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Characterization and developmental gene regulation of a large gene family encoding amastin surface proteins in *Leishmania* spp.

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Abstract

The ability of *Leishmania* amastigotes to survive within the drastic environmental changes encountered in the phagolysosomes of mammalian macrophages is heavily dependent on the developmental regulation of a variety of genes. The identification of genes that are expressed preferentially in the mammalian stage of the parasite should increase our understanding of the molecular mechanisms regulating stage-specific gene expression and of the determinants that control its intracellular survival and contribute to its pathogenesis. We report here detailed sequence characterization and structural organization of the amastin gene family in *Leishmania major* and *Leishmania infantum* and the study of their developmental gene regulation throughout the parasite's life cycle. Amastin surface proteins represent the largest developmentally regulated gene family reported so far in *Leishmania* comprising up to 45 members. All the members of the amastin gene family in both *Leishmania* and *Trypanosoma* species share a similar structural organization and contain a highly conserved 11 amino acid extracellular domain, which is unique to amastin proteins. The majority of the amastin gene homologs are specifically expressed in the amastigote stage of the parasite. Three distinct RNA elements were identified in the 3′-untranslated regions (3′UTR) of the amastin transcripts. The majority of these transcripts contain a conserved 450 nt cis-acting 3′UTR element shown previously to regulate stage-specific gene expression at the level of translation, which suggests that several amastin homologs may be regulated by a similar mechanism of translational control inside the macrophage. These findings further highlight the unique features of gene expression control in *Leishmania*.

Keywords: *Leishmania*; Amastin gene family; Amastin signature sequence; Surface proteins; Stage-specific gene expression; 3′UTR conserved elements

1. Introduction

Leishmania is an early-branching unicellular eukaryote belonging to the order Kinetoplastida and family Trypanosomatidae [1,2]. More than 20 *Leishmania* species cause a large spectrum of clinical manifestations of leishmaniasis ranging from self-resolving skin lesions to life-threatening visceral diseases. Leishmaniasis is endemic in 88 countries and more than 15 million are currently infected. In addition, 397 million people are at risk of acquiring leishmaniasis [3,4]. *Leishmania* parasites exist in two major developmental stages; extracellular flagellated promastigotes in the alimentary tract of the sandfly vector and intracellular amastigotes residing in the phagolysosomes of macrophages in the mammalian host. The cytodifferentiation of *Leishmania* within the phagolysosomes is accompanied by a series of morphological and biochemical changes [5–8] which are often mediated by the differential expression of a variety of genes. To date, over 20 amastigote-specific genes have been characterized in *Leishmania* [9–20].

The *Leishmania* haploid genome content is ∼34 Mb, consisting of 36 chromosomes which range in size from 0.3 to 2.5 Mb and encode ∼8300 genes [21]; (http://www.genedb.org/). *Leishmania* and the related *Trypanosoma*

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species possess unusual mechanisms of gene expression [22]. All protein coding genes in these organisms are transcribed polycistronically by α-amanitin sensitive RNA polymerase II in a divergent or a convergent manner on either DNA strands of the different chromosomes [23–28]. As the result of polycistronic transcription, mRNA synthesis requires posttranscriptional control, which involves processing of the primary polycistronic precursor through trans-splicing and polyadenylation [29–31]. Several experimental evidences support that trans-splicing and polyadenylation are mechanistically coupled in these parasites and share common regulatory signals, mainly polypyrimidine-rich stretches within the intergenic regions [32,33]. Thus, regulation of gene expression in these organisms occurs exclusively at the posttranscriptional level and could alter trans-splicing, polyadenylation, mRNA stability, translation rates or protein stability [22]. Transcripts differentially expressed in the intracellular amastigote form of *Leishmania* are mainly regulated by mechanisms that involve sequences within 3′-untranslated regions (3′UTR) and affect either mRNA stability [13,34–38] or pre-mRNA processing [39] and/or mRNA translation rates [37,40,41]. In trypanosomatids, due to the absence of RNA polymerase II promoters and heavy reliance on posttranscriptional control, there is an even greater dependence on regulatory cis-acting RNA elements and RNA–protein interactions. However, there have been very few well-characterized cis-acting motifs in trypanosomatid 3′UTRs [37,40,42–44] and even fewer documented RNA–protein interactions [45–47]. Identifying the genes that are expressed preferentially during the parasite's intracellular growth and elucidating the mechanisms involved in their stage-specific regulation could enhance our understanding of how molecular determinants control the intracellular life of *Leishmania* and contribute to its pathogenesis. Moreover, these studies could eventually lead to the identification of novel therapeutic targets and the development of more effective drugs or vaccines. Therefore, considerable efforts are being directed toward identifying and characterizing these developmentally expressed genes and the molecular mechanisms underlying their regulation. The recent completion of the *Leishmania major* and *Leishmania infantum* genome sequence projects (http://www.genedb.org/) should significantly contribute to these efforts.

In previous studies, we identified the first amastin gene homolog in *Leishmania* that is related to the *Trypanosoma cruzi* amastin surface proteins [48] and showed that this gene was specifically expressed in the intracellular amastigote stage of the parasite [17]. Furthermore, we showed that regulation of stage-specific expression of the *Leishmania* amastin mRNA was at the level of translation and that involved a 450 nt element within the amastin 3′UTR [40,49,50]. Hybridization of a genomic *L. infantum* cosmid library indicated the presence of other sequences homologous to amastin. In this study we show that *Leishmania* amastigotes express a large number of amastin gene homologs that comprise up to 45 members. We also present data on the sequence and structural organization of these homologs in *L. major* and *L. infantum* and their expression throughout the life cycle of the parasite. Amastin surface proteins represent one of the largest developmentally regulated gene families in *Leishmania* and our data suggest that amastin transcripts are regulated by distinct conserved elements in their 3′UTR.

2. Materials and methods

2.1. Cell culture

The *L. infantum* (MHOM/MA/67/ITMAP-263) and *L. major* Friedlin strains used in this study were described previously [51,52]. Promastigotes were cultured at 25 °C in SDM-79 medium supplemented with 10% heat-inactivated fetal calf serum (Multicell, Wisent Inc.) and 5 μg/ml of hemin. Axenic differentiation of *L. infantum* promastigotes into amastigote like forms was induced as described previously [53,54]. Briefly, axenically grown amastigote forms of *L. infantum* were maintained at 37 °C with 5% CO_2 by weekly sub-passages in MAA/20 medium in 25 cm^2 flasks. Axenically grown amastigotes show morphological, biochemical, and biological characteristics similar to those of in vivo isolated amastigotes [55]. Amastigotes were also isolated from the spleens of gold Syrian hamsters infected intraperitoneally with 5×10^7 *L. infantum* amastigotes as described [9].

2.2. Nucleic acid preparations and hybridization studies

Total RNA of *L. infantum* promastigotes and axenic amastigotes was isolated using TRIzol™ reagent (Gibco BRL). Southern and Northern blot hybridizations were performed following standard procedures [56]. All probes used in these studies were made by PCR amplification using specific primers for each amastin gene homolog. The majority of the probes (14/20) specifically recognized distinct amastin gene homologs as they were carefully chosen within the protein coding regions demonstrating the lowest level of identity. However, due to the high level of sequence identity (>75%) between several amastin gene homologs, the probe corresponding to the LinJ31.0190 amastin homolog could also recognize five more homologs, Ldi-849 probe could recognize four amastin homologs, Ldi-3133 probe could recognize three amastin homologs, Ldi-3651 probe could recognize three amastin homologs, Ldi-1804 probe could recognize three amastin homologs and Ldi-1234 probe could recognize two amastin homologs. To evaluate the copy number of *L. infantum* amastin gene homologs, we performed comparative Southern blot hybridization using probes specific to the different amastin gene homologs and the trypanothione reductase (*TR*) single copy gene probe [57] as a control. Hybridization signals were quantified by densitometric analysis using a PhosphorImager with the ImageQuant 3.1 software. To normalize steady-state levels of the different *L. infantum* amastin transcripts in logarithmic promastigotes, stationary