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M Phase-Specific Phosphorylation of DNA Topoisomerase IIα in HeLa Cells

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Abstract: Using topoisomerase II (topo II) isozyme-specific antibodies, we investigated the phosphorylation of topo IIα in mitotic HeLa cells. Topo IIα was specifically modified in the mitotic cells, resulting in slow migration on SDS-polyacrylamide gel electrophoresis. To characterize the nature of this modification, we treated the nuclear extracts prepared from the mitotic cells with alkaline phosphatase. After the treatment with alkaline phosphatase, the slowly migrated band disappeared and instead a normal (170 kDa) topo IIα band appeared. These results indicate that human topo IIα is modified at a specific site(s) in M phase by phosphorylation, supporting the possibility that M phase-specific phosphorylation of topo II is critical for mitotic chromosome condensation and segregation.
Key words: anti-peptide antibody, cell cycle, DNA topoisomerase II, protein phosphorylation.

DNA topoisomerase II (topo II) is an essential enzyme which regulates the interconversion of DNA topoisomer. Topo II catalyzes the passage of one intact DNA helix through a transient double-stranded break in a second DNA helix in an ATP and Mg^{2+}-dependent manner (reviewed by Wang, 1985; Watt and Hickson, 1994). Topo II participates in many aspects of DNA metabolism, including DNA replication, transcription, and recombination (Bae *et al.*, 1988; Brill and Sternglanz, 1988; Kim and Wang, 1989). In addition, this enzyme is a prominent component of both the interphase nuclear matrix and the mitotic chromosomal scaffold, and has a role in chromosome segregation in both mitosis and meiosis (Earnshaw *et al.*, 1985; Gasser *et al.*, 1986; Uemura and Yanagida, 1986; Rose *et al.*, 1990; Adachi *et al.*, 1991).

The activity of topo II can be modulated *in vitro* by a change in phosphorylation state. The phosphorylation of topo II by either casein kinase II or protein kinase C stimulates the catalytic activity of the enzyme, as measured by DNA relaxation of supercoiled plasmid DNA (Ackerman *et al.*, 1985; Sahyoun *et al.*, 1986; Rottman *et al.*, 1987). Topo II exists as a phosphoprotein in budding yeast (Cardenas *et al.*, 1992), fission yeast (Shiozaki and Yanagida, 1992), Drosophila (Ackerman *et al.*, 1988), chicken (Heck *et al.*, 1989), mouse (Saijo *et al.*, 1990), and HeLa cells (Kroll and Rowe, 1991). Topo II phosphorylation increases as cells enter mitosis (Heck *et al.*, 1989; Burden *et al.*, 1993).

Topo II functions as a homodimer. Recent works have revealed that two isoforms of topo II, designated topo IIα and topo IIβ, are expressed in mammalian cells. In human cells, the topo IIα (molecular mass of 170 kDa) is encoded by a gene on chromosome 17, and the topo IIβ (molecular mass of 180 kDa) is encoded by a gene on chromosome 3 (Tsai-Pflugfelder *et al.*, 1988; Chung *et al.*, 1989; Jenkins *et al.*, 1992). Despite strong sequence homology between the two proteins, the topo IIα and β have quite different biochemical and pharmacological properties (Drake *et al.*, 1989). The isoforms are differentially expressed during the cell cycle, and give distinct intracellular localization patterns. The topo IIα is localized in the nucleoplasm, whereas the topo IIβ is localized in the nucleolus (Negri *et al.*, 1992).

Considering the evidence from the topo II participation in mitotic chromosome organization and segregation (Earnshaw *et al.*, 1985; Gasser *et al.*, 1986; Uemura and Yanagida, 1986; Adachi *et al.*, 1991), from the increased topo II phosphorylation in M phase (Heck *et al.*, 1989; Burden *et al.*, 1993), and from the nucleoplasmic localization of topo II (Negri *et al.*, 1992), it can be speculated that the M phase-specific phosphorylation of topo IIα affects chromosome organization and segregation. In this study, we have investi-

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gated M phase-specific phosphorylation of human topo IIα . Recently, it has been reported that human topo IIβ is phosphorylated at specific sites in M phase (Kimura *et al.*, 1994).

Materials and Methods

Materials

Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Gibco. Keyhole limpet hemocyanin, Freund's adjuvant, phenylmethylsulfonyl fluoride (PMSF), leupeptin, soybean trypsin inhibitor, aprotinin, tosyl-phenyl-chloromethyl ketone (TPCK), sodium fluoride, sodium orthovanadate, β-gylcerophosphate, sodium pyrophosphate, Triton X-100, were obtained from Sigma. Nitrocellulose membrane was obtained from Schleicher & Schuell and Protein A-sepharose from Pharmacia. Alkaline phosphatase and p-nitrophenyl phosphate (PNPP) were obtained from Boehringer Mannheim. The Enhanced Chemiluminescence (ECL) Western blot detection system was supplied by Amersham. All other reagents were of analytical reagent grade. Topo II was purified from calf thymus (Lee *et al.*, 1993).

Cell culture

HeLa cells were grown in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum at 37℃ in 5% CO_2.

Peptide synthesis

Topo II isoform-specific peptides were synthesized using an Applied Biosystems 430A peptide synthesizer and purified by reverse phase chromatography on a preparative HPLC instrument.

Preparation of anti-peptide antibodies

The respective peptides were coupled to keyhole limpet hemocyanin as described by Harlow & Lane (1988). Antibodies against the peptides were raised in Korean White rabbits as follows: Animals were injected subcutaneously with 800 μg of the keyhole limpet hemocyanin-conjugated peptide mixed with Freund's complete adjuvant. Booster injections were given with antigen in Freund's incomplete adjuvant at 3 week intervals. Serum was collected at 14 days after boosting and the antibody titer was determined by immunoblotting. An IgG fraction was prepared using Protein A-sepharose chromatography.

Immunoblotting

Nuclear extracts prepared from HeLa cells or topo II enzyme purified from calf thymus were electrophoresed on 6 or 7.5% polyacrylamide gel in the presence of SDS. After electrophoresis, proteins were transferred to nitrocellulose in a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories, Richmond, USA) and blocked overnight in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk. The membrane was incubated with antibodies against topo II at a 1 : 300 dilution in 0.2% skim milk for 1 h, then washed 3 times in TBST. The blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG and treated with either 4-chloro-1-naphthol (for immunoblot of the purified topo II) or the ECL Western blot detection system (for immunoblot of the nuclear extracts).

Exponentially growing cells and mitotic cells

Exponentially growing HeLa cells in 100 mm-dishes were washed three times with ice-cold phosphate-buffered saline and collected by scraping with a rubber policeman. Mitotic cells were obtained as described by Ashihara and Baserga (1979) with some modification. Briefly, HeLa cells were cultured subconfluently in 100 mm-dishes and mitotic cells were detached by shaking the dishes gently. Detached cells were pelleted at 1000 $\times g$ and washed twice with cold phosphate-buffered saline. This shakeoff procedure was repeated several times at 6 h intervals. The cells were frozen at −70℃ until all samples were ready to be processed further.

Preparation of nuclear extract

Cells were thawed on ice, resuspended in 300 μl of Buffer A (50 mM Tris, pH 7.4, 0.05% Triton X-100, 2 mM EDTA, 60 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 4 mM PNPP, 0.5 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml TPCK, 1 μg/ml leupeptin, 1 μg/ml aprotinin), and incubated on ice for 20 min. The cell suspension was homogenized on ice using 30 strokes in a tissue grinder. As judged by microscopy, this procedure disrupted the native morphology of the cell membrane. The nuclei were precipitated by centifugation at 1500$\times g$ for 5 min and washed once with Buffer A. The nuclear pellet was resuspended in 100 μl of Buffer A containing 360 mM NaCl, and incubated on ice for 20 min. The nuclear suspension was homogenized as above. After a centrifugation at 14000$\times g$ for 10 min, the supernatant was recovered. The protein content of the nuclear supernatant was determined using the Bio-Rad protein assay kit with bovine serum albumin as the standard. For some experiments, mitotic nuclear extracts were prepared in the absence of phosphatase inhibitors (β-glycerophosphate, sodium orthovanadate, sodium pyrophosphate, and PNPP).

Alkaline phosphatase treatment

60 μg protein of the nuclear extracts was treated with 2 units of alkaline phosphatase in 30 μl solution of 100 mM Tris, pH 8.0, 50 mM $MgCl_2$, 0.5 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml TPCK, 1 μg/ml leupeptin, and 1 μg/ml aprotinin for 30 min at 37℃.

Results and Discussion

Eukaryotic chromosome undergoes major structural change through the cell cycle. During the G2/M transition of the cell cycle, the interphase chromatin condenses into compact mitotic chromosome. Topo II is required for the condensation of chromatin and for the separation of the sister chromatid during mitosis (Holm *et al.*, 1985; Uemura and Yanagida, 1986; Uemura *et al.*, 1987; Adachi *et al.*, 1991). Although the important roles of topo II in mitosis have been suggested, the modulation of the enzyme activity during mitosis is not known. If topo II activity is modulated during mitosis, protein phosphorylation may be most responsible for the modulation. As a first step in examining the phosphorylation of topo II in mitotic HeLa cells, we raised topo II isoform-specific antibodies. Anti-topo II antibody, designated BL3, was produced against a peptide corresponding to amino acids 875~886 of the deduced amino acid sequence of the human topo IIα. Anti-topo IIβ antibody, designated BL4, was produced against a peptide corresponding to amino acids 928~940 of the deduced amino acid sequence of the human topo IIβ (Fig. 1) (Tsai-Pflugfelder *et al.*, 1988; Chung *et al.*, 1989; Jenkins *et al.*, 1992). In a previous study, we prepared antibody BL1 against a peptide containing a sequence common to both isozymes near the amino terminal region of topo II (Lee *et al.*, 1993). Although we injected rabbits with a peptide corresponding to amino acids 791~808 of the deduced amino acid sequence of the human topo IIα, which contained the active tyrosine that was covalently linked to the DNA when the topo II transiently broke the DNA, an antibody titer against this peptide was not detected (Fig. 1).

These antibodies were characterized on Western blots containing topo II purified from calf thymus. As shown in Fig. 2, antibody BL3 selectively recognized a 170 kDa protein (topo IIα) and antibody BL4 recognized both a 180 kDa protein (topo IIβ) and a 160 kDa protein, indicating that the 160 kDa protein might be a proteolytic form of topo IIβ (Fig. 2, lanes 3 and 5). Antibody BL1 reacted with all of these bands, indicating that BL1 recognizes both isoforms of topo II (Fig. 2, lane 1). Preimmune rabbit sera for the BL3 and BL4 antibodies did not react with these protein bands (Fig. 2, lanes 2 and 4). These results indicate that both isozymes of topo II are expressed in calf thymus tissue as in other mammalian cells and that the antibodies BL3 and BL4 are specific to topo IIα and topo IIβ, respectively. It has not been reported whether topo IIβ is expressed in calf thymus (Halligan *et al.*, 1985; Lee *et al.*, 1993).

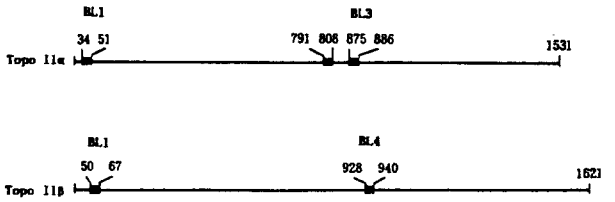

Fig. 1. Recognition sites of anti-peptide antibodies on human topo IIα and β. The thin lines and the black boxes indicate topo II isoforms and recognition sites of the antibodies, respectively. Numbers indicate the coordinates of the topo IIα and β proteins based on Jenkins *et al.* (1992). A detailed description is provided in text.

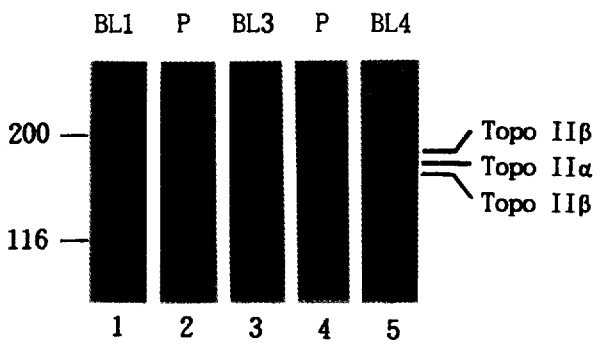

Fig. 2. Immunoblotting of anti-peptide antibodies to purified calf thymus topo II. Purified calf thymus topo II was electrophoresed on 7.5% SDS-polyacrylamide gel, tranferred to membrane strips, and immunoblotted with antibody BL1 (lane 1), preimmune serum for BL3 (lane 2), antibody BL3 (lane 3), preimmune serum for BL4 (lane 4), and antibody BL4 (lane 5). The P indicates preimmune serum. Positions of topo IIα and β are indicated at the right. Molecular markers (in kDa) are shown at the left.

To investigate whether topo II was modified in M phase, we prepared both exponentially growing and mitotic HeLa cells. To eliminate the artifact of mitotic arrest by a chemical reagent, the mitotic cells were obtained by mechanical shakeoff as described under Materials and Methods. Cell-cycle distribution of the mitotic cells was determined by flow cytometry and more than 88% were in M phase (data not shown). Nuclear extracts were prepared from both the exponentially growing and the mitotic cells in the presence of phosphatase inhibitors, and then equal amounts of the nuclear proteins were electrophoresed on SDS-polyacrylamide gel, and finally these were immunoblotted with anti-topo II antibodies (Fig . 3). Antibody BL1 recog-

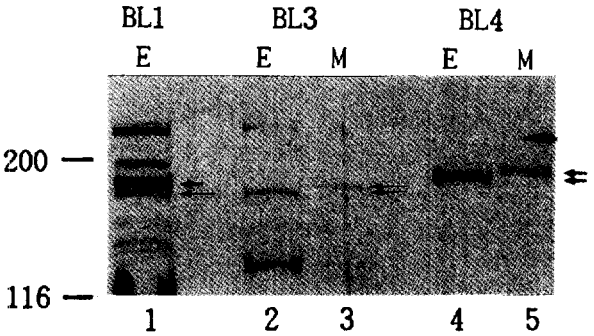

Fig. 3. M phase-specific modification of human topo IIα and β. Equal amounts of nuclear proteins prepared from exponentially growing (lanes 1, 2, and 4) and mitotic (lanes 3 and 5) HeLa cells were electrophoresed on 6% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and probed with antibody BL1 (lane 1), antibody BL3 (lanes 2 and 3), and antibody BL4 (lanes 4 and 5). The E indicates exponentially growing cells; M, mitotic cells; the long and thin arrow, position of topo IIα; the short and thick arrow, position of topo IIβ. Molecular markers (in kDa) are shown at the left.

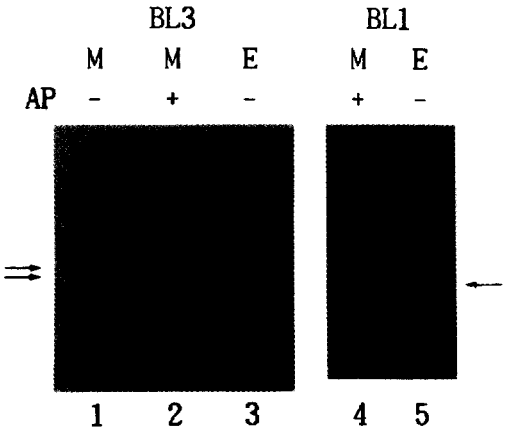

Fig. 4. Characterization of the M phase-specific modification of topo IIα. 60 μg of nuclear proteins prepared from mitotic (lanes 1, 2, and 4) and exponentially growing (lanes 3 and 5) cells were subjected to electrophoresis on 6% SDS-polyacrylamide gel. Mitotic nuclear proteins in lanes 2 and 4 were treated with alkaline phosphatase before the electrophoresis. After electrophoresis the proteins were transferred to nitrocellulose membrane and immunoblotted with antibody BL3 (lanes 1~3) and antibody BL1 (lanes 4 and 5). The M indicates mitotic cells; E, exponentially growing cells; AP, alkaline phosphatase; arrows, positions of topo IIα.

nized both topo IIα (170 kDa) and β (180 kDa) of the exponentially growing HeLa cells, antibody BL3 recognized only topo IIβ, and antibody BL4 only topo IIβ (Fig. 3, lanes 1, 2, and 4). In Fig. 3, the band of topo IIβ protein was darker than that of topo IIα. This result, however, does not mean that topo IIβ is expressed more than topo IIα in the exponentially growing HeLa cells (See Fig. 4, lanes 4 and 5). Instead the reactivity of the antibody BL4 with topo IIβ may be stronger than that of antibody BL3 with topo IIα under this experimental condition.

We found that the antibodies BL3 and BL4 reacted with the altered molecular masses in mitotic HeLa cells, as in Fig. 3. Lanes 3 and 5 in Fig. 3 showed that both topo IIα and β acquired a slower electrophoretic mobility on SDS-polyacrylamide gel. These results indicate that both topo IIα and β are modified in M phase. Such a mobility shift had been recently observed with human topo IIβ in M phase (Kimura *et al.*, 1994). Thus, we examined whether the altered mobility of human topo IIα was due to phosphorylation. Mitotic nuclear extracts were prepared in the absence of phosphatase inhibitors as described under Materials and Methods, and the nuclear extracts were treated with alkaline phosphatase for 30 min at 37℃. The alkaline phosphatase-treated extracts were subjected to SDS-polyacrylamide gel electrophoresis in parallel with untreated nuclear extracts of the mitotic and exponentially growing cells, and immunoblotted with anti-topo II antibodies. Again topo IIα exhibited a reduced electrophoretic mobility in mitotic cells (Fig. 4, lane 1). After the alkaline phosphatase treatment the topo II of the mitotic cells recovered its increased mobility (Fig. 4, lanes 2 and 4), showing that its molecular mass was the same as that of the exponentially growing topo II (Fig. 4, lanes 3 and 5). The alkaline phosphatase treatment did not affect the topo II mobility of exponentially growing cells (data not shown).

Kimura *et al.* (1994) reported that human topo IIβ was phosphorylated at a specific site in M phase. Our study revealed human topo II was also phosphorylated specifically in M phase. This finding was thought as result that in this study several phosphatase inhibitors were used to protect proteins from dephosphorylation by endogenous protein phosphatases during nuclear extract preparation. The mitotic phosphorylation sites of topo IIα may be more susceptible to dephosphorylation by the protein phosphatases. When we were studying the mitotic phosphorylation of topo IIα in HeLa cells, M phase-specific phosphorylation of the topo IIα and β in Chinese hamster ovary cells was reported (Burden and Sullivan, 1994). It would seem likely, therefore, that the specific modification of topo IIα is a common feature in M phase. The M phase-specific phosphorylation of topo IIα reflects either M phase-specifically enhanced protein kinase activity or a M phase-specific inactivation of a protein phosphatase. The topo IIα is localized in the nucleoplasm, whereas the topo IIβ is localized in nucleolus (Negri *et al.*, 1992). We, therefore, suggest that M phase-specific phosphorylation of topo IIα may play a critical role in regulating mitotic chromosome condensation and sister chromatid segregation. Futher study is necessary

to determine which amino acid on topo IIα is phosphorylated, which protein kinase is responsible for the phosphorylation in M phase, and whether M phase-specific phosphorylation of topo IIα affects chromosome organization or segregation.

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