A Mutation in Human Topoisomerase II α Whose Expression Is Lethal in DNA Repair-deficient Yeast Cells*

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Type II DNA topoisomerases are ATP-dependent enzymes that catalyze alterations in DNA topology. These enzymes are important targets of a variety of antibacterial and anti-cancer agents. We identified a mutation in human topoisomerase II α, changing aspartic acid 48 to asparagine, that has the unique property of failing to transform yeast cells deficient in recombinational repair. In repair-proficient yeast strains, the Asp-48 → Asn mutant can be expressed and complements a temperature-sensitive top2 mutation. Purified Asp-48 → Asn Top2α has relaxation and decatenation activity similar to the wild type enzyme, but the purified protein exhibits several biochemical alterations compared with the wild type enzyme. The mutant enzyme binds both covalently closed and linear DNA with greater avidity than the wild type enzyme. hTop2α(Asp-48 → Asn) also exhibited elevated levels of drug-independent cleavage compared with the wild type enzyme. The enzyme did not show altered sensitivity to bisdioxopiperazines nor did it form stable closed clamps in the absence of ATP, although the enzyme did form elevated levels of closed clamps in the presence of a non-hydrolyzable ATP analog compared with the wild type enzyme. We suggest that the lethality exhibited by the mutant is likely because of its enhanced drug-independent cleavage, and we propose that alterations in the ATP binding domain of the enzyme are capable of altering the interactions of the enzyme with DNA. This mutant enzyme also serves as a new model for understanding the action of drugs targeting topoisomerase II.

Type II topoisomerases catalyze changes in DNA topology in an ATP-dependent mechanism (for recent reviews see Refs. 1 and 2). Changes in DNA topology require cleavage of the DNA backbone. For eukaryotic type II topoisomerases, DNA cleavage results from a transesterification reaction between phosphates in the DNA backbone and catalytic tyrosines from each of the two identical subunits of the enzyme, resulting in a protein-bridged DNA double-strand break. ATP binding and hydrolysis are required for the enzyme to carry out catalytic changes in DNA topology, although the high energy cofactor does not play a direct role in the breakage/reunion reaction of the enzyme. Current models suggest that ATP hydrolysis is required for regeneration of the enzyme to complete the catalytic cycle (3, 4), although it has been suggested that ATP hydrolysis may also accelerate strand transfer reactions (5). In addition to an absolute requirement for type II topoisomerase activity in processes such as chromosome segregation (6, 7), these enzymes are targeted by a number of antibacterial and anticancer agents in widespread clinical use. Drugs targeting DNA topoisomerases principally act by increasing the levels of the covalent complex that is an intermediate of the enzyme reaction (reviewed in Refs. 2, 8, and 9).

The overall domain organization of prokaryotic and eukaryotic type II topoisomerases is similar, although the eukaryotic enzyme is a homodimer, and the prokaryotic enzymes are heteromultimers. The subunits of the prokaryotic topoisomerase II are homologous to regions of the monomeric subunit of topoisomerase II. The ATPase domain of the protein occurs in the amino-terminal 400 amino acids of topoisomerase II (10, 11), whereas the central 700 amino acids are required for breakage/reunion reactions and include a CAP homology domain that is involved in DNA binding (12–14), as well as residues involved in binding the divalent cation that is essential for the cleavage and religation reactions. High resolution electron microscopy suggests that the amino-terminal domain sits on top of the breakage reunion domain (15–17), suggesting that conformational changes within the protein are needed for communication between different protein domains (18).

Anticancer agents such as etoposide inhibit type II topoisomerases by stabilizing the covalent enzyme-DNA intermediate of the enzyme reaction (18, 20). The initial ternary complexes formed between drugs such as etoposide and the enzyme-DNA covalent adduct are mainly reversible (21, 22). However, cellular processing of the complexes into an irreversible covalent adduct elevates the intracellular level of DNA damage leading to cell death (8). The result of topoisomerase II inhibition by complex-stabilizing agents is conversion of the normal catalytic activity of an essential enzyme into a cellular toxin; hence these agents are termed topoisomerase poisons.

Although the breakage/reunion domain is likely to be the principal focus of action of topoisomerase II poisons, the nucleotide cofactor also influences the formation of stabilized covalent complexes. Liu and co-workers (23, 24) have suggested that topoisomerase II poisons can be divided into ATP-dependent and ATP-independent classes. Etoposide, which can inhibit both the ATPase and decatenation activities of topoisomerase II, is an example of an ATP-dependent poison, because ATP stimulates the level of trapped covalent
complexes in the presence of the drug. By contrast, the level of
covalent complexes formed in the presence of ATP-inde-
dependent poisons such as aminoflare are not substantially
altered by the presence of nucleotides (23, 24). The influence
of ATP on DNA cleavage stimulation by topoisomerase poi-
ses is consistent with communication between the amino-
terminal ATPase domain, and the domains involved in DNA
binding, cleavage, and strand passage.

In this work we describe a novel mutation in the amino-
terminal domain of human topoisomerase II α that is toxic in
repair-deficient yeast cells. The mutant enzyme has normal
strand passage activity in vitro, but has enhanced DNA binding
for both linear and covalently closed DNA, and forms elevated
levels of drug-independent cleavage complexes. The properties
of the mutant protein suggest that its alteration affects com-
munication between the ATPase and cleavage/relation
domains of the protein. This mutant protein is also a novel tool
for understanding the biochemical mechanisms of drugs targeting
topoisomerase II.

EXPERIMENTAL PROCEDURES

Yeast Strains—Experiments to assess the in vivo effects of topoi-
 somerase II alleles used yeast strains derived from JN362a (MATa,
ura3-52, leu2, trp1, his7, ade1-2, ISE2, rad52-leu2, top2-4). Isogenic
derivatives of this strain with the temperature-sensitive top2-4 allele,
disruptions of the RAD52 gene, and top2-4 rad52:LEU2 double mu-
tants have been described previously (25). Assessment of the comple-
mentation of null alleles of TOP2 was carried out by using a strain
bearing a heterozygous deletion of the yeast TOP2 gene generated from
the Saccharomyces deletion project (BY745 TOP2/top2-4 KANMX4)
obtained from Open Biosystems. Wild type human topoisomerase II α
and the mutant enzyme were purified using the previously described
strain JEL1t1 (trp1, leu2, ura3-52, pbr1-1122, pep4-3, his3::
gAL10GAL4, top1:LEU2) (18, 26).

Plasmids—For experiments assessing the properties of human topoi-
somerase II in yeast, the human enzyme was expressed by using the
vector pMJ1 (27). This vector includes the open reading frame of human
Top1p under the control of the yeast TOP1 promoter. The plasmid
pYX113/TOPII was used for initial experiments for overexpression of
human topoisomerase II α in yeast for enzyme purification (24, 28). However,
this plasmid generates low yields of protein (~1 mg/liter of culture). We
therefore modified the previously described expression plasmid for
human topoisomerase II α to pYEPw68 (29). pYEPw68 expresses human topoisomerase II α from the GAL1 promoter, but the coding sequence in that plasmid
lacks the first 28 amino acids of human topoisomerase II α and contains instead the
first six amino acids of yeast Top2p. Given the close proximity of
Asp-48 to the region deleted in yEPwob6, we modified the plasmid to
replace the first 28 amino acids of human topo II α with the first 6
amino acids of yeast Top2p. The TOPO2α sequence of pCM1 was confirmed by DNA sequencing. pCM1 efficiently expresses hTop2p with yields of ~5 mg of purified protein/ liter of culture (data not shown). Purification of topoisomerase II α was carried out as described previously (28, 30).

Yeast Transformants—Yeast transformation was carried out using
the lithium acetate procedure (31). The yeast plasmid YCP50 (32) was
used as a transformation control. All transformations used 5 μg of plasmid DNA. Plasmid DNA was prepared in Escherichia coli using the
strain DH5α and purified from E. coli using Qiagen kits, following
manufacturer’s instructions.

Oligonucleotide-directed Mutagenesis—The Asp→48 → Asn muta-
tion was introduced into pMJ1, pYX113/TOPII, and pCM1 using the
QuickChange mutagenesis kit (33, 34). The two mutagenic primers
were used: ATTTTGGCCCGCCCAACACCTATCGGTTTC and GAA-
CCATGGTGATTGGTGCGGAGAAAT, where the underlined nucleotides
indicate the changed nucleotides. Primers that change Tyr-
806 to Phe were described previously (35). All mutations were con-
firmed by DNA sequencing.

Measurement of Topoisomerase II Enzymatic Activity—DNA topoi-
somerase II relaxation assays were carried out as described previously (34). Decatenation was carried out by using a similar procedure except
kinetoplast DNA from Crithidia fasciculata was used as the substrate.
The rate of enzyme-catalyzed ATP hydrolysis was measured by using a
coupled enzyme assay as described previously (36). Samples containing
DNA included 100 μg/ml salmon sperm DNA.

Assays for Closed Clamp Formation by Topoisomerase II—Two as-
says were used to assess the formation of salt-stable closed clamps by
topoisomerase II α. Filter binding assays were carried out as described
previously (3). Salt-stable closed clamp formation was also determined
by analytical ultracentrifugation as described previously (35, 37).
Briefly, 6 μg of an 8.6 kb plasmid was incubated at 37 °C with 6 μg of
human topoisomerase II α or D48N mutant protein in a 40-μl reaction
containing 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 100 mM NaCl,
10 mM MgCl2, 0.1 mM EDTA, and 50 μg/ml bovine serum albumin. Some
reactions also contained a cofactor ADPNNP at a concentration of either
50 μM or 0.5 mM. After the reaction mixture was incubated for 15 min,
reactions were terminated by adding 30 μl of saturated CaCl2 solution and
0.1 M of 10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA. The reaction mixture
along with a balance solution containing all ingredients except for DNA
and enzyme were spun at 40,000 rpm at 20 °C for 30 h in an analytical
ultracentrifuge (XL-A ultracentrifuge, Beckman Instruments). The ab-
sorbance at 260 or 280 nm was scanned across the gradient to monitor
the distribution of DNA in the density gradient.

Electrophoretic Mobility Shift Assay—An electrophoretic mobility
shift assay was used to measure the binding of DNA to topoisomerase II.
The assay was performed as described previously (38) with minor
modifications (34). Briefly, a 10-μl reaction mixture containing 10 mM
Tris-HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 2.5% glycerol, 88.5 μg of
supercoiled or linearized pUC18, and the indicated concentrations of
purified Top2p were incubated at 37 °C for 10 min. The samples were
mixed with 1 μl of loading buffer containing 0.67% orange G and 60% su-
corese and subjected to electrophoresis at 6 Vcm in a 1% agarose gel
in 1× TAE buffer (40 mM Tris acetate and 10 mM EDTA). The gel was
stained with ethidium bromide and photographed under UV light.

DNA Cleavage by Topoisomerase II—DNA cleavage assays using plasmid
DNA as a substrate was determined using the procedure described
by Robinson and Osheroff (39) with minor modifications. In this assay,
supercoiled plasmid DNA was incubated with purified enzyme, and
the enzyme reaction was stopped with SDS to trap enzyme covalently
bound to DNA. DNA cleavage was measured by determining the
amount of DNA cleaved by linearized DNA, which was formed following protease K
cleavage of the protein covalently bound to the DNA ends. Cleavage assays
were incubated in a total volume of 100 μl for 15 min at 37 °C and
terminated with 25 μl of 10% SDS. Samples were digested with 400 μg
of proteinase K at 50 °C overnight, except where indicated, phenol-
extracted, precipitated with ethanol, resuspended in TE buffer, and
analyzed by agarose gel electrophoresis.

DNA cleavage sites were examined using 32P-end-labeled pUC18
DNA. Briefly, 5 μg of EcoRI-digested pUC18 was labeled by a fill-in
reaction with [32P]dATP, non-radioactive nucleotides, and Klenow for
15 min at room temperature. After ethanol precipitation, the [32P]-
pUC18 was digested with BamHI for 3 h at 37 °C in order to create a
uniquely end-labeled molecule. After digestion with BamHI, the DNA
was purified over a chroma spin STE-30 column (Clontech). Topoi-
somerase II cleavage reactions containing 150 ng of [32P]-pUC18, 100 mM
KCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 8 mM MgCl2, 2 mM ATP, 6.5
mM 2-mercaptoethanol, and 50 mg/ml acetylated bovine serum albumin
were incubated with various concentrations of protein in a final volume
of 100 μl for 10 min at 37 °C. Some reactions also contained 10 μg/ml
ethoposide. Reactions were terminated with 12.5 μl of water followed by ethanol
precipitation. Samples were resuspended in 10 μl of loading buffer and
subjected to electrophoresis at 6 Vcm in a 1.5% agarose gel for 15 h in
1× TAE buffer. The gel was dried, and labeled fragments were visual-
ized either by exposure to film or with a Storm 860 PhosphorImager
(Amersham Biosciences).

RESULTS

Expression of hTop2α(Asp→48 → Asn) Is Lethal in Yeast
Strains Lacking RAD52—We reported previously (33) that mu-
tations of human topoisomerase II α changing amino acid Tyr-50 to Phe
result in an enzyme that is insensitive to bisdioxopiperazines.
Because this region is conserved in all eukaryotic type II topoi-
somases, we explored the effects of mutating other amino
acids in the vicinity of Tyr-50. We introduced mutations in the
vector pMJ1, which expresses human topoisomerase II α from the
yeast TOP1 promoter, and transfected the mutated plas-
mids into yeast cells carrying a temperature-sensitive top2-4

3 The abbreviations used are: topo, topoisomerase; ADPNNP,
5′-adenyl-β-, γ-imidodiphosphate.
allele and a deletion of the RAD52 gene. One mutation in this region, changing Asp-48 to Asn, could not be transformed into rad52 cells. We carried out quantitative transformation of plasmids containing either wild type hTop2as or hTop2as(Asp-48 \rightarrow Asn) using the isogenic strains JN362a (RAD52) and JN394 (rad52). A typical result is shown in Fig. 1. The RAD52 and rad52 strains could be readily transformed with a yeast vector (yCP50) or with a plasmid expressing wild type hTop2as. However, no transformants were obtained when rad52 cells were transformed with pMJ1(D48N). In our previously published experiments, we could introduce pMJ1-carrying Y50F into rad52 cells. Taken together these results indicated that expression of hTOP2/Asp-48 \rightarrow Asn) was deleterious in recombination-deficient yeast cells. We also transformed pMJ1(D48N) into JN362at2-4 cells. This strain is isogenic to JN362a but carries a temperature-sensitive topoisomerase II allele (top2-4). Transformation of either pMJ1 or pMJ1(D48N) supported growth of JN362at2-4 at 34°C, a non-permissive temperature for the top2-4 allele (data not shown).

To further assess whether pMJ1(D48N) could complement the in vivo function of yeast topoisomerase II, we examined whether pMJ1(D48N) could complement a null allele of yeast topoisomerase II. We introduced pMJ1, pMJ1(D48N), or an empty vector into strain BY4743 (TOP2/top2-\Delta0::KANMX4), a diploid strain carrying a heterozygous deletion of the entire open reading frame of TOP2 (40). The strains bearing each of these plasmids was sporulated, and the resulting ascii were micro-dissected to recover individual haploid spores. Viable spores carrying the top2-\Delta0::KANMX4 allele were obtained with both pMJ1 and pMJ1(D48N) but not with the empty vector (data not shown). There was no obvious difference in the growth rate of cells carrying either the wild type or Asp-48 \rightarrow Asn allele of hTop2as. These results indicate that hTop2as(Asp-48 \rightarrow Asn) can support all of the essential functions of yeast topoisomerase II and strongly suggested that the Asp-48 \rightarrow Asn allele encodes an active protein, a result confirmed below.

We also examined other amino acid changes of Asp-48 for the ability to confer rad52-dependent lethality or the inability to complement a top2-4 allele of yeast Top2. We introduced Asp-48 \rightarrow Glu and Asp-48 \rightarrow Ala mutations into pMJ1, and we examined transformation into JN362t2-4 (RAD52) and JN394 (rad52) cells. Both of these mutations could be introduced into JN394 (rad52) cells, indicating that these alleles did not have the same property as the Asp-48 \rightarrow Asn allele. Furthermore, both alleles could complement the top2-4 allele, suggesting that they did not compromise Top2 activity in vivo (data not shown).

We next determined whether the rad52-dependent lethality of the Asp-48 \rightarrow Asn allele required the ability of the enzyme to cleave DNA. We constructed a double mutant of human topoisomerase II having both Asn substituted for Asp at residue 48 and Phe substituted for the catalytic Tyr-805. Expression of the hTop2as(Asp-48 \rightarrow Asn/Tyr-805 \rightarrow Phe) double mutant in a rad52-deleted yeast strain did not substantially reduce the transformation efficiency (Fig. 1B), indicating that enzyme activity was required for the lethality.

We also examined several other DNA repair-deficient strains to determine what other repair functions were required for cell survival when the Asp-48 \rightarrow Asn allele was expressed. Lethality was observed in mutants deficient in either rad50 or rad54, indicating that other defects in recombination repair beside rad52 resulted in an inability to viably express hTop2as(Asp-48 \rightarrow Asn). By contrast, cells lacking rad2 (defective in excision repair), rad6 (defective in post-replication repair), or rad9 (defective in a DNA damage checkpoint) all could be transformed with pMJ1D48N at efficiencies comparable with wild type cells (data not shown). These data indicate that the major DNA repair pathway required for expression of D48N is recombination repair.

Enzymatic Activity of hTop2as(Asp-48 \rightarrow Asn)—We next introduced the Asp-48 \rightarrow Asn mutation into a vector appropriate for overexpression, and we purified the mutant protein. Fig. 2A shows an SDS gel of the purified hTop2as wild type and hTop2as(Asp-48 \rightarrow Asn)-purified proteins. As shown in Fig. 2B, hTop2as(Asp-48 \rightarrow Asn) has essentially the same activity for relaxation of negatively supercoiled DNA as the wild type enzyme. Similar results were obtained assessing decatenation of kinetoplast DNA (data not shown).

Because the mutation occurs in a region of the protein known to be involved in ATP binding, we also characterized the ATPase activity of the mutant protein. In the absence of DNA, hTop2as(Asp-48 \rightarrow Asn) has the same ATPase activity as the wild type enzyme. Most interesting, the mutant protein was defective in DNA-stimulated ATPase activity, compared with the wild type protein (Fig. 3). However, in results to be reported elsewhere, we observed a similar deficiency in DNA-stimulated ATPase in other mutants such as hTop2as(Tyr-50 \rightarrow Phe) that can be functionally expressed in rad52 cells. Therefore, we do not think that the inability to transform repair-deficient strains relates to the altered ATPase activity of hTop2as(Asp-48 \rightarrow Asn).

We also assessed the ability of the enzyme to form a salt-stable complex with circular DNA in the absence of cofactors, in the presence of ATP, or in the presence of a non-hydrolyzable ATP analog. Closed clamp formation was assessed by analytical ultracentrifugation in CsCl. Fig. 4 displays the results...
obtained without cofactor or in the presence of ADPPNP. Both the wild type and Asp-48 → Asn mutant proteins incubated with DNA but without any cofactor result in a single DNA peak, consistent with a lack of enzyme binding to DNA in CsCl. A series of bands of lower buoyant density is seen when either enzyme is incubated with ADPPNP; however, there is a greater reduction in the free DNA and a greater shift toward lighter densities for samples that included hTop2α(Asp-48 → Asn). Similar results were obtained using a filter binding assay (data not shown). Although greater levels of complexes were seen with ADPPNP and hTop2α(Asp-48 → Asn), there were no detectable complexes in the absence of ATP. Furthermore, there was no difference in the levels of salt-stable complexes detected by ultracentrifugation in the presence of the bisdioxopiperazine ICRF-187 (Fig. 5).

**hTop2α(Asp-48 → Asn) Has a Greater Affinity for Both Covalently Closed and Linear DNA**—Both the filter binding and ultracentrifugation assays detect complexes stable in >2 M salt solutions. To assess whether hTop2α(Asp-48 → Asn) could form salt-labile complexes, we investigated the DNA binding of the protein by using an electrophoretic mobility shift assay (Fig. 6). Fig. 6A shows the results obtained with covalently closed pUC18, and Fig. 6B shows the results with pUC18 that had been linearized by restriction digestion. For supercoiled DNA with wild type hTop2α, all the DNA is shifted to a retarded mobility when 1.6 μg of protein is added. By contrast, addition of 0.4 μg of hTop2α(Asp-48 → Asn) is sufficient to induce the same mobility shift. Most important, a similar differential is seen when the DNA was linearized prior to the binding assay. Consistent with previous reports (41), we observed that wild type protein has a lower affinity for linear DNA, and 1.6 μg of protein was insufficient to completely shift the linear DNA. For hTop2α(Asp-48 → Asn), a complete loss of the unshifted band is seen at about 0.8 μg of protein. Taken together, these results indicate that hTop2α(Asp-48 → Asn) has altered interaction with DNA but that altered interaction is unlikely to be a closed clamp because an altered affinity is seen with both linear and covalently closed DNA.

**D48N-hTop2 Forms Elevated Levels of Cleavage Complexes in Vitro**—We next investigated the ability of the hTop2α(Asp-48 → Asn) mutant to stabilize the DNA-TOP2 covalent complex in the absence of any agent that could stimulate DNA cleavage. In this assay, enzyme is incubated with covalently closed DNA, and cleavage is monitored by the enzyme-mediated formation of protein-linked linear DNA. In Fig. 7, with wild type enzyme, a faint linear band is seen with 1 μg of protein, and a prominent signal is seen at 5 μg of protein. It should be noted that although only a linear band is seen with 5 μg of protein, most of the DNA is intact and is present as catenated circles that do not enter the gel. This was demonstrated by treating the samples again with a low concentration of topoisomerase II, which results in decatenation of the circles and the appearance of a monomer length circle (data not shown). As expected, addition of 10 μg/ml etoposide to samples containing 1 or 5 μg of protein enhanced the formation of linear DNA. Additionally, if the samples were not pretreated with
proteinase K prior to electrophoresis, no linear band was seen (data not shown). A much greater level of drug-independent cleavage was seen with hTop2α(Asp-48 → Asn) protein. A weak signal of linear DNA was seen at 0.2 μg of protein, and a robust signal was seen at 1 μg of protein, a 5-fold lower concentration than what was needed to observe a signal with wild type protein. The substantially lower concentration of hTop2α-(Asp-48 → Asn) protein required to induce the formation of linear DNA indicates that the mutant protein formed elevated levels of covalent complexes, even in the absence of inhibitors.

To further confirm that hTop2α(Asp-48 → Asn) formed elevated levels of covalent complexes in the absence of inhibitors, and to explore the nature of the DNA preference for hTop2α(Asp-48 → Asn)-mediated cleavage of DNA, we exam-
above DNA linearized with EcoRI. The amount of added protein is indicated.

Drug-independent cleavage complexes

Fig. 6. hTop2α(Asp-48 → Asn) protein binds DNA with a greater avidity than the wild type (WT) enzyme. Binding of wild type topoisomerase II or the Asp-48 → Asn mutant protein to covalently closed (A) or linear DNA (B) was assessed by using an electrophoretic mobility shift assay. The amount of added protein for each sample is indicated in micrograms above each lane.

Fig. 7. hTop2α(Asp-48 → Asn) protein forms elevated levels of drug-independent cleavage complexes. Cleavage complex formation was assessed by using a gel cleavage assay. The lane labeled U contains uncut plasmid DNA, and the lane labeled L contains pUC18 DNA linearized with EcoRI. The amount of added protein is indicated above each lane. Lanes labeled with an E also contained 10 μg/ml etoposide. WT, wild type.

Fig. 8. DNA cleavage patterns by hTop2α (Asp-48 → Asn) are similar to those formed with wild type hTop2α. DNA cleavage patterns were assessed by examining DNA cleavage using uniquely end-labeled pUC18 DNA, followed by agarose gel electrophoresis. The amounts of added protein and the presence of etoposide are indicated above each lane. The thick band at the top of the illustration is uncut pUC18 DNA. WT, wild type.

Examination of the cleavage patterns shows no obvious differences, when samples of hTop2α were compared with hTop2α(Asp-48 → Asn) in the absence of etoposide. Similarly, there was also no qualitative difference in the pattern seen between the two proteins when incubated in the presence of etoposide. As has been noted previously, there is a difference in patterns seen in the absence and presence of etoposide (42), and the differences are preserved between hTop2α or hTop2α(Asp-48 → Asn). Taken together, the results of this experiment confirmed that hTop2α(Asp-48 → Asn) showed elevated drug-independent cleavage compared with hTop2α, but the increase in cleavage did not appear to arise from cleavage of a new set of sites by the mutant protein.

**DISCUSSION**

We have described a mutation in human Top2α that confers profound changes in the in vivo and in vitro characteristics of the enzyme. Expression of the Asp-48 → Asn mutant enzyme complements a yeast strain with a temperature-sensitive top2-4 allele, demonstrating that the mutant enzyme is active in vivo. Nonetheless, plasmids expressing the hTop2α(Asp-48 → Asn) fail to transform yeast strains defective in homologous recombination, suggesting that the mutant enzyme generates DNA damage or interferes with DNA metabolism in a way that results in a requirement for recombination pathways. These in vivo effects of the hTop2α(Asp-48 → Asn) enzyme are consistent with the observed biochemical characteristics of the purified protein. Specifically, hTop2α(Asp-48 → Asn) exhibits elevated levels of drug-independent DNA cleavage. The hTop2α(Asp-48 → Asn) mutant therefore is formally similar to the mutations in topoisomerase I described by Bjornsti and co-workers (43, 44), which are also lethal in yeast strains defective in homologous recombination and which also exhibit elevated levels of drug-independent DNA cleavage in vitro.

The hypothesis that enhanced DNA cleavage is responsible for the lethality in recombination-deficient strains is supported by the observation that expression of an enzyme that carries the D48N mutation along with a second mutation that blocks the ability of the enzyme to cleave DNA can be expressed in
recombination-deficient cells. Additionally, the level of DNA cleavage seen with the D48N mutant enzyme is approximately the same as in the presence of 5 μM etoposide, a drug concentration that severely affects the growth of yeast cells expressing human topoisomerase II (27). Thus, the enhanced DNA cleavage exhibited by the D48N mutant should be sufficient to kill recombination-deficient yeast cells.

Three important biochemical properties of the enzyme are altered in the hTop2α(Asp-48 → Asn) protein. First, the enzyme is defective in DNA-stimulated ATPase activity. We do not think that this alteration is responsible for the enhanced DNA binding or elevated DNA cleavage. We have isolated several other mutations in the amino-terminal domain of hTOP2a that have essentially identical alterations in ATPase activity. One example is a mutation that changes Tyr-50 to Phe.2 None of the other mutations are inviable in repair-deficient yeast strains (33, 45), nor do they exhibit enhanced DNA binding in vitro (46).

A property related to the altered ATPase of the enzyme is the enhanced level of the closed clamp form of the enzyme seen in the presence of non-hydrolyzable ATP analogs. This result could arise from an alteration in the ability to form a closed clamp. However, we were unable to detect significant levels of stable closed clamps in the absence of any cofactor or in the presence of differing ATP concentrations.

A second important alteration in the hTop2α(Asp-48 → Asn) mutant enzyme is an enhancement of DNA binding. Because enhanced DNA binding is seen with both linear and covalently closed DNA, we do not think that the altered DNA binding can be explained by the formation of a closed clamp. Furthermore, the gel retardation assays were performed in the absence of any ATP analog. Therefore, we suggest that the intrinsic DNA binding is enhanced in the hTop2α(Asp-48 → Asn) mutant protein.

An important DNA binding domain has been suggested to be the helix-turn-helix domain found in the gyrA homology domain (12). Although no crystal structure of a eukaryotic topoisomerase II containing both the amino-terminal ATPase domain and the helix-turn-helix domain that carries out DNA cleavage has been obtained, electron microscopy studies have suggested that the two domains are not normally in close proximity (17). If the alteration in DNA binding arises from changes in the binding affinity of the helix-turn-helix domain, then the enzyme must normally be quite sensitive to conformational changes arising from ATP binding and hydrolysis. Lindsley and colleagues (5, 36) carried out kinetic studies on the ATPase activity of yeast topoisomerase II and concluded that the ATP hydrolysis by the identical subunits of the holoenzyme occurred in two sequential steps. They proposed that the first ATP hydrolysis might stimulate strand passage by the enzyme. This model would require intimate communication between the ATPase domain and the helix-turn-helix domain. Most interesting, in subsequent work (48), they suggested that etoposide inhibits ATP hydrolysis after the first ATP is hydrolyzed. This again suggests that the ATPase domain can “sense” events occurring in the cleavage religation domain of the enzyme. Our results are consistent with their hypotheses, although they do not demonstrate how the communication may take place.

The considerations discussed above for the enhanced DNA binding by the Asp-48 → Asn mutant also apply to the third major biochemical alteration, elevated drug-independent cleavage. The elevated DNA cleavage could simply be because of enhanced DNA binding by the D48N mutant protein. Alternatively, a defect in communication between the ATPase domain and the DNA binding and cleavage domain could lead both to enhanced DNA binding and elevated DNA cleavage. Distinguishing between these two possibilities will require the identification of additional mutants that separate these two biochemical effects.

An additional question that is raised by the Asp-48 → Asn mutant is to what extent does the enhanced DNA cleavage of the mutant mimic the properties of the wild type enzyme treated with topoisomerase poisons? Topoisomerase II poisons are small molecules that lead to elevated levels of topoisomerase II covalently bound to DNA (2). Because these agents are used clinically as both anti-bacterial and anti-cancer agents, a detailed biochemical understanding of their mechanism of action would be of considerable value. It has been suggested that for many topoisomerase poisons, ATP is required for drug action (23, 24). Gasser and colleagues (49) have suggested that etoposide binds to the ATPase domain of topoisomerase II, although mutations that affect sensitivity to etoposide have not been mapped to the ATPase domain but instead map to amino acids in close proximity to the DNA binding/cleavage domain (20). A detailed understanding of drug binding to topoisomerase II will be required to resolve this question.

In addition to its interesting biochemical properties, the Asp-48 → Asn mutant should also be of great use in elucidating pathways that are required for tolerating and repairing topoisomerase II-mediated DNA damage. The mutations in topoisomerase I have been useful in identifying novel functions required for cell survival in the presence of top I poisons (47, 50, 51). The Asp-48 → Asn mutant expressed in cells clearly shares some properties of cells harboring a wild type enzyme although mutations that affect sensitivity to etoposide have not previously been used to identify new pathways required for repairing DNA damage mediated by topoisomerase II.

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