Plk1-dependent Phosphorylation Regulates Functions of DNA Topoisomerase IIα in Cell Cycle Progression*

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Plk1 (Polo-like kinase 1) has been documented as a critical regulator of many mitotic events. However, increasing evidence supports the notion that Plk1 might also have functions outside of mitosis. Using biochemical fractionation and RNA interference approaches, we found that Plk1 was required for both G1/S and G2/M phases and that DNA topoisomerase IIα (topoIIα) was a potential target for Plk1 in both interphase and mitosis. Plk1 phosphorylates Ser1337 and Ser1524 of topoIIα. Overexpression of an unphosphorylatable topoIIα mutant led to S phase arrest, suggesting that Plk1-associated phosphorylation first occurs in S phase. Moreover, overexpression of the unphosphorylatable topoIIα mutant activated the ATM/R-dependent DNA damage checkpoint, probably due to reduced catalytic activity of topoIIα, and resulted in accumulation of catenated DNA. Finally, we showed that wild type topoIIα, but not the unphosphorylatable mutant, was able to rescue topoIIα depletion-induced defects in sister chromatid segregation, indicating that Plk1-associated phosphorylation is essential for the functions of topoIIα in mitosis.

The cell cycle entails a series of macromolecular events that lead to cell division and the production of two daughter cells, each maintaining genetic information identical to that of the parental cell. DNA replication occurs during the S phase of the cell cycle and the duplicated chromosomes are distributed into two daughter cells during mitosis or the M phase. Precise temporal control of the cell cycle ensures a high fidelity of chromosome duplication/segregation, which occurs only when all covalent DNA links between replicated sister chromatids have been removed. The enzyme that decatenates covalently interlinked DNA molecules to disentangle intertwined chromosomes is DNA topoisomerase II (1). There are two known isoforms of DNA topoisomerase II in mammalian cells, topoIIα and topoIIβ (1). It has been shown that the two isoforms are differentially regulated during cell cycle progression. Although the level of topoIIβ remains fairly constant across different phases of the cell cycle, topoIIα levels rise significantly in S phase, peak in G2/M phase, and then fall rapidly following mitosis (2). In addition, the phosphorylation of topoIIα and topoIIβ is also regulated throughout the cell cycle. Mammalian topoIIα and topoIIβ are hyperphosphorylated at mitosis, and several M phase-specific phosphorylation sites have been identified (2). In Chinese hamster ovary cells, distinct topoII phosphorylation sites have been observed in mitosis and interphase (3).

The role of phosphorylation in regulating topoIIα has been the subject of several publications, but no consistent pattern has emerged (2). In Drosophila, topoIIα was phosphorylated by a number of protein kinases, including casein kinase II, protein kinase C, and Cdc2 kinase. In all cases, phosphorylation stimulated enzyme activity (4). In budding yeast, it was reported that dephosphorylation of topoIIα resulted in a loss of catalytic activity, suggesting that at least some phosphorylation was required for its activity (5). However, studies with mammalian topoIIα yielded conflicting results. In one study, dephosphorylation of topoIIα by A-phosphatase treatment had essentially no effect on its decatenation activity (6). In a separate study, by mutating serine to alanine, Chikamori and colleagues showed that phosphorylation at Ser1106 in topoIIα positively regulated its enzymatic activity, and a kinase likely to be responsible for the phosphorylation was casein kinase II (7).

In addition to cyclin-dependent kinases, Plk1 (Polo-like kinase 1) has also emerged as a key regulator involved in many cell cycle-related events, such as centrosome maturation, bipolar spindle formation, and cytokinesis (8, 9). Sufficient evidence also indicates that Plk1 plays a critical role in chromosome segregation at the onset of anaphase. In budding yeast, Cdc5-associated phosphorylation of cohesin subunit Scc1 strongly enhances its cleavage by separase, which leads to sister chromatid separation (10). Using depletion experiments, Plx1, the Plk1 homolog in Xenopus, was shown to be required for cohesin displacement from chromosome arms in a phosphorylation-dependent manner (11). In human cells, cohesin dephosphorylation-mitotic delay can be rescued by inhibition of topoII, suggesting that the accumulation of catenations by topoII inhibition in preseparated sister chromatids may overcome the reduced tension arising from cohesin depletion (12). Similarly, Plk1-associated phosphorylation of PICH, a centromere protein, also causes it to dissociate from chromatin arms to centromeres and lead to the formation of DNA threads connecting sister kinetochores. Remarkably, these PICH-positive threads are exacerbated by the inhibition of topoII or cohesin, suggesting that they represent stretched centromeric...
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chromatin (13). The data accumulated so far are consistent with the model that topoII may act in the last step of sister kinetochore separation (13). In this paper, we show that topoIIα is also a Plk1 substrate both in vitro and in vivo, and the essential topoIIα functions during cell cycle progression might be regulated by Plk1-associated phosphorylation.

EXPERIMENTAL PROCEDURES

Reagents—The phosphohistone H3 antibody (06-570) and Cdc2 antibody (06-923) were from Upstate Biotechnology, Inc. The rabbit antibodies against TopoIIα and TopoIIβ were obtained from TopoGene. The mouse Plk1 antibody was ordered from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Ki-67 antibody was purchased from BD Transduction Laboratories.

Vector Construction—To specifically deplete endogenous topoIIα in mammalian cells, plasmid pBS/U6-topoIIα was constructed as previously described (14). The targeting sequence of human topoIIα (accession number NM_001067) was GGTTGAATTACGGCCCAAG, corresponding to 1242–1261 of the coding region relative to the first nucleotide of the start codon. Plasmid pBS/U6-topoIIα-1st half (sense strand) was used as a control vector. This control vector produces RNA that cannot form a hairpin structure to generate interfering RNA (RNAi). Plasmid pBS/U6-Plk1 was previously described (14).

Cell Culture and Synchronization—HeLa, U2OS, and hTERT-RPE1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 100 units mL−1 penicillin, and 100 units mL−1 streptomycin at 37 °C in 8% CO2. To synchronize HeLa cells, cells were treated with 2.5 mM thymidine for 16 h, released for 8 h, and then treated with thymidine a second time for 16 h. After two washes with phosphate-buffered saline (PBS), cells were cultured for different times as indicated in each experiment and harvested. Based on our experience, cells enter S phase after 4 h of release and accumulate in G2 phase after 8 h of release into normal medium. Most cells arrest at mitosis after 12 h of release in the presence of 100 ng mL−1 of nocodazole, and 13.5 h of release in the absence of nocodazole results in at least 50% of cells at telophase/cytokinesis. Alternatively, cells were treated with 0.3 mM mimosine for 20 h, 4 mM hydroxyurea for 24 h, or 200 ng mL−1 nocodazole for 12 h to arrest at G1, S, or M phase, respectively.

DNA Transfections—For phenotype analysis of gene depletion in randomly growing cells, HeLa cells were co-transfected with pBS/U6-topoIIα or pBS/U6-Plk1 and pBabe-puro at a ratio of 8:1 using GenePorter reagents. After 2 days of selection for transfection-positive cells with 2 μg mL−1 puromycin, floating cells were washed away with PBS, and the attached cells were incubated until harvesting for phenotypic analysis. To deplete topoIIα in well synchronized cells, cells were treated with thymidine for 16 h, co-transfected with pBS/U6-topoIIα and pBabe-puro, incubated for 8 h, and blocked with the second dose of thymidine in the presence of puromycin for 20 h. After synchronization/selection, the floating cells were removed, and the remaining cells were released into fresh medium for different times. To rescue the topoIIα depletion-induced phenotypes, cells growing on coverslips were co-transfected with pBS/U6-topoIIα and RNAi-resistant GFP-topoIIα (WT or Plk1 unphosphorylatable mutant) at a ratio of 5:1 and subjected to the thymidine block. Upon release into fresh medium for different times, cells were stained with 4’,6’-diamidino-2-phenylinodole (DAPI), and GFP-positive cells were analyzed.

Isolation of Nuclear and Chromosome-binding Fractions—Cytosolic, nuclear, and chromosome-binding fractions from cell lysates were prepared by using a Qproteome nuclear protein kit (Qiagen). Briefly, harvested cells were resuspended in lysis buffer supplemented with detergent solution NP. After centrifugation for 5 min, the supernatant was collected as the cytoplasmic fraction. The nuclei pellet was resuspended in nuclear protein lysis buffer NL and centrifuged for 5 min. After removing the supernatant, the nuclear pellet was resuspended in extraction buffer NX1. After a 10-min spin, the supernatant was collected as the soluble nuclear fraction. The insoluble pellet was resuspended in extraction buffer NX2 supplemented with benzonase and incubated for 1 h with gentle agitation. After another 10-min spin, the supernatant was collected as the chromosome-binding fraction.

Kinase Assay—Cdc2 was immunoprecipitated from cell lysates with a Cdc2 antibody and resuspended in TBMD buffer (50 mM Tris, pH 7.5, 10 mM MgCl2, 5 mM dithiothreitol, 2 mM EGTA, 0.5 mM sodium vanadate, 20 mM p-nitrophenyl phosphate) supplemented with 25 μM ATP and 50 μCi of [γ-32P]ATP. The reaction mixtures were incubated at 30 °C for 30 min in the presence of histone H1 as a substrate and resolved by SDS-PAGE. The gels were stained with Coomassie Brilliant Blue, dried, and subjected to autoradiography.

Mitotic Chromosome Spread—Chromosome spread analysis was performed as described (12). HeLa cells were transfected with GFP-topoIIα (WT or Plk1 unphosphorylatable mutant) and blocked at M phase with nocodazole treatment for 12 h. Mitotic cells were mechanically shaken off of plates, washed with PBS, and swollen in a hypotonic solution (75 mM KCl), followed by spreading using a 1000-rpm spin for 5 min. Spread cells were fixed by paraformaldehyde and subsequently stained with DAPI.

Topoisomerase IIα Activity Assay—TopoIIα enzymatic activity was assayed by measuring the decatenation of kinetoplast DNA (kDNA) as described (15). A standard assay was carried out in a total volume of 20 μl, including 50 mM Tris-HCl, pH 7.9, 88 mM KCl, 10 mM MgCl2, 0.5 mM EDTA, 10 mM ATP, 10 mM dithiothreitol, 100 μg mL−1 bovine serum albumin, and 125 ng of kDNA. The reaction mixture containing equal amounts of topoIIα (WT, S1337A/S1524A, or S1337E/S1524E) was incubated at 37 °C for different times, and the reaction was stopped by the addition of 5 μl of stop solution (5% SDS, 25% Ficoll, and 0.05% bromphenol blue). The samples were resolved by electrophoresis at 115 V using a 1% agarose gel in a Tris acetate-EDTA buffer. Following electrophoresis, the gel was stained with ethidium bromide and photographed under UV illumination.

RESULTS

Plk1 Functions in G1/S Phase—It has been well documented that Plk1 is involved in many mitotic processes, such as mitotic entry, bipolar spindle formation in metaphase, and cytokinesis
However, several recent reports indicate that Plk1 might have additional functions outside of mitosis. For example, Plk1 is required for recovery from the DNA replication checkpoint response, which occurs in S phase (16). Based on immunofluorescence (IF) staining, it has long been believed that Plk1 starts to express in the cytoplasm at late S or early G2 phase (17). To further examine the possible non-M phase functions of Plk1, HeLa cells were synchronized with a double thymidine block protocol (16 h of thymidine treatment and 8 h of release, followed by a second 16-h incubation with thymidine). At different times after release from the block, cells were harvested and fractionated into cytoplasmic, nuclear, and chromosome-binding fractions. Different subcellular fractions were then analyzed by Western blot. Based on FACS analysis, cells were arrested at late G1 after the block, went through S phase at 4–6 h postrelease, reached G2 phase at 8 h postrelease, and entered mitosis at 10 h postrelease (data not shown). As shown in Fig. 1A, Plk1 was clearly detected at early S phase in HeLa cells (4 h after release). Significantly, Plk1 was mainly localized in the nucleus during S phase and G2 phase and was also localized to chromosomes during G2/M phase (Fig. 1A). This apparent inconsistency might be due to the fact that only cytoplasmic fractions were analyzed in the previous study. To determine whether the nuclear localization of Plk1 was a general phenomenon, U2OS, another tumor cell line, and hTERT-RPE1, a nontransformed cell line, were used to further analyze Plk1 localization and expression. Cells were treated with mimosine, hydroxyurea, or nocodazole to block at G1, S, or M phase, respectively. Cytoplasmic and nuclear fractions were isolated and analyzed by Western blot. Nuclear localization of Plk1 during interphase was clearly detected in HeLa cells and U2OS cells but not in hTERT-RPE1 cells (Fig. 1B), indicating that the nuclear localization of Plk1 might be tumor cell-specific. Moreover, two tumor cell lines showed much higher overall levels of Plk1 than that of RPE1 cells, in agreement with the notion that Plk1 overexpression might be correlated with transformation (18). Nuclear localization of Plk1 in HeLa cells was also observed by IF staining, using a modified permeabilization/fixation protocol (Fig. 1C).

Next, RNAi was used to test whether Plk1 was required for cell cycle progression in the early stages, such as G1 and S phases. Plk1 was depleted by using a vector-based RNAi approach in randomly growing cells as previously described.
Upon nocodazole treatment, control cells quickly accumulated at the G2/M phase, as shown by the increase in cell population with 4N DNA content by FACS. In contrast, Plk1-depleted cells were much more resistant to nocodazole treatment (Fig. 1, D and E). To confirm this observation, we also examined the degradation rate of cyclin E, a G1/S marker protein. In control cells, cyclin E was almost completely degraded after 6 h of treatment with nocodazole. However, a significant amount of cyclin E was still detected in Plk1-depleted cells even after 12 h of nocodazole treatment (Fig. 1F), indicating that Plk1 might be required for G1/S phase. Finally, Plk1-depleted cells were also treated with nocodazole for shorter times and stained with a phosphohistone H3 antibody, a mitotic marker. The ratio between phosphohistone H3-positive cells and the cell population with 4N DNA content was used to follow the G2/M transition. For control cells, the percentage of phosphohistone H3-positive cells out of cells with 4N DNA content clearly increased upon nocodazole treatment. However, such an increase was not detected in the Plk1-depleted cells, supporting the notion that Plk1 is required for mitotic entry (Fig. 1G).

To further appreciate the potential involvement of Plk1 in the early stages of cell cycle, we next tried to deplete Plk1 in a synchronized culture. Accordingly, HeLa cells were depleted of Plk1 and treated with thymidine for 24 h to block at the G1/S boundary. Cells were then released into fresh medium for different times in the presence or absence of nocodazole and harvested. The double thymidine block protocol is not ideal for these experiments, since Plk1 depletion also causes dramatic G2/M block. As indicated, Plk1 depletion was very efficient using such a protocol (Fig. 2A). To be consistent with the results obtained with an asynchronous culture as described above, Plk1-depleted cells showed an obvious G1 peak during the entire releasing period, even in the presence of nocodazole, whereas control cells quickly entered mitosis (Fig. 2, B and C).

It has been documented that Plk1 depletion leads to cell cycle arrest, followed by apoptosis in HeLa cells (14). Thus, in addition to the normal 2N, 4N peaks, cells with sub-G1 DNA content (1N peak) were detected at later stages of nocodazole treatment after Plk1 depletion (Fig. 1D). In a synchronized culture after Plk1 depletion, apoptotic cells with 1N DNA content were also detected after 8 h of release from the thymidine block, even in the absence of nocodazole (Fig. 2B), indicating that the apoptotic cell death we observed in Fig. 1D is not due to nocodazole treatment.

**TopoIIα Interacts with Plk1 in Vivo**—To search for a possible Plk1 target during interphase, we turned our attention to DNA topoIIα, which is well known to be overexpressed in tumor cells and has functions in both S and M phases (1). In addition, topoIIα was also found to be one of several potential Plk1 substrates in a yeast two-hybrid screen to search for Plk1-interacting proteins. To test whether topoIIα is a binding partner of Plk1, cells were treated with mimosine, hydroxyurea, or nocodazole to block at G1, S, or M phase, respectively. Soluble nuclear and chromosome-binding fractions were combined and subjected to anti-Plk1 immunoprecipitation (IP), followed by anti-topoIIα Western blot analysis. As
shown in Fig. 3A, topoIIα was co-immunoprecipitated with Plk1 in both hydroxyurea and nocodazole-treated cells, but not in mimosine-treated cells, indicating that the binding between topoIIα and Plk1 occurs during S and G2/M phase in vivo. Both topoIIα and Plk1 were clearly detected in the nuclei of randomly growing cells by Western blot analysis (Fig. 3B). TopoIIβ and Erk2 were used as loading controls to indicate efficient subcellular fractionation. The nuclear co-localization of topoIIα and Plk1 was further confirmed by IF analysis (Fig. 3C).

Based on these data, we hypothesized that topoIIα might be a substrate of Plk1 in both interphase and mitosis.

**TopoIIα Is Required for Cell Proliferation**—To investigate the functions of topoIIα during normal cell cycle progression, we first used vector-based RNAi to specifically deplete topoIIα in HeLa cells. As indicated by Western blot analysis, topoIIα was efficiently depleted with this approach (Fig. 4A). We next determined whether topoIIα depletion influences the proliferation of HeLa cells. Although transfection with the control vector did not affect the growth rate of cells, transfection with the plasmid pBS/U6-topoIIα strongly inhibited cell proliferation (Fig. 4B). We also examined the viability of topoIIα-depleted cells. Transfection with the control vector showed little effect on cell viability, whereas ~10% of topoIIα-depleted cells were still attached to the culture dishes at 6 days post-transfection (Fig. 4C). To characterize the inhibition of cell growth by topoIIα depletion, cell cycle progression was analyzed by FACS. As shown in Fig. 4D, transfection with the control vector did not affect the cell cycle profile, whereas topoIIα depletion induced a slight increase of cell population in G2/M phase and obvious cell cycle arrest at S phase. Alternatively, these results may be due to the possibility that topoIIα-depleted cells undergo an aberrant mitosis, resulting in daughter cells with highly unequal DNA content. Starting from 5 days post-transfection, topoIIα-depleted cells showed a significant sub-G1 population (Fig. 4D), suggesting that these cells were undergoing apoptosis. To further analyze this phenotype in topoIIα-depleted cells, an anti-caspase 3 Western blot was performed (Fig. 4E). Caspase 3, the executioner caspase in apoptosis, was clearly activated in topoIIα-depleted cells, as shown by the cleavage of full-length protein. Finally, a BrdUrd labeling approach was used to confirm the S phase arrest induced by topoIIα depletion. As shown in Fig. 4F, at 3 days post-transfection, topoIIα-depleted cells...
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Depletion of TopoIIα Leads to Multiple Cell Cycle Defects—Considering that TopoIIα is involved in chromosome condensation and segregation (1), we next examined the possible mitotic defects induced by topoIIα depletion. For that purpose, topoIIα was depleted in synchronized cells using the protocol shown in Fig. 5A. TopoIIα-depleted cells showed obvious defects in chromosome behavior during mitosis, especially in sister chromatid separation. As shown in Fig. 5B, topoIIα-depleted cells were eventually able to go through mitosis but with obvious connected DNA bridges between separated sister chromatids through all late mitotic stages, including anaphase, telophase, and cytokinesis (Fig. 5B). To confirm the formation of DNA bridges, topoIIα-depleted cells were treated with either DNase or RNase (Fig. 5C). We found that these bridges were sensitive to DNase but not RNase treatment, indicating that they contain DNA. To further analyze topoIIα depletion-induced phenotypes, mitotic progression was followed by staining with a phosphohistone H3 antibody. Although no dramatic difference between control cells and topoIIα-depleted cells was detected, topoIIα-depleted cells showed a slight delay in mitotic exit (Fig. 5D). Interestingly, phosphohistone H3 staining was positive in the DNA bridges connecting the separating sister chromatids, even long after cell division (Fig. 5E).

We also assessed the percentage of cells expressing the proliferation marker Ki67, which is normally expressed in cells in G₁, S, G₂, and M phases but not in G₀ (19). Almost 100% of control cells were detected as Ki67-positive, whereas only about 33% of topoIIα-depleted cells were Ki67-positive, indicating that a significant portion of topoIIα-depleted cells had exited the cell cycle (Fig. 5F).

Abnormal nuclear morphology was also observed in topoIIα-depleted cells. Based on DAPI staining, cells can be further categorized into three groups: cells with a normal nucleus, cells with a deformed nucleus, and multinucleated cells. For control cells, ~95% contained normal nuclei, ~5% of cells were multinucleated, and very few cells with deformed nuclei were detected. In striking contrast, almost 30% of topoIIα-depleted cells had deformed nuclei, and 20% of topoIIα-depleted cells were multinucleated (Fig. 5G). Taken together, these results indicate that topoIIα is required for chromosome segregation in mitosis.

As a different approach, two topoII inhibitors were also utilized to study the effects on cell cycle progression. Accordingly, HeLa cells were synchronized using the double thymidine block, released for different times in the presence of VP16 or

![Image](https://example.com/image.png)

**FIGURE 5.** Cell cycle defects induced by topoIIα depletion. A, the protocol used to deplete topoIIα in a well synchronized culture. B, representative images of topoIIα-depleted cells during late mitosis/cytokinesis. DNA was stained with DAPI. C, topoIIα-depleted cells were permeabilized and incubated for 15 min with buffer containing RNase or DNase (5 μg ml⁻¹) prior to fixation and stained with DAPI. Histograms showed the percentage of the cells with DNA bridges. D and E, cells were depleted of topoIIα as in A, released for different times, and stained with a phosphohistone H3 antibody. F, cells were depleted of topoIIα as in Fig. 4A and stained with a Ki67 antibody. G, cells were depleted of topoIIα as in Fig. 4A and stained with DAPI. Based on nuclear morphology, topoIIα-depleted cells were categorized into three groups: normal, deformed, and multiple nuclei. Both representative images from each category (top) and quantification results (bottom) are shown. Scale bars for B, C, E, and G, 5 μm. Scale bar for F, 20 μm.

showed a slightly higher percentage of BrdUrd-positive cells compared with that of control cells, indicating that topoIIα is not required for DNA synthesis per se but might be involved in other interphase functions.
ICRF193, and harvested for FACS (Fig. 6). Since a much more stringent synchronization protocol was used here (double thymidine block in Fig. 6 versus a single thymidine block in Fig. 2), FACS profiles of control samples at 0 h points are slightly different, with a better synchronization result after the double thymidine block. Cells treated with VP16 were blocked in S phase over the entire releasing period, probably due to the activation of the DNA damage checkpoint. By inhibiting the religation activity of topoII, VP16 treatment leads to DNA double strand breaks (20). In contrast, ICRF193 is a topoII inhibitor that does not cause DNA damage but arrests the enzyme at a point in its catalytic cycle after strand passage and religation but before release of the passed DNA (21). Cells treated with ICRF193 were able to progress into mitosis and blocked there. Therefore, topoII activity is not essential for DNA replication but is absolutely required for mitosis. That BrdUrd incorporation is not affected in topoII/H9251-depleted cells also supports such a notion (Fig. 4F). However, we do observe an increase of S phase population in topoII-depleted cells, indicating that topoIIα may have additional interphase functions independent of its enzymatic activity (Fig. 4D).

Plk1 Phosphorylates TopoIIα in Vitro—To investigate whether Plk1 directly regulates topoIIα, we first examined whether Plk1 phosphorylates topoIIα in vitro. Purified Plk1-WT or Plk1-KM (kinase-defective mutant) was incubated with purified topoIIα in the presence of [γ-32P]ATP. The reaction mixtures were resolved by SDS-PAGE, followed by autoradiography. As shown in Fig. 7A, wild type Plk1 phosphorylated topoIIα efficiently, whereas the corresponding kinase inactive...
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A

| Construct | Amino acids |
|-----------|-------------|
| GFP-TopoII |             |
| GFP-TopoIIΔ2A | |
| HA-TopoIIα-N | S137A/S1524 |
| Flag-TopoIIα-C | 1263 |
| Flag-TopoIIα-CΔ2A | 1263 |

B

C

| Constructs | Apoptosis |
|------------|-----------|
| TopoIIα    | ++++      |
| TopoIIαΔ2A | -         |
| TopoIIαΔS137A | ++++ |
| TopoIIαΔS1524A | +++ |
| TopoIIαΔN   | -         |
| TopoIIαΔC   | +++       |
| TopoIIαΔCΔ2A | +++ |

D

E

F

FIGURE 8. TopoIIαΔ2A-expressing cells arrest at G1/S phase. A, various topoIIα constructs used in the overexpression study. B, at 2 days post-transfection with GFP-topoIIα or GFP-topoIIαΔ2A, HeLa cells were harvested and analyzed by FACS. C, cells were transfected with different topoIIα constructs, harvested, and analyzed by FACS to determine the degree of apoptosis. D, cells were co-transfected with topoIIα (WT or 2A mutant) and pBabe-puro at a ratio of 7:1. At 1 day post-transfection, puromycin was added for 30 h to select for transfection-positive cells. After the floating cells were removed, attached cells were treated with 200 ng ml⁻¹ nocodazole for 12 h, harvested, and subjected to FACS analysis. A 4N DNA line in the right diagrams was used to visualize the S phase arrest of topoIIαΔ2A-expressing cells, especially after nocodazole treatment. E, after expression of topoIIαΔ2A in D, cells were treated with nocodazole for 6 h, harvested, and analyzed by Western blot. F, after expression of topoIIαΔ2A as in D, cells were treated with nocodazole for 12 h, harvested, and analyzed by an anti-Cdc2 IP/kinase assay using histone H1 as a substrate.

form did not. Considering that most known phosphorylation sites in topoIIα are localized to the C-terminal domain (2), we next purified two overlapping C-terminal fragments of topoIIα (aa 1079–1350 and 1345–1532) for kinase assay in vitro. Both fragments were good substrates for Plk1 (Fig. 7B). To further narrow down the sites, four GST fusion topoIIα fragments (aa 1079–1258, 1259–1350, 1345–1438, and 1439–1532) were purified and subjected to the kinase assay. We found that the fragments containing aa 1259–1350 and 1439–1532 yielded strong phosphorylation signals (Fig. 7C). Using phosphoamino acid analysis, the major phosphate-accepting residue of topoIIα in vivo was shown to be serine (22). Therefore, we generated a series of single or multiple serine to alanine mutations within aa 1259–1350 and 1439–1532 of topoIIα. Kinase assays showed that a single mutation of Ser1337 or Ser1524 to alanine was sufficient to completely abolish the phosphorylation signal within aa 1259–1350 or 1439–1532 of topoIIα, respectively (Fig. 7D and E). It has been reported that the sequence (D/E)(S/T)φX(D/E) (where X represents any amino acid and φ is a hydrophobic amino acid) is an optimal phosphorylation sequence targeted by Plk1 (23). The two phosphorylation sites identified in topoIIα fit very well with this Plk1 target consensus sequence (Fig. 7F). Altogether, these data indicate that Ser1337 and Ser1524 of topoIIα are two major Plk1 phosphorylation sites in vitro.

TopoIIαΔ2A-expressing Cells Showed G1/S Phase Arrest—To probe the possible function of Plk1-associated phosphorylation of topoIIα, we compared the phenotypes resulting from overexpression of topoIIα with different phosphorylation states. A series of constructs were used in this study and are shown in Fig. 8A. FACS profiles indicated that overexpression of topoIIα led to obvious apoptosis, whereas expression of topoIIαΔ2A (S137A/S1524A) did not show any sign of cell death (Fig. 8B). Further analysis of this phenotype showed that apoptosis was due to expression of the C-terminal, but not the N-terminal, domain of topoIIα, and the phosphorylation state of both sites was important in the process (Fig. 8C). We also found that hydroxyurea treatment rescued topoIIα expression-induced apoptosis, indicating that the cell death was probably due to defects in G2/M phase (Fig. 8C). Induction of apoptosis by overexpression of topoIIα was reported previously (24). In that study, topoIIα expression-induced apoptosis was blocked by coexpression of a dominant-negative form of the cyclin-dependent kinase Cdk2 but not by Cdk1. Overexpression of dominant negative forms of Cdk2 and Cdk1 leads to cell cycle arrest in G1 and G2/M phases, respectively. Thus, it was proposed that topoIIα expression-induced cell death is due to a premature mitotic entry (24). Based on these studies, one would predict that treatment of topoIIα-expressing cells with drugs to
block cells at interphase should rescue the cell death. Our experimental results with hydroxyurea are consistent with this prediction.

To further explore the mechanism, FACS profiles of topoIIα- and topoIIα-2A-expressing cells were carefully analyzed (Fig. 8D). Compared with topoIIα-expressing cells, topoIIα-2A-expressing cells showed a lower percentage of cells with 4 n DNA content both in the presence or absence of nocodazole, indicating that the expression of topoIIα-2A probably leads to G1/S phase arrest. In addition, slightly higher cyclin E levels (Fig. 8E) and obvious lower Cdc2 kinase activities (Fig. 8F) were detected in topoIIα-2A-expressing cells, further supporting the notion that expression of topoIIα-2A might lead to G1/S arrest.

Cell Cycle Arrest in TopoIIα-2A-Expressing Cells Might Be Due to Activation of the DNA Damage Checkpoint—To further distinguish whether topoIIα-2A-expressing cells arrest at G1, or S phase, cells transfected with GFP-topoIIα-2A were incubated in medium containing BrdUrd reagent. After 2 h of incubation, cells were stained with an anti-BrdUrd antibody and analyzed by microscopy. Compared with that of control cells, the topoIIα-2A-expressing cells showed a slightly higher percentage of BrdUrd-positive staining, indicating that these cells might have a prolonged S phase (Fig. 9A). In addition, inhibition of mitotic entry was detected in topoIIα-2A-expressing cells, which is probably a secondary effect of S phase arrest (Fig. 9B).

Based on DAPI staining, abnormalities in nuclear morphology were also observed in topoIIα-2A-expressing cells. Almost 30% of topoIIα-2A-expressing cells contained micronuclei, whereas only 5% of topoIIα-expressing cells had this phenotype (Fig. 9C). Next, we tried to test the possibility that the S phase arrest in topoIIα-2A-expressing cells might be due to activation of the DNA damage checkpoint. Accordingly, we performed Western blot analysis with an antibody against phosphohistone H2AX, a marker for DNA double strand breaks (25). Positive phosphohistone H2AX signals were detected in cell lysates from topoIIα-expressing cells, topoIIα-2A-expressing cells, and topoIIα-depleted cells (Fig. 9D). Since both expression of wild type topoIIα and deletion of topoIIα led to apoptosis, but expression of topoIIα-2A did not show any sign of cell death, we propose that the positive phosphohistone H2AX signals in topoIIα-expressing cells and topoIIα-depleted cells were caused by the activation of caspases, which subsequently cleave DNA, whereas the positive phosphohistone H2AX signal observed in the topoIIα-2A-expressing cells is probably due to direct DNA damage. To further confirm that topoIIα-2A expression-induced S phase arrest is due to activation of the DNA damage checkpoint, caffeine, an ATM/R inhibitor, was used to treat the topoIIα-2A-expressing cells. As expected, we found that the addition of caffeine led to obvious cell death in topoIIα-2A-expressing cells as well as that in topoIIα-expressing cells (Fig. 9E). Our initial phenotypic analysis showed that topoIIα expression-induced apoptosis occurs during G2/M phase (Fig. 8C). The results we show here indicate that the addition of caffeine promoted the topoIIα-2A-expressing cells to enter into G2/M phase, further supporting the notion that the S phase arrest in topoIIα-2A-expressing cells might be induced by the ATM/R-mediated DNA damage checkpoint.

Plk1 Is a Positive Regulator of TopoIIα Activity—Considering that the essential functions of topoIIα in cell cycle progression rely on its enzymatic activity, it is intriguing to test whether Plk1-dependent phosphorylation regulates the decatenation activity of topoIIα. Toward that end, HEK293 cells were transfected with GFP-topoIIα with different phosphorylation states (wild type, S1337A/S1524A (unphosphorylatable mutant), and S1337E/S1524E (phospho-mimetic mutant)) and blocked with thymidine at G1 phase. Nuclear and chromosome-binding fractions from these cells were incubated with purified Plk1 under kinase reaction conditions and subjected to anti-GFP IP, followed by a topoII activity assay. The enzymatic activity of topoIIα was analyzed by an ATP-dependent decatenation assay using kDNA as a substrate (15). Compared with that of wild type topoIIα, the enzymatic activity of the topoIIα-2A and -2E mutants was significantly decreased and increased, respectively (Fig. 10, A and B), suggesting the hypothesis that Plk1 might be a positive regulator for the enzymatic activity of topoIIα.
Next, we tried to test whether the activity of topoIIα is cell cycle-dependent. At 16 h post-transfection with GFP-topoIIα, HEK293 cells were treated with thymidine to avoid topoIIα expression-induced cell death, released for different times, and harvested. Nuclear and chromosome-binding fractions from cells enriched at different phases were prepared and subjected

**FIGURE 10.** Plk1 is a positive regulator of topoIIα activity. A, HEK293 cells were transfected with GFP-topoIIα (WT or 2A or 2E mutant), incubated with thymidine for 1 day, and harvested. Soluble nuclear and chromosome fractions were prepared, and the amounts of GFP-topoIIα were quantified by Western blot analysis. The nuclear and chromosome fractions containing equal amounts of GFP-topoIIα were incubated with purified Plk1 under kinase reaction conditions and subjected to anti-GFP IP, followed by incubation with kDNA for different times as indicated. B, histograms quantifying the results of A to show the percentage of catenated kDNA remaining after different incubation times. C, HEK293 cells were transfected with GFP-topoIIα and incubated with thymidine for 1 day. G1, S, and M phase cells were harvested after 0, 4, and 10 h release from the thymidine block, respectively. After the amounts of GFP-topoIIα were quantified by Western blot analysis, the nuclear and chromosome fractions containing equal amounts of GFP-topoIIα were subjected to anti-GFP IP, followed by incubation with kDNA for different times, as indicated. D, HEK293 cells transfected with GFP-topoIIα were released to different cell cycle stages as described in C. Nuclear extracts were incubated with or without purified Plk1, and immunoprecipitated with GFP antibody, followed by topoII activity analysis. To compare the effects of Plk1 on topoIIα activity, the amounts of GFP-topoIIα used for IP from S and M phase cells were 30 and 10% of that from G1 phase cells, respectively. E, HEK293 cells transfected with GFP-topoIIα or GFP-topoIIα-2A were released to different cell cycle stages as described in C. Equal amounts of GFP-topoIIα and topoIIα-2A from different phases of the cell cycle were subjected to anti-GFP IP, followed by topoII activity analysis.
to anti-GFP IP, followed by a decatenation assay. TopoIIα activity was detected in G1 phase, significantly increased at S phase, and reached a peak at M phase, suggesting that topoIIα activity is regulated in a cell-cycle-dependent manner (Fig. 10C). We further examined the effects of Plk1-dependent phosphorylation on the activity of topoIIα prepared from cells at different phases. Accordingly, nuclear extracts from different phases of the cell cycle were incubated with or without purified Plk1 under kinase reaction conditions and subjected to anti-GFP IP, followed by topoII activity analysis. The most dramatic difference after Plk1 incubation was detected in samples prepared from G1 cells, whereas no detectable difference was observed in samples prepared from S phase or M phase cells (Fig. 10D, compare −Plk1 and +Plk1 samples). To capture a potential minor effect of Plk1, the amounts of GFP-topoIIα used from S and M phase cells were reduced, corresponding to about 30 and 10% of that from G1 phase cells, respectively. These data indicated that the Plk1-associated phosphorylation of topoIIα occurs as early as S phase, and the phosphorylation positively regulates its enzymatic activity. Finally, the activity of topoIIα-WT and -2A at different cell cycle stages was also examined (Fig. 10E). Dramatic differences were observed at all stages of the cell cycle, further confirming that topoIIα activity is positively regulated by Plk1-associated phosphorylation during the cell cycle in vivo.

Plk1-dependent Phosphorylation in TopoIIα Is Required for Sister Chromatid Segregation—Dynamic relocation of topoIIα on chromosomes during M phase has been previously described. It was believed that topoIIα was evenly distributed over the whole chromosome at prophase but concentrated to the kinetochore at metaphase (26). Recently, a number of studies showed that Plk1 was involved in the dynamic localization of two chromosome structural proteins, cohesin and PICH, both of which participate in the process of sister chromatid separation (11, 13). Considering that the localization of topoIIα during mitosis was very similar to that of cohesin and PICH, we tested whether Plk1-associated phosphorylation of topoIIα also affects its dynamic distribution on chromosomes. Toward that end, HeLa cells were transfected with GFP-topoIIα (WT or 2A) and mitotic chromosomes were spread. Both topoIIα-WT and topoIIα-2A were observed to evenly spread on the chromosomes, and no significant localization differences were detected between them (Fig. 11A). Thus, Plk1-dependent phosphorylation in topoIIα might not be required for its relocation during mitosis.

Next, we tested whether the Plk1-associated phosphorylation of topoIIα was essential for its function in sister chromatid segregation. For that purpose, HeLa cells were co-transfected with pBS/U6-topoIIα and RNAi-resistant GFP-topoIIα (WT-r and 2A-r) at a ratio of 5:1 to express topoIIα with different phosphorylation states in the absence of endogenous protein (Fig. 11B). Only GFP-positive cells in cytokinesis were analyzed. After topoIIα depletion, ~74% of GFP positive cells in cytokinesis had DNA bridges. Expression of RNAi-resistant topoIIα-WT was able to reduce GFP-positive cells in cytokinesis with DNA bridges to 24%, whereas ~71% of GFP-topoIIα-2A-r-positive cells in cytokinesis still had DNA bridges, indicating that WT topoIIα, but not the 2A mutant, can rescue the topoIIα depletion-induced DNA bridge formation between the separating sister chromatids (Fig. 11C). Taken together, we concluded that Plk1-dependent phosphorylation in topoIIα was required for normal sister chromatid segregation in late mitosis. Finally, to understand the topoIIα-depletion-induced phenotypes as described in Fig. 5G, we monitored the FACS profiles of synchronized HeLa cells after long-term release in the presence of ICRF193. After 24 h of release, cells with S phase arrest, 8N DNA content, and sub-G1 population were accumulated (Fig. 11D), indicating that the abnormal nuclei morphology we observed in topoIIα-2A expression cells might be due to mitotic defects.

**DISCUSSION**

The essential role of Plk1 during mitosis has been well established. However, increasing evidence suggests that Plk1 might have additional functions outside of M phase. Plk1 has been shown to control recovery from G2 phase DNA damage-induced arrest in mammalian cells (27). Although the detailed mechanism is still unclear, the p38 mitogen-activated protein kinase pathway-mediated phosphorylation of Plk1 might play a critical role in this process (28). Moreover, Xenopus Plk1 is essential for the adaptation of a DNA replication checkpoint response, which occurs in S phase (16). Plk1-dependent phosphorylation of a DNA damage adaptor protein, claspin, causes it to dissociate from chromatin and be degraded, which leads to termination of a DNA replication checkpoint response (16, 29, 30). Here, we provide evidence to show that Plk1 is also required for G1/S phase progression even in the absence of stress.

So, what could be a potential substrate for Plk1 in interphase? DNA topoisomerase IIα is a likely candidate for the following reasons. First, both proteins are localized to the nucleus in interphase and detected in chromosome-binding fractions during mitosis. Second, topoIIα was co-immunoprecipitated with Plk1 in both hydroxyurea- and nocodazole-treated cells, the highest binding affinity being observed in S phase. Third, depletion of topoIIα using vector-based RNAi led to defects in both S phase and mitosis. Fourth, Plk1 directly phosphorylates Ser1337 and Ser1524 of topoIIα in vitro. Fifth, overexpression of topoIIα-2A (S1337A/S1524A) led to S phase arrest, reminiscent of the interphase defects induced by Plk1 depletion. Sixth, introduction of alanine mutations in two Plk1 phosphorylation sites inhibited the decatenation activity of topoIIα.

By using direct transfection of double-stranded RNA targeting topoIIα, it was previously shown that topoIIα is involved in sister chromatid segregation, as indicated by the presence of massive chromatin bridges in topoIIα-depleted cells (31, 32). This phenotype is in agreement with what we have described here using the vector-based RNAi approach. In addition, we also found that topoIIα depletion led to S phase arrest and inhibition of cell proliferation. Direct transfection with double-stranded RNA targeting topoIIα did not show any sign of cell growth inhibition, probably due to the relatively low depletion efficiency of that approach (32). In the vector-based RNAi approach, pBabe-puro, containing a puromycin resistance gene, was co-transfected with the vector generating short hairpin RNA. Subsequent selection of transfection-positive cells
with puromycin led to much more efficient topoIIα depletion, thus providing an opportunity to detect additional phenotypes that are not observed using the direct transfection of double-stranded RNA. The S phase defect observed in topoIIα-depleted cells is also supported by the phenotype associated with overexpression of the Plk1 unphosphorylatable mutant. Probably due to a dominant negative effect, the catalytically inactive topoIIα mutant blocks cells at S phase.

As described above, the role of phosphorylation in regulating topoIIα has been controversial (2). In this study, we showed that the phosphorylation of topoIIα by Plk1 substantially increased the catalytic activity of topoIIα. We are confident to draw such a conclusion for the following reasons. First, phosphorylation by casein kinase II, protein kinase C, and Cdc2 stimulate topoII activity in flies and budding yeast (4, 5). Second, phosphorylation at Ser1106 by casein kinase II promotes topoIIα activity in mammalian cells (7). Third, overexpression of the Plk1 unphosphorylatable topoIIα mutant led to cell cycle arrest at S phase, a phenotype that is strikingly different to that associated with overexpression of wild type topoIIα, strongly suggesting that Plk1-associated phosphorylation regulates its catalytic activity.

To our knowledge, we are the first group to report the phenotype for overexpression of a topoIIα unphosphorylatable mutant and analyze phosphorylation-dependent functions in vivo. Fourth, although the C-terminal domain of eukaryotic topoIIα does not contain the catalytically functional regions, such as the ATP binding sites or the active tyrosine for DNA breakage and religation, some previous studies indicated that this region still might be related to the regulation of its enzymatic activity. For example, binding to a PT1342 antibody, which recognizes phospho-Thr1342 in topoIIα, completely abolished topoIIα activity. It was proposed that the catalytically active sites and Thr1342 were close in secondary structure and might interact, although they were separated in the primary structure (33). Two Plk1 phosphorylation sites we identified are located in the C-terminal region of topoIIα, and the phosphorylation states of these two sites affected its activity both in vitro and in vivo (Fig. 10). One possible explanation is that phosphorylation in the C-terminal domain of topoIIα

**FIGURE 11.** Plk1-dependent phosphorylation at Ser1337 and Ser1524 in topoIIα might be required for sister chromatid segregation. A, chromosome spreading of GFP-topoIIα and GFP-topoIIα-2A-expressing HeLa cells. B and C, after co-transfection with pBS/U6-topoIIα and RNAi-resistant GFP-topoIIα (WT or 2A) at a ratio of 5:1 for 16 h, cells were blocked with thymidine for 24 h. At 15 h after release from the thymidine block, cells were harvested and analyzed by Western blot (B) or stained with DAPI (C). The arrows indicate formation of DNA threads due to topoIIα depletion. Scale bar, 5 μm. D, HeLa cells were synchronized with the double thymidine block, released into medium containing ICRF193 for the times indicated, and harvested for FACS. E, a model illustrating the possible function of Plk1-dependent phosphorylation in topoIIα during the cell cycle.
might change its secondary structure, through which the enzymatic activity is regulated. Another possibility is that phosphorylation in the C-terminal domain makes this region negatively charged and therefore subsequently affects its interactions with DNA or other proteins.

In summary, Plk1 first interacts with and phosphorylates topoIIα at Ser1337 and Ser1524 in S phase, and the maximum level of phosphorylation occurs in mitosis. Although Plk1-associated phosphorylation of topoIIα does not affect the dynamic localization of topoIIα in chromosomes, it is required for the essential role of topoIIα in sister chromatid segregation. Over-expression of a Plk1 unphosphorylatable topoIIα mutant leads to ATM/R-dependent activation of the DNA damage checkpoint, leading to S phase arrest, probably due to the DNA damage formation in the previous M phase (Fig. 11E).

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