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

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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,

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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/).

fumigate (XP.754318), *Tetrahymena pyriformis* (P54408) and 38% identity to *Thermoplasma acidophilum* (NP.394733). In phylogenetic analysis, LdTCP1γ exhibited close evolutionary relationship with other kinetoplastids, forming an independent cluster (Fig. 1).

3.2. Recombinant expression and purification of LdTCP1γ protein in *E. coli*

The recombinant protein, rLdTCP1γ, was expressed as a histidine tagged protein in *E. coli* and purified under denaturating conditions using Ni–NTA agarose. Analysis of the purified rLdTCP1γ protein by SDS–PAGE exhibited a major band of 60 kDa protein (Fig. 2A) that corresponded to the molecular weight predicted by the open reading frame plus the size of the epitope tag (six histidines). The specificity of the recombinant protein was validated by western analysis using the anti-histidine monoclonal antibody (Roche). Single 60 kDa band was observed in the induced culture lysate and in the purified fractions (Fig. 2B, lane 1–4). The un-induced culture lysate did not exhibit any corresponding band (Fig. 2B, lane 5). The recombinant protein was then purified to homogeneity by electro-elution and used for antibody generation in mice.

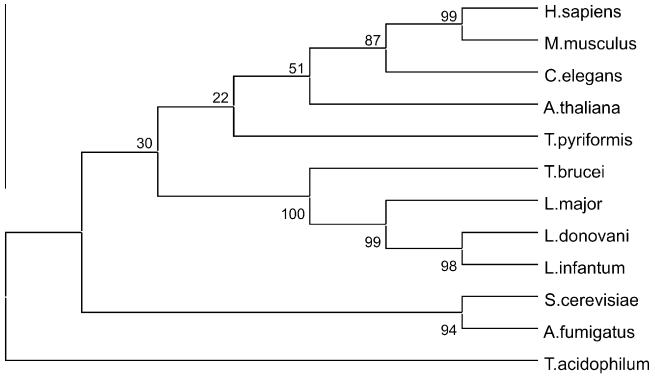

Fig. 1. Phylogenic relationship of LdTCP1γ with TCP1 gamma subunit of other organisms with their accession no. (*Leishmania infantum,* XP.001465819; *Leishmania major,* XP.001683466; *Trypanosoma brucei* XP.847146; *Saccharomyces cerevisiae*, NP.012520; *Aspergillus fumigates,* XP.754318; *Mus musculus,* NP.033966; Homo sapiens, AAH08019; *Caenorhabditis elegans*, NP.494218; *Arabidopsis thaliana*, AAO22566; *Thermoplasma acidophilum*, NP.394733; *Tetrahymena pyriformis*, P54408). The dendogram is generated by comparing full-length amino acid sequences of LdTCP1γ and TCP1 gamma subunit of other organisms using maximum likelihood with 500 numbers of bootstraps replication.

3.3. Endogenous expression of LdTCP1γ in promastigotes

Fig. 2C exhibits the endogenous expression of LdTCP1γ in promastigotes at various stages of growth. Interestingly, the LdTCP1γ antibody reacted with a single ~60 kDa protein in the lysates of *L. donovani* promastigotes. The expression of LdTCP1γ increases gradually as the cells enter from early log phase (day 2) to mid and late log phase (day 3 and 4) respectively (Fig. 2C). Mid (day 3) and late log phase (day 4) promastigotes exhibited almost 2-fold increased expression of LdTCP1γ (lane 2 and 3 respectively) as compared to early log phase promastigotes (day 2, lane 1), while stationary phase (day 5, lane 4) had 2.5-fold less expression than mid log phase parasites (day 3, lane 2). Interestingly, early log phase (day 2) and late stationary phase (day 5) parasites exhibited almost similar LdTCP1γ expression at protein level.

3.4. Subcellular localization of LdTCP1γ

Fig. 3C shows that LdTCP1γ was distributed throughout the cytoplasm of parasite including nucleus, flagellar pocket and flagellum. However, the protein exhibited predominant accumulation near peri-nuclear region of nucleus, flagellar pocket, and apical region. With actin, LdTCP1γ co-localized extensively in cytoplasm, nucleus and peri-nuclear region but not at flagellar pocket and the apical region of the promastigotes (Fig. 3E).

4. Discussion

The role of molecular chaperones (HSP70 and 90 families) in development and pathogenesis of *Leishmania* spp. has been elucidated by various groups [20,21]. However, the role of cytosolic HSP60 family especially chaperonin, T-complex protein-1 (TCP1) is yet to be explored. The eukaryotic chaperonin TRiC (TCP1-ring complex, also called CCT) is about 850–900 kDa complex consisting of two apposed hetero-oligomeric protein rings. Each ring, constituted by eight heterologous subunits (encoded by the essential CCT1–CCT8 genes in budding yeast), contains a central cavity in which unfolded polypeptide substrates attain a properly folded state in an ATP-dependent reaction [22,23]. TRiC is required for the proper folding of an important subset of cytosolic proteins,

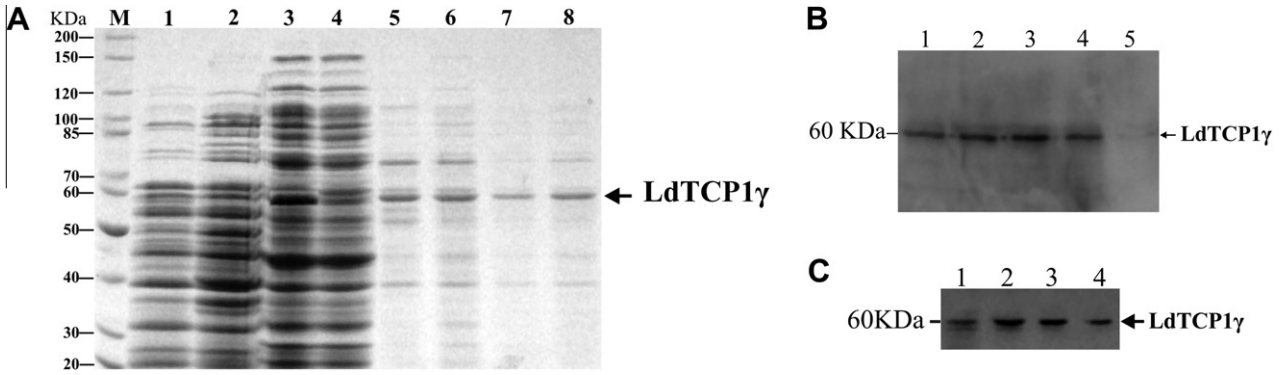

Fig. 2. Expression and purification of rLdTCP1γ in *E. coli.* (A) SDS–PAGE analysis. Lane *M*, molecular weight marker. Lane 1, *E. coli* cell lysate. Lane 2, un-induced cell lysate. Lane 3, induced cell lysate. Lane 4, flow through. Lane 5–8, elutions 1–4. Arrow indicates 60 kDa band of rLdTCP1γ. (B) Western Blot analysis with monoclonal anti-His antibodies. Lane 1, elution1. Lane 2, elution 2. Lane 3, elution 3. Lane 4, induced culture and Lane 5, uninduced culture. Arrow indicates rLdTCP1γ (C) LdTCP1γ expression in different stages of growth of *L. donovani* promastigotes. 2×10^6 promastigotes cells lysate protein from day 2 to day 5 cultures were separated on SDS–PAGE and hybridized with polyclonal anti-LdTCP1γ antibodies. Lane 1: day 2 promastigotes. Lane 2: day 3 promastigotes. Lane 3: day 4 promastigotes and Lane 4: day 5 promastigotes. Arrow indicates LdTCP1γ.

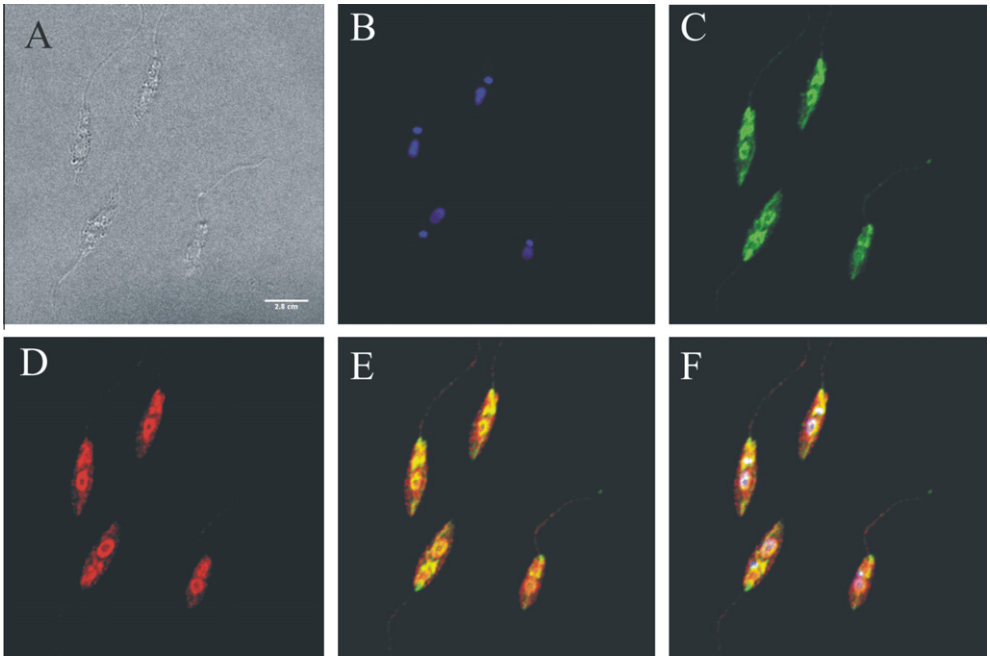

Fig. 3. Indirect immunofluorescence microscopy of *Leishmania donovani* promastigotes by immunostaining with anti-LdTCP1γ and anti-*Leishmania* actin antibodies. Panel A. phase micrograph; B. DAPI staining; C. LdTCP1γ antibody (green); D. Actin antibody (red); E. Merged image of LdTCP1γ and actin (yellow); E. Merged image of DAPI, LdTCP1γ and actin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

including cytoskeleton components, cell cycle regulators, and tumor suppressor proteins [24]. Some of these protein substrates are themselves encoded by essential genes; thus, TRiC is indispensable for eukaryotic cell survival. TCP1 gamma subunit has been shown to play an important role in the biogenesis of cilia in *Tetrahymena*, a protozoan, growth progression [25,26] and in the folding of actin and tubulin [27,28]. However, the gene has not been cloned or characterized in *Leishmania* spp. Recently, by proteome analysis, differential expression of γ-subunit of TCP1 complex was demonstrated in different stages of *L. infantum* promastigotes [16]. We have also observed an up-regulation of this subunit in the resistant field isolate of *L. donovani* by transcriptome analysis (unpublished data). All these reports prompted us to clone and characterize gamma subunit of TCP1 complex of *L. donovani*, as a first step to explore its role in parasite differentiation/biogenesis, if any.

An open reading frame of 1656 bp encoding a polypeptide of 551 amino acids with predicted molecular mass of 60.2 kDa was observed as TCP1γ gene of *L. donovani* (Fig. 3A). Protein domain/signature sequence search by various tools revealed that LdTCP1γ gene sequence has all characteristic features of TCP1γ gene. The two signature sequences of TCP-1/cpn60 chaperonin family [29] between bps 59–75 and 87–95 and highly conserved 106 residues, observed throughout the evolution, were also present in TCP1γ subunit of *L. donovani*. Further, presence of 12 signature residues of apical domain, involved in catalytic activity [30] (Table 1) suggested that cloned LdTCP1γ is a TCP1γ gene. These signature residues are predominated by charged amino residue, which have electrostatic interaction with their substrates (actin and tubulin) and found conserved throughout the species [30,15]. The highly conserved ATP/Mg binding and hydrolysis motifs, present within equatorial domain [31], were also present in LdTCP1γ. Presence of His-pro motif at position 118–119 show that it participates in heteromeric ring-ring interaction [32] with other subunits of CCT or TRiC Complex.

The phylogenetic analysis of LdTCP1γ with other organisms, revealed conservation of the gene from archaea to higher eukaryotes [24,15], sharing almost 50% amino acids identity (Fig 1.). On the basis of evolution, TCP1γ can be divided into two groups, i.e. archaea and eukaryotes. Among eukaryotes, they are further divided into three groups, i.e. euglenozoa, kinetidoplastida and higher eukaryotes (plantae, nematoda and fungi). As per evolutionary tree, kinetoplastids including *L. donovani*, *L. infantum*, *L. major*, and *T. brucei* were grouped into a separate cluster indicating significant differences from mammalian gene(s).

In order to assess and confirm the reported differential expression of TCP1γ [16] in different stages of promastigotes, polyclonal serum was raised against recombinant LdTCP1γ protein expressed in *E. coli* (rLdTCP1γ) (Fig. 2A) and used for western blot analysis. Interestingly, LdTCP1γ exhibited differential expression in log and stationary phase promastigotes of *L. donovani* (Fig. 2C). Mid and late log phase promastigotes showed significant (2.0-fold) upregulation of LdTCP1γ as compared to early log phase parasites. However, LdTCP1γ expression was decreased drastically (2.5-fold) in stationary phase promastigotes. Log phase promastigotes (procyclic) are rapidly dividing and are non-infective while stationary phase or metacyclic promastigotes are non dividing and highly infectious [33,34] parasites. The two stages exhibit differential expression of proteins involved in motility, including para-flagellar rod protein 1D, α and β-tubulin [35,36]. Hence, the differential expression of LdTCP1γ is in accordance with the change in expression pattern of genes reported in metacyclic stage promastigotes.

In higher eukaryotes and yeast, TCP1 gamma subunit localizes in the cytoplasm and its role in the folding of actin and tubulin has also been established [37,38]. Subcellular localization of *Leishmania* TCP1γ was studied in actively dividing promastigotes (day 3) that exhibited increased expression of the gene (Fig. 2C). LdTCP1γ was distributed throughout the cytoplasm of parasite with predominant accumulation near the peri-nuclear region of nucleus, flagellar pocket and apical region (Fig. 3C). With actin, it co-localized in cytoplasm but not at cytoplasmic face of plasma membranes (Fig. 3E), flagellar pocket and apical region. It has been shown earlier that *Leishmania* actin localizes in cytoplasm, vacuolar and cytoplasmic face of the plasma membranes [39]. Co-localization of LdTCP1γ with actin, suggests that TCP1 complex may be involved in folding of actin. Large amount of this protein

concentration in flagellar pocket and in flagella also indicates its possible role in maintaining the motility of parasite in non-clinical stage.

Taken together, we have for the first time reported gene cloning, characterization, and subcellular localization of gamma subunit of T-complex protein-1 (TCP1), a chaperonin from *L. donovani*. The confirmation of decreased endogenous expression of LdTCP1γ in stationary phase promastigotes as compared to log phase promastigotes strongly suggests that this gene may have a role in biogenesis. By exploring the role of LdTCP1γ in metacyclogenesis and by targeting it, new drugs could be developed to control visceral leishmaniasis.

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