6286–6297 *Nucleic Acids Research, 2006, Vol. 34, No. 21* *Published online 10 November 2006*
doi:10.1093/nar/gkl829

'LeishMan' topoisomerase I: an ideal chimera for unraveling the role of the small subunit of unusual bi-subunit topoisomerase I from *Leishmania donovani*

Agneyo Ganguly, Benu Brata Das, Nilkantha Sen, Amit Roy, Somdeb Bose Dasgupta and Hemanta K. Majumder*

Department of Molecular Parasitology, Indian Institute of Chemical Biology 4, Raja S.C.Mullick Road, Jadavpur, Kolkata 700032, India

Received May 24, 2006; Revised October 4, 2006; Accepted October 8, 2006

ABSTRACT

The active site tyrosine residue of all monomeric type IB topoisomerases resides in the C-terminal domain of the enzyme. *Leishmania donovani*, possesses unusual heterodimeric type IB topoisomerase. The small subunit harbors the catalytic tyrosine within the SKXXY motif. To explore the functional relationship between the two subunits, we have replaced the small subunit of *L.donovani* topoisomerase I with a C-terminal fragment of human topoisomerase I (HTOP14). The purified LdTOP1L (large subunit of *L.donovani* topoisomerase I) and HTOP14 were able to reconstitute topoisomerase I activity when mixed *in vitro*. This unusual enzyme, '*LeishMan*' topoisomerase I (*Leish* for Leishmania and *Man* for human) exhibits less efficiency in DNA binding and strand passage compared with LdTOP1L/S. Fusion of LdTOP1L with HTOP14 yielded a more efficient enzyme with greater affinity for DNA and faster strand passage ability. Both the chimeric enzymes are less sensitive to camptothecin than LdTOP1L/S. Restoration of topoisomerase I activity by LdTOP1L and HTOP14 suggests that the small subunit of *L.donovani* topoisomerase I is primarily required for supplying the catalytic tyrosine. Moreover, changes in the enzyme properties due to substitution of LdTOP1S with HTOP14 indicate that the small subunit contributes to subunit interaction and catalytic efficiency of the enzyme.

INTRODUCTION

Eukaryotic DNA topoisomerase I governs the topological state of duplex DNA by transiently cleaving and rejoining one DNA strand during each catalytic cycle (1,2). This ubiquitous enzyme participates in vital cellular processes such as replication, transcription and chromosome segregation. The enzyme has been extensively studied from various sources starting from vaccinia virus (3) to human. The mechanism by which this enzyme alters the DNA topology involves three major steps: (i) nucleophilic attack by the hydroxyl group of the active site tyrosine on the scissile phosphate resulting in covalent attachment of enzyme to the 3′ end of the broken strand, (ii) a topoisomerization step involving strand passage or free rotation, and (iii) religation of the DNA strand and release of the enzyme (1,4).

The most widely studied member of the type IB subfamily is human topoisomerase I, which is a monomeric enzyme comprising 765 amino acids (91 kDa). The enzyme consists of the following four major domains (5): an N-terminal domain (1–214), a core domain (215–635), a linker domain (636–712) and a C-terminal domain (713–765). The core domain is highly conserved and contains the residues directly implicated in DNA binding and catalysis. The C-terminal domain is also highly conserved and harbors the active site tyrosine at position 723. It has been shown earlier that the core and the catalytic domains can interact and reconstitute topoisomerase I activity when separately purified and mixed *in vitro* (6).

Reconstitution of topoisomerase I activity by association of two subunits has recently been evidenced as a natural phenomenon in the kinetoplastid parasites, *Leishmania* (7) and *Trypanosoma* (8). Topoisomerase I in these parasites is a bi-subunit enzyme and the two subunits are synthesized from two different genes, which associate with each other to form a dimeric enzyme within the parasite. We have previously reported the *in vitro* reconstitution of the bi-subunit topoisomerase I from *Leishmania donovani* (9). The enzyme from *L.donovani* consists of two subunits, a larger one comprising 635 amino acids (73 kDa) and a smaller one of 262 amino acids (29 kDa). The large subunit (LdTOP1L) shares 38.4% sequence identity and 54% similarity with human topoisomerase I but is catalytically inactive (10). Interestingly, the

*To whom correspondence should be addressed. Tel: +91 33 2412 3207; Fax: +91 33 2473 5197; Email: hkmajumder@iicb.res.in

small subunit (LdTOP1S) is found to have very little sequence homology with other topoisomerase I, except the SKXXY motif that harbors the active site tyrosine.

In spite of its unusual architecture, the *L.donovani* topoisomerase I exhibits behavioral similarity to other eukaryotic type IB topoisomerases (9). As evidenced from multiple alignment, the large subunit apparently harbors almost all residues implicated in catalysis except the catalytic tyrosine whereas the second subunit harbors the active site tyrosine within the SKXXY motif. This observation gives rise to two obvious questions: (i) whether the second subunit is essential only for catalysis or it contains some putative domain actually necessary for the enzyme to function, and (ii) whether the large subunit actually corresponds to the core domain of eukaryotic topoisomerase I and is able to function when supplied with a linker and C-terminal domain. To address this issue we have taken a novel approach. We have replaced the small subunit of *L.donovani* topoisomerase I with a fragment from the C-terminus of human topoisomerase I (amino acids 636–765). The 14 kDa fragment bearing the linker and C-terminal domain of human topoisomerase I (HTOP14) was successfully able to reconstitute topoisomerase activity when mixed with the large subunit of *L.donovani* topoisomerase I (LdTOP1L) *in vitro*. From our detailed investigation it was found that the hybrid protein '*LeishMan*' topoisomerase I, behaved like a bonafide type IB topoisomerase with somewhat reduced processivity, reduced CPT sensitivity and lesser affinity for DNA compared with the wild-type enzyme. The ability of LdTOP1L to interact with HTOP14 and reconstitute topoisomerase I activity clearly suggests that the second subunit of *L.donovani* topoisomerase I is primarily required for supplying the catalytic tyrosine which can be complemented by the C-terminal fragment of human topoisomerase I.

We further extended our study by fusing LdTOP1L with HTOP14 to generate a monomeric protein and compared its properties with monomeric human topoisomerase I. Our objective was to see whether the *L.donovani* large subunit along with the C-terminus of human topoisomerase I could behave similar to the monomeric human topoisomerase I when fused together. Interestingly, the chimeric fusion protein, although could not mimic human topoisomerase I in its properties, exhibits improved processivity and increased DNA binding compared with *L.donovani* topoisomerase I. Surprisingly, the fusion protein also exhibits lesser CPT sensitivity than *L.donovani* topoisomerase I which tempted us to speculate that the *L.donovani* small subunit harbors some domains necessary for the enzyme to be CPT sensitive. Changes in the enzyme behavior due to the substitution of LdTOP1S with HTOP14 helps in understanding the role of the small subunit in the topoisomerization reaction. Moreover, restoration of topoisomerase activity by the fusion of LdTOP1L with HTOP14 strengthens the possibility of gene fusion as a mechanism of evolution of eukaryotic topoisomerase I.

MATERIALS AND METHODS

Cloning and construction of recombinant plasmids

Cloning in bacterial expression vectors. The full-length large subunit gene (LdTOP1L) and the small subunit gene (LdTOP1S) were cloned previously in the bacterial expression vector pET16b (9). For the construction of HTOP14, region corresponding to amino acids 636–765 were amplified by PCR using plasmid pKM10 as template. The plasmid pKM10 bearing the full-length cDNA of human topoisomerase I (11) was received as a gift from Prof. J.J. Champoux, University of Washington, Seattle, WA. PCR amplification was carried out using sense primer 5′-GGAATTCCATATGC-CACCAAAAACTTTTGAGAAG-3′ containing NdeI site and antisense primer 5′-CGGGATCCCTAAAACTCATAGTCT-TCATC-3′ containing BamHI site. The PCR product was cloned into the NdeI/BamHI site of pET16b vector to create the construct pET16bHTOP14.

To construct the fusion protein, large subunit gene was PCR amplified using the sense primer 5′-CGGGATCCTGA-TGAAGGTGGAGAATAGC-3′ containing BamHI site and antisense primer 5′-CGGAATTCCACCCTCAAAGCTGC-AAGAGG-3′containing EcoR1 site, and pET16bLdTOP1L as template. The antisense primer was designed by omitting the stop coddon of the LdTOP1L ORF. The PCR product was cloned into BamHI/EcoR1 site of pET28c vector to generate *pET28cLdTOP1L*. The sequence corresponding to HTOP14 was PCR amplified using sense primer 5′-CG-GAATTCCCACCAAAAACTTTGAG-3′ containing EcoR1 site and antisense primer 5′-CCCAAGCTTCTAAAACTCA-TAGTCTTC-3′ containing HindIII site, and pET16bHTOP14 as template. The product was cloned into pET28cLdTOP1L as vector in EcoR1/HindIII site to generate the recombinant plasmid *pET28cLdTOP1L-fus-HTOP14*. The amino acids Glu (E) and Phe (F) links the two proteins due to insertion of the EcoR1 restriction endonuclease site. The recombinant constructs were transformed into *Escherichia coli* BL21 (DE3) PlysS cells for the expression and purification of proteins.

Cloning in baculovirus expression vector. The ORFs for LdTOP1L, LdTOP1S and HTOP14 were PCR amplified, and cloned into BamHI/EcoRI site of the baculovirus expression vector pBlueBacHis2A (Invitrogen). To construct the fusion LdTOP1L-fus-HTOP14, the plasmid *pET28cLdTOP1L-fus-HTOP14* was digested with BamHI/HindIII and the resulting insert was subcloned into BamHI/HindIII site of the baculovirus expression vector pBlueBacHis2C. All the recombinant constructs were transfected into Sf-9 cells using BacNBlue transfection kit (Invitrogen) according to the manufacturer's protocol. Recombinants were selected by plaque assay, and were confirmed by PCR. Fresh Sf-9 cells were infected with the recombinant viruses, and high titer viruses were generated for each protein.

Overexpression and purification of proteins from bacterial cells. *Escherichia coli* BL21 (DE3) pLysS cells harboring pET16bLdTOP1L, pET16bLdTOP1S, pET16bHTOP14 and pET28cLdTOP1L-fus-HTOP14 were separately induced at 0.6 OD (280 nm) with 0.5 mM IPTG at 22°C for 12 h. Cells harvested from 1 l of culture were separately suspended in lysis buffer containing 1 mg/ml lysosyme, sonicated and proteins were purified using Ni^{2+}-NTA–agarose column (Qiagen). Proteins were dialyzed and purified using a phosphocellulose column (P11, Whatman) as described previously (9,12). Finally, the purified proteins LdTOP1L, LdTOP1S, HTOP14 and LdTOP1L-fus-HTOP14 were stored at −70°C.

Overexpression and purification from insect cells

Approximately, 1×10^8 Sf-9 cells were infected with recombinant virus bearing *LdTOP1L*, *LdTOP1S*, *HTOP14* and *LdTOP1L-fus-HTOP14* separately. At 48 h post-infection cells were harvested, lysed and loaded onto a Ni^{2+}-NTA–agarose column (Qiagen). Proteins were eluted with 500 mM imodazole, dialyzed and repurified using phosphocellulose column (P11, Whatman) as described previously (9). Finally, the purified proteins LdTOP1L, LdTOP1S, HTOP14 and LdTOP1L-fus-HTOP14 were dialyzed and stored at −70°C.

The wild-type human topoisomerase I (91 kDa) was purified from Sf-9 insect cells infected with the recombinant baculovirus (a gift from Prof. J.J. Champoux). Approximately, 1×10^9 Sf-9 cells were infected with the recombinant virus and cells were harvested after 48 h infection. The cells were lysed and enzyme was purified as described previously (13).

In vitro reconstitution

Purified LdTOP1L was mixed with purified LdTOP1S and HTOP14 separately in 1:1 molar ratio (as determind by Ni^{2+}-NTA co-immobilization assay) following standard protocol (9,12). The total protein concentration was 0.5 mg/ml in the reconstitution buffer containing 50 mM potassium phosphate, pH 7.5, 0.5 mM DTT, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 10% glycerol. The mixture was dialyzed at 4°C for 12 h and the dialyzed proteins were used for all subsequent assays.

Plasmid relaxation assay

DNA topoisomerase I was assayed by decreased mobility of the relaxed isomers of supercoiled plasmid pBluescript (SK^+) DNA in agarose gel. Relaxation assays were carried out separately with LdTOP1L/S, LdTOP1/HTOP14, LdTOP1L-*fus*-HTOP14 and HTOP91, serially diluted in the relaxation buffer (25 mM Tris–HCl, pH 7.5, 5% glycerol, 0.5 mM DTT, 10 mM $MgCl_2$, 50 mM KCl, 25 mM EDTA and 150 μg/ml BSA) and supercoiled plasmid pBluescript (SK^+) DNA (85–95% were negatively supercoiled, with remainder being nicked circles). The reconstituted enzymes LdTOP1L/S and LdTOP1L/HTOP14 were assayed at 50 mM KCl concentration whereas LdTOP1L-*fus*-HTOP14 and HTOP91 were assayed at 150 mM KCl. For all kinetic studies, the reaction mixtures containing the buffer and DNA were heated to 37°C before the addition of the enzymes. The reactions were rapidly quenched using stop solution and kept on ice. The gels were stained with ethidium bromide (EtBr) and the amount of supercoiled monomer DNA band fluorescence were quantified by integration using GelDoc 2000 (Quality One software; Bio-Rad). Initial velocities (nM DNA base pairs relaxed × min^{-1}) were calculated using the following equation:

$$\text{Initial Velocity} = \left([\text{Supercoiled DNA}]_0 - \frac{\text{Int}_t.[\text{Supercoiled DNA}]_0)}{\text{Int}_0}\right) \Big/ t \qquad \mathbf{1}$$

where $[\text{Supercoiled DNA}]_0$ is the initial concentration of the supercoiled DNA, Int_0 is the area under the supercoiled band at time zero and Int_t is the area at reaction time t (14). The effect of DNA concentration on the kinetics of relaxation was examined over a range of 2.5–40 nM supercoiled DNA (0.1–2 μg/25 μl of reaction mixture) at a constant concentration of 10 mM $MgCl_2$ and 0.8 nM enzyme (LdTOP1L/HTOP14 and LdTOP1L-*fus*-HTOP14 separately) at 37°C for 1 min. The data were analyzed by Lineweaver–Burk plot. The intercept of y-axis is $1/V_{max}$, and the turnover (K_{cat}) = V_{max}/enzyme concentration (plasmid molecules relaxed/min/molecule of enzyme).

Cleavage assay

Cleavage assay was carried out as described in (9). Briefly, 50 fmol of pHOT1 supercoiled DNA (containing Topo I cleavage site), 100 fmol of purified enzymes (LdTOP1L/HTOP14 and LdTOP1L-*fus*-HTOP14) 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 containing 0.5 μg/ml of EtBr. EtBr was added to the gel to resolve the more slowly migrating nicked product (Form II) from the supercoiled molecules (Form I).

Preparation of radiolabeled oligonucleotide substrates

The 25mer oligonucleotide ML25 (5′-GAAAAAAGACTTA-GAAAAATTTTTA-3′) was radiolabeled at its 5′ end with [γ^{32}-P]ATP and T4 polynucleotide kinase (Roche) in a suitable buffer. The 5′ end-labeled oligonucleotide was annealed with the complementary sequence MC25 (5′-TAAAAATTT-TTCTAAGTCTTTTTTC-3′) following standard annealing conditions (6) to generate the 25mer duplex DNA substrate. The 14mer oligonucleotide ML14 (5′-GAAAAAAGACT-TAG-3′) was radiolabeled at its 5′ end following similar procedure as mentioned above. The radiolabeled ML14 was annealed to MC25 as described previously (6) to generate the suicidal substrate ML14/MC25.

Competition binding assay with limiting concentration of oligonucleotides

A series of samples were prepared from the enzyme stocks such that they contain increasing concentrations of LdTOP1L/HTOP14 (50 nM, 100 nM, 200 nM and 400 nM) with or without a fixed concentration (100 nM) of LdTOP1L/S. The final salt concentration was kept at 50 mM. In a separate set of experiment, enzyme stocks were prepared with increasing concentrations of LdTOP1L/S (50 nM, 100 nM, 200 nM and 400 nM) and a fixed concentration (100 nM) of LdTOP1L-fus-HTOP14. The salt concentration was kept at 100 mM so as to achieve maximum cleavage for both the enzymes. Each sample was mixed with 7.5 nM 5′-^{32}P end-labeled 25mer duplex DNA substrate and incubated in standard cleavage buffer (25 mM Tris–HCl, pH 7.5, 0.5 mM DTT, 10 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA and 150 μg/ml BSA) for 30 min at 37°C. The samples were then adjusted to 1% SDS, boiled and fractionated by 15% SDS–PAGE.

Cleavage reaction with an excess of oligonucleotide and equal concentrations of enzymes

Equal concentrations (100 nM) of LdTOP1L/S, LdTOP1L/HTOP14 or LdTOP1L-fus-HTOP14 were separately added to two sets of reactions containing either the 5′-^{32}P end-labeled suicidal oligonucleotide ML14/ML25 or the 25mer duplex oligonucleotide. Reactions were carried out at 50 mM, 100 mM and 200 mM KCl for the respective enzymes as indicated in a reaction buffer containing 25 mM Tris–HCl, pH 7.5, 0.5 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA and 150 μg/ml BSA. The samples were incubated at 37°C for 1 h and were adjusted to 1% SDS, boiled and fractionated by 15% SDS–PAGE.

Religation kinetics

Covalent complexes used as substrates for the religation reaction were generated by incubating 10 nM 5′-^{32}P-end-labeled substrate ML14/MC25 with 250 nM enzymes (LdTOP1L/S, LdTOP1L/HTOP14, LdTOP1L-fus-HTOP14 or HTOP91) separately for 4 h at 23°C. Religation was initiated by the addition of 300-fold molar excess of the 11mer acceptor oligonucleotide MR11 (5′-OH-AGAAAAATTTT-3′) as described previously (15). The reactions were stopped at different time points (5 15 30 60 120 and 300 s) by the addition of an equal volume of 1% SDS. The samples were precipitated with ethanol and dissolved in 10 μl of 1 mg/ml trypsin and digested at 37°C for 1 h to remove all but a short topoisomerase I derived peptide from the covalent complex. The samples were analyzed on a 20% sequencing gel followed by autoradiography. The religation product migrates as a 23mer and is well resolved from the oligonucleotide–peptide complex that migrates slower than the uncleaved oligo. The percentage of religation at each time point was quantified using a phosphor imager (Bio-Rad Molecular Imager System) and Quality One software.

Fluorescence polarization assay for protein–protein interaction

One milligram of LdTOP1S and HTOP14 were labeled separately with fluorescien isothiocyanate (FITC) according to the company protocol (Pierce Biotechnology). Briefly, the proteins were incubated with FITC and dimethyl formamide in a phosphate buffer (pH 8.0) and incubated at 16°C for 30 min. The samples were loaded onto desalting columns to remove the free FITC. Samples were prepared by mixing constant concentration of labeled LdTOP1S or HTOP14 (25 nM) with varying concentrations of LdTOP1L (10–250 nM). Fluorescence polarization measurements were carried out at excitation of 495 nm and emission of 518 nm in a F-3010, HITACHI/JAPAN polarization system. Fluorescence polarization was determined by using the following equation:

$$P = \frac{I_{II} - I_{\perp}}{I_{II} + I_{\perp}}, \qquad \mathbf{2}$$

where I_{II} is the intensity of the emission light parallel to the excitation light plane and $I_{\perp}$ is the intensity of the emission light perpendicular to the excitation light plane.

The fractions of the bound proteins LdTOP1L were calculated by the following equation:

$$f_B = \frac{P - P_{min}}{\Delta P} = \frac{F}{K_D + F}, \qquad \mathbf{3}$$

where $\Delta P = (P_{max} - P_{min})$ is the total change in polarization. P_{max} is the maximum polarization at saturation point and P_{min} is the minimum polarization (16). The fractions of bound protein (f_B) were plotted against the protein concentrations (F) and the K_D for the respective interactions were calculated from Equation 3.

RESULTS

DNA relaxation activity of the chimeric proteins

The large subunit of *L.donovani* topoisomerase I (LdTOP1L) contains almost all conserved residues to constitute the core domain of eukaryotic topoisomerase I but lacks the ability to perform relaxation. Although the small subunit (LdTOP1S) supplies the catalytic tyrosine for activity, it is not yet clear whether this subunit has some definite role in governing the enzyme behavior.

This prompted us to check whether activity can be restored by replacing the *L.donovani* small subunit with the C-terminus fragment of human topoisomerase I (HTOP14), which also contains the catalytic tyrosine required for the enzyme to function. With this objective we cloned and overexpressed the recombinant protein constructs shown in Figure 1A. Purified LdTOP1L and HTOP14 were mixed at a molar ratio of 1:1 in a reconstitution buffer. The reconstituted enzyme (LdTOP1L/HTOP14) as well as the fused chimera (LdTOP1L-fus-HTOP14) were examined for plasmid relaxation activity. Both the enzymes (LdTOP1L/HTOP14 and LdTOP1L-fus-HTOP14) exhibited topoisomerase I relaxation activity. This interesting finding further led us to inspect the changes in relaxation properties of the enzymes due to substitution of the small subunit with the C-terminus fragment of human topoisomerase I. That LdTOP1L and HTOP14 also interacts in 1:1 molar ratio was determined by Ni^{2+}-NTA co-immobilization assay (9). Moreover, to confirm that there was no contamination of bacterial topoisomerase I, we assayed the individual proteins (LdTOP1L, LdTOP1S and HTOP14) for relaxation activity. No relaxation was observed with any of the individual protein preparations (Figure 1C, lanes 2–7). Relaxation was only found when the appropriate proteins were mixed and reconstituted (Figure 1C, lanes 8–11).

Time course relaxation assays were performed in a standard assay mixture where the enzyme:DNA molar ratio was 1:4. The velocities for LdTOP1L/HTOP14 and LdTOP1L-fus-HTOP14 were linear for first 5 min of reaction. All subsequent initial velocities were calculated up to 1 min of incubation, well within the linear range of velocity examined. As evident from Figure 2, LdTOP1L/HTOP14 relaxes supercoiled DNA faster than wt-LdTOP1L/S. In the reactions catalyzed by LdTOP1L/HTOP14, the supercoiled band shifted more rapidly towards an intermediate superhelical density (Figure 2B) compared with LdTOP1L/S (Figure 2A). This pattern is indicative of a distributive reaction, in which

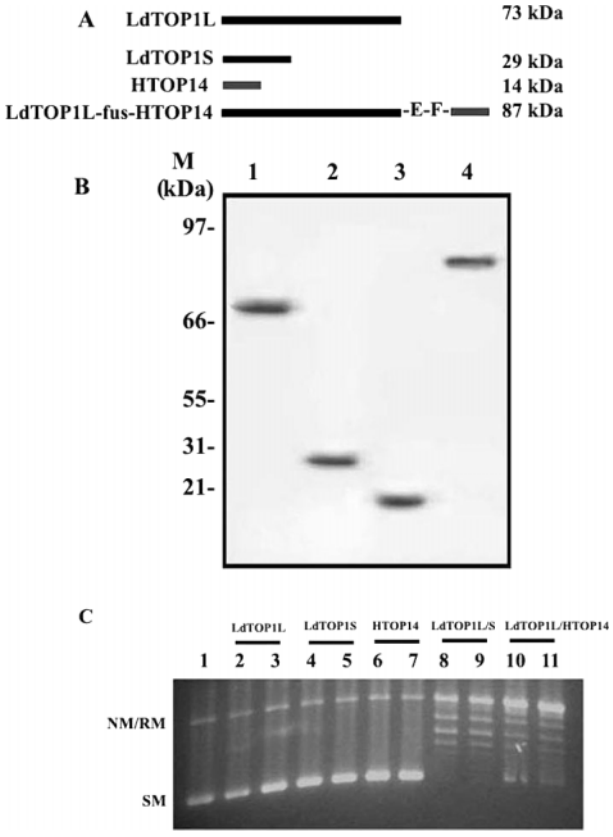

Figure 1. Protein constructs and purification. (**A**) Structure of recombinant proteins. The first line shows the full-length large subunit (dark). The second line shows the small subunit (dark). The third line shows the C-terminal fragment of human topoisomerase I (shaded). The fourth line shows the fusion of large subunit (dark) with the C-terminal fragment of human topoisomerase I (shaded). The amino acids E and F inserted due to the restriction endonuclease site are indicated. (**B**) Coomassie stained 12% SDS–PAGE analysis of recombinant proteins (2 μg/lane). Lanes 1–4: LdTOP1L, LdTOP1S, HTOP14 and LdTOP1L-fus-HTOP14 proteins purified by Ni^{2+}-NTA column, respectively, followed by phosphocellulose column. The position of the standard protein molecular weight markers are indicated on the left. (**C**) Relaxation assay with 0.1 μg and 0.2 μg of LdTOP1L (lanes 2 and 3), LdTOP1S (lanes 4 and 5) and HTOP14 (lanes 6 and 7), respectively. Lanes 8 and 9, relaxation with 0.2 and 0.4 μg of LdTOP1L/S; lanes 10 and 11, relaxation with 0.2 and 0.4 μg of LdTOP1L/HTOP14.

LdTOP1L/HTOP14 dissociates from partially relaxed plasmids more rapidly than LdTOP1L/S during the course of assay. Similar experiments with HTOP91 and LdTOP1L-fus-HTOP14 reveal a different relaxation pattern. In reactions catalyzed by HTOP91 (Figure 2C) and LdTOP1L-fus-HTOP14 (Figure 2D), the supercoiled band persists even after 15 min incubation and appears to be shifted primarily to the position of fully relaxed products with lesser accumulation of partially relaxed intermediates. Relaxation patterns of HTOP91 and LdTOP1L-fus-HTOP14 reveal that both the monomeric enzymes are more processive than LdTOP1L/S. Altogether the enzymes can be arranged in the order of increasing processivity as follows: HTOP91 > LdTOP1L-fus-HTOP14 > LdTOP1L/S > LdTOP1L/HTOP14.

To get a clear picture of the enzyme behavior, the kinetics of relaxation by LdTOP1L/HTOP14 and LdTOP1L-fus-HTOP14 were examined over a range of supercoiled DNA (2.5–40 nM) and the initial velocities were plotted on a

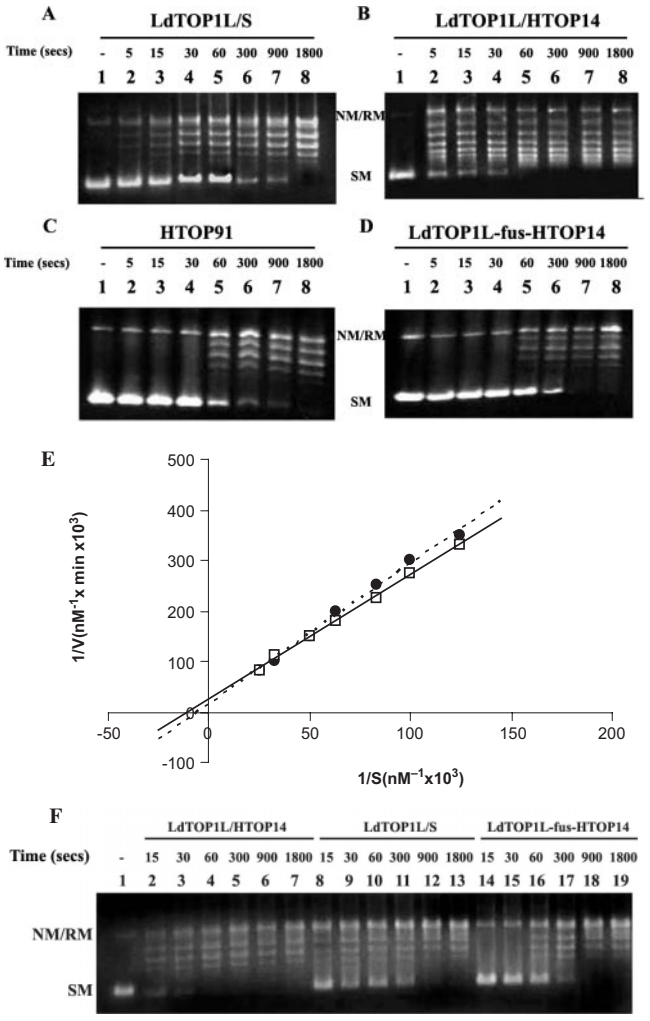

Figure 2. Plasmid relaxation assay. Relaxation of supercoiled pBS (SK^+) DNA with enzymes LdTOP1L/S (**A**), LdTOP1L/HTOP14 (**B**), HTOP91 (**C**) and LdTOPL-fus-HTOP14 (**D**) at a molar ratio of 4:1. Lane 1, 100 fmol of supercoiled pBS (SK^+) DNA. Lanes 2–8 same as lane 1 and incubated with 25 fmol of enzyme at 37°C for varying time periods as indicated in the figures. Reactions were stopped by the addition of 0.5% SDS and samples were electrophoresed on 1% agarose gel. Positions of supercoiled monomer (SM) and relaxed and nicked monomers (RM/NM) are indicated. (**E**) Lineweaver–Burk representation of the kinetics of relaxation of supercoiled pBS (SK^+) DNA by LdTOP1L/HTOP14 (closed circles) and LdTOP1L-fus-HTOP14 (open squares). DNA concentrations range from 2.5 to 40 nM and enzyme concentration was at 0.8 nM. (**F**) Time course relaxation assay with LdTOP1L/HTOP14 (lanes 2–7), LdTOP1L/S (lanes 8–13) and LdTOP1L-fus-HTOP14 (lanes 14–19) purified from baculovirus expression system.

Lineweaver–Burk plot. The enzyme:DNA ratio was kept within the steady-state assumption. The maximal velocity (V_{max}) for LdTOP1L/HTOP14 and LdTOP1L-fus-HTOP14 were 7.14×10^{-8} M and 4×10^{-8} M base pairs of supercoiled DNA relaxed/min/0.8 nM enzyme, which corresponds to turnover numbers of 90 and 50 plasmid molecules relaxed/min/molecule of enzyme, respectively. From a similar set of experiments with LdTOP1L/S and HTOP91, the initial velocities were calculated to be 5.5×10^{-8} M and 2.5×10^{-8} M base pairs of supercoiled DNA relaxed/min/0.8 nM enzyme which correspond to turnover numbers of 68 and 32 plasmid molecules relaxed/min/molecule of enzymes,

respectively. These variations in turnover number clearly reflect the differences in enzyme functionality and their differential affinity for DNA. The increased turnover number of LdTOP1L/HTOP14 compared with LdTOP1L/S indicates that the former dissociates faster from DNA due to its reduced affinity for the substrate. On the other hand, processive action and low turnover number of LdTOP1L-fus-HTOP14 suggest that this enzyme has improved DNA-binding property and remains largely bound to the plasmid DNA throughout the relaxation process. Post-translational modifications such as phosphorylation are known to affect enzymatic activity. All the hybrid enzymes used in the assays are purified from the bacterial system, lacking the post-translational modifications, whereas the wt-HTOP91 is purified from the baculovirus expression system. To confirm that all the above results and interpretations are not skewed due to the absence of post-translational modifications, we simultaneously expressed all the proteins in baculovirus system and compared their kinetic properties. Similar relaxation patterns were observed for all the proteins as compared to those expressed in bacteria (Figure 2F). Morover, the kinetic properties of the enzymes (V_{max}, K_{cat}) calculated were also found to be similar to those of bacterially expressed proteins (data not shown). Therefore in all subsequent experiments, the enzymes purified from bacterial expression system were used.

Non-turnover relaxation experiment

A high turnover number and distributive mode of action of LdTOP1L/HTOP14 compared with LdTOP1L/S clearly indicates that there is a difference in topoisomerization by the two enzymes. This difference can be at the initial cleavage step just after binding to the substrate or during strand rotation. To address this issue, we performed a non-turnover relaxation assay (17). The reaction mixtures contained a 30-fold stoichiometric excess of each enzyme. This excess enzyme eliminates the need for enzyme turnover during the reaction, as complete relaxation of the plasmid can be achieved if each topoisomerase molecule catalyzes only a single DNA strand passage event. Morover, the DNA substrate pBS (SK+) used in the assay has a size of 2.9 kb which corresponds to roughly 14 negative supercoils per DNA molecule. Thus, 30-fold molar excess of the enzyme is used to achieve complete non-turnover condition. It was found that relaxed intermediates start appearing from 15 s of incubation in LdTOP1L/S and relaxed almost 80% of supercoiled plasmids in 10 min (Figure 3A), whereas relaxed intermediates start appearing from 30 s in case of LdTOP1L/HTOP14 (Figure 3B). This result is indicative of a faster strand passage by LdTOP1L/S although it has a lower turnover number. Similarly, almost 100% relaxation was achieved within 45 s with HTOP91 (Figure 3C) whereas the same is achieved in 2 min by LdTOP1L-fus-HTOP14

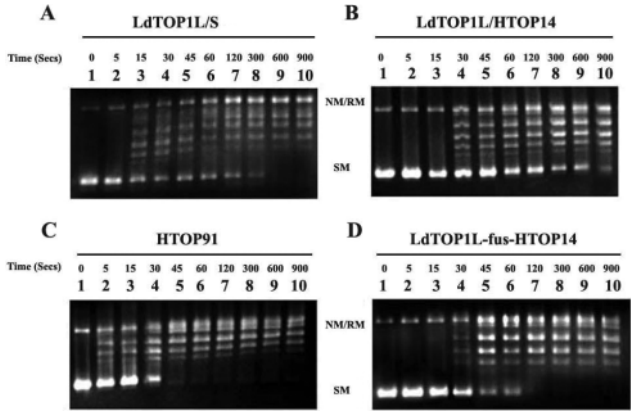

Figure 3. Non-turnover relaxation assay. The non-turnover relaxation of supercoiled pBS (SK^+) DNA was carried out with enzymes LdTOP1L/S (**A**), LdTOP1L/HTOP14 (**B**), HTOP91 (**C**) and LdTOPL-fus-HTOP14 (**D**). Relaxation reactions were carried out from 0 to 900 s (lanes 1–10) using 150 nM enzyme and 5 nM DNA. Positions of supercoiled monomer (SM) and relaxed and nicked monomers (RM/NM) are indicated.

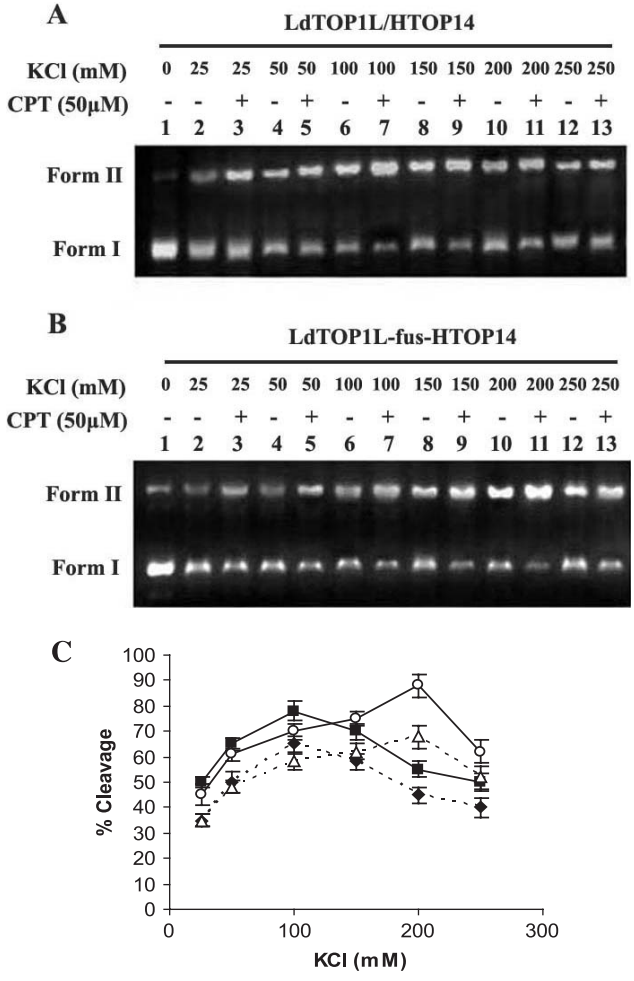

Figure 4. Cleavage assay: SDS-K^+ mediated cleavage of supercoiled pHOT1 DNA as a function of KCl concentration was carried out in the presence and absence of CPT. Cleavage reactions and electrophoresis in agarose gel containing ethidium bromide were performed as described in Materials and Methods. Lane 1, 50 fmol of pHOT1 supercoiled DNA, lanes 2–13, same as lane 1 but incubated with 100 fmol of LdTOP1L/HTOP14 (**A**) and LdTOPL-fus-HTOP14 (**B**) at indicated concentrations of KCl in the presence or absence of 50 μM CPT. The position of supercoiled substrate (Form I) and nicked monomers (Form II) are indicated. (**C**) Graphical representation of 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. Percentage of cleavage without CPT (closed diamonds) and with CPT (closed squares) for LdTOP1L/HTOP14, Percentage of cleavage without CPT (open triangles) and with CPT(open circles) for LdTOPL-fus-HTOP14. Data represented as means ± SD ($n = 3$).

(Figure 3D). The result of this typical experiment goes in favor of our second assumption that the strand rotation is affected due to substitution of LdTOP1S with HTOP14.

Cleavage assay and effect of salt concentration

To assess the effect of salt concentration on the enzyme activity, we performed DNA cleavage assay under varying salt concentrations (Figure 4A and B). Camptothecin, the most established topoisomerase I (18) inhibitor, has been shown to stabilize the cleavable complex (19,20). Cleavage assays were carried out in the presence or absence of fixed concentrations of CPT and the percentages of cleavable complex stabilized were plotted against salt concentrations. The graph in Figure 4C indicates that, in the absence of CPT, the reconstituted enzyme LdTOP1L/HTOP14 exhibits maximum cleavage at 100 mM salt concentration. CPT enhances the cleavage by only 10%. The optimum salt concentration to achieve maximum cleavage by LdTOP1L/S was determined previously to be 50 mM (9). Although, LdTOP1L/HTOP14 exhibits maximum cleavage activity at a higher salt concentration than wt-LdTOP1L/S, it must be noted that the percentage of cleavage at this optimum salt concentration is much less than that of the wt-LdTOP1L/S, both in the presence and the absence of CPT. On the other hand, LdTOP1L-fus-HTOP14 display optimal activity at 200 mM KCl, whereas the optimal condition for wt-Human topoisomerase I (HTOP91) is also 200 mM KCl in the presence of Mg^{2+} (6). Fusing LdTOP1L with HTOP14 yielded a more salt-resistant and processive enzyme. Although CPT-induced cleavage increased in the case of LdTOP1L-fus-HTOP14 compared with LdTOP1L/HTOP14, it is not as high as observed for wt-LdTOP1L/S. These results clearly suggest that the small subunit of *L.donovani* topoisomerase I definitely has some role to play in enhanced DNA binding and CPT sensitivity of LdTOP1L/S.

Effect of camptothecin on relaxation activity

The observation that CPT could not substantially enhance the cleavable complex formation by LdTOP1L/HTOP14 and LdTOP1L-fus-HTOP14 led us to speculate that the chimeric enzymes are less sensitive to CPT than LdTOP1L/S due to the absence of the small subunit (LdTOP1S). To verify this hypothesis, we examined the effect of CPT on the relaxation activity of the chimeric enzymes as well as the wt-LdTOP1L/S. The plasmid DNA and the enzymes were mixed at a molar ratio of 1:2 to circumvent the possible effect due to the slow dissociation rate and enzyme turnover in the presence of CPT. The relaxations of LdTOP1L/S, LdTOP1L/HTOP14 and LdTOP1L-fus-HTOP14 were assessed over minutes and almost complete relaxation was achieved at 1 min for LdTOP1L/S and LdTOP1L/HTOP14 in the absence of CPT. LdTOP1L-fus-HTOP14 was found to relax almost 80% of the supercoiled DNA in 1 min. However, in the presence of CPT, the time required to complete relaxation for LdTOP1L/S is increased ~20-fold (1–20 min; Figure 5B), where as the drug slows the rate of relaxation by LdTOP1L/HTOP14 by only 5-fold (Figure 5D). LdTOP1L-fus-HTOP14 exhibits comparatively higher CPT sensitivity where the time required to complete relaxation increased 10-fold (Figure 5F). This result is consistent with our

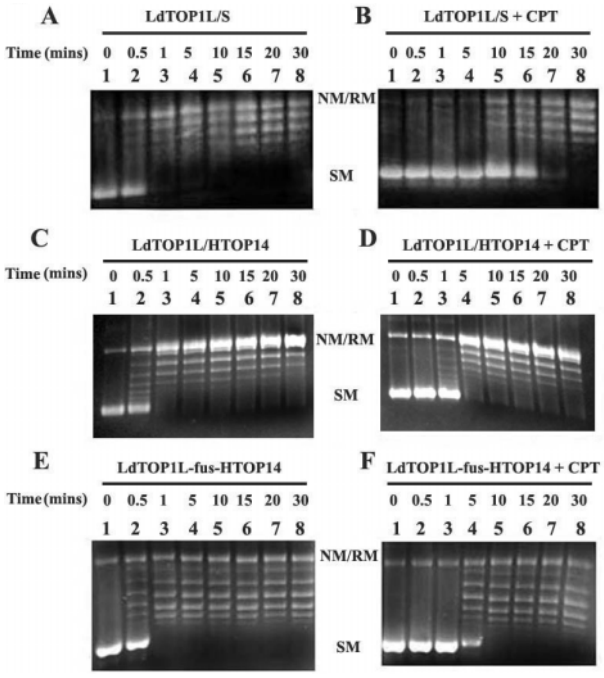

Figure 5. Effect of CPT on relaxation activity of LdTOP1L/HTOP14 and LdTOPL-fus-HTOP14. Relaxation of supercoiled pBS (SK^+) DNA with LdTOP1L/S (**A** and **B**), LdTOP1L/HTOP14 (**C** and **D**) and LdTOPL-fus-HTOP14 (**E** and **F**) at a molar ratio of 1:2 in the presence or absence of CPT. Lane 1 of A, C and E contain 50 fmol of supercoiled pBS (SK^+) DNA. Lanes 2–8, same as lane 1 but incubated with 100 fmol of LdTOP1L/S, LdTOP1L/HTOP14 or LdTOPL-fus-HTOP14, respectively. B, D and F, same as A, C and E, but 50 μM CPT was added in lanes 2–8. Reactions were carried out at 37°C for varying time periods as indicated and stopped by the addition of 0.5% SDS and samples were electrophoresed on 1% agarose gel. Positions of supercoiled monomer (SM) and relaxed and nicked monomers (RM/NM) are indicated.

hypothesis that both the chimeric enzymes exhibit reduced CPT sensitivity than wild-type LdTOP1L/S. One possible explanation for this reduced CPT sensitivity might be attributed to the improper assembling of the two subunits to attain the most favored conformation for maximum stabilization of the cleavabale complex. Since post-translational a modifications are known to affect CPT sensitivity of mammalian topoisomerase I, we checked whether the hybrid enzymes also exhibit any change in their sensitivity to CPT when expressed in baculovirus system. No significant difference in CPT sensitivity was observed between the enzymes expressed in bacterial system and those expressed in baculovirus system (data not shown).

Relative affinity for DNA

From the observations in plasmid relaxation assays performed under multiple turnover and non-turnover conditions, we hypothesized that the hybrid enzyme LdTOP1L/HTOP14 dissociates from DNA faster than the wt-LdTOP1L/S, which in turn dissociates faster than LdTOP1L-fus-HTOP14. To test this hypothesis and to determine the relative affinities of the enzymes for a common 25 bp oligonucleotide substrate, we performed the competition-binding assay (6). In this experiment, the cleaved strand of the duplex substrate was labeled with ^{32}P at its 5′ end. Enzyme mixtures were prepared

such that they contain increasing concentrations of LdTOP1L/HTOP14 (50 nM, 100 nM, 200 nM and 400 nM) with or without a fixed concentration (100 nM) of LdTOP1L/S. Each sample was mixed with 7.5 nM 5′-^{32}P-end-labeled 25mer duplex DNA substrate and incubated for 30 min at 37°C. The samples were then adjusted to 1% SDS, boiled and fractionated by 15% SDS–PAGE followed by autoradiography. In a similar set of experiment, increasing concentrations of LdTOP1L/S (50 nM, 100 nM, 200 nM and 400 nM) were used with or without a fixed concentration (100 nM) of LdTOP1L-fus-HTOP14. The results of this experiment are shown in Figure 6. Based on software (Quantity One, Bio-Rad) analysis and quantification of the amounts of covalent complexes in lanes 5–8 in Figure 6B, it appears that LdTOP1L/HTOP14 must be in a 4-fold molar excess over LdTOP1L/S in order to reduce the substrate binding of LdTOP1L/S by one-half. Given this level of competition, it is possible to calculate that LdTOP1L/HTOP14 binds duplex DNA substrate with an affinity that is at least 4-fold lower than LdTOP1L/S. A titration experiment similar to the one in lanes 3 and 4 of Figure 6B, extending to lower protein concentrations yielded a value for the equilibrium dissociation constant (K_D) of $\sim 2.8 \times 10^{-7}$ M for binding of LdTOP1L/S to the 25mer substrate DNA. Given this value we can estimate the K_D value of $\sim 11.5 \times 10^{-7}$ M for LdTOP1L/HTOP14 binding to the same substrate (21). A similar set of experiment with varying concentrations of LdTOP1L/S and fixed concentration of LdTOP1L-fus-HTOP14 is shown in Figure 6C where it appears that LdTOP1L/S must be in a 2-fold molar excess over LdTOP1L-fus-HTOP14 in order to reduce the substrate binding of LdTOP1L-fus-HTOP14 by one-half.

From the above findings we can estimate the K_D value for LdTOP1L-fus-HTOP14 binding to the same substrate, i.e. $\sim 1.4 \times 10^{-7}$ M. Overall, these results indicate a lower affinity of LdTOP1L/HTOP14 for DNA compared with LdTOP1L/S which is consistent with the findings in plasmid relaxation assay. Moreover, there is a 2-fold increase in DNA binding than LdTOP1L/S when LdTOP1L and HTOP14 are fused.

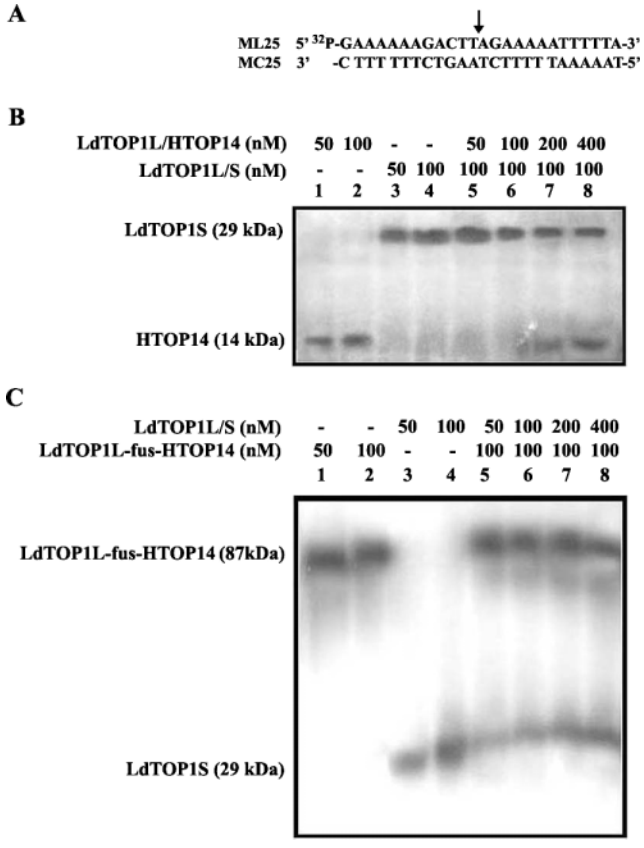

Figure 6. Relative affinity for DNA. (**A**) Radiolabeled 25mer duplex DNA substrate. The duplex 25mer radiolabeled substrate is cleaved at the single site (indicated by the arrow) upon the addition of SDS to topoisomerase I reactions. (**B**) Competition binding experiment with LdTOP1L/S and LdTOP1L/HTOP14. The enzymes alone or as mixtures were incubated with the ^{32}P-end-labeled 25mer duplex DNA substrate (7.5 nM) where the final salt concentrations were 50 mM. Lanes 1 and 2, contain only 50 and 100 nM LdTOP1L/HTOP14; lanes 3 and 4, 50 and 100 nM of LdTOP1L/S. The samples in the lanes 5–8 contain equal quantity of LdTOP1L/S (100 nM) and increasing concentrations (50, 100, 200, 400 nM) of LdTOP1L/HTOP14. (**C**) Competition binding experiment with LdTOP1L/S and LdTOP1L-fus-HTOP14. The experiment was performed similar to that in (B). The samples in lanes 5–8 contain equal quantities of LdTOP1L-fus-HTOP14 (100 nM) and increasing concentrations of LdTOP1L/S (50, 100, 200 and 400 nM). Lanes 1 and 2, contain only 50 and 100 nM LdTOP1L-fus-HTOP14, lanes 3 and 4, 50 and 100 nM of LdTOP1L/S. After 30 min incubation at 37°C, the samples were adjusted to 1% SDS and analysed by 15% SDS–PAGE followed by autoradiography.

Suicidal cleavage activity of the chimeric and the wild-type enzymes

The efficiency with which SDS traps the covalent complex of LdTOP1L/HTOP14 when it is bound to the duplex 25mer is significantly less than that for LdTOP1L/S bound to the identical substrate. SDS-induced trapping of covalent complex increased significantly with the intact enzyme LdTOP1L-fus-HTOP14. This phenomenon was tested using equimolar quantities of the three enzymes with an excess of oligonucleotide substrate (Figure 7B, lanes 2, 4 and 6). Suicidal cleavage experiment was performed at 50, 100 and 200 mM KCl for LdTOP1L/S, LdTOP1L/HTOP14 and LdTOP1L-fus-HTOP14, respectively, so as to achieve maximum cleavage activity by the respective enzymes. SDS denaturation yielded ~3-fold less covalent complex for LdTOP1L/HTOP14 and ~2-fold more covalent complex for LdTOP1L-fus-HTOP14 compared with LdTOP1L/S enzyme. But when equal quantities of the three enzymes were allowed to react separately with an excess of radiolabeled suicide substrate (Figure 7A), the final quantity of covalent complex formed for the three enzymes were almost identical (Figure 7B, lanes 1, 3 and 5). This result indicates that both LdTOP1L/HTOP14 and LdTOP1-fus-HTOP14 have the same molar ratio of functional active sites per quantity of enzyme as that of LdTOP1L/S.

To determine the rate of formation of the covalent complex, we performed a time course assay using the radiolabeled suicidal oligonucleotide substrate. The cleavage activities of the enzymes as determined from the percentage of substrate converted into product was plotted as a function of time (Figure 7C). In suicidal assays for LdTOP1L-fus-HTOP14 and HTOP91, ~80 and 90% of input DNA were covalently bound to the enzymes, respectively, and reached the cleavage plateau in 5 min of incubation. LdTOP1L/S was found to reach the plateau after 30 min of

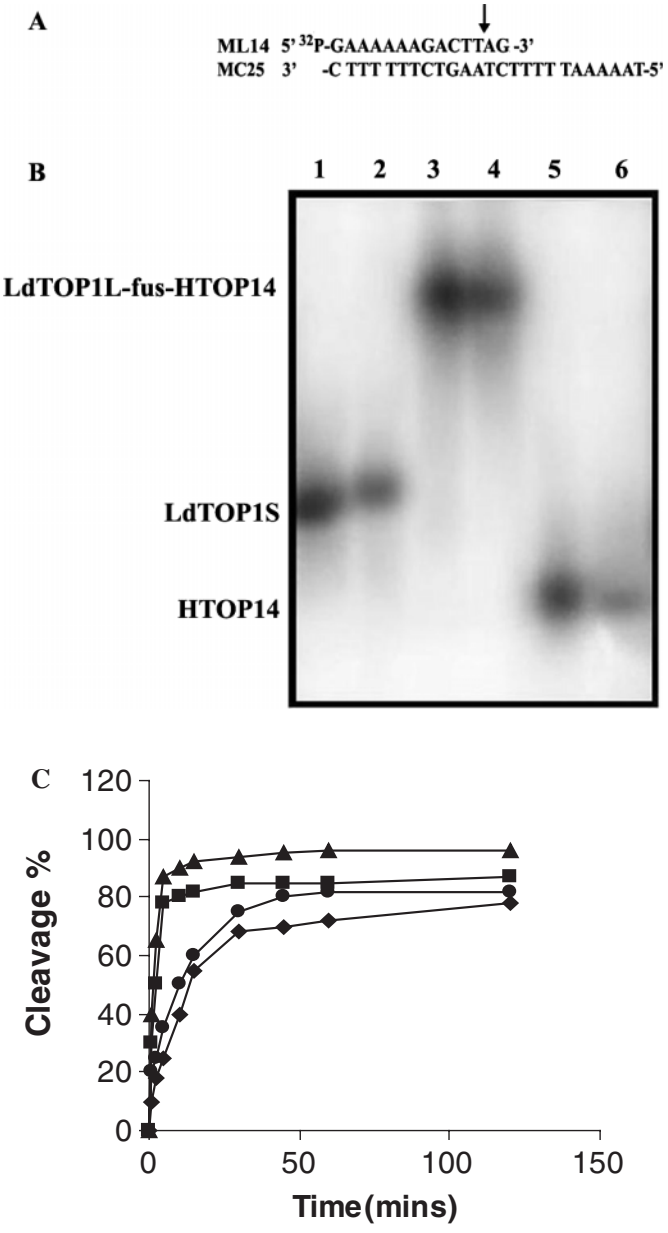

Figure 7. Suicide cleavage and SDS-mediated breakage of duplex substrate using equal quantities of enzyme. (**A**) Radiolabeled suicidal DNA substrate. The radiolabeled substrate is cleaved at the single site (indicated by the arrow) upon the addition of SDS to topoisomerase I reactions. (**B**) Equimolar concentrations of enzymes (LdTOP1L/S, LdTOP1L-fus-HTOP14 or LdTOP1L/HTOP14 were allowed to react with either the suicidal substrate ML14/MC25 (lanes 1, 3 and 5) or the 25mer duplex oligonucleotides (lanes 2, 4 and 6). The final KCl concentrations were 50 mM in lanes 1 and 2, 200 mM in lanes 3 and 4, and 100 mM in lanes 5 and 6. The salt concentrations were optimized according to the enzyme used to achieve the maximum cleavage. After 1 h incubation at 37°C, the reactions were stopped by the addition of 1% SDS, and the samples were analysed by 15% SDS–PAGE and autoradiography. (**C**) DNA cleavage rates for LdTOP1L/S (closed circles) LdTOP1L/HTOP14 (closed diamonds) LdTOP1L-fus-HTOP14 (closed squares) and HTOP91 (closed triangles) with 5′-^{32}P-labeled suicide DNA substrate. The reaction mixtures were incubated with the topoisomerase I variants for different time points at 23°C as described in Materials and Methods. Cleavage products were analyzed by sequencing gel and percentage of cleaved DNA product was plotted as a function of time. The results depicted are average of three independent experiments normalized relative to the highest amount of substrate converted to cleaved product by the respective enzymes.

incubation with almost 75% of the DNA attached to it. LdTOP1L/HTOP14 also completed the same reaction in 30 min of incubation with somewhat lesser amount of DNA attached. The cleavage rate was found to be faster for the fused protein LdTOP1L-fus-HTOP14 and is comparable with the monomeric human topoisomerase I. Hence, replacement of LdTOP1S with HTOP14 has very little effect on the initial step after binding and before strand rotation. Notably, there is an improvement in the initial cleavage rate, in case of the fused enzyme LdTOP1L-fus-HTOP14.

Single turnover religation activity

Religation was studied under single turnover conditions by assaying the ability of the covalent intermediate to attach a 5′-OH-terminated 11mer (MR11) to the cleaved oligonucleotide and to form a 23mer product. The ligation reactions were performed with LdTOP1L/HTOP14 and LdTOP1L-fus-HTOP14, as described in Materials and Methods. Reactions were stopped at varying time points by the addition of an equal volume of 1% SDS. The samples were treated with trypsin to remove all but a short trypsin-resistant peptide

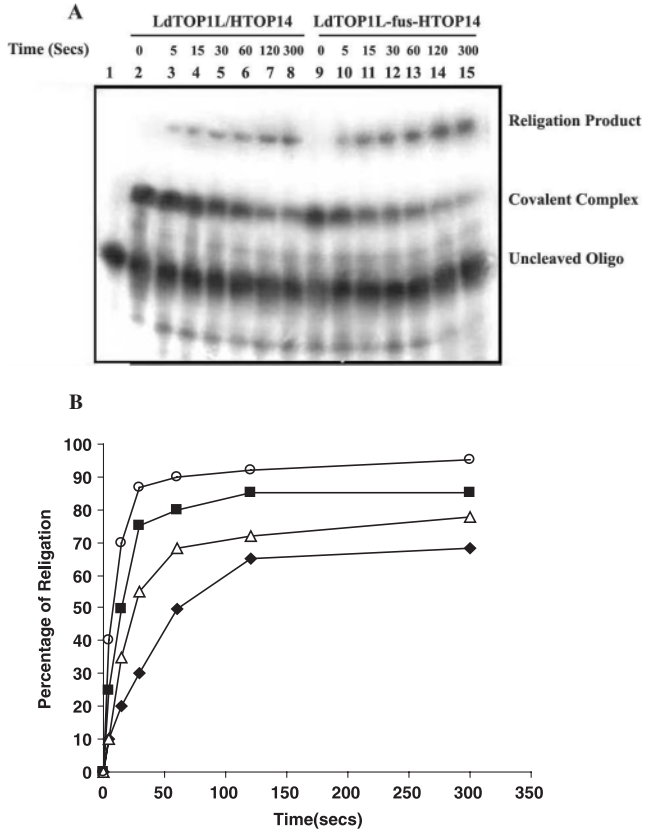

Figure 8. Religation kinetics with recombinant enzymes: the religation reactions were carried out as described in Materials and Methods. (**A**) Sequencing gel showing the time course of appearance of the 23mer religation product for LdTOP1L/HTOP14 and LdTOP1L-fus-HTOP14. (**B**) The relative amount of cleavage product converted to religation product by LdTOP1L/HTOP14 (closed diamonds) and LdTOP1L-fus-HTOP14 (closed squares) in each sample were plotted as a function of time. The relative amount of cleavage product converted to religation product by LdTOP1L/S (open triangles) and HTOP91 (open circles) was calculated from a similar set of control experiments (data not shown) and were also plotted in the same graph.

from the topoisomerase I–DNA complex before analysis in sequencing gel (Figure 8A). The percentage of ligated product formed at each time point was plotted against time. From a similar set of control experiment (data not shown), the percentages of religated products were plotted for LdTOP1L/S and HTOP91 in the same graph (Figure 8B). The results indicate that the reconstituted enzyme LdTOP1L/HTOP14 has a slower rate of religation than the wt-LdTOP1L/S. Although maximum religation was achieved within 60 s by LdTOP1L/S, the chimeric enzyme LdTOP1L/HTOP14 takes 120 s to achieve maximum religation. On the other hand, both the intact enzymes exhibit identical religation kinetics. The slower religation rate of LdTOP1L/HTOP14 can be attributed to the slow strand passage as observed previously from the non-turnover plasmid relaxation assay.

Fluorescence polarization assay for protein–protein interaction

All the experiments performed above suggest that the hybrid protein LdTOP1L/HTOP14 binds less efficiently to DNA and exhibits slower strand passage compared with LdTOP1L/S. This finding indicates that the small subunit directly participates in DNA binding, which might be responsible for the improved DNA binding and strand passage of the wild-type enzyme. From the percentage of positively charged residues and etectrophoretic mobility shift (data not shown) with LdTOP1S, it is clear that the small subunit does not have substantial DNA-binding properties. We therefore propose that the small subunit can specifically interact with the large subunit (LdTOP1L) and exhibits improved DNA binding compared with LdTOP1L/HTOP14. To study the interaction between the two proteins, we used fluorescence polarization assay (16,22). Proteins (LdTOP1S and HTOP14) were labeled with fluorescein isothiocyanate and assays were performed as described in Materials and Methods. Samples were prepared by mixing constant concentration of labeled LdTOP1S or HTOP14 (25 nM) with varying concentrations of LdTOP1L (10–250 nM). Fluorescence polarization measurements were carried out at excitation wavelength of 495 nm and emission wavelength of 518 nm. The fractions of bound protein (f_B) were plotted against the protein concentrations (F) for both LdTOP1S and HTOP14 (Figure 9) and the respective K_D values for the interactions were calculated according to Park and Raines (16). The K_D value for interaction of LdTOP1L and LdTOP1S was 5.98×10^{-8} M whereas the same for LdTOP1L and HTOP14 was 16.23×10^{-8} M. These K_D values are in compliance with our hypothesis that the interaction between LdTOP1L and LdTOP1S is stronger and more specific than that between LdTOP1L and HTOP14. This specific interaction is responsible for more efficient anchoring of the catalytic domain on the DNA which contributes to the improved DNA binding and strand rotation.

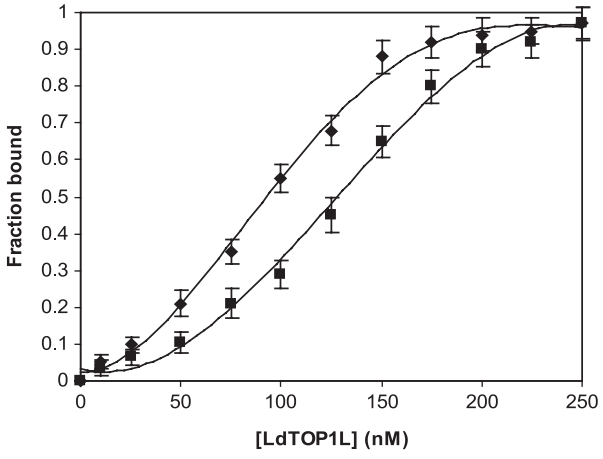

Figure 9. Fluorescence polarization assay of protein–protein interaction: increasing concentrations of LdTOP1L (10–250 nM) were mixed with constant amount of FITC-labeled LdTOP1S or HTOP14. Fluorescence polarization measurements were carried out at excitation of 495 nm and emission of 518 nm. Fractions bound of LdTOP1L when reacted with labeled LdTOP1S (closed diamonds) or HTOP14 (closed squares) were calculated and curves were obtained by fitting the data to the Equation 3 as described in Materials and Methods. Each data point is an average of five measurements.

DISCUSSION

Unique from other eukaryotic topoisomerase I, kinetoplastid topoisomerase I is a bi-subunit enzyme. The large subunit of the enzyme contains almost all conserved residues to constitute the core domain of eukaryotic topoisomerase I but is unable to show any relaxation activity. The catalytic tyrosine required for activity resides on a small subunit, which has a very distinct architecture. The small subunit shares very little sequence homology with other eukaryotic topoisomerase I except the SKXXY motif harboring the catalytic tyrosine. This tempted us to find whether topoisomerase I activity can be reconstituted by replacing the small subunit with a C-terminus fragment (residues 636–765) of human topoisomerase I, which also contains the catalytic tyrosine required for the enzyme to function. Topoisomerase I activity was observed in plasmid relaxation experiments when large subunit of *L.donovani* topoisomerase I (LdTOP1L) and the C-terminal fragment of human topoisomerase I (HTOP14) were mixed in 1:1 ratio *in vitro*. Hence we coined the name '*LeishMan*' (Leish from *Leishmania* and Man from Human) topoisomerase I for this hybrid enzyme, which can serve as a model for studying the functional relationship between the two subunits and understanding the mechanism of evolution of type IB topoisomerase. This finding further prompted us to construct a chimeric monomer (LdTOP1L-fus-HTOP14) by fusing LdTOP1L and HTOP14 to generate a monomeric enzyme structurally equivalent to human topoisomerase I. Our objective was to see whether this chimeric monomer could resemble human topoisomerase I in its properties. Both the enzymes (LdTOP1L/HTOP14 and LdTOP1-fus-HTOP14) were tested for their ability to bind, cleave and relax DNA and the way they respond to topoisomerase I poison camptothecin. The experiments described in this work allow a better understanding of the respective roles of the two subunits of *L.donovani* topoisomerase I.

The first obvious result is the change in relaxation pattern of the enzymes due to the substitution of LdTOP1S with HTOP14. The hybrid enzyme LdTOP1L/HTOP14 exhibits a more distributive pattern than LdTOP1L/S. This result is indicative of the fact that LdTOP1L/HTOP14 readily dissociates from the substrate following the release of one or a few superhelical turns. Champoux and co-workers (6) have shown previously that the intact enzymes have higher affinity for DNA and are more processive than the reconstituted

enzymes. To test this possibility, we examined the relaxation pattern of the chimeric monomer LdTOP1L-fus-HTOP14. As expected the monomeric enzyme was more processive than LdTOP1L/HTOP14. The relative affinities of the enzymes for DNA was examined by competition binding experiments. From the equilibrium dissociation constant (K_D) values calculated, the enzymes can be arranged in increasing order of their affinity for DNA as follows: LdTOP1L/HTOP14 < LdTOP1L/S < LdTOP1L-fus-HTOP14. This difference in affinity among the enzymes for DNA gives rise to several possibilities. The fact that intact enzymes have higher affinity for DNA than the reconstituted enzymes explains the enhanced DNA binding observed for LdTOP1L-fus-HTOP14, but fails to explain the 4-fold difference in DNA binding among the two reconstituted enzymes. The possibility that *L.donovani* small subunit harbors some putative domain that directly contributes in the DNA binding can be ruled out since, enhanced DNA binding is observed with the chimeric monomer LdTOP1L-fus-HTOP14. Therefore, it is justified to speculate that the small subunit of *L.donovani* topoisomerase I harbors some putative domain (not present in HTOP14) that help in more specific interaction with the large subunit which in turn promotes better DNA binding.

To assess the difference in topoisomerization by the chimeric and the wild-type enzymes, we performed non-turnover relaxation assays for all the enzymes including human topoisomerase I (HTOP91). Under non-turnover condition, HTOP91 was found to relax supercoiled plasmids faster than LdTOP1L-fus-HTOP14 whereas LdTOP1L/S relaxes supercoiled DNA much faster than LdTOP1L/HTOP14. Since the reactions were carried out under non-turnover condition, slower rate of relaxation indicates a slower strand passage by the respective enzyme. Thus, we propose that the strand passage and efficiency of the reaction by LdTOP1L/S is largely dependent on the interaction between LdTOP1L and LdTOP1S. Substitution of the small subunit with the HTOP14 directly affects the strand rotation due to lesser degree of interaction between LdTOP1L and HTOP14. Fusion of the two proteins (LdTOP1L and HTOP14) overcomes this problem and yields a catalytically more efficient enzyme.

DNA cleavage assay was performed to determine the effect of salt on the reconstituted and the intact enzymes. LdTOP1L/HTOP14 exhibited optimal cleavage activity at 100 mM KCl where as the chimeric monomer LdTOP1L-fus-HTOP14 exhibits optimal activity at 200 mM KCl. Although the wt-LdTOP1L/S exhibits optimal cleavage at 50 mM KCl (9), the percentage of cleavage achieved by the enzyme at this salt concentration is almost similar to that of LdTOP1L/HTOP14 at 100 mM KCl. This indicates that both the reconstituted enzymes are sensitive to high salt concentration and the intact enzyme LdTOP1L-fus-HTOP14 can tolerate high salt concentration similar to monomeric human topoisomerase I (6). All the cleavage reactions were carried out either in the absence or in the presence of topoisomerase I poison camptothecin (CPT). The presence of CPT enhances the cleavable complex formation by ~40% in the case of LdTOP1L/S (9). In contrast, CPT does not substantially enhance the cleavage in the case of LdTOP1L/HTOP14. LdTOP1L-fus-HTOP14 although exhibits enhanced CPT-mediated cleavage, the amount of cleavable complex stabilized was less than that of LdTOP1L/S. We therefore tested the effect of CPT on the relaxation activity of these enzymes. Both the enzymes bearing HTOP14 as the catalytic domain exhibit reduced CPT sensitivity compared with LdTOP1L/S. There was a 4-fold reduction in CPT sensitivity of LdTOP1L/HTOP14 whereas the fusion protein LdTOP1L-fus-HTOP14 is 2-fold less sensitive to CPT than wild-type LdTOP1L/S. Based on these findings we can only speculate that the small subunit (LdTOP1S) has direct involvement in regulating CPT sensitivity of *L.donovani* topoisomerase I. Mutation of two amino acids (Gly185 and Asp325) on the large subunit has been reported to be responsible for CPT resistance in *L.donovani* (23). Role of the small subunit in regulating CPT sensitivity has not yet been accounted for. As demonstrated by Champoux and co-workers (24), reconstituted human topoisomerase I topo58/12 and topo58/6.3 were found to have reduced CPT sensitivity due to lack of a properly folded, functional linker domain. Moreover, the deletion of a region in the linker domain of human topoisomerase I renders the enzyme insensitive to camptothecin (25). HTOP14, although contains the linker domain of human topoisomerase I, seems to remain misfolded in the case of LdTOP1L/HTOP14. Fusion of LdTOP1L with HTOP14 although recovers CPT sensitivity to some extent, it failed to achieve similar degree of sensitivity as LdTOP1L/S. Thus, we can hypothesize that interaction and assembling of the two subunits as well as proper folding is required to attain the most favoured conformation for maximum stabilization of cleavable complex by CPT.

The rate of cleavable complex formation was measured to determine actually which step of the topoisomerization reaction is affected due to the substitution of LdTOP1S with HTOP14. Both the intact enzymes (LdTOP1L-fus-HTOP14 and HTOP91) reached the cleavage plateau within 5 min of incubation, whereas the reconstituted enzyme LdTOP1L/S and LdTOP1L/HTOP14 reached the plateau after 30 min. The amount of cleavable complex formed by LdTOP1L/HTOP14 is substantially less than that of LdTOP1L/S at the same time point (Figure 7C), which can be attributed to the reduced affinity for DNA. Equal rates of cleavage indicate that the cleavage step is not much affected due to the substitution of LdTOP1S with HTOP14. That intact enzymes has faster cleavage rate than reconstituted ones (6) explains the faster rate of cleavage by LdTOP1L-fus-HTOP14.

To gather stronger evidence in support of our hypothesis that strand rotation and, hence, catalytic efficiency is affected due to the substitution of LdTOP1S with HTOP14 we performed single turnover religation kinetics. A substantially slower rate of religation was observed for LdTOP1L/HTOP14 than LdTOP1L/S, whereas the intact chimeric enzyme exhibited religation kinetics similar to that of human topo I. This slower religation rate indicates that the enzyme LdTOP1L/HTOP14 is catalytically less efficient than LdTOP1L/S.

The two enzymes, LdTOP1L/S and LdTOP1L/HTOP14, bearing two different catalytic domains differ in their DNA-binding properties, strand rotation and catalytic efficiency. The former is catalytically more efficient, having a higher affinity for DNA and faster strand passage. This effect cannot be directly attributed to the presence of a putative domain in LdTOP1S, which directly participates in DNA binding, since fusion of LdTOP1L with HTOP14 yielded a catalytically more efficient enzyme. The only other possibility is that LdTOP1S can interact more specifically than HTOP14 with

LdTOP1L and impart catalytic efficiency to LdTOP1L/S. To test this possibility, we examined the protein–protein interaction by fluorescence anisotropy and calculated the K_D values for the respective interactions. We obtained a 2.7-fold higher K_D value (16.23×10^{-8} M) for the interaction of LdTOP1L and HTOP14 compared with that between LdTOP1L and LdTOP1S (5.98×10^{-8} M). This supports the notion that the small subunit (LdTOP1S) of *L.donovani* topoisomerase I interacts strongly with the large subunit (LdTOP1L) and helps in anchoring the enzyme on the DNA more efficiently. HTOP14 although restored relaxation activity by interacting with LdTOP1L, the interaction is comparatively weaker and hence it is catalytically less efficient. Fusion of LdTOP1L to HTOP14 overcomes the need to interact and hence behaves like a monomeric enzyme with improved DNA-binding affinity.

In conclusion, our study demonstrates the role of the small subunit of *L.donovani* topoisomerase I in governing the property of the enzyme. It is obvious from our study that the unconserved, extended region of the small subunit (amino acids 1–120) governs subunit interaction and CPT sensitivity of the enzyme. Although activity was restored by substitution of the small subunit with HTOP14, it failed to exhibit similar degree of DNA binding and CPT sensitivity as the wild-type enzyme. We therefore propose that the unconserved region of the *L.donovani* topoisomerase I small subunit actually corresponds to the linker domain of eukaryotic topoisomerase IB. In the case of *L.donovani*, it is justified to rename the linker domain as the 'interacting domain' since this part of the enzyme participates in specific interaction with the large subunit rather than linking the two subunits. The above findings and propositions further allow us to speculate that all eukaryotic type IB topoisomerase evolved from the common ancestral bi-subunit enzyme by fusion of the two subunits at genetic level. Thus, the construction of chimeric '*LeishMan*' topoisomerase I offers a valuable model for studying the role of the small subunit of the unusual bi-subunit enzyme in carrying out high-precision DNA transactions to complete topoisomerization of DNA.

ACKNOWLEDGEMENTS

We thank Prof. J.J. Champoux, University of Washington, Seattle, WA, for providing us with the full-length cDNA clone of human topoisomerase I and the recombinant baculovirus containing wild-type human topoisomerase I. We are thankful to Dr S. Roy, Director, Indian Institute of Chemical Biology for his interest in this work. This work was supported by grants from Network Project SMM 003 of Council of Scientific and Industrial Research, Government of India and Department of Science and Technology, Government of India (SP/SO/D11/2000) to H.K.M. A.G. was supported by Senior Research Fellowship from Council of Scientific and Industrial Research, Government of India. Funding to pay the Open Access publication charges for this article was provided by Indian Institute of Chemical Biology, Kolkata, India.

Conflict of interest statement. None declared.

REFERENCES

1. Champoux,J.J. (2001) DNA topoisomerases: structure, function, and mechanism. *Annu. Rev. Biochem.*, **70**, 369–413.
2. Wang,J.C. (1996) DNA topoisomerases. *Annu. Rev. Biochem.*, **65**, 635–692.
3. Stivers,J.T., Harris,T.K. and Mildvan,A.S. (1997) Vaccinia DNA topoisomerase I: evidence supporting a free rotation mechanism for DNA supercoil relaxation. *Biochemistry*, **36**, 5212–5222.
4. Gupta,M., Fujimori,A. and Pommier,Y. (1995) Eukaryotic DNA topoisomerases I. *Biochim. Biophys. Acta*, **1262**, 1–14.
5. Stewart,L., Ireton,G.C. and Champoux,J.J. (1996) The domain organization of human topoisomerase I. *J. Biol. Chem.*, **271**, 7602–7608.
6. Stewart,L., Ireton,G.C. and Champoux,J.J. (1997) Reconstitution of human topoisomerase I by fragment complementation. *J. Mol. Biol.*, **269**, 355–372.
7. Villa,H., Otero Marcos,A.R., Reguera,R.M., Balana-Fouce,R., Garcia-Estrada,C., Perez-Pertejo,Y., Tekwani,B.L., Myler,P.J., Stuart,K.D., Bjornsti,M.A. *et al*. (2003) A novel active DNA topoisomerase I in *Leishmania donovani*. *J. Biol. Chem.*, **278**, 3521–3526.
8. Bodley,A.L., Chakraborty,A.K., Xie,S., Burri,C. and Shapiro,T.A. (2003) An unusual type IB topoisomerase from African trypanosomes. *Proc. Natl Acad. Sci. USA*, **100**, 7539–7544.
9. Das,B.B., Sen,N., Ganguly,A. and Majumder,H.K. (2004) Reconstitution and functional characterization of the unusual bi-subunit type I DNA topoisomerase from *Leishmania donovani*. *FEBS Lett.*, **565**, 81–88.
10. Broccoli,S., Marquis,J.F., Papadopoulou,B., Olivier,M. and Drolet,M. (1999) Characterization of a *Leishmania donovani* gene encoding a protein that closely resembles a type IB topoisomerase. *Nucleic Acids Res.*, **27**, 2745–2752.
11. Madden,K.R. and Champoux,J.J. (1992) Overexpression of human topoisomerase I in baby hamster kidney cells: hypersensitivity of clonal isolates to camptothecin. *Cancer Res.*, **52**, 525–532.
12. Das,B.B., Sen,N., Dasgupta,S.B., Ganguly,A. and Majumder,H.K. (2005) N-terminal region of the large subunit of *Leishmania donovani* bisubunit topoisomerase I is involved in DNA relaxation and interaction with the smaller subunit. *J. Biol. Chem.*, **280**, 16335–16344.
13. Stewart,L., Ireton,G.C., Parker,L.H., Madden,K.R. and Champoux,J.J. (1996) Biochemical and biophysical analyses of recombinant forms of human topoisomerase I. *J. Biol. Chem.*, **271**, 7593–7601.
14. Osheroff,N., Shelton,E.R. and Brutlag,D.L. (1983) DNA topoisomerase II from *Drosophila melanogaster*. Relaxation of supercoiled. DNA. *J. Biol. Chem.*, **258**, 9536–9543.
15. Yang,Z. and Champoux,J.J. (2002) Reconstitution of enzymatic activity by the association of the cap and catalytic domains of human topoisomerase I. *J. Biol. Chem.*, **277**, 30815–30823.
16. Park,S.H. and Raines,R.T. (2004) Fluorescence polarization assay to quantify protein–protein interactions. *Methods Mol. Biol.*, **261**, 161–166.
17. Dickey,J.S. and Osheroff,N. (2005) Impact of the C-terminal domain of topoisomerase II alpha on the DNA cleavage activity of the human enzyme. *Biochemistry*, **44**, 11546–11554.
18. Li,T.K. and Liu,L.F. (2001) Tumor cell death induced by topoisomerase-targeting drugs. *Annu. Rev. Pharmacol. Toxicol.*, **41**, 53–77.
19. Bodley,A.L. and Shapiro,T.A. (1995) Molecular and cytotoxic effects of camptothecin, a topoisomerase I inhibitor, on trypanosomes and Leishmania. *Proc. Natl Acad. Sci. USA*, **92**, 3726–3730.
20. Hertzberg,R.P., Caranfa,M.J. and Hecht,S.M. (1989) On the mechanism of topoisomerase I inhibition by camptothecin: evidence for binding to an enzyme–DNA complex. *Biochemistry*, **28**, 4629–4638.
21. Horovitz,A. and Levitzki,A. (1987) An accurate method for determination of receptor–ligand and enzyme–inhibitor dissociation constants from displacement curves. *Proc. Natl Acad. Sci. USA*, **84**, 6654–6658.
22. Brownbridge,G.G., Lowe,P.N., Moore,K.J., Skinner,R.H. and Webb,M.R. (1993) Interaction of GTPase activating proteins (GAPs) with p21ras measured by a novel fluorescence anisotropy method. Essential role of Arg-903 of GAP in activation of GTP hydrolysis on p21ras. *J. Biol. Chem.*, **268**, 10914–10919.
23. Marquis,J.F., Hardy,I. and Olivier,M. (2005) Topoisomerase I amino acid substitutions, Gly185Arg and Asp325Glu, confer camptothecin resistance in *Leishmania donovani*. *Antimicrob. Agents Chemother.*, **49**, 1441–1446.
24. Stewart,L., Ireton,G.C. and Champoux,J.J. (1999) A functional linker in human topoisomerase I is required for maximum sensitivity to camptothecin in a DNA relaxation assay. *J. Biol. Chem.*, **274**, 32950–32960.
25. Ireton,G.C., Stewart,L., Parker,L.H. and Champoux,J.J. (2000) Expression of human topoisomerase I with a partial deletion of the linker region yields monomeric and dimeric enzymes that respond differently to camptothecin. *J. Biol. Chem.*, **275**, 25820–25830.