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Expression of biopterin transporter (BT1) protein in *Leishmania*

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Abstract

The present work focuses on the growth phase regulated expression of biopterin transporter gene (*BT1*) from the LD1 locus on chromosome 35 of *Leishmania donovani*. Antiserum against recombinant BT1 detected a polypeptide of 45 kDa of equal intensity at lag, log and stationary phases of promastigote growth, both in *L. donovani* strain LSB-7.1 (MHOM/BL/67/ITMAP263), and strain LSB-146.1 (HOM/IR/95/X81), a natural isolate from Isfehan, Iran that caused cutaneous leishmaniasis. However, in both these strains an additional polypeptide of higher molecular mass (50 kDa) was also observed during lag phase only. In addition, polypeptides of 40, 20, 18 and 16 kDa were seen only during the lag and log phases of both strains. Analysis of *L. donovani* single, double and triple (null) *BT1* knockout mutants confirmed that the 45-kDa polypeptide was the *BT1* gene product, as it was absent in the null mutant. These results indicate that 45-kDa BT1 protein in *Leishmania* is consistently and constitutively expressed in all the growth stages of the parasite.

Keywords: Biopterin transporter protein (BT1); BT1 knockout mutant; LD1; Growth phase regulation

1. Introduction

Leishmania are protozoan pathogens that cause leishmaniasis. Globally, millions of people are infected with these parasites and options for drug treatment are limited to the antimonial drugs. Resistance to existing drugs is a major problem. Drug resistance in *Leishmania* leads to amplification of specific genes {Beverley, CA, USA, 1991}. However, amplified DNAs have also been found in the absence of drug pressure. The LD1 locus on chromosome 35 (chr35) is amplified in 15% of the natural isolates [1]. The complete LD1 locus, or parts of it, are amplified, primarily as circular DNA or linear [2]. A number of open reading frames (ORFs) with potential protein coding functions have been identified from the LD1 [2,3]. Sequence analysis and database searches revealed genes encoding ribosomal protein L37 [4–6], a protein with homology to *sfhB* gene of *Escherichia coli* [4], a biopterin transporter [6,7], two GTP-binding proteins [8], and a potential protein kinase (P.J. Myler, unpublished data).

The biopterin transporter gene (*BT1*), initially known as *ORFG*, is amplified in all strains of *Leishmania* showing LD1 amplification [3] (P.J. Myler, unpublished data). It encodes a transmembrane protein with 10–12 putative membrane-spanning domains. These regions are predicted to form amphiphilic α-helix or β-strands typical of type IV integral membrane proteins. Database searches revealed that BT1 has 34% amino acid sequence identity and 64.3% similarity with ESAG10 gene product from the VSG expression site of *Trypanosoma* [6]. A unique type of LD1 amplification was observed in *Leishmania donovani* LSB-51.1, where the *BT1* gene is translocated to the rRNA locus on chromosome 27 (chr27), resulting in substantially increased BT1 transcript levels [9]. We report here expression of a 45-kDa BT1 protein in a *L. donovani* strain and a natural isolate causing cutaneous leishmaniasis.

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2. Materials and methods

2.1. Parasite culture

The stocks used in this study were *L. donovani* strains LSB-51.1 (MHOM/SD/00/Khartoum) and LSB-7.1 (MHOM/BL/67/ITMAP263 clone 10), which cause visceral leishmaniasis (VL) and strain LSB-146.1 (HOM/IR/95/X-81), a natural isolate from Isfehan, Iran that causes cutaneous leishmaniasis (CL). The last was kindly provided by Dr. K.P. Chang (Chicago Medical School). Speciation of LSB-146.1 is not yet done. Promastigotes of the above strains were maintained at 22°C in M199 medium (Gibco/BRL) supplemented with 100 μg ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% heat inactivated FBS (Biological Industries, Israel). In order to examine BT1 expression at different growth phases, stationary cultures of promastigotes were diluted 10-fold in fresh medium and cells harvested after 24 h (lag phase), 72 h (log phase), and 120 h (stationary phase).

Deletion mutants of the *BT1* transporter gene were derived from LSB-51.1 by three rounds of targeted gene replacement with puromycin, hygromycin and phleomycin drug resistance markers, replacing the two *BT1* alleles on chr35 and the one on chr27 [7]. The single knockout mutant (SKO) retained two copies of *BT1*, one on chr35 and the other on chr27, the double knockout mutant (DKO) lacked both copies of *BT1* on chr35 but retained the copy on chr27, and the triple knockout mutant (TKO) lacked all copies of *BT1*. Promastigotes of these strains were maintained at 22°C as described earlier [7].

2.2. Immunoprecipitation of BT1 protein

Promastigotes at lag, log and stationary phase were harvested, washed and resuspended in Hank's balanced salt solution (HBSS) to a cell density of 5×10^7 cells ml^{-1}. 50 μCi of [^{35}S]methionine were added to 1 ml aliquots of the cell suspension which were then incubated for 6 h at 22°C, before the cells were harvested and washed twice with phosphate buffered saline (PBS), pH 7.4. The cell pellets were resuspended in 250 μl of lysis buffer (10 mM Tris, pH 7.4, 0.3% Triton X-100, 150 mM NaCl, 0.02% sodium azide, 100 μg ml^{-1} PMSF, 0.3% NP-40 and 2 mM EDTA) and centrifuged at $2700\times g$ for 5 min at 4°C to remove the cell debris. Incorporation of [^{35}S]methionine was determined by trichloroacetic acid (TCA) precipitable radioactivity in 5 μl of the supernatant. For immunoprecipitation, aliquots of each sample containing 10^6 cpm were diluted to 500 μl with fresh lysis buffer and 10 μl of pre-immune sera were added for 1 h. 30 μl of protein A-Sepharose (Pharmacia) was then added and the suspensions were incubated at room temperature for 1 h, with constant inversion of the tube, before centrifugation at $2700\times g$ for 10 min at 4°C. The cleared supernatants were then immunoprecipitated with 10 μl of rabbit antiserum raised against recombinant protein representing the C-terminal portion of BT1 [7]. After 2 h incubation on ice, 30 μl of protein A-Sepharose was added and the suspensions were incubated for a further 1 h at room temperature with constant shaking. Following centrifugation as above, the pellet was washed twice with lysis buffer, resuspended in equal volume of 2× sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer (0.15 M Tris, pH 6.8, 4.0% SDS, 20% glycerol, 0.06% bromophenol blue, 1.4 M 2-mercaptoethanol) boiled for 10–15 min and loaded on to a 10% polyacrylamide gel [10]. After electrophoresis, the gel was rinsed with dimethylsulfoxide (DMSO) and incubated in five times the gel volume of DMSO for 45 min. The gel was then placed in five times the gel volume of 22% diphenyloxazole in DMSO and incubated for 45 min at room temperature with constant shaking. The diphenyloxazole impregnated gel was kept in a gentle flow of water for 30 min to 1 h. The gel was then dried, exposed to X-ray film and kept at −80°C before developing.

3. Results and discussion

In order to characterize the expression of BT1 protein, polyclonal antisera raised in a rabbit against recombinant BT1 were used to detect [^{35}S]methionine labeled proteins from the lag, log and stationary phases of promastigotes from LSB-7.1 (which causes VL) and LSB-146.1 (which causes CL). The results are shown in Fig. 1. Similar amounts of labeled polypeptide with an apparent molec-

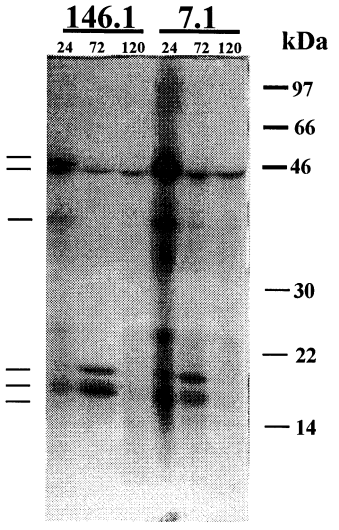

Fig. 1. Expression of BT1 protein in *Leishmania*. Promastigotes from strains LSB-7.1 and LSB-146.1 grown for 24 h (lag phase), 72 h (log phase) or 120 h (stationary phase) were incubated with [^{35}S]methionine for 6 h and the cells were harvested. Protein extracts were mixed with a rabbit antiserum and the protein A-Sepharose adsorbed immune complexes were separated by SDS–PAGE. Molecular mass markers (Pharmacia) are shown to the right, and the sizes of the labeled major polypeptides detected are shown to the left.

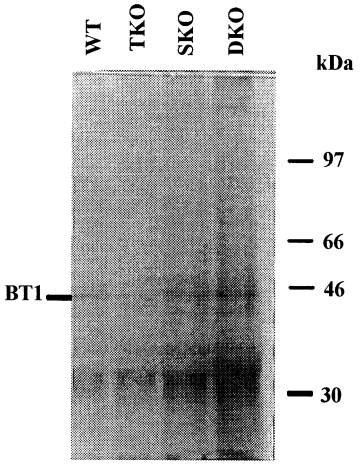

Fig. 2. Expression of BT1 in knockout mutants of *Leishmania*. Log phase promastigotes from LSB-51.1 wild-type (WT), single (SKO), double (DKO) and triple (TKO) knockout mutants were labeled with [^{35}S]methionine for 6 h, and the cells harvested. Proteins were immunoprecipitated and separated by SDS–PAGE. Molecular mass markers (Pharmacia) are shown to the right, and the labeled *BT1* encoded polypeptide is shown to the left.

ular mass of 45 kDa were observed in all the three growth phases of both strains. In contrast, a 50-kDa polypeptide was observed only during lag phase (24 h) in both LSB-7.1 and LSB-146.1. Four lower molecular mass polypeptides (40, 20, 18 and 16 kDa) were also present in lag phase parasites in both strains. The 50- and 40-kDa polypeptides were absent in log (72 h) and stationary (120 h) phase promastigotes, while the abundance of the 20-, 18- and 16-kDa polypeptides was considerably higher in log phase parasites. They were all absent in stationary phase cultures. The level of most of these proteins varied as function of parasites culture age. The 50-, 40-, 20-, 18- and 16-kDa polypeptides are not due to non-specific precipitation. Even after the pre-treatment of the cell lysates with pre-immune sera no residual complexes were observed in any of the samples (data not shown). However one protein of 45 kDa consistently and constitutively expressed in both the strains and also at different stages of growth phase.

The expected size of the BT1 protein predicted from the genomic sequence is 69.7 kDa [6,7]. However, anomalous migration of integral membrane proteins (such as the BT1) in SDS–PAGE is not uncommon [11]. Immunoprecipitation of log phase promastigotes of wild-type LSB-51.1 and the three daughter lines with single (SKO), double (DKO) and triple (TKO) knockouts of the *BT1* gene is shown in Fig. 2. The 45-kDa polypeptide is present in all lines except TKO, which is a null mutant for *BT1*. Thus, the 45-kDa polypeptide is indeed derived from the *BT1* gene. Interestingly, smaller polypeptides ($\sim$40 and 20 kDa) are present in all mutants, indicating that they are not derived from the BT1 gene. They presumably represent polypeptides containing epitopes that cross-react with the anti-BT1 antibodies. The variability of the immunoprecipitation profiles between the various strains might be due to the existence of the cellular proteins with similar epitopes that are not related to BT1 at all.

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