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Cloning, characterization and sub-cellular localization of gamma subunit of T-complex protein-1 (chaperonin) from *Leishmania donovani*

Bhaskar, Neeti Kumari, Neena Goyal *

Division of Biochemistry, CSIR-Central Drug Research Institute, Chattar Manzil Palace, PO Box 173, Lucknow, India

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ABSTRACT

T-complex protein-1 (TCP1) complex, a chaperonin class of protein, ubiquitous in all genera of life, is involved in intracellular assembly and folding of various proteins. The gamma subunit of TCP1 complex (TCP1γ), plays a pivotal role in the folding and assembly of cytoskeleton protein(s) as an individual or complexed with other subunits. Here, we report for the first time cloning, characterization and expression of the TCP1γ of *Leishmania donovani* (LdTCP1γ), the causative agent of Indian Kala-azar. Primary sequence analysis of LdTCP1γ revealed the presence of all the characteristic features of TCP1γ. However, leishmanial TCP1γ represents a distinct kinetoplastid group, clustered in a separate branch of the phylogenic tree. LdTCP1γ exhibited differential expression in different stages of promastigotes. The non-dividing stationary phase promastigotes exhibited 2.5-fold less expression of LdTCP1γ as compared to rapidly dividing log phase parasites. The sub-cellular distribution of LdTCP1γ was studied in log phase promastigotes by employing indirect immunofluorescence microscopy. The protein was present not only in cytoplasm but it was also localized in nucleus, peri-nuclear region, flagella, flagellar pocket and apical region. Co-localization of LdTCP1γ with actin suggests that, this gene may have a role in maintaining the structural dynamics of cytoskeleton of parasite.

1. Introduction

Protozoan parasites of the genus *Leishmania* cause a wide spectrum of diseases (visceral, cutaneous and mucosal) in humans collectively referred to as leishmaniasis, prevalent in 88 countries [1]. Visceral leishmaniasis (VL), also known as kala-azar, is the most severe form of the disease (http://www.dndi.org/diseases/vl.html). With no vaccine in sight, treatment for kala-azar relies primarily on chemotherapy [2]. The drugs recommended for the treatment are far from ideal because of high costs, toxicity and long-term treatment requirements [3]. Increasing incidences of therapeutic failures [4] and emergence of drug resistant parasites [5] against the first-line treatment have made imperative the need to understand parasite biology in order to identify novel chemotherapeutic approaches to fight leishmaniasis.

Leishmania spp. are dimorphic protozoan parasites with an extracellular flagellated promastigote stage that reside in the sand fly vector and an intracellular amastigote stage occurring within mammalian macrophages [6,7]. Promastigotes can further be differentiated in rapidly dividing, non-infective log phase parasites and non-dividing, infective stationary phase or metacyclic promastigotes. During its digenetic life cycle, the *Leishmania* parasite encounters various stresses like heat, pH, nutrient, hypoxia and oxygen radicals [8–10]. Under environmental stress, all organisms examined to date respond with the synthesis of a subset of chaperone molecules, the heat-shock proteins (HSPs) which play an important role in protein folding, assembly, secretion and regulation of other proteins [11]. This fact has led several researchers to investigate the stress response in *Leishmania*. Among various HSPs (HSP70 and 90 families), the expression of HSP100, a HSP104 homolog, is chiefly restricted to conditions of heat stress [12] and expressed only in the amastigote stage of the parasite [13]. HSP100 appears to function as an antagonist of amastigotes to promastigotes differentiation and a promoter of full amastigote development [14]. T-complex protein-1 (TCP1) complex, a HSP60 family protein, is the only identified chaperonin in eukaryotic cytosol which is involved in folding and assembly of wide range of cytosolic proteins [15]. Recently, an up-regulated expression of gamma subunit of TCP1 was reported in log phase parasites with respect to stationary phase *Leishmania infantum* promastigote [16]. Keeping in view, the role of TCP1γ in the biogenesis in other eukaryotes [25] and lack of information on this chaperonin in *Leishmania* parasite, we have, initiated the studies on characterization of the TCP1γ gene of *Leishmania donovani*. In the present study, we report, for the first time, cloning, characterization,

* Corresponding author. Address: Division of Biochemistry, CSIR-Central Drug Research Institute, Chattar Manzil Palace, PO Box 173, Lucknow 226001, UP, India. Fax: +91 522 2623938/2623405.
E-mail address: neenacdri@yahoo.com (N. Goyal).

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expression and intracellular localization of γ-subunit of TCP1complex of *L. donovani*.

2. Materials and methods

2.1. Parasite and culture condition

L. donovani promastigotes (WHO designation MHOM/IN/80/Dd8), originally obtained as a gift from (late) Prof. P.C.C. Garnham and routinely maintained at Central Drug Research Institute in golden hamsters, were used in the present study. Promastigotes were grown in medium 199 (Sigma) supplemented with 10% heat inactivated fetal bovine serum (Gibco) and 1% antibiotic and antimycotic solution (Sigma) [5].

2.2. Cloning of LdTCP1γ ORF and sequence analysis

Genomic DNA was isolated from log phase promastigotes using genomic DNA isolation kit (Qiagen) and used as a template for amplification of full-length ORF of TCP1γ. Forward primer-1(5′ATGAATGGGCAGCAACCGGT3′) and reverse primer-2 (5′CGGC TCTGCAGCACCATCGGG3′) were designed using *L. infantum* JPCM5 T-complex protein-1, gamma subunit sequence (LinJ23.1460). The amplified product was cloned in pCR-TOPO II vector (Invitrogen), to generate construct, pCRII-LdTCP1γ. Total 7 clones were sequenced in both directions to confirm the sequence of 1656 nucleotide long ORF.

2.3. Heterologous-expression of recombinant protein (rLdTCP1γ) in Escherichia coli

The full length open reading frame of LdTCP1γ was PCR amplified using primers 1 and 2 and ligated in pCR-T7/NT TOPO vector (Invitrogen) which adds 6 His tag at the amino terminal of LdTCP1γ subunit to obtain construct pCRT7-His-LdTCP1γ. The expression construct was transformed into *E. coli* BL-21 (DE3) pLysS cells. The recombinant protein (rLdTCP1γ) was expressed by induction of log phase cultures (A600 = 0.4−0.5) with 0.5 mM IPTG for 16 h at 24 °C with vigorous shaking. Protein expressed in bacteria was purified under denatured conditions using Ni–agarose ion exchange column chromatography as per manufacturer's protocol (Qiagen Inc., Valencia, CA) and analyzed on SDS–PAGE [17]. The rLdTCP1γ protein was further electroeluted from gel as previously described [18] and concentrated using Amicon ultra 50 (Millipore). Sample purity was evaluated on coomassie stained 10% SDS–PAGE gel.

2.4. SDS–PAGE and western blotting

The purified recombinant protein was used to immunize Balb/c mice to generate anti-LdTCP1γ antibodies. To compare the endogenous expression of LdTCP1γ gene in various stages of promastigotes, proteins from equivalent number of cells (2×10^6) were analyzed by SDS–PAGE, transferred onto nitrocellulose membrane and processed for western blot analysis with anti-LdTCP1γ antibody as described previously [19].

2.5. Immunofluorescence microscopy

Log phase promastigotes were harvested by centrifugation and washed twice with chilled PBS. Washed cells were allowed to adhere on the ploy-L-lysine (Sigma) coated cover-slip for 15 min at 25 °C. Adhered cells were fixed with 4% (w/v) paraformaldehyde in PBS at 25 °C for 30 min, and washed three times with 0.5% (w/v) glycine containing PBS. Adhered cells were permeabilized with 0.5% (v/v) Triton X-100 (Sigma) and blocked with 1% (w/v) bovine serum albumin in PBS for 1 h at 25 °C. Blocked cells were first incubated with primary antibodies (anti-LdTCP1γ mouse sera and anti-*Leishmania* actin rabbit sera) at 4 °C for 4 h. The cells were then stained with FITC tagged anti-mouse IgG (1:500) and Cy3 tagged anti-rabbit IgG (1:400) at 4 °C for 4 h. Nucleus and kinetidoplast DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) (5 μg/ml). Coverslips were mounted in Prolong Gold Antifade reagent (Invitrogen) and images were acquired by ACS APO 63x/1.30 oil CS objective on Leica TCS SPE, Germany. The images, at excitation 532, 635 and 488 nm, were acquired separately and merged for presentation.

3. Results

3.1. Cloning of LdTCP1γ

An open reading frame of 1656 bp of LdTCP1γ gene was observed that encodes a polypeptide of 551 amino acids with predicted molecular weight of 60.2 kDa. Average hydropathy values suggested that rLdTCP1γ is hydrophilic in nature (data not shown). The protein did not have any trans-membrane domain or signal peptide. The predicted protein has 79 basic amino acids (H, K and R) and 111 acidic amino acids (D, E, N, and Q). The LdTCP1γ Gene has high (61%) GC content. The complete sequence of LdTCP1γ was submitted to GenBank, accession no. JX088118.

Protein domain search by various tools, data bases and Clustal W sequence alignment (data not shown) of LdTCP1γ with other reported TCP1γ subunit sequences, namely, *Leishmania infantum* (XP. 001465819; putative), *Trypanpsoma brucei* (XP. 847146; putative), *Mus musculus* (NP. 033966; characterized) and *Tetrahymena pyriformis* (P54408; characterized) revealed that LdTCP1γ gene sequence has all characteristic domains of TCP1γ [15] namely equatorial domain, 1 and 2; apical domain and intermediate domain, 1 and 2 (Table 1). LdTCP1γ exhibited significant homology with other organisms in all these conserved domains except in intermediate domain 1.The calculated percent similarity shows that LdTCP1γ has highest ⩾98% identity to *L. infantum* (XP.001465819) and *L. major* (XP.001683466) putative TCP1γ gene followed by 82% identity to *Trypanosoma brucei* (XP.847146), 52–58% identity to *Saccharomyces cerevisiae* (NP.012520), *Caenorhabditis elegans* (NP.494218), *Arabidopsis thaliana* (AAO22566), *Homo sapiens* (AAH08019), *Mus musculus* (NP.033966), *Aspergillus*

Table 1
Functional domain(s) and conserved motif(s) analysis of LdTCP1γ.*

	Amino acid position(s) (Ref. No.)
Domain(s):	
Chaperonin superfamily	33–528
Equatorial domain 1	1–139 [15]
Intermediate domain1	140–197 [15]
Apical domain	198–371[15]
Lid	246–277 [31]
Intermediate domain 2	372–404 [15]
Equatorial domain 2	405–551 [15]
Motif(s):	
Chaperonin TCP-1 Signature 2	57–75
Chaperonin TCP-1Signature 3	87–95
ATP/Mg binding and hydrolysis	41–43, 93, 97,158, 394, 412, 452, 496, 498 [31]
His-Pro motif	118–119 [32]
Apical domain	223–225, 248–249, 274, 295–296, 304, 316, 319, 324 [30]

* CD-Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), scanprosite, (http://prosite.expasy.org/scanprosite/).