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Increased Camptothecin Toxicity Induced in Mammalian Cells Expressing *Saccharomyces cerevisiae* DNA Topoisomerase I*

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Christine Hann‡§, Devon L. Evans‡, Jolanta Fertala‡, Piero Benedetti¶‖, Mary-Ann Bjornsti‡, and David J. Hall‡**

From the ‡Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and ¶Consiglio Nazionale delle Ricerche, Istituto di Biologia Cellulare, 00137 Rome, Italy

The yeast *Saccharomyces cerevisiae* has been useful in establishing the phenotypic effects of specific mutations on the enzymatic activity and camptothecin sensitivity of yeast and human DNA topoisomerase I. To determine whether these phenotypes were faithfully reiterated in higher eukaryotic cells, wild-type and mutant yeast Top1 proteins were epitope-tagged at the amino terminus and transiently overexpressed in mammalian COS cells. Camptothecin preferentially induced apoptosis in cells expressing wild-type eScTop1p yet did not appreciably increase the cytotoxic response of cells expressing a catalytically inactive (e*Sctop1Y727F*) or a catalytically active, camptothecin-resistant e*Sctop1vac* mutant. Using an epitope-specific antibody, immobilized precipitates of eScTop1p were active in DNA relaxation assays, whereas immunoprecipitates of eScTop1Y727Fp were not. Thus, the enzyme retained catalytic activity while tethered to a support. Interestingly, the mutant e*Sctop1T722A*, which mimics camptothecin-induced cytotoxicity in yeast through stabilization of the covalent enzyme-DNA intermediate, induced apoptosis in COS cells in the absence of camptothecin. This correlated with increased DNA cleavage in immunoprecipitates of eScTop1T722Ap, in the absence of the drug. The observation that the phenotypic consequences of expressing wild-type and mutant yeast enzymes were reiterated in mammalian cells suggests that the mechanisms underlying cellular responses to DNA topoisomerase I-mediated DNA damage are conserved between yeast and mammalian cells.

Eukaryotic DNA topoisomerase I catalyzes the relaxation of supercoiled DNA through the transient breakage and religation of a single DNA strand in a DNA duplex (reviewed in Refs. 1–3). This enzyme plays a role in a number of essential cellular processes, such as replication, recombination, and transcription (1, 3–5). Furthermore, the naturally occurring antitumor drug camptothecin specifically targets this enzyme by stabilizing the covalent enzyme-DNA intermediate (Refs. 6 and 7; reviewed in Ref. 8). During DNA replication (S phase), these stabilized enzyme-DNA adducts are converted into lethal double-stranded DNA breaks due to their interaction with the DNA replication fork (9–12).

Although this enzyme participates in numerous cellular processes, strains of the yeast *Saccharomyces cerevisiae* deleted for the gene encoding DNA topoisomerase I (*top1*Δ) are viable because other gene products, such as DNA topoisomerase II, can compensate for the loss of *TOP1* (11, 13). These *top1*Δ strains are completely resistant to the cytotoxic action of camptothecin (14–16). However, expression of either *S. cerevisiae* or human DNA topoisomerase I restores the sensitivity of these cells to camptothecin-induced lethality (4, 14–16). These results demonstrate the specificity of camptothecin for eukaryotic DNA topoisomerase I and the utility of using yeast as a model system for the analysis of drug-enzyme interactions. In fact, mutations in yeast and human *TOP1* that render the enzyme resistant to camptothecin (17, 18) or render the enzyme cytotoxic even in the absence of camptothecin (19, 20)[1,2] have been defined using this yeast system.

These results indicate a significant conservation of function between the yeast and human enzymes, consistent with extensive similarities in *TOP1* sequences. Nevertheless, differences between these proteins do exist. For example, expression of a camptothecin-resistant yeast or human DNA topoisomerase I mutant (*top1vac*) has different effects on the viability of yeast strains defective in the repair of double strand DNA breaks (17). *In vitro*, these mutant enzymes exhibit different sensitivities to other DNA topoisomerase I poisons, including saintopin, a DNA intercalator that targets both DNA topoisomerase I and II, and the minor groove binding ligand netropsin (21). The human enzyme plays a direct role in transcriptional activation *in vitro* (22–24) and suppresses the basal level of transcription (22). Although the catalytic activity of human DNA topoisomerase I is dispensable for its role in suppressing transcription, the yeast enzyme could not replace the human enzyme in these assays (22). It is not yet known whether these observations reflect intrinsic differences in enzyme structure or in specific functional domains that mediate enzyme interactions with other cellular factors. However, because these enzymes constitute the cellular targets of clinically important chemotherapeutic agents, it is essential that the mechanisms of DNA topoisomerase I-induced DNA damage be better understood.

Here we investigate the potential differences between yeast and human DNA topoisomerase I with regards to camptothecin-induced cytotoxicity. A family of *S. cerevisiae* mutants were transiently expressed in mammalian COS cells and examined for their effects on drug-induced apoptosis. These as-

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** To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, 233 S. 10th St., Philadelphia, PA 19107. Tel.: 215-503-2035; Fax: 215-923-9162; E-mail: hall@hendrix.jci.tju.edu.

[1] J. Fertala and M.-A. Bjornsti, unpublished results.
[2] P. Fiorani, J. Amatruda, M.-A. Bjornsti, and P. Benedetti, manuscript in preparation.

says included wild-type yeast *TOP1* (e*ScTOP1*), the camptothecin-resistant mutant e*Sctop1vac* (17, 21), a catalytically inactive mutant (e*Sctop1Y727F*) (25), and the lethal mutant e*Sctop1T722A*, which mimics that action of camptothecin in stabilizing the covalent enzyme DNA intermediate (20). Given the difficulties inherent in the selection of cytotoxic phenotypes in mammalian cells, the effects of such lethal mutations on mammalian cell viability have not previously been described. In the studies presented here, expression of yeast wild-type protein enhanced COS cell sensitivity to camptothecin, consistent with earlier observations that overexpression of the human enzyme in mammalian cells enhances their sensitivity to the drug (26). The phenotypic consequences of overexpressing the other classes of yeast *top1* mutants were also faithfully reiterated in these mammalian cells; no appreciable increase in camptothecin-induced apoptosis was observed in cells expressing e*Sctop1vac* or the inactive mutant e*Sctop1Y727F*, whereas e*Sctop1T722A* expression induced an apoptotic response in the absence of the drug. Moreover, when these yeast enzymes were immunoprecipitated, the activities of the bead-bound enzymes correlated with the observed patterns of drug sensitivity and cell lethality. These results highlight the conservation of enzyme function both in inducing DNA damage and in the cellular responses to this DNA topoisomerase I-mediated damage.

MATERIALS AND METHODS

Plasmids, Cell Culture, and Drug Treatment—An eight-amino acid residue epitope tag, recognized by monoclonal antibody M2 (Kodak/IBI), was engineered into the amino terminus of yeast DNA topoisomerase I. To avoid confusion with the endogenous mammalian enzyme, the yeast gene and its protein products are prefixed with an e*Sc* to indicate the epitope tag (*e.g.* e*ScTOP1* and eScTop1p). Complementary oligos encoding the sequence MDYKDDDDKAI were cloned into an *Nco*I site, previously engineered into the sequences encoding the initiating methionine residue in the *eScTOP1* plasmid YCpGAL1-TOP1 (17), to yield YCpGAL1-eScTOP1. The epitope tag (underlined in the sequence above) was immediately amino-terminal to the original methionine in the fusion protein eScTop1p. The eSc*top1Y727F*, eSc*top1 vac*, and eSc*top1T722A* constructs were prepared by swapping a 989-base pair *Bam*HI-Bsu 36I DNA fragment from YCpGAL1-eTOP1 into the backbones of plasmids YCpGAL1-top1Y727F, YCpGAL1-top1 vac (17, 21), and YCpGAL1-top1T722A (20), respectively. The resultant e*Sctop1* constructs were all excised by digestion with MluI and *Pst*I, and the blunted-ended DNA fragments were cloned into the blunt-ended *Eco*RI site of the pMT2 COS cell expression vector (27).

COS cells (African green monkey kidney cells transformed with SV40 T antigen) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. All transfections were performed on subconfluent monolayer cultures. Plasmids (30 μg) were transfected into COS cells by the calcium phosphate procedure. The cells were glycerol shocked 5–6 h after DNA addition.

Camptothecin (Sigma) was resuspended in Me_2SO at a concentration of 10 mM, and aliquots were stored at −20 °C. Prior to adding camptothecin to the tissue culture medium, the drug was serially diluted in fresh Me_2SO to equalize the final Me_2SO concentration in each experiment. Camptothecin was added to the cells over a final concentration range of 0.001–50 μM.

Immunofluorescence, Confocal Microscopy, and Immunoprecipitation—Indirect immunofluorescence was performed as described by Logan *et al.* (28). Briefly, COS cells were plated on 10-cm tissue culture dishes containing glass coverslips at approximately 500,000 cells/plate. The cells were cultured for 24 h and then were transfected with the various e*ScTOP1* expression plasmids (described above). After the glycerol shock, the cells were treated with various concentrations of camptothecin for an additional 24 h. The cells were then washed twice in PBS and fixed with 4% paraformaldehyde in PBS for 20 min followed by two washes in PBS. To permeabilize the cells, the coverslips were then treated with PBS containing 0.2% Triton X-100 for 15 min followed by three 5-min washes in PBS containing 0.2% gelatin (as described in Ref. 29). The M2 monoclonal antibody (Kodak/IBI) was diluted 1:200 in PBS containing 0.2% gelatin. Coverslips containing the fixed and permeabilized cells were placed cell side down on 50 μl of diluted antibody and incubated for 1.5 h at 37 °C. The coverslips were then washed three times in PBS plus 0.2% gelatin and inverted on 50 μl of fluorescein-conjugated goat anti-mouse IgG (Vector Laboratories) diluted to 30 μg/ml in PBS plus 0.2% gelatin. After 30 min at 37 °C, the coverslips were successively washed in PBS plus 0.2% gelatin (10 min), PBS plus 0.2% gelatin plus 0.05% Tween 20 (10 min), and PBS (10 min). The DNA within the nuclei of the cells was then stained with DAPI.[3] The coverslips were rinsed in deionized water, dried, mounted onto glass slides, and analyzed by fluorescence microscopy.

Confocal microscopy was performed on the fluorescein-stained cells, using a Leica TCS 4D confocal microscope with a 100× oil objective lens. The cell thickness was 2.24 μm, and the optical sections taken were 0.24 μm.

For the immunoprecipitations, 15 μg of nuclear extract was incubated at 4 °C with 3 μg of M2 monoclonal antibody (Kodak/IBI) for one hour. Two micrograms of *Staphylococcus* protein A acrylamide beads were added to the extracts for an additional 1.5 h at 4 °C. The beads were then pelleted, washed five times in NTEN (100 mM NaCl, 20 mM Tris pH 8, 1 mM EDTA, 0.5% Nonidet P-40), and resuspended in 50 μl of DNA relaxation assay buffer (see below). The immunoprecipitates were assayed in a plasmid DNA relaxation activity as described below.

Generation of Extracts—Nuclear extracts for Western blots and activity assays were generated as described by Moberg *et al.* (30), by lysing the cells on ice in 0.1% Nonidet P-40, 10 mM Tris (pH 7.9), 10 mM $MgCl_2$, 15 mM NaCl, and the protease inhibitors phenylmethylsulfonyl fluoride (0.5 mM), pepstatin (2 μg/ml), and leupeptin (1 μg/ml). The nuclei were pelleted by centrifugation at 800 × *g* for 10 min, resuspended in extraction buffer consisting of 0.5 M NaCl, 20 mM Hepes (pH 7.9), 20% glycerol, phenylmethylsulfonyl fluoride (0.5 mM), pepstatin (2 μg/ml), and leupeptin (1 μg/ml) for 10 min on ice and then centrifuged at 14,000 × *g* for 8 min to pellet the residual nuclear material. The supernatant fraction was termed nuclear extract.

Western Blot Hybridizations—Proteins in the nuclear extracts were electrophoretically resolved by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters. The blots were then washed in TBST buffer, blocked with 2.5% bovine serum albumin in TBST for 30 min at room temperature and incubated with the primary antibodies for 30–60 min at room temperature in TBST. The blots were incubated with either a rabbit polyclonal antibody specific for yeast DNA topoisomerase I (17, 21) or with the M2 monoclonal. The blots were then incubated with a 1:7500 dilution of secondary antibody (goat anti-rabbit or goat anti-mouse, Vector Laboratories) conjugated to alkaline phosphatase for 30 min at room temperature in TBST. The blot was stained using the Protoblot system from Promega.

DNA Topoisomerase I Activity Assays—DNA topoisomerase I activity was assayed by the relaxation of negatively supercoiled plasmid DNA as described previously (17). Briefly, nuclear extracts were first equalized for any differences in protein concentration and then serially 10-fold diluted in relaxation assay buffer containing 20 mM Tris (pH 7.5), 10 mM Na_2EDTA, 150 mM KCl, and 50 μg/ml gelatin. 2-μl volumes were then incubated in 20-μl reactions containing relaxation buffer and 0.3 μg of pHC624 DNA (2015 base pair) (17) for 1 h at 30 °C (the optimal growth temperature for yeast). The reactions were terminated by the addition of 1% SDS. The extent of plasmid DNA relaxation was assessed following electrophoresis of the reaction products in a 1% agarose gel in 0.1 M Tris-borate buffer at 5 V/cm for 4 h and subsequent visualization by staining with ethidium bromide.

Immunoprecipitates were similarly assayed by incubating the protein-bound beads in a final 100-μl reaction mixture containing relaxation buffer, protease inhibitors (as above), and 0.5 μg of plasmid DNA. Following incubation at 30 °C for 30 min, the beads were pelleted, and the plasmid DNA topoisomers (in the supernatant) were phenol extracted, ethanol precipitated, and resolved in 1% agarose gels as detailed above.

Cleavage Assays—A single 944-base pair DNA fragment representing a high affinity site was 3′-end labeled with ^{32}P-ATP. Approximately 10 ng (5000 cpm) of this labeled fragment was incubated with washed immunoprecipitates, as described above, from cells transfected with either e*ScTOP1*, e*Sctop1T722A*, or the control vector (pMT2). The 50-μl mixtures included 50 mM Tris (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, and 4% Me_2SO. Where indicated, camptothecin was added to a final concentration of 100 μM. After 30 min at 30 °C, the reactions were terminated by the addition of 1% SDS, heating to 75 °C for 10 min, and treatment with 0.4 mg/ml proteinase K. The cleaved DNA fragments were resolved in a 7 M urea, 8% polyacrylamide gel and visualized by autoradiography (as described in Ref. 21).

[3] The abbreviations used are: DAPI, 4′,6-diamidino-2-phenylindole; TBST, 10 mM Tris, pH 8, 150 mM NaCl, and 0.05% Tween 20.

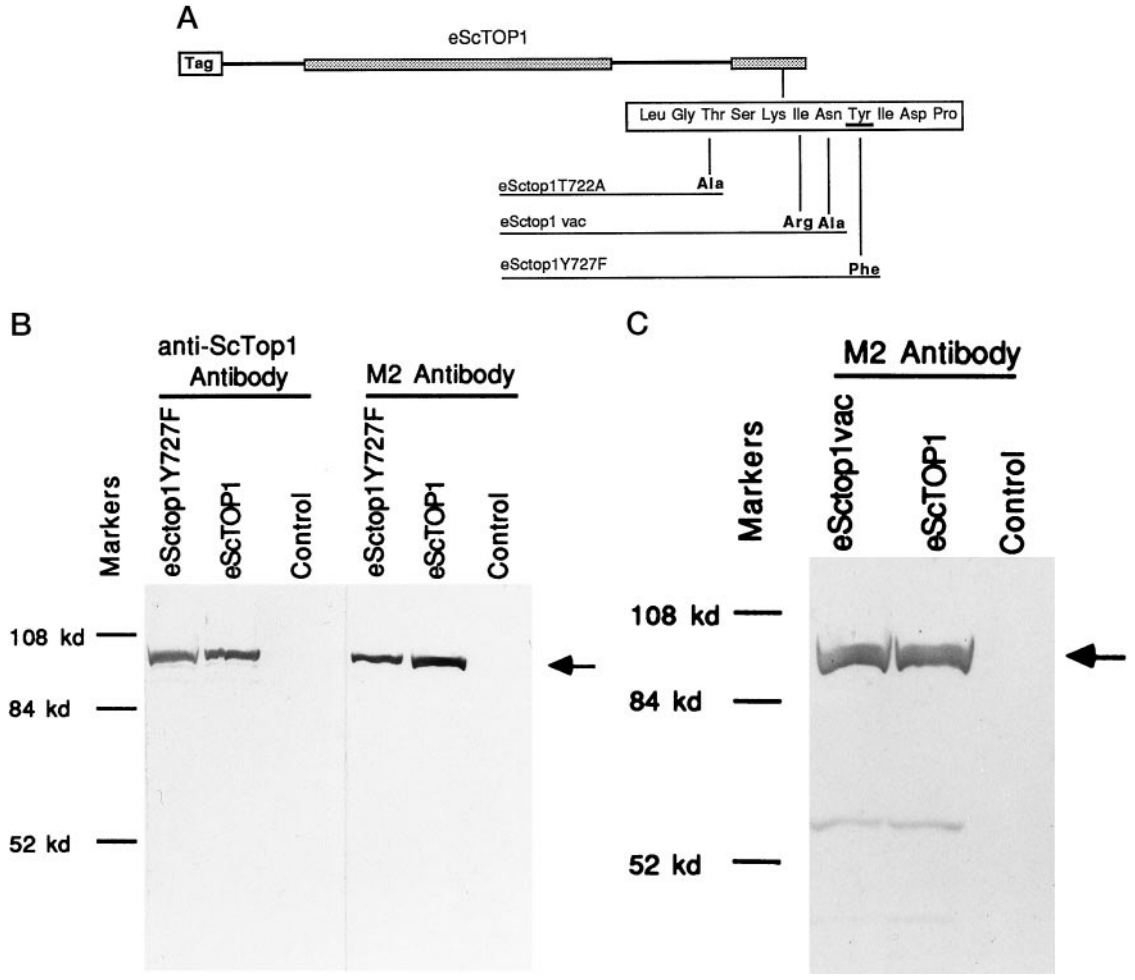

FIG. 1. **eScTop1p, eScTop1Y727Fp, and eScTop1vacp are expressed to high Levels in COS cells following transient transfection.** *A*, schematic identification of the amino acid residues in eScTop1p mutated in eScTop1vacp, eScTop1T722Ap, and eScTop1Y727Fp. The epitope tag, Asp-Tyr-Lys-Asp, was introduced into the amino terminus of the wild-type and mutant proteins. The *shaded areas* in eScTop1p correspond to the two highly conserved domains. The e*ScTOP1*, e*Sctop1vac*, and e*Sctop1Y727F* genes were cloned into the pMT2 expression vector and transiently transfected into subconfluent growing COS cells. As a control, the pMT2 plasmid alone was also transfected into COS cells. 24 h after transfection, nuclear extracts were generated and aliquots (50 μg) were electrophoresed by SDS-polyacrylamide gel electrophoresis. *B*, expression of e*ScTOP1* and e*Sctop1Y727F*. The gels were blotted and probed with a either a monoclonal antibody (M2) directed against the engineered amino-terminal epitope or a rabbit polyclonal antibody directed specifically against the ScTop1 protein. The *arrow* points to the ectopically expressed protein. *C*, expression of e*ScTOP1* and e*Sctop1vac*. The gel was blotted and probed with a monoclonal antibody (M2) directed against the amino-terminal epitope. The *arrow* points to the ectopically expressed protein.

RESULTS

Expression of Epitope-tagged S. cerevisiae DNA Topoisomerase I (eScTop1p) in COS Cells—To investigate the function of yeast DNA topoisomerase I in mammalian cells, an epitope tag was introduced at the amino terminus of the yeast enzyme. The epitope is represented by four amino acids (DYKD) that are specifically recognized by the M2 monoclonal antibody (Kodak/IBI). This tag made it possible to efficiently detect the ectopically expressed yeast enzyme by immunoblotting, immunofluorescence, and immunoprecipitation of the yeast protein from COS cell nuclear extracts. Along with the wild-type ScTop1 protein, several mutant yeast DNA topoisomerase I genes were also tagged (see Fig. 1*A*). One was the catalytically inactive mutant *Sctop1Y727F*, in which the active site tyrosine was changed to phenylalanine (25). In a second yeast mutant enzyme, Top1vacp, the two amino acid residues immediately amino-terminal to the active site tyrosine, Ile^{725} and Asn^{726}, were mutated to the Arg and Ala residues found at the corresponding position in the camptothecin-resistant vaccinia virus DNA topoisomerase I (17, 25). These substitutions render the catalytically active yeast Top1vac enzyme resistant to camptothecin-induced DNA cleavage (4, 17, 21). A third yeast mutant, *top1T722A*, is lethal when overexpressed in yeast cells, even in the absence of camptothecin, as discussed below (20).

The introduction of the epitope tag had no detectable effect on the catalytic activity of partially purified preparations of wild-type and mutant DNA topoisomerase I (data not shown). In addition, the camptothecin-sensitive, camptothecin-resistant, and lethal phenotypes associated with overexpression of *ScTOP1 Sctop1Y727F*, *Sctop1vac*, and *Sctop1T722A*, respectively, were unaffected by the epitope tag in *top1*Δ yeast strains.[4]

To assess the function of the yeast enzymes in higher eukaryotic cells, the genes encoding eScTop1p, eScTop1Y727Fp, eScTop1T722Ap, and eScTop1vacp were cloned into the eukaryotic expression vector pMT2. High levels of gene expression can be achieved when this vector is introduced into COS cells (African green monkey) (27, 31). These pMT2eSctop1 constructs were then transiently transfected into COS cells, along with the vector alone as a negative control. After a period of 24 h, nuclear extracts were electrophoresed in SDS-polyacryl-

[4] C. Hann and M.-A. Bjornsti, unpublished observations.

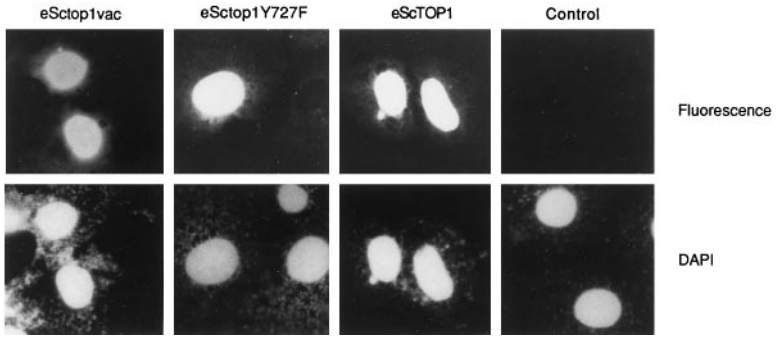

FIG. 2. **eScTop1, eScTop1vac, and eScTop1Y727F enzymes are efficiently targeted to the nucleus in COS cells following transient transfection.** The e*ScTOP1*-, e*Sctop1vac*-, and e*Sctop1Y727F*-expressing plasmids were transiently transfected into subconfluent COS cells growing on glass coverslips. As a control, the pMT2 plasmid alone was also transfected into COS cells. Twenty-four hours after the transfection, the coverslips were processed for indirect immunofluorescence using the M2 monoclonal antibody as a primary antibody followed by a fluorescein-conjugated secondary antibody. The DNA was then stained with DAPI to highlight the nuclei. Shown are the fluorescence micrographs of nuclei staining positive for expression of the epitope-tagged proteins.

amide gels and the proteins were blotted onto nitrocellulose. The epitope-tagged yeast enzymes were visualized by probing the blots with either the M2 monoclonal antibody or an anti-ScTop1 specific polyclonal antibody, followed by an alkaline phosphatase-conjugated secondary antibody. As shown in Fig. 1*B*, the ectopically expressed eScTop1 and eScTop1Y727F proteins were specifically recognized by the M2 and anti-ScTop1p antibodies and were expressed to equivalent levels in COS cells. The proteins were intact and migrated to a position expected for their size (approximately 90 kDa) (17). Similar results were obtained with nuclear extracts of COS cells transfected with pMT2eSctop1vac, shown in Fig. 1*C*. Over a number of experiments, the levels of expression of the wild-type and mutant enzymes appeared to be equal and the enzymes appeared to be of equal stability.

To ensure that the yeast proteins were targeted to the nucleus, indirect immunofluorescence was performed on cells 24 h after transfection. In these experiments, the M2 monoclonal antibody was used as the primary antibody, whereas the secondary antimouse antibody used was conjugated to fluorescein. As can be seen in the photomicrographs in Fig. 2, the eScTop1, eScTop1Y727F, and eScTop1vac proteins accumulate in the nuclei, indicating that the yeast enzymes contain nuclear localization sequences efficiently recognized by the mammalian nuclear transport machinery. The fluorescence appeared to be uniformly distributed throughout the nuclei for all the enzymes shown. At lighter exposures of the fluorescence and at lower levels of expression, these proteins are clearly present in the nucleoli (data not shown), consistent with previous reports (32, 33).

Immunofluorescence studies have indicated that the DNA topoisomerase I may also be localized to other sites within the nucleus such as the nuclear membrane (32, 33). Although the eScTop1 protein in COS cells appears to be distributed uniformly throughout the nucleus, the intensity of the staining precluded any assessment of a perinuclear distribution. To determine whether the protein was targeted to the nuclear membrane, the fluorescent positive eScTop1p cells were viewed by confocal microscopy. Shown in Fig. 3, *a–l*, are 0.24 μM optical sections of a fluorescein positive cell (the same cell stained with DAPI is shown in Fig. 3*m*). The epitope-tagged yeast enzyme appears to be localized near the inner surface of the nuclear membrane. When the fluorescence intensity was measured across the nucleus in a single optical section, in the direction of the *arrow* at the top of Fig. 4, the intensity was maximal at positions corresponding to the nuclear membrane (Fig. 4, *bottom*).

To next assess whether the ectopically expressed yeast enzymes were catalytically active, nuclear extracts of transfected COS cells were prepared, and equal amounts were incubated with negatively supercoiled plasmid DNA in a relaxation assay (as described in Refs. 14 and 17). In the absence of Mg++ and ATP, DNA topoisomerase IIα and β activities are not detectable in this assay. Yeast DNA topoisomerase I activity can be distinguished from endogenous DNA topoisomerase I activity by quantitating the increase in plasmid DNA relaxation in extracts derived from COS cells transfected with vector alone and the various pMT2eSctop1 constructs. As shown in Fig. 5, when serial 10-fold dilutions of the indicated extracts were analyzed, DNA relaxation activity is approximately 10-fold higher in the extracts containing eScTop1p and eScTop1vacp than in the pMT2 control extracts or in the extracts containing the catalytically inactive eScTop1Y727Fp. The relative position of the negatively supercoiled and relaxed plasmid DNA topoisomers are marked in Fig. 5 by the *arrow* and *R*, respectively. The transfection efficiency in these experiments averaged about 10%. Therefore, this 10-fold increase in DNA topoisomerase I activity was due to the expression of e*ScTOP1* and e*Sctop1vac* in only 1/10 of the cell population. This would suggest that there was roughly a 100-fold increase in DNA topoisomerase I activity in the COS cells expressing the yeast enzymes.

The inclusion of the epitope tag allowed us to immunoprecipitate the eScTop1 enzyme from nuclear extracts of transfected COS cells using the M2 monoclonal antibody (data not shown). To further establish that the ectopically expressed yeast enzymes were catalytically active, nuclear extracts of cells transfected with the control plasmid (pMT2) or with plasmids expressing eScTop1p, eScTop1vacp, or eScTop1Y727Fp were immunoprecipitated with the M2 antibody. The immunoprecipitates were immobilized on Staph A-acrylamide beads and extensively washed, and a buffer solution containing negatively supercoiled plasmid DNA was then added to the beads. Following incubation at 30 °C for 30 min, the supernatant was phenol extracted and the DNA analyzed by agarose gel electrophoresis. As seen in Fig. 6, the immunoprecipitates containing eScTop1p and eScTop1vacp were active in relaxing the plasmid DNA, whereas immunoprecipitates prepared from e*Sctop1Y727F*-expressing cells or the pMT2 transfected control did not exhibit any appreciable activity. These data indicate two things: first, that ectopically expressed eScTop1p and eScTop1vacp are catalytically active whereas eScTop1Y727Fp is not; second, that eScTop1p is active even though its amino terminus is tethered to a support, implying that the enzyme need not be free in solution to complete the catalytic cycle.

Expression of Catalytically Active eScTop1p in COS Cells Leads to Increased Sensitivity to Camptothecin—Overexpres-

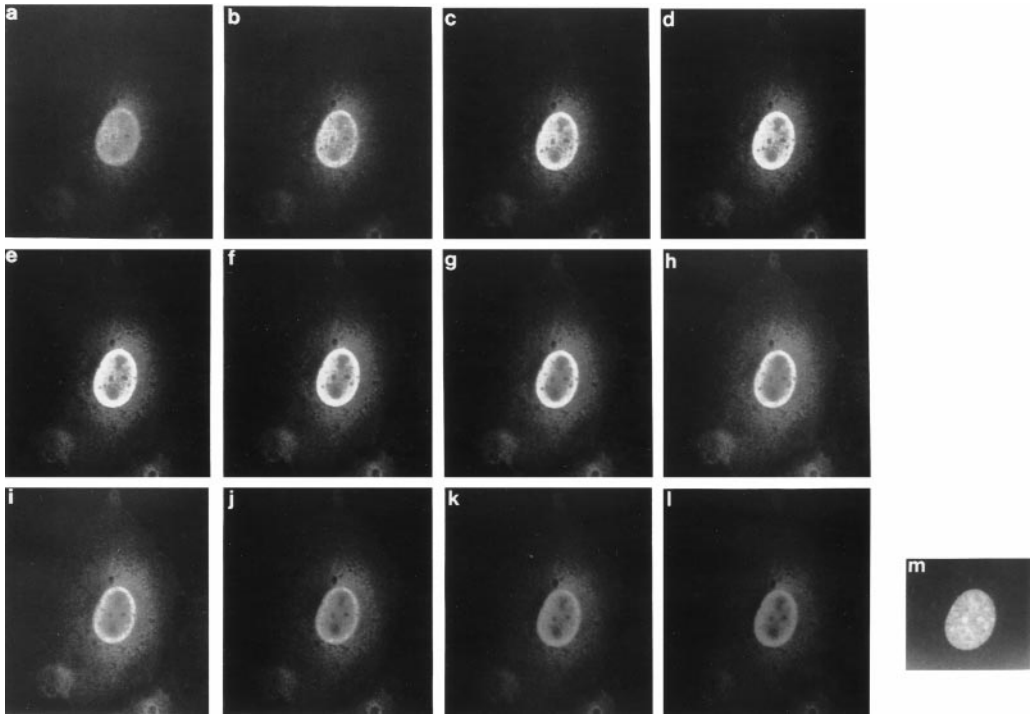

FIG. 3. **Confocal microscopy indicates that the eScTop1 protein is primarily localized to the area adjacent to the nuclear membrane.** A fluorescent positive cell expressing eScTop1 protein (from Fig. 2 above) was processed for laser scanning confocal microscopy. 0.24 μM optical sections were taken starting from the substratum (*a*) and proceeding through to the top of the cell (*l*). Images *e* through *h* represent sections through the center of the nucleus. A DAPI-stained image of the same nuclei is shown in *m*.

sion of human or yeast DNA topoisomerase I in *S. cerevisiae* leads to increased cell lethality, following treatment with camptothecin (14–17). In addition, it has been shown that overexpression of human DNA topoisomerase I in baby hamster kidney cells leads to increased sensitivity to camptothecin (26). To determine the extent of functional similarity between the yeast and human enzymes, we examined the camptothecin sensitivity of COS cells overexpressing eScTop1p. The approximately 100-fold increase in DNA topoisomerase I activity, resulting from the overexpression of the epitope-tagged yeast enzyme, should specifically enhance the camptothecin sensitivity of those cells relative to the untransfected COS cells in the same population. Because the ectopically expressed yeast enzyme is epitope-tagged, it allowed us to identify the number of cells expressing e*ScTOP1* within a given population by immunofluorescence. Treatment of the transfected cells with increasing doses of camptothecin should drive them to apoptosis and be seen as a concentration dependent increase in the percentage of apoptotic immunofluorescent positive cells.

To determine whether yeast DNA topoisomerase I enhanced the sensitivity of COS cells to the cytotoxic action of camptothecin, the cells were plated out onto glass coverslips and then transfected with the e*ScTOP1*-, e*Sctop1vac*-, or e*Sctop1Y727F*-expressing plasmids. Immediately after the glycerol shock, the cells were treated with increasing doses of camptothecin or with a Me_2SO control. Twenty-four hours after camptothecin treatment, the coverslips were processed for fluorescent microscopy, using M2 as a primary antibody, followed by DAPI staining. The altered nuclear morphology of an apoptotic cell, as assessed by DAPI staining, has been well defined (34, 35). Examples of camptothecin-induced apoptotic cells that are both fluorescent positive due to expression of e*ScTOP1* and fluorescent negative are shown in Fig. 7. The well characterized apoptotic features of nuclear blebbing and DNA fragmentation and condensation are evident (34, 35). It is interesting to note that eScTop1p becomes cytoplasmic following camptothecin treatment, which is consistent with the recent report that the enzyme changes its subcellular distribution after drug treatment (32).

The percentage of apoptotic cells, both fluorescent positive and fluorescent negative, was then determined over a range of camptothecin concentrations. As shown for the population of eScTop1p-expressing cells (Fig. 8*A*), the percentage of apoptotic fluorescent positive cells increases significantly with increasing dose of camptothecin. At the highest camptothecin dose this increase is 4-fold over that occurring in the fluorescent negative population (the cells not expressing eScTop1p). Thus, the eScTop1p-expressing cells are preferentially dying in response to increasing dose of camptothecin. It should be noted that cells transfected with the vector alone (*i.e.* pMT2) exhibit the same percentage of apoptotic cells over the range of camptothecin concentrations as the fluorescent negative population in Fig. 8*A* (data not shown).

When an experiment was performed with the catalytically inactive mutant e*Sctop1Y727F* (shown in Fig. 8*B*), the percentage of apoptotic fluorescent positive cells is the same as the percentage of apoptotic fluorescent negative cells at the highest doses of camptothecin. In the no drug control, and at the lower doses, there is a 2-fold increase in apoptotic cells in the immunofluorescent positive population. This slight cytotoxic effect is consistent with our previous observations in yeast, that overexpression of the catalytically inactive ScTop1Y727F protein is somewhat detrimental to cell growth in the absence of camptothecin (20). In mammalian cells, the expression of e*Sctop1Y727F* might interfere with essential *TOP1* functions by displacing the catalytically active endogenous enzyme from protein-DNA complexes that could be involved in transcription or replication, for example. Nevertheless, as these effects are drug independent, the data indicate that overexpression of the catalytically active eScTop1p, but not the catalytically inactive mutant, is required to enhance the cytotoxic action of camptothecin in COS cells. Moreover, expression of the catalytically

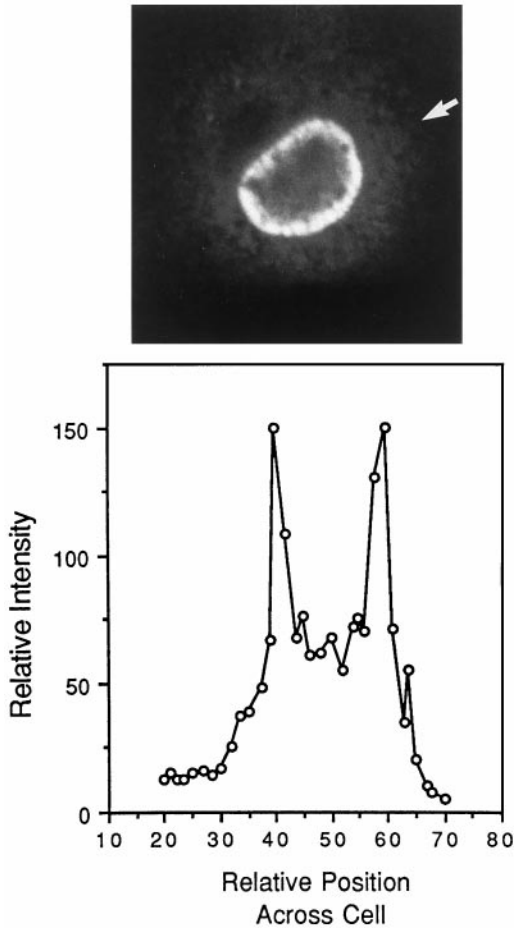

FIG. 4. **The eScTop1 protein exhibits a perinuclear distribution.** An optical section of a fluorescent-positive nuclei (from Fig. 3 above) was processed for densitometry, laterally across the image of the nuclei. The *arrow* next to the nucleus shows the direction of the densitometric scan. The scan was quantitated (relative intensity *versus* relative distance across the cell) and plotted as shown. The peaks of intensity from the scan (*bottom*) correspond to the region overlapping the nuclear membrane (*top*).

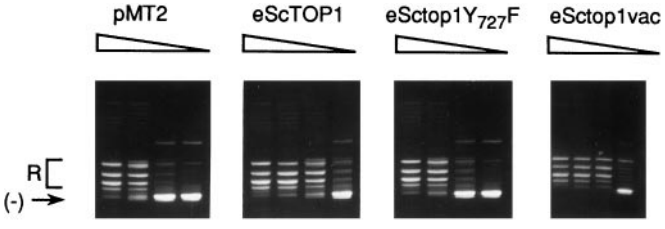

FIG. 5. **Extracts from COS cells transfected with e*ScTOP1* and e*Sctop1vac* exhibit increased DNA relaxation activity.** The e*ScTOP1*-, e*Sctop1vac*-, and e*Sctop1Y727F*-expressing plasmids were transiently transfected into subconfluent growing COS cells. The pMT2 plasmid was used as a control. 24 h after transfection, nuclear extracts were generated and adjusted to give identical protein concentrations (2 μg/μl). Undiluted and serial 10-fold dilutions were then tested in DNA relaxation assays (2-μl aliquots of each). The *arrow* indicates the relative position of negatively supercoiled plasmid DNA (*Sc*), whereas the distribution of relaxed DNA topoisomers is marked with *R*.

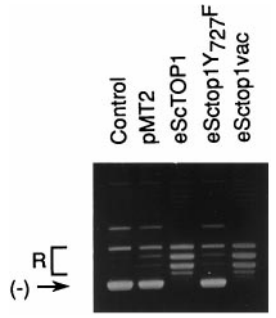

FIG. 6. **Immunoprecipitates of eSc*TOP1* and e*Sctop1vac* extracts are catalytically active.** The e*ScTOP1*-, e*Sctop1vac*-, and e*Sctop1Y727F*-expressing plasmids were transiently transfected into subconfluent growing COS cells. The pMT2 plasmid alone served as control. 24 h after transfection, the ectopically expressed eScTop1, eSctop1vac, and eScTop1Y727F proteins were immunoprecipitated from 15 μg of nuclear extracts. The immunoprecipitate was immobilized on Staph-A acrylamide beads and tested in DNA relaxation assays as in Fig. 5. The *arrow* points to negatively supercoiled DNA (*Sc*), and relaxed DNA topoisomers are labeled *R*.

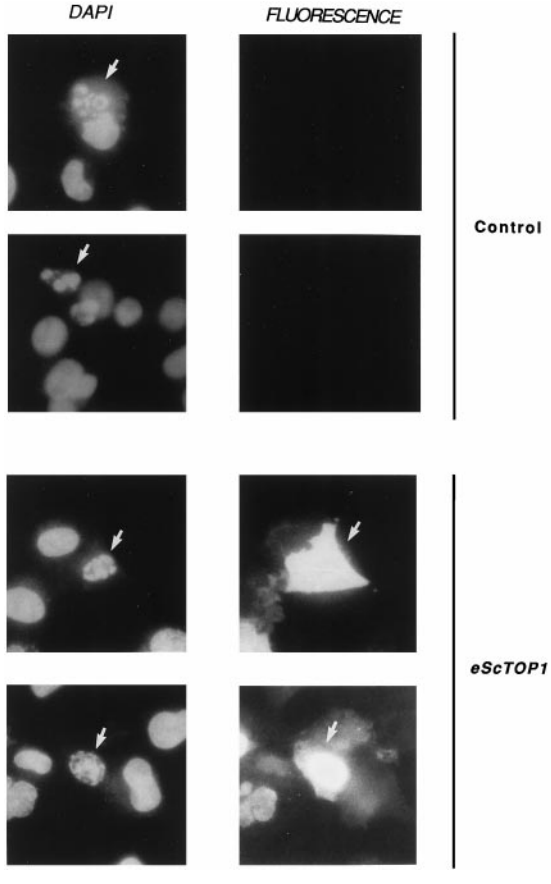

FIG. 7. **Induction of apoptosis in COS cells by camptothecin and assessed by DAPI staining.** COS cells were transfected with either the pMT2 plasmid (*control*) or pMT2eScTOP1 plasmid (e*ScTOP1*). Immediately after the transfection, the cells were treated with 5 μM camptothecin for an additional 24 h. The cells were processed for indirect immunofluorescence followed by DAPI staining. Shown are two independent fields for the pMT2 and pMT2eScTOP1 transfection. Cells scored as apoptotic (and fluorescent positive) are indicated by the *arrows*.

active, camptothecin-resistant e*Sctop1vac* mutant did not increase COS cell sensitivity to the drug; therefore, the percentage of apoptotic fluorescent positive and negative cells was same (Fig. 8*C*). These data indicate that the decreased camptothecin sensitivity of *S. cerevisiae* cells expressing the ScTop1vac enzyme, as demonstrated by Knab *et al.* (17, 21), is reiterated in mammalian cells.

To ensure that the effect of eScTop1p on the camptothecin sensitivity of COS cells is not due to an increase in the cellular form of DNA topoisomerase I, extracts from the control cells and e*ScTOP1* transfected cells were analyzed in immunoblots, with sera from scleroderma patients exhibiting high titer antibodies for mammalian DNA topoisomerase I. These antibodies do not recognize yeast DNA topoisomerase I (17, 21). The

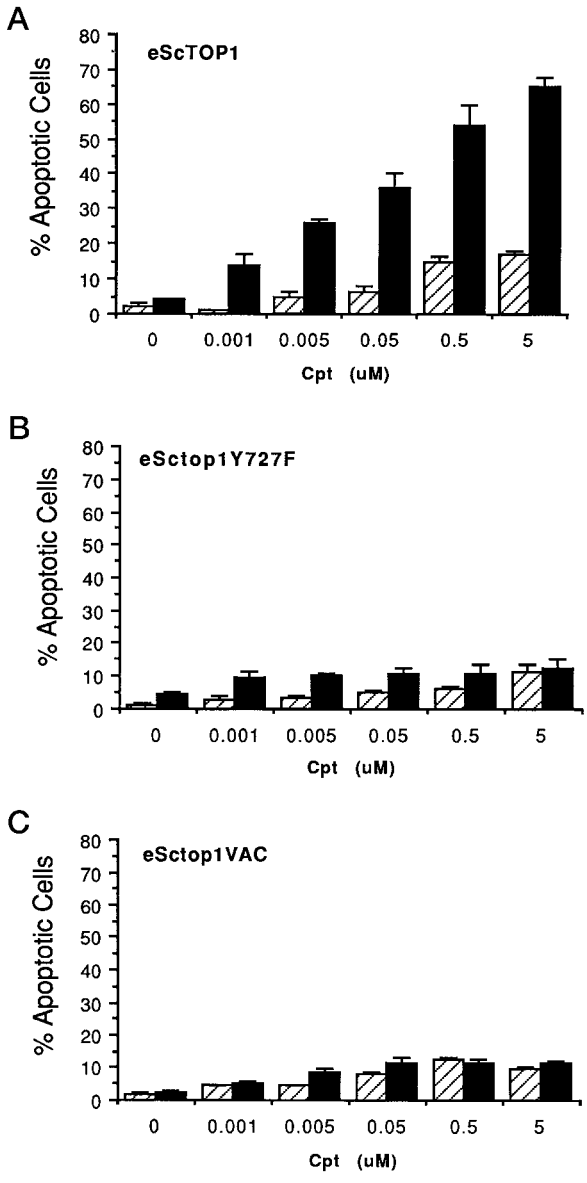

FIG. 8. **COS cells transfected with e*ScTOP1* are preferentially killed in the presence of camptothecin, whereas cells transfected with either e*Sctop1Y727F* or e*Sctop1vac* are resistant to camptothecin.** The e*ScTOP1*-expressing (*A*), e*Sctop1Y727F*-expressing (*B*), and e*Sctop1vac*-expressing (*C*) plasmids were transiently transfected into subconfluent COS cells plated onto glass coverslips. As a control, the pMT2 plasmid alone was also transfected into COS cells. Immediately following the transfection, the cells were treated with the indicated concentrations of camptothecin (in Me_2SO). Control cells were treated with Me_2SO only. 24 h following the transfection, the coverslips were processed for indirect immunofluorescence using the M2 monoclonal antibody as a primary antibody followed by a fluorescein-conjugated secondary antibody. The DNA was then stained with DAPI to highlight the nuclei. The percentage of apoptotic cells (both fluorescent positive and negative) was determined by assessing the morphology of the DAPI stain (as described in the legend to Fig. 7). The *error bars* represent the S.D. from multiple experiments. ▨, fluorescent negative; ■, fluorescent positive.

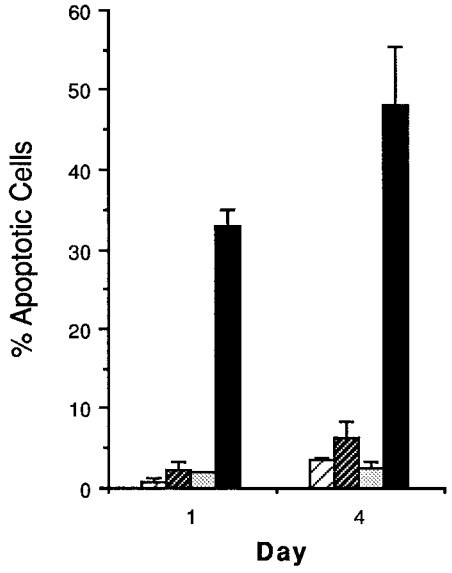

FIG. 9. **Expression of e*Sctop1T722A* in COS cells results in their selective death.** Percentages of apoptotic cells at 1 and 4 days posttransfection. Equal numbers of COS cells (5×10^5 cells/10-cm plate) were seeded onto glass coverslips. The cells were transfected with the indicated plasmids. At 1 and 4 days following the transfection, the coverslips were processed for immunofluorescence. Shown are the percentages of apoptotic cells (both fluorescent positive and fluorescent negative) as assayed by DAPI staining. The error bars represent the S.D. from multiple experiments. ▨, eScTOP1 (fluorescent negative); ▨, eScTOP1 (fluorescent positive); ▨, eSctop1T722A (fluorescent negative); ■, eSctop1T722A (fluorescent positive).

levels of COS cell DNA topoisomerase I were the same in cells transfected with pMT2 or pMT2eScTOP1 (data not shown), indicating that the expression of eScTop1p does not cause a detectable increase in the levels of the COS cell DNA topoisomerase I.

Overexpression of the Lethal Mutant eSctop1T722A in COS Cells Is Cytotoxic in the Absence of Camptothecin—Substitution of alanine for threonine 722 in *S. cerevisiae* DNA topoisomerase I produces a lethal phenotype when the mutant enzyme is overexpressed in yeast, even in the absence of camptothecin (20). To determine whether the mechanism of *top1* mutant-induced DNA damage is also conserved in higher eukaryotes, e*Sctop1T722A* was cloned into the pMT2 vector and transiently expressed in COS cells. eScTop1T722Ap was found to be expressed to the same levels as eScTop1p by Western blot analysis (data not shown). To determine the potential lethality of this mutant in COS cells, cells were plated onto coverslips and transfected with pMT2 constructs expressing e*ScTOP1* and e*Sctop1T722A*. At 1 and 4 days after transfection, the cells were processed for immunofluorescent microscopy. As shown in Fig. 9, the percentage of apoptotic cells is significantly increased in the cells transfected with e*Sctop1T722A* compared with cells transfected with e*ScTOP1*. Transfection of the inactive mutant e*Sctop1Y727F* resulted in a similar number of apoptotic cells as e*ScTOP1* (data not shown). These data indicate that expression of the lethal mutant, e*Sctop1T722A*, induces an apoptotic response in higher eukaryotes, similar to its cytotoxic effect when overexpressed in yeast.

The mechanism by which e*Sctop1T722A* is thought to kill yeast is via enhanced cleavage of DNA, due to an increase in the stability of the covalent enzyme-DNA intermediate (20). To determine whether the eScTop1T722A enzyme was capable of increased DNA cleavage, the protein was immunoprecipitated from nuclear extracts following the transfection. These immunoprecipitates were immobilized on *Staphylococcus* protein A-acrylamide beads, which were then used in a DNA cleavage assay with a 944-base pair ^{32}P-labeled DNA fragment containing a high affinity DNA topoisomerase I binding site (20). Extracts from control transfected cells or from cells transfected with e*ScTOP1* were also used in the assay. As shown in Fig. 10, enhanced cleavage occurred in the immunoprecipitates of the eScTop1T722A enzyme but not in the immunoprecipitates of the eScTop1 protein or the control. These data suggest the increased formation of covalent eScTop1T722Ap-DNA intermediates in nuclear extracts of cells expressing this mutant en-

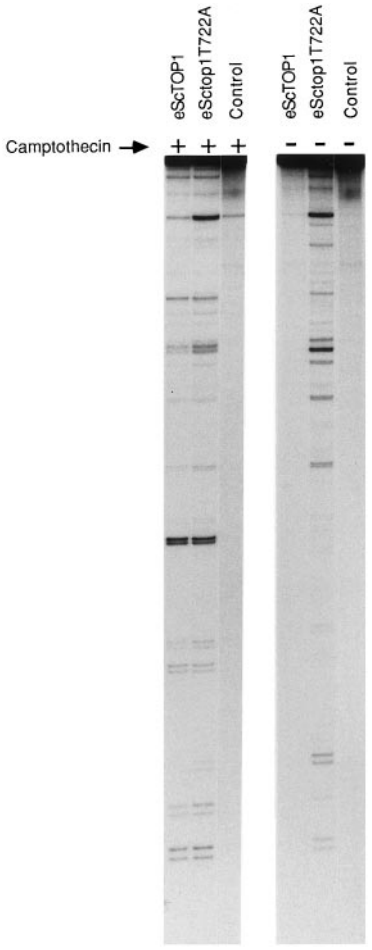

FIG. 10. **Immunoprecipitates of eScTop1T722Ap from nuclear extracts demonstrate increased DNA cleavage in the absence of camptothecin.** The e*ScTOP1*- and e*Sctop1T722A*-expressing plasmids were transiently transfected into subconfluent growing COS cells. The pMT2 plasmid alone served as control. 24 h after transfection, the ectopically expressed eScTop1 and eScTop1T722A proteins were immunoprecipitated from 15 μg of nuclear extracts. The immunoprecipitates were immobilized on Staph-A acrylamide beads and incubated with 5000 cpm of ^{32}P-, 3′-end labeled DNA fragment (containing a high affinity DNA topoisomerase I binding site) and with or without 100 μM camptothecin (where indicated). The cleaved DNA products were trapped with 1% SDS at 75 °C, treated with proteinase K, and resolved in a DNA sequencing gel. An autoradiograph of the gel is shown.

zyme. Camptothecin was also added to the immunoprecipitates to show that the eScTop1 enzyme was sensitive to the action of the drug, resulting in a stabilization of the cleavable complex (see Fig. 10), consistent with the known action of camptothecin on this protein (17).

DISCUSSION

To establish the conservation of eukaryotic DNA topoisomerase I function, we have examined the effects of transiently overexpressing an epitope-tagged *S. cerevisiae* DNA topoisomerase I enzyme (eScTop1p) on the viability of a mammalian cell line. Nuclear extracts of the transfected COS cells contained the ectopically expressed protein as assessed by immunoblotting with either a yeast DNA topoisomerase I antibody or an antibody directed against the epitope tag. The enzyme was also found to be appropriately targeted to the nucleus, as assessed by indirect immunofluorescence.

An important observation was that the ectopically expressed eScTop1 protein enhanced the camptothecin sensitivity of the transfected COS cells because treatment with the drug resulted in preferential killing via apoptosis of those cells expressing the yeast enzyme. Thus, the yeast enzyme appears functional in mammalian cells. Because DNA topoisomerase I has been firmly established as the sole cellular target of camptothecin, overexpression of e*ScTOP1* in COS cells in the presence of camptothecin likely increases the amount of double-stranded DNA breaks, resulting in apoptotic cell death. Therefore, the conservation of structure and function between the yeast and mammalian enzymes is sufficient to produce lethal DNA damage in mammalian cells in response to the yeast enzyme and the drug. These data highlight the reciprocity in action between the yeast and human enzymes, suggesting that regardless of the source of enzyme or cell type, overexpression of eukaryotic DNA topoisomerase I in a eukaryotic cell will lead to death in the presence of camptothecin.

Along these lines, it is apparent that the phenotypes associated with overexpression of *Sctop1* mutants in yeast were also faithfully reiterated in COS cells. The ScTop1Y727F mutant protein is catalytically inactive (25), and *top1*Δ yeast strains expressing the e*Sctop1Y727F* mutant are completely resistant to the effects of camptothecin (4, 17). Although eScTop1Y727Fp was efficiently targeted to the nucleus in COS cells, as measured by indirect immunofluorescence, overexpression of this mutant protein produced no observable changes in DNA relaxation activity in nuclear extracts or in the camptothecin sensitivity of transfected COS cells. As has been reported in yeast (20), overexpression of this protein was slightly cytotoxic in the absence of camptothecin. Whether or not this effect results from interference with endogenous *TOP1* functions has yet to be determined. Nevertheless, these results support the notion that the increased cytotoxic effects of camptothecin on e*ScTOP1*-expressing cells is a direct result of the increased DNA topoisomerase I activity in these cells.

The e*Sctop1vac* mutant is a double mutation of Ile^{725} to Arg and Asn^{726} to Ala (25). When eScTop1vacp is overexpressed in a *S. cerevisiae top1*Δ strain, the cells are completely resistant to the lethal effects of camptothecin (17, 21). Biochemical studies indicate that this catalytically active mutant enzyme is ~20-fold more resistant to camptothecin-induced DNA cleavage than the wild-type enzyme (17). This camptothecin-resistant phenotype is also evident in mammalian cells because the cytotoxic action of camptothecin on COS cells overexpressing e*Sctop1vac* is diminished in comparison to COS cells overexpressing the wild-type eScTop1 enzyme.

One of the more striking DNA topoisomerase I mutants involves a substitution of alanine for threonine at position 722 (20). This substitution (e*Sctop1T722A*) converts the enzyme into a cellular poison when it is overexpressed in yeast, due to the fact that the covalent enzyme-DNA intermediate is stabilized (20). Interestingly, a similar effect on cell viability is evident in COS cells ectopically expressing e*Sctop1T722A*, in contrast to cells expressing e*ScTOP1*. The cells expressing e*Sctop1T722A* die via an apoptotic mechanism, likely the result of the DNA damaging capability of eScTop1T722Ap, as assessed by a DNA cleavage assay (Fig. 10). Further, as in yeast, the cell lethality induced by e*Sctop1T722A* is camptothecin independent. Taken together, these results highlight the conservation in function between yeast and human DNA topoisomerase I and support the use of model systems such as yeast to explore the cytotoxic mode of action of DNA topoisomerase I poisons.

Another novel finding presented in this work concerns the activity of eScTop1p in immobilized immunoprecipitates. Extensively washed Staph-A immobilized immunoprecipitates of the ectopically expressed eScTop1 protein had increased DNA relaxation activity and increased DNA cleavage activity in the presence of camptothecin. This suggests that the enzyme is

functional even though its amino terminus is tethered to a support and is immobilized. Control experiments demonstrated that the bulk of the enzyme remains tethered to the Staph-A beads (data not shown). This approach is currently being exploited to assess the effect of specific amino acid substitutions on DNA binding by the enzyme.

Finally, examination of subcellular location of ectopically produced eScTop1p indicated that it can be targeted to a perinuclear region, as demonstrated by confocal microscopy. This is interesting in light of the evidence that the nuclear membrane participates in DNA replication (36) possibly by anchoring chromosomes. The nuclear membrane would therefore be a potential target for DNA topoisomerase I-mediated relaxation of supercoiled DNA generated during replication. A similar perinuclear distribution of human DNA topoisomerase I was reported in CEM cells following leucine starvation (37). Although the enzyme demonstrates a predominantly nucleolar distribution in proliferating cells (37, 38), it is also seen localized to other sites within the nucleus, such as the nuclear membrane (32, 33). Because at a low level of expression, eScTop1p can be seen localized to the nucleoli (data not shown), it is possible that at a higher level of expression the protein occupies available binding sites near the nuclear membrane. The nature of these sites remains to be determined.

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Increased Camptothecin Toxicity Induced in Mammalian Cells Expressing *Saccharomyces cerevisiae* DNA Topoisomerase I

Christine Hann, Devon L. Evans, Jolanta Fertala, Piero Benedetti, Mary-Ann Bjornsti and David J. Hall

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