Molecular & Biochemical Parasitology 165 (2009) 32–47

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Whole-genome comparative RNA expression profiling of axenic and intracellular amastigote forms of *Leishmania infantum*

Annie Rochette[a,b], Frédéric Raymond[a,b], Jacques Corbeil[a,b], Marc Ouellette[a,b], Barbara Papadopoulou[a,b,*]

[a] *Research Centre in Infectious Disease, CHUL Research Centre, CHUQ, 2705 Laurier Blvd., Quebec, Canada G1V 4G2*
[b] *Department of Medical Biology, Faculty of Medicine, Laval University, Quebec, Canada*

ARTICLE INFO

Article history:
Received 13 September 2008
Received in revised form 13 December 2008
Accepted 23 December 2008
Available online 15 January 2009

Keywords:
Leishmania infantum
DNA microarrays
MRNA abundance
Axenic amastigotes
Intracellular amastigotes

ABSTRACT

Leishmania parasites cycle between the alimentary tract of a sandfly vector as free-living promastigotes and the acidified phagolysosomes of the vertebrate host macrophage as aflagellated amastigotes. The differentiation process can be mimicked in host-free culture by switching promastigotes (e.g. 25 °C, neutral pH) to a phagolysosomal-like environment (e.g. 37 °C, acidic pH and 5% CO_2) for certain, but not all *Leishmania* species. Axenically grown amastigotes have been shown to share several morphological and biochemical characteristics with macrophage-derived intracellular amastigotes. In this study, we used a DNA oligonucleotide full-genome array to compare global RNA expression profiling of *Leishmania infantum* axenic amastigotes to intracellular amastigotes derived from infected macrophages. In general, 40% more genes (518 genes vs. 309 genes) were found upregulated in axenic amastigotes compared to intracellular amastigotes. Comparisons in expression profiling between axenic amastigotes and intracellular amastigotes revealed substantial differences in regulated mRNA abundance. Remarkably, among the differentially upregulated transcripts only 12% were common to both amastigote preparations. The major differences between axenic and intracellular amastigotes were observed in metabolic process, especially in fatty acid metabolism, in intracellular transport and membrane vesicular fusion, in proteolysis, in the number and type of protein kinases and RNA binding proteins and in the response to oxidative stress. These findings highlight the importance of the host macrophage in driving the parasite to specific adaptations, which consequently result in highly regulated changes in gene expression.

1. Introduction

Leishmania are parasitic protozoa spread by the sandfly vector that are responsible for the leishmaniases, a group of diseases affecting more than 12 million people worldwide. The main clinical manifestations of leishmaniasis found in humans are cutaneous, muco-cutaneous, visceral (also known as kala-azar) and post-kala-azar dermal leishmaniasis (PKDL) [1]. Visceral leishmaniasis (VL) caused by the *Leishmania donovani* complex is the most serious form of the disease and is frequently fatal if left untreated. Current treatment is based on chemotherapy, which is difficult to administer, expensive and becoming ineffective due to the emergence of drug resistance [2].

All *Leishmania* species are transmitted by the bite of female phlebotomine sand flies as extracellular, flagellated metacyclic promastigotes and replicate as intracellular, aflagellate amastigotes in mononuclear phagocytes in the mammalian host [3]. During the parasite's differentiation from promastigotes into the obligatory intracellular amastigotes, *Leishmania* is switching hosts and is subjected to rapidly changing environments that include, among other, a rise in temperature, an exposure to oxygen and nitrogen-reactive species, a shift in extracellular pH, an intense extracellular proteolytic activity and a nutritional stress within the phagolysosome of mammalian macrophages. *Leishmania* are able to withstand these conditions and undergo important morphological and physiological changes [4–8] as the result of differential regulation of a variety of genes and proteins. A limited number of differentially expressed genes in either life stage have been characterized [9–16] and more recently DNA microarrays have been helpful in identifying genome-wide differential gene expression profiles [17–19].

Studies over the last decade indicated that shifting promastigotes to an intramacrophage-like environment in host-free culture (e.g. 37 °C and pH 5.5 in 5% CO_2) induced differentiation into amastigote-like forms [20–25]. Axenic amastigotes express several of the known stage-enriched or stage-specific proteins, including A2, amastin, specific proteases, nucleotidases, kinases and

* Corresponding author at: Research Centre in Infectious Disease, CHUL Research Centre, CHUQ, 2705 Laurier Blvd., Quebec, Canada G1V 4G2. Tel.: +1 418 6542705; fax: +1 418 6542715.
E-mail address: barbara.papadopoulou@crchul.ulaval.ca (B. Papadopoulou).

0166-6851/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2008.12.012

phosphatases [9,26–29], and down-regulate the expression of the surface coat lipophosphoglycan (LPG) [30]. The first axenic culture was successfully done with *Leishmania mexicana*. Parasites were taken from a lesion and kept in culture with 20% fetal calf serum at 32–33 °C under acidic pH to retain the amastigote morphology. Ultrastructural, biochemical and infectivity data showed that these cultured forms were similar to lesion amastigotes [31]. Since then, host-free systems to grow amastigotes were successfully developed for several *Leishmania* species such as *L. donovani* [21], *Leishmania infantum* [32], *Leishmania chagasi* [33], *Leishmania amazonensis* [34], *Leishmania panamensis* [35] and *Leishmania braziliensis* [33] but not for *Leishmania major*. Axenic amastigotes are being widely used for investigating different aspects of *Leishmania* biology and also for analyzing global gene expression profiles at the level of mRNA and/or protein during the parasite's differentiation and development without the complications of host cell material [17,18,28,29,35–37].

The recent completion of the genomic sequences of several *Leishmania* species (www.genedb.org) provided the opportunity to study the pattern of whole-genome expression during differentiation and development. A variety of DNA microarrays have been used to study *Leishmania* gene expression profiles in distinct developmental stages [17–19,28,38,39]. Saxena et al. analyzed the *L. donovani* transcriptome during the progression of promastigote to amastigote differentiation in a host-free system. Using a shotgun genomic array designed for *L. major* that covers approximately 40% of the *L. major* protein-coding genes they found that changes in RNA abundance are important during differentiation [28]. Analysis of the *L. mexicana* transcriptome between promastigotes and axenic amastigotes using a whole-genome array designed for *L. major* revealed only 0.2% of changes in differential gene expression [17]. However, no previous studies have examined whole-genome expression profiles between axenic amastigotes and intracellular amastigotes of a parasite species causing visceral leishmaniasis. In this study, we used DNA oligonucleotide full-genome arrays representing all *L. infantum* annotated genes to determine global RNA expression profiling of axenic amastigotes and to assess how it compares to intracellular amastigotes. Our results indicate important differences in mRNA expression patterns between *L. infantum* axenic and intracellular amastigotes with only few differentially expressed genes in common. These findings suggest that in contrast to intracellular amastigotes that are adapted to proliferate within the dynamic and highly complex environment of the macrophage phagolysosome, axenically grown amastigotes regulate mRNA expression only in response to differentiation signals, such as heat shock and extracellular pH drop.

2. Materials and methods

2.1. Leishmania strains, cell culture and differentiation

The *L. infantum* MOHM/MA/67/ITMAP-263 strain used in this study has been described elsewhere [40]. Both parasite life stages were cultured in MAA/20 medium supplemented with 20% heat-inactivated fetal calf serum (Multicell, Wisent Inc.), 5 μg/ml hemin and 1X of penicillin–streptomycin–glutamine (Invitrogen). *L. infantum* promastigotes were cultured at pH 7.0 and 25 °C. Axenic differentiation of *L. infantum* promastigotes into amastigote-like forms was induced as described previously [32]. Axenically grown amastigote forms of *L. infantum* were maintained at 37 °C in the presence of 5% CO_2 by 4–5-days sub-passages in MAA/20 medium in 25 cm^2 flasks. Amastigotes used in the present study were kept for not more than four passages in MAA/20. *L. infantum* intracellular amastigotes were cultured in a human leukemia monocyte cell line (THP-1 cells) as described previously [41]. Briefly, THP-1 cells in the log phase of growth were differentiated in medium containing 20 ng of phorbol myristate acetate/ml (Sigma) and subsequently infected with stationary-phase *L. infantum* promastigotes in flat bottom tissue culture flasks (75 cm^2) at a parasite/macrophage ratio of 15:1. After 2 h, non-internalized parasites were removed by several washes, and infected macrophages were incubated for 4 days.

2.2. RNA extraction and cDNA sample preparation

Total RNA was prepared using RNeasy Plus (Qiagen) as instructed by the manufacturer. RNA was treated with a Turbo DNA-free kit (Applied Biosystems). The quality and quantity of RNA was assessed using RNA 6000 Nano Assay Chips (Agilent Technologies). The presence of three distinct ribosomal peaks (18S, 24Sα and 24Sβ) confirmed successful RNA extraction. To extract *Leishmania* RNA from infected macrophages, macrophages were incubated with cold HEPES-NaCl-0.0125% SDS. After centrifugation at 3000 rpm, macrophages were resuspended in cold HEPES-NaCl and passed 10 times in syringe (27G1/2) to obtain pure amastigotes free of macrophage material. Amastigotes were washed once in cold HEPES-NaCl and total RNA was prepared as described above. Complementary DNA (cDNA) was generated from 5 μg of total RNA using a random primer hexamer (GE Healthcare) and aminoallyl-dUTP mix (Sigma) following the protocol for Superscript III (Invitrogen). After 5 h of incubation at 50 °C, total RNA was hydrolyzed with NaOH and EDTA for 15 min at 65 °C, then pH was neutralized with HCl. cDNA was purified from unincorporated aminoallyl-dUTP using MinElute PCR purification columns (Qiagen), phosphate wash buffer (5 mM KPO_4 pH 8.0, 80% EtOH) and phosphate elution buffer (4 mM KPO_4 pH 8.5).

2.3. Preparation of labeled cDNA and microarray hybridization

We have recently designed a high-density multispecies 70-mer oligonucleotide genome microarray representing the entire genomes of *L. major* and *L. infantum* [19,42]. This microarray includes 8978 70-mer probes that recognize with no mismatches all *L. infantum* genes (8184, GeneDB version 2.0) and 372 control probes for assessing synthesis variability, location of the probe within a given open reading frame and number of mismatches. Probes for DNA microarray hybridizations were prepared with 10 μg of total RNA for each condition. Purified cDNA from either promastigotes or axenic amastigotes or intracellular amastigotes was dried and coupled with Alexa 555 and Alexa 647 dyes (Invitrogen). After coupling, cDNA was purified with MinElute PCR Purification columns (Qiagen) and washed with phosphate washing buffer and phosphate elution buffer. The array was pre-hybridized with 5X complete Denhardt, 30% deionized formamide, 6X SSPE, 0.5% SDS and 0.1 mg/ml ssDNA for 1 h at 42 °C, washed several times and hybridized overnight at 42 °C (2.5X modified Denhardt, 30% deionized formamide, 6X SSPE, 0.5% SDS, 0.1 mg/ml ssDNA and 0.75 mg/ml yeast tRNA) as described [19]. Four biological replicates of all hybridizations were performed to account for sample heterogeneity, variation between slides and variations due to hybridization. To prevent bias by preferential label incorporation into particular sequences, dyes were swapped between the two RNA preparations. All microarray data will be freely available on the GEO NCBI database in the MIAME format; http://www.ncbi.nlm.nih.gov/geo/.

2.4. Fluorescence detection data processing and statistical analysis

The fluorescence signal intensities of four slides hybridized with RNA isolated from *L. infantum* axenic parasites were measured using the Perkin Elmer ScanArray Express scanner. The fluorescence signal intensities of the array features and local background was measured by the GenePix Pro 6.0 image analysis software (Axon