Overexpression of Type I Topoisomerases Sensitizes Yeast Cells to DNA Damage

Received for publication, March 26, 2001, and in revised form, May 10, 2001
Published, JBC Papers in Press, May 15, 2001, DOI 10.1074/jbc.M102674200

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DNA topoisomerases play essential roles in many DNA metabolic processes. It has been suggested that topoisomerases play an essential role in DNA repair. Topoisomerases can introduce DNA damage upon exposure to drugs that stabilize the covalent protein-DNA intermediate of the topoisomerase reaction. Lesions in DNA are also able to trap topoisomerase-DNA intermediates, suggesting that topoisomerases have the potential to either assist in DNA repair by locating sites of damage or exacerbating DNA damage by generation of additional damage at the site of a lesion. We have shown that overexpression of yeast topoisomerase I (TOP1) conferred hypersensitivity to methyl methanesulfonate and other DNA-damaging agents, whereas expression of a catalytically inactive enzyme did not. Overexpression of topoisomerase II did not change the sensitivity of cells to these DNA-damaging agents. Yeast cells lacking TOP1 were not more resistant to DNA damage than cells expressing wild type levels of the enzyme. Yeast topoisomerase I covalent complexes can be trapped efficiently on UV-damaged DNA. We suggest that TOP1 does not participate in the repair of DNA damage in yeast cells. However, the enzyme has the potential of exacerbating DNA damage by forming covalent DNA-protein complexes at sites of DNA damage.

DNA topoisomerases catalyze the interconversion of topological isomers of DNA (1). Topological changes catalyzed by these enzymes are required for a wide variety of cellular processes including transcription, replication, and chromosome segregation (2–4). The importance of topoisomerases in DNA metabolism has frequently led to the suggestion that topoisomerases might play important or essential roles in DNA repair and DNA damage tolerance. However, there has been little direct evidence that topoisomerases play a direct role in the repair of DNA damage in eukaryotic cells (reviewed in Ref. 5).

DNA topoisomerases are the targets of a large number of anti-cancer and anti-bacterial agents (6, 7). These agents stabilize a covalent intermediate where the enzyme is covalently bound to DNA through a phosphotyrosine linkage and, therefore, convert the enzyme into a DNA adduct with protein bound to the site of DNA strand breaks (8). Although the covalent intermediate is reversible, DNA metabolic processes such as replication can convert the intermediate into irreversible DNA damage. Extensive evidence has demonstrated that the DNA damage, rather than inhibition of enzyme activity, is responsible for cytotoxicity (9, 10). Hence these agents have been termed topoisomerase poisons. Thus, topoisomerases clearly have the potential of inflicting cytotoxic DNA damage under appropriate circumstances.

Recent experiments demonstrate that alterations in DNA structure are able to trap topoisomerases on DNA. Topoisomerase I can be trapped by strand discontinuities such as nicks or gaps (11) or by mismatched bases (12). UV damage to DNA also efficiently traps eukaryotic Top1 on DNA (13). Other types of DNA damage such as abasic sites and etheno adenine adducts also stabilize Top1 covalent complexes (14–16). Interestingly, there are two different mechanisms that can lead to topoisomerase I covalent complexes on DNA. UV damage, abasic sites, and mismatches all lead to a covalent complex that is not readily reversible. Other DNA lesions such as oxidized bases or benzaldehyde adducts increase the rate of cleavage of the enzyme at or near the lesion but do not prevent re-ligation (17). This latter mechanism has also been observed for topoisomerase II at abasic sites (18, 19). Other types of DNA damage such as UV damage inhibit topoisomerase II enzymatic activity but do not lead to increased topoisomerase II covalent complexes (20).

If DNA damage is able to trap topoisomerases on DNA in the same way as topoisomerase poisons, then topoisomerases may influence cell survival after DNA damage and may also influence the consequences of DNA lesions. Experiments described here test the hypothesis that the level of topoisomerases affect cell killing after DNA damage. We have taken advantage of the fact that yeast cells can tolerate different levels of both topoisomerase I and topoisomerase II. We have found that topoisomerase I overexpression greatly sensitizes yeast cells to DNA damage due to simple alkylating agents, UV light, or ionizing radiation, but overexpression of topoisomerase II does not affect yeast cell survival after exposure to these agents. These results indicate that topoisomerases can be important survival factors after DNA damage but that the enzymes do not participate directly in repair.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The yeast strains used in this study are derivatives of CH335 (21). CH335leu was constructed by converting CH335 to leu2Δ by one-step gene disruption (22). A top1Δ derivative of CH335leu was constructed by one-step gene disruption (23). The top1Δ disruption removes the entire open reading frame of TOP1 and replaces it with the yeast LEU2 gene. The resulting strain is termed CH335top1Δ. Both strains CH335 and CH335leu were transfected with yCP50 or pGAL7TOP1 (24). The strains carrying yCP50 served as vector controls, whereas strains with pGAL7TOP1, which expresses yeast TOP1 under the control of the yeast GAL1 promoter, were used for experiments where yeast TOP1 was overexpressed. Overexpression of human TOP1 was accomplished using the vector pGAL7TOP1 (25), and expression of Escherichia coli topoisomerase I used the vector...
pGALECTOP1 (26). The three vectors for overexpressing type I topoisomerases in yeast were the gift of Dr. J. C. Wang. A vector carrying the GAL1 fragment of the yeast RAD54 gene. After hybridization, the DNA was washed and resuspended in water at a concentration of about 107 cells/ml. Cells were diluted and plated to SC-/ura/Glu, incubated for 3 days at 30 °C, then plates with an appropriate number of colonies were counted, and surviving fractions compared with unirradiated control plates. The effect of topoisomerase I in the presence of DNA damage was first examined by overexpressing this enzyme in Saccharomyces cerevisiae. A plasmid carrying the yeast topoisomerase I gene under the control of the inducible GAL1 promoter (24) was transformed into yeast strain CH335. The control cells for these experiments were CH335 cells transformed with the centromeric vector yCP50. Actively growing cells were exposed to MMS in SC-ura/GAL. MMS concentrations were selected that reduced the viability of wild type cells (cells not overexpressing Top1p) to about 10–100% after 1–3-h exposures. After exposure to MMS, cells were diluted and plated to SC-/ura/Glu. The results obtained with MMS are shown in Fig. 1. At different concentrations of MMS, cell survival was significantly lower in cells overexpressing Top1p than in cells carrying yCP50. A similar experiment was performed using yeast cells overexpressing human topoisomerase I from the yeast GAL1 promoter. Yeast cells overexpressing hTOP1 were also more sensitive to the killing effects of MMS than cells that did not overexpress TOP1 (data not shown).

RESULTS
Topoisomerase I Overexpression Increases Sensitivity to DNA-damaging Agents—The effect of topoisomerase I in the presence of DNA damage was first examined by overexpressing this enzyme in Saccharomyces cerevisiae. A plasmid carrying the yeast topoisomerase I gene under the control of the inducible GAL1 promoter (24) was transformed into yeast strain CH335. The control cells for these experiments were CH335 cells transformed with the centromeric vector yCP50. Actively growing cells were exposed to MMS in SC-ura/GAL. MMS concentrations were selected that reduced the viability of wild type cells (cells not overexpressing Top1p) to about 10–100% after 1–3-h exposures. After exposure to MMS, cells were diluted and plated to SC-/ura/Glu. The results obtained with MMS are shown in Fig. 1. At different concentrations of MMS, cell survival was significantly lower in cells overexpressing Top1p than in cells carrying yCP50. A similar experiment was performed using yeast cells overexpressing human topoisomerase I from the yeast GAL1 promoter. Yeast cells overexpressing hTOP1 were also more sensitive to the killing effects of MMS than cells that did not overexpress TOP1 (data not shown).

The enhanced sensitivity to DNA-damaging agents was not confined to simple alkylating agents. Similar results were obtained with other types of DNA damage. Yeast cells overexpressing Top1p exposed to either UV light or ionizing ra-

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1 The abbreviations used are: MMS, methyl methanesulfonate; yCP50, yeast extract/peptone/dextrose/adenine medium.
radiation exhibited significantly reduced survival when compared with cells carrying the control plasmid yCP50 (Fig. 2, panels A and B).

Interestingly, a somewhat different pattern of sensitivity was seen with cells that express E. coli topoisomerase I, a type IA enzyme. Cells expressing E. coli topA from the yeast GAL1 promoter had slightly greater sensitivity to MMS than control cells (Fig. 3). The difference in sensitivity at 0.04% MMS was statistically significant, whereas the difference at 0.08% MMS was not statistically significant. Although expression of a type IA enzyme causes a slight increase in sensitivity to DNA-damaging agents, the effect is considerably smaller than seen when eukaryotic type 1B enzymes are overexpressed.

Sensitivity to DNA-damaging Agents Is Unaffected in Cells Expressing Elevated Levels of Inactive Topoisomerase I—We next examined whether the sensitization of cells by TOP1 overexpression requires that the protein be active and able to cleave DNA. We introduced a plasmid carrying a mutant of TOP1 where the active site tyrosine (Tyr-727) was mutated to phenylalanine. The mutant TOP1 gene was also under the control of the GAL1 promoter (31). Unlike the results obtained with the active TOP1 gene, expression of the Y727F mutant did not sensitize cells to MMS (Fig. 4). It is also noteworthy that the Y727F mutant had a similar effect on growth in the absence of MMS as overexpression of the active TOP1 gene (compare Fig. 1 and Fig. 4). This result shows that the observed sensitization by TOP1 overexpression is not due to a reduction in growth rate or decreased plating efficiency when TOP1 is overexpressed.

Sensitivity to DNA-damaging Agents Is Unaffected by the Overexpression of Topoisomerase II—When topoisomerase II is transcribed at the high levels expressed from the GAL1 promoter, cells are unable to successfully undergo cell division. To examine the effects of topoisomerase II overexpression in the presence of DNA-damaging agents, a plasmid containing the topoisomerase II gene in front of the constitutive promoter DED1 was transformed into yeast (10). Cells were then treated with MMS, and survival was measured. The results, shown in Fig. 5, indicate that expression of the yeast TOP2 gene from the DED1 promoter does not lead to an increase in sensitivity to MMS. Similar results were obtained with UV and ionizing radiation (data not shown). These results suggest that the increase in sensitivity to DNA damage that is observed with topoisomerase overexpression is limited to type I topoisomerases.

Induction of the Recombinational Repair Gene RAD54 Is Not Affected by Overexpression of Topoisomerase I—Since topoisomerase I has been implicated in transcription (32, 33), a plausible model for the effects of topoisomerase I on DNA damage response is an alteration in the expression levels of genes required for responding to DNA damage. A set of genes has been identified in yeast whose transcription is increased...
after DNA damage (34, 35). We examined the transcription of one such gene, RAD54, which is inducible by MMS. CH335 cells carrying pGALTOP1 were treated with MMS in either glucose or galactose, and at various times after MMS addition, aliquots were removed for RNA isolation. The RNA was electrophoresed, and after transfer to a nylon membrane, probed with full-length RAD54 DNA. The results are shown in Fig. 6. In glucose in the absence of MMS, RAD54 mRNA is barely detectable. After a 1- or 3-h exposure to 0.05% MMS, there is a clear induction of the RAD54 message. Similarly, RAD54 message is barely detectable in galactose grown cells in the absence of MMS. This suggests that overexpression of TOP1 from the GAL1 promoter by itself is insufficient to elicit the induction of RAD54. Upon the addition of MMS, RAD54 message is induced to an extent similar to that seen with glucose-grown cells. Specific conditions are indicated on the figure.

FIG. 6. Overexpression of TOP1 does not alter DNA damage-inducible gene expression. RNA from cells carrying pGALyTOP1 was isolated from cells grown either in glucose (therefore not overexpressing TOP1) or galactose (therefore overexpressing TOP1). Cells under both conditions were also exposed to MMS for either 1 or 3 h before RNA isolation. After electrophoresis and transfer to a nylon membrane, the blot was probed with a probe for the RAD54 gene.

GAL1 promoter by itself is insufficient to elicit the induction of RAD54. Upon the addition of MMS, RAD54 message is induced to an extent similar to that seen with glucose-grown cells. Similar results were also obtained with the gene for a subunit of the single-stranded DNA-binding protein RPA (data not shown). These results indicate that overexpression of TOP1 does not increase the sensitivity of cells to DNA-damaging agents by changing gene expression.

FIG. 5. Overexpression of yeast topoisomerase II does not confer hypersensitivity to MMS. Yeast cells pre-grown in galactose were exposed to MMS in yeast synthetic growth medium containing galactose using the same conditions shown in Fig. 1. CH335 cells with pDEDTOP2 were exposed to 0% MMS (open squares), 0.04% MMS (open circles), or 0.08% MMS (open triangles). CH335 cells with pDEDTOP2 were exposed to 0% MMS (closed squares), 0.04% MMS (closed circles), or 0.08% MMS (closed triangles). Error bars indicate S.E.; symbols lacking error bars have S.E. less than the size of the symbol.

FIG. 4. Overexpression of an inactive yeast topoisomerase I mutant does not increase sensitivity to MMS. Yeast cells pre-grown in galactose were exposed to MMS in yeast synthetic growth medium containing galactose using the same conditions shown in Fig. 1. CH335 cells with pGALyTOP1Y727F were exposed to 0% MMS (open squares), 0.04% MMS (open circles), or 0.08% MMS (open triangles). CH335 cells with pGALyTOP1Y727F were exposed to 0% MMS (closed squares), 0.04% MMS (closed circles), or 0.08% MMS (closed triangles). Error bars indicate S.E.; symbols lacking error bars have S.E. less than the size of the symbol.
results indicate that wild type levels of topoisomerase I expression do not sensitize cells to DNA damage nor does topoisomerase I play a detectable role in repair for these DNA-damaging agents. These results agree with a previous determination of the sensitivity of top1−/− cells to DNA damage (37).

Trapping of Yeast Topoisomerase I by DNA Damage—Previous reports discussed in the Introduction have shown that mammalian topoisomerase I can be trapped on DNA carrying various types of DNA damage. We next wanted to confirm that yeast topoisomerase I could also be trapped by DNA damage. We first carried out a simple assay to test whether there was preferential nicking of damaged DNA by DNA topoisomerase I. Purified yeast topoisomerase I was incubated with end-labeled damaged DNA that was either unirradiated or UV-irradiated with 1000 J/m2. After incubation at 30 °C, the reaction was stopped with Protein A-Sepharose. 

We also observed that the addition of camptothecin to unirradiated DNA samples increased the level of topoisomerase I-DNA covalent complexes, processes such as DNA replication can convert the (reversible) protein-DNA lesion into an irreversible lesion (38). DNA replication also can convert the single-strand break formed by topoisomerase I into a double-strand break (39). Both the protein-DNA “adduct” and the generation of secondary double-strand breaks could contribute to camptothecin cytotoxicity. It is therefore plausible that electrophoretic mobility shift would be able to readily detect topoisomerase I-DNA covalent complexes. To assess this, we examined the ability of topoisomerase I to reduce the mobility of linear DNA that was either unirradiated or irradiated with different UV doses. Fig. 9A shows the result of the electrophoresis. As can be readily seen, a weak shifted band can be observed in samples containing 6 units of topoisomerase I and damaged DNA. The addition of camptothecin greatly increased the intensity of the shifted band. If instead of camptothecin, UV-irradiated DNA was used, a significant increase in intensity of the shifted band was also observed. The intensities of the bands with unirradiated DNA, DNA irradiated with 1000 J/m2, or 2000 J/m2 UV light are shown in Fig. 9B. The intensity of the band is approximately linear with respect to added topoisomerase I over the range examined for all three DNA samples. The slope of the calculated linear regression is 2.8-fold higher for DNA irradiated with 1000 J/m2 than for unirradiated DNA and 5.3-fold higher for DNA irradiated with 2000 J/m2. Since the reactions were treated with SDS before electrophoresis, the interactions between topoisomerase I and damaged DNA that we detect must be covalent rather than noncovalent. To verify that the shifted bands represent protein-DNA complexes, we also treated one set of samples containing the highest amount of topoisomerase I with proteinase K before electrophoresis. Treatment with proteinase K resulted in complete loss of the shifted band whether complexes were trapped with camptothecin or UV damage. The results of Figs. 8 and 9 taken together demonstrate that UV-damaged DNA can efficiently trap topoisomerase I covalent complexes.

**DISCUSSION**

Anti-cancer drugs such as camptothecin are able to trap a covalent intermediate of the topoisomerase I reaction, and trapping of this intermediate can interfere with DNA metabolism. It is well established that the cytotoxicity of camptothecin depends on its ability to stabilize topoisomerase I-cleavable complexes and that the degree of cytotoxicity correlates with the levels of covalent complexes (9). For camptothecin-induced topoisomerase I-DNA covalent complexes, processes such as DNA replication can convert the (reversible) protein-DNA lesion into an irreversible lesion (38). DNA replication also can convert the single-strand break formed by topoisomerase I into a double-strand break (39). Both the protein-DNA “adduct” and the generation of secondary double-strand breaks could contribute to camptothecin cytotoxicity. It is therefore plausible...
that other agents that increase the level of topoisomerase I-cleavable complexes are likely to be cytotoxic by the same mechanisms. In this paper, we demonstrate that in the presence of DNA damage, topoisomerase I also forms a stable covalent complex similar to the cleavable complexes formed in the presence of the anti-cancer drug camptothecin and that these covalent complexes enhance the cytotoxicity of DNA damage.

Several trivial explanations for the hypersensitivity of cells expressing topoisomerase I to DNA-damaging agents can be readily excluded. Although cells overexpressing topoisomerase I grow more slowly than cells expressing normal levels of this enzyme, cells expressing a catalytically dead topoisomerase I also grow more slowly than wild type cells, but those cells are not hypersensitive to DNA-damaging agents. Since both yeast and human topoisomerase I expression leads to drug hypersensitivity, we directly examined the levels of protein-DNA complexes using an electrophoretic mobility shift assay. Both assays gave quantitatively similar results when similar samples were examined. The potassium/SDS assay indicated a 3-fold increase in covalent complexes with 1000 J/m2 UV versus 5-fold with the mobility shift assay. A potential advantage of the mobility shift assay is the ability to examine DNA with several different types of DNA damage. The shortcoming of the potassium SDS assay is the necessity of separately labeling each DNA that has a different type or amount of DNA damage.

Other recent studies also indicate that DNA damage is able to stabilize topoisomerase I-DNA covalent complexes in vitro. Pedrini and co-workers (13) first showed that purified topoisomerase I stably cleaves UV-damaged DNA at sites at or near UV damage (13). Pommier and co-workers (44) also find that topoisomerase I can form a stable covalent complex at the sites of several different specific DNA lesions. Earlier results had shown that factors such as DNA curvature could stimulate topoisomerase I cleavage (43) and that topoisomerase I could act at sites of DNA breaks. Taken together, these results suggest that topoisomerase I action can be altered by many different changes in DNA structure.

If the normal reaction of topoisomerase I at sites of DNA damage did not lead to further DNA damage, topoisomerase I could act as an efficient sensor of DNA lesions. Because the enzyme acting at damage results in a more complex lesion, the recognition of damage by topoisomerase I does not seem to be useful for promoting cell survival. Results described here that cells completely lacking topoisomerase I are not more sensitive to DNA damage than cells with wild type levels of the enzyme suggest that DNA damage recognition does not appear to be a normal indispensable role for this enzyme.

Osheroff and co-workers (19) find that some types of DNA damage can also lead to trapping of topoisomerase II on DNA. In their studies, abasic sites greatly stimulated topoisomerase II cleavage of DNA. Since we failed to observe decreased survival in cells overexpressing topoisomerase II, the trapping of topoisomerase II by DNA damage either occurs infrequently in vivo, or cells possess an efficient system for preventing topoisomerase II-mediated DNA damage. In results to be presented elsewhere, we have found that topoisomerase II levels increase after DNA damage, which leads us to suggest that trapping of topoisomerase II in vivo is a relatively infrequent event. It may be relatively infrequent for two reasons. First, topoisomerase II cleavage is strongly inhibited by some types of DNA damage such as photoproducts induced by UV light. Second, the DNA repair systems that recognize abasic sites may be much more efficient at binding to abasic sites than topoisomerase II. If so, then cells lacking apurinic endonucleases may become sensitive to topoisomerase II dosage. We are currently testing this hypothesis.

How then do cells deal with the dangerous activity of topoisomerase I when DNA is damaged? In mammalian cells, poly-ADP-ribose polymerase is rapidly activated by DNA strand breaks (44). A major target for poly(A)DP-ribose polymerase is topoisomerase I, and modification of topoisomerase I by this enzyme inhibits topoisomerase activity (45, 46). Yeast cells apparently lack this enzyme, so some other pathway must function to attenuate topoisomerase I activity after DNA damage. The inactivation of topoisomerase I should occur fairly rapidly to prevent the formation of covalent complexes at sites of damage. Either covalent modification or targeted degrada-
stability of topoisomerase I conjugated to small ubiquitin-related modifier remains to be demonstrated (49). Also, down-regulation of topoisomerase I after ionizing radiation has also been reported (50).

There are likely other pathways that control topoisomerase I after DNA damage as well as pathways that can repair the DNA damage arising from topoisomerase I. Nash and co-workers (51, 52) recently describe a yeast protein that can disjoin topoisomerase I covalent complexes. Since DNA damage and not just topoisomerase I poisons such as camptothecin are able to trap topoisomerase I on DNA, the enzyme described by Nash likely functions as one DNA repair system designed to deal with the ability of topoisomerase I to generate covalent complexes at the sites of damage.

Our results connect topoisomerase I to pathways of DNA damage repair and DNA damage tolerance, but the connection we propose is not that topoisomerases participate in repair but, rather, as an impediment to accurate repair. It has also been found that overexpression of topoisomerase I is able to increase nonhomologous integration of transfected DNA in yeast (53). The nonhomologous integration could arise from the action of topoisomerase I at sites of endogenous DNA damage. This may suggest that topoisomerase I could play a significant role in genome destabilization after DNA damage.

Acknowledgments—We thank Dr. Jerrylaine Walker for purifying yeast topoisomerase I and Ms. Mehrpouya for help with cleavage assays. We also thank Drs. Rolf Sternberg (SUNY, Stonybrook, NY), Connie Holm (University of California, San Diego, CA), and James C. Wang (Harvard University) for the gift of strains or plasmids.

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doi: 10.1074/jbc.M102674200 originally published online May 15, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102674200

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