Trapping of Mammalian Topoisomerase I and Recombinations Induced by Damaged DNA Containing Nicks or Gaps

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Trapping of Mammalian Topoisomerase I and Recombinations Induced by Damaged DNA Containing Nicks or Gaps

IMPORTANCE OF DNA END PHOSPHORYLATION AND CAMPTOTHECIN EFFECTS*

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We used purified mammalian topoisomerases I (top1) and oligonucleotides containing a unique top1 cleavage site to study top1-mediated cleavage and recombination in the presence of nicks and short gaps mimicking DNA damage. In general, top1 cleavage was not induced opposite to the nicks, and nicks upstream from the top1 cleavage site suppressed top1 activity. Irreversible top1 cleavage complexes (“suicide products” or “aborted complexes”) were produced in DNA containing nicks or short gaps just opposite to the normal top1 cleavage site. Camptothecin enhanced the formation of the aborted top1 complexes only for nicks downstream from the cleavage site. These aborted (suicide) complexes can mediate DNA recombination and promote illegitimate recombination by catalyzing the ligation of nonhomologous DNA fragments (acceptors). We report for the first time that top1-mediated recombination is greatly enhanced by the presence of a phosphate at the 5’ terminus of the top1 aborted complex (donor DNA). By contrast, phosphorylation of the 3’ terminus of the gap did not affect recombination. At concentrations that strongly enhanced inhibition of intramolecular religation, resulting in an increase of top1 cleavable complexes, camptothecin did not reduce recombination (intermolecular religation). Nicks or gaps with 5’-phosphate termini would be expected to be produced directly by ionizing radiations or by processing of abasic sites and DNA lesions induced by carcinogens or drugs used in cancer chemotherapy. Thus, these results further demonstrate that DNA damage can efficiently trap top1-cleavable complexes and enhance top1-mediated DNA recombination.

DNA rearrangements such as deletions, translocations, inversions, or gene amplifications are involved in genetic diseases and carcinogenesis (1–4). These modifications are based on the cell’s ability to promote recombinations. This process can be initiated after exposure to exogenous DNA damaging agents such as ionizing radiations, environmental carcinogens, and drugs used in cancer chemotherapy (5). Even though their molecular mechanisms are not yet fully understood, two types of recombinations have been described depending on the homology of the DNA molecules joined. In eukaryotes, the nonhomologous (or illegitimate) recombination occurs more frequently than the homologous (or legitimate) recombination (6).

Topoisomerases are ubiquitous enzymes involved in multiple processes including replication, transcription, chromosome segregation, and recombinations (7, 8). DNA topoisomerase I (top1) acts as a monomer and creates transient DNA single-strand breaks via the formation of a covalent bond between the 3’-phosphate of the cleaved strand and a tyrosine residue of the enzyme. Under physiological conditions, these intermediates are referred to as cleavable complexes. The cleavable complexes are readily reversible by top1-mediated religation of the 5’-hydroxyl termini (7, 9, 10).

In mammalian cells, top1 is involved in illegitimate recombination in vitro (11–15) and probably in vivo (16–18). top1 inhibitors such as camptothecin (CPT) and its derivatives, which have recently been introduced in cancer treatment, inhibit the religation step and lead to the conversion of cleavable complexes into DNA double-strand breaks after replication fork collisions (19, 20). We recently reported that top1 can be trapped irreversibly by common DNA endogenous lesions such as DNA mismatches and abasic sites (21). Previous studies also reported that nicks in the vicinity of the top1 cleavage site were able to generate double-strand breaks, leading to suicide or aborted products, where covalently bound top1 is able to reigate various nonhomologous acceptors bearing a 5’-hydroxyl terminus and thus participates into illegitimate recombination (10, 12, 13, 22–24).

In this study, we used purified mammalian top1 and DNA oligonucleotides containing a unique top1 cleavage site derived from the rDNA sequence of Tetrahymena (25, 26) to study top1-mediated DNA damage and recombination induced in DNA substrates containing nicks or gaps in the non-scissile strand (i.e. opposite to the cleaved strand). Such nicks and gaps mimic endogenous DNA lesions or modifications resulting from DNA damage (such as ionizing radiations) or DNA repair (base elimination or excision). Such lesions were introduced at various positions relative to the top1-mediated cleavage site in the oligonucleotide shown in Fig. 1. The results demonstrate for the first time the importance of a phosphate at the 5’ terminus of the DNA break opposite to the top1 linkage for top1-mediated recombinations. We also report the influence of DNA nicks on CPT activity and discuss these results in the context of the known potentiating effect of ionizing radiations on camptothecin activity.

EXPERIMENTAL PROCEDURES

Chemicals—High performance liquid chromatography-purified oligonucleotides were purchased from The Midland Certified Reagent Co.

1 The abbreviations used are: top1, topoisomerase I; CPT, camptothecin; DTT, dithiothreitol.
Trapping of Top1 by DNA Damage and Recombination

(rsb2 (1 volume of rsb1

Pellets were washed four additional times: once with rsb1, twice with rsb1, and finally with detergent. After each wash, pellets were resuspended in 1 ml of buffer."),

were resuspended in 1 ml of hsb buffer (10 mM Tris-HCl, pH 7.4, 500 mM KCl). The supernatants were stored at 2 °C before use.

\textbf{Preparation of Oligonucleotides Containing Nicks or Gaps—Radiolabeled single-stranded DNA oligonucleotides were annealed to the same concentration of unlabeled complementary strands in 1 x annealing buffer (10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA) to create nicks or gaps as indicated by the sequences shown in the figures. Reaction mixtures were heated to 95 °C and slowly chilled overnight to room temperature. The reaction mixtures were then centrifuged through a G-25 Sephadex column and used in the top1 reactions.

\textbf{RESULTS}

Position-dependent Effects of Nicks—Nicks were introduced at various positions in the non-scissile strand of the oligonucleotide shown in Fig. 1. Nicks at positions -5/-4 and -4/-3 suppressed top1-induced cleavage even in the presence of CPT (Fig. 2A). Top1 cleavage was also decreased by the nick at position -3/-2. These results are consistent with the effects of uracil and abasic site incorporations at these positions (21) and confirm the importance of DNA-top1 interactions in this upstream (5') region of the DNA substrate for top1 activity.

Nicks at position -1/-1 and +1/-2 enhanced top1-mediated DNA cleavage independently of CPT (26- and 13-fold, respectively). When a nick was present at the -2/-1 position, cleavage at the normal cleavage site was suppressed, but cleavage occurred at two upstream sites (Fig. 2, open arrowhead) was observed. Cleavage was not reversed by salt for nicks at positions +1/-1 and +1/-2 and was partially reversed for nick at position -2/-1 (Fig. 2, see lanes 3 and 5 for the corresponding panels). These results are consistent with the formation of double-strand breaks associated with suicide (or aborted) top1 intermediate when top1 cleavage occurs opposite to a nick.

When nicks were introduced more downstream (3') from the expected top1 cleavage site, from positions +2 to +6, salt-resistant top1-mediated DNA cleavage was induced by CPT (Fig. 2B, lanes 5). Therefore, nicks at these positions enhanced CPT activity and were converted to double-strand breaks (and suicide products) in the presence of CPT.

\textbf{DNA End Phosphorylation Affects Top1-mediated DNA Cleavage and Recombination in Nicked and Gapped Oligonucleotides—Processing of physiological, environmental, or pharmacological DNA damage frequently leads to loss of bases. In the case of ionizing radiation or abasic site processing, DNA termini are usually 5'-phosphates and 3'-phosphates or phosphoglycolates (5). To investigate the effect of such termini on top1 activity, we used three different oligonucleotide substrates containing either a gap in +1/-1 position (+1/-1 gap) or a two-base gap (bases +1 and +2). We compared top1 activity in such oligonucleotides bearing either a phosphate or anhydroxyl group at the 5' terminus (Fig. 3).

When a hydroxyl was present at the 5' terminus, top1-mediated cleavage at the predicted site was greatly enhanced and was salt-resistant for the oligonucleotides containing a nick.

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and a one-base gap (Fig. 3, arrowhead). However, cleavage was suppressed for the two-base gap oligonucleotide bearing a 5'-hydroxyl. The presence of a phosphate at the 5' terminus increased top1 cleavage in the oligonucleotides with a nick or a one-base gap. 5' end phosphorylation restored top1 cleavage activity for the two-base gap oligonucleotide, probably by repositioning the enzyme on the DNA recognition site (Fig. 3, far right panel; see “Discussion”). In all cases, cleavage was irreversible and led to suicide products and DNA double-strand breaks (Fig. 3, compare lane 2 with lane 3). Interestingly, presence of a phosphate at the 5' terminus markedly stimulated the formation of top1-mediated recombination products with all three substrates (Fig. 3, R band).

To determine the effect of 3' end phosphorylation on top1-mediated cleavage and recombination, the one-base gap oligonucleotide was used as a substrate with the four different combinations for phosphorylation of the DNA termini (Fig. 4). Recombination was readily detected when a phosphate was present at the 5' terminus of the gap (same result as Fig. 3), and several products of different sizes were detected. By contrast, presence of hydroxyl or phosphate at the 3' end of the gap had no effect on top1 activities. Taken together, these results indicate that a phosphate at the 5' terminus of a gap mimicking DNA damage or DNA repair intermediates increases both the cleavage and the recombinogenic activity of top1.

Characterization of the Recombination Products—To further characterize the nature of the different recombination products, a time course was performed with the one-base gap oligonucleotide containing a phosphate at the 5' terminus at the nick or gap. The central portions of the DNA substrates are shown at the top. Labeling was at the 3' end of the upper strand (see Fig. 1). X refers to the 5' terminus of the base at the DNA backbone interruption: either phosphate (P) or hydroxyl (OH). Lane 1, DNA alone; lane 2, +top1 for 15 min at 25 °C and stopped with 0.5% SDS; lane 3: +top1 for 15 min at 25 °C, reversed with 0.5 M NaCl for 1 h at 25 °C, and stopped with 0.5% SDS. Cleavage products are indicated by arrowheads. R, top1-mediated recombination products.
and the 79-mer product, after 1 h. To determine the mechanism of the recombination, the bands were excised from the sequencing gel after 4 h reaction and submitted to purine sequencing (29). Results are shown in Fig. 5C. Arrow 1 (panels B and C) indicates the intermolecular junctions between the 14-mer (donor) top1-linked DNA and the 79-mer product, after 1 h. To determine the mechanism leading to the recombination products is presented in Fig. 5C. Arrow 1 (panels B and C) indicates the intermolecular junctions between the 14-mer (donor) top1-linked DNA fragment generated after cleavage of the 37-mer substrate and the 5' terminus of another uncleaved oligonucleotide (acceptor). The 51-mer resulting product can then serve as a new acceptor and be religated to a second donor, giving rise to the 65-mer product (Fig. 5, B and C, arrow 2). In agreement with this recombination scheme, we did not observe any recombinant when the 5' terminus of the scissile strand was phosphorylated (data not shown).

**Differential Inhibition of top1-mediated Intra- and Intermolecular Religation by Camptothecin—**We further tested whether CPT affected top1-mediated DNA recombination, as it is able to inhibit intramolecular religation (9, 30). For this purpose, we compared the inhibition of intra- and intermolecular religation using increasing concentrations of CPT (Fig. 6). As expected, inhibition of intramolecular religation occurred with low CPT concentrations, resulting in an increase of top1 cleavage (Fig. 6A). To test the effect of CPT on intermolecular religation, we used an unlabeled donor containing the one-base gap (see Fig. 4) with a 5'-phosphate and incubated this substrate with top1 in the presence of a 3'-labeled 23-mer blunt-end acceptor and increasing concentrations of CPT (Fig. 6B). High concentrations of CPT were required to inhibit intermolecular recombination. Both intra- and intermolecular religation products were quantified, expressed as the percentage of the highest rate of religation obtained, and plotted as a function of CPT concentration (Fig. 6C). Complete inhibition of intramolecular religation was observed between 0.3 and 1 μM CPT. By contrast, in this concentration range, intermolecular religation was not significantly inhibited. Inhibition of intermolecular religation required much higher concentrations of CPT, such as 10–100 μM. Previous experiments using a partial double-stranded top1-DNA post-cleavage complex also demonstrated that CPT concentrations in excess of 1 μM were required for inhibition of religation of an exogenous scissile strand (27). Together, these data are consistent with the selective binding of CPT at the enzyme-DNA interface and with its interaction with the +1 base pair 3' to the top1 cleavage site (25, 29, 31).

**DISCUSSION**

Nicks and single-base gaps are frequent DNA lesions that can arise from both spontaneous and environmental DNA modifications. Nicks can be produced directly by ionizing radiations, and single-base loss can result from base mismatch, uracil misincorporation, or modified base processing via the base excision repair pathway. After recognition by a specific DNA glycosylase, misincorporated or modified bases are processed into abasic sites, which upon conversion by AP endonucleases, lead to base elimination (5).

In this study, we systematically investigated the effects of nicks and gaps on top1 activity by studying the effects of such DNA lesions as a function of their position relative to a preexisting top1 site. A first conclusion is that the presence of nicks does not appear to induce novel top1 sites opposite to the nicks. This result is consistent with an earlier study (23) using plasmid DNA and probability calculations showing that the conversion of nicks to double-strand breaks by eukaryotic top1 was due to the random distribution of the top1-cleavable complexes rather than to the induction of top1 breaks by the preexisting nicks. A second conclusion of the present study is that nicks immediately upstream (5') (between positions −5 and −3) from a preexisting top1 site abolish top1 activity. This observation is consistent with our previous findings that top1-cleavable complexes are suppressed by DNA mismatches and abasic sites within the first 4 bases immediately upstream to the top1 cleavage site (21) and by cytosine methylation at the −3 position (32). Furthermore, the base sequence preference for top1 cleavage also indicates a strong preference for 4 base pairs immediately upstream (5') of the top1 cleavage site (29, 33). Together, these observations indicate that DNA structure including DNA backbone integrity immediately upstream from the top1 cleavage site is critical for enzyme activity (34–37).

Conversion of the nicks or short gaps into a DNA double-strand break by top1 was consistently observed for nicks at positions −1/+1 and +1/+2 (opposite from the preexisting top1 site) and when the +1 base was removed opposite to the cleavage site (one-base gap oligonucleotide). Such double-strand breaks represent a unique type of DNA damage, as top1 remains covalently attached to the 3' DNA terminus as an aborted (suicide) intermediate. Thus, our data further demonstrate that top1 can be trapped by DNA damage. Recently, trapping of top1 by DNA mismatches, abasic sites (21), and UV damage (38) has also been reported. At the present time, it is not known whether such a trapping is part of DNA repair (39) or cell death pathways. Top1 suicide (or aborted product) is a very efficient donor for the religation of an exogenous DNA duplex bearing a 5'-hydroxyl end (Figs. 3–5) (12, 13, 18, 22, 24, 27, 40). Religation of blunt-ended DNA duplex molecules is not influenced by the terminal sequence of the acceptor (13). Thus, these data further demonstrate that top1-mediated intermolecular religation can produce illegitimate recombination.

Our data demonstrate for the first time that the efficiency of
the intermolecular religation (recombination) is markedly enhanced by the presence of a phosphate group at the 5′ terminus of the break or gap opposite from the top1 linkage. This is physiologically relevant, as damaged DNA with nicks or gaps generally exhibits 5′-phosphate and 3′-hydroxyl termini. For example, nicks and gaps induced by ionizing radiations are usually 5′-phosphate and 3′-phosphate or phosphoglycolate (5).

Thus, taking into account the high frequency of top1 sites in most genes (for instance see Ref. 33), our study suggests that by random occurrence a fraction of the top1-cleavable complexes might be converted into DNA double-strand breaks and aborted (suicide) top1 intermediates with high recombinogenic potential in the presence of DNA-damaging agents that lead to backbone interruptions. Secondly, from an enzyme-DNA interaction standpoint, the fact that the −1/+1 phosphate opposite from the top1–DNA covalent bond facilitates top1 cleavage, and intermolecular religations suggests that this phosphate is involved in the interaction between the enzyme and the DNA. Further evidence for a structural role of this −1/+1 phosphate can be inferred from the observation that its removal inhibits top1 cleavage in the oligonucleotide with a two-base gap 3′ to the cleavage site (Fig. 3). Transcription bypass by T7 RNA polymerase has also been shown to be sensitive to the phosphorylation of the termini of the gapped DNA template (41).

Finally, the present study provides further insights for the molecular interactions of camptothecin with top1. This is the first report showing that nicks downstream (between bases +2 and +5) from a top1 site can convert camptothecin-induced reversible cleavable complexes into DNA double-strand breaks and abortive (suicide) intermediates. Since this effect was not
detectable in the absence of camptothecin, it seems plausible that camptothecin destabilizes the DNA immediately downstream (3') from the top1 cleavage site. This would be consistent with the selective binding of camptothecin at the enzyme-DNA interface (25, 31) and with its interaction with the +1 base pair immediately flanking the cleavage site (29). The importance of the +1 base pair for camptothecin binding inside the top1-cleavable complexes is further supported by the present data showing that camptothecin is markedly more potent than camptothecin derivatives in the absence and presence of ionizing radiations and camptothecin de-

REFERENCES


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