Phosphorylation-independent Stimulation of DNA Topoisomerase IIα Activity*

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Keiji Kimura†§, Masafumi Saijo†, Masato Tanaka§, and Takemi Enomoto‡

From the †Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo 113, Japan and the §Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan

It has been suggested that casein kinase II phosphorylates DNA topoisomerase IIα (topo IIα) in mouse FM3A cells, by comparison of phosphopeptide maps of topo IIα labeled in intact cells and of topo IIα phosphorylated by various kinases in vitro. The phosphorylation of purified topo IIα by casein kinase II, which attached a maximum of two phosphate groups per topo IIα molecule, had no effect on the activity of topo IIα. Dephosphorylation of purified topo IIα by potato acid phosphatase, which almost completely dephosphorylated the topo IIα, did not reduce the activity of topo IIα. The incubation itself, regardless of phosphorylation or dephosphorylation status, stimulated the enzyme activity in both reactions. topo IIα activity was stimulated by incubation in a medium containing low concentrations of glycerol but not in that containing high concentrations of glycerol, such as the 50% in which purified topo IIα is stored. The stimulation of topo IIα activity by incubation was dependent on the concentration of topo IIα, requiring a relatively high concentration of topo IIα.

DNA topoisomerase II (topo II)1 is an abundant and essential nuclear enzyme that catalyzes the decatenation and the unknotting of topologically linked DNA circles and the relaxation of supercoiled DNA chains (1, 2). The DNA decatenation activity of the enzyme is essential for the condensation of interphase chromatin into metaphase chromosomes (3–8), and is necessary for the segregation of daughter chromosomes (9–13). In addition, topo II appears to play important roles in the organization of nuclei and mitotic chromosomes, since it is a component of the nuclear matrix (14) and the mitotic chromosome scaffold (15, 16).

Topo II exists as a phosphoprotein in intact cells from a variety of species (16–24). The phosphorylation of topo II is regulated in a cell cycle dependent manner, reaching the maximal level during the G2–M phase (19, 22, 23, 25). Casein kinase II may phosphorylate topo II in Drosophila cells and yeast (18, 22, 26). In vitro, topo II is phosphorylated by a number of protein kinases, including casein kinase II (26–30), protein kinase C (17, 28, 31, 32), and Cdc2 kinase (30) and in all cases, phosphorylation stimulates the enzyme activity.

In vertebrate organisms, two isoforms of topo II have been identified, which have been designated topo IIα and topo IIβ, the latter having been discovered later (33). Thus, most reports describing the phosphorylation of topo II of mammalian cells referred to topo IIα, except for a few (16, 24, 34, 35). While it appears that in yeast and Drosophila melanogaster, there is one enzyme which more closely resembles topo IIα. We reported that an unidentified protein kinase, PKII phosphorylated topo IIα, which stimulated enzyme activity (36). However, the effect of phosphorylation on the activity of topo II varied among preparations. In addition, Shiozaki and Yanagida (37) have reported that yeast topo II without the phosphorylated termini had about 4-fold more catalytic activity than intact topoisomerase II, and that dephosphorylated topo II retained enzymatic activity (38).

In this study, to re-evaluate our results and to examine the effect of phosphorylation upon the activity of topo II, we investigated the kinase that phosphorylates topo IIα in mouse FM3A cells, which dominantly express topo IIα. We found that casein kinase II phosphorylated topo IIα in FM3A cells and that phosphorylation of topo IIα by casein kinase II had no effect on the activity of topo IIα under our experimental conditions. More importantly, we found that the incubation itself stimulated the activity of topo IIα.

EXPERIMENTAL PROCEDURES

Buffers—Buffer 1 contained 20 mM potassium phosphate buffer, pH 7.5, 0.1 mM Na2EDTA, 1 mM 2-mercaptoethanol, 0.25 mM phenylmethylsulfonyl fluoride, and 1% ethanol. Buffer 2 consisted of all components of buffer 1, plus 20% ethylene glycol and 0.01% Triton X-100. Buffer 3 consists of all components of buffer 1, plus 50% glycerol and 0.01% Triton X-100.

DNA Topo II Assay—DNA topo II activity was assayed by measuring supercoiled DNA relaxing or DNA unknotting activities. The standard reaction mixture (20 μl) for the DNA relaxation assay consisted of 50 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM MgCl2, 1 mM ATP, 0.5 mM dithiothreitol, 0.5 mM Na2EDTA, 100 μg/ml bovine serum albumin, and 0.225 mg of supercoiled pUC19 DNA. The incubation proceeded at 30°C, and the reaction was stopped with 5 μl of stop solution (5% SDS, 25% Ficoll, and 0.05% bromphenol blue). The sample was incubated for 15 min at 50°C, then loaded on a 0.8% agarose gel in TBE buffer (89 mM Tris borate, pH 8.2, and 2 mM EDTA). After electrophoresis, the gel was stained with ethidium bromide and photographed under UV illumination. DNA unknotting activity was assayed as described above for the relaxing assay except that knotted P4 phage DNA was used as the substrate DNA.

Purification of DNA Topo IIα—All operations were performed at 0–4°C. A total of 8 × 10⁸ frozen FM3A cells were thawed, suspended in buffer 1 at a concentration of 1.25 × 10⁷ cells/ml, and sonicated 4 times for 10 s at each interval of 20 s with a Branson model 185 sonifier. Buffer 1 (0.1 volume) containing 3.5 mM KCl was added dropwise to the sonicate to bring the final concentration of KCl to 0.3 mM. After stirring for 30 min, the sonicate was centrifuged for 30 min at 10,000 × g. The supernatant was recovered as the crude extract and loaded onto a
phosphocellulose column (30 ml) equilibrated with 0.45 M KCl in buffer 2. The flow-through fraction was dialyzed against 140 mM KCl in buffer 2 and applied to a second phosphocellulose column (30 ml) equilibrated with 0.3 M KCl in buffer 2 at 0.5 ml/min. The column was washed with 3 bed volumes of the same buffer and eluted with a linear gradient of 0.3 to 1 M KCl from 0.45 M KCl in buffer 2. The active fractions were pooled and loaded onto a phosphocellulose column (10 ml) equilibrated with 0.3 M KCl in buffer 2. The column was washed with 3 bed volumes of the same buffer and eluted with a linear gradient of KCl from 0.3 to 1 M KCl in buffer 2. The active fractions were pooled and loaded onto a phosphocellulose column (10 ml) equilibrated with 0.3 M KCl in buffer 2. The column was washed with 3 bed volumes of the same buffer and eluted with a linear gradient of KCl from 0.3 to 1 M KCl in buffer 2. The active fractions were pooled and loaded onto a phosphocellulose column (10 ml) equilibrated with 0.3 M KCl in buffer 2. The column was washed with 3 bed volumes of the same buffer and eluted with a linear gradient of KCl from 0.3 to 1 M KCl in buffer 2. Casein kinase activity was eluted from the column between 0.4 and 0.5 M KCl in buffer 2. The active fractions were pooled, dialyzed against 0.2 M KCl in buffer 3, and stored at -20 °C.

Labeling Topo IIα in Intact Cells—Exponentially growing FM3A cells were washed once with phosphate-free RPMI 1640 and incubated into 60-mm plastic dishes at a density of 1.5 × 10⁶ cells/ml with 5 ml of above medium supplemented with 10% dialyzed fetal bovine serum. The cells were incubated for 1 h at 37°C, then labeled with [32P]orthophosphate (60 µCi/ml, 8500–9300 Ci/mmol) for 2 h at 37°C. [32P]Labeled topo IIα was immunoprecipitated with anti-topo II antibody by the procedure of Saigo et al. (23).

Phosphorylation of Topo IIα—Purified topo IIα (60 ng) was incubated with 50 ng of casein kinase II in the reaction mixture containing 20 mM Hepes, pH 7.4, 0.1 mM ATP or 10 mM γ-[32P]ATP (0.1 Ci/mmole), 150 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM bovine serum albumin, 10% dialyzed fetal bovine serum, and 0.2 units of purified protein kinase C at 30°C for 30 min. For phosphopeptide mapping, purified topo IIα (1 µg) was phosphorylated by the indicated kinases as follows. Phosphorylation by casein kinase II proceeded in a reaction mixture containing 20 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, and 10 µM (γ-[32P]ATP (1–5 Ci/mmole) at 30°C for 30 min. Phosphorylation by PKII occurred in a reaction mixture containing 20 mM Hepes, pH 7.4, 10 mM MgCl₂, 1 mM dithiothreitol, and 10 µM (γ-[32P]ATP (1–5 Ci/mmole) at 30°C for 30 min. Protein kinase C phosphorylation proceeded in a reaction mixture containing 20 mM Hepes, pH 7.4, 3 mM MgCl₂, 10 µM (γ-[32P]ATP (1–5 Ci/mmole), 25 μg/ml phosphatidylycerine, and 4 μg/ml dialdehyde/glycerol at 30°C for 30 min.

Phosphatase Treatment—[32P]Labeled topo IIα was resolved by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The stained band of topo IIα was cut and washed with 25% isopropanol alcohol 5 times, with 10% methanol twice, and with double distilled water twice at 30-min intervals. The gel slice was crushed and incubated in 50 μl of medium containing 1 mg/ml Achromobacter protease I and 50 μg/ml NH₄Cl, 0.1 mM dithiothreitol, and 50 mM NH₄Cl, pH 7.8, or 0.1 mM endoproteinase Asp-N and 50 mM Tris-HCl, pH 7.5, at 37°C for 12 h. The crashed gels were removed from the medium by centrifugation for 10 min at 10,000 × g, then the supernatant was recovered and dried. A solution (40 μl) containing 62.5 mM Tris-HCl, pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue was added to dissolve the digested peptides and boiled for 30 s.

Phosphatase activity was measured by phosphoimager an image analyzer (LAS 2000 Fuji Photofilm, Tokyo, Japan).

RESULTS

The Kinase Responsible for Phosphorylating Topo IIα in FM3A Cells—We purified topo IIα and casein kinase II from mouse FM3A cells as described under "Experimental Procedures." The purified topo IIα fraction contained a single 170-kDa band (Fig. 1A) and purified casein kinase II fraction consisted of two major bands at 28 and 43 kDa (Fig. 1B). The purified topo IIα was phosphorylated by protein kinase C, casein kinase II, or PKII. The labeled topo IIα was separated by SDS-PAGE, digested by Achromobacter protease I, and the phosphopeptides were mapped as described under "Experimental Procedures." The purified topo IIα labeled by each kinase had a distinct phosphopeptide profile (Fig. 2, lanes 1–3), indicating that these kinases phosphorylate topo IIα at different sites. By contrast, the phosphopeptide maps of topo IIα phosphorylated by casein kinase II and that of topo IIα labeled in intact cells, which were produced by digestion with Achromobacter protease I, V8 protease, or endoproteinase Asp-N, were similar (Fig. 2, lanes 4–9). Thus it is likely that casein kinase I phosphorylates topo IIα in FM3A cells.

The Effect of Phosphorylation by Casein Kinase II upon Topo IIα Activity—Casein kinase II appeared to phosphorylate topo IIα in intact cells. Thus, we examined the effect of phosphorylation by casein kinase II on the activity of topo IIα. Fig. 3 shows the time course of topo IIα phosphorylation by casein kinase II. At the maximal level, about 2 molecules of phosphate were incorporated into one molecule of topo IIα. Incubating topo IIα with heat-inactivated casein kinase II or in the absence of the kinase resulted in no phosphate incorporation. Topo IIα was incubated with casein kinase II or heat-inactivated casein kinase II for 30 min under the same conditions as those of Fig. 3; then the activity of topo IIα was measured. The levels of activity of topo IIα incubated with casein kinase II or heat-inactivated casein kinase II were considerably higher than that of activity of topo IIα without incubation when topo IIα activity was determined by the DNA relaxing assay (Fig. 4A) or the DNA unknotting assay (Fig. 4B). This indicated that the activity of topo IIα was stimulated during the incubation independently of phosphorylation by casein kinase II, because the heat-inactivated casein kinase II did not phosphorylate topo IIα (Fig. 3).

The Effect of Dephosphorylation on Topo IIα Activity—To determine whether the purified topo IIα had been sufficiently phosphorylated in the cells and that additional phosphorylation scarcely affected the activity of topo IIα, we incubated it with PAP. We first confirmed the dephosphorylating activity of...
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Conditions for Stimulating Topo IIα Activity—Purified topo IIα was stored in a buffer containing 50% glycerol, whereas the concentration of glycerol in the buffer in which topo IIα was preincubated was only 5%. Thus we examined the effect of glycerol concentration on the activation of topo IIα activity. Topo IIα was incubated in the buffer containing various concentrations of glycerol for 30 min. Then the activity of topo IIα was measured by the DNA relaxing (Fig. 8A) or unknotting assays (Fig. 8B) in a reaction mixture containing glycerol of a concentration below 3.75%. Incubation of topo IIα in the buffer containing 75 and 50% glycerol did not stimulate the activity of topo IIα, whereas the activity was enhanced during incubation in the buffer containing 5% or 20% glycerol.

When the concentration of topo IIα was decreased to one-tenth during the incubation for activation, topo IIα was not activated by the incubation, indicating that the activation is dependent on the concentration of topo IIα (Fig. 9).

**DISCUSSION**

Phosphorylation is an important means by which enzymatic activity and protein functions are regulated in the cells. DNA topoisomerase exists as a phosphoprotein in the cells of various species (16-24). We reported that topo II is phosphorylated in mouse FM3A cells and that the phosphorylation of topo II purified from the mouse cells by an unidentified protein kinase, PK II, stimulated the activity of topo IIα (36).

To evaluate these results, we first tried to identify the protein kinase that phosphorylates topo IIα in FM3A cells. The results shown in Fig. 1 indicate that casein kinase II phosphorylates topo IIα in mouse cells. Topo II in Drosophila and yeast cells are phosphorylated by casein kinase II (27, 30). Wells et al. (40) have reported that casein kinase II phosphorylates the C-terminal domain of topo IIα, primarily, the 2 serine residues
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The activity of topo II from Drosophila and yeast cells is stimulated by casein kinase II phosphorylation. Thus we examined whether the phosphorylation of mouse topo IIα by casein kinase II stimulated topo IIα activity. Phosphorylation of topo IIα (−2 phosphates/enzyme) by casein kinase II had no effect on topo IIα activity (Figs. 3 and 4). The inability to stimulate topo IIα activity by phosphorylation may be due to the fact that the topo IIα is sufficiently phosphorylated to exhibit enzyme activity. However, this possibility was excluded because the almost total dephosphorylation of topo IIα by PAP did not decrease topo IIα activity (Fig. 5). Thus phosphorylation of topo IIα had no effect upon the enzyme activity under our experimental conditions. In this context, it is interesting that the C-terminal domain of topo II, which is the site of phosphorylation, is not required for the activity of Schizosaccharomyces pombe and Saccharomyces cerevisiae topo II (38, 41, 42).

The key finding in this study was that the incubation itself stimulates topo IIα activity (Fig. 7). Frere et al. (43) have reported that the human topo IIα 1013–1056 fragment associates into stable two-stranded α-helical coiled-coil structures through hydrophobic interactions. In addition, Lamhasni et al. (44) have reported that yeast topo II exists as a monomer-dimer equilibrium depending on both the enzyme concentration and salt concentration. Vassetteky et al. (45) indicated that multimerization of topo IIα required its phosphorylation. Since topo IIα was stimulated even by the incubation with PAP, multimerization of topo IIα is not required for the stimulation. Thus it seems likely that mutual interaction of topo IIα to form homodimers or a conformational change of topo IIα dimers occurs...
We also reported that treatment of topo IIα with agarose bead-conjugated CIAP reduced topo IIα activity. By contrast, in this study topo IIα activity was not reduced after treatment with potato acid phosphatase, which removes almost all the phosphate groups from topo IIα. FIG. 6 shows that ATP in the reaction mixture for the assay of topo IIα activity degraded rapidly when topo IIα was first incubated with CIAP. Although the cause of the inhibition of topo II activity in our previous study must be studied precisely, one possibility is that topo IIα activity was inhibited by CIAP, which was released from agarose beads and carried over to topo II assay mixture, due to not to the dephosphorylation of topo IIα but to ATP degradation.

The present finding that phosphorylation has no effect on topo IIα activity is incompatible with previous studies on Drosophila and S. cerevisiae topo II (27, 29, 30). This discrepancy must be analyzed in detail in future experiments. Thus, the conclusions of the present work do not at this time appear to be applicable to the regulation of topo II activity from lower eukaryotes.

This study showed that the activity of topo IIα was stimulated by incubation itself. The stimulation was observed under specific conditions: a relatively low concentration of glycerol and high concentrations of topo IIα, which had not been stored for long periods. Thus, there is at present no evidence to suggest that the previously reported conclusion that the activity of topo II is modulated by its phosphorylated state is not valid. We emphasize that the studies on the effect of phosphorylation on the activity of topo II must be done and interpreted very carefully.

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