Internal and Surface-Localized Major Surface Proteases of *Leishmania* spp. and Their Differential Release from Promastigotes^\textsuperscript{\textcopyright}\n
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Major surface protease (MSP), also called GP63, is a virulence factor of *Leishmania* spp. protozoa. There are three pools of MSP, located either internally within the parasite, anchored to the surface membrane, or released into the extracellular environment. The regulation and biological functions of these MSP pools are unknown. We investigated here the trafficking and extrusion of surface versus internal MSPs. Virulent *Leishmania chagasi* undergo a growth-associated lengthening in the *t*\textsubscript{1/2} of surface-localized MSP, but this did not occur in the attenuated L5 strain. The release of surface-localized MSP was enhanced in a dose-dependent manner by MβCD, which chelates membrane cholesterol-ergosterol. Furthermore, incubation of promastigotes at 37°C with Matrigel matrix, a soluble basement membrane extract of Engelbreth-Holm-Swarm tumor cells, stimulated the release of internal MSP but not of surface-located MSP. Taken together, these data indicate that MSP subpopulations in distinct cellular locations are released from the parasite under different environmental conditions. We hypothesize that the internal MSP with its lengthy *t*\textsubscript{1/2} does not serve as a pool for promastigote surface MSP in the sand fly vector but that it instead functions as an MSP pool ready for quick release upon inoculation of metacyclic promastigotes into mammals. We present a model in which these different MSP pools are released under distinct life cycle-specific conditions.

The digenetic protozoa of *Leishmania* spp. shuttle between an extracellular promastigote form in the sand fly vector and an intracellular amastigote form in mammalian hosts, including humans. In the sand fly, the avirulent procyclic promastigotes develop to the virulent metacyclic organisms, a process termed metacyclogenesis that can be mimicked by in vitro cultivation of logarithmic- to stationary-phase promastigotes (36). *Leishmania* causes 1.5 to 2 million new cases of human leishmaniasis annually, with manifestations ranging from self-healing cutaneous sores to life-threatening visceral leishmaniasis (8, 9). Among a few well-characterized *Leishmania* virulence factors is the major surface protease (MSP), also called GP63. MSP plays several important roles during *Leishmania* spp. infection of mammals, including (i) enhancing promastigote phagocytosis by macrophages, (ii) facilitating promastigote evasion of complement-mediated lysis, and (iii) promoting amastigote survival in the phagolysosomes of macrophages (see reference 45 for a review). There is also evidence suggesting that in the sand fly MSP plays a role in the early-stage development of promastigotes (16) and contributes to promastigote adhesion in the guts and salivary glands (see reference 37 for a review).

MSP is encoded by a family of highly conserved genes organized in a tandem array. MSP genes (MSPs) and homologues have been found in all *Leishmania* spp. studied to date, as well as in other trypanosomatids, including the monoxenous insect parasite *Crithidia* and the extracellular mammalian parasite *Trypanosoma brucei* (11, 13, 45). The number of MSPs in individual trypanosomatids ranges from seven in *L. major*, to dozens in *L. braziliensis*, to hundreds in *T. cruzi* (12, 28, 39, 41).

At least 18 MSPs are present in *L. chagasi*, the causative protozoan of visceral leishmaniasis in Latin America (33, 35). During in vitro promastigote growth of virulent strain *L. chagasi* from the logarithmic to the stationary phase, MSP protein abundance increases 14-fold. Concomitantly, the number of MSP isoforms observed on two-dimensional gel electrophoresis (2-DE) increases from 4 to 11 (46–48). In the present study we use “MSP” or “MSPs” when referring to properties of all MSP isoforms and “MSP isoforms” when referring to specific MSP isoforms.

In addition to detecting surface MSP, we and other groups have independently found that MSP is released into the extracellular medium from *Leishmania* spp. and other trypanosomatids (7, 10, 18, 26, 46). Moreover, a subpopulation of internal MSPs has been detected and appears to be stable for several days (42, 47). Collectively, data generated from several laboratories, including our own, have demonstrated the existence of three subpopulations, i.e., surface-localized MSP, internal MSP, and released MSP.

We hypothesize that these three MSP subpopulations are separately trafficked through the cell to interact with the environment, and that internal MSP serves as a pool ready for rapid release after inoculation of metacyclic promastigotes into mammalian skin. We previously showed that the half-life (*t*\textsubscript{1/2}) of surface-localized 63-kDa MSP in virulent *L. chagasi* increases 75% during promastigote growth from the logarithmic to the stationary phase (47). In the present study, we demonstrate that this growth-associated regulation of surface-local-
ized MSP $I_{L5}$ diminished in the attenuated L5 *L. chagasi* strain. Furthermore, we report that the membrane lipid disruption reagent methyl-$eta$-cyclodextrin (M$eta$CD) enhanced the release of surface-localized MSP into the extracellular medium, whereas the internal MSP was released only after environmental exposure to an in vitro extracellular matrix modeling basement membrane, but only at the elevated temperature characteristic of a mammalian host. These data suggest that the different MSP pools are regulated independently and play distinct functions during the life cycle of *Leishmania* spp. A model illustrating the potential relevance of these findings during the parasite life cycle is presented.

**MATERIALS AND METHODS**

**Parasites.** A Brazilian strain of *L. chagasi* (MHOM/BR/00/1669) was continuously passed, by intracardiac injection of amastigotes, in golden hamsters to maintain its virulence. Amastigotes were isolated from the spleens of infected hamsters and transformed to promastigotes at 26°C, in hemoglobin-lactate-modified minimal essential medium with 10% heat-inactivated fetal calf serum (HOMEM; reagents from Gibco, Rockville, MO). Virulent promastigotes were passaged weekly in HOMEM and used within five passages. The attenuated L5 strain of *L. chagasi* has been continuously cultured in vitro in HOMEM for over 9 years (43). Strain L5 differs from the virulent strain in several respects, including (i) less-abundant MSP and the expression of only MSP1 genes (6, 43, 48), (ii) a shorter and simpler lipophosphoglycan (27), and (iii) reduced virulence for rodent models (43). In some experiments, virulent promastigotes were spread on semisolid M199 agar plates to obtain clonal cells (19). A total of 124 clones were established in two independent experiments. Promastigote cultures were started at a cell density of 10$^5$ cell/ml at day 0 of cultivation. Logarithmic- and stationary-phase promastigotes were collected between days 2 and 4 and days 6 and 9, respectively, with phases defined according to cell density and morphology as previously described (49).

**Chemicals and antibodies.** Sulfo-NHS-biotin, streptavidin agarose beads, and growth factor reduced Matrigel matrix were purchased from Pierce (Rockford, IL), Sigma (St. Louis, MO), and BD Biosciences (Bedford, MA), respectively. M$eta$CD, protein G-agarose beads and ProteoLyzing Plus (Sigma-Aldrich, St. Louis, MO), and biotinylated streptavidin agarose (Sigma-Aldrich, St. Louis, MO), and Promix for 0.5 h, and triplicate samples were assayed by a liquid scintillation analyzer for incorporation of radioisotope after total proteins were precipitated with trichloroacetic acid as described previously (3). The relative 35S-labeled reagent M$eta$CD affects the release of surface-localized MSP, stationary-phase promastigotes were incubated in either 0 or 15 mM M$eta$CD for 3 h after surface biotinylation. Triplicate samples of cells were suspended to a density of 2 $\times$ 10$^5$ cells/ml, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS. Both biotinylated proteins and the internal MSP were analyzed.

**MSP release into Matrigel matrix.** Stationary-phase promastigotes in the first passage after being converted from amastigotes isolated from hamsters were surface biotinylated. Triplicate samples of cells were suspended to a density of 2 $\times$ 10$^5$ cells/ml, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS, in ice-cold HBSS. Cultures were then incubated at either room temperature or 37°C for 2 hours. Matrigel matrix solidified under these conditions. The mixtures were transferred to 4°C overnight to liquefy the matrix, and promastigote cells were separated from the liquefied Matrigel matrix by centrifugation. Biotinylated proteins and the nonbiotinylated MSP were collected from both the whole cellular lysate and the liquefied Matrigel matrix.

**Electrophoresis and protein detection.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting were conducted as described previously (46). Autoradiography was achieved by exposing X-ray MR films (Kodak, Rochester, NY). Samples analyzed by 2-DE were separated in the first dimension by isoelectric focusing (IEF) in Immobiline Drystrips pH 4-7 (Amersham) and in the second dimension according to size in SDS–7.5% polyacrylamide gels (48). In the case of cellular lysates of individual clones, the samples were separated in Immobiline Drystrips pH 4-7 (Amersham), after which proteins were transferred to a nitrocellulose membrane and MSP was detected by Western blotting.

**RESULTS**

**MSP expression in clonal *L. chagasi* lines.** Virulent stationary-phase *L. chagasi* promastigotes contain at least 11 isoforms of MSP according to 2-DE immunoblots. However, there have been no documented differences in the function or localization of these MSP isoforms. At least some of the different MSP isoforms are derived from different MSPs (47, 48). We approached the question of differential function by carefully characterizing MSP isoforms. Our prior work documents MSP isoforms in uncloned *L. chagasi* populations (47, 48); Fig. 1 shows 2-DE MSP profiles in clonal *L. chagasi* cell lines. Clonal lines were derived from a stationary-phase promastigote population on semisolid M199 agar plates. Proteins were separated by IEF and transferred to nitrocellulose membranes, and MSPs were detected by immunoblotting with polyvalent antiserum against MSP. An MSP profile similar to that of the entire population was observed for all 124 clones examined; representative examples shown in Fig. 1. Eight bands between...
isolectric points (pl) 5.8 and 6.7 were detected, along with an additional three bands between pl 4.8 and 5.2. Each of these bands formed a spot in the second dimension, as shown in immunoblots of 24 representative clones (Fig. 1). As we anticipated but thought it important to investigate, we did not detect clonal variation in MSP expression during growth in vitro.

Surface-localized MSP isoforms are differently regulated in attenuated and virulent strains. The MSP proteins of *L. chagasi* promastigotes are found in three cellular locations, i.e., internal, surface, and released into the environment (46, 47). The cellular distribution of MSP proteins changes during in vitro growth. We previously showed that an increase in total promastigote MSP content during “in vitro metacyclogenesis” is associated with a decrease in the rate of MSP release into the environment. To study the population of MSP released we contrasted MSP synthesis in, and loss from, virulent promastigotes compared to a nonvirulent attenuated line of parasites (L5) that does not undergo changes in total MSP content during metacyclogenesis (Fig. 2). For the purposes of comparison, we contrasted the 63-kDa MSP, an isoform that is expressed in both promastigote lines.

First, the rate of MSP synthesis in both virulent and attenuated lines, in both logarithmic- and stationary-growth phases, was almost identical (Fig. 2B). L5 or virulent *L. chagasi* promastigotes were metabolically labeled with [35S]methionine, MSPs were immunoprecipitated, and newly synthesized MSPs were detected by autoradiogram. Cytosolic P36, which is constitutively expressed (22, 47), was used as a control. The ratio of MSP to P36 remained constant in the logarithmic- and stationary-phase promastigotes of both L5 and virulent strains.

Second, the $t_{1/2}$ of cellular surface MSP was longer in stationary virulent promastigotes than in logarithmic virulent promastigotes, coinciding with its increased abundance in stationary virulent promastigotes. In contrast, surface MSP was lost at a uniform rate in the growth phases of attenuated L5 parasites (Fig. 2A). Both virulent and attenuated promastigotes were surface labeled by biotinylation during the logarithmic or stationary phase of growth and chased over the next 72 h. Immuno blotting was used to confirm that the indicated bands were indeed MSPs (not shown). The surface MSP of virulent *L. chagasi* promastigotes had a shorter $t_{1/2}$ (52 h) when labeled in logarithmic growth phase than MSP proteins labeled in stationary phase (90 h; Fig. 2A). Whether this is due to the predominant MSP isoforms synthesized in the different growth stages or to other factors inherent in the growth phase of proteins with [35S]methionine-cysteine and immunoprecipitation with polyclonal antisera to MSP or P36. The ratio of MSP to P36 is presented. The data shown are representative of two independent experiments. (C) Release of surface-localized MSP into the extracellular medium. Attenuated L5 or virulent *L. chagasi* promastigotes during logarithmic (L)- or stationary (S)-phase growth were surface biotinylated and resuspended in fresh medium. Extracellular medium was collected 48 h later. MSP was detected in the streptavidin-bead pull-down fraction by Western blotting. Shown is the relative MSP abundance standardized to stationary-phase virulent promastigotes, which was set at one arbitrary unit. The results for one of two independent experiments are shown.
parasites cannot be determined (46, 47). In contrast, the $t_{1/2}$ of surface-localized 63-kDa MSP in the attenuated L5 strain of *L. chagasi* promastigotes remained unchanged during growth from the logarithmic (51 h) to the stationary (52 h) phase. Indeed, the MSP $t_{1/2}$ was almost identical to that of logarithmic-growth-phase virulent strain promastigotes (52 h) (Fig. 2A). In contrast to surface MSP, the internal MSP of both virulent and attenuated L5 strains remained stable throughout promastigote growth (Fig. 2A) (47).

Third, the mechanism differentiating the $t_{1/2}$ of surface MSP in virulent as opposed to attenuated L5 promastigotes was a difference in the rate of MSP shedding into the medium. Surface MSP was labeled by biotinylation in both L5 and virulent strain parasites. Parasites were then incubated in fresh medium, and surface biotinylated MSPs were detected by Western blotting of the streptavidin bead-purified fraction of the spent media after 48 h of incubation. A minimum of fourfold more MSPs was found in the spent media of both the logarithmic and the stationary phases of L5 strain and the logarithmic phase of the virulent strain than the stationary phase of the virulent strain (Fig. 2C). Collectively, these data indicate the increase in surface-localized MSP in the stationary-phase virulent promastigotes is associated with a decrease in the rate of shedding into the environment compared to logarithmic-phase virulent promastigotes. There is no similar growth phase-dependent regulation of MSP in the L5 attenuated strain of *L. chagasi*.

**MβCD enhances the release of the surface-localized MSP isofoms.** The unique retention of surface MSP by virulent stationary-phase promastigotes could be due to its association with surface lipid-containing membrane domains. MβCD depletes lipid rafts from the plasma membranes of a variety of mammalian cells by chelating and transiently removing membrane cholesterol (14, 17, 21, 24, 31, 40). Based on the hypothesis that differential association of MSP with membrane lipids could account for its release by logarithmic promastigotes and retention by stationary promastigotes, we reasoned that membrane lipid disruption with MβCD could enhance MSP release from the *Leishmania* membrane. In replicate experiments, virulent *L. chagasi* promastigotes were treated with 0, 5, 10, or 15 mM MβCD for 48 h. A dose-dependent augmented release of MSP into the extracellular medium was observed. Specifically, control cells (0 mM MβCD) released ca. 35% of MSP, whereas the cells in 15 mM MβCD released ~80% of MSP into the extracellular medium (Fig. 3A and B).

To eliminate the possibility that the enhanced MSP release was due to a toxic effect of MβCD on promastigotes, the rate of promastigote protein synthesis was measured in the absence or presence of MβCD under the experimental conditions. Comparable levels of [35S]-radioisotopes were incorporated into newly synthesized proteins of untreated or MβCD-treated cells (Fig. 3C), indicating that MβCD treatment of promastigotes under these conditions is not detrimental to the cells. Furthermore, the growth curves of untreated or MβCD-treated cells in HOMEM were similar (data not shown). Thus, perturbing the plasma membrane lipid-containing domains of stationary-phase promastigotes accelerates MSP release into the extracellular medium, although it does not appear to harm the promastigotes in culture.

To test the hypothesis that disruption of membrane lipid-
containing domains with MβCD only promotes release of surface-localized MSP, stationary-phase promastigotes were treated with 15 mM MβCD for 3 h after surface biotinylation. Control cells were treated identically but received no MβCD. Spent medium was collected, from which surface-biotinylated proteins were isolated by streptavidin affinity purification. Internal MSP was purified by immunoprecipitation from the streptavidin-cleared fraction of the spent medium. Immunoblots were used to assay for the presence of MSP. As shown in Fig. 3D, nonbiotinylated, internal MSP was not detectable in extracellular medium. In contrast, biotinylated, surface MSP was 34.6% ± 12.8% (n = 3) more abundant in the spent media of the MβCD-treated cells than in controls. Furthermore, no cytoskeletal β-tubulin and cytosolic P36 markers were detected by immunoblotting in the clear fraction of the same spent media after biotin-streptavidin affinity purification and MSP immunoprecipitation (Fig. 3D and data not shown), which eliminates the possibility that MSP release is due to cell lysis. These data indicate that MβCD enhances the release of only surface-localized MSP, a result consistent with the possibility that MSP stabilization in the surface membrane requires an association with cholesterol/ergosterol-containing lipid domains.

Release of internal MSP isoforms is stimulated by the Matrigel matrix, specifically at 37°C. Although surface MSP can be artificially released by disrupting membrane lipid domains, the natural evolution of stationary promastigotes in the sand fly is to a cellular state that retains surface MSP. Metacyclic promastigotes are inoculated by sand flies into mammalian tissues, whereupon they initially encounter an elevated temperature and components of extracellular mammalian environment. We investigated whether MSP would be released under conditions that mimic the in vivo setting. First, we tested whether the highest mammalian body temperature encountered by the parasite, i.e., 37°C, would stimulate internal MSP release. Stationary-phase promastigotes were metabolically labeled, surface biotinylated, and subsequently incubated at 37°C for 24 h to test for release of surface versus internal MSP. Similarly treated control promastigotes were incubated at room temperature. Surface and internal MSPs were immunoprecipitated from the streptavidin bead-enriched or -cleared cellular lysates and detected by autoradiography. Under these conditions, there was no detectable change in internal versus surface MSP in the promastigotes after 24 h at a higher temperature (data not shown). These data suggest that a temperature increase to 37°C is by itself insufficient to stimulate internal MSP release.

We then incubated stationary-phase promastigotes in the Matrigel matrix at 37°C to test whether this combination would stimulate the release of internal MSP. Matrigel matrix is a soluble basement membrane extract of Engelbreth-Holm-Swarm tumor cells, which has been used to study the metastasis of cancer cells (29, 34). One prominent feature of this matrix is that it is a liquid at 4°C but it gels at room temperature and above, forming a reconstituted basement membrane. Consequently, when promastigotes are incubated in the matrix at 37°C, this setting experimentally mimics the site of sand fly inoculation into a mammalian host.

Stationary-phase promastigotes were surface biotinylated prior to incubation in either Matrigel matrix or HBSS. Promastigotes incubated in HBSS released surface MSP but little or no internal MSP into the extracellular medium at room temperature. Neither MSP form, either surface or internal, was substantially released at 37°C (Fig. 4A and B). In contrast, incubation of promastigotes in the Matrigel matrix for 3 h at 37°C stimulated release of mostly internal MSP (Fig. 4A and B). This effect was enhanced by a longer (3 versus 1 h) incubation time.
bation time. Strikingly, the effect of Matrigel on release of internal MSP was significantly lower at room temperature, whereas more surface MSP was released under these conditions (Fig. 4A and B). Furthermore, the level of total internal MSP was significantly higher in parasites incubated in Matrigel compared to HBSS, although there was no change in internal MSP when parasites were incubated at room temperature versus 37°C (Fig. 4C). Hence, it is very unlikely that the specific release of internal MSP stimulated by a combination of Matrigel matrix and 37°C was due to leakiness of intracellular content from damaged promastigotes, even though we cannot formally eliminate this possibility at this time. Overall, these results lead us to conclude that surface MSP is released at room temperature and that this release is inhibited at 37°C, whereas internal MSP is released in response to the presence of Matrigel matrix, specifically at 37°C (Fig. 4).

DISCUSSION

MSPs are among the most abundant cellular proteins in promastigotes of all Leishmania spp. studied to date. Indeed, in L. mexicana, MSPs account for 1 and 0.1% of total proteins in promastigotes and amastigotes, respectively (1). Promastigote cell-associated MSP is predominantly attached to the cell surface by glycosylphosphatidylinositol anchors (4, 5). However, our laboratory and others have observed that as much as one-third of the cell-associated MSP is located intracellularly, as determined by a combination of surface biotinylation, immunoelectron microscopy, and cytofluorometry (42, 47). Furthermore, the internal MSP in L. chagasi is so stable that no reduction in abundance is detected for up to 6 days using pulse-chase analysis (47). We hypothesized that the surface-localized and internal MSPs are regulated separately via different mechanisms. Furthermore, the role and origin of the MSP released by promastigotes into extracellular medium has as yet been uncharacterized. In the present study we showed by using MboCD that the decreased release of surface MSP by the virulent stationary promastigotes is associated with the content of membrane lipids, since MboCD-mediated removal of cholesterol/ergosterol specifically enhanced the release of surface-localized MSP into extracellular medium. This likely reflects changes in the promastigote membrane during metacyclogenesis, in that a lipid-rich membrane retaining MSP in metacyclic parasites could promote retention of high surface levels of this virulence factor. In contrast, exposure to conditions mimicking mammalian tissue with Matrigel at 37°C stimulated the release of internal, but not surface, MSP. These data demonstrate for the first time that the surface-localized and internal MSPs are trafficked out of the promastigote cell in response to different external stimuli.

Phenotypic variation has been found in isoforms of a 235-kDa rhoptry protein between clones of Plasmodium yoelii parasites. This protein is encoded by a multigene family of ~50 genes and may be involved in the selection of red blood cells for invasion by merozoites (2, 15, 20, 30, 38). Because there are at least 11 MSP isoforms in stationary-phase virulent L. chagasi promastigotes (47, 48), we hypothesized that similar variation between L. chagasi parasites could yield clonal isolates that express one or a few MSPs. However, we were not able to document clonal variation in MSP expression by cells expanded from individual clones by using 2-DE immunoblotting. This does not prove that all parasite clones express all MSP isoforms or that individual parasite clones cannot express only one or a few MSP isoforms in vivo. Nonetheless, according to our ability to detect we tentatively conclude that at least some L. chagasi parasites are able to express the majority of MSP isoforms when derived from a single cloned cell.

The three MSP classes of mRNAs (MSPL, MSPS, and MSPC) in L. chagasi are posttranscriptionally regulated. In the case of MSPL mRNA this regulation is known to occur specifically at the level of mRNA stability (6, 32, 44). Regarding MSP regulation at the protein level, we showed herein that the measurable rate of MSP synthesis was very similar throughout promastigote growth in vitro from logarithmic to stationary phase, a finding consistent with our earlier report (47). Therefore, the growth-associated 14-fold increase in the abundance of cell-associated MSP must be posttranslationally regulated. An increase in protein stability, associated with decreased MSP shedding, accounts for a fivefold increase (47). We show here that the internal pool of MSP is extremely stable throughout growth of both attenuated L5 and virulent parasite strains. Consequently, internal MSP appears not to be affected by the growth-associated regulation of MSP stability. We also demonstrate that the t1/2 of surface-localized 63-kDa MSP in the attenuated strain is similar to that of the lower MSP-expressing, logarithmic-phase promastigotes of the virulent strain, regardless of the growth phase (Fig. 2). One plausible explanation for this difference between attenuated and virulent strains during growth is the different rates of MSP shedding. We documented that the rate of MSP shedding by stationary-phase virulent promastigotes is slower than that of logarithmic-phase virulent promastigotes and that MSP shedding by L5 attenuated promastigotes is more rapid than virulent L. chagasi in all growth phases (Fig. 2C).

The biochemical mechanisms by which Leishmania spp. promastigotes regulate MSP release are not well understood. Released MSPs have electrophoretic mobilities similar to those of their cell-associated counterparts (46). At least some surface-localized L. amazonensis MSP is released through autoproteolytic activity, as shown by site-specific mutation and inhibition by a zinc chelator (26). We previously determined that released MSP does not bind to a antibody against the cross-reactive determinant, suggesting it is not released by a phosphatidylinositol-specific phospholipase C (46) similar to the released MSP of L. amazonensis. Released L. amazonensis MSP does not contain ethanolamine, suggesting it lacks a glycosylphosphatidylinositol membrane anchor (26). The data generated here by using lipid chelation suggests that the decreased release of MSP from stationary virulent promastigotes is due to remodeling of the surface membrane such that MSP is retained in association with lipids. We cannot rule out the additional possibility that there may also be recycling and degradation of MSP as a means of decreasing cellular levels of MSP, but this has yet to be tested.

In addition to the above evidence that MSP release by virulent promastigotes requires a specific membrane lipid composition, we approached the mechanisms by which Leishmania spp. promastigotes release MSP using a model of in vivo conditions. The Matrigel matrix contains laminin, collagen IV, entacan, heparin sulfate proteoglycan, growth factors, collage-
might be related to the nutrient requirements of logarithmic growth of the malian host environment. A model for MSP regulation in the masticates, and the lower diagram depicts release from their metacyclic (stationary-phase) counterparts. In the mammalian host, a metacyclic promastigote is depicted. Mφ, macrophage.

The goal of the present study was to address how the three MSP subpopulations (surface, internal, and released) are regulated during metacyclogenesis and in response to the mammalian host environment. A model for MSP regulation in the different promastigote environments is illustrated in Fig. 5. In this model MSP is abundantly released by the dividing, procytic promastigotes in the sand fly gut, as simulated by the logarithmic growth of L. chagasi in culture. This released MSP might be related to the nutrient requirements of Leishmania in the insect gut environment, where residual mammalian blood from the sand fly meal is a main source of nutrients. Indeed, it has been shown that downregulation of MSP in L. amazonensis reduces the parasites' early development in sand flies (16). As procytic promastigotes develop to metacyclic promastigotes, the rate of released surface-localized MSP decreases and the abundance of surface-localized MSP increases (47). Our data suggest that this increase is due to association of metacytic MSP with lipid-containing membrane domains. Internal MSP is not released during metacyclogenesis. However, after inoculation into mammalian subcutaneous tissue by a sand fly vector, metacytic promastigotes encounter a temperature increase, host extracellular matrix, and innate immune mechanisms such as complement, antimicrobial peptides, and phagocytic cells. In response to these stimuli, promastigotes could release internal MSP into mammalian tissue. It is thus logical to consider the possibility that the surface-localized MSP plays a role in the promastigotes' evasion of complement-mediated killing and their phagocytosis and/or internalization by macrophages and other cells. Internal MSP, on the other hand, may play a role in the degradation of extracellular matrix components such as collagens IV and fibronectin, as suggested in a prior report on an L. amazonensis (25). As such, it could facilitate promastigote migration toward cells such as macrophages, dendritic cells, and fibroblasts that are favorable for parasite entry and long-term survival. Thus, it is likely that the many isoforms of MSP protease facilitate parasite survival through different mechanisms in the diverse host and vector environments encountered by the parasite.

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