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Leishmania major: Identification of developmentally regulated proteins in procyclic and metacyclic promastigotes

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2D-PAGE, two-dimensional polyacrylamide gel electrophoresis
MALDI-TOF-MS, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
PNA$^-$, peanut agglutinin
IEF, isoelectric focusing
MOWSE, molecular weight search
(DTT), dithiothreitol

ABSTRACT

The differentiation from procyclic to metacyclic promastigotes (metacyclogenesis) has been correlated with an increased infectivity in a number of *Leishmania* species. We compared the proteomes of procyclic and metacyclic promastigotes of *L. major*. Lysates from either life cycle stage were resolved by 2D-PAGE, followed by Coomassie brilliant blue staining. Spots were analyzed by MALDI-TOF MS. 25 protein spots were found to be differentially expressed during metacyclogenesis. We found that proteins involved in protein synthesis were less abundant in metacyclic promastigotes, while proteins involved in motility, including paraflagellar rod protein 1D, α-tubulin and β-tubulin were more abundant. Also, two mitochondrial enzymes (succinyl-CoA synthetase β subunit and cytochrome *c* oxidase subunit IV) were differentially expressed in both life cycle stages. Down-regulation of proteins related to synthetic pathway in metacyclic promastigotes is consistent with the arrested growth in this life cycle stage, while up-regulation of proteins related to motility in metacyclic promastigotes is in agreement with the high motility observed in this stage.

1. Introduction

Infection by protozoan parasites of the genus *Leishmania* is associated with a wide spectrum of clinical features, ranging from self-healing cutaneous lesions to the fatal, generalized, visceral leishmaniasis. The clinical picture is mostly dependent on the infecting species, but host factors may also determine the course of an infection. Worldwide, it is estimated that 12 million humans are infected with parasites of this genus, and ~350 million humans are at risk of infection (Mauel, 2002).

Although individuals exposed to a natural infection of Old World, cutanotropic *Leishmania* spp. develop immunity against later infections, no successful vaccine has been developed against the leishmaniases to date (Mauel, 2002). This may partly be explained by the complex life cycle of these parasites involving both sandfly vectors and mammalian hosts. *Leishmania* spp. traverse between two developmental stages: (i) the elongated, motile flagellated promastigotes in the alimentary tract of phlebotomine sandflies, and (ii) the round, non-motile, aflagellated amastigotes that reside in phagosomes of mammalian macrophages (Mauel, 2002).

Promastigotes mature in the sandfly and progress through two major, distinct stages during this maturation. Sequential development of promastigotes initially involves the rapid division of non-infective procyclic promastigotes in the midgut of the sandfly. This ultimately leads to the production of the non-dividing, infective, metacyclic promastigotes in the anterior parts of the digestive tract (Sacks and Perkins, 1984; Bates, 1994).

Differentiation of procyclic promastigotes into metacyclic promastigotes (metacyclogenesis) is known to be crucial for infectivity (Sacks and Perkins, 1984; Sacks et al., 1985; da Silva and Sacks, 1987; Bates, 1994). It has been shown that promastigotes of *L. major* recovered on day 3 through 7 post-infection from laboratory infected sandflies, display a progressive increase of infectivity in susceptible BALB/c mice (Sacks and Perkins, 1984).

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An increase of promastigote infectivity was also observed in culture when promastigotes of *Leishmania* passed from the logarithmic phase (day 1–3) to the stationary phase (day 4–6) of the growth cycle (Sacks and Perkins, 1984; Sacks et al., 1985; da Silva and Sacks, 1987; Bates, 1994). The infectivity of stationary phase promastigotes has been attributed to the proportion of the promastigotes that are not agglutinated by peanut agglutinin (PNA^-), a lectin that binds to a surface carbohydrate specific to procyclic promastigotes (Sacks et al., 1985).

The mechanisms underlying the infectivity of the metacyclic promastigotes have been the subject of numerous studies during the past three decades. Several investigations have been devoted to the identification of specific, metacyclic stage antigens (Sacks et al., 1985; Coulson and Smith, 1990; Knuepfer et al., 2001). In this context, it is important to consider that within the *Trypanosomatidae*, including *Leishmania* spp. *and Trypanosoma* spp., any regulation of gene expression occurs exclusively at a post-transcriptional level. Transcription initiates only at a limited number of start points on each chromosome, producing polycistronic precursor transcripts. Individual mRNAs are generated from the precursor by a trans-splicing reaction at the 5′ end that is coupled to polyadenylation at the 3′ end (Clayton, 2002; Liang et al., 2003). Changes in the proteome during differentiation must therefore be controlled by post-transcriptional mechanisms, including (i) modulation of mRNA stability, (ii) regulation of translation, and (iii) post-translational modifications of proteins (Clayton, 2002).

It is known that the transformation of non-infective procyclic to infective metacyclic promastigotes occurs in a nutrient-depleted environment (Sacks and Perkins, 1984), curtailing the resources for the *de novo* synthesis of proteins. Therefore, post-translational modifications can be expected to play a role in the control of the metacyclic stage. In keeping with this concept, only a few proteins were identified as specifically expressed during the metacyclic stage (Sacks et al., 1985; Coulson and Smith, 1990; Knuepfer et al., 2001).

We undertook a straightforward proteome analysis to identify protein species that are either differentially expressed or differentially modified in procyclic and metacyclic promastigotes of *L. major,* the etiologic agent of cutaneous leishmaniasis in Middle East and North Africa. With this approach, we hope to understand the mechanisms by which infective metacylic promastigotes pre-adapt to the mammalian host.

2. Materials and methods

2.1. Parasites

In vitro culture of *L. major* promastigotes (MHRO/IR/75/ER) was initiated on NNN medium, using primary isolates from infected BALB/c mice. They were adapted at 26 °C to RPMI 1640 medium (Invitrogen), containing 10% fetal calf serum (Invitrogen), 1000 U/ml penicillin, and 50 μg/ml streptomycin. They were incubated with repeated dilution for 15 days and then divided into two aliquots. One aliquot was cultured for another 5 days and represented procyclic promastigotes. The other aliquot was cultured from day 15–20 without media refreshment. Metacyclic promastigotes were harvested from this 5-day-old stationary phase culture, as described previously (da Silva and Sacks, 1987; Saraiva et al., 2005). In fact, while log-phase *L. major* promastigotes (procyclic) represent a homogenous, low-infectious population, stationary phase promastigotes are heterogeneous with regard to their infectivity (Bates, 1994; Sacks and Perkins, 1984; da Silva and Sacks, 1987; Sacks et al., 1985). Therefore, in the present study, metacyclic promastigotes were purified using PNA selection from stationary phase cultures (da Silva and Sacks, 1987). In short, PNA (Sigma) was added to washed stationary phase promastigotes to a final concentration of 50 μg/ml. The PNA^- parasites were separated from PNA^+ agglutinated parasites by centrifugation at 200*g* for 5 min. The non-agglutinated metacyclic promastigotes were then collected from the supernatant. Metacyclic and procyclic promastigotes were washed three times in PBS, collected by centrifugation at 3500*g* for 20 min at 4 °C.

The increased infectivity of the PNA^- metacyclic promastigotes was tested in mice. Female BALB/c mice (6–8 weeks) were inoculated subcutaneously in the left hind footpad with either 106 procyclic promastigotes or 106 metacyclic promastigotes. Each group of mice consists of 5 mice. Footpad swelling was measured weekly with metric caliper and its size was defined as the mean of thickness and width of footpad. Animals were killed when lesions became necrotic.

2.2. Protein sample preparation and electrophoresis

Promastigote pellets were resuspended in lysis buffer (1×10^8/100 μl) containing 8 M urea, 40 mM Tris, 4% Chaps, 1× protease inhibitor cocktail (Roche) and incubated for 2 h at room temperature. The cell extracts were then centrifuged at 15 °C for 10 min and the supernatant was collected. The soluble protein extracts were stored at −70 °C in single use aliquots. Protein concentration was determined using the 2D Quant kit. All reagents, except protease inhibitor, were obtained from GE Healthcare.

First dimension isoelectric focusing (IEF) was carried out using the IPGphor 2 IEF system (GE Healthcare). Approximately 500 μg of protein extracts from each developmental stage was applied per IPG strips (18-cm: pH 4.0–7.0; GE Healthcare) by in gel rehydration for 12 h at 30 V. Re-hydration solution contained 8 M urea, 2% Chaps, 50 mM DTT, and 0.5% IPG buffer. The loaded IPG strips were focused at 50–55,000 Vh. After IEF, focused strips were equilibrated for 15 min in equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, and 2% SDS) containing 65 mM dithiothreitol (DTT). This was followed by a 15 min incubation in equilibration buffer containing 135 mM iodoacetamide.

The equilibrated strips were sealed to the top of the 2nd dimension gels using 0.5% agarose in standard Tris–glycine electrophoresis buffer. The second dimension was run as 12% SDS–PAGE, using a twin gel apparatus (Sci Plus) at 30 mA/gel, 15 °C, until the tracking dye reached the bottom of the gel. A pre-stained SDS–PAGE marker (Bio-Rad) was used in the second dimension as molecular mass reference.

Three 2D gels were run for each developmental stage with proteins obtained from two independent experiments.

2.3. Gel staining, imaging, and image analysis

Proteins were visualized by Coomassie brilliant blue staining as previously described (Bente et al., 2003). Gels were incubated overnight in staining solution containing 1.6% *ortho* phosphoric acid (Fluka), 8% ammonium sulfate (Fluka), 0.12% Coomassie blue G 250 (Merck), and 20% methanol (Merck). Destaining was performed in 25% methanol. The gels were then stored in 25% ammonium sulfate.

Gels were scanned using an Image scanner (GE Healthcare) at 300 dpi resolution and analyzed using the Image Master 2D Platinum software version 5 (GE Healthcare). The match analysis was first performed in an automatic mode, and then edited manually where necessary. The image with the greatest number of spots was set as the reference gel. Spots with differences in normalized spot volume (%Vol) greater than twofold between metacyclic and procyclic groups were considered as differentially expressed spots.