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Identification and Characterization of a Deletion Mutant of DNA Topoisomerase I mRNA in a Camptothecin-resistant Subline of Human Colon Carcinoma

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In previous studies, we established two camptothecin (CPT)-resistant sublines, HT-29/CPT and St-4/CPT, from the human colon cancer cell line HT-29 and the human stomach cancer cell line St-4, respectively. Cellular contents of DNA topoisomerase I (topo I) in the resistant cells were eight-fold less than those in the corresponding parental lines. In this study, we have shown expression of two species of the *TOP1* mRNA in HT-29/CPT. The longer mRNA (4.0 kb) is the wild-type *TOP1* mRNA, and the shorter mRNA (3.3 kb) proved to have a deletion of 672 bp (nucleotides 58–729 or 59–730) that caused the in-frame deletion of amino acids 20–243 of human topo I. The deleted region is identical to exons 3–9 of the *TOP1* gene. The expression level of the 3.3-kb mRNA was similar to that of the wild-type mRNA in HT-29/CPT. St-4/CPT expressed only the wild-type *TOP1* mRNA in lesser amounts than did St-4. Mouse NIH3T3 cells transfected with the wild-type *TOP1* cDNA showed higher sensitivity to CPT than the parental cells, whereas those transfected with the deleted *TOP1* cDNA showed levels similar to those of the parental cells. Expression of the exogenous *TOP1* mRNA was confirmed; however, expression of the truncated topo I was not detected in cells transfected with the deleted *TOP1* cDNA. These results suggest that the expression of the deleted *TOP1* mRNA led to the low expression of CPT-sensitive topo I in the resistant cells.

Key words: DNA topoisomerase I — Camptothecin — Drug resistance — Deletion mutant

Camptothecin (CPT) is an antitumor alkaloid isolated from *Camptotheca acuminata*, a tree native to southern China. Although this compound showed strong activity against experimental tumor models, it was found to be ineffective in clinical studies.[1–3] Severe side effects included myelosuppression, vomiting, diarrhea, and hemorrhagic cystitis, and as a result clinical trials were discontinued.[4, 5] Many derivatives of CPT that have less toxicity and higher solubility in water have been synthesized and evaluated. CPT-11, a water-soluble analog of CPT, has broad-spectrum activity against murine tumors and human tumor xenografts, including multidrug-resistant tumors.[6–8] A recent study showed that a half-molecule ABC transporter BCRP/MXR/ABCP is involved in CPT-11 resistance.[9] Glucuronidation of SN-38, an active metabolite of CPT-11, by UDP-glucuronosyltransferase is another important determinant for CPT-11 sensitivity.[10] Clinical studies demonstrated that CPT-11 is effective against various types of cancer.[11–14] A survival advantage of CPT-11 treatment was found especially for colon cancer.[14]

CPT inhibits both DNA and RNA synthesis[15, 16] and induces reversible DNA strand breaks in mammalian cells,[17] due to the inhibition of DNA topoisomerase I (topo I).[18, 19] Eukaryotic DNA topoisomerases are involved in balancing torsional stress arising in DNA during transcriptional and replicational processes.[20] Topo I resolves torsional problems by a mechanism that involves concerted breaking and rejoining of DNA along with strand passage. CPT binds to topo I in the enzyme-DNA complex and thereby inhibits the religation reaction of the enzyme.[18, 21] Crystal structures of human topo I have been reported, and a mode of CPT binding to the enzyme-DNA complex was proposed based on the structural information.[22] One possible mechanism of CPT-induced cell killing is that the drug-stabilized cleavage complex interferes with replication forks, resulting in replication arrest and fork disassembly.[23, 24]

To examine the role of topo I in the mechanism of CPT action and to characterize the resistance mechanism to

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The abbreviations used are: CPT, camptothecin; CPT-11, 7-ethyl-10-[4-(1-piperidino]-1-piperidino)carbonyloxy-camptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; topo I, DNA topoisomerase I; IC_{50}, concentration of drug required for 50% inhibition of cell growth; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT-PCR, reverse-transcription-PCR; HA, hemagglutinin.

CPT, we have established CPT-resistant tumor cell lines of different origins.[25] In previous studies, we demonstrated the quantitative decrease in the cellular content of topo I in three CPT-resistant lines, HT-29/CPT, St-4/CPT and P388/CPT from the human colon cancer cell line HT-29, the human stomach cancer cell line St-4 and the mouse leukemia cell line P388, respectively.[25] In this study, we show that two species of *TOP1* mRNA, the wild-type (4.0 kb) and a deleted-type (3.3 kb), are expressed in HT-29/CPT. Possible involvement of the expression of the deleted *TOP1* mRNA in CPT resistance was examined by means of DNA transfection experiments.

MATERIALS AND METHODS

Cell culture and assay of drug sensitivity Two CPT-resistant sublines HT-29/CPT and St-4/CPT were established from human colon cancer cell line HT-29 and human stomach cancer cell line St-4, respectively, by treating the tumor cells *in vitro* with increasing concentrations of CPT.[25] Cells were grown in DMEM supplemented with 10% fetal bovine serum. The sensitivities of the cultured cell lines to drugs were evaluated in terms of the inhibition of cell growth after incubation at 37°C for 5 days with various concentrations of drugs, as described previously.[26] The number of tumor cells was counted in a Coulter counter, and the IC_{50} values were determined.

Immunoblot analysis Cells were solubilized with 2% SDS, 5% 2-mercaptoethanol, and 50 m*M* Tris-HCl, pH 7.5. The cell lysates (1×10^5 cells equivalent) were subjected to electrophoresis on 5–20% SDS-polyacrylamide gels.[27] After electrophoresis, the protein was transferred onto a nitrocellulose membrane. For the detection of topo I, the blot was incubated with the anti-topo I monoclonal antibody T14C,[25] washed, and then reacted with ^{125}I-labeled anti-mouse IgG (Amersham, Arlington Heights, IL). For the detection of exogenous topo I with the HA-tag, the blot was incubated with the anti-HA polyclonal antibody Y-11 (Santa Cruz Biotechnology, Santa Cruz, CA), washed, and reacted with peroxidase-conjugated anti-rabbit IgG (Amersham). The membrane-bound peroxidase was visualized using an ECL+Plus kit (Amersham).

RNA blot analysis A *TOP1* cDNA clone λh*TOP1*-D2 was kindly provided by Dr. L. F. Liu.[28] Total cellular RNA was extracted after disruption of tumor cells in guanidine thiocyanate solution followed by CsCl density gradient centrifugation. Polyadenylated mRNA was obtained by chromatography on oligodeoxythymidylate cellulose. The mRNA samples (5 μg/lane) were subjected to electrophoresis on a 1% formaldehyde-agarose gel and transferred to a nitrocellulose membrane. The *TOP1* cDNA probe was radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham), and hybridization was carried out as described previously.[29] The membrane filter was then washed and subjected to autoradiography. The equality of the mRNA loading in each lane of the blot was confirmed by control hybridization with a human β-actin cDNA probe, as described.[30]

Isolation of cDNA clones and construction of expression vectors The *TOP1* clones corresponding to 4.0-kb and 3.3-kb *TOP1* mRNAs were isolated from an HT-29/CPT cDNA library in λgt11[31] using the *TOP1* cDNA clone λh*TOP1*-D2 as a probe. Nucleotide sequences of the wild-type (4.0 kb) and the deleted (3.3 kb) *TOP1* cDNA were confirmed by an ABI PRISM 377 DNA Sequencing System (Perkin-Elmer, Foster City, CA). Next, the HA-tag[32] was added upstream from the first ATG codon of the wild-type or the deleted *TOP1* cDNA to differentiate between the endogenous and exogenous topo I in the transfected cells. For this purpose, the 5′-parts of the wild-type and the deleted *TOP1* cDNAs were amplified by PCR using 5HA-S (5′-CGCGCTAGCATGTACCCATACGACGTCCCAGACTACGCTATGAGTGGGGACCACCTCCAC-3′; HA-antigen sequence and nucleotides 1 to 22 from the first ATG of *TOP1* cDNA[33]) and 5HA-AS (5′-CATGATTCGTCTCTTCAGCATGCCCATCTTG-3′; antisense of nucleotides 1104 to 1134 of *TOP1* cDNA) as primers. The resulting PCR products were ligated with the 3′-part of *TOP1* cDNA. The wild-type and the deleted *TOP1* cDNAs with the HA-tag were cloned into a mammalian expression vector, pCALNLw, that has a strong CAG promoter to drive the expression of the cDNA.[34] Nucleotide sequences of these constructs were confirmed.

Transfection Mouse fibroblast NIH3T3 cells (5×10^5 cells/60-mm dish) were cotransfected with the expression construct containing the *TOP1* cDNA (5 μg) and a neomycin-resistant plasmid pMC1neopolyA (0.25 μg; Stratagene, La Jolla, CA) using the SuperFect Transfection Reagent (QIAGEN, Valencia, CA). The cells were replated 2 days after transfection and selected in medium containing 600 μg/ml of G418. G418-resistant colonies were isolated and used in further studies. Integration of the *TOP1* cDNA in the transfectants was examined by genomic PCR using a sense-strand primer p1 (5′-TACGACGTCCCAGACT-3′; the sequence in the HA-tag) and an antisense-strand primer p2 (5′-CAGGAGGCTCTATCTT-3′; antisense of nucleotides 1060 to 1075 of *TOP1* cDNA). Expression of the exogenous *TOP1* mRNA in the transfectants was examined by RT-PCR using a sense-strand primer p1 and an antisense-strand primer p3 (5′-AGAACTCTGCCTCTTG-3′; antisense of nucleotides 2306 to 2321 of *TOP1* cDNA). As an internal standard of RT-PCR, β_2-microglobulin mRNA in the transfectants was amplified using a sense-strand primer (5′-ACCCCCACTGAAAAAGATGA-3′) and an antisense-strand primer (5′-ATATTCAAACCTCCATGATG-3′).

***In vitro* transcription/translation** To examine the topo I activity of the truncated enzyme, the wild-type and the

deleted *TOP1* cDNAs were cloned into a pGEM-4 vector (Promega, Madison, WI) with an SP6 promoter, and the respective topo I proteins were synthesized using *in vitro* transcription/translation systems (Promega).[35, 36] Topo I activity was assayed essentially as described by Ishii *et al*.[37] In brief, the activity was measured by relaxing the supercoiled plasmid DNA at 37°C for 15 min in a 20 μl reaction mixture containing 0.2 μg of supercoiled pT2GN plasmid DNA, 50 m*M* Tris-HCl (pH 7.5), 50 m*M* NaCl, 5 m*M* EDTA, 0.5 m*M* dithiothreitol, 30 mg/ml BSA, 10% glycerol and various concentrations of *in vitro* translation products. The reaction was terminated by adding SDS to 1%. The extent of DNA relaxation was evaluated after electrophoresis on 0.8% agarose gels.

RESULTS

Expression of *TOP1* mRNA in HT-29/CPT and St-4/CPT Degrees of resistance of these CPT-resistant lines to inhibitors of topo I and II are summarized in Table I. HT-29/CPT and St-4/CPT expressed approximately one-eighth the amount of topo I as did the corresponding parental lines (Fig. 1A). To understand the molecular basis of this lowered expression in the CPT-resistant cells, RNA blot analysis was carried out using the human *TOP1* cDNA probe λhTOP1-D2. As shown in Fig. 1B, two species of the *TOP1* mRNA were found in HT-29/CPT. The longer mRNA (4.0 kb) is the wild-type *TOP1* mRNA, and the shorter mRNA (3.3 kb) appears to possess a deletion. The amounts of the two mRNAs in HT-29/CPT were approximately one-half the amount of the wild-type mRNA expressed in the parental HT-29. On the other hand, St-4/CPT expressed only the wild-type *TOP1* mRNA, and at a lower level than in St-4 (Fig. 1B).

Identification of the deleted region in the 3.3-kb mRNA To identify the deleted region of the 3.3-kb mRNA expressed in HT-29/CPT, we carried out a series of gene amplification experiments using various primers with cDNA templates from HT-29 and HT-29/CPT. One set of primers, 2F (5′-TCCCAGATCGAAGCGGATTT-3′; nucleotides 28 to 47 from the first ATG of *TOP1* cDNA[33]) and 5R (5′-GATTCGTCTCTTCAGCATGC-3′; antisense of nucleotides 1112 to 1131) successfully amplified the deleted cDNA fragment in HT-29/CPT (data not shown). The expected size of the PCR fragment was 1104 bp; however, the amplification gave rise to a 432-bp fragment in HT-29/CPT. The difference between the lengths of the PCR fragment and the expected fragment, 0.7 kb, was the same as the difference between the lengths of the two *TOP1* mRNAs expressed in HT-29/CPT (Fig. 1B). The

Table I. Drug Resistance of HT-29/CPT and St-4/CPT

Cell lines	Degree of resistance (*x*-fold)			
	CPT	ADM	VP-16	m-AMSA
HT-29/CPT	6.9[a)]	0.3	1.4	0.2
St-4/CPT	8.0	1.8	3.2	0.9

a) Degree of resistance was calculated by dividing the IC_{50} value of the resistant lines by that of the corresponding parental line.

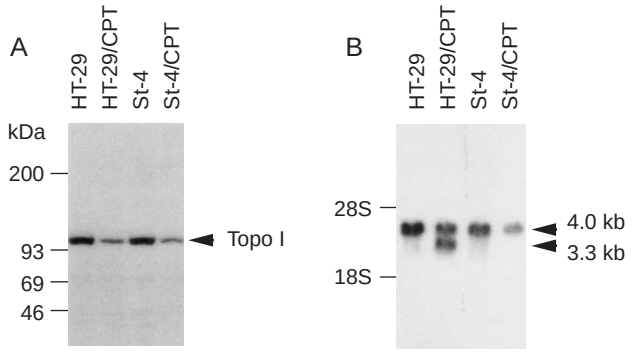

Fig. 1. (A) Immunoblot analysis of whole cell lysates from CPT-sensitive and -resistant cell lines. Cell lysates from 1×10^5 cells were prepared, subjected to SDS-PAGE in 5–20% polyacrylamide gel and subsequently transferred to a nitrocellulose filter. The blot was reacted with the anti-topo I monoclonal antibody T14C and ^{125}I-labeled anti-mouse IgG. (B) RNA blot analysis of mRNA from CPT-sensitive and -resistant cell lines. Polyadenylated mRNA (5 μg) was electrophoresed in a formaldehyde-agarose gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled human *TOP1* cDNA probe λh*TOP1*-D2.

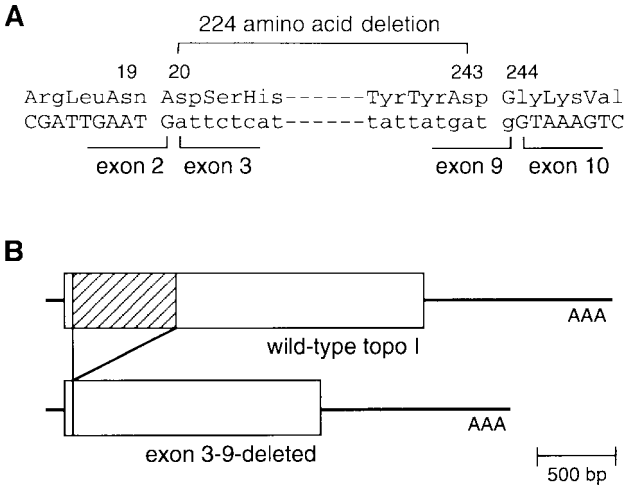

Fig. 2. (A) Nucleotide and amino acid sequences at the break points of the exon 3–9-deleted *TOP1* cDNA. (B) Schematic structures of wild-type and deleted *TOP1* cDNAs. The diagonal region is identical to exons 3–9 of the *TOP1* gene.

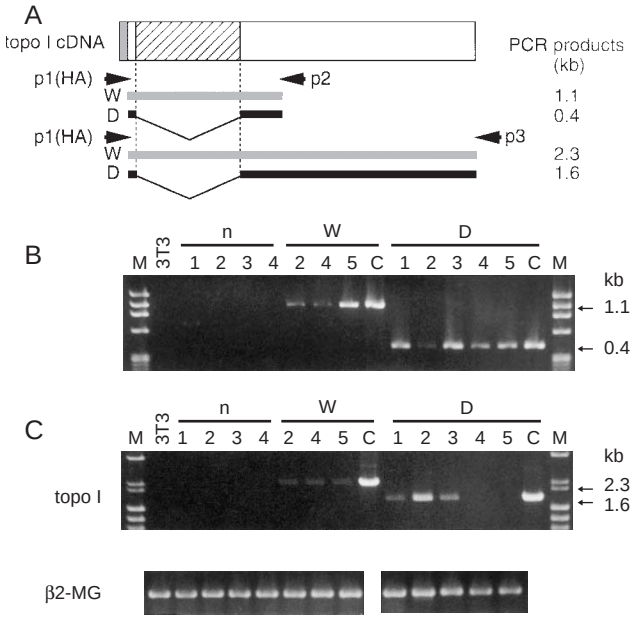

Fig. 3. Integration of the *TOP1* cDNA and expression of the *TOP1* mRNA in NIH3T3 cells transfected with wild-type or deleted *TOP1* cDNA. (A) Positions of PCR primers and expected sizes of the amplified products. (B) Detection of the exogenous *TOP1* cDNA by genomic PCR using p1-p2 primers. (C) topo I: Detection of the exogenous *TOP1* mRNA by RT-PCR using p1-p3 primers. β2-MG: Detection of β_2-microglobulin mRNA as an internal control of RT-PCR. M, DNA size marker ϕX174/*Hae*III. 3T3, parental NIH3T3 cells. n, mock transfectant clones. The numbers 1, 2, 3 and 4 represent clones n1, n2, n3 and n4, respectively. W, clones transfected with the wild-type *TOP1* cDNA. The numbers 2, 4 and 5 represent clones W2, W4 and W5, respectively. C, control PCR product using wild-type *TOP1* cDNA as a template. D, clones transfected with the deleted *TOP1* cDNA. The numbers 1, 2, 3, 4 and 5 represent clones D1, D2, D3, D4 and D5, respectively. C, control PCR product using the deleted *TOP1* cDNA as a template.

432-bp PCR fragment was subcloned into *Sma*I-digested pUC13 vector and sequenced. The nucleotide sequence of the PCR fragment was identical to the reported sequence of *TOP1* cDNA[33] but lacked 672 bp (nucleotides 58–729 or 59–730). This deletion should cause an in-frame deletion of amino acids 20–243 of human topo I (Fig. 2). Since the nucleotides at 58 and 730 are both G, we cannot identify which was deleted. However, it is likely that the region 59–730 was deleted because it is identical to exons 3–9 of the human *TOP1* gene.[38] Therefore, we refer to the short mRNA as exon 3–9-deleted *TOP1* mRNA. In spite of the expression of the truncated mRNA with an in-frame deletion in HT-29/CPT, we could not detect the corresponding 70-kDa protein by western blots with anti-topo I monoclonal antibody T14C (Fig. 1A) or anti-topo I polyclonal antibody Scl-70 (Topogen, Columbus, OH) (data not shown).

Mammalian expression experiments The wild-type and the deleted *TOP1* cDNAs were cloned into a pCALNLw vector and were termed pCAL-TW and pCAL-TD, respectively. Mouse fibroblast NIH3T3 cells were cotransfected with pCAL-derived construct and pMC1neopolyA. The transfectants were selected in medium containing 600 μg/ml of G418. G418-resistant colonies were isolated and used in further studies.

Integration of the *TOP1* cDNA and the expression of the exogenous *TOP1* mRNA in the transfectant cells were examined in a series of PCR experiments. The position of PCR primers and the expected sizes of amplified products are summarized in Fig. 3A. In this series, only the exogenous *TOP1* cDNA and mRNA were amplified since primer p1 codes the HA-tag. First, integration of the exogenous *TOP1* cDNA with the HA-tag was examined by genomic PCR using primers p1 and p2. Transfectant clones with integration of the exogenous *TOP1* cDNA (three clones, W2, W4 and W5, transfected with the wild-

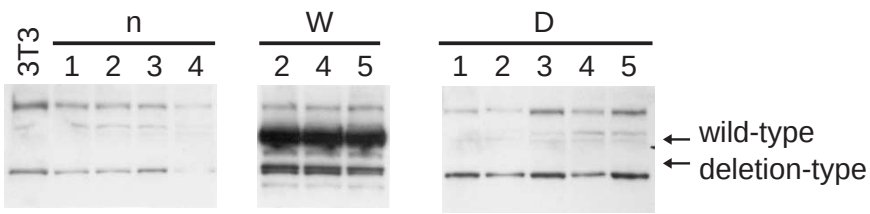

Fig. 4. Immunoblot analysis of whole cell lysates from clones transfected with wild-type or deleted *TOP1* cDNA. Cell lysates from 1×10^5 cells were prepared, subjected to SDS-PAGE in 5–20% polyacrylamide gel and subsequently transferred to a nitrocellulose filter. The blot was reacted with the anti-HA polyclonal antibody Y-11, washed and incubated with peroxidase-conjugated anti-rabbit IgG as described in "Materials and Methods." 3T3, parental NIH3T3 cells. n, mock transfectant clones. The numbers 1, 2, 3 and 4 represent clones n1, n2, n3 and n4, respectively. W, clones transfected with the wild-type *TOP1* cDNA. The numbers 2, 4 and 5 represent clones W2, W4 and W5, respectively. D, clones transfected with the deleted *TOP1* cDNA. The numbers 1, 2, 3, 4 and 5 represent clones D1, D2, D3, D4 and D5, respectively.

type *TOP1* cDNA and five clones, D1, D2, D3, D4 and D5, transfected with the deleted *TOP1* cDNA) were used in further experiments (Fig. 3B). Integration of the exogenous full-length *TOP1* cDNA in these clones were confirmed by another genomic PCR using primers p1 and p3 (data not shown). As shown in Fig. 3C, expression of the exogenous, wild-type *TOP1* mRNA (2.3 kb) was detected in W2, W4 and W5 by RT-PCR using the p1 and p3 primers. Expression of the deleted *TOP1* mRNA (1.6 kb) was detected in D1, D2 and D3, but not in D4 and D5.

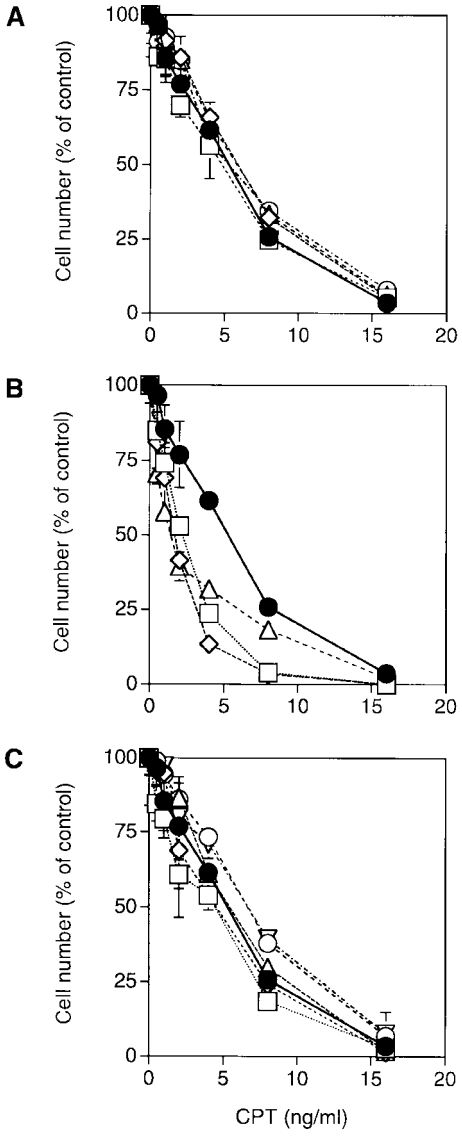

Fig. 5. Sensitivity to CPT of clones transfected with wild-type or deleted *TOP1* cDNA. To evaluate the sensitivity of the transfectants to CPT, cells were cultured in medium containing various concentrations of CPT. After 5 days, viable cells were counted, and the percentage of cell growth was calculated (means±SEM, n=4 per group). Closed circles, CPT sensitivity of the parental NIH3T3 cells. (A) CPT sensitivity of the mock transfectant clones. ● 3T3, □ n1, ◇ n2, △ n3, ○ n4. (B) CPT sensitivity of three clones transfected with the wild-type *TOP1* cDNA. ● 3T3, □ W2, ◇ W4, △ W5. (C) CPT sensitivity of five clones transfected with the deleted *TOP1* cDNA. ● 3T3, □ D1, ◇ D2, △ D3, ○ D4, ▽ D5.

Western blot analysis using anti-HA antibody revealed high-level expressions of the exogenous topo I in all three clones transfected with the wild-type *TOP1* cDNA (Fig. 4). No exogenous protein with the expected size was found in clones transfected with the deleted *TOP1* cDNA (Fig. 4).

CPT sensitivity of transfected clones The sensitivity to CPT of the transfectants was examined using a cell growth inhibition assay (Fig. 5). The mock transfectant clones n1, n2, n3 and n4 showed similar CPT sensitivity to the parental 3T3 cells (IC_{50} values were 4.6–5.5 ng/ml) (Fig. 5A). The IC_{50} values to CPT of W2, W4 and W5 were 2.1, 1.6 and 1.3 ng/ml, respectively, which were approximately 3-fold higher than that of the control cells (Fig. 5B). The IC_{50} values to CPT of D1, D2, D3, D4 and D5 were 4.3 to 6.3 ng/ml, which showed no significant changes in drug resistance from that of the control cells (Fig. 5C). The IC_{50} values of these clones are plotted in Fig. 6.

***In vitro* transcription/translation of topo I** To examine the catalytic activity of the deleted topo I, *in vitro* translation experiments were carried out using pGEM-TW or pGEM-TD with the HA-tagged wild-type or deleted *TOP1* cDNA, respectively. High-level expression of the wild-type topo I (100-kDa protein) was detected by western blot analysis. The deleted topo I protein (70 kDa) was also

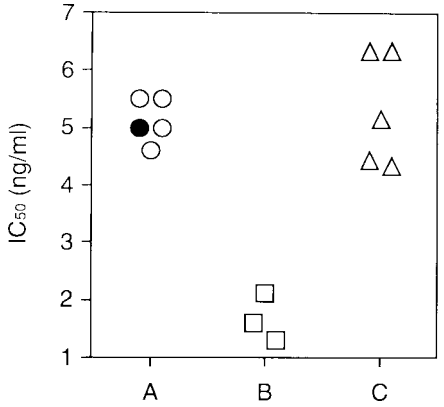

Fig. 6. IC_{50} values for CPT of clones transfected with the *TOP1* cDNA. (A) Closed circle, IC_{50} value of parental NIH3T3 cells; open circles, IC_{50} values of mock transfectant clones. (B) Open squares, IC_{50} values of clones transfected with the wild-type *TOP1* cDNA. (C) Open triangles, IC_{50} values of clones transfected with the deleted *TOP1* cDNA.

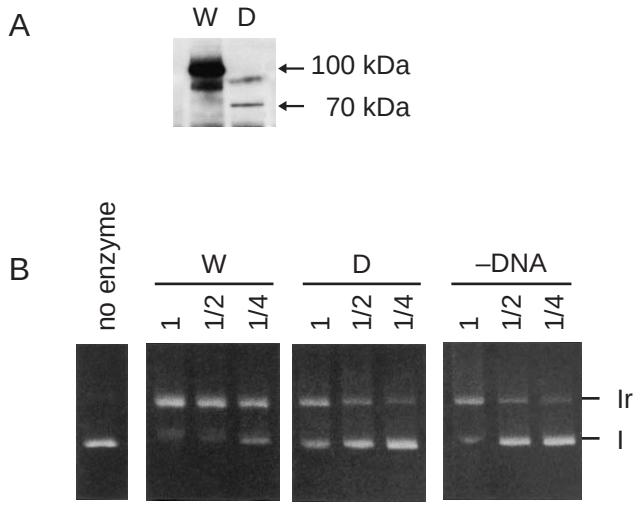

Fig. 7. (A) Detection of *in vitro* translation products by western blot analysis using anti-HA antibody Y-11. W, products from the wild-type *TOP1* cDNA; D, products from the deleted *TOP1* cDNA. (B) DNA relaxation activity of the *in vitro* translation products. Serial dilutions of the *in vitro* translation products were incubated with substrate plasmid DNA as described in "Materials and Methods." no enzyme, substrate plasmid DNA for the assay; W, DNA relaxation activity of *in vitro* translation products from the wild-type *TOP1* cDNA; D, DNA relaxation activity of *in vitro* translation products from the deleted *TOP1* cDNA; −DNA, DNA relaxation activity of *in vitro* translation products without *TOP1* cDNA. The numbers 1, 1/2 and 1/4 represent serial dilutions of *in vitro* translation products. I and Ir represent supercoiled DNA and relaxed closed circular DNA, respectively.

detected, but its expression level was much lower than that of the wild-type protein (Fig. 7A). The 100-kDa protein showed *in vitro* DNA relaxation activity, whereas the 70-kDa protein had no detectable activity (Fig. 7B).

DISCUSSION

In this study, we showed the expression of two species of *TOP1* mRNA in HT-29/CPT cells. The longer mRNA (4.0 kb) was the wild-type *TOP1* mRNA, and the shorter mRNA (3.3 kb) had a deletion that resulted in the internal deletion of 224 amino acids (residues 20−243) of human topo I. The cDNAs corresponding to both mRNAs were isolated and introduced into NIH3T3 cells. Cells transfected with the wild-type *TOP1* cDNA showed higher sensitivity to CPT than did 3T3 cells. On the other hand, transfectant clones with the deleted *TOP1* cDNA showed similar levels of drug resistance to 3T3 cells.

The amounts of the wild-type and mutant mRNAs in HT-29/CPT were the same and together were approximately one-half the amount of wild-type mRNA expressed in the parental HT-29 (Fig. 1B). This suggested that the deleted *TOP1* mRNA in HT-29/CPT cells was transcribed from one of the alleles of the genome. To identify the possible deletion or rearrangement in one of the alleles of the *TOP1* gene, Southern blot analysis was carried out using eight restriction endonucleases. However, we could not identify significant rearrangements or deletions of the *TOP1* gene in HT-29/CPT (data not shown). Therefore, it is likely that certain mutations in the splicing donor/acceptor sites in one of the alleles in HT-29/CPT resulted in a 672-bp deletion of the *TOP1* mRNA. Isolation and analysis of the genomic DNA of HT-29/CPT is ongoing to clarify this point.

In this study we show the expression of truncated *TOP1* mRNA as a mechanism of CPT resistance. Expression of shorter mRNA was reported by Matsumoto *et al.* as a mechanism of resistance to topo II inhibitor.[39] They reported that an acquired 600-bp deletion in one topo IIα allele resulted in a reduced topo IIα protein level of a VP-16-resistant subline MDA-MB-231-VP7. Therefore expression of shorter mRNA may be an infrequent event, but it can happen in other systems.

Quantitative reduction of topo I in resistant cells has been reported in various systems and seems to be the most common mechanism of CPT resistance. Any mechanism which would result in lowered protein expression can be related to CPT resistance. Such mechanisms include low mRNA expression, genomic deletion, alternative splicing, protein instability, and so on. We previously reported the reduction of cellular topo I content in HT-29/CPT, St-4/CPT and P388/CPT.[25] In this study, we found that the expression of *TOP1* mRNA was also decreased in St-4/CPT from the parental St-4. These results suggest that the decrease in the cellular content of topo I in St-4/CPT was caused simply by the reduction in the cellular content of its mRNA. In contrast, the total amount of *TOP1* mRNA in HT-29/CPT appeared similar to the mRNA expressed in the parental HT-29, suggesting that mechanisms related to the transcription efficiency and mRNA stability were not altered in HT-29/CPT. The amount of wild-type *TOP1* mRNA expressed in HT-29/CPT cells was approximately one-half that in parental HT-29; however, the cellular content of topo I in HT-29/CPT was eight-fold less than that in the parental HT-29. Therefore, the expression level of wild-type *TOP1* mRNA in HT-29/CPT does not account for the low amount of the protein. This result suggests that there exists a mechanism other than lowered mRNA expression for the low-level content of topo I in HT-29/CPT. One possible mechanism is an acceleration of topo I degradation in HT-29/CPT. The stability of topo I protein and proteasome function in HT-29/CPT is to be examined to clarify this point.

NIH3T3 cells transfected with the wild-type *TOP1* cDNA showed higher sensitivity to CPT than the control

cells (Figs. 5, 6). *TOP1*-transfected baby hamster kidney cells expressed two- to five-fold higher amounts of wild-type topo I and showed increased cell death in the presence of CPT.[40] In another system, COS cells expressing *Saccharomyces cerevisiae* topo I showed increased CPT sensitivity.[41] These results clearly demonstrate that cellular contents of topo I directly correlate to the sensitivity of cells to CPT.

Mechanisms of CPT resistance other than the quantitative reduction of topo I have been reported in various experimental systems. We previously reported the expression of mutant topo I in a CPT-resistant human T lymphoblastic leukemia cell line, CPT-K5.[42] An amino acid change from Asp to Gly at residue 533 is responsible for the CPT resistance of the enzyme.[43, 44] The expression of mutant (Gly-533) topo I confers a dominant form of CPT resistance in cells expressing wild-type topo I.[45] Maliepaard *et al.* recently reported that a half-molecule ABC transporter BCRP/MXR/ABCP is involved in CPT-11 resistance.[9] Glucuronidation of SN-38, an active metabolite of CPT-11, by UDP-glucuronosyltransferase is another important determinant for CPT-11 sensitivity.[10]

The exon 3–9-deleted topo I of HT-29/CPT lacks amino acid residues 20–243. The deleted region contains the putative nuclear localization signal (residues 141–210).[46] Human topo I with the deletion of the 70-amino-acid region showed topo I catalytic activity *in vitro*. Overexpression of topo I in *S. cerevisiae* is lethal, whereas overexpression of the topo I with the deletion of the 70-amino-acid region did not result in cell death,[46] suggesting that the truncated enzyme is inactive *in vivo* because of a defect in its transport into the nucleus. Therefore, it is not surprising that the CPT sensitivity of the NIH3T3 cells was not affected by the transfection of the deleted *TOP1* cDNA. It is possible to speculate that the exon 3–9-deleted topo I is unstable when it exists in the cytoplasm. In *in vitro* translation experiments, expression of a 70-kDa protein was detected from the deleted *TOP1* cDNA. But, as shown in Fig. 7A, the level of the 70-kDa protein was much lower than that of the 100-kDa protein from the wild-type *TOP1* cDNA. The inefficient expression of the 70-kDa protein supports the notion that the 3–9-exon region of *TOP1* gene is involved in the enzyme's stability. This may be the reason why we could not detect the 70-kDa protein expression in HT-29/CPT or in cells transfected with the deleted *TOP1* cDNA.

Whether or not the exon 3–9-deleted topo I encodes a functional enzyme was not clarified in this study. Vaccinia virus topo I consists of 314 amino acids and has a molecular weight of 32000, which is one-third of the molecular weight of the human enzyme.[47] In addition, the vaccinia virus enzyme does not have the region that corresponds to exons 3–9 of the human enzyme. Therefore, it is possible that the exon 3–9-deleted topo I does, in fact, function. However, DNA relaxation activity of the 70-kDa protein synthesized by *in vitro* translation could not be detected, probably due to the low expression of the protein (Fig. 7B). Large-scale preparation and purification of the 70-kDa protein will be necessary to elucidate this point.

In this study, we identified the exon 3–9-deleted *TOP1* mRNA in CPT-resistant cells. Cells transfected with the deleted *TOP1* cDNA showed no significant changes in CPT sensitivity, probably because of the instability of the truncated topo I protein in the cells. These results suggest that expression of the deleted *TOP1* mRNA leads to low expression of CPT-sensitive topo I in resistant cells. Possible interactions of the truncated topo I with wild-type enzyme and endogenous topo I-interacting proteins could be examined more precisely by means of a double transfection experiment with both the deleted and wild-type *TOP1*.

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