Threonine 1342 in Human Topoisomerase IIα Is Phosphorylated Throughout the Cell Cycle

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To investigate the relationship between the modulation of topoisomerase II activity and its phosphorylation state during the cell cycle, a monoclonal antibody against C-terminal peptide (residues 1335–1350) of topoisomerase IIα containing a consensus sequence of casein kinase II, TDDE and its phosphorylated threonine were prepared. In an enzyme-linked immunosorbent assay, the antibody, named PT1342, recognized the immunogenic phosphopeptide but not the non-phosphorylated form of the peptide. The PT1342 antibody reacted only with a 170-kDa protein from HeLa cells and recognized anti-topoisomerase IIα immunoprecipitants. Furthermore, the antibody did not react with the human topoisomerase IIα mutated at codon 1342 from threonine to alanine, showing that PT1342 was directed only with a 170-kDa protein from HeLa cells and recognized phosphopeptide but not the non-phosphorylated form of the peptide. The PT1342 antibody reacted with topoisomerase IIα throughout the cell cycle, HeLa cells were stained simultaneously for phosphorylated topoisomerase IIα and DNA and analyzed by flow cytometry. Cells in the G2-M phase contained about double the PT1341-reacted topoisomerase IIα than did cells in G1 or S phases. The antibody stained the nuclei in interphase and mitotic chromosomes and its periphery, as seen with anti-topoisomerase IIα antibody. Thus, threonine 1342 in topoisomerase IIα is phosphorylated throughout the cell cycle.

DNA topoisomerases are enzymes that play an important role in DNA replication and transcription by relieving torsional or interlocking constraints of DNA accumulating during processes of macromolecular syntheses (1–3). Topoisomerases I and II relax supercoiled DNA through transient single- and double-strand breaks, respectively, and topoisomerase II unknots or decatenates the knotted or catenated DNA. The latter activity of topoisomerase II is presumably related to chromosomes dynamics in mitosis, where topoisomerase II is absolutely required (4–7). In addition to catalytic activity, topoisomerase II may function to anchor chromosomal DNA loops to the nuclear scaffold (8, 9); topoisomerase II is a major non-histone protein present in nuclear scaffold fractions (10, 11), and DNA sequences that bind preferentially to the nuclear matrix/chromosomal scaffold (MAR/SAR) contain topoisomerase II cleavage consensus (12). In contrast, the role of topoisomerase II in the maintenance of chromosomes was not supported in studies done using Xenopus egg extracts or Xenopus embryos (13, 14). Topoisomerase II has been considered as one of the targets for anticancer drugs (15). Topoisomerase II inhibitors, such as etoposide or 4′-(9-acridinylamino) methane sulfon-f-m-anisidide, stabilize a catalytic reaction intermediate termed “cleavable complex” in which the enzyme binds covalently to 5′-phosphoryl termini of broken DNA (15). There are two known isoforms of topoisomerase II in mammalian cells, topoisomerase IIα and IIβ. They have molecular masses of 170 kDa and 180 kDa, respectively. These isoforms are mapped to different genes on chromosomes 17 and 3 (16), respectively, and have distinct susceptibility to various topoisomerase II inhibitors (17).

Because topoisomerase II plays an important role in the cell cycle, especially in M phase, the activity of the enzyme may be regulated throughout the cell cycle. Indeed, topoisomerase II protein levels increase following cell proliferation and cell cycle progression from G1 to S and G2-M phase (18–20). The phosphorylation of topoisomerase II also changes throughout the cell cycle, in parallel with level of the protein (21–23), and sites of phosphorylation are similar but not identical throughout the cell cycle of budding yeast (24). In mammalian cells, topoisomerase II is phosphorylated at specific sites in G2-M phase; MPM-2 antibody (25), which recognizes phosphoprotein in G2-M phase, reacts with topoisomerase IIα and IIβ (26). Threonine residue of topoisomerase II are phosphorylated in mitosis but not in the other cell cycle phases (27). In Chinese hamster ovary cells, distinct sites of topoisomerase II are phosphorylated in interphase and in mitosis (28).

Topoisomerase II is phosphorylated in vitro by various protein kinases such as casein kinase II, protein kinase C, CDC2 kinase, and Ca2+ calmodulin-dependent protein kinase (29–32). Among these protein kinases, casein kinase II has been given much attention because the major in vivo phosphorylation sites of topoisomerase II correspond to those of casein kinase II in vitro in Drosophila and budding yeast (33, 34), and topoisomerase II was copurified with casein kinase II from budding yeast and from Drosophila (32, 35, 36). Moreover, phosphorylation of topoisomerase II by casein kinase II increased Drosophila enzyme activity and reactivated the Saccharomyces cerevisiae enzyme inactivated by alkaline phosphatase (29, 32, 34), although the fission yeast topoisomerase II

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was not inactivated by phosphatase (37). When the mechanism by which phosphorylation of topoisomerase II stimulates the enzyme activity was examined, the increased catalytic activity of phosphorylated topoisomerase II was found to be due to enhanced ATP hydrolysis (38). Phosphorylation of yeast topoisomerase II also enhances affinity of the enzyme for linear DNA (39). The phosphorylation sites of yeast topoisomerase II are concentrated at the C-terminal region, although some phosphorylation was noted in the N-terminal region of the enzyme (34, 37). Because most catalytic functions of topoisomerase II occur in N-terminal and central regions, the significance of C-terminal phosphorylation, although not well understood, is speculated to remove the negative regulatory function of the C-terminal phosphorylation, although not well understood, is speculated to remove the negative regulatory function of the C-terminal region (40). Phosphorylation of topoisomerase II also influences susceptibility of the enzyme to topoisomerase II inhibitors. One factor that stimulates topoisomerase II activity is a protein kinase; it enhances the 4′-(9-acridinylamino)-methane-sulfonyl fluoride- and anisidide-induced DNA cleavage (41). VP-16-resistant KB cells have hyperphosphorylated topoisomerase II (42).

To closely examine the biological role of phosphorylation of topoisomerase II, we prepared an antibody against the C-terminal phosphopeptide of topoisomerase IIC. Using this specific antibody, we have shown that the threonine at 1342 was phosphorylated throughout the cell cycle. This is the first report that threonine 1342 in topoisomerase IIC is phosphorylated in vivo.

MATERIALS AND METHODS

Cell Culture—HeLa S3 cells were grown in Dulbecco's modified Eagle's medium containing 10% calf serum, penicillin (10 units/ml), and streptomycin (100 μg/ml) under a humidified atmosphere of 5% CO2 in air.

Peptides—A peptide in human topoisomerase IIα, FSDFDEKTDDEDVVPC (PT1342), and its phosphothreonine-containing peptide (PT1342), synthesized as described (43), were purchased from Peptide Institute, Inc., Osaka, Japan.

Immunization and Production of Hybridomas for Phosphopeptide—A phosphopeptide, PT1342, was coupled to keyhole limpet hemocyanin (KLH), as described by Nishizawa et al. (44). A monoclonal antibody was prepared as described previously (45). In brief, PT1342-KLH emulsified in complete Freund's adjuvant was injected intraperitoneally into BALB/c mice. A booster of the PT1342-KLH emulsion was given to the mice at 2-week intervals. Three days after the final boost, the spleen cells were fused with mouse myeloma cell SP2/0-Ag 14, using polyethylene glycol 4000. The hybridomas producing anti-PT1342 antibody were screened after 3 days by enzyme-linked immunosorbent assay in microtiter plates coated with PT1342. The cells from the positive wells were cloned twice to ensure monoclonality. In this manner, the hybridoma clone 3DA producing PT1342 antibody was developed. The hybridoma cell 3DA was grown as ascites tumors in BALB/c mice primed with pristane. A homogeneous IgG fraction was prepared by applying ascites fluid on protein A-gel (Bio-Rad) and eluting it, according to the manufacturer's instructions. Immediately after the elution, IgG protein was dialyzed against PBS.

Preparation of Monoclonal Antibodies against Topoisomerase IIα and IIβ—Topoisomerase IIα purified from HL60 cells or SFDPFEPSSLPRTGRARSE, a peptide in the C terminus of human topoisomerase IIα conjugated with KLH, was injected intraperitoneally into BALB/c mice four times at 14-day intervals. Three days after the final boost, the spleenocytes were fused with the myeloma line P3-X63Ag8,653. The culture medium were screened by enzyme-linked immunosorbent assay. The isolated clones, 4E12, 3H10, and 1H6, produced antibodies that specifically recognized topoisomerase IIα, IIβ, or both IIα and IIβ, respectively.

Site-directed Mutagenesis of Human Topoisomerase IIα—The human topoisomerase IIα expression plasmid YePWOB6 (46) was altered at position A4024G by site-directed mutagenesis, using the Stratagene Chimeras system, according to the manufacturer's instructions. The base change altered the codon 1342 from threonine to alanine. This mutant yeast transformed in vivo, and protein expression was induced with galactose. Recombinant human topoisomerase IIα and IIβ proteins were overexpressed as described previously (47).

Cell Lysis—Exponential-phase HeLa S3 cells were pelleted at 3000 rpm for 5 min at 4°C and lysed on ice for 1 h by resuspension in cold lysis buffer (50 mM Tris-HCl buffer, 350 mM NaCl, 0.1% (v/v) Nonidet P-40, 5 mM Na3EDTA, and 50 mM NaF, pH 7.4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 20 μg/ml each of antipain, aprotinin, chymostatin, leupeptin, and pepstatin A) and then centrifuged at 10,000 rpm for 5 min at 4°C. The supernatants were used for immunoprecipitation and immunoblotting after boiling for 5 min in 3× SDS-PAGE loading buffer.

Immunoprecipitation and Immunoblotting—Topoisomerase IIα was immunoprecipitated from whole-cell lysates, using 1 of anti-topoisomerase II antibody (4E12) per 50 μg of lysate protein. The lysate/antiserum mixture was incubated at 4°C for 1 h. Then 5 mg of protein A-Sepharose in 50 μl of lysis buffer containing protease inhibitors described above were added, and the mixture was shaken at 4°C for 30 min. Protein A-Sepharose-topoisomerase IIα complexes were pelleted at 10,000 rpm for 5 min at 4°C and washed three times with 500 μl of cold lysis buffer containing the protease inhibitors. The immunoprecipitates were resuspended in 30 μl of SDS-PAGE loading buffer (62.5 mM Tris-HCl, pH 6.8, 12.5% SDS, and 1.25% 2-mercaptoethanol) and boiled for 5 min. After centrifugation at 10,000 rpm for 5 min, the resulting supernatant was subjected to electrophoresis on 7.5% polyacrylamide gel and electroblotted onto nitrocellulose membrane. The blots were incubated with 1 h and with PT1342 antibody frequently with horseradish peroxidase-conjugated antimouse IgG serum (Amersham) for 1 h and detected by enhanced chemiluminescence (ECL), using the ECL Western blot detection system (Amersham Corp.).

Band Depletion Assay—Cells were incubated with various concentrations of VP-16 for 1 h, pelleted, and lysed with SDS-PAGE loading buffer, sonicated, and then boiled for 5 min. The samples were electrophoresed, blotted, and detected by ECL as described above, except that anti-topoisomerase IIβ antibody was used as a first antibody.

Alkaline Phosphatase Treatment—KB cells were lysed at 2.5×105 cells/ml and treated with 10 units of calf intestine alkaline phosphatase in a total of 20 μl of alkaline phosphatase buffer (100 mM glycine, 1 mM MgCl2, 0.1 mM ZnCl2, and 1 mM CaCl2, pH 9.8). The reactions were run at 37°C for 30 min and halted by adding 6.67 μl of 8% SDS, 800 μM 2-mercaptoethanol, and 40% glycerol. The samples were then boiled, electrophoresed in a 6% SDS-PAGE gel, and electroblotted onto nitrocellulose membrane as described above. Blots were detected by ECL.

Assay of Topoisomerase II Activity—Topoisomerase II activity was measured by its deactetnation of plasmid DNA in the presence of plasmid DNA cleavage. A reaction mixture contained 10 μl of Tris-HCl buffer, pH 8.0, 120 mM KCl, 10 mM MgCl2, 0.5 mM ATP, 0.5 mM dithiothreitol, 30 μg/ml alkaline phosphatase buffer, 200 ng of kinetoplast DNA, and 2 units of human topoisomerase II (TopoGEN, Inc.). For the inhibition assay by antibody, topoisomerase II was pretreated with PT1342 antibody (1:25 mg/ml) for 1 h and then centrifuged for 30 min at 37°C after adding the reaction mixture. Reactions were halted with 1 μl of 1% SDS and 0.0025% bromphenol blue. Electrophoresis was done on a 0.8% agarose gel in TAE buffer (40 mM Tris acetate-20 mM EDTA) for 60 min at 50 V. Etidium bromide-stained gels were photographed.

Flow Cytometry—Fixation of cells and double staining of cells for DNA and phosphorylated topoisomerase II were done as described by Steven et al. (48). Briefly, cells were trypsinized, washed with PBS, and fixed in PBS containing 1% formaldehyde at 4°C for 24 h. Fixed cells were pelleted and washed with PBS. The cells were suspended in permeabilization medium (5 mM Hapes, pH 7.5, 150 mM NaCl, 4% fetal bovine serum, and 0.1% Triton X-100) for 15 min on ice. Four volumes of PBS were added to the suspension, and then the cells were pelleted. The samples were stained for 3 h at room temperature with 200 μl of PBS containing RNase A (0.5 μg/ml), propidium iodide (PI, 10 μg/ml), and PT1342 antibody (1:1000). After washing the cell pellets with PBS, they were incubated for 20 min in PBS containing secondary antibody (1:1000 diluted fluorescein isothiocyanate-conjugated goat anti-mouse IgG). The cells were washed and resuspended in PBS containing PI and then analyzed. After passing the cells through nylon mesh, flow cytometry was performed on a FACScan (Becton-Dickinson).

Immunofluorescence—Reactions were carried out at room temperature unless stated otherwise. HeLa cells were cultured on glass coverslips, rinsed with PBS, fixed with 4% formaldehyde for 10 min, and then washed with PBS and permeabilized with 0.5% Triton X-100 for 5 min. After washing with PBS, the cells were treated with 1 mg/ml RNase in
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RESULTS

PT-1342 Antibody Reacts with Phosphopeptide but not Non-phosphopeptide—Topoisomerase II α is a ubiquitous enzyme in pro-and eukaryotic cells and has conserved sequences in the N-terminal and in the central regions, while the sequences in the C-terminal region are diverged (49). The sites of phosphorylation of topoisomerase II are mostly located at C-terminal domain (34, 37). In addition, in vivo phosphorylation sites correspond well to those phosphorylated by casein kinase II. Thus, we focused a topoisomerase II peptide in the C-terminal domain containing the consensus recognition site for casein kinase II (TXXE) to examine the relation between modulation of topoisomerase II activity and its phosphorylation. In particular, we chose threonine 1342 as the phosphorylation site of the casein kinase II because we are interested in phosphorylation of topoisomerase II in M phase, and the threonine residue of the enzyme is phosphorylated only in M phase (27). Furthermore, nuclear localization sequence is located near threonine 1342. The phosphopeptide corresponding to 1335–1350 in human topoisomerase II α was synthesized. Threonine 1342 was phosphorylated in vitro by casein kinase II (data not shown). Monoclonal and polyclonal antibodies against the phosphopeptide were prepared and tested by enzyme-linked immunosorbent assay, using phosphopeptide or non-phosphopeptide as an antigen. The polyclonal antibody recognized not only the phosphopeptide but also the non-phosphopeptide. In contrast, as shown in Fig. 1, the monoclonal antibody recognized only the phosphopeptide, not the non-phosphopeptide. The monoclonal antibody was named PT 1342.

PT1342 Antibody Recognizes Human Topoisomerase IIα—To determine whether the PT1342 antibody recognizes topoisomerase II α, HeLa cell extracts were prepared, subjected to electrophoresis, electroblotted, and probed with the PT1342 antibody and the ECL Western blot detection system. The antibody recognized the 170-kDa protein, a size corresponding to topoisomerase II α (Fig. 2a). To confirm that the 170-kDa protein recognized by PT1342 is topoisomerase II α, the protein was first immunoprecipitated with anti-topoisomerase II α antibody. The resulting immunoprecipitated materials were probed with anti-topoisomerase II α and PT1342 antibody. Both antibodies recognized the anti-topoisomerase II α immunoprecipitated protein as a single band of 170 kDa (Fig. 2b). The PT1342 antibody immunoprecipitated protein also reacted with anti-topoisomerase II α antibody, yielding a band of 170 kDa (data not shown). On the contrary, PT1342 antibody did not recognize anti-topoisomerase II β immunoprecipitated protein (data not shown). Topoisomerase II inhibitors, such as VP-16 and 4′-(9-acridinylamino) methanesulfon-m-anisidine, stabilize a covalent DNA-topoisomerase II complex (50). Since topoisomerase II in the complex is resistant to extraction with SDS from cell nuclei, the amount of the topoisomerase II interacting with the inhibitor can be detected by band depletion assay (51). If the PT1342 antibody recognizes topoisomerase II α, the amount of SDS-extracted protein recognized by the antibody will be reduced in VP-16-treated cells. HeLa cells were exposed to increasing concentrations of VP-16 for 1 h, and the SDS-extracted proteins were proved with PT1342 or anti-topoisomerase II α antibody. Both antibodies recognized the protein corresponding to 170 kDa and slightly lower molecules, which may be degradation products (Fig. 3). Anti-topoisomerase II α antibody also reacted with 180-kDa protein that is overlapped with 170 kDa in lane 5 but separated in lanes 6 and 7. The band recognized with PT1342 antibody was reduced in a dose-dependent manner. These results suggest that PT1342 antibody specifically recognizes topoisomerase II α. We next asked whether the PT1342 antibody would recognize the phosphorylated sites of topoisomerase II. Crude extracts of HeLa cells were treated with an alkaline phosphatase, electrophoresed, and tested with PT1342 antibody. The 170-kDa band, detected in non-phosphatase-treated samples, disappeared after phosphatase treatment (data not shown).

Further confirmation that PT1342 was directed against the phosphorylated threonine 1342 was provided by a mutant human topoisomerase II α lacking threonine 1342, produced by site-directed mutagenesis. Western blotting of this mutant topoisomerase II α, alongside recombinant human topoisomerase II α, human topoisomerase II β, and a bacterially expressed GST fusion protein of the human topoisomerase II α C-terminal domain (amino acids 1244–1531),2 confirmed that antibody PT1342 only recognized phosphorylated human topoisomerase II α containing threonine 1342 (Fig. 4).

2 C. A. Austin and L. M. Fisher, unpublished observations.
PT1342 Antibody Inhibits Topoisomerase II Activity—Threonine 1342 is located at the C-terminal region of human topoisomerase IIα. This region is not involved in enzyme activity such as ATPase, DNA breakage, and rejoining (49); the site may be related to regulatory elements of enzyme activity (3, 52). To determine whether PT1342 interacts with topoisomerase II, the effect of the antibody on HeLa topoisomerase II activity was examined using kinetoplast DNA as a substrate. The decatenating activity of the topoisomerase II was completely inhibited by PT1342 (Fig. 5).

Phosphorylation of Topoisomerase II in the Cell Cycle—In mammalian cells, the amount and phosphorylation of topoisomerase IIα increases following cell cycle progression from G1 to S and M phases (19, 23, 27). To clarify whether phosphorylation of threonine 1342 changes during the cell cycle, cells were stained simultaneously with PT1342 antibody for topoisomerase IIα and with PI for DNA and examined using flow cytometry. Fig. 6 shows a bivariate distribution of cells stained for DNA and phosphorylated topoisomerase IIα. Because cells in G2–M are about one-half the numbers of those in G1 and S phase, cells in G2–M contain about double the phosphorylated topoisomerase IIα at threonine 1342.

Localization of the Phosphorylated Topoisomerase II in HeLa Cells—Topoisomerase IIα locates in the nuclei in interphase and associated mitotic chromosomes (53, 54). We examined the subcellular distribution of P-topoisomerase II, using the PT1342 antibody. As shown in Fig. 7, the antigen detected by PT1342 antibody locates in interphase nuclei and mitotic chromosomes and its periphery, thereby confirming that threonine 1342 in topoisomerase IIα is phosphorylated throughout the cell cycle.

DISCUSSION

We obtained evidence that PT1342 antibody recognizes phosphorylated threonine 1342 in topoisomerase IIα. The antibody-recognized protein is present in the nuclei of interphase and is associated with the mitotic chromosome, findings in agreement with the cellular distribution of topoisomerase IIα (53, 54). The phosphorylation of threonine-1342 in human topoisomerase IIα occurs throughout the cell cycle, albeit the extent of the phosphorylation is about twice that in G2–M phase as compared to G1 and S phases (Fig. 6).

Threonine 1342 is located in the C-terminal region of topoisomerase IIα in a casein kinase II consensus sequence. The C-terminal domain of eukaryotic topoisomerase II is the least...
conserved, in contrast with the N-terminal domain, which has a highly conserved sequence. The region does not contain the catalytically functional regions, such as the ATP binding site, or the active tyrosine for DNA breakage and reunion (44). In Schizosaccharomyces pombe, transport of topoisomerase II into nuclei is reduced partially by C-terminal deletion (35). Similar results is the C-terminal domain is involved in nuclear transport functions were obtained by deletion analysis in Drosophila and S. cerevisiae (55, 56). The region is also speculated to be related to dimerization or to the regulation of enzyme activity (49). Recently, Watt et al. (57) have found that a protein related to Escherichia coli RecQ protein interacts with the C-terminal domain of S. cerevisiae topoisomerase II in vivo (57). Unexpectedly, PT1342 antibody completely inhibited topoisomerase II activity; thus, it is most likely that the catalytically active sites and threonine 1342 are close in secondary structure and interact, even though they are separated in the primary structure.

The biological role of casein kinase II is unclear, but it is known that it phosphorylates many growth-related proteins or transcription factors (58): For example, c-Myc, a transforming protein is phosphorylated by casein kinase II, and its phosphorylation inhibits DNA binding of Myb. v-Myb, which lacks the carboxy-terminal domain, is phosphorylated in vivo (59). Unexpectedly, PT1342 antibody completely inhibited topoisomerase II activity (49). Recently, Watt et al. (1993) reported that nuclear translocation of topoisomerase II is essential for mitotic chromosome condensation, segregation, and decondensation but not for other biological events in the cell cycle (7, 61–63). This does not mean that topoisomerase II functions only in M phase. In yeast, deletion mutants in topoisomerase I can survive, but topoisomerase II activity; thus, it is most likely that the catalytically active sites and threonine 1342 are close in secondary structure and interact, even though they are separated in the primary structure.

Using ICRF-193, a specific inhibitor of topoisomerase II, we clarified that this enzyme is essential for mitotic chromosome condensation, segregation, and decondensation but not for other biological events in the cell cycle (7, 61–63). This does not mean that topoisomerase II functions only in M phase. In yeast, deletion mutants in topoisomerase I can survive, but topoisomerase II activity; thus, it is most likely that the catalytically active sites and threonine 1342 are close in secondary structure and interact, even though they are separated in the primary structure.

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