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Transcriptomics throughout the life cycle of *Leishmania infantum*: High down-regulation rate in the amastigote stage

Pedro J. Alcolea [a], Ana Alonso [a], Manuel J. Gómez [b], Inmaculada Moreno [c], Mercedes Domínguez [c], Víctor Parro [b], Vicente Larraga [a,*]

[a] *Departamento de Microbiología Molecular y Biología de las Infecciones, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), Calle Ramiro de Maeztu 9, 28040 Madrid, Spain*

[b] *Laboratorio de Bioinformática y Laboratorio de Ecología Molecular, Centro de Astrobiología, Instituto Nacional de Técnica Aeroespacial "Esteban Terradas" (INTA)-CSIC, Carretera de Ajalvir, Km 4, 28850 Torrejón de Ardoz, Spain*

[c] *Servicio de Inmunología, Centro Nacional de Microbiología, Virología e Inmunología Sanitarias, Instituto de Salud Carlos III, carretera de Majadahonda-Pozuelo, km 2, Majadahonda, Madrid, Spain*

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ABSTRACT

Leishmania infantum is the causative agent of zoonotic visceral leishmaniasis in the Mediterranean Basin. The promastigote and amastigote stages alternate in the life cycle of the parasite, developing inside the sand-fly gut and inside mammalian phagocytic cells, respectively. High-throughput genomic and proteomic analyses have not focused their attention on promastigote development, although partial approaches have been made in *Leishmania major* and *Leishmania braziliensis*. For this reason we have studied the expression modulation of an etiological agent of visceral leishmaniasis throughout the life cycle, which has been performed by means of complete genomic microarrays. In the context of constitutive genome expression in *Leishmania* spp. described elsewhere and confirmed here (5.7%), we found a down-regulation rate of 68% in the amastigote stage, which has been contrasted by binomial tests and includes the down-regulation of genes involved in translation and ribosome biogenesis. These findings are consistent with the hypothesis of pre-adaptation of the parasite to intracellular survival at this stage.

1. Introduction

Leishmania infantum is the etiological agent of zoonotic visceral leishmaniasis in Mediterranean areas. Domestic dogs are the main reservoir of the parasite (Alvar et al., 2004) and an increase in co-infection with HIV has been registered (Pasquau et al., 2005). The dimorphic life cycle of the parasite consists of promastigote development to an infective stage inside the gut of phlebotomine sand-flies followed by transmission to the definitive mammalian host dermis and survival of non-motile amastigotes inside phagocytes (reviewed in Handman, 2001). Promastigote development inside the vector gut was mimicked in axenic culture (Neal and Miles, 1963; Lemma and Schiller, 1964; Steiger and Steiger, 1976, 1977; Berens and Marr, 1978) and the biology of the promastigote stage has been widely studied, but only under these conditions. On the other hand, amastigotes from mammalian phagocytes are not widely recognised as being equivalent to axenic amastigotes, a controversial issue (e.g. Gupta et al., 2001; Debrabant et al., 2004) which was resolved by the finding of considerable differences between axenic and intracellular amastigotes in the gene expression profile (Holzer et al., 2006; Rochette et al., 2009). Therefore, lesion-derived amastigotes and in vitro-cultured macrophage-derived amastigotes are generally more suitable for the study of parasite biology. Nevertheless, noticeable infection rates have been reported for axenic amastigote experimental models (Debrabant et al., 2004).

Comparing expression profiling of amastigotes with logarithmic (Akopyants et al., 2004; Almeida et al., 2004; Holzer et al., 2006; Leifso et al., 2007) and stationary-phase promastigotes (Akopyants et al., 2004; Almeida et al., 2004; Saxena et al., 2007; Srividya et al., 2007; Rochette et al., 2009) has been performed using microarray technology. However, surprisingly gene expression modulation during promastigote differentiation has not aroused much interest as only three reported expression profile analyses have been performed by partial genome microarrays to date. Namely, 188 (Saxena et al., 2003) and 22 (Almeida et al., 2004) differentially regulated genes were found for logarithmic versus stationary-phase promastigotes (heterogeneous populations) and 36 (Akopyants et al., 2004) for procyclic versus metacyclic promastigotes in *Leishmania major*. Subsequently, 22 differentially regulated genes have been found in *Leishmania braziliensis* promastigotes (Depledge et al., 2009). We reported the first procyclic versus metacyclic promastigote transcriptomic analysis by means of whole-genome shotgun DNA microarrays which produced 300

* Corresponding author. Tel.: +34 918373112; fax: +34 915360432.
E-mail address: vlarraga@cib.csic.es (V. Larraga).

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differentially regulated genes in a species causing visceral leishmaniasis (Alcolea et al., 2009). A reduced number of differentially regulated genes has been described elsewhere (Holzer et al., 2006; Cohen-Freue et al., 2007; Leifso et al., 2007), which supports the hypothesis of constitutive gene expression in *Leishmania* spp. with changes in gene expression modulation in a reduced number of genes. In order to assess gene expression profiles throughout the *L. infantum* life cycle, we obtained the results of microarray hybridization assays for transcriptome comparison of stationary versus logarithmic-phase promastigotes (S/L), stationary-phase promastigotes versus intracellular amastigotes (S/A) and logarithmic-phase promastigotes versus intracellular amastigotes (L/A). As a result, a set of differentially regulated genes has been found throughout the life cycle of the parasite in a variety of biochemical and physiological processes (transport, movement and cytoskeletal functions, sugar and lipid metabolism, oxidative phosphorylation, amino acid and polyamine metabolism, DNA replication, gene expression, protein maturation and secretory pathway). The differential regulation pattern of the Hydrophylic Surface Protein/Small Hydrophylic Endoplasmic Reticulum-associated Proteins (HASP/SHERP) cluster is consistent with specificity for the stationary-phase but not the metacyclic stage, except for Hydrophylic Surface Protein B (HASPB), which is up-regulated in amastigotes. Previously, these and other genes were reported to be metacyclic stage-specific, but transcript analysis was only performed for heterogeneous populations in the stationary phase instead of purified procyclic and metacyclic sub-populations (Saxena et al., 2003; Almeida et al., 2004). Consequently, specific markers for the metacyclic stage have yet to be found, so the terms metacyclic and stationary phase promastigote should be used rigorously. For this reason, a comparison between procyclic-metacyclic (Alcolea et al., 2009) and logarithmic-stationary phase promastigote expression profiles is described in this study, which should be useful to distinguish between such terms.

In the context of constitutive genome expression in *Leishmania* spp. (Holzer et al., 2006; Cohen-Freue et al., 2007; Leifso et al., 2007), transcriptomic analysis results suggest a greater down-regulation rate in the amastigote stage with respect to logarithmic-phase promastigotes provided that the set of down-regulated genes is notably greater and contains a wide variety of ribosomal constituents and translation initiation factors. In this study we performed binomial contrasts for gene modulation data sets contained in this manuscript and published elsewhere for several *Leishmania* spp. that confirm a significantly greater down-regulation rate in amastigotes. These findings have led us to conclude that down-regulation prevails over up-regulation in the amastigote stage and supports a previously reported hypothesis (Depledge et al., 2009) about the limited dynamic modulation of gene expression for the adaptation of amastigotes to the intraphagolysosomal environment.

2. Materials and methods

2.1. Promastigote culture and amastigote isolation from in vitro infected macrophages

Leishmania infantum isolate M/CAN/ES/98/10445 (zymodeme MON-1) was cultured in RPMI 1640 supplemented with L-glutamine (Cambrex, Karlskoga, Sweden), 10% heat inactivated foetal bovine serum (HIFBS) (Cambrex) and 100 μg/ml streptomycin – 100 IU/ml penicillin (Cambrex) (complete medium) at 27 °C and a starting density of 2×10^6 promastigotes/ml. Experiments were performed on three replicate early passage (ninth for replicates 1 and 3, and eighth for replicate 2) axenically cultured preinocula, originally obtained from the gut of experimentally infected sandflies, axenized and frozen for storage as previously described (Moreno et al., 2007). Cell density was assessed daily; promastigotes were recovered on days 2 and 7 and washed in PBS by centrifugation at 2000*g* for 10 min.

U937 human monocyte-like cell line was cultured at 37 °C in a 5% CO_2 atmosphere at a starting density of 10^5 cells/ml in complete medium and harvested at 250*g* after 72 h. Cells were then stimulated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) in complete medium for 72 h (Minta and Pambrun, 1985). After washing mildly with RPMI supplemented with L-glutamine (Cambrex), adhered cells were recovered by shaking the flask vigorously 10–20 times in the presence of 0.5 g/l trypsin, 0.2 g/l EDTA (Trypsin-Versene, Cambrex). The trypsin was subsequently inactivated by adding 1 vol. of complete medium after which the cells were harvested. Macrophage infections were performed by incubating 20×10^6 promastigotes/ml:10^6 macrophages/ml at 37 °C for 2 h in complete medium. This incubation was carried out in a water bath and tubes were mildly agitated at 15 min intervals. Next, the mixture was centrifuged at 250*g* for 10 min and incubated in fresh complete medium for 2 h in the presence of 5% CO_2. This step was repeated after 16 h and incubations continued until 72 h. After infected macrophage recovery, amastigotes were obtained by macrophage lysis with 0.5% SDS in RPMI with vigorous agitation for 1 min followed by centrifugation at 13,000*g* for 1 min (Hart et al., 1981). Aliquots of the infected macrophage culture were fixed for modified Giemsa staining and indirect immunofluorescence assays (IFAs) before macrophage lysis. In order to perform IFAs, cells were fixed with acetone:methanol (1:1) at −20 °C for 10 min at a density of 2×10^4/5 μl drop. They were then incubated with SIM 6.11.2.1. monoclonal anti-gp63 IgG, SIM 110 monoclonal IgG antibody against soluble leishmanial antigens (SLA), anti-rabbit complement factor H antibody or PBS at 37 °C for 30 min in a hydration chamber, washed three times by mild agitation in PBS for 10 min, incubated with FITC-conjugated goat anti-mouse IgG (Serotec, Raleigh, NC, USA) and 0.1% Evans' Blue (Fisher, Pittsburgh, PA, USA). Washes were repeated and preparations mounted with 90% glycerol. Negative controls were anti-rabbit complement factor H monoclonal IgG and PBS.

2.2. Microarray hybridization assays, analysis of selected clone sequences and validation

Genomic DNA was isolated from non-infected U937 cells as described (Alcolea et al., 2009) and directly labelled with Cy5 (350 μM dATP, dCTP, dGTP and (1/3 Cy5-dUTP + 2/3 dTTP)) using a GenomiPhi™ DNA amplification kit (GE Healthcare). RNA isolation, quality assessment and amplification, labelled cDNA synthesis, *L. infantum* random genome microarray hybridization, normalization of raw data, statistical analyses, clone sequencing, assembling and mapping of DNA sequences and validation by relative quantitative real time reverse transcription PCR (qRT-PCR) were performed as described previously (Alcolea et al., 2009). Briefly, hybridization experiments were as follows: Cy5-labelled cDNA from stationary-phase promastigotes (day 6) versus Cy3-labelled cDNA from logarithmic-phase promastigotes (day 2) (S/L); Cy5-cDNA from stationary-phase promastigotes (day 6) versus Cy3-cDNA from amastigotes (S/A); and Cy5-cDNA from logarithmic-phase promastigotes (day 2) versus Cy3-cDNA from amastigotes (L/A). U937 Cy5-labelled DNA was hybridized following the same procedures. Locally Weighted Scatter Plot Smoothing (LOWESS) per pin algorithm was used to normalize medians of raw fluorescence intensity values and ratios after background subtraction. The criteria for a given spot to be considered as differentially regulated were: (i) $F \geqslant 1.7$ (Cy5/Cy3 ratio if Cy5 > Cy3) or $\leqslant -1.7$ (Cy3/Cy5 ratio if Cy3 > Cy5), (ii) total relative fluorescence intensity value > 5000 Fluorescence Units (FU) and (iii) $p < 0.05$ (ad-