Intramolecular synopsis of duplex DNA by vaccinia topoisomerase

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Complexes formed by vaccinia topoisomerase I on plasmid DNA were visualized by electron microscopy. The enzyme formed intramolecular loop structures in which non-contiguous DNA segments were synapsed within filamentous protein stems. At high enzyme concentrations the DNA appeared to be zipped up within the protein filaments such that the duplex was folded back on itself. Formation of loops and filaments was also observed with an active site mutant, Topo-Phe274. Binding of Topo-Phe274 to relaxed DNA circles in solution introduced torsional strain, which, after relaxation by catalytic amounts of wild-type topoisomerase, resulted in acquisition of negative supercoils. We surmise that the topoisomerase–DNA complex is a plectonemic supercoil in which the two duplexes encompassed by the protein filaments are interwound in a right handed helix. We suggest that topoisomerase-mediated DNA synopsis plays a role in viral recombination and in packaging of the 200 kbp vaccinia genome during virus assembly.

Keywords: DNA loops/plectonemic supercoils/protein-mediated synopsis/recombination/type I topoisomerase

Introduction

The type IB DNA topoisomerase family includes eukaryotic topoisomerase I, a ubiquitous nuclear enzyme, and the topoisomerases encoded by vaccinia and other cytoplasmic poxviruses (Wang, 1996). These proteins relax supercoiled DNA via a common reaction mechanism that involves: (i) non-covalent binding of the enzyme to duplex DNA; (ii) cleavage of one strand of the duplex to form a covalent DNA–(3′-phosphotyrosyl)protein intermediate; (iii) rotation of the DNA duplex around the protein-induced nick; and (iv) religation by the enzyme–DNA intermediate to restore the original phosphodiester linkage. Most studies of the topoisomerase reaction have dealt with the transesterification steps. One of the first questions to be examined was the site specificity of covalent adduct formation. The cellular topoisomerases cleave with loose sequence preference at a four base motif, 5′-(A/T)(G/C)(A/T)T↓, immediately 5′ of the scissile phosphate (Edwards *et al.*, 1982; Been *et al.*, 1984; Jaxel *et al.*, 1991). In contrast, vaccinia topoisomerase cleaves

duplex DNA with high specificity at sites containing the target sequence 5′-(C/T)CCTT↓ (Shuman and Prescott, 1990; Shuman, 1991). Synthetic model substrates containing single cleavage sites have been used extensively to analyze covalent adduct formation and topoisomerase-catalyzed strand transfer reactions (Stevnsen *et al.*, 1989; Svejstrup *et al.*, 1990; Shuman, 1991, 1992a,b; Christiansen *et al.*, 1993; Christiansen and Westergaard, 1994).

Insights into the non-covalent interaction of type IB topoisomerases with DNA have come from studies of topoisomerase mutants containing phenylalanine in lieu of the active site tyrosine. Elimination of the nucleophilic hydroxyl precludes transesterification (Eng *et al.*, 1989; Lynn *et al.*, 1989; Shuman *et al.*, 1989a) and allows the binding step be studied in isolation (Shuman and Prescott, 1990; Shuman, 1991; Sekiguchi and Shuman, 1994a,b, 1995). The vaccinia active site mutant Topo-Phe274 binds non-covalently to CCCTT-containing duplexes with as little as 10 bp of DNA 3′ of the cleavage site and 10 bp 5′ of the cleavage site (Sekiguchi and Shuman, 1994a). Topo-Phe274 binds to duplex DNA lacking the consensus pentamer with only 7- to 10-fold lower affinity than to CCCTT-containing DNA. This has prompted the suggestion that topoisomerase initially binds to DNA non-specifically and then tracks along the nucleic acid until it encounters a consensus pentamer. Specific contacts between the enzyme and the CCCTT target are presumed to trigger transesterification to the scissile phosphate (Sekiguchi and Shuman, 1996a; Wittschieben and Shuman, 1997).

Madden *et al.* (1995) found that the Y723F active site mutant of human topoisomerase I binds preferentially to positively or negatively supercoiled plasmid DNA compared with relaxed DNA molecules. They proposed that topoisomerase I binds with high affinity at the nodes created by the crossing of two duplex helices. Zechiedrich and Osheroff (1990) had drawn similar conclusions based on their analysis via electron microscopy of the interaction of mammalian topoisomerase I with plasmid DNA. They found a clear preference for topoisomerase I binding at intramolecular crossovers of both circular and linear DNA molecules.

Here we employ electron microscopy to visualize complexes formed by vaccinia topoisomerase on plasmid DNA. We find that binding of either wild-type topoisomerase or the Topo-Phe274 mutant results in formation of intramolecular DNA loops with protein-bound nodes and stems. With increasing topoisomerase concentration the stems expand and the DNA is encompassed within filament-like structures. Biochemical evidence shows that binding of Topo-Phe274 to relaxed circular DNA introduces torsional strain. We suggest a model whereby protein–protein interactions between DNA-bound topoisomerase monomers ‘zip up’ the DNA into a filament with helical twist.

Fig. 1. Electron microscopy of topoisomerase–DNA complexes. (A) Linear pBluescript plasmid DNA. (B) Topoisomerase–DNA complexes formed in a binding reaction mixture containing 17 nM linear DNA and 500 nM wild-type vaccinia topoisomerase.

Results

Visualization of topoisomerase–DNA complexes by electron microscopy

Purified vaccinia topoisomerase was incubated *in vitro* with pBluescript plasmid DNA that had been linearized with *Hind*III. The topoisomerase–DNA complexes were fixed with glutaraldehyde and the fixative and unbound protein were removed by gel filtration. The isolated complexes were mounted on carbon film-coated grids and shadowed with tungsten in parallel with a free DNA control sample. Figure 1A shows an electron micrograph of linear DNA alone. The molecules were of uniform size and were well spread on the grid, although occasional molecules with single crossovers were detected in the free DNA population. Figure 1B shows topoisomerase–DNA complexes that formed in binding reactions containing 17 nM plasmid and 500 nM topoisomerase. All six DNA molecules in the field were decorated with bound protein, which was visible either as single ‘blobs’ on the DNA or

Fig. 2. Topoisomerase-mediated DNA synopsis.

as filament-like clusters. The cluster of bound protein were most often encountered at the base of protein-constrained DNA loops, which were present in every DNA. Topoisomerase-induced DNA looping was exclusively intramolecular and more than one loop was typically present per DNA molecule at this ratio (30:1) of protein to DNA.

The key features of the topoisomerase–DNA complex are exemplified by the molecule shown in Figure 2, in which the ends of the linear DNA are apparent and the path of the duplex is easily discerned. The molecule contains a single node (N) with a protein blob at the crossover point. Synopsis of non-contiguous DNA segments by linear clusters of topoisomerase results in formation of two loops (L) demarcated by topoisomerase stems (S). In the molecule shown in Figure 2 the stems clearly encompass two duplex segments in an inverted orientation. Topoisomerase-mediated synopsis in direct orientation (such that the entering and leaving segments of the loop were on opposite sides of the stem) was also observed, albeit less frequently than loop formation between inverted segments.

Evolution of the complexes as a function of topoisomerase concentration

We showed previously by DNase I footprinting that a single molecule of topoisomerase protected ~25 bp of duplex DNA (Shuman, 1991). Thus, if the topoisomerase bound quantitatively to the plasmid at the 30:1 molar ratio in the reaction mixture (500 nM topoisomerase and 17 nM DNA), we estimate that the bound protein should cover less than half of the linear DNA molecule and, indeed, this is what we observed (Figures 1 and 2). The linear clustering of bound protein suggested that binding might be cooperative. Consistent with this idea, we found that when the topoisomerase concentration was decreased to 200 nM the linear DNA was decorated with several discrete protein blobs that were often associated with a DNA crossover (Figure 3D). Virtually no clusters or stem structures were seen at low protein concentrations.

Increasing the topoisomerase concentration to 1 μ M resulted in formation of filamentous structures with linear

Fig. 3. Evolution of topoisomerase–DNA complexes as a function of topoisomerase concentration. Binding reaction mixtures contained 17 nM linear DNA and wild-type topoisomerase at 1 μ M (A) or 200 nM (D) concentration or Topo-Phe274 at 1 μ M (B) or 500 nM (C) concentration.

Fig. 4. Visualization of topoisomerase bound to circular plasmid DNA. Reaction mixtures contained 5 nM pUC18 plasmid DNA and 500 nM wild-type topoisomerase (A–G). (H) A control mixture contained 5 nM pUC18 DNA without topoisomerase.

or branched morphology (Figure 3A). The contour length of these complexes was clearly less than that of free linear DNA or the sparsely decorated complexes formed at low enzyme concentration (compare Figure 3A and D). The thickness of these structures is similar to that of the stems seen at intermediate concentrations of topoisomerase (Figure 3C). We infer that the structures in Figure 2A evolve by propagation of the synapsed stems such that they encompass most or all of the DNA molecule.

Visualization of complexes formed by Topo-Phe274

Replacement of the vaccinia topoisomerase active site Tyr274 by Phe abrogates covalent adduct formation, but has little impact on binding in solution to linear plasmid DNA or to synthetic 24–60 bp duplex ligands (Shuman and Prescott, 1990; Sekiguchi and Shuman, 1994a). To examine whether the formation of microscopic DNA loops observed for wild-type topoisomerase was contingent on covalent adduct formation, we visualized the complexes formed by purified Topo-Phe274 on linear plasmid DNA. At 500 nM Topo-Phe274 we observed structures similar to those detected with wild-type topoisomerase, i.e. single blobs, linear clusters and protein-induced loops (Figure 3C). At 2-fold higher concentrations of Topo-Phe274 the linear DNA was condensed into filaments indistinguishable from those formed by the wild-type protein (Figure 3B). We surmise that protein-mediated synapsis was not dependent on covalent adduct formation.

Binding of topoisomerase to circular DNA

Wild-type topoisomerase (500 nM) was reacted in solution with supercoiled pUC18 plasmid DNA (5 nM). Microscopic analysis of the plasmid DNA alone showed that the closed circular molecules had the morphology expected of plectonemic supercoils, i.e. they appeared as condensed linear or branched molecules in which the two duplexes were wound tightly about each other (Figure 4H). Incubation with topoisomerase relaxed the supercoils to completion, as gauged by agarose gel electrophoresis of the deproteinized reaction products (not shown). Microscopic analysis of glutaraldehyde-fixed reaction products is shown in Figure 4A–G. The relaxed molecules now appeared dumb-bell shaped. Clusters of topoisomerase were observed at the bases of stem-loop structures arising by protein-mediated synapsis of non-contiguous segments of the relaxed circles. The thickness of the protein-bound stem segments was clearly greater than that of the plectonemically interwound duplexes (Figure 4). Both wild-type topoisomerase and Topo-Phe274 formed similar stem-loop complexes on circular plasmid DNA molecules that had been relaxed to completion prior to the binding reaction (not shown).

Binding of Topo-Phe274 to relaxed closed circular DNA introduces torsional strain

The microscopic images suggested that vaccinia topoisomerase forms higher order structures in which the DNA is compacted. Biochemical evidence that topoisomerase

Fig. 5. Topoisomerase-induced DNA supercoiling. Binding reaction mixtures (20 μ l) containing 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 2.5 mM EDTA, 0.3 μ g supercoiled (SC) or relaxed (R) pUC19 DNA and 100, 200 or 400 ng Topo-Phe274 (proceeding from left to right in each titration series) were incubated for 15 min at 37°C. Where indicated (+) the mixtures were then supplemented with 5 ng wild-type topoisomerase and incubation was continued for another 15 min at 37°C. The reactions were halted by adding SDS to 0.2% and EDTA to 20 mM. The samples were electrophoresed through a 1% agarose horizontal slab gel in TG buffer (50 mM Tris base, 160 mM glycine). The gel was stained in a 0.5 μ g/ml solution of ethidium bromide, destained in water and then photographed under short wavelength UV illumination.

binding alters DNA structure was gleaned from the experiment shown in Figure 5. Here we incubated relaxed pUC19 plasmid DNA (0.17 pmol) with a molar excess of Topo-Phe274 (3, 6 or 12 pmol). The protein-DNA complexes were then treated with a small amount of wild-type topoisomerase (0.15 pmol), which was sufficient to relax 0.3 μ g supercoiled plasmid DNA to completion (Figure 5, lanes SC). The samples were then deproteinized and the topological state of the plasmid was assessed by agarose gel electrophoresis. We observed a concentration-dependent acquisition of superhelical turns upon stoichiometric binding by Topo-Phe274 that depended completely on relaxation of the protein-bound DNA by catalytic amounts of wild-type topoisomerase (Figure 5). Eight or more superhelical turns were introduced per DNA molecule at a topoisomerase:DNA ratio of 70:1 (Figure 5).

The sign of the superhelical reaction products was gauged by subjecting them to 2-dimensional agarose gel electrophoresis (Figure 6; Giaever and Wang, 1988). This technique permits resolution of unit linking number changes along an arc-shaped 2-dimensional distribution of topoisomers. The range of linking numbers resolved is dictated by the concentrations of chloroquine used in each dimension. In the method used here negatively supercoiled pUC19 DNA ran at the lower left end of the topoisomer arc (Figure 6A), whereas relaxed circular DNA ran at the lower right end of the arc (Figure 6C). (Note that the single species of nicked circular pUC19 DNA provides a fixed point of reference for comparison of the samples.) Positively supercoiled DNA runs even further clockwise along the arc than does relaxed DNA under these conditions (Giaever and Wang, 1988). The supercoiled Topo-Phe274 binding reaction products migrated at intermediate positions along the arc (Figure 6B). The product distribution was shifted counterclockwise relative to relaxed circular DNA, hence these molecules were negatively supercoiled. In the specific case shown in Figure 6 the binding of 8 pmol Topo-Phe274 to 0.17 pmol relaxed pUC19 introduced between three and 12 negative turns into the plasmid DNA.

Fig. 6. Two-dimensional gel electrophoresis of supercoiled reaction products. A reaction mixture containing 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 2.5 mM EDTA, 0.3 μ g relaxed pUC19 DNA and 300 ng Topo-Phe274 was incubated for 15 min at 37°C, then reacted for 15 min with 5 ng wild-type topoisomerase. The reaction product (B) was applied to a 1% agarose gel in TBE buffer (90 mM Tris-borate, 2.5 mM EDTA) containing 0.8 μ g/ml chloroquine. Electrophoresis in the first dimension was for 16 h at 47 V. The gel was then soaked for 1 h in TBE containing 4 μ g/ml chloroquine. The gel was rotated through 90° and then electrophoresed in the second dimension in TBE buffer with 4 μ g/ml chloroquine for 16 h at 47 V. The gel was stained for 1 h in a 1 μ g/ml solution of ethidium bromide, then destained in water and photographed under short wavelength UV illumination. Control reactions mixtures containing negatively supercoiled pUC19 DNA (A) or relaxed pUC19 DNA (C) were analyzed in parallel.

Discussion

Zechiedrich and Osheroff (1990) were the first to visualize the preferential binding of eukaryotic type I topoisomerase at intramolecular crossovers. The present study of vaccinia virus topoisomerase confirms their findings regarding the formation of DNA loops by type IB enzymes. We suggest that the loops arise through protein-protein-mediated DNA synopsis rather than through bivalent DNA binding of a single topoisomerase monomer.

Zechiedrich and Osheroff focused on DNA molecules containing what appeared to be a single protein complex at DNA nodes. These structures were prevalent at a 3:1 molar ratio of calf thymus topoisomerase I to plasmid DNA. As pointed out by Wang (1996), an inherent problem in microscopic analysis is that one cannot gauge the number of topoisomerase monomers within the seemingly discrete protein 'blobs'. Therefore, it is unclear if the protein-bound nodes are formed because one topoisomerase monomer simultaneously engages two DNA duplexes or because two (or more) DNA-bound topoisomerases interact with each other. Note that there is no mechanistic imperative for two DNA binding sites on type IB topoisomerases, as there is in the case of type II topoisomerases.

We analyzed the appearance of topoisomerase-DNA complexes formed at topoisomerase:DNA ratios of 12:1, 30:1 and 60:1. We found that vaccinia topoisomerase formed higher-order structures in which non-contiguous duplex segments were synapsed within filamentous protein stems. At high enzyme concentrations the DNA appeared to be zipped up within the protein filaments such that the duplex was folded back on itself.

Biochemical evidence, together with the electron microscopic analysis, suggests that the topoisomerase-DNA complex takes the form of a plectonemic supercoil. Binding of Topo-Phe274 to relaxed DNA circles introduces torsional strain, which, after relaxation by catalytic amounts of wild-type topoisomerase, results in the acquisition of negative supercoils. Explanations for this result include: (i) bound topoisomerase unwinds the DNA duplex; (ii) DNA is wrapped around the topoisomerase to form solenoidal supercoils; (iii) the synapsed duplex

segments form plectonemic supercoils. We showed previously that covalent binding of vaccinia topoisomerase to DNA results in unpairing of the thymine base immediately 5' of the scissile phosphate (Sekiguchi and Shuman, 1996b). However, non-covalent binding by Topo-Phe274 does not elicit any detectable local alteration of DNA structure. Therefore, the supercoiling induced by Topo-Phe274 bound to relaxed plasmid DNA was probably not caused by duplex unwinding. The microscopic findings are most consistent with plectonemic supercoiling. Plectonemic supercoils are made by doubling back of the helical axis, which necessarily brings together distant sites in duplex DNA (Cozzarelli *et al.*, 1990). This is precisely the case in the stem-loop structures formed by topoisomerase on plasmid DNA. Branching is a natural property of plectonemic supercoils, but not of solenoidal supercoiling (Cozzarelli *et al.*, 1990). Branched topoisomerase-DNA complexes are observed microscopically.

We surmise that the two duplex segments within the topoisomerase filaments are interwound in a right handed helix. We presume that the structure of the filaments is dictated by protein-protein interactions between topoisomerase monomers arrayed along each of the duplexes within the synapse. Given that vaccinia topoisomerase is a monomer in solution (Shaffer and Traktman, 1987; Shuman *et al.*, 1988), it is likely that the higher order protein-protein interactions are contingent on topoisomerase binding to the plasmid DNA.

Vaccinia topoisomerase is essential for virus replication (Shuman *et al.*, 1989b). Yet, the role of the enzyme in viral nucleic acid transactions *in vivo* is unknown, because viruses bearing conditional mutations of the topoisomerase have not been isolated. The topoisomerase is a component of the nucleoprotein core of the vaccinia virus particle (Bauer *et al.*, 1977), which contains a 192 kbp duplex DNA genome with hairpin telomeres and inverted terminal repeats (Moss, 1996). The virus core also contains a complete array of enzymes involved in transcription, processing and extrusion of vaccinia early mRNAs (Moss, 1996). The component enzymes have been purified and characterized. However, we know next to nothing about the physical state of the DNA genome within the vaccinia particle.

The unit length DNA genome of the virion is derived from concatameric replication intermediates by a conservative recombination reaction within the inverted repeats flanking the concatemer junction: this resolves the concatemers and generates the imperfectly paired hairpin telomeres (Moss, 1996). Vaccinia topoisomerase, which resolves four-way Holliday junctions *in vitro* (Sekiguchi *et al.*, 1996), is a good candidate for catalyzing the strand exchange reaction. The portion of the viral genome flanking the telomeres is rich in topoisomerase binding sites. We hypothesize that by synapsing distant segments of the genome, including perhaps the subtelomeric repeats, the topoisomerase assists in packaging the DNA into progeny virus particles. The topoisomerase may also establish functional domain boundaries within the virion core, e.g. by isolating individual early transcription units as looped out regions between topoisomerase-bound stems. The intervening DNA is likely to be associated with two highly abundant vaccinia DNA binding proteins of 25 and 11 kDa that constitute 6.5 and 11% of the virion protein

mass respectively (Kao *et al.*, 1981; Yang and Bauer, 1988). The 25 and 11 kDa proteins bind to duplex DNA and single-stranded DNA non-specifically and reportedly do not alter DNA topology when bound to closed circular plasmids (Kao *et al.*, 1981; Yang and Bauer, 1988). At present the topoisomerase is the only poxvirus protein that has the demonstrated capacity to form DNA loops or to condense duplex DNA into an ordered structure.

Materials and methods

Vaccinia topoisomerase

Wild-type vaccinia topoisomerase and the Topo-Phe274 mutant were expressed in *Escherichia coli* BL21 by infection with bacteriophage λ CE6 and purified from soluble lysates by sequential phosphocellulose and SP5PW ion exchange chromatography. The enzyme preparations used in this study were homogeneous with respect to the topoisomerase polypeptide as determined by SDS-PAGE (Sekiguchi and Shuman, 1995). Protein concentration was determined using the BioRad dye reagent with bovine serum albumin as standard.

Electron microscopy

Binding reaction mixtures (100 μ l) containing 20 mM HEPES-NaOH, pH 7.5, 3.4 μ g *Hind*III-cut pBluescript DNA (Stratagene) and vaccinia topoisomerase were incubated at 25°C for 5 min. The samples were fixed by adding 10 μ l 6% glutaraldehyde. Unbound protein was removed by gel filtration through a 2 ml BioGel A15M column equilibrated in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The excluded fractions were collected dropwise. Protein-DNA complexes were mounted onto freshly glow discharged carbon-coated grids, then spread in spermidine and dehydrated serially in 20, 50, 70 and 100% ethanol (Griffith and Formosa, 1985; Bear *et al.*, 1988). The grids were shadowed with a thin coating of tungsten and imaged at 50 000-fold magnification in a Hitachi H600-II transmission electron microscope.

Preparation of relaxed closed circular DNA

A reaction mixture (100 μ l) containing 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 5 mM MgCl₂, 25 μ g supercoiled pUC19 DNA and 0.5 μ g vaccinia topoisomerase was incubated for 30 min at 37°C. The reaction was halted by addition of SDS to 0.2% and EDTA to 20 mM. The products were digested with 30 mg proteinase K for 2 h at 37°C, then extracted twice with phenol and once with chloroform. DNA was recovered by ethanol precipitation and resuspended in 100 μ l 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

Acknowledgements

We thank Dr Stephen Jett for valuable technical advice. This work was supported by NIH grant GM46330 (S.S.), NSF grants MCB-9723969 and MCB-9408231 (D.G.B.) and dedicated health research funds of the University of New Mexico School of Medicine.

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Received on July 1, 1997; revised on August 11, 1997