Protozoan Parasite of Humans: Surface Membrane with Externally Disposed Acid Phosphatase

Abstract. *Plasma membranes isolated from the protozoan parasite* Leishmania donovani *were enriched in acid phosphatase (E.C. 3.1.3.2) activity. Cytochemically, the enzyme was distributed uniformly on the surface of intact cells and was localized on the external face of isolated membranes. Physical characteristics and orientation of the membrane-bound enzyme suggest that the organism is adapted for existence in hydrolytic environments.*

Surface membrane interactions are of paramount significance in host-parasite relationships. For example, how a symbiotic organism survives, is nourished, and multiplies within an infected host, especially on confrontation with host-immune responses, are functions in which the parasite surface membrane must have a significant role. Yet despite their apparent relevance in disease processes, the biochemistry of parasite surface membranes remains largely unexplored.

The trypanosomatid protozoan *Leishmania donovani* is the etiologic agent of human visceral leishmaniasis (kala azar), a debilitating and often fatal disease of man. This organism resides and multiplies, by mechanisms yet unknown, in hydrolytic environments throughout its digenetic cycle; that is, extracellularly in the alimentary tract of the sand-fly vector and intracellularly within the lysosomal system of mammalian macrophages (*1*). We now report on the identification and partial characterization of an acid phosphatase in surface membranes isolated from *L. donovani* promastigotes. We also present cytochemical evidence for the uniform, and highly unusual, distribution of this enzyme on the surface of intact cells, and its localization on the external face of the isolated parasite surface membrane.

A cloned strain of *L. donovani* (*2*) promastigotes was grown in vitro, harvested, and washed as described (*3*). The cells were subjected to homogenization and subcellular fractionation to isolate the surface membranes (*4*). The fractions containing these surface membranes were greater than 95 percent enriched, as ascertained by electron microscopy. The surface origin of these membranes was established unequivocally by their association with subpellicular microtubules, a characteristic of trypanosomatid protozoa. The activity of acid phosphatase (E.C. 3.1.3.2) was measured in a standard reaction mixture containing *p*-nitrophenyl phosphate (PNPP) or other phosphate ester substrates, by determination of released *p*-nitrophenol and inorganic phosphate, respectively (*5*).

The localization of acid phosphatase activity in the fine structure of intact cells and isolated surface membranes was determined as described (*4*) by using a modified Gomori (*6*) medium (*7*) made in 50 m*M* sodium acetate and 0.146*M* sucrose containing 3.3 m*M* Pb $(NO_3)_2$, *p*H 5; β-glycerophosphate was added as substrate. Controls contained, in addition, 10 m*M* sodium fluoride as an enzyme inhibitor. A second control medium lacked substrate.

Acid phosphatase activity was readily detected in live intact cells as well as in homogenates of *L. donovani*. Under identical conditions, living cells hydrolyzed PNPP at a rate equal to one-half of that of disrupted cell homogenates. Similar results were observed with other nonpermeable substrates. The ability to obtain purified surface membrane preparations from *L. donovani* promastigotes permitted us to assess directly the presence of the enzyme in the membrane. The plasma membrane fraction and homogenate obtained during the course of the membrane isolation procedure were assessed for their ability to hydrolyze PNPP. The results of a typical experiment are presented in Table 1. These results show that approximately one-half of the acid phosphatase activity present in the cell homogenate was recovered in the plasma membrane fraction. Cumulative results of six experiments demonstrated a fivefold enhancement in specific activity of the isolated surface membrane fraction compared to the whole cell homogenate, and a 10 percent recovery of cellular protein in the membrane fraction. The data agreed with those in which living cells and cell homogenates were compared for *p*-nitrophenyl phosphatase hydrolase activity.

Intact cells incubated with β-glycerophosphate had an electron-dense, lead phosphate enzyme reaction product uniformly distributed over their entire surface membrane (Fig. 1A). Reaction product was also present on the membrane lining the flagellar reservoir, as well as free within the reservoir lumen. The "free" lumen product might have result-

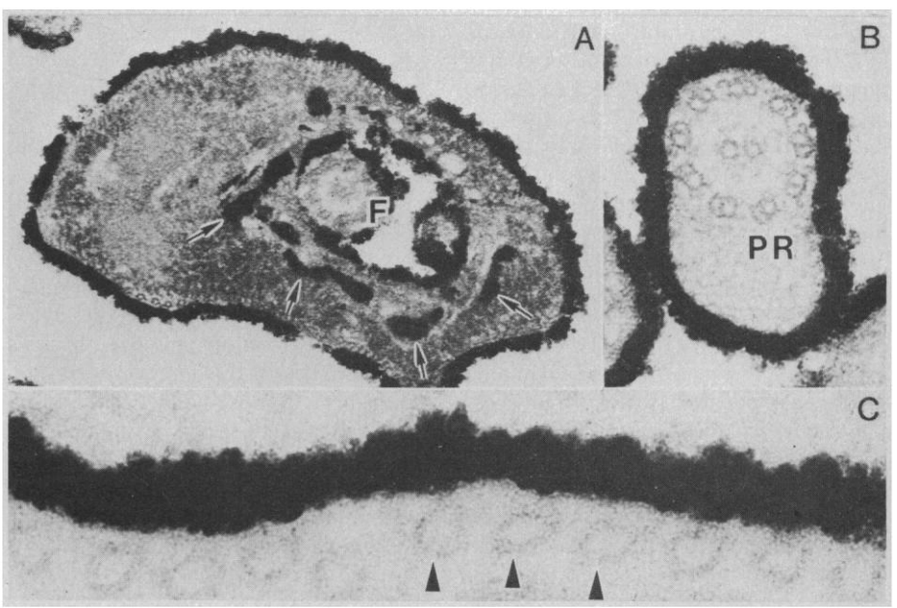

Fig. 1. The cytochemical localization of acid phosphatase reactivity in *L. donovani*. (A) The electron-dense, lead phosphate enzyme reaction product, which is indicative of acid phosphatase activity, is uniformly distributed on the surface membrane of this intact cell. The flagellum (*F*) membrane and the membrane lining the flagellar reservoir also contain reaction product. Intracellularly, the enzyme reaction product is evident within vesicles and vacuoles (lysosomes?) surrounding the flagellar reservoir (arrows) (cross section, ×50,000). (B) Distribution of enzyme reaction product on the surface of an externally disposed flagellum. Flagellar microtubules (electron-dense circles) and a paraxial rod (*PR*) are evident (cross section, ×103,000). (C) Enzyme reaction product uniformly distributed on the external lamina of an isolated *L. donovani* cell surface membrane. Subpellicular microtubules (arrow heads) attached to the membrane cytoplasmic (inner) lamina impart a structural asymmetry to the isolated surface membrane (cross section, ×255,000).

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Table 1. Acid phosphatase activity of homogenates and plasma membranes of *L. donovani* promastigotes. The preparations from a typical experiment were assayed as described (5) with PNPP as substrate. Percentage recovery is the ratio of the total enzyme activity in the plasma membrane fraction relative to the total enzyme activity in the homogenate × 100. The relative specific activity is the cumulative result of six experiments and is expressed as the ratio of specific activity in the membrane fraction to the specific activity in the homogenate ± standard deviation.

Enzyme source	Specific activity	Percentage recovery	Relative specific activity
Homogenate	41.9	100	1.00
Plasma membranes	212.0	51	4.98 ± 0.58

ed from soluble secreted enzyme within the reservoir or from mechanical dislodgement of membrane deposits. Acid phosphatase reaction product was also irregularly present on the flagellar membrane within the reservoir, and on externally disposed portions of flagellar membranes (Fig. 1B). Intracellular acid phosphatase products were almost exclusively distributed in the anterior region (that is, the flagellar end) of cells. Within this area, most enzyme reaction product was localized in vacuoles and vesicles surrounding the flagellar reservoir (Fig. 1A). Such vesicles might represent lysosomes or phagolysosomes because endocytosis occurs within the flagellar reservoir. These vesicles might also represent newly synthesized membrane fated for fusion and insertion into pellicular, flagellar, or reservoir membranes. Another possibility is that such vesicles contain "soluble" enzyme fated for exocytosis and secretion (*8*). Current observations do not permit differentiation or exclusion of any of these hypotheses. Enzyme reaction product was uniformly absent from controls incubated either in sodium fluoride or in the absence of β-glycerophosphate substrate.

Because the subpellicular microtubules remain attached to the cytoplasmic lamina of the isolated *L. donovani* surface membranes, they impart a structural asymmetry to the membranes. Isolated membranes incubated with β-glycerophosphate had an electron-dense lead phosphate reaction product uniformly distributed on their surface (Fig. 1C). Enzyme reaction product was uniformly absent both from the cytoplasmic lamina of these membranes as well as their attached microtubules. The enzyme was therefore asymmetrically oriented within the isolated membranes, presumably with its active site oriented externally in the outer lamina. This observation correlates well with both the cytochemical and biochemical results obtained with intact cells. Enzyme reaction product was uniformly absent in all isolated control membranes.

The membrane-bound enzyme had a *p*H optimum of 5. No alkaline phosphatase activity was discerned in either the homogenates or the subcellular fractions of this organism. Enzyme activity was completely abolished by the addition of sodium fluoride at concentrations equal to or greater than 0.5 m*M*. Activity of the enzyme was neither inhibited nor stimulated by the addition of EDTA, divalent cations, or K^+. The enzyme was active with the following phosphomonoester substrates, which are listed in the order of decreasing activity: fructose-1,6-diphosphate, fructose-6-phosphate, β-glycerophosphate, glucose-6-phosphate, glucose-1-phosphate. The membrane-bound acid phosphatase was differentiated from several other membrane-bound enzymes (that is, Mg^{2+} stimulated adenosinetriphosphatase and a 5′- and a 3′-nucleotidase) on the bases of *p*H optima, as well as sensitivity to inhibitors and metal cofactor requirements.

Acid phosphatase is generally considered as a mammalian lysosomal (marker) enzyme both from biochemical (*9*) and cytochemical (*10*) standpoints. However, "acid phosphatases' have been reported as plasma membrane constituents (*11*). Previously, plasma membrane fractions obtained from several trypanosomatids were enriched for acid phosphatase activity (*12*), although the cytochemical distribution and the physical characteristics of this enzyme were not established. In closely allied trypanosomatid genera and species, acid phosphatase was localized in the flagellar reservoir region (*13*). In contrast, our experiments demonstrate that this enzyme, in addition to being distributed over the entire surface membrane of *L. donovani* promastigotes, is localized intracellularly and within the flagellar reservoir region. Our results not only identify an unusual distribution for this enzyme, but also provide further demonstration of leishmanial membrane asymmetry. In addition to the morphological asymmetry identified by the subpellicular microtubules, there is a functional asymmetry—that is, the active site of the membrane acid phosphatase is accessible to substrate in the extracellular milieu only.

Although the physiologic role of the surface membrane–bound acid phosphatase has not been established, its activity may enable the organism to obtain necessary nutrients from organic phosphates in its environment that would otherwise not be available. Alternatively, the enzyme might play a role in protecting the parasite from the host by inhibiting the digestion of the protozoan in the alimentary tract of the sand-fly vector, and possibly intracellularly in the lysosomal system of macrophages in the mammalian host.

We have examined only the promastigote stage of *L. donovani*; the acid phosphatase content of the amastigote stage remains unknown. Within macrophages, *L. donovani* amastigotes reside and multiply within the host cell lysosomal system, and acid phosphatase has been visualized cytochemically within such parasite-containing phagolysosomes (*1*). This activity may be partially of parasite origin and not due totally to host enzyme as previously assumed. The acid hydrolytic activity in the amastigotes of *L. donovani* will have to be analyzed before the contribution of this enzyme to disease pathogenesis can be determined.

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5. Subcellular fractions were routinely assayed in reaction mixtures containing 50 m*M* sodium acetate, *p*H 5.0, and 5 m*M* substrate (either PNPP or other phosphate esters); incubation was at 42°C for 30 minutes. Intact cells were assayed in mixtures containing tris-maleate buffered saline, *p*H 7.0, containing 5 m*M* substrate, and incubated at 30°C for various times. The reactions in mixtures containing PNPP were terminated by addition of 0.02*M* NaOH, and released *p*-nitrophenol was measured spectrophotometrically at 410 nm. Enzyme activities with other phosphate esters were determined by liberation of inorganic phosphate [L. F. Leloir and C. E. Cardini, *Methods Enzymol.* **3**, 840 (1957)]. Enzyme units and specific activities were expressed as nanomoles of *p*-nitrophenol or inorganic phosphate liberated per minute and enzyme units per milligram of protein, respectively.
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Activation of the Transforming Potential of a Normal Cell Sequence: A Molecular Model for Oncogenesis

Abstract. *The molecularly cloned, long terminal repeat (LTR) of the Moloney sarcoma virus (M-MSV) provirus has been covalently linked to* c-mos, *the cellular homolog of the M-MSV–specific sequence,* v-mos. *These newly constructed clones lack any M-MSV–derived sequences other than the LTR, but in DNA transfection assays they transform cells as efficiently as cloned subgenomic M-MSV fragments containing both* v-mos *and LTR. Cells transformed by LTR:*c-mos *hybrid molecules contain additional copies of* mos *DNA, and several size classes of polyadenylated RNA's with sequence homology to* mos. *The activation of the transforming potential of* c-mos *by the proviral LTR suggests a model whereby LTR-like elements could activate other normal cell sequences with oncogenic potential.*

The transforming retroviruses (that have short periods of latency) (*1–3*) provide an appropriate system for studying the activation of the oncogenicity of normal cellular genes. One such virus, Moloney murine sarcoma virus (M-MSV), is a recombinant between a cellular sequence, *mos*, and Moloney leukemia virus (M-MuLV) (*4–6*). The M-MSV–specific sequence is termed v-*mos* as part of the viral genome while the cellular homolog in normal mouse DNA is termed c-*mos*. We isolated biologically active recombinant DNA clones containing the entire M-MSV proviral genomes of the m1 and HT1 strains (*7, 8*) and used the cloned v-*mos* sequence to identify its cellular homolog and isolate it from normal mouse cell DNA (*9*).

The M-MSV long terminal repeat (LTR) enhances the transforming efficiency of M-MSV v-*mos* (*10, 11*). The proviral LTR contains putative transcriptional control elements (*12–15*), suggesting that the LTR may provide initiation and termination signals for v-*mos* messenger RNA (*10–12*). Cloned cellular DNA containing c-*mos* does not transform cells, although by restriction and heteroduplex analysis c-*mos* and v-*mos* are indistinguishable (*9*). However, c-*mos* was activated by replacing all sequences 5′ to c-*mos* with nontransforming sequences derived from the 5′ end of the m1 MSV provirus, including an LTR (*9, 16*). This result does not establish whether the LTR alone is sufficient to activate the transforming potential of c-*mos*. Therefore, we constructed recombinant DNA clones with an LTR inserted into mouse genomic DNA at various positions 5′ to c-*mos*, and tested their transforming activity. Some of these results have been described (*11*).

The recombinant clones used in our study (Fig. 1) were constructed with the use of LTR sequences from plasmid pm1sp (Fig. 1) (*7, 8, 13*) and c-*mos* sequences from plasmid pMSI (Fig. 1) (*9*). This plasmid is inactive in DNA transfection-transformation assays (*9*), while cloned intact m1 MSV proviral DNA (not shown) can induce 53,000 foci per picomole of DNA (*10*). Clones containing both c-*mos* and LTR sequences (pTS series) also transform. Their specific activities are lower than that of intact cloned M-MSV provirus but equivalent to M-MSV subgenomic fragments that contain v-*mos* and a single LTR (*10*). Clones lacking an LTR are either inactive (pMSB7), or exhibit very low activity (pMSB30; see below). Thus the LTR alone provides all the functions required for efficient activation of the transforming potential of c-*mos* and is capable of activating this sequence even if it is inserted at a distance of about 1500 base pairs (bp) in front of c-*mos* (Fig. 1, pTS101). Furthermore, the entire LTR is not required for c-*mos* activation since the pTS clones lack the 115 bp at the 3′ terminal of LTR, which includes a puta-

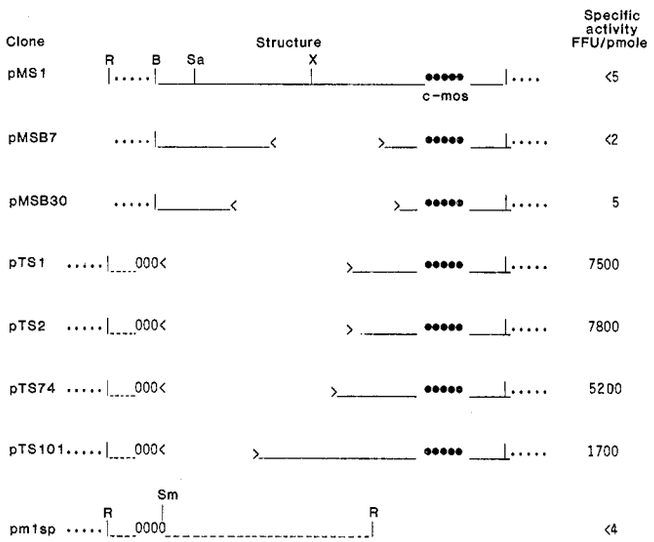

Fig. 1. Physical maps and specific activities of recombinant clones containing *c-mos*; (●●●) *c-mos* (*9*); (000) LTR (*12, 13*); (——) normal mouse DNA sequences (*9*); (····) plasmid pBR322 sequences; (- - -) normal mink sequences (*7, 8*). Restriction endonuclease sites are labeled *R*, Eco RI; B, Bgl II or Bam HI; Sa, Sac I; X, Xba I; Sm, Sma I. The pointed brackets (< >) approximately enclose the Bal 31 deletions. To generate the clones shown, plasmid pMSI was opened at either the Sac I or Xba I site and subjected to limited digestion with exonuclease Bal 31 to remove varying amounts of normal mouse cellular sequences. The resulting DNA was either closed again without further treatment (as in pMSB7 and pMSB30), or it was digested with Eco RI and linked to an Eco RI–Sma I fragment from plasmid pm1sp to generate the four pTS clones. The plasmid pMSB7 has ~ 1000 bp of normal mouse sequence removed by this procedure, whereas pMSB30 has ~ 1300 bp removed. In pTS1 and pTS2, the LTR is placed ~ 600 bp upstream to the 5′ end of c-*mos*, while in pTS74 and pTS101 the LTR is ~ 800 and ~ 1500 bp upstream, respectively. Cloned DNA was linearized by treatment with either Eco RI or Bam HI and then was used to transfect NIH 3T3 cells as described (*10*). Specific activities represent the number of foci induced per 2.5×10^5 cells transfected, and were calculated from at least four replicate determinations. The lower limits represent the limit of detectability (no foci observed) in these determinations.

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