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Internal and surface subpopulations of the major surface protease (MSP) of *Leishmania chagasi*

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Abstract

Major surface protease (MSP) facilitates *Leishmania* promastigote evasion of complement-mediated lysis in the mammalian host and enhances host macrophage phagocytosis of the promastigotes. We previously showed that the steady-state abundance of MSP protein increases 14-fold during in vitro cultivation of *L. chagasi* promastigotes from logarithmic to stationary phase, despite the fact that the total amount of *MSP* mRNA does not increase. Furthermore, 10 major MSP isoforms are differentially expressed in different promastigote growth phases, and attenuation of parasites by long-term in vitro cultivation influences MSP isoform expression. Herein, we report that although about two-thirds of newly synthesized MSP becomes surface localized, the rest of the MSP does not reach the promastigote surface. This internal MSP is stable without detectable decrease in abundance up to 6 days after biosynthesis. Furthermore, surface-localized MSP is released at different rates from logarithmic and stationary phase virulent *Leishmania* promastigotes. These data are consistent with the hypothesis that the major mechanism regulating MSP abundance is the rate of loss of surface-localized MSP from the promastigote surface, and that internally localized MSP is very stable.
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Keywords: *Leishmania chagasi*; Major surface protease (MSP); GP63; Protein translation; Membrane remodeling

1. Introduction

Major surface protease (MSP) is the most abundant surface glycoprotein of *Leishmania* spp. promastigotes. Because it is a protease of approximately 63 kDa, it is commonly called GP63 or leishmanolysin. Each *L. major* promastigote in stationary phase is estimated to have 500,000 copies of MSP, constituting about 1% of the organism's total protein [1]. MSP has been found on all species of *Leishmania* studied to date. *Leishmania* are intracellular in mammalian hosts, but MSP homologues have also been detected in the monogenetic insect protozoa *Crithidia fasciculata* and *Herpetomonas samuelperssoai*, as well as the extracellular protozoan *Trypanosoma brucei* and the digenetic protozoan *T. cruzi* (see [2] for a review). MSP is a zinc metalloprotease belonging to M8 metzincin family, with an active site sequence motif of HEXXHXXGX$_{(n)}$H. MSP plays vital roles in the different stages of *Leishmania* life cycle. It promotes promastigote evasion of complement-mediated lysis in the mammalian host, it enhances phagocytosis of promastigotes through

Abbreviations: BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; HIFCS, heat inactivated fetal calf serum; HOMEM, hemoflagellate modified minimal essential medium; IP, immunoprecipitation; IR, intergenic region; LPG, lipophosphoglycan; MSP, major surface protease; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; p*I*, isoelectric point; 2DE, two-dimensional electrophoresis; $T_{1/2}$, half-life; RT, room temperature; TCA, trichloroacetic acid; UTR, untranslated region

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macrophage receptors such as CR3, it promotes degradation of host cytosolic MARCKS-related protein, and it is capable of cleaving surface CD4 and intracellular peptides presented by MHC Class I molecules (reviewed in [2]). Whether it functions in the intracellular amastigote is debated since purified MSP can protect [^{125}I]-bovine serum albumin (BSA) from intracellular degradation, but MSP seems not to be necessary for intracellular survival of *L. major* or *L. mexicana* [3–5].

Leishmania chagasi has more than 18 *MSP* genes arranged in a tandem head-to-tail array that are classified into three categories (*MSPL*, *MSPS*, *MSPC*) according to unique sequences in their 3' untranslated regions (UTRs) and the differential expression of their mRNAs [6,7]. Steady-state levels of MSP proteins increase about 14-fold during promastigote growth in vitro from logarithmic to stationary phase [8,9]. Concomitantly, the number of major MSP isoforms detected by two-dimensional Western blot increases from four in the former to 10 in the latter growth phase [10]. Furthermore, MSP is released into the promastigote extracellular medium in both membrane-associated and -free forms [9]. Of the 10 major MSP isoforms in stationary phase promastigotes, only four are released extracellularly [10]. MSPs have also been found to be released into the extracellular medium of *Crithidia*, *Leptomonas* and several *Leishmania* spp., including *L. major*, *L. donovani*, *L. infantum*, *L. tropica* and *L. amazonensis* [11,12]. They are released from both newly isolated clinical strains and laboratory-adapted isolates [11]. Experiments using surface biotinylation, cytofluorimetry and immunoelectron microscopy show that three-fourths of *L. mexicana* MSP occurs on the cell surface, whereas the remainder is located intracellularly [13]. It is not clear whether the internal MSP subpopulation is trafficking en route from the site of synthesis in the endoplasmic reticulum to the cell membrane, whether there is a stable internal subpopulation of MSP, or a combination of both. In this study we report the existence of a distinct subpopulation of MSP that remains inside the cell without detectable loss up to 6 days after synthesis. In contrast, the major mechanism through which cell surface MSP is lost from the cell is through shedding into extracellular medium, a process that is more pronounced in logarithmic than stationary phase promastigotes. We hypothesize that developmentally regulated remodeling of the promastigote cell surface may influence the rate through which membrane-bound, but not internal, MSP is lost from the promastigote cell.

2. Materials and methods

2.1. Parasites

A Brazilian strain of *L. chagasi* (MHOM/BR/00/1669) was continuously passed in hamsters to maintain its virulence as previously described [9]. An attenuated strain of *L. chagasi* (L5) has been continuously passed in vitro in hemoflagellate-modified minimal essential medium (HOMEM) containing 10% heat inactivated fetal calf serum (HIFCS) at 26 °C for over 9 years, resulting in reduced infectivity for mice [14], decreased MSP abundance and an altered pattern of *MSP* mRNA expression [7,14,15]. An attenuated strain of *L. donovani* (Ldon1S) was purchased from ATCC (Manassas, VA). Promastigote cultures were seeded at 1×10^6 cell ml^{-1} in HOMEM at day 0, and cells were collected in logarithmic (days 3–4) or stationary growth phase (days 7–8) as previously defined by cell density and morphology [16].

2.2. Antibodies and chemicals

Polyclonal rabbit and sheep antisera to MSP were raised against purified *L. chagasi* MSP as previously described [14]. Monospecific rabbit serum to a cytosolic protein P36 of *L. amazonensis*, kindly provided by K.-P. Chang, the Chicago Medical School, was generated to gel-purified recombinant P36 [17]. Monoclonal antibody (mAb) to α-tubulin (AB-1) was purchased from Oncogene (San Diego, CA). Peroxidase conjugated anti-rabbit, -sheep and -mouse antisera were purchased from CalBioChem (San Diego, CA), Kirkegaad & Perry Laboratories (Gaitherburg, MA) and Bio-Rad Laboratories (Richmond, CA), respectively. NHS-Sulfo-Biotin was from Pierce (Rockford, IL). Protein G-agarose beads were from CalBioChem. Streptavidin-agarose beads and peroxidase-conjugated ExtrAvidin were purchased from Sigma (St. Louis, MO).

2.3. Metabolic labeling

Two methods were utilized. First, promastigotes (2×10^7 cells ml^{-1}) were metabolically labeled for 1 h in rich medium, i.e., methionine and cysteine-free RPMI 1640-10% HIFCS containing 50 μCi ProMix ml^{-1} ([^{35}S]-methionine and [^{35}S]-cysteine, Amersham Pharmacia Biotech, Piscataway, NJ), at room temperature (RT) as previously described [9]. Alternatively, promastigotes were pulsed at a density of 1×10^8 cells ml^{-1} in nutrient-poor medium, i.e., Hanks' balanced salt solution (HBSS, GIBCO BRL, Rockville, MD) containing 100 μCi ProMix ml^{-1}, at RT for 0.5 h [11]. Excess cold L-methionine (final concentration of 1 mM) was added to the media at the end of pulsing. In some experiments, promastigotes, after being pulsed in HBSS, were surface-biotinylated by incubation in 0.25 volume of 2 mg ml^{-1} Sulfo-NHS-Biotin dissolved in phosphate-buffered saline (PBS), pH 7.4, for one additional hour at RT. Biotinylation was performed either at the same time as cold methionine was added, or after a time delay to measure the time taken for nascent MSP to reach the cell surface. In cases where there was a time delay, radioisotope-labeled promastigotes were resuspended in RPMI 1640-10% HIFCS in the interim before biotinylation in HBSS. 0.1 volume of 1 M NH_4Cl was added to stop surface biotinylation. Cells were collected by centrifugation ($1400 \times g$, 13 min) and washed twice in RPMI 1640-10% HIFCS by centrifugation. Cells were finally dispersed in the

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