

## Hypophosphorylation of Topoisomerase II $\alpha$ in Etoposide (VP-16)-resistant Human Carcinoma Cell Lines Associated with Carboxy-terminal Truncation

Yoshihito Matsumoto,<sup>1,2,3</sup> Hiroshi Takano,<sup>2</sup> Katsuzo Kunishio,<sup>1</sup> Seigo Nagao<sup>1</sup> and Tito Fojo<sup>2</sup>

<sup>1</sup>Department of Neurological Surgery, Kagawa Medical University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793 and <sup>2</sup>Medicine Branch, Bldg 10, Rm 12N226, DCS, NCI, NIH, 9000 Rockville Pike, Bethesda, MD 20892, USA

**Topoisomerase II $\alpha$  is a target for many chemotherapeutic agents in clinical use. To define mechanisms of resistance and regions crucial for the function of topoisomerase II $\alpha$ , drug-resistant cell lines have been isolated following exposure to topoisomerase II poisons. Two resistant sublines, T47D-VP and MCF-7-VP, were isolated from human carcinoma cell lines following exposure to 300 or 500 ng/ml etoposide (VP-16). Cytotoxicity studies confirmed resistance to etoposide and other topoisomerase II poisons. KCl-sodium dodecyl sulfate (K-SDS) precipitation assays using intact cells showed reduced DNA-topoisomerase II complex formation following VP-16 or amsacrine (m-AMSA). RNase protection analysis identified a deletion of 200 base pairs in the topoisomerase II $\alpha$  cDNA of T47D-VP and "AA insertion" in the topoisomerase II $\alpha$  cDNA of MCF-7-VP. Reduced topoisomerase II $\alpha$  mRNA and protein levels were observed in both cell lines. It was somewhat surprising to find that nuclear extracts from T47D-VP and MCF-7-VP cells had comparable topoisomerase II activity to that of parental cells. Analysis of the extent of phosphorylation demonstrated that topoisomerase II $\alpha$  from the resistant cells was relatively hypophosphorylated compared to that of parental cells. In these cell lines, hypophosphorylation secondary to loss of a portion of the C-terminal domain of topoisomerase II $\alpha$  mediated the restored activity, despite a fall in topoisomerase II $\alpha$  mRNA and protein, and this resulted in cross resistance to topoisomerase II poisons.**

**Key words:** Topoisomerase II — Nuclear localization — Acquired drug resistance — Etoposide — Topoisomerase II poisons

The epipodophyllotoxins, VP-16 and VM-26, are useful antineoplastic agents with activity against both hematologic malignancies and solid tumors.<sup>1)</sup> The anticancer activity of these agents is thought to result at least in part from stabilization of the cleavable complex, an intermediate in which topoisomerase II is covalently bound to DNA in a step that precedes DNA cleavage. This mechanism of action is shared by other antitumor agents including the anthracyclines, the aminoacridines, and the ellipticines.<sup>2)</sup>

Topoisomerase II exists in cells as a phosphoprotein; this posttranslational modification occurs primarily on serine residues in the carboxyl domain of the enzyme.<sup>3,4)</sup> Studies using synchronized cells and *in vitro* phosphorylation of purified topoisomerase II $\alpha$  have demonstrated a correlation between phosphorylation and topoisomerase II catalytic activity, suggesting that phosphorylation may be an important mechanism for regulating topoisomerase II function.<sup>5,6)</sup> For example, the extent of phosphorylation of topoisomerase II increases as cells enter the G2/M phase of the cell cycle, when the activity of the enzyme is

greatest.<sup>7,8)</sup> More recently, peptide mapping of topoisomerase II $\alpha$  at various times after synchronization of Chinese hamster ovary cells revealed that CKII $\alpha$  was a major kinase responsible for phosphorylation of topoisomerase II $\alpha$ .<sup>7)</sup> However, several reports suggest that protein kinase C (PKC) may also play a role in phosphorylation and/or regulation of topoisomerase II $\alpha$ .<sup>7)</sup> In all these cases, phosphorylation *in vitro* enhanced topoisomerase II activity, as measured by decatenation assays.<sup>9)</sup>

Analysis of truncated forms of yeast topoisomerase II has provided intriguing insights about the importance of the C-terminal domain and its phosphorylation. Drastic truncations up to amino acid 922 in the *S. cerevisiae* TOP2 gene do not allow rescue of top2ts or deletion strains.<sup>10)</sup> In contrast, C-terminal truncation of the *S. pombe* topoisomerase II to amino acid 1198, which removes most of the potential CKII phosphoacceptor sites, partially complements an *S. pombe* top2ts mutation and a null mutant, allowing conditional growth, yet fails to complement another top2ts mutation.<sup>11)</sup> Surprisingly, protease digestion studies show that a proteolytic core of the enzyme that extends only to amino acid 1220 retains the full decatenation activity of topoisomerase II. Similarly, a truncation of the *S. cerevisiae* enzyme to amino acid 1167, produces a topoisomerase II dimer that is fully active *in vitro*, but

<sup>3</sup> To whom correspondence should be addressed at: Department of Neurological Surgery, Kagawa Medical University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793.

E-mail: mizaya@kms.ac.jp

which complements a top2 disruption poorly *in vivo*.<sup>12)</sup> Paradoxically, when purified, these C terminally truncated enzymes are highly active *in vitro*,<sup>4)</sup> while the full-length, dephosphorylated topoisomerase II is nearly inactive.<sup>13)</sup> Since phosphorylation by CKII suffices to reactivate the full-length enzyme, the C-terminal 259 amino acids is a negative regulatory domain that can be neutralized by phosphorylation. Without this inhibitory domain (e.g., truncated eukaryotic enzymes), topoisomerase II should not require phosphorylation to be active, and should be insensitive to the stimulatory effects of the kinases.

Using a single clone selection process, we have isolated and characterized two human carcinoma cell lines with resistance to topoisomerase II agents. An RNase protection assay identified an acquired mutation. The present study describes the characterization of two etoposide-selected cell lines in which aberrant splicing leads to deletion of an exon, resulting in a C-terminally truncated enzyme, which exhibits impaired phosphorylation.

## MATERIALS AND METHODS

**Cell lines** T47D-VP and MCF-7-VP cells were isolated from parental T47D and MCF-7 human breast cancer cells using a cloning ring to isolate colonies growing in 300 and 500 ng/ml etoposide.

**Cytotoxicity assays** Cytotoxicity assays were performed as previously described.<sup>14)</sup> Briefly, 500 to 1000 cells were plated in 96-well dishes and incubated overnight prior to the addition of drug, followed by an additional 5-day incubation. After fixation with trichloroacetic acid, the dishes were stained with 0.4% sulforhodamine B (SRB) dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 10 mM unbuffered Tris base. The density was determined with a 96-well microtiter plate reader.

**KCl-sodium dodecyl sulfate (K-SDS) precipitation assay for protein-DNA complexes** The *in-vitro* formation of covalent topoisomerase-DNA complexes was quantitated as previously described.<sup>2)</sup> Briefly, the DNA in logarithmically growing cells was labeled by adding [methyl-<sup>3</sup>H]thymidine to a final concentration of 5  $\mu$ Ci/ml medium. After an overnight incubation, cells were trypsinized and total radioactivity determined. Cells were then diluted in fresh medium to a final concentration of  $1 \times 10^5$  cpm/ml, and 1 ml was aliquoted into each well of a 24-well plate prior to an overnight incubation. The following day, cells were treated with a range of drug concentrations for 30 min at 37°C, washed with cold phosphate-buffered saline (PBS) once and lysed by adding 1 ml of prewarmed lysis solution (1.25% SDS, 5 mM EDTA, 0.4 mg/ml salmon sperm DNA, pH 8.0, 65°C). After shearing chromosomal DNA by passing the lysate through a 22 gauge needle, the sample was transferred to a tube con-

taining 0.25 ml of 325 mM KCl. The sample was vortexed vigorously for 10 s, cooled on ice for 10 min and centrifuged at 3000 rpm for 10 min at 4°C. The pellet was resuspended in 1 ml of wash solution (100 mM KCl, 1 mM EDTA, 0.1 mg/ml salmon sperm DNA, 10 mM Tris-HCl, pH 8.0) and placed at 65°C for 10 min with occasional mixing. After cooling on ice for 10 min the sample was centrifuged and the pellet was washed again. The pellet was resuspended in 0.2 ml of water at 65°C and added to 10 ml of scintillation fluid to determine the number of counts precipitated.

**RNase protection assay:** Total RNA (20  $\mu$ g) was hybridized with  $2 \times 10^5$  cpm of antisense RNA probes synthesized using a modification of the method of Melton *et al.*<sup>15)</sup> RNase protection analysis was performed on these hybrids as described.<sup>16)</sup> Following heat denaturation of the RNA probe and sample RNA at 85°C for 5 min and overnight hybridization at 45°C, the samples were digested with an RNase mixture at 30°C for 1 h before treatment with a freshly prepared mixture of proteinase K and 10% SDS for 15 min at 37°C. The samples were phenol and chloroform-extracted, ethanol-precipitated, lyophilized, and resuspended in gel loading buffer for separation on a 6% polyacrylamide gel. Samples were electrophoresed at 55 mA for 2–3 h followed by autoradiography for 1–3 days.

**Cloning of topoisomerase  $\alpha$  cDNA by reverse transcription polymerase chain reaction (RT-PCR)** Synthetic oligonucleotides corresponding to the published cDNA sequence of human topoisomerase  $\alpha$  were used to isolate by RT-PCR specific products for direct cloning into pGEM-3z vectors (Promega, Madison, WI). The identity of the cDNA clone was confirmed by direct sequence analysis prior to use as a probe for northern analysis. The sequences of the oligonucleotides used in the RT-PCR were as follows. Topoisomerase II $\alpha$ : –91–5'TGTGGAGAAGCGGCTTGGTC3' and 449–5'TAGTTACTAGAAGT-TAGGAGCTG3'.

**Northern blotting** Total RNA (20  $\mu$ g/lane) was separated on a 6% formaldehyde gel and transferred to Hybond N+ with  $10 \times$  SSC. The membranes were hybridized at 42°C overnight with the radiolabeled probe in Hybrisol I (Oncor, Gaithersburg, MD).

**Separation of cytosolic and nuclear fractions** Separation of cytosolic and nuclear fractions was performed as previously described.<sup>2)</sup> Cells were centrifuged at 9000g, sequentially washed with 10 ml of PBS and 10 ml of PBS containing 1.5 mM MgCl<sub>2</sub> and resuspended in 150  $\mu$ l of buffer containing 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 30 mM Tris (pH 7.5), with 1 mM phenylmethanesulfonyl fluoride (PMSF), 10  $\mu$ g/ml antipain, aprotinin, chymostatin, leupeptin, and pepstatin A in 1% Triton-X 100 (v:v). The samples were vortexed, placed on ice for 5 min, and centrifuged at 13 000g for 1.5 min at 4°C. A 150  $\mu$ l aliquot of

supernatant (cytosolic fraction) was incubated with 1.5  $\mu$ l of 10 $\times$  DNase/RNase solution (1 unit/ml DNase I, 2.5 mg/ml RNase) and 17  $\mu$ l 2% SDS/100 mM dithiothreitol (DTT) for 60 min at 4°C. The pellet (nuclear fraction) was resuspended in 135  $\mu$ l of 50 mM Tris (pH 7.5), 5 mM MgCl<sub>2</sub>, 1 unit/ml DNase I, 0.25 mg/ml RNase A with 10 mg/ml antipain, aprotinin, chymostatin, leupeptin and pepstatin A, and 15  $\mu$ l of 2% SDS/100 mM DTT for 60 min at 4°C. Both fractions were vortexed every 15 min during the incubation. Following the 60-min incubation, an equal volume of 2 $\times$  sample buffer (4% SDS, 0.2 M DTT and 20% glycerol) was added and the samples were boiled for 5 min, then separated by SDS-polyacrylamide gel electrophoresis (PAGE).

**Immunoblotting** Cell extracts, cytosolic and nuclear fractions were separated on 6% PAGE. Following transfer to a difluoride membrane, the blots were incubated with antibody against human DNA topoisomerase II (1:500), rinsed with Tris-buffered saline (TBS)-0.5% Tween-20, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG, followed by enhanced chemiluminescence detection.

**DNA topoisomerase II activity assay** The standard reaction mixture contained 50 mM Tris-HCl (pH 7.5), 85 mM KCl, 10 mM MgCl<sub>2</sub>, 5 mM DTT, 0.5 mM EDTA, 0.03 mg/ml bovine serum albumin, and 1 mM ATP. The decatenation reaction of catenated DNA was carried out with serial dilutions of nuclear extract and 0.1 mg of kinetoplast DNA (TopoGEN, Columbus, OH) in a final volume of 20  $\mu$ l at 30°C for 30 min. The reaction was terminated by adding 2 ml of 1.2  $\mu$ g/ml proteinase K in 22 mM EDTA followed by incubation for 15 min at 37°C. Then, 5 ml of 0.05% bromophenol blue in 50% glycerol was added to the reaction mixture. Samples were separated by electrophoresis through a 1% agarose gel. After staining with ethidium bromide, gels were photographed under UV illumination.<sup>1)</sup>

**DNA topoisomerase II phosphorylation** Phosphorylation and immunoprecipitation were performed using a modification of a technique previously described.<sup>1)</sup> Cells (5 $\times$ 10<sup>5</sup>) were plated in 100 mm dishes and allowed to attach overnight. The plates were rinsed with PBS, and 10 ml of phosphate-free, serum-free minimum essential medium (MEM) was added. After 4 h, <sup>32</sup>P orthophosphate was added to a final concentration of 100 mCi/ml and the incubation was allowed to proceed for an additional 1 h prior to preparation of nuclear extracts (see above). The nuclear extracts were incubated with antibody against human topoisomerase II $\alpha$  at 4°C overnight in 800  $\mu$ l of 20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 10 mg/ml aprotinin. Protein A-Sepharose was added to the reaction mixture followed by incubation for 2 h at room temperature with gentle rocking. The mixture was centrifuged at 12 000g for 20 s at

4°C; the supernatant was removed, and 1 ml of 50 mM Tris-HCl, 150 mM NaCl, 2.5 M KCl, 0.5% NP-40 buffer was added. This procedure was repeated twice and the mixture was then washed with 1 ml of 50 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 0.05% SDS buffer, and centrifuged at 12 000g for 20 s at 4°C, after which the supernatant was removed. The pellets were then resuspended in loading buffer and boiled. The supernatants were subjected to electrophoresis on 6% SDS polyacrylamide gel and

Table I. Relative Resistance

|          | VP-16 | m-AMSA | MTX | ADR | VCR |
|----------|-------|--------|-----|-----|-----|
| T47D     | 1     | 1      | 1   | 1   | 1   |
| T47D-VP  | 126   | 142    | 109 | 27  | 1   |
| MCF-7    | 1     | 1      | 1   | 1   | 1   |
| MCF-7-VP | 18    | 8      | 7.8 | 9.8 | 1.5 |

Cytotoxicity assays were performed as described.<sup>14)</sup> Drug exposure was continuous for 5 days. Degree of resistance was calculated dividing the IC<sub>50</sub> values of the resistant lines by those of the corresponding parent lines.

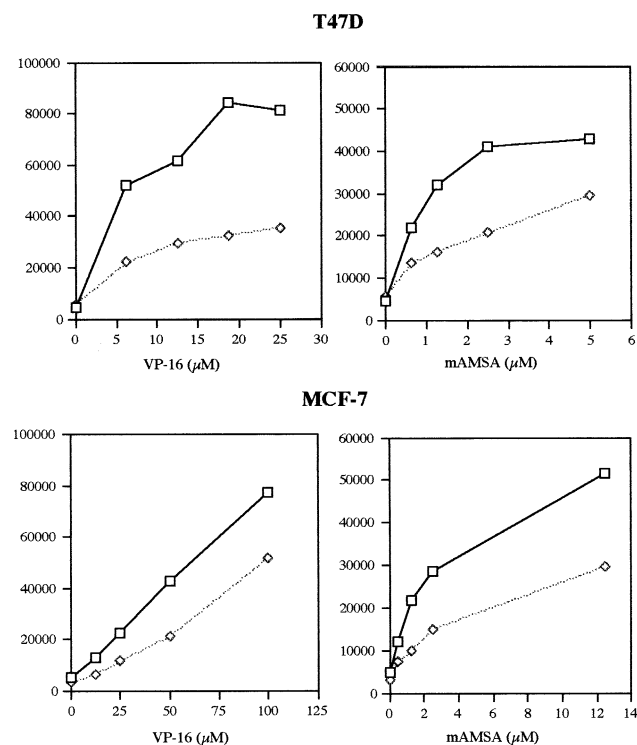


Fig. 1. K-SDS precipitation. Intact parental and T47D-VP cells were exposed to increasing concentrations of drug, after which precipitable protein counts were determined.  $\square$ , parental cell lines, T47D and MCF-7;  $\diamond$ , VP-16 resistant cell lines, T47D-VP and MCF-7-VP.

transferred to an Immobilon-P membrane. Following autoradiography, immunoblotting for topoisomerase II $\alpha$  protein was performed on the same membrane.

**RESULTS**

T47D-VP and MCF-7-VP cells were isolated from parental T47D and MCF-7 human carcinoma cells by using a cloning ring to isolate colonies growing in 300 and 500 ng/ml etoposide. Table I depicts the cross resistance profile of the two sublines to various chemotherapeutic agents. Cross resistance to four topoisomerase II poisons was observed in the two sublines, but there was little resistance to the anti-microtubule agent, vincristine.

Parental T47D and MCF-7 cells do not express MDR-1, and none was found in T47D-VP or MCF-7-VP cells by northern analysis (not shown). Expression of the multidrug

resistance-associated protein (MRP) in parental T47D and MCF-7 cells and the resistant T47D-VP and MCF-7-VP cells was also examined by northern analysis and expression levels were found to be comparable (not shown).

A functional role of topoisomerase II in the observed cross resistance was demonstrated by K-SDS precipitation assay, as shown in Fig 1. Treatment of intact cells with increasing concentrations of either m-AMSA or etoposide increased the formation of K-SDS-precipitable cleavage complexes to a greater extent in parental cell lines than in resistant cell lines, confirming the cytotoxicity studies.

In order to screen a large number of cell lines for acquired mutations in topoisomerase II $\alpha$ , an RNase protection assay was developed. Fragments covering the entire coding sequence of topoisomerase II $\alpha$ , were isolated by restriction enzyme digestion of a full-length cDNA and subcloned in pGEM3z or pBluescript 2 KS+vector. Anal-

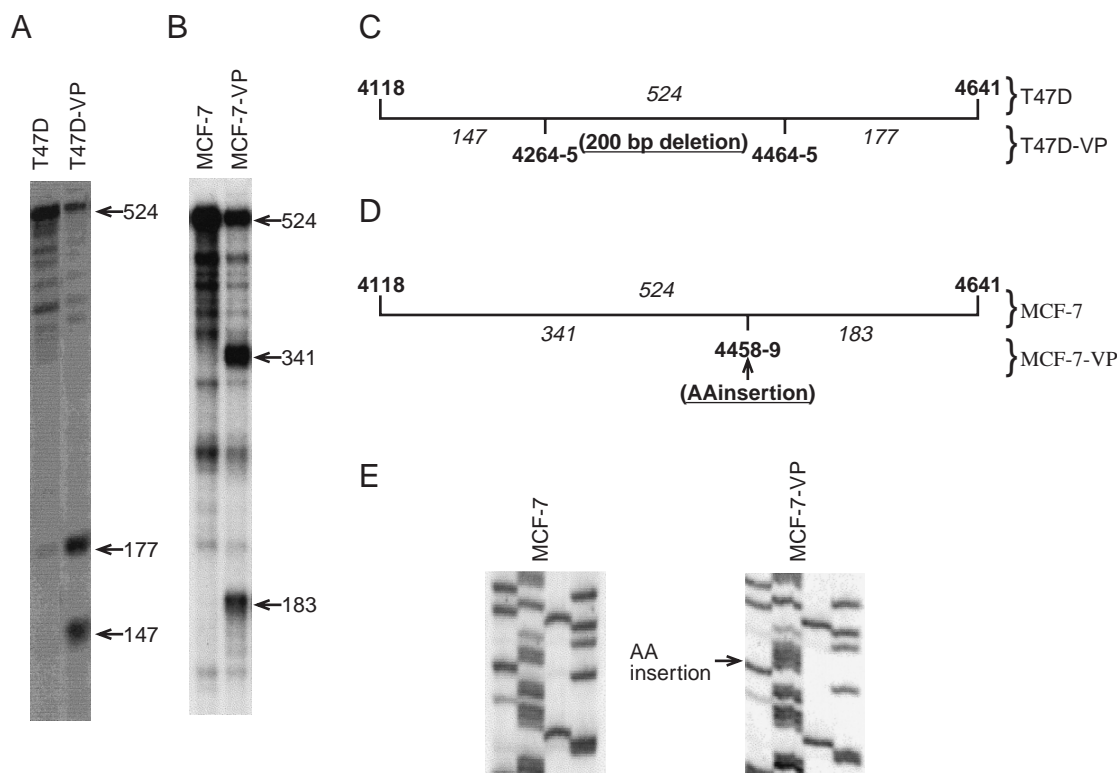


Fig. 2. RNase protection analysis in T47D-VP and MCF-7-VP. As shown in panel A and in the schematic in panel C, full-length protection of a 524-base-pair fragment was observed with RNA from parental T47D cells and also with that from T47D-VP cells. In addition, when the source of the RNA was T47D-VP, 177- and 147-base-pair fragments were also identified, suggesting that a deletion or mutation was present (panel A). The amplified fragments were sequenced using Sequenase Version 2. This demonstrated a 200 bp deletion in T47D-VP RNA between 4265 and 4464. As shown in panel B and in the schematic in panel D, full-length protection of a 524-base-pair fragment was observed with RNA from parental MCF-7 cells and also with that from MCF-7-VP cells. In addition, when the source of the RNA was MCF-7-VP, 341- and 183-base-pair fragments were also identified, suggesting that a deletion or mutation was present (panel B). The amplified fragments were sequenced using Sequenase Version 2. This demonstrated an "AA insertion" in MCF-7-VP RNA between 4458 and 4459 (panel D, E).

ysis of RNA from T47D-VP and MCF-7-VP cells demonstrated complete identity of sequence with all probes, except the fragment covering residues 4118 to 4641. As shown in panel A of Fig. 2, compared to RNA from parental T47D cells, which protects a full-length 524-base-pair fragment, smaller fragments were protected by T47D-VP RNA, consistent with a large deletion. The amplified fragments were subcloned in pGEM3z vectors and sequenced using Sequenase Version 2. This demonstrated a 200-base-pair deletion in T47D-VP RNA beginning with residue 4265 and extending to residue 4464 (amino acid 1422–1487) (panel B), explaining the 177- and 147-base-pair fragments obtained in the RNase protection analysis (panel A).

Fig. 2 summarizes the results obtained with the fragment corresponding to residues 4118–4641. As shown in panel A and in the schematic in panel B, full-length protection of a 524-base-pair fragment was observed with RNA from parental MCF-7 cells and also with that from MCF-7-VP cells. In addition, when the source of the RNA was MCF-7-VP, 341- and 183-base-pair fragments were also identified, suggesting that a deletion or mutation was present. The amplified fragments were subcloned in pGEM3z vectors and sequenced using Sequenase Version 2. This demonstrated an “AA insertion” in MCF-7-VP RNA between 4458 and 4459 (amino acid 1486) (panel C).

Having identified and characterized the 200 bp deletion and “AA insertion,” an explanation was sought for how the acquired phenotype could lead to drug resistance. A truncated topoisomerase II $\alpha$  protein could be unstable and rapidly degraded, so immunoblotting and northern analysis were performed using a specific topoisomerase II $\alpha$  antibody and a cDNA probe encoding residues –91 to 449, as

shown in Fig. 3. Comparing parental cells and the drug-resistant T47D-VP cells, the aberrantly spliced message coded for a protein of 159 kDa. This latter size is consistent with a 200 bp deletion. Northern analysis showed decreased mRNA expression in T47D-VP and MCF-7-VP compared to parental cells, consistent with the results of western analysis in whole cell extracts, suggesting that these truncated topoisomerase II $\alpha$  proteins were not unstable (Fig. 3).

Finally we addressed the question of how T47D-VP and MCF-7-VP cells could survive with a small amount of topoisomerase II $\alpha$ . Expression of topoisomerase II $\beta$  was

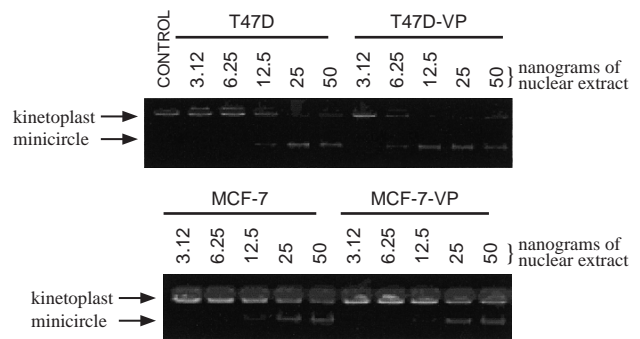


Fig. 4. Decatenation of kinetoplast DNA by nuclear extract. In the absence of nuclear extract, catenated kinetoplast DNA remains in the well of the gel. Comparable topoisomerase activity is demonstrated by comparable decatenation of kinetoplast DNA to monomer minicircles, using increasing amounts of nuclear extracts.

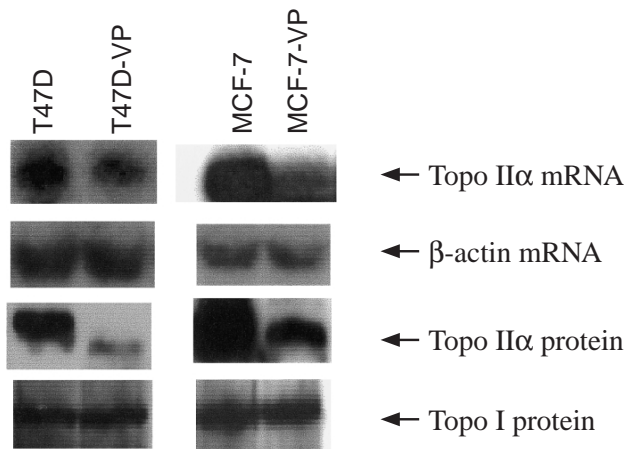


Fig. 3. Northern blotting and immunoblotting of topoisomerase II $\alpha$ .  $\beta$ -Actin mRNA and topoisomerase I protein were used as internal controls.

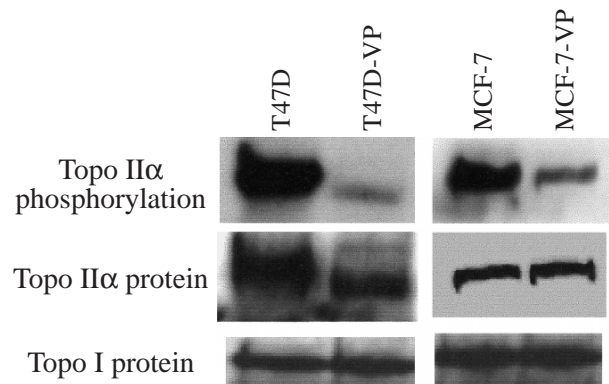


Fig. 5. Phosphorylation of topoisomerase II $\alpha$  from parental and resistant cells. Following labeling, immunoprecipitation and electrophoresis, the labeled proteins were transferred to a nylon membrane and exposed for 48 h. The autoradiogram is shown in the upper panel. The same filter was then hybridized with an anti-topoisomerase II antibody and developed for chemiluminescence as shown in the middle panel. Topoisomerase I protein was used as an internal control (the lower panel).

similar in both parental and resistant cells, precluding a compensatory increase in this isoform as an explanation (not shown). Although the immunoblotting studies demonstrated a small quantity of topoisomerase II $\alpha$  in the resistant cell lines, surprisingly, when topoisomerase II activity was measured in decatenation assays using crude nuclear extracts, similar levels of activity were observed in the crude nuclear extracts of T47D-VP and MCF-7-VP cells and those of the parental cells (Fig. 4). This high level of activity in spite of the low topoisomerase II levels prompted us to examine the level of phosphorylation. As shown in Fig. 5, the truncated protein is relatively hypophosphorylated compared to that in parental cells; this was not unexpected, since the C-terminal region has been shown to be the major site of phosphorylation. This excludes hyperphosphorylation as an explanation, while raising the possibility that absence of the C-terminal domain results in activation.

## DISCUSSION

Topoisomerase II is essential for cell proliferation and is known to exist as a phosphoprotein in cells from both lower and higher eukaryotic species. Post-translational modification of proteins by phosphorylation is one of the most important means by which enzymatic activity is regulated in human cells. This modification can lead to inhibition or stimulation of activity and can alter affinity for a target protein or DNA. Previous studies have shown that topoisomerase II $\alpha$  is a phosphoprotein *in vivo* and that a major site of phosphorylation is located at serine 1524.<sup>5)</sup> This site can be phosphorylated by casein kinase II. The proposal that the extreme C-terminal domain is the major target for regulatory phosphorylation of the eukaryotic topoisomerase II enzyme is hard to reconcile with the apparently dispensable nature of this region as shown by several studies utilizing C-terminally truncated versions of the enzyme. On the other hand, PT1342 antibody, which recognizes phosphorylated threonine 1342 in topoisomerase II $\alpha$ , completely inhibited topoisomerase II activity.<sup>17)</sup> The most plausible explanation for these apparently contradictory data is that the C-terminal domain after threonine 1342 has a negative regulatory role, which is relieved by phosphorylation. Thus, in the truncated versions of the enzyme lacking a large proportion of the C-terminal domain, no stimulatory phosphorylation is required and the enzyme is consequently fully functional *in vivo*.<sup>18)</sup> A similar explanation has been advanced for the effects of phosphorylation on the activity of the mammalian DNA ligase I.<sup>12)</sup>

The present study describes the characterization of multidrug-resistant sublines derived from two parental carcinoma cell lines by exposure to VP-16. The results

demonstrate the adaptation that occurs during the course of *in vitro* selection. The adaptation observed in both T47D-VP and MCF-7-VP was a reduction in topoisomerase II $\alpha$  levels. However, topoisomerase II activity was fully maintained, as a result of hypophosphorylation owing to the loss of a part of the C-terminal domain of the enzyme, including a major site of phosphorylation at serine 1524.

The results described in the present study support and extend previous observations. The initial change observed was a decrease in the expression of topoisomerase II $\alpha$ . Previous studies have documented reduced expression of topoisomerase II $\alpha$  with drug selection and this has been proposed as a mechanism of drug resistance to topoisomerase poisons.<sup>1, 19–22)</sup> We ourselves have found decreased expression of topoisomerase II $\alpha$  in 50 of 53 single step isolates, including the two described in the present communication, suggesting that reduced expression is a common adaptation. While reduction of topoisomerase II $\alpha$  levels effectively diminishes the main intracellular target of VP-16 and other topoisomerase poisons, and can confer broad cross resistance to three agents (m-AMSA, adriamycin and mitoxantrone), such a reduction also leads to a decrease in topoisomerase II activity.

It was somewhat surprising to find that nuclear extracts from T47D-VP1 and MCF-7-VP8 cells had comparable topoisomerase II activity to that of parental cells. Analysis of the extent of phosphorylation demonstrated that topoisomerase II $\alpha$  from the resistant cells was hypophosphorylated compared to that of the parental cells. This was not unexpected, given the available evidence, which suggests that the C-terminal region is the principal area for this modification, and the fact that the deletion and truncation resulted in a loss of 17 serines and 7 threonines. The results demonstrate unequivocally that phosphorylation sites exist elsewhere, since the protein was clearly phosphorylated. While a conclusive explanation for the relatively high activity of the truncated topoisomerase cannot be provided, two possibilities can be considered: (1) phosphorylation sites outside of the C-terminal domain can bring about a greater degree of activation; or (2) the C-terminal region has a negative effect on activity which can be relieved by phosphorylation, or better still by truncation, a hypothesis that has been previously suggested,<sup>18)</sup> and argued against.<sup>13)</sup>

In these cell lines, hypophosphorylation secondary to loss of a part of the C-terminal domain of topoisomerase II $\alpha$  resulted in maintained activity, despite decreases in topoisomerase II $\alpha$  mRNA and protein, which resulted in cross resistance to topoisomerase II poisons.

(Received March 7, 2001/Revised April 25, 2001/Accepted May 8, 2001)

## REFERENCES

- 1) Matsumoto, Y., Takano, M. and Fojo, T. Cellular adaptation to drug exposure: evolution of the drug-resistant phenotype. *Cancer Res.*, **57**, 5086–5092 (1997).
- 2) Takano, H., Kohno, K., Ono, M., Uchida, Y. and Kuwano, M. Increased phosphorylation of DNA topoisomerase II in etoposide resistant mutants of human cancer KB cells. *Cancer Res.*, **51**, 3951–3957 (1991).
- 3) Campain, J. A., Gottesman, M. M. and Pastan, I. A novel mutant topoisomerase II $\alpha$  present in VP-16-resistant human melanoma cell lines has deletion of alanine 429. *Biochemistry*, **33**, 11327–11332 (1994).
- 4) Crenshaw, D. W. and Hsieh, T. S. Function of the hydrophilic carboxyl terminus of type II DNA topoisomerase from *Drosophila melanogaster*. *J. Biol. Chem.*, **268**, 21335–21343 (1993).
- 5) Wells, N. J., Addison, C. M., Fry, A. M., Ganapathi, R. and Hickson, I. D. Serine 1524 is a major site of phosphorylation on human topoisomerase II $\alpha$  protein *in vivo* and is a substrate for casein kinase II *in vitro*. *J. Biol. Chem.*, **270**, 29746–29751 (1994).
- 6) Wells, N. J., Fry, A. M., Guano, F., Norbury, C. and Hickson, I. D. Cell cycle phase-specific phosphorylation of human topoisomerase II $\alpha$ . *J. Biol. Chem.*, **269**, 28357–28363 (1995).
- 7) Alghisi, G. C., Roberts, E., Cardenas, M. E. and Gasser, S. M. The regulation of DNA topoisomerase II by casein kinase II. *Cell. Mol. Biol. Res.*, **40**, 563–571 (1994).
- 8) Pepperkok, R., Lorenz, P., Ansoerge, W. and Pyerin, W. Casein kinase II is required for transition of G0/G1, early G1, and G1/S phase of the cell cycle. *J. Biol. Chem.*, **269**, 6986–6991 (1994).
- 9) Hochhauser, D., Stanway, C. A. Harris, A. L. and Hickson, I. D. Cloning and characterization of the 5'-flanking region of the human topoisomerase II $\alpha$  gene. *J. Biol. Chem.*, **267**, 18961–18965 (1992).
- 10) Cardenas, M. E., Dang, Q., Glover, C. V. C. and Gasser, S. M. Casein kinase II phosphorylates the eukaryote-specific C-terminal domain of topoisomerase II *in vitro*. *EMBO J.*, **11**, 1785–1796 (1992).
- 11) Caron, P. R., Watt, P. and Wang, J. C. The C-terminal domain of *Saccharomyces cerevisiae* DNA topoisomerase II. *Mol. Cell. Biol.*, **14**, 3197–3207 (1994).
- 12) Vassetzky, Y. S., Dang, Q. I., Benedetiti, P. and Gasser, S. M. Topoisomerase II forms multimer *in vitro*: effects of metals,  $\beta$ -glycerophosphate, and phosphorylation of its C-terminal domain. *Mol. Cell. Biol.*, **14**, 6962–6974 (1994).
- 13) Shiozaki, K. and Yanagida, M. Functional dissection of the phosphorylated termini of fission yeast DNA topoisomerase II. *J. Cell Biol.*, **119**, 1023–1036 (1992).
- 14) Matsumoto, Y., Sasaoka, N., Tsuchida, T., Fujiwara, Y., Nagao, S. and Ohmoto, T. Fluorescent dye Rhodamine 6G as a molecular probe to study drug resistance of C6 rat glioma cells. *J. Neurooncol.*, **13**, 217–222 (1992).
- 15) Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. and Green, M. R. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.*, **12**, 7035–7056 (1984).
- 16) Mickley, L. A., Bates, S. E., Richert, N. D., Currier, S., Tanaka, S., Foss, F. and Fojo, T. Modulation of the expression of a multidrug resistance gene (mdr-1/P-glycoprotein) by differentiating agents. *J. Biol. Chem.*, **264**, 18031–18040 (1989).
- 17) Ishida, R., Iwai, M., Marsh, K. L., Austin, C. A., Yano, T., Shibata, M., Nozaki, N. and Hara, A. Threonine 1342 in human topoisomerase II $\alpha$  is phosphorylated throughout the cell cycle. *J. Biol. Chem.*, **271**, 30077–30082 (1996).
- 18) Wassermann, R. A., Austin, C. A., Fisher, L. M. and Wang, J. C. Use of yeast in the study of anticancer drugs targeting DNA topoisomerases: expression of a functional recombinant human DNA topoisomerase II alpha in yeast. *Cancer Res.*, **53**, 3591–3596 (1993).
- 19) Takano, H., Ise, T., Nomoto, M., Kato, K., Murakami, T., Ohmori, H., Imamura, T., Nagatani, G., Okamoto, T., Ohta, R., Furukawa, M., Shibao, K., Izumi, H., Kuwano, M. and Kohno, K. Structural and functional analysis of the control region of the human DNA topoisomerase II alpha gene in drug-resistant cells. *Anticancer Drug Des.*, **14**, 87–92 (1999).
- 20) Beck, W. T., Kim, R. and Chen, R. Novel actions of inhibitors of DNA topoisomerase II in drug-resistant tumor cells. *Cancer Chemother. Pharmacol.*, **34** (Suppl.), S14–S18 (1994).
- 21) Larsen, A. K., Escargueil, A. E. and Skadanowski, A. Resistance mechanisms associated with altered intracellular distribution of anticancer agents. *Pharmacol. Therap.*, **85**, 217–229 (2000).
- 22) Mirski, S. E., Gerlach, J. H. and Cole, S. P. Sequence determinants of nuclear localization in the alpha and beta isoforms of human topoisomerase II. *Exp. Cell Res.*, **251**, 329–339 (1999).