Identification and Characterization of a Protein-tyrosine Phosphatase in *Leishmania*

**IN INVOLVEMENT IN VIRULENCE**

Received for publication, June 29, 2006, and in revised form, August 21, 2006 Published, JBC Papers in Press, September 27, 2006, DOI 10.1074/jbc.M606256200

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*Leishmania* parasites are eukaryotic protozoans responsible for a variety of human diseases known as leishmaniasis, which ranges from skin lesions to fatal visceral infections. *Leishmania* is transmitted by the bite of an infected sandfly where it exists as promastigotes and, upon entry into a mammalian host, differentiate into amastigotes, which replicate exclusively in macrophages. The biochemical pathways enabling *Leishmania* to differentiate and survive in the mammalian host are poorly defined. We have therefore examined the role of protein-tyrosine phosphorylation, which is essential in regulating cell function in higher eukaryotes. Using the recently completed *Leishmania* genome, we have identified and cloned a *Leishmania* protein-tyrosine phosphatase (PTP) gene (LPTP1) by virtue of its homology with the human protein-tyrosine phosphatase 1B gene (hPTP1B). The enzyme activity of recombinant LPTP1 was confirmed using a combination of PTP-specific substrates and inhibitors. We further demonstrate, by creating LPTP1 null mutants through gene targeting, that LPTP1 is necessary for survival as amastigotes in mice, but it is dispensable for survival as promastigotes in culture. Human PTPs, including the PTP1B enzyme, are actively pursued drug targets for a variety of diseases. The observations with the LPTP1 mutants in mice suggest that it may also represent a drug target against the mammalian amastigote stage. However, *in silico* structure analysis of LPTP1 revealed a striking similarity with hPTP1B in the active site suggesting that, although this is an attractive drug target, it may be difficult to develop an inhibitor specific for the *Leishmania* LPTP1.

Leishmaniasis is a disease caused by infection with *Leishmania* protozoan parasites that results in a spectrum of clinical manifestations ranging from self-healing cutaneous lesions to fatal visceral disease (reviewed in Ref. 1). There are over two million new cases of leishmaniasis each year and over 12 million people currently suffering from this infection in 88 tropic and subtropic countries (2, 3). During its life cycle, *Leishmania* alternates between promastigotes in the sandfly vector and amastigotes in the mammalian host. Once transmitted to the mammalian host through the bite of an infected sandfly, the promastigotes differentiate into nonflagellated intracellular amastigotes, whereupon they multiply exclusively in the phagolysosome organelle of infected macrophages. Amastigotes are responsible for the diverse pathologies associated with leishmaniasis, which depends to a large extent on the *Leishmania* species (reviewed in Ref. 1). The biochemical changes associated with differentiation from promastigotes to amastigotes and with the long term survival of amastigotes in the mammalian host are poorly understood, and consequently the biological role of protein phosphorylation remains largely unknown in *Leishmania*.

Protein phosphorylation is among the most important regulatory biochemical changes in higher eukaryotic cells. Phosphorylation of tyrosine residues is controlled by protein-tyrosine kinases and protein-tyrosine phosphatases (PTPs). Particularly, protein-tyrosine phosphorylation and dephosphorylation regulate multiple central processes including cellular phenotypic functions, differentiation, proliferation, and cell death (reviewed in Ref. 4). The overall protein phosphorylation pattern in *Leishmania* parasites and related trypanosomatids has been shown to change during differentiation associated with different life cycle stages, suggesting that protein kinases and phosphatases play a role in these processes (5). More recently, it has been established that *L. major* promastigote extracts contain protein-tyrosine phosphatase activity, although the corresponding gene(s) has not yet been identified (6).Because of the central role of tyrosine phosphorylation in higher eukaryotic cell function, we have begun to investigate the potential contribution of protein-tyrosine phosphorylation to the *Leishmania* life cycle and virulence. We had previously observed that heterologous expression of a prototype human protein-tyrosine phosphatase 1B (hPTP1B) in *Leishmania donovani* mediates partial differentiation toward the amastigote stage, induces expression of amastigote-specific proteins,
and increases virulence in BALB/c mice (7). These observations argue that PTPs play a role in amastigote survival in the mammalian host and provide strong justification for the characterization of endogenous Leishmania PTP genes as detailed within.

In the present study, we describe the identification of a PTP gene from Leishmania major, Leishmania infantum, and L. donovani that has extensive sequence and correspondence structural similarity with the human PTPIB gene product. These Leishmania genes have been designated LmPTP1, LiPTP1, and LdPTP1, respectively. The enzyme activity of LmPTP1 has been confirmed with relevant substrates and inhibitors. We have also developed LdPTP1 heterozygous and homozygous null mutant knock-out clones that proliferated in a similar fashion to wild type promastigotes in culture but were severely impaired with respect to survival as amastigotes in BALB/c mice. Through in silico structural analysis, we also show that the L. infantum PTPI (LiPTPI) and human PTPIB (hPTP1B) shared remarkable structural conservation in the active site; however, notable differences outside this region are also present. This observation suggests that from a phenotypic perspective, the PTPI represents an attractive drug target. However, from a structural perspective, it may be difficult to develop a small molecule specific to the active site of LmPTP1.

**EXPERIMENTAL PROCEDURES**

Parasite Cultures—The L. donovani 1S/CI2D and L. major Friedlin V9 promastigotes were routinely cultured at pH 7.2 and 27 °C in Medium 199 medium (Invitrogen) supplemented with 10% fetal bovine serum. L. donovani differentiation into amastigotes was performed by shifting to amastigote culture medium (37 °C, pH 5.5 in RPMI 1640 plus 10% fetal bovine serum) overnight, which mimics the temperature and pH of the host macrophage phagolysosome.

Cloning, Sequencing, and Tagging of L. major PTPI—The 1.5-kb L. major DNA fragment homologous to the human PTPIB was identified in the L. major data base by BLAST search (Entry GenDB LmjF36.5370; www.genedb.org). Based on the sequence obtained from the database, two primers were designed to PCR amplify the LmPTPI gene and to incorporate a sequence encoding a His tag at the N terminus, and the amplified product was then ligated into the mammalian expression vector pcDNA3. The primers used were HsF 5′-agcatcGTGCCGATGCAGTCCCCTTCCGTGTTGGCCGC-CCGCTCTCGTGTTGGCCGCATCATCATCATCATCAT-ATATGGAGATGGAAAAGGAGATTTCGAG-3′ with an EcoRI site and the A2HisR (hPTP) 5′-gaattcATATGGAGATGGAAAAGGAGATTTCGAG-3′ with a BamHI site.

The L. donovani LdPTPI gene was PCR-amplified from L. donovani 1S/CI2D genomic DNA with the same primers used above for L. major and sequenced (GenBank™ bankit827438 DQ862810). In brief, the amplified fragment was cloned into the TOPO TA cloning vector (Invitrogen) and termed pLdPTPI-TOP. and M13 reverse and M13 forward primers were used in sequencing reactions with the MegaBace500 (Molecular Dynamics of GE Healthcare). Sequencing reactions were performed by a DYEnamic ET Terminator Cycle sequencing kit with Thermo Sequenase II DNA polymerase, and post-reaction cleanup was achieved by extensive ethanol precipitation before adding formamidine loading solution.

Transfection of Cos7 Cells and Purification of A2His-tagged LmPTPI and hPTPIB—The Cos7 cell line was transiently transfected using Lipofectamine reagent according to the manufacturer's protocol (Invitrogen). Briefly, a 100-mm dish of Cos7 cells (∼8 × 105 cells) was transfected with a total of 8 μg of DNA (6 μg of pcDNA3-LmPTP1 and 2 μg of a β-galactosidase expression plasmid) in 20 μl of Lipofectamine. The following day (∼20 h after transfection), the cells were washed with cold medium and lysed with Nonidet P-40 lysis buffer (150 mM NaCl, 1% Nonidet P-40, 20 mM Tris pH 8.0) with protease inhibitors (Roche Applied Science complete mixture tablets) on ice for 30 min. The cell lysates were centrifuged, and β-galactosidase assays were performed to determine the levels of transfection efficiency in each dish and to normalize the amount of protein used in each assay. His-bind® resin (Novagen) was used for purification of the A2- His-tagged LmPTPI and hPTPIB according to the manufacturer’s protocol. Briefly, His-bind® resin was activated in 1-binding buffer (8 × 4 mM NaCl, 160 mM Tris-HCl, 40 mM imidazole, pH 7.9) with 0.1% Nonidet P-40. 60 μl of activated His-bind® resin was added to 200 μl of cell lysates and incubated with agitation for 3 h at 4 °C. His-bind® resin was thoroughly washed five times in 1.0 ml of 150 mM NaCl, 1% Nonidet P-40, 20 mM Tris, pH 8.0 buffer with protease inhibitors (Roche Applied Science complete mixture tablets), and half of the His-bind® resin with the purified A2- His-tagged LmPTP1 or hPTPIB was used for activity assays, and the other half used for Western blot analysis with anti-A2 tag monoclonal antibodies.

Protein-tyrosine Phosphatase Activity and Inhibition Assays—The 4-p-nitrophenylphosphate (pNPP) assay was used for detection of total phosphatase activity as described previously (7). Briefly, 30 μl of His-bind® resin containing purified PTP proteins was washed once in 1.0 ml of 150 mM NaCl, 1% Nonidet P-40, 20 mM Tris, pH 8.0, Roche Applied Science complete protease inhibitor buffer and placed in a 96-well plate. 180 μl of reaction buffer (50 mM Heps, pH 7.5, 0.1% β-mercaptoethanol containing 10 mM fresh pNPP) was added to each well, and the plate was incubated at 37 °C overnight. The plates were read at 405 nm.

The Malachite green phosphatase activity assay with the insulin receptor (IR) phosphopeptide is a more specific assay
for hPTP1, because the IR is a major substrate of this enzyme (9, 10). The assay was performed according to the manufacturer’s instructions (Sigma; PTP101, nonradioactive phosphotyrosine phosphatase assay).

Two inhibitors, 1 mM of sodium orthovanadate (Na$_3$VO$_4$), and 10 μM of potassium bisperoxo(1,10-phenanthroline)oxovanadate (V)(bpV (phen)) were used to further confirm the activity of LmPTP1 and the hPTP1B as the control. bpV (phen) is a member of a class of potent and specific PTP inhibitors (11, 12). For the inhibition assays, His-bind$^\text{®}$ resin containing LmPTP1 and hPTP1B was washed five times with 1.0 ml of 150 mM NaCl, 1% Nonidet P-40, 20 mM Tris, pH 8.0, Roche Applied Science complete protease inhibitor buffer and then incubated with inhibitors for 1 h at 4°C. His-bind$^\text{®}$ resin containing the PTPs was washed four times with 1.0 ml of 150 mM NaCl, 1% Nonidet P-40, 20 mM Tris, pH 8.0, Roche Applied Science complete protease inhibitor buffer and then assayed for phosphatase activity using pNPP as substrate, as detailed above.

Disruption of the LdPTP1 Genes from L. donovani—The L. donovani PTP1 gene disrupted strains were generated by homologous gene targeting as outlined in Fig. 4A. A HindIII and XbaI fragment containing the L. donovani PTP1 gene from the pLdPTP1-TOPO plasmid described above was subcloned into a pBlue script vector. The resulting pBSLdPTP1 plasmid was digested with BclI to remove the 357-bp catalytic region of the LdPTP1 gene.

The fragment containing the hygromycin resistance gene was removed from the pSPY hygromycin vector (13) with BamHI and BglII and inserted into the BclI site within the LdPTP1 sequence, generating the plasmid pBSLdPTP Hyg. The linear fragment containing the hygromycin gene and the LdPTP flanking sequences was then electroporated into L. donovani. Transfectants were initially selected, in the first round targeting, with 50 μg/ml hygromycin to obtain the heterozygous single LdPTP1 knock-out mutant. The double knock-out homozygous null mutant for the LdPTP1 gene was achieved by increasing the hygromycin concentration to 200 μg/ml in selection culture medium.

Complementation of the double knock-out null mutant with a plasmid containing the LdPTP1 gene was carried out as follows. The following oligonucleotide primers LdPTP1F1 5′-cccagacctTCACCTTTGGTGAGCCTTTGT with a HindIII site and the reverse LdPTP1R 5′-cagagtttCAGAGGTGACAGCCA-
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GTCATA with a BglIII site were used to amplify a 3140-bp fragment from L. donovani genomic DNA that contained the LdPTP1 gene open reading frame including 655-bp upstream and 1000-bp downstream flanking sequences. The 3140-bp fragment was then inserted into HindIII and BamHI sites of plasmid pSPY-Neo (13) to generate the complementing plasmid pSPYNeoLdPTP1 as shown in Fig. 7A.

Southern Blotting—For Southern blot analysis, 10 μg of Leishmania genomic DNA was digested with restriction enzymes PstI and SstI and separated in a 0.7% agarose gel. Hybridization and washing were performed as previously described (14). The LdPTP1 active domain encoding DNA (357-bp BclI fragment from nucleotides 677–1034) was used as a probe for DNA from the single (+/−) and double knock-out (−/−) clones described above to demonstrate the disruption of the LdPTP1 gene (Fig. 4B). Southern blot using the hygromycin gene demonstrated specific targeting into the LdPTP1 gene (Fig. 4C). The L. major 1.5-kb LmPTP1 gene containing fragment was used in the Southern blot to confirm the presence of the episomal LdPTP1 added back to the double knock-out null clone LdPTP1−/− (Fig. 7B).

Infection of BALB/c Mice and Recovery of Amastigotes—Female BALB/c mice (Charles River) weighing 20–25 g (n = four mice/group) were injected via tail vein with 1.5 × 10^6 late log phase promastigotes in 100 μl of phosphate-buffered saline, as described previously (15). After 4 weeks of infection, the mice were examined for L. donovani parasite burden by counting the number of amastigotes in the Giemsa-stained liver imprints. Liver parasite burden, expressed as Leishman-Donovan units, was calculated by multiplying the number of amastigotes/1000 cell nuclei × liver weight (g). Spleen parasite burden were determined by limiting dilution in 96-well plates as previously detailed (16).

Proliferation in Culture—Parasite growth was evaluated by determination of the optical density at 600 nm of diluted cultures (starting from 10^6 cell/ml) grown in 96-well plates from days 0–8 (promastigotes) or 0–5 (amastigotes).

LiPTP1 Catalytic Domain Modeling—The alignment between the L. infantum PTP1 amino acid sequence and human PTP1B obtained with ClustalW (Fig. 1) in conjunction with the PTP1B crystal structure (Protein Data Bank code 1SUG) were used in Modeler 8 (version 2, default configuration) (17) to create the L. infantum PTP1 homology model. A 20-residue stretch of amino acids present in the Leishmania PTP1 sequence that was outside of the enzyme active site was not present in the human enzyme sequence and, as a result, was not modeled (residues 32–51). In addition, the construct used to elucidate the human enzyme structure did not contain any residues beyond position 319. Therefore, these two regions were removed from the Leishmania homology model.

RESULTS

We began this study by performing a BLAST search of the L. major data base (Ref. 18; www.genedb.org) for sequences that could represent PTP genes by virtue of their homology with the human PTP1B gene. In total, nine potential PTP genes were identified in the Leishmania genome. The one with the greatest identity with human PTP1B was LmjF36.5370 located on chromosome 36, which we have designated LmPTP1. The L. infantum PTP1 (LiPTP1) sequence was also identified in this manner from the L. infantum data base (Lin36.5860; www.genedb.org). Based on these sequences, we designed PCR primers to amplify, clone, and sequence the L. donovani LdPTP1 homolog (GenBank™ bankit827438 DQ862810) as detailed under “Experimental Procedures.” Alignment comparison of the various Leishmania PTP1 and hPTP1B proteins revealed they share ~40% sequence identity, including a number of important conserved amino acid residues within the hPTP1B signature catalytic domain ([(U/V)HCXGXAR(S/T/G)], which contains the essential cysteine and arginine residues required for enzyme activity (boxed region in Fig. 1) (reviewed in Ref. 4). In addition to the conserved catalytic domain, LPTP1s share relevant accessory motifs with hPTP1B including the adjacent signature WPD and the Q residues (boxed region indicated with bold type in Fig. 1), which play a role in maintaining the conformation of the active site. The LPTP1s and hPTP1B also share a proline-rich region from amino acids 325–340 that are responsible for SH3 domain protein-protein type interactions and cellular localization in hPTP1B (4). Southern blot analysis of genomic DNA from L. major demonstrated that LmPTP1 was a single copy gene in the haploid genome.6 Included in Fig. 1 is the closest PTP sequence homolog from Saccharomyces cerevisiae, which is more divergent from hPTP1B than is the LPTP1s.

6 M. Nascimento, W.-W. Zhang, and G. Matlashewski, unpublished data.
Although the *Leishmania* PTP1 sequences shown in Fig. 1 suggest that they encode for a hPTP1B homolog, it was necessary to validate this experimentally using relevant enzyme substrates and inhibitors. We designed primers to amplify, clone, and insert the LmPTP1 gene into a eukaryotic expression vector (pcDNA3) for expression in transfected simian Cos7 cells. To detect and partially purify the LmPTP1 from Cos7 cells, a 10-amino acid epitope tag derived from the *L. donovani* A2 protein, followed by a His tag encoding sequence, were inserted at the 5’ end of the LmPTP1 gene (Fig. 2A). In this

![Graphs and images showing enzymatic activity and inhibition for LmPTP1 and hPTP1B](image)

**FIGURE 3.** LmPTP enzymatic activity and inhibition following expression in Cos7 cells. LmPTP1 and hPTP1B purified on the His-bind resin were assayed on PTP substrates: pNPP (A) and IR phosphopeptide (B) as indicated. Also shown is a Western blot confirming equal levels of PTP protein used for each substrate enzyme assay. For the enzyme inhibition assays, LmPTP1 and hPTP1B purified on the His tag resin were assayed in the absence (−) or presence (+) of the PTP inhibitors; Na₃VO₄ (C) and bpV (phen) (D) as indicated. Also shown is a Western blot confirming equal levels of PTP protein used in the presence (+) or the absence (−) of the inhibitors in each assay. Note that both inhibitors impaired the enzyme activity to levels similar to that derived from the control transfected cells (control pcDNA3). The values reported are the average means of three independent experiments, and the results in *bar graphs* are the means ± S.E. Microsoft Excel was used to calculate the Student’s test. *, *p* ≤ 0.05; **, *p* ≤ 0.01, statistical difference from control.
manner, the Cos7 cell expressing A2-His$_7$-tagged LmPTP1 could be detected with anti-A2 monoclonal antibodies (8) and partially purified from cell lysates by affinity chromatography with His-bind$^\text{R}$ resin. We also generated the same construct using the human hPTP1B gene as a positive control for subsequent comparison.

Expression of the A2-His$_7$ tagged LmPTP1 and hPTP1B genes in Cos7 cells was analyzed by Western blot analysis with anti-A2 monoclonal antibodies 24 h after transfection. As shown in Fig. 2B, the hPTP1B and LmPTP1 proteins were detectable at the predicted molecular masses of 50 and 55 kDa, respectively (lanes 2 and 3). The control empty pcDNA3 vector did not produce bands (lane 1), confirming the specificity of the Western blot for A2-tagged PTPs. The A2-His$_7$-tagged proteins were subsequently partially purified from the transfected Cos7 cell lysate using His-bind$^\text{R}$ resin and washed extensively, followed by Western blot analysis with anti-A2 monoclonal antibodies. As shown in Fig. 2B (lanes 5 and 6), the washed His tag-binding resin contained approximately equal amounts of hPTP1B and LmPTP1. These data confirmed that it was possible to express, detect, and partially purify similar levels of hPTP1B and LmPTP1 from transfected Cos7 cells.

We next determined whether we could detect PTP enzyme activity in the washed His-bind$^\text{R}$ resin containing the extracted hPTP1B and LmPTP1 from the transfected Cos7 cells. Two protein phosphatase substrates were used for these assays including pNPP and a specific tyrosine phosphatase substrate, IR phosphopeptide (Fig. 3, A and B). For this assay, the hPTP1B and LmPTP1 expression constructs were transfected into Cos7 cells, purified on His-Tag resin, assayed for activity, and further subjected to parallel Western blot analysis to confirm similar levels of hPTP1B and LmPTP1 in each assay. As shown in Fig. 3 (A and B), both LmPTP1 and hPTP1B enzyme activities were detected on the His-bind$^\text{R}$ resin with the pNPP and IR phosphopeptide substrates when compared with the control (His tag-binding resin from control pcDNA3-transfected cells). To confirm that the activity detected in the samples shown was due to PTP activity, we determined whether it was possible to specifically inhibit this activity using the protein phosphatase inhibitor Na$_3$VO$_4$ and a more specific protein-tyrosine phosphatase inhibitor bpV (phen) (12, 19). As shown in Fig. 3 (C and D), both the LmPTP1 and hPTP1B were inhibited with Na$_3$VO$_4$ and bpV (phen) (white bars). Accompanying Western blots confirmed there were similar levels of LmPTP1 and hPTP1B assayed in the presence (+, white bars) and absence (−, black bars) of these inhibitors. Importantly, the inhibitors did not affect the background activity observed on His tag-binding resin from the control vec-
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Because Leishmania are diploid organisms, the two alleles of the catalytic domain of the LdPTP1 gene were targeted for deletion as summarized in Fig. 4A. The BclI restriction enzyme fragment containing the LdPTP1 catalytic domain (nucleotides 677–1034; Fig. 1) was replaced with a BamHI-BglII fragment containing the selectable marker gene conferring hygromycin (Hyg) resistance. The resulting plasmid, termed pBsLdPTP1hyg, was linearized and targeted into the LdPTP1 site of the L. donovani genome by transfection, and transformants were selected for hygromycin resistance. The first round of gene targeting (heterozygous deletion) was carried out using 200 \mu g of hygromycin selection, and the second round (homozygous deletion) was carried out using 200 \mu g of hygromycin. In this manner, both LdPTP1 alleles could be targeted with one selectable marker. Cultures were then subjected to serial dilution to isolate individual LdPTP1 knock-out clones.

Southern blot analyses were performed to confirm the heterozygous and homozygous targeted deletion of the catalytic domain of the LdPTP1 gene in the cloned L. donovani mutant cultures. For this analysis, the 357-bp BclI restriction enzyme fragment, which was deleted by gene targeting (heterozygous deletion) was carried out using 200 \mu g of hygromycin selection, and the second round (homozygous deletion) was carried out using 200 \mu g of hygromycin. In this manner, both LdPTP1 alleles could be targeted with one selectable marker. Cultures were then subjected to serial dilution to isolate individual LdPTP1 knock-out clones.

For the analysis, the 357-bp BclI restriction enzyme fragment, which was deleted by gene targeting (Fig. 4A), was used as the hybridization probe. As shown in Fig. 4B, the BclI LdPTP1 gene fragment encoding the catalytic domain was eliminated from the L. donovani clones as indicated by the absence of the band containing this sequence in the null mutant double knock out (–/–) cultures and further by a 50% reduction in the single knock out clone (+/–), as compared with wild type L. donovani (+/+). PCR analysis of the LdPTP1 gene in these mutant clones confirmed the Southern blot data showing deletion of the catalytic domain (data not shown). We
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Further confirmed the accurate replacement of the Hygromycin resistance gene specifically into the LdPTP1 locus on the same clones by performing Southern blot analysis with a probe specific for the hygromycin resistance gene. As shown in Fig. 4C, the hygromycin resistance gene was only present in the specific site for the LdPTP1 gene. Taken together, these Southern blot analyses demonstrated that the active site region of the LdPTP1 gene was specifically and completely disrupted in the null mutant clones, thereby confirming their suitability for subsequent phenotypic analysis.

Initially, the phenotype of the LdPTP1 null mutants was compared with that of the wild type L. donovani using well established in vitro axenic culture protocols for promastigotes and amastigotes. As observed in Fig. 5A, the two individual homozygous LdPTP1 null mutant clones (Ld1PTP1−/−, Ld2PTP1−/−), cultured under promastigote conditions (26 °C, pH 7.2), proliferated at a slightly slower rate compared with wild type L. donovani (LdWT). The heterozygous single knock-out clone (LdPTP1+/−) proliferated at a similar rate to the null mutant clones. Under amastigote culture conditions (37 °C, pH 5.5), the homozygous null mutants (Ld1PTP1−/−, Ld2PTP1−/−) also demonstrated a slight reduction in proliferation compared with the single knock-out clone (LdPTP1−/−) and the wild type culture (LdWT) (Fig. 5B). With respect to promastigote morphology, there did not appear to be any difference between the null mutant clones and the wild type cultures as shown in Fig. 5C.

The most stringent assay for L. donovani virulence is its ability to survive in the visceral organs in a mammalian host. We therefore compared the ability of the LdPTP1 null mutant clones and the parental wild type L. donovani to survive in the liver 4 weeks following injection in the tail vein of BALB/c mice. As shown in Fig. 6, the two LdPTP1 null mutant clones (Ld1PTP1−/− and Ld2PTP1−/−) displayed significantly reduced virulence compared with the wild type parasites as indicated by both the number of amastigotes per nuclei in liver imprints (upper panel) and by calculating the Leishman-Donovan units determined by multiplying the level of infection by liver weight (lower panel). Both LdPTP1 null mutant clones displayed the same phenotype with an approximately 80–90% reduction in virulence as determined by their ability to survive in the liver. The single knock-out clone LdPTP1+/− showed a clear intermediary reduction in virulence, consistent with reduced expression of LdPTP1. These results argue that the LdPTP1 gene plays a significant role in parasite survival in the mammalian host.

We next determined whether it was possible to restore the virulence of the null mutant clone (Ld1PTP1−/−) by complementation with the wild type LdPTP1 gene. This was performed by introducing the LdPTP1 gene including its flanking regulatory sequences into a plasmid (Fig. 7A). The LdPTP1 plasmid containing plasmid (pSPYneoLdPTP1) was transfected into the null mutant clone (Ld1PTP1−/−), and transformants were selected in G418 and cloned by limiting dilution. For subsequent infections in mice, the wild type L. donovani promastigotes that had been in culture for several months and used to generate the null mutants characterized in Fig. 6 were no longer available. Nevertheless, the heterozygous single knock-out clone LdPTP1+/− also represented a relevant control because it had retained one wild type PTTP1 allele (Fig. 4B), was more virulent than the null mutant clones (Fig. 6), and had undergone the same selection procedure as the null mutant clone, Ld1PTP1−/−. Southern blot analysis with the entire LdPTP1 gene probe confirmed that the pSPYneoLdPTP1 plasmid had been successfully introduced into the null mutant clone (Fig. 7B, lanes 5 and 6).

We compared the virulence of the mutant Ld1PTP1−/− containing the complementary pSPYneoLdPTP1 plasmid to the parental null mutant Ld1PTP1−/− and the heterozygous mutant (LdPTP1+/−), which retained one intact endogenous LdPTP1 allele. In addition, a newly established L. donovani culture was used for comparison. As shown in Fig. 7 (C and D), adding back the plasmid-derived LdPTP1 gene to the Ld1PTP1−/− null mutant increased virulence in the liver (lanes 4) in comparison with the parental Ld1PTP1−/− null mutant (lanes 3). The Ld1PTP1−/− parasites containing the add back LdPTP1 was similar in virulence to the heterozygous LdPTP1+/− clone (Fig. 7, C and D, lanes 2), which retained one functional endogenous allele for the LdPTP1 gene. It is also noteworthy that the infection levels shown in Fig. 7 (C and D), for both the heterozygous LdPTP1+/− (lanes 2) and null mutant Ld1PTP1−/− (lanes 3) were almost identical to the previous experiment shown in Fig. 6. As expected, the newly thawed culture of wild type L. donovani (Fig. 7, C and D, lanes 1) was considerably more virulent than the previous L. donovani culture that was used to generate the original LdPTP1 null mutants and which had been maintained in culture for several months (Fig. 6, A and B, lanes 1).

To further examine the ability of the add back LdPTP1 gene to restore virulence to the null mutant, we compared infection levels in the spleen. As shown in Fig. 7E, adding back the plasmid derived LdPTP1 gene to the Ld1PTP1−/− null mutant resulted in increased spleen parasite levels (lane 4) compared with the parental Ld1PTP1−/− null mutant (lane 3) and was similar in virulence to the heterozygous single allele knock-out clone (+/−, lane 2). Taken together, these infection levels demonstrated that the LdPTP1 gene plays a critical role in parasite virulence in the mammalian host.
results in the mouse liver and spleen demonstrate that adding back the LdPTP1 gene on a plasmid partially complemented the virulent phenotype in the null mutant LdPTP1−/− clone, thus confirming the importance for LdPTP1 in amastigote survival in the mammalian host.

The preceding observations provide an argument that inhibition of Leishmania PTP1 in amastigotes may have therapeutic potential and therefore may represent a drug target. With this in mind, in silico homology modeling was performed to compare the three-dimensional structures of the L. infantum PTP with the hPTP1B (Fig. 8A). This was performed to determine whether there were significant differences in the active sites between these enzymes that could be exploited to develop specific small molecule inhibitors. The structure of the complex between a tyrosine-phosphorylated peptide substrate (sequence etdy(Pt)rkggkgll) and human PTP (HEPTP, Protein Data Bank code 1G1G), was used to model the position of the peptide ligand into the L. infantum homology via superposition. Enzyme residues within eight angstroms of the ligand were used to perform the superposition. The hydrogen bonding pattern seen in the substrate peptide-human HEPTP complex between the phosphate group and the backbone of the protein appears to be very well conserved in the L. infantum PTP1 homology model, and a high level of structural conservation between the human and Leishmania enzymes is concentrated in and around the active site (Fig. 8, A and B). Thus, it seems likely that the active sites of the two proteins are similar, although there are significant differences in residues further away from the active site, including residues 32−51 (Fig. 8, A and B). Two differences close to the active site are worth mentioning. First, although the L. infantum enzyme has a proline residue at position 67, the human enzyme has an arginine. Second, residue 205 in the L. infantum sequence is a glutamine, whereas in all human tyrosine phosphatases there is a phenylalanine residue at this position. These differences affect the hydrophobicity of the substrate-binding pocket and thus, potentially, the preferred substrate. This altered specificity may provide scope for designing an inhibitor that binds more tightly to the L. infantum enzyme than to the human homologs.

**DISCUSSION**

Among the most important signaling mechanisms in eukaryotic cells are those that involve protein-tyrosine phosphorylation and dephosphorylation (4). It was therefore important to identify a prototype PTP-related gene in the Leishmania genome, determine its role in the parasite life cycle, and determine its potential as a drug target. We were particularly interested in identifying a Leishmania PTP1B-type gene because we had previously observed that overexpression of the human PTP1B increased the virulence of L. donovani and appeared to mediate certain aspects of differentiation toward the amastigote stage (7). The present study confirmed that Leishmania parasites do have a functional PTP1B-like gene, which we termed LPTP1. Deletion of the L. donovani PTP1 gene resulted in attenuated amastigotes in BALB/c mice but did not significantly impair promastigote survival in culture. These results argue that Leishmania PTP1 plays a significant role in biochemical pathways associated with amastigote survival in the mammalian host.
On the other hand, we did not obtain evidence that *Leishmania* PTP1 was directly involved in differentiation into amastigotes as previously suggested (7), because the LdPTP1^−/−^ knock-out mutant promastigotes were capable of morphologically differentiating into and proliferating as amastigotes in culture. In addition, we did not observe any changes in the expression of the amastigote-specific protein A2 in the LdPTP^−/−^ null mutant clones (data not shown).

This study further supports the recent conclusion that *L. major* has PTP enzyme activity (6) and provides insight into the primary and three-dimensional structural features of the *Leishmania* PTP1 gene. Comparison of the primary structure of the human PTP1B and the *Leishmania* PTP1 proteins revealed striking similarities. Firstly, the 10-amino acid active site containing the central cysteine residue is highly conserved with only a two-amino acid difference in this region. As revealed in Figs. 1 and 8, human PTP1B and the *Leishmania* PTP1 enzymes share additional important motifs, including the invariant Asp residue in the WPD loop and the Gln residue in the Q loop, which are also involved in the catalysis of the cysteinyl-phosphate catalytic intermediate (4). The *in silico* three-dimensional structural analysis in the presence of a substrate peptide further confirms the close similarities between the *Leishmania* and human PTPs. This is further supported by the observations in Fig. 3, which showed that when assayed under identical conditions in the presence of the same substrates and inhibitors, the *Leishmania* PTP1 and human PTP1B had similar levels of activity. Outside of the active site, the proline-rich region at the C-terminal section of human PTP1B is involved in its association with substrates containing SH3 domains (4, 20); this proline enrichment of the C-terminal region was also present in the *Leishmania* PTP1 sequence. Subcellular localization of the human PTP1B enzyme is largely mediated through the highly hydrophobic C-terminal region, which anchors this enzyme to the endoplasmic reticulum (21–23). Likewise, the last 35 amino acids of *Leishmania* PTP1 are also highly hydrophobic, suggesting a potential association with cell membranes similar to the human PTP1B protein. This is consistent with the recent report that PTP activity is associated with *L. major* membrane fractions (6).

To define the role of the *Leishmania* PTP1 gene in the parasite life cycle stages and virulence, the active site of the enzyme was deleted by gene targeting. It is noteworthy that although LdPTP1 is a single copy gene, it was relatively easy to develop both heterozygous and homozygous PTP1 gene knock-outs in promastigotes, indicating that the enzyme does not play a major role in the survival of promastigotes in culture. Morphologically, the LdPTP1 mutant promastigotes were similar to wild type cultures. However, the LdPTP1 mutants were severely attenuated in comparison with the wild type *L. donovani* with respect to survival in the liver and spleen of BALB/c mice. Future studies will be necessary to identify the endogenous targets (parasite or host) of *Leishmania* PTP1 to define the biochemical pathways involved in mediating parasite survival in the mammalian host. Although there is no secretion leader sequence on the *Leishmania* PTP1 enzyme, its strong homology with human PTP1B makes it tempting to speculate that it might also target host cell substrates. This would be similar to other obligate macrophage pathogens including *Yersinia* spp., *Salmonella typhimurium*, and *Mycobacterium tuberculosis*, all of which secrete PTP enzymes into the host cell that play significant roles in the virulence of these pathogens (reviewed in Refs. 24 and 25).

This study represents an example of how recent tools including the *Leishmania* genome and *in silico* protein structure analysis can be combined with more traditional molecular biological approaches including gene targeting and virulence analysis in mouse models to define potential drug targets. We have focused on PTPs because of the central role of tyrosine phosphorylation in the biology of higher eukaryotic cells, making this among the most intensely studied areas of cell biology and drug development. This study revealed a strikingly high conservation between the *Leishmania* and human PTPs. Because the active sites of these enzymes are highly conserved, it is difficult to conclude that *Leishmania* PTP1 represents a viable target for drug development. Nevertheless, there may be subtle differences in charge within the active site, suggesting that screening a library of PTP inhibitors for *Leishmania* PTP1-specific inhibitors may be justified. The combination of the technologies used in this study should, however, prove beneficial in defining additional drug targets against this important human parasite.

Acknowledgments—We thank Dr. Michael Tremblay of the McGill Cancer Center for helpful advice during the course of this research and Dr. Samantha Grunheid and Keyrillos Rizg for help with microscopy.

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