Identification of Mammalian DNA Topoisomerase I as an Intracellular Target of the Anticancer Drug Camptothecin

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ABSTRACT

Camptothecin, a plant alkaloid with antitumor activity, has been shown to be a potent inhibitor of nucleic acid synthesis and a strong inducer of DNA strand breaks in mammalian cells. Previous studies have shown that camptothecin inhibits purified mammalian DNA topoisomerase I by trapping a reversible enzyme-DNA “cleavable complex” (Hsiang et al., J. Biol. Chem., 260: 14873-14878, 1985). Our present studies, using L1210 cells and SV40-infected monkey cells, have shown that camptothecin-induced strand breaks are protein linked. The linked protein is most likely DNA topoisomerase I as revealed by immunoblot analysis, using antibodies against purified mammalian DNA topoisomerase I. Brief heating of camptothecin-treated cells to 65°C resulted in a rapid reduction of the number of protein-linked DNA breaks. Reversal of the camptothecin-induced topoisomerase I-DNA complex by heat was also observed in an in vitro system by using purified mammalian DNA topoisomerase I. Our results suggest that camptothecin interferes with DNA topoisomerase I both in cultured mammalian cells and in the purified system by trapping a reversible enzyme-DNA cleavable complex.

INTRODUCTION

Camptothecin, a plant alkaloid, was isolated from stem wood of Camptotheca acuminata (family Nyssaceae) (1). Its strong antitumor activities have prompted investigations to determine its intracellular target and its mode of action. Earlier studies have shown that camptothecin is a strong inhibitor of both DNA and RNA synthesis (2–6). DNA synthesis is rapidly inhibited by camptothecin and only partially recovered upon drug removal (3, 7). At high camptothecin concentrations, DNA synthesis is irreversibly inhibited and S-phase cells cannot progress into the G2 phase of the cell cycle (8, 9). Inhibition of RNA synthesis by camptothecin appears to be more severe for high molecular weight RNAs (e.g., rRNA and heterogeneous nuclear RNA) than for low molecular weight RNAs (4S and 5S RNAs). The inhibition is rapid and reversible (3, 4). In addition to its inhibition of nucleic acid synthesis, camptothecin has also been found to induce “reversible” DNA strand breaks in Hela cells (10). It is not clear whether the induction of DNA strand breaks is related to the inhibition of nucleic acid synthesis.

Recently, mammalian DNA topoisomerase I has been suggested as a possible intracellular target of camptothecin (11). Using purified calf thymus DNA topoisomerase I, it has been demonstrated that camptothecin can inhibit the enzyme by trapping an enzyme-DNA intermediate, termed the “cleavable complex” (11). Although the exact chemical nature of this cleavable complex is still unclear, it has been shown that treatment of drug-induced cleavable complexes with protein denaturants results in single-strand DNA breaks and the concomitant covalent linking of a topoisomerase I polypeptide to the 3’ phosphoryl end of each strand break (11). Treatment of the cleavable complexes with high concentrations of salts (e.g., 0.5 M NaCl) prior to the addition of protein denaturants results in rapid “reversal” of the cleavage reaction (11). Despite these studies in the purified system, there has been little evidence that topoisomerase I is involved in the action of the drug in cultured mammalian cells. Our present study demonstrates that camptothecin also interferes with the breakage-reunion reaction of intracellular mammalian DNA topoisomerase I by trapping reversible cleavable complexes on chromosomal DNA.

MATERIALS AND METHODS

Materials. Media and sera for cell culture work were purchased from Gibco. Rabbit antiserum against purified calf thymus DNA topoisomerase II was prepared as described previously (12). Purified calf thymus DNA topoisomerase I was used to prepare rabbit antiserum by using the same procedure as described for the preparation of topoisomerase II-specific antiserum. Camptothecin, both lactone form (NSC 94600) and sodium form (NSC 100880), and m-AMSA (NSC 249992) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. VM-26 (teniposide) was a gift from Bristol-Myers Co. Sodium camptothecin (100 mM) was dissolved in H2O while camptothecin lactone, VM-26, and m-AMSA (10 mM) were dissolved in dimethyl sulfoxide. All drugs were aliquoted and stored at −20°C.

Cells and Virus. Mouse leukemic cells (L1210) were grown in Fisher’s medium. Monkey kidney cells (BSC) were grown in Eagle’s minimal essential medium. All media were supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine. The infection of BSC cells with SV40 virus was carried out as described previously (13). Confluent BSC cells were infected with an SV40 viral stock (strain 776) at a multiplicity of infection of 10–30 plaque-forming units per cell. Virus-infected cells were maintained in Eagle’s minimum essential medium with 2% fetal bovine serum.

K⁺-SDS Coprecipitation Assay. K⁺-SDS coprecipitation assays, both in the purified system and in cultured mammalian cells, were done as described previously (14, 15). Immunoblot Analysis of Whole Cell Lysates. Immunoblot analyses of whole cell lysates, using antiserum against purified calf thymus DNA topoisomerase(s), were done as described previously (12). Briefly, 15 ml of L1210 cells (3 x 10⁶ cells/ml) were treated with various topoisomerase III inhibitors for 30 min at 37°C in a CO2 incubator.

Cells were pelleted and prepared for electrophoresis by resuspending in 0.5 ml of a 2x SDS sample buffer (20% glycerol, 10% β-mercaptoethanol, 6% SDS, 125 mM Tris, pH 6.8). Following heating in a boiling water bath for 3 min, proteins in boiled lysates (10⁶ cells/lane) were separated electrophoretically in a 7.5% SDS polyacrylamide gel and electroblotted onto a nitrocellulose filter. The filters were then hybridized with antiserum against purified calf thymus DNA topoisomerase(s). Topoisomerase bands were detected by incubation with 125I-labeled protein A as described previously (12).

DNase I Digestion of Whole Cell Lysates. Logarithmically growing L1210 cells (3 x 10⁶ cells/ml) were treated with camptothecin lactone (100 μM in 0.5% dimethyl sulfoxide) at 37°C for 30 min. Cells (4 x 10⁶ cells/sample) were pelleted and lysed with 500 μl of a buffer containing 1% SDS, 20 mM EDTA, 10 mM Tris, pH 8.0, and 3 μM aprotinin.

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1 Recipient of the ACS faculty research award. To whom requests for reprints should be addressed, at Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.

2 The abbreviations used are: m-AMSA, 4'-(9-acridinylamino)methanesulfonanisidide; SDS, sodium dodecyl sulfate; K⁺-SDS, potassium-sodium dodecyl sulfate.

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Topoisomerase I Cleavage Assays. Topoisomerase I cleavage assays were done as described previously (11).

RESULTS

Camptothecin Induces Protein-DNA Cross-Links in Cultured Mouse L1210 Cells. If topoisomerase I is responsible for camptothecin-induced DNA fragmentation in cultured mammalian cells, one would expect to observe a concomitant increase in the number of covalent protein-DNA cross-links following camptothecin treatment. To test this hypothesis, L1210 cells were treated with camptothecin and the level of protein-linked DNA was measured using the K⁺-SDS coprecipitation assay. As shown in Fig. 1 increasing amounts of protein-linked DNA were observed following treatment of L1210 cells with increasing concentrations of camptothecin. VM-26 and m-AMSA were used as positive controls. The large amounts of protein-linked DNA induced by VM-26 and m-AMSA are consistent with their known effect on the intracellular DNA topoisomerase II (13, 16).

Camptothecin Induces Protein-linked DNA Breaks on Intracellular SV40 DNA. The ability of camptothecin to induce DNA fragmentation and protein-DNA cross-links in L1210 cells suggested a possibility that the DNA lesion might be protein-linked breaks. To test whether camptothecin induces protein-linked DNA breaks, we examined the effect of camptothecin on intracellular SV40 DNA in SV40-infected BSC cells. As shown in Fig. 2, the amount of form II (nicked) SV40 DNA isolated from camptothecin-treated, SV40-infected BSC cells increased with increasing concentrations of camptothecin, indicating that camptothecin induces primarily single-strand DNA breaks on intracellular SV40 DNA (Fig. 2, Lanes A-E). In contrast, VM-26 (100μM), a drug which interferes with the breakage-reunion reaction of mammalian DNA topoisomerase II, induced both form II (nicked) and form III (linear) SV40 DNA, indicating that VM-26 induces both single- and double-strand DNA breaks (Fig. 2, Lane F) (13, 17). The specific association of these single- and double-strand breaks with tightly (most likely covalently) linked proteins were demonstrated by their sensitivity to phenol extraction prior to proteinase K treatment. Electrophoresis and Southern blotting were done as described in Fig. 2.

Fig. 1. Camptothecin induces protein-DNA cross-links in L1210 cells. Logarithmically growing mouse L1210 cells (2 x 10⁶ cells/ml) were labeled with [3H]thymidine (0.7 μCi/ml) for 18 h. Cells were then washed three times with phosphate-buffered saline and treated with camptothecin (lactone form), m-AMSA, or VM-26 for 30 min. Protein-DNA cross-links in drug-treated cells were measured by using the K⁺-SDS coprecipitation procedure as described in "Materials and Methods." The total acid-precipitable cpm per assay were determined to be 8.6 x 10⁶. Proteinase K treatment (400 μg/ml at 50°C for 2 h) of the cell lysates prior to the K⁺-SDS coprecipitation abolished the precipitable counts.

Fig. 2. Camptothecin induces single-strand breaks on intracellular SV40 DNA. SV40-infected BSC cells at 36 h postinfection, were treated with camptothecin (Lanes A-E, 0.2, 1, 5, 25, 125 μM, respectively), VM-26 (Lane F, 100 μM), or 0.5% dimethyl sulfoxide (Lane G) (the final concentration of dimethyl sulfoxide in all drug-treated cells was 0.5%). After 30 min of drug treatment, cells were lysed at 37°C with 2 ml of 1% SDS, 10 mM Tris, pH 7.6, and 20 mM EDTA. Proteinase K treatments (400 μg/ml) were done at 50°C for 4 h. DNA samples were then extracted with phenol, ethanol precipitated, and electrophoresed on a 1% agarose gel. SV40 DNA was detected by Southern blotting by using a 32P-labeled SV40 DNA probe. SV40 (I), (II), and (III) depict supercoiled, nicked, and linear forms of SV40 DNA, respectively.

Fig. 3. Camptothecin-induced DNA breaks on intracellular SV40 DNA are protein linked. SV40-infected BSC cells at 36 h postinfection, were treated with 0.5% dimethyl sulfoxide (Lanes A and B) 25 μM camptothecin (Lanes C and D), or 100 μM VM-26 (Lanes E and F) at 37°C for 30 min as described in Fig. 2. Cell lysates were extracted with phenol prior to (Lanes A, C, and E) or after (Lanes B, D, and F) proteinase K treatment. Electrophoresis and Southern blotting were done as described in Fig. 2.
Camptothecin Promotes Specific Covalent Association of DNA Topoisomerase I with Chromosomal DNA. The possibility that topoisomerase I was responsible for camptothecin-induced protein-linked DNA breaks was further supported by the following experiment. Proteins in whole cell lysates from drug-treated L1210 cells were separated by a 7.5% SDS polyacrylamide gel, electrophoresed, and immunoblotted with either topoisomerase I-specific (Fig. 4, left) or topoisomerase II-specific (Fig. 4, right) antisera. As shown in Fig. 4, left, the M, 100,000 topoisomerase I protein band decreased significantly in intensity following treatment of L1210 cells with camptothecin (Fig. 4, left, Lanes B and C). Treatment of L1210 cells with 25 µM camptothecin cause a 90% reduction of the intensity of DNA topoisomerase I protein band (Fig. 4, left, Lane B). The decrease in the intensity of this M, 100,000 topoisomerase I was specific since the protein pattern as detected by Coomassie blue staining (data not shown) and the topoisomerase II protein band (M, 170,000) as detected by immunoblotting with topoisomerase II-specific antisera did not change (Fig. 4, right, Lanes B and C). Similarly, more than 90% of intracellular DNA topoisomerase II molecules were specifically eliminated from the gel following treatment of L1210 cells with the topoisomerase II-specific inhibitor, VM-26 (Fig. 4, right, Lanes D and E). The specific disappearance of topoisomerases in gel can be explained by the covalent linking of topoisomerase to chromosomal DNA, which are too large to enter the SDS polyacrylamide gel. To test this hypothesis, cell lysates were digested with DNase I before loading onto the SDS polyacrylamide gel. Following DNase I digestion, the intensity of the M, 100,000 topoisomerase I returned to the same level as control cell lysates (Fig. 5, left, compare Lanes B and D). This result therefore suggests that intracellular DNA topoisomerase I is specifically linked to chromosomal DNA in the presence of camptothecin.

Camptothecin-induced DNA Damage is Reversible both in Cultured L1210 Cells and in a Purified System. Using purified mammalian DNA topoisomerase I, it has been demonstrated that camptothecin-induced, protein-linked DNA breaks can be rapidly reversed by the addition of high concentrations of salt (e.g., 0.5 M NaCl) prior to treatment with protein denaturants (11). To test whether a brief heating at 65°C can also reverse this type of DNA damage in the purified system, the following two experiments were performed; Fig. 6 shows the experiment using gel mapping to monitor the reversibility of camptothecin-induced strand breaks upon brief heating at 65°C following a 30-min incubation at 37°C. The reversibility of DNA strand breaks upon heating was evidenced by the time-dependent decrease in DNA fragmentation. Within 3 min at 65°C, the majority of DNA breaks induced by camptothecin and topoisomerase I disappeared. The residual DNA fragments, although still evident in the gel, were considerably less in amounts compared to the amounts produced before heating (Fig. 6, Lanes F–R). Fig. 7, right, shows the experiment using the K+-SDS coprecipitation procedure to monitor the reversibility of camptothecin-induced protein-DNA cross-links upon brief heating at 65°C. The amounts of protein-linked DNA decreased following a similar time course. These two experiments together indicate that camptothecin-induced topoisomerase I-mediated DNA breaks can be reversed by heat, consistent with the cleavable complex hypothesis (11). To test whether the cellular DNA damage induced by camptothecin can also be reversed by heat, the K+-SDS coprecipitation procedure was used to measure the
amounts of camptothecin-induced protein-linked DNA upon brief heating. As expected, a brief heating at 65°C rapidly reduced the amounts of protein-linked DNA (Fig. 7, left). The similarity of heat reversibility of the topoisomerase I-DNA cross-links provides further evidence that topoisomerase I is the intracellular target of camptothecin. In addition, camptothecin appears to trap the same reversible cleavable complex in cultured mammalian cells as in the purified system (11).

DISCUSSION

Our present results strongly suggest that DNA topoisomerase I is an intracellular target responsible for the DNA damage induced by camptothecin. The induction of topoisomerase I-linked DNA breaks and the reversibility of the DNA damage in camptothecin-treated cells provide strong evidence that camptothecin interferes the breakage-reunion reaction of cellular topoisomerase I causing the accumulation of reversible enzyme-DNA cleavable complexes. The inhibition of DNA and RNA synthesis in cultured cells may be related to the drug interference with the breakage-reunion reaction of DNA topoisomerase I.

The chemical nature of camptothecin-induced cleavable complexes is still unclear. A number of topoisomerases have been shown to form such cleavable complexes (18-20). The cleavable complex was first reported for Escherichia coli DNA topoisomerase I (21). E. coli topoisomerase I forms alkali-cleavable complexes with either single-stranded DNA or negatively supercoiled DNA. Exposure of the alkali-cleavable complex to strong protein denaturant results in topoisomerase-linked, single-strand breaks. Dissociation of alkali-cleavable complexes with salt plus Mg\(^{2+}\) results in intact DNA and active topoisomerase I (21). Similar complexes have also been detected for mammalian DNA topoisomerase I and II (14, 22). It is interesting that a number of potent antibiotic and antitumor agents are found to stimulate the formation of these cleavable complexes. Bacterial DNA gyrase has been shown to form large amounts of cleavable complexes in the presence of quinolone antibiotics (19). Mammalian DNA topoisomerase II has also been shown to form cleavable complexes in the presence of a number of clinically important antitumor drugs such as VP-16 (etoposide), Adriamycin, and mitoxantrone (20). These bactericidal and cytotoxic drugs appear to kill cells by a similar mechanism related to the formation of drug-induced cleavable complexes.

Camptothecin-induced, topoisomerase I-mediated, cleavable complexes share many similarities with other topoisomerase-cleavable complexes. Like many other topoisomerase II-targeting drugs (13, 16, 23-24), the induction of these reversible cleavable complexes may be the key cellular event leading to rapid cell death and other cellular responses such as sister chromatid exchanges and chromosomal aberrations (25). The reversibility of the cleavable complexes suggests that cellular processes surrounding these drug-induced cleavable complexes may be crucial in the expression of the drug effect. Further studies are necessary to establish this new type of DNA damage and its biological consequences. These basic studies are also likely to establish DNA topoisomerase I as a new therapeutic target in cancer chemotherapy.

A recent report that a camptothecin-resistant topoisomerase I is present in camptothecin-resistant human lymphoblastic leukemia cells provides further evidence that topoisomerase I is the cellular target of camptothecin (26).

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REFERENCES

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