Role for topoisomerases in the release of DNA into the detergent-soluble fraction of eukaryotic cells

(replication/4'-(9-acridinylamino)methanesulfon-m-anisidide/DNA topoisomerase/teniposide/camptothecin)

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ABSTRACT  Detergent-soluble DNA is the fraction (2-4%) of DNA that is released into the supernate upon mild detergent lysis. It is nonmitochondrial in origin. It labels efficiently with deoxy[3H]ribonucleosides and the labeling is prevented by inhibitors of polymerase α and ribonucleotide reductase. In previous publications we have characterized detergent-soluble DNA from splenocytes of immunologically activated mice. In this publication we show that incorporation of [3H]thymidine into detergent-soluble DNA is prevented by pretreatment with novobiocin, 4'- (9-acridinylamino)methanesulfon-m-anisidide (m-AMSA), and teniposide (VM26), three inhibitors of type II topoisomerases. Camptothecin, an inhibitor of type I topoisomerases, also reduces incorporation of [3H]thymidine but only to 50% of control levels. In addition to affecting incorporation of [3H]thymidine, preincubation with the topoisomerase II inhibitors m-AMSA and VM26 alters the amount of DNA recovered in the detergent-soluble fraction. At low concentrations of m-AMSA the amount of detergent-soluble DNA increases somewhat, whereas at higher drug concentrations a marked decrease is observed. Treatment with VM26 results in diminished amounts of DNA being released into the detergent-soluble fraction as well. However, maximal inhibition of detergent-soluble DNA release by VM26 requires the presence of camptothecin. Therefore, we suggest that topoisomerases play an important role in making a small part of lymphocyte chromatin detergent labile. Furthermore, these results are consistent with recent studies demonstrating a role for topoisomerases in yeast replication. Thus, the newly synthesized portion of detergent-soluble DNA may arise as DNA replication intermediates not yet stabilized into mature chromatin.

Topoisomerases are a group of enzymes that interconvert various topological isomers of DNA without altering the nucleotide sequence (1-3). Type II topoisomerases result in 2n changes in linking number through transient double-stranded breaks and covalent insertion of the enzyme at the 5' end of DNA. The energy for rejoining the broken strand(s) comes from an enzyme-associated ATPase activity (4). Type I topoisomerases produce unit changes in linking number by means of single-stranded breaks and covalent insertion of the enzyme at the 3' end of DNA (5). Because of the topological constraints of DNA replication, some sort of topoisomerase involvement is likely (6). It is now accepted that resolution of intertwined pairs of newly replicated DNA molecules involves type II DNA topoisomerases (7, 8) and that topoisomerases are required for replication in yeast (9).

Topoisomerases are frequently assayed in the presence of detergent to stabilize the protein-DNA intermediate and to prevent resealing of the DNA strand(s). In this publication we demonstrate that three inhibitors of type II topoisomerases with distinctly different mechanisms inhibit the release of rapidly labeled DNA into the detergent-soluble fraction of dividing cells. Maximal inhibition of release by one of the type II topoisomerase inhibitors requires the presence of an inhibitor of type I topoisomerase as well. We propose that DNA in the detergent-soluble fraction arises at least in part from replication intermediates involving topoisomerases, intermediates not yet fully stabilized into mature chromatin.

A preliminary report of this work has appeared (27).

MATERIALS AND METHODS

Cell Preparation and Incorporation of [3H]Thymidines. Splenocytes were freshly obtained from concanavalin A-stimulated, outbred CD-1 male mice, 6-8 weeks of age, as described in earlier publications (10, 11). The cells were suspended at a final concentration of 5 x 10⁶ per ml in phosphate-buffered saline (PBS) containing 5 mM glucose and 0.1% bovine serum albumin (PBS/Glc/Alb).

After cells were incubated with or without inhibitor for the period described in the text, purified [3H]thymidine [60-90 Ci/mmol, depending on the lot (1 Ci = 37 GBq)] was added to a final concentration of 6 µCi/ml for 60 min. Cells were washed twice to remove exogenous [3H]thymidine and lysed rapidly in the indicated buffer without bovine serum albumin but containing 25 mM EDTA, 350 µg of phenylmethylsulfonyl fluoride per ml, and 0.5% sterile Nonidet P-40 at 4°C. The detergent supernatant fraction was separated from the detergent-insoluble fraction by Microfuge centrifugation at 4°C. Pellets and supernates were stored at -20°C. In some cases the experiment was continued by incubating cells in the absence of drug before final washing and lysis.

Chemicals. Chemicals were obtained from previously listed sources (11, 12). Novobiocin was purchased from Sigma. The anticancer drug 4'-(9-acridinylamino)methanesulfon-m-anisidine (m-AMSA) was the gift of Michael Mattern or was obtained through the courtesy of Ven Narayanam (Department of Health and Human Services). VM26 was the gift of Leroy Liu or was obtained through the courtesy of Dale Stringfellow (Bristol Myers Co.). Camptothecin was obtained from Ven Narayanam. [3H]Thymidine, obtained either from Dupont-NEN Products (Boston, MA) or from Amersham, was purified before use as described (10).

DNA Isolation and Analysis. DNA, isolated from the detergent-soluble fraction and purified as described earlier (11, 12), was resolved by means of electrophoresis using native 1.2% agarose gels. Standards obtained from New England Biolabs were a DNA digested with HindIII or φX174 replicative form (RF) DNA digested with Hae III. The amount of DNA in each lane was determined from the photograph of the ethidium bromide staining pattern by scanning densitometry using the Shimadzu TLC Scanner

Abbreviation: m-AMSA, 4'-(9-acridinylamino)methanesulfon-m-anisidide.

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was compared with the area under the curve for known amounts of φX174 RF DNA digested with Hae III run on the same gel. To determine the location of the incorporated radiolabel, the gel was cut into 2-mm slices, melted in 1.0 ml of deionized, distilled H₂O, dissolved in 5.0 ml of Scintiverse (Fisher, Medford, MA), and assayed for radioactivity scintillation spectrometry. Alternatively, the gel was processed for fluorography by using New ENHANCE (Dupont–NEN Products, Boston, MA) or Amplify (Amer sham). The incorporation of [³H]thymidine was determined by scintillation spectrometry after chromatin in the detergent-insoluble fraction was sheared repeatedly in 8 M urea containing 2% sodium dodecyl sulfate.

RESULTS

Novobiocin Reversibly Inhibits Incorporation of [³H]Thymidine into DNA in the Detergent-Soluble Fraction.

Novobiocin inhibits topoisomerase II activity by preventing ATP hydrolysis required for strand rejoining (13–15). When splenocytes were preincubated or coincubated with 100–200 μM novobiocin, incorporation of [³H]thymidine into detergent-soluble DNA was reduced. However, the amount of DNA in the detergent-soluble fraction remained unchanged (Fig. 1). If cells were first preincubated with novobiocin for 30 min and then washed free of the drug for 1 hr before the addition of [³H]thymidine, incorporation returned to the level of control cells incubated without drug (data not shown). Novobiocin inhibited incorporation of [³H]thymidine into the detergent-insoluble fraction to a lesser extent than into the detergent-soluble fraction. For example, at 50 μM novobiocin, incorporation into DNA in the detergent-insoluble fraction was 95% of control, whereas incorporation into DNA in the detergent-soluble fraction was 32% of control. At 200 μM novobiocin, incorporation into DNA in the detergent-insoluble fraction was 37% of control, whereas incorporation into DNA in the detergent-soluble fraction was 4% of the control value.

**m-AMSA Inhibits the Appearance of DNA in the Detergent-Soluble Fraction in a Biphasic Fashion.** Novobiocin inhibits not only topoisomerase II but also other enzymes with ATPase-associated activity such as DNA polymerase α (16) and DNA ligase (17). To further implicate topoisomerase II, we turned to other inhibitors with different modes of action. The drug m-AMSA inhibits topoisomerase II by forming a ternary complex with the enzyme and DNA so that the enzyme dissociates poorly from DNA (18, 19). When splenocytes were preincubated for 30 or 60 min with m-AMSA at concentrations of 1 μM or greater, incorporation of [³H]thymidine into DNA recovered in the detergent-soluble fraction was inhibited (Figs. 2 and 3). At each concentration, [³H]thymidine incorporation into DNA from the detergent-soluble fraction was inhibited to a greater extent than incorporation into the detergent-insoluble fraction. In contrast to the incorporation data, the amount of DNA recovered in the detergent-soluble fraction of splenocytes was increased by 10–30% at low concentrations of m-AMSA (1–5 μM), whereas treatment with m-AMSA at 50 μM and 100 μM resulted in diminished amounts of DNA in the detergent-soluble fraction (see Fig. 2). At 20 μM, the amount of DNA recovered in the detergent-soluble fraction depended on the experiment. In addition, as the amount of detergent-soluble

![Fig. 1. Effect of novobiocin on detergent-soluble DNA from splenocytes of immunologically activated mice. After splenocytes (5 × 10⁹ per ml) were incubated in the presence or absence of 200 μM novobiocin for 60 min at 37°C, [³H]thymidine was added to a final concentration of 6 μCi/ml. One hour later the cells were washed free of exogenous [³H]thymidine in buffer with or without novobiocin and lysed. DNA isolated from the detergent-soluble fraction was resolved on native 1.2% agarose gels. The distribution of incorporated radiolabeled [³H]thymidine was determined by fluorography. Lanes 1–4, ethidium bromide staining patterns; lanes 5–8, fluorographs. Lanes 1 and 3, preincubation in the absence of novobiocin; lane 2, preincubation for 60 min with novobiocin before addition of [³H]thymidine; lane 4, simultaneous addition of [³H]thymidine and novobiocin.](image)

![Fig. 2. Effect of m-AMSA on detergent-soluble DNA from splenocytes of immunologically activated mice. Splenocytes from concanavalin A-stimulated mice were exposed to the topoisomerase II inhibitor m-AMSA for 1 hr at 37°C. [³H]Thymidine was added to a final concentration of 6 μCi/ml (120 nM) and the incubation was continued for an additional 60 min. DNA isolated from the detergent-soluble fraction was resolved on native 1.2% agarose gels. Lanes 1–4, ethidium bromide staining patterns; lanes 5–8, fluorography. Lane 1, 0 μM m-AMSA; lane 2, 5 μM m-AMSA; lane 3, 20 μM m-AMSA; lane 4, 100 μM m-AMSA. Scanning densitometry indicated that the amount of DNA in lanes 2, 3, and 4 was 134%, 29%, and 12% of the control, respectively.](image)
VM26 Alters Incorporation of $^{3}H$H Thymidine and the Amount of DNA Accessible to Detergent Solubilization, but Maximal Inhibition of Release Requires an Inhibitor of Topoisomerase I. m-AMSA is able to intercalate into the DNA helix. Another agent that interacts directly with type II topoisomerases without intercalation is teniposide (VM26). Pretreatment of splenocytes with VM26 at concentrations equal to or greater than 1 $\mu$M resulted in decreased incorporation of $^{3}H$H thymidine into DNA from detergent-soluble and detergent-insoluble fractions (Fig. 3). Although $^{3}H$H thymidine incorporation was diminished by 85% or more by 50–100 $\mu$M VM26, the amount of DNA accessible to detergent solubilization decreased only by 35% (Fig. 4). The size distribution of DNA remaining in the detergent-soluble fraction did not change.

Because type I and type II topoisomerases can substitute in some reactions for each other in yeast (7–9), we examined the effects of camptothecin, an inhibitor of type I topoisomerase (19), alone and together with VM26. The effects of camptothecin (lactone form) on incorporation of $^{3}H$H thymidine into DNA from the detergent-soluble and detergent-insoluble fractions as a function of concentration are shown in Fig. 3. Below 10 $\mu$M, camptothecin was as effective on a molar basis as VM26 in inhibiting $^{3}H$H thymidine incorporation. However, inhibition was never more than 55%. Furthermore, treatment with 125 $\mu$M camptothecin resulted in only 25% inhibition of release of DNA into the detergent-soluble fraction.

Simultaneous treatment with VM26 and camptothecin, however, gave striking results (Fig. 4). Pretreatment with 100 $\mu$M VM26 and 125 $\mu$M camptothecin together resulted in 86% inhibition of $^{3}H$H thymidine incorporation into detergent-soluble DNA and 71% reduction in the amount of DNA accessible to detergent solubilization. The same treatment resulted in 73% inhibition of $^{3}H$H thymidine incorporation into DNA in the detergent-insoluble fraction.

DNA diminished, the smaller size classes were lost preferentially. In Fig. 2, for example, DNA of 1400 base pairs and smaller decreased from 55% of the total detergent-soluble DNA from control cells to 14% of the total detergent-soluble DNA isolated from splenocytes treated with 100 $\mu$M m-AMSA. At the same time there was a tendency to accumulate DNA of the largest size classes. The drug m-AMSA has been shown to induce strand breaks in mouse L1210 cells in a reversible manner (18). In contrast, the effects of m-AMSA on detergent-soluble DNA were not reversible when cells were washed and incubated for 1 hr in PBS/Glc/Alb without drug before the addition of $^{3}H$H thymidine.
DISCUSSION

In this report we present data showing that three different inhibitors of type II topoisomerases—namely, novobiocin, m-AMSA, and VM26—markedly inhibit the incorporation of [3H]thymidine into DNA that can be dissociated from chromatin by detergent solubilization. In contrast, an inhibitor of type I topoisomerases, camptothecin, partly inhibits incorporation of [3H]thymidine into detergent-soluble DNA. Furthermore, m-AMSA and VM26 both alter the amount of DNA recovered in the detergent-soluble fraction as well. Since the three topoisomerase II inhibitors act by distinct mechanisms (20, 21), secondary effects on other enzymes are unlikely. Therefore, it appears that topoisomerases are involved in rendering certain DNA available to release into the detergent-soluble fraction.

Detergent-soluble DNA is that fraction of DNA released from chromatin by detergent treatment of dividing cells (11, 12). Nonmitochondrial in origin (11), it can be isolated from a wide variety of cell types, including splenocytes from immunologically active mice (11, 12), erythroblasts from phenylhydrazine-treated anemic mice, various mouse and human lymphocyte lines (T and B), murine erythroleukemia cells, Chinese hamster ovary cells, HeLa cells, and, finally, Xenopus laevis and Drosophila melanogaster (unpublished data). It is metabolically active since it is labeled more efficiently with [3H]thymidine than is bulk chromatin, which remains in the detergent-insoluble fraction (11, 12, 23). Detergent-soluble DNA has been observed in the past by a number of investigators (24–26). Its origin has been described as cytoplasmic, membranous, or artifactual, the result of cell death and/or a calcium, magnesium-activated endonuclease activity unrelated to cell division. Although cell death may result in nuclear fragmentation, the results presented here suggest most detergent solubility arises from the ongoing interaction of topoisomerases with chromatin. The observation that camptothecin augments the effect of VM26 suggests that the remaining metabolically inactive detergent-soluble DNA may be generated by type I topoisomerase activity or a combination of the two topoisomerase types. We add that lysis under conditions that would be expected to completely inhibit known DNA nuclease—e.g., lysis with sodium dodecyl sulfate or lysis in the presence of diethylcarbamate—also generates DNA in the detergent-soluble fraction with the same size distribution as that obtained under conditions described here (E. Palome, J.C.T., and P.R.S., unpublished data). Hence, detergent-soluble DNA is unlikely to be generated by nonspecific, "promiscuous" nuclease.

About 2–4% of total DNA from splenocytes of mice that have been immunologically activated is recovered in the detergent-soluble fraction (11). Therefore, we suggest that this DNA is transiently located between two nearby topoisomerase molecules. The DNA is released into the soluble fraction when the enzyme–DNA interaction is interrupted by detergent treatment. We cannot exclude the possibility that additional longer fragments are created that are detergent insoluble simply because of their size. Although detergent-soluble DNA represents <5% of the total cellular DNA and its specific activity is increased over that of DNA recovered from the detergent-insoluble fraction (11, 12), this percentage of total DNA is too great to contain only recently replicated intermediates not yet stabilized into chromatin. Possibly, topoisomerases are inserted well in advance of replication in preparation for unwinding. These fragments would not yet have incorporated radiolabeled thymidine and would decrease the observed specific activity of detergent-soluble DNA. Even taking this possibility into account, the amount of DNA recovered in the detergent-soluble fraction is at least an order of magnitude too great to be accounted for solely by replicating regions. Hence, in splenocytes, release of DNA into the detergent-soluble fraction probably reflects topoisomerase activity not only in replicating regions but also in nonreplicating regions. Indeed, topoisomerase I participates in yeast DNA replication and ribosomal RNA synthesis when topoisomerase II has been rendered inactive (9). When both topoisomerase classes are completely inactivated, cell growth and division in yeast finally stop.

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