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ScienceDirect
Gene 416 (2008) 1 – 10
GENE
www.elsevier.com/locate/gene

Characterization and localization of ORFF gene from the LD1 locus of *Leishmania donovani*

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Received 16 August 2007; received in revised form 21 November 2007; accepted 10 January 2008
Available online 26 January 2008
Received by F.G. Alvarez-Valin

Abstract

The *Leishmania* genome project has identified new genes at a rapid rate. The 32.8-megabase haploid genome of *Leishmania major* (Friedlin strain) is published and the comparative analysis of genome sequences of two other species, *Leishmania infantum* and *Leishmanai braziliensis* has been done. The haploid genome of *Leishmania major* (Friedlin strain) has around 8272 protein-coding genes, of which only 36% can be ascribed a putative function. Out of these open reading frames around 910 *Leishmania major* genes have no orthologs in the other two Tritryp genomes. These "*Leishmania* -restricted" genes hold a potential as novel drug targets and potential vaccine candidates. Open reading frame, *ORFF*, is a single copy gene located on the chromosome 35 as a part of the multigene LD1 locus. Indirect immunofluorescence study and creation of ORFF-GFP fusion showed that ORFF is localized in the DNA containing compartments of *Leishmania donovani*, the nucleus and the kinetoplast. In order to characterize ORFF gene of *L. donovani*, we have created ORFF over-expressors and single allele deletion mutants by homologous replacement strategy. ORFF is likely to be an important gene for the parasite growth since results from over-expression studies and characterization of *ORFF* heterozygous knockout mutants reveal marked alterations in the cell cycle phenotype compared to the wild-type parasites. Flowcytometry based cell cycle analysis showed selective increase in the DNA synthetic phase of the ORFF over-expressors and a subversion of the same in heterozygous knockouts of ORFF suggesting its potential role in cell cycle progression.

Keywords: Heterozygous knockout mutants; Over-expression; *Leishmania donovani*; Cell cycle; ORFF-GFP fusion

1. Introduction

Leishmania are protozoan parasites of the order Kinetoplastida that cause a variety of diseases in humans, from dermal lesions to visceral infections. They are widespread in the tropics and subtropics. Current options for disease control and treatment are limited.

The *Leishmania* genome project has identified new genes at a rapid rate. The haploid genome of *Leishmania major* (Friedlin strain) is sequenced with around 8272 protein-coding genes, of which 36% can be ascribed a putative function (Ivens et al., 2005). Out of these open reading frames around 910 *Leishmania major* genes have no orthologs in the other two Tritryp genomes. By sequence homology, the products of many of these protein-coding genes are predicted to participate in a variety of physiological activities and regulatory processes and only a small fraction of these genes (~200) are differentially distributed among different species of the parasite (Peacock et al., 2007). However, functions of a substantial fraction of these protein-coding genes are still unknown. Studies aimed at defining the function of protozoan parasite components have often used approaches that genetically alter the cell to either over-express or under-express the protein of interest in order to determine whether this has any biological

Abbreviations: BT1, Biopterin transporter1; DAPI, 4', 6-diamidino-2-phenylindole; FACS, Fluorescence activated cell sorter; FCS, Fetal calf serum; GFP, Green Fluorescent Protein; *HYG*, Hygromycin; *NEO*, Neomycin; ORFF, Open reading frame F; ORFG, Open reading frame G; PBS, phosphate-buffered saline; PI, Propidium Iodide; SAM, S-Adenosylmethionine; WT, wild-type.

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doi:10.1016/j.gene.2008.01.011

effect. In the present study we attempted to gain insight into the characterization of the *ORFF* gene from the LD1 locus of *Leishmania donovani* by over-expressing the ORFF protein and correlating it with the consequences of disruption of the single copy of the ORFF gene.

The multigene LD1 locus is present near one telomere on chromosome 35 in all *Leishmania* isolates examined and is amplified as 100–200 copies of 55-kb circular molecules or 20–60 copies of 200–450-kb linear chromosomes (Ghosh et al., 1999; Lemley et al., 1999; Lodes et al., 1995; Myler et al., 1994; Tripp et al., 1992). This sequence is amplified in approximately 15% of all *Leishmania* strains examined (Tripp et al., 1991). The amplification occurs in the absence of an intentional drug selection, suggesting that it has an important role. While the region that is amplified varies among strains however two of the genes included in the LD1 locus, ORFF and *BT1* (biopterin transporter gene) are invariably a part of the amplification unit. It has been demonstrated earlier that the amplification and consequent over-expression of one of the genes encoded by LD1, *BT1* (biopterin transporter gene) confers a significant growth advantage in both naturally isolated and recombinant cell lines (Lemley et al., 1999). The ORFF gene is localized 365-bp upstream of the BT1 and is ~1086 nucleotide in size and has a S-adenosyl methionine (SAM)-dependent methyltransferase motif (Sunkin et al., 2001). Full length ORFF (Gene ID: L38571) predicts a 361 amino acid sequence of 39.7 kDa, while the shortened (and more likely to be translated) ORFF predicts a 286 amino acid protein of 31.6 kDa (Myler et al., 1994). Our earlier studies show that ORFF is localized in the nucleus (Ghosh et al., 1999; Madhubala et al., 2002). This gene is universally amplified in all strains of *Leishmania* showing LD1 amplification, suggesting that it may provide a selective advantage for the parasite survival. Recombinant ORFF protein (rORFF) has been found to be a differential diagnostic and a promising vaccine candidate (Raj et al., 1999; Dole et al., 2001; Sukumaran et al., 2003; Tewary et al., 2004a,b, 2005). Even though the protein product of this gene contains a SAM-dependent methyltransferase motif (Sunkin et al., 2001), it has too little sequence homology to ascribe a specific function to this protein. This indicates that it may be a *Leishmania* specific gene product.

In order to characterize ORFF gene, we have generated ORFF-over-expressing strain and ORFF single knockout mutants of *L. donovani* by homologous replacement using either neomycin or hygromycin disruption cassettes. These transgenic parasites were further characterized to study the role of ORFF in cell cycle progression. Our present data suggests that ORFF is localized in the DNA containing cellular compartments and has a possible role in the regulation of cell cycle progression of the parasite.

2. Materials and methods

2.1. Materials

[α^{32}P]-dCTP (3000 Ci/mmol) was acquired from Amersham Biosciences. All restriction enzymes and DNA modifying enzymes were obtained form MBI Fermentas. The pX63-NEO and pX63-HYG vectors that encompass the neomycin phosphotransferase (*neo*) and hygromycin phosphotransferase genes (*hyg*) respectively were kindly provided by Dr. Stephen Beverley (Harvard Medical School). Probes for *neo* and *hyg* were obtained by excision of pX63-NEO and pX63-HYG with SpeI. The DNA probe used in the present study included a ~750-bp *ORFF* specific EK1 probe. Hygromycin and Geneticin (G418) were obtained from Sigma. The other materials used in this study were of the highest purity and were commercially available.

2.2. Parasite cell culture

All parasite cell lines used in this study were derived from an Indian isolate of *L. donovani* AG83 (MHOM/IN/1983/AG83). Promasigotes were grown at 22 °C in M199 medium (Sigma U.S.A.) supplemented with 100 units/ml penicillin (Sigma, U.S.A.), 100 μg/ml streptomycin (Sigma, U.S.A.) and 10% fetal bovine serum (FBS, Hyclone). Recombinant parasites were routinely maintained in the same medium supplemented with either hygromycin B or Geneticin (G418).

2.3. Plasmid constructs

2.3.1. ORFF over-expression construct (psp72αhygroα-ORFF$^+$)

ORFF gene was PCR amplified from *L. donovani* genomic DNA and cloned into *Leishmania* specific shuttle vector psp72αhygroα, kindly provided by Dr. Marc Ouellette, Centre de Recherche en Infectologie du Centre De Recherche du, CHUL and Department of Microbiologie, Faculte de Medecine, Universite Laval, Quebec, Canada. Sequence of the sense primer was 5'-GC*TCTAGA*GCGGCTATGCTTCCTGTGAG-3' with a flanking XbaI site (underlined) and that of antisense was 5'CCC*AAGCTT*T-TAACAGACGCCACCACG3' with a flanking HindIII site (underlined). The conditions for PCR were as follows: 94 °C for 10 min, then 30 cycles of 94 °C for 1 min, 66 °C for 1 min and 72 °C for 1 min. Final extension was carried out for 10 min at 72 °C. The expected 1.1 kb PCR product was cloned into the XbaI and HindIII sites of the shuttle vector psp72αhygroα containing hygromycin phosphotransferase as the selection marker. The clones obtained were verified for the correct insert by double digestion and confirmed by automated sequencing.

2.3.2. ORFF-GFP fusion construct (pGEM-7zfαNeoα-ORFF$^+$)

ORFF gene was amplified using the primers 5'-CCC*AAGCT-T*ATGCAAAGCGACGCACG-3' and 5'-CCC*AAGCTT*CTCTG-CAACCGCAGACG-3' with a flanking HindIII site (underlined). The conditions for PCR were as follows: 94 °C for 10 min, then 35 cycles of 94 °C for 1 min, 66 °C for 1 min and 72 °C for 1 min. Final extension was carried out for 10 min at 72 °C. The 858-bp PCR product was digested with HindIII and cloned in the vector pGEM-7zfαNeoα (containing neomycin phosphotransferase as the selection marker) with GFP at the carboxy terminal of ORFF gene product. The vector was kindly provided by Dr. Marc Ouellette, Centre de Recherche en Infectologie du Centre De Recherche du, CHUL and Department of Microbiologie, Faculte de Medecine, Universite Laval, Quebec, Canada The clone was checked for the correct orientation of the insert by restriction enzyme digestion and confirmed by sequencing.