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Preclinical study

Effects of mutations in the F361 to R364 region of topoisomerase I (Topo I), in the presence and absence of 9-aminocamptothecin, on the Topo I–DNA interaction

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Steady-state levels and rates of DNA binding and release of wild-type and mutant topoisomerase I (Topo I) proteins were quantified by surface plasmon resonance analysis. The proteins were constructed and expressed as GST fusion proteins. The Topo I mutations analyzed were F361S, R362L and R364G, all altering a highly conserved region of wild-type eukaryotic Topo I. The R362L and R364G mutations resulted in much lower steady-state levels of DNA binding than wild-type. This was due to a large increase in the k_d. The F361S mutation increased the steady-state levels of the protein–DNA interaction by increasing the k_a 2-fold, while having little effect on the k_d. The F361S mutation has been shown to confer resistance to camptothecin and its analogs. The camptothecin analog 9-aminocamptothecin decreased greatly the overall k_d of the wild-type Topo I, but had little effect on the F361S mutant. Both the wild-type and the F361S mutant exhibited decreased steady-state levels in the presence of the drug, and this was attributable to decreased association. [© 1999 Lippincott Williams & Wilkins.]

Key words: 9-Aminocamptothecin, DNA binding, kinetic analysis, surface plasmon resonance, Topoisomerase I mutants.

Introduction

Eukaryotic topoisomerase I (Topo I) resolves topological predicaments in DNA by regulating the superhelical state of the DNA molecule.[1] These predicaments arise because activities such as transcription and replication cause local supercoiling of DNA.[2,3] Supercoiling of DNA, in turn, affects a wide range of DNA-associated events.[4–7]

Topo I-mediated relaxation of DNA involves a number of sequential and probably concerted events. These include DNA recognition, a complex non-covalent Topo I-DNA interaction, subsequent formation of a covalent DNA-Topo I phospho-tyrosyl (Topo I Y723) intermediate, relaxation of the DNA substrate, resolution of the patent DNA duplex and finally dissociation of Topo I from the DNA. Previous work[8] and recently solved crystal structures of Topo I-DNA non-covalent and covalent complexes reveal an elaborate interaction between Topo I and DNA that differs depending on the transitional state examined (i.e. covalent or non-covalent[9,10]). This indicates that Topo I interaction with DNA is temporally and structurally complex. Although crystal structures are revealing, transitional events leading to catalytic activity and release of the enzyme need to be described by different approaches. Surface plasmon resonance techniques allow the characterization of Topo I-DNA interactions in real time.

Of particular importance in Topo I catalysis are the events surrounding the reformation of the DNA duplex. This is because a new class of anti-cancer drugs, analogs derived from the Chinese elm natural product camptothecin, act by stabilizing the covalent DNA-Topo I complex.[11] Camptothecin binds the complex (with both DNA and protein bonds) at the DNA cleavage site and prevents the Topo I from facilitating DNA ligation.[9] The drug stabilized covalent enzyme-DNA intermediates lead to lethal double-stranded DNA breaks during replication and other potentially lethal DNA lesions which result in cancer cell selective toxicity.[12,13] These drugs are effective

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against several cancers that are resistant to other chemotherapies.[11] How this class of compounds functions is dependent on the interaction of Topo I and DNA.

Determining the effects of individual amino acid mutations on Topo I-DNA binding and release provides an avenue for determining the function of those amino acids, and their respective protein domains, in Topo I-DNA interactions. Reported here are studies of the interaction between DNA and a particular set of Topo I mutants involving the 'lip'[9,10] region from amino acid 361 to 364.[11,14]

Material and methods

Topo I and mutants

Wild-type GST fusion Topo I and mutants were constructed, expressed and purified according to Li *et al.*[11,14] These preparations were uniformly between 80 and 90% pure. These were stored at −80°C until the day of use and then stored at −20°C until experiments were run.

Biotinylation of DNA

Salmon testis DNA was obtained from Sigma (St Louis, MO) as were the chemical reagents listed below. *Sau*3A restriction endonuclease and Klenow fragment DNA polymerase were obtained from New England Biologicals (Boston, MA). The CM5 sensor chip and biotin-14-dATP were obtained from Pharmacia & Upjohn (Uppsala, Sweden) and Amersham Life Sciences (Arlington Heights, IL), respectively. Streptavidin was obtained from Pierce Life Science (Rockford, IL). Salmon testis DNA was cut with *Sau*3A. The restriction enzyme-digested DNA was extracted with phenol/isoamylalcohol/chloroform (24/1/25), precipitated with ethanol and redissolved in TE (10 mM Tris, pH 7.4, 1 mM EDTA). The DNA was biotinylated using Klenow polymerase in reaction buffer (50 mM Tris, pH 7.2, 100 mM $MgSO_4$, 0.1 mM dithiotheitol, 1 mM dCTP, dGTP and dTTP, and biotinylated dATP) for 2 h at 37°C. The DNA was ethanol precipitated as above. Biotinylation was assessed by agarose gel retardation analysis.

Activation and derivitization of the Biacore CM5 chip

The BIAcore 2000 allows injection of mobile ligand over four flow cells simultaneously. Three flow cells were derivatized with streptavidin using the following method, the fourth left blank. The CM5 flow-cell chip was activated with a mixture of *N*-ethyl-*N*'(dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinamide (NHS) as recommended by BIAcore, Pharmacia (Uppsala, Sweden).[15] Streptavidin then spontaneously reacted with the activated flow-cell surface when applied at 100 μg/ml in 10 mM sodium acetate, pH 6.0, at a flow rate of 10 μl/min. Approximately 500 RU were added to lanes 1, 2 and 3. The surface was then deactivated with 1 M ethanolamine, pH 8.0. Biotinylated DNA, 100 μg/ml in HEPES-buffered saline (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% Tween detergent) was applied to the flow cell until the desired level of resonance units (about 250 and 500 RU to lanes 1 and 2) was achieved.

Topo I application to flow-cells

Topo I wild-type and mutants were diluted in running buffer (HBS, 150 mM NaCl, 8.7 mM Mg^{2+}, 3.7 mM EDTA, 10 mM HEPES, pH 7.4 and 0.005% Tween detergent) and applied to the biosensor flow cell in 100 s injections. Experiments with 9-aminocamtothecin (9AC, a gift from Dr JA Holden, University of Utah, Department of Pathology) were performed in the presence of 10 mM $MgCl_2$ because, in the presence of increased Mg^{2+}, dissociation is greatly accelerated and thus yields a greater signal/time ratio. A constant level of 10 μM 9AC was present in the running buffer during these experiments. Flow-cells were regenerated between experiments by 5 min washing with a 60 s injection of 0.5 M NaCl and a 5 s injection of 0.005% SDS. Dissociation data were modeled for 100 or 150 s after a 100 or 120 s Topo I injection.

Analysis of data

Data were analyzed as described previously[16] using commercially supplied BIAeval software.[17] The best fits obtained for several concentrations of injections were used for comparative analysis. Association rate constants are presented as pseudo-first-order values to provide a meaningful rate comparison. Satisfactory analysis of data was indicated by small measures of error (e.g. SE, χ^2, etc.[17]) and by obtaining consistent rate constants (within 2 SE) across a range of Topo I concentrations.

Results

Plasmon resonance analysis of Topo I–DNA interaction

DNA was used as the immobilized ligand as described previously.[16] Briefly, salmon testis DNA was cut to completion by a restriction endonuclease that cuts about every 256 bp. This relaxed, random sequence, linear, duplex DNA likely contained a complete set of the DNA binding domains for Topo I. The association and dissociation of Topo I with the immobilized DNA fragments were easily detected and quantified using plasmon resonance technology.

The analysis of Topo I-DNA binding presented here rendered information about Topo I-DNA binding and release in the absence of influence of *in vivo* conditions such as superhelical state, other DNA binding proteins, etc. This abstraction allowed direct functional analysis of particular Topo I residues involved in real-time Topo I-DNA interactions over a random DNA substrate. Modeling such interactions is complex, and it is likely that attempts to model associative or dissociative interactions involving more than two rate constants would be of limited value in understanding and defining molecular events involved in catalysis. Both association and dissociation of 100 kDa human Topo I with DNA[16] have been accurately modeled using both BIAeval software[17] and global analysis[18,19] (data not shown). However, it has been demonstrated[16] that Topo I-DNA association in the protocol used here is complicated by cooperative binding effects and is not adequately modeled by the BIAeval software. Nevertheless, association data obtained with the GST-Topo I proteins could be simplified by describing the overall rates with a single rate constant. Likewise, even though dissociation was best described as bi-phasic (with two rate constants),[16] it too could be described by a 'pseudo-first-order' rate constant. Using such an approach, analysis of the interactions between wild-type protein and DNA yielded a k_a of 1.1×10^5 Ms^{-1} and a k_d of 9.58×10^{-4} s^{-1}. These pseudo-first-order rate constants provide a straightforward way to compare the relative rates of association and dissociation amongst the proteins. These analyses also showed that the wild-type fusion protein yielded binding data indistinguishable from that of normal Topo I.

Comparison of wild-type and F361S

Mutation from phenylalanine to serine at position 361 increased the overall k_a of the enzyme for DNA from the 1.1×10^5 Ms^{-1} seen with wild-type protein to 1.84×10^5 Ms^{-1} (Figure 1 and see Table 3) with little affect on dissociation (see Table 2). Steady-state binding levels thus increased significantly over the wild-type construct (Figure 1). As stated above, dissociation of DNA from wild-type or F361S was modeled accurately by a formula describing two

Table 1. Dissociation constants (k_d), standard error of k_d and χ^2 (goodness of fit) of indicated proteins from the curves in Fig. 1 modeled with the equation A+B↔AB

Curve	k_d	SE (k_d)	R_0	χ^2
WT	9.58×10^{-4}	2.56×10^{-5}	910	69.7
F361S	9.01×10^{-4}	2.67×10^{-5}	1.15×10^{-3}	133
R364G	1.48×10^{-3}	1.2×10^{-5}	471	3.2
R362L	1.91×10^{-3}	1.17×10^{-5}	389	1.92

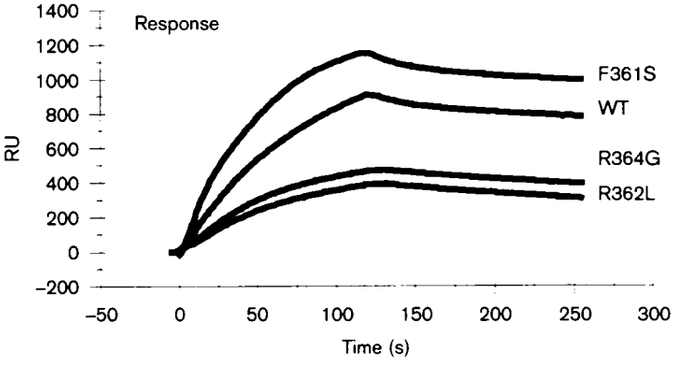

Figure 1. Representative sensorgram compiled from injections of indicated proteins. Injection concentrations were approximately 100 nM.

Table 2. Wild-type and F361S curves in Fig. 1 model with the equation $A_j+B_i \leftrightarrow A_iB_j$

Cuves	R_1	k_{d1}	SE (k_{d1})	R_0	k_{d2}	SE (k_{d2})	t_0	χ^2
WT	78.8	0.0396	1.65×10^{-3}	910	5.44×10^{-4}	2×10^{-5}	119	2.03
F361S	86.8	0.0439	6.33×10^{-4}	1.15×10^{-3}	4.79×10^{-4}	6.38×10^{-6}	122	0.46

Table 3. Association constants for the curves in Fig. 1 modeled with the equation $AB \leftrightarrow A+B$[17]

Curve	k_a	SE (k_a)	R_{eq}	SE (R_{eq})	Concentration (nM)
WT	1.1×10^{-5}	981	1.22×10^{-3}	4.09	1×10^{-7}
F361S	1.84×10^{-5}	1.71×10^{-3}	1.27×10^{-3}	3.77	1×10^{-7}
R364G	1.52×10^{-5}	1.79×10^{-3}	562	1.81	1×10^{-7}
R362L	1.31×10^{-5}	1.05×10^{-3}	430	1.21	1×10^{-7}

independent modes of DNA release by enzyme (χ^2 for the fits generated with the bi-phasic model of dissociation were far superior to those for uni-phasic dissociation; *cf.* Tables 1 and 2). The fact that F361S and wild-type have almost identical dissociation rates suggests little or no involvement of the phenylalanine in the DNA release function.

Comparison of wild-type and F361S in the presence of 9-AC

9AC is thought to stabilize Topo I cleavable complexes. Cleavable complexes are the covalent DNA-Topo I intermediates that lead to cell death.[12,13] When 9AC was co-injected with the wild-type enzyme dramatic changes occured in Topo I-DNA interactions. 9AC greatly slowed the overall dissociation of the wild-type-DNA complex. This was the anticipated effect of cleavage complex stabilization (Figure 2). The slowing was apparent in both k_{d1} and k_{d2}, although the effect was more pronounced on k_{d2} (data not shown). Normally, with a slowed rate of dissociation, a higher level of steady-state binding would be expected. However, plasmon resonance revealed a previously unsuspected effect of 9AC on Topo I-DNA interactions. DNA-protein association was greatly slowed in the presence of drug. As a result the steady-state levels of wild-type DNA binding were decreased when drug was co-injected. In contrast, when 9AC was co-injected with the Topo I F361S mutant little effect was seen on the dissociation rate constant (Table 4). This was consistant with the drug resistance of this protein. However, 9AC did slow the overall k_a for F361-DNA association (Table 4), and thus steady-state binding levels of F361S and DNA

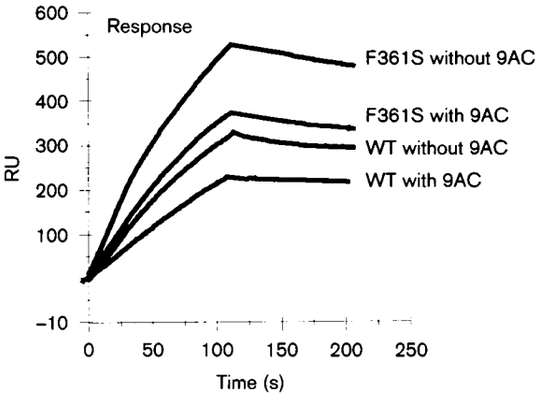

Figure 2. Representative sensorgram of 100 nM injection of wild-type and F361S Topo I with and without 9-AC (10 μM).

were decreased in a manner similar to that seen for wild-type, though the effect was less pronounced.

Effects of R364G and R362L mutations

Mutations in these two residues yielded major changes in the Topo I-DNA interaction. All the mutant proteins showed increases in association rates over wild-type (Figure 1). As discussed earlier, the F361S mutation produced the greatest increase, almost 2-fold over wild-type. The R364G and R362L mutations increased association rates by 1.4 and 1.2 times, respectively. Despite faster association rates, both arginine mutations resulted in greatly decreased steady-state levels of protein bound to DNA (Figure 1). These lower steady-state binding levels result from greatly increased

Table 4. Constants derived from Fig. 2 modeled with the equation A+B↔AB[17]

Curve	k_d	SE (k_d)	k_a	SE (k_a)
F361S without 9AC	1.02×10^{-3}	1.23×10^{-5}	4.40×10^{-4}	714
F361S with 9AC	1.00×10^{-3}	1.94×10^{-5}	3.12×10^{-4}	454
WT without 9AC	1.03×10^{-4}	3.06×10^{-5}	3.39×10^{-4}	432
WT with 9AC	5.19×10^{-4}	7.12×10^{-6}	1.03×10^{-4}	556

overall dissocciation rates. The dissociation rates for the R364G and R362L mutations increased, 1.5 and 2.0 times the wild-type protein (Table 1). Additionally, the Topo I-DNA dissociation with these proteins was best fit by a model describing a single mode of dissociation. These results imply that, functionally, the R364G and R362L mutations result in proteins that have a predominating single fast rate of DNA release. This probably implies release without strand scission and rejoining since these two mutants are greatly impaired in topoisomerization (although some activity could be shown in plasmid relaxation assays, R362L being slightly more active than R364G[11,14]).

Discussion

Plasmon resonance detection was ideal for the functional analysis of the GST-Topo I proteins used here. It is routinely used to quantify ligand-receptor interactions in complex solutions. In this case the expressed fusion proteins were between 80 and 90% pure. The preparations contained non-specific *Escherichia coli* proteins as well as some specific peptides smaller than the fusion proteins which were thought to be N-terminal fragments of the GST-Topo Is. Plasmon resonance detection was particularly applicable because non-DNA binding proteins are not detected at all and because non-specific binding that contributes to the baseline signal is subtracted before the data are 'fit'. Two convincing facts argue for the veracity of the data obtained in this study. First, the DNA dissociation data obtained with the wild-type and F361 proteins used here were quantitatively indistinguishable from data obtained with highly purified normal human Topo I (as determined in Pond *et al.*[16]). Second, 9AC, a molecule only known to interact with Topo I and DNA, had the specific effects one would predict from published data obtained in non-quantitative experimental systems.[11,12,14,20] Thus, since the contaminating proteins would have been present in all the preparations equally, it cannot reasonably be argued that the differences in the k_as and k_ds reported here are due to impurities present in the fusion protein preparations.

The DNA binding data obtained with the GST-Topo I fusion proteins was consistent with that obtained with that previously obtained with normal human enzyme.[16] As was observed with normal Topo I, the complexity of the DNA association data precluded kinetic modeling by BIAeval software. Through the use of 'global analysis' software,[18,19] association data obtained with higher concentrations of the GST-enzyme (data not shown) could be accurately modeled. Unfortunately, neither uni- or bi-modal models of association nor formulae representing an inducible conformational change could fit data obtained with lower concentrations of protein. It is therefore suggested that the inability to model association data obtained at lower protein levels might be due, in part, to the inadequate 'signal-to-noise' ratio. In any case, only pseudo-first-order k_as are presented here.

Dissociation of GST-Topo I and DNA, however, was found to be identical to that found with normal human enzyme.[16] The bi-phasic nature of the normal enzymes' dissociation has not yet be explained in molecular terms. It is, however, consistent with the previous description by Caserta and colleagues of calf thymus Topo I catalysis as bi-phasic.[21] It was hypothesized in that work that the two distinct catalytic rate constants might reflect the enzyme's interaction with two classes of substrates sites, preferred or less preferred. Topo I association with and dissociation from preferred sites in the DNA would be represented by the faster k_a and k_d. This hypothesis is consistent with surface plasmor resonance data[16] for Topo I-DNA association and dissociation, where both association and dissociation are best characterized by bi-phasic interactions.

Data presented here show that replacement of phenylalanine with serine at position 361 in Topo I results in increased steady-state DNA binding levels. There a number of plausible reasons for this finding: In the 3-D crystal structure of Topo I determined by Champoux *et al.*[9,10] F361 is in a lip domain that appears to clamp onto and maintain an intimate relationship with the target DNA. F361 and the neighboring R364 appear to directly contact the DNA

on several local sites depending on the transition state examined. Even though previous work showed no sequence specificity in the general association of Topo I with DNA,[16,22] there could still be a limited number of possible binding domains in a given DNA substrate.[22] If loss of the phenylalanine at position 361 caused a decrease in Topo I selectivity for binding such domains, an increase in the overall k_a might be expected. Alternatively, structural data[9,10] and modeling data obtained with global fitting[15] of normal 100 kDa human Topo I (data not shown) are both consistent with protein conformation changes as part of DNA binding. A bulky phenylalanine at position 361 might interfere or impede such a conformational change. If so, it is possible that its mutation to S361 could facilitate the conformation change and so increase the k_a. Since the F361S mutant is almost as active as the wild-type in a plasmid relaxation assay and since the k_ds describing dissociation appear normal, F361S's relationship with the DNA appears to be normal once it is bound. This implies that catalytic manipulations of the DNA by the enzyme are not altered once bound. Thus, the lip region of the protein may be involved in cleavage site selection, or in 'decision' making about whether to cleave the DNA or not. Apparently inconsistent with this conclusion were the previous observations that F361S was significantly impared in a cleavage/ligation assay and yet exhibited similar DNA binding in a mobility shift assay, when compared with wild-type.[11,14] Unlike the current study, these earlier experiments used short oligonucleotides synthesized to contain a single high-efficiency cleavage site as substrate. A decrease in the cleavage of one specific oligonucleotide site by F361S would not preclude an increased affinity of F361S for a greater number of lower efficiency cleavage sites in a random DNA substrate. Also, F361S and wild-type proteins might bind a specific cleavage sequence with similar affinities and yet exhibit differing affinities for DNA overall. So, it is suggested here that loss of F361 either decreases energetic barriers in binding-dependent protein conformation changes or allows less selective but still active association with DNA.

The effects of 9AC on the steady-state levels of protein-DNA binding were surprising. It was assumed that by slowing dissociation, via stabilization of the cleavable complex, steady-state levels of DNA-bound protein would increase. This was not seen in either the wild-type or F361S proteins. The presence of 9AC appears to impede the association of Topo I and DNA. How this occurs is open to speculation but may be consistant with the previous report of Westergaard and colleagues in which they showed inhibition of Topo I-DNA cleavage (as well as ligation) in the presence of camptothecin.[20] Whether by association with the DNA or enzyme, or by inserting itself in the associating complex (making it a trinary versus a binary event), the overall effect appears to be the reduction of the overall rate of association. The effect of 9AC on wild-type dissociation was as expected, it greatly prolonged the residence of wild-type protein on the DNA. Consistent with this was the observation that DNA dissociation from the camptothecin-resistant, but catalytically active, F361S was not affected.

The large decrease in steady-state binding levels of the R362L and R364G mutants is also interesting. Previous work has shown greatly decreased plasmid relaxation activity (about 40-fold) for both enzymes, compared to wild-type.[11,14] The published structures[9,10] of the Topo I-DNA non-covalent complex show R364 to bind via its guanidinium moitey to the N3 atom of the +2 guanine base in the minor groove, the ribose oxygens on the +2 and +3 nucleosides on the cleaved strand, and/or main chain N interactions with the phosphate between the -1 and -2 bases on the uncut strand. Even though the R362L and R364G mutations showed slightly increased association rates with DNA, the predominant effect was on dissociation, consistent with this region of the lip domain being heavily involved in stabilizing the enzyme-DNA complex. It was surprising that the R362L had an effect similar to the R364G. It was shown to have similar catalytic deficiencies,[11,14] but, acording to the published crystal structures,[9,10] lacks the extensive contacts with DNA that have been shown for R364. Either R362 makes extensive contacts with the DNA substrate which have not yet been elucidated or it perturbs the interactions of the nearby R364 to result in a smilar phenotype. In contrast to the normal protein, the DNA dissociation of the R364 or R362 mutants was of first order. This observation could be consistent with the the hypothesis discussed above, if the R mutants only bound to preferred binding sites in DNA. Such sites which might not require the added stabilization of the 'upper lip' arginines. It is even possible that Topo I-DNA association, catalysis and dissociation could occur without the hypothesized 'clamp' conformational change. Consistent with this is the fact that HP1 integrase, an enzyme capable of DNA cleavage and ligaiton, is 90% homologous to the core subdomain III and carboxyl domain of Topo I, but has no similar 'upper lip' (core subdomains I and II).

In summary, the observed effects of Topo I mutation in the region of F361 to R364 illustrate that specific amino acid residues in the lip region of core subdomain I play specific and important roles in the association and/or dissociation of Topo I and DNA during catalysis. The camptothecin drugs appear to have two antagonistic effects on Topo I-DNA interaction. They slow association, but prolong dissociation via inhibition of catalysis.

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