Major Surface Protease (MSP, or GP63) of Trypanosomatids, One Size Fits All?

Running title: Trypanosomatid major surface protease

Chaoqun Yao*

Department of Veterinary Sciences and Wyoming State Veterinary Laboratory, University of Wyoming, Laramie, WY 82070

*Correspondent: Dr. Chaoqun Yao, Department of Veterinary Sciences and Wyoming State Veterinary Laboratory, University of Wyoming, Laramie, WY 82070. Phone: (307)742-6638; Fax: (307)721-2051; Email: cyao@uwyo.edu
Abstract

Major surface protease (MSP, or GP63) is the most abundant glycoprotein localized to the plasma membrane of *Leishmania* promastigotes. It plays several important roles in the pathogenesis of leishmaniasis. These include, but are not limited to: 1) evading complement-mediated lysis, 2) facilitating macrophage (Mø) phagocytosis of promastigotes, 3) interacting with extracellular matrix, 4) inhibiting natural killer cellular functions, 5) resisting to antimicrobial peptide killing, 6) degrading cytosolic proteins of Mø and fibroblasts, and 7) promoting survival of the intracellular amastigotes in Mø. MSP homologues have been found in all other trypanosomatids studied to date including heteroxenous members of *Trypanosoma cruzi*, the extracellular *T. brucei*, unusual intraerythrocytic *Endotrypanum* spp., and phytoparasitic *Phytomonas* spp. and numerous monoxenous species. They very likely perform different roles than those in *Leishmania* spp. Multiple MSPs in individual cells may play distinct roles at some time points and collaborative roles at the others in trypanosomatid life cycles; they may play redundant roles at most time. The cellular locations and the extracellular release of MSPs are also discussed in connection with MSP functions in leishmanial promastigotes.
Trypanosomatids are a very diverse group of protozoa including many species of medical, veterinary and/or economical importance. *Leishmania* spp. cause disease on the continents of Asia, Africa, Europe and America; *Trypanosoma cruzi* is the etiology of Chagas’ disease in South and Central America; and *T. brucei* infections result in African sleeping sickness. Four genera of heteroxenous trypanosomatids transmitted by insect vectors infect a wide range of hosts including humans, animals and plants. Six genera of monoxenous trypanosomatids parasitize numerous species of insects. Due to this diversity, the life cycle of *Leishmania* spp. is presented as a model to which other heteroxenous and monoxenous groups will be briefly contrasted.

During their life cycle, *Leishmania* spp. shuttle between a flagellated promastigote stage in the sand fly vector and a non-motile amastigote stage in a mammalian host. During blood meals on infected animals, the sand fly vectors ingest amastigote-laden macrophages (Mø). These amastigotes transform to procyclic promastigotes in the sand fly gut, multiply by binary fission and eventually develop into metacyclic promastigotes, the infective stage for mammalian hosts including humans. The sand fly vectors, upon taking another blood meal, inoculate the metacyclic promastigotes into dermis where they are phagocytized by Mø of the mammalian hosts. Amastigotes, transformed from metacyclic promastigotes, multiply in parasitophorous vacuoles (PV) of the host’s Mø. Multiplication of amastigotes eventually leads to rupture of the infected Mø and release of amastigotes that infect additional Mø, perpetuating the cycle of replication (38).
Other trypanosomatids divert this model of the life cycle in different ways. (A) *T. cruzi* is transmitted to humans by the reduviid (or kissing) bug by fecal contamination of skin wounds or mucous membranes while taking a blood meal. In the human host, *T. cruzi* exists in amastigote and trypomastigote forms. The intracellular amastigote stage multiplies within virtually any cell. The extracellular trypomastigotes are disseminated in the blood to all parts of the body (38). (B) *T. brucei* is transmitted to humans by a tsetse fly during blood meals. This parasite lives extracellularly as trypomastigotes in blood, lymph and cerebrospinal fluid (90). (C) Two species of *Endotrypanum* spp. are parasites of forest-dwelling tree sloths in South America. Both *E. schaudinni* and *E. monterogeli* have an unusual intraerythrocytic stage (53, 75) as trypomastigotes and epimastigotes, respectively, even though both use sand flies as vectors (24). (D) *Phytomonas* spp., transmitted between plants by phytophagous insects, infect several plants of great economic importance in widespread geographic regions (13). (E) All monoxenous trypanosomatids are parasites of various insects. They consist of six genera including *Blastocrithidia, Crithidia, Leptomonas, Herpetomonas, Rhynchoidomonas*, and *Phytomonas* (87).

*Leishmania* spp. harbor a zinc metalloprotease, discovered in the mid 1980s, on their surface (14, 15, 36, 37, 88). This enzyme, accounting for about 1% of the total protein in promastigotes of *L. major* and *L. mexicana* (2, 10) and being potentially important during different stages of the life cycle, soon became the object of much investigation (8, 70, 71, 107). Although referred to here as major surface protease (MSP), this zinc metalloprotease has also been termed GP63, surface acid protease, promastigote surface protease, EC 3.4.24.36 and leishmanolysin.
MSPs of *Leishmania* spp. belong to the M8 family of endopeptidases, sharing several characteristics with mammalian matrix metalloproteases. Similarities include degradation of the extracellular matrix, cell surface localization, protease activity requiring \( \text{Zn}^{2+} \), and inhibition of protease activity by chelating agents and \( \alpha-2 \)-macroglobulin (for review, see Ref. (70). Many, but not all, of the *Leishmania* MSPs are bound to the outer leaf of the plasma membrane of promastigotes by a glycosylphosphatidylinositol (GPI) anchor (9). MSPs are encoded by multiple *MSP* genes that may vary in sequence, especially in the untranslated regions. *MSP* genes of all *Leishmania* spp studied to date are usually in tandem array and are often differentially expressed during various life-cycle stages (72, 91, 92, 101). This heterogeneity in *MSP* genes without affecting enzymatic domains makes stage-specific regulation possible.

Unlike other eukaryotic cells, trypanosomatids do not have classic promoters for RNA polymerase II. Long arrayed protein-coding genes are transcribed to polycistronic RNA precursors. Mature mRNAs derive from a coupled process in which trans-splicing of capped splicing leader at the 5' end of one gene is coupled to the polyadenylation addition of the upstream gene (20). Redundancy in certain genes including *MSP* genes appears to be a mechanism to affect mature mRNA production, and therefore, protein abundance.

The genomes of *L. major*, *L. infantum*, and *L. braziliensis* contain 4, 5 and 33 *MSP* genes in tandem array on chromosome 10 with an additional 2, 3 and 6 *MSP*-like genes located on chromosome 28 and/or 31, respectively (49, 84). These numbers differ significantly from the earlier reported 7 and \( \geq 18 \) *MSP* genes in tandem array detected by partial digestion of genomic DNAs and Southern blotting of *L. major* and the South American *L. chagasi*, respectively (91,
The latter species is considered synonymous with *L. infantum* by many authors (60, 65, 66, 76, 89). This divergence in number of MSP genes appears related to the different parasitic strains used in these studies [MHOM/IL/81Friedlin zymodeme MON-103 vs. NIH S (MHOM/SN/74/Seisman) of *L. major*; JPCM5 (MCAN/ES/98/LLM-877) vs. (MHOM/BR/00/1669) of *L. infantum*], although the biological significance remains to be assessed. Alternatively, variation might be caused by technical challenges in genome sequencing. Identical tandem-arrayed genes may not have been able to be correctly resolved for gene copy number. In contrast, Southern blots of the partially digested genomic DNA subjected to resolution by electrophoresis are capable of detecting gene copy numbers of identical tandem-arrayed genes.

Another GPI-anchored molecule, lipophosphoglycan (LPG), is the dominant molecule on the surface of *Leishmania* spp. promastigotes. Intriguingly, protective roles for LPG and MSP overlap, including resistance to complement-mediated lysis (CML) and facilitating phagocytosis by host Mo. LPG also functions in the binding to and release of the parasite from the midgut of the sand fly vector, accounting for retrograde migration of the metacyclic promastigote to the sand fly proboscis (6, 16). Since roles for both MSP and LPG overlap, they may function synergistically in the pathogenesis of leishmaniasis or they may function redundantly with the failure of one being compensated for by the other.

**A. MSPs’ role in the pathogenesis of leishmaniasis**

Two important roles of MSP in the pathogenesis of leishmaniasis were comprehensively reviewed (107). These include resistance to CML and facilitating phagocytosis of extracellular
promastigotes by host Mø (107), briefly summarized here to avoid redundancy. MSPs of promastigotes bind to C3 with high affinity. Proteolysis of the active component C3b to the inactive C3bi inhibits CML (11, 93). MSP\textsuperscript{-} null mutant promastigotes of L. major were generated by deleting all seven tandem MSP genes on chromosome 10. Mutant cells derived from either sand flies or in vitro cultures exhibited increased sensitivity to CML. In contrast, mutant cells expressing a cloned MSP gene were significantly resistant to CML (52).

MSPs facilitate phagocytosis of promastigotes by host Mø. This is supported by observations from multiple labs collectively showing that preincubation of Mø with purified MSPs or presence of antibodies specific to MSP significantly block phagocytosis of promastigotes of L. amazonensis, L. braziliensis, L. Mexicana, and L. chagasi (14, 63, 94, 103). Nevertheless, MSP proteolytic activity is not an absolute requirement for phagocytosis. This is supported by evidence generated from mutants lacking MSP activity (104) or MSP-low cells overexpressing a mutated inactive form of MSP in the zinc binding motif (E265D) (12). Mø receptors known to be involved in the phagocytosis of Leishmania spp. promastigotes include: i) C3bi receptor CR3 that bind Leishmania spp. promastigotes (77, 104, 105), ii) C3b and C3bi receptor CR1, and iii) the scavenger receptor MARCO, the latter two bind to L. major promastigotes (29, 40). In addition, mannose receptors, fibronectin receptors, TLR2 and 9, and the receptors for advanced glycoconjugates are also implicated in Leishmania recognition of host Mø (99).

The remainder of this section will focus on new developments and advancements in the last several years. These include:
a. Interactions with host extracellular matrix

When inoculated into a subcutaneous location by a sand fly vector, metacyclic promastigotes come into contact with host extracellular matrix. In an in vitro model system, Matrigel containing soluble extracts of extracellular matrix and basement membranes of Engelbreth-Holm-Swarm tumor cells, genetically altered L. amazonensis expressing high levels of MSP migrated more efficiently through the extracellular matrix compared with control promastigotes (68). Enhanced migration of promastigotes ultimately correlates with the ability of MSP to degrade components of the extracellular matrix such as fibronectin and collagen IV (68). The release of internal, but not surface-oriented, MSPs from promastigotes of L. chagasi incubated with extracellular matrix increased at normal mammalian body temperature (37°C) but not at room temperature. Conversely, the release of surface-localized MSPs increased at room temperature, but was inhibited at 37°C (106). Release of MSP at 37°C could facilitate close contact between promastigotes and host cells such as Mø and fibroblasts that are favorable for parasite entry and/or long-term survival.

Both surface-localized and released MSPs of promastigotes participate in degradation of fibronectin, one component of the extracellular matrix. In contrast, degradation of fibronectin by MSPs released from amastigotes requires a cysteine protease B, while degradation at the surface of amastigotes remains MSP-dependent (55). This difference in degradation of extracellular matrix components between promastigote and amastigote stages may reflect their biological niche. Promastigotes are predominantly extracellular whereas amastigotes are mainly intracellular parasites. Degradation of extracellular matrix components has additional effects.
Leishmania-induced degradation of fibronectin decreased production of reactive oxygen intermediates by infected Mø, facilitating survival of amastigotes in infected Mø (55).

b. Inhibition of natural killer (NK) cellular function

NK cells are a crucial component of innate immunity against diverse pathogens, including parasites, via cytotoxic activity and early cytokine production. NK cells are important in the early stages of host protective immunity against *L. major* infection in mouse models (61). In the resistant strain C57BL/6 mice, depletion of NK1.1+ cells prior to *L. major* infection led to rapid parasite spreading with kinetics similar to those seen in susceptible BALB/c mice. In susceptible mice, rapid dissemination of parasites occurred within 24 h from the inoculation site in the subcutaneous footpad to the popliteal lymph node, spleen, lung, liver and bone marrow (61). Using a MSP−/− null mutant of *L. major*, Lieke and colleagues found that MSPs bound directly to human NK cells, inhibited proliferation, and suppressed interferon-γ responses (62). These authors have identified MSPs as important players in suppression of NK cell during *Leishmania* infection (62). By diminishing the functions of NK cells and other components of innate immunity, promastigotes of *Leishmania* increase their chances of survival and ability to establish a successful infection in mammalian hosts.

c. Resistance to antimicrobial peptide (AMP) killing

AMPs, also called host defense peptides, are another important component of innate immunity. AMPs are short cationic peptides of diverse structure found in a wide variety of organisms including mammals. The antimicrobial activity of AMPs is capable of killing a wide spectrum of microorganisms including bacteria, fungi, parasites, enveloped viruses and
transformed cells. These peptides directly disrupt the membrane potentials of microorganisms leading to cell death by necrosis or apoptosis (44). By comparing \( MSP^{-/-} \) null mutants to wild type parasites of \( L. major \), Kulkarni et al., convincingly demonstrated that the promastigotes of \( MSP^{-/-} \) null mutants were effectively killed in a dose-dependent manner by AMPs such as pexiganan whereas the wild type parasites were not. By reintroducing an \( MSP \) gene into the mutant parasites, promastigotes survival was comparable to the wild type cells (56). These authors also showed that MSP-associated enzymatic degradation of AMPs by wild type promastigotes resulted in inactive fragments, which led to resistance to AMP-mediated apoptotic killing (56). More recently, these same investigators defined a further mechanism of AMP-mediated apoptotic death in \( Leishmania \) spp: calcium-dependent, caspase-independent mitochondrial toxicity. At the same time, they described a distinct group of AMPs such as protegrin-1 that induce non-apoptotic cell death of \( Leishmania \) spp. (57).

d. Promoting intracellular amastigote survival in Mø

Compared to metacyclic promastigotes, MSPs in amastigotes are reduced dramatically, accounting for 1% and 0.1% of the total proteins in these two stages of \( L. mexicana \), respectively (2). It remains debatable whether MSP functions favor survival of the intracellular amastigotes inside PV of host Mø. Supporting evidence includes: i) purified MSP of \( L. amazonensis \) protects liposome-encapsulated \([^{125}\text{I}]\) bovine serum albumin from degradation inside PV of Mø (17); ii) genetic rescue of MSP-deficient \( L. amazonensis \) increases early survival of the parasite in Mø (67); iii) lower levels of MSP induced by specific antisense mRNA reduces the early intracellular survival of \( L. amazonensis \) amastigotes in Mø (18); and iv) coating MSP-low
attenuated cells of *L. amazonensis* with proteolytically active MSP protects these cells from degradation inside Mø (97).

Alternatively, null mutants argue that MSP do not promote survival of amastigotes in Mø. As described earlier, an *MSP<sup>-</sup>* null mutant of *L. major* was generated by deletion of all seven *MSP* genes in tandem array on chromosome 10. MSP proteins were below detectable levels by flow cytometry (52). A second mutant of *L. mexicana* was developed by knocking out the gene for GPI:protein transamidase. The null mutant lacked GPI-anchored MSP and other GPI-anchored proteins but GPI linked non-proteins such as LPG were unaffected (46). Both mutants were capable of surviving as amastigotes in Mø *in vitro* or caused lesions in mouse models *in vivo*, albeit with a delay in lesion formation or smaller lesions compared to the wild type controls (46, 52). These results are unexpected but not completely surprising given following reasons. First, two additional *MSP*-like genes are each located in chromosome 28 and 31 in *L. major* (49). The antibody used in flow cytometry experiments may not react with the products of these *MSP*-like genes, leading to the conclusion of undetectable levels of MSP.

Secondly, it was elegantly shown that majority of MSP of amastigotes of *L. chagasi* is not GPI anchored (47). Consequently, the null mutant lacked GPI-anchored MSPs would not have much affects on this stage. Thirdly, *Leishmania* spp. exhibit reduced virulence and decreased expression of a number of virulence factors during the months of *in vitro* cultivation required for the generation of knockout mutants. Thus, there may have been additional undetected defects in both the wild type and mutant parasites used in the above experiments. Finally, the data were generated in different *Leishmania* species, and it is possible that the role of MSP in intracellular...
survival of amastigotes varies between species, e.g., MSP of *L. amazonensis* promotes amastigote survival in host Mø and MSP of *L. major* and *L. mexicana* does not.

e. Degrading cytosolic proteins of host Mø and fibroblasts

MSP also appears to have other activities during host infection such as degradation of MARCKS-related proteins (21, 22) and the intracellular peptides presented by MHC Class I molecules (39) of infected Mø. Interestingly, these activities require the presence of MSP in the cytosol of infected Mø. How MSP localizes to the cytosol of host cells is unclear, given that amastigotes of *Leishmania* spp. reside inside morphologically distinct PV, unique forms of phagolysosomes (late endosome) of infected Mø (54). Regardless, lines of evidence derived from i) cell fractionation, ii) radioisotope labeling, iii) confocal microscopy and iv) co-immunoprecipitation suggest leishmanial elongation factor-1α (evidence from all four lines) and fructose-1,6-bisphosphate aldolase (evidence from lines i and iv only) are exported from PV into the cytosol of infected Mø (80, 81). A model for the release of cysteine peptidase B into the cytosol of host Mø has been proposed (78). In this model, activated cysteine peptidase B is first released from flagellar pockets of amastigotes into PV. Next, vacuoles containing the peptidase bud from PV and release their contents into the cytosol of host Mø (78). Immunoelectron microscopy has shown that MSP in amastigotes of both *L. chagasi* and *L. mexicana* are predominantly localized to the flagellar pocket (47, 73). Therefore, it is possible that MSP endopeptidase uses a similar approach to reach the cytosol of host Mø as does cysteine peptidase B.
Although Mø are the primary host cells harboring amastigotes following initial infection, fibroblasts are important host cells in chronic and persistent leishmanial infection (7). Recently, by employing cellular and in vitro assays using protease inhibitors, MSP null mutants, and recombinant enzymes, Halle et al., found that leishmanial MSPs degraded several signaling proteins of fibroblasts infected with L. major. These signaling proteins included the phosphorylated adaptor protein p130Cas, the protein-tyrosine phosphatase-PEST, cotactin, T-cell protein-tyrosine phosphatase, caspase-3 and p38 (45). These authors concluded that MSPs play a central role in several molecular cascade events that likely contribute to leishmanial infectivity (45). Analogous to infected Mø, degradation of these signaling proteins requires MSP localization to the cytosol of infected fibroblasts.

**B. MSP homologues in other Trypanosomatidae**

MSP homologues have been discovered in many trypanosomatids. These parasites infect different insects, mammalian or plant hosts, and their life cycles differ considerably from that of the Leishmania spp. Although not as well studied, the functions of these MSPs in trypanosomatids are well adapted to sustain the unique life cycle of each parasite. The following discusses the potential roles of these MSPs homologues in trypanosomatids.

**T. cruzi:** Like Leishmania, the American trypanosome T. cruzi has an intracellular amastigote stage in mammalian host cells, but T. cruzi amastigotes reside in the host cell cytosol whereas those of Leishmania spp. reside in PV. The T. cruzi genome has a minimum of 425 MSP gene homologues including 251 pseudogenes (33). The gene sequence predictions indicate that some MSPs have C-terminal hydrophobic regions that are likely replaced by a GPI anchor.
Northern blot analyses indicate that some of these MSP genes are differentially expressed during different life-cycle stages. Immunofluorescent staining and phosphatidylinositol-specific phospholipase C (PI-PLC) digestion indicate that at least one group of MSP homologues are linked by a GPI anchor to the surface of all *T. cruzi* life-cycle stages, including metacyclic trypomastigotes, the infective stage for humans. These MSP homologues have zinc metalloprotease activity. MSP-specific antisera partially block the *in vitro* invasion of mammalian cells by trypomastigotes (23). Recently, Kulkarni and colleagues demonstrated that a *TcMSP* gene was expressed as a 55 kDa intracellular protein without N-glycosylation and a 61-kDa ecto-glycoprotein in metacyclic trypomastigotes and epimastigotes, respectively, indicating expression was post-translationally regulated during different life-cycle stages (58). These authors further showed that antibodies against the corresponding antigens partially blocked infection of host myoblasts by trypomastigotes (58). These data collectively demonstrate that MSPs play an important role during attachment and/or entry of host cells by the American trypanosomes.

*T. brucei*: The African trypanosome, *T. brucei*, is exclusively an extracellular organism and, as such, MSP activity for host cell attachment and/or entry is irrelevant. The *T. brucei* genome contains fourteen MSP homologues that can be grouped into three classes based on their differential expression in various life-cycle stages and the presence or absence of a GPI-anchor addition site (5, 32, 59). Zinc metalloproteases encoded by the *T. brucei* MSP B (*TbMSP-B*) and located on the surface of the procyclic insect stage are required to remove ectopically expressed variant surface glycoproteins (VSG) (42, 59). Recently, they have been demonstrated to participate, in concert with PLC, in the removal of the VSG coat when bloodstream stage...
trypanosomes differentiate to procyclic trypanosomes. Bloodstream stage trypanosomes of the
double null mutant *TbMSP-B*<sup>-/-</sup>/*PLC*/<sup>-/-</sup> fail to differentiate into the procyclic form with most VSG
remaining on the cell surface. In contrast, those of single null mutants of *TbMSP-B*<sup>-/-</sup> or *TbPLC*<sup>-/-</sup>
differentiate successfully with VSG being released into the extracellular medium in a non-
truncated form reminiscent of wild type controls (41). A further detailed analysis provides
evidence for a coordinated and inverse regulation of the activities of MSP-B and PLC during
synchronous differentiation. MSP-B upregulation is inversely reflected by PLC downregulation
with the latter playing only a quantitatively minor role during differentiation (43).

*Endotrypanum* spp.: The trypanosomatid parasites of the forest-dwelling tree sloths in
South America have an unusual intraerythrocytic stage (53, 75). As such, the *Endotrypanum*
MSP homologues apparently cannot perform all the same functions as *Leishmania* MSPs,
particularly those involved in Mø entry (for review, see Ref. (107). MSP homologues were
found in one *Endotrypanum* sp. using an *L. mexicana* MSP gene probe in Southern blots (74).
Nevertheless, the role(s) of MSP homologues during the *Endotrypanum* life cycle, and whether
or not this includes attachment and/or entry into host erythrocytes remains to be elucidated.

*Phytomonas* spp.: *Phytomonas* spp. infect fruits, seeds, and plants of great economic
importance in widespread and diverse geographic regions. These digenetic parasites are
transmitted between plant hosts by phytophagous insects (13). A 67-kDa metalloprotease has
been isolated and purified from *P. françai*, a parasite of cassava causing a disease known as
“chochamento das raizes”, meaning “empty roots” (1). This metalloprotease along with another
62-kDa protein shares common epitopes with the leishmanial MSP and is localized on the cell
They play a role in the protozoan binding to the extracted gut of *Aedes aegypti*, possibly through a 50-kDa ligand (64). *P. serpens*, the parasite of tomato fruits, has metalloprotease activity in the 70 and 94 kDa range exclusively in the extracellular stage (100). A further study shows that a 63-kDa protein reacting with MSP antibody is attached by a GPI anchor to the cell surface. An anti-MSP antibody raised against a *L. amazonensis* MSP significantly reduces binding of the parasite to the salivary glands of the insect *Oncopeltus fasciatus*, suggesting MSP homologues in these parasites play a role in establishment of phytomonads in the insect salivary gland (27).

**Monoxenous trypanosomatids**: Increasing evidence suggests that monoxenous trypanosomatids are capable of infecting and surviving in immunocompromised individuals such as AIDS patients and even immunocompetent persons, leading to visceral or cutaneous lesions resembling leishmaniasis. Monoxenous trypanosomatids from three genera, *Crithidia*, *Herpetomonas*, and *Leptomonas*, have been positively identified in such human hosts (19). Further, *C. deanei* and *H. roitmani* are phagocytized by and survive in mouse fibroblasts *in vitro* (95). These data collectively raise the question concerning the roles of monoxenous trypanosomatids as opportunistic pathogens in immunocompromised and even in immunocompetent populations of humans. Therefore, it is relevant to discuss and compare these parasitic protozoa with the well recognized human pathogens.

Genes encoding MSP homologues have been cloned and sequenced in *C. fasciculata*. They form a multicopy family comprised of approximately 7 genes. The deduced amino acid sequences show a great degree of conservation when compared with leishmanial MSP (48).
Metalloprotease activity has been detected using fibrinogen as a substrate suggesting that the genes are functional in these protozoa (35). Immunoreactive MSP-like proteins have been detected in *Leptomonas seymouri*, *C. luciliae* (51), *C. deanei* (28), *C. guilhermeri* (64), and *B. culicis* (25) using antibodies to leishmanial MSPs in immunoblots. In addition, two 50- and 58-kDa metalloproteases of *B. culicis* possess a GPI anchor (25). Even more interesting, two 67- and 62-kDa proteins in the total cellular lysates of *C. deanei* and *C. guilhermei* share common epitopes with leishmanial MSP and are localized on the cell surface (64). These MSP-like proteins function in the binding of the protozoa to the extracted gut of *A. aegypti* possibly through a ligand of 50 kDa (64). Similar results are also obtained when *Leptomonas collosoma*, *Leptomonas samueli*, and *Leptomonas wallacei* protozoa are used (85). In adhesion experiments of *H. megaseliae* to the extracted guts, it was clearly and convincingly shown that MSP-like proteins play a role in the attachment of these monoxenous parasites to the gut of their original host *Megaselia scalaris* (82). Metalloproteases have also been detected in *H. samuelpessoaai* (35), *H. anglusteri*, and *H. roitmani* (98), although their identities have yet to be confirmed.

The data presented so far collectively support the likelihood that all members of trypanosomatids have MSP, MSP homologues, or MSP-like proteins. The data also indicate that the protease activities of these metalloproteases contribute to the life cycles of these organisms in many different ways, either directly or indirectly. It may be that the wide substrate specificity and functionality at various pH levels of these proteases facilitate the adaptation of these protozoa to different environments.
C. “Abnormal” MSPs of leishmanial promastigotes: Functions of the released and internal MSPs

As indicated above, most of the Leishmania MSP is bound to the plasma membrane of promastigotes through a GPI anchor (9). Several groups, including ours, have reported that some MSPs of several Leishmania spp. are intracellularly localized in promastigotes and/or are released into the culture medium (34, 51, 69, 102, 108, 109) (Fig 1).

**MSP release.** In contrast to the rapid loss or replacement of the VSG coat from T. brucei bloodstream forms, release of Leishmania MSP is slow and accompanied by a loss of cell-associated MSPs (4, 108). Release of transfected L. amazonensis MSPs from the promastigote surface is dramatically reduced when MSP proteolytic activity is abrogated by site mutagenesis (E265D mutant) or inhibited with a zinc chelator, 1,10-phenanthroline, indicating that autoproteolysis contributes to this release (69). Two lines of evidence indicate that some released MSPs either have not acquired or have lost a GPI anchor. First, released MSPs of L. amazonensis contain neither a GPI anchor nor ethanolamine. Second, treatment of released L. amazonensis MSP with PI-PLC fails to expose the CRD motif, characteristic of PI-PLC-treated GPI anchors (69). This contrasts to L. chagasi in which some of the released MSPs exist as either membrane-bound vesicles or large aggregated micelles (108), supporting the concept that release potentially occurs through several distinct mechanisms. MSPs lacking a GPI anchor because of defective synthesis of GPI precursors are released with a half life (T½) of 2 h, which is significantly slower than the transport rate of 40 min expected of wild type MSP to the cell surface (79). Similarly, MSPs lacking a GPI anchor due to deletion of the genes encoding GPI:protein transamidase are secreted in a manner dependent upon N-glycosylation and are
inhibited by tunicamycin (34). The mechanism of MSP release from this mutant parasite may
indicate that N-glycosylation is required for trafficking of MSPs forward through the
endoplasmic reticulum (ER) and Golgi to the parasite surface, where they are usually retained
via an intact membrane anchor. Release of membrane-bound N-glycosylated and GPI-anchored
MSPs from wild-type promastigotes may involve autoproteolysis and/or proteolysis of a related
molecule required for release.

MSP release occurs in newly isolated clinical as well as laboratory-adapted strains of
Leishmania spp. A pulse-chase experiment revealed that recent clinical isolates of L. infantum
and L. tropica promastigotes release maximal amounts of MSP after 12 h in culture, with release
continuing for at least 24 to 48 h post-chase. In contrast, MSP release from L. amazonensis
promastigotes in culture is detected as early as 4 h after the start of the chase, peaking at 12 h at
which time approximately 60-70% of the newly synthesized proteins are found in the
extracellular medium (69). The release of MSPs from L. major promastigotes reaches a peak at
day 4 and then decreases, whereas MSP release from L. donovani promastigotes continually
increases until day 6 after suspension in liquid culture (51). These observations collectively
support a notion that promastigotes of different Leishmania species release MSPs at different
rates. Release of MSPs may be an intrinsic property of Leishmania spp. that relates to their
pathogenesis.

Many of the numerous monoxenous trypanosomatids also release MSP-like proteins or
metalloproteases into the extracellular medium. These include C. guilhermei (30, 83), C. deanei
(26, 28), C. desouzai, C. oncopelti, C. fasciculata (26), C. luciliae, Leptomonas seymouri (51),
H. anglusteri and H. roitmani (98), and B. culicis (25, 31). Interestingly, significantly greater enzymatic activity of metalloproteases is detected from an aposymbiotic strain of B. culicis compared with a strain carrying bacterial endosymbionts, suggesting a role for MSP-like proteins in digestion of nutrients (25). In addition, release of MSP homologues is found in plant parasites P. françai (1) and P. serpens (100).

In short, release of MSP and its homologues from the insect stages into their environments has been observed across all trypanosomatids examined to date including monoxenous insect parasites, digenetic plant parasites and digenetic mammalian parasites. Nutrient acquisition via degradation of gut content by the endopeptidase activity of these released MSPs is likely of major biological significance while these protozoa reside inside the guts of the insect host. They may also enhance the survival of digenetic protozoa in their hosts. We have proposed a model (Fig 1) that considers the three subpopulations of MSP in Leishmania spp. including surface localized, intracellular and released MSPs (106).

Internally located MSP: In contrast to extracellular release, some MSPs remain at an intracellular location in leishmanial promastigotes. Using surface biotinylation, fluorescence microscopy, and immunoelectron microscopy, approximately one-fourth of all cell-associated MSPs were shown to reside internally in L. mexicana. These internal MSPs are distributed among the tubulovesicular compartment (46%), ER (5%), the Golgi complex (1.9%), and the tubular cluster/translucent vesicle complex (3.6%) (102). They could represent both transient MSPs trafficking from ER to the plasma membrane as well as mature, stable MSPs situated at their final destination. To confirm the existence of the latter, L. chagasi MSPs were localized...
using pulse-chase metabolic labeling and immunoprecipitation to detect all MSPs and surface
biotinylation to mark only surface-localized MSPs. Thus after removal of surface MSPs with
streptavidin, internal MSPs were detected in immunoprecipitates by autoradiography. These
experiments revealed that one fourth of the nascent MSPs remained internally for up to six days
following biosynthesis, strongly supporting the existence of a stable internal subpopulation of
MSPs. It appeared that only the mature 63-kDa form but not the 59-kDa protein accounts for
this subpopulation of internal MSPs (106, 109). We have not formally ruled out the less likely
possibilities that: 1) some surface-MSP isoforms are inaccessible to surface biotinylation due to
the presence of densely packed LPG, a situation reminiscent of the failure of immunogold to
label some MSPs on the cell surface of L. major promastigotes (86); and 2) MSPs are constantly
internalized with biotin removal. The second explanation is also unlikely because of the
following reasons: (i) one of the eleven major MSP isoforms detected on western blots of total L.
chagasi proteins separated by two-dimensional SDS-PAGE is not surface-biotinylated (110), (ii)
intracellular MSP does not co-localize with the endosome marker FM4-64 by confocal
microscopy, and is instead found in a subregion of ER (111), and (iii) quantitation of MSPs by
immunoelectron and fluorescence microscopy agrees with results of surface biotinylation (102,
109). Together, these data suggest that a subpopulation of MSPs resides continuously at an
internal location of promastigotes. Consistent with this possibility is the fact that the MSPC
class and its homologues lack a C-terminal hydrophobic signal for GPI anchor addition. Our
hypothesis is that the internal population of MSPs serves as a pool for quick release upon
inoculation of metacyclic promastigotes into the mammalian host by sand fly vectors, rather than
a pool for promastigote surface MSP in the sand flies (106). A model outlining the function of
MSPs in Leishmania spp. is presented in Fig 1.
Concluding remarks

MSPs and homologues have been documented in all trypanosomatids examined to date, ranging from monoxenous insect parasites to heteroxenous plant and mammalian parasites. They are encoded by a family of genes varying from several to hundreds in number in each species. Their designations in the insect stages of promastigotes include surface localized, intracellular located and released into surrounding environments, although whether these are the products of different MSP genes or different post-translational modification of the same genes remains to be elucidated. One function conserved across the phylogenetic tree of various trypanosomatids appears to be nutrient acquisition in the gut of various insects. Provided that endopeptidases like MSPs do not really cleave protein substrates extensively, this very likely requires a coordinated action from other enzymes. In addition, MSPs also serve a variety of roles in protecting these parasites from innate defenses of different hosts ranging from insects, plants to mammals. Consequently, collaborative efforts should be continuing in the development of MSP vaccination for domestic animals and humans, despite of the marginal and/or moderate protections of MSP immunized individuals against leishmaniasis in most cases. Hypothetically, a major surface protein like MSP may have been evolutionally selected to become antigenically less provocative for the parasite benefit of escaping host immune surveillance. Nevertheless, liposome-encapsulated MSPs have been shown to confer significant protections against *L. major* infection in susceptible BALB/c mice (50). It is also worthy of investigating whether MSP vaccination will effectively hamper the generation of metacyclic promastigotes in the sand fly vectors that will eventually leads to interruption of leishmanial transmission. Finally, crystal structure of one *L. major* MSP was determined more than a decade ago (96). Structural
modeling is an effective approach to search for peptidomimetic inhibitors of these endopeptidases. In fact, low micromolar concentrations of some peptidomimetics were found toxic for cultured bloodstream trypanosomes, and inhibitory for *in vitro* cleavage of a synthetic peptide substrate by purified *L. major* MSP (3). The peptidomimetic approach is highly potential in addressing the proposed MSP functions and in developing chemotherapeutic drugs against leishmaniasis.
ACKNOWLEDGMENT

The author is grateful to Drs. Donald L. Montgomery and Kenneth W. Mills of University of Wyoming for critically reading this manuscript. Support for this work comes from a Veterans’ Affairs Merit Review grant and a startup package from University of Wyoming.
REFERENCES


Mooney, S. Moule, D. M. Martin, G. W. Morgan, K. Mungall, H. Norbertczak, D.
Rajandream, C. Reitter, S. L. Salzberg, M. Sanders, S. Schobel, S. Sharp, M.
Aken, D. Walker, D. Wanless, S. Wang, B. White, O. White, S. Whitehead, J.
Woodward, J. Wortman, M. D. Adams, T. M. Embley, K. Gull, E. Ullu, J. D. Barry,
A. H. Fairlamb, F. Opperdoes, B. G. Barrell, J. E. Donelson, N. Hall, C. M. Fraser,
S. E. Melville, and N. M. El-Sayed. 2005. The genome of the African trypanosome

7. Bogdan, C., N. Donhauser, R. Doring, M. Rollinghoff, A. Diefenbach, and M. G.
Rittig. 2000. Fibroblasts as host cells in latent leishmaniosis. J. Exp. Med. 191:2121-
2130.
1986. Leishmania and Trypanosoma surface glycoproteins have a common
membrane and soluble forms of the major surface protein of Leishmania promastigotes. J.


influence of the endocymbiont and similarities with virulence factors of pathogenic trypanosomatids. Parasitology 130:413-420.


response and protection assay of recombinant major surface glycoprotein of Leishmania (rgp63) reconstituted with liposomes in BALB/c mice. Vaccine 24:5708-5717.


64:273-282.


Chaoqun Yao completed MD from Tongji Medical University, Wuhan, PR China, and PhD from University of Georgia followed by postdoctoral training at Washington State University and University of Iowa. It was in graduate school that he developed a strong interest in molecular pathogenesis and host-parasite interaction of intracellular parasites of medical/veterinary importance. Dr. Yao was promoted from an Assistant to an Associate Research Scientist at University of Iowa and was simultaneously appointed as a Research Health Science Specialist at Iowa City VA Medical Center. He is currently an Assistant Professor in the Department of Veterinary Sciences and a Parasitologist in Wyoming State Veterinary Laboratory, University of Wyoming and an Adjunct Assistant Professor at University of Washington. In his work he has used two model organisms, i.e., *Trichinella* and *Leishmania*, the intracellular parasites of mammals including human beings.
**Figure Legend**

**Figure 1.** MSP subpopulations and possible functions of *Leishmania* spp.  

**A.** Model of MSP release from *Leishmania* promastigotes. MSP proteins are synthesized and post-translationally modified with a GPI anchor (blue circles) in the endoplasmic reticulum and are designated for the surface of plasma membrane. Some of these are released from the cell surface with the removal of their GPI anchors (green circles) during *in vitro* cultivation. The intracellular MSPs (red circles) do not have a GPI anchor and are not released *in-vitro* culture, but are released upon promastigotes inoculation into mammalian hosts *in vivo*. Adapted from Fig 10 of (69) and Fig 9 of (102).

**B.** MSP release and functions in the sand fly vector and the mammalian hosts (reprint with modification from Figure 5 of Eukaryotic Cell 2007, 6:1905-1912 (106) with permission). Solid and open circles represent the surface-localized and internal MSP, respectively. Arrows depict MSP release into the extracellular environment with their width proportional to amount of MSP released. The MSP release in the procyclic and metacyclic promastigotes within the sand fly panel is depicted in the upper and the lower diagram, respectively. A metacyclic promastigote is depicted in the mammalian host panel.
A

- Multivesicular tubule
- Tubular vesicle
- Flagellar pocket
- Endoplasmic reticulum
- Golgi
- Kinetoplast
- Tubular vesicle

B

- Sand fly
- Procyclic
- Metacyclic
- Mammalian host
- MSP functions
  1. Nutrient acquisition
  2. Attachment to gut wall
  3. Evading complement-mediated lysis
  4. Binding to and uptake by macrophage
  5. Interacting with extracellular matrix
  6. Resisting antimicrobial peptide killing
  7. Evading natural killer cell killing