Kinetoplast-associated DNA topoisomerase in *Crithidia fasciculata*: crosslinking of mitochondrial topoisomerase II to both minicircles and maxicircles in cells treated with the topoisomerase inhibitor VP16

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ABSTRACT

The mitochondrial DNA of the trypanosomatid *Crithidia fasciculata* consists of thousands of copies of a 2.5 kb minicircle and a small number of 37kb maxicircles catenated into a single enormous network. Treatment of *C. fasciculata* with the type II DNA topoisomerase inhibitor VP16 produces cleavable complexes of a type II DNA topoisomerase with both minicircles and maxicircles. A combined Southern and Western blot analysis of the cleaved DNA species released from the network by SDS treatment has identified topoIImt, the kinetoplast-associated topoisomerase, in covalent complexes with linear forms of minicircle and maxicircle DNAs. These results directly implicate topoIImt in the topological reactions required for the duplication of the kinetoplast network.

INTRODUCTION

The mitochondrial DNA in kinetoplastid protozoa consists of two circular DNA species, minicircles and maxicircles. These circular DNAs are present in the single mitochondrion of these organisms as a highly catenated network of topologically interlocked circles (1). The maxicircles encode mitochondrial gene products and their expression has recently been shown to involve a novel form of RNA processing termed RNA editing (2, 3). In this process U residues are posttranscriptionally inserted or deleted at multiple precise sites in several mRNA species under the apparent direction of small RNA species termed guide RNAs. Such guide RNAs have been shown to be encoded in maxicircles and in some cases also in minicircles (4, 5). No protein products have been found to be encoded in minicircles.

Networks of the trypanosomatid *Crithidia fasciculata* contain 5,000–10,000 minicircles (2.5 kb) and 20–40 maxicircles (37 kb). Replication of this highly catenated structure involves the release of individual minicircles from the network followed by

their replication and subsequent reattachment to the growing network (6). Each of these steps in minicircle replication likely involves DNA topoisomerases. Replication of the maxicircles and the eventual cleavage of the double size network to yield two daughter networks may also require the action of DNA topoisomerases (7).

In *C. fasciculata* a type II topoisomerase was found to be enriched in kinetoplast extracts and was immunolocalized to the kinetoplast (8, 9). This enzyme (topoIImt) is an ATP dependent topoisomerase with properties similar to those of nuclear type II topoisomerases of higher eukaryotes. TopoIImt is inhibited by both intercalating and nonintercalating inhibitors of mammalian type II topoisomerases and forms a cleavable complex with its DNA substrate. The DNA in these complexes undergoes double strand cleavage upon the addition of SDS resulting in the covalent attachment of topoIImt to the 5' termini of the DNA (8). The catalytic properties of topoIImt and its localization to the kinetoplast suggest that topoIImt may be involved in one or more of the topological interconversions required for the duplication of the kinetoplast network.

Type II topoisomerases have been identified in other trypanosomes (10, 11) and a second type II enzyme, distinct from topoIImt, has been partially purified from *Crithidia* (12, 13). It is unknown whether any of these enzymes are present in kinetoplasts. However, the observation of linear minicircle DNA covalently complexed to protein in sodium dodecyl sulfate lysates of *Trypanosoma equiperdum* cells treated with type II topoisomerase inhibitors indicates the presence of a type II topoisomerase in the kinetoplast of this trypanosome (14). Here we demonstrate the production of both minicircle and maxicircle DNA protein complexes in *C. fasciculata* cells treated with the topoisomerase inhibitor VP16 and show that the protein moiety in these complexes reacts specifically with monoclonal antibodies against topoIImt. These results demonstrate the direct action of topoIImt on both the maxicircle and minicircle components of kinetoplast DNA.

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MATERIALS AND METHODS

Drug treatment of permeabilized cells

C. fasciculata Cf-C1 was grown at 28°C in BHI medium (Difco) containing 10 µg/ml hemin and 100 µg/ml streptomycin to a density of $2-4 \times 10^7$ cells/ml. Cells were harvested and resuspended in fresh medium at 2×10^8 cells/ml. VP16 was added at various concentrations followed by the immediate addition of digitonin to a concentration of 0.001%. At 4 min. after drug addition the cells were harvested and resuspended in 0.02M Tris pH 8, 2mM EDTA and 0.2M NaCl at a density of 7×10^8 cells/ml.

Cell lysis and gel electrophoresis

The cells were immediately lysed by the addition of an equal volume of 40mM Tris pH 8, 75mM EDTA, 1% SDS. RNase A was added to 250 µg/ml and incubated at 37°C for 40 min. The lysates were centrifuged in 0.2ml aliquots through a 3ml bed of Sephacryl S400 SF equilibrated in 30mM Tris pH 8, 38.5mM EDTA, 0.1M NaCl, 0.5% SDS and then concentrated 25-fold using an Amicon 30 microconcentrator. Samples for electrophoresis on 1% agarose-SDS gels (15) contained 8% glycerol and 0.01% bromophenol blue and represented 2.5×10^8 cell equivalents. Electrophoresis buffer contained 80mM Tris pH 7.5, 5 mM Na acetate, 1mM EDTA and 0.03% SDS. Agarose gels (7.5 cm in length) in electrophoresis buffer were run at 9 volts with buffer replacement every twelve hours.

Transfer of complexes to nitrocellulose

Gels were washed in distilled water for 3–5 hr. followed by 15 min in 0.25M HCl, 15 min (twice) in 1.5M NaCl, 0.5M NaOH and 15 min (twice) in 1.5M NaCl, 0.5M Tris pH 7.5. The complexes were transferred to nitrocellulose by vacuum blotting in $20 \times$ SSC. The membranes were washed with TBS (25mM Tris pH 8.0, 137mM NaCl, 2.7mM KCl) after UV crosslinking of the DNA to the filters and then blocked overnight in 3% powdered milk, 5% goat serum, TBS, 0.05% Tween 20.

Immunological analysis

Antibodies used for immunodetection of blotted DNA-protein complexes were rabbit anti-topoIImt (9) at 1:500 dilution or anti-topoIImt monoclonal antibodies 2E7, 1G11 and 1H2 as 1:100 dilutions of cell culture supernatants (16). A control monoclonal antibody 45.6 of undefined specificity was used at 10 µg/ml. A rabbit anti-mouse IgG (Pierce) was used at 10 µg/ml as bridging antibody with the monoclonals. Immune complexes were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG (Boehringer-Mannheim).

DNA probes

Specific hybridization probes were prepared using the following DNA's: minicircles—half-length minicircle fragment released from M13 CFK120 (17) by *EcoRI* and *HindIII* digestion, maxicircles—2 kb maxicircle fragment released from pCFK16 (18) by *EcoRI* and *HindIII* digestion, nuclear DNA—7.9 kb *EcoRI* fragment of *C. fasciculata* genomic DNA from the $\lambda 7$ clone containing the *Cfa TOP2* gene (16). Individual restriction fragments were gel purified and labeled with [α - 32 P] dATP to a specific activity of $1-2 \times 10^9$ cpm/mg using the Stratagene random primer kit according to the manufacturer's instructions.

Hybridization

After immunological screening, nitrocellulose filters were washed three times (1 min each) with 0.1M glycine pH 2.8 followed by a 5 min wash with 0.1M Tris pH 9.0, equilibration in $6 \times$ SSPE and prehybridization for 2.5 hr at 42°C in a solution containing $6 \times$ SSPE, 0.5% SDS, 100 µg/ml sonicated salmon sperm DNA, 50 µg/ml yeast t-RNA, 50% formamide and $5 \times$ Denhardt's solution (19). Membranes were hybridized for 21 hr. at 42°C in prehybridization solution containing 250 ng of labeled probe. Filters were washed to a final stringency of $0.1 \times$ SSPE, 0.1% SDS at 65°C. Autoradiography was at -70°C using Kodak XAR-5 film and a Dupont Quanta III intensifying screen. Exposure times of 0.5–1.5 hr. were required for minicircle and maxicircle probes. Exposure for 18 hr. was required for the nuclear DNA probe. Stripping of radioactivity from filters was accomplished by boiling the filter four times for 5 min in $0.1 \times$ SSPE, 0.1% SDS. Filters were exposed to autoradiography to confirm the complete removal of all radioactivity prior to hybridizing with a different radioactive probe.

RESULTS

The covalent attachment of type II topoisomerases to their target DNA provides a means of establishing the identity of a topoisomerase that acts upon the DNA contained within an organelle. The recent availability of monoclonal antibodies against topoIImt, the kinetoplast associated topoisomerase of *C. fasciculata*, has permitted us to identify topoIImt in complexes with kinetoplast minicircle DNA following treatment of *C. fasciculata* with the drug VP16. The lack of sensitivity of *C. fasciculata* to topoisomerase inhibitors, including VP16, was overcome by permeabilization of the cells with 0.001% digitonin. Under the conditions described in Materials and Methods the cells retain their structural integrity and viability.

DNA-protein complexes produced in permeabilized cells treated with VP16 were electrophoresed thru an agarose gel and then transferred to a nitrocellulose membrane for both nucleic acid hybridization and immunological probing. As shown in Fig. 1 (lanes b–e) treatment of cells with VP16 leads to the production of a major DNA-protein complex in SDS lysates which co-migrates on an agarose gel with minicircle DNA-protein complexes generated *in vitro* by cleavage of kinetoplast DNA with purified topoIImt in the presence of VP16 (lane f). Immunoprobings of the blot with the monoclonal antibody 2E7 shows a strong signal associated with the minicircle complexes that increases with increasing drug concentration. The DNA contained in the *in vivo* complexes hybridizes with minicircle DNA. Minicircle DNA species migrating just ahead of the minicircle-topoIImt complexes in lanes b–e co-migrate with free minicircles released from kinetoplast DNA networks treated with topoIImt (lane a) and are possibly released from the networks as an indirect consequence of the cleavage of adjoining minicircles. Treatment of the complexes with proteinase K prior to electrophoresis results in the co-migration of the cleaved DNA with linear minicircle DNA produced by restriction enzyme cleavage of kinetoplast networks (data not shown).

In addition to the minicircle-protein complexes, a minor species that just enters the gel can be seen in the immunoblot in Fig. 1 (lanes c–e). This species also increases in amount with increasing drug concentrations. The DNA component present in this complex hybridizes with a maxicircle probe indicating that this

Fig. 1. *In vivo* crosslinking of topoIImt to *C. fasciculata* kinetoplast minicircle DNA. *C. fasciculata* cells grown in BHI medium were treated with VP16 for 4 minutes at various concentrations in the presence of 0.001% digitonin. DNA-protein complexes were prepared as described in Materials and Methods, electrophoresed through 1% agarose-SDS gels for 38 hr, transferred to nitrocellulose membranes and probed with the anti-topoIImt monoclonal antibody mAb 2E7 followed by nucleic acid hybridization with minicircle DNA. Following autoradiographic exposure, the nitrocellulose filter was stripped and probed with maxicircle DNA. Lanes b–e contain complexes from cells treated with increasing concentrations of VP16 (lane b, 0; lane c, 100 μ M; lane d, 333 μ M; lane e, 1 mM). Marker lanes—lane a, free minicircle DNA prepared from kDNA by treatment with purified topoIImt *in vitro*; lane f, kDNA treated with topoIImt in the presence of 1 mM VP16; lane g, linear minicircle DNA produced by XhoI digestion of kDNA. Migration positions of linear minicircle DNA (closed triangle), linear minicircle-topoIImt complexes (open triangle) and maxicircle-topoIImt complexes (arrow) are indicated.

species represents maxicircle DNA complexed to topoIImt and will be examined further in Fig. 3. The material remaining at the top of the gel in lane f and which hybridizes with minicircle DNA represents network DNA which was not decatenated in the *in vitro* reaction (lane f).

Similar results were obtained using monoclonal antibodies 1G11 and 1H2 and also with rabbit polyclonal antibodies. In the blots shown in Fig. 2 free topoIImt migrates as a smear between

Fig. 2. Immunological detection of minicircle-topoIImt complexes with polyclonal and monoclonal antibodies. DNA-protein complexes were prepared from cells treated with 1mM VP16 as in Fig. 1, electrophoresed through 1% agarose-SDS gels for 17.5 hr, transferred to nitrocellulose membranes and probed with rabbit antiserum against topoIImt (RαTII) or with mouse monoclonal antibodies 1G11, 1H2, or 2E7 against topoIImt or the mouse monoclonal antibody 45.6. Lane a, complexes produced *in vivo*; lane b, complexes produced *in vitro*; lane c, purified topoIImt. Migration positions are indicated as in Fig. 1.

Fig. 3. Crosslinking of topoIImt to both maxicircle and minicircle DNA in drug-treated cells. DNA-protein complexes were prepared from untreated *C. fasciculata* cells (lane a) or from cells treated with 1 mM VP16 (lane b). Complexes were electrophoresed through a 0.6% agarose-SDS gel for 38 hr, transferred to nitrocellulose and probed with a pool of monoclonal antibodies (1G11, 1H2, and 2E7) followed by nucleic acid hybridization with *C. fasciculata* maxicircle DNA or nuclear DNA. Marker lane—lane c, linear maxicircle DNA produced by PstI digestion of kDNA.

the position of the DNA-protein complexes and that of purified topoIImt.

Further resolution of the maxicircle-topoIImt complexes was obtained by electrophoresis on a 0.6% agarose gel as shown in Fig. 3. In addition to the abundant minicircle-topoIImt complexes, a lower mobility species detected by immunoprob- ing with pooled monoclonal antibodies against topoIImt hybridizes specifically with maxicircle DNA and is only observed in cells treated with VP16 (lane b). Because of the large size (37kb) of the maxicircle DNA, the maxicircle-topoIImt complexes migrate at the same position as unit-length linear maxicircles released from networks by cleavage with PstI (lane c). Nuclear DNA migrates more slowly in the gel and was identified by hybridization to a probe for CfaTOP2, the nuclear gene encoding topoIImt.

The immunological detection of topoIImt in association with both minicircle and maxicircle components of kinetoplast DNA but not nuclear DNA is consistent with the immunolocalization of topoIImt to the kinetoplast using polyclonal antibodies (9) and the monoclonal antibodies described here (data not shown).

DISCUSSION

The purification and analysis of mitochondrial replication proteins is complicated by the low abundance of these proteins and by the similarity of their catalytic properties to those of their nuclear counterparts. Substantial progress has been made recently in defining some of the genes and proteins involved in the replication of mitochondrial DNA (20–22). So far, most mitochondrial replication proteins have distinctive biochemical properties that distinguish them from their nuclear counterparts. In yeast, the genes encoding the mitochondrial RNA and DNA polymerases have been sequenced and found to be more closely related to bacteriophage polymerases than to the corresponding nuclear enzymes (23, 24).

Because of the presence of thousands of closed circular DNA molecules catenated into a single network structure in trypanosomes, it was expected that these organisms would be a rich source of organelle-associated type II DNA topoisomerase. Replication of kinetoplast minicircle DNA has been shown to occur free of the kDNA network by a mechanism in which individual minicircles are decatenated from the network, replicated and then rejoined to the perimeter of the network structure (6, 7). Eukaryotic type II DNA topoisomerases catalyze an ATP-dependent double-strand passage reaction and are capable of catenating and decatenating circular DNA molecules. Type II topoisomerases must therefore play an essential role in the duplication of kinetoplast DNA.

In order to provide more direct evidence for the action of topoIImt, the *C. fasciculata* kinetoplast-associated topoisomerase, on kinetoplast DNA, we have exploited the ability of type II DNA topoisomerase inhibitors to trap type II enzymes in cleavable complexes with their DNA substrates (25, 26). Drug-induced cleavage of kinetoplast minicircles was observed in previous studies with *Trypanosoma equiperdum* treated with VP16 but the enzyme responsible for mediating the cleavage reaction has not yet been identified (14). The detection of topoIImt covalently bound to linear forms of both minicircles and maxicircles in detergent lysates of drug-treated *Crithidia* indicates that topoIImt is specifically responsible for cleavage of both components of the kinetoplast DNA structure and must act upon both DNA species *in vivo*. These results provide direct support for a role of topoIImt in kDNA duplication but do not exclude the possible involvement of one or more additional topoisomerases in the overall process. For example, the enzyme responsible for releasing unreplicated minicircles from the kDNA network might be distinct from the enzyme that rejoins newly replicated minicircles to the growing network.

Analysis of the structure of partially replicated kDNA networks supports a model in which newly replicated minicircles are reattached to the perimeter of the network while unreplicated minicircles are released from the central (unreplicated) region of the growing network (7). It is not yet known whether topoIImt is involved in one or both of these steps in the duplication of individual minicircles or what role it may play in the segregation of daughter networks. The recent cloning of CfaTOP2 (16), the gene encoding topoIImt, and the development of methods for gene

replacement in parasitic protozoa (27) provide a means for further elucidation of the precise role of topoIImt in kinetoplast duplication.

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