Molecular cloning of a cDNA of a camptothecin-resistant human DNA topoisomerase I and identification of mutation sites

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ABSTRACT

Camptothecin (CPT), a plant alkaloid with antitumor activity, is a specific inhibitor of eukaryotic DNA topoisomerase I. We have previously isolated and characterized a CPT-resistant topoisomerase I isolated from a CPT-resistant human leukemia cell line, CPT-K5. cDNA clones of topoisomerase I were isolated from the CPT-resistant and the parental CPT-sensitive cell lines, respectively. Sequencing of the clones identified two mutations in the cDNA isolated from the resistant cells, which cause amino acid changes from aspartic acid to glycine at residues 533 and 583 of the parental topoisomerase I. When the CPT-K5 topoisomerase I was expressed in *E. coli* as a fusion protein with Staphylococcal Protein A fragment, the activity was resistant to CPT at a dose level up to 125 μM, whereas the parental fusion protein was sensitive to CPT as low as 1 μM. The resistance index (>125) of the CPT-K5 fusion topoisomerase I is similar to that of the native CPT-K5 topoisomerase I. These results indicate that either or both of the two amino acid changes identified in the mutant enzyme is responsible for the resistance to CPT.

INTRODUCTION

Topoisomerases are enzymes that catalyze the topological changes of DNA. Eukaryotic enzymes relax supercoiled DNA by transient protein-linked cleavages of either one (type I) or both (type II) of DNA strands (1). In eukaryotes, these enzymes are implicated to be involved in the replication of DNA and chromosomal segregation, as well as in the transcription of specific genes (2).

Recent studies have identified the DNA topoisomerases as targets of a number of anticancer drugs (3). The majority of these therapeutics acts on DNA topoisomerase II; however, a plant alkaloid camptothecin (CPT) acts specifically on eukaryotic DNA topoisomerase I (4). CPT, an alkaloid isolated from *Camptotheca acuminata*, has a strong antitumor activity against a wide range of experimental tumors (5). CPT inhibits RNA and DNA synthesis and causes rapid and reversible fragmentation of DNA in mammalian cells (6–8). The inhibitory effect of CPT on topoisomerase I catalysis was shown to be related to the drug-mediated stabilization of a cleavable complex (3,9).

Previously, we reported the isolation and characterization of a CPT-resistant cell line (CPT-K5) from a human acute lymphoblastic leukemia cell line (RPMI8402) (10). It was shown that CPT-K5 cells possess a CPT-resistant DNA topoisomerase I with the same molecular weight and proteolytic map as the wild-type enzyme. The CPT-K5 enzyme had other altered enzymatic properties such as its higher efficiency of recognition of specific sequences and higher stability of cleavable complexes (11). Thus, the enzyme site(s) or domain(s) affected by CPT and the mutation conferring resistance to the drug are determinants responsible for strand passage and/or religation steps. The relationship between the mutation site(s) and the active site of the enzyme is to be clarified. Several alternative mechanisms might be possible for the acquisition of the resistance: such as mutation of the structural gene, post-translational modification or expression of a 'pseudo'-gene whose product is resistant to CPT

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(12). As a first attempt to address this problem, we have attempted cloning and sequencing of the cDNAs of the wild-type and CPT-K5 topoisomerase I.

Here we report the molecular cloning of cDNAs of topoisomerase I from the mutant and the wild-type cells. Comparison of the deduced amino acid sequences revealed amino acid changes at two positions at about one-third from the carboxyl terminus of the enzyme. Expression of the mutant enzyme in *E. coli* revealed that the two amino acid changes found in the mutant enzyme are responsible for the resistance to CPT. Comparison of the amino acid sequence with those of other eukaryotic topoisomerase I, i.e. those of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, showed that the region in which the mutations occurred is highly conserved (13).

MATERIALS AND METHODS

Material

E. coli K12 N4830-1 (λ,ΔBam,N+,cI_{857},ΔH1), expression vector pRIT2T and heparin-Sepharose were purchased from Pharmacia. λZAP phage vector was purchased from Stratagene. CPT was obtained from Yakult Honsha Co., Ltd.(Tokyo).

Cell culture

Human T-cell-derived acute lymphoblastic leukemia cells RPMI 8402 (wild-type), and a camptothecin-resistant line, CPT-K5, were grown in RPMI 1640 medium containing 10% fetal bovine serum as described (10).

Construction and screening of cDNA libraries

Total cellular RNA was isolated from wild-type and CPT-K5 cells by the method of Chomczynski and Sacchi (14). Poly(A)$^+$ RNA was enriched with oligo(dT) cellulose chromatography. λgt11 cDNA libraries were constructed with a kit of Amersham according to the manufacturer's protocol, using oligo(dT) primers or random primers. Screening of the libraries was carried out according to standard procedures. The probe used for the screening was a 3.0 kb EcoRI fragment excised from a cDNA clone (phtop1) of human topoisomerase I isolated from a human brain stem cDNA library. This clone (phtop1) was screened immunologically with anti- topoisomerase I autoimmune serum from a Scleroderma patient (15) by the method of Young and Davis (16) (Keene *et al.*, unpublished data). Primer extended cDNA libraries were constructed using a 14mer synthetic oligonucleotide primer corresponding to HaeIII site at the 5′ end of K3 clone (5′-GGCCACGGAAAAGT-3′) and λZAP as a vector. About 1×10^4 recombinant phages were screened, and two additional clones were isolated as shown in Fig.1.

Construction of an expression vector of topoisomerase I and its expression in *E.coli*

The cloned cDNA was inserted into the EcoRI site of expression vector pRIT2T in frame so that a fusion protein comprised of 30 kDa N-terminal portion of Protein A (Protein A′) of *Staphylococcus aureus* linked to the C-terminal two-thirds of topoisomerase I (amino acid residues 163−765) is to be expressed in *E. coli* (N4830-1) lysogenic for temperature sensitive (ts) cI phage. A plasmid expressing the mutant cDNA was constructed by exchanging the HindIII-HindIII fragment containing the mutation sites, as depicted in Fig. 5. Fifty ml each of the culture of the transformed cells was shifted up from 32°C to 42°C when O.D. at 600 nm was 0.75 to induce the expression of the fusion protein. Cells were harvested 2 hrs after induction, and extracts were prepared as described by Thrash *et al.* (17). Partial purification of the fusion topoisomerase I was performed as follows (18). The crude extract was applied onto a heparin-Sepharose column equilibrated with extraction buffer and then the enzyme activity was eluted with a KCl linear gradient (0.5 to 0.9 M). The activity was recovered at 0.6−0.7 M KCl.

DNA topoisomerase I assays

Assays for bacterial and human topoisomerase I were performed as described (19) with a slight modification. Bacterial topoisomerase I activity was assayed in a total volume of 50 μl containing 0.1 μg of supercoiled ColEl DNA, 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, BSA (30 μg/ml) and 10% glycerol. The mixtures were incubated at 37°C for 15 min. Human topoisomerase I activity was assayed as described above with the exception of EDTA (5 mM) and the absence of Mg^{2+}. In both cases, the reaction was terminated by the addition of SDS to 1% and heating at 65°C for 5 min. The extent of DNA relaxation was assayed by electrophoresis in a 1.0% agarose gel as described (18). One unit of topoisomerase I represents the amount of enzyme necessary to relax 50% of 0.3 μg of supercoiled ColEl DNA for 15 min at 37°C.

Predicted conformation of topoisomerase I at the mutation sites

Conformation of topoisomerase I was predicted by the method of Nishikawa and Ooi (20). In brief, a spatial location of an amino acid residue in a protein is defined by the number of α-carbon (C^α) atoms located within a sphere of a radius of 14Å centered at the C^α atom of a given residue. This number, N_{14}, called the 'contact number', corresponds approximately the number of residues interacting with the residue. This quantity is a measure of the exposure of a residue to solvent, and is closely related to the distance from the center of a mass of a protein. Correlation between the computed quantities and experimental ones obtained from the x-ray crystallographic data is as high as 0.5 on the average over 92 different proteins of known three dimensional structures (20).

Other procedures

Blot hybridization was performed according to standard procedures (21). cDNA sequences were determined by the dideoxynucleotide chain-termination method (22) following subcloning cDNA inserts into M13 or pUC19. Immunoblot analysis was carried out as described (10,23).

RESULTS

Genomic organizations and transcripts of DNA topoisomerase I genes in the wild-type and the CPT-K5 cells

A Southern blot of the wild-type and the CPT-K5 cellular DNA digested with several restriction enzymes was probed with a 0.42 kb HindIII-TaqI fragment (from nucleotide 1661 to 2081; numbered according to Ref. 24) of phtop1 (Fig. 2). As shown in Fig. 1 (a), the genomic organization of topoisomerase I in the mutant cells seems to be similar to that of the wild-type cells suggesting that there is no detectable genomic disorders such as gene amplification or chromosomal rearrangement around topoisomerase I gene of the mutant cell. A Northern blot analysis showed that the expression level of topoisomerase I in the mutant cells is similar to that in the wild-type cells with the size of 4.2 kb (Fig. 1 (b)). These results indicate that the mutation in the

CPT-K5 topoisomerase I gene is moderate, most probably point mutation(s) in the structural gene. This is supported by the fact that the peptide maps of the wild-type and the mutant enzyme are indistinguishable (10). To address this question, cloning and sequencing of cDNA had been initiated.

Molecular cloning of cDNAs encoding CPT-K5 and wild type DNA topoisomerase I

Both cDNA libraries of CPT-K5 and its parental cells (RPMI8402, wild-type) were constructed in a λgt11 expression vector. From 6×10^4 recombinant phages of the CPT-K5 library and 2×10^4 phages of the wild-type library, several clones with inserts ranging 0.3−3.0 kb were isolated as probed with a human topoisomerase I cDNA, that was isolated from a λgt11 expression library of human brain stem cells by immunological screening with an anti-human topoisomerase I autoimmune serum (Keene *et al.*, unpublished data, 15). Fig. 2 shows restriction maps of these cDNA clones, W3, K3 and K10. Since even the longest cDNA did not cover the whole coding sequence that was previously reported by D'Arpa *et al.* (24), primer extended cDNA libraries were constructed and screened as described in 'Materials and Methods'. Additional cDNA clones extended toward 5'-end were isolated from those libraries (Fig. 2, W5 and K4). However, even the longest clone from the mutant libraries, K4, still lacked 69 nucleotides (corresponding to 23 amino acid residues) of 5'-end of the coding sequence, as revealed by sequencing.

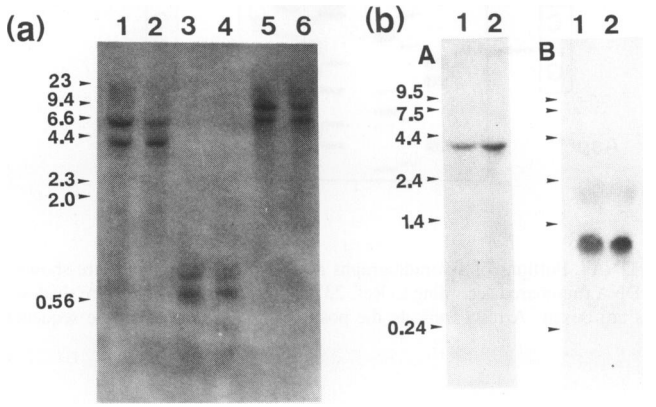

Figure 1. Genomic organizations and transcripts of topoisomerase I genes in the wild-type and CPT-K5 cells. (a) Southern blot hybridization of the wild-type (lanes 1, 3 and 5) and CPT-K5 (lanes 2, 4 and 6) DNA. 5 μg of each genomic DNA was digested with HindIII (lanes 1 and 2), HaeIII (lanes 3 and 4) or BamHI (lanes 5 and 6). A 0.42 kb HindIII-TaqI cDNA fragment of phtop1 was used as a probe. Arrow heads indicate the positions of markers with indicated sizes in kb. (b) Northern blot hybridization of the total RNA (15 μg) isolated from the wild-type (lane 1) and the CPT-K5 (lane 2) cells. A 2.0 kb EcoRI-EcoRV fragment of phtop1 was used as a probe (panel A), and the same filter was probed with chiken β-actin gene as an internal control (panel B). Arrow heads indicate the positions of markers with indicated sizes in kb.

Identification of mutation sites of the CPT-K5 topoisomerase I

Nucleotide sequencing of the inserts following its subcloning was carried out according to the standard procedures. Differences found between the wild-type and the CPT-K5 cDNA sequences were two nucleotide substitutions changing Asp-533 (GAC) and Asp- 583(GAC) of the wild-type topoisomerase I to Gly(GGC) (numbered according to D'Arpa *et al.* (24) (Fig.3). These nucleotide substitutions may not be due to artifacts during cloning and/or subcloning procedures because two independent cDNA clones (K3 and K10) isolated from independent libraries gave the same result. The nucleotide sequence encoding the wild-type topoisomerase I was identical to the corresponding region of a 3.6 kb topoisomerase I cDNA isolated from a human placental library (24), except for two base differences: one, A at Asp-583(GAC) in our wild-type sequence is replaced by G to change to Gly-583(GGC) in the placental sequence, and the other, a neutral mutation, A at Thr-591(ACA) in our wild-type sequence

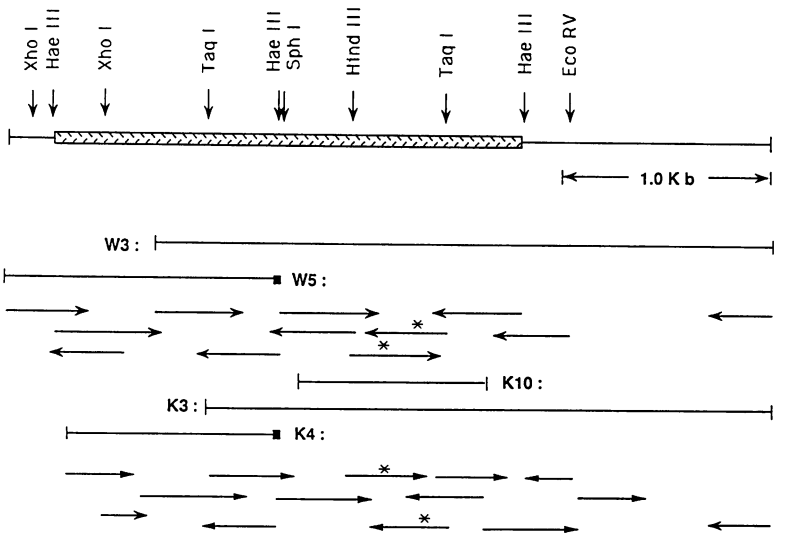

Figure 2. Restriction map and sequencing strategy of cDNA clones of topoisomerase I isolated from the wild-type and CPT-K5 libraries. Clones W3 and W5 were isolated from the wild-type library, and clones K3, K4 and K10 were isolated from the CPT-K5 library. Selected restriction sites are indicated. Horizontal arrows indicate the regions and directions of sequencings of various subclones which were obtained by subcloning various restriction fragments into M13 or pUC19. Some sequencings were carried out by using synthetic oligonucleotides as a primer. The hatched box represents a putative coding sequence of human topoisomerase I (23). Clones W5 and K4 were isolated from primer-extended cDNA libraries as described in 'Materials and Methods'. The primer is shown by closed boxes. Clone K10 was isolated from a cDNA library constructed using random primers. Asterisks indicate two mutation sites described in the text. kb, kilobases.

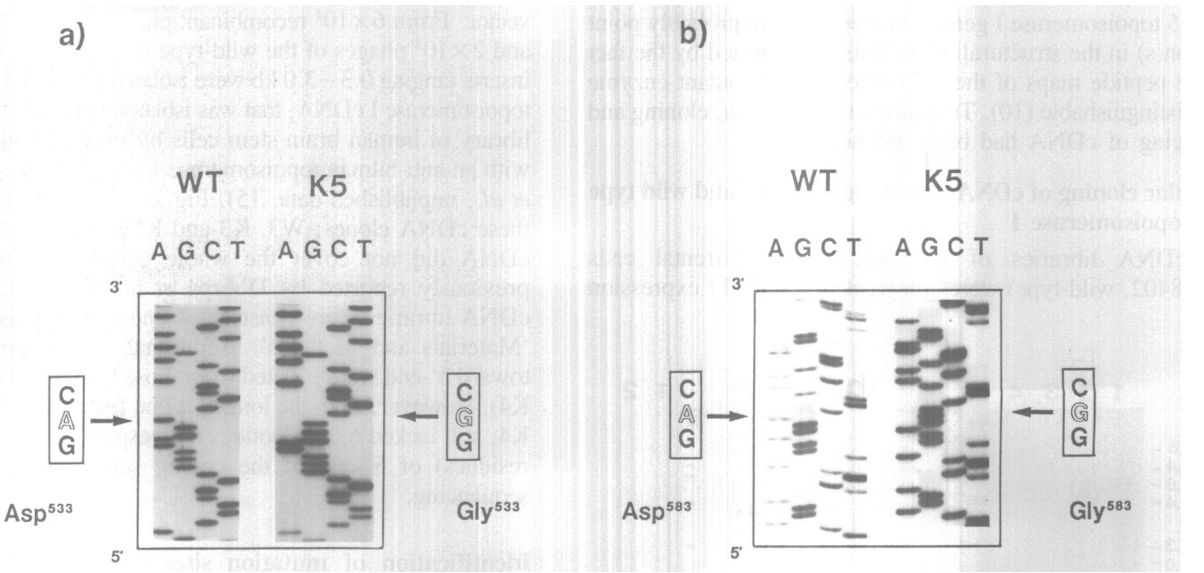

Figure 3. Sequence differences between the wild-type and CPT-K5 topoisomerase I cDNAs. Portions of autoradiographs around the mutation sites are shown. A to G transitions occurred at nucleotide 1809 (a) and nucleotide 1959 (b) in the CPT-K5 cDNA (numbered according to Ref. 23). Nucleotides substituted are shadowed. Triplet codons for amino acids in the wild-type (Asp) and the CPT-K5 (Gly) cDNAs are boxed. Arrows indicate the positions of differences on the sequencing gels (shown by asterisks in Fig. 2).

replaced by G to change Thr-591(ACG) in the latter sequence. The sequences of these sites of the cDNA used as a probe for the screening was the same as those of our wild-type one. Amino acid sequences of yeast, human and vaccinia virus type I topoisomerases surrounding the mutation sites are summarized in Fig. 4 (17,24–26). This comparison shows that the region in which two mutations occurred is highly conserved.

Expression of cloned topoisomerase I cDNA in E. coli as a fusion protein

To know whether the two mutations found in the CPT-K5 cDNA are responsible for the resistance, we tried to express the mutated cDNA in *E. coli*. To express the wild-type topoisomerase I, a 3.0 kb cDNA (encoding the protein from the residue-163 over to 3'noncoding region) was excised with EcoRI from clone W3 and then inserted into pRIT2T expression vector in which the protein encoded by the cDNA was fused to the 30 kDa N-terminal portion of *S. aureus* Protein A (pRITWT). To express the mutated protein, a 2.0 kb HindIII-HindIII fragment of pRITWT was replaced by that of clone K3 which contains the two mutation sites as shown in Fig. 5, (pRITK5). After induction, the crude extracts were made as described in 'Materials and Methods'. Topoisomerase I activities of these lysates were measured (Fig. 6). The lysates from both *E. coli* cells showed similar DNA relaxation activity in the presence or absence of Mg^{2+} which is essential for the activity of the bacterial topoisomerase I (17) (Fig. 6,lanes 4 to 6). The relaxing activities of lysates were not due to nuclease activities of the extracts, as shown by conversion of the product relaxed form I to positively supercoiled DNA in the presence of ethidium bromide. Furthermore, the activity in the lysate did relax positively supercoiled DNA, which *E. coli* topoisomerase I is devoid of this activity (data not shown, 1). These results indicate that the relaxation activities detected in the lysate harboring pRITWT or pRITK5 are due to eukaryotic topoisomerase I activities. Total activities recovered in the lysates of 100 ml culture were 2×10^4 units in both pRITWT and pRITK5 transformants. Assuming the specific activity of the

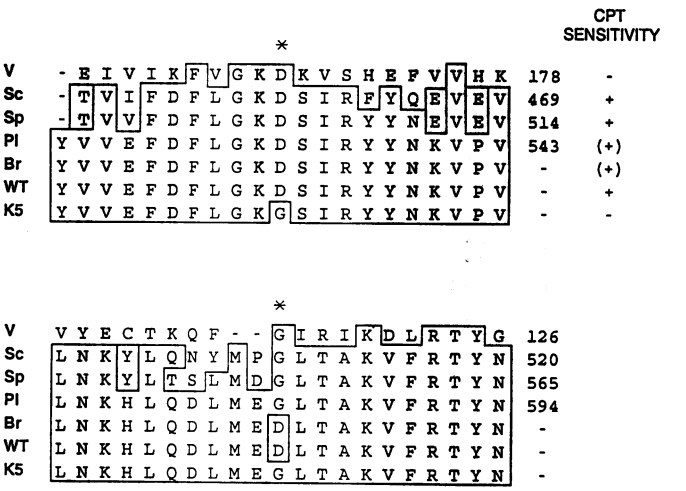

Figure 4. Alignment of predicted amino acid sequences for eukaryotic and vaccinia virus type I topoisomerases surrounding the mutation sites. V, vaccinia virus; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Pl, human placenta; Br, human brain stem; WT, wild-type; K5, CPT-K5. Residues that are identical are boxed. Dashed lines indicate gaps inserted into the sequence in alignment. Asterisks indicate the positions of mutation sites identified in the CPT-K5 cDNA. Amino acids are designated by the single-letter code. The numbers at the right side of the sequences represent the residue number at the right end of each protein. CPT sensitivity is shown by + (sensitive), − (resistant) and (+) (presumably sensitive).

fusion protein being the same as that of the native enzyme, i.e. 2 units per ng protein, ~0.1% of the whole proteins in the lysates consists of the fusion protein. This low level of expression might be due to instability and/or toxicity of these fusion proteins in the host cells. Immunoblot analysis showed that lysates exhibiting the eukaryotic topoisomerase I activity contained an immunoreactive polypeptide whose size corresponded to that of the expected fusion protein (100 kDa) whereas the control lysate

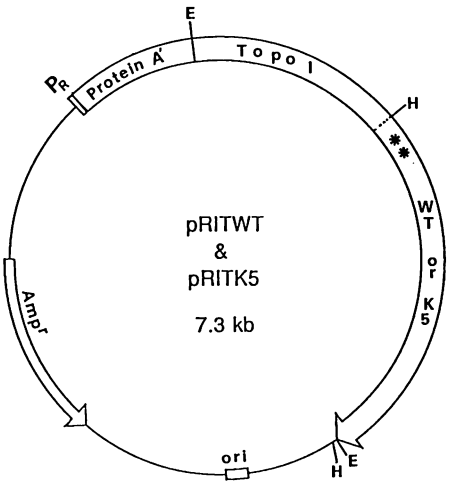

Figure 5. Constructs of plasmids pRITWT and pRITK5. Plasmids expressing fused protein which consists of N-terminal fragment of Protein A and C-terminal two-thirds of topoisomerase I was constructed as described in 'Materials and Methods'. Asterisks indicate the mutation sites in the CPT-K5 cDNA. E, EcoRI; H, Hind III. Only Hind III sites used to construct a mosaic topoisomerase I cDNA are indicated.

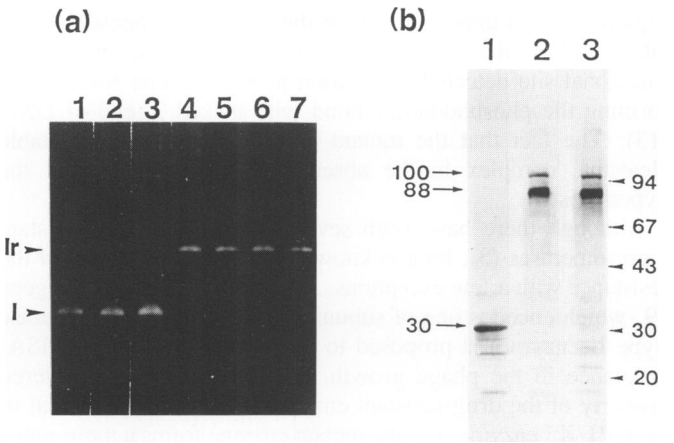

Figure 6. Expression of human topoisomerase I in transformants harboring the plasmids pRITWT and pRITK5. The fusion protein was induced from the lambda phage promoter P_R by inactivation of ts cI_{857} repressor. (a) Topoisomerase I activities in the heat- induced lysates of bacteria harboring pRIT2T (lanes 2 and 3), pRITWT (lanes 4 and 5) and pRITK5 (Lanes 6 and 7) in the absence (lanes 2, 4 and 6) and presence (lanes 3, 5 and 7) of Mg^{2+}. Two microliters of each extract was used for the assay. Lane 1, substrate ColE1 DNA only. I and Ir represent form I and relaxed form I DNA, respectively. (b) Immunoblot analysis of lysates with anti-human topoisomerase I IgG. Aliquots (10 μl) of each lysate were subjected to SDS-polyacrylamide gel electrophresis followed by electroblotting onto a nylon membrane. The colormetric detection of immune complexes was performed as described (10). Lysates of bacteria harboring pRIT2T (lane 1), pRITWT (lane 2) and pRITK5 (lane 3) were analyzed. The positions and sizes ($M_r \times 10^{-3}$) of marker proteins are indicated by the arrow heads. Arrows indicate the positions of major immunoreactive polypeptides with $M_r \times 10^{-3}$.

harboring only the vector showed no such band (Fig. 6(b)). The lysates also contained several additional immunoreactive polypeptides (probably proteolytic products of the 100 kDa polypeptide). It is to be ascertained whether the 88 kDa immunoreactive protein possesses an enzymatic activity. The major band with low molecular weight (30 kDa) observed in the

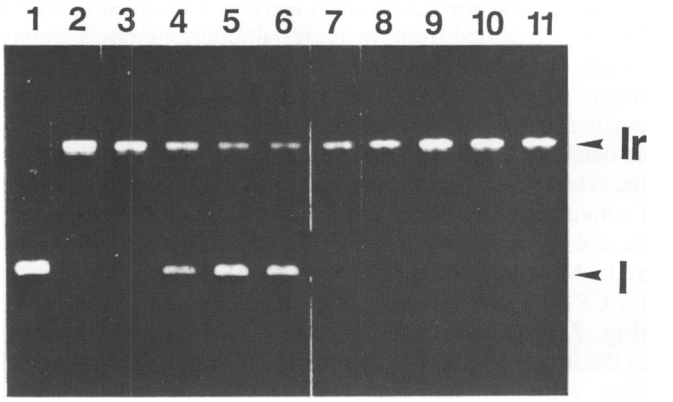

Figure 7. Effect of CPT on the partially purified fusion topoisomerase I. Fusion enzymes in the crude lysates were partially purified with heparin-Sepharose as described in 'Materials and Methods.' and the relaxation activities were assayed in the absence (lanes 2 and 7) or presence of CPT at 1.0 (lanes 3 and 8), 5.0 (lanes 4 and 9), 50 (lanes 5 and 10), and 125 μM (lanes 6 and 11). Lanes 2–6, the wild-type fusion topoisomerase I; lanes 7–11, the CPT-K5 fusion topoisomerase I. Lane 1, substrate ColEl DNA only.

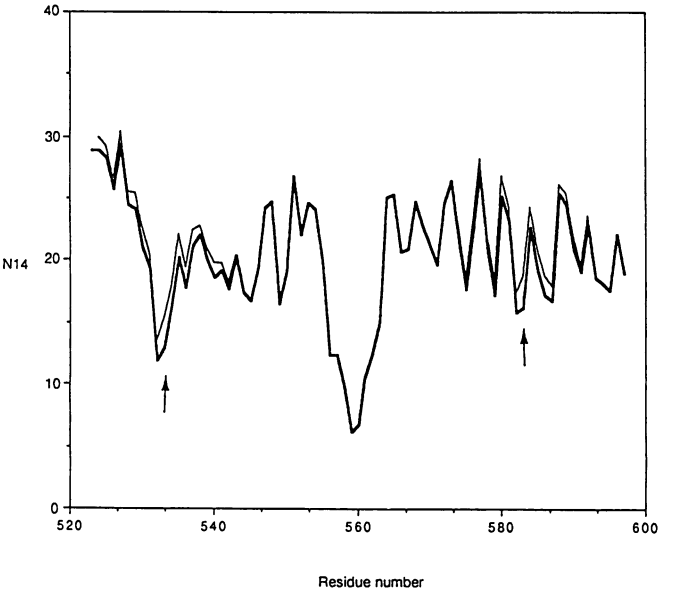

Figure 8. Prediction of the conformational change at the mutation sites of the CPT-K5 enzyme. The amino acid sequences were analyzed by the method of Nishikawa and Ooi (19) as briefly described in 'Materials and Methods'. The computed profiles of the quantity, N_{14}, were plotted along the residue number of the wild-type (thick line) and the CPT-K5 (thin line) enzymes around the mutation sites. Arrows indicate the positions of the mutation sites, residues 533 and 583, respectively.

lysate of the vector-transformed *E. coli* cells seemed to be a Protein A′ which binds to IgG (27). These results indicate that the fusion protein of topoisomerase I linked to Protein A′ was expressed in *E. coli*, and exhibited the eukaryotic topoisomerase I activity.

CPT resistance of topoisomerase I activities of the fusion proteins

To assess the resistance to CPT of the fusion protein, the topoisomerase I activity was partially purified from the lysates by chromatography on heparin-Sepharose as described in

'Materials and Methods.' Each activity was eluted at 0.6–0.7 M KCl, separated from the bacterial topoisomerase I eluting at a higher salt concentration. Immunoblot analysis showed that two major polypeptides (100 kDa and 88 kDa) in both partially purified active fractions reacted with the anti-topoisomerase I IgG as detected in the crude lysates (data not shown). Fig 7 shows the effect of CPT on the partially purified fusion topoisomerase I activities. When added to the active fraction (2 units/assay) of the wild-type fusion protein, CPT inhibited the relaxation activity at 1 μM or higher (Fig. 7, lanes 3–6) whereas the activity of the CPT-K5 was not inhibited at all concentrations up to 125 μM (Fig. 7, lanes 7–11). From these results, the resistance index of the fusion CPT-K5 topoisomerase I is calculated to be >125. This value is consistent with that of the native CPT-K5 topoisomerase I (10).

Prediction of conformational change of human topoisomerase I at the mutation sites

The conformation of a protein has been amply discussed in relation to the hydrophobicity–hydrophilicity of amino acids, to helix-turn structure, etc. (28,29). We have employed the method of Nishikawa and Ooi (20) to predict a conformation of human DNA topoisomerase I and the effect of the mutations on it. N_{14} value was calculated for each amino acid residue along the entire length of the protein. In Fig. 7, the values around the mutation sites were plotted. The result predicted that the region containing the mutations was protruding toward the surface of the molecule, and the mutation replacing aspartic acid to glycine makes the region recess from the surface.

DISCUSSION

We have isolated cDNAs of DNA topoisomerase I from a CPT-resistant human lymphoblastic cell line (CPT-K5) possessing a CPT-resistant topoisomerase I, and from its parental cells (RPMI8402). We identified two mutations in the CPT-K5 cDNA by sequence analyses. When the mutant topoisomerase I was expressed in *E. coli* as a fusion protein linked to Protein A′, the fused protein exhibited the same degree of resistance to CPT as the native CPT-K5 resistant enzyme. These results indicate that the mutations found in the conserved region located near the C-terminus are responsible for the resistance of the CPT-K5 enzyme to CPT; the finding that the mutated topoisomerase I expressed in *E. coli* maintained the same degree of resistance to the drug excluded the possibility of the change(s) of post-translational modification of the enzyme being the cause of the resistance.

We found mutations at two sites in the CPT-K5 cDNA. However, it is not clear which mutation is responsible for the resistance. The amino acid residues corresponding to the position 533 of human topoisomerase I are aspartic acid for all species compared (Fig. 4), with the exception of glycine in the CPT-K5 topoisomerase I, whereas the residues corresponding to the position 583 are variable, i,e. glycine for all the enzymes compared except for the wild-type and brain stem topoisomerase I. Taking into account the fact that yeast enzymes are of glycine-type and is sensitive to CPT, one could argue that the differences at residue 583 (Asp or Gly) among the human enzymes are the result of a polymorphism of the genome unrelated to the CPT-resistance, and the mutation at this position as well as that at 533 may have occurred in the course of establishing the CPT-K5 cell line. If this is correct, the key mutation responsible for the resistance might be the one at 533 (Asp→Gly). In order to clarify this point, *in vitro* mutagenesis to create single mutation at either site is under investigation.

Although we have not yet compared the N-terminal region corresponding to 23 amino acids between the wild-type and the CPT-K5 topoisomerase I cDNAs, the finding that the resistance was expressed as a fusion protein lacking N-terminal 162 amino acid residues suggests that this region is not involved in the acquisition of CPT-resistance. It was shown earlier that the N-terminal region is not involved in the relaxation activity by the findings that a 68 kDa proteolytic product exhibits the relaxation activity *in vitro* (30) and CPT-resistance as well (31). It is tempting to speculate that the N-terminal region plays a role in the interaction with regulatory molecules and/or specific sequences(s) of DNA.

One model for inhibition by CPT is that CPT binds avidly to some specific site(s) on the covalent topoisomerase I-DNA complex, thereby stabilizing the complex and blocking the resealing step of the reaction cycle (3). Perhaps the mutations in the CPT-K5 enzyme confer resistance by altering this drug-binding site(s) of the complex. This hypothesis is supported by the prediction on the secondary structure of the enzyme that the mutation sites seem to be facing toward the outer surface of the protein (Fig. 8). Furthermore, the finding that the region, where mutations were detected, is within the most conserved region along the sequence of the enzyme strongly suggests that this region plays an important role in the catalysis, especially in the latter half of the nicking and closing reactions, the second functional site detected in addition to the tyrosine residue-723 forming the phosphodiester bond with nicked 3′-end of DNA (13). The fact that the mutant enzyme forms a more stable cleavable complex in the absence of CPT (11) favors the hypothesis.

Although there have been several reports on drug-resistant topoisomerases (3), little is known about the mechanism of the resistance with a few exceptions. An amber mutation in the gene 39, which encodes one of subunits of T4 phage topoisomerase (type II enzyme) is proposed to be responsible for *m*-AMSA-resistance in the phage growth (32). In this case, an altered property of the drug-resistant enzyme is very similar to that of the CPT-K5 enzyme, i.e. the mutant enzyme forms a more stable cleavable complex in the absence of the drug. This suggests that the stability of the DNA-protein cleavable complex in the absence of the drug is closely related to the drug resistance of these enzymes. Recently, Shuman *et al.* showed that vaccinia virus topoisomerase I is resistant to CPT (33), although amino acids corresponding to the mutation sites in the CPT-K5 enzyme are of the sensitive type (Fig. 4). According to the prediction of the tertiary structure of vaccinia virus topoisomerase I by the method of Nishikawa and Ooi (20), the protein assumes quite different conformation around these sites from others (data not shown). This difference might account for the resistance to CPT of the vaccinia virus topoisomerase I.

Recently one of the CPT derivatives, CPT-11, has proved to be remarkably effective as an antitumor agent with reduced side effects (34). In view of the clinical usefulness of CPT derivatives, the identification of the mutation sites of CPT resistant topoisomerase I is potentially useful for modeling novel derivatives acting against CPT resistant tumor cells. Further studies of the resistance mechanism to CPT will not only provide useful information for clinical intervention in cancer chemotherapy but also a better understanding of the mechanism of action of this important enzyme.

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