A Decatenation-Supercoiling Assay Using Kinetoplast DNA Suitable for Prokaryotic and Eukaryotic Type II Topoisomerases

ABSTRACT

A versatile assay for eukaryotic and prokaryotic type II topoisomerases is described based upon detection of kinetoplast DNA (kDNA). Because type II topoisomerases can decatenate DNA but only DNA gyrase can negatively supercoil the products, this assay discriminates between type II topoisomerases that are supercoiling proficient (DNA gyrase) and supercoiling deficient (eukaryotic topo II). The decatenation assay is specific for topo II activity even in crude extracts containing excess topo I. The method is suitable for rapid screening of topo II inhibitors and should be ideal for identification of novel type II enzymes that possess supercoiling activities. Electrophoretic separations are possible within a short time frame (<30 minutes) because of the large size difference between catenated DNA and decatenated products.

INTRODUCTION

A single theme is common to all topos regarding their reaction mechanism: These enzymes adjust the topological state of DNA by breaking and resealing DNA strands, resulting in alterations in DNA linking number (3,11,12). There are two classes of topoisomerases; type II enzymes transiently break both strands of DNA in concert, and type I enzymes transiently break one strand at a time. Given that these enzymes are important targets for novel anticancer and antibacterial therapeutics, assays that high differentiate between the enzyme classes are important in drug discovery (1,3,6). For example, in vitro, type II enzymes efficiently resolve catenated DNAs, such as kinetoplast DNA (4). Another important difference exists between eukaryotic topo II and DNA gyrase (a bacterial topoisomerase II). DNA gyrase uses ATP hydrolysis to negatively supercoil DNA whereas eukaryotic topoisomerase II only relaxes supercoiled DNA. Both enzymes share the same property of being proficient in decatenation reactions in vitro; however, only gyrase has the ability to supercoil the decatenated DNA products. We have modified the original assay described by Marini et al. (4) and show that decatention assays can be used to infer whether a given type II topoisomerase is supercoiling proficient (gyrase) or not (eukaryotic topo II). Additionally, the assay is suitable for identification of novel topo II inhibitors. Because the assay is rapid, it can be adapted for high volume screening of potential inhibitors.

MATERIALS AND METHODS

Enzymes and DNAs

Homogeneously purified human topoisomerase I (TG2005H-RC1) and topoisomerase II (TG2000H) were used for these studies (9,10). Kinetoplast DNA (TG2013) was also used in these experiments. For details on how we certify enzyme activity (units sold) please see Fig. A1 below.

Enzyme assays

Eukaryotic topoisomerase II was assayed by decatenation of KDNA and monitoring the appearance of a 2.5 kilobase (KB) DNA. Reactions contained 0.1 ug KDNA (final volume of 20ul), 50 mM Tris-HCl (pH 8), 120 mM KCl, 10 mM MgCl2, 0.5 mM each of dithiothreitol, ATP and 30 ug BSA/ml (topo II reaction buffer TG4040). The reactions were incubated for 15 min at 37o C and terminated with 0.1 vol of stop buffer (5% sarkosyl, 0.025% bromophenol blue, 50% glycerol). One unit of topoisomerase II is defined as the amount of enzyme required to fully decatenate 0.1 ug of KDNA in 15 min at 37o C. Assays designed to detect topoisomerase II inhibitors were carried out in topo II cleavage buffer (30 mM Tris-HCl, pH 7.6, 3 mM ATP, 15 mM 2-mercaptoethanol, 8 mM MgCl2, 60 mM NaCl) in a final volume of 20 ul. Reactions were incubated with 4 units of enzyme in the presence or absence of the indicated inhibitor for 30 min at 37o. The reactions were terminated with 2 ul of 10% sodium dodecyl sulfate, followed by

proteinase K treatment for 15 min at 37o. After addition of 0.1 vol of loading dye (50% glycerol, 0.025% bromophenol blue) samples were extracted once with an equal volume of chloroform:isoamyl alcohol (24:1). Following a brief centrifugation in a microfuge, the blue upper layer was loaded directly onto an agarose gel. Assays for DNA gyrase were carried out on reconstituted holoenzyme (equal concentrations of A and B subunits were mixed). Reactions (in a final volume of 30 ul) contained 50 mM Tris-HCl (pH 7.6), 20 mM KCl, 10 mM MgCl2, 2 mM dithiothreitol, 1.5 mM ATP, 5 mM spermidine, 1.5 ug bovine serum albumin and 0.2 ug KDNA (7). The decatenation products were analyzed on 1% agarose gels run either without or with 0.5 ug ethidium bromide/ml as specified. Electrophoretic analysis of KDNA was performed using standard agarose gel electrophoresis units. Separations designed to resolve supercoiled and relaxed DNA monomers (gyrase assays) were performed at 50 volts. Eukaryotic topo II products were separated at 100 volts which allowed rapid resolution of catenated networks from the minicircles. In gels containing ethidium bromide, appearance of the monomer DNA species was conveniently monitored with a hand-held UV light source; typically, electrophoresis was continued until the bromophennol blue dye front had migrated 50-75% down the gel. Following electrophoresis, gels lacking ethidium were stained with 0.5 ug ethidium bromide/ml for 20 min. and photographed. Gels containing ethidium bromide were destained in water (20-30 minutes at room temperature) prior to photography.

RESULTS AND DISCUSSION

Kinetoplast DNA (KDNA), the mitochondrial DNA of Crithidia fasciculata, is a catenated network of DNA rings, the majority of which are 2.5 KB monomers (8). Type II topoisomerase, but not topo I, has the ability to decatenate KDNA and generate the monomer DNA (4); therefore, decatenation is a highly specific assay for topo II. Furthermore, since KDNA networks are large (relative to the monomers) separation is achieved after only a few minutes of electrophoresis. We modified the original method described previously (4) by running gels in the presence of an intercalator (ethidium bromide) to monitor the appearance of monomers with a hand-held UV light source and to resolve various DNA forms (linear, nicked circular DNA, supercoiled DNA and relaxed DNA monomers).

A typical reaction is outlined in Figure 1. This gel contains ethidium bromide which allows one to clearly resolve nicked (OC monomers) and circular (relaxed and supercoiled). The kDNA networks are too large to enter the gel, however, purified eukaryotic topoisomerase II yields monomeric DNA rings. In contrast, topoisomerase I did not produce any change in the kDNA networks. The monomer DNA products of eukaryotic topo II are composed of two species, open circular or (OC) DNA and covalently closed circular (CCC), relaxed DNA. Catenated KDNA generally contains some level of OC DNA since the nicking is not caused by the purified topo II (data not shown). In the absence of ethidium bromide, the OC and CCC DNAs are resolved and the relaxed DNA species are present as a gaussian distribution of topoisomers. Inclusion of ethidium bromide in the gel system consolidates the topoisomers into a single band. As noted above, the monomers contained OC DNA due to pre-existing nicks in the KDNA substrate. (It is concievalbe that nicking occurs during the purification of KDNA since ethidium bromide is used in the purification.) Despite the presence of nicks, topoisomerase I cannot decatenate the networks (not shown). In contrast to the eukaryotic topo II reaction products, gyrase produced decatenated CCC DNA monomers that were supercoiled (last lane on right, Figure 1). The decatenation assay can be used to confirm the presence of a typical eukaryotic type II topoisomerase activity in crude extracts. Topo II will produce monomer species that are OC or CCC DNA, but not linear DNAs. Expected KDNA reaction products for different enzymes are described in Table 1. Formation of linear DNA or degradation products might indicate the presence of a nuclease activity (refer to Table 1). Therefore, in crude extracts a potent DNase I contaminant might interfere. Similarly, in crude extracts, a potent topo I activity might reverse gyrase mediated supercoiling of kDNA monomers. For example, we find that topo II relaxation is able to prevail over gyrase mediated supercoiling (not shown). We conclude that crude extracts containing topo I or II relaxation activity, gyrase supercoiling (but not decatenation) could be masked. To avoid this problem one can use extracts from cells that lack topo I activity, such as null mutations in yeast topoisomerase I or rely on specific topoisomerase inhibitors to suppress a potent relaxation activity. The decatenation assay is also suitable for screening and identification of novel topoisomerase II poisons that stabilize cleavable complexes and lead to nicked or fragmented DNA; however, this should be carried out using purified

enzyme (crude extracts may give misleading results). The assay is carried out using purified enzyme and a cleavage buffer optimized to detect formation of cleavable complexes. Cleavage buffer promotes the enzyme into a processive mode (5); thus, decatenation activity is a bit less robust in this buffer. As shown in Figure 1 eukaryotic topo II alone will produced nicked and circular (relaxed) DNA (see Table 1). Inclusion of VM-26 or VP-16 (topo II poisons) in the reaction resulted in formation of linears and OC DNA since the inhibitors stabilize single strand and double strand cleavage intermediates). These results require the use of proteinase K in order to resolve topo cleavage complexes since it is necessary to remove the covalently trapped protein off the DNA prior to electrophoresis; failure to do so may shift the band position due to the presence of associated protein. KDNA decatenation assays for drug screening are practical provided that appropriate controls are run (markers, extract alone controls, etc.); however, the investigator must be careful to perform additional controls to ensure that the kDNA is not being nicked or cleaved in a topo II independent fashion. For example, it is conceivable that some test compounds or solvents, may nick DNA. Controls will reveal these cleavage products to be artifactual. Formation of linear or nicked monomer DNA due to the presence of a drug such as etoposide or teniposide is immediately obvious by comparison to control reactions lacking drugs (for more information go to Complications).

There are several advantages to this type of assay:

1. It is fast, allowing high throughput of test samples;

2. The results are clear cut and unambiguous;

3. Topo II activity is robust with kDNA, thus, the enzyme goes farther with this type of assay, and;

4. Decatenation assays can also be used to detect inhibitors that block catalytic activity but do not stabilize cleavable complexes.

REFERENCES

Drlica, K., and R.J. Franco. 1988. Inhibitors of DNA topoisomerases. Biochemistry 27:2253-2259.

Hajduk, S.L., V.A. Klein, and P.T. Englund. 1984. Replication of kinetoplast DNA maxicircles. Cell 36:483-492.

Liu, L.F. 1989. DNA topoisomerase poisons as antitumor drugs. Ann. Rev. Biochem. 58:351-375.

Marini, J.C., K.G. Miller, and P.T. Englund. 1980. Decatenation of kinetoplast DNA by topoisomerases. J. Biol. Chem. 255:4976-4979.

Muller, M.T., J.R. Spitzner, J.A. DiDonato, V.B. Mehta, and K. Tsutsui. 1988. Single-strand DNA cleaves by eukaryotic topoisomerase II. Biochemistry 27:8369-8379.

Osheroff, N. 1989. Biochemical basis for the inhibition of type I and type II topoisomerases with DNA. Pharmacol. Ther. 41:223-241.

Otter, R., and N.R. Cozzarelli. 1983. Escherichia coli DNA gyrase. Methods Enzymol. 100:171-180.

Ryan, K.A., T.A. Shapiro, C.A. Rauch, and P.T. Englund. 1988. Replication of kinetoplast DNA in trypanosomes. Annu. Rev. Microbiol. 42:339-358.

Spitzner, J.R., and M.T. Muller. 1988. A consensus sequence for cleavage by vertebrate DNA topoisomerase II. Nucleic Acids Res. 16:5533-5556.

Trask, D.K., and M.T. Muller. 1983. Biochemical characterization of topoisomerase I purified

from avian erythrocytes. Nucleic Acids Res. 11:2779-2800.
Wang, J.C. 1985. DNA topoisomerases. Annu. Rev. Biochem. 54:665-697.
Wang, J.C. 1991. DNA topoisomerases: Why so many? J. Biol. Chem. 266:6659-6662.

IMPORTANT NOTE about TopoGEN's Activity determinations.

For QC activity determinations on topo II provided by TopoGEN, the following conditions apply. Looking at a typical topo II prep (above) you can see that the activity is significantly higher than 2 units/ul (unit defination, 1 unit fully decatenate the 0.2 ug kDNA in 30 min at 37o C). The typical preparation, when fresh, may be anywhere from 6 to 32 units/ul; however we certify at LEAST 2units/ul. For this reason, the customer actually obtains higher activity (=more topo II) than he or she pays for. For example, if you purchase 500 units, you will receive 250 ul of enzyme (@2 u/ul). In fact you are really obtaining from 1000 to as much as 4000 units in your order. We designed it this way because topo II is somewhat unstable and gradual activity loss is unavoidable during shipping; therefore, we send you much more activity than you ordered to account for the unavoiadable loss. For this reason, the actual activity (units/ul) often varies from lot to lot (one lot may be at or near 2 units/ul and the next lot may be much greater than 2units/ul); however, the activity will never be less than 2/ul as we certify.

For Information on Topoisomerase II assay and Drug Screening Kits, please contact TopoGEN Technical Services at 614-451-5810. Additional reagents and products used in the preceding article are available from TopoGEN as follows:

Human Topoisomerase II, 170 kDa Form
- TG2000H-1 250 Units
- TG2000H-2 500 Units

Human Topoisomerase I, 105 kDa Form
- TG2005H-RC1 500 Units

Kinetoplast DNA
- Catenated DNA TG2013-1 25 ug
- Catenated DNA TG2013-2 50 ug
- Linear DNA Marker TG2017-1 10 ug
- Decatenated Marker TG2020-1 10 ug

FIGURE A1: TYPICAL QC OF TOPO II LOT OF ENZYME

Marker 1:16 1:8 1:4 1:2 Undilute

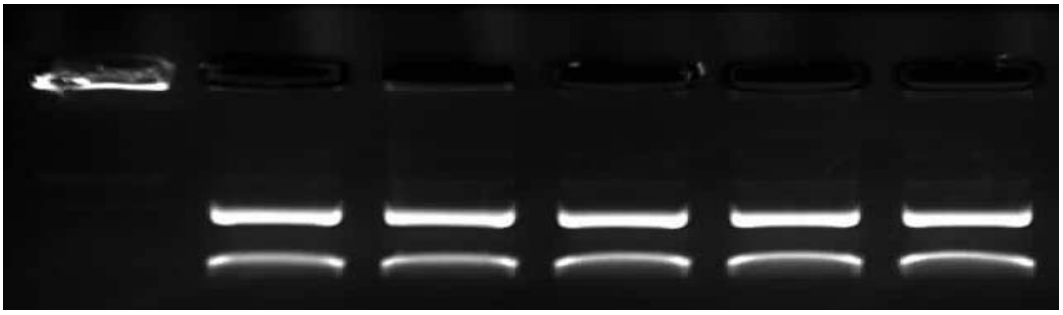

FIGURE 1: TOPO II/kDNA

Topo II and DNA gyrase reaction products with kDNA substrate. The data shown below are based upon the following concept:

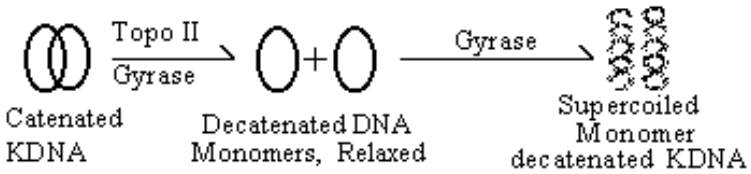

Figure 1. Different decatenation products with eukaryotic topo II and DNA gyrase.

Shown below is an idealized view of kDNA reactivity with eukaryotic topo II and prokaryotic DNA gyrase. From Left to right:

- kDNA Marker: Most of the kDNA will be high MW catenanes that fail to enter the gel
- Topo II Lest activity: Low enzyme and high enzyme activity levels are shown. At low enzyme activity, some kDNA may remain in the wells; at higher levels, all of the kDNA is released as either OC nicked monomers or relaxed circularized monomer.
- Linear kDNA Marker: Produced by incubating with a restriction enzyme that cuts kDNA monomers once.
- Decatenated kDNA Marker: Shows relative positions of OC nicked and circular monomers
- DNA gyrase products: Since gyrase is a topo II, it will decatenate kDNA; however, it will then supercoil the circular monomers.