Type II Topoisomerase activities are required in both G₁ and G₂/M phases of the dinoflagellate cell cycle

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SUMMARY

Dinoflagellates genomes are large (up to 200 pg) and are encoded in histoneless chromosomes that are quasi-permanently condensed and circular. This unique combination of chromosomal characteristics presents additional topological and cell cycle control problems for a eukaryotic cell, potentially requiring novel topoisomerase activities. The heterotrophic dinoflagellate *Cryptocodinium cohnii* was used in this study. The topoisomerase II activities throughout its cell cycle were investigated by DNA flow cytometry following enzyme deactivation. Fluorescence microscopy was also used for studying the chromosome morphology of treated cells.

Two classes of topoisomerase II inhibitors were applied in our study, both of which caused G1 delay as well as G2/M arrest in the *C. cohnii* cell cycle. At high doses, the topoisomerase poisons amsacrine and ellipticine induced DNA fragmentation in *C. cohnii* cells. Topoisomerase II activities, as measured by the ability to decatenate kinetoplastid DNA (kDNA), are normally detected throughout the cell cycle in *C. cohnii*. Our results suggest that the requirement of type II topoisomerase activities during the G1 phase of the cell cycle may relate to the unwinding of quasi-permanently condensed chromosomes for the purpose of transcription.

Keywords: topoisomerase, chromosomes, dinoflagellate, cell cycle

INTRODUCTION

The genomes of the dinoflagellates are large and circular, as well as quasi-permanently condensed (Soyer-Gobillard and Moreau, 2000), presenting very unusual chromosome topology that probably requires novel topoisomerase activities. Dinoflagellate chromosomes are also devoid of nucleosome structures (Bodansky et al., 1979) and histone proteins (Rizzo, 1981; Rizzo et al., 1984). Chromosomal proteins rich in basic amino acids have been isolated (Rizzo, 1981; Rizzo and Nooden, 1974) but the protein to DNA ratio in dinoflagellate chromosomes is lower than that of the bacteria. At high concentrations (over 200 mg/ml), DNA exists in liquid crystal states in vitro (Leforestier and Livolant, 1992). Dinoflagellate genome sizes range from 3.2 to over 200 pg per cell. For *Amphidinium carterae*, a dinoflagellate with one of the smallest known genome, the concentration of DNA was estimated to be 200 mg/ml (Bohrmann et al., 1993). From a physiochemical standpoint, these properties make it likely that dinoflagellate DNA/chromosomes occur in liquid crystal states.

Under conventional transmission electron microscopy, dinoflagellate chromosomes show characteristic periodic structures, including bands, arches, and crenelations. Several models have been proposed to account for these structures. The early one of “superhelices of coiled DNA filaments” was basically disproved by better ultrastructural preservation and more detailed procedures for cryosubstitution and cryosectioning of vitrified specimens (Gautier et al., 1986). Bouligand (1968) proposed, and later confirmed by Gautier et al, (1986) that the dinoflagellate chromosomes are highly compacted “plywood”-like structures. The geometry of the chromosomes, constructed from TEM cross-sections, corresponds to the twisted nematic or cholesteric forms of liquid crystals. The strong birefringence of the dinoflagellate chromosomes is also in

agreement with a highly compact and ordered structure of liquid crystals. Freeze-fracture-etch replicas of concentrated DNA solutions were almost identical to TEM images of dinoflagellate chromosomes (Rill et al., 1989), providing strong support for the cholesteric DNA packing model proposed by Bouligand and Livolant.

In most eukaryotic cells, DNA synthesis usually occurs at the end of the chromosomal condensation-decondensation cycle, which is partly mediated by topoisomerases. Ultrastructural and confocal microscopy studies have revealed the uniqueness of the dinoflagellate chromosomal behavior during the cell cycle (Rizzo, 1981). Chromosomes appear to be loosely condensed in the early G₁ phase. The boundaries between chromosomes and the nucleoplasm are ruffled because numerous structures protrude toward the nucleoplasm from the chromosomes. In late G₁ phase, the chromosomes remain loosely condensed, but the material inside them appears more densely packed into arch domains. The ruffled aspect of the chromosome and nucleoplasm boundaries is absent, and there are very few protruding structures (Bhaud et al., 2000). During the S phase, the chromosomes are transiently unwound (Spector et al., 1981). The chromosomes in the G₂ phase are tightly condensed and show a double number of arches when seen under electron microscopy. This quasi-permanently condensed liquid crystal state of the dinoflagellate chromosomes set unique topological requirements for the topoisomerases.

The major known function of topoisomerase II is in the decatenation of replicated DNA after the S phase. The expression of topoisomerase II is cell cycle dependent with both protein and catalytic activity peaking at G₂/M. Studies on *top2* mutant of *Saccharomyces cerevisiae* revealed that topoisomerase II activity was required specifically at the time of mitosis (Holm et al., 1985). They showed that the

temperature-sensitive *top2* were defective in chromosome segregation when they were grown in restrictive temperature. In the parasitic protozoa *Leishmania infantum*, analysis of protein expression showed topoisomerase II is cell cycle regulated, present in the proliferative stage but absent in the non-replicating promastigotes (Hanke *et al.*, 2003). In addition, both the antibodies and the topoisomerase II drug prevented or hindered the segregation of chromatin daughter sets at the anaphase stage of mitosis in drosophila embryos (Buchenau *et al.*, 1993). Furthermore, immunodepletion of topoisomerase II from *Xenopus* egg extracts or incubation of these extracts with topoisomerase II inhibitors suggests that topoisomerase II is required for chromatin condensation (Larsen *et al.*, 1996) and chromosome segregation at the metaphase/anaphase transition (Bakshi *et al.*, 2001). In mammalian cells, upon treating with topoisomerase II inhibitors, the cell cycle was arrest in the G₂/M phase. In yeast and *Drosophila*, there is only one form of topoisomerase II. In contrast, there are two forms of topoisomerase II, the α isoform and β isoform, in mammalian cells (Bakshi *et al.*, 2001; Champoux, 2001; Cortes *et al.*, 2003; Porter *et al.*, 2004; Wang, 2002). Topoisomerase II α but not topoisomerase II β is essential for cell proliferation (Bakshi *et al.*, 2001). Topoisomerase II α plays a major role at mitosis in mammalian cells, as in other eukaryotes, and its activity is cell cycle regulated, peaking at G₂/M phase. The amount of topoisomerase II β , accounting for 20-30% of total topoisomerase II, does not change significantly throughout the cell cycle (Larsen *et al.*, 1996; Prosperi *et al.*, 1992; Taagepera *et al.*, 1993; Woessner *et al.*, 1991). Topoisomerase II β does not seem to be involved in activities specific for a given phase of the cell cycle as is the case for topoisomerase II in other eukaryotic cells or the α isoform. More recently it has been shown that β isoform is only associated with bulk DNA not undergoing replication

(Larsen *et al.*, 1996). Topoisomerase II levels are also correlated with the mitotic activity in plant tissues. In *Arabidopsis thaliana*, both protein and RNA levels increased with the proliferative state of the cells (Xie *et al.*, 1994). Higher level of topoisomerase II expression was also observed in actively dividing and proliferative tissues in pea (Reddy *et al.*, 1999). Topoisomerase II inhibitor ICRF-193 abrogated chromosome condensation in cultured alfalfa and tobacco cells (Gimenez-Abian *et al.*, 2002). Available evidences suggest that the major role of topoisomerase II (except the β isoform) in eukaryotes is to ensure proper decatenating of the sister chromatids at the G₂/M phase.

Cell cycle-phased topoisomerase II activities can be regulated by expression, mRNA, and posttranslational levels (Isaacs *et al.*, 1998). The transcriptional activation of topoisomerase II is apparently similar to most other S-phase specific genes like DNA polymerase α (Hochhauser *et al.*, 1992; Hu *et al.*, 2002). The reduced level of topoisomerase II protein after G₂/M is attributed to downregulation of transcription and decreased stability of both its mRNA and protein (Goswami *et al.*, 1996; Heck *et al.*, 1988; Heck *et al.*, 1989; Ramachandran *et al.*, 1995). When dephosphorylated, topoisomerase II from budding yeast and from Swiss 3T3 cells ceases its catalytic activity (Dang *et al.*, 1994; Saijo *et al.*, 1992). Phosphorylation of topoisomerase II in the fission yeast enhances its nuclear import (Shiozaki *et al.*, 1992).

There are many known inhibitors of topoisomerases. Amsacrine and ellipticine are two specific inhibitors targeting topoisomerase II that stabilize the enzyme-DNA covalent intermediate (Wasserman *et al.*, 1994). In addition to topoisomerase II inhibition, amsacrine and ellipticine are known to cause breakage of topoisomerase II-dependent DNA strands in other eukaryotic cells (Larsen *et al.*, 2003). Because DNA damage is known to affect cell cycle progression, the delay of cell cycle observed in response to

topoisomerase II inhibitors could be a consequence of DNA damage (Clarke *et al.*, 1993). Another class of type II inhibitors, the bis-dioxopiperazines ICRF-193, traps the enzyme in a closed clamp form (Roca *et al.*, 1994), which does not stabilize the cleavable complexes between topoisomerase II and the cut DNA. A recent study (Skoufias *et al.*, 2004) showed that ICRF-193 only caused DNA decatenation inhibition but not DNA damage. In that experiment, cell cycle arrest relating to ICRF-193 was not accompanied by recruitment of Mad2 or Bub1 to kinetochores, nor by phosphorylation of the histone H2AX; therefore, cell cycle arrest by ICRF-193 was not due to activation of the spindle assembly checkpoint, nor was it a response to DNA damage. Both types of topoisomerase inhibitors will be used in our study.

Topoisomerase activities in dinoflagellates have never been described, but the dinoflagellate cell extract provoked an immunologically positive reaction from an anti-human topoisomerase II polyclonal antibody (Minguez *et al.*, 1994). Due to the unique chromosomal and mitotic features of dinoflagellates, it is questionable whether their topoisomerases play the same role as their counterparts in other eukaryotes. This study investigated topoisomerase activity during the dinoflagellate cell cycle and tested the effects of topoisomerase II inhibitors on cell cycle progression and chromosome morphology in synchronized *C. cohnii* cells.

MATERIALS AND METHODS

Chemical sources, Cell culture and cell cycle synchronization

Most of the chemicals, including the topoisomerase II inhibitors amsacrine and ellipticine, were obtained from Sigma. ICRF-193 was obtained from BIOMOL Research Laboratories Inc. The decatenation assay kit was purchased from TopoGEN Inc. Anti-lamin A + C monoclonal antibodies were purchased from Abcam Limited.

C. cohnii strain 1649 was obtained from the Culture Collection of Algae at the University of Texas in Austin, and was cultured in MLH medium (Tuttle and Loeblich, 1975). Cell cycle synchronization was carried out by the colony release-filtration method (Wong and Whiteley, 1996). When *C. cohnii* colonies on MLH agar plates were washed with fresh medium, only motile G₁ cells were released. The medium containing the newly released cells was subjected to a filtration step (10 µm filter) to increase the proportion of G₁ cells to beyond 90%. Synchronized G₁ cells will progress through the cell cycle synchronously and a new cell cycle normally starts at 10 - 12 hours after synchronization (T=12).

Topoisomerase inhibitors

To investigate the effects of topoisomerase II deactivation on *C. cohnii* cell cycle progression, three inhibitors were added at T=0 and T=7, corresponding to the early G₁ phase and early G₂/M phase respectively. Three topoisomerase inhibitors are used, including those that cause DNA damage (amsacrine and ellipticine) and one that do not cause DNA damage (ICRF-193, see Introduction section).

Flow cytometric studies

Samples (50 ml at approximately 10^4 cells per ml) were harvested at 2-hour intervals as the cell cycle progressed. Cells were collected by centrifugation and fixed in 70% ethanol for at least 24 hours at 4°C. After fixation, the cells were resuspended in PBS (phosphate buffered saline: 80 mM Na_2HPO_4 , 20 mM NaH_2PO_4 , and 100 mM NaCl, pH 7.5) together with RNaseH (5 $\mu\text{g/ml}$) and incubated at 37°C for 1 hour. The cells were then stained with propidium iodide (2.5 $\mu\text{g/ml}$) at 4°C overnight. Flow cytometry analysis was performed using with a Beckon-Dickson Vantage cell sorter.

Fluorescence Microscopy analysis

To visualize the chromosome morphology, *C. cohnii* cells were fixed with 2% PFA (paraformaldehyde) prior to DNA staining. After fixation, the samples were stored in 4°C until needed. Before staining, the cells were first washed in PBS and then exposed to SYTOX Green (final concentration at 50 μM in PBS) for 10 minutes at room temperature. Stained-cells were directly mounted for microscopic examination.

Transmission electron microscopy

Control cells and ICRF-193 treated cells of *C. cohnii* were fixed, embedded in resin and examined at ultrastructural level. The standard glutaraldehyde-osmium tetroxide cell fixation protocol was used for specimen preparation. *C. cohnii* cells were collected by centrifugation at 3000 g for 10 minutes. They were fixed for DNA preservation in a primary fixative (4% glutaraldehyde/0.1 M PIPES buffer) for at least 1 hour at 4°C. After washing the pre-fixed cells twice in 0.1 M PIPES buffer for 15 minutes each in order to remove any free unreacted glutaraldehyde, the cells were subjected to a secondary

fixative (1% osmium tetroxide/0.1 M PIPES buffer) for 1 hour at 4°C in the dark. The post-fixed cells were subjected to a series of washing and then followed by dehydration in ethanol. Finally, the cells were embedded in epoxy resin (from Sigma). Sections were stained with 1 % uranyl acetate (dissolved in 50 % methanol) and then 0.25 % lead citrate (dissolved in 0.1 N NaOH) and examined in a JEOL 100CX transmission electron microscope.

Analysis of DNA fragmentation by gel electrophoresis

C. cohnii cells were collected at 2-hour interval. DNA extraction was performed using a cetyltrimethylammonium bromide (CTAB)-based method (Sambrook and Russell, 2001). Electrophoresis of the extracted-genomic DNA was performed in 1% agarose gel for 1 hour at 100 volts. The DNA smear pattern was observed under a UV lamp after staining with ethidium bromide.

Cellular extracts preparation for topoisomerase activities

C. cohnii cells were harvested at 3-hour intervals as the cell cycle progressed, and the dry cell pellets were stored in liquid nitrogen for no more than a week. On the day of assaying, the cells were resuspended in extraction buffer [1X topoisomerase II assay buffer without adenosine tri-phosphate (ATP), bovine serum albumin (BSA) or dithiothreitol (DTT), and 1mM phenylmethylsulfonylfluoride (PMSF), 1 µg/ml aprotinin and pepstatin A, and 10 µg/ml leupeptin]. The cells were treated by ultrasonication at high power until all the cells and nuclei were lysed under microscopic examination. Supernatant, which was obtained after a 15 minutes centrifugation at 16,000 xg at 4°C, were concentrated by centrifuging through a Centricon YM-100[®] according to the

procedures recommended by the manufacturer (Millipore Corporation). We determined the total protein concentration for each extract by using the Bio-Rad Protein assay according to the manufacturer's protocol (Bio-Rad Laboratories). Concentrated cellular extract can be immediately subjected to the decatenation assay, or stored in liquid nitrogen for no more than a week.

Decatenation assays

Kinetoplast DNA (kDNA) is a network of concatenated DNA circles extracted from the mitochondria of the trypanosome *Crithidia fasciculata* (Osheroff and Bjornsti, 1999). The network consists of several thousands of minicircular DNA. Because topoisomerase II can make a double strand cut on DNA molecules and then reseal the break, the enzyme thereby has the ability to decatenate the networks of kDNA into individual minicircles. The decatenated minicircles exist in two forms: a nicked, open circular (OC) form of 2.5 kb, and a relaxed closed form of 1.5 kb. Thus, the activities of topoisomerase II can be measured by investigating the presence of decatenated form(s) of kDNA using a commercially available decatenation assay kit (Topogen Corporation).

The assay was performed according to the protocol provided by the manufacturer. In brief, the kDNA (180 ng) was incubated with 2 µl of cell extracts in 20 µl of 1X reaction buffer (0.05 M Tris-Cl pH 8.0, 0.12 M KCl, 0.01 M MgCl₂, 0.5 mM DTT, 0.5 mM ATP, 1 mM EDTA and 0.03 µg/µl BSA) at 28°C for 20 minutes. A preincubation of the kDNA in the 1X reaction buffer at 28°C for 10 minutes was required. The reaction was stopped by 4 µl of 5X stop buffer with loading dye. The assay products were loaded into a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide. Electrophoresis was carried out for 2 hours at 70 volts, and the gel was destained in water for at least 30 minutes before

observation under UV lamp. Nuclei extracts were applied in the decatenation assay, but their results were no different from using cellular extracts. Western blot was performed according to the method of Harlow and Lane (1988). Nuclear lamins A and C were used as loading controls in consideration of the fact that we were purifying a nuclear enzyme and the dinoflagellate permanent nuclear envelopes were previously proven to contain nuclear lamin (Minguez et al., 1994).

Single cell gel electrophoresis

The cell preparation and gel electrophoresis were performed with minor modifications of the comet assay methods established for mammalian cells (Hajji et al., 2003; Petersen et al., 2000; Singh et al., 1988). Cells were embedded in low melting agarose (LMA) in PBS without Ca^{2+} and Mg^{2+} by using a three-layer technique. Firstly, microscope slides were precovered with 150 μl of 1% LMA. The gel was carefully and evenly spread on the slide with a coverslip (18 x 18 mm) and left to solidify. The coverslip was then carefully removed. Cells that had been treated with DMSO, amsacrine and ICRF-193 were harvested 3 hours after treatment and the cell pellets were resuspended in 0.7% LMA, which had been pre-warmed to 37°C. Then, 80 μl of the cell suspension was spread evenly with a coverslip on top of the precovered gel and left to solidify for 15 minutes at 4°C. Finally, after the coverslip was removed, 100 μl of the pre-warmed 0.7% LMA was layered on top and left to solidify for 15 minutes.

Immediately after this preparation, the slides were first immersed in PBS containing 3 U cellulase immediately for 1 hour to induce cellulose degradation in order to weaken the thick cell wall of the *C. cohnii*. The slides were then immersed in lysis solution (2.5 M NaCl, 100 mM ethylene-diaminetetraacetic acid [EDTA], 1% N-laurylsarcosine, 10

mM Tris-base, pH 10, with 10% DMSO and 1% Triton X-100 added just before use) containing 1.5 mg/ml proteinase K for at least 1 hour. The DNA was then allowed to unwind for at least 20 minutes in an electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13.2) at 4°C before electrophoresis was run at 20 volts for 20 minutes at 4°C using an ordinary electrophoresis tank. After electrophoresis, the gels were washed in neutralizing buffer (400 mM Tris-HCl, pH 7.5) for 5 minutes, and the DNA was stained with 10 mM SYTOX Green in PBS for at least 15 minutes.

RESULTS

Amsacrine and ellipticine caused G₁ delay and G₂/M arrest

Our experiments employed various concentrations of amsacrine and ellipticine to investigate the effect of topoisomerase II deactivation on cell cycle progression. Synchronous *C. cohnii* cells in the G₁ phase were exposed to different doses of amsacrine or ellipticine. DNA flow cytograms of the control cells, which were treated only with the vehicle DMSO, confirmed that synchronous cell cycle progression was accomplished 12 hours after synchronization. As shown in the flow cytograms (Fig. 1A), 5 μ M amsacrine and 1 μ M ellipticine effectively delayed the synchronous cells at G₁ phase and S phase entry if they were added at the beginning of the G₁ phase. Percentage of G₁ cells in amsacrine and ellipticine-treated cultures displayed approximately 2 hours delay when compared to that in the control culture (Fig. 1c).

To investigate the effects of the inhibitors on mitosis, the same doses of amsacrine and ellipticine were added seven hours after synchronization (at T=7), when most of the cells already accomplished S phase and at the beginning G₂/M phase. Flow cytograms (Fig. 1B) showed that both drugs also have the ability to arrest *C. cohnii* cell cycle progression at the G₂/M phases. Cell cycle progression of the control cells (re-entered G₁ at T=12), which were treated with the vehicle DMSO, was not affected.

High concentrations of amsacrine and ellipticine induced DNA fragmentation

When higher doses of amsacrine and ellipticine (50 mM and 10 mM respectively) were applied, a sub-G₁ area (Fig. 2A), which indicating possible DNA damage (Bartova *et al.*, 1997; Cheng *et al.*, 2003), was observed. Fig. 2B shows DNA-stained photos of the control and amsacrine-treated cells. The rod-shaped chromosomes that observed in

control cell could not be seen in amsacrine-treated cells, showing a smear nucleus instead of individual chromosomes inside the nucleus. This observation is probably caused by DNA damage in the chromosomes. An agarose gel electrophoresis of the genomic DNA of both control and drug-treated cells were performed to determine whether genomic DNA fragmentation occurred (Fig. 2C). For the control cells, most of the genomic DNA was in the upper region in the gel due to its large molecular size. In contrast, smears of genomic DNA with lower molecular size were observed in amsacrine-treated cells. This result indicated that DNA fragmentation might occurred in the amsacrine-treated cells, which is consistent with the evidence of the DNA flow cytograms in Fig.2a.

ICRF-193 delayed S phase entry and arrested cells at G₂/M

In this study, the other topoisomerase II inhibitor, ICRF-193 that has a different inhibitory mechanism, was applied to examine its influence on cell cycle progression in synchronous *C. cohnii* cells. Both flow cytograms and percentage of G₁ cells measurement displayed a dose-dependent G₁ delay in cells incubated with ICRF-193 (Fig. 3). When the same doses of ICRF-193 were applied to the cells at the onset of G₂/M, the cell cycle was severely blocked at this phase, whereas the control cells did not affect cell cycle progression.

Chromosome morphology of *C. cohnii* cells upon topoisomerase II deactivation

Fluorescence microscopy was applied to our chromosome morphology study, following Bhaud et al (Bhaud *et al.*, 2000). Cells were harvested for DNA fluorescence staining at specific time points after synchronization. We employed SYTOX Green, a DNA stain that gives a better resolution of chromosome morphology. The corresponding

DNA-stained fluorescence photomicrographs of control cells at different time points are shown in Fig. 4A. Condensed chromosomes can be observed throughout the *C. cohnii* cell cycle. The nuclei appeared spherical in early G₁ cells. During the interphase (from T=2 to T=6), the chromosomes appeared to be small rods inside the round nuclei with a defined nucleolus. Nuclei enlargement appeared between 2-4 hours after cell synchronization. Mitotic cells started to appear at 6 hours, and they can be categorized into four main groups as defined by Bhaud et al (2000). Most of the cells accomplished mitosis at T=10, but the new daughter cells still remained inside the mother cell wall. They were released and forming early G₁ cells thereafter, thus most of the cells at T=12 are early G₁ cells.

Since ICRF-193 acts as a catalytic inhibitor of topoisomerase II without causing DNA damage, it was applied to deactivate the topoisomerase II in *C. cohnii* cells (Fig. 4B). Cells treated with 5 μ M of ICRF-193 appeared not to divide at any point in the entire cell cycle. Although clear rod-shaped chromosomes were still observed at T=2, they became unclear (indistinct outline) by T=4. Thereafter, the chromosomes became progressively more obscure, showing a cotton-like structure.

Ultrastructural changes in the *C. cohnii* chromosomes upon ICRF-193 treatment

In the control cells (Fig. 5a-c), which were treated with the vehicle DMSO, the chromosomes exhibited either ellipsoid or round shape with well-defined entities. Inside the chromosomes, arches and bands were clearly displayed (Fig. 5c). These patterns are consistent with the ultrastructure of dinoflagellate chromosomes previously described, corresponding to a twisted cholesteric state of liquid crystal. Periphery of the ellipsoid chromosome exhibited crenulation pattern, which are also characteristics of

dinoflagellate chromosomes.

Comparing to the control cells, the ultrastructure of the chromosomes after ICRF-193 treatment became irregular (Fig. 5D-F). The well-defined entities were not clear as that of the control *C. cohnii* cells. The crenulation pattern of the chromosome periphery disappeared, and the characteristic arches and band patterns disappeared. The alignment of the DNA filaments was aberrant and forming an uneven pattern. The DNA filaments inside the chromosome were not as compressed as that of control, and seemed to be stretched and diffused. In summary, the ultrastructure of *C. cohnii*'s chromosomes became totally unorganized upon ICRF-193 induced topoisomerase II deactivation.

Topoisomerase II activities during the *C. cohnii* cell cycle

In the presence of topoisomerase II activities, catenated kDNA is decatenated into two forms: a nicked open circular (OC) form and a relaxed closed circular (CC) form. Because only topoisomerase II can perform both double strands breakage and re-ligation processes, the presence of either or both forms indicates the presence of topoisomerase II activity. As shown Fig.6, OC form kDNA and linear DNA were produced when the kDNA was incubated with the semi-purified cell extracts from *C. cohnii*. This suggests that topoisomerase II activity is present in *C. cohnii* cell extract. To confirm that the OC form of kDNA observed in the decatenation assay was produced by topoisomerase II, we challenged the *in vitro* assay with topoisomerase II inhibitors. At a concentration of 2mM, either ICRF-193 or amsacrine produced an inhibitory effect on topoisomerase II activity, as indicated by the absence of OC form kDNA and a nearly 50% decrease in the intensity of the linear bands. The vehicle (DMSO) did not inhibit the decatenating ability of the cell extract, similar to the result of cell extract and study as only semi-

purified enzymes were used. Purified human topoisomerase II was reported to have an activity of 0.3 ng kDNA/min/nM (Durrieu *et al.*, 2000). We can only approximately estimate the percentage of decatenated kDNA per unit of protein extracts. On this basis, *C. cohnii* extract has an activity of 281 ng kDNA/min/mg protein, compared to 0.008 ng kDNA/min/mg protein in another alveolate *Plasmodium berghei* (Riou *et al.*, 1986).

To investigate topoisomerase II activities in the dinoflagellate *C. cohnii* cell cycle, we prepared semi-purified cellular extracts obtained at different time points. The variations in topoisomerase II activities during the cell cycle are shown in Fig. 7A. The cell extracts used in the assay were equalized to have the same amount of protein, and anti-lamin A and C monoclonal antibodies were used to monitor the loading quantity (Fig. 7B). In the immunoblot, two bands can be seen at approximately 62 kDa and 75 kDa. The two nuclear lamins remained unchanged throughout the *C. cohnii* cell cycle. Because dinoflagellates have a permanent nuclear envelope throughout the whole cell cycle, the lamin proteins (which are the major component of the nuclear envelope) should be present throughout the whole cell cycle. After normalizing the DNA bands' intensity, a curve representing the cell cycle variation in topoisomerase II activity was plotted (Fig. 7C). Topoisomerase II activities were present throughout the whole cell cycle in *C. cohnii*, but total activity was lowest at T=0. The decrease in activity at this time point might not be a response to the synchronization procedure as G₁ cells were selected and separated by its phase-dependent mobility and by size physically. No chemicals had been added into the synchronous culture.

DNA damage determination in drug-treated cells

Some researchers have reported that ICRF-193 can cause DNA strand breakage in mammalian cells (Hajji *et al.*, 2003), while others found no DNA damage (Skoufias *et al.*, 2004), or noted that the little damage they observed was only due to the high pH variation in their study (Boos *et al.*, 2000). When DNA in a mammalian cell has been damaged, it typically shows a comet shape after a single cell gel electrophoresis in strong alkaline solution. This method is sensitive and easy for mammalian cells because their membrane can be easily lysed.

We conducted electrophoresis of single cells to determine whether ICRF-193 would induce DNA damage in *C. cohnii*. In dinoflagellate cells, a weakened cell wall was still present after cellulose treatment; thus, the DNA could not migrate out of the cell and remained inside after electrophoresis. The untreated control cells (Fig. 8A) showed round condensed DNA the same size as their nuclei. The cells treated with amsacrine (Fig.8C) experienced DNA fragmentation, with DNA dispersed throughout the whole cell; thus, no round shape could be observed. The cells treated with ICRF-193 (Fig.8B) showed the round DNA shape after single cell electrophoresis, identical to what was observed in the control cells. We observed similar patterns at other concentrations (1 and 5 μ M) and confirmed these results in three experimental runs. ICRF-193, unlike the topoisomerase poison amsacrine, did not cause observable DNA damage in *C. cohnii* cells.

DISCUSSION

Topoisomerase II activities are normally required at the G₂/M phase of the eukaryotic cell cycle. Conversely, topoisomerase II activities are required and are detected during both the G₁ and the G₂/M phases of the dinoflagellate cell cycle. The only other known exception to the eukaryotic G₂/M pattern is the topoisomerase II β that has no observable cell cycle variation in mammalian cells. However, topoisomerase II β is dispensable and its level only account for 20-30% of the mammalian enzyme (Akimitsu et al., 2003; Chaly et al., 1996; Woessner et al., 1991). One possible function of topoisomerase II in the dinoflagellate G₂/M phase is to promote chromosome segregation during mitosis, as it does in other eukaryotes. However, what is the role of topoisomerase II during the G₁ phase, or before the S phase entry?

Because dinoflagellate chromosomes appear condensed throughout the cell cycle, a question naturally arises: How can genes encoded in such a structure be transcribed and replicated? In higher eukaryotes, these events depend mostly on the dynamic structure of chromatin and nucleosomes (Wolfe, 1993). In dinoflagellates, extrachromosomal DNA loops (formed by local untwisting of DNA filaments at the chromosome peripheries) are a possible site where transcription and replication can occur. In *C. cohnii* cells, numerous structures protruding from the chromosome core toward the nucleoplasm have been observed during the G₁ phase (Bhaud et al., 2000).

Our fluorescence photomicrographs show that structural abnormalities started to appear between 2-4 hours after the beginning of the cell cycle in *C. cohnii* cells that had been treated with the type II inhibitor ICRF-193 (Fig.4). The rod-shaped chromosomes became blurred (indistinct outline). TEM photos also showed irregular chromosomal ultrastructural in ICRF-193 treated cells. This abnormality caused by topoisomerase II

inhibition during the G₁ phase suggests that the type II enzyme probably participates in DNA loop formation or organization in dinoflagellate chromosomes. The effect is consistent with topoisomerase II having a role in the transcription and replication of the condensed chromosomes that are characteristic of dinoflagellates. However, we cannot completely exclude the possibility that the cell cycle delay may alternatively be caused by indirect transcription inhibition by the presence of trapped topoisomerase II molecules on the DNA as opposed to a direct requirement for the enzyme in G₁/S progression.

In most eukaryotic cells, topoisomerase II is essential during the final stages of DNA replication, in order to facilitate chromosome untangling, condensation, and mitotic segregation. Consequently, expression levels of topoisomerase II increase several-fold during the late S and G₂/M phases. However, in our study, topoisomerase II activity was nearly the same as it was during the G₁ phase. Besides the special requirement of topoisomerase II during the G₁ phase, the unique type of mitosis in dinoflagellates might explain the lack of upregulation during the G₂/M phase. Bouligand et al (1968) proposed a DNA liquid crystal structure in the nuclei of dinoflagellates (Spector, 1984). The same authors also suggested that the chromosome separation and segregation in dinoflagellates probably depend on liquid crystalline states (Bouligand et al., 2001). They proposed that the two replicated filaments are immiscible with the rest of the DNA. This immiscibility occurs in the context of an ordered liquid, with the DNA closely layered in a regular twist, a situation proposed to strongly minimize entangling after replication and therefore strongly facilitates segregation. The liquid crystal DNA structure during mitosis may overcome the relatively low level of topoisomerase II activity during dinomitosis.

Figures

Fig. 1. Effects of amsacrine and ellipticine on synchronous *C. cohnii* cell cycle progression. Flow cytograms of propidium iodide-stained synchronous *C. cohnii* cells treated with amsacrine and ellipticine. *T* time in hour that the samples were harvested after cell cycle synchronization. Vehicle dimethyl sulfoxide (DMSO; control), amsacrine (5 µM) and ellipticine (1 µM) were added at *T*=0 (**a**) and *T*=7 (**b**). Samples were collected at 2-h intervals as the cell cycle progressed. Note that the cell cycle was completed and was back to G₁ phase 12 h after synchronization in control cells. For amsacrine- and ellipticine-treated cells, G₁ delay was observed when the drugs were added at *T*=7. (**c**) Percentage of G₁ cells in control and amsacrine- and ellipticine-treated cells for drugs at *T*=0

Fig. 2. Both amsacrine and ellipticine induced DNA fragmentation at high concentrations. (a) A sub- G_1 peak appeared at $T=4$ when the synchronous *C. cohnii* cells treated with higher doses of amsacrine (50 μM) and ellipticine (10 μM). (b) Photos of control and amsacrine-treated cells. After treating with amsacrine, a smear of condensed DNA instead of individual chromosomes was observed inside the nuclei. (c) Agarose DNA gel showing DNA fragmentation from *C. cohnii* cells treated with 50 μM amsacrine. $M1$ and $M2$ are DNA marker λ and 100 b.p., respectively. Genomic DNA extracted from untreated control and amsacrine-treated cells harvested at $T=0$ to 6 are shown. Note that DNA smears, with smaller sizes than control cells, appeared in lanes 8 to 10 only. Bar, 10 μm

Fig. 3. Effects of different concentrations of ICRF-193 on *C. cohnii* cell cycle progression. Synchronous *C. cohnii* cells were treated with DMSO (control) and ICRF-193 (1, 5, and 10μM) at $T=0$ (a) and $T=7$ (b). Samples were collected at 2-h intervals. Both G_1 and G_2/M delays observed. Percentage of G_1 (C) cells in control and different concentrations of ICRF-193 treatment at $T=0$ and $T=7$

Fig. 4. Chromosome morphology of *C. cohnii* cells upon ICRF-193 treatment. Photomicrographs showing control cells (a) and ICRF-193 treated cells (b) at different time points in a cell cycle. *BF*, bright field. *FL1*, fluorescent field with focusing plane 1 (focused to nucleolus). *FL2*, another fluorescent field with an upper focusing plane.

Compared to the chromosomes in control cells, diffuse chromosomes were observed in ICRF-193 treated cells. *Bar* 10 μm .

Fig. 5 Chromosomal ultrastructure of *C. cohnii* cells upon ICRF-193 treatment. (a)

Chromosomes of control cells. Photos from the top to the bottom have increasing magnifications. At low magnification, ellipsoid or round chromosomes could be observed. Characteristic arch and band patterns could be observed at higher magnification. **(b)** Chromosomes of ICRF-193-treated cells. Chromosomes with irregular shapes were observed. The DNA filaments seemed to be diffused upon topoisomerase II deactivation

Fig. 6. In vitro topoisomerase II assays by decatenation of kinetoplastid DNA. Markers *D* and *L* represent decatenated kDNA and linear DNA, respectively. The upper band in marker *D* represents nicked and open circular (OC) forms of decatenated kDNA. Note that both linear DNA and OC forms of decatenated kDNA were produced by *C. cohnii* cell extract. kDNA was incubated with asynchronized *C. cohnii* cell extracts together with the topoisomerase II inhibitors or the vehicle DMSO. Both ICRF-193 and amsacrine inhibit the kDNA decatenating ability from the cell extract, while DMSO had no effects

Fig. 7. Topoisomerase II activities throughout *C. cohnii* cell cycle. **(a)** A DNA agarose gel shows a decatenation assay that determines the topoisomerase II activities in *C. cohnii* cells from different time point in the cell cycle. **(b)** An immunoblot with anti-lamin A + C monoclonal antibodies as loading controls of the cellular extracts. **(c)** Topoisomerase II activity variation in *C. cohnii* cell cycle. The relative band intensity was calculated by: (decatenated kDNA band intensity + linear band intensity)/ lamin band intensity in immunoblot. Topoisomerase II activities can be detected throughout the whole cell cycle in *C. cohnii*, but its activity was the lowest during the G₁ phase (T=0 and T=12).

Fig. 8. Fluorescence photomicrographs of DNA-stained *C. cohnii* cells after whole-cell gel electrophoresis. *Left* photomicrographs of single cells at high magnification (x100). *Right* photographs with lower magnification. **(a)** Control cells. Note that a smaller round condensed DNA with shape and size similar to the nuclei was observed inside the *C. cohnii* cells. **(b)** ICRF-193-treated (5µM) cells. A small and condensed DNA shape, which was similar to that in control cells, was observed inside the cells. **(c)** Amsacrine-treated (10µM) cells. Note the larger and more diffused DNA shape; this possibly implied that DNA inside was dispersed throughout the whole cells. The cells were stained with the DNA staining dye SYTOX Green. *Bar* 10 µm

References:

- Akimitsu N, Adachi N, Hirai H, Hossain MS, Hamamoto H, Kobayashi M, Aratani Y, Koyama H, Sekimizu K (2003) Enforced cytokinesis without complete nuclear division in embryonic cells depleting the activity of DNA topoisomerase II α . *Genes Cells* 8: 393-402
- Bakshi RP, Galande S, Muniyappa K (2001) Functional and regulatory characteristics of eukaryotic type II DNA topoisomerase. *Crit Rev Biochem Mol Biol* 36: 1-37
- Bhaud Y, Guillebault D, Lennon J, Defacque H, Soyer-Gobillard MO, Moreau H (2000) Morphology and behaviour of dinoflagellate chromosomes during the cell cycle and mitosis. *J Cell Sci* 113: 1231-1239
- Bodansky S, Mintz LB, Holmes DS (1979) The mesokaryote *Gyrodinium cohnii* lacks nucleosomes. *Biochem Biophys Res Commun* 88: 1329-1336
- Bohrmann B, Haider M, Kellenberger E (1993) Concentration evaluation of chromatin in unstained resin-embedded sections by means of low-dose ratio-contrast imaging in STEM. *Ultramicroscopy* 49: 235-251
- Boos G, Stopper H (2000) Genotoxicity of several clinically used topoisomerase II inhibitors. *Toxicol Lett* 116: 7-16
- Bouligand Y, Norris V (2001) Chromosome separation and segregation in dinoflagellates and bacteria may depend on liquid crystalline states. *Biochimie* 83: 187-192
- Buchenau P, Saumweber H, Arndt-Jovin DJ (1993) Consequences of topoisomerase II inhibition in early embryogenesis of *Drosophila* revealed by *in vivo* confocal laser scanning microscopy. *J Cell Sci* 104 (Pt 4): 1175-1185

- Chaly N, Chen X, Dentry J, Brown DL (1996) Organization of DNA topoisomerase II isotypes during the cell cycle of human lymphocytes and HeLa cells. *Chromosome Res* 4: 457-466
- Champoux JJ (2001) DNA topoisomerases: structure, function, and mechanism. *Annu Rev Biochem* 70: 369-413
- Clarke DJ, Johnson RT, Downes CS (1993) Topoisomerase II inhibition prevents anaphase chromatid segregation in mammalian cells independently of the generation of DNA strand breaks. *J Cell Sci* 105: 563-569
- Cortes F, Pastor N, Mateos S, Dominguez I (2003) Roles of DNA topoisomerases in chromosome segregation and mitosis. *Mutat Res* 543: 59-66
- Dang Q, Alghisi GC, Gasser SM (1994) Phosphorylation of the C-terminal domain of yeast topoisomerase II by casein kinase II affects DNA-protein interaction. *J Mol Biol* 243: 10-24
- Durrieu F, Samejima K, Fortune JM, Kandels-Lewis S, Osheroff N, Earnshaw WC (2000) DNA topoisomerase IIa interacts with CAD nuclease and is involved in chromatin condensation during apoptotic execution. *Curr Biol* 10: 923-926
- Gautier A, Michel-Salamin L, Tosi-Couture E, McDowall AW, Dubochet J (1986) Electron microscopy of the chromosomes of dinoflagellates in situ: confirmation of Bouligand's liquid crystal hypothesis. *J Ultrastruct Res* 97: 10-30
- Gimenez-Abian JF, Weingartner M, Binarova P, Clarke DJ, Anthony RG, Calderini O, Heberle-Bors E, Moreno Diaz de la Espina S, Bogre L, De la Torre C (2002) A topoisomerase II-dependent checkpoint in G2-phase plant cells can be bypassed by ectopic expression of mitotic cyclin B2. *Cell Cycle* 1: 187-192

Goswami PC, Roti Roti JL, Hunt CR (1996) The cell cycle-coupled expression of topoisomerase II α during S phase is regulated by mRNA stability and is disrupted by heat shock or ionizing radiation. *Mol Cell Biol* 16: 1500-1508

Hajji N, Pastor N, Mateos S, Dominguez I, Cortes F (2003) DNA strand breaks induced by the anti-topoisomerase II bis-dioxopiperazine ICRF-193. *Mutat Res* 530: 35-46

Hanke T, Ramiro MJ, Trigueros S, Roca J, Larraga V (2003) Cloning, functional analysis and post-transcriptional regulation of a type II DNA topoisomerase from *Leishmania infantum*. A new potential target for anti-parasite drugs. *Nucleic Acids Res* 31: 4917-4928

Harlow E, Lane DP (1988). *Antibodies laboratory manual*, pp. 488-490. Cold Spring Harbor Laboratory Press, USA.

Heck MM, Hittelman WN, Earnshaw WC (1988) Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. *Proc Natl Acad Sci USA* 85: 1086-1090

Heck MM, Hittelman WN, Earnshaw WC (1989) *In vivo* phosphorylation of the 170-kDa form of eukaryotic DNA topoisomerase II. Cell cycle analysis. *J Biol Chem* 264: 15161-15164

Hochhauser D, Stanway CA, Harris AL, Hickson ID (1992) Cloning and characterization of the 5'-flanking region of the human topoisomerase II α gene. *J Biol Chem* 267: 18961-18965

Holm C, Goto T, Wang JC, Botstein D (1985) DNA topoisomerase II is required at the time of mitosis in yeast. *Cell* 41: 553-563

Hu Q, Bhattacharya C, Maity SN (2002) CCAAT binding factor (CBF) binding mediates cell cycle activation of topoisomerase II α . Conventional CBF activation domains are not required. *J Biol Chem* 277: 37191-37200

Isaacs RJ, Davies SL, Sandri MI, Redwood C, Wells NJ, Hickson ID (1998) Physiological regulation of eukaryotic topoisomerase II. *Biochim Biophys Acta* 1400: 121-137

Larsen AK, Escargueil AE, Skladanowski A (2003) From DNA damage to G2 arrest: the many roles of topoisomerase II. *Prog Cell Cycle Res* 5: 295-300

Larsen AK, Skladanowski A, Bojanowski K (1996) The roles of DNA topoisomerase II during the cell cycle. *Prog Cell Cycle Res* 2: 229-239

Leforestier A, Livolant F (1992) Distortion of DNA cholesteric liquid crystal quenched at low temperature : geometrical analysis and models. *J Phys* 2: 1853-1880

Minguez A, Franca S, Moreno Diaz de la Espina S (1994) Dinoflagellates have a eukaryotic nuclear matrix with lamin-like proteins and topoisomerase II. *J Cell Sci* 107: 2861-2873

Petersen AB, Gniadecki R, Wulf HC (2000) Laser scanning cytometry for comet assay analysis. *Cytometry* 39: 10-15

Porter AC, Farr CJ (2004) Topoisomerase II: untangling its contribution at the centromere. *Chromosome Res* 12: 569-583

Prosperi E, Sala E, Negri C, Oliani C, Supino R, Astraldi Ricotti GB, Bottiroli G (1992) Topoisomerase II alpha and beta in human tumor cells grown *in vitro* and *in vivo*. *Anticancer Res* 12: 2093-2099

Ramachandran C, Mead D, Wellham LL, Sauerteig A, Krishan A (1995) Expression of drug resistance-associated *mdr-1*, *GST pi*, and topoisomerase II genes during cell cycle traverse. *Biochem Pharmacol* 49: 545-552

Reddy MK, Nair S, Tewari KK, Mudgil Y, Yadav BS, Sopory SK (1999) Cloning and characterization of a cDNA encoding topoisomerase II in pea and analysis of its expression in relation to cell proliferation. *Plant Mol Biol* 41: 125-137

Rill RL, Livolant F, Aldrich HC, Davidson MW (1989) Electron microscopy of liquid crystalline DNA: Direct evidence for cholesteric-like organization of DNA in dinoflagellate chromosomes. *Chromosoma* 98: 280–286

Riou J, Gabillot M, Philippe M, Schreve J, Riou G (1986) Purification and characterization of plasmodium berghei DNA Topoisomerases I and II: Drug action, inhibition of decatenation and relaxation, and stimulation of DNA cleavage. *Biochemistry (Mosc)* 25: 1471 - 1479

Rizzo PJ (1981) The enigma of the dinoflagellate chromosomes. *J Protozool* 38: 246-252

Rizzo PJ, Choi J, Morris RL (1984) The major histone-like protein from the nonphotosynthetic dinoflagellate *Cryptothecodinium cohnii* (Pyrrophyta) is present in stationary phase cultures. *J Phycol* 20: 95–100

Rizzo PJ, Nooden LD (1974) Partial characterization of dinoflagellate chromosomal proteins. *Biochim Biophys Acta* 349: 415-427

Roca J, Ishida R, Berger JM, Andoh T, Wang JC (1994) Antitumor bisdioxopiperazines inhibit yeast DNA topoisomerase II by trapping the enzyme in the form of a closed protein clamp. *Proc Natl Acad Sci USA* 91: 1781-1785

Saijo M, Ui M, Enomoto T (1992) Growth state and cell cycle dependent phosphorylation of DNA topoisomerase II in Swiss 3T3 cells. *Biochemistry* 31: 359-363

Sambrook J, Russell DW (2001). Preparation and analysis of eukaryotic genomic DNA. New York: Cold Spring Harbor Laboratory Press.

Shiozaki K, Yanagida M (1992) Functional dissection of the phosphorylated termini of fission yeast DNA topoisomerase II. *J Cell Biol* 119: 1023-1036

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175: 184-191

Skoufias DA, Lacroix FB, Andreassen PR, Wilson L, Margolis RL (2004) Inhibition of DNA decatenation, but not DNA damage, arrests cells at metaphase. *Mol Cell* 15: 977-990

Soyer-Gobillard MO, Moreau H (2000). Dinoflagellates. In: Lederberg (ed) *Encyclopedia of Microbiology*, pp. 42-54. Academic Press, San Diego.

Spector DL (1984). Dinoflagellate Nuclei. In *Dinoflagellates*, (ed. Spector), pp. 107-147. Orlando: Academic Press.

Spector DL, Vasconcelos AC, Triemer RE (1981) DNA duplication and chromosome structure in the dinoflagellates. *Protoplasma* 105: 185-194

Taagepera S, Rao PN, Drake FH, Gorbsky GJ (1993) DNA topoisomerase II alpha is the major chromosome protein recognized by the mitotic phosphoprotein antibody MPM-2. *Proc Natl Acad Sci USA* 90: 8407-8411

Tuttle RC, Loeblich AR (1975) An optimal growth medium for the dinoflagellate *Cryptothecodinium cohnii*. *Phycologia* 14: 1?

Wang JC (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* 3: 430-440

Wasserman RA, Wang JC (1994) Mechanistic studies of amsacrine-resistant derivatives of DNA topoisomerase II. Implications in resistance to multiple antitumor drugs targeting the enzyme. *J Biol Chem* 269: 20943-20951

Woessner RD, Mattern MR, Mirabelli CK, Johnson RK, Drake FH (1991) Proliferation- and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. *Cell Growth Differ* 2: 209-214

Wolfe SL (1993). *Molecular and cellular biology*. California: Eadsworth Publishing Company.

Wong JTY, Whiteley A (1996) An improved method for the cell cycle synchronization of the heterotrophic dinoflagellate *Cryptothecodinium cohnii*. J Exp Mar Biol Ecol 197: 91-99

Xie S, Lam E (1994) Abundance of nuclear DNA topoisomerase II is correlated with proliferation in *Arabidopsis thaliana*. Nucleic Acids Res 22: 5729-5736