Aggregates of Oligo(dG) Bind and Inhibit Topoisomerase II Activity and Induce Formation of Large Networks*

In Kwon Chung and Mark T. Muller‡
From the Department of Molecular Genetics, Ohio State University, Columbus, Ohio 43210-1292

DNA cleavage by eukaryotic type II DNA topoisomerase (EC 5.99.1.3) was strongly inhibited by an oligonucleotide containing 10 dGua residues. Catalytic activities of topoisomerase II, as measured by relaxation and decatenation reactions, were also inhibited by oligo(dG)₁₀. Inhibition was specific to oligo(dG)₁₀; other oligonucleotides, nucleotides, or single-stranded DNAs tested did not influence the activity of topoisomerase II. Oligo(dG)₁₀ did not inhibit other activities such as restriction enzymes. Although the enzyme neither binds nor cleaves oligo(dG)₁₀, inhibition can be explained by the finding that topoisomerase II binds tightly with aggregated oligo(dG) structures (estimated to contain between 20 and 30 molecules of monomeric oligo(dG)₁₀) that form spontaneously prior to addition of enzyme. These aggregated oligo(dG)-topoisomerase complexes are large networks that can be pelleted by a 20-min centrifugation step in a Microfuge. Western blotting with a monoclonal antibody confirmed that topoisomerase II is trapped in these pellets. The ability of the enzyme to form large DNA-protein networks could be a biochemical mechanism by which topoisomerase II might promote or participate in chromosome condensation in vivo prior to mitosis.

Type II topoisomerases transiently break and reseal DNA strands, thereby allowing adjustments in DNA topology. These enzymes are well conserved (1, 2) and are essential for completion of mitosis in yeast and higher organisms (3–6). Topoisomerase II is also a major component of mitotic chromosome and interphase nuclear scaffolds (7–10) and is present at a stoichiometry of ~3 copies (on average) per loop in human metaphase scaffolds (10). The location and abundance of topoisomerase II at the base of scaffold loops strongly suggest a structural role, possibly in anchoring DNA domains (9, 10); however, our biochemical results suggest that topoisomerase II may also participate in chromosome condensation.

The enzymatic activity of topoisomerase II in vivo is controlled in several ways, notably, through the accessibility of the underlying DNA sequence (11, 12), through the inherent affinity of topoisomerase II for any given site (1, 13), and by post-translational modifications (14–17). We reported that topoisomerase II is highly reactive toward sequences containing purine/pyrimidine repeats (13) and AT-rich repeats similar to those found in the scaffold-associated regions (10). More recently, we observed that non-B DNA structures can also significantly influence topoisomerase II activity at a given site. In this report, we have examined the interaction between topoisomerase II and oligo(dG)₁₀, which is structurally polymorphic. Monomeric single-stranded oligo(dG)₁₀ were neither inhibitors nor cleavage substrates for the enzyme; however, self-annealed oligo-(dG)₁₀ complexes were effective inhibitors of topoisomerase II. Inhibition was due to the stable formation of high molecular weight networks of self-annealed oligo(dG) complexes and topoisomerase II.

MATERIALS AND METHODS

Enzymes and DNA and Assays—Topoisomerase II was purified to homogeneity from chicken erythrocytes as described previously (18), and the same procedures were used to purify the yeast and human enzymes (the latter from human placenta). Preliminary experiments were carried out with the phenyl-Sepharose fraction (18), which contained minor lower weight bands due to proteolysis. Experiments were reproduced using topoisomerase that had been further purified by gel filtration over a fast protein liquid chromatography Superose 6 column (Pharmacia LKB Biotechnology Inc.). This fraction contained a single band of 155-160 kDa. One unit of topoisomerase activity is the amount of enzyme required to convert 50 ng of DNA substrate into product during a 15-min incubation at 30 °C in a 20-µl reaction (1 unit in a 20-µl reaction corresponds to 0.7 nM purified protein). Topoisomerase II assays (relaxation of plasmid DNA, decatenation of kinetoplast DNA, cleavage of radioactive fragments) were carried out as described previously (1, 18). DNA manipulation, including subcloning procedures, plasmid purification, fragment end-labeling, and DNA sequencing were all according to standard laboratory procedures as described (19, 20). Kinetoplast DNA was isolated from Crithidia fasciculata as described elsewhere (18). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. Radioactive nucleotides were from Du Pont-New England Nuclear; T₄ polynucleotide kinase was purchased from United States Biochemical Corp., and restriction enzymes were from Bethesda Research Laboratories.

Precipitation Assays and Ion Strength Gel Electrophoresis—Reactions (final volume of 20 µl) were carried out under conditions that allow topoisomerase II activity (cleavage buffer (30 mM Tris-HCl, pH 7.6, 80 mM KCl, 8 mM MgCl₂, 15 mM 2-mercaptoethanol, 3 mM ATP, 30 µg/ml bovine serum albumin), 15,000 cpm of end-labeled oligo(dG)₁₀ (4 ng), and 25 µg/ml sonicated salmon sperm DNA as carrier). Note that although ATP was included, it was not required for the precipitation assay described below. Additionally, the precipitation assay does not require salmon sperm DNA; however, recovery of the oligo(dG)-topoisomerase II networks after several washing steps was improved in its presence. The reactions were preincubated for 30 min at room temperature; during this period, oligo(dG)₁₀ self-anneals (see Fig. 3). Reactions were initiated by addition of topoisomerase II and then incubated for 30 min at 30 °C. When total reaction products were subjected to electrophoretic analysis, the samples were mixed with 2 µl of 50% glycerol and directly loaded onto a 10% low ionic strength polyacrylamide gel (6.7 mM Tris-HCl, pH 7.9, 33 mM

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‡ To whom correspondence should be addressed: Dept. of Molecular Genetics, Ohio State University, 484 W. 12th Ave., Columbus, OH 43210-1292. Tel.: 614-292-1914; Fax: 614-292-4702.

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sodium acetate, acrylamide-bisacrylamide weight ratio of 80:1. For fractionation of the reaction products, samples were centrifuged in a Microfuge (12,500 × g, 20 min, 4 °C), and the pellets were washed once with 200 μl of 4 °C buffer followed by resuspension in 20 μl of the same buffer. Pellets and supernatants were then mixed with 2 μl of 50% glycerol and loaded onto a 10% low ionic strength polyacrylamide gel. The gel was prerun for 1 h at 20 mA, and sample electrophoresis was carried out at 20 mA with constant buffer recirculation until the bromphenol blue marker migrated 12 cm (4 h).

Western Blotting—The pellets (resuspended in the same original volume of cleavage buffer) and supernatants recovered from the Microfuge (see above) were incubated with 1 μl of 50 μg/ml DNase I for 10 min at room temperature and spotted onto a slot blot containing a nitrocellulose filter (Schleicher & Schuell). Each sample well was washed once with 400 μl of 25 mM sodium phosphate buffer, pH 6.5, and filters were air-dried. The nitrocellulose filters were incubated for 3 h in 3% (w/v) nonfat dry milk in TBS (20 mM Tris-HCl, pH 8.0, 100 mM NaCl) at room temperature. The filters then were incubated overnight at room temperature with 1% nonfat dry milk in TBS containing anti-topoisomerase II monoclonal antibody (1:500 dilution of B53B ascitic fluid, courtesy of J. R. Spitzner) and washed with several changes of TBS containing 0.5 ml/liter Tween 20 over a 30-min interval. The filters were then incubated with rabbit anti-mouse immunoglobulin (diluted 1:10,000) in 1% nonfat dry milk in TBS and 2.5 μg of iodinated protein A (Amersham Corp.).

RESULTS

Inhibition of Topoisomerase II by Oligo(dG)—The topoisomerase II consensus sequence is degenerate and reflects the high frequency of topoisomerase II cleavages in any given DNA sequence in vitro (21). From the consensus sequence, we predicted that some sequences, such as long runs of guanines, should be immune to cleavage by topoisomerase II; these predictions were verified by constructing various cleavage sites. We also tested single-stranded DNAs and oligonucleotides and found that topoisomerase II did not cleave these DNAs. Single-stranded oligonucleotides also did not compete for topoisomerase cleavage of duplex DNAs; however, the following competition experiment shows that oligonucleotides containing contiguous guanine residues behaved differently. Topoisomerase II was incubated with pRYG, which contains a 54-base pair sequence of purine/pyrimidine repeats from a region 5’ of the human β-globin gene (13). Topoisomerase II makes a number of cleavages throughout this sequence (agents that stabilize topoisomerase II cleavage complexes are not required). Addition of an oligo(dG)10 suppressed cleavage at all sites in pRYG, with the weaker sites being suppressed at lower concentrations of competitor (Fig. 1). We estimate that 50% inhibition was reached with 0.12 μM oligo(dG)10 (Fig. 1, lane 5); and at 0.625 μM, cleavage was completely suppressed. No other oligonucleotide showed the effect when tested at 1.25 μM, a concentration that is 10-fold higher than the 50% inhibitory concentration of oligo(dG)10 (Fig. 1, lanes 8–12); thus, inhibition is specific for oligo(dG)10. Oligo(dG)10 inhibited the catalytic activity of topoisomerase II as measured by decatenation of kinetoplast DNA (Fig. 2A) or by relaxation of supercoiled plasmid (Fig. 2B). Decatenation activity was reduced by 50% in the presence of 0.62 μM oligo(dG)10 (50 ng), and total inhibition was observed at 1.2 μM (100 ng). Other oligonucleotides (Fig. 2A, lanes 12–16) tested at 1.2 μM (100 ng) had no affect on decatenation activity. The products of decatenation were analyzed on an ethidium bromide-containing agarose gel to resolve open circular (labeled Monomers in Fig. 2A) and covalently closed (fastest migrating band just below monomers) DNA products. The open circular DNA monomers are derived from nicked DNAs in the kinetoplast DNA preparation. The data show that oligo(dG)10 inhibits
decatenation and that both open circular and closed circular DNA products are reduced equally; therefore, oligo(dG)10 is not stimulating formation of a nicked DNA intermediate.

Relaxation of supercoiled DNA was also suppressed by oligo(dG)10, but not by other oligonucleotides tested (Fig. 2B).

Topoisomerase II Binding to Oligo(dG)—It is well established that G-rich sequences can form non-B DNA structures, such as tetrameric aggregates and four-stranded helices (22–31). Contiguous runs of guanine residues are unique among all other bases in their ability to engage in intramolecular base-stacking interactions; thus, inhibition of topoisomerase II by oligo(dG) may be related to these novel structures. We evaluated topoisomerase binding to oligo(dG) conformers by mobility shift experiments (32, 33). Oligo(dG)10 was end-labeled and incubated with increasing amounts of purified topoisomerase II, and the samples were analyzed on a low ionic strength polyacrylamide gel. In the absence of protein (Fig. 3A, lane 1), 80% of the oligonucleotide migrated as single-strand oligo(dG)10; the remainder was present as either a higher molecular weight smear of aggregates (brackets) or a

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2 The abbreviations used are: TBS, Tris-buffered saline; SARs, scaffold-associated regions.
fraction that failed to enter the gel. The oligo(dG) aggregated structures are intra- and intermolecular complexes that form spontaneously in vitro. With increasing topoisomerase II, there was a perceptible loss of the aggregated, smeared material attendant by the appearance of shifted complexes in the wells. The protein-DNA complexes trapped in the wells are large networks that can be recovered by centrifugation at 12,500 × g for 20 min and are not extracted with subsequent rounds of washing and centrifugation. In contrast, the small amount of oligonucleotide recovered from the pellet of reactions lacking topoisomerase II is variable and is reduced further with additional washing steps (Fig. 3A, lane 6). As shown in Fig. 3A, the pellets contained high molecular weight oligonucleotide in a complex, but not the intermediate mobility aggregates or single-stranded oligo(dG)₁₀ (Fig. 3A, lanes 2–5 with lanes 7–10). Immunoblotting experiments show that topoisomerase II is present in the pellet of oligo(dG) networks (see below). Moreover, proteinase K treatment of networks released the oligo(dG) aggregates from the pellet (data not shown); and since these experiments were conducted with purified protein, we conclude that topoisomerase II is responsible. The amount of monomeric single-strand oligonucleotide in the supernatant did not change with increasing topoisomerase II; and single-strand oligo(dG)₁₀ was not recovered from the pellet, which shows that the enzyme did not bind monomeric oligo(dG)₁₀. Collectively, the results suggest that the oligo(dG) aggregates (Fig. 3, brackets) are the immediate precursors that bind topoisomerase II and are then recovered by centrifugation.

The oligo(dG) aggregates contain between 20 and 30 copies of oligo(dG)₁₀ based upon electrophoretic mobility on polyacrylamide gels with markers and based upon sizing the aggregates by gel filtration on fast protein liquid chromatography. Since the aggregates can be isolated from a preparative polyacrylamide gel and do not revert to monomers under conditions used to assay topoisomerase II, we conclude that they are quite stable. As shown in Fig. 3B, the purified oligo(dG) aggregates are efficiently gel-shifted by topoisomerase II; in contrast, monomeric oligo(dG)₁₀ was not. A control shows that under these conditions, topoisomerase does not bind appreciably to oligo(dC)₁₀ (Fig. 3C). Furthermore, other single-stranded DNAs (denatured restriction fragments, salmon sperm DNA, and other oligonucleotides) did not bind or inhibit the topoisomerase as the oligo(dG) aggregates did (data not shown; see also Fig. 1). Mobility shifts were also eliminated when oligo(dG)₁₀ was hybridized to oligo(dC)₁₀ (Fig. 3B, lanes 1–5).

Chicken, Human, and Yeast Topoisomerases Are Inhibited by Oligo(dG)₁₀—We have also determined that DNA recognition sites for eukaryotic type I topoisomerases share considerable homology. For these reasons, it was of interest to test the generality of our results by comparing the human and yeast enzymes for inhibition by oligo(dG) aggregates. As shown in Fig. 4A, purified yeast (Saccharomyces cerevisiae) and purified human topoisomerases II were both inhibited by oligo(dG)₁₀. In contrast, six different restriction enzymes were

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not inhibited by 2.5 μM oligo(dG)₁₀ (data not shown).

As noted above, topoisomerase II binds preferentially to an aggregated form of oligo(dG) that contains ~25 oligo(dG)₁₀ molecules. We have not demonstrated in fact that this species is the inhibitor; however, this was tested in the experiment shown in Fig. 4B. In this experiment, oligo(G) aggregates were purified from a preparative polyacrylamide gel and tested for their inhibitory activity before and after boiling, which converts the aggregates to monomers. If oligo(dG)₁₀ is melted just prior to addition of topoisomerase, the inhibitory activity associated with the oligonucleotide is abolished (compare lanes 3 and 4). The data suggest that hydrogen bonding of oligo(dG)₁₀ is required for inhibition.

Relative Affinity of Topoisomerase II and Oligo(dG)₁₀ Aggregates—Topoisomerase II is like other DNA-binding proteins in that it binds to DNA in two modes, specifically and nonspecifically (34). In a typical mobility shift experiment, a combination of these two modes is measured; by including heterologous competitor DNAs, one favors detection of specific binding (32, 33). From mobility shift data, we determined the binding affinity of topoisomerase II for oligo(dG) aggregates relative to a duplex DNA. The difference in DNA binding affinity between oligo(dG)₁₀ aggregates and other DNAs must be large because of the inhibitory effect of the oligonucleotide on cleavage activity (Fig. 1). As shown in Fig. 3B, we were unable to induce mobility shifts with double-stranded oligo(dG·dC) under conditions where oligo(dG)₁₀ aggregates were efficiently shifted; however, oligo(dG·dC) is not cleaved by topoisomerase II (13, 21). We therefore compared binding of topoisomerase to oligo(dG) aggregates and pRYG, a duplex DNA fragment containing numerous topoisomerase II recognition sites (see Fig. 1). Binding reactions were performed in the presence of 500 ng of salmon sperm competitor DNA (Fig. 5A). A fragment from pRYG was selected that was approximately the same size as the oligo(dG) aggregates (300 base pairs); and from the specific activities of the labeled fragments, the concentrations of DNAs were adjusted to equality. Approximately 50% of the oligo(dG) aggregates were shifted at a topoisomerase concentration of 0.7 nM (Fig. 5A, lane 4). In contrast, we did not observe a mobility shift with the pRYG fragment at the maximum amount of topoisomerase tested (2.8 nM). The stringency of the binding was adjusted by decreasing the competitor DNA to 100 ng and repeating the analysis with more protein (Fig. 5B). Binding to the oligo(dG) aggregates is clearly favored over binding to pRYG. At the highest concentration of topoisomerase (8.4 nM) (Fig. 5B, lane 10), ~10% of pRYG was bound to protein. Densitometry of the autoradiographs was carried out to compare binding affinities of the two DNAs (percent of fragment band-shifted at a given enzyme concentration). For example, 8.4 nM topoisomerase was required to band shift a 0.1 nM concentration of the pRYG fragment (0.4 ng), whereas between 0.1 and 0.2 nM enzyme was sufficient to band shift this amount of oligo(dG) aggregates. From these data, we estimate that there is a 40-50-fold binding preference for oligo(dG) aggregates over pRYG.
was achieved by boiling and quick cooling just prior to use. pUC19 DNA was incubated with topoisomerase I\textsubscript{1} along with native and denatured oligo(dC),\textsubscript{10} as control.

of topoisomerase I\textsubscript{1} from different sources in the presence of enzymes.

above the equilibrium constant (calculated to be in the nanomolar range) (see Fig. 5C). Likewise, the above antitumor drugs did not affect oligo(dG)-topoisomerase interaction. This observation coupled with the fact that oligo(dG)-enzyme network formation can readily occur under conditions that do not support enzymatic activity (reactions lacking MgCl\textsubscript{2} and ATP) further indicate that network activity is distinct from DNA cleavage activity.

Monomeric oligo(dG)o\textsubscript{10} was neither recognized nor cleaved by topoisomerase II. Furthermore, inhibition of topoisomerase activity by purified oligo(dG) aggregates was reversed when the aggregates were heat-denatured prior to adding enzyme. Since the monomeric form of oligo(dG) was not present in the oligo(dG)\textsubscript{o10}-topoisomerase II networks that were retrieved by low speed centrifugation, we conclude that topoisomerase II binds only the aggregated forms of the oligonucleotide. We estimate that the binding energy for oligo(dG) aggregates is 40-fold greater than that for pRG, a DNA possessing multiple enzyme cleavage sites (13); thus, topoisomerase II binds strongly to the oligo(dG) aggregates.

Cooperative binding and aggregation of topoisomerase II with duplex DNA elements containing scaffold-associated regions (SARs) which are AT-rich sequences were recently reported by Adachi et al. (36). Their results are similar to ours in that the large DNA-topoisomerase II complexes form only with target sequences above a critical size (200–300 nucleotides). These investigators also showed that topoisomerase II activity was selectively directed toward the SAR-containing DNA fragments. The networks we detected with oligo(G)-containing sequences were inhibitory, and we have not been able to demonstrate activity of topoisomerase II in these complexes. From these collective results, we conclude that more than one type of sequence or DNA structure can induce high molecular weight network formation with purified topoisomerase II; however, the DNA sequence will dictate whether the complexed enzyme will retain activity (SARs) or become inactivated (guanine repeats). The finding that more than one type of DNA sequence (SARs and oligo(dG) conformers) can induce networks is remarkable and suggests an in vivo role for such activity. Topoisomerase II could facilitate or participate in chromosome condensation through such a mechanism; likewise, its location at the base of DNA loops (9, 10) places it at a strategic site to organize such events. Additional observations support this speculation. First, aggregation is most likely a feature common to all eukaryotic type II enzymes (Drosophila, yeast, human, and chicken), and conserved features generally indicate functional significance. In contrast, bacterial topoisomerase II (DNA gyrase) did not display inhibition by oligo(dG) aggregates.\textsuperscript{4} Second, an in vitro system for analysis of chromosome condensation has been reported using interphase nuclei and mitotic extracts from various species (including chicken); these experiments reveal that chromosome condensation correlates with the level of endogenous topoisomerase II activity in target interphase nuclei (37, 38). Like the oligo(dG) binding activity reported here, the chromosome condensation reaction is not species specific, again indicating conservation of function. Our observations are compatible with a structural role for topoisomerase II in chromosomes.

Why the oligo(dG) aggregates attract topoisomerase II into networks is unknown. It is conceivable that the oligo(dG) aggregate may simply be a polyanionic network that serves as an ionic exchanger of free and bound topoisomerases. Al-

\textsuperscript{4}I. K. Chung and M. T. Muller, unpublished data.
FIG. 5. Comparison of topoisomerase II binding to oligo(dG) aggregates and pRYG. A and B, aggregates of oligo(dG)$_{10}$ were purified from a preparative gel, and the specific activity was determined. The 300-base pair fragment from pRYG described in the Fig. 1 legend was also prepared. An equal mass of each fragment (4 ng) was reacted with purified topoisomerase II at the concentrations specified above each lane. Molarity concentrations were calculated based upon aggregated molecular weight by assuming that each oligo(G) aggregate (G-Agg.) contained 25 copies of monomeric oligo(dG)$_{10}$. The reactions contained between 1 and 2 nM concentrations of the oligo(dG) aggregate or the pRYG fragment. A, all reactions contained 500 ng of salmon sperm competitor DNA and the concentrations of topoisomerase II indicated. Lanes 1-6, oligo(dG) aggregates; lanes 7-12, pRYG fragment. B, all reactions contained 100 ng of salmon sperm competitor DNA and the concentrations of topoisomerase II indicated. Lanes 1-5, oligo(dG) aggregates; lanes 6-10, pRYG DNA. Reactions (20 µl) were incubated for 30 min at 30 °C, mixed with 2 µl of 50% glycerol, and loaded onto a 5% low ionic strength gel (see "Materials and Methods"). These two gels were run for different lengths of time (A, 3 h; B, 1.5 h). C, Western blot of topoisomerase II in the oligo(dG) complexes. Pellet and supernatant fractions described in the Fig. 3 legend were applied to a slot blot device containing a nitrocellulose filter. Lane 1, negative control; lane 2, topoisomerase II (8.4 nM) without oligonucleotides; lanes 3-5, 3.6 µM oligo(dG)$_{10}$ (300 ng) incubated with 2, 6, and 12 units of topoisomerase II, corresponding to 1.4, 4.2, and 8.4 nM enzyme, respectively; lanes 6 and 7, reactions incubated with 8.4 nM topoisomerase II in the presence of 3.6 µM oligo(dC)$_{10}$ (300 ng) and oligo(dA)$_{10}$, respectively. The top row contained pellet and the bottom row supernatant fractions. The blot was probed with a monoclonal antibody directed against avian topoisomerase II.
though we cannot rule this out, it seems unlikely since other DNA-binding proteins (restriction enzymes) are not affected by oligo(dG) aggregates. Additionally, single-stranded DNA of mixed sequence that also has a high density of negative charge does not mimic oligo(dG) aggregates. We have evidence that topoisomerase II cleaves tetrastrand parallel DNA (27, 30), but not the monostrand equivalent; therefore, non-B DNA structures can direct enzymatic activity. The oligo(dG) aggregates are recognized by topoisomerases. Additionally, single-stranded DNA (26, 31) to form larger cohering structures that are recognized by topoisomerases.

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