Cloning and Characterization of a Leucyl Aminopeptidase from Three Pathogenic Leishmania Species*

Rory E. Morty‡ and Jennifer Morehead

From the Section of Microbial Pathogenesis, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut 06536

Aminopeptidases are emerging as exciting novel drug targets and vaccine candidates in parasitic infections. In this study, we describe for the first time an aminopeptidase from three highly pathogenic Leishmania species. Intronless genes encoding a leucyl aminopeptidase (lap) were cloned from Leishmania amazonensis, Leishmania donovani, and Leishmania major, which encoded 60-kDa proteins that displayed homology to leucyl aminopeptidases from Gram-negative bacteria, plants, and mammals. The lap genes were present as a single copy in each genome, and lap mRNA was detected by reverse transcription-PCR in all life-cycle stages of L. amazonensis. Lap assembled into catalytically competent 360-kDa hexamers and demonstrated potent amidolytic activity against synthetic aminopeptidase substrates containing leucine, methionine, and cysteine residues, representing the most restricted substrate specificity of any leucyl aminopeptidase described to date. Optimal activity was observed against L-Leucyl-7-amido-4-methylcoumarin ($K_{i}$ values of 3 and 44 nM, respectively). Actininonin was a tight binding competitive inhibitor ($K_i \approx 1$ nM), whereas arphamenine A ($K_i \approx 70$ μM) and l-leucinol ($K_i \approx 100$ μM) were non-tight binding competitive inhibitors. Lap was not secreted by Leishmania in vitro and was localized to the parasite cytosol.

Protozoan parasites of the genus Leishmania cause visceral, cutaneous, and mucosal diseases in humans, collectively referred to as leishmaniasis. Leishmaniasis is prevalent in 88 countries, with 12 million people currently infected, a further 350 million at risk, and 2 million new cases reported per year. No vaccine exists, and therapies are inadequate (1). There is a pressing need for the identification of novel leishmanial virulence factors, drug targets, and vaccines to improve our understanding, prevention, and treatment of leishmaniasis.

The peptidases of parasitic protozoans (for review, see Ref. 2) are becoming increasingly important as virulence factors, drug targets, and vaccine candidates in parasitic infections. However, only three peptidases from Leishmania, lysosomal cysteine peptidases (3), a cell-surface metallopeptidase (4), and a cytosolic serine oligopeptidase (5) have received attention.

Aminopeptidases, which catalyze the removal of N-terminal amino acid residues from peptides and proteins (6), are emerging as novel and exciting anti-parasite targets. Vaccination of sheep with Fasciola leucyl aminopeptidase (Lap)3 elicited high anti-Lap titers that conferred protection against fascioliasis and fascioliasis-related liver damage (7). Synthetic broad-spectrum aminopeptidase inhibitors (8) and 1,2-aminoalcohol inhibitors of Lap (9) have shown promise as drugs against malaria, and the aminopeptidase inhibitor arphamenine A has activity against Trypanosoma brucei, a kinetoplastid protozoan that is a close relative of Leishmania (10).

Despite these observations, no aminopeptidase has ever been studied in any kinetoplastid protozoan parasite, including Leishmania. To address this, we report here the cloning, genetic analysis, and biochemical characterization of a novel aminopeptidase from three highly pathogenic Leishmania species. This aminopeptidase, which is responsible for the bulk of soluble leucyl aminopeptidase activity in Leishmania extracts, appears to represent the most phylogenetically distant branch of the M17 family of leucyl aminopeptidases (EC 3.4.11.1). Leishmania Laps exhibit the most restricted substrate specificity of any Lap described to date, confining activity almost exclusively to three amino acid residues, leucine, cysteine, and methionine. In addition to providing important new data on the enzymology of this interesting family of peptidases, the data we present here considerably expand our knowledge of the peptidolytic capacity of Leishmania, which thus far has been limited to three peptidases, by introducing a newly identified peptidase from these parasites. These data will also serve as a solid foundation for subsequent studies on the potential of this aminopeptidase as a drug target and vaccine candidate in Leishmania infections.

MATERIALS AND METHODS

Parasite and Bacterial Strains—Virulent Leishmania major V1 (MHOM/IL/80/Freidlin) and Leishmania amazonensis PH8 (IFLA/BR/100/H11015, IFLA/BR/100/H9262, and IFLA/BR/100/H11547) were non-tight binding competitive inhibitors. Lap was not secreted by Leishmania in vitro and was localized to the parasite cytosol.

Protozoan parasites of the genus Leishmania cause visceral, cutaneous, and mucosal diseases in humans, collectively referred to as leishmaniasis. Leishmaniasis is prevalent in 88 countries, with 12 million people currently infected, a further 350 million at risk, and 2 million new cases reported per year. No vaccine exists, and therapies are inadequate (1). There is a pressing need for the identification of novel leishmanial virulence factors, drug targets, and vaccines to improve our understanding, prevention, and treatment of leishmaniasis.

The peptidases of parasitic protozoans (for review, see Ref. 2) are becoming increasingly important as virulence factors, drug targets, and vaccine candidates in parasitic infections. However, only three peptidases from Leishmania, lysosomal cysteine peptidases (3), a cell-surface metallopeptidase (4), and a cytosolic serine oligopeptidase (5) have received attention.

Aminopeptidases, which catalyze the removal of N-terminal amino acid residues from peptides and proteins (6), are emerging as novel and exciting anti-parasite targets. Vaccination of sheep with Fasciola leucyl aminopeptidase (Lap)3 elicited high anti-Lap titers that conferred protection against fascioliasis and fascioliasis-related liver damage (7). Synthetic broad-spectrum aminopeptidase inhibitors (8) and 1,2-aminoalcohol inhibitors of Lap (9) have shown promise as drugs against malaria, and the aminopeptidase inhibitor arphamenine A has activity against Trypanosoma brucei, a kinetoplastid protozoan that is a close relative of Leishmania (10).

Despite these observations, no aminopeptidase has ever been studied in any kinetoplastid protozoan parasite, including Leishmania. To address this, we report here the cloning, genetic analysis, and biochemical characterization of a novel aminopeptidase from three highly pathogenic Leishmania species. This aminopeptidase, which is responsible for the bulk of soluble leucyl aminopeptidase activity in Leishmania extracts, appears to represent the most phylogenetically distant branch of the M17 family of leucyl aminopeptidases (EC 3.4.11.1). Leishmania Laps exhibit the most restricted substrate specificity of any Lap described to date, confining activity almost exclusively to three amino acid residues, leucine, cysteine, and methionine. In addition to providing important new data on the enzymology of this interesting family of peptidases, the data we present here considerably expand our knowledge of the peptidolytic capacity of Leishmania, which thus far has been limited to three peptidases, by introducing a newly identified peptidase from these parasites. These data will also serve as a solid foundation for subsequent studies on the potential of this aminopeptidase as a drug target and vaccine candidate in Leishmania infections.

MATERIALS AND METHODS

Parasite and Bacterial Strains—Virulent Leishmania major V1 (MHOM/IL/80/Freidlin) and Leishmania amazonensis PH8 (IFLA/BR/100/H11015, IFLA/BR/100/H9262, and IFLA/BR/100/H11547) were non-tight binding competitive inhibitors. Lap was not secreted by Leishmania in vitro and was localized to the parasite cytosol.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF424691, AF424692, and AF424693.

‡ To whom correspondence should be addressed: Zentrum für Innere Medizin, Medizinische Klinik und Poliklinik II, Justus Liebig University, Arne West 123 (Raum 6–11), D-35392 Giessen, Germany. Tel.: 49-641-994-2303; Fax: 49-641-994-2309; E-mail: rory.motty@innere.med.uni-giessen.de.

1 The abbreviations used are: Lap, leucyl aminopeptidase; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; AMC, 7-amino-4-methylcoumarin; Mes, 4-morpholineethanesulfonic acid; AMT, acetate-Mes-Tris; Cbz, N‘-carbobenzyloxy; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; ORF, open reading frame; RT, reverse transcription; Tricine, N‘,2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
A metal-binding (M) and active-site (A) residues are boxed. Sequences were obtained from the GenBank/EBI data base under the following accession numbers: X63444 (Arabidopsis thaliana), U50151 (Lyseopteris esculcentum), S85367 (Bos taurus), AF061738 (Homo sapiens), AE000496 (Escherichia coli), U32584 (Haemophilus influenzae), V. cholerae, U32584 (Rickettsia prowazekii), U32584 (Escherichia coli), accession numbers: X63444 (Arabidopsis thaliana), AE000496 (Escherichia coli), U32584 (Haemophilus influenzae), V. cholerae, U32584 (Rickettsia prowazekii), U32584 (Escherichia coli), accession numbers: X63444 (Arabidopsis thaliana), AE000496 (Escherichia coli), U32584 (Haemophilus influenzae), V. cholerae, U32584 (Rickettsia prowazekii), U32584 (Escherichia coli), accession numbers: X63444 (Arabidopsis thaliana), AE000496 (Escherichia coli), U32584 (Haemophilus influenzae), V. cholerae, U32584 (Rickettsia prowazekii), U32584 (Escherichia coli), accession numbers: X63444 (Arabidopsis thaliana), AE000496 (Escherichia coli), U32584 (Haemophilus influenzae), V. cholerae, U32584 (Rickettsia prowazekii), U32584 (Escherichia coli). Promastigotes were grown in M199 medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum, 0.1% (m/v) hemin, 10 mM succinate, 10 mM adenine, 0.1% (m/v) hemin, 20% (v/v) fetal bovine serum, pH 5.4 at 31 °C. Amastigotes were grown in M199 medium supplemented with 0.25% (m/v) glucose, 0.5% (m/v) trypticase, 40 mM succinate, 10 mM adenine, pH 7.5, at 27 °C.

**Cloning, Sequencing, and Analysis of the lap Open Reading Frame**—Using the published sequence of the L. major Freidlin chromosome (GenBank/EKI accession number AL117384), forward (5'-GCT TCG GAT TCC CAG CGG-3') and reverse (5'-GCT TCG GAT TCC CAG CGG-3') primers were synthesized complementary to non-coding sequences flanking a L. major ORF designated " probable cytosolic aminopeptidase." PCR amplification from Leishmania genomic DNA was carried out by an initial denaturation (95 °C, 1 min), annealing (50 °C, 1 min), and primer extension (55 °C, 2 min) followed by a final extension step (72 °C, 10 min). PCR products were cloned into pCR2.1 (Invitrogen) as described above, with forward primer (5'-ATG CTC GCA GCA GCA CAG-3') and reverse (5'-GAT TCG GAT TCC CAG CGG-3') primers. Plasmids were sequenced using M13 forward and reverse (5'-CCT GGC TGC CGA GTC CGG-3') primers and log-(density 1, gap penalty 3, gap window 5, the scale at the bottom measures distance between sequences. The units indicate the number of substitution events (%).

**Stage-specific lap expression was determined by reverse transcription (RT)-PCR.** Total RNA from 3 x 10^6 L. amazonensis amastigotes and log-phase (- 1 x 10^6 cells/ml) and stationary-phase (density = 5 x 10^6 cells/ml) promastigotes was isolated with TRIzol® reagent (Invitrogen) per the manufacturer’s instructions. Reverse transcription (RT) was undertaken with Thermoscript™ reverse transcriptase (Invitrogen). RT was undertaken with Thermoscript™ reverse transcriptase (Invitrogen) per the manufacturer's instructions, primed with 2.5 μl oligo(dT)20 containing 500 ng of mRNA template. PCR reactions, carried out as described above, contained forward (5'-ATG TCT GGG CCC TGT GTG CTG CCG CGG-3') and reverse (5'-TAA AGG CTT GTG GGC CAC GGA GAA-3') primers and 2 μl of cDNA generated in the RT reaction (or 2 μl of an RT reaction to which no reverse transcriptase had been added as negative control).

**Hyperexpression of Recombinant Lap**—The lap genes were amplified by PCR from Leishmania genomic DNA with PfuTurbo polymerase (Stratagene) as described above, with forward primer (5'-GTC ACC CTT
Bacteria were harvested by centrifugation (7,000 g, 11,000 S-300 HR gel filtration column (16 ml)) polyhistidine-tagged fusion proteins were expressed by induction of addition of L-Leu-AMC. The pH stability was determined by incubating massie Blue-stained 12% Tris-Tricine SDS-PAGE gel (11). manufacturer’s specifications. In some instances, the polyhistidine tag recombinant Lap was determined using molecular masses of 62.424, 83.689, and 126.947 kDa by Southern blot. Southern blot of Lap copy number by Southern blot.

mM acetic acid, 50 mM Mes, 100 mM Tris-HCl (12), pH 4–11 before the addition of L-Leu-AMC. The pH stability was determined by incubating Lap (200 nM, 37 °C) in these AMT buffers (1 h) before the addition of L-Leu-AMC. The pH stability was determined by incubating Lap (200 nM, 37 °C) in these AMT buffers (1 h) before assaying residual activity. The Lap pH profile was determined by incubating Lap (200 nM, 37 °C, 5 min) in constant ionic strength acetate/Mes/Tris (AMT) buffers (50 mM acetate, 50 mM Mes, 100 mM Tris-HCl (12), pH 4–11) before the addition of L-Leu-AMC. The pH stability was determined by incubating Lap (200 nM, 37 °C) in these AMT buffers (1 h) before assaying residual activity.

Enzymatic Analysis of Recombinant Enzymes—The molecular mass of active Lap was determined with Lap (2 μg), preincubated in 50 mM Tris-HCl, 1 mM MnCl₂, pH 8 (12 h, 4 °C), and loaded onto a Sephacryl S-300 high-performance gel filtration column (16 x 800 mm, 0.8 ml min⁻¹), preequilibrated in 50 mM Tris-HCl, 1 mM MnCl₂, pH 8. Fractions (0.5 ml) were assayed for L-Leu-AMC amidolytic activity (described below). The column was calibrated with thyroglobulin (669 kDa), ferritin (443 kDa), IgG (160 kDa), bovine serum albumin (68 kDa), and cytochrome c (12.4 kDa) standards.

Substrate specificity of recombinant Lap was determined using fluorescently labeled substrates by preincubation of Lap (1 nm, 37 °C, 1 min) in 50 mM Tris-HCl, 0.5 mM MnCl₂, pH 8, followed by the addition of substrate. Initial steady-state velocity was determined by continuous assay for a steady-state velocity was determined by continuous assay for a second (50 mM Tris-HCl, 1 mM MnCl₂, pH 8, followed by the addition of substrate. Initial steady-state velocity was determined by continuous assay for a

FIG. 2. Expression of Lap mRNA and protein in Leishmania. A, RT-PCR from L. amazonensis amastigotes (Am) and log-phase (Pr (log.)) and stationary-phase (Pr (stat.)) promastigote mRNA. An RT reaction to which no reverse transcriptase had been added (–RT) and plasmid pNA159 (containing the full-length L. amazonensis lap gene) served as positive and negative control templates, respectively, in the PCR reaction. B, soluble Leishmania extracts were resolved by Tris-Tricine SDS-PAGE under reducing conditions, blotted, probed with polyclonal rabbit anti-porcine cytosol leucyl aminopeptidase serum, and developed using enhanced chemiluminescence as described under “Materials and Methods.” Recombinant L. amazonensis Lap from which the polyhistidine affinity tag had been removed (75 ng) served as a positive control.

activity of a 5-μl aliquot in pH 8 AMT buffer. Ionic strength dependence was investigated by assaying Lap activity in pH 8 AMT buffer over the ionic strength range 0.01–0.5.

Metal ion dependence of Lap was investigated by assaying Lap activity after preincubation of Lap (1 nm, 37 °C, 30 min) in 50 mM Tris-HCl, pH 8, containing a metal chloride (0.01–10.0 mM). Metal cations were also tested for their ability to re-activate metal-depleted Lap (apo-Lap) by incubating L. amazonensis Lap (10 mg) with 1,10-phanthanthrolene (10 mM) in 50 mM Tris-HCl, pH 8 for 16 h. Pheanthanthrolene was removed by dialysis against 50 mM Tris-HCl, pH 8 (4 x 4 h, 4 °C). Apo-Lap was concentrated in Centricon YM-10 spin concentrators (Millipore), and aliquots (containing 125 ng protein) were incubated with metal chlorides (1–5 mM, 30 min) before assaying activity against L-Leu-AMC in 50 mM Tris-HCl, pH 8 at 37 °C.

Inhibition of Lap by peptidase inhibitors was determined by prein-
cubation of Lap (2 nm, 37 °C) with inhibitors in 50 mM Tris-HCl, 0.5 mM MnCl₂, pH 8 for 5 min before the addition of substrate. In the presence of metal ion chelators, MnCl₂ was omitted from the assay buffer. The Km for non-tight binding reversible competitive inhibitors was determined as described in Salvesen and Nagase (13). The enzyme-catalyzed hydrolysis of the Lap-AMC assay was monitored continuously to establish an uninhibited rate of substrate hydrolysis (vₒ). Slow binding inhibition was evaluated as described in Salvesen and Nagase (13). The enzyme-catalyzed hydrolysis of L-Leu-AMC was monitored at 37 °C over 800 s. Progress curves that are obtained using the Ed/M/K ratio ratio (S) were fitted to this equation with SigmaPlot® (Jandel Scientific) to obtain the apparent inhibition constant in the presence of substrate (Kᵦ/Kᵦ/Kᵦ). The overall inhibition constant, Kᵦ, was obtained from the equation v = vₒ/(1 + [I]/Kᵦ), where [I] denotes inhibitor concentration. For tight binding inhibitors (where the (vₒ/Ei) ratio > 0.01 (14)), the method of Williams and Morrison (15) was employed. Briefly, Lap (5 nm) was incubated in 50 mM Tris-HCl, 0.5 mM MnCl₂, pH 8 at 37 °C alone or with inhibitor (at a concentration of 0.5–2.5 × (Ei)) before assay residual activity against Lap-AMC (20–50 μM). A plot of velocity in the presence of inhibitor (vᵦ) at inhibitor concentration [I] was fitted to the general integrated equation P = vₒ [E]/(Kᵦ + [I]) × (1 + [I]/Kᵦ) (22) using non-linear regression with SigmaPlot® (Jandel Scientific) to obtain the apparent inhibition constant in the presence of substrate (15). In both tight and non-tight binding cases, the true Km was calculated from Kᵦ = Kᵦ/Kᵦ × [S]/Kᵦ, where [S] denotes substrate concentration. Slow binding inhibition was evaluated as described in Bienvenue et al. (16). L. amazonensis Lap (125 ng of protein) was prewarmed to 37 °C in 50 mM Tris-HCl, 0.5 mM MnCl₂, pH 8, added to a solution of apstatin (0–1.25 μM) or bestatin (0–50 μM) in 50 mM Tris-HCl, 0.5 mM MnCl₂, 0.2 μM-Lap-AMC, pH 8, and the reaction was monitored at 37 °C over 800 s. Progress curves that are obtained using this procedure can be described by the general integrated equation P = vₒ × (Kᵦ × [I]/(Kᵦ + [I]) × (1 + [I]/Kᵦ)), where Kᵦ represents the amount of product at time = 1, vₒ represents the initial velocity, the primary steady-state constant, and k represents the apparent first-order rate constant for the establishment of the equilibrium between the initial (Ei) and mature (Eᵦ) enzyme-inhibitor complexes (17). Progress-curve data were fitted to this equation with SigmaPlot® (Jandel Scientific), permitting the determination of vₒ and k. The overall inhibition constant, Kᵦ, is described by the equation v = vₒ/(1 + [I]/Kᵦ) + [S], where v is the maximum velocity, Kᵦ and [S] are the Michaelis constant and the concentration of the substrate employed, and [I] is the inhibitor concentration (17). The Kᵦ was, thus, obtained from a secondary plot of vₒ versus [I].

Preparation of Leishmania Extracts and Culture Supernatants—Leishmania (1×10⁶ cells) were harvested by centrifugation (1,000 × g, 10 min, 4 °C), resuspended in 100 mM Tris-HCl, 1 mM AEBSF, 10 μM E-64, and 10 μM peptatin A, pH 8 (2 ml, 4 °C), sonicated on ice with a Branson Sonifier 250 sonicator (duty cycle = 20%, output = 2; 2 × 30 s), and centrifuged (15,000 × g, 30 min, 4 °C) to yield a crude soluble extract. Leishmania were evaluated for Lap secretion as described in Morty et al. (18). Mid-log-phase promastigotes L. amazonensis (5 × 10⁵ cells/ml) were maintained in M199 medium described above. At 30 min intervals, aliquots (1 ml) were removed and centrifuged (1,500 × g, 2 min, 25 °C). The Triton X-100 solubilized pellets and cell-free supernatants were tested for Lap activity against Lap-AMC.

Antibodies, Western Blotting, and Immunofluorescence—Polyclonal rabbit anti-porcine Lap (19) was from Pio Colepicolo (Universidade de São Paulo, Brazil). Polyclonal rabbit anti-Vibrio cholerae Lap (20) was from Claudia Toma (University of the Ryukus, Japan). Antibodies were evaluated for Lap-neutralizing activity by preincubating Lap (2 nm) in 50 mM Tris-HCl, 0.5 mM MnCl₂ containing 100 μg/ml pre-immune or anti-Lap rabbit IgG (5 min, 37 °C) before the addition of Lap-AMC.

For Western blots, soluble Leishmania extracts (15 μg) or purified Lap (25 μg) were resolved on 12% reducing Tris-Tricine SDS-PAGE gels (11), transferred to Immobilon™-P (Millipore) membranes, blocked in 5% (v/v) nonfat milk in 0.1% (v/v) Tween 20 in 20 μM Tris-HCl, 150 mM NaCl, pH 7.4, and incubated with rabbit anti-porcine Lap serum (1: 5000, 2 h). Blots were washed (3 × 5 min), incubated with peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry; 1:10,000, 30 min), washed again (1 × 30 min), and developed using the ECL™ system (Amersham Bioscience).

For immunofluorescence, log-phase L. amazonensis were air-dried on poly-l-lysine-coated glass coverslips, fixed with 3.8% (v/v) paraformaldehyde in phosphate-buffered saline (PBS) (1 h, RT), permeabilized with 0.2% (v/v) Triton X-100 in 2% (v/v) bovine serum albumin in PBS (5 min), incubated with pre-immune or anti-porcine Lap rabbit sera (1:1000, 1 h), washed with 2% (v/v) bovine serum albumin in PBS (3 × 5 min), and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Molecular Probes; 1:2000, 1 h). Coverslips were washed with 2% (v/v) bovine serum albumin in phosphate-buffered saline (1 × 30 min), mounted, and examined with an Axiovert 135 fluorescent microscope (Zeiss) at 100× magnification.

Inductively Coupled Plasma-atomic Emission Spectroscopy—Purified Leishmania Lap was dissolved in distilled, deionized H₂O (10 mg/ml) and submitted to Huffman Laboratories, Inc. (Golden, CO) for determination of manganese and zinc by inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

RESULTS

Cloning, Sequencing, and Analysis of Leishmania lap Genes—The lap genes isolated from Leishmania consisted of an ORF of 1688 base pairs, encoding polypeptides of 565 amino acids with predicted molecular masses of 60 129.00, 60 041.30, and 60 274.10 Da for L. amazonensis, L. donovani, and L. major, respectively, with predicted pl values of 9.1–9.2. Nucleotide sequences have been deposited in the GenBank™/EBI Data Base under accession numbers AF424691 (L. major Lap), AF424692 (L. amazonensis Lap) and AF424693 (L. donovani Lap). Their encoded polypeptide chains contained a M17 Lap family signature sequence (21), and sequence alignments with other members of the M17 Laps indicate conservation of predicted catalytically important residues (Fig. 1A). The encoded Laps from Leishmania Lap shared significant (92–96%) identity to each other (Fig. 1B); however, a phylogenetic comparison of these three full-length Leishmania Lap sequences with eight other M17 Laps indicated that the Leishmania enzymes were the most evolutionarily divergent members of this family, including homologues from prokaryotic and eukaryotic organisms (Fig. 1B). The E. coli and human Laps exhibit 30% identity, whereas L. amazonensis Lap exhibits 26% and 24% identity to E. coli and human Laps, respectively.

Leishmania genomic DNA digested with endonucleases that do not cut within the lap ORF (Hind III, BglIII, and SmaI)
yielded single bands on a Southern blot when probed with full-length lap, whereas endonucleases that cut once within the lap ORF (AvaI, PstI, XhoI, AccI) yielded two bands (Fig. 2). Thus, lap genes are present as a single copy per Leishmania genome.

Messenger RNA for Leishmania lap was detected by RT-PCR in amastigote and log- and stationary-phase promastigotes (Fig. 3A). Similarly, Lap protein was detected by Western blot in amastigote and log- and stationary-phase promastigote L. amazonensis using antisera raised against porcine Lap (Fig. 3B). These data indicate that lap genes are expressed at the translational and transcriptional level in all life-cycle stages of Leishmania.

Enzymatic Properties of Recombinant Leishmania Lap—The full-length Leishmania lap genes were expressed in E. coli as catalytically active (Table I), polyhistidine-tagged recombinant enzymes with a yield of 7–10 mg/liter of bacterial culture. The observed molecular mass of the recombinant Laps (~65 kDa; Fig. 4) compared well with the calculated mass of the affinity-tagged translational products of the lap genes (~62.5 kDa).

The Lap activity eluted in a single, well resolved peak from a Sephacryl S-300 HR column at a molecular mass corresponding to 376 kDa (Fig. 5A). No activity was eluted at an elution volume corresponding to 62 kDa (the molecular mass of the translation products of Leishmania lap genes), although Lap monomers were detected in this fraction by Western blot (data not shown). These data may indicate that the translation product associates into catalytically competent homohexamers and suggests that Lap monomers may not be catalytically active. A similar observation has been reported for plant Laps (22).

Optimal amidolytic activity was observed against L-Leu-AMC ($k_{cat}/K_m = 60 \text{ s}^{-1}\text{mM}^{-1}$) (Table I). Substrates L-Cys-AMC and l-Met-AMC were also efficiently hydrolyzed, although at a lower rate, with $k_{cat}$ values generally 20% of those seen for L-Leu-AMC. Substrates l-Ala-AMC and l-Ile-AMC were hydrolyzed considerably less efficiently ($k_{cat}/K_m \approx 3 \text{ s}^{-1}\text{mM}^{-1}$), and very poor activity was detected against l-Trp-AMC ($k_{cat}/K_m \approx 1 \text{ s}^{-1}\text{mM}^{-1}$). No activity was observed against any other l-amino acid amide, Cbz-l-Leu-$\beta$-naphthylamine, or Cbz-Gly-Gly-Leu-$\beta$-naphthylamine. Removal of the N-terminal polyhistidine tag from the catalytically active polyhistidine tag did not alter the kinetic profile (Table I), illustrating that the tag did not interfere with the catalytic capacity.

Amidolytic activity against l-Leu-AMC was optimal at pH 8.5, with strong activity still detectable up to pH 10 (Fig. 5B). Activity rapidly declined under mildly acidic conditions (pH 6), although Lap was stable over a broad pH range (pH 4–11) (Fig. 5C). Amidolytic activity was also influenced by ionic strength, with increasing ionic strength accompanied by reduced catalytic capacity (Fig. 5C), possibly by interfering with ionic enzyme-substrate interactions or disturbing ionic interactions that hold the hexameric complex together.

The Laps exhibited enhanced activity in the presence of several metal ions with the order of preference manganese $>$ magnesium $>$ cobalt $>$ nickel (Table II). Calcium, cupric, and...
ferrous ions were toxic, abrogating activity at 1 mM, whereas the zinc ion had little effect at 1 mM and was potentially inhibitory at 10 mM. Titration of Mn(II) illustrated that the manganese anion optimally enhanced Lap activity at 0.5 mM (hence, this concentration was routinely used in Lap assays) and became inhibitory above 10 mM (Fig. 5D). After incubation with the metal-ion chelator 1,10-phenanthroline and subsequent removal of the chelator, metal-depleted Lap (apo-Lap) retained negligible activity (<0.01%). Incubation with Mn(II) at 1 and 5 mM (30 min, 37°C) reactivated the apo-Lap by up to 55% (data not shown), with Mg(II) having less of an effect (6–8% reactivation at 5 mM). Both metal ion chelators EDTA (at 1 mM) and 1,10-phenanthroline (at 0.1 mM, but not its non-chelating analogue, 1,7-phenanthroline) were potent (89–98% inhibition) inactivators of Lap, whereas the calcium-selective chelator EGTA was 0.05:1. These data suggest that zinc is the natural metal co-factor of \textit{Leishmania} Lap, with two zinc atoms binding each Lap subunit.

Biphasic reaction progress curves were observed for the hydrolysis of L-Leu-AMC by Lap in the presence of the inhibitors bestatin and apstatin (Fig. 6), suggesting a slow binding inhibition mechanism (23). Both bestatin and apstatin were very potent Lap inhibitors, with overall inhibition constant ($K_i$) values of 3 and 44 nM respectively. In contrast, reaction progress curves for L-leucinol, arphiomenine A, and actinonin remained linear for 600 s, and initial (60 s) reaction velocities indicated an elevated $K_m$ without changing the $V_{max}$ suggesting simple competitive inhibition (data not shown). Actinonin proved to be a tight binding inhibitor ($[E]/K_i > 0.01$) with a $K_i = 0.8–2.9$ nM for the three Lapas (Table III), with weaker inhibition observed for arphiomenine A ($K_i \approx 70$ μM) and L-leucinol ($K_i \approx 100$ μM).

\textbf{Subcellular Localization of Lap—Polycoidal antiserum raised against porcine Lap specifically reacted with \textit{Leishmania} Lap in soluble parasite extracts and with recombinant Lap on Western blots (Fig. 3B). Immunochemical studies with these antibodies on permeabilized, fixed \textit{Leishmania} amastigotes and from log-phase \textit{L. amazonensis} promastigotes yielded a diffuse, uniform fluorescence throughout the parasite cytosol, with no apparent localization of the Lap to the cell membrane or to intracellular structures (Fig. 7). No Lap activity was detected in \textit{Leishmania} cell-free culture supernatants, with 100% of the activity remaining cell-associated (data not shown). Taken together with the very poor activity of Lap at even mildly acid pH (which makes its presence in the lysosome unlikely), these data suggest that Lap is a cytosolic enzyme that is not secreted by \textit{Leishmania}. This localization is consistent with the cytosolic location of plant Laps (24).

**Leucyl Aminopeptidase Activity of Leishmania Soluble Extracts—Anti-porcine Lap IgG potently inhibited recombinant \textit{Leishmania} Lap (125 ng) activity against L-Leu-AMC, L-Cys-AMC, and L-Met-AMC. Up to 96% of Lap activity was inhibited in the presence of 100 μg/ml-1 anti-Lap IgG relative to Lap activity determined in the presence of 100 μg/ml-1 pre-immune IgG (data not shown), providing a tool with which Lap activity in cell extracts could be selectively neutralized.

Aminopeptidase activity against L-Leu-AMC, L-Cys-AMC, and L-Met-AMC was detected in crude soluble extracts from \textit{L. amazonensis} amastigotes and from log-phase \textit{L. amazonensis}, \textit{L. donovani}, and \textit{L. major} promastigotes (Table IV). Anti-

---

**Table II**

\textbf{Influence of divalent metal cations on \textit{Leishmania amazonensis} leucyl aminopeptidase activity}

| Metal     | Concentration | Relative activity $^a$ |
|-----------|---------------|-----------------------|
| None      |               | % (100)               |
| Mn(II)    | 0.1           | 440 ± 14              |
|           | 1.0           | 953 ± 10              |
|           | 10.0          | 97 ± 5                |
| Mg(II)    | 0.1           | 348 ± 13              |
|           | 1.0           | 557 ± 7               |
|           | 10.1          | 614 ± 11              |
| Co(II)    | 1.0           | 381 ± 11              |
|           | 10.0          | 226 ± 16              |
| Cu(II)    | 0.1           | 26 ± 2                |
|           | 1.0           | 7 ± 1                 |
| Ni(II)    | 0.1           | 148 ± 5               |
|           | 1.0           | 433 ± 4               |
|           | 10.0          | 870 ± 5               |
| Ca(II)    | 0.1–1.0       | 0 ± 0                 |
| Fe(II)    | 1.0–10.0      | 0 ± 0                 |
| Zn(II)    | 1.0           | 113 ± 10              |
|           | 10.0          | 3 ± 3                 |

$^a$ Similar data (within 5% agreement) were obtained for the \textit{L. donovani} and \textit{L. major} Laps. Data reflect the mean relative activity ±S.D. ($n = 3$).

---

**Table III**

\textbf{Inhibitor profile of \textit{Leishmania} leucyl aminopeptidase activity}

| Inhibitor $^a$ | Concentration | L. \textit{amazonensis} Lap | L. donovani Lap | L. \textit{major} Lap |
|----------------|---------------|----------------------------|----------------|----------------------|
|                | mM            | %                          | %              | %                    |
| Actinonin      | 0.01          | $K_i = 1.1 \pm 0.05$ nM    | $K_i = 0.8 \pm 0.2$ nM | $K_i = 2.9 \pm 0.9$ nM |
| Apstatin       | 0.1           | $K_i = 6.75 \pm 1.76$      | $K_i = 5.04 \pm 0.12$ | $K_i = 3.39 \pm 0.80$ |
| Arphiomenine A | 0.01          | $K_i = 2.00 \pm 0.77$      | $K_i = 1.19 \pm 0.04$ | $K_i = 1.09 \pm 0.09$ |
| Bestatin       | 0.01          | $K_i = 6.61 \pm 0.51$      | $K_i = 6.24 \pm 0.08$ | $K_i = 5.09 \pm 1.12$ |
| Dithiothreitol | 0.05          | $K_i = 98.31 \pm 192$     | $K_i = 101.34 \pm 2.37$ | $K_i = 97.89 \pm 1.99$ |
| EDTA           | 5.0           | $K_i = 9.44 \pm 1.07$     | $K_i = 8.28 \pm 0.91$ | $K_i = 11.12 \pm 1.16$ |
| EGTA           | 1.0           | $K_i = 43.22 \pm 4.03$    | $K_i = 44.24 \pm 2.76$ | $K_i = 14.38 \pm 2.92$ |
| GSH            | 1.0           | $K_i = 93.10 \pm 1.31$    | $K_i = 88.44 \pm 1.00$ | $K_i = 89.29 \pm 3.67$ |
| L-Leucinol     | 1.0           | $K_i = 109 \pm 14$ μM     | $K_i = 118 \pm 7$ μM | $K_i = 102 \pm 11$ μM |
| 1,7-Phenanthroline | 0.1 | $K_i = 94.44 \pm 4.04$ | $K_i = 93.49 \pm 5.03$ | $K_i = 92.23 \pm 4.45$ |
| 1,10-Phenanthroline | 0.1 | $K_i = 3.32 \pm 0.11$ | $K_i = 2.28 \pm 0.08$ | $K_i = 2.19 \pm 0.81$ |
| Phosphoramidon | 0.01          | $K_i = 92.90 \pm 5.60$    | $K_i = 94.44 \pm 4.41$ | $K_i = 96.65 \pm 2.29$ |
| Puromycin      | 0.1           | $K_i = 82.63 \pm 1.92$    | $K_i = 80.08 \pm 2.24$ | $K_i = 85.55 \pm 1.12$ |
| dL-Thiorphan   | 0.01          | $K_i = 92.92 \pm 3.03$    | $K_i = 93.39 \pm 3.39$ | $K_i = 95.56 \pm 1.02$ |

$^a$ No inhibition was observed with AEBSF (1 mM), antipain (10 μM), 3,4-dichloroisocoumarin (1 mM), E-64 (10 μM), or pepstatin A (10 μM). Data reflect the mean % or $K_i \pm$ S.D. ($n = 3$).

$^b$ The slow binding inhibition by apstatin and bestatin are illustrated in detail in Fig. 6.
porcine Lap IgG was used to determine the extent to which these aminopeptidase activities were attributable to Lap. The l-Leu-AMC activity of Leishmania-soluble extracts was almost completely (90–95%) inhibited by anti-Lap IgG (Table IV), indicating that Lap was responsible for the bulk, if not all, l-Leu-AMC-hydrolyzing activity in Leishmania-soluble extracts. In contrast, L-Cys-AMC and L-Met-AMC hydrolytic activity of soluble Leishmania extracts was inhibited by 14–40 and 14–35%, respectively (Table IV), suggesting that other, unidentified aminopeptidases in addition to Lap are responsible for l-Cys-AMC and l-Met-AMC hydrolytic activity in extracts.

**DISCUSSION**

Peptidases of parasitic protozoa are currently the subject of intense investigation in the hope of identifying novel virulence factors, drug targets, and vaccine candidates. Only two Leishmania peptidases have received considerable attention to date. Lysosomal cysteine peptidases (for review, see Ref. 3) are virulence factors (25) that have been validated as drug targets (26) and vaccine candidates (27). A membrane-bound zinc metallopeptidase (gp63 or leishmanolysin) is also a virulence factor that shows promise as a vaccine (for review, see Ref. 4). Oligopeptidase B (5) and the proteasome (28) have also received attention. Although not evaluated in leishmaniasis, homologues of gp63 (29) and oligopeptidase B (30, 31) are promising drug targets in African trypanosomiasis caused by the closely related kinetoplastid protozoan T. brucei.

Aminopeptidases have not been studied in kinetoplastid protozoans including *Leishmania*. However, Laps have emerged recently as novel and exciting vaccines and drug targets in other parasites. Vaccination with worm-derived Laps protect against challenge infections (7). Synthetic Lap inhibitors show promise as drugs against malaria (8, 9) through inhibition of malarial aminopeptidases involved in terminal stages of hemoglobin degradation (32), which takes place in the parasite cytosol (33). Furthermore, arphamenine A (which we show here inhibits Leishmania Lap) has activity against kinetoplastid protozoans closely related to *Leishmania* (10). These reports prompted us to identify and characterize aminopeptidases from *Leishmania*.

We report here the cloning, genetic analysis, and biochemical characterization of a novel aminopeptidase from three highly pathogenic *Leishmania* species. In contrast to the gp63 (4) and cysteine peptidase (25) genes of *Leishmania*, the intronless lap genes were present as a single copy per genome and were expressed in all *Leishmania* life-cycle stages. Their encoded proteins exhibited 20–30% identity with members of the M17 cytolic Lap family of metallopeptidases. This represents the first identification of a M17 Lap from any protozoan parasite. To date, the only other aminopeptidase conclusively identified in protozoans is an aminopeptidase N homologue, a member of the M1 family of zinc metallopeptidases (EC subclass 3.4.11), from *Plasmodium falciparum* (34). The Leishmania Laps described here lack the canonical HEXXH_\_XE and GAMEN motifs that define the M1 family of zinc-dependent aminopepti-

![Fig. 6. Slow binding inhibition of Leishmania Lap by apstatin and bestatin.](image)

**Fig. 6.** Slow binding inhibition of *Leishmania* Lap by apstatin and bestatin. *L. amazonensis* Lap (125 ng) was added to 2 ml of 50 mM Tris-HCl, 0.5 mM MnCl\_2, 5 mM l-Leu-AMC, pH 8, containing either apstatin (0–125 \mu M) (A) or bestatin (0–50 \mu M) (B), and the reaction was monitored spectrophotometrically at 37 °C over 800 s. FITC, fluorescein isothiocyanate. AFU, arbitrary fluorescence units.

**Fig. 7.** Immunocytochemical localization of *Leishmania amazonensis* Lap. Log-phase promastigote stage *L. amazonensis* cells were air-dried on poly-l-lysine-coated glass coverslips, fixed, permeabilized, and stained with pre-immune or anti-porcine Lap rabbit sera. Samples were examined with an Axiosvert 135 fluorescent microscope at 100× magnification.

![Table IV](image)

**Table IV**

| Species       | Specific activity a | Specific activity a |
|---------------|---------------------|---------------------|
|               | l-Leu-AMC | l-Cys-AMC | l-Met-AMC |
|               | + pre-immune | + anti-Lap | + pre-immune | + anti-Lap | + pre-immune | + anti-Lap |
| *L. amazonensis* (Am) | 267 ± 12 | 12 ± 4 | 294 ± 9 | 180 ± 1 | 1025 ± 68 | 889 ± 118 |
| *L. amazonensis* (Pr) | 386 ± 14 | 38 ± 11 | 511 ± 24 | 368 ± 44 | 4934 ± 129 | 3204 ± 198 |
| *L. donovani* (Pr) | 542 ± 79 | 60 ± 2 | 644 ± 110 | 557 ± 56 | 6121 ± 223 | 5098 ± 288 |
| *L. major* (Pr) | 850 ± 111 | 76 ± 18 | 998 ± 112 | 801 ± 118 | 7089 ± 300 | 6111 ± 298 |

* Specific activity was determined in the presence of either 100 \mu g · ml\^\_1 pre-immune IgG (+ pre-immune) or 100 \mu g · ml\^\_1 anti-Lap IgG (+ anti-Lap). Data reflect the mean specific activity ±S.D. (n = 3).
Leucyl Aminopeptidase from Leishmania

dases (21), indicating that they are not related to the P. falciparum aminopeptidase.

Intriguingly, Leishmania Laps represent the most evolutionarily distant group of the M17 Laps, clustering into their own branch of the M17 Lap phylogenetic tree that diverges even before the mammalian and bacterial Laps diverge from each other. Polyclonal rabbit antisera raised against V. cholerae Lap did not recognize Leishmania Lap on a Western blot (data not shown), whereas antiserum to porcine Lap did, indicating that Leishmania Lap is antigenically more similar to other eukaryotic Laps than it is to bacterial Laps. We see no evidence of post-translational processing of Leishmania Lap, since Laps were detected by Western blot at a molecular mass that corresponds to the size of the translational product of the full coding sequence. These data contrast with reports that plant (24) and mammalian (20) Laps, which are processed from a 55- to a 34-kDa sequence. These data contrast with reports that plant (24) and mammalian (20) Laps, which are processed from a 55- to a 34-kDa sequence.

The sequence of post-translational processing of these Laps is likely that these Laps are responsive to the size of the translational product of the full coding sequence. These data contrast with reports that plant (24) and mammalian (20) Laps, which are processed from a 55- to a 34-kDa sequence. These data contrast with reports that plant (24) and mammalian (20) Laps, which are processed from a 55- to a 34-kDa sequence.

It is likely that the activation of Leishmania Lap we see in this study with Mn(II), Mg(II), Co(II), and Ni(II) results from substitution of the site 1 Zn(II) with these metal ions. Indeed, substitution of the site 1 Zn(II) of porcine kidney Lap with Mn(II), Mg(II), and Co(II) has been shown to activate that enzyme by elevating the \( k_{cat} \) (44). Similarly, the phenanthroline-generated apo-Lap we generated in this study probably retained the site 2 zinc and was reactivated by treatment with Mn(II) and Mg(II) that bound at the site 1 and restored catalytic activity to the enzyme.

Metallopeptidase inhibitors phosphoramidon, thiorphan, or puromycin did not inhibit Leishmania Laps, setting them apart from thermolysin, enkephalinase, and cysteolic alanyl aminopeptidases. Neither was activity influenced by reducing agents. The leucine derivative \( L - \) leucinol inhibited Leishmania Lap \( (K_i \approx 100 \mu M) \), although 5-fold less efficiently than the inhibition reported against bacterial Laps \( (16) \), with arphamine A exhibiting similar potency \( (K_i \approx 70 \mu M) \). The broad-spectrum aminopeptidase inhibitor bestatin and aminopeptidase P inhibitor apstatin were potent, slow competitive inhibitors of Leishmania Laps. This mechanism of inhibition by bestatin has been reported for bacterial and bovine Laps \( (45, 46) \), and the \( K_i^* \) reported here for Leishmania Lap \( (3 \text{ nM}) \) closely approximated that observed for the inhibition of bovine lens Lap by bestatin \( (1.3 \text{ nM}) \) (46). However, although the inhibition of porcine Lap by apstatin has been observed \( (47) \), the mechanism was not investigated, and we report here for the first time a slow, competitive binding mode of action by apstatin. The peptide deformylase inhibitor actinin was a tight \(([E]/K_i \approx 3) \) and fast competitive inhibitor \((K_i \approx 1 \text{ nM}) \) of Leishmania Laps, approximating the potency of actinin inhibition of peptide deformylase \((K_i = 10 \text{ nM}) (48)\).

It thus appears that Leishmania Laps have evolved into their own subgroup of M17 Laps, demonstrating a unique, restricted substrate specificity that includes cysteine residues.

The presence of Lap in all life-cycle stages of Leishmania together with its apparent potential as a drug target in other parasitic infections strongly suggests that it represents a drug target in Leishmania. Because the Laps described here appear to be entirely responsible for leucyl aminopeptidase activity in Leishmania extracts, their selective targeting by inhibitors may interfere with parasite viability. This contrasts with the methionyl and cysteinyl aminopeptidase activities that seem to be undertaken by at least one other aminopeptidase in addition to Lap. Furthermore, the identification in this study of potent Leishmania Lap inhibitors, which are well tolerated in experimental animals (in mice, the oral LD\(_{50}\) of bestatin is \( \approx 4 \text{ g} \cdot \text{kg}^{-1} \) (49), and the intraperitoneal LD\(_{50}\) of arphamine A is \( >500 \text{ mg} \cdot \text{kg}^{-1} \) (50)), will provide a good starting point to explore the anti-Leishmania effects of Lap inhibitors.

Acknowledgments—We thank David Sacks and Albert Descoëttes for strains, Claudia Toma and Pio Colecipolo for generous gifts of anti-Lap antibodies, J. Joseph Blum, Anne LeFurgey, and Peter Ingram for advice on metal determinations, Ron Keil and Huffman Laboratory for performing the ICP-AES, Jorge E. Galan, Jonathan C. Kagan, David R. Liston, and Craig R. Roy for expert advice, John D. Lonsdale-Eccles for critical reading of the manuscript, and Oliver Eickelberg and Norma W. Andrews for outstanding support.

REFERENCES

1. Handeman, E. (2001) *Clin. Microbiol. Rev.* 14, 229–243
2. Rosenthal, P. J. (1999) *Adv. Parasitol.* 43, 105–159
3. Mettram, J. C., Brooks, D. R., and Coombs, G. H. (1998) *Curr. Opin. Microbiol.* 1, 455–460
4. Bouvier, J., Schneider, P., and Etges, R. (1995) *Methods Enzymol.* 248, 64–663
5. Morty, R. E., Lonsdale-Eccles, J. D., Morehead, J., Canel, E. V., Mentele, R., Auerswald, E. A., Coetzer, T. H. T., Andrews, N. W., and Burleigh, B. A. (1999) *J. Biol. Chem.* 274, 26149–26156
6. Taylor, A. (1993) *FASEB J.* 7, 290–298

\( ^2 \) J. J. Blum, personal communication.
Cloning and Characterization of a Leucyl Aminopeptidase from Three Pathogenic Leishmania Species
Rory E. Morty and Jennifer Morehead

J. Biol. Chem. 2002, 277:26057-26065.
doi: 10.1074/jbc.M202779200 originally published online May 2, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M202779200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 13 of which can be accessed free at http://www.jbc.org/content/277/29/26057.full.html#ref-list-1