Ubiquitin-dependent Destruction of Topoisomerase I Is Stimulated by the Antitumor Drug Camptothecin*

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Topoisomerase I (TOP1) relaxes superhelical DNA through a breakage/rejoining reaction in which the active site tyrosine links covalently to a 3’ phosphate at the break site as a transient intermediate. The antitumor drug camptothecin (CPT) and its analogs inhibit the rejoining step of the breakage/rejoining reaction, which traps the enzyme in covalent linkage with DNA (the cleavable complex). Little is known about the fate of cellular TOP1 trapped in the cleavable complex. We have analyzed TOP1 in mammalian cell lines treated with CPT. When CPT-treated cells were lysed with either SDS or alkali and analyzed by Western blotting, greater than 90% of the TOP1 was linked to DNA. Nuclease treatment of the cell lysate to remove the covalently linked DNA from TOP1 revealed a distinct ladder of higher molecular weight bands having properties indicative of multi-ubiquitin (Ub) conjugates of TOP1. Approximately 5–10% of TOP1 was present as these conjugates within minutes of CPT treatment. Consistent with ubiquitination, TOP1 was not modified in ts85 cells at the restrictive temperature for its thermolabile ubiquitin-activating enzyme (E1). Because conjugation with ubiquitin can mark proteins for destruction by the 26S proteasome, we analyzed TOP1 protein levels during prolonged CPT treatment. TOP1 protein levels were reduced to about 25% during CPT treatments of 2–4 h resulting from increased destruction, with the half-life dropping from 10–16 h down to 1–2 h. The destruction of TOP1, like the formation of Ub-TOP1 conjugates, was not observed in ts85 cells at the restrictive temperature. The destruction of TOP1 was also prevented in cells treated with MG-132 and lactacystin, specific inhibitors of the 26S proteasome. Finally, the multi-Ub conjugates of TOP1 were observed whether or not aphidicolin was included in cotreatment with CPT, indicating that replication fork activity was not involved in making TOP1 a substrate for ubiquitination. These results demonstrate that independent of DNA replication, the TOP1 cleavable complex is ubiquitinated and destroyed in cells treated with antitumor drugs that block the rejoining step of the TOP1 reaction.

DNA topoisomerase I (TOP1) is a vital DNA metabolic enzyme as well as a molecular target of antitumor drugs. TOP1 relaxes DNA supercoils by cleaving a single strand of duplex DNA and passing the complimentary DNA strand through the cleaved strand before religation (1). In a transient reaction intermediate, termed the cleavable complex, the enzyme links covalently to DNA through tyrosine 723 (human TOP1) leaving a DNA break having a free 5’ hydroxyl end. Stopping the TOP1 reaction with strong protein denaturants produces a very low yield of TOP1 linked covalently to DNA. The rarity of this product is a reflection of the transient nature of the “cleaved” reaction intermediate. Antitumor drugs that act on TOP1 inhibit the DNA religation step through a reversible mechanism. The inhibition of religation can be observed as an increase in the yield of the enzyme-DNA covalent complex produced when whole cells or cell-free reactions are rapidly denatured (2).

The TOP1 cleavable complex can block both DNA replication and transcription. In vitro, the reversible TOP1 cleavable complex blocks the progression of transcribing TT RNA polymerase (3). In cells TOP1 protein and activity localizes to actively transcribed regions of the DNA (4–6). On the ribosomal gene repeats, the CPT-trapped TOP1 cleavable complex has been shown to cause RNA polymerase I to redistribute to the 5’ end of the gene, suggesting that it acts as a “roadblock” to the transcribing RNA polymerase (7, 8). The TOP1 cleavable complex also causes the replication fork to generate a double-strand DNA break and to arrest, which has been shown both in cell-free systems and in cells (9–11). The interaction of the replication fork with the cleavable complex is the predominant cytotoxic mechanism of CPT as evidenced by the protective effect of DNA synthesis inhibitors against the double-strand break formation as well as the cytotoxicity (9, 13–15).

Although the TOP1 cleavable complex obstructs DNA metabolism, little is known about the molecular mechanisms for managing it. We therefore analyzed TOP1 trapped in the cleavable complex in cells treated with camptothecin. We found the TOP1 cleavable complex multi-Ub conjugated and destroyed by a 26S proteasome-dependent mechanism.

**EXPERIMENTAL PROCEDURES**

Cell Cultures—Stock cultures of the mouse mammary carcinoma cell line ts85 (temperature-sensitive for ubiquitin-activating enzyme) (16), were maintained in a humidified atmosphere of 5% CO2 at 30 °C in Dulbecco’s minimum essential medium containing penicillin-streptomycin and 10% fetal bovine serum. Stock cultures of FM3A, the parental cell line of ts85, and Chinese hamster ovary cells (CHO, line AA8).
American Type Culture Collection) were maintained under identical conditions at 37 °C. Cells were shifted to the restrictive temperature by transferring the culture dishes to a 42 °C incubator.

Drug Treatment, Processing of Cell Lysates, and Western Blotting—Cells were treated with CPT (0.8 to 25 μM, 1% Me2SO) for the indicated times. To analyze Ub-TOP1 conjugates, treated cells were pelleted, chilled on ice, and lysed with ice-cold 0.2 M NaOH, 2 mM EDTA. Cell lysates were sonicated and then neutralized with 1/3 volume of 2 M HCl. 1/3 volume of 10% Nonidet P-40, 1 M Tris, pH 7.4, 0.1 M MgCl2, 0.1 M CaCl2, 10 mM dithiothreitol, 1 mM EGTA, and 100 μg/mI of each leupeptin, pepstatin, and aprotinin was then added immediately. Bal31 nuclease (200 units/ml) or Staphylococcus nuclease (10 μg/ml) was added to the neutralized lysates for a 30-min incubation at room temperature where indicated. Reactions were stopped by the addition of SDS-PAGE sample buffer (final concentration, 50 mM Tris-HCl, pH 6.8, 15% sucrose, 12 mM EDTA, 3% SDS, 10% β-mercaptoethanol). For analysis of cleavable complexes by the band depletion method, CPT-treated cells were pelleted and lysed immediately with SDS-PAGE sample buffer. Immunoprecipitation of TOP1 was as described (17), except nuclei were prepared in the presence of 5 mM N-ethylmaleimide for 15 min on ice, followed by the addition of 10 mM cysteine. In some experiments (see Fig. 4B), cell pellets were chilled on ice for 5 min and then lysed with an ice-cold high salt solution (50 mM Tris, pH 7.4, 0.8 M NaCl, 0.5% Nonidet P-40, 5 mM MgCl2, 5 μg/μl leupeptin, 5 μg/μl pepstatin, 5 μg/μl aprotinin) containing 5 mM N-ethylmaleimide. This lysate was then diluted with an equal volume of water containing 10 mM cysteine before the addition of 1/3 volume of 3 × SDS-PAGE sample buffer.

SDS-PAGE was performed with 6% acrylamide gels. Western blotting was performed using TOP1 antiserum obtained from scleroderma patients (18). Detection of primary antibodies was accomplished using 125I-protein A (19) and the Bio-Rad Phosphor Imaging System (GS-250) for quantitation of the various topoisomerase forms within their linear ranges of detectability as determined using serial dilutions of cell lysates. Chemiluminescence was also used for detection of primary antibodies (ECL, Amersham Corp.) in conjunction with densitometry (Bio-Rad GS-7000).

RESULTS

Multi-Ub Conjugates of TOP1 in Cells Treated with CPT—To analyze TOP1 trapped in the cleavable complex, CHO cells were treated with CPT for various times and then rapidly lysed with SDS. CPT is known to cause TOP1-mediated DNA cleavage and the covalent linkage of TOP1 to the 3’ ends of DNA upon SDS lysis (2). This covalent TOP1-DNA cleavable product has slower mobility during SDS-PAGE compared with free TOP1 (20). In CHO cells exposed to 25 μM CPT and immediately lysed with SDS, more than 90% of the TOP1 disappeared from its location at 100 kDa on Western blots where it is present as two differently phosphorylated forms (17), and it coincidentally reappeared as a higher molecular mass smear (Fig. 1). Against the background smear of TOP1 immunoreactivity, a number of distinct bands having a definite periodicity are visible. This ladder pattern of bands was recognized by several different TOP1 antisera (data not shown) that were previously shown to recognize different regions on TOP1 (21). To further analyze the ladder of TOP1-immunoreactive bands, cell lysates were digested with endonucleases to remove the DNA covalently linked to TOP1 prior to SDS-PAGE. After treatment with Staphylococcus or Bal31 nuclease, most of the background smear above TOP1 disappeared; the majority of TOP1 reappeared at 100 kDa; and a ladder of at least six TOP1-immunoreactive bands became more distinct. These bands accounted for approximately 5–10% of cellular TOP1 (Fig. 1).

The periodicity of the bands suggests that TOP1 is modified by multiples of the same molecule, which is reminiscent of multi-Ub conjugation. To analyze the suspected multi-Ub conjugated TOP1, we employed the murine cell line, ts85, which contains a thermolabile ubiquitin-activating enzyme (E1) that causes a deficiency in protein ubiquitination at the restrictive temperature (22). ts85 and its wild-type parental cell line

![Image](376x575 to 496x729)

**FIG. 1.** A ladder of TOP1-immunoreactive bands, suggestive of Ub-TOP1 conjugates, is present in CHO cells treated with CPT. CHO cells exposed to either the solvent control Me2SO (−) or CPT (+) for 10 min were pelleted and immediately lysed. The lysates were treated with either Bal31 or Staphylococcus (Staph.) nuclease or were not treated before SDS-PAGE and Western blotting with TOP1 antiserum (see “Experimental Procedures”). Top1* is a phosphorylated, gel-shifted form (17).

FM3A were incubated for 90 min at their permissive temperatures, or at 42 °C, the restrictive temperature for ts85. CPT was then added for 10 min at the corresponding temperatures, and cells were immediately lysed with SDS. Under these conditions, the quantity of cleavable complexes formed was about 90% in FM3A and ts85 at both the permissive and restrictive temperatures (measured as the quantity of TOP1 depleted on the Western blot, data not shown). When the lysates were treated with Staphylococcus nuclease to digest covalently associated DNA away from TOP1, very distinct TOP1 conjugates could be seen in the parental cell line FM3A that had been incubated at either 37 or 42 °C. These conjugates were also present in ts85 incubated at the permissive temperature but were absent in ts85 incubated at the restrictive temperature for E1, where ubiquitin conjugation to cellular proteins is known to be deficient (Fig. 2). These results suggest that the ladder of TOP1-immunoreactive bands is composed of multi-Ub conjugates of TOP1.

Ubiquitination-dependent Destruction of TOP1—To determine whether Ub-TOP1 conjugates were destroyed, we quantified the steady state levels of TOP1 during CPT treatment using Western blotting. In these experiments, FM3A cells were treated with CPT for 0.25–4 h. The CPT was then washed away, and the cells were incubated in medium without CPT for 30 min at 37 °C to allow ample time for the cleavable complexes to dissociate before the cells were lysed with SDS. Using this protocol, CPT treatment caused the TOP1 level to drop by an average of 25% after 15 min and by 80% after 2 h in cells that were treated at either the permissive or the restrictive temperature. A reduction in TOP1 level in CPT-treated cells was previously published in a study with another cell line (23). TOP1 levels were similarly reduced in ts85 incubated at the permissive temperature but remained unchanged in ts85 at the restrictive temperature for the thermolabile E1 (Fig. 3). This result suggests that the destruction of TOP1 in CPT-treated cells requires a functional E1.

Although the TOP1 level remained constant in ts85 during CPT treatment at the restrictive temperature, the phosphorylation state of the enzyme changed (17); the slow migrating phosphorylated form diminished and the fast migrating form intensified (Fig. 3). This change in phosphorylation state was independent of CPT treatment, suggesting that it is an effect of E1 inactivation.

We then determined whether the Ub-associated drop in
TOP1 level during CPT treatment was due to reduced de novo synthesis of TOP1 or its proteolytic destruction. To block de novo synthesis of TOP1, FM3A cells were treated with the protein synthesis inhibitor cycloheximide, which by itself did not appreciably affect TOP1 levels during a 2-h treatment (data not shown). In cells treated with CPT together with cycloheximide, the reduction in TOP1 level was not different than after CPT treatment alone, suggesting that TOP1 levels were reduced as a result of increased proteolytic destruction (data not shown).

To analyze the relationship between cleavable complex formation, multi-Ub TOP1 conjugate formation and TOP1 destruction, the CPT dose dependence of each of these effects was determined. CPT concentrations in the range of 0.8–10 μM were added to FM3A cells. To analyze the dose dependence of cleavable complex formation, cells treated with CPT for 10 min were immediately lysed to detect the amount of TOP1 covalently linked to DNA. Although this method produces variable results, probably due to variability in the shearing of DNA before being treated with CPT (25 μM) for 10 min. Cells were then lysed and treated with nuclease before SDS-PAGE and Western blotting with TOP1 antiserum (see "Experimental Procedures").

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FIG. 2. In ts85 at the restrictive temperature the CPT-induced TOP1 ladder is not formed, suggesting that it is composed of Ub-TOP1 conjugates. The murine cell line ts85, which contains a thermolabile ubiquitin-activating enzyme, or its parental cell line FM3A was incubated for 90 min at their normal growth temperatures (perm.) or at the restrictive temperature for ts85 (restrict.) before being treated with CPT (25 μM) for 10 min. Cells were then lysed and treated with nuclease before SDS-PAGE and Western blotting with TOP1 antiserum (see "Experimental Procedures").

FIG. 3. CPT treatment causes a reduction in the TOP1 steady state level that is dependent on a functional ubiquitin-activating enzyme. A, ts85 and FM3A cells were incubated for 90 min at their normal growth temperatures or at the restrictive temperature for ts85 before being treated with CPT (25 μM) for the times indicated. To measure the steady state level of TOP1 after each CPT treatment time, cells were washed free of CPT and incubated for 30 min before lysis to reverse cleavable complexes. SDS-PAGE and Western blotting with TOP1 antiserum were performed as described under "Experimental Procedures." Serial 2-fold dilutions were used as standards for quantitation. B, plot of the densitometric scan of the Western blots shown in A.

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(FIG. 4, A and C). These same CPT treatment conditions caused about 5–10% of TOP1 to be conjugated with Ub (Fig. 4, B and C). For analysis of the destruction of TOP1, cells were washed free of CPT after a 2-h exposure and were then incubated for 30 min at 37 °C to allow time for the cleavable complexes to reverse before lysis with SDS. The 2-h CPT treatment caused the destruction of about 70% of TOP1 at the highest dose (Fig. 4, D and E). Thus, the concentration of CPT required to trap TOP1 in the cleavable complex (Fig. 4, A and C) closely paralleled the concentration required to stimulate the multi-Ub conjugation of TOP1 (Fig. 4, B and C) and the destruction of TOP1 (Fig. 4, D and E). This result suggests that TOP1 trapped in the cleavable complex is conjugated with ubiquitin and destroyed.

To analyze the effect of CPT treatment on the half-life of TOP1, FM3A or ts85 cells were metabolically labeled with [35S]methionine and either were not treated or were treated with CPT during the chase period. Cells were then lysed at 2 and 4 h post-chase, and TOP1 was salt-extracted from nuclei, immunoprecipitated, and subjected to SDS-PAGE and autoradiography. In untreated FM3A and ts85 cells at the permissive temperatures, no more than 20% of TOP1 disappeared after the 4-h chase, corresponding to a half-life of TOP1 greater than 10 h. At the restrictive temperature, the rate of destruction of TOP1 in untreated cells was not detectably different than at permissive temperature. After treatment with CPT, however, the TOP1 half-life dropped to about 1.2 h in FM3A cells at both temperatures (data not shown). In ts85 at permissive temperatures, the half-life of TOP1 was similarly shortened (Fig. 5, A and B). In ts85 cells at restrictive temperature, the half-life of TOP1 did not change (Fig. 5, A and B). These results confirm the requirement for a functional E1 enzyme in the CPT-induced destruction of TOP1 and show an 8-fold shortening of the TOP1 half-life during CPT treatment.

Ub-protein conjugates are substrates for proteolysis by the 26S proteasome. To determine if the destruction of TOP1 might
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FIG. 5. CPT treatment reduces the half-life of TOP1 dependent on a functional ubiquitin-activating enzyme. ts85 cells metabolically labeled with [35S]Met and [35S]Cys (Express labeling kit, NEN Life Science Products) were chased for 90 min at either the permissive or the restrictive temperature before treatment with 25 μM CPT (+) or solvent control (−) for the indicated times at the corresponding temperatures. TOP1 was then salt-extracted from nuclei and immunoprecipitated before SDS-PAGE (see "Experimental Procedures").

be mediated by the 26S proteasome, FM3A cells were preincubated with either of the specific proteasome inhibitors MG-132 or lactacystin (24–27). These inhibitors blocked the CPT-induced destruction of TOP1 (Fig. 6). This result indicates that the multi-Ub-TOP1 conjugates are proteolyzed by the 26S proteasome. Although the proteasome inhibitors prevented the destruction of TOP1, accumulation of Ub-TOP1 conjugates was not observed.

DISCUSSION

In our experiments, the multi-Ub-TOP1 conjugates were formed within minutes, similar to the kinetics for the formation of the TOP1 cleavable complex (within 2–5 min, data not shown), suggesting that TOP1 trapped by CPT in the cleavable complex is recognized as a substrate for ubiquitination. CPT binds very specifically to TOP1 only in the presence of DNA (28). The production of this ternary cleavable complex composed of TOP1-DNA-CPT is the only known activity of CPT. Thus it is unlikely that another type of CPT-TOP1 complex without DNA might be a substrate for ubiquitination. Furthermore, the R isomers of two CPT derivatives that do not produce the cleavable complex did not cause Ub-TOP1 conjugation, whereas their S isomers that produce the cleavable complex did stimulate Ub-TOP1 conjugation (data not shown). Also, conjugation of Ub to topoisomerase II-α was not stimulated by CPT, further indicating the specificity of the ubiquitin conjugation to TOP1 in cells treated with CPT (data not shown). TOP1 in rodent cells can be seen by SDS-PAGE as a doublet of electrophoretically distinct forms that differ in phosphorylation state. One of these phosphorylated forms diminishes when transcription is inhibited (17). Whether these forms are differentially ubiquitinated is currently unknown.

Modification of proteins by multi-Ub chains makes them substrates for proteolysis by the 26S proteasome. This proteolysis pathway is an integral and essential part of cellular processes (29–32). The 76 amino acid ubiquitin molecule is linked though its carboxyl terminus to Lys residue(s) (isopeptide bond) of substrate proteins in a three-step mechanism: 1) the carboxyl terminus of ubiquitin links to the ubiquitin-activating enzyme (E1) through a thiol ester in an ATP-requiring step; 2) ubiquitin is then transferred via transacylation to the active site cysteine of a conjugating enzyme (E2), which in some cases can directly conjugate ubiquitin to proteins in vitro; or 3) a ubiquitin-ligating enzyme (E3) that binds the ubiquitin-charged E2 enzyme as well as the substrate protein and in some cases can form a thiol ester with ubiquitin before transferring it to the substrate (33, 34) is also required for the specific conjugation to many cellular substrates. The conjugation of ubiquitin to free ubiquitin, or to ubiquitin conjugated to substrate proteins can produce free multi-Ub chains or multi-Ub conjugates of substrate proteins. The subunits of multi-Ub are linked from the carboxyl terminus of one ubiquitin to the next though Lys48, most frequently, although other linkages have been identified (35–38).

In our experiments, an apparent Ub-TOP1 conjugate in untreated cells (detected by multiple TOP1 antisera; data not shown) co-migrated with the first conjugate in CPT-treated cells (see Figs. 1 and 2). The role of this Ub-TOP1 conjugate is unknown, but it may be an intermediate in the multi-Ub conjugation that may mark this long-lived protein (39) for proteolytic destruction during normal turnover. Alternatively, it may be similar to the stably monoubiquitinated histones H2A and H2B, which have been implicated to be preferentially associated with transcribed DNA (40, 41). Monoubiquitinations of small basic proteins in vitro have been shown to be catalyzed by a group of ubiquitin-carrier enzymes in a reaction that does not require a ubiquitin-protein ligase activity. Some of these monoubiquitinating ubiquitin-carrier enzymes have been shown by genetic studies to be involved in a variety of fundamental cellular processes (30); however, the function of the monoubiquitinations is unknown. TOP1, like the ubiquitinated histones H2A and H2B, is highly basic (calculated isoelectric point of 10.05) (42) and localized to transcribed regions (8), and therefore it may be monoubiquitinated for similar reasons.

The structural characteristics that stimulate the ubiquitination of the TOP1 cleavable complex at present remain unknown. Polypeptide elements that stimulate ubiquitination have been studied by inserting random polypeptides into β-galactosidase. These studies have identified clusters of hydrophobic residues that appear to be critical for stimulating ubiquitin conjugation and destruction (43). One possibility is that a normally buried hydrophobic region of TOP1 becomes exposed when the enzyme is trapped in the cleavable complex.

In our studies, six TOP1 immunoreactive bands were consistently observed between the 100-kDa TOP1 and the 200-kDa (prestained myosin) molecular mass standard (Fig. 1). Analysis of the apparent molecular masses of the first four of these bands (determined from Rf) showed differences between successive bands of between 14 and 17 kDa instead of the 8-kDa shifts known to result from the conjugation of ubiquitin monomers to substrate proteins. A possible explanation for this result is that only even-numbered Ub-TOP1 conjugates are present, which might suggest that their formation is favored or...
that odd-numbered ubiquitin conjugates are preferentially deubiquitinated or destroyed. The possibility that odd-numbered conjugates may be preferentially destroyed may be less likely because analysis of the binding of Ub-lysozyme conjugates to the 26S proteasome subunit 5 showed that efficient avidity required at least four ubiquitins, providing no basis for the preferential binding of odd-numbered Ub conjugates (38, 44).

Another possibility we consider less likely is that the conjugates are actually ubiquitin cross-reactive protein (UCRP). Although the size of UCRP (15 kDa) roughly coincides with the size of the TOP1 band shifts, previous studies indicate that the activation and conjugation of UCRP to cellular proteins probably occurs by a pathway distinct from the ubiquitin pathway; the affinity of E1 for UCRP is much lower than for ubiquitin, and moreover, UCRP concentrations are significantly lower than ubiquitin concentrations in cells; evidence is also lacking for the presence of multi-UCRP chains and for the destruction of proteins conjugated with UCRP (34). In addition, treatment of U937 cells with type I interferons increases the conjugation of UCRP to numerous cellular proteins (45), but this treatment did not affect the abundance of the TOP1 conjugates (data not shown). However, our experiments cannot eliminate the possibility that UCRP or another UCRP-like protein (46) that can form multiple conjugates may be responsible for the multi-Ub conjugates of TOP1.

Finally, the electrophoretic mobility shifts of 14–17 kDa could result from an even-numbered ubiquitin conjugates being preferentially formed. The crystal structure of a diubiquitin conjugate (Ub$_2$) shows that the molecule exhibits a 2-fold symmetry dictating that a third ubiquitin molecule must attach in a different way to Ub$_2$ (47). Thus, the rate of addition of odd-numbered Ub units may be slow relative to even-numbered additions resulting in the predominance of even-numbered conjugates at steady state. Another possible explanation for the apparently even-numbered ubiquitin conjugates of TOP1 is that they are produced by the sequential addition of two ubiquitin molecules in the form of Ub$_2$. Precedence for this type of mechanism is provided by experiments demonstrating activation of Ub$_2$ by E1, its transfer to E2, and its ligation to histones H2A/H2B through an E3-independent mechanism in vitro (48). In these experiments Ub$_2$ and Ub$_2$ conjugated as effectively as ubiquitin monomers. Free multimeric chains of ubiquitin have been found in cells providing support for the hypothesis that chains of multi-Ub may be conjugated in a single step to substrate proteins in vivo (48).

In our experiments about 90% of cellular TOP1 was trapped in the cleavable complex after CPT treatment (25 μM), but only 5–10% was conjugated to ubiquitin. Several hypotheses can account for this small fraction of Ub-TOP1 conjugates despite the great majority of TOP1 being in the cleavable complex. These include: 1) the TOP1 cleavable complexes may be saturating with respect to the ubiquitin conjugating components; 2) only a small fraction of cleavable complexes may be sequestered to regions where they can be Ub-conjugated; and 3) the Ub-TOP1 conjugates may be in a competition between Ub-dependent destruction and ubiquitin chain removal by ubiquitin-specific proteases that might function when the cleavable complex, in rapid reversible equilibrium with the noncleavable form, dissociates. Finally, a combination of these possibilities may account for the small fraction of Ub-TOP1 despite the large fraction of TOP1 in the cleavable complex. Interestingly, when the proteasome was inhibited, the Ub-TOP1 conjugates did not accumulate. Whatever the predominant pathway, the enzymes for ubiquitination and ubiquitin-dependent proteolysis are proposed to be present in complexes (32, 49), which may suggest that the ubiquitin system enzymes that act on TOP1 may be closely associated with active chomatin.

It is possible that the TOP1 cleavable complex becomes a substrate for ubiquitin conjugation only after being acted on by DNA metabolizing enzymes, which could be the rate-limiting step in the Ub-TOP1 conjugation. The active DNA replication fork is known to convert the TOP1 cleavable complex into a two-strand break and to arrest at the site of the breakage (9, 10, 12, 50, 51). To test the possibility that the replication fork might produce the TOP1 substrate for ubiquitination, cells were treated with aphidicolin, an inhibitor of replicative DNA polymerases, before and during CPT treatment. Aphidicolin treatment prevents the double-strand breaks and the lethality produced by CPT, but it had no effect on the quantity of Ub-TOP1 conjugates (data not shown). We also tested whether transcription might be necessary for converting TOP1 into a substrate for ubiquitination. Neither of the transcription inhibitors actinomycin D or 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole affected the quantity of Ub-TOP1 conjugates in CPT-treated cells (data not shown).

We speculate that a pathway for the repair of protein-DNA adducts may be responsible for the Ub-dependent destruction of TOP1. We have recently observed the Ub-dependent destruction of topoisomerase II following treatment of cells with specific inhibitors that trap this enzyme in a similar cleavable complex. We are continuing studies to understand the role of ubiquitin conjugation in the regulation of topoisomerases and in the response to antitumor inhibitors that trap the cleavable complex.

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