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Identification of Yeast DNA Topoisomerase II Mutants Resistant to the Antitumor Drug Doxorubicin: Implications for the Mechanisms of Doxorubicin Action and Cytotoxicity

SANDHIYA PATEL, ANTJE U. SPRUNG, BEATRICE A. KELLER, VICTORIA J. HEATON and L. MARK FISHER

Molecular Genetics Group, Department of Cellular and Molecular Sciences, St. George's Hospital Medical School, University of London, London SW17 0RE, UK.

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SUMMARY

Doxorubicin is a therapeutically useful anticancer drug that exerts multiple biological effects. Its antitumor and cardiotoxic properties have been ascribed to anthracycline-mediated free radical damage to DNA and membranes. Evidence for this idea comes in part from the selection by doxorubicin from stationary phase yeast cells of mutants (petites) deficient in mitochondrial respiration and therefore defective in free radical generation. However, doxorubicin also binds to DNA topoisomerase II, converting the enzyme into a DNA damaging agent through the trapping of a covalent enzyme-DNA complex termed the 'cleavable complex.' We have used yeast to determine whether stabilization of cleavable complexes plays a role in doxorubicin action and cytotoxicity. A plasmid-borne yeast *TOP2* gene was mutagenized with hydroxylamine and used to transform drug-permeable yeast strain JN394t2–4, which carries a temperature-sensitive *top2–4* mutation in its chromosomal *TOP2* gene. Selection in growth medium at the nonpermissive temperature of 35° in the presence of doxorubicin resulted in the isolation of plasmid-borne *top2* mutants specifying functional doxorubicin-resistant DNA topoisomerase II. Single-point changes of Gly748 to Glu or Ala642 to Ser in yeast topoisomerase II, which lie in and adjacent to the CAP-like DNA binding domain, respectively, were identified as responsible for resistance to doxorubicin, implicating these regions in drug action. None of the mutants selected in JN394t2–4, which has a rad52 defect in double-strand DNA break repair, was respiration-deficient. We conclude that topoisomerase II is an intracellular target for doxorubicin and that the genetic background and/or cell proliferation status can determine the relative importance of topoisomerase II- versus free radical-killing.

Doxorubicin (Adriamycin) is a versatile anthracycline antitumor agent (Fig. 1) that is widely used in cancer chemotherapy (1). The drug is active against a range of cancers including solid tumors, leukemias, and lymphomas (2). Doxorubicin is a DNA intercalator (3) that in cultured mammalian cells inhibits DNA and RNA synthesis (4, 5), produces single-stranded DNA breakage (6, 7) and membrane damage (8), and causes inhibition *in vitro* of the nuclear enzyme DNA topoisomerase II (9). These pleiotropic effects have made it difficult to establish the exact molecular basis of either the antitumor activity of doxorubicin or the dose-limiting cardiotoxicity associated with myofibrillar degeneration and mitochondrial damage in patients.

Cardiotoxicity has been ascribed to the one-electron reduction of the anthracycline quinone moiety, generating a semiquinone free radical that, with molecular oxygen, produces highly reactive chemical species including superoxide ($0_2^{\cdot-}$), hydrogen peroxide and hydroxyl radicals (7). These radicals can damage membranes by lipid peroxidation. Genetic evidence for the involvement of superoxide generation and free radical damage in doxorubicin cytotoxicity has come from studies in the yeast *Saccharomyces cerevisiae* (10). First, overexpression of superoxide dismutase gives partial protection against doxorubicin killing. Second, yeast selected for doxorubicin resistance was found to exhibit the petite phenotype associated with defective mitochondrial respiration (10, 11). It is assumed that mitochondrial respiration provides redox intermediates needed for free radical formation and doxorubicin-mediated cytotoxicity in yeast. However, it is not clear whether free radical damage (to membranes or DNA) or effects on DNA topoisomerase II underlie the antitumor properties of the drug.

Studies *in vitro* have shown that doxorubicin inhibits the activity of the nuclear enzyme DNA topoisomerase II, inducing double-stranded DNA breakage (12, 13). Topoisomerase II is an essential homodimeric eukaryotic enzyme that mediates chromosome segregation by catalyzing the ATP-de-

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ABBREVIATIONS: CAP, catabolite activator protein; SC-URA, synthetic complete medium lacking uracil; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; kb, kilobase pair(s); MLC, minimal lethal concentration.

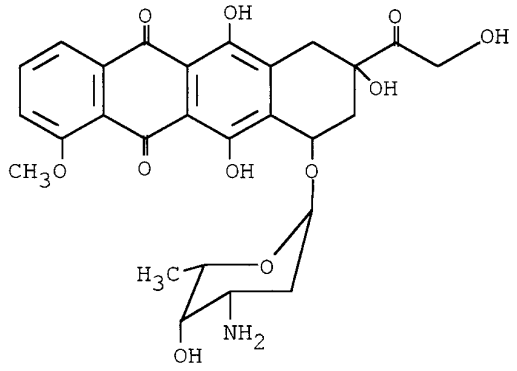

Fig. 1. Chemical structure of doxorubicin.

pendent transport of a DNA duplex (called the T segment) through a transient enzyme-bridged, double-strand break in another DNA segment (called the 'gate' or G segment) (9). It seems that many antitumor agents, including doxorubicin, etoposide and amsacrine interfere with enzymatic DNA breakage-reunion trapping topoisomerase II on DNA in a covalently bound state (14). The ternary complex of enzyme, drug, and DNA has been termed the 'cleavable complex' because its disruption with detergent leads to double strand DNA breakage and covalent attachment of each subunit in the topoisomerase II homodimer, one to each 5′ phosphate end via a phosphotyrosyl linkage. For several anticancer agents, drug cytotoxicity has been correlated with the intracellular formation of cleavable complexes (13–15). Although little is known about how doxorubicin interacts with topoisomerase II, it seems that the enzyme could be an intracellular target of the drug.

Recently, several elegant yeast systems have been developed to test whether topoisomerase II is a target for antitumor agents (16–18). This approach relies on drug-permeable, temperature-sensitive DNA topoisomerase II mutants bearing either *top2–1* or *top2–4* mutations that allow growth at 25° but not at 35°. One method exploits the fact that if topoisomerase II is the lethal target of a cleavable complex forming drug, then a *top2–1* mutant will exhibit much higher drug resistance at 30° than at 25°, reflecting the lower activity of target at the higher temperature. A variant technique involves showing that overexpression of yeast topoisomerase II from a plasmid-borne yeast *TOP2* gene sensitizes the yeast to drug killing. By using both approaches, it has been established that amsacrine and etoposide each target topoisomerase II (17, 18). The one study reported to date on doxorubicin was somewhat equivocal: a drug-permeable *top2–1* yeast mutant was shown to exhibit partial rather than high-level drug resistance at 30° (19).

To examine doxorubicin action on topoisomerase II and its role in cytotoxicity, we have exploited a third yeast system. We used a randomly mutagenized *TOP2* plasmid library to rescue the lethal phenotype of a drug-permeable yeast *top2* strain bearing a temperature-sensitive mutation in its single essential chromosomal *TOP2* gene. Consistent with the concept that topoisomerase II is a drug target *in vivo*, we show that doxorubicin-resistant *TOP2* mutants can be selected at 35° and that two different *TOP2* alleles encode drug-resistant proteins bearing single point mutations that map in or near a putative DNA binding region related in fold to the *Escherichia coli* CAP (20).

Materials and Methods

Yeast strains, plasmids and drugs. *S. cerevisiae* strains JN394t2–4 *(MATa ISE2 ura3–52 top2–4 rad52::LEU2)*, which carries the *top2–4* mutant allele in place of the wild-type topoisomerase II gene and JEL1 (*a leu2 trp1 ura3–52 prb1–1122 pep4–3* Δ *his3::PGAL10 -GAL4)*, a protease-deficient strain with a *GAL1* promoter-linked *GAL4* gene were provided by Professor J. C. Wang (Harvard University, Cambridge, MA). Yeast cells were grown in SC-URA to select for plasmids carrying URA3 as a marker. Yeast transformation was carried out using the modified lithium acetate method of Schiestl and Gietz (21). To test for the respiration-deficient phenotype (i.e., the inability to grow on ethanol and glycerol as carbon source), yeast transformants were streaked on to YEPG plates (1% yeast extract, 2% peptone, 2% ethanol, 2% glycerol, and 2% agar).

Plasmids YCpDEDWOB10, which carries the yeast *TOP2* gene under the control of the DED1 promoter, and YEpTOP2-PGAL1, which expresses the yeast topoisomerase II from a galactose-inducible promoter, were obtained from Dr. C.A. Austin (University of Newcastle-upon-Tyne, UK). Supercoiled pBR322 was purified as described previously (22).

Doxorubicin was from Farmitalia Carlo Erba (Milan, Italy) and was obtained through the Pharmacy Dept., St George's Hospital, London. Amsacrine was from the Drug Synthesis and Chemistry Branch, Development Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Etoposide was purchased from Sigma (Poole, Dorset, UK). Etoposide and amsacrine were dissolved in DMSO; doxorubicin was dissolved in water. Oligonucleotides were made at St. George's Hospital Medical School on an Applied Biosystems Synthesizer (Norwalk, CT). The Sequenase version 2.0 sequencing kit, [α-^{35}S]dATP (3000Ci/mmol) and [γ-^{32}P]dATP (3000 Ci/mmol) were from Amersham International (Little Chalfont, Bucks, UK). Dialysis type VS 0.025-μm filters were from Millipore (Bedford, UK)

***In vitro* mutagenesis.** Hydroxylamine mutagenesis was used to create mutations in the yeast *TOP2* gene. The method of Sikorski and Boeke (23) was followed with minor modifications. Supercoiled plasmid YCpDEDWOB10 (10 μg) was reacted with 500 μl of 100 mM hydroxylamine in 0.25 M KPO_4, pH 6.0, and 5 mM EDTA at 75°. At 20- or 40-min time points, the reaction was stopped by incubation on ice. Excess hydroxylamine was removed by dialysis against 10 mM Tris·HCl, ph 7.5, and 1 mM EDTA, using Millipore type VS 0.025 μm filters. The DNA was then precipitated with isopropanol and transformed into *E. coli* XL-1 Blue cells. For each of the 20- and 40-min mutagenized DNA, 20,000 transformants were pooled and grown separately for 10 hr in 500 ml of Terrific Broth medium. Plasmid DNA for each was isolated using alkaline lysis and purified by cesium chloride density gradient.

Selection of doxorubicin-resistant mutants. Drug-resistant mutants were isolated using the method described previously (24). The 20- and 40-min mutagenized pool of YCpDEDWOB10 was used independently to transform JN394t2–4. A total of 20,000 transformants was pooled for each case and suspended in SC-URA medium to different cell densities (absorbance, 0.8, 1.0, 2.0, 3.0) Doxorubicin was added to a final concentration of 100 μg/ml to each culture and the cells were incubated for 48 hr at 35°, after which each culture was diluted 1:1 with SC-URA, and fresh doxorubicin was added to a final concentration of 100 μg/ml. The cells were incubated at 35° for a further 48 hr, after which they were plated to SC-URA plates and incubated at 35° for 5 days. Colonies that were obtained were replica-plated at 35° on SC-URA plates containing 100 μg/ml doxorubicin. Drug cytotoxicity assays were performed on JN394t2–4 retransformed with plasmids recovered from the drug-resistant clones as follows (25). Cells (1.5 ml) from 72-hr cultures were pelleted and suspended in 0.2 ml lysis buffer (2% Triton X-100, 1% SDS, 10 mM Tris·HCl, pH 8.0, 1 mM EDTA). The cells were vortexed for 2 min in the presence of 0.2 ml of phenol/chloroform/isoamyl alcohol (25:24:1)

and 0.3 g of acid-washed glass beads. After a 5-min spin in a microfuge, 2.5 μl of the supernatant was used to transform *E. coli* strain DH5*α* by electroporation (26). Plasmid DNA was purified and restriction analysis was carried out to eliminate gross rearrangements or large deletions.

To confirm that the drug resistance of JN394t2–4 carrying either of the two mutant *TOP2* alleles was caused by the identified point mutation, a 2.7-kb *Kpn*I-*Spe*I restriction fragment from each of the mutant *TOP2* alleles was gel-purified and used to replace the corresponding fragment in wild-type YCpDEDWOB10. The presence of each mutation in the chimeric constructs was confirmed by DNA sequencing. The chimeric constructs were used to transform JN394t2–4 for drug cytotoxicity studies.

Drug cytotoxicity assays. Drug sensitivity of JN394t2–4 carrying wild-type or mutagenized YCpDEDWOB10 to doxorubicin, amsacrine, or etoposide was determined as described previously. Briefly, cells were grown in SC-URA liquid medium at 35°. Cells were diluted to an A_{600nm} of 0.4, drug (0–100 μg/ml) or solvent (0.05% final if DMSO) was added, and the cultures were incubated at 35°. After 24-hr incubation, aliquots were removed, diluted, and plated onto SC-URA plates. Plates were incubated at 25° for 5 days, after which the number of surviving colonies were counted. Drug sensitivity was measured as percentage of relative survival (cell number at 24 hr relative to time zero).

DNA sequence analysis. DNA sequence of the wild-type *TOP2* gene of YCpDEDWOB10 and the mutant alleles in plasmids YCpDEDWOB10-R1 and YCpDEDWOB10-R2 was determined by the dideoxy chain-termination method (27) using the Sequenase version 2.0 kit. For all three alleles, the entire coding region of the *TOP2* gene was sequenced using a panel of 20-mer oligonucleotide primers made to sequences spaced at 200-bp intervals and spanning the entire gene. The single point mutations identified in the two mutant alleles were confirmed by sequencing the noncoding strand.

Overexpression and protein purification. To allow purification of the mutant topoisomerasee II proteins, the 2.2-kb *Kpn*I-*Avr*II restriction fragment from each of the mutant YCpDEDWOB10 alleles was gel-purified and used to replace the corresponding fragment in YEpTOP2PGAL1. The presence of each mutation in the expression plasmids was confirmed by DNA sequencing. YEpTOP2-PGAL1 and the two constructs carrying drug-resistance mutations were used to transform the yeast strain JEL1 for overexpression and purification of the respective topoisomerase II proteins. Wild-type and mutant proteins were purified following the method of Worland and Wang (28). In the final purification step, the phosphocellulose column was eluted with a linear gradient of KCl (0.25–1.0 M) and the topoisomerase II activity eluted at approximately 0.5 M KCl. Active fractions were flash frozen in liquid nitrogen and stored at −70°.

Topoisomerase II assays. Enzyme activity was determined by the ATP-dependent relaxation of supercoiled plasmid pBR322. The assay contained 50 mM Tris·HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA, 1 mM ATP, 30 μg/ml bovine serum albumin and 0.4 μg of supercoiled pBR322 DNA (total volume 20 μl). One unit is the amount of enzyme required to relax 50% of the DNA in 30 min at 37°. Reactions were stopped by addition of loading buffer containing 25% glycerol, 5% SDS and 0.25 mg/ml bromphenol blue and examined by electrophoresis in 0.8% agarose gels run in Tris/borate/EDTA buffer (89 mM Tris, 89 mM boric acid and 2.5 mM EDTA). Gels were stained with ethidium bromide and photographed under UV transillumination.

For DNA cleavage, reaction mixtures (total volume 20 μl) contained 10 mM Tris·HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/ml bovine serum albumin, 1 mM ATP, 100 ng of wild-type or mutant yeast topoisomerase II, a 4333-bp *Eco*RI-*Hin*dIII fragment of pBR322 DNA labeled with ^{32}P at the *Eco*RI end (2000 cpm; Cerenkov) and amsacrine or etoposide (final DMSO concentration of 2.5%). Reactions were incubated at 37° for 20 min and then treated with 1% SDS, followed by digestion with proteinase K at 42° for 60 min. DNA samples were analyzed by electrophoresis in 1% agarose in Tris/borate/EDTA buffer. Gels were blotted overnight on to DE81 paper (Whatman, Maidstone, UK) before autoradiography.

Results

***TOP2* plasmid mutagenesis and isolation of doxorubicin-resistant yeast transformants.** To examine the role of topoisomerase II as a putative intracellular target for doxorubicin, we made use of yeast strain JN394t2–4, which carries a temperature-sensitive *top2–4* mutation that allows growth at 25° but not at 35°. At the nonpermissive temperature, the chromosomally encoded host topoisomerase II is totally inactive (29, 30). The strain requires uracil for growth, has an *ISE2* mutation that makes it drug permeable and a *rad52* double-strand DNA break repair deficiency. Importantly, the lethal phenotype of the JN394 *top2–4* mutation can be rescued by transformation with a plasmid-borne *TOP2* gene that allows growth at 35°. Thus, were topoisomerase II to be the principal intracellular target for a drug, then it should be possible to isolate mutant *TOP2* plasmids that confer drug resistance at 35°. For complementation, we utilized YCpDEDWOB10, a low copy *E. coli*-yeast shuttle plasmid (carrying *URA3* and ampicillin resistance genes for selection) in which the yeast *TOP2* gene is constitutively expressed from a yeast *DED1* promoter (Fig. 2A).

Supercoiled YCpDEDWOB10 was subjected to random mutagenesis *in vitro* by incubation with hydroxylamine, a chemical mutagen that induces C to T transitions in DNA. Plasmid DNA was recovered and amplified by transformation and ampicillin selection of *E. coli* XL-1 before transformation of yeast JN394t2–4 and selection at 25° on SC-URA. Approximately 20,000 URA+ yeast transformants were pooled and grown in SC-URA medium at 35° for 96 hr in the presence of 100 μg/ml doxorubicin (a drug concentration at least 10 times that required to inhibit growth of JN394t2–4 carrying the wild-type plasmid). This selection was carried out separately for plasmid pools derived from 20- and 40-min periods of mutagenesis, after which viable cells were identified by plating on SC-URA plates and incubating at 35°. One hundred and fifty colonies derived from plasmid mutagenized for 20 min, and 500 from the 40-min mutagenesis were tested for resistance by replica-plating to SC-URA plates containing 100 μg/ml doxorubicin and incubation at 35°. In contrast to control JN394t2–4 containing wild-type YCpDEDWOB10, virtually all the colonies from the drug selections were able to grow in the presence of doxorubicin. Thus, the drug-selected colonies must carry a functional plasmid-encoded yeast *TOP2* allele.

Decreased doxorubicin cytotoxicity is plasmid-mediated and does not involve defective mitochondrial respiration. Several yeast clones from each of the two independent liquid selections were grown and their plasmid DNAs were transformed into *E. coli* and purified on a large scale. Yeast JN394t2–4 was retransformed with these plasmids and cytotoxicity assays were carried out as follows. Mid-log phase cultures grown in SC-URA at 35° were diluted and then incubated at 35° in the absence or presence of various concentrations of doxorubicin for 24 hr, a suitable exposure time chosen from pilot-cell survival studies (data not shown). Cells were then diluted appropriately, plated on SC-URA plates, and incubated at 25° for 5 days. The number of surviving colonies was counted and results expressed as

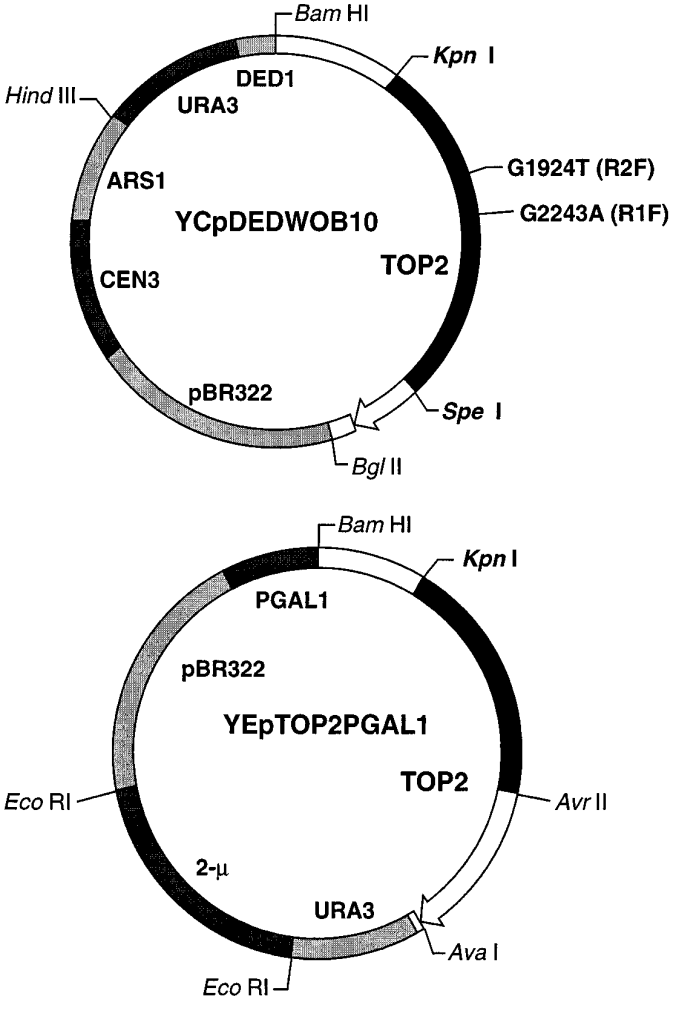

Fig. 2. Structures and restriction maps of yeast-*E. coli* shuttle plasmids that allow expression of the yeast *TOP2* gene driven by either the DED1 (*top*) or PGAL1 (*bottom*) promoters. Sites for *Ava*I, *Avr*II, *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I and *Spe*I are indicated; *bold*, unique sites. *Filled section*, fragment exchange of *Kpn*I-*Spe*I and *Kpn*I-*Avr*II *TOP2* fragments was used to express *TOP2* alleles bearing doxorubicin resistance mutations at nucleotide positions 1924 and 2234 in *TOP2*.

percentage of relative cell survival (cell number at 24 hr relative to time zero).

Fig. 3 shows survival curves for JN394t2–4 transformed with the wild-type *TOP2* plasmid and two representative retransformants that contain YCpDEDWOB10-R1 and YCpDEDWOB10-R2 (in which the plasmids R1 and R2 were derived from 40-min and 20-min mutagenized DNA, respectively). In the absence of drug, the wild-type plasmid supported a 10–20-fold increase in viable counts at 24 hr (Fig. 3A). Inclusion of doxorubicin led to inhibition of yeast growth and cell killing at higher concentrations. Even 1 μg/ml of drug was somewhat growth inhibitory; 5 μg/ml or greater resulted in cell killing (Fig. 3A). Only 2% of cells survived 24 hr at 100 μg/ml of doxorubicin (not shown). The MLC (the concentration required to kill the yeast as opposed to inhibit growth) is a useful index in comparing drug inhibition. For JN394t2–4 carrying the wild-type *TOP2* plasmid, the MLC was 4–5 μg/ml. By contrast, although in the absence of drug, R1 and R2 supported growth of JN394t2–4 similar to that of the wild-type plasmid, these transformants were much more resistant to doxorubicin (Fig. 3, B and C). For the R1 transformant, growth inhibition was seen at 10 μg/ml and the MLC was approximately 75 μg/ml (Fig. 3B). The R2 transformant seemed somewhat more sensitive than R1 to drug with a doxorubicin MLC of 25–50 μg/ml (Fig. 3C). The cell viability of the R1 and R2 transformants at 100 μg/ml doxorubicin was 30% and 15%, respectively. Moreover, these resistance profiles are similar to those of the original R1 and R2 clones derived directly from the liquid culture selection (not shown). The results are consistent with the idea that plasmids R1 and R2 confer, respectively, 10–15-fold and some 5–10-fold greater resistance to doxorubicin at 35° and account largely for the resistance phenotype obtained by liquid selection.

It is known that doxorubicin can select for petites that through defects in mitochondrial respiration are resistant to drug action. Petites constitute about 2% of the population of a yeast culture and their transformation with a *TOP2* plasmid might allow their isolation in our screening protocol. To exclude this possibility, we tested both the original liquid selected transformants and retransformed JN394t2–4 cells for their ability to grow on medium containing glycerol and ethanol as carbon source. All the doxorubicin-resistant transformants grew on this medium, which suggests they were not defective in respiration. Moreover, other experiments suggested a role for *TOP2* in resistance. Thus, it was found that whereas the R1 and R2 plasmids conferred a dominant resistance phenotype at 35°, the JN394t2–4 transformants were unable to grow on drug plates containing doxorubicin at 10 μg/ml when examined at 25° (not shown), a temperature at which the drug susceptible host topoisomerase II is active. It is known for *TOP2* alleles that resistance to cleavable complex forming agents is recessive to sensitivity. Thus, the genetic data indicated that the *TOP2* genes of the R1 and R2 plasmids were directly implicated in the resistance phenotype.

Drug-resistant *TOP2* alleles specify single amino acid changes at Gly748 or Ala642 in yeast topoisomerase II. The nucleotide sequences of the *TOP2* genes for YCpDEDWOB10, R1 and R2 were determined and compared. Sequence analysis of the wild-type *TOP2* gene revealed two differences from that originally reported. The nucleotide sequence indicated that codon 548 specifies Pro (instead of Leu), which is consistent with the presence of a Pro at this position in all other eukaryotic type II topoisomerases. Secondly, there was no codon for Asn74, again in agreement with other known type II enzymes. It seems that yeast topoisomerase II is a 1428-residue (rather than 1429-residue) polypeptide. However, to facilitate comparison with earlier studies and with the crystal structure of yeast topoisomerase II (see below), we have retained the earlier numbering system. The R1 and R2 *TOP2* alleles were identical in sequence to that of the wild-type plasmid except for a single point change in each case. Plasmid R1 carried a G2243 to A mutation in *TOP2* consistent with the known mutagenic effects of hydroxylamine. This change resulted in Gly-to-Glu mutation at residue 748 (G748E) located only 35 amino acids away from the catalytic Tyr783 engaged in DNA breakage-reunion. Although all other eukaryotic topoisomerases have Asn at the position equivalent to Gly748, the residue lies in a region that is highly conserved among these proteins (Fig. 4A). For

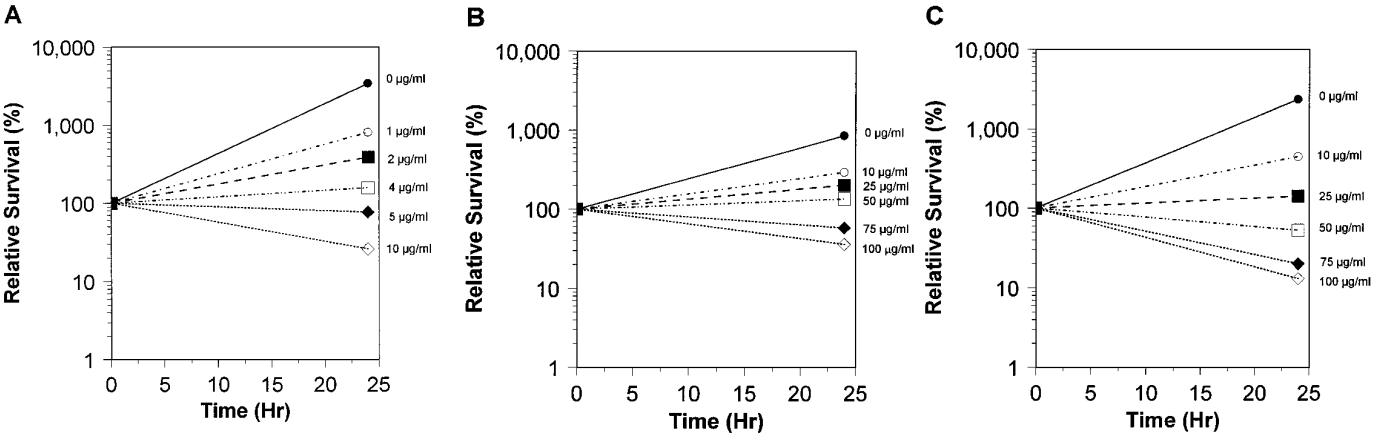

Fig. 3. Effects of doxorubicin on the viability at 35° of yeast strain JN394top2–4ts transformed with a wild-type *TOP2* plasmid YCpDEDWOB10 (A) or with plasmids R1 (B) or R2 (C), two mutant YCpDEDWOB10 derivatives. Viable counts were determined after growth of JN394t2–4 transformants for 24 hr (in the absence or presence of doxorubicin at the concentrations indicated) and are expressed as a percentage of viable counts present at time zero.

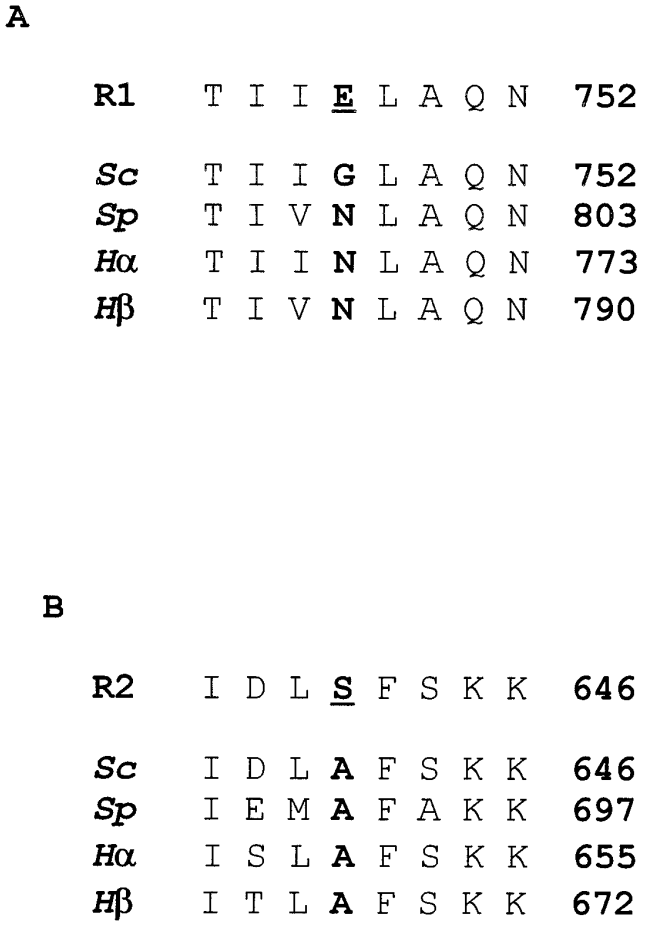

Fig. 4. Doxorubicin-resistance mutations map to conserved regions of yeast topoisomerase II. G748E and A642S amino acid changes specified by the *TOP2* allele in plasmid R1 (A) and R2 (B), respectively. *Sc*, *Sp*, *Hα* and *Hβ* denote topoisomerase sequences of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and the human α and β isoenzymes, respectively (33). *Bold,* residues equivalent to G748 and A642 in yeast *S. cerevisiae*; *Numbers at right,* amino acid position in the primary sequence.

the R2 *TOP2* allele, a mutation of G1924 to T resulted in an Ala-to-Ser substitution at position 642 (A642S). A642 is homologous in all eukaryotic type II topoisomerases (Fig. 4B). Although hydroxylamine acts predominantly at cytosine residues, it can also mediate side-reactions at other DNA bases, which could explain the observed G-to-T transversion (29).

G748E or A642S changes in topoisomerase II decrease the cytotoxicity of a variety of antitumor agents. To confirm unequivocally that the single point *TOP2* mutations were responsible for the drug resistance, a fragment exchange was carried out in which a sequenced 2.7-kb *Kpn*I-*Spe*I *TOP2* fragment from each mutant allele was used to replace the corresponding fragment in the *TOP2* gene of the wild-type plasmid (Fig. 2A). The resulting *TOP2* constructs, YCpDEDWOB10-R1F and YCpDEDWOB10-R2F, were isolated and were shown by DNA sequencing to carry the respective mutations. These plasmids were used to transform JN394t2–4 and the drug resistance phenotypes of the transformants were determined at 35° for a number of agents (Table 1). The mutant and wild-type alleles supported similar growth rates (not shown). Both mutant alleles conferred substantial resistance to doxorubicin, with an MLC of 50 μg/ml compared with 6 μg/ml for the wild-type allele (i.e., an 8-fold increase in resistance for both mutants). These results show conclusively that the G748E and A642S topoisomerase II alterations encoded by R1F and R2F plasmids are responsible for resistance. It follows that doxorubicin targets topoisomerase II in yeast.

Interestingly, plasmids R1F and R2F also conferred substantial resistance to amsacrine (each >16-fold) and to etoposide (each >7-fold). The results indicate that G748E and A642S changes mediate resistance to a number of cleavable complex forming drugs.

Overexpression and properties of the G748E and A642S topoisomerases II. Wild-type yeast topoisomerase

TABLE 1

Cross resistance profile at 35° of JN394t2-4 transformed with wild-type and mutant *TOP2* alleles

Plasmid	Mutation in topo somerase II	MLC		
		Doxorubicin	Amsacrine	Etoposide
			μg/ml	
YCpDEDWOB10		6	6	15
YCpDEDWOB10-R1F	G748E	50	>100	>100
YCpDEDWOB10-R2F	A642S	50	>100	>100

II was overexpressed using the multicopy YEpTOP2PGAL1 plasmid in which the yeast *TOP2* gene is under the control of an inducible *GAL1* promoter (Fig. 2B). Overexpression plasmids for the two mutant proteins were constructed by replacing a 2.2-kb *Kpn*I-*Avr*II restriction fragment from YEpTOP2PGAL1 with a corresponding fragment isolated from the mutant plasmids YCpDEDWOB10-R1 or YCpDEDWOB10-R2 (Fig. 2B)—the resulting proteins will be referred to as G748E topoisomerase II and A642S topoisomerase II, respectively. Each plasmid was transformed into a protease-deficient yeast strain (JEL1) and protein expression was achieved by induction with galactose to a final concentration of 2%. Each recombinant protein was purified essentially by the method of Worland and Wang (28). Briefly, the procedure involves cell lysis and ammonium sulfate precipitation of Polymin P extracts, followed by phosphocellulose chromatography. Topoisomerase II activity eluted at ~0.5 M KCl. The protein preparations were followed by SDS-polyacrylamide gel electrophoresis (Fig. 5). For preparation of the wild-type enzyme, a 160-kDa band was present in the cell extracts of induced JEL1 containing wild-type plasmid (Fig. 5, *lane 3*) that was absent in extracts from uninduced cells (Fig. 5, *lane 2*). The 160-kDa topoisomerase II protein in the peak fractions from the wild-type, G748E, and A642S protein purifications was >90% homogenous (Fig. 5, *lanes 4, 5,* and *6*, respectively). The catalytic activities of the purified proteins were assayed by relaxation of supercoiled pBR322 DNA. For all three proteins, relaxation was totally dependent on the presence of ATP, which indicated that the preparations were free of contaminating topoisomerase I activity. The specific activities of the mutant and wild-type proteins were similar at ~10^6 units/mg. Approximately 0.7 mg of purified topoisomerase II protein was obtained per liter of culture medium.

The recessive phenotype of doxorubicin resistance at 25° conferred by the R1 and R2 plasmid *TOP2* alleles is consistent with the drug acting via cleavable complex formation. Therefore, the ability of the mutant proteins to promote drug-induced DNA cleavage was examined using as substrate a linear *Eco*RI-*Hin*dIII fragment of pBR322 that had been labeled with ^{32}P at the 5′-*Eco*RI end. Equal amounts (100 ng) of wild-type or mutant topoisomerases II were incubated with radiolabeled DNA and 1 mM ATP in the absence or presence of anticancer drugs. DNA cleavage was induced by the addition of SDS followed by proteinase K digestion, and the DNA products were separated and examined by agarose gel electrophoresis-autoradiography. For doxorubicin, despite repeated attempts using a variety of enzyme, drug, and DNA concentrations, it proved extremely difficult to induce measurable DNA cleavage using yeast topoisomerase II. [It is known that doxorubicin-promoted DNA breakage by the mammalian topoisomerase II is also highly sensitive to conditions (13).[1]] As the mutant *TOP2* genes exhibited cross-resistance to amsacrine and etoposide, we decided to compare drug-induced DNA breakage by wild-type and mutant yeast proteins in the presence of these agents. The results are shown in Fig. 6. In the absence of amsacrine, there was no observable DNA breakage by any of the three yeast proteins (Fig. 6A, *lanes 2, 7* and *12*). The effects of amsacrine at 1, 10, 50 and 500 μg/ml are shown for the wild-type enzyme (*lanes 3–6*) and for the mutants (*lanes 8–11* and *13–16*). DNA cleavage was observed for the wild-type enzyme with ~50% of the labeled DNA cleaved at 10 and 50 μg/ml amsacrine (Fig. 6A, compare *lanes 2, 4* and *5*). The same drug levels promoted DNA breakage by the two mutant proteins, although much less efficiently. From inspection of the band intensities in Fig. 6A, it can be estimated that DNA cleavage by the mutant proteins was some 2–3-fold less efficient (Fig. 6A, compare *lanes 9, 10* and *14, 15* with lanes *4, 5*). Similar but more pronounced effects were seen for etoposide (Fig. 6B). Inclusion of etoposide at 0.5, 5, 50, and 500 μg/ml (Fig. 6B, *lanes 3–6*) resulted in substantial dose-dependent DNA cleavage by the wild-type enzyme. Breakage was detectable at 0.5 μg/ml (Fig. 6B, *lane 3*): more than 90% of the DNA was cut at 5 μg/ml (Fig. 6B, *lane 4*). By contrast, comparable levels of cleavage by either of the mutant proteins required 50 μg/ml etoposide (Fig. 6B, compare *lanes 4, 10* and *15*). Thus, 10-fold greater etoposide concentrations were needed with either mutant enzyme to induce comparable levels of DNA cleavage. It seems that both mutant proteins are defective in cleavable complex formation.

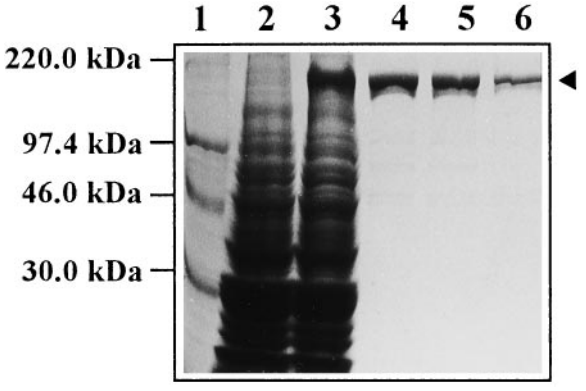

Fig. 5. Purification of wild-type, mutant G748E and mutant A642S yeast topoisomerase II proteins overexpressed in *S. cerevisiae* strain JEL1 bearing the corresponding *TOP2* alleles in plasmid YEpTOP2PGAL1. The 5% polyacrylamide-SDS gel shows the cleared lysate from yeast cells (carrying a plasmid-borne wild-type *TOP2* gene) uninduced (*lane 2*) and induced with galactose (lane 3). The peak fractions from the phosphocellulose columns of wild-type, G748E- and A642S-topoisomerase II protein preparations are in *lanes 4–6*. *Arrowhead,* topoisomerase II. Protein size markers (*lane 1*) and their molecular masses are shown.

Discussion

Progress in understanding doxorubicin action has been hindered by the presence in mammalian cells of two genetically distinct topoisomerase II isoforms, α and β (30, 31), by the recessive phenotype of topoisomerase II-mediated drug resistance, and by the multiple biological effects induced by the drug, including oxygen free-radical damage (10). Therefore, to examine the importance and nature of topoisomerase II-doxorubicin interactions in drug cytotoxicity, we have employed the yeast *S. cerevisiae* as a eukaryotic system whose essential single-copy *TOP2* gene allows the application of molecular genetic techniques. We isolated several dominantly acting *TOP2* resistance alleles and, by DNA sequence analysis and enzymatic characterization, showed that single point mutations of either G748E or A642S in topoisomerase II were sufficient to confer doxorubicin resistance and cross-resistance to other antitopoisomerase II agents. These re-

[1] Patel, S, V. J. Heaton and L. M. Fisher, unpublished observations.

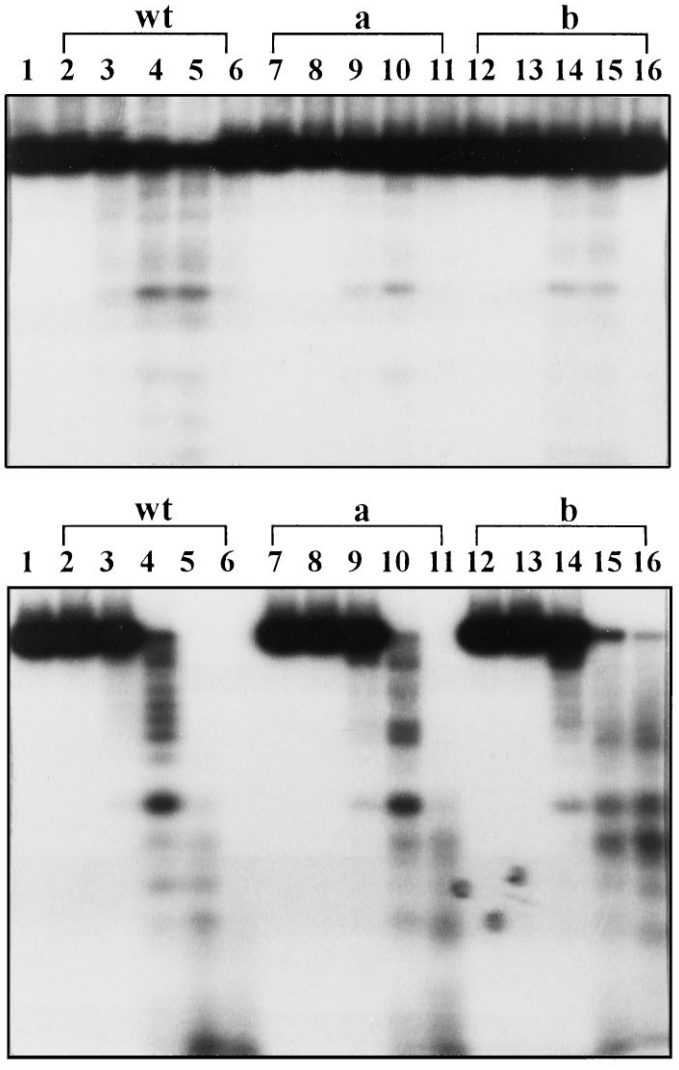

Fig. 6. Comparison of DNA cleavage promoted by wild type and drug-resistant topoisomerase II proteins. A *Hin*dIII-*Eco*RI DNA fragment from plasmid pBR322 was 5′-end ^{32}P-labeled at its *Eco*RI site and incubated with wild-type (*wt*), A642S (*a*) or G748E (*b*) proteins (each 100 ng) in the absence or presence of various concentrations of amsacrine (*top*) or etoposide (*bottom*) at 37° for 20 min. All reactions contained 2.5% DMSO. After incubation with SDS and proteinase K, samples were analyzed by electrophoresis in 1% agarose before autoradiography. *Top*, *lanes 2–16* contained enzymes. Each set of lanes, *2–6, 7–11,* and *12–16*, contained amsacrine at 0, 1, 10, 50, and 500 μg/ml, respectively. *Lane 1*, no enzyme addition plus 500 μg/ml amsacrine. *Bottom*, as in *top*, except etoposide replaced amsacrine in lanes *2–6, 7–11,* and *12–16* at 0, 0.5, 5, 50, and 500 μg/ml, respectively. *Lane 1*, no enzyme addition plus 500 μg/ml etoposide.

sults establish for the first time that doxorubicin targets topoisomerase II *in vivo*.

Doxorubicin elicits pleiotropic cellular effects, which suggests that it could have more than one cytotoxic action. Indeed, our results contrast with previous studies in yeast, which have implicated superoxide radicals and mitochondrial respiration in doxorubicin cytotoxicity (10, 11). The main evidence was that doxorubicin challenge of a wild-type yeast resulted in the isolation of drug-resistant petite mutants that exhibited deficient mitochondrial respiration. Furthermore, it could be shown that transfection of a cytochrome oxidase subunit IV gene into a petite mutant defective in this function, restored both respiration and wild-type doxorubicin susceptibility. From these results, it was suggested that doxorubicin-promoted free-radical generation (dependent on redox intermediates provided by mitochondrial respiration) is responsible for drug-induced killing (10). It is noteworthy that although we also used a *MATa* (i.e., haploid) yeast strain in our experiments, we did not isolate any mutants that were respiration deficient.

How can these striking differences in mutant selection be explained? If, as seems likely, both topoisomerase II and free radical mechanisms can induce cell killing, then, in principle, both the genetic background of the yeast and the selection conditions, particularly the growth status of the yeast, could influence which pathway predominates and therefore which type of resistant mutant is selected. First, in the JN394t2–4 system that we have used, potentially enhanced enzyme levels (arising from the plasmid-borne *TOP2* gene driven by a constitutive *DED1* promoter) in concert with the *rad52* double-strand break repair deficiency could favor killing by drug bound to topoisomerase II. Thus, the genetic background of the JN394t2–4 host may predispose somewhat to selection of topoisomerase II mutants compared with wild-type yeast and could obscure detection of other killing pathways. Second (our preferred hypothesis), the different growth conditions used in the experiments could critically influence the genetic outcome. We note that our topoisomerase II mutants were obtained by challenge of proliferating yeast cells in growth medium, whereas the respiration mutants were isolated from nondividing yeast cells in water. It is now known that transit through the cell cycle is essential for the cytotoxicity in yeast of drugs such as etoposide and amsacrine thought to act exclusively by targeting topoisomerase II (32). For example, yeast cells arrested in G_1 are resistant to killing by etoposide and amsacrine. It seems that active cellular processes occurring in S-phase (but probably not transcription), convert the ternary drug-topoisomerase II-DNA complex into a cytotoxic lesion, most likely an irreparable double-stranded DNA break (32). Because we used proliferating yeast cells, mutations in topoisomerase II that disrupt cleavable complex formation would affect the predominant killing mechanism and afford resistance to doxorubicin. For nondividing cells, killing through doxorubicin-induced free radicals would likely predominate, thus explaining the selection of respiration mutants. The dual killing mechanisms of doxorubicin and especially the role of cell proliferation are important factors that have not been previously considered. Indeed, it is interesting to speculate that topoisomerase II may be the predominant factor mediating doxorubicin cytotoxicity against dividing tumor cells whereas free radical damage could underlie its side-effects against the highly aerobic, terminally differentiated cardiac cells. Irrespective of these various considerations, the dominance of *TOP2*-mediated resistance to doxorubicin in JN394t2–4 allows the isolation of resistance alleles and the identification of residues in topoisomerase II potentially involved in drug action and resistance.

The G748E and A642S mutations in topoisomerase II encoded by the *TOP2* plasmids R1 and R2 each conferred an 8-fold increase in resistance to doxorubicin *in vivo*. Interestingly, the same mutations also conferred >16- and >7-fold cross-resistance to amsacrine and etoposide, respectively (Table 1). By overexpressing and purifying the mutant proteins from yeast (Fig. 5), it could be shown that each was

catalytically active but was defective in mediating DNA breakage stimulated by amsacrine or etoposide (Fig. 6). It seems that the mechanistic basis underlying resistance to doxorubicin, amsacrine, and etoposide *in vivo* is likely to be a reduction in drug induced cleavable complex formation.

One advantage of studying doxorubicin action on topoisomerase II from yeast is that the structure of a 92-kDa fragment of the enzyme (residues 410-1202) has recently been solved (20). This region constitutes the DNA breakage-reunion domain of the enzyme that contains catalytic Tyr783 and is likely to be involved in cleavable complex formation. We have used the available coordinates to display the high resolution structure of this fragment and to locate the positions of doxorubicin-resistance mutations (Fig. 7). The fragment crystallizes as a heart-shaped dimer that forms a 5.5-nm hole into which the transported DNA helix (T segment) is passed during catalysis of strand crossing. The catalytic tyrosines (Fig. 7, *yellow*) that interact with the G DNA segment lie at the top of the protein structure within a subdomain (residues 702–790) that forms a structure similar to that of the CAP of *E. coli*, a known DNA binding protein. Interestingly, the G748E mutation lies in this CAP-like region at one end of a helix designated A′α4, which is analogous to the DNA recognition helix of the CAP and which binds to the major groove of DNA. G748 also lies close to the catalytic tyrosine; the crystal structure data suggests that its mutation to Glu in the doxorubicin-resistant protein directly disrupts formation of the cleavable complex. It is significant that helix A′α4 also contains G738, the mutation of which to Asp confers partial resistance to etoposide and cross-resistance to the fluoroquinolone CP-115,953 but not amsacrine (24). Strikingly, G738 and G748 bracket S741, the amino acid that is homologous to S83 of *E. coli* GyrA, a residue whose alteration confers resistance to fluoroquinolones in bacteria. Recently, it has been found (33) that an S741W change in yeast topoisomerase II conferred resistance to fluoroquinolones but hypersensitivity to etoposide. Thus, it seems that residues 738–748 including G748 play a critical role in the binding of a variety of structurally dissimilar drugs.

Residue A642 lies in a region of the 92-kDa fragment (residues 632–682) that was not resolved in the crystal structure presumably because of conformational mobility. However, this stretch of polypeptide connects the bottom of helix B′α8 (Fig. 7B, *red helix to left of Y783*) to helix A′α1 (Fig. 7B, *end-on below arrow for G748*). Although the connecting peptide is not shown in Fig. 6B, it seems that A642 lies spatially close to the CAP-like DNA binding domain implicated in cleavable complex formation. Thus, the crystal structure suggests that both A642 and G748 may be directly involved in doxorubicin action. Consistent with this idea, we found these residues to be frequently mutated in the resistant alleles we examined: of eight *top2* plasmids we isolated, three had the A642S mutation and five had the G748E change (results not shown). Interestingly, recent work selecting for resistance to amsacrine has identified A642T or A642G mutations in yeast topoisomerase II as conferring high level resistance (29). Similar to the doxorubicin-resistant A642S mutant protein, the purified A642G mutant protein also promoted significantly less DNA cleavage in the presence of mAMSA or etoposide compared with wild-type yeast topoisomerase II. These results indicate that mutations at a small subset of residues including A642 and G748 can confer resistance to different anticancer agents, suggesting a commonality in the mode of drug interaction with topoisomerase II.

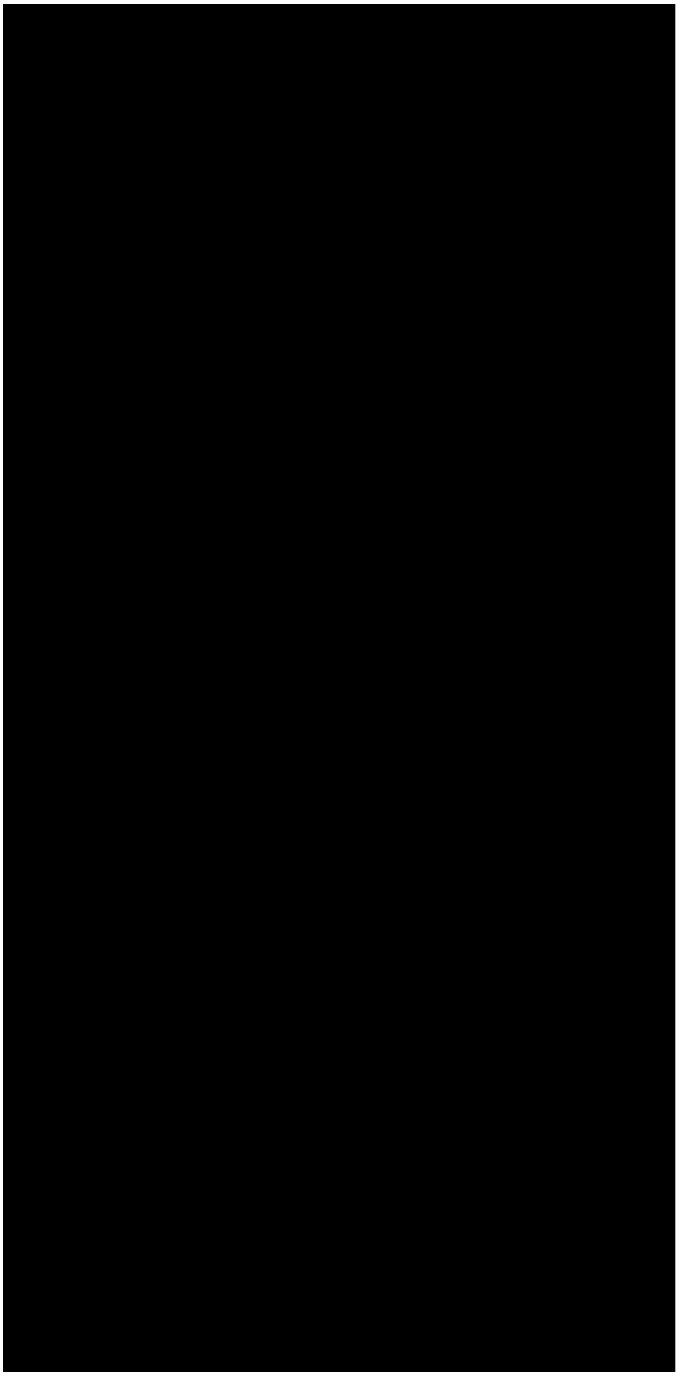

Fig. 7. Doxorubicin resistance mutations in yeast DNA topoisomerase II lie in or adjacent to the CAP-like DNA binding region of the protein. A, Crystal structure of a 92-kDa fragment (residues 410-1202) containing the DNA breakage-reunion region of the yeast enzyme. The subunits of the dimer are colored *blue* and *red*. The positions of the catalytic tyrosines (Y783) involved in DNA breakage-reunion are shown in *yellow*. *Arrows,* position of G748 (*yellow*) at the end of helix A′α4, the putative DNA recognition helix of a CAP-like subdomain. Mutation of this residue to glutamate confers resistance to doxorubicin. B, Expanded view of the CAP-like putative DNA binding domain. Symbols as in A. Residue A642, whose mutation also confers doxorubicin resistance, lies in a region (residues 632–682) that is close to the CAP domain but is not resolved in the crystal structure (see text).

Finally, the catalytic domains of mammalian topoisomer-

ases II, including human α and β isoforms, share close sequence homology with the yeast protein (34). Moreover, though efflux pumps and other factors can be associated with drug resistance (35–36), it is known that doxorubicin can induce DNA breakage in mammalian cells and that changes in the structure or activity of topoisomerase II can influence cell sensitivity to the drug (14, 36–39). Though genetic proof is lacking for the mammalian systems, these observations point to topoisomerase II as an important antitumor target for doxorubicin. Recently, both human *TOP2α* and *TOP2β* constructs were shown to complement in yeast JN394t2–4 (30, 40). Thus, the approaches described in this paper should be applicable in defining the molecular basis of doxorubicin binding to its topoisomerase target(s) in tumor cells.

Acknowledgments

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Send reprint requests to: Prof. Mark Fisher, Department of Cellular and Molecular Sciences, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK. E-mail: lfisher@sghms.ac.uk