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Involvement of Amino Acids 361 to 364 of Human Topoisomerase I in Camptothecin Resistance and Enzyme Catalysis

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ABSTRACT. Camptothecins are antineoplastic drugs that specifically target the enzyme DNA topoisomerase I. Prior work has identified a human topoisomerase I mutation, F361S, that confers resistance to camptothecin. We now demonstrate that substitutions in the 361–364 region can alter DNA cleavage/ligation by the enzyme. The defective catalysis exhibited by certain mutants likely relates to an impaired interaction with DNA, since these enzymes are more sensitive to the inhibitory effects of DNA binding ligands. Moreover, studies with peptides and fusion proteins suggest that the 361–364 region may bind DNA directly. The finding that the 361–364 region is involved in both enzyme catalysis and camptothecin resistance suggests that this region is part of the active site of human topoisomerase I and that camptothecin may interact with the enzyme at this site. BIOCHEM PHARMACOL 53;7:1019–1027, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. topoisomerase; camptothecin; DNA binding; DNA cleavage; drug resistance; enzyme mutations

Eukaryotic type I topoisomerases are capable of relaxing both negatively and positively supercoiled DNA. This function is believed to be important in cellular processes requiring access to DNA, such as transcription and replication [1–5]. The catalytic activity of eukaryotic type I topoisomerases may be devided into a series of steps involving: (i) non-covalent binding to DNA, (ii) cleavage of a single strand with generation of a covalent 3′-phosphotyrosyl bond and a free 5′-hydroxyl, (iii) passage of the intact strand through the nick, and (iv) religation [6]. In the case of the human enzyme, the tyrosine at position 723 has been implicated in the formation of the 3′-phosphotyrosyl bond [7]. However, little is known about the involvement of other regions of human (topo I) § in the catalytic cycle.

The availability of compounds that impair topo I catalytic activity provides an opportunity to evaluate functional regions of the molecule. The alkaloid camptothecin is known to inhibit topo I [8, 9], and there is considerable evidence that this drug binds to a normally transient topo I–DNA covalent complex, resulting in inhibition of the religation step of the catalytic cycle [10, 11]. Studies of cell lines resistant to camptothecin have led to identification of mutant forms of topo I with reduced catalytic activity [12, 13]. Other studies of resistant cell lines have led to the discovery of mutations that confer resistance to camptothecin apparently without altering the ability of the enzyme to relax supercoiled DNA [14–16]. The present work concerns one such mutant, in which the phenylalanine at position 361 is replaced with serine (F361S) [16]. This region of the enzyme is highly conserved among eukaryotic species and is homologous to sequences in certain nucleic acid-binding proteins (Table 1), suggesting that it may be involved in DNA binding during catalysis. To explore the function of this region, additional mutants were made in which one or both of the conserved arginine residues at positions 362 and 364 were replaced with non-polar amino acids. Studies with the three mutants indicate that the 361–364 region is involved in DNA cleavage/ligation by the enzyme and suggest that this region interacts with DNA directly.

MATERIALS AND METHODS

Drugs

The sodium salt of camptothecin was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. The drug was dissolved in water to a stock concentration of 100 mM, di-

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§ *Abbreviations:* topo I; topoisomerase I; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactoside.

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Table 1. Sequence homology between the 359 and 367 region of human topoisomerase I and nucleic acid-binding proteins

Protein	Region of homology									
Topo I	359 - G	L	F	R	G	R	G	N	H	P
E2F1 (mouse)	101 - G	P	F	R	G	R	G	R	H	P
E2F1 (human)	106 - G	P	A	R	G	R	G	R	H	P
SSB1 (*S. cerevisiae*)	150 - G	G	F	R	G	R	G	N	F	R
HMG-I (human)	21 - T	E	K	R	G	R	G	R	P	R

vided into aliquots, and stored at −20°. Hoechst dye 33342 and distamycin (Sigma, St. Louis, MO) were dissolved in DMSO at stock concentrations of 10 mM, divided into aliquots, and stored at −20°.

Topo I Fusion Protein Expression Vectors

pGEX-TOP1 is an inducible prokaryotic expression vector in which coding sequences for GST are linked to the amino terminus of himan topo I [16]. pGEX-MTOP1 is identical except that this vector contains a point mutation resulting in substitution of serine for phenylalanine at position 361 [16]. Additional mutants in this region were constructed by oligonucleotide-based mutagenesis using primers in which codon 362 was altered from CGT to CTT (yielding an R362L mutant), codon 364 from CGC to GGC (yielding an R364G mutant), or both codons altered together (yielding an R362L/R364G mutant). The polymerase chain reaction and standard cloning techniques were used to replace the *NdeI/SphI* fragment in pGEX-TOP1 with a fragment containing these mutations. Similar techniques were utilized to make plasmids coding for GST-fusion proteins containing either amino acids 346–376 of human topo I, or amino acids 346–376 with R362L/R364G substitutions. In all cases, the resulting plasmids were sequenced to confirm the presence of the mutation(s).

Expression and Purification of Topo I Fusion Proteins

The protease-deficient *Escherichia coli* strain BLR (Novagen, Madison, WI) was utilized for production of fusion proteins. Cultures were grown to an $O.D._{600}$ of 0.5 to 0.7, and IPTG was added to 0.1 mM. After incubation at 25° for 4 hr, bacteria were harvested and resuspended in lysis buffer [PBS containing 1 mg/mL lysozyme, 5 mM EDTA (pH 8.0), 10 μg/mL leupeptin, 0.1 mM pepstatin, and 1 mM DTT]. Following a 15-min incubation on ice, the suspensions were frozen in liquid N_2 and stored at −80° until used. After thawing, Triton X-100 was added to 1%, PMSF was added to 1 mM, and nucleic acid was precipitated with 1 M NaCl/6% polyethylene glycol (mol. wt 8000). The supernatants were removed, equilibrated to 250 mM NaCl, and added to glutathione-Sepharose 4B beads (Pharmacia, Piscataway, NJ) in PBS containing 250 mM NaCl and 1% Triton X-100. The slurries were incubated while rotating for 1 hr at 4°. After collection by centrifugation, the beads were washed three times with PBS/1% Triton, and once with PBS. Bound fusion proteins were eluted with a solution of 200 mM NaCl, 50 mM glutathione, 75 mM Tris–HCl, pH 8.0, and concentrated using Centricon-100 ultrafiltration devices (Amicon, Beverly, MA). After measurement of protein concentration (Biorad Protein Assay, Biorad, Melville, NY), glycerol was added to 40%, and the proteins were divided into aliquots and stored at −20°. The purity of fusion proteins was assessed by SDS–PAGE followed by silver staining (BioRad Silver Stain Plus). The typical yield of this procedure is 1 mg of 70–90% pure fusion protein (assessed by silver staining of SDS–polyacrylamide gels) from each liter of bacterial culture.

Immunoblotting

Solutions of purified fusion proteins were subjected to electrophoresis in 4–15% gradient SDS–polyacrylamide gels and then were transferred to nitrocellulose filters. The blots were incubated in 5% dry milk in PBS with 0.1% Tween 20 (PBST) for 1 hr at 20°, followed by washing with PBST and incubation with a polyclonal human topo I antibody (Topogen, Inc., Columbus, OH) for 1 hr at 20°. The blots were washed with PBST and incubated with an anti-human IgG peroxidase conjugate (Amersham, Arlington Heights, IL) for 1 hr at 20°. After additional washing, the blots were developed using an enhanced chemiluminescence technique (ECL Detection System, Amersham) and Kodak X-Omat XAR film.

Plasmid Relaxation Assays

Topo I enzyme activity was measured by a DNA relaxation assay using supercoiled plasmid DNA. Unless stated otherwise, reaction mixtures (20 μL final volume) contained 1 μg supercoiled DNA, 10 mM Tris–HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, and various amounts of recombinant topo I fusion protein. In experiments using distamycin or Hoechst 33342, the mixtures contained 2 μL of drug diluted in water immediately prior to use. Reactions were performed at 37° for 30 min, and were terminated by the addition of 2.2 μL of a 10× loading buffer (5% SDS, 0.3% bromophenol blue, 16% Ficoll 400, and 10 mM NaH_2PO_4). Samples were loaded onto a 0.8% agarose gel and subjected to electrophoresis for 16 hr at 3 V/cm. The electrophoresis buffer consisted of 30 mM NaH_2PO_4, 36 mM Tris–HCl, and 1 mM EDTA, pH 7.8. Unless otherwise stated, the gels also contained 4 μM chloroquine to separate nicked, relaxed, and supercoiled DNA. After staining with ethidium bromide, gels were photographed with Kodak Ektapan film. One unit of topo I catalytic activity was defined as the amount of enzyme necessary to relax 50% of 1 μg supercoiled DNA in 30 min at 37°.

DNA Cleavage Assays

A ^{32}P-labeled 500 bp linear DNA substrate was prepared from the pGEX-TOP1 plasmid using the polymerase chain

reaction and a 5′-end-labeled oligonucleotide primer. Oligonucleotide substrates were either synthesized on an Applied Biosystems DNA synthesizer model 394 or were purchased from Bio-Synthesis, Inc. (Lewisville, TX) (Table 2). Oligonucleotides were purified by gel electrophoresis and labeled at the 5′-end prior to hybridization using T4 polynucleotide kinase and [γ-^{32}P]ATP. For hybridizations, equimolar amounts of end-labeled oligos and unlabeled complementary oligos were incubated in 250 mM NaCl for 1 hr at 45°.

Cleavage reactions were performed with approximately 10 pmol of labeled DNA substrate and various amounts of recombinant topo I in a 20-μL reaction volume containing 20 mM Tris–HCl, pH 7.5, and 5 mM EDTA. For experiments assessing intermolecular ligation, reaction mixtures also contained 10 nmol of unlabeled acceptor oligonucleotide. Reactions were incubated for 20 min at 30° and terminated by the addition of SDS to 0.5%. Proteinase K was then added to 150 μg/mL and the mixtures were incubated at 37° for 1 hr followed by phenol/chloroform extraction, ethanol precipitation, and resuspension in 2 μL of 10 mM Tris–HCl, pH 7.5, 1 mM EDTA. After the addition of 1 μL of loading buffer (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanole), samples were heated to 70° for 2 min and analyzed using denaturing polyacrylamide gels. Gels were exposed for 16–48 hr to Kodak XAR film using an intensifying screen.

Electrophoretic Mobility-Shift and Immunodepletion Assays

A duplex oligonucleotide (Table 2) was prepared and end-labeled as described above. Approximately 10 pmol (1×10^5 cpm) of the labeled duplex oligonucleotide was incubated with recombinant fusion proteins or peptides for 30 min at 20° in a buffer containing 12 mM Tris–HCl, pH 7.8, 1 mM EDTA, and 6% glycerol. The reaction products were analyzed by electrophoresis in either a 6% polyacrylamide gel or a 1% agarose gel containing 22 mM Tris–HCl, pH 8.0, 22 mM boric acid, and 0.5 mM EDTA. Gels were exposed overnight to Kodak XAR film.

Immunodepletion experiments were performed by incubating 4 μg of the topo I fusion protein with 14 μL of a 50% slurry (in PBS) of protein A-linked Sepharose beads with or without 1 μL of either a polyclonal antibody to human topo I or a polyclonal antibody to human topo II (Topogen). The mixtures were incubated for 1 hr at 37°, followed by centrifugation. The supernatant was collected and used in mobility-shift assays as described above.

Table 2. Oligonucleotides

Cleavage/Ligation assays	Mobility-Shift assays
5′-caaagtcaggctgatga (cleavage oligo)	5′-caaagtcaggctgatgagcctctt
5′-aagaggctcatcagcctgactttg (complementary oligo)	3′gtttcagtccgactactcggagaa
5′-gagcctctt (acceptor oligo)	

RESULTS

Effects of Amino Acid Substitutions in the Region of 361–364 of Human Topo I on Enzyme Activity and Sensitivity to Camptothecin

Replacement of phenylalanine with serine at position 361 in human topo I has been shown recently to confer camptothecin resistance while having little apparent effect on the plasmid relaxation activity of the enzyme [16]. To determine whether other alterations in this region could affect enzyme activity, additional mutants were constructed in which one or both of the highly conserved arginines at positions 362 and 364 were replaced with leucine and glycine, respectively. These mutants were expressed in *E. coli* as GST-fusion proteins and purified using glutathione-Sepharose affinity chromatography. Silver staining of purified proteins separated by SDS–PAGE indicated predominant bands at the expected size of approximately 120 kDa (Fig. 1). The 120 kDa bands were immunoreactive with a topo I antibody (data not shown). Serial dilutions of the purified proteins were tested in standard plasmid relaxation assays using a high substrate:enzyme molar ratio. The results indicate that under these conditions the recombinant wild-type protein possesses a specific activity of approximately 0.5×10^5 U/mg (data not shown). The activity of the F361S mutant was similar to that of the wild-type enzyme (Fig. 2). While the relaxation activities of the R362L and R364G proteins appeared slightly less than that of the wild-type enzyme, the activity of the R362L/R364G mutant was impaired markedly (Fig. 2). These findings indicate that substitutions in the 361–364 region of topo I can affect plasmid relaxation activity.

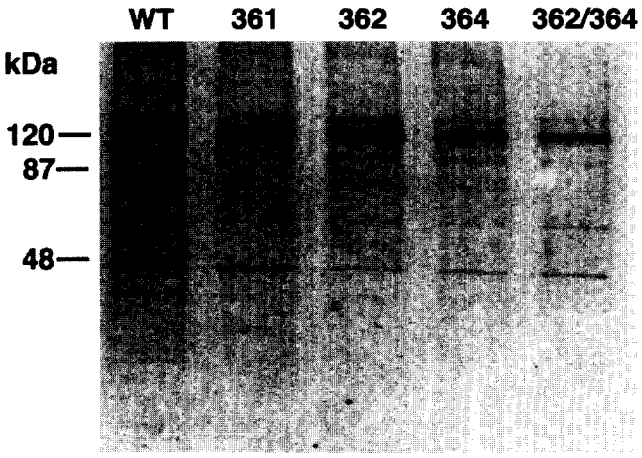

FIG. 1. Expression of wild-type and mutant GST-human topo I fusion proteins. Proteins were expressed in *E. coli* and purified as described in Materials and Methods. The purified proteins (4 μg) were separated on an SDS–polyacrylamide gel that was subsequently silver stained. WT refers to the wild-type enzyme; the mutant proteins are labeled according to the altered amino acid(s).

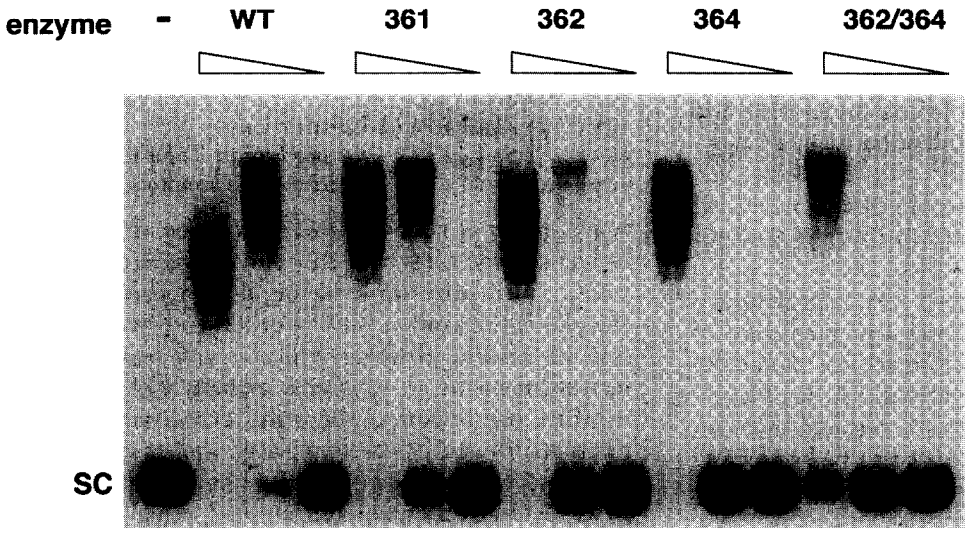

FIG. 2. Plasmid relaxation activity of wild-type and mutant human topo I proteins. One microgram of supercoiled plasmid DNA was incubated with recombinant wild-type or mutant topo I protein for 30 min at 37°. Reactions were analyzed by agarose gel electrophoresis followed by ethidium bromide staining. Topoisomerase activity was evidenced by conversion of supercoiled plasmid (labeled SC) to relaxed, lower-mobility forms. Lanes are grouped according to enzyme, with lanes in each group corresponding to 133, 33, and 8 ng of enzyme, except for the R362L/R364G enzyme, in which 10-fold greater quantities of protein were used.

Next, the ability of stoichiometric quantities of enzyme to nick linear duplex DNA was assayed in the absence or presence of camptothecin. In the absence of drug, little DNA nicking was detected using the wild-type, F361S, R362L, or R364G enzyme (Fig. 3). In the presence of 1 μM camptothecin, enhanced nicking was observed using both the wild-type and R362L mutants. These findings indicate that the R362L substitution has little effect on sensitivity to camptothecin. At 50 μM but not 1 μM camptothecin, enhanced nicking was observed with the F361S mutant, which is consistent with the known relative resistance of this mutant to the drug. By contrast, little nicking was seen with the R364G mutant even at high concentrations of camptothecin (Fig. 3). Similarly, DNA nicking was not

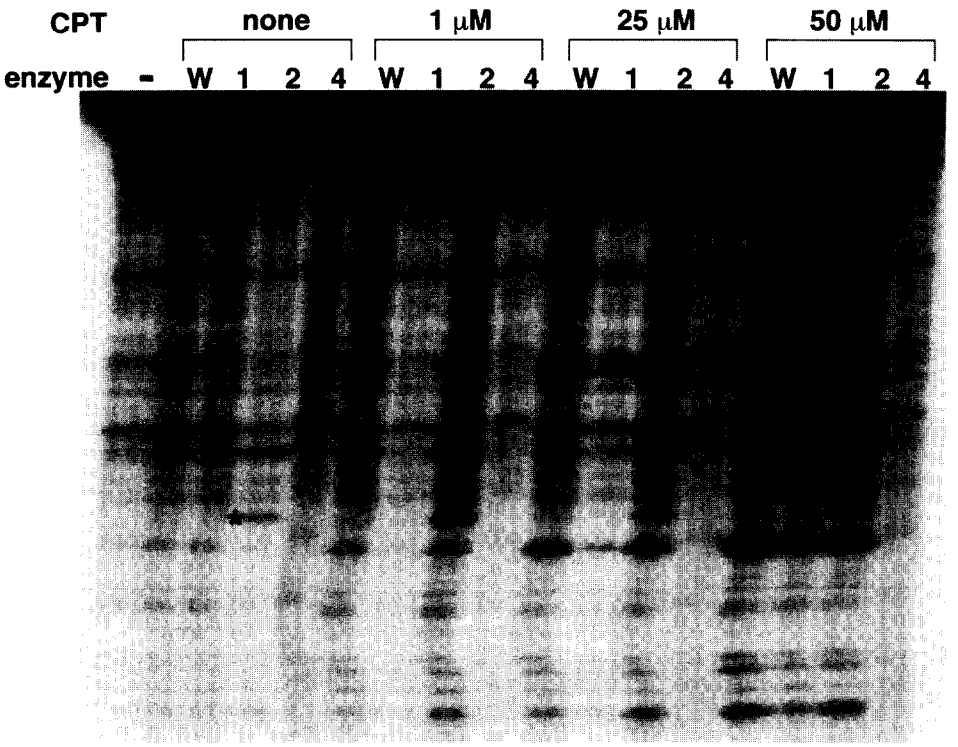

FIG. 3. DNA cleavage by wild-type and mutant human topo I proteins in the presence or absence of camptothecin. A labeled 500 bp PCR product was generated as described in Materials and Methods and incubated with wild-type or mutant enzyme for 20 min at 30°. Reaction products were treated with proteinase K, ethanol precipitated, and visualized using a 6% sequencing gel. The intact DNA substrate is represented by the dark band at the top of the gel. Lanes are grouped according to camptothecin (CPT) concentration. The asterisk (*) indicates the difference in the cleavage patterns of the R362L mutant and the wild-type enzyme in the absence of camptothecin and at low concentrations of this drug. Enzyme quantities were normalized so that reactions contained similar relaxation activity [1 μg for the wild-type (W), F361S (1), and R362L (2) proteins; 5 μg for the R364G (4) protein].

detected with the R362L/R364G enzyme in the absence or presence of camptothecin (data not shown). These findings indicate that formation of the topo I-DNA-camptothecin ternary complex is reduced greatly using these enzymes. Interestingly, in the absence of camptothecin and at low concentrations of this drug, the cleavage pattern of the R362L mutant was different from that of the wild-type enzyme (Fig. 3, asterisk). Taken together, these results indicate that substitutions in the 361–364 region can affect both camptothecin sensitivity and DNA cleavage by topo I.

Effects of Mutations in the 361–364 Region on the Ability of Topo I to Cleave and Intermolecularly Ligate DNA

To clarify the functional defect(s) in the mutant enzymes, we evaluated the effects of the mutations on the ability of topo I to cleave and intermolecularly ligate oligonucleotide substrates (Fig. 4). This assay was chosen because it enables quantitation of cleavage/ligation activity without the need for cleavage stabilizers such as camptothecin [17, 18]. Furthermore, since this assay is performed using excess enzyme, accumulation of the ligation product is independent of enzyme processivity. In the absence of acceptor oligonucleotide, incubation of the duplex oligonucleotide substrate with wild-type or mutant enzyme did not yield any detectable cleavage or religation products (Fig. 4). By contrast, addition of acceptor oligonucleotide to reactions with the wild-type enzyme resulted in the appearance of three higher molecular weight bands corresponding to intermolecular ligation products (Fig. 4). The occurrence of multiple ligation products in this assay has been observed by others and is presumably related to cleavage at different sites in the labeled oligonucleotide [18]. Analyses of the cleavage/ligation activity of the mutant enzymes indicated distinct phenotypes relative to the wild-type enzyme. While the activity of the R362L enzyme appeared only slightly less than that of the wild-type enzyme, the activities of the F361S, R364G, and R362L/R364G mutants were greatly diminished, with no intermolecular ligation products detectable using the latter two enzymes (Fig. 4). These findings indicate that the 361–364 region in topo I is involved in the DNA cleavage/ligation process. Moreover, these data provide an explanation for the impaired plasmid relaxation activity exhibited by enzymes containing substitutions in the 361–364 region.

Effects of Substitutions in the 361–364 Region on Non-Covalent DNA Binding by Topo I

The reduced DNA cleavage/ligation seen with mutants in the 361–364 region could be explained by a decreased affinity of the enzyme for DNA. To evaluate this possibility, we performed mobility-shift assays using a labeled duplex oligonucleotide substrate. Binding of recombinant wild-type protein to the oligonucleotide was demonstrated by the appearance of both a smear and a high-molecular weight complex during polyacrylamide gel electrophoresis (Fig. 5A). This pattern is typical for non-specific protein-DNA interactions [19]. Immunodepletion experiments indicated that the altered oligonucleotide mobility could be prevented by immunoprecipitation with a topo I but not a topo II antibody (Fig. 5A). Furthermore, immunoblotting with a topo I antibody confirmed that the high-molecular weight complex contains topo I, and recombinant GST protein alone did not alter the mobility of the oligonucleotide (data not shown). These data indicate that the observed alteration in oligonucleotide mobility is due to binding by topo I. Oligonucleotide binding by the F361S, R362L, and R364G fusion proteins was similar to that of the wild-type enzyme (Fig. 5B). In contrast, binding of the R362L/R364G mutant to the oligonucleotide was slightly less than that of the other proteins (Fig. 5B), suggesting that more extensive structural alterations in the 361–364 region may impair DNA binding by topo I.

Effects of Mutations in the Region of Amino Acid 361 on the Sensitivity of Topo I to Inhibition by DNA Minor Groove Binding Drugs

Although single amino acid substitutions in the 361–364 region did not impair the ability of topo I to bind an oligonucleotide under the conditions of the mobility-shift assay, we questioned whether these mutant enzymes possessed a defect in DNA binding that might be detectable using other methods. Recent work indicates that DNA minor groove ligands inhibit topoisomerase I catalysis [20–22]. We therefore analyzed the effects of these drugs on the plasmid relaxation activity of the mutant proteins. Concentrations of up to 10 mM Hoechst 33342 had no detectable effect on the electrophoretic migration of supercoiled DNA in our assays (Fig. 6 and data not shown). Relaxation of supercoiled DNA by the wild-type enzyme was inhibited partly by 10 μM Hoechst 33342, whereas a 5 μM concentration of this drug had little effect on enzyme activity (Fig. 6). In contrast, the catalytic activities of the F361S, R364G, and R362L/R364G mutants were inhibited greatly by 5 μM Hoechst 33342, and completely inhibited by a 10 μM concentration of this drug (Fig. 6). Similar results were obtained using distamycin (data not shown). These results indicate that certain mutations in the 361–364 region of topo I confer an increased sensitivity to DNA binding ligands and are consistent with a role for this region in DNA binding during topo I catalysis. Interestingly, the sensitivity of the R362L mutant to Hoechst 33342 was similar to that of the wild-type enzyme (Fig. 6). These results are concordant with the relatively preserved activity of the R362L mutant in the DNA cleavage/ligation assays and suggest that this amino acid substitution has a minimal effect on the interaction between the enzyme and DNA.

DNA Binding by Peptides and Fusion Proteins Containing the 361–364 Region of Topo I

To determine whether the 361–364 region is capable of binding DNA directly, we constructed a peptide containing amino acids 360–370 (LFRGRGNHPKM) of human topo I. As a control, we made a similar peptide containing sub-

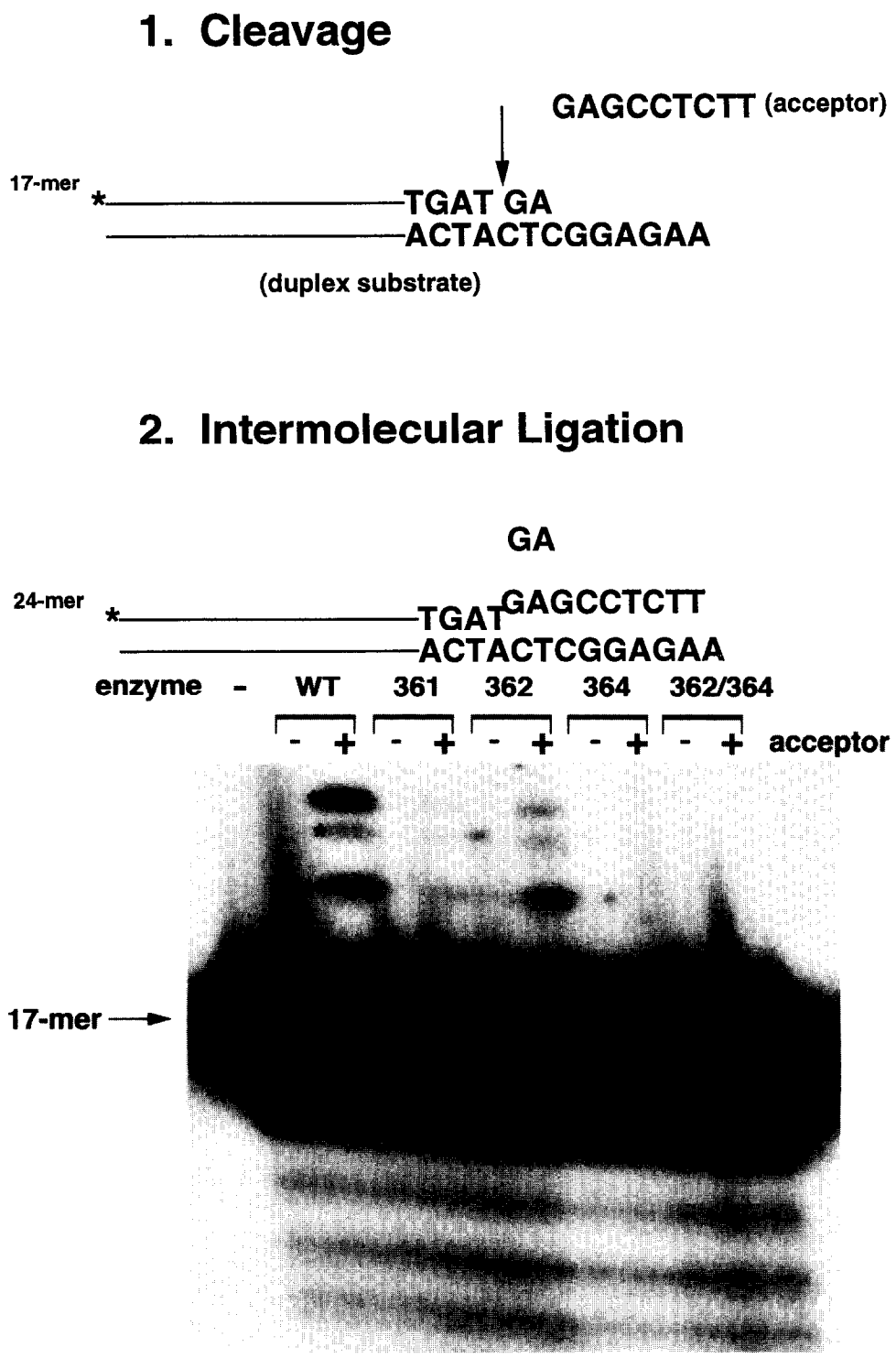

FIG. 4. Intermolecular ligation activity of wild-type and mutant human topo I proteins. A 5′-end-labeled duplex oligonucleotide (see Table 2) was prepared as described in Materials and Methods. Reactions were performed as indicated in the Fig. 3 legend, with the exception that certain reactions contained a 200-fold molar excess of acceptor oligonucleotide. Top panel: Schematic illustration of cleavage of the duplex oligonucleotide followed by acceptor oligonucleotide ligation. The position of the ^{32}P label is represented by an asterisk and topo I cleavage by an arrow. Bottom panel: Comparison of wild-type and mutant proteins. Reaction products were separated on a 12% sequencing gel. Lanes correspond to reactions with 2 μg of the indicated enzyme; the presence or absence of the acceptor oligonucleotide and the migration of the labeled 17-mer are indicated. The asterisks denote intermolecular ligation products.

stitutions corresponding to the R362L/R364G mutant (LFLGGGNHPKM). Mobility-shift assays were performed as for the intact proteins, with the exception that the reaction products were analyzed using 1% agarose rather than polyacrylamide gels. In the presence of the wild-type peptide, altered oligonucleotide mobility was manifested by the appearance of both a band migrating near the sample well and a smear of labeled material migrating near the unbound oligonucleotide (Fig. 7A). These findings suggest that the peptide-oligonucleotide binding is of variable stoichiometry, and that the predominant complex is a high-molecular weight aggregate. By contrast, similar quantities of the mutant peptide had little, if any, effect on the mobility of the oligonucleotide (Fig. 7A).

To confirm these results, we constructed GST fusion proteins containing amino acids 346–376 of either wild-type topo I or the R362L/R364G mutant. In mobility-shift assays, DNA binding was apparent using 100 or 50 pmol of

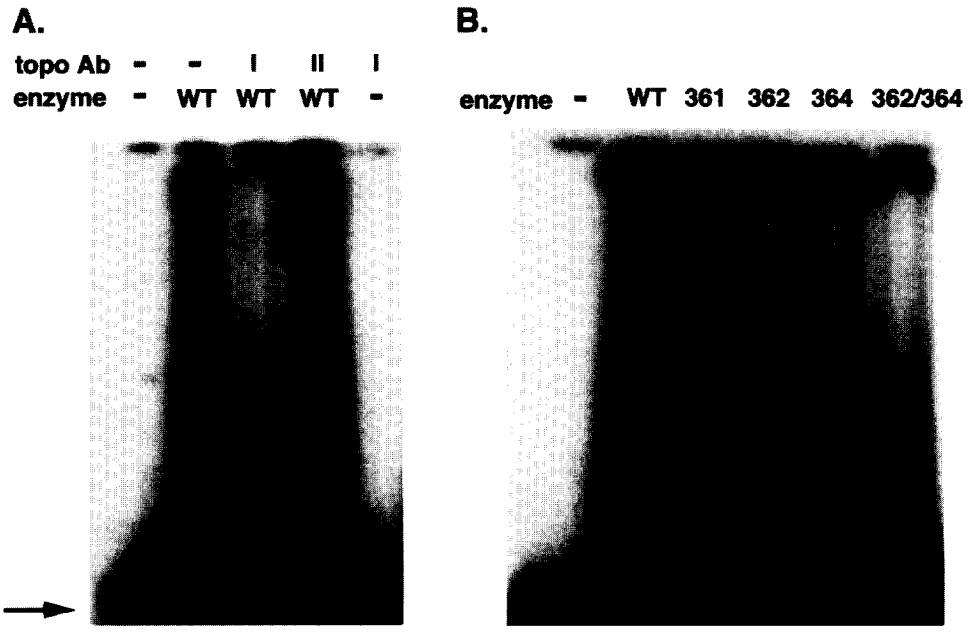

FIG. 5. Oligonucleotide binding by wild-type and mutant human topo I enzymes. A 5′-end-labeled duplex oligonucleotide (see Table 2) was incubated with wild-type or mutant topo I enzyme for 30 min at 20°. Reaction products were analyzed on a 4% nondenaturing polyacrylamide gel. Migration of the oligonucleotide in the absence of protein is indicated by the arrow. (A) Effect of immunodepletion with a topo I or topo II antibody. Four micrograms of wild-type (WT) enzyme was immunoprecipitated with protein A-linked beads alone or with the beads and a topo I or II antibody, followed by incubation with the labeled oligonucleotide. (B) Comparison of oligonucleotide binding by 2 μg of wild-type or mutant topo I protein. The mutant proteins are labeled according to te altered amino acid(s).

a GST-fusion protein containing amino acids 346–376 of wild-type topo I (Fig. 7B). In contrast, DNA binding was not detectable using the same amounts of the mutant fusion protein (Fig. 7B). Since the predicted pIs of these wild-type and mutant fusion proteins are 8.66 and 8.04, respectively, it is unlikely that these results are simply due to a loss of net positive charge in the mutant fusion protein. These findings are consistent with the results obtained with the 360–370 peptides, and suggest not only that this region is capable of binding DNA, but that this binding is dependent upon R362 and/or R364.

DISCUSSION

We have used GST-fusion proteins to study human topo I function and interaction with camptothecin. The specific activity of purified GST-topo I is 0.5×10^5 U/mg, which is similar to the 1×10^5 U/mg activity of human topo I expressed in a baculovirus system [23] but clearly lower than the 2×10^8 U/mg activity reported for topo I purified from HeLa cells [24]. Since phosphorylation of topo I has been reported to increase catalytic activity, it is possible that the lower specific activity of the bacterially expressed protein relates to a lack of phosphorylation [25–27]. Nevertheless, the activity of recombinant eukaryotic topo I expressed in bacteria is sufficient to allow *in vitro* studies of mutants [12, 28–30]. Indeed, our prior studies using GST-fusion proteins demonstrated that a single amino acid substitution at position 361 conferred resistance to camptothecin [16]. By additional analyses we now show that the region containing amino acids 361–364 is involved in topo I catalysis.

Our studies with linear DNA and oligonucleotides indicate that certain substitutions in the 361–364 region of topo I affect DNA cleavage/ligation by the enzyme. In the

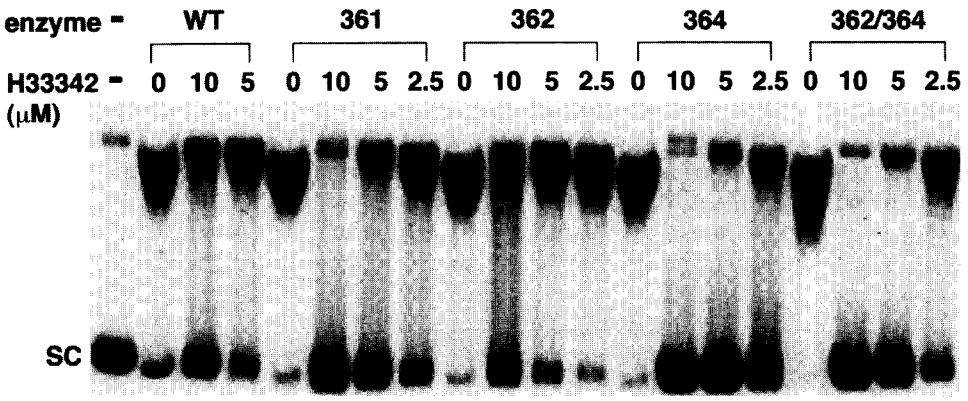

FIG. 6. Effect of Hoechst 33342 on the plasmid relaxation activity of wild-type and mutant human topo I proteins. Plasmid relaxation activity was analyzed as described in the legend of Fig. 2. Enzyme quantities were normalized so that reactions contained similar relaxation activity (50 ng for the wild-type, F361S, and R362L proteins; 250 ng for the R364G protein; and 2 μg for the R362L/R364G protein).

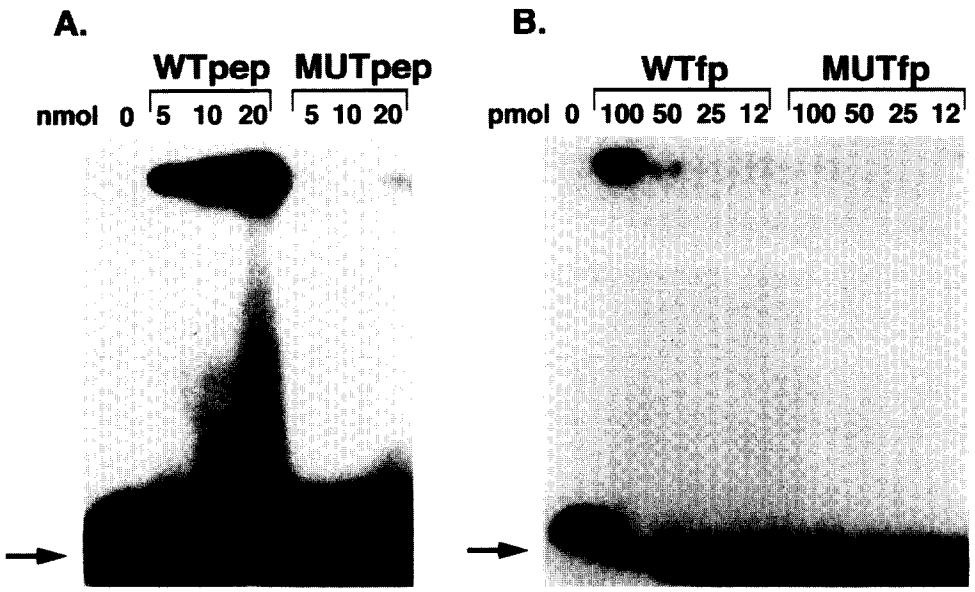

FIG. 7. Oligonucleotide binding by fragments of human topo I containing amino acids 361–364. Peptides were synthesized containing amino acids 360–370 of wild-type topo I (WTpep) or the R362L/R364G mutant (MUTpep). GST-fusion proteins were constructed containing amino acids 346–376 of the wild-type enzyme (WTfp) or the R362L/R364G mutant (MUTfp). Oligonucleotide binding was detected as described in the legend of Fig. 5, with the exception that 1% agarose gels were used rather than polyacrylamide. Migration of the oligonucleotide in the absence of protein is indicated by the arrow. (A) Analysis of oligonucleotide binding by peptides. (B) Analysis of binding by GST-fusion proteins.

F361S, R364G, and R362L/R364G mutants, cleavage/ligation of oligonucleotides was impaired greatly. Since the mutant proteins did not exhibit enhanced DNA cleavage, it is likely that the defect in these proteins relates more to the cleavage step than the religation step. Interestingly, the F361S mutant acts in a more distributive manner than the wild-type enzyme (data not shown). This finding may explain the preserved activity of this mutant in plasmid relaxation assays, which are performed using excess substrate. Similar alterations in enzyme distributivity may explain the finding that the R364G and R362L/R364G mutants lack detectable oligonucleotide cleavage/ligation activity despite being active in plasmid relaxation assays.

The alteration of DNA cleavage/ligation activity in topo I mutants containing substitutions in the 361–364 region presumably relates to modification of an interaction between the enzyme and DNA. Indeed, data with peptides and fusion proteins containing the 361–364 region support a direct interaction between this region and DNA. In addition, relative to the wild-type enzyme, the catalytic activities of enzymes with certain substitutions in this region are inhibited more easily by DNA minor groove ligands. Although our mobility-shift assays did not demonstrate impaired oligonucleotide binding in single amino acid mutants that were defective catalytically, this assay does not detect the covalent topo I–DNA reaction intermediate (data not shown). Therefore, it is possible that the 361–364 region interacts with DNA in a transition state during topo I catalysis that is not detected by the mobility-shift assay. Indeed, prior work supports a two-step model for the interaction between topo I and DNA in which the initial association may involve non-specific DNA binding [31–33]. Taken together, these results suggest that the role of the 361–364 region is not in an initial non-specific binding of DNA by topo I but in an interaction with DNA required for cleavage/ligation.

The hypothesis that the 361–364 region binds DNA is strongly supported by recent data obtained from crystallization of a 26 kDa fragment of *Saccharomyces cerevisiae* topo I [34]. Residues 293–296 in this fragment are homologous to amino acids 361–364 in the human protein. These residues are part of a long β-hairpin-like loop that is protected from lysine modification in the presence of DNA, indicating that this loop is capable of binding DNA directly. Given the extensive homology between the yeast 26 kDa fragment and the corresponding region in the human protein, it is likely that the human protein has a similar structure, and that the 361–364 region is part of a loop that binds DNA.

Since alterations in the 361–364 region of topo I are known to confer resistance to camptothecin, these amino acids may also be part of the camptothecin-binding site on topo I [15, 16]. A hypothetical model for an interaction between the 361–364 region, DNA, and camptothecin may be developed using recent data indicating that hydrophobic side chain intercalation is a common mechanism of protein-DNA interaction [35]. Examples of this type of DNA binding include minor groove intercalation by F99 and F190 in the yeast TATA box-binding protein and by I13 in the high-mobility group domain protein SRY [35]. Using this model, it may be hypothesized that F361 is part of a minor groove intercalating wedge, with nearby basic residues (R362 and R364) forming salt bridges with the DNA backbone. If this intercalation occurs at the DNA cleavage site, the model is consistent with the proposed stacking of camptothecin at this site, since the presence of an intercalating phenylalanine would presumably stabilize drug binding [36]. Thus, although it requires confirmation by structural studies, this model explains the observation that mu-

tations in the 361–364 region of topo I affect both catalysis and sensitivity to camptothecin.

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