Characterization of an altered DNA catalysis of a camptothecin-resistant eukaryotic topoisomerase I

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ABSTRACT

We investigated topoisomerase I activity at a specific camptothecin-enhanced cleavage site by use of a partly double-stranded DNA substrate. The cleavage site belongs to a group of DNA topoisomerase I sites which is only efficiently cleaved by wild-type topoisomerase I (topo I-wt) in the presence of camptothecin. With a mutated camptothecin-resistant form of topoisomerase I (topo I-K5) previous attempts to reveal cleavage activity at this site have failed. On this basis it was questioned whether the mutant enzyme has an altered DNA sequence recognition or a changed rate of catalysis at the site. Utilizing a newly developed assay system we demonstrate that topo I-K5 not only recognizes and binds to the strongly camptothecin-enhanced cleavage site but also has considerable cleavage/religation activity at this particular DNA site. Thus, topo I-K5 has a 10-fold higher rate of catalysis and a 10-fold higher affinity for DNA relative to topo I-wt. Our data indicate that the higher cleavage/religation activity of topo I-K5 is a result of improved DNA binding and a concomitant shift in the equilibrium between cleavage and religation towards the religation step. Thus, a recently identified point mutation which characterizes the camptothecin-resistant topo I-K5 has altered the enzymatic catalysis without disturbing the DNA sequence specificity of the enzyme.

INTRODUCTION

Eukaryotic DNA topoisomerases are involved in balancing torsional stress arising in DNA during transcriptional and replicational processes (1,2). DNA topoisomerase I takes part in resolving such torsional problems by a mechanism which includes concerted single-stranded cleavage-religation reactions on duplex DNA (1,2). Putative sites of catalytic action of topoisomerase I on duplex DNA have been mapped by the SDS-mediated cleavage assay *in vivo* as well as *in vitro* (3,4,5,6). Comparisons of the underlying DNA sequences have revealed only a weak consensus (3,4).

The cytotoxic plant alkaloid, camptothecin, influences the catalytic activity of eukaryotic DNA topoisomerase I (7), most likely by binding to topoisomerase I in the enzyme-DNA complex (8,9) and thereby inhibiting the religation reaction of the enzyme (10,11,12). Several types of cleavage sites have been identified by using camptothecin in mapping studies (13,14). We have previously classified such sites into three categories according to their response to drug treatment (13,14). The first category, designated class A, includes cleavage sites that are only slightly affected by camptothecin. The sites in the second category, class B, are characterized by a high degree of cleavage-enhancement due to drug action. Interestingly, comparisons of the underlying DNA sequences at the various drug-induced cleavage sites have not revealed any major discrepancies to the general consensus sequence for topoisomerase I-mediated cleavage sites, except for a preference for 5'-T↑ G-3' at the position of cleavage (15). These data are in agreement with the results obtained by Jaxel et al. (16) that camptothecin enhances cleavage 3−4 fold more at TG-dinucleotides relatively to TA or TC in oligodeoxynucleotide's of otherwise identical sequences. The third category of cleavage sites affected by camptothecin, class C, have been characterized by a substantial decrease in degree of cleavage when the drug is present (10,14).

A mutant camptothecin-resistant eukaryotic DNA topoisomerase I (topo I-K5) has been isolated (17), and recently, the amino acid substitution responsible for the enzyme's drug resistance have been identified (18). We have previously performed a comparative biochemical analysis of the wild-type topoisomerase I (topo I-wt) with topo I-K5 and shown that the camptothecin-promoted stimulation of cleavage at certain sites seen with topo I-wt is not observed with topo I-K5 (10). These observations raised the important question whether the mutation in topo I-K5 changed the enzyme's DNA sequence recognition or the rate of catalysis.

To address this question we have developed a new system to investigate topoisomerase I activity at strongly camptothecin-enhanced cleavage sites. The technique takes advantage of a previously described method to study the different stages of topoisomerase I catalysis at specific sites by use of suicide DNA substrates (12,19). We find that both topo I-K5 and topo I-wt

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recognize the strongly camptothecin-enhanced cleavage site, and that topo I-K5 exerts considerable cleavage/religation activity at the site.

EXPERIMENTAL PROCEDURES

Materials

T4 polynucleotide kinase was purchased from Boehringer (Mannheim) and [γ-^{32}P]ATP was from ICN. Camptothecin lactone form (NSC 94600) was obtained from the National Cancer Institute, U.S.A. The compound was dissolved in Me$_2$SO (Merck) at 10 mM and stored in aliquots at $-20°C$.

Cell cultivation and purification of topoisomerase I

Human T-cell-derived acute lymphoblastic leukemia RPMI 8402 cells, and a camptothecin-resistant derivative line, CPT-K5, were grown in RPMI 1640 medium containing 10% fetal calf serum as previously described (17). Type I DNA topoisomerases from both cell lines were purified to a specific activity of 5×10^6 units/mg topoisomerase I by a modification (20) of the method of Ishii et al. (21). One unit of topoisomerase I represents the amount of enzyme necessary to relax 50% of 1 μg of supercoiled pBR322 in 30 min at 30°C in the assay buffer described previously (10,13). The specific activity of both the topo I-wt and topo I-K5 enzymes were equal as described previously (13).

Synthetic oligodeoxynucleotides

All synthetic oligodeoxynucleotides were synthesized on a DNA synthesizer model 381A from Applied Biosystems and purified by polyacrylamide gel electrophoresis. The synthetic oligodeoxynucleotide, B51, contains the strong camptothecin-enhanced site b (underlined) and was constructed by hybridization of the non-cleaved strand (77mer): 5'-ATTCGAGCTCGGTA CCCGGGGATCGATCGTCTTTTTTCATGCACCATTCC TTGCGGCGGCAGAGTCGACCTGCAGGC-3' with the cleaved strand (51mer): 5'-GCCTGCAGGTCGACTCTGCC GCCGCAAGGAATGGTGCATGAAAAAAGACGA-3'. The flanking sequences were derived from M13mp18 (19). The synthetic 42mer oligonucleotide: 5'-GCATGAAAAAAGACG-ATCGATCCCCGGGTACCGAGCTCGAAT-3', is complementary to the noncleaved strand of B51 from the 5' end to the position of the cleavage and has a 5'-OH-end.

Labelling and hybridisation of synthetic oligodeoxynucleotide

Oligodeoxynucleotide 51mer was labelled at the 5' end of the cleaved strand prior to hybridisation using T4 polynucleotide kinase and [γ-^{32}P]ATP (23). For hybridisation, 1 pmol labelled 51mer was mixed with 2 pmol of the complementary 77mer non-cleaved strand in 10 mM Tris–HCl, pH 7.6, 40 mM NaCl, 5 mM MgCl$_2$ and 5 mM CaCl$_2$; the mixtures were heated to 68°C for 2 min and allowed to cool slowly to room temperature.

Purification and labelling of restrictions fragment

The plasmid pNC1 (20) was digested with the restriction endonucleases *Hin*dIII and *Pvu*II, according to suppliers instructions. The fragment was isolated and labelled at the recessed 3' ends using Klenow polymerase and [α-^{32}P]dATP (3000 Ci per mmol) (13). DNA sequencing reactions were performed using the procedures described by Maxam & Gilbert (24).

DNA-polymerase-generated sequencing markers

Equimolar amounts of 77mer (B51) template and 5'-end-labelled 37-mer primer, derived from M13mp18 (19) were annealed in the reaction mixture as described above. Dideoxy primer-extensions were performed as described (25).

DNA cleavage by topoisomerase I

In a standard cleavage reaction, 4 fmol of 5' end labelled partly double-stranded B51 was incubated with 20 units topoisomerase I (either topo I-wt or topo I-K5) in 10 mM Tris–HCl, pH 7.6, 60 mM NaCl, 5 mM MgCl$_2$, 5 mM CaCl$_2$, 0 to 80 μM camptothecin, 6% Me$_2$SO for 15 min at 30°C in 50 μl reaction volume. The cleavage reactions were terminated either by the addition of SDS to 1% or addition of NaCl to 400 mM before ethanol-precipitation. The 3' end-labelled *Hin*dIII-*Pvu*II fragment of pNC1 (3 fmol) was incubated with 20 units topoisomerase I under the above mentioned reaction conditions for 30 min at 30°C and cleavage trapped by addition of SDS to 1% before precipitation. Precipitated DNA was redissolved in 6 μl of TE (10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA) containing 0.5 mg/ml trypsin and incubated at 37°C for 30 min. Following trypsin-digestion, 6 μl of gel-loading buffer was added and samples applied to denaturing polyacrylamide gel electrophoresis.

Gel electrophoresis and autoradiography

Denaturing polyacrylamide gel electrophoresis and autoradiography were performed as described previously (23).

Quantification of cleavage and religation products

The relative frequency of cleavage and religation was determined by densitometric scanning of the autoradiograms using a Shimadzu dual wavelength thin-layer chromatoscanner, model CS903. The frequency was determined by normalization of the absorbency in the band generated either by cleavage or foreign religation to the total absorbency in the lane (25).

RESULTS

Sequence-dependent effect of camptothecin on topoisomerase I-mediated DNA cleavage

The influence of camptothecin on the DNA cleavage properties of a camptothecin resistant (topo I-K5) and wild type (topo I-wt) topoisomerase I was analyzed by the SDS-mediated cleavage assay (13). A 1.6 kb *Hin*dIII-*Pvu*II restriction fragment of the plasmid pNC1 (20) was employed as substrate (right panel of Fig. 1). The fragment, which contains a single copy of the hexadecameric topoisomerase I recognition sequence (6), was 3'-end labelled at the *Hin*dIII-site and used as substrate in cleavage reactions with the two enzymes. The transiently nicked topoisomerase I-DNA intermediates were trapped by addition of SDS and the position of cleavage determined by denaturing polyacrylamide gel electrophoresis (Fig. 1).

In absence of camptothecin, topo I-wt and topo I-K5 generate one major band resulting from cleavage at the strong recognition sequence (site a, lanes 2 and 5). In presence of camptothecin, the level of cleavage at this site is only slightly affected for topo I-wt (compare lane 2 with lanes 3–4) whereas for topo I-K5 the level of cleavage is unaltered (compare lanes 5 and 6). However, the general cleavage patterns generated by topo I-wt and topo I-K5 differ significantly at sites other than the hexadecameric
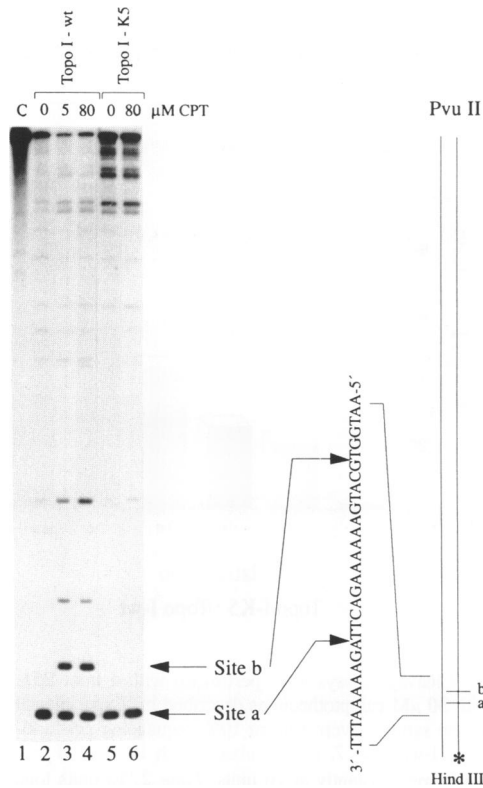
Figure 1. Sequence-dependent effect of camptothecin on topoisomerase I DNA cleavages. 3 fmol 3'-end labelled 1.6. kb *Hind*III-*Pvu*II fragment of pNC1 was incubated with topo I-wt (*lanes 2−4*) or topo I-K5 (*lanes 5−6*) at different concentrations of camptothecin as described in 'Experimental procedures'. Reactions were terminated by addition of SDS to 1%. After precipitation and trypsin-digestion the samples were run on 10% sequencing gels and visualized by autoradiography. *Lane 1*: DNA substrate. In the right panel, a schematic illustration of the 3'-end labelled *Hind*III-*Pvu*II fragment is presented. The position of topoisomerase I-mediated cleavages in this region is indicated by **a** and **b**, while the asterisk denotes the site of the radioactive label. The mid-panel illustrates the DNA sequence of the region. The arrows indicate the position of cleavage at site a and site b.

Figure 2. Sequence-specific topoisomerase I-mediated DNA cleavage at the strongly camptothecin-enhanced cleavage site on a partly double-stranded DNA substrate. Topoisomerase I-mediated cleavage reactions were performed on B51 substrate with topo I-K5 (*lanes 5−10*) or topo I-wt (*lanes 11−16*) at the indicated concentrations of camptothecin as described in 'Experimental procedures'. Reaction products were terminated by addition of SDS to 1%. After precipitation and trypsin-digestion the samples were run on 10% sequencing gels and visualized by autoradiography. Sequencing reactions were performed and run in parallel to determine the cleavage position (*lanes 1−3*). *Lane 4*, B51 substrate DNA. The lower panel is a schematic illustration of the partly double-stranded oligodeoxynucleotide employed in this experiment. The position of cleavage is indicated by an arrow, while the asterisk denotes the site of the radioactive label. Ⓟ denotes a phosphorylated 5'-end of the non-cleaved strand.

sequence. Thus, several additional cleavage sites are observed with topo I-wt in presence of low or high concentrations of camptothecin (lanes 3 and 4). The additional cleavage sites (of which site b is the most enhanced) have been categorized as class B sites (10,15). The mid-panel in Figure 1 shows the underlying DNA sequence and the arrows indicate the positions for topo I-wt generated cleavages (sites a and b) in this region of the fragment. When topo I-K5 was used, no additional cleavages were observed even at high concentrations of camptothecin (compare lanes 5 and 6). These results demonstrate that topo I-K5 escapes the general camptothecin-elicited alteration of the cleavage pattern observed with topo I-wt.

Sequence specific topoisomerase I-mediated cleavage on a synthetic oligodeoxynucleotide

To further investigate the molecular basis for the apparent change in DNA sequence recognition of topo I-K5 we utilised a technique by which it is possible to study topoisomerase I catalysis on partly double-stranded DNA substrates at specific sequences (12,19). In the bottom panel of Figure 2, a schematic illustration of the partly double-stranded oligodeoxynucleotide B51 is shown. The
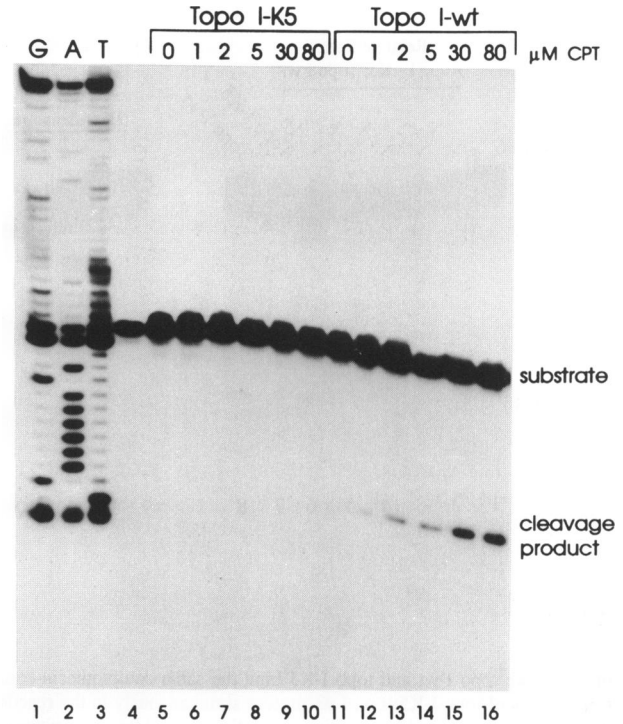
DNA substrate does not include site a and thus only contains the strongly camptothecin-enhanced cleavage site (site b) which was revealed with topo I-wt on duplex DNA. The products of topoisomerase I action on B51 are shown in Figure 2 for topo I-K5 (lanes 5 to 10) and topo I-wt (lanes 11 to 16) in the presence of 0 to 80 μM camptothecin. Topo I-wt mediated cleavage is observed on the cleaved strand when 1 μM of camptothecin is present in the reaction mixture and is further stimulated to saturation by increasing the concentration of the drug to 30 μM. It should be noted that the migration of the cleavage band is shifted 4 nucleotides upwards in the gel due to a small trypsin-resistant residue of the covalently bound topoisomerase I as described by Svejstrup et al. (19). Thus the position of cleavage is at the predicted position between T and G as indicated by the
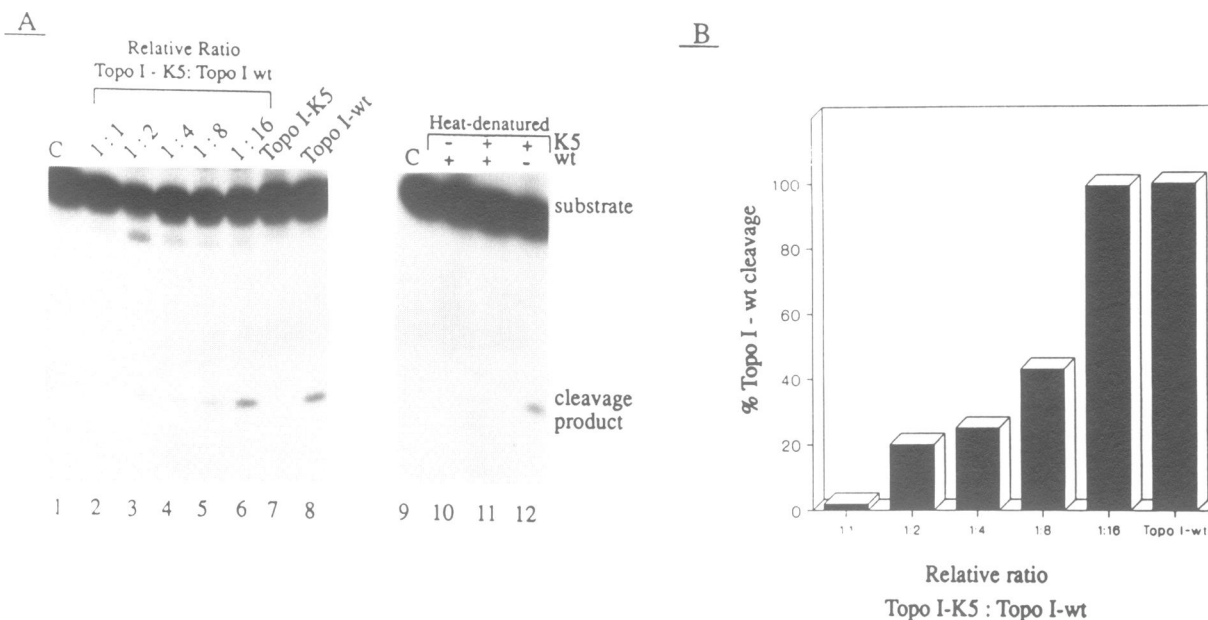
Figure 3. Both topo I-wt and topo I-K5 bind the same camptothecin-enhanced cleavage site. A, Cleavage assays were performed with 4 fmol B51 substrate and different ratios of topo I-K5:topo I-wt present simultaneously in the reaction mixture together with 30 μM camptothecin as described in 'Experimental procedures'. Reactions were terminated by addition of SDS to 1%. After precipitation and trypsin-digestion the samples were run on 10% sequencing gels and visualized by autoradiography. *Lanes 1* and *9*, B51 substrate DNA; *lane 8*, B51 incubated with 10 units of topo I-wt. *lane 7*, B51 incubated with 10 units of topo I-K5. *Lanes 2−6*, B51 incubated with different ratios of topo I-K5:topo I-wt, keeping the total amount of enzyme constantly at 20 units. *Lane 2*, 10 units topo I-K5 and 10 units topo I-wt (1:1); *lane 3*, 6.6 units topo I-K5 and 13.4 units topo I-wt (1:2); *lane 4*, 4 units topo I-K5 and 16 units topo I-wt (1:4); *lane 5*, 2.2 units topo I-K5 and 17.8 units topo I-wt (1:8); *lane 6*, 1.2 units topo I-K5 and 18.8 units topo I-wt (1:16). *Lanes 10−12*, B51 incubated in the presence of 30 μM camptothecin and either native or heat-denatured enzymes as indicated by−and +, respectively. *Lane 10*, 10 units native topo I-K5 and 10 units heat-denatured topo I-wt. *lane 11*, 10 units of heat-denatured topo I-K5 and 10 units of heat-denatured topo I-wt. *lane 12*, 10 units native topo I-wt and 10 units heat-denatured topo I-K5. B, Histogram based on densitometric scanning of the results in *A*. Each bar represents topo I-wt cleavage frequency in reactions containing different ratios of topo I-K5:topo I-wt (*A, lanes 2−6*) expressed as percentage of the cleavage frequency obtained in the sample containing only topo I-wt (*A, lane 8*).

arrow in the bottom panel of Fig. 2. In absence of drug no cleavage is observed (lane 11). When topo I-K5 was tested both in the absence or presence of even a high concentrations of camptothecin, no cleavage products were detected (lanes 5 to 10).

The data show that the interaction of topo I-wt and topo I-K5 with the synthetic B51 mimics the cleavage response observed on long double-stranded DNA fragments, both in absence and presence of camptothecin.

The drug-resistant topoisomerase I binds to the strongly camptothecin-enhanced cleavage site

Although no cleavages are observed with topo I-K5 on B51, the possibility that the enzyme is able to recognize and bind the camptothecin-enhanced cleavage site could not be excluded. To test this possibility we performed a competition binding assay at the camptothecin-enhanced cleavage site. In this experiment advantage was taken from the finding that topo I-wt, but not topo I-K5, cleaves B51 in presence of camptothecin as demonstrated in Fig. 2. The B51 substrate was used in reactions where different amounts of topo I-wt and topo I-K5 were present simultaneously in the presence of 30 μM camptothecin (Fig. 3). When 10 units of topo I-wt (equivalent to 5 times saturation level) were incubated with B51 substrate in the absence of topo I-K5, the cleavage product was observed at the predicted position (lane 8). As expected, when 10 units topo I-K5 were used in absence of topo I-wt, no cleavage product was detected (lane 7). Surprisingly, when 10 units of each enzyme (equal relaxation-units) were mixed and added to the reaction mixture, the previously observed topo

I-wt mediated cleavage was quenched (lane 2). Only by decreasing the relative ratio of topo I-K5:topo I-wt to 1:16, the normal level of cleavage was obtained (lanes 2−6). In a control experiment where 10 units of topo I-wt were mixed with 10 units of heat-denatured topo I-K5, no quenching of topo I-wt cleavage product was observed (lane 12) excluding the possibility of non-specific protein−protein interactions. Thus, topo I-K5 is apparently able to recognize and bind efficiently to the strongly camptothecin-enhanced cleavage site. To quantify the relative binding efficiency of the enzymes at this site, the gel presented in Figure 3A was scanned and the results obtained are shown in Figure 3B. As can be seen, the reduction of topo I-wt mediated DNA cleavage in presence of topo I-K5 reveals that the mutant enzyme binds the B51 substrate approximately 10-fold more efficiently than topo I-wt.

The drug-resistant topoisomerase I has catalytic activity at the strongly camptothecin-enhanced cleavage site

As shown above, the topo I-K5 mutation has apparently not changed the DNA sequence specificity of the enzyme. The enzyme binds to the cleavage site, but, as demonstrated by the experiments of figure 1 and 2, seems to have no cleavage activity at the site. However, the inability to detect cleavage activity of topo I-K5 at the site could be caused by an altered cleavage/religation equilibrium of the enzyme, making it impossible to detect an eventual cleavage by the utilized assays. To investigate this possibility, we developed a new system to detect potential cleavage/religation activity at site b (see Fig. 4).
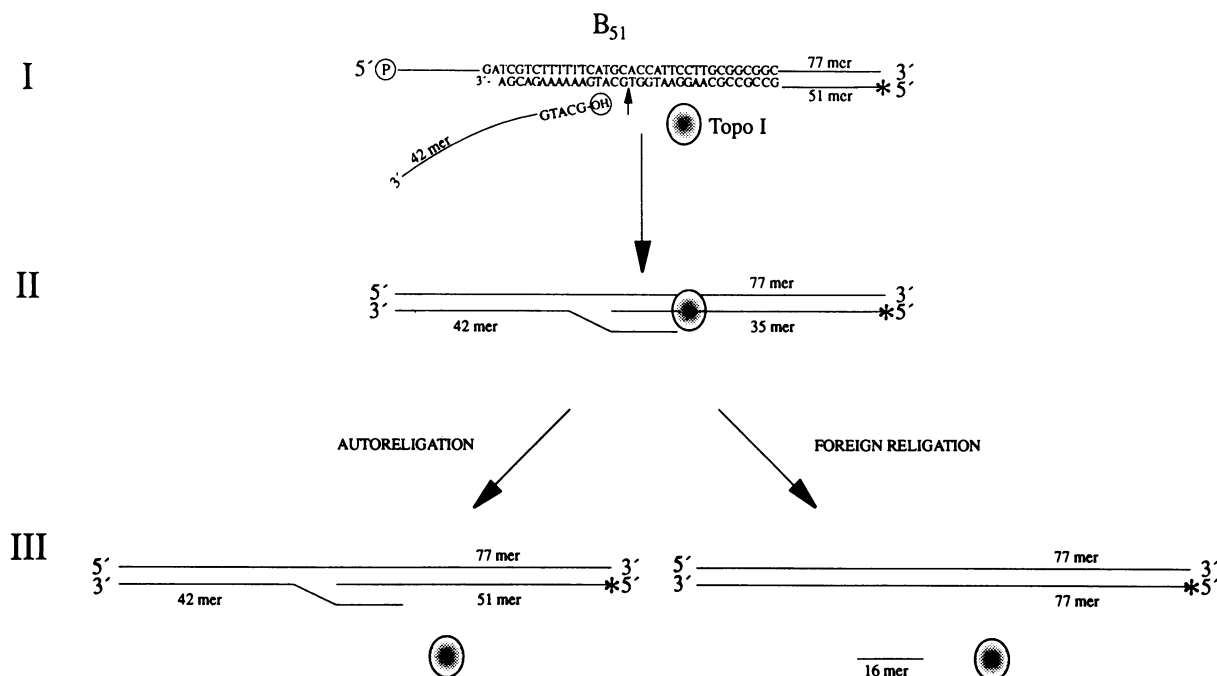
Figure 4. Experimental procedure for revealing topoisomerase I activity at a specific camptothecin-enhanced cleavage site. I, the B51 DNA substrate containing the strongly camptothecin-enhanced cleavage site is incubated with 30-fold molar excess of single-stranded 42 mer and topoisomerase I (filled sphere). The 42mer is complementary to the single-stranded region of the non-cleaved strand of B51 to the position of cleavage and has a free 5'-OH-end. II, after cleavage, the covalently bound topoisomerase I is able to religate to the 5'-OH-end of DNA in the nick or to the single-stranded 42mer when present in 30-fold molar excess. III, the products of the two religation reactions will either be the initial substrate band, that is, B51 (autoreligation), or a new 77mer band (foreign religation). The asterisk denotes the site of the radioactive label and the arrow indicates the position of topoisomerase I cleavage. (P) and (OH) denote a phosphorylated, and a hydroxy 5'-end of the non-cleaved strand and the religation substrate, respectively.

When topoisomerase I is incubated with B51 in presence of excess amounts of single-stranded religation substrate (42mer), it is possible to visualize the product of a religation activity, and hereby, as religation requires an initial nicking event, cleavage activity at the site can be identified. The 42mer religation substrate is complementary to the single-stranded region of the non-cleaved strand of B51 and further up to the cleavage position. In this system, a topoisomerase I cleavage at the specific site can be detected as a product of religation to the 42mer (foreign religation). Such religation will result in a new 77mer religation product. The 42mer is present in large excess in the reaction mixture to efficiently compete with religation to the 5'-OH end of the topoisomerase I-mediated nick (autoreligation). In all experiments a 30-fold molar excess of 42-mer was used, equivalent to twice the religation saturation level (data not shown).

When topo I-wt is investigated using this assay, no cleavage product is detected when the reaction is performed in absence of camptothecin (Fig. 5A, lane 2). However, a band with a size corresponding to 77 bases is observed in the upper part of the gel. This shows that topoisomerase I has cleaved at the strongly camptothecin enhanced cleavage site and then religated at least part of the nicked strands to the 42mer religation substrate. The foreign religation product accumulates in a time-dependent manner (Fig. 5A, compare lanes 2 and 3) showing that topo I-wt has catalytic activity at the cleavage site even in the absence of camptothecin. When topo I-K5 is used, the 77mer foreign religation product is observed, independent of camptothecin presence (Fig. 5B, lanes 3 and 4), showing that this enzyme has catalytic activity at the camptothecin-enhanced cleavage site also.

This observation is in clear contrast to the previous inability to detect topo I-K5 cleavage at this site by the SDS-mediated cleavage assay (Figure 1 and 2). When the 42-mer religation substrate is blocked by phosphorylation at the 5'-end, no foreign religation is detected neither with topo I-wt nor with topo I-K5 in the presence or absence of camptothecin (data not shown).

In experiments performed with topo I-wt in the presence of camptothecin, both the expected cleavage product and the 77mer religation product is observed (Fig. 5A, lane 6). With topo I-K5, no cleavage product is observed even in the presence of drug (Fig. 5B, lane 4). Furthermore, the amount of foreign religation product formed by topo I-wt is approximately 5-fold higher compared to the amount observed in absence of drug (compare Fig. 5A, lanes 3 and 6). This result, though unexpected, is in agreement with the fact that camptothecin inhibits the reclosure of topoisomerase I-mediated nicks in DNA (12,16). Inhibition of the autoreligation reaction might allow the 42mer to enter the enzyme−DNA complex, and due to its large excess be preferentially religated to the nicked strand.

The experiment also shows that the amount of 77mer religation product is 3−4 times higher with topo I-K5 compared to topo I-wt in the presence of camptothecin (Fig. 5B, compare lanes 2 and 4). The difference in amounts of foreign religation product formed by topo I-K5 and topo I-wt in the absence of drug is even larger (Fig. 5B, compare lanes 1 and 3). Densitometric analysis of the gels shows that topo I-K5 produces approximately 10-fold more religation product than topo I-wt.

This experiment clearly demonstrates that nicking-closing activity takes place with both the wild type and the resistant
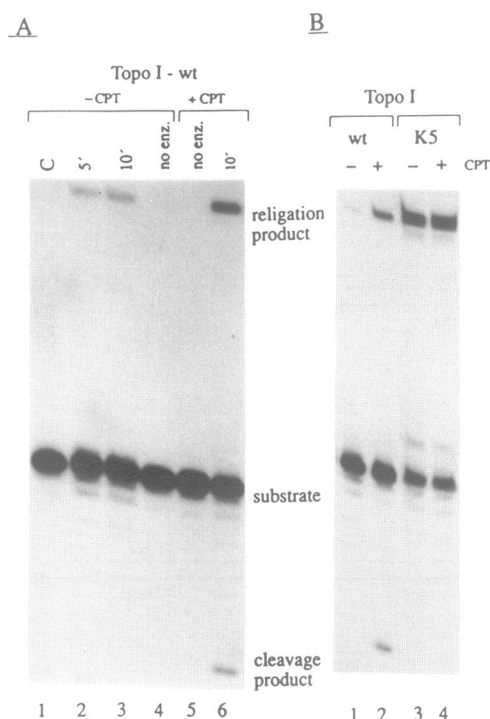
Figure 5. Both topo I-wt and topo I-K5 are catalytically active at the strongly camptothecin-enhanced cleavage site. **A.** Topoisomerase I was incubated with 4 fmol B51 substrate for 5 or 10 min as described in 'Experimental procedures' in the presence of 120 fmol single-stranded 42mer. Reactions were stopped by NaCl to 400 mM and the reaction products analyzed on a 10% sequencing gel after ethanol-precipitation and trypsin-digestion before visualization by autoradiography. Reactions were performed with topo I-wt in the absence (*lanes 2 and 3*) or presence (*lanes 6*) of 30 μM camptothecin for 5 min and 10 min, respectively. *Lane 1*, substrate DNA. In *lanes 4* and *5* the DNA substrate was incubated in absence of topoisomerase I. **B.** Reactions performed as *A* for topo I-wt (*lanes 1* and *2*) and topo I-K5 (*lanes 3* and *4*) either in absence (*lanes 1* and *3*) or in presence (*lanes 2* and *4*) of 30 μM CPT but incubated for 30 min.
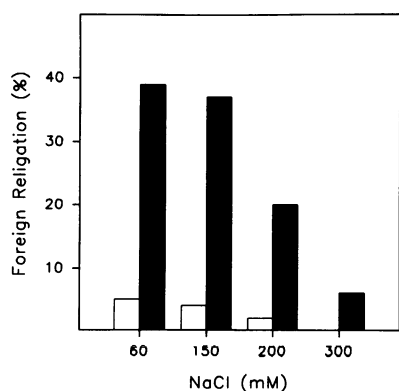

Figure 6. Topo I-K5 has a higher salt-stability of cleavable complex formation than topo I-wt. 4 fmol 5' end-labelled B51 was incubated with 30 units topo I-wt or 30 units topo I-K5 in the presence of 120 fmol 42mer at different concentrations of NaCl for 30 min. All reactions were performed in the absence of camptothecin and prepared for analysis as described in 'Experimental procedures'. Following ethanol-precipitation and trypsin-digestion the reaction products were analyzed on a 10% sequencing gel and visualized by autoradiography. Each bar represents the amount of foreign religation product (i.e. 77mer) formed at any salt-concentration expressed as the percentage of the total amount of DNA in the lane. The open bars represent data obtained with topo I-wt, the solid bars represent the data obtained using topo I-K5.
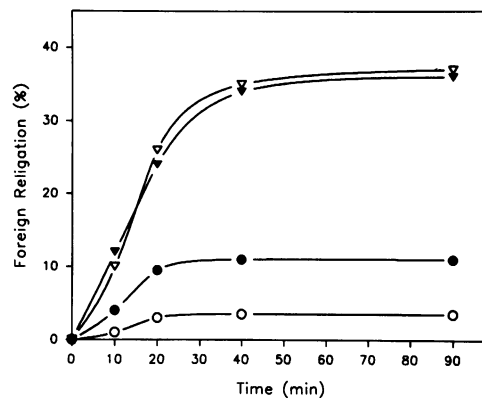

Figure 7. Topo I-K5 has a higher rate of catalysis than topo I-wt. Topoisomerase I was incubated with 4 fmol B51 substrate and 120 fmol 42mer in the absence or presence of 30 μM camptothecin as described in 'Experimental procedures'. After 0 to 90 minutes of incubation the reactions were terminated by addition of NaCl to 400 mM before ethanol precipitation. Following trypsin-digestion the reaction products were analyzed on a 10% sequencing gel and visualized by autoradiography. By using results obtained by densitometric scanning of the autoradiogram, the amount of foreign religation product (i.e. 77mer) at any point was expressed as percentage of the total absorbancy generated by all bands in the lane. The percentage of foreign religation was plotted against time of sampling. (○) Topo I-wt incubated with the DNA substrate in absence of camptothecin; (●) Topo I-wt, 30 μM camptothecin; (▽) Topo I-K5, no camptothecin; (▼) Topo I-K5, 30 μM camptothecin.

enzyme at the camptothecin-enhanced cleavage site. Thus, topo I-wt and topo I-K5 not only share similar sequence specificity but also have cleavage/religation activity at the strongly camptothecin enhanced cleavage site. The inability to trap topo I-K5 cleavage complexes at site b on duplex DNA by SDS treatment might be explained by a shift in the cleavage/religation-equilibrium favouring the religation step.

The camptothecin resistant topoisomerase I has a higher salt stability in cleavable complex formation

It has been suggested that topoisomerase I-mediated DNA cleavage at high ionic strength might reflect a relatively higher DNA binding affinity of topoisomerase I at the cleaved sites (26). Previously, we have shown that topo I-K5 cleavable complexes are less sensitive to increased ionic strength than topo I-wt on site a in the *Hind*III-*Pvu*II fragment (10). To investigate whether cleaved complexes formed by topo I-K5 on the strongly camptothecin-enhanced cleavage site (site b) also have a higher salt-stability, the cleavage/foreign religation assay (described in Fig. 4) was employed. The B51 substrate was incubated in the absence of camptothecin with topoisomerase I for 20 min at 30°C in presence of 42mer at different concentrations of NaCl. Reactions were terminated by addition of NaCl to 400 mM. Following ethanol-precipitation and trypsin-digestion, the products were analyzed on 10% sequencing gels and visualized by autoradiography. The histogram depicted in Fig.6 represents the values obtained by densitometric scanning of the religation products formed in each salt-concentration expressed as the percentage of the total absorbancy generated by all bands in the lane. With topo I-wt, almost the normal level of religation product is observed for NaCl concentrations up to 150 mM. Increasing the ionic strength to 300 mM NaCl abolishes any formation of the religation product indicating that topo I-wt clevage/religation activity is completely inhibited under these conditions. For topo I-K5, in contrast to topo I-wt, a religation product is formed even

at 300 mM NaCl which is not seen for other topoisomerases (13). This result demonstrates that the mutant enzyme has relatively less sensitivity to increased ionic strength in cleavable complex formation at site b. When this result is considered together with our previous findings for site a (10), it shows that topo I-K5, in general, has a higher stability in cleavable complex formation compared to topo I-wt and thereby supports the conclusion that the camptothecin resistant topoisomerase I has a higher DNA affinity than the wild type counterpart.

Comparative time course for the religation-reaction of topo I-wt and topo I-K5

The observed difference in affinity to DNA and salt-stability of cleavable complex formation between the two enzymes might correlate to a changed rate of cleavage/religation catalysis at the strongly camptothecin-enhanced cleavage site. To investigate the relative catalytic activity of topo I-wt and topo I-K5, we performed a time course experiment using the cleavage-religation assay.

Topo I-wt or topo I-K5 were incubated with B51 substrate and excess amounts of 42mer in the absence or presence of 30 μM camptothecin. Samples were withdrawn from the reaction mixture at different times (0 to 90 min) and ethanol-precipitated. Following trypsin-digestion, the reaction products were analyzed on sequencing gels and visualized by autoradiography. The values obtained by densitometric scanning of the religation products formed at each time point was expressed as percentage of the total absorbency generated by all bands in the lane, and plotted versus time (Fig. 7). With topo I-wt, the initial rate of foreign religation was calculated to be 0.004 fmol of B51 substrate processed per min in absence of camptothecin. The maximum amount of religation product formed, equivalent to 3% of the input DNA substrate, was reached after 20 min of incubation. However, in presence of camptothecin the maximal level of foreign religation was increased 3−4-fold together with a similar increase in the initial rate velocity. When this result is considered together with the religation experiments presented in Figure 5B, it strongly indicates that camptothecin inhibits autoreligation stronger than foreign religation, perhaps because camptothecin interacts with the 5'-end of the DNA nick in the topo I-wt-DNA complex (16).

With topo I-K5, a maximum level of foreign religation product equivalent to 35% of the input DNA substrate was reached after 40 min of incubation. From the plot, the initial rate of foreign religation was calculated to be 0.05 fmol B51 substrate processed per min. As expected, camptothecin has no effect neither on the maximal level nor on the initial rate of topo I-K5 catalysis.

In conclusion, the experiments show that topo I-K5 has acquired an approximately 10-fold higher rate of cleavage/religation activity than the wild-type enzyme. The 10-fold higher rate of catalysis correlates with a 10-fold higher DNA affinity of the enzyme, indicating that the higher catalysis might be a result of improved DNA binding.

DISCUSSION

The antitumor drug, camptothecin, enhances topoisomerase I-mediated cleavages with high efficiency at some sequences (type B sites) while having little effect on DNA cleavage at other sequences (type A sites) (10). The camptothecin-resistant form of topoisomerase I, topo I-K5, escapes the general cleavage enhancement elicited by the drug (13,15). To investigate whether the altered drug response of topo I-K5 is due to a change in the

enzyme's DNA sequence recognition, an alteration in the DNA binding, or in the rate of catalysis of the enzyme at the various DNA cleavage sites, we utilized a new cleavage/foreign-religation technique based on a typical camptothecin-enhanced cleavage site (site b). The technique was utilized to show that not only topo I-wt but also topo I-K5 actually has cleavage/religation activity at the strongly camptothecin-enhanced cleavage site in the absence of drug. For topo I-wt, these data directly demonstrate that camptothecin does not act to induce cleavage but rather to slow down a normally very fast religation. Further, it was found that the mutant enzyme binds to DNA with approximately 10 times higher affinity than the wild-type enzyme, and that it has a higher salt-stability of cleavable complex formation at the camptothecin-enhanced cleavage site. The rate of religation activity of topo I-K5 at the site was also shown to be approximately 10-fold higher than that of the wild type enzyme. Thus, the results demonstrate that topo I-wt and topo I-K5 share similar sequence specificities but differ significantly in the mode of catalysis at the recognized sites.

Two amino acid substitutions (Asp → Gly) at residues 533 and 583 of topo I-K5 have recently been identified (18). However, only the residue at position 533 is altered compared to a previously published amino acid sequence for human topoisomerase I (27), suggesting that it is the substitution at position 533 which determines the phenotype of topo I-K5. Consistent with this suggestion, it has recently been found that a human topoisomerase I mutated only at position 533 (Asp → Gly) is resistant to camptothecin (T.Andoh, personal communication).

As proposed by Jaxel et al. (16), camptothecin might enter the topoisomerase I-DNA complex after DNA cleavage and interact with the 5'-OH end of the nick thereby inhibiting the religation step. However, our data makes it unlikely that camptothecin enters the topo I-K5-DNA complex as the level of foreign religation product remains unaltered after camptothe cin addition (Fig. 5). The finding that topo I-K5 has a higher DNA affinity might explain the apparent ability of this enzyme to exclude campothecin from its complex with DNA. Recently, it has been demonstrated that a mAMSA-resistant T4-phage topoisomerase II also forms a more stable cleavable complex than the respective wild-type enzyme (28). Such apparent similarities between drug-resistant topoisomerases indicates that the stability of enzyme−DNA binding might be generally closely related to the mechanism of drug resistance. Taken together, it therefore seems reasonable to suggest that the (Asp→Gly) mutation at position 533 of topo I-K5 has improved the DNA binding ability of the enzyme and thereby made it resistant to camptothecin.

It has been discussed if the relative intensities of gel bands generated from end-labeled DNA fragments after SDS-mediated cleavage by topoisomerase I reflect the relative catalytic activity of the enzyme at different DNA sequences (29). For the camptothecin-resistant topoisomerase I we have previously identified a category of sites on duplex DNA which escape SDS-mediated cleavage detection (13). In this study, we have demonstrated that topoisomerase I actually has considerable cleavage/religation activity at a site belonging to this category (e.g. site b). Thus, our data suggest that a correlation between gel-band intensities and the level of catalytic activity should be evaluated with great care. However, it still remains to be elucidated whether topo I-K5 has swivelase activity at site b, or if the rate of catalysis is so fast that the enzyme is not able to have relaxation-activity at this site. The above mentioned note of caution is likely to have implications in the determination of

topo I-wt cleavage sites on duplex DNA also. Thus, it is possible that some categories of topoisomerase I cleavage sites exist which escape detection by SDS and/or camptothecin treatment due to a very rapid cleavage/religation equilibrium of the enzyme at these sites.

A further indication to the mode of camptothecin action comes from our observation that religation by topo I-wt to foreign DNA (foreign religation) seems favored compared to religation of the introduced nick (autoreligation) in the presence of camptothecin and might indicate that camptothecin is able to interact with the 5'-OH end of the topoisomerase I-mediated nick. Thus, our results indicate that camptothecin influences strongly on the autoreligation process of topoisomerase I while the enzyme is retained in an active state capable of religating to 5'-OH ends of foreign DNA. Such an interaction is in agreement with the model proposed by Jaxel et al. (16) and might explain the increased frequency of sister chromatid exchange (SCE) observed in cells treated with camptothecin (30). Furthermore, the data demonstrates the possible involvement of topoisomerase I in illegitimate recombinational processes.

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