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Exploration of the Role of the C-Terminal Domain of Human DNA Topoisomerase IIα in Catalytic Activity

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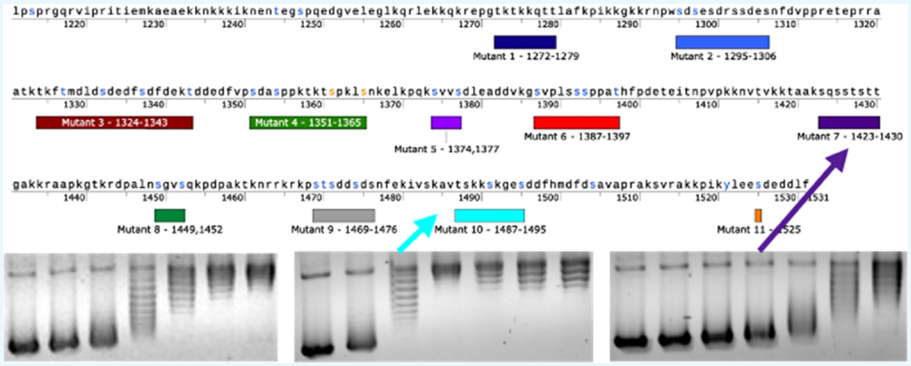

ABSTRACT: Human topoisomerase IIα (TOP2A) is a vital nuclear enzyme involved in resolving knots and tangles in DNA during replication and cell division. TOP2A is a homodimer with a symmetrical, multidomain structure. While the N-terminal and core regions of the protein are well-studied, the C-terminal domain is poorly understood but is involved in enzyme regulation and is predicted to be intrinsically disordered. In addition, it appears to be a major region of post-translational modification and includes several Ser and Thr residues, many of which have not been studied for biochemical effects. Therefore, we generated a series of human TOP2A mutants where we changed specific Ser and Thr residues in the C-terminal domain to Ala, Gly, or Ile residues. We designed, purified, and examined 11 mutant TOP2A enzymes. The amino acid changes were made between positions 1272 and 1525 with 1−7 residues changed per mutant. Several mutants displayed increased levels of DNA cleavage without displaying any change in plasmid DNA relaxation or DNA binding. For example, mutations in the regions 1272−1279, 1324−1343, 1351−1365, and 1374−1377 produced 2−3 times more DNA cleavage in the presence of etoposide than wild-type TOP2A. Further, several mutants displayed changes in relaxation and/or decatenation activity. Together, these results support previous findings that the C-terminal domain of TOP2A influences catalytic activity and interacts with the substrate DNA. Furthermore, we hypothesize that it may be possible to regulate the enzyme by targeting positions in the C-terminal domain. Because the C-terminal domain differs between the two human TOP2 isoforms, this strategy may provide a means for selectively targeting TOP2A for therapeutic inhibition. Additional studies are warranted to explore these results in more detail.

INTRODUCTION

The regulation of DNA topology is a critical element of cellular survival. The proper maintenance of DNA in a cell ensures that fundamental processes like transcription, replication, and cell division are able to take place without being impeded by DNA topology.[1,2] Cells employ two families of enzymes to carry out this maintenance: type I and type II topoisomerases. Topoisomerases break one (type I) or two (type II) strands of the double helix to alleviate topological strain (i.e., relaxation) or to detangle chromosomes.[2,3] In the case of type II topoisomerases, the mechanism involves the passage of an intact DNA strand through a temporary double-stranded break in another segment of DNA.

Due to the critical role that these enzymes play in transcription, replication, and mitosis, anticancer agents have been developed against type I (e.g., irinotecan) and type II (e.g., etoposide and doxorubicin) topoisomerases.[4] These agents have activity in a broad range of cancers including both solid and hematological malignancies.[5,6] While these are called topoisomerase inhibitors by the medical profession, they act by

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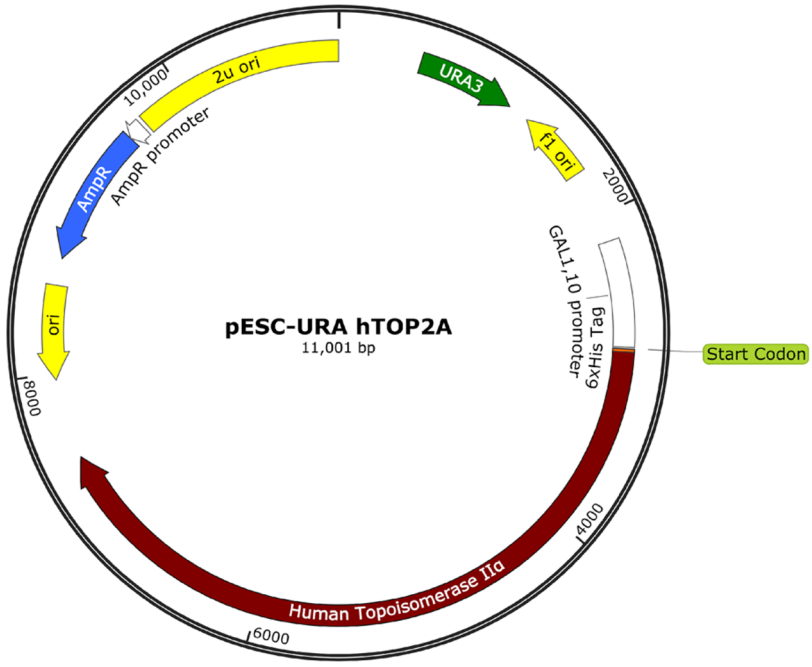

Figure 1. Construct map for the pESC-URA hTOP2A plasmid. The map displays key features of the construct generated by GenScript. The map produced using SnapGene.

blocking the ability of topoisomerases to reseal the temporary strand breaks generated during the process of the catalytic cycle.[7,8] This mechanism is referred to as "poisoning" and these agents are known within the topoisomerase field as topoisomerase poisons to distinguish them from a separate mechanism known as catalytic inhibition.[7,8] This blocking of ligation by topoisomerase poisons leads to single- and double-stranded breaks, which are intended to overwhelm cancer cells and cause cell death.

Some cancer treatments that target the type II enzymes, human topoisomerase IIα (TOP2A) and IIβ (TOP2B), are associated with life-threatening adverse events such as cardiotoxicity and secondary leukemia.[9,10] Of the two isoforms of topoisomerase II, TOP2A appears to be associated with replication and cell division, while TOP2B is associated with transcription and chromatin topology.[1,3] There is also evidence that TOP2B may be involved in some of the off-target toxicities mentioned above.[11−14] Thus, from a therapeutic perspective, TOP2A would seem to be a better target for anticancer agents than TOP2B. Finding ways to differentiate between the isoforms is of importance to the field and may open doors to the development of selective inhibitors, which so far are very rare.[4,15] NK314 represents the first-known agent to show a preference for TOP2A over TOP2B. This compound induces topoisomerase II-mediated double-strand breaks but the exact mechanism of action and TOP2A selectivity has not been worked out.[15] NK314 does appear to hit other targets such as DNA-PK.[16] More recently, a novel class of 6-amino-tetrahydroquinazoline compounds was found to serve as catalytic inhibitors (rather than poisons) of topoisomerase II and displayed a 100-fold selectivity against TOP2A over TOP2B.[17] While NK314 is being tested with clinically approved agents, neither of these approaches has reached the clinic.[18]

TOP2A and TOP2B are homodimeric enzymes with multiple folded protein domains.[1,19] While much of the enzyme is very similar between the two human isoforms, the C-terminal domain (CTD) amino acid sequence diverges significantly.[3,19] Published studies indicated that the C-terminal domain influences localization and catalytic activity.[19−24] Very little structural information is available regarding the CTD of these enzymes. This region is considered a "low complexity sequence" and may be an intrinsically disordered region (IDR).[22,25,26] IDRs have increasingly been recognized as critical components of protein structure allowing flexibility for recognition motifs, post-translational modification (PTM) sites, and coupled binding and folding.[27,28] While the TOP2A CTD has not been examined as an IDR directly, this region is home to dozens of post-translational modifications (PTMs), some of which have known effects on the cellular or biochemical functions of the enzyme.[19,29] However, many previous studies of these residues focused on single amino acid changes and several other positions have not been studied for their biochemical effects on enzyme catalysis.[19,29]

The published results indicate that phosphorylation of Ser/Thr residues in the CTD plays a significant role in regulating TOP2A.[19,30−39] Based upon the studies of PTMs of TOP2A and the intrinsically disordered nature of the CTD, it is reasonable to infer that at least some of the Ser and Thr residues in the CTD are involved in interactions with DNA and/or proteins.[19] However, single-residue studies could have missed important functions that involved multiple residues, as may be the case if this region is an IDR.[27,28,40] Further, if this region is an IDR, we predict that there may be multiple subdomains within the larger region that could play roles in protein−protein and DNA−protein interactions. Therefore, we set out to examine the impact of specific changes to groups of Ser and Thr residues in the CTD of TOP2A to determine the effect(s) these positions have on TOP2A catalytic function. We hypothesize that any regions that disrupt or alter the function of TOP2A in DNA binding and/or enzyme catalysis may represent potential drug-targeting sites that could be exploited by novel anticancer therapeutics.

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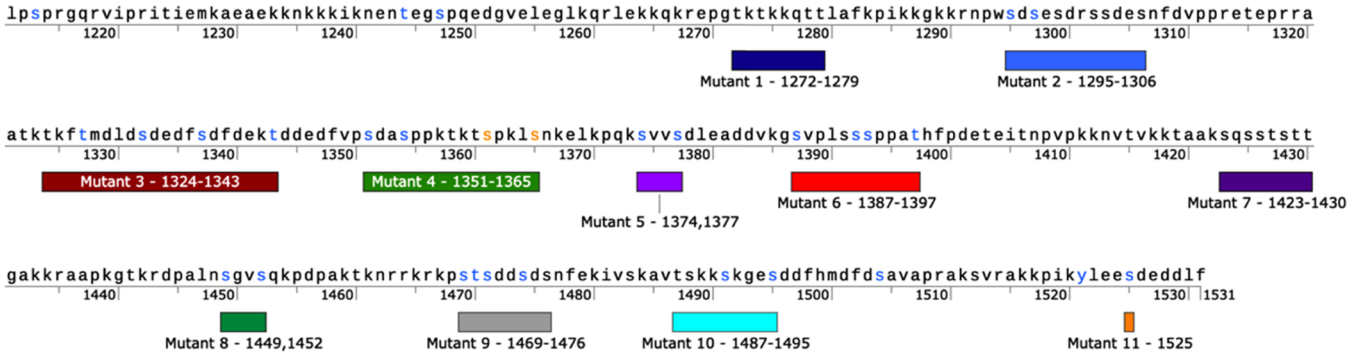

Figure 2. Location of mutants in the CTD of human TOP2A. Residues 1210−1531 are shown. Bars represent the region modified for each mutant. See Table 1 for specific amino acid changes made in each mutant. Positions shown in blue represent amino acids that are modified in association with mitosis according to the Phosphosite database (phosphosite.org). Positions in orange represent sites known to be involved in the stability of the protein.

For most of the mutant enzymes, we selected focused regions with multiple Ser/Thr residues in close proximity and mutated these regions separately. These mutated enzymes were purified and examined for biochemical activity including DNA relaxation, cleavage, binding, and decatenation. Our studies indicate that mutations of these regions did impact overall biochemical activity, but the effects were specific to each mutant. Several mutants altered DNA relaxation and/or decatenation without altering DNA binding or cleavage by TOP2A.

RESULTS

Development and Testing of an N-Terminally His-Tagged TOP2A Construct. Previous work from our lab involved using a C-terminally His-tagged TOP2A construct.[41] To avoid any negative impact of the His-tag on the CTD, we set out to design an N-terminally His-tagged TOP2A construct. We utilized GenScript to clone the TOP2A cDNA sequence (NM_001067.3) into the pESC-URA expression vector with a 6-His-tag in frame with the N-terminus of the protein (Figure 1). The protein from this expression construct was expressed and purified. It should be noted that a recent study indicates that key PTM sites in human TOP2A are modified in a *Saccharomyces cerevisiae* expression system.[42]

Selection of Mutation Sites Across the CTD. We selected regions of the CTD of TOP2A with 2−7 Ser and/or Thr residues and mutated these positions to Ala, Gly, or Ile (Figure 2). In one case, we mutated a single Ser residue (Mutant 11), which was already known to have a regulatory role during mitosis. As mentioned above, there is evidence that TOP2A is phosphorylated at native PTM sites even in the yeast expression system. The intention was to prevent these positions from being phosphorylated while also allowing for conformational flexibility by replacing mutated positions with nonbulky amino acids. Specific changes were selected based upon codon degeneracy to minimize the number of nucleotide changes within a given region. We generated 11 mutants covering a range of differently sized modifications from as few as one amino acid change up to seven amino acids (Figure 2). These included many residues (blue font) that have been shown in cellular studies to be important in mitosis.[26,30,31,33−39,43−47] The specific residue changes are outlined in Table 1.

Mutations Have a Variable Effect on the Plasmid Relaxation Activity. The N-terminally His-tagged TOP2A retains overall catalytic activity in DNA relaxation, indicating that the enzyme generated from the pESC-URA hTOP2A construct is functional and the N-terminal His-tag does not appear to interfere with the catalytic cycle since the enzyme is able to complete relaxation efficiently (Figure 3) and has comparable plasmid DNA cleavage and relaxation activity when compared to the C-terminally His-tagged TOP2A (Figure S1). Several of the mutant enzymes appear to relax supercoiled DNA as well as or slightly faster than the WT TOP2A (Table 1). In a few cases (to a lesser extent with Mutants 2, 3, and 7 and a greater extent with Mutants 4 and 9), there is a noticeable decrease in relaxation activity. Mutants 4 (1351−1365) and 9 (1469−1476) showed the least activity and failed to fully relax the plasmid DNA by 30 min (Figure 3).

Processive DNA relaxation by topoisomerase II is characterized by the enzyme binding to DNA and removing most or all of the supercoils from a substrate before releasing (see WT gel in Figure 3).[48] Based upon the data, Mutants 4 and 9 appear to have shifted toward a more distributive mode of relaxation. In other words, the enzyme appears to be dissociating and not relaxing substrates to completion. While

Table 1. TOP2A C-Terminal Domain Mutations and Relaxation Activity

enzyme	positions mutated	plasmid DNA relaxation[a]
WT	NA	∼10 min
mutant 1	T1272A, T1274A, T1278A, T1279A	∼5 min
mutant 2	S1295A, S1297A, S1302G, S1303G, S1306G	∼15 min
mutant 3	T1324A, T1327A, S1332A, S1337A, T1343A	∼15 min
mutant 4	S1351A, S1354G, T1358A, S1361A, S1365G	>30 min
mutant 5	S1374G, S1377A	∼5 min
mutant 6	S1387I, S1391G, S1392A, S1393A, T1397G	∼5 min
mutant 7	S1423G, S1425A, S1426G, T1427I, S1428G, T1429I, T1430A	∼15 min
mutant 8	S1449A, S1452A	∼10 min
mutant 9	S1469A, T1470I, S1471A, S1474A, S1476A	>30 min
mutant 10	T1487A, S1488I, S1491A, S1495I	∼2.5 min
mutant 11	S1525A	∼5 min

[a]Time represents the approximate time at which all supercoiled DNA are relaxed.

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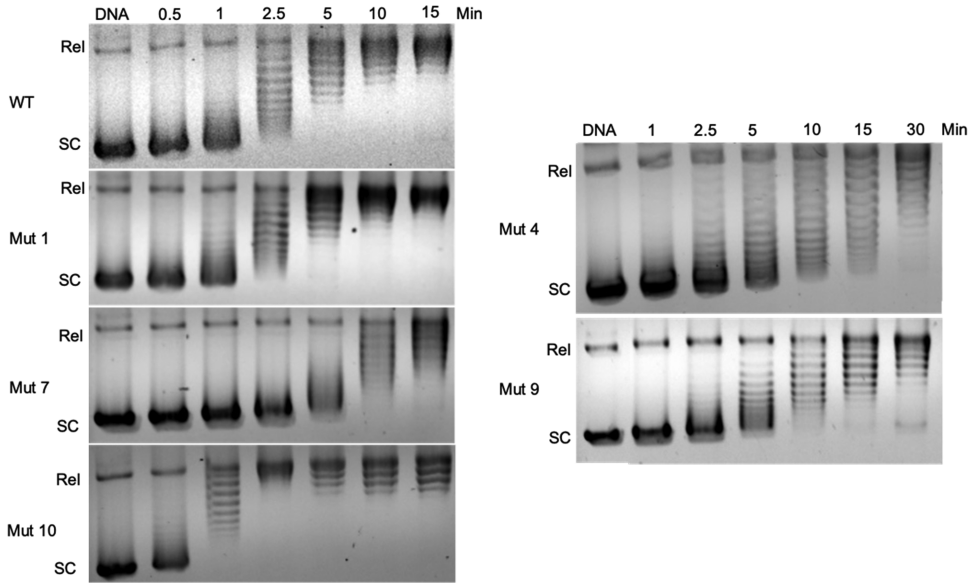

Figure 3. Relaxation of plasmid DNA by human WT TOP2A and mutant TOP2A from the pESC-URA hTOP2A construct. Gel images show the migration of supercoiled plasmid (SC) in the absence of topoisomerase II (DNA) as well as in the presence of TOP2A at increasing time points (0.5−15 min on the left; 1−30 min on the right). The relaxed plasmid (Rel) migrates more slowly than SC plasmid. Wild type (WT) is shown at the top left followed by mutants (Mut) 1, 7, and 10. Mutants 4 and 9 are shown at the right on a 30 min time scale. Gel images are representative of four or more experiments.

these results give a broad picture of activity, additional assays were needed to more fully characterize the effects in question.

Several TOP2A CTD Mutants Display Increased Plasmid DNA Cleavage. To address the ability of TOP2A CTD mutants to cleave DNA, we performed plasmid DNA cleavage assays. These assays enable the monitoring of double-strand breaks (DSBs) and single-strand breaks (SSBs) in a plasmid DNA substrate. The DSB DNA cleavage results are shown in Figure 4 (upper panel).

As mentioned above, WT TOP2A displays cleavage activity that is similar to both an untagged and a C-terminally His-tagged construct previously used in our published work (see Figure S1 for comparison with C-terminally His-tagged TOP2A).[49,50] All of the mutants retain or display slightly enhanced DNA cleavage activity. Only Mutant 11 displayed a statistically significant increase in DNA cleavage (Figure 4, upper panel).

As with all type II topoisomerases, TOP2A has two active sites, which enable the enzyme to generate DSB during the catalytic cycle. Since both DSB and SSB can be monitored in this DNA cleavage assay, we calculated the ratio of DSB/SSB to determine whether there is a change in the level of coordination between the active sites (Figure 4, lower panel). As seen in Figure 4, WT TOP2A has a ratio of less than 0.5, suggesting that the enzyme is more likely to form a single-strand break than a double-strand break in the absence of perturbing factors, which may serve to protect the genome from unnecessary and potentially dangerous DSBs.[51,52] An increase coordination would result in a higher ratio of DSB/SSB when compared to WT. Again, while some of the mutants displayed an increase in the DSB/SSB ratio, these changes were not statistically significant. These results imply that for this set of the enzymes there are no significant changes in the coordination of DNA cleavage between the two active sites.

To test whether these enzymes could still be impacted by a known topoisomerase II-targeting drug, we examined DNA cleavage activity in the presence of the anticancer agent etoposide. As seen in Figure 5, etoposide enhanced the DNA cleavage of all of the mutants when compared to the absence of the drug. There does appear to be some enhancement with several mutants that goes beyond what is seen with WT TOP2A. For example, Mutants 1, 3, and 4 all display statistically significantly enhanced cleavage activity in terms of percent DNA cleaved when compared to WT TOP2A. This may indicate that these regions serve a role in interacting with the DNA and perhaps regulate binding and/or catalytic activity. Mutant 11 at the 200 μM etoposide also displays a statistically significant increase though to a lesser extent than the mutants mentioned above.

To determine whether the decreased relaxation activity of mutants 4 and 7 was due to the enzyme losing catalytic activity, we performed an inactivation assay. During the inactivation assay, the enzymes were incubated at 37 °C without the plasmid DNA for 0−15 min and then mixed with the DNA and 100 mM etoposide before being incubated for an additional 6 min. While there is a trend toward decreasing DNA cleavage activity, the decrease is not statistically significant even after 15 min of incubation (Figure S2). These results indicate that the enzymes are still active even after a period of incubation.

CTD Mutations do not Significantly Affect Plasmid DNA Binding Activity. To clarify whether changes in DNA cleavage and relaxation were due to alterations in DNA binding, we performed plasmid DNA binding experiments using Electrophoretic Mobility Shift Assays (EMSAs). In the presence of TOP2A, the plasmid DNA will migrate more slowly due to the binding to the enzyme. The shift toward the top of the gel is proportional to the concentration of TOP2A added in the experiment.[53,54] This experiment is run in the absence of Mg^{2+}, which is required for cleavage and ligation. Therefore, any gel shift represents noncovalent interactions.

Gels were quantified by measuring the level of DNA found migrating at the same level as the unbound supercoiled (SC) DNA. Most of the enzymes bind as well as or slightly better

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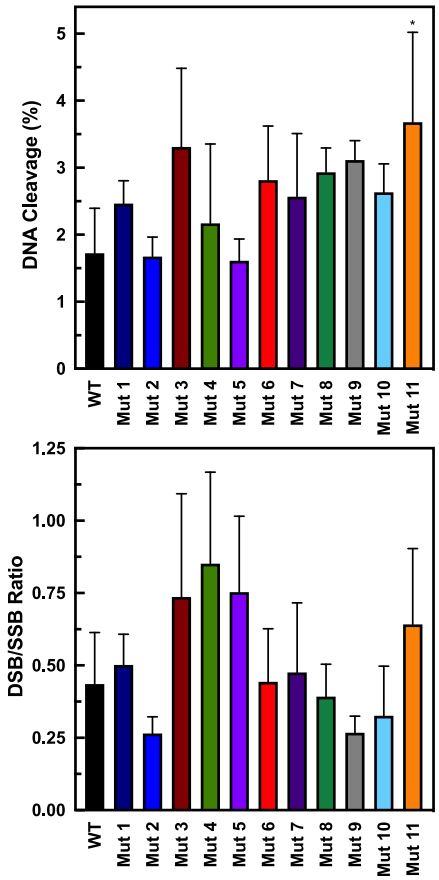

Figure 4. Impact of mutations in the CTD on DNA cleavage and coordination of cleavage. Upper panel: Percent DNA cleavage is shown for reactions with WT or mutant hTOP2A and the plasmid DNA. Lower panel: DSB/SSB ratios were generated by taking the ratio of DNA in the linear/DSB band and dividing by the DNA in the nicked/SSB band. Error bars represent the standard deviation of the mean for three or more experiments. Asterisks (*) represent statistical significance: *p = 0.0127 when compared to WT.

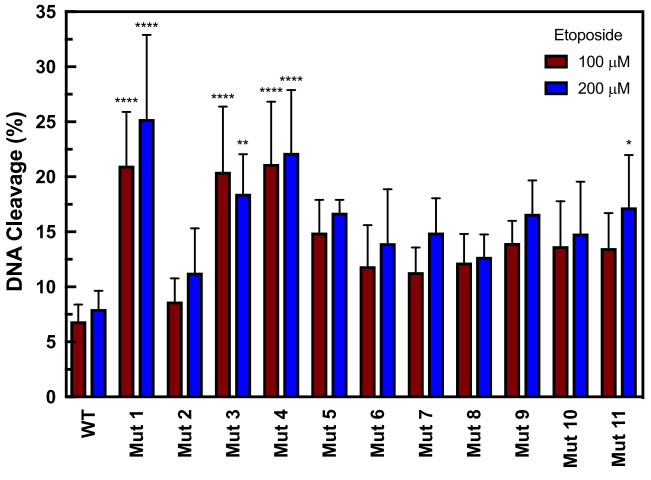

Figure 5. Enhancement of TOP2-mediated DNA cleavage in the presence of the etoposide. Percent of DS-cleaved DNA in the presence of WT or mutant TOP2 with the100 μM (dark red) or 200 μM (blue) etoposide. Error bars represent the standard deviation of the mean for three or more experiments. Statistical significance was determined by analysis of variance (ANOVA) followed by multiple comparisons of means (*p = 0.0186; **p = 0.0061; ****p < 0.0001).

than WT TOP2A (Figure 6). Therefore, their DNA binding does not account for the changes in activity.

Some Mutants Display Altered Decatenation Activity. Since binding does not appear to account for the decreased relaxation activity seen with Mutants 4 and 9, we decided to measure the ability of these enzymes to decatenate DNA. While relaxation is one measure of catalytic activity, the ability to unlink catenated DNA circles is another important measure as it relates to the decatenation function of TOP2A. Further, this function does not require the enzyme to be processive. Therefore, we performed decatenation assays using catenated kinetoplast DNA (kDNA) minicircles. The results for the selected mutants are shown in Figure 7. Mutant 1 displays a slightly increased rate of decatenation compared with WT. Mutant 9 appears to decatenate fairly efficiently, in contrast to the delayed relaxation timeline seen in Figure 3. Mutant 9 appears to be slightly faster than WT at the 1 min time point. Whereas Mutants 4 and 7 appear to be somewhat delayed in decatenation activity compared to WT. Due to the slightly decreased relaxation seen with Mutants 2 and 3, we also examined these enzymes for decatenation activity and found that they are also delayed in terms of decatenation. Mutants 10 and 11, which had faster relaxation, also displayed faster decatenation activity (Figure S3).

DISCUSSION

Alignment of TOP2A and TOP2B Offers Insight Into Potential Targets for Selective Targeting of the CTD of TOP2A. To selectively target the CTD of TOP2A, it is critical not only to find regions that impact catalytic function but also to find regions that are distinct between the two isoforms. Therefore, we aligned the CTD of TOP2A and TOP2B to compare the locations of the mutants with the corresponding regions in TOP2B. As seen in Figure 8, some of the regions align well between the two isoforms. For example, the regions around Mutants 10 and 11. On the other hand, mutants 4, 7, and 8 include regions where TOP2A and TOP2B differ in sequence composition. Mutant 4 does appear to contain some Ser/Thr positions that align with TOP2B, but there is an eight amino acid stretch in TOP2B not found in TOP2A within this region. Interestingly, the region around Mutant 9 contains some key differences between TOP2A and TOP2B.

While the analogous regions of TOP2B have not been characterized using this approach, this data still provides some starting points for further analysis. Mutants 4 and 9 will require an additional examination to determine why these changes result in the activity changes we are observing. In addition, it will be interesting to determine whether these results stem from individual positions within the mutants or are a product of the combination of changes. Additional studies are currently being planned to examine these factors.

Exploration of Ser/Thr Residues in the CTD of TOP2A in DNA Binding and Catalysis. The TOP2A CTD includes a large number of possible sites for post-translational modification along with several functional domains such as Nuclear Localization Sequences (NLS) and a Chromatin Tether (ChT) domain.[19,23,32,55,56] This section will review the mutations that resulted in the largest changes in enzyme activity and will explore whether the residues mutated are known to have a role in the biochemical and/or biological functions of TOP2A. A summary of this information is found in Table 2.

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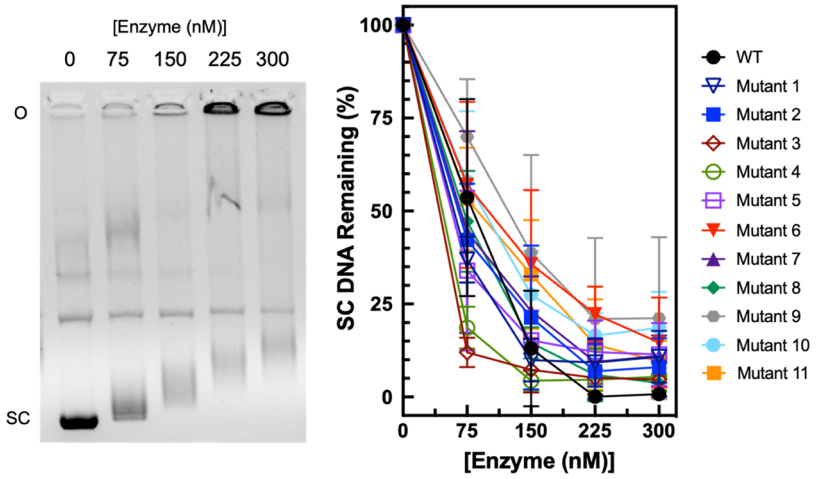

Figure 6. Plasmid DNA binding by TOP2A. DNA binding measured by the shifting of the SC DNA band in the presence of increasing concentrations of WT or mutant TOP2A. Left, a representative gel of WT TOP2A binding is shown. The unbound DNA is primarily in the supercoiled (SC) band, while the bound DNA shifts upward at increasing concentrations of TOP2A. At the highest concentrations, most of the DNA is retained in the origin of the gel. Right, quantification of the supercoiled DNA band for each mutant is shown. Error bars represent the standard deviation of the mean for three or more experiments.

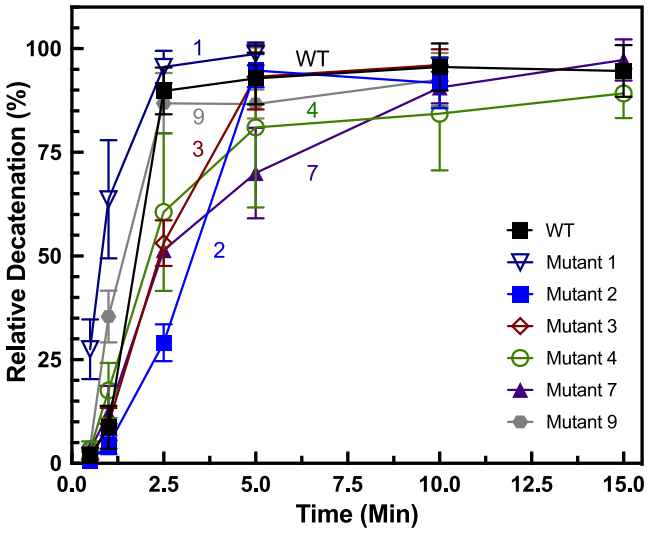

Figure 7. Decatenation of kDNA. Decatenation of catenated kinetoplast DNA (kDNA) circles by wild-type (WT) TOP2A and TOP2A Mutants 1, 2, 3, 4, 7, and 9 is shown. Error bars represent the standard deviation of four or more independent experiments.

Between residues 1272 and 1279 (Mutant 1), there are four Thr residues (1272, 1274, 1278, 1279), which we mutated to Ala residues. Interestingly, there is no published evidence on the role of these residues in catalysis or the regulation of TOP2A. Data from Mutant 1 demonstrates that the enzyme remains active and appears to cleave DNA at a higher level in comparison to WT TOP2A, especially in the presence of the etoposide (Figure 5). While it is hard to assign an exact function to these positions, they may be involved in interacting with the DNA.

In the region 1324−1343 (Mutant 3), five residues were changed (T1324A, T1327A, S1332A, S1337A, T1343A). While mutations to this region did not eliminate activity, there is evidence of increased DNA cleavage activity in the presence of the etoposide, indicating that these residues are not required for activity but may serve a regulatory function. In other words, the loss of this region leading to an increased DNA cleavage activity may indicate that there is a loss of regulation due to these mutations. Moreover, the S1337 within this region has been shown to undergo phosphorylation by Plk1, and these modifications are related to sister chromatic segregation.[38] Additionally, T1343 was found to be phosphorylated by CKII and PLK3, and phosphorylation of this site increased during the G2/M phase in HeLa cells.[31,33,37] It is unclear whether these phosphorylation events are needed to mediate protein−protein or protein−chromatin interactions.

Mutations between 1351 and 1365 (Mutant 4) included changing five amino acids (S1351A, S1354G, T1358A, S1361A, S1365G). Our evidence suggests that this region is critical for relaxation activity. While this mutant was unable to efficiently relax supercoiled DNA, it appears that the changes enabled the protein to achieve higher levels of DNA cleavage in the presence of the etoposide (Figure 5). In fact, a study conducted in PLC5 cells and HDAC-inhibitor-treated mice suggested that the area between S1361 and E1368 plays a role in regulating the stability of the enzyme.[61] Another study using HeLa cells concluded that simultaneous phosphorylation at S1354 and S1361 inhibited trypsin-mediated cleavage between the two residues.[43] This finding may indicate that combined phosphorylation at S1354 and S1361 blocks trypsin's access to the cleavage site, signifying the flexible nature of the CTD. Taken together, this is a critical region for the function of the protein. While it is unclear what is causing the increase in cleavage combined with a decrease in relaxation, decatenation assays show that Mutant 4 decatenation is similar to wild-type activity with a nonsignificant delay.

Mutations between 1469 and 1476 (Mutant 9) included five Ser/Thr residues (S1469A, T1470I, S1471A, S1474A, S1476A). Interestingly, mutations to this region decreased plasmid DNA relaxation activity, shifted the relaxation mode toward a more distributive form, and increased the rate of decatenation. Thus, this region may play an important part in stabilizing the interaction with DNA. Consistent with this, evidence from experimental studies have demonstrated that this region may have several roles in regulating the interactions and activity of the protein. S1469 exhibited cell cycle-dependent phosphorylation in cells and a purified system.[34]

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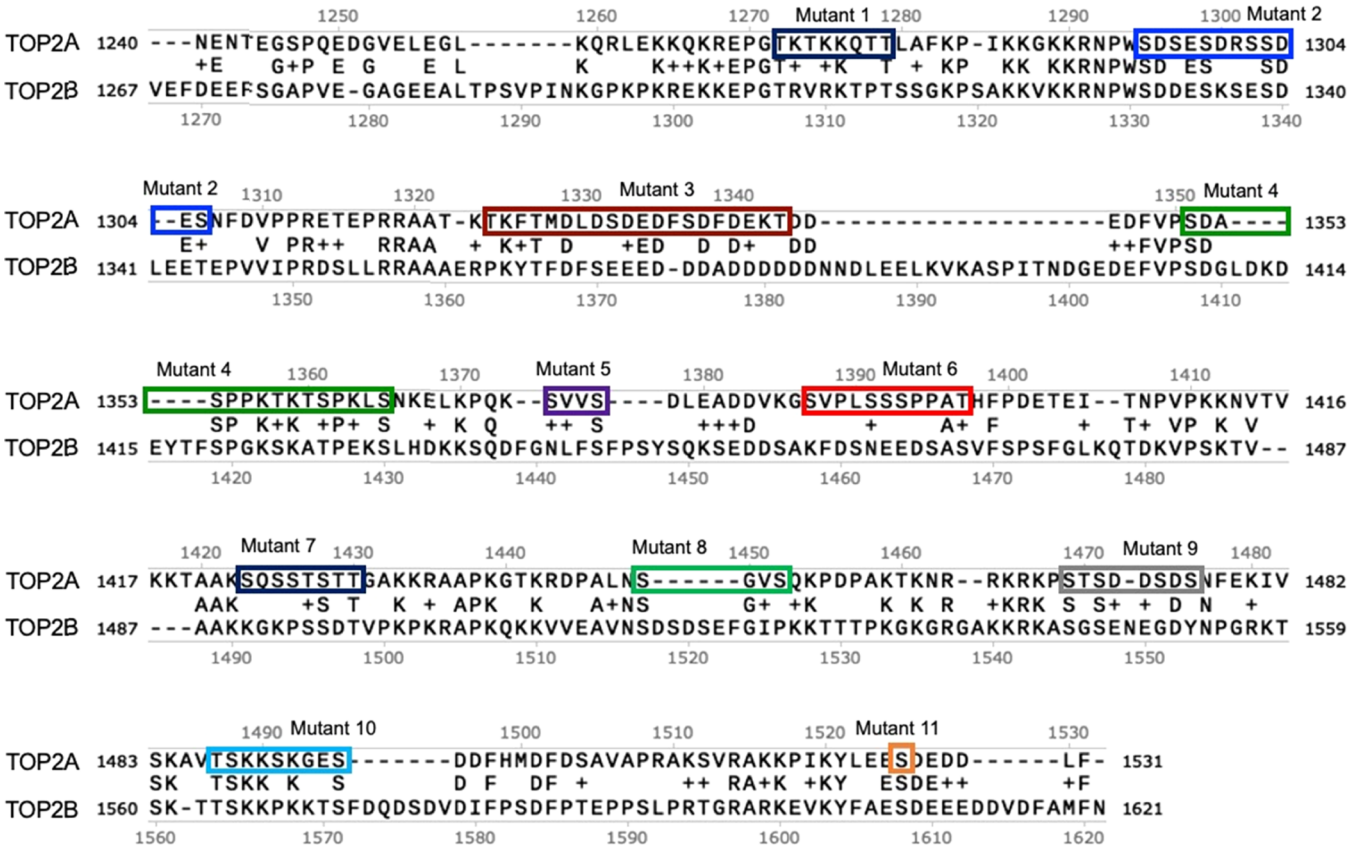

Figure 8. Sequence alignment of CTD of TOP2A and TOP2B showing locations of CTD TOP2A mutants and sites of consensus. Alignment generated from TOP2A sequence NP_001058.2 and TOP2B sequence NP_001059.2 using SnapGene (Needleman–Wunsch global alignment).

Post-translational modification at this residue is mediated by interactions with CKII and protein phosphatase 2 A (PP2A).[26,36] T1470, S1471, and S1474 are also phosphorylated in association with mitosis.[57] Mutant 9 is also within the second NLS and the DTHCT region.[55,56,63] The DTHCT region, first recognized in 2004, shares common sequence features with areas in DNA gyrase B, topoisomerase IV, and the HATPase C-terminus (abbreviation derived from underlined letters).[63] While the significance of the DTHCT region has not been examined to date, it does overlap with the ChT and contains several known phosphorylation sites.

The only mutant with a single-residue change in our study is S1525A (Mutant 11), which has been shown by numerous studies to have an impact on TOP2A's regulatory and mitotic functions. Our results demonstrate that S1525A does not inhibit DNA binding or catalytic activity. However, S1525 is a phosphorylation target for CD7/DBF4, Plk1, CKII, and p38γ (MAPK12).[36,38,47,65] Cell cycle-dependent phosphorylation at this site is involved in the enzyme's localization to centromeres and regulates the G2/M decatenation checkpoint, where phosphorylated S1525 was required to bind MDC1.[64] Modifications at S1525 may also have an effect on regulating protein stability.[65] Notably, this residue is part of the chromatin tether domain.[23] Taken together, this site plays a key role in cellular regulation and localization but the residue does not appear to be critical for the catalytic activity or DNA binding in a purified system.

Mutant 7 displayed reduced DNA relaxation and decatenation efficiency without a difference in DNA binding or cleavage (Table 1). The remaining mutations did not significantly impact the activity of the enzyme. Many of these sites, including those in Mutant 7, have not yet been studied or exist within larger motifs. In a few cases, however, detailed information is available. For example, S1377 within Mutant 5 was shown to be phosphorylated in vitro by CKII and MAPK in a non-cell cycle-dependent manner.[30,43] In Mutant 6, S1393 is phosphorylated by p34-cdc2 (MAPK).[43] Several of the remaining mutants are contained within known TOP2A domains. Mutants 2 and 10 span identified NLSs.[55,56] Mutants 8 and 10 are part of the DTHCT region.[63] Given that changes in these residues did not significantly alter TOP2A catalytic activity in our studies, the functions of these residues may be more clearly defined by studies using cellular systems.

Considerations for the Involvement of Ser/Thr Residues in the CTD of TOP2A in DNA Binding and Catalysis. Taken together, our results described above demonstrate that positions within the CTD have an impact on the catalytic activity of TOP2A. While some regions appear to possibly deregulate the control of catalytic activity (e.g., Mutants 1, 3, and 4), other mutations have a lesser impact on enzyme activity (e.g., Mutants 5, 6, and 8). Mutant 9 displays decreased relaxation without losing DNA cleavage or decatenation activity. Thus, the CTD is involved in DNA interactions and participates in regulating the activity of the enzyme.

There are several important limitations of the present study. For example, it should be noted again that a lack of a response to specific mutations does not imply that the positions are unimportant, especially in the context of a cell. Clarification of those effects will require experiments within cellular model

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Table 2. CTD Mutation Effects with Corresponding Phosphorylation Events and Functional Regions

TOP2A CTD mutant	region of mutation(s)	effects of mutations on biochemical activity	phosphorylation notes from literature[a]	known domains/functions (AA in TOP2A)	references
mutant 1	1272–1279	increased etoposide-induced DNA cleavage; faster relaxation and decatenation	T1272/T1274 (unknown effect; HTP[b])	NLS (1259–1296)	55–57
mutant 2	1295–1306	slightly slower relaxation and decatenation	S1295 and S1297 in mitosis (HTP)	NLS (1259–1296)	55, 56, 58, 59
mutant 3	1324–1343	increased etoposide-induced DNA cleavage; slightly slower relaxation and decatenation	T1327 (mitosis); S1332 and S1337 (PLK1, S-phase/mitosis); and T1343 (CKII, PLK3; G2/M)	none	31, 33, 37, 38, 45, 60
mutant 4	1351–1365	significantly delayed plasmid DNA relaxation and decatenation; increased etoposide-induced DNA cleavage	S1354/S1361(GSK2B)/S1365(CKII) [enzyme stability; ubiquitin-dependent degradation]	regulates stability/degradation (1361–1368)	43, 58, 60, 61
mutant 5	1374–1377	no significant effects	S1377 (CKII; mitosis associated from HTP)	none	30, 43, 60
mutant 6	1387–1397	no significant effects	S1387/S1392/S1393/T1397 (mitosis associated from HTP); S1393 (p34-cdc2)	none	43, 58, 60, 62
mutant 7	1423–1430	slightly delayed plasmid DNA relaxation	(unknown effect; HTP)	none	57
mutant 8	1449–1452	no significant effects	S1449/S1452 (mitosis associated from HTP)	DTHCT (1435–1521)	57, 60, 62, 63
mutant 9	1469–1476	decreased plasmid DNA binding, relaxation, and cleavage; slightly faster decatenation	S1469 (CKII; cell cycle-dependent phosphorylation); S1469/T1470/S1471/S1474 (mitosis associated from HTP)	NLS (1454–1497), DTHCT (1435–1521)	26, 34, 36, 55, 56, 60, 63
mutant 10	1487–1495	faster relaxation and decatenation	S1491/S1495 (mitosis associated from HTP)	NLS (1454–1497), DTHCT (1435–1521)	32, 55, 56, 60, 63
mutant 11	1525	a small increase in etoposide-induced DNA cleavage; faster relaxation and decatenation	S1525 (CD7/DBF4, PLK1, CKII, and p38g [MAPK12]; G2/M decatenation checkpoint)	ChT (1500–1531)	23, 36, 38, 47, 64, 65

[a]Kinase(s) and known features or cell cycle phase in parentheses. [b]HTP = high-throughput experiment.

systems. This present study is also limited by the specific groupings of residues examined. Given that the CTD covers around 400 amino acids, it will require more studies to explore the entire region. Further, it is also possible that combinations of regions are involved in complex interactions where the elimination of a single region may be unable on its own to significantly impact DNA binding or catalytic activity. Nonetheless, these results identify important roles for the CTD in DNA binding and catalysis.

If this CTD region is intrinsically disordered as has been suggested in the literature and identified through computational algorithms (data not shown), then this may clarify how mutations within various portions have such diverse effects on TOP2A activity. It is likely that the flexible, disordered nature of this region facilitates complex interactions and regulation of the enzyme. In other words, the large number of TOP2A binding partners and PTMs may be best explained by viewing the CTD as a large IDR with multiple functional subregions. It will take additional work to explore the roles of these subregions, but it is clear that a number of them serve critical roles in DNA interactions and catalysis. Cellular studies will also be needed to better understand how these changes impact cellular functions.

CONCLUSIONS

We set out to examine whether targeted point mutations in the CTD of TOP2A could impact catalytic activity and DNA binding. In particular, we focused on the alteration of Ser/Thr residues with the idea that these positions are potentially involved in the regulation of the enzyme. While it is clear that some positions and regions are controlling interactions with proteins and the chromatin, other studies have not explored the impact of these positions on basic catalytic functions.[19,29] Our results support the hypothesis that the CTD of TOP2A has a role in regulating enzyme activity and DNA interactions. Again, these roles for the CTD are consistent with roles identified for other IDRs.[27,28] While further investigation is needed and additional studies are underway, these results indicate that several regions of the CTD serve regulatory functions for TOP2A.

METHODS

Enzymes and Materials. A wild-type human TOP2A expression construct was designed by cloning the cDNA sequence for TOP2A (NM_001067.3) immediately behind a 6x-His-tag sequence and removing the N-terminal Met of the TOP2A sequence. The sequence was placed immediately downstream of a Gal promoter within the pESC-URA expression vector (Figure 1). Synthesis and sequencing of the construct were carried out by GenScript (Piscataway, NJ). Mutant enzymes were designed to remove Ser and/or Thr residues from the sequence in groups of 2–7 (or as a single mutant in one case). The specific amino acid changes are documented in Table 1. Attempts were made to preserve the nucleotide sequence whenever possible. Mutagenesis and sequencing to validate changes were carried out by GenScript (Piscataway, NJ). Sequencing data are available upon request.

Wild-type and mutant human topoisomerase II (TOP2A) were individually expressed in *S. cerevisiae* JEL1Δtop1 cells using the pESC-URA-TOP2A expression construct. Enzymes were purified, as described previously.[53] The enzymes were stored at −80 °C as a 1 mg/mL (4 μM) stock in 50 mM Tris−

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HCl, pH 7.7, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 750 mM KCl, and 5% glycerol.

Negatively supercoiled pBR322 DNA was prepared from *Escherichia coli* using a Plasmid Mega Kit (Qiagen) with some modifications of the manufacturer's protocol. Resuspension, lysis, and neutralization buffer volumes were increased to 200 mL and an additional isopropanol precipitation was performed prior to loading on the column. The etoposide (Sigma) was stored at 4 °C as a 20 mM stock solution in 100% dimethyl sulfoxide (DMSO). Working solutions were prepared by diluting the stock solution to 2 mM etoposide in 10% DMSO.

Topoisomerase IIα-Mediated Relaxation of Plasmid DNA. Reaction mixtures contained 7.5 nM wild-type topoisomerase IIα, 5 nM negatively supercoiled pBR322 DNA, and 1 mM ATP in 20 μL of 10 mM Tris−HCl, pH 7.9, 175 mM KCl, 0.1 mM Na_2EDTA, 5 mM $MgCl_2$, and 2.5% glycerol. Assays were started by the addition of TOP2A, and DNA relaxation mixtures were incubated for up to 30 min at 37 °C. DNA relaxation reactions were carried out in the presence of 1% DMSO (control) or etoposide. DNA relaxation was stopped by the addition of 3 μL of stop solution (77.5 mM Na_2EDTA, 0.77% sodium dodecyl sulfate (SDS)). Samples were mixed with 2 μL of agarose gel loading buffer, heated for 2 min at 45 °C, and subjected to gel electrophoresis in 1% TBE agarose gels. The agarose gel was then stained in ethidium bromide for 15−30 min. DNA bands were visualized by UV light using a Bio-Rad ChemiDoc MP Imaging System and Image Lab Software (Hercules, CA). DNA relaxation was monitored by the conversion of the supercoiled plasmid DNA to relaxed topoisomers.

Topoisomerase IIα-Mediated Cleavage of Plasmid DNA. Plasmid DNA cleavage reactions were performed using the procedure of Fortune and Osheroff.[66] Reaction mixtures contained 75 nM of wild-type or mutant TOP2A and the 5 nM negatively supercoiled pBR322 DNA in 20 μL of 10 mM Tris−HCl, pH 7.9, 100 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, and 2.5% glycerol. Unless stated otherwise, assays were started by the addition of the enzyme, and DNA cleavage mixtures were incubated for 6 min at 37 °C. DNA cleavage reactions were carried out in the absence of the compound (1% DMSO solution as a control), or in the presence of the etoposide, DNA cleavage complexes were trapped by the addition of 2 μL of 5% SDS followed by 2 μL of 250 mM Na_2EDTA, pH 8.0. Proteinase K was added (2 μL of a 0.8 mg/mL solution), and reaction mixtures were incubated for 30 min at 37 °C to digest TOP2A. Samples were mixed with 2 μL of Nucleic Acid Sample Loading Buffer (Bio-Rad, Hercules, CA), heated for 2 min at 45 °C, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris−acetate, pH 8.3, and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. Double-stranded DNA cleavage was monitored by the conversion of the negatively supercoiled plasmid DNA to linear molecules. DNA bands were visualized by UV light and quantified using a Bio-Rad ChemiDoc MP Imaging System and Image Lab Software (Hercules, CA). The results were analyzed and plotted using GraphPad Prism 8 (La Jolla, CA). Relative DNA cleavage was calculated by setting DNA cleavage levels in the presence of DMSO to 1. Statistical analysis was performed using a one-way ANOVA followed by a Tukey's Post-Test Analysis.

Topoisomerase IIα Binding of the Plasmid DNA. Plasmid DNA binding reactions were performed using a modification of a previously used procedure.[53] Reaction mixtures contained ∼75−300 nM of wild-type or mutant TOP2A and 5 nM negatively supercoiled pBR322 DNA in 20 μL of 10 mM Tris−HCl, pH 7.9, 150 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, and 2.5% glycerol. Unless stated otherwise, binding assays were started by the addition of the enzyme and were incubated for 6 min at 37 °C. Binding reactions were stopped by the addition of 2 μL of Nucleic Acid Sample Loading Buffer 4 (Bio-Rad, Hercules, CA) and subjected to electrophoresis in 1% agarose gels in 40 mM Tris−acetate, pH 8.3, and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. DNA binding was monitored by electrophoretic mobility shift. DNA bands were visualized by UV light and quantified using a Bio-Rad ChemiDoc MP Imaging System and Image Lab Software (Hercules, CA).

Decatenation of Kinetoplast DNA (kDNA) by Topoisomerase IIα. kDNA decatenation reactions were carried out, as previously described.[52] Briefly, reactions contained 100 nM TOP2A (WT or mutant as specified), 2 nM kDNA, and 1 mM ATP in 20 μL of reaction buffer (10 mM Tris−HCl, pH 7.9, 175 mM KCl, 5 mM $MgCl_2$, 0.1 mM NaEDTA, 2.5% glycerol). Reactions were incubated for 0−30 min before stopping with stop solution (0.5% SDS, 77 mM EDTA). Nucleic acid sample dye (Bio-Rad) was added to the samples before electrophoresis in a 1% TBE gel with ethidium bromide. kDNA bands were visualized by UV light and quantified using a Bio-Rad ChemiDoc MP Imaging System and Image Lab Software (Hercules, CA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c02083.

Figure S1: Plasmid DNA cleavage and relaxation controls. Figure S2: Stability of DNA cleavage over time. Figure S3: Decatenation of kDNA (PDF)

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■ ABBREVIATIONS

ANOVA, analysis of variance; ChT, chromatin tether domain; CTD, C-terminal Domain; DSB, double-stranded DNA break; DTHCT, **D**NA gyrase B, **t**opoisomerase IV, and the **H**ATPase **C**-**t**erminus; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; PP2A, protein phosphatase 2A; NLS, nuclear localization sequence; SSB, single-stranded DNA break; TOP2, topoisomerase II

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