Expression of Human Topoisomerase I with a Partial Deletion of the Linker Region Yields Monomeric and Dimeric Enzymes That Respond Differently to Camptothecin*

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Gregory C. Ireton, Lance Stewart‡, Leon H. Parker§, and James J. Champoux¶

From the Department of Microbiology, University of Washington School of Medicine, Seattle, Washington 98195-7242

Eukaryotic type I topoisomerases are capable of relaxing both positively and negatively supercoiled DNA in the absence of an energy-donating cofactor (1). The enzyme acts by introducing a transient break in one of the strands of DNA that allows superhelical tension to be relieved by strand rotation at the site of the nick (2). Cleavage occurs by a transesterification reaction involving nucleophilic attack by an active site tyrosine on a DNA phosphodiester bond resulting in the formation of a covalent DNA 3′-phosphotyrosyl linkage. In the religation phase, a similar transesterification reaction involves attack by the free DNA 5′-hydroxyl that reseals the phosphodiester bond and releases the enzyme from the DNA (1). Topoisomerase I has been implicated in various biological processes including DNA replication, transcription, and recombination (3, 4).

Topoisomerase I is the sole target of the anti-tumor drug camptothecin (5–10), a cytotoxic plant alkaloid originally identified as a potent inhibitor of eukaryotic DNA replication and transcription (11, 12). Camptothecin poisons topoisomerase I by binding to the transient enzyme-DNA covalent complex and slowing the religation step of the nicking-closing cycle (13–18). The drug is specifically toxic to cells in S phase (19, 20), and several studies support a model for camptothecin cytotoxicity in which the collision of a replication fork with a topoisomerase I covalent complex results in a potentially lethal DNA break (21–28).

As suggested by its mode of action, the cytotoxic effects of camptothecin require the presence of active topoisomerase I in the cell. The topoisomerase I gene, TOP1, is dispensable in the yeasts Saccharomyces cerevisiae (29) and Schizosaccharomyces pombe (30), and null mutants that are permeable to the drug are resistant to its cytotoxic effects (5, 6). Drug resistance is restored when such strains are transformed with plasmids that express either the yeast TOP1 gene (5) or a cDNA encoding human topoisomerase I (10).

From previous studies involving homology comparisons, limited proteolysis, hydrodynamic measurements, and the analysis of the crystal structure of the protein, it has been possible to identify four domains within human topoisomerase I (91 kDa) (2, 31–35). The unconserved NH2-terminal domain (residues 1–214) is highly charged, contains four putative nuclear localization signals (36), is extremely sensitive to proteases, and is dispensable for in vitro enzymatic activity (34, 37–40). A form of the enzyme missing the first 174 amino acids (topo70D) (Fig. 1, panel A) has been described that retains all of the in vitro enzymatic properties normally ascribed to the full-length enzyme (34). The conserved core domain (residues 215–635) has a globular structure and is relatively protease-resistant. A short, unconserved protease-sensitive linker region (residues 636–712) connects the core to the highly conserved and protease-resistant COOH-terminal domain (residues 713–765).

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‡ Present address: Emerald BioStructures, Inc., 7869 N.E. Day Rd. W., Bainbridge Island, WA 98110.
§ Present address: Dept. of Molecular Biology, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080-4990.
¶ To whom correspondence should be addressed: Dept. of Microbiology, Box 357242, University of Washington, Seattle, WA 98195-7242. Tel.: 206-543-8574; Fax: 206-543-8297; E-mail: champoux@u.washington.edu.

1 The abbreviations used are: topo70, NH2-terminal truncation of human topoisomerase I lacking the first 174 amino acids; topo70D, mutant form of topo70 in which tyrosine 720 has been replaced with phenylalanine (Y720F); topo12, 108 amino acid COOH-terminal fragment of human topoisomerase I composed of amino acids 658 to 765; topo6.3, 53 amino acid COOH-terminal fragment of human topoisomerase I composed of amino acids 713 to 765; topo70A, mutant form of topo70 in which amino acids 680 to 688 have been deleted; topo70A, LPhe, topo70AL with the Y723F mutation; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; kbp, kilobase pair; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; GST, glutathione S-transferase.
The COOH-terminal domain contains the active site tyrosine at position 723.

We have previously shown that human topoisomerase I activity can be reconstituted in vitro by mixing a 58-kDa core fragment corresponding to residues 175–659 (topo58) with any one of a series of COOH-terminal fragments ranging in size from 12 (topo12, residues 658–765) to 6.3 kDa (topo6.3, residues 713–765) (Fig. 1, panel A) (41). The core and COOH-terminal fragments bind to form a 1:1 complex, and both topo58/12 and topo58/6.3 display nearly the same plasmid relaxation activity under standard assay conditions as the full-length enzyme or topo70 (41). The reconstituted enzymes (topo58/12 and topo58/6.3) differ from intact topo70 by exhibiting a reduced affinity for DNA and a cleavage-religation equilibrium that is strongly biased toward religation (41).

To define further the role of the linker region, we have characterized a form of the enzyme produced by a deletion in the coding region that removes approximately the distal 40% (residues Asp660 to Lys688) of the coiled-coil linker region (2). We show here that during the purification of the resulting topo70L protein, both the anticipated monomer and a smaller amount of an unusual dimeric species are found. Although both forms are enzymatically active, they differ with respect to their processivities and their responses to camptothecin in a plasmid relaxation assay.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Site-directed Mutagenesis**

Plasmids were constructed using conventional cloning techniques (42) and propagated using the Escherichia coli strain TOP10F (merA, Δmrr-hsdRMS-merC), Δ(mrr-hsdRMS)M15, ΔacX74, deoR, recA1, araD139, 3·ara, leu2-3,112, galU, galK, rpsL, endA1, nupC (F′ lacIq Tn10 tet’’’)(Invitrogen). Plasmid DNA was rescued from the strain using the helper phage M13K07. The single-stranded DNA was subjected to oligonucleotide-directed mutagenesis following the protocol provided by the manufacturer (Life Technologies, Inc.). The wild type and active site mutant topo70 were generated with the Bac-to-Bac expression system according to the protocol provided by the manufacturer (Life Technologies, Inc.). The wild type and active site mutant topo70L proteins were purified from baculovirus-infected insect cells as described previously (34).

**Recombinant Baculoviruses and Purification of Proteins from Infected Insect Cells**

The isolation of topo70-expressing recombinant baculovirus, the culture and infection of Sf9 insect cells, and the purification of the various proteins have been described previously (34). The generation of the topo58/6.3 and topo58/12 recombinant proteins is described elsewhere (34). The topo58/6.3 coding sequences were amplified from pADH1B-topo70L (URA) as a BamHI-HindIII fragment and ligated into pFastBac1 (Life Technologies, Inc.). A similar plasmid containing the GAL4 DNA binding domain sequence at the junction between the coding regions from pADH1B-topo70L (URA) was inserted into pGBT9 at the BamHI end (end-filled) and PstI sites in the polylinker. The resulting plasmid encodes a fusion protein comprised of the GAL4 DNA binding domain (GBD) fused to the active site mutant (Y723F) form of topo70. pGBT-topo58—An ~2.9-kbp BamHI (end-filled) to PstI fragment from pAE-topo58 (34) was inserted into the polylinker of pGBT9 to generate pGBT-topo58 which encodes a fusion protein comprised of the GAL4 DNA binding domain fused to topo58.

**Recombinant GST-Topo12**

The generation of plasmid pGST-topo12 and the purification of the GST-topo12 fusion in the BL21 (Novagen) strain of E. coli were described previously (41). The GST-topo12 protein is comprised of GST followed by a factor Xa cleavage site, two extraneous amino acids (Gly-Ile), and residues Leu658–Phe665 of topoisomerase I (Fig. 1, panel A). The purification of topo12 by factor Xa cleavage of the GST fusion protein and subsequent purification by Mono S column (5/10) chromatography were described previously (41).

**Yeast Transformations and Camptothecin Sensitivity Assays**

Yeast cells were transformed with the various expression plasmids using the lithium acetate procedure (45, 46). Transformants were selected.
lected on synthetic dextrose (SD) medium lacking the appropriate supplements to maintain plasmid selection. At least four independent clones were selected from each transformation.

Camptothecin sensitivity assays were performed using S. cerevisiae strain JNS-104 (MATa rad52::LEU2 trp1 ade2-1 his3 ura3-52 is1 top1 796-265) containing form of camptothecin. The material and that corresponds in size to the topo58 core domain was purified from infected Sf9 insect cells. One of the final steps in the purification procedure involved elution from a Mono S (H5/R) cation exchange column using 50–200 mM KPO4 gradient. Unlike topo70 that elutes as a single species from the column (34), the topo70L proteins eluted as two distinct peaks at ~140 and ~170 mM KPO4, irrespective of the status of the active site residue 723 (wild type or Y723F). The material from each pool was re-chromatographed over the Mono S column, and in each case, the material was eluted as a single peak at the same KPO4 concentration as in the first chromatographic analysis (data not shown).

The topo70L proteins present in the low and high salt pools migrated with identical mobilities in SDS-PAGE and, as expected, both migrated slightly faster than topo70 (Fig. 1, panel B, lanes 1–3). Note that a band representing less than ~5% of the material and that corresponds in size to the topo58 core domain was present in both pools (Fig. 1, panel B, compare lanes 2 and 3 with lane 4). It is likely that this material was generated by proteolytic breakdown during protein expression and purification.

**RESULTS**

**Purification of Topo70L Proteins**—To determine the biochemical consequences of deleting a portion of the linker region of human topoisomerase I, recombinant baculoviruses were constructed that express the topo70 form of the protein deleted for amino acids 660–688 (topo70L) (Fig. 1, panel A). Both topo70L and the catalytically inactive topo70L Phe (Y723F mutation) were purified from infected Sf9 insect cells. One of the final steps in the purification procedure involved elution from a Mono S (H5/R) cation exchange column using a 50–200 mM KPO4 gradient. Unlike topo70 that elutes as a single species from the column (34), the topo70L proteins eluted as two distinct peaks at ~140 and ~170 mM KPO4, irrespective of the status of the active site residue 723 (wild type or Y723F). The material from each pool was re-chromatographed over the Mono S column, and in each case, the material was eluted as a single peak at the same KPO4 concentration as in the first chromatographic analysis (data not shown).

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**Enzyme Activity of Purified Topo70L Proteins**—To examine the effect of the linker deletion on enzyme activity, the two purified topo70L protein pools were assayed for activity by the 2-fold serial dilution plasmid relaxation assay (34). Under standard reaction conditions (150 mM KCl) both the low and high salt pools of topo70L were found to be enzymatically active with activity levels reduced only 2–4-fold relative to the wild type topo70 (Fig. 2, compare panels A, C, and D). Trace amounts of insect cell topoisomerase I are known to co-purify with recombinant human topo70 that has been expressed in the baculovirus/insect cell system (34). To estimate the contribution of the contaminating insect cell enzyme to the activity exhibited by the mutant enzymes, the relaxing activity of the catalytically inactive Y723F mutant form of topo70L (low salt pool) was determined. Although a low level of insect topoisomerase I contamination was detectable (Fig. 2, panel B, lane 1), the activity was ~200-fold lower than that of the topo70L low or high salt forms.

An important aspect of topoisomerase activity relates to the ability of the enzyme to remain associated with a given substrate molecule through multiple rounds of cleavage and religation (50). In a plasmid relaxation assay, processive relaxation results in the production of completely relaxed molecules and few topological isomers with intermediate superhelicities. Alternatively, distributive activity results in the gradual relaxation of the plasmid DNA with the production of many partially

**Assays for Topoisomerase Activity**

The standard reaction mixtures for topoisomerase relaxation assays contained 150 mM Tris hydrochloride, pH 7.5, 150 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.1 mg/ml bovine serum albumin (Reaction Buffer). Enzyme Dilution Buffer contained 10 mM Tris hydrochloride, pH 7.5, 1 mM EDTA, 1 mM DTT, and 0.1 mg/ml bovine serum albumin. For the serial dilution assays, protein samples were serially diluted 2-fold in Dilution Buffer, and reactions were initiated by the addition of 5 μl of diluted enzyme to 15 μl of Reaction Buffer containing 0.5 μg of supercoiled pBR322 DNA (Stratagene). After 10 min at 37 °C the reactions were stopped by the addition of 5 μl of Stop Dye (2.5% SDS, 25% Ficoll 400, 0.03% bromphenol blue, 0.03% xylene cyanol, 25 mM EDTA), and one-half of each sample was analyzed by electrophoresis in an 0.8% agarose gel. For the time course assays, the enzyme was diluted to 3 ng/μl in Dilution Buffer, and the reactions were initiated by the addition of 12 μl of diluted enzyme to 228 μl of Reaction Buffer containing 35 ng/μl of plasmid DNA. Reactions were incubated at 37 °C, and at the indicated time points 20-μl aliquots were removed into 5 μl of Stop Dye to terminate the reaction. The DNA bands were visualized by ethidium bromide staining and photographed with UV illumination.

**Proteolysis and Native Continuous Gel Electrophoresis**

Subtilisin (Roche Molecular Biochemicals) was diluted to the indicated concentration in 10 mM Tris hydrochloride, pH 7.5, 1 mM EDTA. Purified proteins stored at ~20 °C were diluted to 1.25 mg/ml in Storage Buffer. 6 μl of subtilisin at 4 μg/ml was aliquoted into tubes; for the no digestion control reactions, 1 μl of 30 mM phenylmethylsulfonyl fluoride (PMSF) was also added to the tubes. 4 μl of diluted protein was added to the subtilisin and subtilisin plus PMSF tubes to begin the proteolysis reaction, and the samples were incubated for 20 min at room temperature. Digestion was stopped by the addition of 1 μl of 30 mM PMSF, and reactions were placed on ice. Half of the sample (5.5 μl) was removed into 4 μl of Native Load Buffer (20% glycerol, 5 mM histidine, 6 mM MOPS, pH 6.6, 0.04% methyl green) and analyzed by Native-PAGE, and 4 μl of SDS Load Buffer (20% glycerol, 5% SDS, 5% v/v 2-mercaptoethanol, 100 mM Tris hydrochloride, pH 8.0, 0.05% bromphenol blue) was added to the remaining reaction to be analyzed by SDS-PAGE.

**SDS-polyacrylamide gel electrophoresis** was performed according to Laemmli (48). Native protein analysis was carried out by continuous gel electrophoresis through 5% polyacrylamide using 25 mM histidine, 30 mM MOPS running buffer, pH 7.2, and either 10 μg/ml camptothecin (final concentration, 0.05% Me2SO) or 0.05% Me2SO alone as a control. Plates were incubated at 30 °C until colonies were clearly visible on the Me2SO control plates, usually 4–5 days. The numbers of colonies on each plate visible to the unaided eye (>0.2 mm) were counted.

**Immunoblot Analysis of Yeast Extracts**

Expression of the recombinant fragments of human topoisomerase I in the yeast cells was confirmed by immunoblot analysis of extracts prepared by disruption with glass beads according to the procedure described by Jazwinski (47). Western blot transfer and immunoblot analysis using polyclonal rabbit anti-human topoisomerase I serum were carried out as described previously (33).

**Gel Filtration, Sedimentation, and SDS-induced Oligonucleotide Breakage Assays**

The gel filtration and glycerol gradient sedimentation procedures used to estimate the apparent molecular mass of the low and high salt pools of topo70L were carried out as described previously (34). The 25-mer oligonucleotide (5′GAAAAAGACTTAGAAAAATTTTATAG) was purified, 5′-end-labeled, and used as the substrate for the SDS cleavage assays as described previously (41). Briefly, 20-μl samples were prepared so that each contained 317 nM purified enzyme. Reactions were initiated by the addition of 2.5 μl of 5′-32P-end-labeled single-stranded (suicide cleavage) or duplex 25-mer (SDS cleavage) DNA substrate to give a final concentration of 1.43 μM. The final reaction conditions were 138 mM KCl, 12 mM NaCl, 10 mM Tris hydrochloride, pH 7.5, 1 mM EDTA, 1 mM DTT, 5.5% Me2SO, with or without 50 μM camptothecin. After a 45-min incubation at room temperature, the reactions were adjusted to 1% SDS, boiled, and fractionated by 8–17% SDS-PAGE. Gels were dried, and the radiolabeled cleavage products were visualized by autoradiography using Kodak XAR film, and quantified by PhosphorImager analysis using ImageQuant software (Molecular Dynamics).

**Native Continuous Gel Electrophoresis**

Native protein analysis was carried out by continuous gel electrophoresis through 5% polyacrylamide using 25 mM histidine, 30 mM MOPS running buffer, pH 6.6, run from anode to cathode (49). Native gels were pre-run for 1–1.5 h at 4 °C at 200 V, and after loading the samples the gels were run for 1 h at 200 V and 4 °C. Proteins were visualized by Coomassie Blue or Silver staining.
relaxed topoisomers. It has been established previously that wild type topo70 is highly processive at 150 mM KCl, whereas the reconstituted enzymes are distributive under the same conditions (41). Interestingly, relaxation by the topo70D
low salt pool appeared distributive, whereas the high salt form appeared processive (Fig. 2, compare panels C and D). These results suggest that the high salt form of the topo70DΔL enzyme possesses a higher affinity for DNA than the low salt form.

To elucidate further the structures of the two forms of topo70DΔL, the low and high salt pools were analyzed by native continuous gel electrophoresis. Because the isoelectric point for...
human topoisomerase I is >9.3, non-standard Native-PAGE procedures were used; the gels were buffered at pH 6.6 and run from the anode to the cathode (see “Experimental Procedures”). On a 5% continuous native gel, the topo70 ΔL low and high salt forms were clearly resolved (Fig. 4, panel A, lanes 5 and 7) with the slower mobility of the high salt form being consistent with a greater molecular size. The larger size of the high salt-eluting form is likely due to non-covalent association of monomers, since the two forms are essentially pure and co-migrate with the expected molecular size in denaturing SDS-PAGE (Fig. 4, panel B). Further support for the view that the high salt pool is dimeric comes from the observation that the topo70 ΔL low salt pool exhibited a mobility only slightly slower than that of the reconstituted topo58/12 and topo58/6.3 proteins (Fig. 4, panel A, compare lanes 1, 2, and 5) which are most likely monomeric (41). In addition, these native gel analyses revealed both low and high salt pools to be single species, with no interconversion between the two forms and no apparent higher molecular weight forms. Taken together, these observations strongly suggest that the low and high salt-eluting species are monomeric and dimeric forms of topo70 ΔL, respectively.

**Subtilisin Sensitivity of the Topo70 ΔL Monomer and Dimer**—Two alternative hypotheses can be presented to explain why a deletion in the linker region could lead to dimer formation. The first is that linker deletion alters the protein structure to expose regions on the protein that interact, but the normal intramolecular protein folding and domain structure within each polypeptide remain intact. For example, two core domains, two COOH-terminal domains, or one core domain and one COOH-terminal domain could interact with each other. Alternatively, one might imagine that removal of part of the linker prevents or alters the normal intramolecular association of core and COOH-terminal domains to allow the COOH-terminal region of one molecule to bind to the core of the other and vice versa. This second hypothesis is consistent with the observation that separately expressed core and COOH-terminal domains can interact to reconstitute active enzyme (41). To distinguish between these possibilities, we examined the limited subtilisin digestion patterns of the proteins by Native-PAGE.

We have previously shown that subtilisin clips within the linker region of the wild type protein to produce fragments corresponding to the core and COOH-terminal domains of the protein and that the digestion products remain associated and retain topoisomerase activity (33, 41). When the monomeric and dimeric forms of topo70ΔL were digested with subtilisin under similar conditions and the products analyzed by SDS-PAGE, it can be seen that both were nearly completely cleaved to produce an ~58-kDa core fragment that had the same mobility as recombinant topo58 (Fig. 4, panel B, lanes 1, 2, 6, and 8) (the smaller COOH-terminal fragments ran off the gel). Under these same conditions only approximately 50% of topo70 was cleaved to the ~58-kDa core fragment (Fig. 4, panel B, lane 4). Two-fold serial dilution assays revealed topo70ΔL monomer to be ~32-fold more sensitive and topo70ΔL dimer to be ~8-fold more sensitive to the protease than the intact topo70 (data not shown). When the same digestion products were analyzed by native gel electrophoresis, the digested topo70ΔL monomer migrated with the same mobility as the untreated control protein (Fig. 4, panel A, lanes 5 and 6) indicating that, similar to the case for the full-length protein (41), the core and COOH-terminal domains remain associated after cleavage within the linker domain. However, limited proteolysis reduced the dimer to a form with the same mobility in the native gel as the monomeric protein (Fig. 4, panel A, lanes 5–8). Digested topo70 migrated as a smear presumably due to incomplete subtilisin cleavage. Were dimer formation to have resulted from an interaction between two core domains or two linker domains, then simple proteolytic cleavage of the dimer at the core: COOH-terminal domain junction would not have been expected to produce a species that co-migrated with the monomeric form of the protein. The simplest interpretation of these data is that dimer formation involves “3D domain swapping” (51) in which interactions between core and COOH-terminal domain on different molecules substitute for the usual intramolecular binding of these same two domains. Limited protease cleavage in what remains of the linker would be expected to reduce the dimer to a monomer that is nearly equivalent to the reconstituted topo58/6.3 (see “Discussion”).

**Reconstitution of Topoisomerase Activity by Fragment Complementation between Topo70ΔL.Phe and a COOH-termina l Fragment**—We previously showed that topoisomerase activity can be restored by fragment complementation between separately expressed COOH-terminal and core proteins (41). Given that the topo70ΔL dimer appears to be formed from an intramolecular interaction between core and COOH-terminal fragments, we tested whether domain swapping between both monomers occurred to form two stable core-COOH-terminal complexes or whether one of the core domains remained at least partially free to complex with an exogenously added COOH-terminal domain. To test this latter possibility, we examined the ability of a wild type COOH-terminal domain fragment (topo12) to reconstitute enzyme activity with the dimeric form of topo70ΔL.Phe. Parallel analyses with the monomeric form of topo70ΔL.Phe were carried out to evaluate the ability of
an isolated COOH-terminal domain to bind to the core of the monomer.

Activity assays were performed by mixing either the monomeric or the dimeric form of topo70LPhe with a 5-fold molar excess of the COOH-terminal fragment, topo12. Two-fold serial dilution assays showed topoiso- merase relaxation activity was reconstituted with topo12 and both forms of topo70LPhe to a level only 4–8-fold less than that of the corresponding low and high salt forms of wild type topo70L (Fig. 2, panels C–F) and well above the control level of both forms of topo70LPhe alone (Fig. 2, panel B, and data not shown). This level of reconstituted activity was >100-fold above that seen previously in control mixtures of nondeleted topo70Phe and various COOH-terminal fragments (41). Importantly, this high level of enzyme activity rules out the possibility that the observed activity arises from reconstitution between the topo12 and the trace amount (<5%) of topo58 core fragment present in the purified topo70LP preparations (Fig. 1, panel B, lanes 2 and 3). These

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**Fig. 3. Gel filtration and glycerol gradient sedimentation.** Panel A, proteins and size standards were chromatographed on a Superose 12 column using fast protein liquid chromatography, and elution profiles were monitored by UV absorbance at 280 nm. The following recombinant proteins were analyzed: full-length topoiso- merase I (F.L. topo I), topo70, topo58, and topo70LP low salt and high pools. Molecular mass standards (Sigma) were apoferritin (443 kDa), β-amylase (200 kDa), bovine serum albumin (BSA, 66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa). The results are presented graphically as log (molecular mass) versus the ratio of observed elution volume (Ve) to the excluded volume (Vo). The calculated apparent molecular masses for full-length topoiso- merase I, topo70LP high salt pool, topo70LP low salt pool, topo70, and topo58 are ~350, ~350, ~100, ~91, and ~82 kDa, respectively. Panel B, proteins were analyzed by sedimentation through 10–30% glycerol gradients. Fractions were collected and analyzed by SDS-PAGE and silver staining. Molecular mass standards (Sigma) were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). The calculated apparent molecular masses for full-length topoiso- merase I, topo70LP high salt pool, topo70LP low salt pool, topo70, and topo58 are ~70, ~130, ~70, ~70, and ~60 kDa, respectively.
results suggest that the core-COOH-terminal domain interactions in the topo70ΔL monomer and in at least one-half of the dimer are sufficiently weak that an exogenous COOH-terminal domain can gain access to its binding pocket on the core to reconstitute activity.

Sensitivity of Topo70ΔL Proteins to Camptothecin—It was of interest to determine whether the deletion within the linker alters the sensitivity of the enzyme to the topoisomerase I inhibitor, camptothecin. Time course plasmid relaxation assays were carried out for topo70 and topo70ΔL monomeric and dimeric forms in the presence and absence of 50 μM camptothecin. Whereas the relaxation activities of both the wild type enzyme and the topo70ΔL dimer were inhibited ~10-fold under these conditions, the monomeric form of topo70ΔL was not significantly inhibited (Fig. 5, panels A–F).

Camptothecin Enhancement of Breakage on a Duplex Oligonucleotide DNA Substrate—Since the topo70ΔL dimer is inhibited by camptothecin to the same extent as the wild type, it seemed unlikely that the lack of inhibition in the case of the monomer was due to a failure of the drug to bind to the enzyme-DNA complex. To examine directly whether the linker deletion affected camptothecin binding to the monomer, we performed SDS-induced DNA breakage assays in the absence and presence of camptothecin for the two forms of topo70ΔL using a 25-base pair oligonucleotide containing a highly preferred binding sequence (52). The cleaved strand of the duplex substrate was labeled with 32P at its 5’ end and added to reaction mixtures containing a 5-fold molar excess of enzyme. Following incubation at room temperature, SDS was added to trap the covalent enzyme-DNA intermediates, and the complexes were analyzed by SDS-PAGE.

To quantify the amount of active enzyme in each preparation, equal concentrations of enzyme were exhaustively reacted with a 4.6-fold molar excess of a DNA suicide oligonucleotide substrate having a specific activity identical to that of the duplex substrate. Quantitation by PhosphorImager analysis showed equal amounts of active enzyme for topo70 and topo70ΔL monomer, whereas the dimer preparation was ~70% active as topo70 (Fig. 6, lanes 1, 4, and 7). When the SDS cleavage activities of topo70 and topo70ΔL dimer in the absence of camptothecin were normalized to the relative relaxation activity of the two preparations, the amount of cleavage was found to be the same (Fig. 6, lanes 2 and 8), whereas the normalized SDS cleavage value for the monomer was ~8-fold less (Fig. 6, lane 5). Thus, similar to the reconstituted enzymes (41), the cleavage-religation equilibrium for the monomer appears to be shifted toward religation. When camptothecin was included in the assays, SDS cleavage was enhanced in all three cases (Fig. 6, lanes 3, 6, and 9). The fold enhancement for topo70 and topo70ΔL dimer was similar (~2.5-fold), whereas the enhancement factor for the monomer was approximately 5-fold (Fig. 6, lanes 2 and 3, 5 and 6, and 8 and 9). Thus we conclude that the lack of camptothecin sensitivity for topo70ΔL monomer in plasmid relaxation assays is not due to a failure of the drug to bind the enzyme-DNA complex.

Camptothecin Sensitivity of Yeast Strains Expressing Linker Deletion Mutants—To ascertain whether the form of human topoisomerase I containing a partial deletion of the linker region was resistant or sensitive to the effects of camptothecin in vivo, plasmids expressing full-length topoisomerase I, topo70, topo70Phe, and topo70ΔL (pADH1B-topo1(URA), pADH1B-topo70(URA), pADH1B-topo70Phe(URA), and pADH1B-topo70ΔL(URA)) were individually introduced into the top1 null strain of yeast, JN2-134 (10). JN2-134 possesses a mutation that reduces its ability to repair DNA damage (rad52) and is permeable to multiple drugs (ise1), including camptothecin. Immunoblot analyses confirmed that all four forms of the protein were expressed (Fig. 7, lanes 1–4).

Camptothecin sensitivity assays were performed by spreading exponentially growing cells on either buffered medium plates containing camptothecin or control plates without the drug. The percent viability was calculated from the number of colonies on the plate with the drug relative to the number of colonies on the control plate. The results indicated that cells expressing topo70 or full-length topoisomerase I were equally sensitive to camptothecin, with percent viability values in both cases being ≤3 × 10−4 (Table I, lines 1 and 2). This result indicates that the NH2-terminal domain is dispensable for camptothecin sensitivity in vivo. As expected, cells expressing topo70Phe survived exposure to the drug (Table I, line 3). However, cells expressing Topo70ΔL were completely resistant to the effects of camptothecin (Table I, line 4). To rule out the possibility that this phenotype was due to the production of an inactive enzyme in yeast cells, activity assays were performed on whole cell extracts. When normalized for the amount of full-length protein produced based on the immunoblot analysis, extracts from cells expressing full-length topoisomerase I, topo70, and topo70ΔL were found to have equal enzyme activities (data not shown).

**DISCUSSION**

The crystal structure of human topoisomerase I reveals that the 77-residue linker region connecting the core domain to the COOH-terminal domain consists of an antiparallel coiled-coil.

![Fig. 4. Limited proteolysis and analyses by Native- and SDS-PAGE](image-url)
that protrudes from the remainder of the protein (2). Here we have characterized a form of the enzyme in which the distal 40% of the linker has been deleted, and we find that ~25% of the purified protein is a dimer that chromatographs differently than the monomer. By the same criterion, the wild type enzyme does not dimerize (34). Since we have not observed any interconversion between monomers and dimers upon prolonged storage at −20 °C, it seems likely that dimerization either occurs in vivo in the insect cell or during the purification process.

Based on the observation that limited proteolysis reduces the dimer to a form that co-migrates with the monomer in a native gel, and on our previous observation that isolated core and COOH-terminal domains can interact to reconstitute enzyme activity, we conclude that the dimer is most likely formed by 3D domain swapping as described by Bennett et al. (51). One possible configuration for the dimer involves a symmetrical association of two monomers in which the COOH-terminal domain of each occupies the core-binding site on the other (Symmetrical Dimer, Fig. 8). Alternatively, the two subunits might associate in an asymmetrical manner with one COOH-terminal domain occupying the core-binding site on the other partner in the same way that the two domains associate in the wild type protein that would leave a core domain and a COOH-terminal domain free (Half-open Dimer, Fig. 8). In this latter case, the two free domains would have to be arranged three-dimensionally so as to preclude the formation of higher oligomeric chains.

Protein dimerization based on domain swapping has now been observed for a number of proteins including diphtheria toxin and RNase A (51, 53). Notably, it was hypothesized that the removal of amino acids between the two domains prevents the normal domain-domain interactions in the monomer generating what is referred to as an open monomer which in turn promotes dimerization (51). We propose that the shortening of the human topoisomerase I linker likewise reduces the flexibility of the linker and constrains the core and COOH-terminal domains of the topo70ΔL monomer such that dimerization can occur under some conditions, but leaves the structure flexible enough so that the monomer retains nearly as much activity as the wild type enzyme. One possibility that is consistent with this suggestion is diagrammed schematically in Fig. 8 in which it is proposed that an equilibrium exists between a partially open monomer that is enzymatically active and a hypothetical completely open monomer that allows dimer formation. Consistent with this hypothesis is the finding that the activity of topo70ΔLPhe monomeric protein can be restored by exogenously added wild type COOH-terminal domain. Recovery of activity by the addition of the wild type COOH-terminal domain to the topo70ΔLPhe dimer might indicate that the dimer assumes a half-open configuration (Fig. 8) with one of the core-binding pockets for the COOH-terminal domain readily accessible to the exogenously added wild type COOH-terminal domain. However, it is also possible that a symmetrical dimer is formed in which the domain-domain interactions are weakened to the same extent as proposed above for the monomer, resulting in a configuration that allows access to an exogenous wild type COOH-terminal domain.

The properties of the monomeric form of topo70ΔL are very similar to what we have previously found for the enzyme reconstituted by mixing topo58 with COOH-terminal fragments topo6.3 or topo12 (see Fig. 1). In all three cases, the cleavage-religation equilibrium is shifted toward religation, the enzyme

![Fig. 5. Sensitivity of topo70ΔL proteins to camptothecin.](image-url)
exhibits a reduced processivity under standard reaction conditions reflecting a reduced affinity for DNA, and the relaxation activity is not inhibited by camptothecin (18). It should be noted that, unlike most camptothecin-resistant mutants that fail to bind the drug (56–61), the loss of linker function in these altered enzymes renders the relaxation activity insensitive to camptothecin without affecting drug binding. Since these characteristics are shared by reconstituted topo58/12 in which the linker domain is simply disconnected from the core and reconstituted topo58/6.3 which is missing the linker altogether, we have proposed that it is the loss of linker function that leads to these properties (18). With respect to the reconstituted enzymes, we concluded previously that besides contributing to DNA binding, the linker region normally slows the religation step and is required for camptothecin inhibition, possibly by directly slowing the rotation of the helix during the lifetime of the nicked intermediate (18). Given these similarities, it seems likely that deletion of the distal portion of the linker region has the same effects on the enzyme activity for the monomeric form of the mutant protein as eliminating the linker altogether (topo58/6.3), and there is no reason to believe that, in the reconstituted form of the enzyme that is missing the linker altogether (topo58/12), the docking between the two domains would be aberrant.

How can one account for the fact that the dimeric form of topo70ΔL is similar to the wild type enzyme with respect to DNA affinity, the cleavage-religation equilibrium, and sensitivity to camptothecin? One explanation for the functional difference between wild type topo70 and the monomeric form of topo70ΔL might be the inability of the COOH-terminal domain to dock properly with the core domain in the mutant protein as depicted schematically in Fig. 8. If the dimer were to assume a half-open configuration (Fig. 8), the remaining COOH-terminal interaction with the core domain would likely be identical to the properly docked COOH-terminal domain in the wild type protein and thus the dimer could function like the wild type enzyme. This explanation seems unlikely since, as described above, the topo70ΔL monomer displays the same properties as the reconstituted form of the enzyme that is missing the linker altogether (topo58/6.3), and there is no reason to believe that, in the absence of a linker, the docking between the two domains would be aberrant.

A more likely explanation for the enzymatic differences between the dimeric and monomeric forms of topo70ΔL is that
some feature of the dimer substitutes for the lack of a functional linker in the monomer. We have proposed previously that the positively charged amino acid side chains on the DNA-proximal surface of the linker may mediate contacts with the DNA either before or during the relaxation process (2). This hypothesis is supported by the finding that the reconstituted enzymes (41) and topo70ΔL all exhibit reduced DNA binding. If contacts between the linker and the DNA are important for prolonging the nicked state as well as for sensitivity to camptothecin, then a surrogate linker could be provided by the presence of the second DNA-binding site in the dimer or by some aspect of the interface between the two subunits of the dimer. In this regard, it is possible that the two partial linker regions present in the dimer interact to provide the surrogate linker function. A complete understanding of the properties of the dimer must await a detailed structural analysis of the protein with bound DNA.

Yeast cells expressing topo70ΔL are no more sensitive to the killing effects of camptothecin than control cells lacking topoisomerase I, whereas survival rates upon expression of the wild type enzyme are reduced several orders of magnitude. Based on the observation that the dimeric form of topo70ΔL is indistinguishable from wild type topo70 in vitro and therefore would be expected to produce a camptothecin-sensitive form of the enzyme in vivo, we conclude that the predominant if not the sole form of the mutant enzyme in yeast cells is the monomer. One possible explanation for the lack of camptothecin sensitivity of cells expressing topo70ΔL relates to the level of SDS-induced cleavage observed for the linker deletion mutant. Given that the cleavage-religation equilibrium of the mutant enzyme is shifted toward religation, it may be that camptothecin simply restores the cleavage-religation equilibrium to near that of the wild type enzyme in the absence of camptothecin. The resultant level of cleaved complexes in the drug-treated cells would then be below the threshold required for cell killing. If this explanation is correct, then one might expect to find naturally occurring camptothecin-resistant mutants that have disabled the linker.

The observation that yeast cells expressing topo70 are sensitive to camptothecin indicates that the first 174 NH₂-terminal amino acids of the human enzyme are not required for the cytotoxic effects of the drug, and this is the same region of the protein that has been shown previously to be dispensable for enzyme activity in vitro (34, 38–40). At a minimum, cell killing in the presence of camptothecin requires that an active form of the topoisomerase has access to the DNA, and thus the one remaining nuclear localization signal in topo70 (34, 36) is apparently sufficient for nuclear import of the human enzyme in yeast cells.

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