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Purification and Characterization of an Altered Topoisomerase II from a Drug-Resistant Chinese Hamster Ovary Cell Line†

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ABSTRACT: The cytotoxicity and DNA damage induced by the epipodophyllotoxins and several intercalating agents appear to be mediated by DNA topoisomerase II. We have purified topoisomerase II to homogeneity both from an epipodophyllotoxin-resistant Chinese hamster ovary cell line, Vpm^R-5, and from the wild-type parental cell line. Immunoblots demonstrate similar topoisomerase II content in these two cell lines. The purified enzymes are dissimilar in that DNA cleavage by Vpm^R-5 topoisomerase II is not stimulated by VP-16 or *m*-AMSA. Furthermore, the Vpm^R-5 enzyme is unstable at 37 °C. Thus, the drug resistance of Vpm^R-5 cells appears to result from the presence of an altered topoisomerase II in these cells. Purified topoisomerase II from VPM^R-5 and wild-type cells has the same monomeric molecular mass as well as equivalent catalytic activity with respect to decatenation of kinetoplast DNA. Etoposide (VP-16) inhibits the activity of both enzymes. Noncovalent DNA–enzyme complex formation, assayed by nitrocellulose filter binding, is also similar, as is protection from salt dissociation of this complex by ATP and VP-16. The data suggest a model in which the drug-resistant cell line, Vpm^R-5, has religation activity which is less affected by drug than that of the wild-type cells. Drug effect on DNA religation and catalytic activity are dissociated mechanistically. In addition, under certain circumstances, the "cleavable complex" observed following denaturation of a drug-stabilized DNA–enzyme complex may not adequately reflect the nature of the antecedent lesion. As the first mammalian cell line bearing an altered topoisomerase II, this line will provide a useful system for further examination of the actions of topoisomerase II.

DNA type II topoisomerases (EC 5.99.1.3) allow the interconversion of DNA topoisomers by introducing a transient enzyme-bridged double-strand DNA break. This reaction is thought to consist of five phases: (i) recognition and binding of the enzyme to its DNA substrate; (ii) double-stranded cleavage of DNA, forming a covalent phosphotyrosyl bond at the 5′ end of each strand; (iii) passage of a second DNA duplex through the break; (iv) religation of the DNA; and (v) the ATP-dependent turnover of topoisomerase II. Further details of this reaction mechanism and the role of such an enzyme in cellular processes have recently been reviewed (Wang, 1985; Vosberg, 1985; Maxwell & Gellert, 1986).

In addition to its enzymatic function(s) in normal cellular processes, recent evidence suggests that topoisomerase II is an intracellular target for several antineoplastic agents, specifically, the epipodophyllotoxins and intercalating drugs (Ross et al., 1984; Chen et al., 1984; Tewey et al., 1984a; Ross, 1985). These drugs allow topoisomerase binding and cleavage of DNA to proceed as outlined above but appear to block the religation step. This is manifested as increased DNA scission when the DNA–enzyme complex is denatured. Although a general correlation between drug-induced DNA strand break frequency and cytotoxicity exists, definitive evidence for a

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causal relationship is lacking. One experimental model that would establish such a relationship is a drug-resistant cell line bearing an altered topoisomerase II that is no longer affected by drug. To date, no such line has been reported. We have previously reported on a Chinese hamster ovary (CHO)[1] line, Vpm^R-5, that bears many characteristics suggestive of such a model. This cell line was selected in the presence of VM-26 during an acute exposure to the drug (Gupta, 1983). The resistance of Vpm^R-5 to VP-16-, *m*-AMSA-, mitoxantrone-, and adriamycin-induced cytotoxicity correlates well with decreased drug-induced DNA damage measured by alkaline elution (Glisson et al., 1986a,b). Neither alterations in drug uptake nor changes in catalytic activity as assayed in crude nuclear extracts account for the resistance of this cell line (Glisson et al., 1986b). However, crude nuclear extracts of the Vpm^R-5 cell line demonstrate a marked resistance to drug-stimulated DNA cleavage. Thus, these results suggest that Vpm^R-5 cells contain a qualitatively different topoisomerase II enzyme.

We have purified topoisomerase II from wild-type and Vpm^R-5 CHO cells to homogeneity by FPLC and confirmed that enzyme from the latter cell line is qualitatively distinct. Although of similar molecular weight and specific activity to wild-type topoisomerase II, Vpm^R-5 enzyme is markedly resistant with respect to drug-induced DNA cleavage activity. Thus, this represents the first altered mammalian topoisomerase II to be described as well as the first instance in which cellular resistance to topoisomerase II active drugs can be ascribed to such an altered enzyme. Additional studies with this enzyme have raised important questions about many aspects of its interaction with epipodophyllotoxins and intercalating agents.

Materials and Methods

Materials

Wild-type and Vpm^R-5 CHO cells were grown in suspension cultures in 15-L spinner flasks (Bellco Glass, Inc., Vineland, NJ) at 37 °C in α-MEM medium with 5% fetal calf serum (Gibco Laboratories, Grand Island, NY) in the presence of 5% CO_2. Penicillin (100 IU/mL) and streptomycin (100 μg/mL) were added to the culture media. In suspension culture, wild-type and Vpm^R-5 cells have doubling times of 14–15 and 19–20 h, respectively. They were harvested at log phase densities (3–5 × 10^5/mL) for enzyme purification.

[^{3}H]Thymidine (20 Ci/mmol) and Aquassure were obtained from New England Nuclear (Boston, MA), and [α-^{32}P]dATP (6000 Ci/mmol) was obtained from Amersham International (Amersham, U.K.). Bristol-Myers Co. (Syracuse, NY) supplied the VP-16, and *m*-AMSA was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. Nonidet P-40 and proteinase K were obtained from Sigma Chemical Co. (St. Louis, MO). The restriction enzymes, *Hin*dIII and *Eco*RI, were supplied by Bethesda Research Laboratories (Gaithersburg, MD). All other reagents and chemicals were of the finest quality available.

The FPLC used for enzyme purification was obtained from Pharmacia, Inc. (Piscataway, NJ). Prepacked Mono Q, Mono S, and Superose 12 columns were also obtained from Pharmacia. LKB (Gaithersburg, MD) supplied the Ultrogel hydroxylapatite resin. Schleicher and Schuell, Inc. (Keene, NH) supplied the nitrocellulose filters (25-mm diameter, 0.45-μM pore size) used in the filter binding assays.

Methods

A FPLC-based procedure used for the purification of topoisomerase II from P388 leukemia cells has been previously reported (Drake et al., 1987). The same general purification scheme is employed below; major modifications are described in appropriate detail.

Buffers. (1) Nuclear Extraction Buffers (All Titrated to pH 7.5 with either KOH or HCl): buffer A (0.15 M NaCl, 10 mM KH_2PO_4); buffer B (10 mM Tris, 1.5 mM $MgCl_2$, 10 mM NaCl, 4 mM DTT, 1 mM PMSF); buffer C (50 mM Tris, 25 mM KCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$, 0.25 M sucrose, 4 mM DTT, 1 mM PMSF); buffer D (same as buffer C with 0.6 M sucrose); buffer E (50 mM Tris, 25 mM KCl, 3 mM $MgCl_2$, 4 mM DTT, 1 mM PMSF); buffer F (50 mM Tris, 2 M NaCl, 4 mM DTT, 10 mM Na_2EDTA, 1 mM PMSF); buffer G [50 mM Tris, 1 M NaCl, 18% PEG (w/v), 4 mM DTT, 1 mM PMSF].

(2) FPLC Buffers (Made with HPLC-Grade H_2O, Titrated to the Appropriate pH with either KOH or HCl, Filtered with 0.2-μM Filters, and Degassed under Vacuum Overnight at 4 °C): buffer H [0.2 M KH_2PO_4, 4 mM DTT, 10% glycerol (w/v), 0.5 mM PMSF], pH 7.0; buffer I (same as buffer H with 1.0 M KH_2PO_4); buffer J [20 mM Tris, 4 mM DTT, 10% glycerol (w/v), 1 mM Na_2EDTA, 0.5 mM PMSF], pH 7.5; buffer K (same as buffer J with 0.5 M NaCl); buffer L [20 mM MES, 4 mM DTT, 10% glycerol (w/v), 1 mM Na_2EDTA, 0.5 mM PMSF], pH 6.5; buffer M (same as buffer L with 0.8 M NaCl).

Purification of Topoisomerase II. The following is a generalized procedure for purifying topoisomerase II from the nuclei of CHO cells. The amounts of buffers, resins, and reagents have been normalized for 1 × 10^{10} cells. All operations were carried out at 2–4 °C.

(1) Extraction of Topoisomerase II from CHO Nuclei. Suspension cultures of log phase wild-type and Vpm^R-5 cells were pelleted by continuous centrifugation at 4000*g* in a Sorvall KSB-R continuous flow system. The pellet was resuspended in 400 mL of buffer A and centrifuged at 2500*g* for 15 min. The cells were washed twice more with 400 mL of buffer A and then swollen by resuspending them in 200 mL of buffer B for 30 min. Release of nuclei was accomplished by gently triturating the cells with 20 mL of 10% NP-40 (v/v) for 15 min followed by Dounce homogenization with 20 strokes of a tight pestle. Nuclei were collected by centrifugation at 2000*g* for 15 min and resuspended in 46 mL of buffer C. This solution was carefully layered over 14 mL of buffer D (7.7-mL aliquots layered over 2.3 mL of buffer D), and the nuclei were collected at 1500*g* for 20 min in a swinging bucket rotor. The nuclei collected from the sucrose step gradient were resuspended in 20 mL of buffer E, and the total volume was measured. Nuclear topoisomerase II was extracted with an equal volume of buffer F for 45 min, and DNA was subsequently precipitated by the addition of an equal volume of buffer G (final PEG concentration was 6%). After 30 min, the solution was adjusted to 10% glycerol (w/v) and centrifuged at 100000*g* for 60 min. The supernatant contains topoisomerase II activity.

(2) Hydroxylapatite Column Chromatography. The PEG supernatant from above was loaded on a hydroxylapatite column (1.6 × 40 cm; 80-mL bed volume/1 × 10^{10} cells) that had been washed with buffer I and equilibrated with buffer

[1] Abbreviations: CHO, Chinese hamster ovary; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; PEG, polyethylene glycol; MES, 2-(*N*-morpholino)ethanesulfonic acid; kDNA, kinetoplast DNA; Me_2SO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; FPLC, fast protein liquid chromatography; VP-16, 9-(4,6-*O*-ethylidene-β-D-glucopyranosyl)-4′-demethylepipodophyllotoxin; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; kDa, kilodaltons.

H. The column was then washed with 1.5 bed volumes of buffer H and subsequently eluted with a phosphate gradient of buffers H to I (see Figure 2) as previously described (Drake et al., 1987). The fractions collected were assayed for topoisomerase II activity by decatenation of kDNA. Active fractions were pooled and immediately applied to an anion-exchange column.

(*3*) *Anion-Exchange Column Chromatography.* The pooled fractions from above were diluted with buffer J and loaded on a Mono Q anion-exchange column (0.5 × 5 cm) as previously described (Drake et al., 1987). The column was then washed with 25 mL of buffer J and eluted with a NaCl gradient of buffer J to K as shown in Figure 3 (total gradient volume was 80 mL). One-milliliter fractions were collected at a flow rate of 0.3 mL/min, and enzyme activity was determined by decatenation of kDNA. Topoisomerase II purity of active fractions was assessed at this stage by SDS–polyacrylamide gel electrophoresis (4% stacking, 10% separating). If enzyme purity was less than that required, topoisomerase II enriched fractions were pooled and further purified by cation-exchange chromatography.

(*4*) *Cation-Exchange Column Chromatography.* The pooled fractions from above were diluted to a NaCl concentration of 0.05 M with buffer L and immediately applied to a Mono S cation-exchange column (0.5 × 5 cm) that had been equilibrated with buffer L. The column was washed with 25 mL of buffer L, and topoisomerase II was eluted with a linear NaCl gradient of buffers L to M (total gradient volume was 70 mL). One-milliliter fractions were collected at a flow rate of 0.25 mL/min, and enzyme activity was determined as before. In most experiments, SDS–polyacrylamide gels demonstrated an enzyme purity of >98% at this stage. Therefore, no further purification was necessary, and enzyme aliquots were stored at −70 °C to be used in subsequent analyses.

(*5*) *Gel Filtration Column Chromatography.* Calibrated gel filtration with a Superose 12 column (1 × 30 cm) was used to determine the native protein molecular weight. Purified topoisomerase II from either the anion- or cation-exchange step from above was loaded on the Superose 12 column and eluted isocratically with buffer K at 0.25 mL/min. Peak enzyme activity was determined by decatenation of kDNA.

Topoisomerase II Assays. (*1*) *Catalytic Activity.* Topoisomerase II catalytic activity was quantified by decatenation of kDNA networks isolated from *Crithidia fasciculata* as previously described (Marini et al., 1980; Sullivan et al., 1986). Agarose gel electrophoresis of the released minicircles was used to assay enzyme activity of column fractions during purification of the enzyme; this assay was also used for the heat lability studies of the enzyme. To expedite this assay, 1% agarose gels were run at 150–200 V for 1 h and processed as before (Sullivan et al., 1986). One unit of enzyme activity is defined as the amount of protein required to fully decatenate 1 μg of kDNA in 30 min at 30 °C. A more accurate determination of enzyme activity was made by quantifying ^{3}H-labeled minicircles released from kDNA networks. Kinetoplast DNA was labeled with [^{3}H]thymidine as previously described (Simpson & Simpson, 1974; Englund, 1978). Specific activities of 10000–15000 cpm/μg of kDNA were routinely obtained. A total assay volume of 40 μL contained the decatenation buffer [50 mM Tris (pH 7.5), 85 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM Na_2EDTA, 30 μg/mL BSA, 1 mM ATP] as well as 1–15 ng of purified topoisomerase II, 1 μg of [^{3}H]kDNA, and 0–100 μM VP-16 dissolved in Me_2SO. After incubation at 30 °C for 30 min, the reactions were stopped by the addition of 5 μL of 2.25% SDS. The reactions were then centrifuged at room temperature for 10 min at 13000*g* in a Fisher microcentrifuge. Two-thirds (30 μL) of the supernatant, which contains the released minicircles, was then placed in 2 mL of H_2O and counted in 2 mL of Aquassure. Decatenation was quantified, after adjusting to 100% cpm of the supernatant minus Me_2SO controls without enzyme, as the percent decatenation of available cpm. A similar assay using [^{3}H]kDNA substrate has recently been reported (Sahai & Kaplan, 1986).

(*2*) *Quantitative Precipitation of the Covalent Topoisomerase II–DNA Complex.* The quantitative precipitation of covalent DNA–enzyme complexes was performed as previously described (Liu et al., 1983; Nelson et al., 1984). The 3′ end of pBR322 DNA or pUC18 DNA was labeled with [α-^{32}P]dATP. These assays were performed in the presence and absence of 1 mM ATP with purified wild-type and Vpm^R-5 enzymes at 0–200 μM VP-16. Quantitation of precipitated covalent complexes is expressed as the percent precipitation of available cpm after correcting for Me_2SO-treated controls.

(*3*) *Mapping of DNA Topoisomerase II Cleavage Sites.* This procedure is a modification of that previously reported (Chen et al., 1984; Tewey et al., 1984b). The 3′-end-labeled pUC 18 DNA that had been linearized with *Hin*dIII (see above) was restricted with *Eco*RI to remove a 51-base-pair fragment. This uniquely end labeled pUC18 DNA was used in the following cleavage reactions. A 30-μL reaction mixture contained the decatenation buffer described above without KCl, 50 ng of [^{32}P]pUC18 DNA, various amounts of purified enzyme, and either 200 μM VP-16 or 0.75 μM *m*-AMSA. Control reactions had either no enzyme or no drug (with appropriate amounts of Me_2SO, in which VP-16 and *m*-AMSA were dissolved). The reactions were incubated at 37 °C for 30 min and stopped by the addition of 3 μL of 10% SDS. To this solution was added 2.5 μL of proteinase K [1.5 mg/mL in 10 mM Tris (pH 8.0) and 300 mM Na_2EDTA], and it was further incubated at 50 °C for 30 min. After the addition of 4.5 μL of 0.1% bromophenol blue in 50% glycerol, the reactions were electrophoresed for 18 h at 50 V in 1.4% agarose gels. The gels were dried, and autoradiography on XAR-5 Kodak film was performed.

(*4*) *Nitrocellulose Filter Binding Assay of DNA–Enzyme Complexes.* The interaction between purified topoisomerase II and DNA was studied by nitrocellulose filter binding of DNA–enzyme complexes. The association of DNA and enzyme was determined in a total reaction volume of 40 μL that contained decatenation buffer with and without 1 mM ATP, 50 ng of ^{32}P-3′-end-labeled pUC (or pBR322) DNA that had been linearized with *Hin*dIII, various amounts of purified enzyme, and 50 μM VP-16. At specific time points, up to 30 min, 40-μL reactions were spotted on nitrocellulose filters that had been presoaked in 20 mM Tris (pH 7.5), 1 mM Na_2EDTA, 4 mM DTT, 50 mM NaCl, and 5 mM $MgCl_2$. The filters, which were in a Millipore vacuum manifold, were immediately washed with three 3-mL room temperature aliquots of the above buffer under vacuum (at a flow rate of approximately 3 mL/min). The washed filters were placed in 5 mL of H_2O and counted under 5 mL of Aquassure.

To study the dissociation of the complex formed above and the effect of ATP and VP-16 on this dissociation, topoisomerase II and DNA were allowed to associate as described above for 30 min. At this time, $^1/_9$ volume of 5 M NaCl was added to give a final concentration of 0.5 M NaCl. At 1, 3, 10, and 20 min post NaCl addition, the amount of complex that binds to nitrocellulose filters was determined as above.

Table I

purification step	protein (mg)	units[b]	specific activity (units/mg)	activity recovered (%)	fold purification
Purification of Topoisomerase II from Wild-Type CHO Cells[a]					
(1) PEG supernatant	69.7	1.76×10^5	2.53×10^3	(100)	1
(2) hydroxylapatite pool	4.1	1.03×10^5	2.51×10^4	58.5	10
(3) Mono Q pool	0.112	8.00×10^4	7.14×10^5	45.5	282
Purification of Topoisomerase II from Vpm^R-5 CHO Cells[a]					
(1) PEG supernatant	54.1	1.44×10^5	2.66×10^3	(100)	1
(2) hydroxylapatite pool	13.5	1.32×10^5	9.78×10^3	91.7	4
(3) Mono Q pool	0.528	4.8×10^4	9.09×10^4	33.3	34
(4) Mono S pool	0.024	2.4×10^4	1.00×10^6	16.7	376

[a] The purification results are normalized to 1×10^{10} log phase cells. For the wild-type purification, the hydroxylapatite pool was determined by decatenation activity, for the Vpm^R-5 by immunoreactivity. [b] One enzyme unit is defined as the amount of protein required to fully decatenate 1.0 μg of kDNA at 30 °C in 30 min.

Both the association and dissociation reactions were done in duplicate or triplicate for several different purified enzyme preparations. Association is expressed as percent complex formation of available cpm (assuming 100% binding of the complex by nitrocellulose). Dissociation is expressed as the ratio of percent complex formation of available cpm at specific times post NaCl addition to percent complex formation of available cpm at time 0 (i.e., after 30 min of association). This normalizes the association after 30 min of incubation to 100%.

(*5*) *Immunoreactive Topoisomerase II.* The enzyme content of nuclear extracts from above was assayed by Western blot using a polyclonal mouse anti-HeLa cell topoisomerase II antibody as previously described (Sullivan et al., 1987).

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gels were run as previously described (Laemmli, 1970) and were stained with either Coomassie Brilliant Blue or silver. Protein concentrations were determined by the Bio-Rad protein assay.

Results

Enzyme Purification. (*1*) *Nuclear Extraction of Topoisomerase II.* Release of nuclei was accomplished by homogenizing cells in the presence of the nonionic detergent NP-40 and was demonstrated by fluorescence microscopy of acridine-orange-stained preparations. The subsequent sucrose step-gradient centrifugation of released nuclei was found to facilitate the purification process, probably by removing contaminating cytoplasmic proteins. Nuclear topoisomerase II was then extracted with 1.0 M NaCl. We have previously observed (Sullivan et al., 1987) that this procedure leaves no residual enzyme in the extracted nuclei. PEG fractionation of the nuclear extract at this stage was found to be advantageous for several reasons. Precipitation of DNA precludes occlusion of the fine-bore tubing and filters of the FPLC instrument, and it prevents decrease of the binding capacity of the hydroxylapatite column by loading it with DNA. In addition, fewer column chromatography steps were necessary to purify the enzyme when the extracts were treated with PEG, probably because PEG precipitates contaminating proteins. An immunoblot of nuclear extracts from wild-type and Vpm^R-5 cells is shown in Figure 1; these blots show approximately equal enzyme concentrations.

(*2*) *Column Chromatography.* The PEG supernatant was loaded on a hydroxylapatite column and eluted with a phosphate gradient (Figure 2). The 0.2 M KH_2PO_4 wash (buffer H) removes a large protein peak that is devoid of topoisomerase II activity. Assays for topoisomerase II content (Western blot) and activity (decatenation) yielded an interesting result. The peak of immunoreactive enzyme was found to elute at approximately 0.4 M phosphate, while the peak

FIGURE 1: Western blot electrophoresis of wild-type and Vpm^R-5 CHO cell nuclear extracts. Nuclear extracts were prepared as described under Methods, and equivalent amounts of protein (25 μg/lane) were electrophoresed in a 7.5% SDS–polyacrylamide gel. After transfer to nitrocellulose, topoisomerase II was detected with a polyclonal mouse anti-HeLa topoisomerase II antibody.

catalytic activity eluted somewhat later at approximately 0.7 M phosphate. This may be due to contaminating inhibitory proteins present in the former, which decrease decatenation activity (Tse et al., 1984; Hsieh & Brutlag, 1980). We have observed that there is a 10–15-fold purification by hydroxylapatite chromatography if fractions are pooled by peak decatenation activity (see wild-type purification in Table I), whereas only a 4–6-fold purification was observed if fractions are pooled by content (see Vpm^R-5 purification in Table I). In addition, it is easier to purify the enzyme to homogeneity when starting with the decatenation pool.

The hydroxylapatite pool was then diluted to a phosphate concentration of 0.1 M and loaded on an anion-exchange column (Figure 3). It was necessary to dilute the phosphate buffer to ensure binding of the enzyme to the Mono Q resin. A Coomassie-Blue-stained SDS–polyacrylamide gel of the active fractions from an anion-exchange column (Figure 4) demonstrates that topoisomerase II is efficiently concentrated by this chromatography step (see fractions 50 and 51 in Figure 4) and purified approximately 300-fold (Table I). For both the wild-type and Vpm^R-5 CHO cell lines, only a single band (of molecular mass 170 kDa) is detected by immunoblotting at all stages of purification with a polyclonal mouse anti-HeLa cell topoisomerase II antibody (Sullivan et al., 1987), with a polyclonal rabbit anti-calf thymus topoisomerase II antibody (provided by Dr. Leroy Liu, Johns Hopkins University), and,

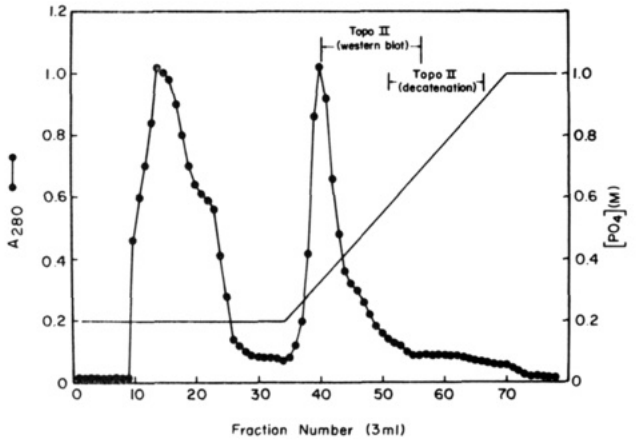

FIGURE 2: Hydroxylapatite column chromatography of a wild-type CHO cell PEG supernatant. The PEG supernatant (110 mL, 98 mg of protein) derived from 50 L of log phase CHO cells (1.7×10^{10} total cells) was loaded on a hydroxylapatite column and eluted with the phosphate gradient as shown. A large protein peak is reproducibly eluted with the 0.2 M KH_2PO_4 wash, and it is devoid of topoisomerase II activity. The peak of enzyme content, determined by immunoblotting with a polyclonal rabbit anti-calf thymus topoisomerase II antibody (kindly provided by Dr. Leroy Liu, Johns Hopkins University), is eluted at approximately 0.4 M KH_2PO_4. The peak of decatenation activity is eluted by approximately 0.7 M KH_2PO_4.

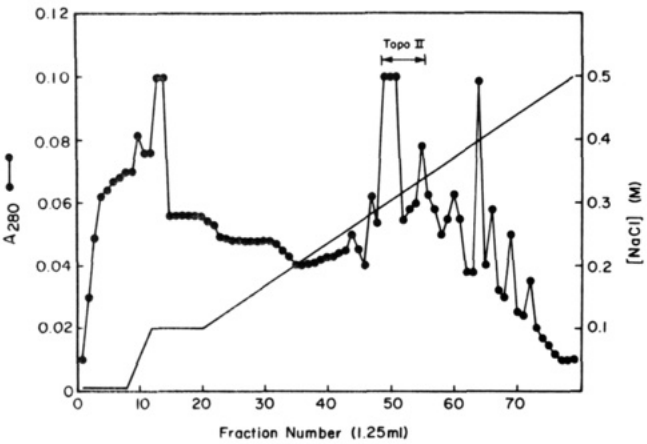

FIGURE 3: Anion-exchange (Mono Q) column chromatography of pooled hydroxylapatite fractions from wild-type CHO cells. On the basis of peak decatenation activity, the fractions from the hydroxylapatite column (Figure 2) were pooled, diluted to a phosphate concentration of 0.1 M, and loaded on an anion-exchange column (total protein was 4.8 mg). The column was eluted with the NaCl gradient shown, and topoisomerase II was found to elute at 0.3 M NaCl. If necessary, the enzyme was further purified by cation-exchange column chromatography as described under Methods.

finally, with a polyclonal rabbit antibody (generated in this laboratory) directed against the recombinant 70-kDa C-terminus of HeLa topoisomerase II (provided by Dr. Leroy Liu, Johns Hopkins University). These results (not shown, except Figure 1) are in contrast to the two bands (170 and 180 kDa) seen in P388 cells (Drake et al., 1987).

To obtain homogeneous topoisomerase II (Figure 5), it is generally necessary to further purify the Mono Q pool with cation-exchange chromatography. Topoisomerase II, when purified by PEG fractionation, hydroxylapatite chromatography, and anion- and cation-exchange chromatography, has been enriched 375–450-fold (Table I), and it behaves the same throughout this purification procedure, whether it is isolated from wild-type or Vpm^R-5 cells. Both purified enzymes are stable for at least 6 months in 10% glycerol at −70 °C. They have the same native molecular mass of 300 kDa (determined

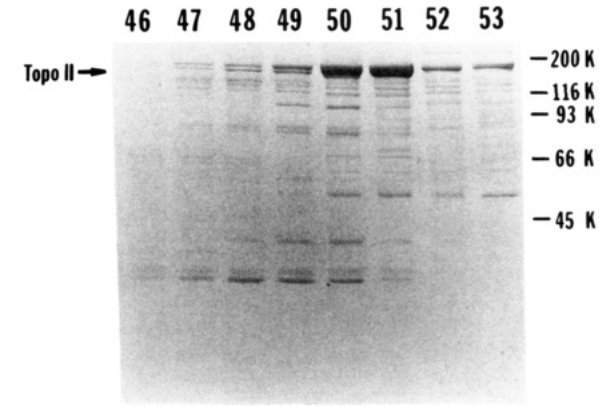

FIGURE 4: SDS–polyacrylamide gel electrophoresis of wild-type topoisomerase II eluted from an anion-exchange column. An equivalent volume (50 μL) of fractions 46–53 from a Mono Q column (see Figure 3) was electrophoresed on a 10% acrylamide gel and stained with Coomassie Blue. Peak catalytic activity (determined by decatenation of kDNA) was found in fractions 50 and 51; the heavy band at 170 kDa in these two fractions was identified as topoisomerase II by immunoblotting.

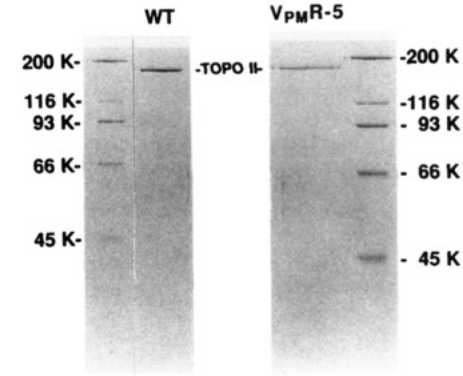

FIGURE 5: SDS–polyacrylamide gel electrophoresis of purified wild-type and Vpm^R-5 topoisomerase II. Topoisomerase II was purified as outlined in Table I from wild-type and Vpm^R-5 cells and electrophoresed on a 10% acrylamide gel. Five micrograms of Mono-Q- purified wild-type enzyme and 3 μg of Mono-S-purified Vpm^R-5 enzyme were loaded; the gels were stained with Coomassie Blue.

by calibrated gel filtration chromatography; data not shown) as well as the same monomer molecular mass of 170 kDa (Figure 5).

Catalytic Activity of Purified Topoisomerase II. The catalytic activity of purified wild-type and Vpm^R-5 enzymes was measured by decatenation of [^{3}H]kDNA networks (see Methods). In the absence of drug, approximately 10 ng of enzyme is required for both to fully decatenate 1 μg of kDNA (Figure 6). We have observed, as well as others (Osheroff et al., 1983; Glisson et al., 1984), that this reaction is strongly inhibited by NaCl or KCl concentrations greater than 150 mM; it is also inhibited by ATP concentrations greater than 1.5 mM. In the range of 5–100 μM VP-16, similar concentration-dependent inhibition of decatenation activity of the two enzymes was observed with total inhibition at 100 μM VP-16.

The decatenation reaction was also used to study the stability of homogeneous preparations of wild-type and Vpm^R-5 enzymes (Figure 7). Equal amounts of wild-type and Vpm^R-5 topoisomerase II were preincubated in a water bath at 37 °C for 0–120 min. At specific time points, aliquots were removed, and decatenation was analyzed by agarose gel electrophoresis. We observed that wild-type enzyme loses catalytic activity by 90 min of preincubation at 37 °C, while Vpm^R-5 decatenation activity is abolished by 20 min of preincubation. Thus, the

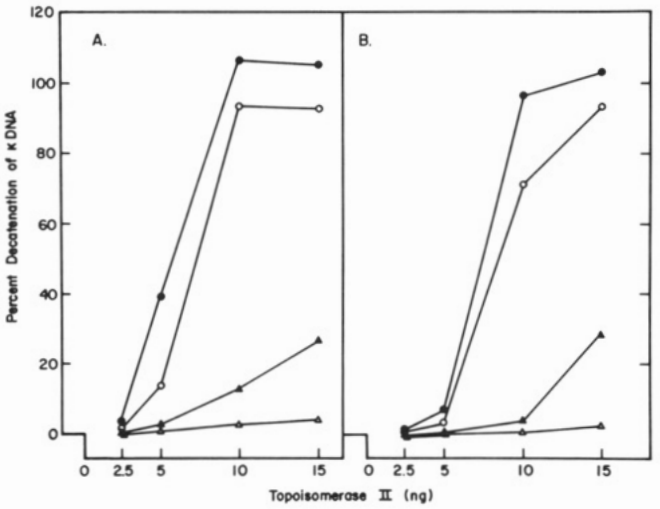

FIGURE 6: Decatenation of [^{3}H]kDNA networks by purified wild-type and Vpm^R-5 topoisomerase II enzymes. Topoisomerase II was purified to homogeneity from the sensitive and resistant CHO cell lines, and its catalytic activity was measured by decatenation of [^{3}H]kDNA networks as described under Methods. The wild-type (A) and Vpm^R-5 (B) enzymes were found to have equal specific activity, which was inhibited similarly by the epipodophyllotoxin VP-16. (●) No drug; (○) 5 μM VP-16; (▲) 25 μM VP-16; (△) 100 μM VP-16.

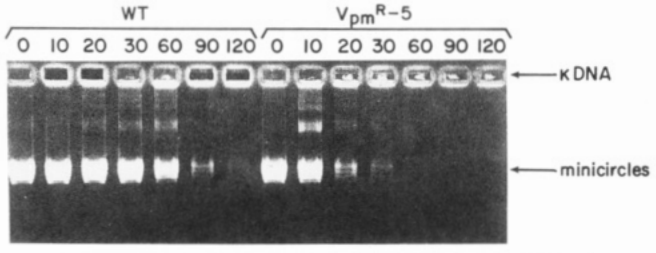

FIGURE 7: Thermal stability of purified wild-type and Vpm^R-5 topoisomerases. Equal concentrations of Mono-S-purified wild-type and Vpm^R-5 enzymes (300-ng total/5-μL Mono S pool) were diluted 1:10 with buffer J to give a final reaction volume of 50 μL containing buffer J with 30 mM NaCl. These solutions were preincubated at 37 °C for the time (minutes) indicated at the top of the gel. At these times, a 5-μL aliquot (30 ng of topoisomerase II) was removed and a decatenation assay was performed as described under Methods. Parallel (undiluted) enzyme samples were preincubated as above; silver-stained SDS–polyacrylamide gels of these samples demonstrated no proteolysis of topoisomerase II from either cell line (data not shown).

Vpm^R-5 enzyme is less stable than the wild-type enzyme, suggesting that it is structurally altered.

Characterization of DNA Binding Properties of WT and Vpm^R-5 Topoisomerase II. We next examined the interaction of purified wild-type and Vpm^R-5 enzymes with DNA using a nitrocellulose filter binding assay. Association of DNA and enzyme was first studied as described under Methods. When association was quantified as a function of time, from 1 to 30 min post enzyme addition, we found no time dependence of complex formation. Maximal complex formation was achieved within 1 min of enzyme addition for both wild-type and Vpm^R-5 enzymes. The presence or absence of both ATP and 50 μM VP-16 had no effect on the amount of complex formed at 30 min at 30 °C; the same amount of complex was formed regardless of these perturbations.

The dissociation of the complex formed above by 0.5 M NaCl is shown in Figure 8. After 30 min of incubation at 30 °C, with and without both 1 mM ATP and 50 μM VP-16, NaCl was added (time 0), and the amount of complex remaining was quantified. In the absence of ATP, with or without drug, the complex is almost totally dissociated for both wild-type and Vpm^R-5 enzymes. In the presence of ATP,

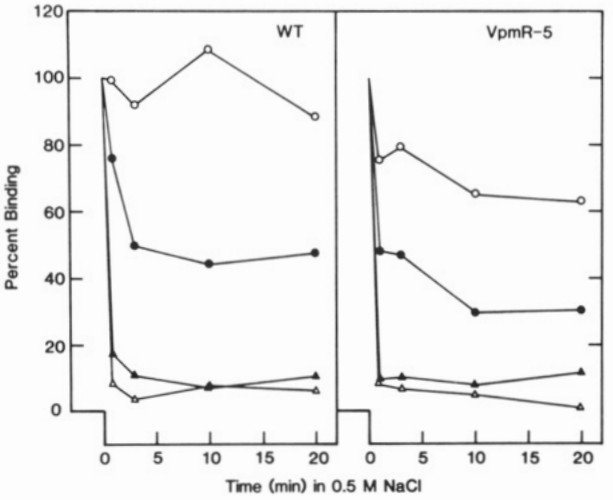

FIGURE 8: Salt dissociation of DNA–topoisomerase II complexes in the presence and absence of both VP-16 and ATP. The complex formed between 3′-[^{32}P]DNA and purified wild-type and Vpm^R-5 enzymes was dissociated by 0.5 M NaCl; undissociated complexes were quantified by a nitrocellulose filter binding assay as described under Methods. (○) 1 mM ATP and 50 μM VP-16; (●) 1 mM ATP without VP-16; (△) 50 μM VP-16 without ATP; (▲) without ATP and VP-16.

approximately 50% of the complex remains for wild-type enzyme and approximately 40% of the Vpm^R-5 enzyme–DNA complexes remain undissociated. The presence of 50 μM VP-16 appears to prevent the 50% and 60% dissociation of the wild-type and Vpm^R-5 complexes, respectively. The nature of the complex formed in the presence of ATP and VP-16 is unknown.

Covalent Enzyme–DNA Complex Formation. In the presence of SDS, topoisomerase II becomes covalently linked to the 5′ end of DNA by a phosphotyrosyl bond (Liu et al., 1983). The covalent complexes can be quantified by the K/SDS assay as described under Methods. It has been previously shown (Glisson et al., 1986b) in crude nuclear extracts of wild-type and Vpm^R-5 cells that VP-16 induces covalent complex formation in a dose-dependent manner for wild-type enzyme; Vpm^R-5 demonstrated minimal cleavage. Using homogeneous topoisomerase II from both cell lines in the presence and absence of 1 mM ATP, we have confirmed these initial observations (Figure 9). In the presence of ATP, VP-16-induced covalent complex formation between DNA and wild-type enzyme reaches a maximum at low drug concentrations (5 μM). In the absence of ATP, drug-induced complex formation proceeds in a more graded fashion, achieving the same maximum at 50 μM VP-16. Purified Vpm^R-5 enzyme, with or without ATP, was not stimulated by VP-16 to form covalent complexes until very high levels of VP-16 (150–200 μM), in the presence of ATP, were used (see inset, Figure 9). The cleavage observed by the Vpm^R-5 enzyme in the absence of drug is quantitatively similar to that of wild-type enzyme. Importantly, this basal cleavage by the altered enzyme is not reduced upon drug addition. These results, with purified wild-type and Vpm^R-5 enzymes, have also been observed with *m*-AMSA (data not shown).

Topoisomerase II Cleavage Sites of pUC DNA. Agarose gel mapping of drug-induced DNA cleavage sites by purified wild-type and Vpm^R-5 enzymes was done as described under Methods (Figure 10). By use of high drug concentrations, there was sufficient DNA cleavage by Vpm^R-5 enzyme to allow identification of individual cleavage sites. This autoradiogram demonstrates that the cleavage patterns for both enzymes with a given drug are qualitatively the same; however,

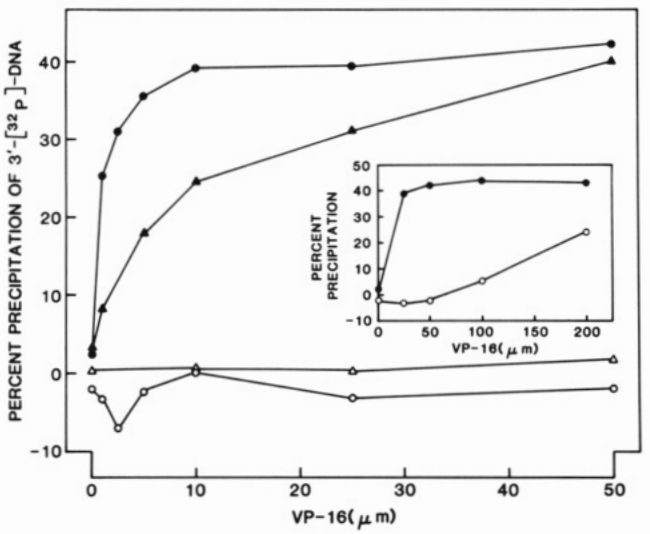

FIGURE 9: Quantitative precipitation of covalent DNA–topoisomerase II complexes in the presence of VP-16. VP-16-induced cleavage of 3′-^{32}P-labeled DNA by purified wild-type and Vpm^R-5 enzymes was quantified by the K/SDS procedure described under Methods. Equivalent amounts of purified wild-type and Vpm^R-5 enzymes (100 ng) were used. The presence (○) or absence (△) of 1 mM ATP had no effect on drug-stimulated cleavage by Vpm^R-5 topoisomerase II. Maximal VP-16-induced cleavage was observed at low drug concentrations in the presence of ATP (●) with wild-type enzyme, whereas a more linear response to VP-16-induced cleavage was found when wild-type topoisomerase II was incubated with DNA in the absence of ATP (▲). (Inset) Drug-stimulated DNA cleavage over a more extended range of etoposide concentrations (0–200 μM). The resistance of the Vpm^R-5 enzyme is relative. ATP was present in these experiments.

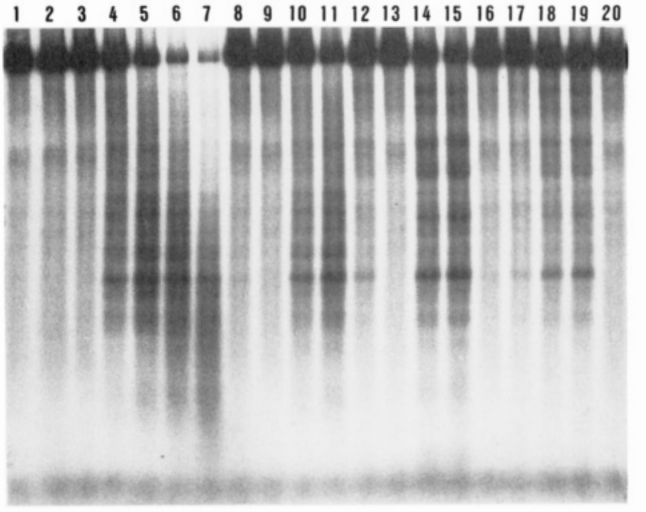

FIGURE 10: Separation of the drug-induced cleavage products from 3′-[^{32}P]pUC DNA treated with purified wild-type and Vpm^R-5 enzymes by agarose gel electrophoresis. Cleavage of labeled DNA by homogeneous preparations of topoisomerase enzymes in the presence of 200 μM VP-16 (lanes 4–11) and 0.75 μM *m*-AMSA (lanes 12–19) was done as described under Methods. (Lanes 1 and 20) Control with no enzyme; (lane 2) control with 36 ng of wild-type enzyme without drug; (lane 3) control with 36 ng of Vpm^R-5 enzyme without drug; (lanes 4 and 12) 9 ng of wild-type enzyme; (lanes 5 and 13) 18 ng of wild-type enzyme; (lanes 6 and 14) 36 ng of wild-type enzyme; (lanes 7 and 15) 54 ng of wild-type enzyme; (lanes 8 and 16) 9 ng of Vpm^R-5 enzyme; (lanes 9 and 17) 18 ng of Vpm^R-5 enzyme; (lanes 10 and 18) 36 ng of Vpm^R-5 enzyme; (lanes 11 and 19) 54 ng of Vpm^R-5 enzyme.

the patterns observed with VP-16 and *m*-AMSA with respect to sites of cleavage and intensity are different. Differences in sites of drug-induced cleavage have previously been reported for calf thymus topoisomerase II (Tewey et al., 1984a,b).

Discussion

The modified purification procedure described herein allows for the reproducible preparation of homogeneous wild-type and Vpm^R-5 CHO cell topoisomerase II. We have been able to demonstrate that the drug resistance of the Vpm^R-5 CHO cell line is associated with the presence of an altered topoisomerase II. This is the first description of a mammalian cell line containing such an altered enzyme. Definitive evidence for a qualitative alteration in nuclear topoisomerase II in other cell lines resistant to topoisomerase II inhibitors is lacking. Further purification and characterization of topoisomerase II from the drug-resistant Chinese hamster lung cell line, DC3F/9-OHE (Salles et al., 1982; Pommier et al., 1986; Charcosset et al., 1988), and from the human cell line, HL-60/AMSA (Bakic et al., 1986), will determine if an altered enzyme confers resistance in these cells. Recent evidence (Deffie et al., 1989) suggests that the resistance to adriamycin by the P388/ADR/3 and P388/ADR/7 cell lines (Goldenberg et al., 1986) is most likely due to a quantitative reduction of enzyme in the resistant cell lines and not due to the presence of an altered topoisomerase II. Finally, the drug resistance of the human lymphoblastic cell lines, CEM/VM-1 and CEM/VM-1-5, does not appear to be due to a relative decrease in immunoreactive topoisomerase II (Danks et al., 1988). The resistance of these two lymphoblastic cell lines may be due to an intrinsically altered enzyme or an intracellular modulator of topoisomerase II activity but, again, this will be definitively determined when purified topoisomerase II is characterized from these cell lines.

The two major differences between the purified wild-type and Vpm^R-5 topoisomerases involve their stability and the cleavage of DNA in response to drug. The Vpm^R-5 enzyme is significantly more labile than the wild-type enzyme (Figure 7). This behavior suggested that the Vpm^R-5 cell line may express a temperature-sensitive phenotype. Unfortunately, we have not been able to demonstrate this experimentally. Drug stimulation of DNA cleavage, assayed as total cleavage by SDS precipitation of covalent topoisomerase–DNA complexes, is markedly reduced in the Vpm^R-5 cell line. This was true whether the epipodophyllotoxin VP-16 or the intercalator *m*-AMSA was used. That these cells are drug resistant and contain an altered enzyme is perhaps the best evidence to date establishing topoisomerase II as the site of cytotoxicity for both epipodophyllotoxins and intercalating agents.

It is of interest to compare the results of our nitrocellulose filter binding studies with those of other investigators. The initial association of purified topoisomerase II, both Vpm^R-5 and wild type, with linear DNA is very rapid and, in low-salt conditions, very stable. This phenomenon was also described for DNA gyrase (Higgins & Cozzarelli, 1982; Peebles et al., 1978) and topoisomerase II from *Drosophila melanogaster* (Osheroff et al., 1983). In the presence of drug, the DNA–enzyme complex is resistant to dissociation by 0.5 M NaCl but only if ATP is present. This effect was seen regardless of the source of enzyme, indicating that, in some fashion, VP-16 affects the behavior of Vpm^R-5 enzyme, even if DNA cleavage does not result. The ability of VP-16 to confer salt resistance on the DNA–enzyme complex has also been described for oxolinic acid, the enzyme in this case being DNA gyrase. We are not aware of previous reports of the ATP requirement for this drug effect, however.

The results presented above, pertaining to nitrocellulose filter binding and DNA cleavage, suggest that more than one DNA–topoisomerase II complex exist and that the behavior of this complex is determined by the method used to study it.

The nitrocellulose filter binding data indicate that one type of DNA–enzyme complex is formed in the absence of ATP, which is easily dissociated by NaCl. In the presence of ATP, a salt-resistant complex is formed; its salt dissociation is further protected by VP-16. In contrast, the covalent complex isolated upon the addition of SDS, measured experimentally as DNA cleavage or as DNA strand breaks by alkaline elution, is not dependent on the presence of ATP, especially at higher drug concentrations. Thus, the cleavable complex quantified by the addition of SDS may be an artifact of the experimental procedure, whereas the ATP-dependent complex measured by nitrocellulose filter binding may more accurately reflect a reaction intermediate in the reaction mechanism of topoisomerase II.

Our investigations suggest a model for the DNA–topoisomerase II interaction for both the sensitive wild-type and resistant Vpm^R-5 CHO enzymes. In the absence of topoisomerase II active drugs, both enzymes behave the same; i.e., they bind DNA, cleave one double strand, allow strand passage of another duplex DNA, ligate the broken strand, and, in the presence of ATP, dissociate from DNA. This is supported by the similar nitrocellulose filter binding data and the equivalent catalytic activity. In the presence of VP-16, both enzymes exhibit an altered form of DNA interaction in the form of a more salt-resistant complex. In addition, decatenation by the two enzymes is inhibited equally by drug. This inhibition of catalytic activity may be the result of interference with the strand passing event, inhibition of ATP hydrolysis (enzyme turnover), or stabilization of a "post-cleavable complex" as described (Osheroff, 1986) for a nonhydrolyzable congener of ATP. Increased DNA cleavage by wild-type enzyme in the presence of VP-16 is observed as previously reported (Ross et al., 1984). This is generally believed to occur because of drug interference with the religation step, although definitive evidence for this is lacking. No such stimulation of DNA cleavage by drug is seen with VPM^R-5 enzyme. Therefore, the most likely explanation for the resistance of the enzyme to drug is that religation is not significantly altered by drug. In other words, the Vpm^R-5 enzyme actually breaks and reseals DNA at a more normal rate in the presence of VP-16. As a result, the drug–enzyme complexes are not excessively trapped when detergent is added to the reaction mixture.

In summary, our data suggest that epipodophyllotoxins and intercalating agents inhibit both religation and catalytic activity in wild-type CHO cells. The drug-resistant Vpm^R-5 cell line is as sensitive to the inhibition of catalytic activity as wild-type cells; however, the religation event does not appear to be drug sensitive. Finally, since the action of both intercalating agents and epipodophyllotoxins is affected by the presumed enzyme alteration, it is likely that they share a common site and mode of interaction with the enzyme.

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