Mutations of Human Topoisomerase IIα Affecting Multidrug Resistance and Sensitivity†

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ABSTRACT: Two mutations, R450Q and P803S, in the coding region of the human topoisomerase IIα gene have been identified in the atypical multidrug resistant (at-MDR) cell line, CEM/VM-1, which exhibits resistance to many structurally diverse topoisomerase II-targeting antitumor drugs such as VM-26, doxorubicin, m-AMSA, and mitoxantrone. The R450Q mutation mapped in the ATP utilization domain, while the P803S mutation mapped in the vicinity of the active site tyrosine of human topoisomerase IIα. However, the roles of these two mutations in conferring multidrug resistance are unclear. To study the roles of these two mutations in conferring multidrug resistance, we have characterized the recombinant human DNA topoisomerase IIα containing either single or double mutations. We show that both R450Q and P803S mutations confer resistance in the absence of ATP. However, in the presence of ATP, the R450Q, but not the P803S, mutation can confer multidrug resistance. The R450Q enzyme was shown to exhibit impaired ATP utilization both for enzyme catalysis and for its ability to form the circular protein clamp. Interestingly, an unrelated mutation, G437E, which is also located in the same domain as the R450Q mutation, exhibited multidrug hypersensitivity in the absence of ATP. However, in the presence of ATP, the G437E enzyme is only minimally hypersensitive to various topoisomerase II drugs. In contrast to the R450Q enzyme, the G437E enzyme exhibited enhanced ATP utilization for enzyme catalysis. In the aggregate, these results support the notion that the multidrug resistance and sensitivity of these mutant enzymes are due to a specific defect in ATP utilization during enzyme catalysis.

Human type II DNA topoisomerases catalyze an ATP-dependent strand-passing reaction (*1*−*3*). It does so by transiently breaking one of the two crossing DNA double helices (*4*, *5*). Coupled to transient DNA cleavage, each subunit of the topoisomerase II (TOP2) homodimer is covalently linked to the 5′-phosphoryl ends of the transiently broken DNA strands (*5*). The crossing DNA segment is thought to be transported through the transient break and the entire protein−protein interface (*6*). Two type II DNA topoisomerases, hTOP2α and hTOP2*β*, have been identified and characterized in human cells (*7*−*9*). hTOP2α is a cell proliferation marker, with its highest level shown in the late S and G2/M phase and its level dramatically reduced in the G1/G0 phase of the cell cycle. hTOP2*β*, on the other hand, experiences little fluctuation during the cell cycle and is present in both proliferating and quiescent cells (*10*, *11*). hTOP2α is involved in DNA replication (*12*, *13*), and chromosome condensation and segregation (*14*−*19*). Inhibition of TOP2 activity by ICRF193 can cause M-phase arrest of yeast and mammalian cells, consistent with a proposed role of TOP2 as a major component of mitotic protein scaffold (*20*).

Both human TOP2α and TOP2*β* have been shown to be molecular targets for many structurally diverse antitumor drugs such as etoposide (VP-16), teniposide (VM-26), adriamycin, mitoxantrone, and mAMSA (*21*, *22*). These anticancer drugs are known to abort the cleavage/religation cycles of TOP2, resulting in stimulation of the covalent cleavage intermediate, the reversible cleavable complex (*23*).

A human leukemic cell line selected for resistance to VM-26 (CCRF-CEM/VM-1) confers an "atypical" multidrug resistance phenotype (*24*). This cell line is only cross-resistant to TOP2 poisons that stabilize the reversible cleavable complexes (*25*). Nuclear extracts isolated from the resistant cells exhibited reduced TOP2 catalytic activity and a reduced capacity to form cleavable complexes in the presence of VM-26 (*25*). Two point mutations have been identified and mapped to R450Q and P803S on the hTOP2α gene in this resistant cell line (*26*, *27*). The R450Q mutation is located in the B′ domain (*28*), while the P803S mutation is located only two amino acids away from the active tyrosine (Y805). It is unclear whether both mutations are present in the same

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allele or in separate alleles. The roles of these two mutations in conferring the multidrug resistant phenotype are also unclear. However, recent studies in a yeast system have demonstrated that both mutations appear to be required for maximum resistance to multiple TOP2 drugs (*29*).

To study the roles of these two mutations in conferring multidrug resistance, we have constructed hTOP2α cDNA clones with either single or double mutations in a yeast expression system. Using partially purified recombinant mutant hTOP2α, we have characterized their catalytic activity, cleavage activity, and protein clamp forming activity in vitro. We showed that in the absence of ATP, the VM-26-induced DNA cleavage activity of mutant hTOP2α with either a single (R450Q or P803S) or double (R450Q/P803S) mutation was greatly reduced. However, in the presence of ATP, only the R450Q mutation is responsible for conferring multidrug resistance to drug-induced DNA cleavage. The P803S mutation had no effect on drug-induced DNA cleavage in the presence of ATP. This ATP effect of the R450Q mutation was also mirrored by impaired ATP utilization for enzyme catalysis and the formation of protein circular clamp. To further characterize the relationship between the mutation in the B′ domain and drug resistance, we also created another mutation G437E which is 13 amino acids away from R450Q in a "Walker consensus sequence" (*30*). Surprisingly, in the absence of ATP, this mutation confers multidrug hypersensitivity rather than multidrug resistance in the drug-stimulated DNA cleavage assay. In contrast to the R450Q mutation, the G437 mutation exhibits more efficient ATP utilization for enzyme catalysis.

MATERIALS AND METHODS

Materials. The yeast strain BCY123 (MATa *pep4*::HIS3 *prb1*::LEU2 *bar1*::HISG *lys*::GAL1/10-GAL4 *can1 ade2 trp1 ura3 his3 leu2-3,112*) and the plasmid YEpWob6 containing the wild-type hTOP2α cDNA were obtained from J. C. Wang (Harvard University, Cambridge, MA). Plasmids pMJ1/R450Q and pMJ1/P803S containing R450Q and P803S mutations, respectively, were constructed as described previously (*29*). CEM and CEM/VM-1 cell lines were obtained from W. Beck (Cancer Center, University of Illinois at Chicago, Chicago, IL). P4 DNA was isolated as described previously (*31*, *32*). VM-26 (teniposide) was obtained from Bristol-Myers Squibb Co. ICRF193 was obtained from T. Andoh (Soka University, Tokyo, Japan).

P4 Unknotting Assay. The TOP2 catalytic activity was measured by the P4 unknotting assay using knotted P4 DNA as described previously (*32*, *33*). The TOP2 enzymatic activity was estimated by 2-fold serial dilution of the enzyme. For each reaction (25 μL), 200 ng of P4 DNA was incubated with the enzyme in the reaction buffer [40 mM Tris (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, and 30 μg/mL BSA]. After incubation at 37 °C for 30 min, each reaction was terminated by addition of 5 μL of a stop solution (5% sucrose, 10 mM EDTA, 1% sarkosyl, and 0.05% bromophenol blue) and then the mixture loaded onto a 0.8% agarose gel. The electrophoresis was carried out at room temperature for 4 h at 1.6 V/cm in TPE buffer (0.09 M Tris-phosphate and 0.002 M EDTA). The gel was viewed under UV light after ethidium bromide staining.

TOP2-Mediated DNA Cleavage Assay. The TOP2-mediated DNA cleavage assay using ^{32}P end-labeled linear YEpG plasmid DNA was performed as described previously (*34*). Unless otherwise indicated, 1 mM ATP was included in all cleavage assays.

Construction of Mutant hTOP2α Overexpression Plasmids. To construct plasmids overexpressing mutant hTOP2α, the R450Q and the P803S mutations in plasmids pMJ1/R→Q and pMJ1/P→S (*29*), respectively, were swapped into the hTOP2α cDNA in YEpWob6 (*35*). Briefly, the fragment containing the R450Q mutation was excised from pMJ1/R→Q by digestion with *Nde*I and *Kpn*I. The fragment was then used to replace the corresponding hTOP2α cDNA fragment in YEpWob6, resulting in YEpM1. The P803S mutation was similarly introduced from a *Nde*I−*Kpn*I fragment in pMJ1/P→S into hTOP2α cDNA in YEpWob6, resulting in YEpM2. YEpM3 was similarly constructed by introducing both the R450Q and P803S mutations into hTOP2α cDNA in YEpWob6 (Figure 1A). The mutation G437E was generated by PCR-based site-directed mutagensis. Three rounds of PCR were carried out to introduce the G437E mutation into hTOP2α cDNA in YEpWob6. The resulting plasmid is named YEpM4 (Figure 1A). The following four primers were used (the mutations are marked in bold): primer A, 5′-AAT TGT CGA CGA ATT CGA CAG GTT-3′ *Sal*I; primer B, 5′-CGA GTT TGG GAA TT**T** CCT TGG TTC-3′; primer C, 5′-GAA TCA AGG **A**AA TTC CCA AAC TCG-3′; and primer D, 5′-CAC CAT GTA GCC TGG TAC CAA ACT-3′ *Kpn*I

Purification of Mutant hTOP2α from Yeast Cells. Overexpression and purification of mutant enzymes were carried out as described previously (*35*) with slight modifications. Plasmids containing either a single or double mutation were separately transformed into a protease-deficient yeast strain BCY123. The BCY123 harboring the plasmid was inoculated into 100 mL of SD medium (synthetic uracil drop-out medium) containing 2% glucose. When the cell density reached an OD_{600} of about 0.7, the culture was diluted with 1 L of fresh glucose-free SD medium containing 3% glycerol and 1% lactic acid. When the OD_{600} of this culture reached about 0.7, galactose was added to a final concentration of 2%. After galactose induction for 12 h, yeast cells were collected by centrifugation. The cell pellet was washed once with prechilled water followed by washing with buffer I [50 mM Tris-HCl (pH 7.7), 1 mM EDTA, 1 mM EGTA, 10% glycerol/25 mM sodium fluoride, 1 mM sodium bisulfite, 1 mM PMSF, 1 mM β-mercaptoethanol, 1 mM benzamidine, 5 μg/mL leupeptin, and 5 μg/mL pepstatin A]. After washing, the cell pellet was resuspended in buffer I (1 mL of buffer I per gram of the cell pellet) and frozen in liquid nitrogen. Frozen cells were thawed on ice and mixed with half a volume of acid-washed glass beads (Sigma). The mixture was vigorously vortexed 10 times (vortexing for 20 s and chilling for 40 s at 0 °C) at the maximum speed. The cell lysate was centrifuged at 13000*g* for 15 min at 4 °C. The supernatant was collected and subjected to high-salt PEG (5% PEG and 1 M NaCl) precipitation so nucleic acids could be removed as described previously (*36*). The high-salt PEG supernatant was loaded onto a hydroxyapatite (Bio-Rad Bio-gel HTP) column. After loading, the column was washed with 0.2 M potassium phosphate buffer (pH 7.0) and eluted with a linear salt gradient (0.2 to 0.7 M KP_i). The fractions containing activity were pooled and stored at −70 °C. The

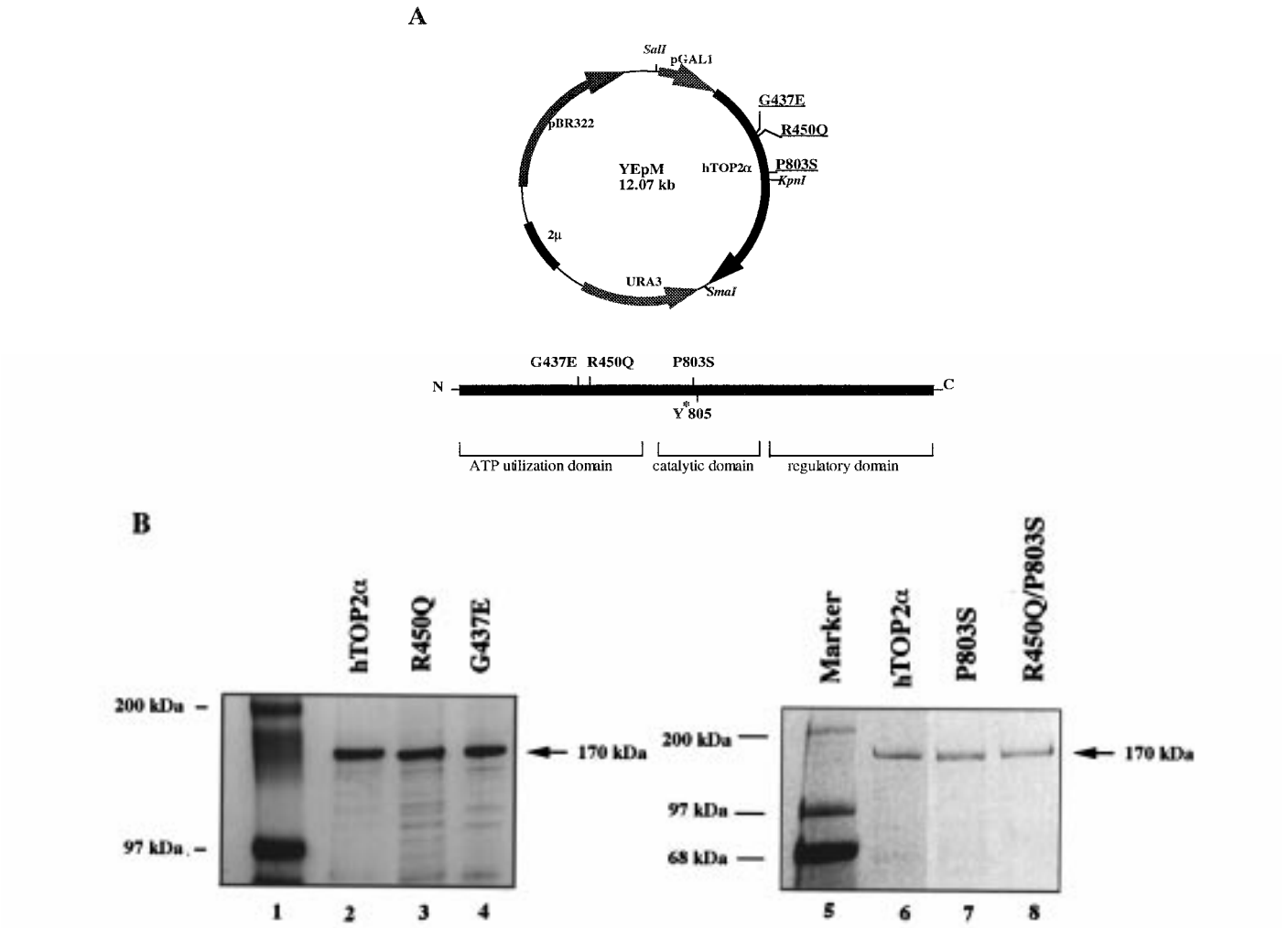

FIGURE 1: Overexpression of mutant hTOP2α in the yeast system. (A) The plasmid map for the hTOP2α overexpressing plasmid, YEpM. Plasmids, YEpM1 (R450Q), YEpM2 (P803S), YEpM3 (R450Q/P803S), and YEpM4 (G437E), were constructed from the same expression vector, YEpWob6, as described in Materials and Methods. YEpWob6 contains wild-type hTOP2α cDNA under the control of the pGAL1 promoter. The three mutations are also denoted in the hTOP2α putative domains. (B) SDS−PAGE of partially purified recombinant hTOP2α. Fifteen microliters of each protein sample (wild type, R450Q, and G437E) was loaded onto a 6% SDS gel (Bio-Rad Mini-gel system) (see lanes 2−4, respectively). The same amount of protein for the wild-type enzyme (hTOP2α), P803S, and R450Q/P803S (see lanes 6−8, respectively) was also loaded onto a 4 to 20% gradient SDS gel (Ready gradient gel from Bio-Rad). Lanes 1 and 5 contained the protein molecular weight markers.

peak fractions of the wild-type and mutant enzymes were analyzed on SDS−PAGE (Figure 1B).

Purification of Enzymes from CEM and CEM/VM-1 Cells. CEM and CEM/VM-1 cells were cultured in the RPMI1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin sulfate at 37 °C in a CO_2 incubator. Ten liters of cells was used for each purification following the procedure described previously (*36*). Nuclei isolation and high-salt PEG precipitation were performed as described previously (*36*). The PEG supernatant was loaded directly onto a hydroxyapatite column, and peak fractions eluted from a linear salt gradient (0.2 to 0.7 M KP_i) were pooled. The pooled fractions were diluted and loaded onto a Bio-Rex 70 column. Peak fractions from the Bio-Rex 70 column eluted with the same salt gradient were pooled, dialyzed against 30 mM KP_i (pH 7.0), 50% glycerol, and 1 mM DTT, and stored at −70 °C.

Filter Binding Assays. The formation of protein clamp was assayed by glass fiber filter retention of circular DNA using a previously published method (*1*, *37*). An equimolar mixture of radiolabeled linear and circular DNA samples was used in each filter binding assay. The radiolabeled DNA substrate was generated by nick translation of nicked pHC624 (*38*) carried out in the presence of [^{3}H]dCTP (2.5 Ci/mmol) for 1 h at 14 °C, followed by ligation with T4 DNA ligase in the presence of 5 μg/mL ethidium bromide for 3 h at 25 °C. The mixture was then phenol extracted and run through a Sephadex G-50 spin column (Amersham Pharmacia Biotech) to remove the unincoporated nucleotides. To generate the linear DNA substrate, the labeled supercoiled DNA was digested with the restriction enzyme *Eco*RI. Labeled DNA substrate (0.05 pmol, 7000 cpm) was incubated in a 25 μL reaction mixture containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 50 μg/mL BSA, and 15 nM wild-type or mutant hTOP2 proteins. The reactions were carried out in the presence 1 mM of ATP with or without 1 mM ICRF193. Following incubation at 37 °C for 15 min, the NaCl concentration in the reaction mixture was made 1 M by using a 5 M NaCl stock solution. The mixture was then filtered through a GF/C glass fiber filter. A portion (2% of the filtrate by volume) of the protein-free DNA recovered from filtrate and a portion

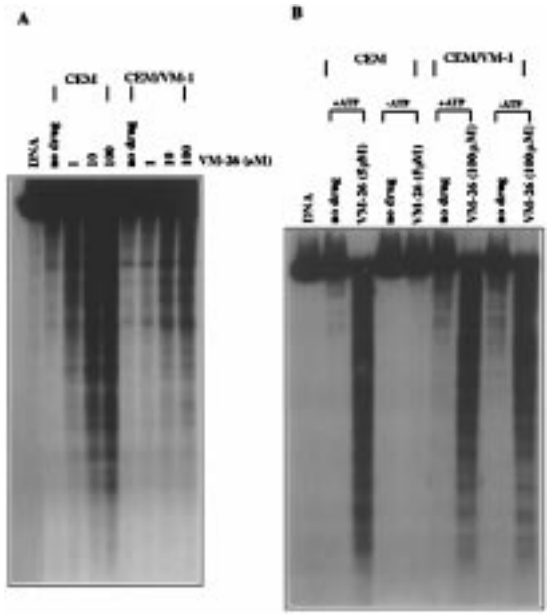

FIGURE 2: Mutant TOP2 purified from CEM/VM-1 cell lines exhibits VM-26 resistance with a reduced dependence on ATP. (A) Cell extracts prepared from CEM/VM-1 cells exhibited a reduced level of DNA cleavage in the presence of VM-26. Equal amounts of cell extracts from CEM and CEM/VM-1 cells were used in the cleavage assay. The cleavage reactions were carried out in the presence of 1 mM ATP with different concentrations of VM-26 as indicated. (B) DNA cleavage induced by partially purified mutant TOP2 from CEM/VM-1 cells exhibits minimal ATP stimulation in the presence of VM-26. Wild-type and mutant TOP2 were partially purified from CEM and CEM/VM-1 cells, respectively, as described in Materials and Methods. The cleavage assays in the presence of either 5 or 100 μM VM-26 were performed to monitor drug sensitivity. Equal amounts of enzymes were used in each reaction. The reaction mixtures containing 1 mM ATP were labeled +ATP at the top of the lanes. The reaction mixtures without ATP were labeled −ATP at the top of the lanes.

(10% of the wash by volume) of the protein-bound DNA recovered from filter by subsequent washing with 1% SDS were analyzed by agarose gel electrophoresis. All samples were treated with 100 μg/mL proteinase K at 45 °C for 15 min before being loaded onto a 1.6% agarose gel containing 1 μg/mL ethidium bromide. At the end of gel electrophoresis, the gel was dried and autoradiographed.

RESULTS

TOP2 Isolated from the Multidrug Resistant Cell Line CEM/VM-1 Is Resistant to VM-26. TOP2-mediated DNA cleavage in the presence of VM-26 was performed in a standard cleavage assay using ^{32}P end-labeled YEpG DNA. As shown in Figure 2A, cell extracts prepared from CEM/VM-1 cells exhibited a much (about 10−100-fold) reduced level of DNA cleavage in the presence of VM-26 as compared to the levels of those prepared from the wild-type CEM cells, consistent with the previous report (*25*). TOP2 was also partially purified from both CEM and CEM/VM-1 cells (see Materials and Methods). Partially purified TOP2 from CEM/VM-1 cells, as expected, was resistant to VM-26 (data not shown). Strikingly, wild-type and mutant TOP2 exhibited quite different DNA cleavage properties in the presence of VM-26. DNA cleavage by wild-type TOP2 was strongly ATP-stimulated (Figure 2B). In the presence of 1 mM ATP, TOP2-mediated DNA cleavage induced by VM-26 was stimulated more than 50-fold (Figure 2B). By contrast, under the same conditions, DNA cleavage by mutant TOP2 was only minimally affected by 1 mM ATP (Figure 2B). The altered ATP effect on TOP2-mediated DNA cleavage in nuclear extract prepared from the mutant CEM/VM-1 cell line has been reported previously (*39*).

*Both the R450Q Mutation and the P803S Mutation of hTOP2*α *Confer Resistance to VM-26 in the Absence of ATP.* To characterize mutant hTOP2α in CEM/VM-1 cells, recombinant enzymes were constructed and overexpressed in yeast. The two putative mutations, R450Q and P803S, were introduced into hTOP2α either singly or in combination (see Materials and Methods for construction and expression). In the absence of ATP, both R450Q hTOP2α and P803S hTOP2α exhibited about 10−100-fold greater resistance to VM-26 than wild-type hTOP2α (Figure 3; see the −ATP lanes). R450Q/P803S hTOP2α exhibited several-fold greater resistance to VM-26 than either R450Q or P803S mutant hTOP2α in the absence of ATP (see Figure 3 and Table 1).

The R450Q Mutation but Not the P803S Mutation Confers Resistance to VM-26 in the Presence of ATP. Surprisingly, in the presence of 1 mM ATP, P803 mutant hTOP2α exhibited wild-type sensitivity to VM-26 (Figure 3). By contrast, the R450Q mutant enzyme exhibited about 300-fold greater resistance to VM-26 than the wild-type enzyme (Figure 3). The double mutant (R450Q/P803S) enzyme exhibited resistance to VM-26 (about 300-fold) similar to that of the R450Q enzyme. The R450Q mutant enzyme was also shown to be cross-resistant to amsacrine (mAMSA), adriamycin (ADR), and mitoxantrone (MTX) in the presence of 1 mM ATP (Figure 4).

*R450Q Mutant hTOP2*α *Exhibits Reduced Catalytic Activity and Requires Higher ATP Concentrations for Catalysis.* Previous studies have shown that mutant TOP2 in the nuclear extracts prepared from CEM/VM-1 cells requires higher ATP concentrations for catalysis (*39*). To test whether the R450Q enzyme behaves similarly, the catalytic activity of the R450Q enzyme was assayed using the P4 unknotting assay. As shown in Figure 5, in the presence of 1 mM ATP, the R450Q enzyme is about 4-fold less active than the wild-type enzyme. However, in the presence of 10 μM ATP, the R450Q enzyme was about 40-fold less active than the wild-type enzyme. Our preliminary studies using extensively purified enzymes have also demonstrated that the R450Q mutant enzyme has reduced DNA-dependent ATPase activity (T. Khalifah, T. Hu, T. S. Hsieh, and L. F. Liu, unpublished results).

The R450Q Enzyme Exhibits Impaired Ability To Form the Circular Clamp in the Presence of ATP and ICRF193. Wild-type TOP2 can form a circular clamp upon ATP binding and can be locked in that conformation with the nonhydrolyzable ATP analogue, AMPPNP (*1*). ICRF193, a derivative of bisdioxopiperazine, binds TOP2 and inhibits ATP hydrolysis, resulting in inhibition of TOP2 catalytic activity (*40*). In the presence of ICRF193 and ATP, TOP2 is arrested in a circular clamp conformation similar to that formed in the presence of AMPPNP (*41*). The interlocking between the TOP2 circular clamp and circular plasmid DNA has been demonstrated to be stable even in 1 M NaCl using a filter binding assay (*1*). The amount of the interlocked TOP2−DNA complexes can be estimated from salt-resistant complexes by washing the filter with SDS (*1*). Using such a filter binding assay, we showed that the amount of

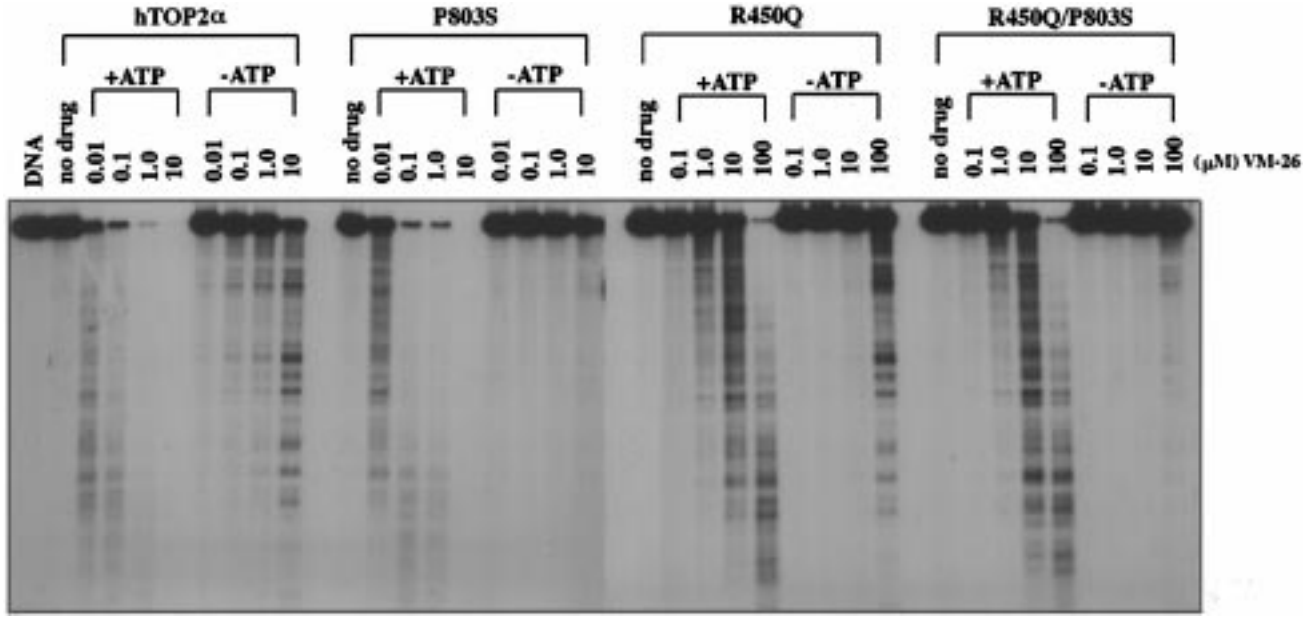

FIGURE 3: Recombinant mutant hTOP2α exhibiting both VM-26 resistance and altered ATP dependence. Wild-type (hTOP2α), P803S, R450Q, and R450Q/P803S enzymes were partially purified and used in the DNA cleavage assay. The concentrations of VM-26, and the presence (+ATP) or absence (−ATP) of 1 mM ATP, are denoted at the top of the lanes. Equal amounts of enzymes (based on protein content) were used in each reaction (0.2 μg of each enzyme).

Table 1: Effect of ATP on TOP2-Mediated DNA Cleavage in the Presence of VM-26

	hTOP2α	P803S	R450Q	R450Q/P803S
relative CC_{20}[a] (with ATP)	1	2	300	300
relative CC_{20} (without ATP)	1	100	50	100
ATP stimulation[b] (-fold)	100	>1000	10	100

[a] CC_{20} is defined as the VM-26 concentration necessary to cleave 20% of the plasmid DNA. The relative CC_{20} is the ratio of CC_{20}(mutant) to CC_{20}(wild-type). [b] ATP stimulation is estimated as the -fold of increased drug concentration in the absence of ATP for achieving the same extent of cleavage in the presence of ATP.

interlocked TOP2−circular DNA complexes in the presence of 1 mM ATP and 1 mM ICRF193 was reduced 60% for the R450Q enzyme compared to that of the wild-type enzyme (see the bands marked RC in Figure 6).

G437E hTOP2α Confers Multidrug Hypersensitivity in the Absence of ATP. The R450Q mutation is located in the B′ domain and appears to be part of the Walker consensus motif $(R/K)X_{2-3}GXL\Phi_2(D/E)$ (amino acids 450−461) (*28*). Close inspection of the amino acid sequence surrounding this region has identified another Walker consensus motif (amino acids 434−444). To test whether mutations within this putative Walker consensus motif can also confer multidrug resistance, we constructed the G437E mutant hTOP2α. Surprisingly, the G437E mutant enzyme exhibited multidrug hypersensitivity rather than multidrug resistance (Figure 7). In the absence of ATP, the G437E enzyme was more than 100-fold more sensitive to VM-26 than the wild-type hTOP2α (Figure 7; see the −ATP lanes marked VM-26). Similarly, in the absence of ATP, the G437E mutant enzyme was more than 100-fold more sensitive to adriamycin (ADR) and mitoxantrone (MTX) and about 10−100-fold more sensitive to amsacrine (mAMSA) than the wild-type hTOP2α (Figure 7; see the −ATP lanes marked ADR, MTX, and mAMSA). Strikingly, in the presence of 1 mM ATP, the G437E enzyme and the wild-type enzyme exhibited almost identical sensitivity to all these TOP2 drugs (Figure 7; see the +ATP lanes).

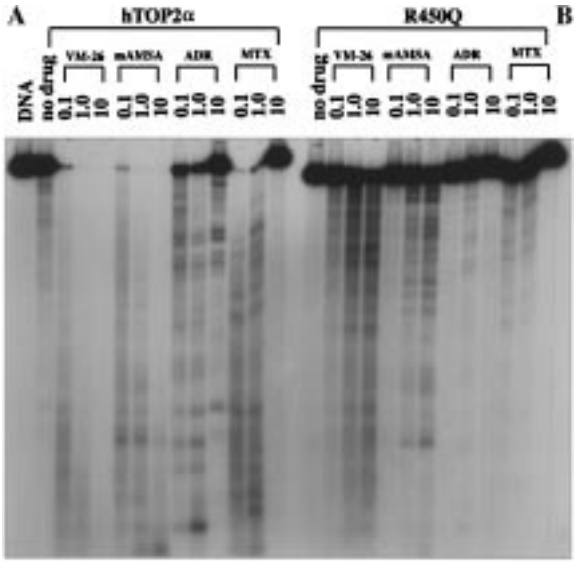

FIGURE 4: R450Q enzyme exhibiting reduced catalytic activity at low ATP concentrations. The catalytic activities of the wild-type enzyme (hTOP2α) and the mutant R450Q enzyme were monitored by the P4 unknotting assay as described in Materials and Methods. Equal amounts of the two enzymes were used, and the activities of the two enzymes were compared using 2-fold serial dilution of the enzymes. The ATP concentrations for the reactions shown in the gels were 1 mM (A) and 10 μM (B), respectively.

Apparently, ATP had a much reduced DNA cleavage stimulatory effect on the G437E mutant hTOP2α (less than 10-fold stimulation) than on the wild-type enzyme (100- to >1000-fold stimulation depending on the drug) (Figure 7).

The G437E Mutant Enzyme Is Catalytically More Active Than the Wild-Type Enzyme at Low ATP Concentrations. Figure 8A shows that in the presence of 1 mM ATP, the G437E enzyme and the wild-type enzyme had about the same specific activity. However, in the presence of 10 μM ATP, the G437E enzyme was about 2-fold more active than the wild-type enzyme (Figure 8B), suggesting that the G437E

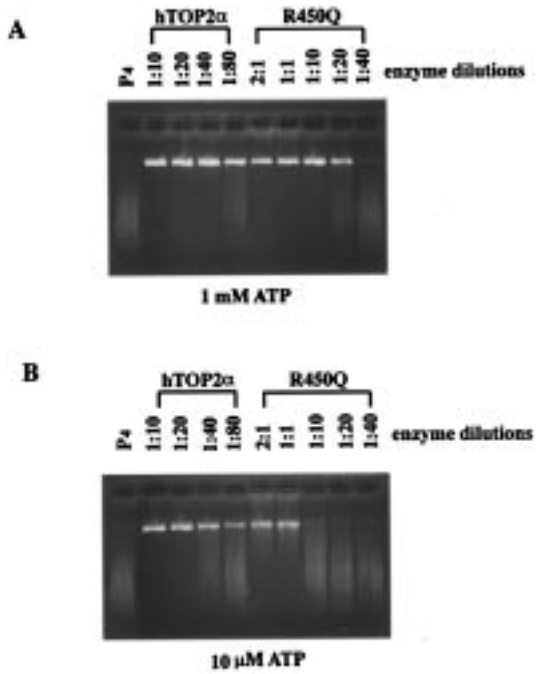

FIGURE 5: R450Q mutant enzyme conferring multidrug resistance. The DNA cleavage assay was performed in the presence of 1 mM ATP using either the wild-type enzyme (hTOP2α) or the R450Q mutant enzyme. The concentrations of teniposide (VM-26), amsacrine (mAMSA), adriamycin (ADR), and mitoxantrone (MTX) were as indicated at the top of each lane.

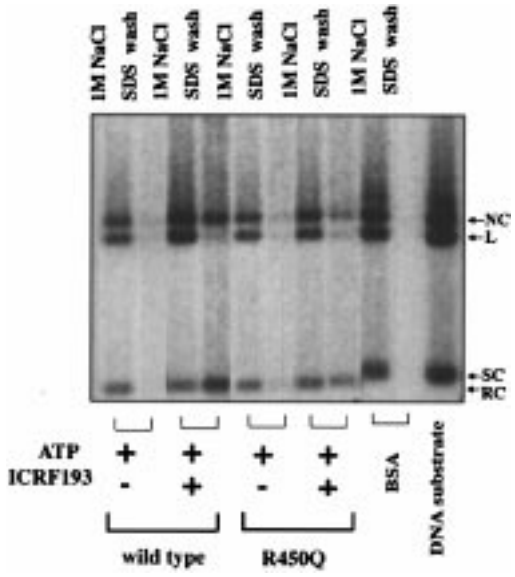

FIGURE 6: R450Q enzyme exhibiting reduced clamp formation ability in the presence of ATP and ICRF193. The filter binding assay for the detection of the circular protein clamp form of hTOP2α in the presence of ICRF193 and ATP was performed as described in Materials and Methods. Equal amounts of the wild-type and mutant enzymes were used in this experiment. The symbols NC, L, SC, and RC denote nicked circular, linear, supercoiled, and relaxed circular DNA, respectively.

enzyme, in contrast to the R450Q enzyme, is more efficient in ATP utilization for catalysis.

DISCUSSION

Previous studies have identified two mutations, R450Q and P803S, in the coding region of the hTOP2α gene in the atypical multidrug resistant cell line, CEM/VM-1 (*27*, *28*). On the basis of our current studies with recombinant mutant hTOP2α enzymes, it appears that one or both of these two mutations in hTOP2α are likely to be responsible for atypical multidrug resistance in CEM/VM-1 cells.

In the absence of ATP, both the P803S and the R450Q mutations can confer resistance to VM-26. This in vitro result is consistent with results obtained from previous in vivo studies with the yeast system (*29*). The molecular basis for multidrug resistance in the absence of ATP is unclear. It seems possible that these mutations affect either a fundamental step in enzyme catalysis such as DNA binding or a common step in drug interaction with the enzyme−DNA complex such as drug intercalation into the DNA duplex. However, in the presence of 1 mM ATP, only the R450Q mutation confers multidrug resistance. The P803 mutant hTOP2α is as sensitive to VM-26 as the wild-type hTOP2α in the presence of 1 mM ATP. In the presence of 1 mM ATP, the R450Q mutation appears to be dominant since the double mutant, like the R450Q mutant, is resistant to VM-26. ATP is known to stimulate TOP2-mediated DNA cleavage (*33*, *42*). However, the mechanism for the stimulatory effect of ATP on DNA cleavage is not known. Our recent studies have demonstrated that the DNA cleavage stimulatory effect of ATP is drug-dependent. DNA cleavage induced by drugs such as VM-26 and mMASA is very sensitive to ATP (more than 50-fold stimulation) and is also strongly inhibited by ADP. On the other hand, DNA cleavage induced by drugs such as amonafide and β-lapachone is much less sensitive to ATP and is also much less inhibited by ADP (*43*, *44*; H. M. Wang and L. F. Liu, unpublished results). Both yeast and human TOP2 are known to undergo conformational change to form circular protein clamp upon ATP binding (*1*, *33*, *41*). The cleavage-stimulatory effect of ATP could be due to the circular clamp conformation which is more favorable for the accumulation of the cleaved intermediate (the TOP2−drug−DNA cleavable complex) in the presence of many TOP2 poisons such as VM-26 and mAMSA.

In the case of the R450Q mutant enzyme, the affinity for ATP is probably reduced by the mutation, which could explain why ATP only has a minimal cleavage-stimulatory effect on the R450Q mutant enzyme. For the P803S mutant enzyme, the mutation probably does not alter the ATP affinity for this enzyme. Consequently, ATP has the same cleavage-stimulatory effect on this enzyme as that of the wild-type enzyme (Table 1). Consistent with the cleavage assay results, the ability of the R450Q mutant enzyme to form the circular clamp in the presence of ATP and ICRF193 is defective (Figure 6). However, the ability of the P803S mutant enzyme to form the circular clamp is similar to that of the wild-type hTOP2α (data not shown). It seems plausible that the ATP-stimulated TOP2-mediated cleavage in the presence of various drugs is related to the ATP-stimulated conformational change of TOP2.

We also studied another mutation, G437E, which is 13 amino acids upstream from the R450Q mutation on hTOP2α. The G437E mutation was generated by site-directed mutagensis and is not a mutation originally identified in the CEM/VM-1 cell line. The G437E mutation is also located in the B′ domain. By aligning with the yeast TOP2 crystal structure (*45*), it maps in a loop structure immediately before the B′β1β sheet in the Rossman fold structure (a putative

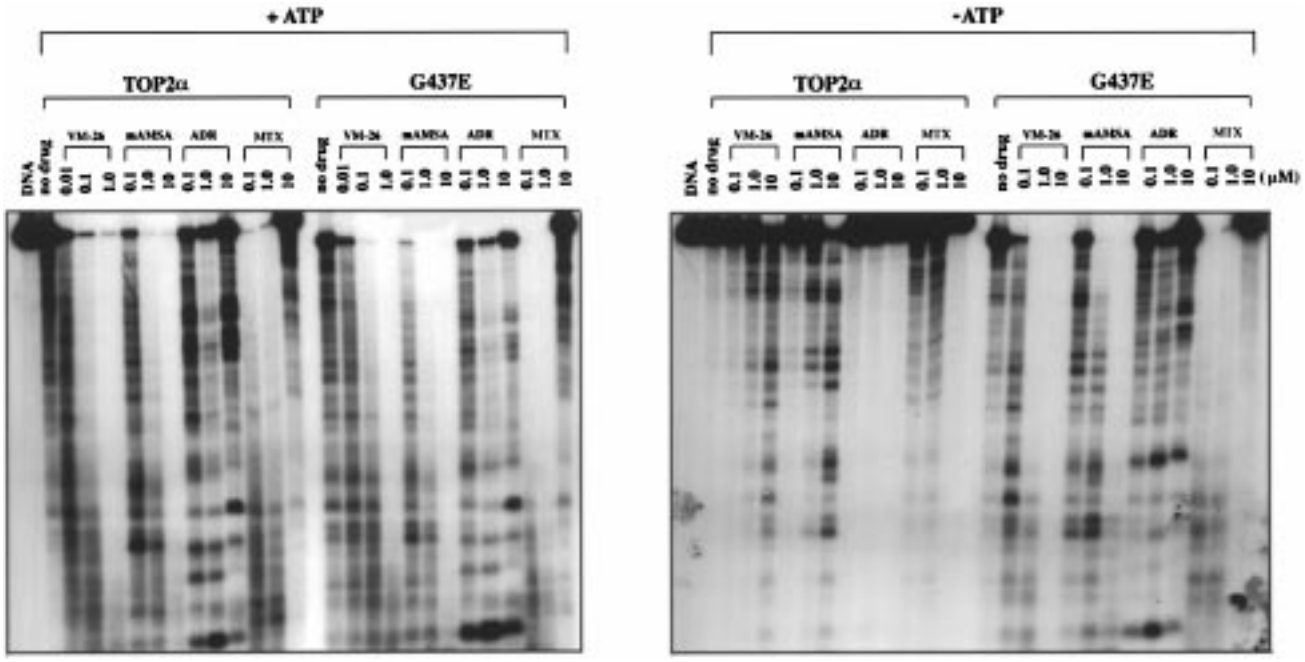

FIGURE 7: G437E enzyme conferring multidrug hypersensitivity in the absence of ATP. The cleavage assay was performed as described in the legend of Figure 4 except that another set of reactions which did not contain ATP (denoted as −ATP) was included; 0.2 μg of each enzyme was used in the reaction. The concentrations of various drugs are denoted.

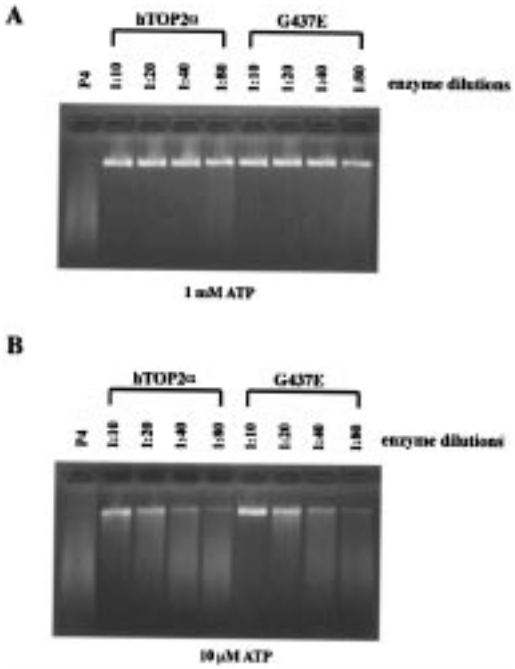

FIGURE 8: G437E enzyme with a reduced ATP requirement for catalysis The catalytic activities of the wild-type enzyme (hTOP2α) and the mutant G437E enzyme were monitored by the P4 unknotting assay as described in Materials and Methods. Equal amounts of the two enzymes were used, and the activities of the two enzymes was compared using 2-fold serial dilution of the enzymes. The ATP concentrations for the reactions shown in the gels were 1 mM (A) and 10 μM (B), respectively.

nucleotide binding domain) (*46*). One would expect that the G437E mutation in the same domain gives a phenotype similar to that of the R450Q mutation which maps in a loop next to the B′β1β sheet. Surprisingly, the G437E mutant enzyme confers multidrug hypersensitivity instead of multidrug resistance (Figure 7). This mutant enzyme has a higher level of background cleavage than the wild-type enzyme (lanes with "no drug" in Figure 7). We have also observed that the G437E enzyme requires a lower ATP concentration for its catalytic reaction than the wild-type enzyme (Figure 8). These results suggest that the Rossman fold structure identified in the yeast TOP2 crystal structure may somehow affect ATP utilization and multidrug sensitivity and resistance. The simplest explanation is that this Rossman fold structure represents an ATP binding site. However, recent studies have demonstrated that the N-terminal fragments of yeast TOP2 which do not contain this Rossman fold exhibit DNA-stimulated ATPase activity (*47*). It seems possible that this Rossman fold may allosterically affect the ATPase activity of TOP2, which in turn may affect multidrug sensitivity and resistance. Further studies are necessary to determine the role of this Rossman fold in ATP utilization during TOP2 catalysis and multidrug sensitivity and resistance.

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