Cell-cycle-dependent phosphorylation and activity of Chinese-hamster ovary topoisomerase II

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Cell-cycle-dependent protein levels and phosphorylation of DNA topoisomerase II in relation to its catalytic and cleavage activities were studied in Chinese-hamster ovary cells. Immunoreactive topoisomerase II protein levels were maximal in G_2-phase cells, intermediate in S- and M-phase cells, and minimal in a predominantly G_1-phase population. When the phosphorylation of topoisomerase II *in vivo* was corrected for differences in specific radioactivity of intracellular ATP, the apparent phosphorylation of S- and M-phase topoisomerase II was altered significantly. Relative phosphorylation *in vivo* was found to be greatest in M-phase cells and decreased in the other populations in the order: S > G_2 > asynchronous. Phosphoserine was detected in every phase of the cell cycle, with a minor contribution of phosphothreonine demonstrated in M-phase cells. Topoisomerase II activity measured *in vivo* as 9-(4,6-*O*-ethylidene-β-D-glucopyranosyl)-4′-demethylepipodophyllotoxin (VP-16)-induced DNA double-strand breaks (determined by neutral filter elution) increased in the order: asynchronous < S < G_2 < M. Topoisomerase II cleavage activity, assayed *in vitro* as the formation of covalent enzyme–DNA complexes, was lowest in S phase, intermediate in asynchronous and G_2-phase cells, and maximal in M phase. Topoisomerase II decatenation activity was 1.6–1.8-fold greater in S-, G_2- and M-phase populations relative to asynchronous cells. Therefore DNA topoisomerase II activity measured both *in vivo* and *in vitro* is maximal in M phase, that phase of the cell cycle with an intermediate level of immunoreactive topoisomerase II but the highest level of enzyme phosphorylation. The discordance between immunoreactive topoisomerase II protein levels, adjusted relative phosphorylation, catalytic activity, cleavage activity and amino acid residue(s) modified, suggests that the site of phosphorylation may be cell-cycle-dependent and critical in determining catalytic and cleavage activity.

INTRODUCTION

DNA topoisomerases are ubiquitous enzymes which interconvert topological isomers of DNA. Type II topoisomerases (EC 5.99.1.3) are homodimeric ATP- and Mg^{2+}-dependent nuclear enzymes, the mechanism of which involves: (1) double-stranded DNA binding; (2) sequential cleavage of the DNA strands with covalent attachment of the 5′ end of each of the cleaved strands to a tyrosyl residue of a topoisomerase II monomer; (3) passage of a second DNA duplex through the gap in the cleaved DNA; (4) re-ligation of the cleaved DNA; and (5) ATP-dependent enzyme turnover (Osheroff et al., 1991). The functions of topoisomerase II *in vivo* are likely to involve roles in DNA replication (Brill et al., 1987), transcription (White and Preston, 1988), meiosis (Rose et al., 1990), recombination (Christman et al., 1988), and DNA repair (Hickson et al., 1990). Topoisomerase II is known to be essential for chromosome condensation and separation of daughter chromosomes during mitosis (Uemura et al., 1987; Downes et al., 1991). This enzyme is also known to be a structural component of both mitotic chromosomal scaffolds (Earnshaw et al., 1985) and the interphase nuclear matrix (Berrios et al., 1985).

Topoisomerase II has also been found to be the target for a variety of anti-neoplastic agents (Ross and Bradley, 1981). These drugs appear to exert their cytotoxic effect by inhibiting re-ligation of the cleaved DNA (Osheroff, 1989; Robinson and Osheroff, 1990), resulting in an increase in apparent DNA damage. One proposed mechanism of resistance to topoisomerase II agents involves altered modulation of topoisomerase II activity, perhaps a change in the phosphorylation state. Phosphorylation of topoisomerase II *in vitro* by various kinases has been shown to increase the activity of the enzyme (as measured by relaxation of supercoiled pBR322 DNA) from organisms such as sponge, *Drosophila*, quail and mouse (Ackerman et al., 1985; Rottmann et al., 1987; Schroeder et al., 1989; Saijo et al., 1990). Incorporation of ^{32}P into topoisomerase II occurs in a number of organisms (Ackerman et al., 1988; Heck et al., 1989; Matthes et al., 1990; Saijo et al., 1990; Ganapathi et al., 1991; Kroll and Rowe, 1991; Takano et al., 1991; Cardenas et al., 1992), and studies suggest that casein kinase II and protein kinase C may be involved in modulating topoisomerase II activity (Sahyoun et al., 1986; Rottmann et al., 1987; Ackerman et al., 1988; Darkin-Rattray and Ralph, 1991; Cardenas et al., 1992; DeVore et al., 1992). Topoisomerase II activity varies across the cell cycle (Chow and Ross, 1987; Estey et al., 1987; Markovits et al., 1987), and ^{32}P incorporation into topoisomerase II *in vivo* has been shown to increase (after correcting for changes in topoisomerase II protein levels) as cells progress from G_1 to G_2/M (Heck et al., 1989; Saijo et al., 1990), or specifically in M phase (Cardenas et al., 1992). One study alone has distinguished G_2 and M phases when investigating topoisomerase II cleavage and catalytic activity (Estey et al., 1987), and a single investigation

Abbreviations used: *m*-AMSA, 4′-(9-acridinylamino)-methanesulphon-*m*-anisidide; CHO, Chinese-hamster ovary; ECL, enhanced chemiluminescence; kDNA, kinetoplast DNA; αMEM, α Minimal Essential Medium; PVDF, poly(vinylidene difluoride); VP-16, 9-(4,6-*O*-ethylidene-β-D-glucopyranosyl)-4′-demethylepipodophyllotoxin.

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has addressed sites of phosphorylation across the cell cycle (Cardenas et al., 1992). The work performed in this paper examines the phosphorylation state of topoisomerase II and its effect on cell-cycle-dependent catalytic and cleavage activities of the enzyme. In addition, the phosphorylation of topoisomerase II is corrected for the differences in specific radioactivity of the intracellular ATP pools, and the topoisomerase II assays are compared between chemically synchronized cells versus cell populations from centrifugal elutriation.

EXPERIMENTAL

Materials

Wild-type Chinese-hamster ovary (CHO) cells were grown in monolayer at 37 °C in α Minimal Essential Medium (αMEM; Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 5% heat-inactivated fetal-bovine serum (Gibco) in the presence of 5% CO_2. Phosphate-free αMEM (Gibco) was also supplemented with 5% heat-inactivated fetal bovine serum. Penicillin (100 i.u./ml) and streptomycin (100 μg/ml) were added to both types of media. Sigma Chemical Co. (St. Louis, MO, U.S.A.) supplied thymidine, aphidicolin, demecolcine, phosphotyrosine, phosphoserine, phosphothreonine, the luciferase assay kit, cellulose acetate and polyethyleneimine–cellulose plates, ninhydrin, aprotinin, leupeptin, chymostatin, pepstatin A, antipain, 9-(4,6-*O*-ethylidene-β-D-glucopyranosyl)-4′-demethylepipodophyllotoxin (VP-16), BSA and Nonidet P-40. $H_3{}^{32}PO_4$ (230–246 Ci/μmol) and [α-^{32}P]dATP (5 Ci/μmol) were obtained from New England Nuclear Research Products (Boston, MA, U.S.A.). ICN Radiochemicals (Irvine, CA, U.S.A.) supplied [*methyl*-^{3}H]thymidine (35 Ci/mmol) and [2-^{14}C]thymidine (50 mCi/mmol). Immobilon-P poly(vinylidene difluoride) (PVDF) membrane was supplied by Millipore Corp. (Bedford, MA, U.S.A.), and nitrocellulose was obtained from Schleicher and Schuell (Keene, NH, U.S.A.). Donkey anti-(rabbit horseradish peroxidase) antiserum was supplied by Amersham Corp. (Arlington Heights, IL, U.S.A.). Pharmacia–LKB Biotechnology AB (Uppsala, Sweden) supplied Protein A–Sepharose CL4B.

Cell synchrony

S- and G_2-phase CHO-cell populations were obtained by a modification of a thymidine/aphidicolin double block (Heintz et al., 1983). Exponential-phase CHO cells were treated with 2 mM thymidine for 12 h, incubated in fresh medium for 6 h, and finally exposed to aphidicolin (5 μg/ml) for 12 h. Cells harvested at 3 h after removal of aphidicolin were > 75% S phase, and those at 6 h were > 80% G_2 phase. M-phase cells were obtained by harvesting the non-adherent cells from a culture exposed to demecolcine (0.2 μg/ml for 8 h) applied at 5 h after release of the thymidine/aphidicolin double block. Cell-cycle distribution was determined by flow-cytometric analysis of 10000 cells on a FACScan instrument (Becton Dickinson, San Jose, CA, U.S.A.) or by estimation of mitotic index as previously described (Worton and Duff, 1979); 200 cells were scored for each mitotic-index determination. Cells were synchronized by centrifugal elutriation using a Beckman JE-6B elutriator rotor (with a standard 4.2 ml chamber) and 3×10^8 cells per run. The rotor was run at 2000 rev./min and the cells were loaded at a flow rate of 8 ml of cold buffer A (150 mM NaCl, 5 mM potassium phosphate, pH 7.4)/min. Nine fractions each of 100 ml were collected, beginning at a flow rate of 18.5 ml/min, and increasing by 1.5 ml/min each to collect fractions 2, 3, 8 and 9, and then by 0.75 ml/min each to collect fractions 4, 5, 6 and 7. Cell-cycle distribution was determined by flow cytometry.

Metabolic labelling with ^{32}P

Monolayer exponential-phase CHO cells (9×10^6 per 75 cm^2 flask) were washed with 2×5 ml of phosphate-free αMEM and incubated for 5 min at 37 °C in 2 ml of phosphate-free αMEM. Cells were pulse-labelled with 1 mCi of $H_3{}^{32}PO_4$/ml for 30 min at 37 °C and harvested by treatment with trypsin. Non-adherent (M-phase) CHO cells were centrifuged at 250 *g* for 5 min at room temperature, washed with 2×5 ml of phosphate-free αMEM containing demecolcine (0.2 μg/ml), resuspended in 2 ml of phosphate-free αMEM containing demecolcine, and returned to the flask from which they were taken (the adherent cells in the flask were washed as above with demecolcine included in the medium). The flask containing both adherent and non-adherent cells was incubated for 5 min at 37 °C and pulse-labelled as above. Labelling of synchronous cells was carried out during the last 30 min of the synchronization.

Cell lysis

Exponential-phase CHO cells were pelleted at 900 *g* for 10 min at 4 °C, washed with 2×5 ml of cold buffer A, and finally lysed by resuspension in cold lysis buffer [50 mM Tris, 350 mM NaCl, 0.1% (v/v) Nonidet P-40, 5 mM Na_2EDTA, 50 mM NaF, pH 7.4] containing protease inhibitors (2 mM phenylmethanesulphonyl fluoride and 20 μg/ml each of antipain, aprotinin, chymostatin, leupeptin and pepstatin A) and 0.1 mM Na_3VO_4 (for ^{32}P-labelled cells). Cells were lysed at $(0.5–2.0) \times 10^4$ cells/μl of lysis buffer, incubated on ice for 30 min, and then centrifuged at 13000 *g* for 5 min at 4 °C. Supernatants were stored at −70 °C (^{32}P-labelled cells) or were immediately assayed for topoisomerase II activity (non-labelled cells). Variation of total cellular protein during the cell cycle was determined by dividing the total amount of lysate protein from a lysis experiment by the number of cells lysed.

Immunoprecipitation of topoisomerase II

The rabbit antiserum generated against recombinant HeLa topoisomerase II has been described previously (Sullivan et al., 1989). Topoisomerase II was immunoprecipitated from whole cell lysates by using 5 μl of topoisomerase II antiserum per 100 μg of lysate protein. This amount of antiserum was determined to be optimal by titrating the amount of antiserum in immunoprecipitation assays until no immunoreactive topoisomerase II remained in the supernatant. The lysate/antiserum mixture was incubated at 4 °C for 1 h. Then 10 mg of pre-hydrated Protein–A Sepharose (in 100 μl of lysis buffer containing 2 mM phenylmethanesulphonyl fluoride) was added (per 100 μg of lysate protein) and the mixture was rocked at 4 °C for 20 min. Protein A–Sepharose–topoisomerase II complexes were pelleted at 13000 *g* for 30 s at 4 °C and washed with 3×1 ml of cold lysis buffer containing 2 mM phenylmethanesulphonyl fluoride. The immunoprecipitates were resuspended in 75 μl of SDS/PAGE sample-loading buffer (4% SDS, 200 mM dithiothreitol and 20% glycerol) and boiled for 5 min. The Sepharose was removed by centrifugation at 13000 *g* for 10 s, and 75 μl of the resulting supernatant was subjected to SDS/PAGE on a 7.5% gel (Laemmli, 1970).

Western-blot and ^{32}P detection

Proteins were electrophoretically transferred from SDS/PAGE gels to nitrocellulose (or PVDF for phosphoamino acid analysis)

in a Trans-Blot cell (Bio-Rad Laboratories, Richmond, CA, U.S.A.) at 1 °C overnight at 70 V in 25 mM Tris/192 mM glycine/20% methanol, pH 6.5. Electroblotted proteins were detected by enhanced chemiluminescence (ECL) using the ECL Western-blot detection system (Amersham). The primary antibody used with the ECL system was the anti-topoisomerase II antiserum described above and the secondary antibody was donkey anti-(rabbit horseradish peroxidase). After immunodetection of topoisomerase II, the blots were stripped of antibodies by placing them in 75 ml of stripping buffer (0.2 M glycine, 0.5 M NaCl, 0.02% NaN_3, pH 2.8) for 5 min, followed by two 15 min washes with 100 ml of TBS (20 mM Tris, 500 mM NaCl, pH 7.5). The blots were air-dried and exposed to film at −70 °C to detect ^{32}P. Control samples of unlabelled immunoprecipitates were also run to verify that a residual ECL signal did not contribute to the ^{32}P signal.

ATP pool assays

Specific radioactivity of ATP was determined by labelling cells as described above or mock-treating parallel flasks with an equivalent amount of H_3PO_4. Cells from both labelled and control flasks were added to releasing reagent from the Sigma luciferase assay kit to release ATP from the cells. Portions from labelled samples were chromatographed on polyethyleneimine–cellulose plates as previously described (Colby and Edlin, 1970), with the first dimension as 0.2 M LiCl for 2 min, then 1.0 M LiCl for 6 min, and finally 1.6 M LiCl to 13 cm from the origin. The second dimension was 0.5 M formic acid/0.5 M sodium formate for 0.5 min, then 2 M formic acid/2 M sodium formate for 2 min, and finally 4 M formic acid/4 M sodium formate to 13 cm from the origin. Spots corresponding to ATP were excised and counted for radioactivity in a scintillation counter to determine ATP-associated c.p.m./cell. The control samples were used to determine pmol of ATP/cell with the luciferase assay as described by the manufacturer.

Phosphoamino acid analysis

Phosphoamino acids were separated and detected by the method of Hunter and Sefton (1980) as modified by Kamps and Sefton (1989). Immunoprecipitated topoisomerase II was electrophoresed and transferred to a PVDF membrane. The topoisomerase II was detected by autoradiography, excised, and hydrolysed on the membrane with 6 M HCl at 110 °C for 1 h under N_2. Thin-layer electrophoresis was performed on cellulose acetate plates in pH 1.9 buffer (2.5% formic acid, 7.8% acetic acid, both v/v) for 45 min at 1.5 kV in the first dimension and pH 3.5 buffer (0.5% pyridine, 5% acetic acid, both v/v) for 30 min at 1 kV in the second dimension. Unlabelled phosphoamino acid standards were added to the samples and detected with ninhydrin, and [^{32}P]phosphoamino acids were detected by autoradiography at −70 °C.

Topoisomerase II catalytic activity

The catalytic activity of topoisomerase II present in whole cell lysates was measured by the decatenation of ^3H-labelled kinetoplast DNA ([^3H]kDNA) networks isolated from *Crithidia fasciculata* as previously described (Sullivan et al., 1989) except that the final salt concentration was 100 mM NaCl, and the assay samples contained 0.5 μg of [^3H]kDNA. Various amounts of lysate (0.1–1.0 μg) were used.

Precipitation of covalent topoisomerase II–DNA complexes

Topoisomerase II cleavage activity was quantified by K^+/SDS precipitation of covalent complexes as previously described (Liu et al., 1983). All assays of cell lysates were diluted with lysis buffer to a final NaCl concentration of 105 mM. Assays which included a topoisomerase II inhibitor contained 100 μM VP-16.

Neutral filter elution

Drug-induced DNA double-strand breaks were measured as previously described (Bradley and Kohn, 1979). Control flasks were treated with solvent alone, and drug-induced DNA damage was quantified after 1 h exposure to 25 μM VP-16.

Miscellaneous techniques

Protein concentrations were determined by using the Bio-Rad protein assay with BSA as the standard. Densitometry was performed on a Bio-Rad model 620 video densitometer. Autoradiography was performed using Kodak X-Omat AR film (Eastman Kodak Corp., Rochester, NY, U.S.A.), pre-flashed as previously described (Voytas, 1988). Exposures were in Kodak X-Omatic cassettes with intensifying screens.

Calculations

Specific relative phosphorylation (Table 2) was calculated by dividing the densitometric signal for ^{32}P by the densitometric signal for immunoreactive topoisomerase II protein level. The asynchronous population was assigned an arbitrary value of 1.00, and the values for the other populations were then scaled to this reference. The amount of topoisomerase II/cell was calculated by multiplying the relative topoisomerase II protein level (which was determined per 100 μg of lysate protein) by the relative total lysate protein per cell. Relative phosphorylation was corrected for differences in specific radioactivity of ATP in each cell population (to give adjusted relative phosphorylation; Table 3) by dividing relative phosphorylation by c.p.m./pmol of ATP for each population, and then multiplying by the c.p.m./pmol of ATP for the asynchronous population. The specific radioactivity of ATP (Table 3) was calculated by first determining ATP-associated c.p.m./cell and pmol of ATP/cell, then dividing c.p.m./cell by pmol/cell.

Statistics

Statistical analysis was performed on four sets of data: c.p.m./pmol of ATP, total lysate protein/cell, immunoreactive topoisomerase II protein levels, and topoisomerase II phosphorylation *in vivo*. An initial two-way analysis of variance, with experiments treated as blocks, was highly significant on each set (maximum $P < 0.02$). Scheffé contrasts were used to determine specific significant differences between means. Additional analyses included scattergrams, boxplots and correlation analysis.

RESULTS

Cell synchrony

S- and G_2-phase populations of CHO cells were obtained by synchronization with a thymidine/aphidicolin double block; M-phase populations were obtained by harvesting the non-adherent cells resulting from a thymidine/aphidicolin double block followed by a demecolcine block. The cell-cycle distribution of each

Table 1 Cell-cycle distribution of asynchronous and synchronous populations of CHO cells

Data are presented as means ± S.E.M. ($n = 4$ independent synchronization experiments): n.d., not determined.

	Percentage of cells in each phase			
Cell population	G_1	S	G_2	M
Asynchronous	58 ± 2.6	25 ± 2.0	18 ± 0.8*	n.d.
S	4 ± 0.3	78 ± 3.0	19 ± 3.0*	n.d.
G_2	6 ± 2.3	9 ± 4.5	85 ± 6.7	< 1†
M	n.d.	n.d.	n.d.	94 ± 0.4†

* Measured as G_2/M by flow cytometry.
† Determined by mitotic index.

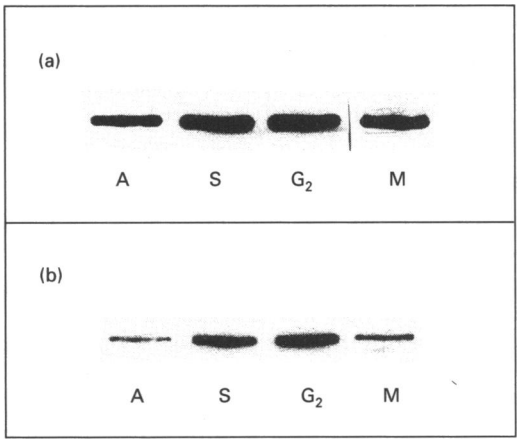

Figure 1 Detection of immunoreactive and ^{32}P-labelled topoisomerase II

(**a**) Western blot of topoisomerase II immunoprecipitates (from 100 μg of lysate protein) of asynchronous (A), S-, G_2- and M-phase cell lysates. (**b**) ^{32}P autoradiogram of topoisomerase II immunoprecipitates (also from 100 μg of lysate protein) from the same phases of the cell cycle as above.

population (as well as asynchronous cells) was determined by flow cytometry or mitotic-index determination (Table 1). The asynchronous population of cells was 58% G_1 phase, the S population of cells was 78% S phase, the G_2 population was 85% G_2 phase, and the M population was 94% M phase. Cells obtained by centrifugal elutriation (results not shown) were predominantly G_1 phase in fractions 1 and 2 (90% and 65% respectively), predominantly S phase in fractions 3 and 4 (55% and 60% respectively), and predominantly G_2 phase in fractions 5–9 (45–70%).

Cell-cycle-dependent topoisomerase II phosphorylation

Asynchronous and synchronous populations of CHO cells were metabolically labelled with [^{32}P]P_i and lysed. Topoisomerase II was quantitatively immunoprecipitated from equal amounts of lysate protein, subjected to SDS/PAGE on a 7.5% gel, and blotted to detect immunoreactive topoisomerase II and ^{32}P-labelled topoisomerase II. A Western blot developed with the ECL method is shown in Figure 1, as is a ^{32}P-labelled autoradiogram. Relative topoisomerase II protein levels were

Table 2 Variation of topoisomerase II protein levels and relative phosphorylation across the cell cycle

Autoradiogams represented by Figure 1 were quantified by densitometry and referenced to asynchronous-cell levels. Relative phosphorylation levels were determined by dividing the ^{32}P densitometric value by the Western-blot densitometric value and then comparing this with that for asynchronous cells, which was assigned a value of 1. Data are given as means ± S.E.M. ($n = 5$ independent labelling experiments).

	Relative topoisomerase II	
Cell population	Protein levels	Phosphorylation levels
Asynchronous	1.00†	1.00‡
S	1.27 ± 0.06*	1.28 ± 0.06‡
G_2	1.65 ± 0.19*	1.44 ± 0.18‡
M	1.26 ± 0.11†	0.95 ± 0.15

* Two-way analysis of variance (using phases blocked by experiments) for asynchronous, S- and G_2-phase cells showed significant differences due to phases at $P \leqslant 0.02$.
† Statistical analysis demonstrates $P = 0.058$ relative to asynchronous cells.
‡ Two-way analysis of variance for asynchronous, S and G_2 statistically significant at $P \leqslant 0.001$.

quantified by densitometry; the asynchronous topoisomerase II protein level is given a value of 1. Relative topoisomerase II phosphorylation was determined by dividing the intensity of the ^{32}P signal (obtained from densitometry) by the intensity of the protein signal, and then assigning the asynchronous phosphorylation level of value of 1. These data are shown in Table 2. Topoisomerase II protein levels increase as cells traverse S phase, reach a maximum of 1.65 in G_2 phase, and decrease to levels similar to those of S-phase cells in mitosis (statistical treatment by two-way analysis of variance shows $P \leqslant 0.02$). Qualitatively similar results (not shown) were obtained with cells synchronized by centrifugal elutriation. It should be noted that these changes in topoisomerase II protein levels during the cell cycle are based on equal amounts of lysate protein, whereas total cellular protein increases relative to asynchronous cells during the cell cycle. Cells in S phase have a relative total protein content of 1.57 ± 0.17-fold more total protein than asynchronous cells, cells in G_2 phase have 1.74 ± 0.21-fold more total protein than asynchronous cells, and M-phase cells have 1.27 ± 0.14-fold ($P \leqslant 0.001$ by two-way analysis of variance) more total protein than asynchronous cells (results not shown). Thus on a per-cell basis the relative topoisomerase II protein levels are S = 1.99, G_2 = 2.87 and M = 1.60. Relative protein levels of the β-isoform of topoisomerase II (180 kDa) were not found to vary significantly with respect to the cell cycle (results not shown).

Relative phosphorylation of topoisomerase II follows the same trend after correction of the observed ^{32}P autoradiogram signals for the variation in topoisomerase II protein levels/μg of total cellular protein (i.e. the difference in phosphorylation actually shown in Figure 1b is partially due to the variation in topoisomerase II protein levels which is represented in Figure 1a). Maximum relative phosphorylation of 1.44 occurs in G_2 phase, whereas a decrease to the asynchronous level of phosphorylation occurs in M phase ($P \leqslant 0.001$ by two-way analysis of variance). These results have been confirmed in cells synchronized by centrifugal elutriation (results not shown).

ATP pool assays

ATP-associated c.p.m./cell was determined by t.l.c., and pmol of ATP/cell was determined by the luciferase assay. From these

Table 3 Specific radioactivity of ATP in each cell population

Data are expressed as means ± S.E.M. ($n = 3$ labelling experiments). See Experimental section for details.

Cell population	Sp. radioactivity (c.p.m./pmol of ATP)	Adjusted relative phosphorylation
Asynchronous	3990 ± 270*	1.00
S	2060 ± 53*	2.48
G_2	3820 ± 460	1.50
M	718 ± 190*	5.28

* One-way analysis of variance with pooled data yielded $P \leqslant 0.0001$.

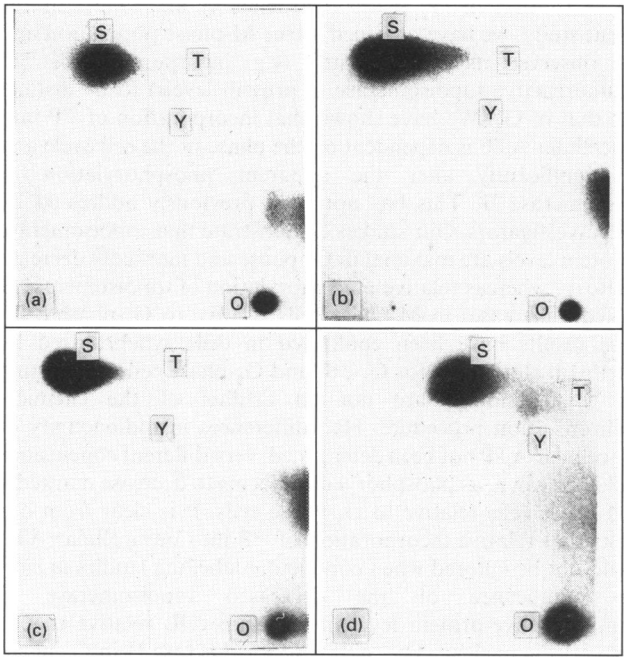

Figure 2 Phosphoamino acid analysis of immunoprecipitated topoisomerase II throughout the CHO cell cycle

Immunoprecipitated ^{32}P-labelled topoisomerase II was transferred to Immobilon-P and hydrolysed *in situ* with 6 M HCl. The hydrolysates were separated by two-dimensional thin-layer electrophoresis along with unlabelled phosphoamino acid standards (S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine); O, origin. Exposure times were equal for all of the autoradiograms shown. (**a**) Asynchronous; (**b**) S phase; (**c**) G_2 phase; (**d**) M phase.

data the specific radioactivity of ATP was calculated for each population of cells (Table 3). The c.p.m./pmol of ATP was not significantly different in G_2-phase cells relative to asynchronous cells, but was substantially less in S-phase cells and M-phase cells ($P \leqslant 0.0001$ by one-way analysis of variance). Also shown in Table 3 are the values for the specific relative phosphorylation corrected for these differences in the radioactivity of the ATP pools. The phosphorylation of topoisomerase II increases in the order: asynchronous < G_2 < S < M. The calculated adjusted

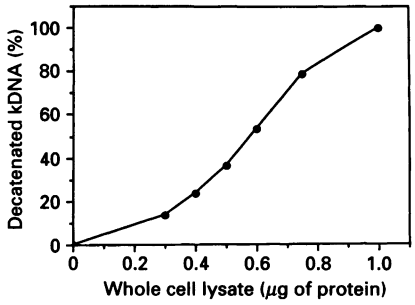

Figure 3 Topoisomerase II catalytic activity present in whole cell lysates

A catalytic-activity assay is shown with a lysate prepared from asynchronous cells. Activity was measured as the decatenation of [^{3}H]kDNA networks.

Table 4 Catalytic activity of topoisomerase II across the cell cycle

One unit of activity is the amount of asynchronous lysate protein required to decatenate 50% of 0.5 μg of kDNA in 30 min at 30 °C. Data were obtained from experiments represented in Figure 4 and are presented as means ± S.E.M. (n = number of independent lysate preparations).

Cell population	Enzyme activity (units/μg of lysate protein)
Asynchronous	1.73 ± 0.15 ($n = 6$)
S	2.70 ± 0.37 ($n = 7$)
G_2	2.80 ± 0.24 ($n = 5$)
M	3.13 ± 0.10 ($n = 4$)

relative phosphorylation is implied to be statistically significant by the fact that the values used in the calculations have been shown to be statistically significant.

Phosphoamino acid analysis

Immunoprecipitated topoisomerase II was transferred to PVDF, the ^{32}P-labelled band corresponding to topoisomerase II was excised and hydrolysed, and the phosphoamino acids were detected by thin-layer electrophoresis (Figure 2). The major amino acid residue modified in each population is serine, with a faint phosphothreonine signal detected in mitotic cells.

Topoisomerase II catalytic activity

Whole-cell lysates were used in assays to measure decatenation of [^{3}H]kDNA. An assay with an asynchronous lysate is shown in Figure 3. One unit of topoisomerase II catalytic activity is defined as the amount of asynchronous lysate protein required to decatenate 50% of 0.5 μg of kDNA in 30 min at 30 °C. The specific catalytic activity was determined for each population of cells (Table 4). Decatenation activity is 1.73 units/μg of lysate protein in asynchronous cells, increases as cells go through S phase and G_2 phase, and reaches a maximum of 3.13 units/μg of lysate protein in M-phase cells. Differences in catalytic activity between S-, G_2- and M-phase cells do not appear to be significant, and thus phosphorylation *in vivo* reaches a maximum in the same phase as the catalytic activity (M phase), but does not correlate with the catalytic activity of topoisomerase II *in vitro* in S and G_2 phase.

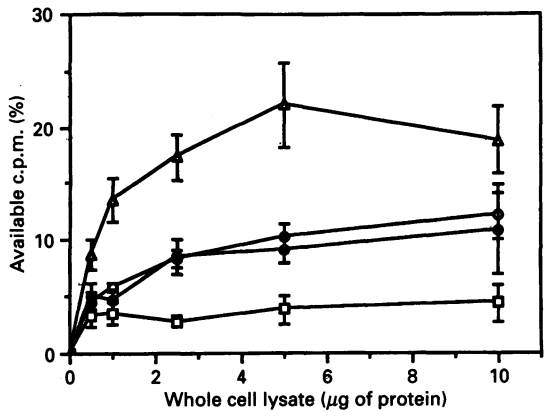

Figure 4 Cell-cycle-dependent topoisomerase II cleavage activity

Topoisomerase II–[^{32}P]DNA covalent complexes were formed in the presence of 100 μM VP-16 and precipitated with K^+/SDS. Results are expressed as percentage of available label (c.p.m.) precipitated versus the amount of lysate, and are presented as means ± S.E.M. ($n = 4$ independent lysate preparations for each population): ●, asynchronous; □, S phase; ○, G_2 phase; △, M phase.

Table 5 Cell-cycle-dependent VP-16-induced DNA double-strand breaks

Data are expressed as means ± S.E.M. ($n = 4$ independent drug treatments for each population). Drug-induced DNA damage was converted into rad equivalents by using a standard dose–response curve of radiation-induced DNA damage.

Cell population	Rad equivalents
Asynchronous	3100 ± 400
S	5700 ± 700
G_2	11000 ± 500
M	17600 ± 700

Topoisomerase II cleavage activity

Topoisomerase II–DNA covalent complexes formed in the presence of VP-16 were precipitated with K^+/SDS. The concentration of VP-16 was held constant (100 μM) and the amount of lysate for each cell population was varied (0.5–10 μg of lysate protein). Results are expressed as the percentage of total available [3′-^{32}P]pUC DNA precipitated for each amount of lysate (Figure 4). When data for 5 μg of lysate protein are used for purposes of comparison, covalent complex formation is comparable for the asynchronous and G_2-phase populations (10%), minimal for S-phase cells (4%), and at a maximum of 22.0% for M phase. Topoisomerase II cleavage activity *in vitro* is thus maximal in M phase, when phosphorylation is the greatest.

Drug-induced DNA damage

Synchronous and asynchronous cells were treated for 1 h with 25 μM VP-16, and the resulting DNA damage was quantified as DNA double-strand breaks by neutral filter elution. These experiments give a measure of topoisomerase II activity *in vivo*. The drug-induced DNA damage was converted into rad equivalents by using a standard dose–response curve of radiation-induced DNA damage (Table 5). Drug-induced DNA damage is lowest in asynchronous cells (3100 rad equivalents), increases as cells go through S and G_2 phases, and reaches a maximum of 17600 rad equivalents in M-phase cells. Thus, as observed with decatenation and cleavage activities, drug-induced DNA damage is greatest in M phase, when phosphorylation is greatest.

DISCUSSION

We have investigated cell-cycle-dependent topoisomerase II protein levels, phosphorylation and activity in mammalian cells and found the degree of phosphorylation to correlate directly with enzyme activity *in vivo* in M phase relative to asynchronous cells. Topoisomerase II protein levels and phosphorylation have been shown to be cell-cycle-dependent in a number of systems. These previous studies found either that both protein levels and phosphorylation of topoisomerase II increase as cells traverse the cell cycle, i.e. G_1 through S and then to a maximum in G_2/M (Chow and Ross, 1987; Heck et al., 1988, 1989; Kaufmann et al., 1991; Woessner et al., 1991; Saijo et al., 1992), or that G_1- and M-phase topoisomerase II protein levels are equivalent but topoisomerase II in M phase is 6–10-fold hyperphosphorylated relative to that in G_1 phase (Cardenas et al., 1992). In the present study, we have obtained a true M-phase population and have observed its characteristics (e.g. incorporation of ^{32}P, immunoreactive topoisomerase II protein levels) to be distinct from that of G_2. We have shown that incorporation of ^{32}P into intracellular ATP is dependent on the phase of the cell cycle and can significantly alter the apparent phosphorylation of topoisomerase II. This has not been previously addressed by other investigators. Our studies demonstrate that topoisomerase II protein levels are maximal in G_2 phase and markedly decrease in mitosis, whereas relative phosphorylation of topoisomerase II markedly increases in M-phase cells relative to G_2-phase cells. These results have been confirmed in cells synchronized by centrifugal elutriation for G_1-, S- and G_2-phase cells, suggesting that these findings are not an artifact of the chemical synchronization procedure. Had differences in radioactivity of intracellular ATP not been determined, very different conclusions would be drawn, as phosphorylation seems to decrease markedly in M-phase cells relative to G_2-phase cells. It is clear from our results that relative incorporation of ^{32}P into intracellular ATP should not be ignored when conducting labelling studies *in vivo*. The significance of the decreased topoisomerase II immunoreactive-protein level in M-phase cells relative to G_2-phase cells is unclear. The 'extra' topoisomerase II in G_2 phase is likely to have a G_2-specific function (perhaps involved in early events in the condensation of chromatin), whereas the M-phase topoisomerase II is probably the minimum required to separate daughter chromosomes at mitosis.

Topoisomerase II is phosphorylated on a serine residue throughout the cell cycle, with a minor phosphorylation occurring on a threonine residue in mitosis. Other researchers have also observed phosphorylation *in vivo* of topoisomerase II in exponential-phase asynchronous cells on serine residues in *Drosophila* Kc cells (Ackerman et al., 1988), mouse FM3A cells (Saijo et al., 1990), HeLa cells (Kroll and Rowe, 1991) and human epidermoid carcinoma KB cells (Takano et al., 1991). Two other studies note, in addition to phosphoserine, the modification *in vivo* of a threonine residue (Cardenas et al., 1992; Saijo et al., 1992). This is the first report to show a phosphothreonine modification occurring only in mitosis.

Topoisomerase II activity varies *in vivo* across the cell cycle when measured as VP-16-induced DNA damage in BALB/c 3T3 cells (Chow and Ross, 1987), VP-16- and 4′-(9-acridinylamino)-methanesulphon-*m*-anisidide (*m*-AMSA)-induced DNA–protein cross-links in NIH 3T3 cells (Markovits et al., 1987), and *m*-AMSA-induced DNA damage in HeLa cells (Estey et al.,

1987). These studies were discordant in that they found that topoisomerase II activity reaches a maximum in S phase (Markovits et al., 1987), G_2/M phase (Chow and Ross, 1987), or M phase (Estey et al., 1987). Cell-cycle-dependent topoisomerase II decatenation activity has also been measured *in vitro* in two of these systems (Estey et al., 1987; Markovits et al., 1987) and correlates with the observations of enzyme activity *in vivo*. However, cell-cycle-dependent phosphorylation was not investigated in these systems, but has been studied in Swiss 3T3 cells (Saijo et al., 1992), MSB-1 chicken lymphoblastoid cells (Heck et al., 1989), and yeast cells (Cardenas et al., 1992) and found to be greatest in G_2/M phase (Heck et al., 1989; Saijo et al., 1992) or specifically in M phase (Cardenas et al., 1992), although specific radioactivity of intracellular ATP was apparently assumed to be the same for different populations of cells in each of these systems. The lack of agreement seen between maximal topoisomerase II activity (S, G_2/M, or M) and phosphorylation (G_2/M or M) necessitates determining both in a single system. In the experiments reported herein, we observe no significant effect on topoisomerase II catalytic activity as cells traverse the cell cycle from S phase to M phase, but we can infer an increase in catalytic activity after the cells exit from G_1 phase (compare asynchronous catalytic activity with S-, G_2- and M-phase catalytic activity and note that asynchronous cells are a combination of these phases and predominantly G_1). This suggests the possibility that the phosphorylation occurring in G_1 phase is at a different site(s) from that occurring in the other phases. DNA cleavage activity (both *in vivo* and *in vitro*) was found to vary in a cell-cycle-dependent manner, but cleavage *in vivo* did not correlate with cleavage activity *in vitro* (compare Figure 4 and Table 5). Quantification of VP-16-induced DNA double-strand scission is the most relevant measure of topoisomerase II activity to correlate with the phosphorylation data, as both are occurring *in vivo*, whereas the correlation of activity *in vitro* with phosphorylation *in vivo* is likely to be affected by the artificial conditions of the assays. In contrast with our work and that cited previously, a study of C3H 10T1/2 mouse embryo fibroblast cells showed no cell-cycle variation in topoisomerase II activity as measured by PM2 DNA catenation (Tricoli et al., 1985).

Experiments *in vitro* suggest that phosphorylation of topoisomerase II increases enzyme activity, and we observed a correlation between relative topoisomerase II phosphorylation and activity (by all measures) in asynchronous and M-phase cells. The phosphorylation *in vivo* of topoisomerase II in S and G_2 phase does not correlate with measurements of activity. One possible explanation for the lack of correlation of topoisomerase II phosphorylation and activity in S and G_2 phase is that different amino acid residues are modified in different phases of the cell cycle, and the different sites modified have vastly different effects on activity. The presence of a faint but detectable phosphothreonine in mitosis supports the model of cell-cycle-dependent sites of phosphorylation. Also supporting this model are the recent findings that distinct sites of phosphorylation of topoisomerase II are modified to different degrees in G_1 phase, as opposed to M phase, in yeast (Cardenas et al., 1992), and that topoisomerase II can be phosphorylated *in vitro* simultaneously by protein kinase C and casein kinase II (DeVore et al., 1992), with such modification decreasing cleavage activity of the enzyme (hence a possible explanation for our observations of decreased topoisomerase II phosphorylation and increased cleavage activity *in vivo* in G_2-phase cells relative to S-phase cells). The lack of a direct correlation between topoisomerase II phosphorylation and protein levels with different activity assays in S and G_2 phases is an indication of the complexity of topoisomerase II regulation. It should be noted that cells appear to be most sensitive to the cytotoxic effects of topoisomerase II drugs in S phase, when drug-induced DNA damage and covalent complex formation appear to be at a minimum (Chow and Ross, 1987; Markovits et al., 1987). Topoisomerase II activity alone may not be sufficient for drug-induced cytotoxicity, but may require a particular subcellular environment which could itself be cell-cycle-dependent. Alternatively, a specific sub-population of topoisomerase II that mediates the effects of drugs may exist at a maximal level in S phase. Additional studies in this area are necessary to address the question of cell-cycle-dependent sites of phosphorylation, to identify the kinase(s) responsible for phosphorylation of topoisomerase II *in vivo* in CHO cells, and to determine the effect on enzyme activity of the subcellular localization of topoisomerase II.

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