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Mutation E522K in Human DNA Topoisomerase IIβ Confers Resistance to Methyl *N*-(4′-(9-acridinylamino)-phenyl)carbamate hydrochloride and Methyl *N*-(4′-(9-acridinylamino)-3-methoxy-phenyl) methane sulfonamide but Hypersensitivity to Etoposide

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ABSTRACT

Human cells express two isoforms of topoisomerase II, α and β, that are both targeted by anticancer drugs. To investigate acridine resistance mediated by topoisomerase IIβ, we used a forced molecular evolution approach. A library of mutated topoisomerase IIβ cDNAs was generated by hydroxylamine mutagenesis and was transformed into the yeast JN394 top2–4. Methyl *N*-(4′-(9-acridinylamino)-phenyl)carbamate hydrochloride (AMCA) selection identified a resistant transformant able to grow in media containing 76 μg/ml AMCA. Topoisomerase IIβ with a glutamic acid-to-lysine substitution at position 522 was responsible for the ~10-fold resistance to AMCA. The transformant was cross-resistant to methyl *N*-(4′-(9-acridinylamino)-3-methoxy-phenyl) methane sulfonamide (mAMSA) and mAMCA but hypersensitive to etoposide and ellipticine. In vitro, the βE522K protein was unable to support acridine-stimulated DNA cleavage, suggesting that resistance to these acridines is caused by reduced drug-stimulated DNA cleavage. However, βE522K showed DNA cleavage with etoposide, and the cleavable complexes formed with etoposide showed greater stability, thus accounting for the hypersensitivity to etoposide. Drug-independent cleavage of an oligonucleotide by βE522K was reduced compared with the wild-type enzyme. Decatenation and relaxation activities were reduced to 52 and 61% of the wild-type levels, which may explain the slower growth of yeast strain JN394top2–4 expressing βE522K at the nonpermissive temperature. This study confirms that topoisomerase IIβ is a target for AMCA and that resistance to AMCA can be mediated by a point mutation at Glu522 in topoisomerase IIβ. Residue 522 lies within a Rossmann fold in the B′ subfragment of topoisomerase II, a region previously implicated in drug interactions.

Type II DNA topoisomerases (topo) are essential enzymes that catalyze topological changes in DNA by passing one double helix through another and religating the cleaved helix, in a reaction coupled to ATP hydrolysis. The type II topoisomerases are targets for anticancer agents that interrupt enzymatic DNA breakage-reunion by DNA topo II and have been termed "poisons". In their presence, topo II binds and cleaves the DNA, but subsequent religation and enzyme turnover is inhibited because of stabilization of the normally transient intermediate, in which the active site tyrosines within the enzyme are covalently bound to the DNA in a "cleavable complex". Accumulation of these complexes triggers cell death. Atypical drug resistance to agents that target topoisomerases occurs by altering the level and/or function of topoisomerase reducing its ability to form cleavable complexes, resulting in drug resistance (Kaufmann, 1998).

Methyl *N*-(4′-(9-acridinylamino)-phenyl)carbamate hydrochloride (AMCA) and methyl *N*-(4′-(9-acridinylamino)-2-methoxy-phenyl)carbamate hydrochloride (mAMCA) are carbamate analogs of methyl N-(4′-(9-acridinylamino)-3-methoxy-phenyl) methane sulfonamide (mAMSA) with the same polycyclic core but different side chains. AMCA and mAMCA are distinguished from mAMSA by their high cytotoxicity toward noncycling cells (Finlay et al., 1994; Turnbull et al., 1999). Both AMCA and mAMCA can stimulate the cleavage activity of calf thymus topo II or human topo IIα and -β in vitro on end-labeled DNA substrates. AMCA and mAMCA promoted cleavage at different sites than those promoted by mAMSA. The presence of a carbamate side chain on AMCA and mAMCA rather than a methoxy group on mAMSA is responsible for this

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ABBREVIATIONS: topo, DNA topoisomerase; AMCA, methyl *N*-(4′-(9-acridinylamino)-phenyl)carbamate hydrochloride; mAMSA, methyl N-(4′-(9-acridinylamino)-3-methoxy-phenyl) methane sulfonamide; mAMCA, methyl *N*-(4′-(9-acridinylamino)-2-methoxy-phenyl)carbamate hydrochloride; MLC, minimum lethal concentration.

altered cleavage-site specificity (Baguley et al., 1997). It was hypothesized that the activity of AMCA and mAMCA against noncycling cells may be caused by targeting DNA topoisomerase IIβ (Finlay et al., 1994). To test this hypothesis, a drug-permeable yeast strain (JN394 top2–4) was transformed with plasmids expressing either human topo IIα or human topo IIβ. mAMCA was the most cytotoxic in this system, whereas AMCA and mAMSA were 4- or 5-fold less cytotoxic. Comparable IC_{50} values were seen with yeast transformants expressing either topo IIα or topo IIβ; 4 and 3.5 μM for mAMCA, 22 and 20 μM for AMCA, and 15 and 19 μM for mAMSA, respectively. These yeast data showed that AMCA, mAMCA, and mAMSA could target either topo IIα or topo IIβ (Turnbull et al., 1999). Further evidence that AMCA and mAMCA were targeting topo IIβ comes from a study using a pair of murine embryo fibroblast (MEF) cell lines with or without topo IIβ using clonogenic cytotoxicity assays. The MEFs lacking topo IIβ were more resistant to cell killing by mAMSA, mAMCA, and AMCA than the MEFs containing both topo IIα and topo IIβ. This confirmed that topo IIβ is a target for mAMSA, mAMCA, and AMCA but not the only target, because the cells containing only topo IIα were also killed (Errington et al., 1999). These studies show that topo IIβ is a drug target for these agents and that topo IIβ plays a role in cell killing by these acridines. Indeed, it has been suggested that topo IIβ is the preferred target of the acridine mAMSA (Dereuddre et al., 1997; Herzog et al., 1998)

To determine the regions within human topo IIβ protein that are important for targeting by the acridines, we used a forced molecular evolution approach. A powerful yeast system was used to select for mutations within human DNA topo IIβ. The strain used was JN394top2–4, which permits growth at 25°C but not at 35°C. Complementation with a plasmid-borne functional TOP2 gene rescues growth at 35°C and allows selection and analysis of putative drug resistance mutations. This system has been used to select for drug resistance mutations in yeast topo II and human topo IIα but it has not previously been reported for human topo IIβ. We previously constructed a complementing plasmid YEphTOP2βKLM that encodes human TOP2β (Meczes et al., 1997). Herein, we report the selection of AMCA-resistant transformants using a library of randomly mutated TOP2β plasmids and the subsequent characterization of a mutated protein βE522K bearing one of the four selected mutations. This is the first report of a mutated protein (βE522K) resistant to AMCA. It is interesting that the βE522K protein showed a comparable level of cross-resistance to the acridines mAMSA and mAMCA but was hypersensitive to etoposide and ellipticine.

Materials and Methods

Chemicals and Drugs

mAMSA, AMCA, and mAMCA were supplied by Prof. B. C. Baguley, Auckland Cancer Society Research Center, University of Auckland (Auckland, New Zealand). Etoposide, mitoxantrone, doxorubicin, and ellipticine were purchased from Sigma. DNA sequencing was carried out by Lark Technologies Inc (Essex, UK). Oligonucleotides were ordered from Invitrogen (Paisley, UK). Radiochemicals were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK) and enzymes from New England Biolabs (Hertfordshire, UK) or Promega (Southampton, UK). kDNA was purchased from TopoGEN (Columbus, OH).

Hydroxylamine Mutagenesis and Selection in the Presence of AMCA

Mutations in the plasmid YEphTOP2βKLM (Meczes et al., 1997) containing htopo IIβ were introduced by hydroxylamine mutagenesis and selected for drug resistance using methods described previously (Nitiss, 1994; Patel et al., 2000). Hydroxylamine mutagenesis was at 75°C for 40 min. Mutagenized plasmid DNA was transformed into the yeast strain JN394top2–4 to select for mutations that confer in vivo drug resistance to AMCA. The yeast transformants were then grown in the presence of 76 μg/ml AMCA for 96 h at 35°C. The amount of AMCA that was used for the selection of drug resistant mutations was 10 times the IC_{50} value in the yeast strain JN394top2–4 (Turnbull et al., 1999). The drug-resistant yeast transformants were selected by plating onto uracil minus plates contain-

TABLE 1

The drug sensitivity profile of JN394 top2–4 expressing βwt or βE522K in the presence of different cytotoxics at 35°C

The yeast strain JN394 top2–4 was transformed with recombinant plasmid encoding either βwt or βE522K. The transformed strains were tested for drug sensitivity to different concentrations of topo II poisons. Equal amounts of each transformant as determined by optical density were plated in a series of dilutions onto adenine-supplemented yeast extract/peptone/dextrose with drug and were grown at 35°C for 5 days. After 5 days, the extent of growth was assessed by comparing the number of yeast colonies to the wt. The results shown here are the amount of growth of yeast colonies: ++++, very good growth (e.g., growth at all dilutions); +++, good growth; ++, reasonable growth; +, poor growth (e.g. growth at just the most concentrated plating); and −, no growth. Comparisons of the level of growth from several dilutions on plates containing several different drug concentrations allowed approximate fold resistance values to be determined. The experiment was performed in duplicate.

Plasmid	Protein	1 μg/ml	5 μg/ml	10 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml	Resistance Factor
AMCA									~10-fold
YEphtop2βKLM	βwt	++++	+/−	+/−	−	−	N.D.	N.D.	
YEphtop2βE522K	βE522K	+++	+++	+++	+	+/−	N.D.	N.D.	
mAMSA									~10-fold
YEphtop2βKLM	βwt	+++++	+++	+/−	−	−	N.D.	N.D.	
YEphtop2βE522K	βE522K	++++	++++	++++	++++	++++	N.D.	N.D.	
mAMCA									~10-fold
YEphtop2βKLM	βwt	++++	−	−	−	−	N.D.	N.D.	
YEphtop2βE522K	βE522K	+++	+++	++	+	+	N.D.	N.D.	
Doxorubicin									~2-fold
YEphtop2βKLM	βwt	N.D.	++++	++++	+/−	−	N.D.	N.D.	
YEphtop2βE522K	βE522K	N.D.	+++	+++	+	+	N.D.	N.D.	
									Hypersensitive
Etoposide									~2-fold
YEphtop2βKLM	βwt	N.D.	N.D.	++++	++++	++++	+++	++	
YEphtop2βE522K	βE522K	N.D.	N.D.	++++	++	++	+	-	
Ellipticine									~2 fold
YEphtop2βKLM	βwt	N.D.	++++	+++	+	−	−	N.D.	
YEphtop2βE522K	βE522K	N.D.	+	+	−	−	−	N.D.	

N.D., not determined.

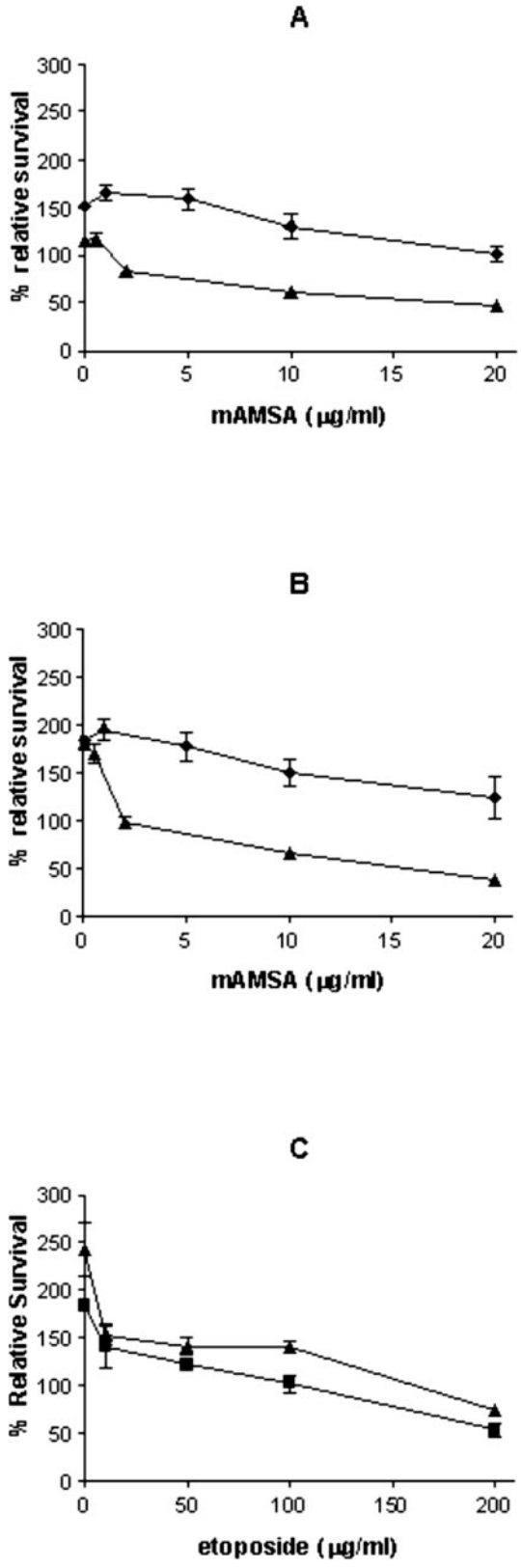

Fig. 1. Effects of mAMSA and etoposide on the viability of yeast strain JN394 top2–4 transformed with plasmids encoding recombinant βwt or βE522K at 35°C. Viable counts were determined after growth of yeast transformants in the absence or presence of mAMSA or etoposide at the concentrations indicated for 0, 6, or 24 h. The values are expressed as a percentage of viable counts measured for time 0, percentage of relative survival. The results are the average of three independent experiments. Error bars represent the standard error from the mean of three experiments. A, 6-h exposure to mAMSA, JN394 top2–4 expressing βwt (▲) and JN394 top2–4 expressing βE522K (♦). B, 24-hour exposure to mAMSA, JN394 top2–4 expressing βwt (▲) and JN394 top2–4 expressing βE522K (♦). C, 24-hour exposure to etoposide, JN394 top2–4 expressing βwt (▲) and JN394 top2–4 expressing βE522K (■)

ing 76 μg/ml of AMCA and grown for 3 to 5 days at 35°C. The DNA from the drug-resistant colonies was isolated, and its restriction pattern was compared with YEpHTOP2βKLM to exclude the possibility of any gross rearrangements or large deletions. Finally, the selected mutagenized plasmids were retransformed into the yeast to verify that the AMCA resistance was plasmid-borne. The selected clones were sequenced on both strands to identify the position of any mutation(s).

In Vivo Characterization

The 1674-bp BamHI-PmaCI restriction fragment of the mutagenized plasmid that contained the point mutation was exchanged with the corresponding fragment in the nonmutagenized YEphTOP2βKLM. DNA sequencing was used to confirm the presence of a mutation in the resulting plasmid. This fragment exchange was carried out to confirm that the drug resistance phenotype resulted from the point mutation and not from other mutation(s) in the noncoding region of the plasmid. The in vivo analysis of the mutagenized plasmid was performed using the fragment-exchanged plasmids. After the selection of drug-resistant clones, the drug sensitivity was determined in two ways; the first method involved continuous exposure on agar plates containing the drug being analyzed and growth at 35°C for 3 to 5 days (Wasserman and Wang, 1994). Yeast cultures of known optical density were serially diluted and plated from microtiter trays using a replicator consisting of an aluminum block with an array of cylindrically shaped prongs. Determination of the concentration of drug that the βE522K and βwt yeast transformants can grow on gives -fold resistance values. This method allows drugs to be analyzed rapidly, enabling determination of the cross-resistance profile with many different drugs. The second method employed short-term exposure to the drug in liquid culture followed by growth in drug-free plates to determine the minimum lethal concentration (MLC) (Nitiss, 1994).

In Vitro Characterization

Protein production. Recombinant htopo IIβ proteins were expressed in the yeast strain JEL1Δtop1bearing plasmid YEphTOP2βKLM or YEphTOP2βE522K, and purification of the recombinant proteins was performed as described previously (Austin et al., 1995).

Strand Passage Assays. Decatenation and relaxation assays were carried out in relaxation buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM dithiothreitol, 100 mM KCl, and 30 μg/ml bovine serum albumin) with 1 μg of supercoiled pBR322 plasmid DNA or 400 ng of kinetoplast DNA, 2 mM ATP, and 10 mM $MgCl_2$, as described previously (Austin et al., 1995; West et al., 2000).

Surface Plasmon Resonance. The method has been described previously (Leontiou et al., 2003)

DNA Cleavage Assays. Cleavage of a 40-bp oligonucleotide and a 4.3-kb linearized pBR322 was performed as described previously (Marsh et al., 1996).

Reversal of Cleavage Assays. The fragment from 375 to 939 bp of pBR322 was amplified by PCR. Cleavage was carried out as described by West et al. (2000). Reversibility experiments were carried out by adding 25 mM EDTA to inhibit further cleavage and then 500 mM NaCl to promote religation. The cleavage products were then stabilized by the addition of 0.5% SDS. The reaction was incubated at 37°C for 0, 2, 5, 10, 20, and 30 min to measure the stability of cleavable complexes (Osheroff and Zechiedrich, 1987; Strumberg et al., 1999a).

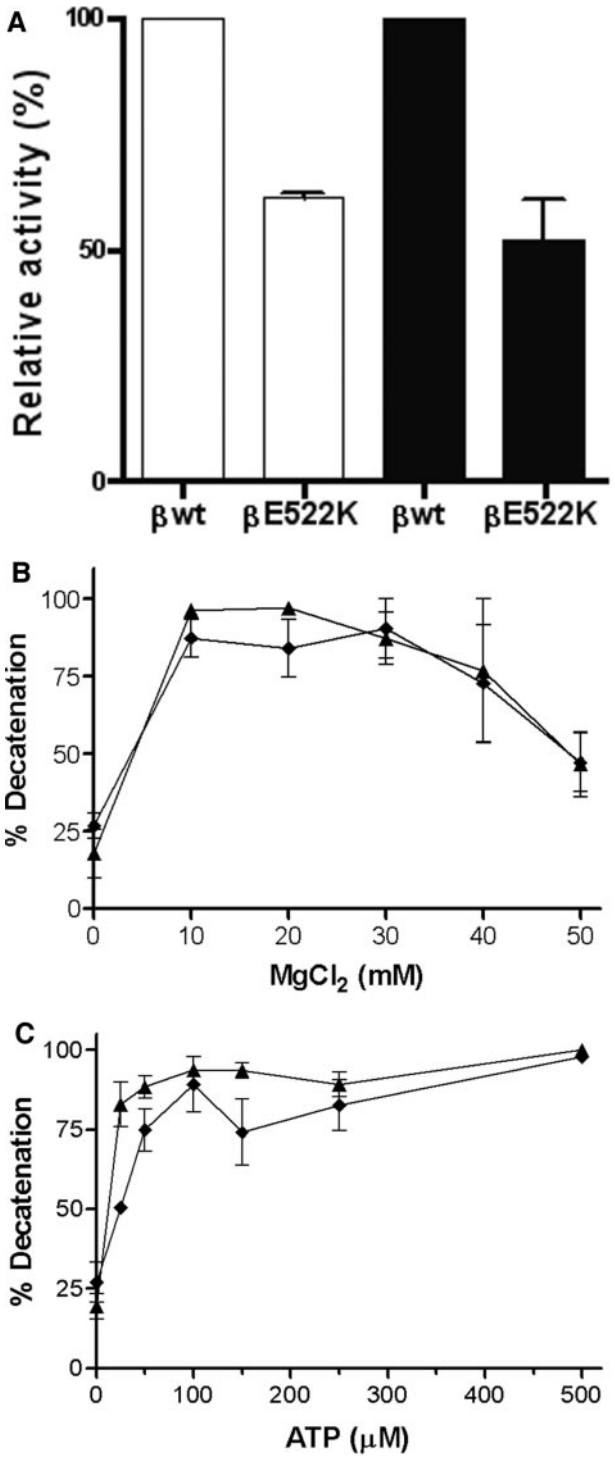

Fig. 2. Strand passage activity. A, histograms of the relative relaxation activity (□) and relative decatenation (■) for βwt and βE522K. βwt is set to 100%. Means were calculated from at least three experiments; error bars represent 1 S.D. from the mean. B, effect of Mg^{2+} concentration on decatenation by βwt (▲) and βE522K (♦). Means and standard deviations are derived from at least three experiments. C, effect of ATP concentration on decatenation by βwt (▲) and βE522K (♦). Means and standard deviations are derived from at least three experiments.

Results

Selection and in Vivo Characterization of AMCA-Resistant Yeast Transformants. A forced molecular evolution approach was used to identify mutations in DNA topo IIβ that caused resistance to AMCA. The wild-type topo IIβ (βwt) cDNA was mutagenized by exposure to hydroxylamine. This library of randomly mutated plasmids was then transformed into the yeast strain JN394 top2–4, in which growth at the nonpermissive temperature is dependent upon a functional plasmid-borne human topo II. Drug-resistance transformants were selected by exposure to 200 μM (76 μg/ml) AMCA in liquid culture for 96 h [i.e., 10 times the IC_{50} for yeast bearing human topo II (Turnbull et al., 1999)]. The surviving yeast were then plated on drug-free plates and grown for five to seven days at 35°C. More than 1000 colonies grew, and these were replica-plated onto plates containing AMCA at 10 or 76 μg/ml and grown for 5 days at 35°C. About 300 colonies were able to grow on the drug plates containing 10 μg/ml, but only 49 survived on drug plates containing 76 μg/ml. Plasmids were successfully rescued from 29 of the most resistant yeast transformants. Yeast strain JN394 top2–4 was retransformed with these plasmids, and the transformants were grown on plates containing AMCA. Eleven transformants were able to grow on plates containing 10 μg/ml AMCA, confirming that their drug resistance was plasmid-borne. Five were also able to grow on plates containing 40 μg/ml AMCA and two on plates containing 76 μg/ml AMCA.

The topo IIβ cDNA inserts in 11 plasmids confirmed to confer AMCA resistance were sequenced. Two clones had no changes in the coding sequence, suggesting that although the resistance was plasmid-borne, the hydroxylamine-induced mutation must be elsewhere in the plasmid, possibly in the gal promoter, for example, because decreased expression of topo IIβ would also confer resistance to the topo II poison AMCA. These two plasmids were not investigated further. In the remaining nine plasmids, single point changes of C to T were detected in the sequence of topo IIβ. The four plasmids only able to grow on 10 μg/ml all bore the same point change at base 1648, resulting in a glycine-to-arginine substitution of amino acid residue 550 (G550R). Three plasmids able to grow on 40 μg/ml (but not 76 μg/ml) all bore the same point change at base 1981, causing an aspartic acid-to-asparagine substitution at amino acid residue 661(D661N). Two plasmids conferring resistance to 76 μg/ml had different point mutations, one at base 1786, causing a substitution of alanine for a threonine at amino acid residue 596 (A596T), and the other at base 1564, causing a change from glutamic acid to lysine at amino acid residue 522, βE522K. Herein, we report the detailed characterization of βE522K that conferred ~10-fold resistance to AMCA. βA596T was not characterized further, and characterization of βD661N and G550R will be reported elsewhere.

Fragment exchange into an unmutagenized vector of the 1674-bp region that contained the C-to-T change, resulting in the βE522K mutation, was performed. The fragment-exchanged construct was transformed into the JN394 top2–4 strain. The temperature-sensitive mutation in the endogenous topo II gene in this strain permits growth of the yeast cells at 25°C but not 35°C. βE522K was able to complement the endogenous yeast enzyme at 35°C, confirming that this mutated protein is functional in vivo (data not shown). However, the JN394 top2–4 strain transformed with βE522K grew more slowly than this yeast strain transformed with βwt. βE522K was able to grow on plates containing AMCA,

confirming that the drug-resistant phenotype was a result of the E522K mutation and that AMCA targets topo IIβ in vivo.

The drug-resistance phenotype of the mutated protein was compared with the wild type by growing JN394 top2–4 expressing βwt or βE522K on adenine-supplemented yeast extract/peptone/dextrose plates containing different drugs, including AMCA, mAMCA, mAMSA, etoposide, ellipticine, and doxorubicin. The level of growth on drug plates containing increasing concentrations of drug is shown in Table 1. βE522K was found to be ~10-fold resistant to mAMSA, AMCA, and mAMCA and ~2-fold resistant to doxorubicin but was ~2-fold hypersensitive to etoposide and ellipticine (Table 1).

The level of drug resistance to mAMSA and hypersensitivity to etoposide was further quantified using the minimum lethal concentration method (Nitiss, 1994), to allow direct comparisons with previously reported mutations resistant to mAMSA and etoposide. JN394 top2–4 were transformed with plasmid encoding βwt or βE522K and were grown in the presence of different concentrations of mAMSA for 6 and 24 h as shown in Fig. 1, A and B, respectively. JN394 top2–4 transformed with plasmid encoding βwt or βE522K in the presence of different concentrations of etoposide as shown in Fig. 1C. The data shown in Fig. 1 are the percentage of relative survival versus drug concentration. The drug concentration that gave 100% survival was taken as the MLC.

The MLC after 6 h was 20 μg/ml for JN394 top2–4 containing βE522K (Fig. 1A) and 1.4 μg/ml for JN394 top2–4 containing βwt (Fig. 1A); thus, βE522K is ~14-fold resistant to mAMSA after a 6-h exposure. The MLC after a 24-h mAMSA exposure was >20 μg/ml for the βE522K transformant (Fig. 1B) and 2 μg/ml for the βwt transformant (Fig. 1B), so βE522K is more than 10-fold resistant when exposed to mAMSA for 24 h. The percentage of relative survival in the presence of etoposide is shown in Fig. 1C. The MLC for the βE522K transformant is 100 ± 21.8 μg/ml (Fig. 1C) and for the βwt transformant, it is 163 ± 7.6 μg/ml (Fig. 2A), this demonstrates that βE522K is 1.6-fold hypersensitive to etoposide compared with the wild-type enzyme, this difference is statistically significant ($p < 0.05$ in a t test).

βE522K Shows Decreased Strand Passage Activities Compared with the βwt Protein. Wild-type and mutated proteins were expressed in yeast, and the enzymes were purified to >95% homogeneity. The strand passage activities of βwt and βE522K proteins were assayed. βE522K showed reduced relaxation (61.2%) and decatenation (52.1%) compared with the wild-type protein. The effect of KCl, $MgCl_2$, and ATP on decatenation was also measured, and the glutamic acid-to-lysine mutation at residue 522 did not alter the salt or energy cofactor optima of the enzyme (Fig. 2 and data not shown).

DNA Binding Properties of βE522K. The DNA binding properties of βwt and βE522K proteins were tested using surface plasmon resonance (Fig. 3). Three substrates were analyzed: a 40-bp linear oligonucleotide also used for the cleavage experiments, a synthetic four-way junction substrate, and a bent DNA substrate. The binding data were fitted to a 1:1 Langmuir binding model (Leontiou et al., 2003). Both the βwt and βE522K were able to bind to all three DNA substrates with comparable K_d values (Table 2). These K_d values indicate that the activation energies for βE522K and βwt binding to DNA are not significantly different; thus, βE522K does not have a reduced binding affinity for these DNA substrates.

DNA Cleavage Properties of βE522K Protein. The cleavage properties of the βwt and βE522K proteins were tested by incubating the protein with a ^{32}P-end-labeled 40-bp linear DNA substrate. SDS was added to stop the reaction, which denatures the topo II protein, thus "fixing" the reaction at that point in the equilibrium; the denatured protein is removed by digestion with proteinase K, and the cleaved DNA products were then analyzed by polyacrylamide gel

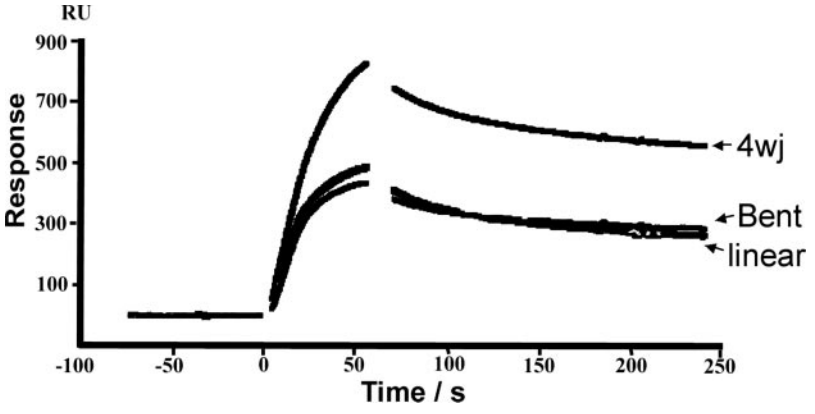

Fig. 3. DNA binding of human topo IIβE522K by surface plasmon resonance. Binding of βE522K (42 nM) to three different DNA substrates, a linear 40-bp oligonucleotide, a four-way junction, and a bent DNA fragment. The experiment was carried out at 25°C, the association phase was 60 s, and the dissociation phase was 180 s. Resonance units are plotted versus time.

TABLE 2

Surface plasmon resonance analysis of E522K DNA binding

The average values of association (k_a) and dissociation (k_d) rate constants for binding of βE522K to three different DNA substrates. The values of the rate constants shown here are the mean value from 12 experiments; the S.D. from the mean value is shown in parenthesis. The equilibrium constants K_a and K_d were estimated using the experimental values of the association (k_a) and dissociation (k_d) rate constants. The average K_d for βwt is shown. This βwt* data was previously reported in Leontiou et al. (2003).

DNA substrate	k_a	K_d	K_a	K_d	βwt × K_d
	$M^{-1}s^{-1} \times 10^6$	$s^{-1} \times 10^{-3}$	$M^{-1} \times 10^8$	$M \times 10^{-9}$	
40 bp	1.40 (± 0.61)	2.30 (± 0.46)	6.08	1.64	1.73
4wj	1.26 (± 0.25)	1.79 (± 0.05)	7.04	1.42	2.85
Bent	1.38 (± 0.57)	1.47 (± 0.32)	9.38	1.06	1.86

electrophoresis. The natural divalent cation for DNA cleavage is magnesium. For mechanistic analysis, cleavage can be enhanced by the presence of calcium ions instead of magnesium; however, calcium cannot support strand passage (Osheroff and Zechiedrich, 1987). DNA cleavage is also increased in the presence of topo II poisons that stabilize cleavable complexes. The ability of the βwt and βE522K proteins to cleave a 40-bp linear DNA substrate was initially tested (Fig. 4). This substrate was selected because it contains one mAMSA site known to produce two cleavage products of 21 and 15 nucleotides (Marsh et al., 1996). However, cleavage of this substrate is not promoted by AMCA (data not shown).

The drug-independent cleavage by βwt and E522K enzymes was tested in the presence of magnesium or calcium ions. The drug-dependent cleavage in the presence of mAMSA was also analyzed to determine the response of the enzymes to this drug.

To calculate the relative cleavage under these conditions, the amount of end-labeled cleaved product in several experiments was quantified by filmless autoradiographic analysis using a BAS-1500 (Fujifilm, Tokyo, Japan). The amount of cleavage with wild-type protein in the presence of mAMSA and magnesium was taken as 100%, and the cleavage under different conditions was calculated relative to this.

The βwt protein cleaved the DNA substrate weakly in the presence of magnesium ions (Fig. 4, lane 2), and addition of mAMSA enhanced the levels of cleavage by 12-fold (100%) (Fig. 4, lane 3). Cleavage was increased by 5-fold in the presence of calcium ions (42 ± 7.7%) (Fig. 4, lane 4) compared with magnesium alone (7.8 ± 2.4%) (Fig. 4, lane 2),and was highest in the presence of calcium and mAMSA (376.4 ± 97.6%) (Fig. 4, lane 5), approximately 50-fold higher than with magnesium and no drug. Cleavage by βE522K was very weak in the presence of magnesium ions in this assay (3.8 ± 0.6%) (Fig. 4, lane 7), and cleavage was not enhanced when mAMSA was added to the reaction (3.7 ± 0.7%) (Fig. 4, lane 8). In the presence of calcium ions, weak cleavage was observed (11.5 ± 2.8%) (Fig. 4, lane 9), but this was 4-fold less than seen with βwt plus calcium (42 ± 7.7%) (Fig. 4, lane 4). The cleavage by βE522K in the presence of calcium was not further enhanced by mAMSA (12.9 ± 3.8%) (Fig. 4, lane 10) and was thus ~30-fold lower than βwt under the same conditions (376.4 ± 97.6%) (Fig. 4, lane 5). From this, we concluded that the cleavable complex with the mutant enzyme was trapped less efficiently by mAMSA or calcium.

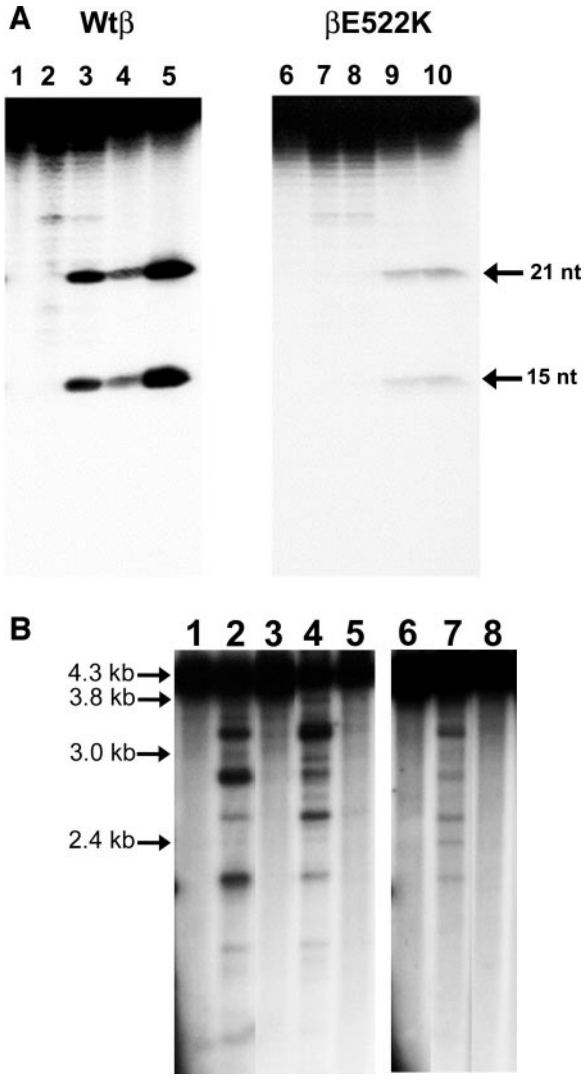

Fig. 4. Cleavage of the ^{32}P-labeled 40-bp DNA substrate by βwt and βE522K. A, cleavage of an oligonucleotide was assessed under a variety of reaction conditions. Lanes 1 and 6 contained no protein; lanes 2 to 5 and 7 to 10 contained 1.2 μg of protein plus Mg^{2+} (lane 2 and 7), Mg^{2+} and mAMSA (lane 3 and 8), Ca^{2+} (lanes 4 and 9), and Ca^{2+} and mAMSA (lanes 5 and 10). Lanes 1 to 5 show βwt cleavage; lanes 6 to 10 show βE522K cleavage. Cleavage generated two products of 21 and 15 nucleotides (nt) that were quantified by filmless autoradiographic analysis (Fujifilm BAS-1500). This figure shows a single experiment that is typical of three others. B, cleavage of the ^{32}P-labeled, 4.3-kb pBR322 probe in the presence of mAMSA, mAMCA, or AMCA. Lanes 1 and 6 contain no protein, lanes 2, 4, and 7 contain βwt protein with mAMSA, mAMCA, or AMCA, respectively, and lanes 3, 5, and 8 contain βE522K protein with mAMSA, mAMCA, or AMCA, respectively.

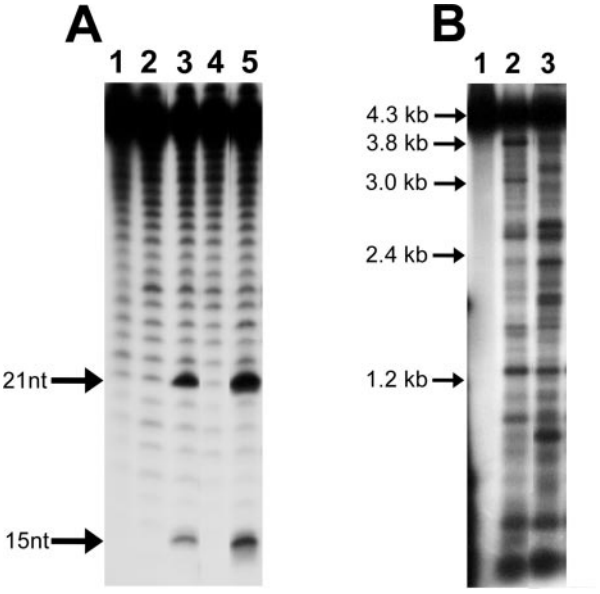

Fig. 5. Cleavage properties of βwt and βE522K in the presence of etoposide. A, cleavage of the ^{32}P-labeled 40-bp oligonucleotide substrate in the presence of etoposide. Cleavage was carried out using 1.2 μg of protein in the presence of 100 μg/ml of etoposide and 10 mM $MgCl_2$. Lane 1, no protein control; lanes 2 and 3, βwt protein without and with etoposide; lanes 4 and 5, βE522K protein without and with etoposide, respectively. B, cleavage of the ^{32}P-labeled, 4.3-kb pBR322 probe in the presence of etoposide; lane 1 contains no protein, lane 2 contains βwt and etoposide, and lane 3 contains βE522K and etoposide.

The cleavage ability of βE522K and βwt in the presence of etoposide was also tested (Fig. 5). Etoposide can stabilize cleavage by βwt on the 40-bp DNA substrate used in this study (Fig. 5A). The results showed that βE522K was able to cleave in the presence of etoposide (Fig. 5A, lane 5) at levels similar to those of the wild-type enzyme (Fig. 5A, lane 3). Because βE522K without drug cleaves the oligonucleotide substrate half as much as the wild-type enzyme without drug (Fig. 5A, lanes 4 and 2, respectively), the relative stimulation of cleavage by E522K with etoposide is greater than that seen with the wild-type enzyme, in line with the 1.6-fold hypersensitivity seen in vivo (Fig. 1C). The cleavage with etoposide shows that βE522K is not defective in all drug-dependent cleavage. The reduced mAMSA promoted cleavage, but cleavage with etoposide suggests βE522K may have a reduced affinity for mAMSA but not for etoposide.

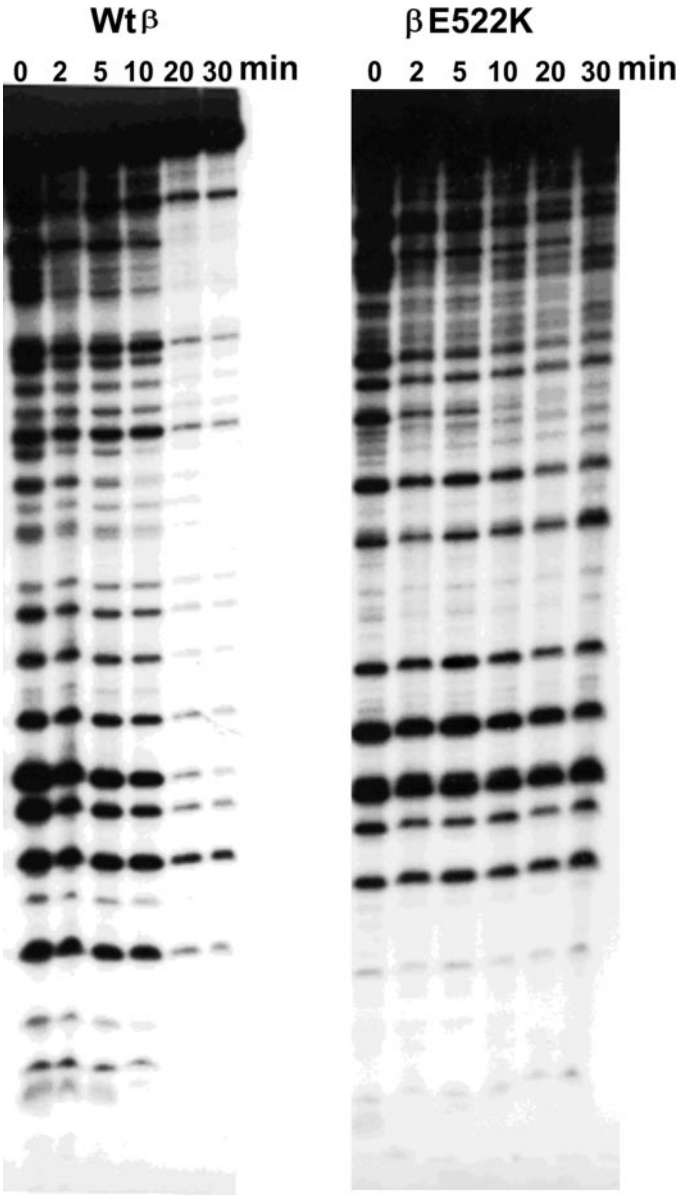

Fig. 6. Reversibility of cleavable complexes in the presence of etoposide. Protein (1.2 μg) was incubated with the 500-bp ^{32}P-labeled pBR322 substrate for 30 min at 37°C in the presence of 10 mM MgCl. Then, 25 mM EDTA and 500 mM NaCl were added, and the reactions were incubated for the indicated time points. Reversibility reactions were terminated with the addition of 0.5% SDS.

Cleavage of the ^{32}P-end-labeled 4.3-kb pBR322 fragment was carried out to test the cleavage pattern given by βE522K enzyme compared with βwt. βE522K was unable to cleave in the presence of mAMSA, mAMCA, or AMCA (Fig. 4B, lanes 3, 5 and 8, respectively), whereas βwt was able to cleave under these conditions (Fig. 4B, lanes 2, 4, and 7, respectively). In contrast, in the presence of etoposide, the βE522K protein was able to cleave the 4.3-kb pBR322 substrate (Fig. 5B, lane 3) but with a different cleavage pattern compared with the βwt (Fig. 5B, lane 2).

βE522K Forms More Stable Cleavable Complexes in the Presence of Etoposide Compared with βwt. βE522K cleavage in the presence of etoposide and the in vivo data shows that βE522K is hypersensitive to etoposide poisoning. To investigate the hypersensitivity to etoposide, the stability of the cleavable complexes of βE522K compared with βwt was examined. The ability of salt to reverse complexes was used. The enzyme was incubated with a 500-bp pBR322 substrate for 30 min. Thereafter, EDTA was added to inhibit further cleavage, followed by NaCl to shift the equilibrium toward the religation reaction (Osheroff and Zechiedrich, 1987). The reversibility reaction was incubated for 0, 2, 5 10, 20, or 30 min (Fig. 6). Etoposide-induced cleavable complexes with the βwt were stable for 10 min after salt addition. However, after a 20-min incubation, a substantial reduction in the level of cleavable complexes was seen, as reported previously for βwt (Errington et al., 2004). In contrast, βE522K cleavable complexes remained stable in the presence of etoposide for at least 30 min. Thus, βE522K formed more stable cleavable complexes than βwt in the presence of etoposide, which may explain the hypersensitivity of βE522K to etoposide.

Discussion

βE522K is ~10-fold resistant to AMCA and ~10-fold cross-resistant to mAMSA and mAMCA but ~2-fold hypersensitive to etoposide and ellipticine, which confirms that AMCA targets β in vivo. The selection procedure is dependent upon the mutated proteins' being functional in vivo. However, the yeast expressing βE522K grew more slowly than those expressing βwt, possibly because of βE522K's reduced decatenation activity. The in vitro DNA binding properties of βE522K were similar to βwt; therefore, it is unlikely that reduced DNA binding accounts for the altered catalytic properties. In addition, the profile of resistance to some drugs but hypersensitivity to others suggests that βE522K alters the region(s) of the enzyme involved in drug interactions.

In vitro βE522K showed no cleavage stimulation by acridines, unlike βwt, suggesting that resistance is caused by a reduction in cleavable complex formation. Mutation E522K also reduced drug-independent cleavage of an oligonucleotide substrate with either magnesium or calcium. DNA cleavage, however, was stimulated by etoposide. The cleavable complexes with βE522K and etoposide were more stable than those with βwt and etoposide, accounting for βE522K's hypersensitivity to etoposide. The longer-lived complexes with etoposide may result from an alteration in the cleavage-religation equilibrium, but because βE522K cleavage with mAMSA did not reverse more quickly than βwt, this possi-

bility was discounted. The reversibility experiments suggest that acridine binding is reduced and that etoposide binding is increased. Hypersensitivity to etoposide has been seen previously in the quinolone-resistant yeast topo II Ser740Trp (Hsiung et al., 1995; Strumberg et al., 1999b), whereas yeast topo II H1012Y was resistant to etoposide and CP115,953 but hypersensitive to ellipticine and mAMSA (Elsea et al., 1995).

Topo II has an N-terminal ATPase domain, a breakage-reunion domain composed of B′ and A′ subfragments and a C-terminal domain. Position 522 is within the B′ subfragment, a region highly conserved from prokaryotes to humans. The homology between bacterial DNA gyrase (Gyr B), bacteriophage topoisomerase II (T4 Gn 39 and Gn 60 subunits), yeast topo II, and the two related human isoforms α and β is shown in Fig. 7.

In addition to βE522K, other drug resistance mutations are located in the B′ subfragment, and their positions are indicated in Fig. 7. For example, R486K in htopo IIα (PLRGK) has been described to cause drug resistance to mAMSA in human cells (Bakic et al., 1986; Hinds et al., 1991; Lee et al., 1992; Kubo et al., 1996). Random mutagenesis of the human α isoform and selection in yeast in the presence of mAMSA also identified R486K and E571K (Patel et al., 2000). These mutations were at least 25- and 100-fold resistant to mAMSA and ~3-fold resistant to etoposide, whereas βE522K is ~10-fold resistant to mAMSA and ~2-fold hypersensitive to etoposide. Mutations in yeast topo II at K478A (PLRG**K**) and A642G/T render yeast resistant to mAMSA (Wasserman and Wang, 1994). Mutation E457K in T4 topo II subunit Gn39 conferred mAMSA resistance; E457K is homologous to βE522K. This enzyme also displayed hypersensitivity to etoposide; however, unlike βE522K, it was resistant to ellipticine (Huff and Kreuzer, 1990; Freudenreich et al., 1998). Mutations in Gyrase B at D426N (EG**D**SA) or K447E (PLRG**K**) confer resistance to the quinolone nalidixic acid (Yoshida et al., 1991). Two cell lines resistant to teniposide have mutations in the B′ subfragment, R493Q in the Chinese hamster cell line VpmR (Chan et al., 1993) and R449Q in human topo IIα in the CCRF CEM VP1 cell line (Danks et al., 1993). This clustering of mutations in the B′ subfragment led to the suggestion that this region contained a drug binding pocket (Huff and Kreuzer, 1990), in agreement with the data presented in this article.

The high similarity between human topo IIβ and yeast topo II around position 522 has allowed us to use the yeast crystal structure to model the human topo IIβ enzyme to investigate the structural effect of mutating glutamic acid 522 to a lysine. Using the alignment of the human and yeast enzymes, the computer program Modeler was used to model residues 420 to 1063 of the βwt monomer onto the T2O yeast crystal structure (Fig. 8) (Berger et al., 1996; Protein Data Bank code 1bgw). The computer program Modeler is conservative in that modeled structures are based very closely on the template structure (Sali and Blundell, 1993).

The B′ subfragment contains a βαβαβαβα fold, or a "Rossmann fold" (Berger et al., 1998); glutamic acid 522 lies in the middle of the second α helix (α2) of this Rossmann fold. This fold contains three conserved motifs, EGDSA, PL(R/K)GK, and IMTD(Q/A)DXD. Residues in these motifs are conserved with type IA topoisomerases, DnaG-type primases, OLD family nucleases, and RecR proteins and has led to the further designation of this fold as the Toprim domain (Aravind et al., 1998).

This Rossman fold is involved in metal ion coordination. Mutations in βE477and βK505 in the Rossman fold alter the magnesium optima for decatenation. Glu477 and Lys505 are proposed to interact with the β3α3 loop (**DQDQD**GS) containing the acidic residues Asp557, Asp559, and Asp561, enabling these acidic residues to coordinate the metal ions required to catalyze DNA cleavage. Two possible locations for magnesium binding were modeled, termed position A and position B (Fig. 8A) (West et al., 2000). Mutations in DNA gyrase confirm that these aspartic acid residues are involved in metal ion binding and that at least two metal ions are required for DNA cleavage in addition to the metal requirement for ATPase activity (Noble and Maxwell, 2002). The unaltered magnesium optima of βE522K decatenation (Fig. 2) indicates that E522K does not affect binding of magnesium ions needed to catalyze DNA cleavage.

The B′ subfragment is a dynamic region that occupies different positions depending upon the point in the catalytic cycle; thus, it has variable interactions with the rest of the protein. Based on a crystal structure with an alternative conformation, T2M, it has been suggested that during DNA cleavage, the conformation of the protein changes so that the B′ subfragment containing the Rossman fold bearing the

```
                      1        2                           3 4    5           6
HsTop2β  456  KLDDANDAEEKHSLECTLILTEGDSAKSLAVSGLGVIGRDRYGVFPLRGKILNVREASHKQIMENAEINN
HsTop2α  440  KLDDANDAGGRNSTECTLILTEGDSAKTLAVSGLGVVGRDKYGVFPLRGKILNVREASHKQIMENAEINN
ScTop2   428  KLEDANKAGTKEGYKCTLVLTEGDSALSLAVAGLAVVGRDYYGCYPLRGKMLNVREASADQILKNAEIQA
EcGyrB   406  KLADCQ...ERDPALSELYLVEGDSA...GGSAKQGRNRKNQAILPLKGKILNVEKARFDKMLSSQEVAT
T4Gn39   401  KANLC....GKDADT.TLFLTEGDSA...IGYLIDVRDKELHGGYPLRGKVLNSWGMSYADMLKNKELFD
              ---------------------------------------------------------------  ------

                                      7                                       8
HsTop2β  526  IIKIVGLQYKKSYDDAESLKTLRYGKIMIMTDQDQDG.SHIKGLLINFIHHNWPSLLK..HGFLEEFITP
HsTop2α  510  IIKIVGLQYKKNYEDEDSLKTLRYGKIMIMTDQDQDG.SHIKGLLINFIHHNWPSLLR..HRFLEEFITP
ScTop2   498  IKKIMGLQHRKKYEDT...KSLRYGHLMIMTDQDHDG.SHIKGLIINFLESSFLGLLD.IQGFLLEFITP
EcGyrB   470  LITALGCGIGRDEYNP...DKLRYHSIIIMTDADVDG.SHIRTLLLTFFYRQMPEIVE..RGHVYIAQPP
T4Gn39   463  ICAITGLVLGEKAFEEKEDGEWFTFELNGDTIIVNENDEVQINGKWITVGELRKNL
T4Gn60    10  SVDMKKYKLQNNVRRSIKSSSMNYANVAIMTDADHDGLGSIYPSLLGFF.SNWPELFE..QGRIRFVKTP
              ----------------------------------------------------------------------
```

Fig. 7. Alignment of topoisomerase II enzymes from different species in the region of Glu522. Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*; Ec, *E. coli* Gyrase B; T4 topo II subunits 39 and 60. Conserved motifs are shown in bold. Residue Glu522 is a highly conserved residue; it is underlined and labeled 6 on this figure. Numbers above the alignment indicate the positions of the following mutations in the B′ subfragment 1, HsαR449Q (Danks et al., 1993); 2, EcGyrB D426N (Yoshida et al., 1991); 3, Hsα R486K (Patel et al., 2000); 4, Sc K478A (Wasserman and Wang, 1994) and EcGyrB K447E (Yoshida et al., 1991) 5, CHαR493Q (Chan et al.,1993); 6, HsβE522K (reported here) and Gn39E457K (Freudenreich et al., 1998); 7, HsβG550R; and 8, Hsα E571K (Patel et al., 2000). The region encoding the Rossmann fold or Toprim domain is indicated by a dashed line beneath the alignment.

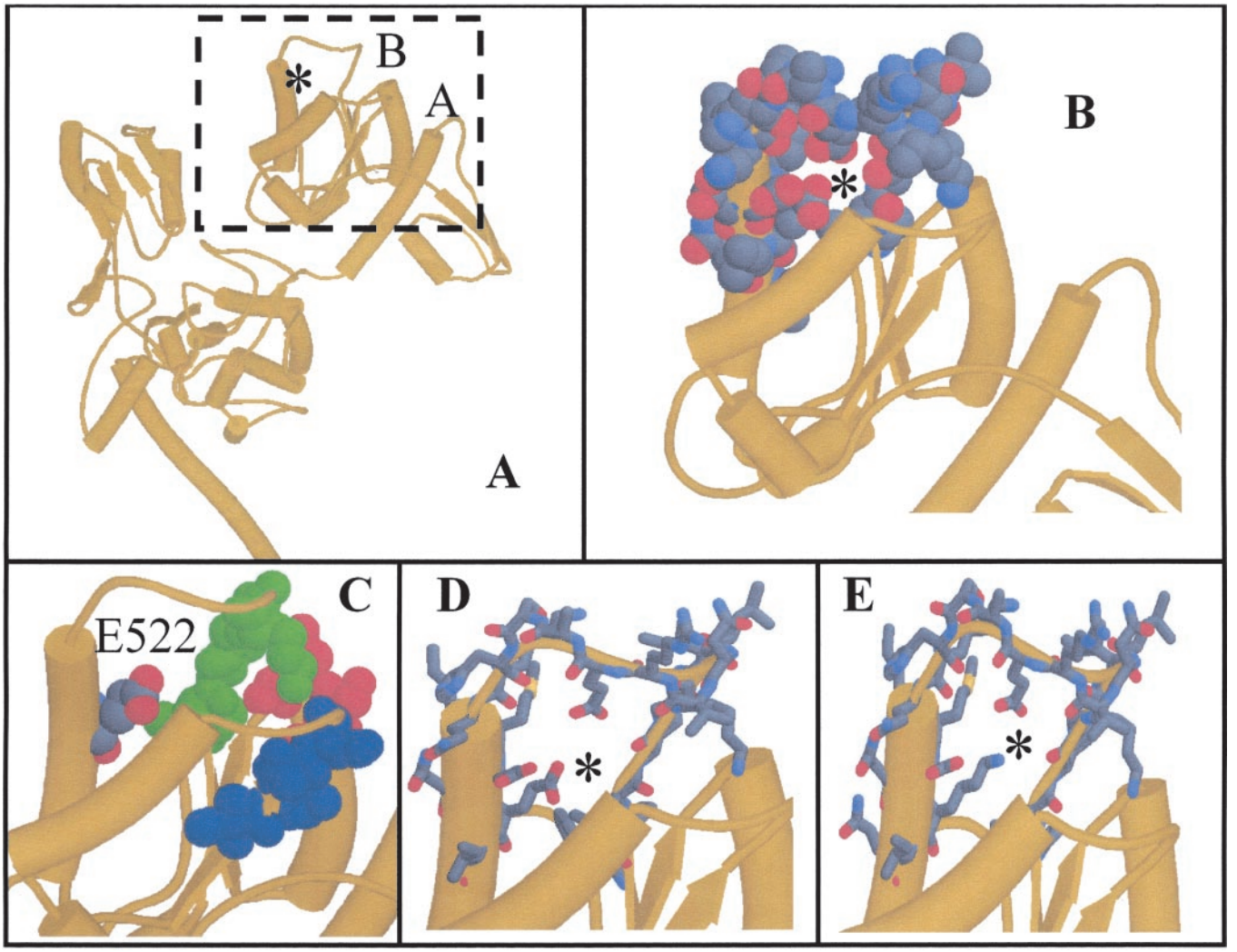

Fig. 8. Structural context of mutation βE522K. Human topo IIβ was modeled onto the yeast topo II T2O structure; position 522 is marked with an asterisk. A, a diagram of one monomer of human topo IIβ showing position 522 and two sites (A and B) predicted to bind metal ions in a previous paper (West et al., 2000). The dashed oblong defines the region displayed in B to D. B, van der Waals representation of residues 60 to 77 (equivalent to β505 to 523, where 76 = β522), in context showing the loop-like structure with a central pore and the exposed carboxyl and carbonyl groups likely to bind a metal ion in this pocket. C, βGlu522 and the three highly conserved motifs within this region are shown in van der Waals spheres and are color coded: EDGSA in red, PLRGK in green, and IMTDQ in blue. D, side chains in the loop region showing the context of the mutation site. E, loop region showing the possible conformation of the lysine in βE522K.

IMTD(Q/A)DXD motif moves nearer the active site tyrosine in the A′ subfragment to form the DNA cleavage site and drug binding pocket (Morais Cabral et al., 1997; Fass et al., 1999; Liu and Wang, 1999). Unfortunately, we cannot determine the position of Glu522 in this T2M structure because the region containing the yeast homolog of Glu522 (Glu494) is not resolved in the T2M yeast crystal structure (Fass et al., 1999; Protein Data Bank code 1bjt).

Glu522 is a highly conserved residue. Mutation to a lysine does not alter DNA binding (Fig. 3) but does result in several functional changes, including resistance to cleavage promoted by acridines but good cleavage by etoposide. The B′ subfragment potentially has a drug binding pocket (Huff and Kreuzer, 1990). Mutation of glutamic acid to lysine could alter the structure and affect the drug binding, making it less able to bind acridines and more able to bind etoposide. There are several possible ways in which Glu522 could contribute to the structural conformation in this region. Glu522 could form a salt bridge with an oppositely charged residue; loss of this salt bridge by mutation to lysine would result in a structural change, altering the drug binding of βE522K. On the other hand, Glu522 might contribute to the correct positioning of a structurally important divalent cation. When the human topo IIβ sequence is modeled onto the yeast T2O structure, Glu522 is directed into a pore that is bordered by negative charges, forming a potential metal ion binding site (Fig. 8, B and D) (Creighton, 1993). Mutation of Glu522 to lysine would change the charge lining this pore, altering its ability to bind a metal ion. The possible importance of a structural noncatalytic metal ion in this negatively charged pore is suggested by the yeast enzyme structure. In the yeast enzyme, there is a glutamic acid at the residue equivalent to Glu522 (ScE494), but the glutamic acid at the position equivalent to β519 is a lysine in the yeast (ScK491). ScE494 and ScK491 could form a salt bridge across the middle of the pore. Hence, the human enzyme that lacks this stabilizing interaction may compensate with a bound metal ion. Because the mutagenesis screen selected only active drug-resistant phenotypes, the lysine in

βE522K may play a stabilizing role via a salt bridge analogous to the yeast enzyme while simultaneously altering the drug-binding pocket.

In this study, mutation βE522K was selected because it conferred in vivo drug resistance to AMCA, an agent that is active on noncycling cells. βE522K is cross-resistant to mAMSA and mAMCA but hypersensitive to etoposide and ellipticine. Cleavable complexes formed with etoposide were more stable with βE522K, whereas resistance to the acridines was caused by a reduction in the number of cleavable complexes. We conclude that βE522K has altered drug affinities for these agents and that drug binding is reduced for the acridines and increased for etoposide. These results indicate that the structurally similar acridines (mAMSA, AMCA, and mAMCA) have a similar mechanism of targeting the human topo IIβ enzyme.

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