0026-895X/94/010058-09$03.00/0

MOLECULAR PHARMACOLOGY, 46:58-66, 1994.

Reduced Phosphorylation of Topoisomerase II in Etoposide-Resistant Human Leukemia K562 Cells

MARY K. RITKE, WILLIAM P. ALLAN, CHERYL FATTMAN, NURTEN N. GUNDUZ, and JACK C. YALOWICH

Department of Pharmacology and the Pittsburgh Cancer Institute (M.K.R., W.P.A., C.F., J.C.Y.), and Department of Surgery (N.N.G.), University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Received December 30, 1993; Accepted April 28, 1994

SUMMARY

In this report we examine biochemical and genetic alterations in DNA topoisomerase II (topoisomerase II) in K562 cells selected for resistance in the presence of etoposide (VP-16). Previously, we have demonstrated that the 30-fold VP-16-resistant K/VP.5 cell line exhibits decreased stability of drug-induced topoisomerase II/DNA covalent complexes, requires greater ATP concentrations to stimulate VP-16-induced topoisomerase II/DNA complex formation, and contains reduced mRNA and protein levels of the *M*, 170,000 isoform of topoisomerase II, compared with parental K562 cells. K/VP.5 cells grown in the absence of VP-16 for 2 years maintained resistance to VP-16, decreased levels of topoisomerase II, and attenuated ATP stimulation of VP-16-induced topoisomerase II/DNA binding, compared with K562 cells. Sequencing of cDNA coding for two consensus ATP binding sites and the active site tyrosine in the K/VP.5 topoisomerase II gene indicated that no mutations were present in these domains. In addition, single-strand conformational polymorphism analysis of restriction fragments encompassing the entire topoisomerase II cDNA revealed no evidence of mutations in the gene for this enzyme in K/VP.5 cells. Nuclear extracts from K562 (but not K/VP.5) cells contained a heat-labile factor that potentiated VP-16-induced topoisomerase II/DNA covalent complex formation in isolated nuclei from K/VP.5 cells. Immunoprecipitated topoisomerase II from K/VP.5 cells was 2.5-fold less phosphorylated, compared with enzyme from K562 cells. Collectively, our data suggest that acquired VP-16 resistance is mediated, at least in part, by altered levels or activity of a kinase that regulates topoisomerase II phosphorylation and hence drug-induced topoisomerase II/DNA covalent complex formation and stability.

The nuclear enzyme topoisomerase II is the target of several clinically effective anticancer drugs (1, 2). Exposure of cells to topoisomerase II-inhibitory drugs results in the accumulation of DNA strand breaks as a consequence of the stabilization of a covalent topoisomerase II/DNA complex, the accumulation of DNA strand breaks, and eventual apoptotic cell death (1–5). Acquired resistance to topoisomerase II inhibitors is usually characterized by a reduction of topoisomerase II/DNA covalent complexes in resistant compared with sensitive cells, which is attributed to a reduction of enzyme levels (6–10) or to a change in topoisomerase II biochemical activities (10–16).

We have selected a VP-16-resistant K562 cell line (K/VP.5) by continuous exposure of K562 cells to 0.5 μM VP-16 (10, 16). K/VP.5 cells are cross-resistant to a variety of other topoisomerase II inhibitors and show reduced levels of topoisomerase II protein and mRNA (10, 16). Compared with K562 cells, K/VP.5 cells or nuclei show 1) reduced levels of DNA damage after exposure to VP-16, 2) more rapid repair of DNA damage after removal of VP-16, 3) more rapid dissociation of drug-induced covalent topoisomerase II/DNA complexes, and 4) reduced ATP stimulation of VP-16-induced topoisomerase II/DNA binding (16). These observations suggest that selection for resistance to VP-16 in K/VP.5 cells results in an alteration or mutation in topoisomerase II that affects the stability of topoisomerase II binding to DNA.

Multiple targets for mutations or alterations in topoisomerase II that confer drug resistance may be present in cells exposed to topoisomerase II-inhibitory agents; of most interest have been the nucleotide binding domains and the active site tyrosine. ATP stimulates the binding of topoisomerase II to DNA, and hydrolysis of ATP is required for enzyme turnover after strand passage (17). Although ATP binding sites have not been conclusively identified for topoisomerase II, the cDNA sequence of the human enzyme reveals three conserved nucleotide binding domains, i.e., motif A, motif B, and DNBS (18, 19). Mutations in or near motif B and DNBS have been reported in cells resistant to topoisomerase II inhibitors (18–

This work was supported by American Cancer Society Grant DHP-49. M.K.R. is the recipient of a Pittsburgh Cancer Institute Postdoctoral Fellowship.

ABBREVIATIONS: topoisomerase II, DNA topoisomerase II (*M*, 170,000 isoform); VP-16, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside) (etoposide); VM-26, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-ethenylidene-β-D-glucopyranoside) (teniposide); mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (amsacrine); DNBS, dinucleotide binding site; SSB, single-strand break; SSCP, single-strand conformational polymorphism; kb, kilobase pair(s); PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMEM, Dulbecco's minimal essential medium; RF, restriction fragment; bp, base pair(s); TBS, Tris-buffered saline.

23). VM-26-resistant CCRF-CEM cells that contain a point mutation in motif B also showed an increased ATP requirement for topoisomerase II strand-passing activity (18, 19). Thus, consensus ATP binding sites of topoisomerase II may represent potential sites of mutation under the selective pressure of exposure to topoisomerase II-inhibitory drugs. A point mutation has also been identified near the active site tyrosine-804 codon of topoisomerase II in CCRF-CEM cells selected for resistance to VP-16 (24). Finally, a mutation of topoisomerase II that contributes to VP-16 and mAMSA resistance in yeast was recently identified in a domain with a currently unknown function (25).

Topoisomerase II is phosphorylated almost exclusively on serine residues *in vivo* (26, 27). Both the levels and the extent of phosphorylation of topoisomerase II increase as cells enter G_2/M of the cell cycle (28). It was reported that a kinase activity copurifies with topoisomerase II (29, 30). In addition, topoisomerase II has been found to serve as an *in vitro* substrate for several kinases, including casein kinase II (31), protein kinase C (26, 32), and $p34^{cdc2}$ kinase (26). Peptide analysis of ^{32}P-labeled yeast topoisomerase II suggested that the pattern of phosphorylation of *in vivo* phosphorylated topoisomerase II most closely resembles that of *in vitro* phosphorylation by casein kinase II (26, 30). Topoisomerase II from human KB cells selected for resistance in the presence of VP-16 was found to be hyperphosphorylated, compared with parental KB cells (33). More recently, topoisomerase II from mAMSA-resistant HL-60 cells, although translated and phosphorylated at a 2–3-fold slower rate, was less rapidly degraded and dephosphorylated, compared with sensitive HL-60 cells (34). These studies suggest that mutation of a critical phosphorylation site of topoisomerase II or of a topoisomerase II-associated kinase might alter the sensitivity of cells to topoisomerase II-inhibitory drugs.

In this paper we demonstrate that resistance to VP-16 in K/VP.5 cells is stable in the absence of selecting agent, and we further characterize the alterations in topoisomerase II that may be responsible for drug resistance. Although the stability of resistance to VP-16 and biochemical changes in topoisomerase II implicate a mutation in resistant K/VP.5 cells, we found no evidence of sequence changes in topoisomerase II cDNA from K/VP.5 cells, compared with K562 cells. However, we did find that immunoprecipitated topoisomerase II from K/VP.5 cells is less phosphorylated than that from K562 cells. In addition, we report that nuclear extracts from K562 cells (but not K/VP.5 cells) contain a heat-labile factor that potentiates drug-induced binding of topoisomerase II to DNA. Our data are most consistent with an alteration in a regulator of topoisomerase II function, such as a protein kinase that may limit drug-induced topoisomerase II/DNA binding and DNA damage by decreasing topoisomerase II phosphorylation in resistant cells.

Materials and Methods

Drugs and chemicals. VP-16 was provided by Bristol-Myers Squibb, Co. (Wallingford, CT) and mAMSA was provided by the Drug Investigational Branch of the National Cancer Institute. Radiolabeled dCTP, dATP methionine, and P_i were obtained from DuPont-New England Nuclear (Wilmington, DE). Electrophoresis reagents were obtained from GIBCO/BRL (Gaithersburg, MD) or Bio-Rad (Richmond, CA). Restriction enzymes were obtained from New England Biolabs (Beverly, MA) or Boehringer Mannheim Corp. (Indianapolis, IN). Unless indicated otherwise, all other reagents were obtained from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Cells. K/VP.5 cells were selected by first periodic and then continuous exposure of K562 cells to 0.5 μM VP-16 for 1 year. The cell line was cloned by limiting dilution and is 30-fold resistant to the growth-inhibitory effects of VP-16. More detailed characteristics of the cloned cell line are presented in a separate manuscript (16). K/VP.5rev cells are K/VP.5 cells maintained in the absence of VP-16 for >600 days. K562, K/VP.5, and K/VP.5rev cells were maintained as suspension cultures in DMEM (Irvine Scientific Co., Irvine, CA) containing 10% fetal bovine serum and 2 mM glutamine (Hazelton Biochemicals, Inc., Lenexa, KS). Enzyme purification and all experiments were performed using exponentially growing cells (cell density of 4–8 × 10^5/ml). K/VP.5 cells were removed from drug at least 3 days before use in experiments.

Drug-induced growth inhibition. Exponentially growing K562, K/VP.5, and K/VP.5rev cells were adjusted to 1 × 10^5 cells/ml and incubated with various concentrations of VP-16 for 48 hr, after which cells were counted with a model ZBF Coulter counter (Coulter Electronics, Hialeah, FL). The extent of growth beyond the starting concentration of 1 × 10^5 cells/ml for drug-treated versus control cells was expressed as percentage inhibition of control growth. The 50% growth-inhibitory concentration for each drug in each cell line was calculated from replicate dose-response curves generated from separate experiments.

Flow cytometry. Mid-logarithmic phase cells were washed twice with saline (0.15 M NaCl) and fixed for 4–16 hr in buffer containing 50% ethanol, 2.5% DMSO, 0.02 M trisodium citrate, and 0.125 M sucrose. Fixed cells were washed three times with phosphate-buffered saline (0.15 M NaCl, 0.7 mM KH_2PO_4, 4.3 mM K_2HPO_4, pH 7.4) and stained with 50 μg/ml propidium iodide in buffer containing 3.4 mM trisodium citrate, 0.3% Nonidet P-40, and 0.1 mg/ml RNase A. Nuclei were stained on ice for 15–30 min before analysis of DNA content with a 742 Epics flow cytometer (Coulter Electronics). Data were analyzed using an M Cycle computer program (Phoenix Flow Systems, San Diego, CA), which quantitates overlapping G_1, S, and G_2/M peaks.

Isolation of nuclei and nuclear extracts. Cells were washed in ice-cold buffer A (1 mM KH_2PO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, pH 6.4) (35). The cells were suspended in 1 ml of buffer A, lysed by the addition of 9 ml of buffer B (0.3% Triton X-100 in buffer A), and incubated on ice for 30 min. After lysis, 40 ml of buffer A were added and nuclei were pelleted by centrifugation at 1000 rpm for 10 min in an IEC model HN-SII tabletop centrifuge. The density of nuclei was adjusted to 1 × 10^6/ml in cold buffer A. After equilibration at 37° for 15 min, nuclei were treated with VP-16 in the presence or absence of 1 mM ATP. Topoisomerase II-containing 1 M NaCl extracts were prepared from nuclei as described previously (11). Protein concentrations were determined using the Bio-Rad protein assay reagent.

Alkaline elution analysis of single-strand DNA damage. Drug-mediated DNA damage was assessed using the alkaline elution technique for high frequency SSBs (36). K562 and K/VP.5 cells were labeled for 48 hr with [2-^{14}C]thymidine (0.02 μCi/ml), after which nuclei were isolated as described above. Nuclei were suspended in buffer A to a final density of 1 × 10^6/ml and were incubated for 30 min at 37° with various concentrations of VP-16. The final solvent concentration for all conditions was 0.4% DMSO. K562 cells (5 × 10^5) containing [^{3}H]DNA were irradiated (1500 rad) on ice using a ^{137}Cs source (Mark Irradiator; J. L. Sheppard and Associates, Glendale, CA). These irradiated cells were added as internal standards to 7.5 × 10^5 drug-treated and ^{14}C-labeled K562 or K/VP.5 cells. Cells were washed twice with phosphate-buffered saline, layered onto a polyvinyl chloride filter (pore size, 0.45 μm; Gelman Sciences, Inc., Ann Arbor, MI), and lysed with a solution of 2% SDS, 10 mM disodium EDTA, and 0.5 mg/ml proteinase K. The DNA was eluted from the filter with tetrapropylammonium hydroxide, pH 12.1, at a flow rate of 0.16 ml/min, with a fractional interval of 5 min. The frequency of VP-16-induced DNA SSBs was quantitated as the fraction of [^{14}C]DNA retained on the filter when 60% of the ^{3}H-labeled internal standard DNA remained. A

calibration curve relating the frequency of VP-16-induced DNA SSBs to a corresponding effect of radiation (radiation-equivalent DNA damage) using ^{14}C-labeled cells was obtained by plotting radiation dose versus [^{14}C]DNA retention at 60% retention of the [^{3}H]DNA internal standard.

Topoisomerase II/DNA binding assays. pBR322 DNA (obtained from GIBCO/BRL Laboratories, Bethesda, MD) was linearized by digestion with *Cla*I restriction enzyme. Linearized pBR322 DNA was labeled at the 3′ end by the addition of 100 μCi of [α-^{32}P]dCTP, 1 mM levels each of dATP, dGTP, and dTTP, and 5 units of Klenow fragment of DNA polymerase I, followed by incubation at 30° for 30 min. Labeled DNA was resolved from unincorporated nucleotides by passage through 1-ml spin columns of Sephadex G50 (Pharmacia Biotechnology, Piscataway, NJ) in TE (10 mM Tris·HCl, 1 mM EDTA, pH 8.0). The amount of labeled DNA recovered was measured by absorbance at 260 nm. The specific activity of 3′-end-labeled DNA was usually 0.5–1.0 × 10^6 cpm/μg, and labeled DNA was used within 2 weeks of preparation. Nuclear extracts or purified topoisomerase II were incubated with 0.2 μg of ^{32}P-labeled pBR322 DNA in the presence or absence of VP-16. VP-16-induced topoisomerase II/DNA covalent complexes were isolated by SDS/KCl precipitation and were quantitated by scintillation counting (16).

Topoisomerase II/DNA covalent complex formation in intact cells and nuclei was measured as described by Zwelling *et al.* (13). Briefly, cells were prelabeled for 24 hr with [*methyl*-^{3}H]thymidine and [U-^{14}C] leucine, after which cells or isolated nuclei (see above) were treated with 25–100 μM VP-16 or 0.1% DMSO. Topoisomerase II/DNA complexes were isolated and quantitated relative to ^{14}C-labeled protein by scintillation counting of KCl/SDS precipitates (16).

Purification of topoisomerase II. Fast protein liquid chromatography (Pharmacia Biotechnology, Inc.) purification of topoisomerase II from 1 M NaCl nuclear extracts of 3 × 10^9 cells was carried out as reported by Drake *et al.* (6). The enzyme strand-passing activity of the Mono S column fractions (0.2–0.6 M NaCl) was monitored by decatenation of [*methyl*-^{3}H]thymidine-labeled *Crithidia fasciculata* mitochondrial DNA (12). Minicircles released by topoisomerase II were quantitated in duplicate by scintillation counting. Decatenation was quantitated after subtraction of counts found in controls lacking topoisomerase II. The purity of fractions containing maximum decatenation activity was assessed by silver staining of protein resolved by 8% SDS-PAGE. The fractions with the highest decatenation activity showed predominantly a 170-kDa band, although several additional bands were sometimes apparent (data not shown).

Reverse transcription of RNA. RNAs were purified by phenol extraction of 3 M guanidinium thiocyanate lysates of 1 × 10^7 midlogarithmic phase K562 and K/VP.5 cells. Ethanol-precipitated RNA was dissolved in sterile water and quantitated spectrophotometrically (A_{260} measurements). Total cell RNA purified from HL-60 and HL-60/AMSA cells was provided by Dr. L. Zwelling (M.D. Anderson Hospital, Houston, TX). cDNA was synthesized from total RNA by reverse transcription using avian myeloblastosis virus reverse transcriptase (10,000 units/ml; Promega, Madison, WI). Each 50-μl reaction contained 1–5 μg of RNA, 50 mM Tris·HCl, pH 8.3, 75 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 36 nM random hexamers (Boehringer Mannheim Biochemicals), 0.5 mM levels each of dATP, dCTP, dGTP, and dTTP, and 15 units of avian myeloblastosis virus reverse transcriptase. cDNA synthesis was carried out at 42° for 30 min, after which samples were boiled for 5 min and then chilled on ice. Each 50-μl first-strand cDNA reaction provided sufficient product for 10 PCR amplifications.

Primers used for amplification of topoisomerase II fragments. Primers were selected either from those used previously to clone topoisomerase II (20) or with the assistance of the OLIGO computer program (Wojciek Rychlik National Biosciences, Inc., Plymouth, MN). Primers were obtained from Genosys (The Woodlands, TX) and used without further purification. PCR products and the primers used to obtain them were as follows [nucleotide numbering corresponds to that of Tsai-Pflugfelder *et al.* (37), where the adenine of the published ATG start codon is nucleotide 1]: PCR product I (1.18 kb, spanning nucleotides −27–1153): 3′ primer, TTGGGTTGTAAAGTCATGTT (20); 5′ primer, AGTACCGGGCCCTTCACGAC; PCR product II (0.38 kb, spanning nucleotides 1312–1671): 3′ primer, GAAGAGAGGGCCAGTTGTGA; 5′ primer, CCCAAACTCGATGATGCCAA; PCR product III (0.54 kb, spanning nucleotides 1033–1573): 3′ primer, CGAAGCGTCTTCAATGAATC; 5′ primer, AAGGGTGGTGTTGCAGTAAA; PCR product IV (1.10 kb, spanning nucleotides 1414–2518): 3′ primer, ACCATTCAGGCTCAACACGC; 5′ primer, CTTGGTGTGGTTGGGAGAGA; PCR product V (1.21 kb, spanning nucleotides 2343–3549): 3′ primer, CCTTGGCTTCAACAGCCTCC; 5′ primer, CCTCTTGCAGCCCATTGGTC (20); PCR product VI (1.29 kb, spanning nucleotides 3353–4643): 3′ primer, [GTCG]ACCAGTCTTGGGCTTGGTAA; 5′ primer, CAGATTCTGGACCAACCTTC (20).

PCR. Reverse-transcribed cDNA (5 μl) was added to 45 μl of a cocktail containing 50 mM Tris·HCl, pH 8.3, 75 mM KCl, 1.5 mM $MgCl_2$, 0.2–0.4 μM levels each of two primers, 0.2 mM levels of each deoxynucleoside triphosphate, and 1.5 units of Amplitaq polymerase (Cetus, Norwalk, CT). In some reactions, 5 μl of pBSHTOPII plasmid DNA (100 pg) were substituted for reverse-transcribed cDNA. This plasmid, containing a full length HeLa topoisomerase II cDNA, was obtained from Dr. L. F. Liu (University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, NJ). Reactions were carried out in a BIOSYCLER thermocycler (Integrated Separations System, Natick, MA) programmed as follows: three cycles of 5 min at 95°, 1 min at 60°, and 1 min at 72°, followed by 27 cycles of 1 min at 95°, 0.5 min at 60°, and 1 min at 72°. To confirm that PCR fragments contained one product of the correct size, 5 μl (10%) of each reaction were electrophoresed through 1–1.5% agarose in 0.5× TBE (1× TBE is 89 mM Tris-borate, 0.25 mM EDTA, pH 8.0). Gels were stained with 0.5 μg/ml ethidium bromide in water and visualized by UV illumination. Fragments were sized using *Hinf*I-digested pSP65 and/or *Pvu*II-digested λ phage DNA as markers.

Sequence analysis. For sequencing from single-strand DNA templates, PCR products were diluted 1/1000 and 5 μl were subjected to 30 additional cycles of asymmetric PCR under the conditions described above, except that one primer was added to a final concentration of 5 nM (38). Single-strand cDNA, obtained by asymmetric PCR of topoisomerase II cDNA, was separated from excess primers and deoxynucleotides by passage through Chroma Spin+ TE-30 columns (Clontech Laboratories, Inc., Palo Alto, CA). Sequencing of cDNA (about 0.4 pmol) was accomplished by dideoxynucleotide chain termination using a Sequenase 2.0 kit (United States Biochemicals, Cleveland, OH), as recommended by the supplier. The primer that was limiting in asymmetric PCR was used in subsequent sequencing reactions. DNA strands were labeled with 1–2 μCi of [^{35}S] dATP-αS (1000 Ci/mmol; NEN) and resolved by electrophoresis through 6% polyacrylamide/bisacrylamide (29:1) gels in 8 M urea/1× TBE, at 1600 V (45–50°).

RF/SSCP. For RF/SSCP analysis, 2–5 μCi of [α-^{32}P]dCTP (3000 Ci/mmol) were added to each PCR mixture (see above). PCR products were then digested with restriction enzymes to obtain fragments of <400 bp. Typically, 5 μl of PCR product were digested in a final volume of 50 μl. Enzymes were inactivated by heating to 90° for 15 min or by the addition of 1 μl of 0.5 M Na_2EDTA, pH 8.0. Enzyme-digested samples from PCR (1–5 μl) were diluted 1/5 in sequencing gel loading buffer (90% formamide, 1× TBE, 2.5 μg/ml bromphenol blue, 2.5 μg/ml xylene cyanol), heated for 5 min at 90°, and then placed in an ice-water bath for 5–10 min. Samples (2–8 μl) were electrophoresed at 40 W through 5–6% polyacrylamide gels (30:1 acrylamide/bisacrylamide) in 1× TBE, at either room temperature or 4°, for 3–8 hr. After electrophoresis, gels were transferred to Whatman filter paper, dried under vacuum, and autoradiographed overnight.

Antibodies. Antibodies to recombinant human topoisomerase II were prepared to a previously described human recombinant topoisomerase II peptide (39). The plasmid harboring the carboxyl-terminal one third of human topoisomerase II (pZII-06) and the bacterial host, *Escherichia coli* BL21(DE_3), were provided by Dr. L. F. Liu. The 70-

kDa recombinant protein from bacterial lysates was resolved by and purified from 7.5% SDS-PAGE (11- × 14- × 3-cm³) gels. New Zealand rabbits were injected with 100 μg in RIBI adjuvant (RIBI Immunochemical Research, Inc., Hamilton, MT) at 2–3-week intervals. High titer antisera (1/5000 to 1/10,000) appeared after the second boost (three total injections); for immunoprecipitations (see below), antisera after the fourth or fifth boost were used.

Immunoprecipitation of radiolabeled topoisomerase II. Exponentially growing cells (5–7 × 10^7) were pelleted, washed with saline, and then resuspended in either methionine-free DMEM containing 5% fetal bovine serum or phosphate-free DMEM containing 1% fetal bovine serum, to a final concentration of 1 × 10^6/ml. Resuspended cells were incubated for 30 min in deficient medium before the addition of 30 μCi/ml EXPRE^{35}S^{35}S (containing [^{35}S]methionine and [^{35}S]cysteine; NEN) or 100 mCi/ml ^{32}Pi. After 4 hr of labeling, cells were pooled, pelleted, and washed, and whole-cell or nuclear lysates were prepared essentially as described by Saijo *et al.* (28). For each immunoprecipitation, 1.5 mg of Protein A-Sepharose and 10 μl of normal or anti-topoisomerase II rabbit sera were adjusted to a final volume of 200 μl with STx-TBS (0.1% SDS and 0.1% Triton X-100 in TBS) and incubated at 4° for 4 hr or overnight. Antibody-bound beads were pelleted and washed three times with STx-TBS to remove unbound antibody and other serum proteins. Cell or nuclear lysate protein (100 μg) was diluted 1/5, added to antibody-bound Protein A-Sepharose beads, and incubated on a rocker platform overnight at 4°. The beads were washed five times with 1 ml of STx-TBS, resuspended in 30 μl of SDS-PAGE sample buffer, and electrophoresed through 7.5% SDS-PAGE gels (0.15 mA/cm²). Immunoprecipitated topoisomerase II was localized by autoradiography and the ^{32}P$_i$ and ^{35}S cpm were quantitated from excised bands by liquid scintillation counting. The levels of topoisomerase II phosphorylation were determined relative to the amount of topoisomerase II protein, as determined by [^{35}S]methionine incorporation.

Results

Stable resistance and altered ATP dependency for VP-16-induced topoisomerase II/DNA complex formation. K/VP.5 cells grown in the absence of VP-16 for >600 days (K/VP.5rev) maintained their resistance to VP-16 (Fig. 1). The 48-hr 50% growth-inhibitory concentration for VP-16 was 0.044 ± 0.005 μM in K562 cells, 1.51 ± 0.12 μM in K/VP.5 cells, and 1.70 ± 0.13 μM in K/VP.5rev cells (mean ± standard error for seven to 20 determinations; $p < 0.001$ for resistant cells, compared with K562 cells). The K/VP.5rev cells continue to express a reduced level of topoisomerase II protein, compared with K562 cells (data not shown), similar to the 5-fold reduction in topoisomerase II levels demonstrated in K/VP.5 cells (10, 16).

The effects of ATP on VP-16-induced DNA SSBs in K562 and K/VP.5 nuclei were examined (Fig. 2). Addition of ATP to K/VP.5 nuclei resulted in less enhancement of VP-16-induced DNA damage, compared with K562 nuclei. In the absence of ATP, 100 μM VP-16 was required in K/VP.5 nuclei to yield the same SSB frequency as 25 μM VP-16 in K562 nuclei. Maximum stimulation of VP-16-induced SSBs occurred at 1 mM ATP in K562 nuclei. Enhancement of VP-16-induced SSBs was progressively reduced at higher ATP concentrations. Substrate inhibition of VP-16-induced SSBs at ATP concentrations greater than 1 mM has been reported previously (40).

ATP stimulation of VP-16 (25–100 μM)-induced topoisomerase II/DNA covalent complexes was also reduced in isolated nuclei from resistant cells, compared with sensitive cells (Fig. 3). ATP (1 mM) stimulated VP-16-induced topoisomerase II/DNA binding 7–16-fold in K562 nuclei but <3-fold in K/VP.5 and K/VP.5rev nuclei. ATP (0.3–1 mM) stimulation of VP-16-induced topoisomerase II binding to ^{32}P-labeled pBR322 DNA

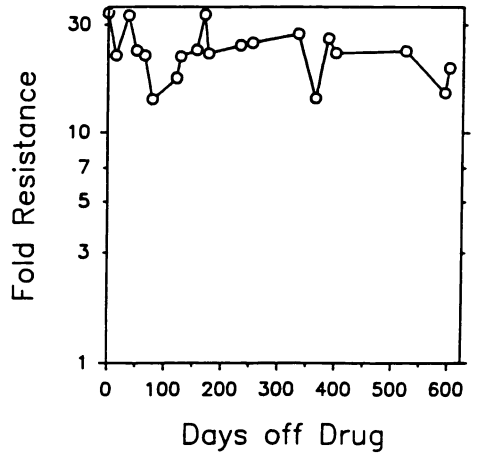

Fig. 1. Stability of VP-16 resistance in K/VP.5 cells maintained in the absence of VP-16 (K/VP.5rev) for 2 years. Resistance to VP-16 was determined periodically by calculating the 50% growth-inhibitory concentrations for VP-16 in K562 versus K/VP.5rev cells from replicate dose-response curves. *Points* represent the fold resistance of K/VP.5rev cells, compared with K562 cells.

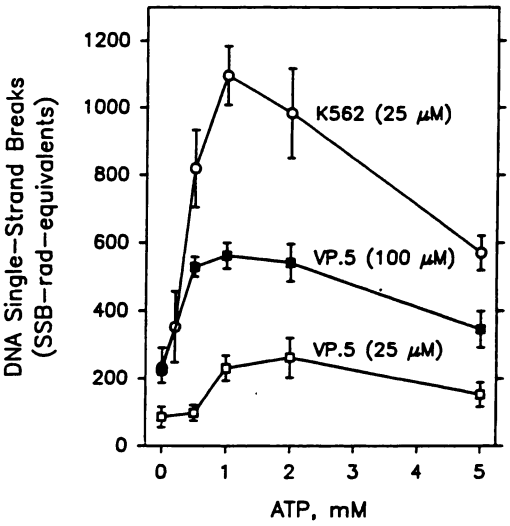

Fig. 2. Effects of ATP on VP-16-induced DNA SSBs in K562 and K/VP.5 nuclei. Isolated nuclei were incubated with 25 or 100 μM VP-16 in the presence of ATP (0–5 mM) for 30 min at 37°. SSBs were quantitated by alkaline elution (see Materials and Methods). *Points* represent the mean ± standard error from five to seven experiments.

was also reduced up to 4-fold for K/VP.5 nuclear extracts, compared with K562 nuclear extracts (Fig. 4). In contrast to results obtained using nuclear extract topoisomerase II, ATP stimulation of VP-16-induced covalent binding of topoisomerase II to ^{32}P-labeled pBR322 DNA was identical using purified enzyme from the two cell lines (Fig. 5). VP-16-induced binding of pBR322 DNA and nuclear extract topoisomerase II was attenuated in the presence of 5 mM ATP (Fig. 4). This attenuation of drug-induced topoisomerase II/DNA complexes was not observed in our previous report using isolated nuclei from K562 and K/VP.5 cells (16), suggesting that topoisomerase II binding to purified DNA (Fig. 4) is less stable and/or may be more sensitive to disruption by excess ATP.

Results of flow cytometric analysis of the DNA content of exponentially growing K562 and K/VP.5 cells showed that equivalent amounts of cells were distributed between the G_1, S, and G_2/M phases for both cell lines. For mid-logarithmic phase

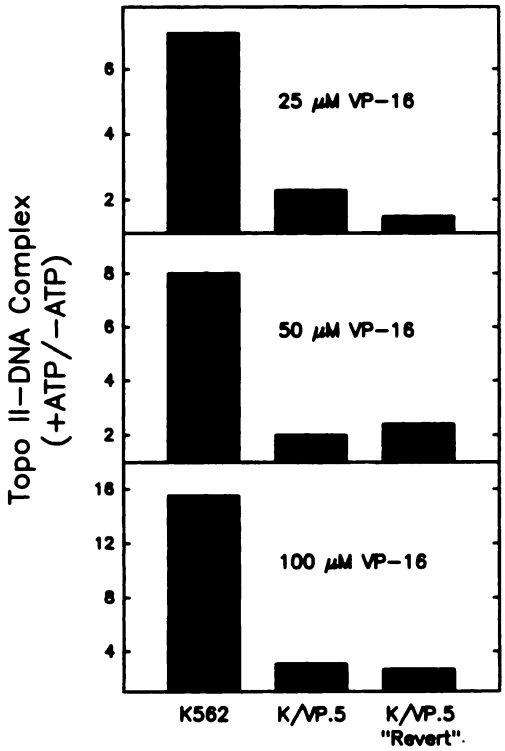

Fig. 3. Effect of ATP on VP-16-induced topoisomerase II/DNA covalent complexes in isolated K562, K/VP.5, and K/VP.5rev (*K/VP.5"revert"*) nuclei. Cells grown in the absence of drug for at least 3 days were prelabeled with [*methyl*-^{3}H]thymidine and [U-^{14}C]leucine. Isolated nuclei were then incubated with 25, 50, or 100 μM VP-16, in the presence or absence of 1 mM ATP, for 30 min at 37°. Topoisomerase II/DNA covalent complexes were isolated by KCl/SDS precipitation. Bound DNA was quantitated by scintillation counting. Results are expressed as the ratio of drug-induced topoisomerase II/DNA (*Topo II-DNA*) complex formation in the presence and absence of ATP. *Bars* represent the average results from two independent experiments.

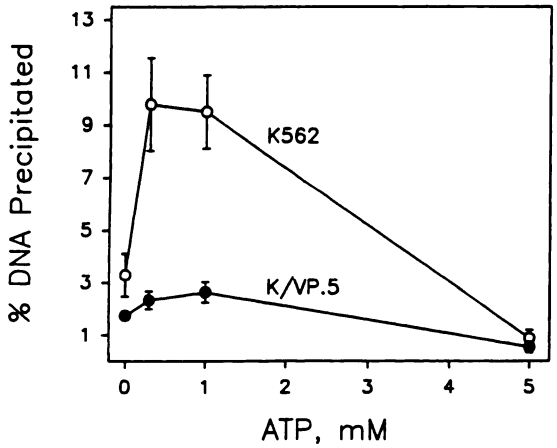

Fig. 4. ATP stimulation of VP-16-induced covalent binding of nuclear extract topoisomerase II to pBR322 DNA. Nuclear extract protein (1 μg) from K562 and K/VP.5 cells was incubated with ^{32}P-3′-end-labeled pBR322 DNA (0.2 μg) for 30 min at 37°, in the presence of 200 μM VP-16 and 0–5 mM ATP. Covalent topoisomerase II/DNA complexes were isolated by KCl/SDS precipitation and bound DNA was quantitated by scintillation counting. Results are expressed as percentage of total ^{32}P-labeled pBR322 precipitated, so that data from different experiments could be pooled. *Points* represent the mean ± standard error from four independent experiments.

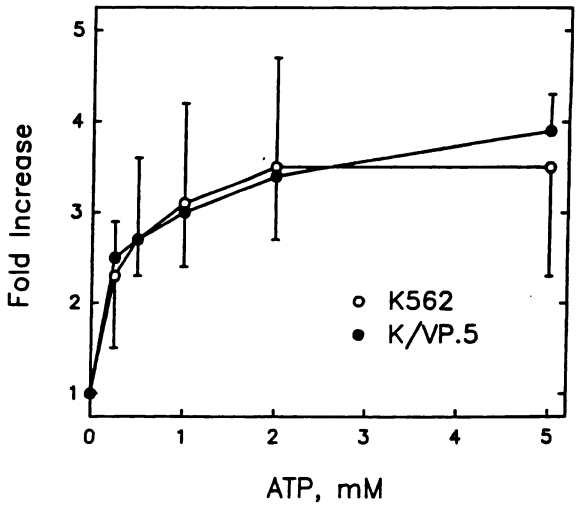

Fig. 5. ATP stimulation of VP-16-induced covalent binding of purified topoisomerase II to pBR322 DNA. Purified topoisomerase II from K562 and K/VP.5 cells was incubated with ^{32}P-3′-end-labeled pBR322 DNA (0.2 μg) for 30 min at 37°, in the presence of 100 μM VP-16 and 0–5 mM ATP. Bound DNA was isolated by KCl/SDS precipitation and quantitated by scintillation counting. Results are expressed as fold increase, relative to complexes isolated in the absence of ATP. *Points* represent the mean ± standard error from three independent experiments.

K562 cells, 46.6 ± 4.6% were in G_1 phase, 43.8 ± 2.8% were in S phase, and 9.6 ± 3.6% were in G_2/M phase (mean ± standard error from four experiments). Similarly, mid-logarithmic phase K/VP.5 cells were 41.7 ± 10.5% in G_1 phase, 48.5 ± 8.2% in S phase, and 9.8 ± 2.7% in G_2/M phase (mean ± standard error from four experiments). Thus, differences in the levels of topoisomerase II and ATP stimulation of drug-induced binding of topoisomerase II to DNA were not attributable to a change in cell cycle distribution. Taken together, the results described above indicate that acquired resistance to VP-16 in K/VP.5 cells is stable and is associated with a qualitative alteration in topoisomerase II function as well as a quantitative reduction of enzyme expression.

Sequence analysis of topoisomerase II cDNA from K562 and K/VP.5 cells. The altered ATP dependency for VP-16-induced topoisomerase II/DNA binding in K/VP.5 nuclear extracts and the stability of VP-16 resistance in these cells suggested the presence of a mutation in a functional domain of topoisomerase II, such as the active site or an ATP-binding consensus sequence in the resistant cell topoisomerase II gene. Using single-strand DNA templates from asymmetric PCR, we sequenced K562 and K/VP.5 topoisomerase II cDNA in regions that include the ATP-binding consensus sequences of motif B (nucleotides 1345–1380) and DNBS (nucleotides 1396–1482). Fig. 6A indicates that no mutation is present in K/VP.5 topoisomerase II cDNA at nucleotide 1346, where a guanine to adenine base change was demonstrated in VM-26-selected resistant CCRF-CEM cells (19). Fig. 6B indicates that no mutation is present in K/VP.5 topoisomerase II cDNA at nucleotide 1457, where a guanine to adenine base change was demonstrated in mAMSA-selected resistant HL-60 cells (20). Other mutations in topoisomerase II cDNA have been reported in or near ATP-binding consensus sequences in a VP-16-resistant Chinese hamster ovary cell line (nucleotide 1478, within the DNBS) (22) and mitoxantrone-resistant Chinese hamster ovary cells (nucleotide 1277) (23). However, no mutations were observed in K/VP.5 topoisomerase cDNA in the

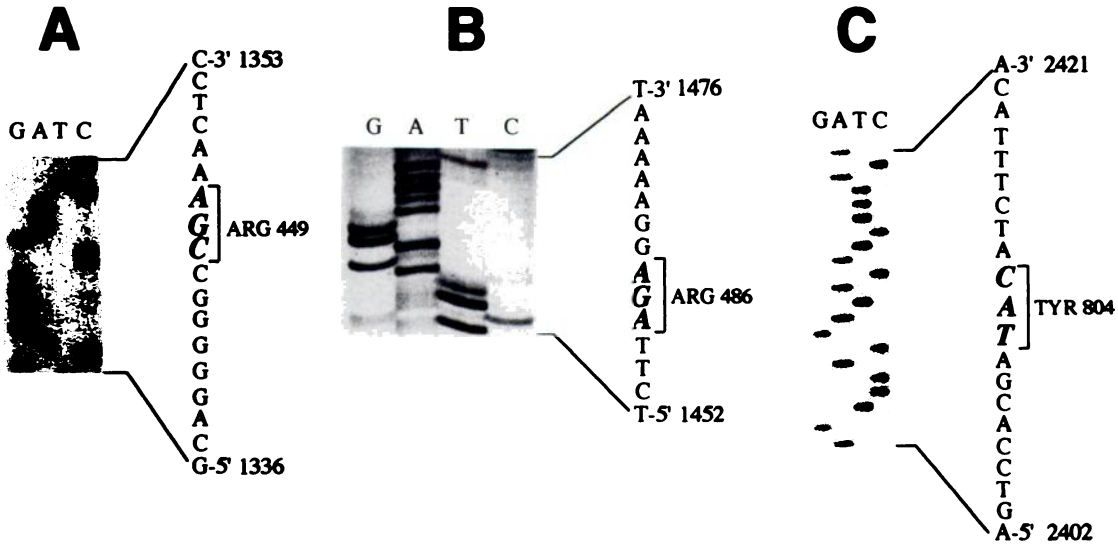

Fig. 6. Sequence analysis of nucleotide binding motif B (A), DNBS (B), and the active site tyrosine-804 region (C) of K/VP.5 topoisomerase II cDNA. Single-strand DNA templates (about 0.4 pmol) were obtained by asymmetric PCR of K/VP.5 topoisomerase II cDNA and were used as templates for dideoxynucleotide chain termination sequencing reactions. For the nucleotide sequence 1336–1353 (A), the oligonucleotide 5′-1033–1052–3′ was used to prime sequencing reactions. For the nucleotide sequence 1452–1476 (B), the oligonucleotide 5′-1312–1331–3′ was used. For the nucleotide sequence 2402–2421 (C), the oligonucleotide 5′-2518–2498–3′ was used. The arginine codons indicated are codons previously reported to be mutated in VM-26-resistant (A) or mAMSA-resistant (B) cells (18–20). The tyrosine codon indicated (C) is the active site tyrosine-804 (37).

region of nucleotides 1093–1602 (data not shown). Sequence information was also obtained for nucleotides 2239–2541, a region that includes the active site tyrosine codon (nucleotides 2246–2248) (Fig. 6C). The sequence of this >300-bp region of topoisomerase II cDNA was identical to that previously reported for HeLa cell topoisomerase II (37).

RF/SSCP analysis of topoisomerase II cDNA from K562 and K/VP.5 cells. SSCP analysis of PCR-derived cDNA has previously been used to identify point mutations in topoisomerase II cDNA from drug-resistant cell lines (18). We developed a modification of the SSCP technique to rapidly screen for mutations throughout the entire coding region of topoisomerase II from K562 and K/VP.5 cells. Large PCR fragments (usually 0.8–1.3 kb) first were digested with restriction enzymes to produce fragments of <400 bp (see Fig. 7) and then were denatured, and the single-strand DNA fragments were electrophoresed.

To ensure that the electrophoretic conditions used were appropriate for detecting point mutations, we performed RF/SSCP analysis using not only K562 and K/VP.5 topoisomerase II cDNA but also cDNA from HL-60 cells and mAMSA-resistant HL-60 (HL-60/AMSA) cells (20). The point mutation in the nucleotide-binding motif DNBS (nucleotide 1457) identified by sequencing of HL-60/AMSA topoisomerase II cDNA (20) has been shown by others to be readily detectable by standard SSCP analysis (18). The electrophoresis conditions used for RF/SSCP analysis in this study also allowed the reproducible detection of this point mutation in a 359-bp PCR topoisomerase II fragment from HL-60/AMSA cells (Fig. 7A, lanes A from duplicate samples). There was no mutation detectable by SSCP analysis in fragment A from K/VP.5 (Fig. 7, *lanes V*), compared with K562 (Fig. 7, *lanes K*), topoisomerase II cDNA, consistent with the sequencing data obtained for these fragments (see above). In another experiment, a 541-bp fragment was synthesized by PCR and digested with *Pvu*II to produce two fragments, both of <400 bp (Fig. 7A, fragments B and C). The HL-60/AMSA point mutation at nucleotide 1457 was detectable in fragment C, i.e., 3′ to the *Pvu*II cleavage site at nucleotide 1282 (Fig. 7A, fragment C). No mutation was detected in K/VP.5 cDNA, compared with K562 cDNA, in either fragment B or C. For Fig. 7B a larger, 1.1-kb, topoisomerase II cDNA fragment was restriction enzyme digested to yield four fragments of <400 bp. Fragments A and B of Fig. 7B, which overlap fragments A and C of Fig. 7A, also showed no evidence of mutation, as determined by their identical electrophoretic mobilities. RF/SSCP analysis of PCR products spanning the entire cDNA of K/VP.5 and K562 cell topoisomerase II revealed no evidence of mutations in the coding sequences (data not shown). These results suggest that a putative mutation or mutations contributing to stable VP-16 resistance most likely resides in a gene distinct from topoisomerase II whose product may serve to regulate topoisomerase II function in the presence of inhibitory drugs such as VP-16. Sequence analysis of K/VP.5 topoisomerase II cDNA is underway and will definitively reveal whether a point mutation in K/VP.5 cell topoisomerase II is related to the qualitative and/or quantitative topoisomerase II alterations documented previously and in the present work.

Effects of nuclear extract protein on VP-16-induced topoisomerase II/DNA complexes in K562 and K/VP.5 nuclei. In the absence of added nuclear extract, VP-16-induced topoisomerase II/DNA complex formation is reduced 4-fold in K/VP.5 nuclei, compared with K562 nuclei (Fig. 4) (16). After normalization of VP-16 effects in K562 and K/VP.5 nuclei, addition of nuclear extract (10–100 μg) from K562 cells potentiated the formation of VP-16 (20 μM)-induced topoisomerase II/DNA complexes in K/VP.5 but not K562 nuclei (Fig. 8A). After K562 nuclear extracts were heated for 10 min at 65°, the VP-16-potentiating activity was eliminated (data not shown). In contrast, nuclear extract from K/VP.5 cells had no effect on drug-induced topoisomerase II/DNA complex formation in nuclei from either cell line (Fig. 8B). These data suggest that K562 nuclear extracts contain a modulating factor for VP-16-

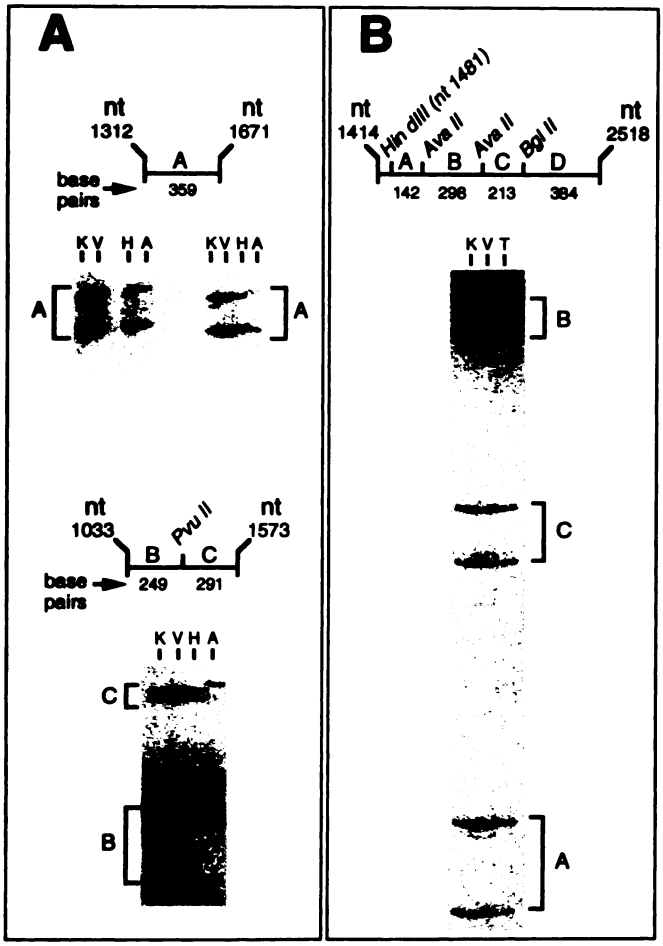

Fig. 7. SSCP and RF/SSCP analysis of consensus ATP binding sites (motif B and DNBS) of topoisomerase II cDNA from K562 (*lanes K*), K/VP.5 (*lanes V*), HL-60 (*lanes H*), HL-60/AMSA (*lanes A*), or HeLa (*lane T*) cells. cDNA synthesized from total RNA by reverse transcription (*lanes K, V, H,* and *A*) or HeLa topoisomerase II cDNA cloned in a plasmid (*lane T*) was amplified by PCR in the presence of [α-^{32}P]dCTP. The positions of the restriction sites used to obtain fragments B and C (A, *bottom*) and fragments A-C (B) are indicated above the linear maps. The sizes of fragments A, B, and C (A and B) are shown below the linear maps. Single-strand DNA fragments were resolved by electrophoresis (600 V) for 4–6 hr. HL-60/AMSA cDNA (A, *lanes A*) harbors a previously identified point mutation at nucleotide 1457 (18, 20) that is detectable by SSCP analysis of fragment A (A, *top*, duplicate experiments shown) or RF/SSCP analysis of fragment C (A, *bottom*).

induced topoisomerase II/DNA binding that is altered and/or limiting in K/VP.5 cells.

Phosphorylation of topoisomerase II in K562 and K/VP.5 cells. Topoisomerase II was immunoprecipitated from lysates of [^{35}S]methionine- and $^{32}P_i$-labeled cells and nuclei and was resolved by SDS-PAGE (Fig. 9). The single 170-kDa band was excised from the gel and quantitated by scintillation counting. To account for the different levels of topoisomerase II in K562 cells, compared with K/VP.5 cells, relative levels of phosphorylation of topoisomerase II were determined by normalizing the ^{32}P cpm to ^{35}S cpm. The results revealed a 2.59 ± 0.48-fold reduction in phosphorylation of K/VP.5, compared with K562, topoisomerase II from whole-cell lysates (mean ± standard error of six determinations; $p = 0.007$, paired Student's t test). Similarly, phosphorylation of topoisomerase II from K/VP.5 nuclear lysates was reduced 2.29 ± 0.35-fold, compared with K562 (mean ± standard error of six determinations; $p = 0.003$, paired Student's t test).

Discussion

Results presented in this study indicate that acquired resistance to VP-16 in K/VP.5 cells is stable and is associated with qualitative alterations in topoisomerase II function. Reduced ATP stimulation of VP-16-induced topoisomerase II/DNA binding in K/VP.5 cells was also observed in resistant cells maintained for almost 2 years in the absence of VP-16 (Fig. 3). These results suggest that resistance to VP-16 in K/VP.5 cells may be due to a mutation that affects drug-induced topoisomerase II binding to DNA. The 4-fold decrease in ATP stimulation of VP-16-induced topoisomerase II/DNA binding in K/VP.5 nuclear extracts, compared with K562 nuclear extracts (Fig. 4), is comparable to the 2–5-fold reduction of VP-16-induced DNA damage and topoisomerase II/DNA covalent complex formation observed in nuclei from resistant cells (Figs. 2 and 3). In contrast, the differential ATP stimulation of drug-induced topoisomerase II/DNA binding in sensitive versus resistant cells was no longer present after purification of topoisomerase II from these cells (Fig. 5), suggesting that a stabilizing or modulating factor for topoisomerase II from K562 cells was lost during enzyme purification. Consistent with this hypothesis, casein kinase II has recently been shown to bind to and phosphorylate topoisomerase II in yeast (41). It is possible that casein kinase II or another protein kinase utilizing ATP as a substrate is responsible for the changes in VP-16-induced topoisomerase II/DNA binding observed in K/VP.5 cells, compared with K562 cells.

Although the stability of resistance and the altered ATP stimulation of VP-16-induced topoisomerase II/DNA binding in K/VP.5 cells suggested mutational alteration(s) in topoisomerase II, sequencing of consensus ATP binding sites B and DNBS, the active site tyrosine region, and RF/SSCP analysis of the entire topoisomerase II cDNA revealed no mutations in the gene for this enzyme (Figs. 6 and 7). Together with the loss of differential ATP stimulation of drug-induced topoisomerase II/DNA binding using purified enzyme (Fig. 5), these results support the idea that a modulating factor for topoisomerase II, as opposed to the topoisomerase II enzyme itself, has been functionally altered or reduced in resistant K/VP.5 cells, compared with K562 cells.

The potentiation of VP-16-induced topoisomerase II/DNA complexes in K/VP.5 nuclei using K562 nuclear extracts (Fig. 8A) further supports the presence of a topoisomerase II-modulating factor in nuclear extracts. Potentiation of drug-induced topoisomerase II/DNA binding did not occur with the addition of K562 nuclear extract to K562 nuclei (Fig. 8A), suggesting that this effect was not merely due to the addition of more topoisomerase II. Furthermore, K/VP.5 nuclear extract had no effect on VP-16-induced topoisomerase II/DNA binding in either K562 or K/VP.5 nuclei (Fig. 8B), ruling out the presence of a more active phosphatase in K/VP.5 nuclear extracts. Supporting this conclusion, preliminary experiments performed in this laboratory indicate that the rates of dephosphorylation of topoisomerase II are identical for K562 and K/VP.5 topoisomerase II (data not shown). Finally, the heat-labile nature of the potentiating activity of K562 nuclear extracts suggests that this factor is a protein.

After accounting for the reduced levels of topoisomerase II in resistant cells, enzyme immunoprecipitated from K/VP.5 cells and nuclei was found to be 2.5-fold less phosphorylated, compared with enzyme from K562 cells (Fig. 9). These results contrast with those reported by Takano *et al.* (33), who showed

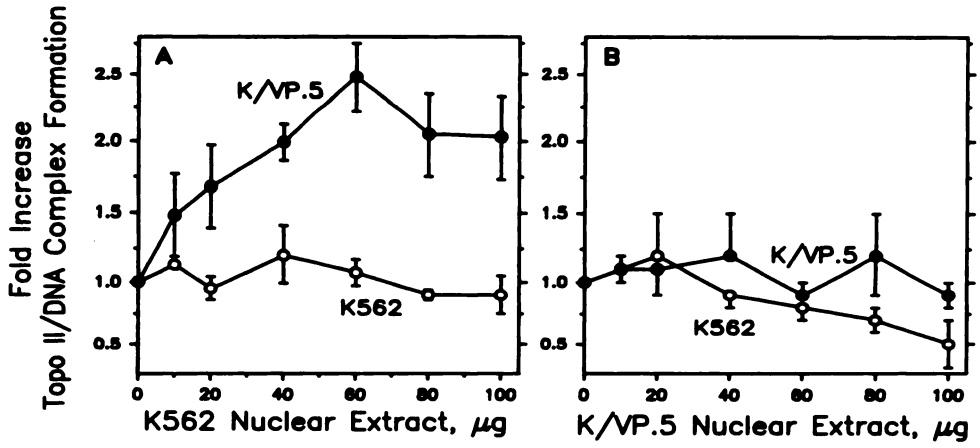

Fig. 8. Effects of nuclear extract protein on VP-16-induced topoisomerase II/DNA covalent complexes in K562 and K/VP.5 nuclei. Nuclei were isolated from K562 or K/VP.5 cells that had been prelabeled for 24 hr with [*methyl*-^{3}H]thymidine and [^{14}C]leucine. K562 (A) or K/VP.5 (B) nuclear extract (10–100 μg) was added to 1 × 10^6 K562 or K/VP.5 nuclei, in the presence of 20 μM VP-16 and 1 mM ATP, and incubated at 37° for 20 min. VP-16-induced covalent topoisomerase II/DNA covalent complexes were isolated by KCl/SDS precipitation and quantitated by scintillation counting. The results are expressed as fold increase of topoisomerase II/DNA (*Topo II/DNA*) covalent complexes formed in the presence and absence of nuclear extract. *Points* represent the mean ± standard error from three to 10 experiments.

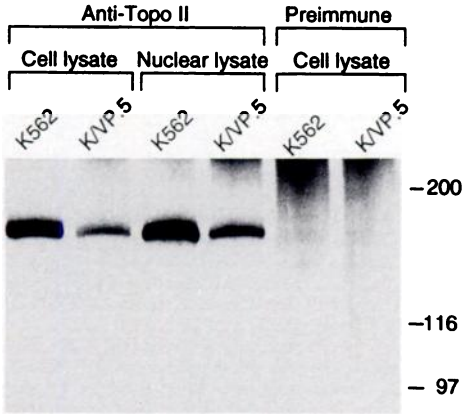

Fig. 9. Phosphorylation of topoisomerase II from K562 and K/VP.5 cells. Topoisomerase II (*Topo II*) was immunoprecipitated from whole-cell or nuclear lysates (100 μg of protein) from K562 and K/VP.5 cells that had been prelabeled for 4 hr with [^{35}S]methionine and $^{32}P_i$. Immunoprecipitated topoisomerase II was resolved on SDS-PAGE gels, localized by autoradiography, excised from gels, and quantitated by scintillation counting. *Numbers to the right*, positions of molecular weight markers.

that topoisomerase II immunoprecipitated from VP-16-resistant KB cells was hyperphosphorylated. Conversely, Ganapathi *et al.* (34) showed that the rates of both synthesis and phosphorylation of topoisomerase II from mAMSA-resistant HL-60/AMSA cells were 2–3-fold slower, compared with parental HL-60 cells. Therefore, it is not clear to what extent topoisomerase II phosphorylation is a determinant of drug sensitivity.

Our results demonstrating reduced ATP stimulation of VP-16-induced topoisomerase II/DNA binding in association with a less phosphorylated enzyme in drug-resistant K/VP.5 cells suggest that a kinase may be responsible for altered topoisomerase II activity in resistant K/VP.5 cells. The importance of a topoisomerase II-associated kinase has been suggested by several studies. First, phosphorylation of topoisomerase II increases as cells approach the G_2/M phase of the cell cycle, where topoisomerase II levels and activities peak (28). Second, *in vitro* phosphorylation of topoisomerase II with casein kinase II or protein kinase C increases the catalytic activity of the enzyme by increasing the rate of hydrolysis of ATP, thus escalating enzyme turnover (30–32). Third, a kinase activity having characteristics of casein kinase II copurifies with topoisomerase II (29, 30). Fourth, casein kinase II and topoisomerase II in yeast have been shown to form a stable complex that increases topoisomerase II phosphorylation and enhances catalytic activity 2–3-fold (41). These observations suggest that kinase-mediated phosphorylation of topoisomerase II is an important mechanism for regulating enzyme activity.

It has been proposed that the carboxyl terminus of topoisomerase II, where *in vivo* phosphorylation sites have been localized (26), is a conformation-dependent negative regulatory domain that is neutralized by phosphorylation (42). The negative effects of this domain are presumably exerted through a conformational state that ultimately affects interaction of the enzyme with DNA (30, 42). Therefore, the decrease in drug-induced topoisomerase II/DNA binding in K/VP.5 cells could be attributable to destabilizing effects on the drug-induced topoisomerase II/DNA complex, which in turn may result from the failure of a kinase to phosphorylate topoisomerase II and neutralize these negative domains. Consistent with this hypothesis, we have shown that VP-16-induced covalent protein/DNA complexes are less stable (i.e., disappear more rapidly after drug removal) in K/VP.5, compared with K562, nuclei (16).

We conclude that the qualitative and quantitative changes in topoisomerase II in VP-16-resistant K/VP.5 cells are most likely the result of an alteration in a kinase, resulting in decreased phosphorylation of the enzyme. Studies are underway to identify and characterize this putative kinase. The net effects of a hypophosphorylated topoisomerase II could be expected to have an impact on 1) the levels of the enzyme (by affecting its post-translational stability), 2) the ATP-dependent activity of the enzyme, and/or 3) the stabilization of drug-induced enzyme binding to DNA. Our studies indicate that post-translational modification of topoisomerase II may represent an important determinant for acquired resistance to topoisomerase II-inhibitory drugs.

Acknowledgments

The authors thank Dr. Guillermo G. Romero for assistance in developing immunoprecipitation protocols and Dr. Jay D. Hunt III for helpful advice concerning PCR techniques. Thanks are also due to Dr. Leonard A. Zwelling for

providing purified HL-60 and HL-60/AMSA RNA and to Dr. Mary K. Danks for constructive suggestions regarding the SSCP procedure.

References

1. Liu, L. F. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* **58**:351–375 (1989).
2. Corbett, A. H., and N. Osheroff. When good enzymes go bad: conversion of topoisomerase II to a cellular toxin by antineoplastic drugs. *Chem. Res. Toxicol.* **6**:585–597 (1993).
3. Kaufmann, S. H. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. *Cancer Res.* **49**:5870–5878 (1989).
4. Walker, P. R., C. Smith, T. Youdale, J. Leblanc, J. F. Whitfield, and M. Sikorska. Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res.* **51**:1078–1085 (1991).
5. Rubin, E., S. Kharbanda, H. Gunji, and D. Kufe. Activation of the C-*jun* protooncogene in human myeloid leukemia cells treated with etoposide. *Molec. Pharmacol.* **39**: 697–701 (1991).
6. Drake, F. H., J. P. Zimmerman, F. L. McCabe, H. F. Bastus, S. R. Per, D. M. Sullivan, W. E. Ross, M. R. Mattern, R. K. Johnson, S. T. Crooke, and C. K. Mirabelli. Purification of topoisomerase II from amsacrine-resistant P388 leukemia cells. *J. Biol. Chem.* **262**:16739–16747 (1987).
7. Webb, C. D., M. D. Latham, R. B. Lock, and D. M. Sullivan. Attenuated topoisomerase II content directly correlates with a low level of drug resistance in a Chinese hamster ovary cell line. *Cancer Res.* **51**:6542–6549 (1991).
8. Cole, S. P. C., E. R. Chanda, F. P. Dicke, J. H. Gerlach, and S. E. L. Mirski. Non-P-glycoprotein-mediated multidrug resistance in a small cell lung cancer cell line: evidence for decreased susceptibility to drug-induced DNA damage and reduced levels of topoisomerase II. *Cancer Res.* **51**:3345–3352 (1991).
9. Long, B. H., L. Wang, A. Lorico, R. C. C. Wang, M. G. Brattain, and A. M. Casazza. Mechanisms of resistance to etoposide and teniposide in acquired resistant human colon and lung carcinoma cell lines. *Cancer Res.* **51**:5275–5284 (1991).
10. Ritke, M. K., and J. C. Yalowich. Altered gene expression in human leukemia K562 cells selected for resistance to etoposide. *Biochem. Pharmacol.* **46**:2007–2020 (1993).
11. Danks, M. K., C. A. Schmidt, M. C. Cirtain, D. P. Suttle, and W. T. Beck. Altered catalytic activity of and DNA cleavage by DNA topoisomerase II from human leukemic cells selected for resistance to VM-26. *Biochemistry* **27**:8861–8869 (1988).
12. Sullivan, D. M., M. D. Latham, T. C. Rowe, and W. E. Ross. Purification and characterization of an altered topoisomerase II from a drug-resistant Chinese hamster ovary cell line. *Biochemistry* **28**:5680–5687 (1989).
13. Zwelling, L. A., M. Hinds, D. Chan, J. Mayes, K. Lan Sie, E. Parker, L. Silberman, A. Radcliffe, M. Beran, and M. Blick. Characterization of an amsacrine-resistant line of human leukemia cells: evidence for a drug-resistant form of topoisomerase II. *J. Biol. Chem.* **264**:16411–16420 (1989).
14. Sullivan, D. M., L. Eakildsen, K. R. Groom, C. D. Webb, M. D. Latham, A. W. Martin, S. R. Wellhausen, P. E. Kroeger, and T. C. Rowe. Topoisomerase II activity involved in cleaving DNA into topological domains is altered in a multiple drug-resistant Chinese hamster ovary cell line. *Mol. Pharmacol.* **43**:207–216 (1993).
15. Campain, J. A., R. Padmanabhan, J. Hwang, M. M. Gottesman, and I. Pastan. Characterization of an unusual mutant of human melanoma cells resistant to anticancer drugs that inhibit toposiomerase II. *J. Cell. Physiol.* **155**:414–425 (1993).
16. Ritke, M. K., D. Roberts, W. P. Allan, J. Raymond, V. V. Bergoltz, and J. C. Yalowich. Altered stability of etoposide-induced topoisomerase II/DNA complexes in resistant human leukemia cells. *Br. J. Cancer* **69**:687–697 (1994).
17. Osheroff, N. Eukaryotic topoisomerase II: characterization of enzyme turnover. *J. Biol. Chem.* **261**:9944–9950 (1986).
18. Danks, M. K., M. R. Warmoth, E. Friche, B. Granzen, B. Y. Bugg, W. G. Harker, L. A. Zwelling, B. W. Futscher, D. P. Suttle, and W. T. Beck. Single-strand conformational polymorphism analysis of the M_r 170,000 isozyme of DNA topoisomerase II in human tumor cells. *Cancer Res.* **53**:1373–1379 (1993).
19. Bugg, B. Y., M. K. Danks, W. T. Beck, and D. P. Suttle. Expression of a mutant DNA topoisomerase II in CCRF-CEM human leukemic cells selected for resistance to teniposide. *Proc. Natl. Acad. Sci. USA* **88**:7634–7638 (1991).
20. Hinds, M., K. Diesseroth, J. Mayes, E. Altschuler, R. Jansen, F. D. Ledley, and L. A. Zwelling. Identification of a point mutation in the topoisomerase II gene from a human leukemia cell line containing an amsacrine-resistant form of topoisomerase II. *Cancer Res.* **51**:4729–4731 (1991).
21. Lee, M.-S., J. C. Wang, and M. Beran. Two independent amsacrine-resistant human myeloid leukemia cell lines share an identical point mutation in the 170-kDa form of human topoisomerase II. *J. Mol. Biol.* **223**:837–843 (1992).
22. Chan, V. T. W., S. W. Ng, J. P. Eder, and L. E. Schnipper. Molecular cloning and identification of a point mutation in the topoisomerase II cDNA from an etoposide-resistant Chinese hamster ovary cell line. *J. Biol. Chem.* **268**:2160–2165 (1993).
23. Rizvi, N. A., S. W. Ng, D. W. Sullivan, J. P. Eder, L. E. Schnipper, and V. T. W. Chan. Identification of a point mutation in topoisomerase II (TOPII) cDNA in a mitoxantrone-resistant Chinese hamster ovary (CHO) cell line, MXN_4. *Proc. Am. Assoc. Cancer Res.* **34**:1984 (1993).
24. Patel, S., and L. M. Fisher. Novel selection and genetic characterisation of an etoposide-resistant human leukaemic CCRF-CEM cell line. *Br. J. Cancer* **67**:456–463 (1993).
25. Jannatipour, M., Y.-X. Liu, and J. L. Nitiss. The *top2-5* mutant of yeast topoisomerase II encodes an enzyme resistant to etoposide and amsacrine. *J. Biol. Chem.* **268**:18586–18592 (1993).
26. Cardenas, M. E., Q. Dang, C. V. C. Glover, and S. M. Gasser. Casein kinase II phosphorylates the eukaryotic-specific C-terminal domain of topoisomerase II *in vivo*. *EMBO J.* **11**:1785–1796 (1992).
27. Kroll, D. J., and T. C. Rowe. Phosphorylation of DNA topoisomerase II in a human tumor cell line. *J. Biol. Chem.* **266**:7957–7961 (1991).
28. Saijo, M., M. Ui, and T. Enomoto. Growth state and cell cycle dependent phosphorylation of DNA topoisomerase II in Swiss 3T3 cells. *Biochemistry* **31**:359–363 (1992).
29. Saijo, M., T. Enomoto, F. Hanaoka, and M. Ui. Purification and characterization of type II DNA topoisomerase from mouse FM3A cells: phosphorylation of topoisomerase II and modification of its activity. *Biochemistry* **29**:583–590 (1990).
30. Cardenas, M. E., R. Walter, D. Hanna, and S. M. Gasser. Casein kinase II copurifies with yeast topoisomerase II and restores decatenation activity. *J. Cell Sci.* **104**:533–543 (1993).
31. Corbett, A. H., R. F. DeVore, and N. Osheroff. Effect of casein kinase II-mediated phosphorylation on the catalytic cycle of topoisomerase II: regulation of enzyme activity by enhancement of ATP hydrolysis. *J. Biol. Chem.* **267**:20513–20518 (1992).
32. Corbett, A. H., A. W. Fernald, and N. Osheroff. Protein kinase C modulates the catalytic activity of topoisomerase II by enhancing the rate of ATP hydrolysis: evidence for a common mechanism of regulation by phosphorylation. *Biochemistry* **32**:2090–2097 (1993).
33. Takano, H., K. Kohno, M. Ono, Y. Uchida, and M. Kuwano. Increased phosphorylation of DNA topoisomerase II in etoposide-resistant mutants of human cancer KB cells. *Cancer Res.* **51**:3951–3957 (1991).
34. Ganapathi, R., L. Zwelling, A. Constantinou, J. Ford, and D. Grabowski. Altered phosphorylation, biosynthesis and degradation of the 170-kDa isoform of topoisomerase II in amsacrine-resistant human leukemia cells. *Biochem. Biophys. Res. Commun.* **192**:1274–1280 (1993).
35. Filipski, J., and K. W. Kohn. Ellipticine-induced protein-associated DNA breaks in isolated L1210 nuclei. *Biochim. Biophys. Acta* **698**:280–286 (1982).
36. Kohn, K. W., L. C. Erickson, R. A. G. Ewig, and C. A. Friedman. Fractionation of DNA from mammalian cells by alkaline elution. *Biochemistry* **15**:4629–4637 (1976).
37. Tsai-Pflugfelder, M., L. F. Liu, A. A. Liu, K. M. Tewey, J. Whang-Peng, T. Knutsen, K. Huebner, C. M. Croce, and J. C. Wang. Cloning and sequencing of cDNA encoding human DNA topoisomerase II and localization of the gene to chromosome region 17q21–22. *Proc. Natl. Acad. Sci. USA* **85**:7177–7181 (1988).
38. Gyllenstein, U. B., and H. A. Erlich. Generation of single stranded DNA by the polymerase chain reation and its application to direct sequencing of the HLA-DQ*alpha* locus. *Proc. Natl. Acad. Sci. USA* **85**:7652–7656 (1988).
39. Hwang, J., S. Shyy, A. Y. Chen, C.-C. Juan, and J. Whang-Peng. Studies of topoisomerase-specific antitumor drugs in human lymphocytes using rabbit antisera against recombinant human topoisomerase II polypeptide. *Cancer Res.* **49**:958–962 (1989).
40. Fry, D. W. Cytotoxic synergism between trimetrexate and etoposide. *Biochem. Pharmacol.* **40**:1981–1988 (1990).
41. Bojanowski, K., O. Filhol, C. Cochet, E. M. Chambaz, and A. K. Larsen. Topoisomerase II and casein kinase II associate in a molecular complex that is catalytically active. *J. Biol. Chem.* **268**:22920–22926 (1993).
42. Cardenas, M. E., and S. M. Gasser. Regulation of topoisomerase II by phosphorylation: a role for casein kinase II. *J. Cell Sci.* **104**:219–225 (1993).

Send reprint requests to: Jack C. Yalowich, Department of Pharmacology, University of Pittsburgh School of Medicine, W1308 Biomedical Science Tower, Pittsburgh, PA 15261.