Reconstitution and functional characterization of the unusual bi-subunit type I DNA topoisomerase from *Leishmania donovani*

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Abstract ***Leishmania donovani*** **topoisomerase I is an unusual bi-subunit enzyme. The activity of the enzyme has been detected when the genes of the individual subunits were co-expressed in yeast [J. Biol. Chem. 278 (2003) 3521]. Here, we report for the first time, the in vitro reconstitution of the two recombinant proteins, LdTOP1L and LdTOP1S, corresponding to the large and small subunits and localization of the active enzyme in both the nucleus and kinetoplast. The proteins were purified from bacterial extract and the activity was measured by plasmid DNA relaxation assay. LdTOP1L and LdTOP1S form a direct 1:1 heterodimer complex through protein–protein interaction. Under standard relaxation assay condition (50 mM KCl and 10 mM Mg^{2+}), reconstituted enzyme (LdTOP1LS) showed reduced processivity as well as 2-fold reduced affinity for DNA compared to eukaryotic monomeric rat liver topoisomerase I (RLTOP1). Cleavage assay at various salt concentrations reveals that Camptothecin (CPT) enhanced the formation of "cleavable complex" at low salt. Interaction between the two subunits leading to the formation of an active complex could be explored as an insight for development of new therapeutic agents with specific selectivity.**

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1. Introduction

DNA topoisomerases are ubiquitous enzymes that govern the topology of DNA inside the cells. The enzymes are classified as type I topoisomerase making transient single strand breaks and type II topoisomerases, which make transient double strand breaks and require ATP as co-factor. The type I DNA topoisomerase is further divided into type IA (bacterial) and type IB (eukaryotic) classes that are distinct from one another in sequence, structure, reaction mechanism and susceptibility to inhibitors [1–3].

Eukaryotic type IB topoisomerase is monomeric and consists of four domains [4]. The unconserved amino terminal domain contains putative signals for nuclear localization of the enzyme and is highly sensitive to proteolysis and dispensable for in vitro activity [5]. The largest core domain is essential for enzyme activity and shows high phylogenic conservation, particularly the amino acid residues closely interacting with DNA. The third domain is known as the linker, which is poorly conserved and is variable in length. Finally, the carboxy terminal domain is highly conserved and contains the SKINYL motif. Cleavage occurs by trans-esterification reaction involving nucleophilic attack by an active tyrosine (Tyr^{723} in human Topo I) on a DNA phosphodiester bond resulting in the formation of a covalent DNA 3′ phosphotyrosyl linkage. In religation phase a similar trans-esterification reaction involves attack by the free DNA 5′ hydroxyl that releases the enzyme from DNA [5,6].

DNA topoisomerase I of kinetoplastid protozoan parasite *Leishmania donovani* is distinct from other eukaryotic counterparts with respect to its biological properties and preferential sensitivity to many therapeutic agents [7]. The first DNA sequence of topoisomerase IB like gene from kinetoplastid *L. donovani* was reported by Broccoli et al. [8], but the overexpressed protein in *Escherichia coli* failed to show any relaxation activity in vitro or complement a mutant deficient in topoisomerase I activity. The consensus SKXXY motif harboring the active site tyrosine was absent in this protein. Recently, the existence of bi-subunit topoisomerase I in kinetoplastid parasites *L. donovani* [9] and *Trypanosoma brucei* [10] has been reported. The two subunits are synthesized from two different genes, which associate with each other to form a dimeric topoisomerase I enzyme within the parasite.

Camptothecin (CPT), an important class of antitumor agent [11], is an uncompetitive inhibitor that traps the enzyme–DNA covalent complex and slows the religation step of the nicking closing cycle [12,13]. In *T. brucei*, *Trypanosoma cruzi* and *L. donovani*, CPT promotes protein–DNA complex formation with nuclear as well as kinetoplast DNA [14].

From our laboratory, we have previously reported ATP independent, type 1 topoisomerase activity from cell extract of *L. donovani* promastigotes [15], which relaxes negative supercoils in topological steps of unity. The enzyme is sensitive to CPT and is a target for potential chemotherapy of antileishmanial drugs [16,17].

In this paper we for the first time describe the in vitro reconstitution of the two recombinant proteins, LdTOP1L and LdTOP1S, corresponding to the large and small subunits and the localization of the active enzyme in both the nucleus and kinetoplast.

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Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription polymerase chain reaction

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2. Materials and methods

2.1. Parasite and culture condition

Leishmania donovani D1700 parasites are maintained as promastigotes as described [16].

2.2. Construction of recombinant plasmid

2.2.1. Large subunit (LdTOP1L). Total cellular RNA was isolated from *L. donovani* D1700 using RNA isolation kit (Roche Applied Science) according to the manufacturer's protocol. Reverse transcription polymerase chain reaction (RT-PCR) was performed [18] with gene-specific primers corresponding to a single open reading frame (ORF) of 1905 bp (LdTOP1L gene, GenBank Accession No. AF303577) using sense primer 5′-GGGAAT TCCATATGCCGTTGC CAGGGGGAGGGACC-3′ containing an *Nde*I site created at the initiation codon of the ORF, and an anti-sense primer 5′-CGGGATCCCTACACCCTCAAAGCTGCAAGAGG-3′, with a *Bam*HI site immediately downstream from the termination codon. The RT-PCR amplified fragment was cloned in the *Nde*I/*Bam*HI site of bacterial expression vector *pET16b* (6× His-tag). The resultant construct *pET16bLdTOP1L* was transformed in *E. coli* BL21 (DE3) pLysS as described [19].

2.2.2. Small subunit (LdTOP1S). Genomic DNA isolated from *L. donovani* promastigotes was subjected to PCR [9] with sets of gene specific primers corresponding to a single ORF consisting of 786 bp (LdTOP1S gene, GenBank Accession No. AY062908), using sense primer 5′-GGGAATTCCCATATGCAGCCTGTTCAAAGTCCT-3′ containing an *Nde*I site created at the initiation codon of the ORF, and an anti-sense primer 5′-CGGGATCCCTCAA-AAATCCAAGTTCT CGGC-3′ with a *Bam*HI site immediately downstream from the termination codon. The PCR amplified fragment was cloned in *Nde*I/*Bam*HI site of bacterial expression vector *pET16b*. The resultant construct *pET16bLdTOP1S* was transformed in *E. coli* BL21 (DE3) pLysS.

The same PCR was performed with a different set of sense and anti-sense primers containing *Bam*HI/*Eco*RI restriction sites and the amplified fragment was cloned in *pGEX-5X2* expression vector under the restriction of *Bam*HI/*Eco*RI site. The resultant construct *pGEX-GST-LdTOP1S* was transformed into *E. coli* BL21 (DE3) [18].

2.3. Over-expression and purification procedures

pET16bLdTOP1L was induced at $OD_{600} = 0.6$ with 0.5 mM IPTG at 22 °C for 12 h. Cells harvested from 1 l of culture were lysed by lysozyme/sonication in resuspension buffer A (50 mM NaH_2PO_4, pH 7.8, 150 mM NaCl, 10 mM imidazole and Cocktail protease inhibitor) and then cleared by centrifugation. Supernatant containing 285 mg protein was loaded onto a Ni^{2+}–NTA agarose column (packed volume 2 ml, Qiagen), pre-equilibrated with resuspension buffer A. The column was washed with buffer A containing 40 mM imidazole and was eluted in the same buffer A containing 300 mM imidazole. For further purification, the fractions were pooled from Ni^{2+} column and dialyzed. The dialysate containing 20.5 mg protein was loaded onto a 5 ml packed phosphocellulose column (P11 cellulose, Whatman). The column was washed with 10 ml of wash buffer (50 mM Tris–HCl, pH 7.5, 0.5 mM EDTA and 20% glycerol) containing 600 mM KCl and finally eluted with the same buffer containing 800 mM KCl. The eluted fractions of LdTOP1L were pooled and stored at −70 °C. *E. coli* BL21 (DE3) pLysS cells harboring *pET16bLdTOP1S* were over-expressed, solubilized, purified and stored at −70 °C as above.

Escherichia coli BL21 (DE3) cells harboring *pGEX-GST-LdTOP1S* were over-expressed, solubilized and purified through glutathione *S*-transferase (GST)–Sepharose column as described by the manufacturer's protocol (Amersham Pharmecia, Bulk GST purification module).

2.3.1. Purification of rat liver topoisomerase I. Eukaryotic monomeric rat liver topoisomerase I (RLTOP1) was purified as described by Champoux and McConaughy [20]. The purified proteins were estimated by Bio-Rad Protein Estimation Kit according to the manufacturer's protocol.

2.4. Reconstitution of LdTOP1L and LdTOP1S in vitro

Purified LdTOP1L was mixed with purified LdTOP1S at a molar ratio of 1:1 and a total protein concentration of 0.5 mg/ml in reconstitution buffer (50 mM potassium phosphate, pH 7.5, 0.5 mM DTT, 1 mM EDTA, 0.1 mM PMSF and 10% glycerol). The mix was dialyzed overnight at 4 °C and the dialyzed fractions were used for the plasmid relaxation activity.

2.5. Plasmid relaxation assay

The type I DNA topoisomerase was assayed by decreased mobility of the relaxed isomers of supercoiled pBluescript (SK^+) DNA in an agarose gel. Relaxation assay was carried out as described [16] with LdTOP1L, LdTOP1S, LdTOP1LS and RLTOP1 serially diluted in the relaxation buffer (25 mM Tris–HCl, pH 7.5, 5% glycerol, 0.5 mM DTT, 10 mM $MgCl_2$, 2.5 mM EDTA and 150 μg/ml BSA), supercoiled pBluescript (SK^+) DNA (85–95% were negatively supercoiled with the remainder being nicked circles) and 50 mM KCl. The amount of supercoiled monomer DNA band fluorescence after ethidium bromide (EtBr) staining was quantitated by integration using Gel Doc 2000 (Bio Rad-Quality one software). Initial velocities (nM DNA base pairs relaxed × min^{-1}) were calculated by the equation

$$\text{Initial velocity} = \frac{([\text{supercoiled DNA}]_0 - Int_0 \cdot [\text{supercoiled DNA}]_0)/t}{Int_t}$$

where $[\text{supercoiled DNA}]_0$ is the initial concentration of supercoiled DNA, Int_0 is the area under the supercoiled DNA band at time zero, and Int_t is the area at the reaction time t [21]. The effect of DNA concentration on the kinetics of relaxation was examined over a range of 2.5–40 nM supercoiled pBluescript (SK^+) DNA (0.1–2 μg/25 μl reaction mix) at constant concentration of 10 mM $MgCl_2$ and 0.9 nM enzyme (LdTOP1LS or RLTOP1) at 37 °C for 1 min. The data were analyzed by Lineweaver–Burk plot. Intercept of Y-axis is $1/V_{max}$ and turnover number = V_{max}/enzyme concentration (plasmid molecules relaxed/min/molecule of enzyme).

2.6. Analysis of topoisomerase I DNA interaction by electrophoretic mobility shift assay

The labeling and annealing of the 25-mer duplex of oligonucleotide 1 (5′-AAAAAGACTTAGAAAAATTTTTAAA-3′) and oligonucleotide 2 (5′-TTTAAAAAT-TTTTCTAAGTCTTTTT-3′), containing a topoisomerase I binding motif, have been carried out as described [22]. DNA binding assay was carried out by incubating the labeled oligo 1/oligo 2 (1 nM) in 25 μl of reaction mix (10 mM Tris–HCl, pH 7.5, 3 mM $CaCl_2$, 10 mM NaCl, 0.1 M sucrose and 5% glycerol). For LdTOP1LS and RLTOP1, the protein concentrations used in the assay range from 2 to 0.06 μM at 15 °C for 15 min. The reaction mixtures were electrophoresed in 6% non-denaturating polyacrylamide gel at 4 °C in 0.167× TBE buffer (45 mM Tris–borate, 1 mM EDTA) and autoradiographed. Quantitation of the unbound oligonucleotide was done by film densitometry. The K_d was estimated from the protein concentration at which one half of duplex oligonucleotide was bound to the protein [23].

2.7. Ni^{2+}–NTA agarose co-immobilization binding assay

Protein complexes of hexa histidine-tagged LdTOP1L and GST-LdTOP1S (10 μg) were mixed with pre-equilibrated Ni^{2+}–NTA agarose bead (Qiagen) in 100 μl reconstitution buffer and incubated at 4 °C for 2.5 h with gentle shaking. The beads were pelleted by centrifugation and washed twice with 500 μl of reconstitution buffer containing 20 mM imidazole so that all the unbound proteins were removed. The protein samples were eluted from the beads with 50 μl reconstitution buffer containing 250 mM imidazole and analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [24]. Molar ratio was calculated by densitometric scanning using standard proteins in Gel Doc 2000 (Bio Rad-Quality one software).

2.8. Cleavage assay

Cleavage assay was carried out as described in [16]. Briefly, 50 fmol of pHOT1 supercoiled DNA (containing Topo I cleavage site), 100 fmol of reconstituted LdTOP1LS and varying concentrations of KCl were incubated in the presence and absence of CPT (50 μM) at 37 °C for 30 min. The DNA samples were electrophoresed in 1% agarose gel. EtBr at a final concentration of 0.5 μg/ml was included in the gel to resolve the more slowly migrating nicked product (Form II) from the supercoiled molecules (Form I).

2.9. Antibody production and Western blotting analyses

A 1.2 kb fragment was PCR amplified from the full-length 1.9 kb clone of LdTOP1L using the sense primer 5′-CGGGATCC TGCTCTGCGACTTCGACCT-TCGAGCCCAT-3′and an anti-sense primer 5′-CCGCTCGAGCAGGATCTCCTC-GAACTCCTT-3′ that codes for the amino acid 125–528 of LdTOP1L gene. The amplified fragment was sub-cloned in *pGEX-5X2* expression vector under the restriction site of *Bam*HI/*Xho*I site and the resultant construct *pGEX-GST-ΔLdTOP1L* was transformed into *E. coli* BL21 (DE3). The GST fusion protein (71 kDa) contained 26 kDa GST from *Schistosoma japonicum* in addition to ≈45 kDa peptide from LdTOP1L. The over-expressed protein was solubilized and purified through GST–Sepharose column as described previously. The purified 71 kDa (pGEX-GST-ΔLdTOP1L) and 29 kDa (LdTOP1S) recombinant proteins (100 μg each) were subcutaneously injected in rabbit separately for raising antibodies [25]. This serum was then used for Western blot analyses [19] and immunofluorescence experiments.

2.10. Immuno-fluorescence microscopy

Immunofluorescence experiments were carried out using *L. donovani* promastigotes (10×10^6 parasites). Cells were adhered to poly-L-lysine coated slides, as described in [18] and probed separately with antibody against LdTOP1L and LdTOP1S (1:25 dilution), followed by reaction with fluorescein–isothiocyanate (FITC)-conjugate goat-derived anti-rabbit IgG (1:100 dilution). To locate the nucleus and the kinetoplast, the fixed cells were stained with EtBr (0.25 μg/ml). The cells were viewed with a TCS-SP Leica confocal microscope system equipped with a krypton–argon mixed laser [19].

3. Results

3.1. Purification of recombinant proteins

The schematic representations of all recombinant constructs are shown in Fig. 1A. The over-expressed proteins from *E. coli* BL21 (DE3) pLysS cells harboring plasmid *pET16bLdTOP1L* and *pET16bLdTOP1S* were purified separately through Ni^{2+}–NTA agarose column. Subunits were further purified through phosphocellulose column which gave rise to proteins of 73 and 29 kDa as analyzed by SDS–PAGE (Fig. 1B). The yields of purified proteins are 20 mg of individual subunits per litre of culture, which is equivalent to 0.15 pg of individual subunits per cell. The over-expressed protein from *E. coli* BL21 (DE3), harboring the plasmid *pGEX-GST-LdTOP1S* when purified through GST–Sepharose, appears to be a protein of 55 kDa (26 kDa from S. *japonicum* glutathione *S*-transferase and 29 kDa LdTOP1S) as analyzed by SDS–PAGE (Fig. 1B).

3.2. Reconstitution of relaxation activity with two subunits and DNA binding affinity

Reconstitution of the enzyme activity was examined by plasmid relaxation assay. The purified subunits of LdTOP1L and LdTOP1S were mixed at a molar ratio of 1:1, in reconstitution buffer and dialyzed overnight at 4 °C as described in Section 2. The reconstituted enzyme (LdTOP1LS) showed topoisomerase I relaxation activity (specific activity $= 0.5 \times 10^6$ U/mg, where 1 U is the amount of enzyme required to fully relax 0.5 μg of negatively supercoiled pBS (SK^+) DNA in 15 min at 37 °C).

Purified subunits, LdTOP1L and LdTOP1S, failed to show the generation of topological isomers when assayed separately (Fig. 2A, lanes 4 and 5). To discriminate between *L. donovani* topoisomerase I activity and contaminating bacterial type IA activity, plasmid DNA relaxation assays were performed in standard assay mix containing 10 mM Mg^{2+} with crude extracts of induced and uninduced bacterial cells harboring

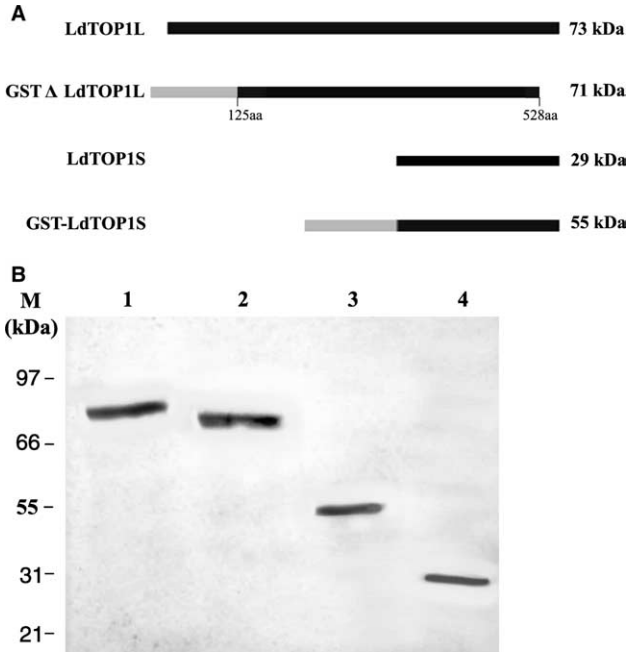

Fig. 1. Protein constructs. (A) Structure of recombinant *L. donovani* topo I proteins. First line shows the larger subunit (dark) as core DNA binding, second line shows the truncated ΔLdTOP1L that starts from amino acid 125 to 528 corresponding to the most conserved region in eukaryotic type I DNA topoisomerase (dark) with a GST tag (light shaded). Third line shows smaller catalytic subunit. Fourth line, same as third line but with a GST tag. (B) Coomassie-stained 10% SDS–PAGE analysis of the purified recombinant proteins with 5 μg per lane. Lanes 1 and 4, LdTOP1L and LdTOP1S proteins purified through Ni^{2+}–NTA followed by phosphocellulose column. Lanes 2 and 3, GST-ΔLdTOP1L and GST-LdTOP1S purified through GST–Sepharose column as described in Section 2. The position and molecular masses of protein standards are indicated on the left.

pET16b. No relaxation activity was observed with uninduced or induced fractions of crude bacterial extract harboring *pET16b* (Fig. 2A, lanes 2 and 3), while reconstituted LdTOP1LS showed the relaxation of supercoiled DNA. The bi-subunit *L. donovani* topoisomerase I is active without Mg^{2+} but in the presence of 100–150 mM KCl. (Fig. 2A, lanes 8 and 9). At 50 mM KCl, Mg^{2+} enhanced the activity, with an optimum activity obtained at 10 mM concentration (Fig. 2A, lanes 10–14).

To investigate the relaxation activity of reconstituted bi-subunit LdTOP1LS, we compared it with that of the eukaryotic monomeric RLTOP1. Time course relaxation experiments were performed in a standard assay mix containing 10 mM $Mg^{2+}$and 50 mM KCl at 37 °C where the plasmid DNA and the enzymes were mixed at a molar ratio of 3:1. The velocity for both enzymes is linear for the first 5 min of the reaction. All the subsequent initial velocities during kinetic studies were calculated for the time point up to 1 min, well within the linear range for the velocity examined. It was found that LdTOP1LS relaxes supercoiled pBS (SK^+) faster than monomeric RLTOP1 (Fig. 2B). The lack of topoisomer intermediates indicates that monomeric RLTOP1 completely relaxes a substrate in processive fashion. However, under this condition the situation with reconstituted LdTOP1LS displays a lower processivity relative to that observed with RLTOP1.

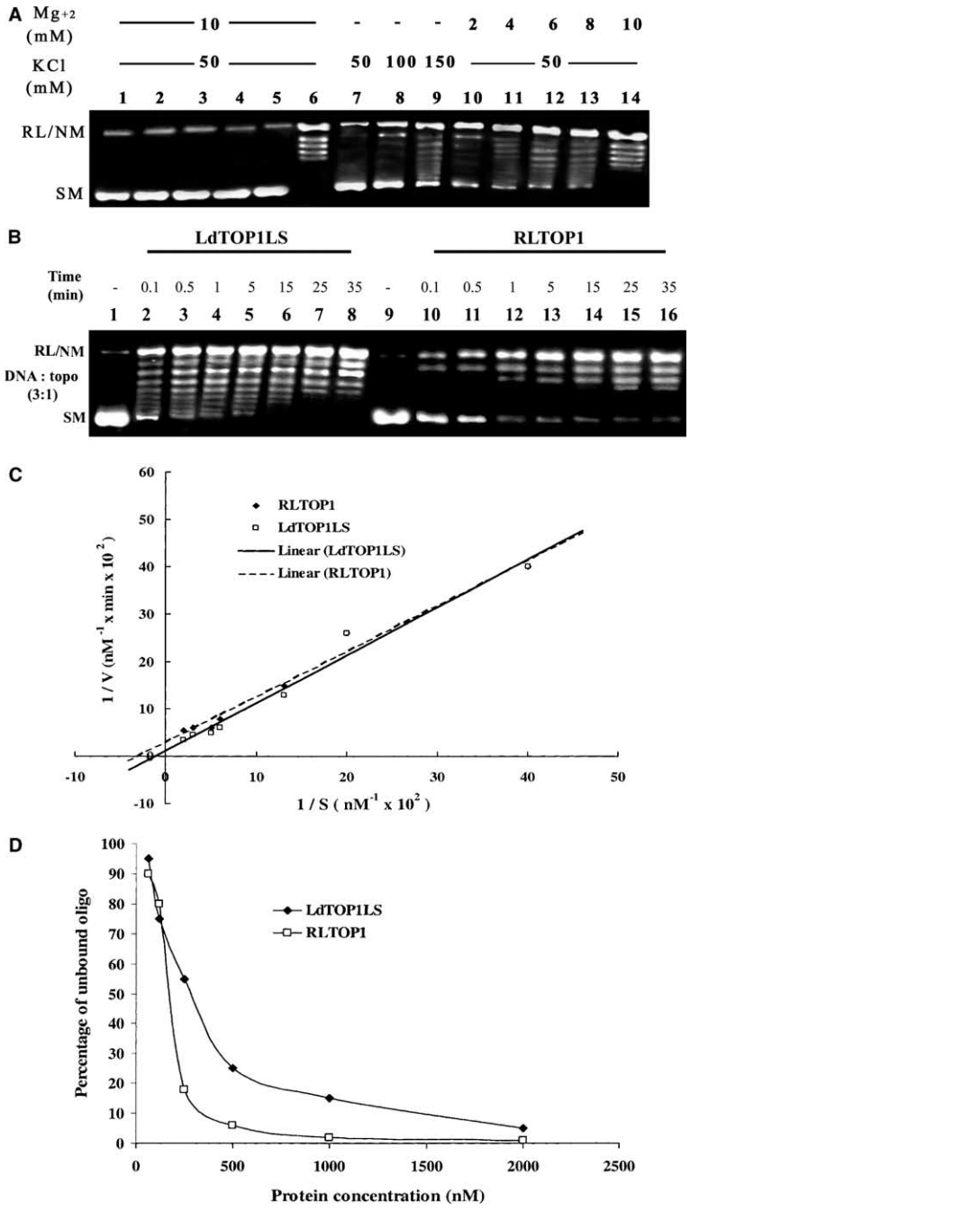

Fig. 2. Reconstitution of *L. donovani* topo I activity and comparison with RLTOP1. (A) Relaxation of 0.5 μg supercoiled pBS (SK^+) DNA (lane 1). Lanes 2 and 3, same as lane 1 but incubated with induced and uninduced bacterial extract containing 0.1 μg protein from cells harboring pET16b. Lanes 4 and 5, same as lane 1 but incubated with 40 fmol of purified LdTOP1L and LdTOP1S, respectively. Lane 6 same as lane 1 but incubated with 20 fmol of reconstituted LdTOP1LS. Lanes 7–9 same as lane 1 but incubated with 20 fmol of LdTOP1LS in topoisomerase I relaxation buffer without Mg^{2+}, but with indicated concentrations of KCl. Lanes 10–14 same as lane 1 but incubated with 20 fmol of LdTOP1LS in topoisomerase I relaxation buffer containing indicated concentrations of KCl and Mg^{2+} at 37 °C for 15 min. Samples were electrophoresed in 1% agarose gel. (B) Relaxation of supercoiled pBS (SK^+) DNA with reconstituted LdTOP1LS and RLTOP1 at a molar ratio of 3:1. Lanes 1 and 9, 60 fmol of pBS (SK^+) DNA. Lanes 2–8, same as lane 1 but incubated with 20 fmol of LdTOP1LS. Lanes 10–16, same as lanes 2–8 but incubated with RLTOP1 instead of LdTOP1LS at 37 °C for different time periods as described in the figure. Samples were electrophoresed in 1% agarose gel. Positions of supercoiled monomer (SM) and relaxed and nicked monomer (RL/NM) are indicated. (C) Lineweaver–Burk representation of the kinetics of relaxation of supercoiled pBS (SK^+) DNA by LdTOP1LS and RLTOP1. DNA concentrations ranged from 2.5 to 40 nM, $MgCl_2$ at 10 mM and enzyme at 0.9 nM concentrations. (D) DNA binding assays. The native gel shift assay was carried out as described in Section 2. The percentage of unbound duplex oligonucleotide present in the gel was quantified by film densitometry and plotted against the protein concentrations. The binding profiles for LdTOP1LS and RLTOP1 are indicated in the figure.

The kinetics of relaxation by LdTOP1LS and RLTOP1 was examined over a range of supercoiled pBS (SK^+) DNA (2.5–40 nM) and the initial velocity was plotted in Lineweaver–Burk plot (Fig. 2C). The maximal velocity (V_{max}) for LdTOP1LS was 6.6×10^{-8} M base pairs of supercoiled DNA relaxed/min/0.9 nM of enzyme which corresponds to a turnover number of

about 73 plasmid molecules relaxed/min/molecules of enzyme. The maximal velocity (V_{max}) for RLTOP1 was 3.3×10^{-8} M base pairs of supercoiled DNA relaxed/min/0.9 nM of enzyme which corresponds to a turnover number of about 36 for RLTOP1 plasmid molecules relaxed/min/molecules of enzyme. These results indicate that LdTOP1LS is two times faster than RLTOP1 in plasmid DNA relaxation activity.

We compared the DNA binding affinity of bi-subunit LdTOP1LS and monomeric RLTOP1 for the duplex oligomer containing the high affinity topoisomerase IB binding site [22] by native gel mobility shift assay. The binding assays yielded a K_d value of 3.1×10^{-7} M for the interaction of LdTOP1LS with the DNA substrate, which is about 2-fold higher than the value measured for the interaction of RLTOP1 with DNA (Fig. 2D). These results indicate a lower affinity for DNA of LdTOP1LS compared to RLTOP1, which is consistent with the reduced processivity of LdTOP1LS in the plasmid relaxation assay.

3.3. *LdTOP1L and LdTOP1S forms a 1:1 complex*

LdTOP1L and *LDTOP1S* in nuclear run-on assay indicate that the abundance of nascent RNA transcripts derived from both the genes was similar, as described by Villa et al. [9]. Hence, a protein–protein interaction at post-translational level should occur to reconstitute an active DNA topoisomerase I in *L. donovani* cells.

The protein–protein interaction between 6× His-tagged LdTOP1L and GST-LdTOP1S in vitro can be shown through Ni^{2+}–NTA agarose co-immobilization assay [24]. In this experiment, purified LdTOP1L was mixed with GST-LdTOP1S in reconstitution buffer for favoring the assembly between the two proteins, and the mixture of proteins was allowed to bind to Ni^{2+}–NTA agarose beads (Fig. 3A, lane L) and incubated for 2.5 h at 4 °C. Excess of unbound proteins was removed by washing with 20 mM imidazole buffer (Fig. 3A, lane W), and finally bound proteins were eluted with 250 mM imidazole. Both LdTOP1L and LdTOP1S were eluted as shown by SDS–PAGE (Fig. 3A, lane E). Densitometric scanning of the two proteins indicate a direct 1:1 molar interaction between LdTOP1L and LdTOP1S in the reconstituted enzyme.

Furthermore, the molecular size of reconstituted enzyme LdTOP1LS (both subunits 6× His-tagged) was determined by gel permeation chromatography using "spin column" as described by Nath et al. [26]. LdTOP1LS (2 μg) was applied onto 1 ml of gel filtration matrix Sephadex G-100 pre-equilibrated with reconstitution buffer under mild centrifugal force. Proteins eluted in the void volume are recovered near quantitatively, while others are partly or almost completely retained depending on molecular size. In the calibration curve of "spin columns" showing recovery of standard proteins versus their molecular weight using the above gel filtration matrix, reconstituted LdTOP1LS appears to be a heterodimer (Fig. 3B).

3.4. *Cleavage assay and salt dependency*

Camptothecin, the most established topoisomerase I inhibitor, has been shown to stabilize the cleavable complex. Here, we investigated the characteristics of the reconstituted bi-subunit LdTOP1LS in cleavage assay. Cleavage assays were performed under various KCl concentrations in the presence and absence of CPT (Fig. 4A). The results show that in the absence of CPT maximum cleavage occurred at 50 mM KCl (Fig. 4, lane 4) and a salt above 150 mM reduced the cleavage

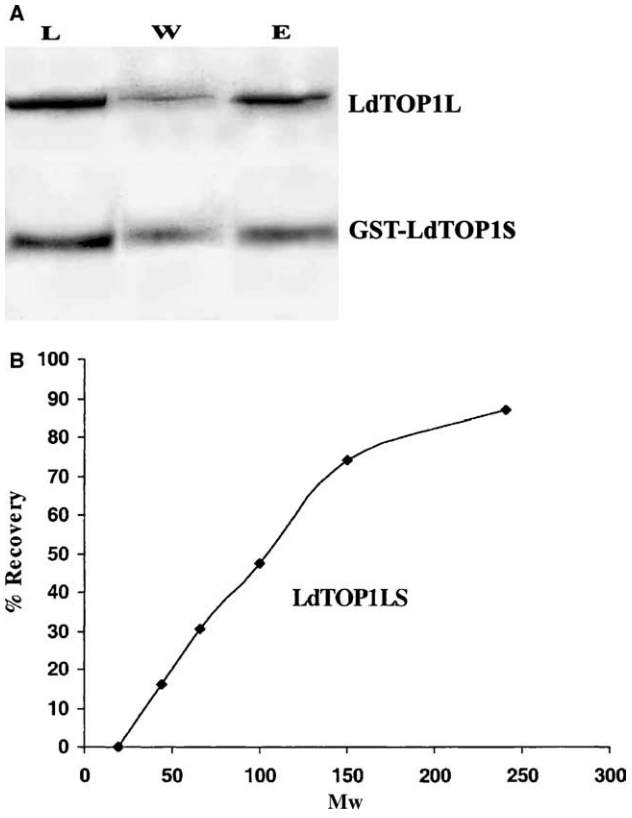

Fig. 3. (A) Ni^{2+}–NTA agarose co-immobilization binding between LdTOP1L and LdTOP1S. Lane L, 6× His-LdTOP1L and GST-LdTOP1S reconstituted complexes before loading onto Ni^{2+}–NTA agarose beads. Lane W, excess unbound proteins after washing with 20 mM imidazole. Lane E, bound proteins eluted with 250 mM imidazole. Proteins were electrophoresed in 10% SDS–PAGE. The positions of the proteins in the gel are indicated with respect to the corresponding molecular weight of the subunits. (B) Calibration curve of "spin columns" showing recovery of standard proteins and LdTOP1LS versus their molecular weight using Sephadex G-100 gel-filtration matrix. Proteins used were myoglobin (19.7 kDa), ovalbumin (43.5 kDa), bovine serum albumin (66.7 kDa), LdTOP1LS (100 kDa), alcohol dehydrogenase (150 kDa) and catalase (240 kDa) indicated in the figure. Variations of results were within ±5% ($n = 3$).

by 35% (Fig. 4B). At 300 mM KCl, the cleavage reaction is completely inhibited.

The fact that cleavage is enhanced in the presence of the drug suggests that CPT binds to the covalent complex between the enzyme and DNA (Fig. 4B). CPT enhanced the formation of cleavable complex by 40% with respect to the extent of cleavage observed at 50 mM KCl in the absence of the drug. At 150 mM KCl, CPT induced cleavage is as low as 30% (Fig. 4B) compared to the cleavage at 50 mM KCl with CPT. However, at 300 mM KCl CPT induced cleavage is negligible (Fig. 4A, lane 9). Hence, cleavage assay at various salt concentrations reveals that LdTOP1LS is active at low salt.

3.5. *Antibody production, Western blot and immunolocalization study*

To gain an insight into the intracellular localization of *L. donovani* topoisomerase I protein, antibody was raised in rabbit separately against the two recombinant subunits LdTOP1L and LdTOP1S.

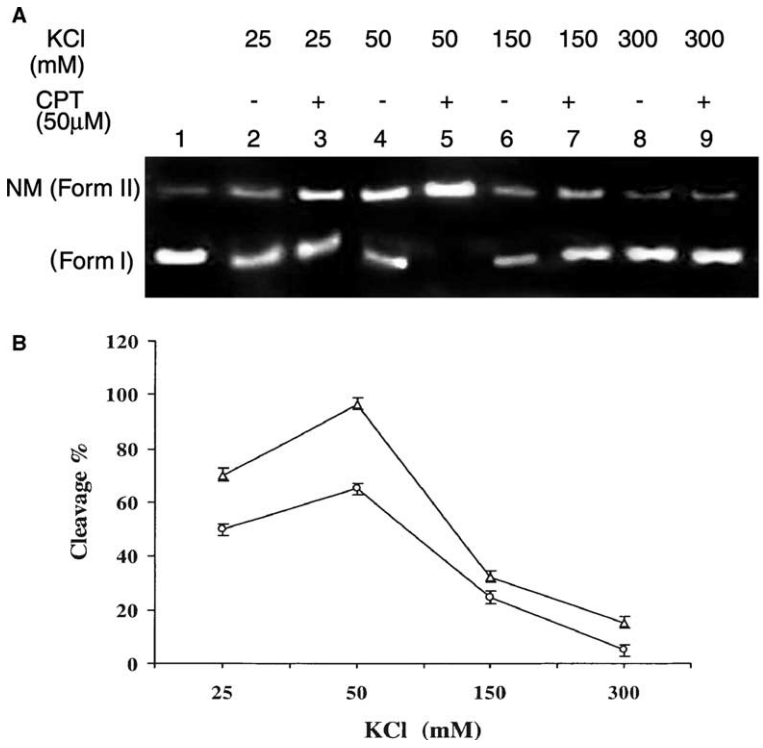

Fig. 4. SDS–K^+ mediated cleavage of supercoiled pHOT 1 DNA substrate as function of KCl concentration in the presence and absence of CPT. (A) Cleavage reactions and electrophoresis in agarose gel were performed as described in Section 2. Lane 1, 50 fmol of pHOT I supercoiled DNA. Lanes 2–9, same as lane 1 but incubated with equal amounts (100 fmol) of LdTOP1LS at the indicated concentration of KCl in the presence or absence of CPT (50 μM). Positions of supercoiled substrate (Form I) and nicked monomers (Form II) are indicated. (B) Graphical representation of the extent of covalent complex formation plotted as a function of KCl concentrations. Cleavage % = Form II DNA/(Form I + Form II) DNA × 100 was determined by densitometry. (○) Percent of cleavage without CPT, (△) percent of cleavage with CPT. Data presented are means ± S.D. ($n = 3$).

The 1.2 kb sequence of *LdTOP1L* full-length gene was chosen because it corresponds to one of the most conserved region in eukaryotic type I DNA–topoisomerase. Following IPTG induction of transformed bacteria carrying the *pGEX-GST-ΔLdTOP1L* construct, an additional band of 71 kDa was observed after SDS–PAGE as compared to uninduced bacterial extracts. The 71 kDa recombinant polypeptide was further purified (Fig. 1B) as described in Section 2 and a polyclonal antibody was raised against the purified protein in rabbit. The antiserum recognizes 71 kDa GST fusion protein as well as 73 kDa bands on Western blots with bacterial crude extracts containing protein pGEX-GST-ΔLdTOP1L and pET16bLdTOP1L. In the case of size fractionated parasite proteins, the antiserum could detect a band of anticipated size of LdTOP1L in *L. donovani* promastigotes extracts. To examine any possible cross-reactivity of the antibody with other eukaryotic type 1B topoisomerase, purified RLTOP1 was used as a negative control in immunoblot experiments (Fig. 5A, lane 4).

In case of the smaller subunit LdTOP1S, the 29 kDa purified protein (Fig. 1B) was injected in rabbit for raising polyclonal antibody. The antiserum recognizes the 29 kDa LdTOP1S in the induced bacterial crude extract harboring pET16bLdTOP1S construct as well as a band of anticipated size of LdTOP1S in *L. donovani* promastigotes extract (Fig. 5B).

The polyclonal anti ΔLdTOP1L antibody was used to probe fixed parasites in order to determine the intracellular localization of the LdTOP1 enzyme. When antiserum against recombinant LdTOP1L was used in double antibody immunofluorescence, it recognizes nuclear as well as kinetoplast antigen (Fig. 5C, panel b), while no fluorescence was observed when pre-immuned serum was used as the first antibody (Fig. 5C, panel a).

The polyclonal antibody against LdTOP1S when used to determine the sub-cellular localization of *L. donovani* topoisomerase I protein, the fluorescence patterns indicated that the antiserum also recognizes a nuclear as well as a kinetoplast antigen (Fig. 5C, panel c).

These results strongly suggest that the bi-subunit *L. donovani* topoisomerase I is associated with both the nucleus and the kinetoplast.

4. Discussion

Existence of unusual bi-subunit topoisomerase I has been recently reported in the kinetoplastid parasites *L. donovani* [9] and *T. brucei* [10]. The bi-subunit topoisomerase I enzyme was purified and characterized from the whole cell extract of *T. brucei* [10]. Villa et al. [9] described active *L. donovani* topoisomerase I when both the large and small subunits were co-expressed in yeast.

We report here for the first time the synthesis of two recombinant proteins, LdTOP1L and LdTOP1S, corresponding to large and small subunits of type I DNA topoisomerase of *L. donovani* in *E. coli* and in vitro reconstitution of enzymatic activity by mixing the two proteins purified from the bacterial extracts. The enzyme is Mg^{2+} independent, but Mg^{2+} enhanced the relaxation activity and binds with eukaryotic type

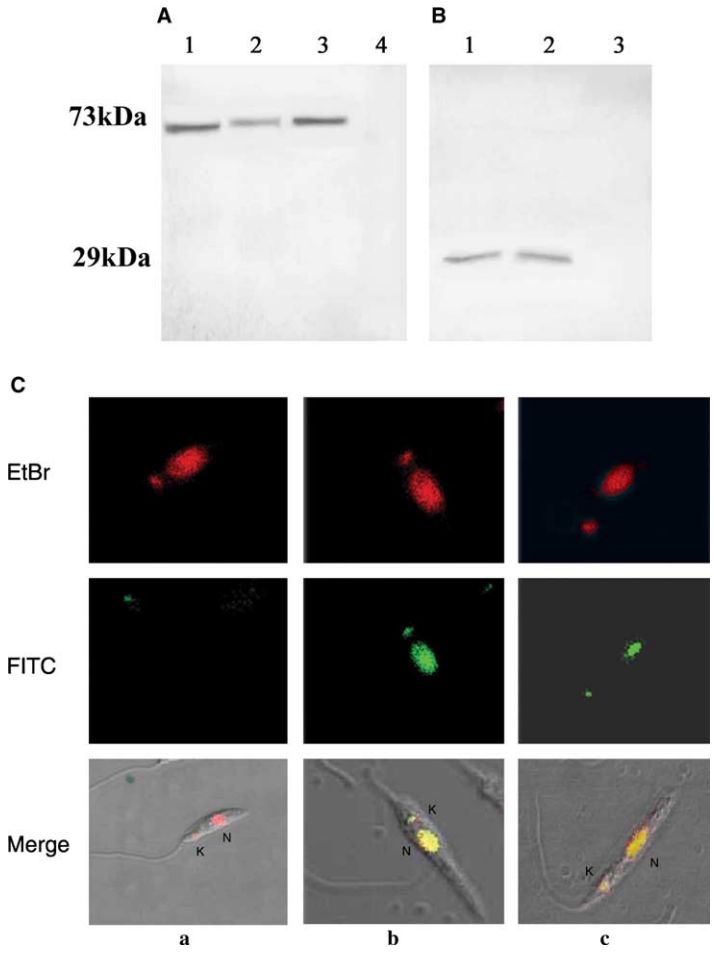

Fig. 5. Western blot analysis and immunofluorescent localization study. (A), Crude bacterial extract of induced pGEX-GST-ΔLdTOP1L (lane 1), pET16bLdTOP1L (lane 3), a leishmanial promastigotes cell extract (lane 2), and purified RLTOP1 as negative control (lane 4), show bands of respective molecular weights indicated in the figure, when reacted with anti-GST-ΔLdTOP1L antibody as described in Section 2. (B) Bacterial crude extract of induced pET16bLdTOP1S (lane 1), a leishmanial promastigotes cell extract (lane 2), and purified RLTOP1 as negative control (lane 3), reacted with anti-LdTOP1S antibody. The position of the bands indicates the molecular weight of the subunit. (C) Immunolocalization studies. Late log phase *L. donovani* promastigotes were fixed. No fluorescence was observed when pre-immune serum was used as primary antibody and FITC-tagged secondary antibody (Panel a) as described in Section 2. Panel b, same as panel a but probed with anti-LdTOP1L. Panel c, same as panel a but probed with anti-LdTOP1S primary antibody. Parasite cells were also stained with EtBr to locate the nucleus and kinetoplast and the area of the overlapping FITC and EtBr stain is shown in merged pictures. Cells were viewed at an original magnification of 100× under a Leica DM IRB inverted microscope. The nucleus (N) and kinetoplast (K) are indicated.

1B specific recognition site [22]. Relaxation assay with individually purified subunits revealed no topological inter conversion of the supercoiled DNA as observed in case of reconstituted enzyme.

At molar excess of plasmid DNA over enzyme, the rate-limiting step for relaxation of total substrate population is enzyme–DNA dissociation rate [27]. The reconstituted enzyme exhibits a reduced processivity under standard assay condition (10 mM Mg^{2+} and 50 mM KCl) when compared with eukaryotic monomeric RLTOP1 that appears to be highly processive at the same conditions. LdTOP1LS has a higher dissociation rate to yield a higher turnover number (73 plasmid molecules relaxed/min/molecule of enzyme) than that of RLTOP1 (36 plasmid molecules relaxed/min/molecule of enzyme), but LdTOP1LS shows 2-fold reduced DNA binding affinity (K_d of 3.1×10^{-7}M) than that of RLTOP1 (K_d of 1.6×10^{-7} M). These results are consistent with the reduced processivity of reconstituted LdTOP1LS than monomeric RLTOP1 where reconstituted LdTOP1LS appears to leave intermediate substrate after removing only a few supercoils at a time. While at the same condition, the relaxation mode of monomeric RLTOP1 is known to be highly processive, going through multiple rounds of relaxation before dissociating from its substrate (Fig. 2B). These findings are in keeping with that of the plasmid relaxation activity by wild type monomeric human topoisomerase I (topo 70), which has been shown to be highly processive, while the relaxation activity by the human reconstituted topo I (58/12) is distributive under same reaction conditions [28].

Cleavage assay with LdTOP1LS reveals an optimal cleavage at 50 mM KCl in the presence or absence of CPT, which binds to the covalent complex of enzyme and DNA [12,13]. At 300 mM KCl, CPT cannot induce stabilization of the cleavable complex. Hence, cleavage assay at various salt concentrations reveals that the enzyme is active at low salt. One possible explanation for such a divergent characteristic of LdTOP1LS is its multimeric nature, where the DNA binding core domain and the catalytic domain are in the separate subunits as

observed in case of reconstituted human topo 58/12 [28]. The reduced activity of LdTOP1LS at high salt may be due to reduced binding with DNA [29] or may be due to inhibition of association between the two subunits.

The small subunit (LdTOP1S) containing active SKXXY motif binds with the large subunit (LdTOP1L) to form an active complex at a molar ratio of 1:1 by protein–protein interaction as evidenced by Ni^{2+}–NTA agarose co-immobilization assay and is a heterodimer as determined by gel permeation chromatography.

Finally, a precise insight into the intracellular localization of the expressed topoisomerase I protein in *L. donovani* was gained by indirect immunofluorescence studies. Polyclonal antibody raised against the conserved portion of LdTOP1L and full-length LdTOP1S showed a nuclear as well as kinetoplast localization of *L. donovani* topoisomerase I. Previous studies in kinetoplastids predict that type I activity could be found in the nucleus and mitochondria [14]. Multiple putative nuclear localization signals are found in the N-terminus of the large subunit but not in the small subunit sequence. Therefore, the complex is likely to assemble in the cytosol before nuclear translocation. However, no specific amino acid residues for eukaryotic mitochondrial import signal could be identified in the two subunits. We have previously reported the dual localization of *L. donovani* topoisomerase II in the nucleus and in the kinetoplast though no specific residues for kinetoplast import were detected [18]. Some mitochondrial precursor proteins of *C. fasciculata* lacking N-terminal targeting signals have been shown to be targeted to mitochondria by means of an internal sequence [30]. This type of putative internal sequence for mitochondrial targeting might also operate in bi-subunit topoisomerase I of *L. donovani* to determine the kinetoplast localization of the protein. Western blot analysis of the whole cell lysate of *Leishmania*, using the LdTOP1L or LdTOP1S specific antibody separately, shows single bands reflecting their corresponding sizes. Thus, the observed immunofluorescence, due to separate subunits of *L. donovani* topoisomerase I antigen in the nucleus and in the kinetoplast, is due to the existence of same topoisomerase I protein in both the organelles.

A future prospect of this work lies in exploiting the specific subunit interactions leading to form an active complex that could be explored to delineate the common and distinguishing features of the host and the parasite enzymes. This information will provide an insight for the development of newer therapeutic agents with specific selectivity.

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