We report the functional characterization in *Leishmania amazonensis* of a soluble pyrophosphatase (LaVSP1) that localizes in acidicalcosomes, a vesicular acidic compartment. LaVSP1 is preferentially expressed in metacyclic forms. Experiments with dominant negative mutants show the requirement of LaVSP1 functional expression for metacyclogenesis and virulence in mice. Depending on the pH and the cofactors Mg\(^2+\) or Zn\(^{2+}\), both present in acidicalcosomes, LaVSP1 hydrolyzes either inorganic pyrophosphate (\(K_m = 92 \mu M, k_{cat} = 125 s^{-1}\)), triplypolyphosphate (\(K_m = 1153 \mu M, k_{cat} = 131 s^{-1}\)), or polyphosphate of 28 residues (\(K_m = 123 \mu M, k_{cat} = 8 s^{-1}\)). Predicted structural analysis suggests that the structural orientation of the residue Lys\(^78\) in LaVSP1 accounts for the observed increase in \(K_m\) compared with the yeast pyrophosphatase and for the ability of trypanosomatid VSP1 enzymes to hydrolyze polyphosphate. These results make the VSP1 enzyme an attractive drug target against trypanosomatid parasites.

Leishmania is a parasitic protozoan causing an endemic disease affecting 88 countries on four continents (www.who.int/tdr/). Treatment with pentavalent antimony-based drugs has been used for decades. However, toxicity and loss of efficacy with the emergence of resistant strains render necessary the development of alternative therapies.

**EXPERIMENTAL PROCEDURES**

**Parasites**—The *Leishmania* strain used in this study was *L. amazonensis* MHOM/BR/1987/BA125 (BA125) (15). Promastigotes were cultured in closed flasks, under gentle agitation at 24 °C (15) in AM medium, i.e. RPMI 1640 medium plus 25 mM Hepes, pH 7.5, 2 mM glutamine, 2 mg/ml dextrose, 1 mM sodium pyruvate, 1× minimum Eagle’s medium essential and nonessential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin, 8% fetal calf serum (all ingredients were purchased from Eurobio).

**Phylogenetic Analysis**—Amino acid sequences of family I soluble pyrophosphatases (sPPases) were downloaded from GenBank™ data bases or from GeneDB (www.genedb.org/) for trypanosomatid sequences. The pyrophosphatase (PPase) domains were aligned using the multiple alignment options in CLUSTAL X (16) followed by manual adjustments. The alignment of the sPPase domains has been deposited at EMBL with accession number AEN_000878. Phylogenetic trees were generated by the neighbor joining method (17) and maximum parsimony heuristic options as implemented in PAUP version 4.0b10 (Alvitec). Bootstrapping was also carried out using PAUP. The accession numbers of the various sequences used in that study are as follows: 1, BAB22922; 2, AAG36781; 3, AAP24964; 4, XP215416; 5, BAB25754; 6, CAG89198; 7, CAG99536; 8, NP013994; 1, 9, P19117; 10,
Cloning of *L. amazonensis* VSP1 Gene—A PCR amplification on genomic DNA was performed using the degenerated set of oligonucleotides LmCPP02 (5'-ATGTGGTCTGCGCTACATGTA-3') and LmCPP03 (5'-TGYGGTGGRTGRTG-3') designed from a partial sequence of the *Leishmania major* VSP1 (AL354096). The amplified DNA fragment of 300 bp was sequenced and used as a probe to screen a cosmids library using the pCOSTL vector (gift from John Kelly, London School of Hygiene and Tropical Medicine). The complete sequence of LaVSP1 gene was obtained by direct cosmid sequencing.

Expression in Escherichia coli of His-tagged Native LaVSP1 (nLaVSP1) and Mutated LaVSP1 (mLaVSP1) Proteins—The LaVSP1 gene was PCR-amplified with the following primers: Mut1 (5'-CGGCCCTCGAGCACCTCCTCCTTCTTCTTAATCTT-3') and Mut3 (5'-CGGCCCTCGAGCACCTCCTCCTTCTTCTTAATCTT-3') designed from the sequence of the *L. amazonensis* LaVSP1 (BA125) gene. The mutated LaVSP1 gene was obtained by overlapping PCR method using the following set of primers: Mut1/Mut2 (GATTTC-GACGCCCTCTTATTGGGTGCTTGTG-3') and Mut3/Mut4 (5'-CGACAAACGCACACCTAAGAGGCGTCGAAATC-3'). The resulting protein was mutated by a D325E substitution. Both native and mutated proteins were expressed in *E. coli* (BL21) and purified as described below.

Overexpression of mLaVSP1 or nLaVSP1 Proteins in *L. amazonensis*—Expressions of the mLaVSP1 and nLaVSP1 proteins in *L. amazonensis* were accomplished by cloning their respective genes in the pFHTLG (gift from Ken Stuart, Seattle Biomedical Research Institute) (18), between the BglII and AvrII restriction sites and transfection of *E. coli* (BL21) and purified as described above.

**Macrophage Infection by the Metacyclic Form**—3×10^5 cells were lysed with SDS and a pool of proteinase inhibitors, boiled for 5 min, and then submitted to 12.5% SDS-PAGE with standard markers (Promega). The proteins were transferred onto Immobilon P membranes (Amersham Biosciences). Rabbit immune serum (1:5,000 dilution) and goat anti-rabbit IgG/alkaline phosphatase (1:10,000 dilution) were used. Staining was done with ECL Western blotting detection reagents as described by the manufacturer (Amersham Biosciences). The membranes were scanned to produce initial images, and the protein bands were quantified using NIH Image software.

**Metacyclic Promastigote Purification—Leishmania** cells were harvested when in stationary phase and then washed twice in PBS; the cell suspension was adjusted to 5×10^5/ml and incubated 30 min at room temperature with a 1:50 dilution of monoclonal antibody 3A1, kindly provided by Dr. Elvira Maria. Saraiva (24). This antibody is directed against the *L. amazonensis* promastigote forms. The cell suspension was then centrifuged at 250× g for 5 min at 4°C. The supernatant was washed twice in PBS, and unagglutinated, i.e. metacyclic, promastigotes were counted using a Malassez hemacytometer.

**Macrophage Infection by the Metacyclic Form**—Macrophages were obtained from BALB/c mice as described (25) by *in vitro* differentiation of bone marrow precursor cells in 24-well plates containing 12-mm diameter round glass coverslips for light microscopy studies. Precursors were deposited in 60-mm cultures dishes. The cells were cultured in RPMI 1640 medium (Eurobio) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Eurobio), 40 μg/ml gentamicin sulfate, 20% L-929 fibroblast-conditioned medium. After 5 days at 37°C in a 5% CO_2 nonadherent cells were removed, and adherent macrophages were further harvested and incubated in culture medium containing only 2% of...
conditioned medium at the concentration of 10⁶ cell/ml in 24-well plates containing 12-mm-diameter round glass coverslips (18–24 h before the addition of the parasites). Macrophages were infected by stationary phase promastigotes, metacyclic promastigotes, at a ratio of four parasites/macrophage. The parasites and macrophages were brought into contact by incubation at 4 °C for 4 h before being washed with Dulbecco’s PBS (Seromed) to remove free parasites. Macrophages were fixed either immediately or 1–72 h after infection. The cells were fixed with methanol and stained with Giemsa for 15 min at room temperature. The coverslips were finally mounted on microscope slides with Mowiol (Calbiochem). The percentage of infected macrophages (of 600–700 cells) and the mean number of amastigotes/infected macrophage (of 150–200 cells) were determined using an inverted microscope.

**Infectivity Tests—Leishmania** promastigotes were cultured for up to 8 weeks after recovery from footpad lesions and harvested in early stationary phase of growth (5–6 days after last passage). 10⁶ promastigotes were injected into BALB/c mouse footpads, and lesion diameters were determined weekly with a caliper.

**Immunofluorescence—**12-mm-diameter round glass coverslips were treated for 30 min in 10 μg/ml poly-L-lysine (>300 kDa; Sigma) in 24-well tissue culture plates (Costar) (15). *Leishmania* promastigotes (10⁶ cells/well) were added, and the plates were centrifuged at 130 × g for 10 min, fixed with 2% paraformaldehyde for 1 h, and sequentially incubated for 10 min with 50 mM NH₄Cl for 30 min with 10% mouse serum (Jackson laboratories) plus 10% goat serum (Eurobio), 1 h with 1:100 dilution of mouse anti-poly-His抗体 (Qiagen) and 1:1,000 dilution of rabbit anti-VP1 immune serum or 1:1,000 dilution of rabbit anti-exopolyphosphatase immune serum in 0.25% (w/v) gelatin (Sigma) and 1 h with 10 μg/ml fluorescein-conjugated goat anti-rabbit Ig antibody (Jackson Laboratories) and 10 μg/ml rhodamine-conjugated goat anti-mouse Ig antibody (Jackson Laboratories) in 0.25% (w/v) gelatin. The coverslips were mounted on microscope slides with Mowiol (Calbiochem). Cells preparations were examined under a conventional Zeiss Axiosplan 2 fluorescence microscope. The images were acquired with a Princeton Instruments camera and analyzed with Metaview® (Universal Imaging).

**Acidocalcisomes, PolyP Metabolism, Pharmacological Target**

6-Diamino-2-phenylindol (DAPI) Accumulation Observed by Fluorescence Microscopy—12-mm-diameter round glass poly-L-lysine-treated coverslips (>300 kDa; Sigma) were used in 24-well tissue culture plates (Costar). *Leishmania* promastigotes (10⁶ cells/well) were washed twice Dulbecco’s PBS and incubated for 10 min at 30 °C in PBS with 10 μg/ml DAPI. Then cells were added in the 24-well tissue culture plates containing the 12-mm-diameter round glass coverslips treated and centrifuged at 130 × g for 10 min. The coverslips were mounted on microscope slides without Mowiol nor resin. PolyP and DNA were detected using the DAPI filter from Zeiss (set 02) and by exposing for 200 ms for polyP detection. DAPI has a fluorescence emission maximum at 456 nm. PolyP shift DAPI fluorescence to a higher wavelength, with a maximum of 525 nm (26).

**RESULTS AND DISCUSSION**

**LaVSP1 Gene Cloning and Phylogenetic Analysis—**Cloning and sequencing of *LaVSP1* were performed as described under “Experimental Procedures.” A search in the *L. major* genome sequence data base (www.genedb.org) with BLAST algorithms, using the *LaVSP1* protein sequence, indicated the existence of only one copy of *VSP1*, which localized on chromosome 11 (LmjF11.0210). The *LaVSP1* and *LmVSP1* protein sequences only differ in 15 amino acids (ALIGN_000878), and none of them correspond to amino acids involved in the catalytic center (27).

**LaVSP1** gene codes for a protein of 443 amino acids (28). Sequence comparison analysis (www.ncbi.nlm.nih.gov/BLAST) revealed that *LaVSP1* exhibits 64% sequence identity with the *TbVSP1* (ALIGN_000878). In addition to the pyrophosphatase domain, *LaVSP1* has a large N-terminal extension region of 160 amino acids, as previously observed for *TbVSP1* (28). This region contains a calcium-binding type II EF-hand domain (COG5126, FRQ1) between positions 111 and 173.

Phylogenetic protein analysis of *LaVSP1* and the cytosolic soluble pyrophosphatase of *Leishmania* was performed with other family I sPases of various bacteria and eukaryotes (Fig. 1). Both *Leishmania* acidocalcisomal (*LaVSP1*) and cytosolic proteins cluster with the animal/fungus PPase family (27), which also contains the plastid enzymes from plants. The mitochondrial and cytosolic PPases of the animal/fungus family probably duplicated and diverged after the speciation (Fig. 1). In contrast, the cytosolic enzymes from plants do not cluster with the other eukaryotic enzymes, including the plastid PPases, suggesting that each plant isoform has a different origin. The origin of the two trypanosomatid PPases is not clear. Indeed, the trypanosomatid isoforms show a paraphyletic organization, and the acidocalcisomal PPases are closer to the animal/fungus PPases than to the cytosolic PPases. This observation suggests that the trypanosomatid acidocalcisomal and cytosolic PPases diverged before the trypanosomatid and animal/fungus speciation, giving rise to two different deep branches of PPases. Alternatively, they may have a different origin as proposed for the plant isoforms. One may speculate that trypanosomatid cytosolic and/or acidocalcisomal PPases have a plant origin, as previously hypothesized for many other metabolic enzymes from trypanosomatids (29). However, this view is not strongly supported by the topology of the PPase tree because the plastid and VSP1 PPases do not constitute a monophyletic group. In any case, this paraphyletic organization, well supported by the bootstrap values, suggests that trypanosomatid VSP1 and cytosolic PPases may carry different functions.

*TbVSP1* expression, in both the insect and mammalian hosts, its acidocalcisome localization, and its ability to hydrolyze both PP₁ and polyP (30) are the representative cellular and biochemical features of *TbVSP1*. We therefore analyzed *LaVSP1* for its expression throughout the *L. amazonensis* life cycle, its cellular localization, and biochemical specificities.

**Differential Expression and Localization of LaVSP1—**Relative expression level quantification between LACK (*Leishmania* homologue of receptors for activated C kinase) (31) and *LaVSP1* proteins was estimated at different life cycle stages of *L. amazonensis*. Fig. 2 shows that *LaVSP1* expression levels were regulated throughout the parasite life cycle. Namely, expression in metacyclic forms was three times greater than in the exponentially growing promastigotes and six times greater than in the amastigote stage. We determined by indirect immunofluorescence *LaVSP1* localization in genetically modified *L. amazonensis* promastigotes (Ba125/nLaVSP1) expressing a recombinant His₅-tagged nLaVSP1 using an anti-His₅ monoclonal antibody. The results shown in Fig. 3D indicate that nLaVSP1 localized to vesicle-like structures of various sizes mainly distributed around the nucleus. Such cellular organization is typical of acidocalcisome distribution (32). Next, to refine this localization, a rabbit antisera directed against the T. brucei vacular proton translocating pyrophosphatase (*TbVSP1*) was used in a colocalization study (Fig. 3) with nLaVSP1 as an acidocalcisome marker on the Ba125/nLaVSP1 strain (30) (Fig. 3). The results shown in Fig. 3 indicated that LaVSP1 and nLaVSP1 colocalized. In addition, LaVSP1 was also found in some vesicles where LaVSP1 was not detected (Fig. 3B).
In a second step to confirm that the structures containing LaVSP1 indeed corresponded to acidocalcisomes, we performed a second experiment where we studied the colocalization of nLaVSP1 and the L. major exopolyphosphatase (LmPPX1). The latter has been reported to localize in acidocalcisomes (33). Fig. 4 shows that LaVSP1 localized in acidocalcisomes where the levels of LaPPX1 were low. However, this finding is in agreement with Fig. 3B and previously reported data showing that Leishmania acidocalcisomes contain heterogenous levels of VP1 and VSP1 (33, 34).

To explain these data we may argue that different acidocalcisome populations with different structures and protein content (4, 34–37) account for the partial colocalizations observed. Alternatively, LaVSP1 may also localize in another structure as well. It has been shown in D. discoideum (38) and in Trypanosoma cruzi (4) that acidocalcisomes and the contractile vacuole complex are functionally linked and possess common markers such as a vacuolar H\(^+\)-ATPase, an H\(^+\)-pyrophosphatase, and a Ca\(^{2+}\)-ATPase (38). In any case additional experiments will be required to investigate the acidocalcisome heterogeneity.

**Substrate Specificities of LaVSP1**—If PPases display nearly absolute specificity for PP\(_i\) in the presence of Mg\(^{2+}\), when a transition metal such as Zn\(^{2+}\), Co\(^{2+}\), or Mn\(^{2+}\) is used, this specificity is lost. We previously observed for TREVSP (3) that depending on the cofactors used in the buffer (Mg\(^{2+}\) or Zn\(^{2+}\)) and the pH buffer (7.5 or acidic), the enzyme had

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**FIGURE 1.** Phylogenetic analysis of LaVSP1 generated with the neighbor joining method. The phylogeny presented is based on the alignment of the entire sPPase domain (alignment is available under accession number ALIGN_000878). This consensus tree was rooted on the bacterial sequences. The bold numbers next to each node indicate bootstrap values as percentages of 100 replicates. A similar pattern was also obtained by the maximum parsimony method (numbers in italics below nodes indicate bootstrap values as percentages of 100 replicates). The species name of each sequence is indicated, and the numbers in parentheses refer to the accession numbers indicated under "Experimental Procedures." The capital letters on the right indicate the subcellular localization, when known. A, acidocalcisome; C, cytosol; M, mitochondria; P, plastid.

**FIGURE 2.** Expression analysis by Western blotting of LaVSP1 throughout the L. amazonensis life cycle. LaVSP1 and LACK expressions were quantified at the amastigote stage (AM), metacyclic stages (META), and different life cycle stages of procyclics (PRO). exp shows the exponential growth phase, stat shows the stationary growth phase, and stat-lt shows the late stationary growth phase. Rabbit antiserum directed against TbVSP1 (28) and LACK (gift from J.-C. Antoine, Institut Pasteur Paris) were used to probe the LaVSP1 and LACK proteins, respectively. The values in the figure indicate the ratios of LaVSP1/LACK expression levels over the ratios of LaVSP1/LACK expression levels at the exponential growth stage. Therefore, the value is 1 for the expression of LaVSP1 at the exponential growth stage.
Acidocalcisomes, PolyP Metabolism, Pharmacological Target

FIGURE 3. Colocalization analysis by indirect immunofluorescence of LaVSP1 and LeVP1 in L. amazonensis. A, phase contrast. B, overlay of C and D. C, fluorescence image of L. amazonensis promastigotes probed with a rabbit antiserum directed against T. brucei vacuolar proton pyrophosphatase (TVP1) (30). D, fluorescence image of the nLaVSP1 probed with monoclonal antibodies specific for the poly-His5 sequence.

FIGURE 4. Colocalization analysis by indirect immunofluorescence of LaVSP1 and LaPPX1 in L. amazonensis. A, phase contrast. B, overlay of C and D. C, fluorescence images of L. amazonensis promastigotes probed with a rabbit antiserum directed against LmPPX1 (33). D, fluorescence image of the recombinant LaVSP1 probed with monoclonal antibodies specific for the poly-His5 sequence.

substrate specificity for either PPi or polyP. Because Mg\textsuperscript{2+} or Zn\textsuperscript{2+} are abundant in acidocalcisomes (32), biochemical analysis of LaVSP1 was carried out in the presence of either Mg\textsuperscript{2+} or Zn\textsuperscript{2+}.

We observed that LaVSP1 specifically hydrolyzed PPi, in a reaction buffer containing Mg\textsuperscript{2+} with an optimum pH of 7 (Fig. 5). The replacement of Mg\textsuperscript{2+} for Zn\textsuperscript{2+} in the reaction buffer resulted in the hydrolysis of polyP\textsubscript{3} and polyP\textsubscript{28}, in addition to PPi, with optimum pH levels of 5.5, 6.5, and 5.5, respectively. The $k_{\text{cat}}$ and $K_m$ values for PPi hydrolysis, at pH 7.2 in the presence of Mg\textsuperscript{2+}, were estimated to be 125 s\textsuperscript{-1} and 92 $\mu$M, respectively (Fig. 5). The calculated catalytic constants for polyP\textsubscript{3} and polyP\textsubscript{28} were 131 and 8 s\textsuperscript{-1} for the $k_{\text{cat}}$ values and 1153 and 123 $\mu$M for the $K_m$ values, respectively (Fig. 5). The $k_{\text{cat}}$ value for PPi hydrolysis using Zn\textsuperscript{2+} as a cofactor was estimated to be 49 s\textsuperscript{-1}. Interestingly, the high $K_m$ value of 1153 $\mu$M for polyP\textsubscript{3} hydrolysis could be correlated to high level of polyP\textsubscript{3} found in parasitic protozoa (39), which can reach 54.3 mM in T. cruzi epimastigotes (40).

polyP\textsubscript{3} is one of the final products of the short and long chain polyP hydrolysis (41). We previously proposed that polyP\textsubscript{3} could be further hydrolyzed, leading to reduced polyP\textsubscript{3} accumulation in acidocalcisomes and to PPi production (28). A conventional exopolyphosphatase, LmPPX1, was characterized in Leishmania acidocalcisomes. In the presence of Mg\textsuperscript{2+}, LmPPX1 reached a maximum activity for pH values ranging from 7.0 to 8 (30). These data indicate that at pH 5–7.5, in the presence of Zn\textsuperscript{2+}, short chain polyP and PPi, hydrolysis could be carried out by the LaVSP1 protein. This biochemical analysis suggests that LaVSP1 could play a role in the acidocalcisome polyphosphate metabolism.

Implication of LaVSP in the Regulation of the Polyphosphate Metabolism in Acidocalcisomes—Because of their N-terminal domain, VSP1 proteins form functional oligomers. We therefore decided to use the dominant negative mutant strategy to study the role of LaVSP1 in the acidocalcisome polyphosphate metabolism. To attain such a goal, promastigote dominant negative mutants were obtained by overexpressing a mutated recombinant LaVSP1 (mLaVSP1). The His\textsubscript{6}-tagged recombinant D325E-LaVSP1 (mLaVSP1) mutant was expressed in E. coli and purified by immobilized metal affinity chromatography. This mutation was selected because the corresponding D120E variant in yeast had no activity (42). First, we confirmed that the recombinant protein could not hydrolyze PPi (data not shown). Secondly, the mutated gene was cloned into the pFHLTG vector to obtain a tetracycline-dependent expression of mLaVSP1 in the L. amazonensis BA125 strain (BA-Ind/mLaVSP1), previously transfected with the pTupactet vector (18). Western blot analysis (Fig. 6, A and B) showed that LaVSP1 expression in noninduced cells was four times less than that in the induced parasites. The same samples analyzed with an anti-His\textsubscript{6} monoclonal antibody indicated expression of mLaVSP1 in the noninduced cells (Fig. 6B). However, this level of expression is much lower than that in...
Acidocalcisomes, PolyP Metabolism, Pharmacological Target

induced cells and was quantified as representing only a third of the endogenous native protein level. DAPI staining, which allowed the detection of polyP in acidocalcisomes (40), revealed a much lower accumulation of polyP in the induced than in the noninduced BA-Ind/mLaVSP1 cells (Fig. 6, C and D). These data confirm a role for LaVSP1 in the acidocalcisome polyphosphate metabolism.

LaVSP1 Is Not Essential for Leishmania Promastigote Growth and Macrophage Infection but for Metacyclogenesis—WT, noninduced, and induced mLaVSP1 promastigotes grew in vitro with an equivalent doubling time of around eight h, and at the stationary phase they reached the same cell density and presented the same viability. However, metacyclic promastigotes, a nondividing stage, which differentiate during the stationary phase (43), were shown to express the highest level of LaVSP1 during the parasite life cycle stage (Fig. 2). We therefore analyzed the importance of LaVSP1 for metacyclogenesis. As monitored by purification with the 3A1 monoclonal antibody (24), metacyclic promastigotes appeared after entry into stationary phase and peaked after 3–4 days (Fig. 7). Morphological change (43) was also obvious and distinguished them from procyclic promastigotes. Fig. 7 shows that Tet-induced promastigotes produced 10 times less metacyclics when compared with either the noninduced or WT cells. This phenotype reminded what was observed for spt2– promastigote mutants that failed to differentiate into metacyclic promastigotes. These mutants died following an autophagic process (44), and their acidocalcisomes were devoid of long chain polyP (45). Is polyP depletion in acidocalcisomes responsible for that defect? An answer can be found in the ability of polyP to respond to cellular stresses such as pH and nutrient changes (40, 46), which are known to be crucial for parasite differentiation (47, 48).

In vitro infections of bone marrow macrophages were performed with purified metacyclic promastigotes. The results indicated that soon (up to 5 h) after macrophage infection with either noninduced, induced BA125Ind/mLaVSP1 or WT cells, 80–90% of macrophages appeared able to infect, differentiate, and survive inside macrophages. Virulence toward Mice—To obtain a constitutive high level of mLaVSP1 expression, WT BA125 were only transfected with the pFH6TG-D330E/mLaVSP1 construction (Fig. 8, inset). Overexpression under the same conditions of mLaVSP1 was used as a control (BA125/ nLaVSP1). Two independent transfections were performed, and we did not clone the transfected cells. For both transfections, only mice inoculated with BA125/mLaVSP1 late stationary phase promastigotes displayed cutaneous lesions (Fig. 8). We did not attribute this virulence defect to metacyclogenesis deficiency only because metacyclic promastigotes (up to 500) were sufficiently abundant in the inoculum to develop lesions. This information indicates that LaVSP1 is essential to maintain virulence in mice and that acidocalcisomes and their polyP metabolism as previously suggested (3, 49) may represent interesting drug targets.

According to the in vitro macrophage infection study, the virulence defect of BA125/mLaVSP1 in mice could be explained by different environmental conditions found in the two systems. Several characteristics reveal the presence of a hypoxic microenvironment in lesions (50) and indicate that macrophages exposed to hypoxia showed a reduction in the percentage of infected cells and the number of intracellular parasites/cell
Acidocalcisomes, PolyP Metabolism, Pharmacological Target

FIGURE 7. Growth and metacyclogenesis analysis of BA125Ind/mlaVSP1. BA125 WT (open circle), noninduced (gray triangle), and induced (black square) BA125Ind/mlaVSP1 were inoculated into RPMI 1640 medium at 2 x 10⁶ cells/ml, and cell density was measured. WT (open bar), noninduced (gray filled bar), and induced (black filled bar) BA125Ind/mlaVSP1 were grown to stationary phase, and the percentage of metacyclic promastigotes was determined at 144, 168, and 196 h after inoculation. For each point at least three independent repeats have been performed.

FIGURE 8. Virulence of BA125/mlaVSP1 in mouse infections. Two sets of infections with different mutant strains obtained by independent transfections were performed. One set of data contained five BALB/C mice infected in both footpads with 10⁶ late stationary phase promastigotes BA125 WT (black squares), Leishmania overexpressing a native LaVSP1 (BA125/mlaVSP1, black diamonds), and Leishmania overexpressing a mutated LaVSP1 (BA125/mlaVSP1, black triangles). Lesion sizes were monitored weekly. The inset represents a Western blot showing the expression of recombinant His-tagged native and mutated LaVSP1 from protein lysates of 3 x 10⁶ cells, monoclonal antibodies directed against the poly-His tag (Qiagen) were used. IND, induced.

FIGURE 9. Structural analysis. A, structures of the PP binding site of the Y-PPase (gray) superimposed with that of R78Y-PPase (red) (55). B, structures of the PP binding site of R78Y-PPase (red) superimposed with a predicted structure of the PP, binding sites of L. amazonensis (blue). C, structures of the PP, binding site of R78Y-PPase (red) and Y-PPase (gray) superimposed with a predicted structure of the PP, binding sites of L. amazonensis (blue) and T. cruzi (green) VSP1. Pi2 is represented in gray, and Pi1 is absent from the mutant structure (55). Metal ions are indicated by black dots. M3 is absent because it is the most labile metal ion in the wild type product complex (55).

Moreover, observations on the kinetics of infection showed that hypoxia did not depress L. amazonensis phagocytosis but induced macrophages to reduce intracellular parasitism (51). Polyphosphates were shown to regulate the mammalian TOR (target of rapamycin) kinase (52) involved in signaling pathways implicated in cellular stress responses such as hypoxia (53). Thus BA125/mlaVSP1 might fail to develop in mice because of their polyphosphatase metabolism deficiency, which did not allow parasite to survive under the hypoxic conditions found in the lesions.

**Predicted Structure Analysis of TbVSP1, LaVSP1, and T. cruzi VSP1 (TcVSP1)**—The animal/fungus and acidocalcisome PPases differ by the ability of the former ones to hydrolyze polyP (Fig. 5). This substrate specificity difference may offer the opportunity to develop molecules specifically inhibiting the polyP activity of the acidocalcisomcal PPases. Despite the primary sequence homology observed between trypanosomatid and the animal/fungus PPases (27), we looked for structural differences existing between the catalytic center of the two sets of PPases.

Based on x-ray crystallographic analyses, it has been shown that 14 or 15 charged or polar residues are conserved in structural alignment between the E. coli apo PPase and the yeast PPase (Y-PPase) (42, 54). Assuming that PP, and polyP could be bound by PPases according to different modes, we focused our attention on the two phosphate-binding subsites, P1 and P2. In Y-PPase, P1 is formed by Arg⁷⁸, Tyr⁹³, Lys⁹⁵, and metal ions M₃ and M₄, whereas P2 interacted with Lys⁷⁸, Tyr⁹³, and metal ions, M₁–M₄ (54). The SWISS-MODEL (swissmodel.expasy.org) was used as an automated protein structure homology modeling server to identify theoretical structural differences of the phosphate-binding sites of LaVSP1 and Y-PPase. The data presented in Fig. 9A indicate a major difference in the positioning of Arg⁷⁸. Two other significant changes concern the side chains of Lys⁵⁶ and Glu⁵₈. Because such structural differences, depending on the P1 and P2 phosphate-binding subsites were previously reported for the yeast R78K variant (55), a predictive structure homology between LaVSP1 and R78K was investigated. According to the P1 and P2 phosphate-binding subsites, the SWISS-MODEL predicts a better structural homology between LaVSP1 and R78K (Fig. 9B) than between LaVSP1 and Y-PPase. In particular, the R78K and LaVSP1 resting enzymes favor the out position of the loop 71–78 (55). These changes are consistent with the reduced affinity for PP, of the R78K variant (Kₘ = 27 μM) and LaVSP1 (Kₘ = 92 μM) compared with that of the WT Y-PPase (Kₘ = 1.45 μM). The need for LaVSP1 and R78K to overcome an unfavorable equilibrium constant in binding substrate could account for the observed increase in Kₘ (55).
Of course, to confirm that hypothesis it would have been interesting to develop variants. However nature often offers natural variants. In the case of VSP1 proteins, we analyzed the Arg78 position for TbVSP1 and TcVSP1 (GenBank™ accession number DQ908844) proteins. The SWISS-MODEL predicted (Fig. 9C) a perfect structure homology between LaVSP and TbVSP1. However, TcVSP1 significantly differed from the two others at position Arg78, which is now completely oriented outside the catalytic center (Fig. 9C). Interestingly, TcVSP1 did not hydrolyze PPi, but P3 and polyP (data not shown), which correlates with TcVSP1 structural differences. In conclusion, to hydrolyze polyP the active site underwent significant distortion to accommodate the larger polyP sizes, suggesting that PPi and polyP are bound by VSP1 by different modes. As a matter of fact the Arg78 residue was shown to be directly involved in binding and optimum orientation for catalysis of one phosphate group of PPi.

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