Evidence for the Reversibility of Cellular DNA Lesion Induced by Mammalian Topoisomerase II Poisons*

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Like many intercalative antitumor drugs, the non-intercalative antitumor drug epipodophyllotoxin VM-26 (teniposide) induces topoisomerase II-linked DNA breaks as revealed by cell lysis with a strong protein denaturant such as sodium dodecyl sulfate or alkali. We show that the majority of topoisomerase II-linked DNA breaks reflect the formation of reversible topoisomerase II-DNA cleavable complexes in drug-treated cells by demonstrating the reversibility of this unusual type of DNA damage at elevated temperatures (e.g. 65 °C).

Mammalian DNA topoisomerase II is the primary cellular target of a number of potent antitumor drugs with diverse and unrelated chemical structures (for a review, see Ref. 1). Examples of these drugs, referred to as topoisomerase II poisons, include acridines (e.g. 4′-(9-acridinylamino)-methanesulfon-m-anisidine), anthracyclines (e.g. adriamycin and daunomycin), anthracenediones (e.g. mitoxantrone and bisantrene), ellipticines (e.g. ellipticine and 2-methyl-9-hydroxy-ellipticinium acetate), and actinomycins (e.g. actinomycin D), all of which are DNA intercalators and epipodophyllotoxins (e.g. demethyllepidodendrillotoxin ethylenidene-β-D-glucoside and VM-26) which do not show significant DNA binding and are not DNA intercalators (1). In mammalian cells, these drugs produce a common cellular DNA lesion which is detected as protein-linked DNA breaks upon cell lysis with SDS or alkali (2-4). The cellular protein which is covalently linked to broken DNA strands has been identified to be topoisomerase II (5, 6). How topoisomerase II mediates the drug action has recently been suggested from studies using purified mammalian DNA topoisomerase II (7-9). All these drugs interfere with the breakage-reunion reaction of topoisomerase II by trapping a tight (possibly covalent) enzyme-DNA complex, termed the "cleavable complex," which is presumed to be the key covalent intermediate or one related to it in the topoisomerase strand-passing reaction pathway (10). The formation of this putative enzyme-DNA-drug ternary complex is reversible and apparently aberts the strand-passing activity of topoisomerase II (10). Exposure of this otherwise reversible enzyme-DNA-drug-cleavable complex to a strong protein denaturant such as SDS or alkali leads to the formation of topoisomerase II-linked single- and double-strand DNA breaks (7-9). Detailed characterization of the cleaved product induced by protein denaturants has shown that a topoisomerase II polypeptide is covalently linked to the 5′-phosphoryl end of the broken DNA strand via a tyrosyl phosphate bond (10). The 3′-hydroxyl end, in the case of a double-strand break, is 4-base recessed (10). These studies suggest that the major cellular DNA lesion induced by topoisomerase II poisons in mammalian cells may be the reversible topoisomerase II-DNA cleavable complexes, and topoisomerase II-linked DNA breaks may be produced by SDS or alkali treatment during cell lysis. We have obtained additional evidence in the present studies that the cellular DNA lesion induced by topoisomerase II poisons is indeed reversible, consistent with the cleavable complex hypothesis.

EXPERIMENTAL PROCEDURES

Materials—DNA topoisomerase II from HeLa cells was purified to homogeneity by slight modification of the published procedures (11). pBR322 DNA was purified by phenol deproteinization of cleared lysates followed by CsCl/ethidium isopycnic centrifugation and gel filtration on an A-50cm column. VM-26 (teniposide) was a gift from Bristol-Myers Co. Media and fetal bovine serum for cell culture work were purchased from GIBCO. Rabbit antisera against purified calf thymus DNA topoisomerase I and II were prepared as described previously (11, 12).

Cells and Virus—Mouse erythroleukemia cells (MELC) were grown in RPMI medium 1640. Monkey kidney cells (BSC-1) were grown in Eagle's minimum essential medium. All media were supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The infection of BSC-1 cells with SV40 virus was carried out as described previously (9). Confluent BSC-1 cells were infected with an SV40 viral stock (strain 776) at a multiplicity of infection of 10–30 plaque-forming units/cell. Virus-infected cells were maintained in Eagle's minimal essential medium with 2% fetal bovine serum.

Topoisomerase II Cleavage Assays—Topoisomerase II cleavage assays were done as described previously (7-10). Briefly, pBR322 DNA was linearized with EcoRI and then end-labeled at the 3′ termini with the large fragment of Escherichia coli DNA polymerase I in the presence of [α-32P]dCTP and three other unlabeled deoxyribonucleotides. Topoisomerase II cleavage reactions were done in reaction mixtures (20 μl each) containing Tris (40 mM, pH 7.5), KCl (100 mM), MgCl₂ (10 mM), dithiothreitol (0.1 mM), EDTA (0.5 mM), bovine serum albumin (30 μg/ml), HeLa DNA topoisomerase II (30 ng), and end-labeled [32P]pBR322 DNA (10 ng). Reactions were incubated at 37 °C for 30 min. For heat reversal, the incubated reaction mixture was then heated to 65 °C for specified times. Reaction were terminated by 10% SDS (1 μl) and proteinase K (1.7 mg/ml, 1 μl), and further incubated at 37 °C for 1 h. DNA samples were analyzed on a 1% agarose gel in TBE buffer (89 mM Tris borate (pH 8.3) and 0.5 mM EDTA).

Immunoblot Analysis of Topoisomerase Contents—Immunoblot analysis using rabbit antisera against human topoisomerase I and II was done with minor modifications as described previously (12, 13).

Potassium-SDS Co-precipitation Assay—Potassium-SDS co-precipitation assays, both in the purified system and in drug-treated cells, were done as described previously (6, 10).

RESULTS AND DISCUSSION

Exposure of topoisomerase I-DNA cleavable complexes to an elevated temperature (e.g. 65 °C) has been shown to abolish subsequent DNA cleavage upon addition of a strong protein...
denaturant (12). This apparent reversal of DNA cleavage at an elevated temperature, which has also been observed for ColE1 relaxation complex (14), is another unusual property of the reversible topoisomerase I-DNA cleavable complex. To test whether topoisomerase II-DNA cleavable complexes induced by topoisomerase II poisons exhibit a similar property toward elevated temperatures, the mammalian topoisomerase II poison, VM-26 (teniposide), was selected for this study. In the presence of purified HeLa topoisomerase II, VM-26 induced large amounts of cleavable complexes, which upon addition of SDS led to fragmentation of end-labeled pBR322 DNA (Fig. 1, lane P). Brief exposure of the cleavable complexes to 65 °C prior to the addition of SDS resulted in time-dependent reduction in subsequent DNA cleavage upon the addition of SDS (Fig. 1, lanes Q–T). Within 3 min, the majority of smaller DNA fragments had been converted into larger DNA molecules (Fig. 1, compare lanes P and R). The residual DNA fragments, although still evident in the gel, were considerably less than those produced before heat treatment (Fig. 1, compare lanes P and R). This effect was also studied by a potassium-SDS co-precipitation assay using the same 32P end-labeled pBR322 DNA (Fig. 2B). The amounts of protein-linked DNA in the precipitate were similarly reduced following heating the reaction mixture of 65 °C (Fig. 2B). These results suggest that the reversibility of the VM-26-induced topoisomerase II-DNA cleavable complexes can be demonstrated by incubation of the complex at an elevated temperature.

The possibility that VM-26-induced DNA lesion in cultured mammalian cells could be reversed by a brief heat treatment at an elevated temperature was tested using MELC. Previous studies have shown that topoisomerase II-linked DNA fragments can be selectively precipitated from lysates of VM-26-treated cells using the potassium-SDS co-precipitation assay (6). As shown in Fig. 2A, the amounts of protein-linked DNA in lysates of VM-26-treated MELC were rapidly reduced following exposure of drug-treated cells to 65 °C (Fig. 2A). Different type of assay which monitors the unbound cellular topoisomerase II in cell lysates was also performed to test the effect of elevated temperatures on the possible formation of topoisomerase II-DNA cleavable complexes in VM-26-treated cells (Fig. 3A). As shown previously, the majority of cellular topoisomerase II molecules which are trapped covalently onto chromosomal DNA in VM-26-treated cells cannot enter a 7.5% SDS-polyacrylamide gel and therefore cannot be detected by immunoblot analysis using topoisomerase II antisera. Reversal of DNA cleavage in drug-treated cells at an elevated temperature is expected to release covalently trapped topoisomerase II molecules from chromosomal DNA and to result in the recovery of unbound topoisomerase II which can be detected by immunoblot analysis. Fig. 3B shows the results of such an assay. The recovery of unbound topoisomerase II is dependent on the temperature. There appears to be a sharp transition temperature around 52 °C above which the majority (70%) of trapped topoisomerase II molecules were recovered (Fig. 3C). The trapping and recovery of topoisomerase II molecules in drug-treated cells was specific as the cellular level of topoisomerase I was unaffected by VM-26 and elevated temperatures (Fig. 3B). The rate of recovery of unbound topoisomerase II in VM-26-treated cells at 65 °C was also measured by the same method (Fig. 4A). Within 1 min at 65 °C, recovery is already complete (Fig. 4C). Again, the cellular topoisomerase I level did not change under such conditions (Fig. 4B).

SV40 virus-infected cells have been successfully used as an experimental system for studying the DNA lesion induced by topoisomerase II poisons (9). Both linearized (from III) and nicked (form II) SV40 DNA, which have been shown previously to represent topoisomerase II-linked DNA breaks, were produced in VM-26-treated cells (Fig. 5, lane B). Brief heating (to 65 °C) of the SV40 virus-infected cells in the presence of VM-26 rapidly abolished both linearized and nicked SV40 DNA in cell lysates (Fig. 5, lanes B–F).

Together, our results provide strong evidence supporting the hypothesis that the unusual cellular DNA lesion induced by topoisomerase II poisons is the formation of reversible topoisomerase II-DNA cleavable complexes. However, the mechanism of reversal of DNA cleavage at elevated temper-
treated with 100 μM VM-26 for 30 min at 37 °C in a 95% air:5% CO₂ incubator. Shifting of VM-treated MELC to elevated temperatures is unclear. The narrow range of the transition temperature and the rapid rate of reversal suggest a possible selective denaturation of the protein structure of the cleavable complex. This is in contrast to the use of strong protein denaturants such as SDS and alkali which trap the cleavable complexes, presumably by nonspecific denaturation of the entire protein molecule. The reversibility of the DNA lesion induced by topoisomerase II poisons raises the question on cellular repair responses to such an unusual DNA damage. Our present results, however, do not rule out the possibility that a small fraction of the VM-26-induced topoisomerase II-DNA cleavable complexes has been converted into an irreversibly cleaved form by interaction with certain cellular processes within 30 min of drug treatment. Further studies are necessary to establish the cellular events following the formation of the topoisomerase II-DNA cleavable complex induced by topoisomerase II poisons.

FIG. 5. Heat reversal of VM-26-induced cleavage of intracellular SV40 DNA. The infection of monkey kidney cells (BSC-1) with SV40 virus was carried out as described under “Experimental Procedures.” At 36 h post-infection, viral infected cells were treated with VM-26 (100 μM, 30 min). After removing the culture media by decanting, cells were heated to 65 °C in water bath for specified times prior to lysing with 1% SDS, 10 mM Tris, pH 8.0, and 20 mM EDTA. Protein kinase K treatment (400 μg/ml) was done at 50 °C for 4 h. DNA were then extracted with phenol, ethanol-precipitated, and electrophoresed in a 0.7% agarose gel. SV40 DNA was detected by Southern blotting using a 32P-labeled SV40 DNA probe. SV40 (I), (II), and (III) depict supercoiled, nicked, and linear forms of SV40 DNA, respectively. Lane A, control, no drug or heat treatment. Lanes B–F, cells were treated with VM-26 (100 μM, 30 min at 37 °C) followed by heating to 65 °C for 0, 1, 3, 5, and 10 min, respectively, prior to the addition of lysis buffer.

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