured by inhibitor titration with PPACK (H-D-Phe-Pro-Arg-CMK) and NanoDrop 1000.

Protease activity was measured using peptide substrates containing C-terminal AMC (7-amino-4-carbamoyl-methylcoumarin) 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 *K*ₘ determination VFRSLK was serially diluted from 100-0.05 μM and RVRR was serially diluted from 20-0.025μM. Enzyme samples were mixed with substrate in 50 mM Tris-HCl, pH 7.5 with 0.2% DMSO in 150 μL total volume. Hydrolysis of the substrates was measured at 25°C using a FlexStation microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Excitation/emission for AMC and ACC were 355/460 nm and 380/460 nm, respectively. *V*ₘₐₓ values were calculated using the accompanying SoftMax Pro v4.8 software.

**Southern & 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+ (GE Healthcare) as per the manufacturer’s instructions for alkali transfer. Southern blot probes were 32P-labeled using Rediprime II Random Prime Labeling System (GE Healthcare) as per the manufacturer’s instructions. Hybridization and washing 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 knockout 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, *hyγ* (confering hygromycin B resistance) (20), *pac* (confering puromycin resistance, used for *L. donovani* only) (21), or *sat* (confering 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 *Leishmania*’s life cycle. To target these knockout cassettes to the SUB 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 SUB ORF and 3’ of the SUB 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 (GeneDB): *L. donovani* 5’ flank forward (5’-CTC ACT AGT CGC CGT TCC GTC 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 TCC GCT GTA GC-3') and reverse (5'-CTC TCT AGA CGT GCC CTG ATC TGC GGC AGC AGT-3') (0.7 kb fragment); L. major 5' flank forward (5'-CTC ACT AGT TCG TTG GAG AGG CCA ACG CGC -3') and reverse (5'-CTC TCT AGA GGA GTA GGA AGA GGT GAC CGT C -3') (0.8 kb fragment) primers (SpeI sites, in bold, and XbaI sites, underlined, were included for cloning). These constructs were maintained and amplified in the pGEM-9Zf(-) vector (Promega). For targeted gene deletion, 50 µg of the targeting constructs were excised from their vectors using the flanking restriction endonucleases SpeI 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 transfect log-phase Leishmania promastigotes by electroporation (2.25 kV/cm, 500 µF) 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 knockout and wildtype 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 10^8 day 4 L. donovani axenic amastigotes from wildtype or SUB -/- cultures were pelleted and washed 3x 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 Company, Hillsboro, OR) with a 4k x 4k UltraScan CCD camera (Gatan Inc., Pleasanton, CA).

Hamster and Mouse Infections—Hamsters were infected intraperitoneally with 10^9 day 4 SUB knockout or wildtype 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 paraffin for histology. Sections were cut at 5 µm and stained with either Wright-Giemsa or hematoxylin and eosin by the UCSF Morphology Core using standard protocols. Psamomma bodies were identified by microscopy and counted. Mice were infected with metacyclic L. major promastigotes purified from day 4 SUB +/- and wildtype 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 in the left hind footpad with 5x10^6 metacyclic promastigotes in 50 µL 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 wildtype and SUB knockout L. donovani. Approximately 10^9 cells were pelleted, washed 3x 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% NP40, 1 mM DTT, and 1x Halt EDTA-free protease inhibitor cocktail (Pierce, Rockford, IL). 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 MWCO dialysis membranes.
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 IPG IEF 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 IEF strips were run in the second dimension on 17 x 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 (GE Healthcare). 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 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 autoinjector (Sunnyvale, CA) and Eksigent software to run at a 5-50% acetonitrile gradient in 0.1% formic acid with a 350 nL/min flow rate. Data were analyzed in Analyst 2.0 software (Applied Biosystems) with the Mascot script 1.6b20 (Matrix Science, London, UK). Analyst processing options for peak finding in spectrum were 0.5% default threshold, 400 Gaussian filter, and a Gaussian filter limit of 10; for TOF auto-centroiding: 20 ppm merge distance, 10 ppm minimum width, 50% percentage height, and 100 ppm maximum width. Default parameters were used except that “no deisotoping” was selected and precursor mass tolerance for grouping was set to 0.2. Database searches were performed using ProteinProspector v. 5.3.0 (http://prospector.ucsf.edu) using the Batch-Tag and Search Compare modules (30). Searches were performed on the SwissProt databank (December 16, 2008) to evaluate sample purity followed by searching TriTrypDB v. 1.0 beta (http://www.tritrypdb.org, downloaded January, 2009).

Hydroperoxide Sensitivity Assay—These assays were performed as was previously described (31). Stationary phase Leishmania promastigotes were split into 2 mL M199 at 2x10^6 per mL in the presence of different concentrations tert-butylhydroperoxide (t-BOOH, Sigma). Culture densities were determined after 5 to 6 days by Coulter Counter. Relative density was calculated by normalizing to untreated controls.

RESULTS

L. donovani SUB uses a non-canonical catalytic triad. The gene encoding Leishmania 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 weight 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 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 pare change. L. infantum SUB also has Glu in place of the Asp, indicating that this adaptation may be specific to parasites within 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.

In order to determine the subfamily of Leishmania subtilisin, the catalytic core sequence was compared to 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 (SREBPs), 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 to the X-33 background strain. Slight activity against the RR substrate was only detected in one *L. major* clone. In this clone the RVRR *V*ₘₐₓ was over 6 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 endogenous 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 *Kₐₚ* and *Kₘ* 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, while *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-fold and 62-fold lower for RVRR and VFRSLK, respectively) when compared to *L. major* SUB. This difference could potentially be due to *L. donovani*’s non-canonical catalytic triad.

Subtilisin (SUB) +/- parasites have defects in promastigote to amastigote differentiation. The published *L. major* and *L. infantum* genomes (GeneDB) indicate that *Leishmania* *spp.* 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.

Wildtype, SUB +/−, and +/- *L. donovani* promastigotes were cultured at 27°C in M199. These parasites replicated at a rate comparable to wildtype 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. Wildtype 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. Wildtype and SUB +/- axenic amastigotes from the low dilution differentiation were processed for transmission electron microscopy. Wildtype axenic amastigotes had typical amastigote morphology—a rounded cell body measuring approximately 3 µm in diameter, no external flagellum, and a condensed electron-dense kinetoplast (Fig. 4A). SUB +/- axenic amastigotes exhibited many abnormalities (Fig. 4B-D). While 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 wildtype 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 apicomplexan
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 wildtype and SUB -/- parasites. Gels were run in triplicate (Fig. 5). On average, gels had approximately 325 well-defined spots, a value typical for 2-D gels of this size. These protein species represent up to 4% of the predicted 8,195 protein-coding genes from the *Leishmania donovani* complex (34). Spots that differed in intensity between the wildtype and SUB -/- 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 *Leishmania donovani* complex, this family of peroxidases is comprised of three cytoplasmic tryparedoxin peroxidases encoded in a multi-gene array, TXNPx1-3 (TRYP1-3), and a mitochondrial peroxidoxin, Prx (35). Interestingly, *L. major* encodes seven cytoplasmic tryparedoxin peroxidases, 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. Wildtype *L. donovani* had high levels of TXNPx1 and 3 forming a single “wildtype doublet” of spots. The level of TXNPx2 was low and Prx was not detectable. In the SUB -/- parasites, the TXNPx1/3 wildtype doublet decreased (to about 35% of the wildtype 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 wildtype TXNPx1/3. TXNPx2 spot density was not significantly different between the wildtype and SUB -/-. The range of mass spec amino acid coverage was nearly complete for all TXNPx spots (Supplemental Fig. 1), with the notable exception of the wildtype doublet spots 4 and 5 for both WT and SUB -/-.

**SUB knockout 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 wildtype and SUB deficient *Leishmania* to oxidative damage, a hydroperoxide sensitivity assay was performed (Fig. 6). As expected, SUB knockout *Leishmania* was significantly more sensitive to hydroperoxide compared to wildtype. In 100 µM 1-bOOH, wildtype parasites were over 60% viable while SUB knockout 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 wildtype 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 to 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 to 8 weeks delayed compared to the wildtype infections.

For the visceral leishmaniasis infection model, male Golden Syrian hamsters were infected intraperitoneally with wildtype 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 wildtype- and knockout-infected animals. All wildtype-infected hamsters’ spleens contained psammoma body calcifications (Fig. 7B&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. 7D&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 ROSs 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 peroxidases 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 *Leishmania 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 *Leishmania 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*_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 knockout to be generated. Knockout 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* wildtype...
and SUB knockouts. We uncovered five sets of protein spots that differed considerably between wildtype and SUB -/- parasites. All five of these sets were identified as members of the trypanothione peroxidase family, the terminal peroxidases of the trypanothione reductase system. Both the cytoplasmic trypanothione peroxidases (TXNPx1, 2, & 3) and the mitochondrial peroxidoxin (Prx) were identified. Wildtype *Leishmania* had high amounts of TXNPx1 & 3 forming a single doublet of spots. Knocking out SUB resulted in a reduction of this wildtype doublet and the appearance of higher molecular weight mutant doublets. TXNPx2 was unchanged following SUB knockout. Peptide analysis of these spots revealed that all the wildtype TXNPx1 & 3 spots did not contain the C-termini of the proteins, while the mutant spots retained these termini (Supplemental Fig. 1). Subtilisin is therefore putatively responsible for C-terminal processing of the wildtype trypanothione peroxidases 1 & 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.

While the trypanothione peroxidases are primarily cytoplasmic they, 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 TXNPx 1 & 3 C-termini encode a PTS1 (SKL & SKQ, Supplemental Fig. 1); however, TXNPx2 lacks this tripeptide targeting sequence. In addition, TXNPx1 & 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 & 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 trypanothione peroxidase pathway.

Proteomic analysis of SUB -/- parasites also showed increased levels of Prx compared to wildtype. The function of mitochondrial Prx is believed to be complementary to that of cytosolic TXNPx (31), thus the increase in Prx may be a compensatory change due to the reduction of functional TXNPx. This hypothesis is supported by the fact that both alleles of the *SUB* gene could not be deleted in *L. major*. While *L. donovani* complex parasites encode three cytosolic trypanothione peroxidase genes in the TXNPx gene array, *L. major* encodes seven cytosolic trypanothione 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 to wildtype 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 knockout parasites had clearly reduced virulence. Our research has shown that subtilisin promotes survival of *Leishmania* amastigotes by serving as a maturase for the trypanothione reductase system, thus aiding in redox homeostasis and protecting the parasite from oxidative stresses in the host macrophage. Since parasite proteases are known to be viable chemotherapeutic targets (3,4), *Leishmania* subtilisin represents a new potential target for rational drug design.

**REFERENCES**


**FOOTNOTES**

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The abbreviations used are: SUB, subtilisin; AMC, 7-amino-4-methylcoumarin; Site-1, Membrane-bound transcription factor peptidase; SREBP, sterol regulatory element binding protein; TR, trypanothione reductase; TS₂ & TS(SH)₂, oxidized and reduced trypanothione; TXN, tryparedoxin; TXNPx, tryparedoxin peroxidase; Prx, peroxidoxin ; t-bOOH, tert-butylhydroperoxide.

**FIGURE LEGENDS**

Table 1. *L. donovani* SUB was compared to the other known trypanosomatid SUB protein sequences using ClustalW2. The catalytic cores of each protein were determined by aligning them with
the Pfam Subtilase family core (PF00082). Subtilisins have the catalytic triad Asp-His-Ser. *L. donovani* SUB has a non-canonical catalytic triad with a 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. *L. infantum* has a truncated C-terminus. All the trypanosomatid SUB proteins fall between 126-191 kDa.

Table 2. The catalytic cores of *L. donovani* and *L. major* SUBs were recombinantly expressed in *P. pastoris* X-33. Isolated and concentrated proteases were tested for activity against the synthetic fluorogenic substrates RVRR and VFRSLK. \( K_m \) and \( k_{cat} \) parameters were calculated for each substrate using both enzymes. Both enzymes had a higher rate of catalysis for the RVRR substrate over VSRSLK. In addition, *L. major* SUB had a much higher rate of catalysis compared to *L. donovani* SUB for both substrates.

Table 3. Spots that differed in intensity between the wildtype and SUB -/- parasites were selected for peptide sequencing by mass spectrometry. Nine distinct spots were found to be tryparedoxin peroxidases, the terminal peroxidases of the trypanothione reductase system. In the *Leishmania donovani* complex, this family of peroxidases is comprised of three cytoplasmic tryparedoxin peroxidases, TXNPx1-3 (TRYP1-3), and a mitochondrial peroxidoxin.

Fig. 1. The catalytic cores of the *Leishmania* subtilisins were compared to the cores of other Clan SB, family S8 family members, as determined by Pfam. Core sequences were aligned using ClustalW2 (EMBL-EBI). 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.

Fig. 2. Successful deletion of both genomic copies of the SUB gene was determined by Southern blot analysis. Digested genomic DNA from wildtype, single-copy, and double-copy knockouts were analyzed at the SUB locus for the presence of either the wildtype locus or the knockout cassette. The blot was stripped and re-probed for the core of the SUB gene itself to ensure that the gene was not relocated to another site in the genome.

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

Fig. 4. Wildtype axenic amastigotes (A) had normal rounded cell bodies measuring approximately 3 µm in diameter (scale bar = 1 µm) with no external flagellum and a condensed electron-dense kinetoplast. SUB +/- axenic amastigotes (B-D) exhibited many abnormalities. While 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 membrane (B). Additionally, many of these cells were binucleated (C). Unlike the wildtype amastigotes, the SUB +/- cells also had multiple flagellar cross sections including flagella appearing in the cytoplasm (B), outside of their expected location within the flagellar pocket. (N = nucleus, K = kinetoplast, FP = flagellar pocket, F = flagellum)

Fig. 5. Wildtype *L. donovani* had high levels of TXNPx1 and 3 forming a single doublet of spots (WT spots 4 & 5). The level of TXNPx2 was low (spot 3) and Prx was not detectable (spot 1). In the SUB -/- parasites, the wildtype TXNPx1/3 doublet decreased (SUB -/- spots 4 & 5) and two new TXNPx1/3
doublets were present at a higher molecular weight (spots 6 & 7) and at a higher molecular weight with a lower pI (spots 8 & 9). Prx levels (spots 1 & 2) were elevated in these parasites.

Fig. 6. *Leishmania major* promastigote replication was measured in the presence of varying concentrations of t-bOOH. Wildtype (black squares) and SUB deficient (dark gray squares) were grown to stationary phase and counted on a Multisizer 3 COULTER COUNTER. Values are expressed at the percent culture density relative to the untreated controls.

Fig. 7. BALB/c mice (n=5) were infected subcutaneously in the left hind footpads with wildtype (solid circle) or SUB +/- (empty circle) parasites. Footpad swelling was measured weekly and the footpad thickness was plotted over time. Footpad size was significantly larger (p=0.001) in WT infections after 40 days PI. Error bars indicate the S.D. between the 5 mice in each group. The mice were sacrificed at 180 days post infection. No swelling was observed in the right hind (uninfected) footpads. Male Golden Syrian hamsters (n=3) were infected intraperitoneally with wildtype or SUB -/- *L. donovani*. At 200 days post-infection, the hamsters were sacrificed and their spleens were sectioned, H & E stained, and histologically examined. All wildtype-infected hamsters’ spleens (B & C) contained psammoma body calcifications (arrows). No psammoma bodies were observed in the spleens of hamsters infected with SUB deficient *Leishmania* (D & E).
Table 1

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Table 3

**Two-Dimensional Gel Analysis of WT and SUB -/- Leishmania**

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Leishmania subtilisin is a maturase for the trypanothione reductase system and contributes to disease pathology
Ryan K. Swenerton, Giselle M. Knudsen, Mohammed Sajid, Ben L. Kelly and James H. McKerrow

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