

Swapping of The N-Terminal Domain of Human Topoisomerase 1B with the Corresponding *Plasmodium Falciparum* Counterpart Strongly Impairs Enzyme Activity

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Abstract

Background: DNA topoisomerases 1B are a class of ubiquitous enzyme that solves the topological problems associated with biological processes such as replication, transcription and recombination. Numerous sequence alignment of topoisomerase 1B from different species shows that the lengths of different domains as well as their amino acids sequences are quite different. In the present study a hybrid enzyme, generated by swapping the N-terminal of *Plasmodium falciparum* into the corresponding domain of the human, has been characterized.

Methods: The chimeric enzyme was generated using different sets of PCR. The *in vitro* characterization was carried out using different DNA substrate including radio-labelled oligonucleotides.

Results: The chimeric enzyme displayed slower relaxation activity, cleavage and re-ligation kinetics strongly perturbed when compared to the human enzyme.

Conclusions: These results indicate that the N-terminal domain has a crucial role in modulating topoisomerase activity in different species.

Keywords: N-terminal domain, *Plasmodium falciparum* topoisomerase 1B, Topoisomerase 1B.

Introduction

DNA topoisomerases are vital enzymes that are responsible for maintaining the topological state of the DNA inside the cell by catalyzing relaxation of DNA supercoils (1, 2). These supercoils are generated as a consequence of biological process like DNA replication, transcription, recombination that requires separation of double stranded DNA (21, 22). Human topoisomerase 1B (hTop1) is a monomeric 91 kDa enzyme consisting of 765 amino acids that are divided into four domains namely: the N-terminal (1-214), the core (215-635), the linker (636-712) and the C-terminal domain (713-765) (3, 4, 18). The catalysis of supercoiled DNA by hTop1 is initiated by transiently breaking one strand through a nucleophilic attack of

the tyrosine residue present in the active site of the enzyme on the scissile phosphate breaks in the DNA strand creating a phosphotyrosine linkage between the tyrosine and the 3' phosphate end of DNA (5, 23). Once cleaving, the enzyme covalently holds one end of the duplex DNA thereby allowing 5'-end of the cleaved site to rotate around the non-cleaved strand. DNA relaxation occurs by the "controlled rotation" mechanism that is supported by various ionic interactions (6). After accomplishing a full rotation, there is a second nucleophilic attack of the phosphotyrosine bond at the 5'-OH group of the cleaved strand enabling the enzyme to reseal the DNA that subsequently results in the dissociation of the

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enzyme from the relaxed DNA (7, 8). Human topoisomerase 1B has a significant medical interest since it is the cellular target of several natural compounds (9, 19). One of the most important of such compounds is camptothecin (CPT) that reversibly stabilizes the DNA/protein cleavable complex (10, 11, 12). The N-terminal structure of hTop1 remains poorly understood because it is the only part of the enzyme still not crystallized. This domain contains various nuclear localization sequences (NLSs) and is found to be essential for the *in vivo* function of the enzyme (35). Different studies suggest that phosphorylation can modulate enzyme activity and CPT sensitivity of hTop1 and that residues starting from 191-206 are required for DNA binding and enzyme processivity (13, 17). The close interaction of Trp-205 to residues in the flexible hinge region have suggested that Trp-205 plays an important role for the rate controlling motion within the hinge region (17) which is involved in the control rotation. Interestingly, alignment of the human and *Plasmodium falciparum* topoisomerase 1B (pfTop1) enzyme indicates that this tryptophan is also present in the pfTop1. The *Plasmodium falciparum* N-terminal domain is 62 amino acids shorter than the human counterpart and its amino acids composition is quite variable as compared to hTop1 N-terminal (16). Alignment of the two sequences shows that pfTop1 shares 42% identity with the hTop1 (27). In spite of the low sequence conservation between the two homologs, they share a common and quite well conserved three-dimensional arrangement, as previously reported in Arnò *et al* (20). Indeed, the structure of pfTop1 obtained through homology modelling using as a template, the structure of hTop1 shows that the core of the two proteins is very well conserved, except for the presence of two polar insertions and for a longer linker domain when compared to htop1 (data not shown). Because of the extremely disordered nature of the N-terminus, this domain has never been solved so we could not provide a model for the pfTop1 N-terminal. In the present study, we have produced and characterized a chimeric enzyme generated by swapping 154 amino acids residues N-terminal domain (1 to 154) of pfTop1 into 215 amino acids residues N-terminal domain (1 to 215) of hTop1, hereafter called hTop1(pf-N-term). The chimera enzyme activity was strongly impaired suggesting the important role of the N-terminal in controlling Top1 activity in different species.

Materials and methods

Chemicals, yeast strains and plasmids

Anti-FLAG M2 monoclonal affinity gel, FLAG peptide and Anti-FLAG M2 monoclonal antibody were purchased from Sigma-Aldrich. Human topoisomerase 1B and hTop1(pf-N-term) were expressed using *Saccharomyces cerevisiae* Top1 null strain EKY3 (*ura3-52, his3Δ200, leu2Δ1, trp1Δ63, top1: TRP1, MATα*) (20). Single copy plasmid YCpGAL-e-hTop1, described previously (29, 30) was used to express the enzyme under galactose inducible promoter. The N-terminal sequence of the epitope-tagged construct YCp-GAL-e-hTop1 contains FLAG: DYKDDDDY indicated as 'e' and is recognized by the M2 monoclonal antibody. To generate hTop1 (pf-N-term) the first step was to obtain only N-terminal (1-153) domain of FLAG-tagged pfTop1 by using Pyes2.1 (topo TA expression kit by Thermo Fisher) pfTop1 as the template and two sets of primers were used: 5'-ATGGACTACAAGGACGAC-3' and 5'-ATCGGTTTGATCATCAATCTT-3'. In the next step, a fragment containing core domain (215-635), linker domain (636-712) and C-terminal domain (713-765) of hTop1 was generated using YCp-GAL-e-hTop1 as template and two sets of primers 5'-AAGATTGATGATCAAACCGATATCAAGTG GAAATTCCTAGAACATAAAGGT-and 5'-GGCTGTCAGTTCTTTTAGCTGCTGCTGTA GCGTGATGGAGGCATTGTATGT-3'. In the final step two primers overlapping to each of the above-mentioned segments were designed and used: 5'-CAGCTAAAAGAACTGACAGCC-3' and 5'-CTAAAACTCATAGTCTTCATCAGCCATGT C-3'. The three separate PCR products were ligated to obtain the chimeric enzyme. The products were obtained by using a Taq DNA polymerase (Sigma-Aldrich) and the specific band was purified from 0.7% agarose gel in 1X TBE buffer (48 mM Tris, 45.5 mM boric acid, 1 mM EDTA) using the gel extraction kit QIAEX II purchased by Qiagen. The hTop1(pf-N-term) construct was cloned into the pYES2.1/V5-His-TOPO expression vector (Invitrogen), according to the provided manufacturer protocol. The cloning reaction was transformed into XL10-Gold *E. coli* cells (Agilent Technologies), and a positive clone was identified by sequencing the extracted plasmid DNA.

Protein purification

In order to purify hTop1 and hTop1(pf-N-term), they were cloned in a single copy plasmid YCpGAL1, under a galactose inducible promoter. By using lithium acetate procedure, hTop1 and hTop1(pf-N-term) were transformed in top1 null EKY3. The cells were then grown overnight on SC-Uracil (synthetic complete medium lacking uracil) with 2% dextrose. In the following step, they were diluted 1:100 in SC-uracil containing 2% raffinose, at OD600 =1.0, the cells were induced with 2% (v/v) galactose for 6 hours. Cells were then harvested and proteins were purified as described in Wang et al (35). Protein level and integrity of the proteins were assessed by performing immunoblot with the monoclonal anti-FLAG M2 antibody (Sigma-Aldrich) The *in-vitro* experiments have been performed using three units of purified hTop1(pf-N-term) and hTop1.

Unit determination: One unit of enzyme is defined as the amount of enzyme that catalyzes the relaxation of 0.5 µg of negatively supercoiled pBlue-script KSII (+) DNA in 15 minutes at 37 °C in a total reaction volume of 30 µl.

DNA relaxation assays

The activity of hTop1 and hTop1(pf-N-term) was evaluated by decreasing mobility of the relaxed isomers of supercoiled pBlue-Script KSII (+) DNA in an agarose gel. The relaxation activity of hTop1 and hTop1(pf-N-term) was assayed in 30 µl of reaction volume containing 0.5 µg of negatively supercoiled pBlue-script KSII (+) DNA present in both dimeric and monomeric form containing reaction buffer (20 mM Tris-HCl pH 7.5, 0.1 mM Na₂ EDTA, 10 mM MgCl₂, 50 µg/ml acetylated bovine serum albumin and 150 mM KCl). The reactions were stopped using 0.5% Sodium dodecyl sulfate (SDS) at each time-course point at 37 °C. The samples were resolved in 1% agarose gel running buffer containing 48 mM Tris, 45.5 mM boric acid, 1 mM EDTA. DNA was visualized by staining of the gel with 0.5 µg/ml ethidium bromide and destained with water. The gel image was photographed using a UV transilluminator.

Cleavage kinetics using CL14/CP25, CL14A/CP25T, CL14/CP25G oligonucleotide substrates

For analyzing cleavage kinetics, oligonucleotide suicide substrates namely CL14 (5'-

GAAAAAAGACTTAG-3'), CL14-A (5'-GAAAAAAGACATAG-3') or CL-14-G (5'-GAAAAAAGACGTAG-3') were used. All the oligonucleotide substrates were radiolabeled with (γ-32P) ATP at their 5'-end. The complementary strand of these oligonucleotides, respectively CP25 (5'-TAAAAATTTTTCTAAGTCTTTTTTC-3'), CP25-T(5'-TAAAAATTTTTCTATGTCTTTTTTC-3') or the CP25-C (5'-TAAAAATTTTTCTACGTCTTTTTTC-3') were phosphorylated with unlabeled ATP at their 5'-end. All the oligonucleotides were annealed as described previously (32). The suicide cleavage reactions were carried out by incubating 20 nM of the substrate with 3 units of hTop1 and hTop1(pf-N-term) in 10 mM Tris pH 7.5, 5 mM MgCl₂, 5 mM CaCl₂ and 150 mM KCl at 25 °C in a final volume of 60 µl. Aliquots containing 5 µl were removed at different time intervals and 0.5% (w/v) SDS was added to stop the reactions. The samples were then subjected to ethanol precipitation. After this step, the samples were digested by adding 5 µl of 1 mg/ml of trypsin and incubated at 37 °C for 60 minutes (min). The samples were evaluated by denaturing polyacrylamide gel electrophoresis (7 M urea/ 20%) in TBE running buffer (48 mM Tris, 45.5 mM Boric Acid, 1 mM EDTA).

Kinetics of Cleavage using CL14-U/CP25 oligonucleotide substrate

Oligonucleotide CL14-U (5'-GAAAAAAGACTUAG-3') was radiolabelled with (γ-32P) ATP at its 5'-end and annealed with CP25 to obtain the CL14-U/CP25 suicide substrate. Three units of hTop1 and hTop1(pf-N-term) were incubated with 20 nM suicide substrate in 20 mM Tris-HCl pH7.5, 0.1 mM Na₂EDTA, 10 mM MgCl₂, 5 µg/mL acetylated Bovine serum albumin, and 150 mM KCl. Five µl of aliquots were removed at different time points and 0.5% (w/v) SDS was used to stop the reactions. The samples were analyzed by denaturing polyacrylamide gel electrophoresis (7 M urea/ 20%) in TBE running buffer (48 mM Tris, 45.5 mM Boric Acid, 1 mM EDTA). In both cleavage experiments (with radiolabelled oligonucleotide CL14 and CL14-U) the percentage of the cleaved substrate (Cl1) was determined by Phosphor Imager and Image Quant

software and normalized on the total amount of radioactivity in each lane.

Kinetics of religation

Analyses of religation kinetics were carried out by using oligonucleotide substrate CL14 (5'-GAAAAAAGACTTAG-3') which was radiolabelled with (γ -³²P) ATP at its 5'-end. The complementary strand of this oligonucleotide namely CP25 (5'-TAAAAATTTTTCTAAGTCTTTTTTC-3') was phosphorylated with unlabeled ATP at its 5'-end. The CL14 strand of the oligonucleotide was annealed with a 2-fold molar excess of CP25 to obtain partially duplex CL14/CP25 suicide substrate. This radiolabeled oligonucleotide (CL14/CP25) was incubated with 3 units of hTop1 and hTop1(pf-N-term) for 60 min at 25 °C and then for additional 30 min at 37 °C in 20 mM Tris HCl pH 7.5, 0.1 mM Na₂EDTA, 10 mM MgCl₂, 50 µg/ml acetylated BSA and 150 mM KCl. After the formation of the cleavage complex (Cl1), five µl aliquot was removed representing time 0. In the next step, 200-fold molar excess of R11 oligonucleotide (5'-AGAAAAATTTT-3') over duplex CL14/CP25 was added to initiate religation kinetics. Aliquots containing five µl were removed at different time points and the reaction was stopped by addition of 0.5% SDS. The samples were then subjected to

ethanol precipitation. After this the samples were digested by adding five µl of one mg/ml of trypsin and incubated at 37 °C for 60 min. Since trypsin doesn't digest hTop1B completely therefore, there is a trypsin resistant peptide that remains attached to the substrate causing the 12nt (CL1) oligo to run slower than the uncleaved band in the gel. The samples were then analyzed by using 7 M urea/ 20% denaturing polyacrylamide gel electrophoresis running buffer containing 48 mM Tris, 45.5 mM Boric Acid, 1 mM EDTA.

Results

Relaxation assay

The relaxation activity of wild type and hTop1(pf-N-term) enzymes has been assessed by incubating three units of each enzyme with 0.5 µg of a negative supercoiled plasmid DNA at 37 °C. The reactions were carried out in a time course experiment starting from 0.5 to 60 min, the products were resolved by agarose gel electrophoresis (Fig. 1). The supercoiled DNA is completely relaxed by the hTop1 enzyme after 0.5 min (Fig. 1, lane 1) while the hTop1(pf-N-term) relaxed the DNA after four min (Fig. 1, lanes 12 and 16). This result indicates that the hTop1(pf-N-term) enzyme has slower relaxation kinetics when compared to the wild type protein.

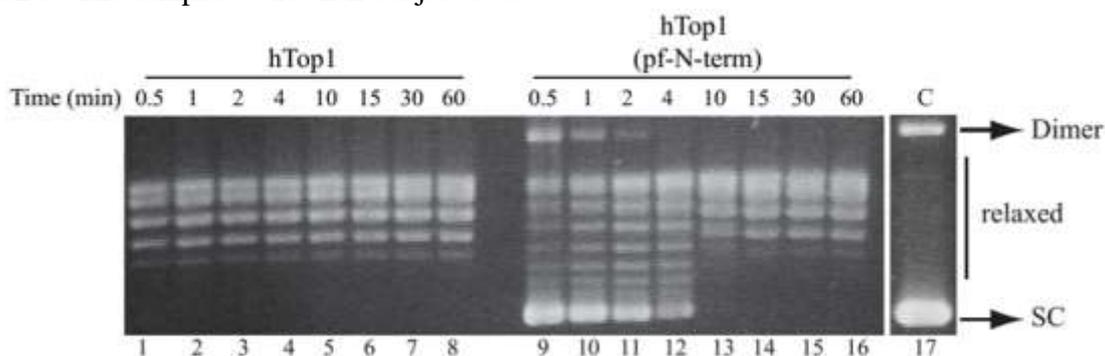


Fig. 1. Relaxation of the negatively supercoiled plasmid in a time course experiment in presence of hTop1 (lane 1-8) and hTop1(pf-N-term) (lane 9-16) protein is not added in lane 17. The reaction products were resolved in an agarose gel and visualize after staining in ethidium bromide. The two forms of plasmid DNA are indicated as Dimer and supercoiled (SC).

Cleavage with CL14/CP25 suicide substrate

The cleavage activity of the hTop1 and hTop1(pf-N-term) was analyzed by comparing the two enzymes in a time course experiment using a CL14/CP25 suicide cleavage substrate. In detail, a 5'-end radiolabelled oligonucleotide CL14 (5'-GAAAAAAGACTTAG-3') has been annealed to the CP25 (5'-TAAAAATTTTTCTAAGTCTTTTTTC-3')

complementary strand to produce a duplex with an 11 base 5' single-strand overhang (Fig. 2). In this experiment the religation step is precluded because the AG-3' dinucleotide is too short to be religated, leaving the enzyme covalently attached to the 12 oligonucleotide 3'-end. Three units of wild type and hTop1(pf-N-term) enzymes were incubated with 20

Enzyme Activity Damage of hTop1 by N-Terminal Swap

nM suicide substrate in a time course experiment. The reactions were stopped at increasing time intervals starting from 0.5 min to 30 min. The samples were precipitated by using 100% ethanol and then digested by trypsin, after this step, the products were resolved in a denaturing urea polyacrylamide gel (Fig. 2A). As evident from Fig. 2A the cleavage rate is almost absent for hTop1(pf-N-term) (Fig. 2A, lanes 10-17) when

compared to the wild type protein (lanes 2-9). Percentage of the cleaved fragment (CL1), normalized to the total radioactivity amount in each lane has been plotted against time for the hTop1(pf-N-term) (Fig. 2B, triangle) and the hTop1 enzyme (Fig. 2B square) confirming the lower cleavage kinetics of the chimeric protein.

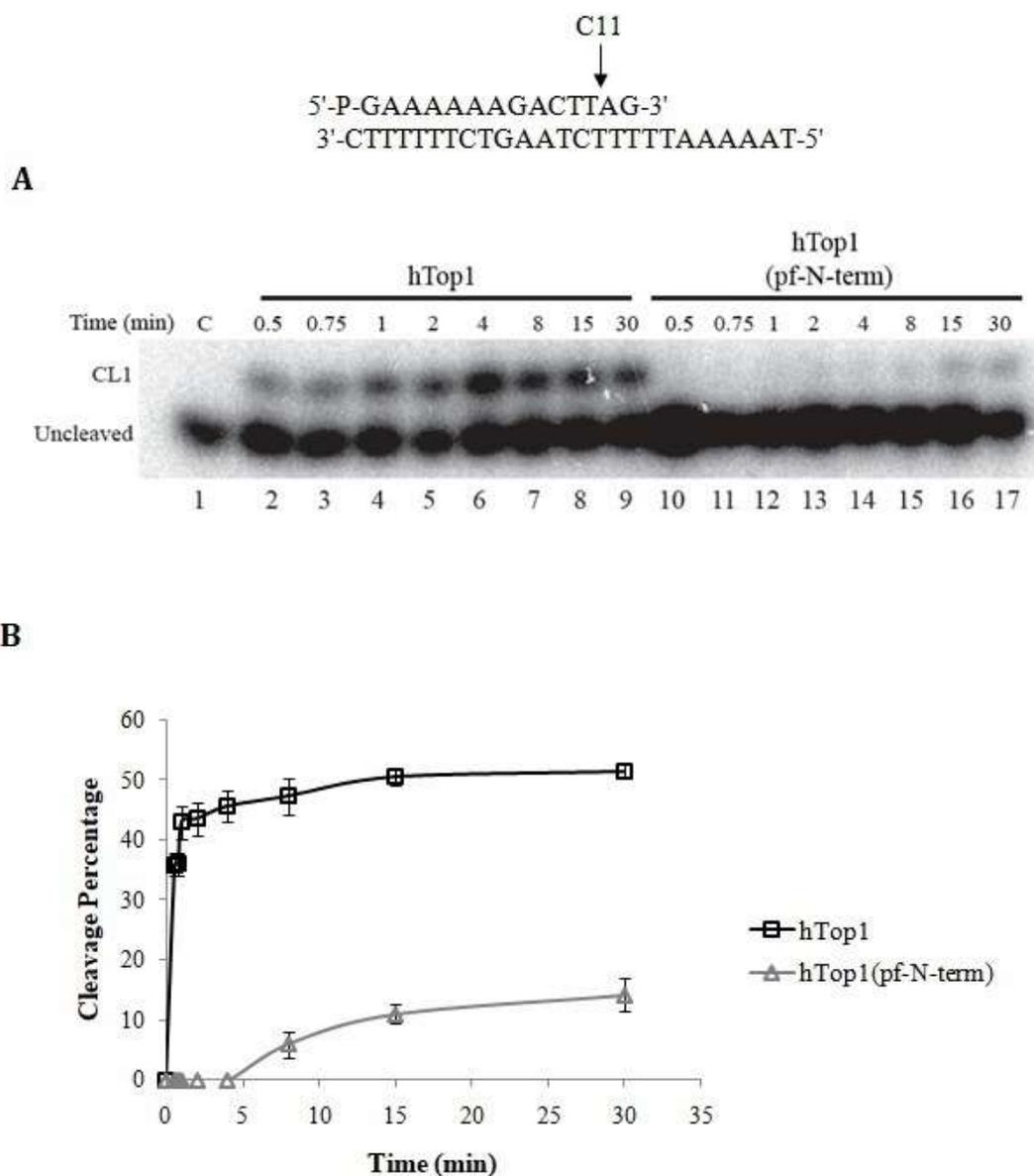


Fig. 2. Cleavage kinetics using suicide substrate. (A) Time course (0.5-30 min) of the cleavage reaction of the purified hTop1 (lanes 2-9) and hTop1(pf-N-term) (lane 10-17) with the CL14/CP25 substrate as shown at the top of the Figure, lane 1 no protein added. The arrow indicates the preferred cleavage site (C11) for hTop1 enzyme. (B) Percentage of cleaved suicide substrate plotted against time for hTop1 (square) and hTop1(pf-N-term) (triangle). Three independent experiments were performed to obtain average value and the standard deviation for error bar.

Cleavage with CL14-U/CP25

In order to better evaluate the cleavage rate, a different cleavage substrate. CL14-U (5'-GAAAAAAGACTUAG-3') oligonucleotide has been used, containing uracil at the preferred hTop1 cleavage site. This oligonucleotide, having the deoxyribose-thymine (dT) in position 12 substituted by a ribo-Uracil (rU), has been annealed to the CP25 (5'-TAAAAATTTTTCTAAGTCTTTTTTC-3') complementary strand, to produce a duplex with an 11-base 5'- single-strand extension. With this oligonucleotide the enzyme does not remain trapped over the substrate as, after cutting at the preferential site, the 2'-OH of the ribose attacks the 3'-

phosphotyrosyl linkage between the enzyme and the ribonucleotide, releasing the enzyme and leaving a 2', 3'-cyclic phosphate end (Fig. 3A). Three units of each enzyme were incubated with 20 nM of radiolabelled ribo modified CL14-U/CP-25 substrate and reactions were stopped at different time intervals from 0.5 min to 30 min. As evident from Fig. 3B hTop1(pf-N-term) enzyme shows the same cleavage rate as hTop1 (Fig. 3B, compare lane 10-17 with lane 2-9). The cleavage percentage has been evaluated by plotting the cleaved products, normalized to the total amount of radioactivity in each lane as a function of time (Fig. 3C).

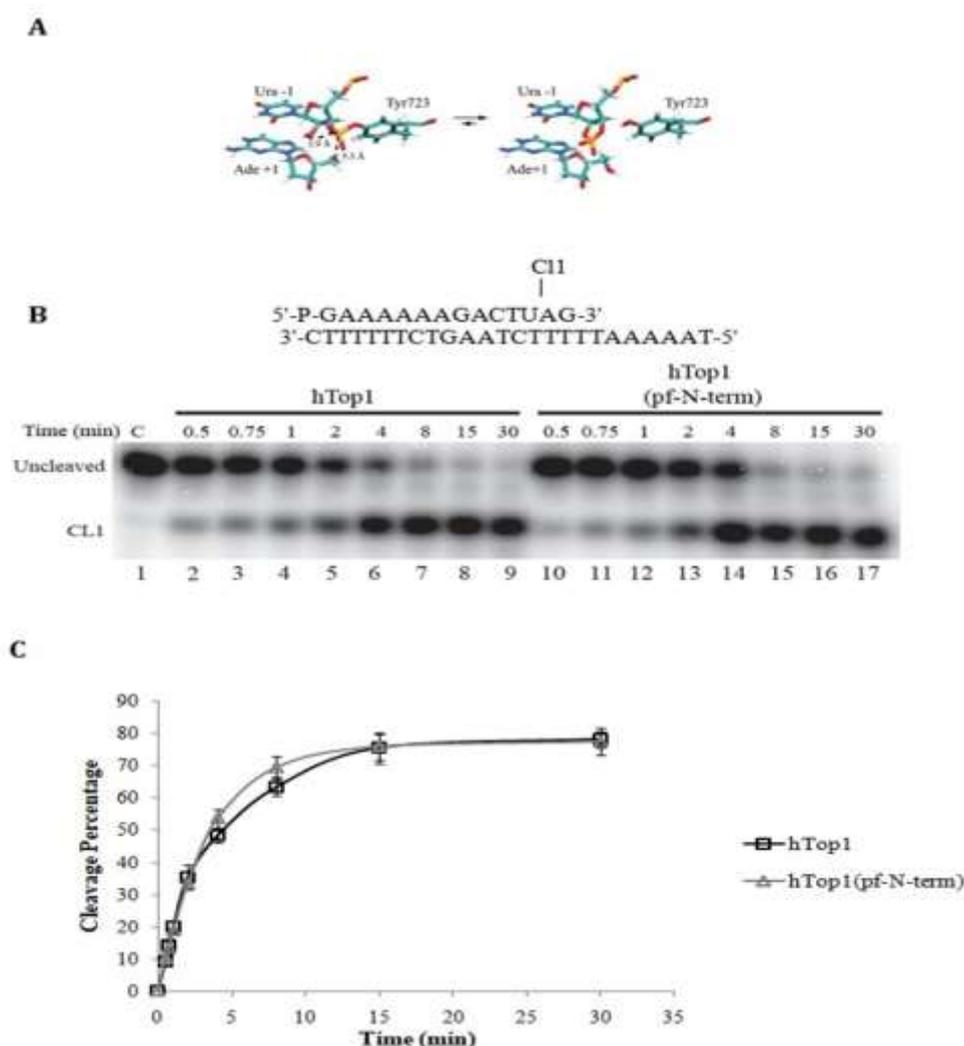


Fig. 3. (A) Sequence of the CL14-U/CP25 substrate and hTop1 cleavage site containing a scissile ribonucleoside monophosphate is highlighted. (B) Time course (0.5-30 min) experiment of the cleavage response of purified hTop1 (lanes 2-9) and hTop1(pf-N-term) (lanes 10-17) with the CL14-U/CP25 substrate (shown in Figure A). No protein is added in lane 1. CL1 represents the DNA substrate cleaved by the enzymes at the preferred cleavage site. (C) Percentage of cleaved product plotted against time for the reaction with hTop1 (square) and hTop1(pf-N-term) (triangle). Data shown are means \pm SD from three independent experiments.

Religation Kinetics

Assessment of the religation step was carried out by incubating three units of each enzyme with 20 nM suicide substrate CL14/CP25 to obtain cleavage complex. A 200-fold molar excess of complementary R11 oligonucleotide (5'-AGAAAAATTTT-3') was then added to the cleavage complex to initiate religation. Aliquots removed at different time intervals from were

0.25 min to two min and reactions were stopped by adding 0.5% SDS. The reaction products were analyzed by denaturing PAGE (Fig. 4). The result indicates that the wild type efficiently religates the substrate (Fig. 4, lanes 2-5), whereas in the case of the hTop1(pf-N-term) the amount of the religated products over the time are very low (Fig. 4 lane 7-10).

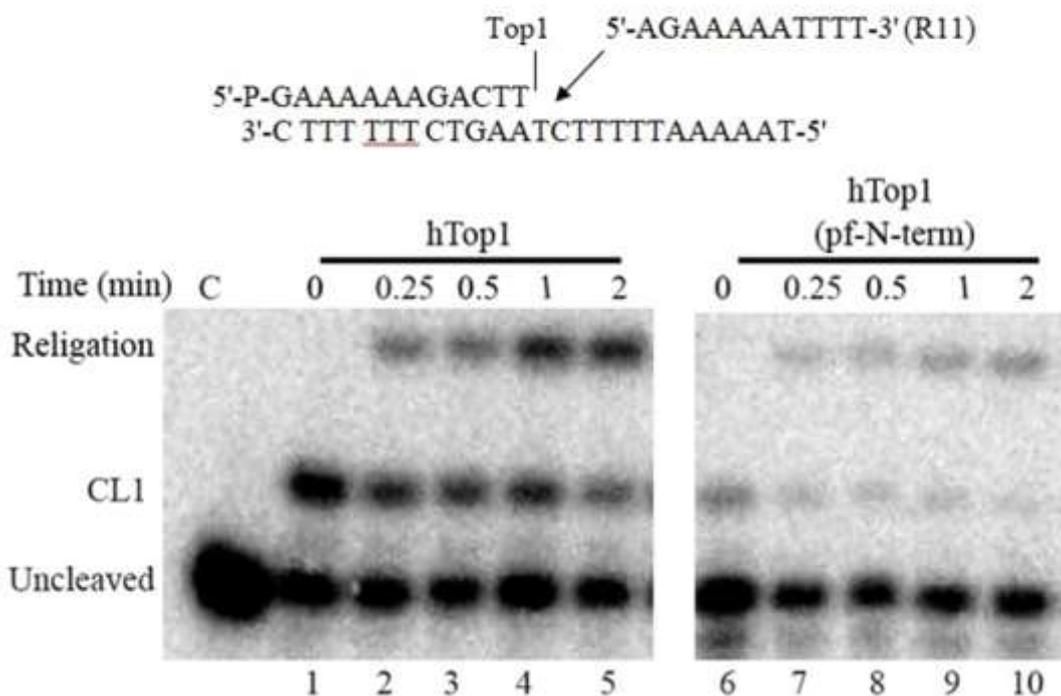


Fig. 4. Religation kinetics observed by incubating the hTop1 and hTop1(pf-N-term) suicide cleavage complex (CLI) with the R11 complementary oligonucleotide (shown at the top of the figure) in presence of hTop1(lanes 2-5) and hTop1(pf-N-term) (lane 7-10); The lanes 1 and 6 represent the time 0 for hTop1 and hTop1(pf-N-term) a mediated reaction, respectively. “CLI” represents the DNA segment cleaved at the preferred enzyme site; “Religation” depicts restored fully duplex oligonucleotide representing the ultimate product of the religation kinetics.

Discussion

In this study, we have characterized the hTop1(pf-N-term) chimeric enzyme constructed by swapping N-terminal of pfTop1 into its human counterpart. Replacement of N-terminal of pfTop1 into hTop1 dramatically alters the enzymes function *in vitro*. hTop1(pf-N-term) displays slower relaxation activity when compared to wild type protein (Fig. 1) indicating that some steps of the catalytic cycle are perturbed by the insertion of the pf-N-terminal domain. In detail the hTop1(pf-N-term) has a slower cleavage rate compare to the wild type protein (Fig. 2). Since the sequence used in the cleavage assay is a sequence recognized especially by hTop1, two other oligonucleotides

(namely CL14A and CL14G) have been tested in the same experiment obtaining the similar results (data not shown). Analyzing the cleavage rate by using CL14 suicide substrate can be misleading because the protein, that remains attached to the DNA, can in part allow the religation of the dinucleotide. Based on this consideration we have decided to uncoupled cleavage and religation performing a cleavage kinetic using CL14-U substrate that does not allow the religation of the cut dinucleotide. Surprisingly this experiment has shown that the hTop1(pf-N-term) cleaves at the same rate as hTop1 and reach the same plateau value suggesting a high religation rate of the chimeric

enzyme (Fig. 3). To confirm this hypothesis, religation efficiency of the chimeric enzymes has been analyzed by a time course experiment with the CL14/CP25 substrate (Fig. 4). As shown in Figure 4 the religation analysis indicates that the presence of N-terminal of pfTop1 strongly impairs the religation step. Every eukaryotic form of DNA topoisomerase 1 contain highly charged and substantial N-terminal domain (15). Since there is no crystallographic structure of this domain, various studies have been performed in the past years based on different deletion of amino acid residues in the N-terminal domain (24, 25, 28). Previous findings indicate that the N-terminal 191-214 residues are vital for DNA binding and enzyme processivity thereby playing a significant function in controlling the motion within the hinge region that is involved in control rotation (14, 26). Tryptophan-205 and perhaps other residues situated between position 191-206 coordinates the restriction of free strand rotation during the topoisomerization stage of catalysis. Moreover, tryptophan-205 appears to be essential for the function of the large part of the N-terminal domain in direct DNA interaction. The results indicated that the tryptophan anchor stabilized the N-terminus of the functional domain and prevented the loss of Top1 structure and function. Our study shows that complete removal of the N-terminal domain of hTop1 and substituting it with pfTop1 counterpart makes the chimeric enzyme unable to recognize CL14/CP25 substrate that contains the preferential binding site of hTop1. Interestingly, a previous study (33) shows that hTop1 lacking 1-175 amino acids from the N-terminal domain and containing only 176-215 amino acid residues was able to recognize and cleave CL14/CP25 substrate indicating the importance of N-terminal domain especially from residues 176-215 in recognizing the substrate. Interestingly experiment performed in order to uncouple cleavage and religation using radiolabelled oligonucleotide CL14-U/CP25 containing uracil at position 12 instead of thymine revealed a surprising result, a chimeric enzyme lacking hTop1 N-terminal and containing N-terminal domain of pfTop1 was able to recognize, bind and cleave DNA substrate containing uracil. It is important to note that the level of uracil in the *Plasmodium falciparum* genome especially in the ring, trophozoite, and schizont intraerythrocytic stages is around 7–10 uracil per million bases, which is significantly higher

compared to other organisms ranging from bacteria to mammals having low levels of DNA in uracil ranging from 0.1–1 uracil per million bases, or even lower (34). The promoter for pfTop1 becomes active in the late trophozoite stage and schizont stage (16), the higher level of uracil DNA in the trophozoite and schizont stages can be attributed to the recognition of substrate containing uracil by chimeric enzyme containing N-terminal domain of pfTop1. The approach of replacing domain between organisms can be useful to study the dynamics of domains in the catalytic activity of the enzyme. Interestingly in a previous study, we showed that chimeric enzyme, generated by substituting the hTop1 linker domain with the equivalent domain of pfTop1 displayed a comparable relaxation but a faster religation rate (20). The linker substituted chimeric enzyme also displayed altered drug reactivity compared to wild type *in vitro* as it was found to be less sensitive to CPT than hTop1 (20). We also studied the consequence of inserting N-terminal domain in the above-mentioned chimeric enzyme generating chimera namely hTop1(pf-N-term/linker) containing N-terminal and linker of pfTop1, and core and C-terminal domain from hTop1. The enzyme was found to be slow in all the enzyme kinetics compared to hTop1 suggesting that insertion of two domains strongly perturbs the enzyme activity (data not shown). Wright et al suggested that improper coordination of N-terminal and linker domain may lead to abnormal Top1-protein interactions thereby impairing cell growth. They demonstrated this by showing the functional interaction of N-terminal and linker domain by swapping domains between human and yeast (31). The present study can be helpful in developing species selective drug targeting topoisomerase 1B by understanding the structural dynamics of different domains of the enzyme.

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