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Phosphorylation of the α- and β-Isoforms of DNA Topoisomerase II Is Qualitatively Different in Interphase and Mitosis in Chinese Hamster Ovary Cells†

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ABSTRACT: Qualitative differences between interphase and mitotic topoisomerase II were studied in Chinese hamster ovary cells. Differences in sites of phosphorylation of *in vivo* ^{32}P-labeled topoisomerase IIα were observed between mitosis and interphase by one-dimensional phosphopeptide mapping of partial tryptic digests. Two-dimensional phosphopeptide mapping of complete trypsin digests revealed two phosphopeptides unique to interphase and three phosphopeptides unique to mitosis. A reduced electrophoretic mobility on denaturing gels (≈190 kDa) was observed for the β-isoform of topoisomerase II in mitosis relative to interphase. Treatment of lysates with alkaline phosphatase demonstrated that this was due to phosphorylation of mitotic topoisomerase IIβ. The existence of interphase- and mitosis-specific sites of phosphorylation of topoisomerase IIα, along with the electrophoretic mobility shift caused by phosphorylation of topoisomerase IIβ in mitosis, demonstrates qualitative differences between interphase and mitosis in the phosphorylation state of both isoforms of topoisomerase II.

DNA topoisomerase II (EC 5.99.1.3) is a eukaryotic homodimeric enzyme which exists in two isoforms in vertebrate organisms; the α-isoform (topo IIα)[1] has a molecular mass of 170 kDa, and the β-isoform (topo IIβ) has a molecular mass of 180 kDa. These two isoforms are products of different genes (Chung *et al.*, 1989) and have differing sensitivities to antineoplastic drugs (Drake *et al.*, 1989). Topo IIα is likely involved in a number of cellular functions and is essential for chromosome condensation and separation of daughter chromosomes during mitosis (Uemura *et al.*, 1987; Downes *et al.*, 1991). Topo IIβ has as yet no defined cellular function, although localization of topo IIβ to the nucleolus (Negri *et al.*, 1992) and to the mitotic chromosomal scaffold (Taagepera *et al.*, 1993) has been reported.

Topo IIα exists as a phosphoprotein *in vivo* (Ackerman *et al.*, 1988; Heck *et al.*, 1989; Matthes *et al.*, 1990; Saijo *et al.*, 1990, 1992; Ganapathi *et al.*, 1991, 1993; Kroll & Rowe, 1991; Takano *et al.*, 1991; Cardenas *et al.*, 1992; Shiozaki & Yanagida, 1992; Burden *et al.*, 1993) and has been shown to be phosphorylated in a cell cycle-dependent manner, with maximal phosphorylation occurring in G_2/M (Heck *et al.*, 1989; Saijo *et al.*, 1992) or specifically M phase (Cardenas *et al.*, 1992; Burden *et al.*, 1993). Distribution of phosphate among multiple sites in topo II varies between G_1 and M

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[1] Abbreviations: topo IIα, topoisomerase IIα; topo IIβ, topoisomerase IIβ; CHO, Chinese hamster ovary; MEM, minimal essential medium; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence.

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phase in the yeast *Saccharomyces cerevisiae* (Cardenas *et al.*, 1992), with a phosphothreonyl residue present in both phases in addition to a phosphoserine. We have previously reported the existence of a phosphothreonine in CHO topo IIα solely in M phase (Burden *et al.*, 1993). In contrast to topo IIα, much less is known about topo IIβ. Topo IIβ protein levels do not seem to vary during the cell cycle (Woessner *et al.*, 1991; Kimura *et al.*, 1994). Immunodetection experiments using an antibody specific for a phosphoepitope suggest topo IIβ exists *in vivo* as a phosphoprotein (Taagepera *et al.*, 1993). Recently, it was reported that topo IIβ from mitotic HeLa cells has a reduced electrophoretic mobility relative to interphase cells (Kimura *et al.*, 1994). The reason for this shift in apparent molecular mass is not known. The experiments presented herein examine phosphorylation of both isoforms of topo II during the cell cycle in CHO cells and collectively demonstrate that topo IIα and topo IIβ are qualitatively different in mitosis than they are in interphase.

EXPERIMENTAL PROCEDURES

Materials. Wild-type CHO cells were grown in monolayer at 37 °C in αMEM (Gibco BRL, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum (Gibco) in the presence of 5% CO_2. Phosphate-free MEM (Gibco) was also supplemented with 5% heat-inactivated fetal bovine serum. Penicillin (100 IU/mL) and streptomycin (100 μg/mL) were added to both types of media. Sigma Chemical Co. (St. Louis, MO) supplied trypsin, cellulose acetate plates, thymidine, aphidicolin, demecolcine, Nonidet P-40, aprotinin, leupeptin, chymostatin, pepstatin A, antipain, benzamidine, and soybean trypsin inhibitor. [^{32}P]H_3PO_4 (8500−9120 Ci/mmol) was obtained from New England Nuclear Research Products (Boston, MA). Protein A−Sepharose CL4B was supplied by Pharmacia-LKB Biotechnology AB (Uppsala, Sweden). Worthington Biochemical Corp. (Freehold, NJ) supplied calf intestine alkaline phosphatase.

Cell Synchrony and Metabolic Labeling. Cells were synchronized using a thymidine/aphidicolin double block (for S and G_2 phase cells) followed by a demecolcine block (for M phase cells) as previously described (Burden *et al.*, 1993). A 12-h demecolcine block (taking only nonadherent cells) was used to obtain M phase populations for the β-isoform experiments. M phase cells were also obtained by mitotic shakeoff as follows. Exponentially growing CHO cells in 150-cm^2 flasks were washed with 20 mL of αMEM at 37 °C and then incubated with 4 mL of αMEM at 37 °C for 10 min. The flasks were then shaken for 5 s on a Bellco orbital shaker (Vineland, NJ) at setting 6. The medium from this shakeoff was discarded, and another 4 mL of αMEM was added. This procedure was repeated for an additional three 10-min time points. The subsequent 10-min time points (to a total time of 6 h) were performed as were the first four except that the period of shaking was for 3 s and the medium was removed from the flask and saved. The medium saved from each time point was added to 22 mL of ice-cold buffer (150 mM NaCl, 5 mM potassium phosphate, pH 7.4), and the cells were then removed by centrifugation at 250*g* for 5 min at 4 °C and resuspended in 22 mL of ice-cold buffer. The next time point was then added to the 22 mL of buffer containing the cells from previous time points, and the procedure was repeated. After time points were collected for 6 h, the cells were lysed (see below). Metabolic labeling with [^{32}P]H_3PO_4 for 30 min at 1 mCi/mL was performed as previously described (Burden *et al.*, 1993).

Cell Lysis and Topo IIα Immunoprecipitation. Cells were lysed and topo IIα was immunoprecipitated as previously described (Burden *et al.*, 1993). The lysis buffer was supplemented with 1 mM benzamidine and 10 μg/mL soybean trypsin inhibitor for the β-isoform experiments.

Proteolytic Digests. Topo IIα was labeled with ^{32}P *in vivo* and immunoprecipitated. The resulting ^{32}P-topo IIα was electrophoresed in a 6% SDS−PAGE gel (Laemmli, 1970) after being boiled for 5 min in the presence of 2% SDS, 100 mM dithiothreitol, and 10% glycerol. After electrophoresis, the gel was fixed, dried, and exposed to film. ^{32}P-Topo IIα was detected by autoradiography, and the bands were excised from the gel. Subsequent preparation of the samples for protease digestion was as previously described (Boyle *et al.*, 1991). Samples for partial proteolytic digests (topo IIα from ≈2 × 10^7 cells) were treated by addition of 50 or 100 ng of trypsin and then incubation at 37 °C for 30 min. Reactions were terminated by addition of 40 μL of 8% SDS, 400 mM dithiothreitol, and 40% glycerol, followed by boiling for 5 min. Samples were electrophoresed in a 15% SDS−PAGE gel, the gel was fixed and dried, and phosphopeptides were detected by autoradiography. For complete trypsin digests, ^{32}P-topo IIα (from ≈5 × 10^7 cells) was incubated for 6 h with 10 μg of trypsin at 37 °C. Next, an additional 10 μg of trypsin was added and the incubation continued at 37 °C overnight (8−12 h). We have found that ≈50% of the Cerenkov cpm present in the sample are made soluble by the initial protease treatment and that at least three additional treatments with protease do not increase this amount (data not shown), so we believe this protocol gives complete digestion of the topo IIα. After digestion, the samples were electrophoresed on a cellulose acetate thin-layer plate (20 cm × 20 cm, 250-μm thickness) in the first dimension using pH 1.9 buffer (2.5% formic acid, 7.8% acetic acid, both vol %) for 30 min at 1.0 kV. Ascending chromatography was performed in the second dimension using phosphochromatography buffer (37.5% 1-butanol, 25% pyridine, 7.5% acetic acid, all vol %) until the solvent front was ≈3 cm from the top of the plate. The plate was then dried and exposed to film to detect the phosphopeptides.

Treatment of Lysates with Alkaline Phosphatase. Asynchronous and M phase CHO cells were lysed at 2.5 × 10^5 cells/μL, and lysate from 5 × 10^6 cells was treated with 10 or 100 units of calf intestine alkaline phosphatase in a total of 200 μL of alkaline phosphatase buffer (100 mM glycine, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM $CaCl_2$, pH 9.8). Reactions were run at 37 °C for 30 min and were stopped by the addition of 66.7 μL of 8% SDS, 400 mM dithiothreitol, and 40% glycerol. The samples were then boiled for 5 min, electrophoresed in a 6% SDS−PAGE gel, and transferred to PVDF as previously described (Burden *et al.*, 1993), and the β-isoform was detected by enhanced chemiluminescence using the ECL Western blot detection system (Amersham Corp., Arlington Heights, IL). The primary antibody used (antiserum IIA) was the generous gift of Dr. Leroy Liu and has been described previously (Kaufmann *et al.*, 1991). The secondary antibody was donkey anti-rabbit horseradish peroxidase (Amersham). Experiments involving prior treatment of the lysate with SDS were conducted by adding 1.5 volumes of 10% SDS to lysates, boiling for 5 min, and then using the lysates in alkaline phosphatase reactions. The

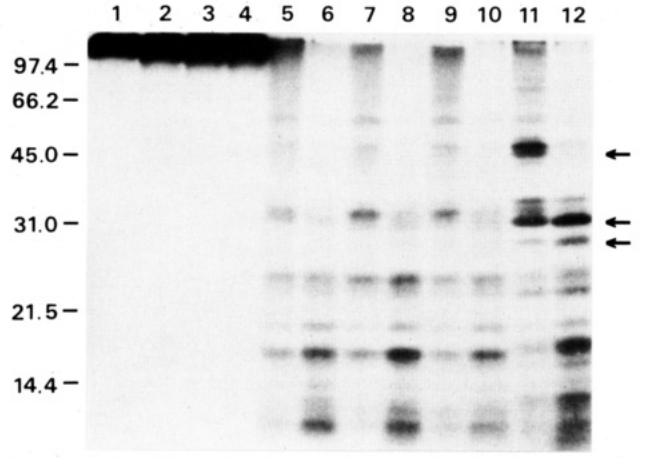

FIGURE 1: Partial tryptic digests of *in vivo* ^{32}P-labeled topo IIα. Synchronous and asynchronous populations of CHO cells were labeled with ^{32}P, and immunoprecipitated topo IIα was gel-purified. The topo IIα preparations were treated in the absence (lanes 1−4) or presence of 50 ng (lanes 5, 7, 9, and 11) or 100 ng (lanes 6, 8, 10, and 12) of trypsin. Populations are of equal Cerenkov cpm, and lanes are assigned as follows: asynchronous, lanes 1, 5, and 6; S phase, lanes 2, 7, and 8; G_2 phase, lanes 3, 9, and 10; M phase, lanes 4, 11, and 12. Arrows indicate phosphopeptides present predominantly in mitosis. Molecular mass estimated from marker proteins is given in kilodaltons.

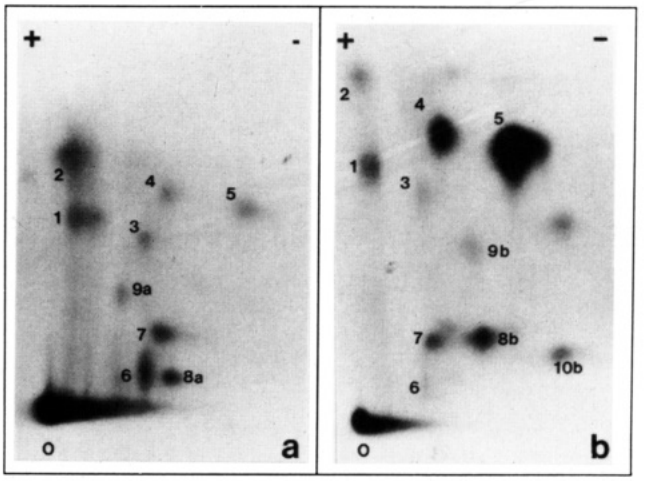

FIGURE 2: Two-dimensional phosphopeptide maps of complete tryptic digests of *in vivo* ^{32}P-labeled topo IIα. Topo IIα was labeled *in vivo* with ^{32}P, was then gel-purified, and completely digested with trypsin, and the resulting phosphopeptides were separated in two dimensions on thin-layer plates as described under Experimental Procedures. Separation in the horizontal dimension was by electrophoresis, with the cathode (−) to the right; separation in the vertical dimension was by ascending chromatography. o indicates the origin; a, phosphopeptide map resulting from digestion of interphase (G_2 phase) *in vivo* ^{32}P-labeled topo IIα; b, phosphopeptide map resulting from digestion of mitotic *in vivo* ^{32}P-labeled topo IIα.

alkaline phosphatase was active under both sets of conditions (±SDS) as judged by assaying its ability to cleave *p*-nitrophenyl phosphate (data not shown).

RESULTS

Partial Proteolytic Digests of in Vivo ^{32}P-Labeled Topo IIα. Topo IIα was partially digested with trypsin as described under Experimental Procedures. Lanes 1−4 of Figure 1 show control incubations in the absence of trypsin, and lanes 5−12 show incubations in the presence of 50 or 100 ng of trypsin. Little difference is observed between asynchronous, S phase, and G_2 phase topo IIα phosphopeptide patterns; M phase, however, exhibits a clear preference for a site(s) of phosphorylation on proteolytic fragments migrating at ≈45, ≈32, and ≈28 kDa (arrows) which is not seen in the other populations. This suggests that the sites of phosphorylation of topo IIα are cell cycle-dependent in CHO cells, supporting observations we have previously made (Burden *et al.*, 1993).

Tryptic Phosphopeptide Maps of Topo IIα during the Cell Cycle. Two-dimensional phosphopeptide maps of complete trypsin digests of ^{32}P-topo IIα were performed using four different labeling experiments for each phase of the cell cycle (asynchronous, S, G_2, M). Figure 2a shows a representative phosphopeptide map for topo IIα phosphorylated *in vivo* in G_2 phase CHO cells. No obvious differences other than minor variations in relative intensities were observed between the phosphopeptide maps for asynchronous, S phase, and G_2 phase topo IIα (data not shown). Similar to the findings with the partial trypsin digests, M phase topo IIα is clearly phosphorylated primarily at a preferred site on the basis of relative intensities (phosphopeptide 5; Figure 2b), and three reproducibly detectable sites present in M phase were observed which do not appear in interphase (phosphopeptides 8b, 9b, and 10b; Figure 2b). There are also two sites present in interphase (phosphopeptides 8a and 9a; Figure 2a) that disappear in mitosis. These differences were confirmed by performing a mixing experiment in which tryptic phosphopeptides of asynchronous and M phase topo IIα were prepared separately; equal cpm (by Cerenkov counting) of each were then mixed, electrophoresed, and chromatographed. It was found (on two separate occasions using two different ^{32}P-labeled populations of each asynchronous and M phase cells) that there are indeed three phosphopeptides present in M phase topo IIα that are not present in interphase populations, while two phosphopeptides that are present in interphase disappear in mitosis. Thus, it appears that there are sites of phosphorylation of topo IIα in CHO cells that are uniquely modified in interphase and mitosis.

Elucidation of the Mitosis-Specific Modification of Topo IIβ. The β-isoform of topo II was detected by Western blot analysis of CHO cell lysates. Lanes 1 and 2 of Figure 3 show asynchronous and mitotic (mitotic index >0.90) lysates, respectively. As was observed in HeLa cells (Kimura *et al.*, 1994), the mitotic topo IIβ exhibits a markedly reduced electrophoretic mobility (apparent molecular mass ≈190 kDa) on SDS gels relative to the asynchronous topo IIβ, an observation confirmed by immunodetection of a mixture of asynchronous and M phase lysates (lane 3). It is unlikely that this difference is an artifact of the demecolcine block since it was also observed in M phase cells obtained by mitotic shakeoff (data not shown). We treated lysates with alkaline phosphatase to see if the change in mobility was due to phosphorylation. Lanes 4 and 5 (Figure 3) show asynchronous and mitotic cell lysates, respectively, that were incubated for 30 min at 37 °C in the absence of alkaline phosphatase (controls). Treatment with the phosphatase had no apparent effect on the electrophoretic mobility of asynchronous topo IIβ (or topo IIα; lanes 6 and 7). Lanes 8 and 9 of Figure 3 show mitotic cell lysates treated with phosphatase. The β-isoform in the control sample (lane 5) has decreased electrophoretic mobility, whereas the phos-

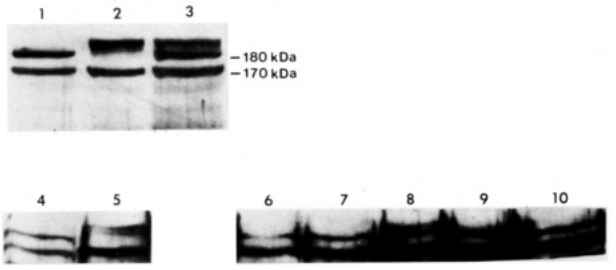

FIGURE 3: Topo IIβ is phosphorylated in mitosis. Cell lysates obtained from asynchronous and mitotic populations of CHO cells were immunoblotted with a topo II antiserum described previously (Kaufmann *et al.*, 1991). Lane 1, 100 μg of asynchronous lysate protein; lane 2, 100 μg of M phase lysate protein; lane 3, 50 μg of each asynchronous and M phase lysate protein. Asynchronous and mitotic cell lysates were also treated with calf intestine alkaline phosphatase. Lanes 4, 6, and 7, asynchronous lysates from 5×10^6 cells (each) of the untreated control (lane 4) or lysates treated with 10 units (lane 6) or 100 units (lane 7) of phosphatase. Lanes 5, 8, and 9, mitotic lysates from 5×10^6 cells (each) of the untreated control (lane 5) or lysates treated with 10 units (lane 8) or 100 units (lane 9) of phosphatase. Lane 10, a mixture of half each from duplicate samples of lanes 7 and 9.

phatase-treated lysates show topo IIβ migrating at apparent molecular masses intermediate between untreated M phase topo IIβ and asynchronous topo IIβ (after treatment with 10 units) or migrating at the apparent molecular mass of the asynchronous topo IIβ (after treatment with 100 units). The intermediate forms observed (in three separate experiments) with the 10-unit treatment are presumed to be partially dephosphorylated topo IIβ. Lane 10 of Figure 3 is a mixing experiment of asynchronous and mitotic phosphatase-treated lysates showing that the 100-unit treatment of the mitotic β-isoform does in fact restore its apparent molecular mass to that of the asynchronous topo IIβ. It is possible that the treatment of the lysates with the phosphatase causes activation of some unknown enzyme by dephosphorylation, and this activated enzyme then removes the modification on the mitotic topo IIβ. We believe this is unlikely since the same results shown in Figure 3 were obtained when the lysates were made 6% in SDS and boiled for 5 min prior to treatment with the phosphatase (data not shown). It thus appears that the mitotic topo IIβ has a retarded electrophoretic mobility due to phosphorylation.

DISCUSSION

We have investigated qualitative properties of topo IIα and topo IIβ as they vary between interphase and mitosis. Previously, topo IIα has been shown to be phosphorylated *in vivo* in a cell cycle-dependent fashion (Heck *et al.*, 1989; Cardenas *et al.*, 1992; Saijo *et al.*, 1992; Burden *et al.*, 1993), with the distribution of phosphate among multiple sites varying between G_1 and M phase in yeast (Cardenas *et al.*, 1992). CHO topo IIα is primarily phosphorylated on a seryl residue throughout the cell cycle with a minor phosphothreonine occurring in mitosis (Burden *et al.*, 1993). Except for these previous two experiments, there have been no other published accounts of sites of phosphorylation of topo II during the cell cycle. The results herein demonstrate that topo IIα has sites of phosphorylation that are unique to interphase and mitosis. The sites of phosphorylation of topo IIα present only in interphase or only in mitosis would prove interesting to investigate in order to determine their effect(s) on the function of the enzyme, especially since two recent reports (Ganapathi *et al.*, 1993; Ritke *et al.*, 1994) suggest that a perturbation in the phosphorylation of topo IIα may be involved in the resistance of human leukemia cells to VP-16 and *m*-AMSA.

Topo IIβ has an altered apparent molecular mass in mitosis (Figure 3), and it is likely that the modification responsible for this is phosphorylation. This is the first report to show that topo IIβ is posttranslationally modified *in vivo* in a cell cycle-dependent manner; one other study has suggested topo IIβ is a phosphoprotein (Taagepera *et al.*, 1993). Immunoreactive protein levels of topo IIβ have been shown to remain constant during the cell cycle (Woessner *et al.*, 1991; Kimura *et al.*, 1994), with an altered β-isoform observed in mitosis (Kimura *et al.*, 1994). Topo IIβ may localize to the nucleolus (Negri *et al.*, 1992), and it is interesting to note that topo IIβ is qualitatively different in mitosis, that phase of the cell cycle in which the nucleolus is disassembled. It is tempting to speculate that the phosphorylation of topo IIβ in mitosis may be involved in the stabilization of the enzyme during nucleolar disassembly.

Qualitative differences exist between interphase and mitotic forms of topo II in CHO cells. Our experiments show that topo IIα has interphase- and mitosis-specific sites of phosphorylation and suggest that topo IIβ is posttranslationally modified *in vivo* and is hyperphosphorylated in mitosis.

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