Molecular Dissection of the Functional Domains of a Unique, Tartrate-resistant, Surface Membrane Acid Phosphatase in the Primitive Human Pathogen *Leishmania donovani*

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The primitive trypanosomatid pathogen of humans, *Leishmania donovani*, constitutively expresses a unique externally oriented, tartrate-resistant, acid phosphatase on its surface membrane. This is of interest because these organisms are obligate intracellular protozoan parasites that reside and multiply within the hydrolytic milieu of mammalian macrophage phago-lysosomes. Here we report the identification of the gene encoding this novel *L. donovani* enzyme. In addition, we characterized its structure, demonstrated its constitutive expression in both parasite developmental forms, and determined the cell surface membrane localization of its translated protein product. Further, we used a variety of green fluorescent protein chimeric constructs as reporters in a homologous leishmanial expression system to dissect the functional domains of this unique, tartrate-resistant, surface membrane enzyme.

*Leishmania donovani* is an important protozoan pathogen of humans that causes severe and most often fatal visceral disease in the tropics and subtropics worldwide (1). This organism has a digenetic life cycle that consists of two major developmental forms: 1) extracellular flagellated promastigotes that reside and multiply in the alimentary tract of their sandfly vectors and 2) obligate intracellular nonflagellated amastigotes that reside and multiply within the phago-lysosomal system of infected human macrophages.

Acid phosphatases (AcPs)3 are phosphomonoesterases that hydrolyze substrates under low pH conditions and are generally considered to be typical marker enzymes of lysosomes (2, 3). Previously, it was shown that *L. donovani* promastigotes (4, 5) and tissue-derived amastigotes possess a unique, externally oriented, surface membrane, tartrate-resistant acid phosphatase.2 The presence of this enzyme on the parasite cell surface is an interesting observation considering that amastigotes of all pathogenic leishmanial species reside and multiply within host cell phago-lysosomes. Moreover, to date, no other tartrate-resistant surface membrane AcP from any source has been reported in the literature.

The tartrate-resistant surface membrane AcP of *L. donovani* (MAcP) has a broad substrate specificity hydrolyzing glycerol phosphates and mono- and di-phosphorylated sugars (5), inositol phosphates, and phosphorylated proteins (6). Although the biochemical properties of this enzyme have been partially characterized, its biological function(s), as with virtually all acid phosphatases (3), remain to be elucidated. Understanding and investigating the role(s) that this AcP plays in parasite growth and survival would be facilitated by the characterization of the gene(s) that encodes this unique enzyme. To date, however, no such leishmanial genes have been reported. Thus, in the current study we identified the gene encoding this novel *L. donovani* enzyme and characterized its structure, expression, and localization in both developmental forms of the parasite. Further, a variety of green fluorescent protein (GFP) chimeric constructs was used as reporters in a homologous leishmanial expression system to dissect the functional domains of this unique, tartrate-resistant, surface membrane enzyme.

**EXPERIMENTAL PROCEDURES**

*Reagents—* All chemicals used, unless otherwise noted, were of analytical grade and were purchased from Sigma. Similarly, enzymes and DNA molecular mass standards were purchased from Roche Molecular Biochemicals. Protein molecular mass standards were purchased from Amersham Biosciences.

*Parasite Cultures—* *L. donovani* promastigotes ([1S, clone 2 D] from the 1S strain World Health Organization designation: (MHOM/SD/62/1S-CL2D) were grown at 26 °C in chemically defined 199([1S, clone were grown at 37 °C as described by Joshi et al. (8)). Axenic amastigotes forms of this *L. donovani* clone were grown at 37 °C as described by Joshi et al. (8). All of the cultures were harvested at log phase (2–3 × 10⁷ cells ml⁻¹) by centrifugation as described (9). The cell pellets were resuspended in the appropriate buffers for isolation of nucleic acids, for preparation of surface membrane fractions, or for transfection experiments. Tissue-derived amastigotes of this *L. donovani* strain were isolated from spleens of infected hamsters (*Mesocricetus auratus*; LVG strain, Charles River Laboratories, Inc., Wilmington, MA) as described previously (10).

Preparation of Parasite Surface Membrane-enriched Fractions—Cell
lysates of both promastigotes and axenic amastigotes were prepared from washed cell pellets by the addition of lysis buffer (10 mM Tris-HCl, 25 mM EDTA, 25 µg/ml leupeptin, pH 8.0) to a final concentration of 5 x 10^6 cells ml^-1 and disruption in a prechilled, tightly fitting Dounce homogenizer (5). The lysates were centrifuged at 8000 x g for 30 min at 4°C, and washed cell pellets by the addition of lysis buffer (10 mM Tris-HCl, 1% Lys-Phe-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-Pro-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Leishmanial Surface Membrane Acid Phosphatase

(CLONTECH) using primers forward GFP 5'-TGTTGAGCGGCCTCC-
AGCGCTCATGGTGAGCAAGGGCC (Eco47 and MluI restriction
endonuclease sites in bold type) and reverse GFP 5'-CCAAAGCGCT-
TTGTCAGCTGTCGATC (Eco47 restriction endonuclease site in bold type). The resulting PCR product was cloned into the pCR2.1
vector (Invitrogen), resulting in the plasmid construct pCR2.1 GFP.

Third, the MacP signal peptide plus its peptide cleavage site (Arg26)
was PCR-amplified from Cos AcP-101 template with primers forward
MacPSP 5'-CATGACGTCCTGATTGCGCTGACAGGTC-
ATC (SpeI restriction endonuclease site in bold type) and reverse
MacPSP 5'-TGTTGAGCGGCCTCATGGTGACAGGGCC (MluI restric-
tion endonuclease site in bold type). The resulting PCR product was
cloned into the pCR2.1 vector (Invitrogen) to produce pCR2.1 MacPSP.

The first chimera that contained GFP and the putative TM domain
of MacP was constructed by subcloning the gel-purified Eco47-digested
GFP fragment from pCR2.1 GFP into the Eco47-linearized pCR2.1 MacPTM
construct, which resulted in the plasmid construct pCR2.1 GFP::TM. The orientation of the GFP fragment was verified using gel electrophoresis
analysis with the appropriate restriction endonucleases.

The second chimera containing the MacP SP, GFP, and the putative
MacP TM domain was produced by subcloning the gel-purified MluI
fragment of pCR2.1 GFP::TM into the MluI-linearized pCR2.1 MacPSP,
which resulted in the plasmid construct pCR2.1 MacPSP::GFP::TM. The orientation of the GFP::TM fragment was verified using gel electrophoresis analysis with the appropriate restriction
endonucleases. The second chimera containing the MacP SP, GFP, and the putative MacP TM domain was produced by subcloning the gel-purified MluI
fragment of pCR2.1 GFP::TM into the MluI-linearized pCR2.1 MacPSP,
which resulted in the plasmid construct pCR2.1 MacPSP::GFP::TM. The orientation of the GFP::TM fragment was verified using gel electrophoresis analysis with the appropriate restriction
endonucleases. The final construct pKS NEO MacPSP::GFP::TM was verified by sequence analysis as described above and was subse-
quently transfected into L. donovani promastigotes for functional
domain analysis studies. Other MacP-GFP chimeric expression plasmids
including pKS NEO MacPSP::GFP and pKS NEO GFP were con-
structed using the same multi-step PCR-based cloning strategy above
as appropriate.

Transfection of GFP Chimeric Constructs into Leishmania—For
transfection experiments, harvested promastigotes cells were suspen-
ded in electroporation buffer to 10⁶ cells ml⁻¹ as described previ-
ously (22). 500 µl of cell suspension was added to a 2-mm gap electro-
poration cuvette (BTX Inc., San Diego, CA). Immediately prior to
 electroporation, 20 µl of purified plasmid DNA (1 mg ml⁻¹ in sterile 10
mm Tris-EDTA quality biological, Inc., cat. 80462) was added to the
cell suspension. Cells were electroporated using a BTX Inc. ECM-600 electroporation system. Electroporation conditions were a single pulse at 475 V, 800 microfarads, and 13 ohms. The electroporated cells were incubated on ice for 10 min and trans-
fected into 5 ml of culture medium as described above and incubated at
26 °C for 24 h. The transfected cells were subsequently harvested by
centrifugation and resuspended in culture medium containing
15 µg ml⁻¹ G418 (Geneticin, Invitrogen). Transfected cells were se-
lected for growth in increasing concentrations of G418 over a period of
several weeks and maintained at 200 µg ml⁻¹ drug. The drug-resistant
cells were used in subsequent experiments.

Microscopy—Transfected promastigotes were washed three times in
phosphate-buffered saline by centrifugation as described above. Fluores-
cence images of such live cells were acquired using a Zeiss AxioPlan
microscope (Carl Zeiss, Inc., Thornwood, NY), which was equipped with
epifluorescence, a cooled CCD camera (Photometrics, Tucson, AZ), and
the appropriate fluorescein isothiocyanate excitation/barrier filters. Transfected parasites were also examined in indirect immunofluores-
cence assays using anti-Bip and anti-GFP antibodies essentially as
described by Debrabant et al. (22). Such cells were examined by confocal
microscopy using a Zeiss LSM 410 system with fluorescein isothiocya-
нате and rhodamine excitation/barrier filters. The fluorescent images
obtained in these channels were collected separately. All of the captured
images were processed using Adobe Photoshop 5.5 (Adobe Systems, San
Jose, CA).

RESULTS

Identification of the MacP ORF—Previously we identified and characterized the genes that encode the 110- and 130-kDa
isoforms of the L. donovani histidine secretory AcPs (SacP-1 and
SacP-2) (13). In the current study, results of Southern
analysis of L. donovani gDNA digested with PstI revealed three
restriction fragments (5.2, 3.9, and 3.0 kb) that hybridized with an
L. donovani AcP gene probe (DIG-300) (Fig. 1). The two
larger PstI fragments, of 3.9 and 5.2 kb, contained the SacP-1
and SacP-2 genes described above (13). The third PstI frag-
ment, of 3.0 kb, was subcloned, sequenced, and found to contain
a partial ORF, very similar but not identical in sequence to
either SacP-1 or SacP-2. To obtain a full-length ORF corre-
sponding to the 3.0-kb PstI fragment, an L. donovani
gDNA cosmid library was screened with the DIG-300 probe, which
is common to all three PstI restriction fragments. Seventeen
positive clones were identified and analyzed by restriction endonucle-
ase digestion with PstI followed by Southern hybridiza-
tion with the DIG-300 probe. Three of the 17 cosmid clones (Cos
AcP-101, -102, and -103) contained a 3.0-kb PstI restriction
fragment. Sequence analysis revealed that these cosmids con-
tained a full-length (948 bp) ORF corresponding to the 3.0-kb
PstI genomic fragment.

Comparison of the L. donovani AcP-deduced Proteins—Sequence
analysis showed that the 948-bp ORF encoded a deduced
protein with a calculated molecular mass of 35,192 Da and
predicted isoelectric point of 8.23. Analysis of the deduced
protein sequence showed that it contained five potential N-
linked glycosylation sites (Asn24, Asn26, Asn113, Asn219, and
Asn245) (Fig. 2A) and one predicted myristoylation site (Gly37).
In addition, the deduced protein possessed five putative phos-
phorylation sites by several different mechanisms (i.e. casein kinase II: Ser43, Ser154, and Ser248 and protein kinase C: Thr107
and Thr167). Moreover, the most abundant residues of this
deduced protein were alanine and leucine, which constitute 23% of its aa content.

The deduced protein can be divided into three structural
domains. Region I consists of a 23-aa putative signal peptide
(Met1–Ala23) based on the Von Heijne algorithm (23) (Fig. 2). The sequence of this putative signal peptide including its pep-
tidase cleavage site is identical to those present at the N-
terminal of the SacP-1 and SacP-2-deduced proteins (13).
Therefore, cleavage at this site would result in Arg24 as the
N-terminal aa of the mature protein. In addition, the two
SacP-deduced proteins of L. donovani (SacP-1 and SacP-2)
and the 948-bp ORF-deduced protein above are identical in aa
sequence for 251 residues beyond the signal peptide (Region II)
(Fig. 2B). The presence of a conserved histidine AcP signature
sequence (i.e. a catalytic site consensus sequence) within
Region II (Val27–Arg39) (Fig. 2) indicates that the 948-bp ORF-
deduced protein is a member of this highly conserved family
of enzymes. However, the second histidine AcP signature se-
sequences (Ala$^{285}$–Thr$^{296}$) present in SAcP-1 and SAcP-2 is absent in the 948-bp ORF-deduced protein. The third region consists of the C terminus of this deduced protein. Based on the Kyte-Doolittle algorithm (24), Region III of 948-bp ORF-deduced protein contains a stretch of 29 hydrophobic aa residues (Leu$^{274}$–Tyr$^{315}$) that could function as a transmembrane anchor domain (Fig. 2). This is followed by a short 13-aa putative cytoplasmic tail (Arg$^{303}$–Tyr$^{315}$). Cumulatively, these observations suggested that the 948-bp ORF represented a membrane-anchored member of the histidine acid phosphatase family; thus, this gene was designated as the *L. donovani* MAcP.

**Characterization of the MAcP Gene Locus—Comparison of both nucleotide and deduced aa sequences revealed that the AcPs from *L. donovani* belong to a highly conserved multi-gene family. To determine whether MAcP constituted a distinct single gene or a multi-gene locus, we designed a specific oligo probe, DIG-111 (Fig. 3A), that would only recognize MAcP and not SAcP-1 or SAcP-2. This oligo probe was used in Southern hybridization analysis of *L. donovani* gDNA. The results of these analyses with both single (AatII, NcoI, and NotI) and double restriction endonuclease (AatII and NcoI; AatII and NotI; and NcoI and NotI) digestions demonstrated that within an ~6-kb region of *L. donovani* gDNA, only single restriction fragments were observed to hybridize with the DIG-111 probe (Fig. 3B). One would expect that multiple restriction fragments would have hybridized with this probe, if the MAcP locus had been present in more than one copy. Thus, the hybridization results indicated that the 6 kb surrounding and including the MAcP locus were present only once within the *L. donovani* genome. These results were confirmed by Southern hybridization of the three cosmids clones, Cos AcP-101, -102, and -103, each of which was shown to contain a single MAcP ORF (data not shown).

**Identification of the MAcP Transcript—** Transcription of MAcP was assayed in a two-step process involving RT and PCR amplification (Fig. 4). First, cDNA of promastigote, axenic amastigote, and tissue-derived amastigote mature mRNA was generated with oligo(dT) and reverse transcriptase. Second, an aliquot of each of these cDNAs was used as template in PCR with gene-specific primers (Fig. 4A), and the expected 250-bp amplified product should contain an internal sequence specific to the MAcP.

The results of these experiments showed that only a single amplified product of 250 bp was obtained using aliquots of promastigote cDNA as template (Fig. 4B, Pro lane). Similarly, a 250-bp amplification product was obtained with aliquots of cDNA from both axenic amastigotes (Fig. 4B, Ax Am lane) and tissue-derived amastigotes (Fig. 4B, Tis Am lane). These RT-PCR results were confirmed using several different RNA-cDNA preparations. In control reactions, in which cDNAs were not amplified products were obtained (data not shown). Moreover, in control reactions in which forward or reverse primers were omitted from the reaction mix, no amplified products were obtained (data not shown). The specificity of the RT-PCR-amplified products was verified by sequencing the gel-purified PCR-amplified reaction products. Sequence analysis revealed that the primer pair specifically amplified a single product, which corresponded to the specific MAcP gene sequence. Together, these data demonstrated that MAcP is actively transcribed by *L. donovani* promastigotes, axenic amastigotes, and tissue-derived amastigotes. Thus, the MAcP appears to be constitutively transcribed throughout the parasite developmental life cycle.

**Localization of MAcP by Western Analysis—** SDS-PAGE and Western blot analysis were used to determine the cellular localization of the protein product encoded by the MAcP. *L. donovani* isolated surface membrane fractions from promastigotes and axenic amastigotes as well as whole cell lysates of tissue-derived amastigotes were probed in Western blots using the anti-MAcP peptide Ab. The results of these assays showed...
that the anti-MAcP peptide Ab reacted with a single ~53-kDa protein in the isolated promastigote surface membranes (Fig. 5, lane 1). These results are consistent with those reported previously concerning the apparent molecular mass of a partially purified tartrate-resistant AeP from the surface membranes of *L. donovani* promastigotes (25). The MAcP peptide Ab also recognized a single ~64-kDa protein in surface membranes of axenic amastigotes (Fig. 5, lane 2) and an ~60-kDa protein in lysates of tissue-derived amastigotes (Fig. 5, lane 3). The ~4-kDa difference in apparent molecular mass between the MAcP protein present in isolated axenic amastigote surface membranes and in lysates of tissue-derived amastigotes might reflect differences in their post-translational modifications (e.g. type and/or amount of glycosylation, phosphorylation, etc.) because of the diverse environments in which these organisms were grown (i.e. in vitro cell culture versus hamster spleen macrophages in vivo). Alternatively, this difference could result from proteolytic degradation/hydrolysis of the MAcP protein during the isolation and processing of tissue-derived amastigotes.

In parallel Western blots (data not shown) these proteins were also recognized by a rabbit polyclonal anti-*L. donovani*...
secretory AcP Ab (No. 172). These data are consistent with all three of the L. donovani AcP-deduced proteins (SAcP-1, SAcP-2, and MAcP) having a common N terminus and verifies that the MAcP protein is a member of a family of conserved AcPs in L. donovani. In contrast, the anti-MAcP peptide Ab did not react with the promastigote 110- and 130-kDa SAcP-1 and SAcP-2 secretory proteins, further demonstrating the specificity of this antibody for the unique C-terminal domain of the MAcP protein. NRS controls showed no reactivity with any leishmanial protein (data not shown).

Taken together, these Western blot results demonstrated that the MAcP protein is produced by tissue-derived amastigotes and that it is expressed in the surface membranes of both in vitro grown promastigotes and axenic amastigotes. Further, these results showed that the MAcP protein expressed by promastigotes and amastigotes differed in apparent molecular mass. The latter may reflect their developmental differences in post-translational processing of this surface membrane enzyme.

**Immunoprecipitation of MAcP Activity—**Surface membranes isolated from L. donovani promastigotes and axenic amastigotes were detergent-solubilized and assayed for total acid phosphatase activity using p-nitrophenyl phosphate as substrate in the presence or absence of 5 mM sodium tartrate. The results of these assays demonstrated that both parasite developmental forms possessed comparable levels of MAcP specific activity (Table I). Further, these assays showed that all of the measurable MAcP activity present in these solubilized surface membranes was resistant to inhibition by sodium tartrate. Aliquots of such solubilized surface membranes were reacted with the anti-MAcP peptide Ab (antibody raised against a portion of the unique C-terminal peptide sequence of MAcP (i.e., aa residues Lys294–His295)) or NRS in a protein A-Sepharose 4B/CL bead-based assay (9, 18). Immunoprecipitates from these were assayed for AcP activity in the presence or absence of 5 mM sodium tartrate. The results from these assays (Table I) showed that the anti-MAcP peptide Ab immunoprecipitated ~20% of the total AcP activity present in the detergent-solubilized surface membranes of each parasite developmental form. Further, these results showed that all of the activity immunoprecipitated by the anti-MAcP peptide Ab was tartrate-resistant. The relatively low amount of enzyme activity immunoprecipitated in these assays might be due to insertion of the C-terminal hydrophobic domain of MAcP into detergent micelles, thus making this epitope only partially available for interaction and recognition by the anti-MAcP peptide Ab.

The results of these immunoprecipitation assays in conjunction with our Western blot results demonstrated that the anti-MAcP peptide Ab specifically reacted with a single tartrate-resistant parasite surface membrane AcP present in both parasite developmental forms. Such expression is in agreement with our RT-PCR results, which indicated that the MAcP gene is actively transcribed by both L. donovani promastigotes and axenic amastigotes.

**Episomal Expression of GFP Chimeras in L. donovani Promastigotes—**Chimeric proteins containing specific domains of the MAcP fused with GFP as a reporter were episomally expressed in L. donovani promastigotes using the pKS NEO leishmanial expression vector. These chimeric constructs were used to demonstrate that the N-terminal region of the MAcP functions as a signal peptide and that its C-terminal region functions as a membrane anchor. All chimeric constructs and control plasmids were transfected into L. donovani promastigotes, which were subsequently grown in increasing concentrations of G418 (to a final concentration of 200 μg ml⁻¹). These transfectants were subsequently analyzed in Western blots and by epifluorescence microscopy.

The first plasmid construct, pKS NEO MAcP::GFP::TM, encoded a protein (Fig. 6A, map 1) containing the putative N-terminal signal peptide domain (Met1–Arg23) of MAcP and cytoplasmic tail (Tyr315). Thus, the MAcP peptide Ab specifically reacted with a single tartrate-resistant parasite surface membrane AcP present in both parasite developmental forms. Such expression is in agreement with our RT-PCR results, which indicated that the MAcP gene is actively transcribed by both L. donovani promastigotes and axenic amastigotes.

**TABLE I**

| Parasite developmental form | Total MAcP activitya | % MAcP activity immunoprecipitatedb |
|-----------------------------|-----------------------|-------------------------------------|
| Promastigotes               | 380 ± 32              | 18.4 ± 1.6                          |
| Axenic Amastigotes          | 450 ± 40              | 19.6 ± 1.7                          |

a Assayed using p-nitrophenyl phosphate as substrate and specific activity expressed as nmol p-nitrophenol liberated min⁻¹ mg⁻¹ of detergent solubilized isolated surface membrane protein. Identical values were obtained in the presence or absence of 5 mM sodium tartrate. The data shown represent the mean results of triplicate assays for each sample from three separate experiments.

b Activity immunoprecipitated using α-MAcP peptide antibody in a protein A-Sepharose 4B/CL bead-based assay using the formula: (Activity Bound/Activity Bound + Activity Unbound)× 100. The results were normalized by subtracting values obtained with NRS (preimmune serum) from those obtained with the α-MAcP peptide antibody. Identical values were obtained in these assays in the presence or absence of 5 mM sodium tartrate. The data shown reflect the mean results of triplicate assays for each sample from three separate experiments.
this expressed GFP chimeric protein contained the putative MACP signal peptide at its N-terminal end and both the MACP putative transmembrane (TM) anchor domain and cytoplasmic tail at its C terminus. This construct was used to determine whether the C-terminal MACP sequence would function to target and anchor the GFP in the cell surface membrane of transfected parasites.

The second plasmid construct, pKS NEO MACPSP::GFP (Fig. 6A, map 2), contained the N-terminal putative signal peptide sequence of the MACP as above and the full-length GFP reporter (i.e. it lacked the entire C-terminal domain of the MACP). This construct was used to determine whether the N-terminal MACP sequence functioned as a signal peptide that would target the nascent protein into the endoplasmic reticulum.

The third plasmid construct (Fig. 6A, map 3), pKS NEO GFP, contained no sequences from the MACP gene. This construct was used as a GFP reporter control for these transfection studies.

Surface membranes were isolated from both pKS NEO MACPSP::GFP::TM and pKS NEO control transfecteds. These were subjected to SDS-PAGE and Western blot analyses with the anti-GFP Ab and anti-MACP peptide Ab. In such blots, the anti-GFP Ab and anti-MACP peptide Ab each reacted very strongly with a single ~32-kDa protein that was only present in the surface membranes of pKS NEO MACPSP::GFP::TM transfectants (Fig. 6B, lanes 2 and 2′, respectively) and not in those from pKS NEO control transfecteds (Fig. 6B, lanes 1 and 1′, respectively). The apparent molecular mass of this ~32-kDa chimeric protein reflects the sum of its GFP and MACP C-terminal domain (42 amino acid residues) components (i.e. ~28 and ~4.2 kDa, respectively). In addition, the anti-MACP peptide Ab also reacted with the single ~53-kDa endogenous MACP protein present in the surface membranes of both the pKS NEO MACPSP::GFP::TM and pKS NEO control transfectants (data not shown). Further, preimmune rabbit serum, normal mouse serum, and ascites controls showed no reactivity in these Western blots. The results of these assays demonstrated that the pKS NEO MACPSP::GFP::TM episomal plasmid was readily translated and expressed as a ~32-kDa MACPSP::GFP::TM chimeric protein in these parasites.

L. donovani promastigotes transfected with the above expression plasmids were also examined by epifluorescence microscopy. Such observations revealed that promastigotes transfected with pKS NEO MACPSP::GFP::TM demonstrated bright GFP cell surface fluorescence (Fig. 6C). In contrast, pKS NEO control transfectants showed no surface fluorescence. These results indicated that the MACPSP::GFP::TM chimeric protein was in fact targeted to and expressed on the cell surface membrane of these parasites. Further, in conjunction with our Western blot data, these results demonstrated that the MACP transmembrane domain was present on the GFP chimeric protein and that it functioned to anchor this protein in the parasite cell surface.

In contrast to the above information, promastigotes transfected with pKS NEO MACPSP::GFP showed only diffuse intracellular fluorescence, reflecting the processing of GFP in the endoplasmic reticulum of these cells. The latter was confirmed by its colocalization (Fig. 6D, panel 3, Merge) with Bip (a resident endoplasmic reticulum protein) in indirect immunofluorescence assays using both anti-GFP (Fig. 6D, panel 1) and anti-Bip (Fig. 6D, panel 2) antibodies. Cell lysates and cell-free culture supernatants from promastigotes transfected with the pKS NEO MACPSP::GFP plasmid or control pKS NEO plasmid were also subjected to SDS-PAGE and Western blot analyses. Such blots were probed with the anti-GFP antibody and matched control reagents. The results of these assays showed that the lysates of pKS NEO MACPSP::GFP but not pKS NEO control transfectants contained a single ~28-kDa protein that reacted with the anti-GFP Ab (Fig. 6E, lanes 2 and 1, respectively). Further, these results also showed that pKS NEO MACPSP::GFP but not pKS NEO control transfectants secreted/released a single, soluble, ~28-kDa GFP into their culture supernatants (Fig. 6E, lanes 2′ and 1′, respectively). The ~28-kDa protein expressed by these transfecteds presumably reflects the apparent molecular mass of the mature GFP alone (i.e. lacking the MACP signal peptide domain). Cumulatively, these results demonstrated that the N-terminal end of the MACP (Met3–Ala23) functions as a signal peptide to translocate the nascent GFP into the endoplasmic reticulum. Further, in the absence of a C-terminal membrane anchor, the mature soluble ~28-kDa GFP is released from these cells into their culture supernatants, presumably via default into the secretary pathway.

Promastigotes transfected with the pKS NEO GFP plasmid (i.e. devoid of any MACP sequences) were also examined in Western blots and by epifluorescence microscopy. Lysates of such cells showed that they contained a single ~28-kDa protein that reacted with the anti-GFP Ab in Western blots; however, no GFP was detected in the cell-free supernatants from these parasites (data not shown). Examination of these cells by epifluorescence microscopy revealed that GFP was diffusely distributed throughout their cytoplasm. These observations are in agreement with our conclusions above concerning the functions of the N- and C-terminal domains of the MACP.

**DISCUSSION**

In the current report we identified a new member (MACP) of the AcP multi-gene family in *L. donovani* by Southern hybridization of a gDNA cosmid library. The MACP gene is present in single copy and encodes a distinct but highly conserved deduced protein of 315 aa. Sequence analysis of gene-specific RT-PCR using RNA isolated from both developmental forms of this parasite demonstrated that *L. donovani* promastigotes, axenic amastigotes, and tissue-derived amastigotes actively transcribed MACP mRNA. In Western blots, the MACP peptide Ab reacted with a single polypeptide in promastigote surface membranes, axenic amastigote surface membranes, and whole cell lysates of tissue-derived amastigotes. Further, this Ab immunoprecipitated the tartrate-resistant MACP activity present in surface membranes of both *L. donovani* promastigotes and axenic amastigotes. Cumulatively, the results of these studies demonstrated that MACP is constitutively transcribed and translated by both promastigotes and amastigotes into an active tartrate-resistant AcP protein, which is expressed in the surface membranes of both of these parasite developmental forms.

Previously, we identified the genes encoding the two major secretory isoforms (SAcP-1 and SAcP-2) of the histidine AcPs in *L. donovani* (13). In the current study we used GFP chimeras to show that the MACP-deduced protein possesses a functional 23-aa signal peptide sequence at its N terminus. Further, we showed that this signal peptide is identical to those of SAcP-1 and SAcP-2. In addition, the N termini of these three leishmanial AcPs (SAcP-1, SAcP-2, and MACP) all contained 251 conserved aa residues. Moreover, within this region, these proteins contain a signature sequence (Val27–Arg39) that is characteristic of a catalytic domain present in all histidine AcPs (13, 26). Downstream of this catalytic domain, however, these three leishmanial AcPs have diverged evolutionarily presumably by gene duplication and polymorphisms. As such, SAcP-1 and SAcP-2 contain regions rich in serine and threonine repeat units (13) that are absent in MACP.

The MACP-deduced protein possesses a unique 41-aa C-ter-
minal domain that is absent from SAP-1 and SAP-2. Further, based on the Kyte-Doolittle algorithm (24), the MACP contains a sequence of 29 hydrophobic aa residues (Leu274-Tyr302). In the current study GFP chimeraux were used to show that this C-terminal domain functions to anchor the MACP protein into the surface membrane of these parasites.

Recently, the mechanism by which tartrate inhibits the enzymatic activity of the tartrate-sensitive histidine AcPs was delineated by LaCount et al. (27). In that report, the authors showed that in addition to the conserved sequence motif Arg-His-Gly-Xaa-Arg-Xaa-Pro, aa residues corresponding to Ala79, His257, and Asp258 of the human prostatic AcP are involved in the binding of tartrate to the active site of these enzymes. In that regard, several genes for tartrate-sensitive leishmanial AcPs have been identified. These include the two L. donovani secretory AcPs, SAP-1 and SAP-2 (13); the two released AcPs, SAP1 and SAP2 (28); and one membrane-bound AcP, MBAP (29) of Leishmania mexicana. The deduced proteins of all of these leishmanial AcPs contain residues, which correspond to those involved in tartrate binding (27). Thus, conservation of these residues accounts for these leishmanial AcP enzymatic activities being inhibited by tartrate. In the current report, we identified a constitutively expressed gene (MACP) that encodes a unique tartrate-resistant surface membrane AcP of L. donovani. Sequence analyses of this MACP demonstrated that it lacked the critical residues corresponding to His257 and Asp258 of the human prostatic AcP. Thus, the absence of these residues in this leishmanial MACP is consistent with the tartrate resistance of this parasite surface membrane enzyme.

Acid phosphatases are generally considered to be typical marker enzymes of lysosomes. In fact, a family of tartrate-resistant acid phosphatases (TRAPs) has been identified and shown to be conserved membrane components of mammalian lysosomes (2, 3). All TRAPs contain five conserved signature sequences (5); however, none of these signature sequences were present in the L. donovani MACP, indicating that this parasite enzyme is not a TRAP per se. Thus, the cumulative results of this study have identified and characterized, for the first time, the gene encoding the unique tartrate-resistant surface membrane histidine acid phosphatase of L. donovani. To date, no other tartrate-resistant surface membrane acid phosphatase has been reported in the literature. Molecular dissection of the functional domains of this novel parasite enzyme demonstrated that both the N-terminal signal peptide and the C-terminal anchor domains were required to target the MACP to the surface membrane of this pathogen. Because L. donovani amastigotes reside and multiply within the phago-lysosomal system of mammalian macrophages, the MACP may afford this parasite a survival advantage in such hostile hydrolytic environments. The availability of the MACP gene therefore should facilitate studies concerning the biological role of this enzyme in parasite survival.

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Molecular Dissection of the Functional Domains of a Unique, Tartrate-resistant, Surface Membrane Acid Phosphatase in the Primitive Human Pathogen *Leishmania donovani*

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