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Yeast *Saccharomyces cerevisiae* as a model system to study the cytotoxic activity of the antitumor drug camptothecin

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Abstract. Eukaryotic DNA topoisomerase I catalyzes the relaxation of positively and negatively supercoiled DNA and plays a critical role in processes involving DNA, such as DNA replication, transcription and recombination. The enzyme is encoded by the *TOP1* gene and is highly conserved in its amino acid sequence and sensitivity to the anti-neoplatic agent camptothecin. This plant alkaloid specifically targets DNA topoisomerase I by reversibly stabilizing the covalent enzyme-DNA intermediate. Presumably, it is the interaction of these drug-stabilized adducts with other cellular components, such as replication forks, that actually produces the DNA lesions leading to cell death. A conservation of the mechanism(s) of camptothecin-induced cell killing is also implicit in studies of the yeast *Saccharomyces cerevisiae*, where the camptothecin sensitivity of *ΔTOP1* yeast cells can be restored by plasmids expressing either yeast or human *TOP1* sequences. This genetically tractable system is currently being exploited to describe the specific molecular interactions required for the cytotoxic action of camptothecin. The results of mutational analyses of yeast and human DNA topoisomerase I are presented, as well as a genetic screen designed to identify genes, other than *TOP1*, that are required for the cytotoxic activity of camptothecin.

Key words: Camptothecin – Drug resistance – DNA topoisomerase I

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Introduction

DNA contains the genetic information necessary for the growth, development, and reproduction of most organisms. This information is deciphered and transmitted during the processes of gene expression and DNA replication during cell division. However, the helical structure of duplex DNA, that is, the right-handed wrapping of one single polynucleotide strand around the other, can pose topological problems when the DNA is replicated or transcribed (reviewed in [1–5]). For example, the progressive unwinding of the DNA template during DNA replication and the subsequent segregation of the intertwined daughter DNA molecules require changes in the linkage of the DNA strands and helices. Similarly, the transcription process itself can result in the generation of local domains of positive and negative supercoiling [5, 6].

Changes in DNA topology are catalyzed by enzymes called DNA topoisomerases [2–4, 7, 8]. These enzymes function through a mechanism involving the transient breakage and rejoining of phosphodiester bonds in the DNA backbone and the formation of a covalent enzyme-DNA intermediate [9, 10]. The enzyme becomes linked to one end of the cleaved DNA strand through a phosphotyrosine linkage. Two types of DNA topoisomerases are distinguished on the basis of the number of DNA strands they transiently cleave; type I enzymes nick one strand of a DNA duplex, whereas type II enzymes break both.

In eukaryotes, DNA topoisomerase I and DNA topoisomerase II have been purified from many sources, and the genes encoding these enzymes, called *TOP1* and *TOP2*, respectively, have been cloned [8, 9, 11–15]. Each enzyme appears to be highly conserved in terms of its mechanism of action and primary amino acid sequence (see Fig. 1) [2–4, 8, 9, 15, 16]. The eukaryotic type I enzyme in particular catalyzes the relaxation of positively and negatively supercoiled DNA and becomes linked to a 3′ phosphoryl group via a tyrosine residue. This so-called active-site tyrosine has been mapped to residue Y_{727} in the yeast *Saccharomyces cerevisiae* enzyme (Fig. 1) [16, 17], which corresponds to residue Y_{723} in the human enzyme [16].

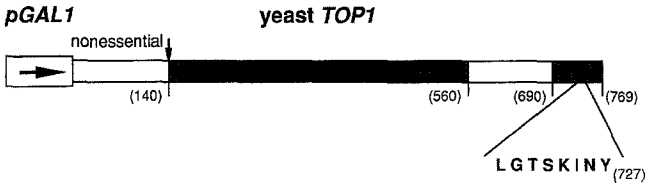

Fig. 1. Schematic representation of the yeast *TOP1* gene under control of the galactose-inducible *GAL1* promoter. Conserved regions of the protein, encoded by the *TOP1* gene, are indicated by *shading*, and the *numbers* correspond to the yeast residues flanking these conserved domains. The relative position of the active-site residue (*Tyr 727*) is indicated. The residues N-terminal to Tyr 727 are highly conserved from yeast to human cells. Identical amino acids are highlighted in *boldface*

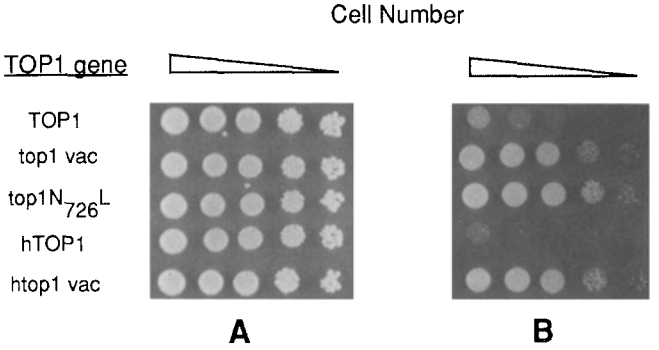

Fig. 2. Substitution of the conserved amino acid residues preceding the active site tyrosine render human and yeast DNA topoisomerase I resistant to camptothecin. (The conserved amino acids are indicated in *boldface* in Fig. 1.) In the mutants *top1 vac* and *htop1 vac*, the Ile and Asn residues N-terminal to the active-site tyrosine in the yeast and human enzymes, respectively, were mutated to the Arg and Ala found at the corresponding position in the vaccinia virus enzyme. The yeast mutant Top1 $N_{726}L$ enzyme contains an Asn_{726}-to-Leu substitution. The various wild-type and mutant *TOP1* sequences were cloned under a galactose-inducible *GAL1* promoter on a single copy plasmid and the plasmids were transformed into a *top1* yeast strain. Exponentially growing cultures of individual transformants were serially diluted and 5-μl volumes were spotted onto selective agar plates containing galactose and either DMSO (**A**) or DMSO and 10 mg camptothecin/ml (**B**)

Except for conservative Ile-to-Leu changes, the residues preceding the active site tyrosine are identical in other eukaryotic enzymes. DNA topoisomerase I functions as a swivelase during DNA replication [2–4, 8], is thought to be involved in regulating RNA transcription [2–4, 8, 18, 19], presumably relaxes localized DNA supercoiling during transcription [2–6, 20], and plays a role in DNA recombination [21, 22].

DNA topoisomerases have also been shown to be the cellular targets of a number of antitumor drugs, including doxorubicin and etoposide, which act on DNA topoisomerase II, and camptothecin, which targets DNA topoisomerase I (reviewed in [23–27]). Camptothecin is a plant alkaloid that specifically and reversibly stabilizes a DNA topoisomerase I-DNA intermediate called the cleavable complex [28–30]. The drug appears to exert its cytotoxic effects by interfering with the religation of the phosphodiester backbone bond catalyzed by DNA topoisomerase I [31]. Camptothecin-induced cytotoxicity is also highly S-phase-specific despite the relatively constant amounts of Top1 protein and camptothecin-induced DNA breaks detected during the cell cycle [32–34]. Drug treatment of mammalian cells produces an irreversible inhibition of DNA synthesis and induces DNA fragmentation, recombination, and G2 delay [35]. Genetic and biochemical studies suggest that in proliferating cells the camptothecin-stabilized enzyme-DNA complex presents an insurmountable barrier to the progression of the DNA replication forks, resulting in the formation of double-stranded DNA breaks, leading to cell death [32, 33]. However, the specific molecular interactions required for the cytotoxic action of camptothecin and how these double-stranded DNA breaks result in cell killing remain obscure.

The chemical synthesis of camptothecin and numerous analogues have identified the structural moieties within the conjugated ring system of camptothecin that are necessary for its activity [36–38]. The antitumor activity of these analogues strongly correlates with their ability to induce enzyme-bound nicks in DNA, and several analogues have entered phase I and II clinical trials [37]. However, despite intensive investigation, a similar determination of the structural motifs in DNA topoisomerase I required for the formation of the drug-stabilized complex have not been forthcoming. Recent applications of the budding yeast *S. cerevisiae* to the study of camptothecin-induced cytotoxicity [39–44] provide a powerful new experimental system with which to address these questions. (See the article by Nitiss et al., this issue, for a discussion of related questions concerning the mode of action of drugs that target DNA topoisomerase II.)

Studies with the yeast *S. cerevisiae*

The utility of the yeast *S. cerevisiae* in elucidating complex eukaryotic processes such as DNA replication, cell proliferation, and gene expression is well established (see [45] for a discussion). In part, this is due to the well-defined genetics of this eukaryotic microorganism, which includes a large number of selectable markers, and the availability of DNA vectors that can be efficiently transformed and stably maintained in yeast cells. The relatively high frequency of homologous recombination in yeast also provides a means of integrating DNA sequences into specific chromosomal locations. Thus, it is possible to construct congenic yeast strains that differ at only one genetic locus and to assess the phenotypic consequences of specific genetic alterations.

Budding yeast also has a sexual cycle that allows for the propagation and maintenance of stable haploid and diploid strains. Yeast strains of different mating types can be selectively mated and induced to undergo meiosis, and the individual products of a single meiosis can be recovered and analyzed. Thus, the effects of specific mutations in a given gene sequence, for example, *TOP1*, can easily be assessed in any genetic background.

Yeast DNA topoisomerase I, as described above, resembles DNA topoisomerase I from other eukaryotic sources in its mechanism of action, primary amino acid sequence (Fig. 1), and sensitivity to the antitumor drug camptothecin [2–5, 8, 16]. Recent studies have shown that in the fruit fly *Drosophila melanogaster* the *TOP1* gene is required during development [46]. However, in yeast the *TOP1* gene is nonessential, presumably because in this single-celled organism, DNA topoisomerase II can compensate for the loss of DNA topoisomerase I [11].

On the basis of these results, Nitiss and Wang [39] and Eng and co-workers [40] demonstrated that camptothecin sensitivity in yeast requires the expression of DNA topoisomerase I. Deletion of the *TOP1* gene renders the cells camptothecin-resistant. However, when the *TOP1* gene is reintroduced into these cells on a multicopy plasmid, camptothecin sensitivity is restored. In addition, the effects of camptothecin on increasing DNA recombination and inducing DNA damage-inducible gene expression similarly require a functional DNA topoisomerase I [39]. These results provide direct evidence that DNA topoisomerase I is the sole cellular target of camptothecin. Moreover, since the yeast *TOP1* gene can be deleted without compromising cell viability, the cytotoxic action of camptothecin is not simply the result of inhibiting DNA topoisomerase I activity. Rather, the actual trapping of the enzyme on the DNA converts the complex into a cellular poison that causes cell death.

To assess the sensitivity of *TOP1+* yeast cells to camptothecin, a drug-permeability mutant, *ise1*, was introduced into these strains as a means of increasing drug uptake [39]. Furthermore, deletion of the *RAD52* gene also enhanced the camptothecin sensitivity of these *ise1*, *TOP1* yeast cells [39, 40]. As *rad52* cells are deficient in the recombinational repair of double-stranded DNA breaks, these data support the notion that the S-phase cytotoxicity of camptothecin results from the conversion of enzyme-linked DNA nicks into double-stranded DNA breaks.

Using an *ise1*, Δ*rad52*, *top1* yeast strain, we showed that expression of human DNA topoisomerase I was also sufficient to restore camptothecin sensitivity in yeast. Moreover, expression of either the yeast *TOP1* gene or the human *TOP1* cDNA from the galactose-inducible, glucose-repressible *GAL1* promoter on a single-copy vector resulted in galactose-inducible camptothecin sensitivity [41]. Thus, consistent with other studies of DNA replication and gene expression, the mechanisms involved in eliciting the cytotoxic activity of camptothecin appear to share functions conserved from yeast to mammalian cells.

The relatively high levels of *TOP1* gene expression obtained from the *GAL1* promoter in these ARS/CEN vectors was sufficient to sensitize *top1* yeast cells to camptothecin [41, 43] and to arrest drug-treated cells in G2 (E. A. Kauh and M.-A. Bjornsti, unpublished results), thus obviating the need for the *ise1* and *rad52* mutations. This proved critical in later analyses of several yeast *top1* mutants (see below). However, the characterization of human camptothecin-resistant *top1* mutants has continued to be carried out in the *top1*, *ise1*, Δ*rad52* yeast strain.

Camptothecin resistance and cell lethality in yeast cells expressing DNA topoisomerase I mutants

Several camptothecin-resistant cell lines have been reported, where the decrease in drug sensitivity was attributable to specific amino acid substitutions in the enzyme, resulting in decreased enzyme activity or sensitivity of the enzyme to camptothecin-induced DNA nicking [47–49]. The latter group includes a D533-to-G change in human DNA topoisomerase I [47] and a G505-to-S substitution in the Chinese hamster enzyme, corresponding to G503 in the human Top1 protein [49]. Another substitution (T729 to A) is presumed to decrease the activity of human DNA topoisomerase I; however, confirmation of this result awaits site-directed mutagenesis [48]. The characterization of camptothecin-resistant *top1* mutants (*top1-Cpt^R*) in higher eukaryotes is complicated by the presence of two *TOP1* alleles, the lack of congenic cell lines that differ at only one genetic locus, and the likelihood that *TOP1* is essential, as it is in *Drosophila*. Thus, it is impossible to rule out completely other mutations affecting the camptothecin sensitivity of these cells, and the phenotypic consequences of single amino acid substitutions in the *TOP1* gene must be inferred from biochemical experiments. However, in yeast *top1* strains, the effect of specific mutations in plasmid-borne yeast or human *TOP1* genes on camptothecin sensitivity can be directly assessed and then further substantiated using biochemical techniques.

Using this approach, amino acid substitutions in both yeast and human DNA topoisomerase I have recently been reported that result in catalytically active Cpt^R enzymes [42–44]. In one case, specific residue changes were engineered by design, that is, by oligonucleotide-directed mutagenesis [44]. In mutant *top1 vac*, the isoleucine and asparagine residues preceding the active-site tyrosine in yeast (I_{725}, N_{726}) were mutated to the arginine and alanine residues found at the corresponding position in the camptothecin-resistant enzyme encoded by vaccinia virus (Fig. 1) [16, 50, 51]. Similar substitutions were also engineered in the human *TOP1* cDNA to give mutant *htop1 vac*. As shown in Fig. 2, expression of either mutant enzyme in *top1* yeast strain JCW1 rendered the cells resistant to camptothecin, whereas cells expressing the corresponding wild-type enzyme were sensitive to the drug [44]. Moreover, the mutant proteins were much less sensitive to camptothecin-induced DNA nicking in vitro than the wild-type proteins despite the observation that the catalytic activity of both mutant enzymes was indistinguishable from that of the respective wild-type proteins. In the case of the other *Cpt^R top1* mutant, *top1N$_{726}$L*, the decrease in the drug sensitivity of cells expressing this mutant could be attributed to a decrease in enzyme catalytic activity [16, 44]. Additional amino acid substitutions resulting in catalytically active *Cpt^R* enzymes have also been generated via degenerate oligonucleotide-directed mutagenesis of yeast *TOP1* and are currently being further characterized in vitro (J. Fertala and M.-A. Bjornsti, unpublished results).

A second method has been to randomly mutagenize the human *TOP1* cDNA by gap mismatch repair, then select for catalytically active mutant enzymes and assess their sensitivity to camptothecin in *top1* yeast [42, 43]. This ap-

proach has successfully been used to identify a camptothecin-resistant human enzyme resulting from a single G_{363}-to-C substitution [43]. Yeast cells expressing this human *top1* mutant are completely resistant to the cytotoxic action of camptothecin, even though the enzyme has been shown to be catalytically active in vivo and in vitro. This approach has recently been expanded to include human *TOP1* sequences mutagenized by hydroxylamine. Additional catalytically active Cpt^R mutants have been isolated, and the responsible mutations are being defined (P. Fiorani et al., unpublished results).

Mutations in yeast DNA topoisomerase I have also been identified that appear to stabilize the covalent enzyme-DNA intermediate by mimicking the action of camptothecin ([52]; M. Megonigal et al., unpublished results). These *top1* mutants either severely inhibit cell growth when overexpressed or cause cell death. In all cases the observed effects on cell growth were independent of *RAD52* function. When one of the mutations responsible for the yeast *top1* lethal phenotype (T_{722} to A) was introduced into the corresponding position in the human *TOP1* gene, similar results were also obtained; the human *top1* mutant was lethal when overexpressed in a *top1* yeast strain (M.-A. Bjornsti and P. Benedetti, unpublished results). These data are consistent with the results described for the camptothecin-resistant *top1* mutants and reinforce the idea that similarities in amino acid sequence between the eukaryotic type I enzymes are reflected in similarities in enzyme function and drug sensitivity.

Further biochemical and genetic studies of the effects of these mutations on enzyme activity and the physiological responses of cells expressing these mutant proteins will illuminate the molecular interactions required to induce DNA damage and the cellular mechanisms concerned with converting this DNA damage into a lethal event.

Future directions

One goal of the experimental approaches described above is to identify the protein domains and the specific amino acid residues in DNA topoisomerase I that are required for the productive interaction of camptothecin with the enzyme-DNA complex. A large body of work has identified the key structural features of camptothecin necessary for its antitumor activity [36–38]; however significantly less is known about the structure of DNA topoisomerase I. On the basis of sequence comparisons, Caron and Wang [53] have proposed that DNA topoisomerases catalyze the transient breakage and religation of DNA in a cleft formed by different protein domains. The wide distribution of amino acid substitutions known to affect the camptothecin sensitivity of DNA topoisomerase I is consistent with this model [42–44, 47–49]. However, significantly more information on the distribution of residues essential for catalytic activity, DNA binding, and camptothecin binding is necessary for an understanding of the possible mechanisms of camptothecin resistance and how analogues may be designed to overcome drug resistance. These data will prove particularly useful for drug design once the crystal structure of the protein becomes available.

A second direction in delineating the mechanisms involved in camptothecin-induced cell killing is a genetic one and makes use of the well-defined genetics of yeast. The basic premise is to identify mutations in genes other than *TOP1* that render cells resistant to the cytotoxic activity of camptothecin and its clinically relevant analogues. A genetic screen to identify mutant strains of yeast that express wild-type DNA topoisomerase I yet are resistant to camptothecin is presently under way, and several such mutants have thus far been isolated (E. Kauh and M.-A. Bjornsti, unpublished results). Further characterization of these mutant strains and the cloning of the mutated gene sequences that confer camptothecin resistance will help to delineate the physiological processes involved in the cytotoxic action of these chemotherapeutic drugs.

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