Phosphorylation of Serine 1106 in the Catalytic Domain of Topoisomerase IIα Regulates Enzymatic Activity and Drug Sensitivity*

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Topoisomerases alter DNA topology and are vital for the maintenance of genomic integrity. Topoisomerases I and II are also targets for widely used antitumor agents. We demonstrated previously that in the human leukemia cell line, HL-60, resistance to topoisomerase (topo) II-targeting drugs such as etoposide is associated with site-specific hypophosphorylation of topo IIα. This effect can be mimicked in sensitive cells treated with the intracellular Ca²⁺ chelator, 1,2-bis(2-aminophenoxo)ethane-N,N,N’,N’-tetraacetic acid (BAPTA-AM). Here we identify Ser-1106 as a major phosphorylation site in the catalytic domain of topo IIα. This site lies within the consensus sequence for the acidotrophic kinases, casein kinase I and casein kinase II. Mutation of serine 1106 to alanine (S1106A) abrogates phosphorylation of phosphopeptides that were found to be hypophosphorylated in resistant HL-60 cells or sensitive cells treated with BAPTA-AM. Purified topo IIα containing a S1106A substitution is 4-fold less active than wild type topo IIα in decatenating kineoplast DNA and also exhibits a 2–4-fold decrease in the level of etoposide-stabilized DNA cleavable complex formation. Saccharomyces cerevisiae (JN394724) cells expressing S1106A mutant topo IIα protein are more resistant to the cytotoxic effects of etoposide or amssacrine. These results demonstrate that Ca²⁺-regulated phosphorylation of Ser-1106 in the catalytic domain of topo IIα modulates the enzymatic activity of this protein and sensitivity to topo II-targeting drugs.

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1 The abbreviations used are: topo, topoisomerase; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; DOX, doxorubicin; m-AMSA, amssacrine; WT, wild type; VP-16, etoposide; PVD, polyvinylidene difluoride; Tricine, N,N,N’,N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Ni NTA, N’N’-nitrilotriacetic acid; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption-ionization-time-of-flight; CID, collisionally induced dissociation; LC-tandem MS, liquid chromatography-tandem/mass spectrometry; kDNA, kinetoplast DNA.
decreased levels of protein kinase C (9, 10, 13). Decreasing intracellular calcium transients, which mimics the resistant phenotype, also results in site-specific hypophosphorylation of topo IIα (9, 10). A majority of the phosphorylation sites in topo IIα protein is located within the C-terminal region (9). Casein kinase II has been recognized as the major kinase interacting with and phosphorylating several sites in topo IIα, including Ser-1342, Ser-1376, Ser-1469, and Ser-1524 in human topo IIα (14–21). In addition to casein kinase II, protein kinase C has been shown to phosphorylate Ser-29 (8) and a proline-directed kinase (24) sites Ser-1212, Ser-1246, Ser-1353, Ser-1360, and Ser-1392 (9).

Despite the extensive knowledge of sites in topo IIα that are phosphorylated, very little is known about the significance of these phosphorylation sites in regulating topo IIα function. In this study, we employed a combination of in vivo phosphorylation and proteomic approaches to identify the site-specific phosphorylation of topo IIα, which affects enzymatic activity and confers resistance to topo II-targeting drugs. Our results demonstrate that Ser-1106 is a major phosphorylation site in the catalytic domain of topo IIα, hypophosphorylation of which correlates with drug resistance in the human leukemia cell line, HL-60. Mutation of serine 1106 to alanine leads to a decrease in the enzymatic activity and sensitivity to topo II-targeting drugs, thereby establishing the functional significance of the Ser-1106 phosphorylation site in topo IIα.

**EXPERIMENTAL PROCEDURES**

**Materials**—The topo II-targeting drugs VP-16 and m-AMSA were obtained from Sigma and the NCI, National Institutes of Health, respectively. Stock solutions of these drugs were prepared in dimethyl sulfoxide (DMSO) (Sigma) and stored frozen at −20 °C. The stock solutions of DMSO were prepared in synthetic dropout liquid medium lacking leucine. The overnight cultures were transferred to YPAD without leucine and incubated at 30 °C with shaking (250 rpm) in synthetic dropout liquid medium lacking leucine.

**Cell Culture**—Cultures of wild type HL-60 cells (HL-60/S) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine for 1 h at 37 °C. Cells were then labeled with 100 μCi/ml of carrier-free [32P]orthophosphoric acid (PerkinElmer Life Sciences) for an additional 2 h. In experiments involving treatment with BAPTA-AM, HL-60/S cells were incubated with 20 μM BAPTA-AM during the 2-h labeling period. The HL-60 yeast cells, expressing WT or S1106A-MT human topo IIα protein, were incubated overnight at 30 °C with shaking (250 rpm) in synthetic dropout liquid medium lacking uracil. The overnight cultures were transferred to YPAD without leucine and incubated at 30 °C with shaking to a cell density corresponding to 0.6 units (A650 nm). Following centrifugation, cells were resuspended into 200 μl of YPAD medium without phosphate containing 5 μCi of [32P]orthophosphoric acid and incubated at 30 °C for 1 h with shaking.

**Purification of Human Topo IIα**—HL-60 cells were lysed by 30 °C for 30 min in RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% Triton-X, 0.1% SDS, and 10 mM 2-mercaptoethanol) supplemented with a mixture of proteases and phosphatase inhibitors (10, 11). The lysate was centrifuged at 100,000 × g for 30 min, and top IIα present in the supernatant was incubated overnight at 4 °C with a topo IIα-specific polyclonal antibody (25) and protein A–agarose. The antigen-antibody complex was dissociated in lithium dodecyl sulfate sample buffer (Invitrogen), and top IIα was purified by gel electrophoresis in Tris-acetate buffer. Following transfer to nitrocellulose membrane (0.45 μm), the topo IIα protein band, visualized by staining with 0.25% Coomassie Brilliant Blue R-250, was excised and used for phosphopeptide mapping.

Recombinant topo IIα from yeast cells was isolated after freezing the cells in liquid nitrogen. The frozen cell pellet stored at −80 °C was lyzed by gentle rotation at 4 °C for 40 min in 2–3 volumes of Y-PER lysis buffer (Pierce) supplemented with 40 mM imidazole, 20 mM β-mercaptoethanol, and a mixture of proteases and phosphatase inhibitors. The cell lysate was centrifuged at 13,000 × g for 10 min at 4 °C, and top IIα protein in the supernatant was purified by Ni2+-nitritrolaticric acid (Ni-NTA)-agarose column (Qiagen, Valencia, CA) chromatography. After washing, the column was washed twice with wash buffer (200 mM imidazole, 20 mM sodium phosphate, 0.5 mM NaCl, pH 7.4), the protein was eluted with six successive 1-ml portions of the elution buffer (200 mM imidazole, 20 mM sodium phosphate, 0.5 mM NaCl, pH 7.4). The fractions from eluates 2–4 were pooled and concentrated at 3000 × g for 15 min using an Amicon Ultra-4, 30K NMWL concentrator (Millipore Inc., Milford, MA). The retentate was recovered and stored at −20 °C in 40% glycerol. Total protein content was determined using the Bio-Rad protein assay reagent (Bio-Rad), and purity was assessed by SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250. To determine the relative amount of WT and S1106A-MT protein present in purified preparations, Western blot analysis was also carried out using polyclonal topo IIα antibody and horseradish peroxidase-labeled secondary antibody.

**Phosphopeptide Mapping of Topo IIα**—[32P]-Labeled top IIα from HL-60 or BJ201 cells was purified by SDS-PAGE and transferred to nitrocellulose membrane as described above. The 170-kDa band of top IIα on the nitrocellulose membrane, identified by staining with Coomassie Brilliant Blue R-250, was excised and subjected to phosphopeptide mapping by CNBr or trypsin. CNBr digestion was carried out at 47 °C for 90 min in the presence of 250–400 μl of a solution containing 160 μg/ml CNBr in 70% formic acid. Following the incubation, the peptides released into the supernatant were concentrated by evaporation in a Savant SpeedVac and separated by one-dimensional gel electrophoresis on Tris-Tricine gel gels. The peptide bands were excised and subjected to phosphopeptide mapping by CNBr digestion followed by phosphoamidation of a PVDF membrane and subjected to autoradiography or Cyclone image analysis (PerkinElmer Life Sciences) to compare the phosphopeptide profiles of top IIα obtained from HL-60 cells treated under different conditions or yeast cells transformed with WT or S1106A-MT top IIα.

See the referenced text for detailed methods and results.
For tryptic digestion, the 170-kDa topo II protein bound to the nitrocellulose membrane was incubated overnight with 1:1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (0.5 μg) in 50 μl of 1% ammoc acidinate. Two-dimensional phosphate peptide mapping (11, 18, 26) was carried out by electrophoresis with pH 1.9 buffer in the horizontal dimension and chromatography in the vertical dimension using phosphochromatography buffer (n-butyl alcohol/pyridine/acetic acid/destilled water, 6:4:5:1.4, v/v). Image density of the phosphopeptides was quantified with a Cyclone imager.

N-terminal Edman Sequencing and Mass Spectrometry—CNBr phosphopeptides were separated by gel electrophoresis, transferred to a PVDF membrane, and stained with Coomassie Brilliant Blue R-250. The stained bands were excised and subjected to N-terminal Edman sequencing (27, 28). The N-terminal sequencing of the phosphate fragments was carried out on a Procise, model 492, protein sequencer (Applied Biosystems, Foster City, CA) fitted with a 140-cg microgradient system, 785A programmable absorbance detector, and a 610A (version 2.1) data analysis system. The internal peptide sequences were searched and identified by BLASTP program of the NCBI. To determine whether the sequenced peptides were phosphorylated, the membrane was autoradiographed before and after cutting out the stained bands.

LC-tandem MS analysis was carried out following tryptic digestion of the 170-kDa topos II protein or CNBr peptides of interest, which were excised from SDS-polyacrylamide gels (29, 30). This procedure was accomplished using a Finnigan LCQ-Deca ion trap mass spectrometry system equipped with an electrospray ion source interfaced to a 10-cm × 50-μm inner diameter C18 capillary high pressure liquid chromatography column. The digests were analyzed by LC-tandem MS with data-dependent acquisition methods to map as many peptides as possible from the protein. In these analyses, the LC-tandem MS data were searched versus the topoisomerase sequence using the program Sequest. Phosphopeptides were recognized by combinations of characteristic losses of 98 Da for H₃PO₄ from phosphoserine and phosphothreonine in the collisionally induced dissociation (CID) spectra. The MALDI-TOF analyses were carried out using a Micromass TofSpec 2E matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry system. Digests desalted using ZipTips were eluted with a solution of a-cyano-4-hydroxycinnamic acid as the matrix before spotting on the target plate.

The data were analyzed using CID spectra to search NCBI nonredundant data base with the search program TurboSequest. Spectra from samples analyzed by MALDI-TOF were internally calibrated using trypsin autolysis peptides, giving mass accuracy that was generally better than 25 ppm. These spectra were used for data base searches with the program MASCOT.

Decatation of Kinetoplast DNA (kDNA) by Topo IIa—Topo IIa enzymatic activity was assayed by measuring the decatation (31) of kDNA in vitro. A standard assay carried out in a total volume of 20 μl included 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 bovine serum albumin, and 300 ng of kDNA. The reaction mixture containing varying amounts of WT or S1106A-MT topo IIa was incubated at 37 °C, and at timed intervals the reaction was stopped by the addition of 5 μl of stop solution (5% SDS, 25% Ficoll, and 0.05% bromophenol blue). The samples were resolved by electrophoresis at 115 V using a 1% agarose gel in Tris acetate electrode buffer. Following electrophoresis, the gel was stained with ethidium bromide and photographed under UV illumination, and the amount of decatentated minicircles of kDNA was quantified using an AlphaInnotech Image analyzer (AlphaImager R544, Alpha Innotech Corp., San Leandro, CA). We have confirmed that the JN394-2 yeast cells are not viable at 35 °C and growth at 35 °C occurs only following transformation with WT pYepWob6 or S1106A-MT pYepWob6 plasmid. Colony growth of the JN394 t2-4 transformed with 20 μg BAPTA-AM were analyzed by two-dimensional phosphopeptide mapping and autoradiography.

Drug Sensitivity Test in Yeast JN394-2 Strain—S. cerevisiae strain JN394-2-4 transformed with either WT or S1106A-MT topo IIa pYepWob6 (22) plasmid was cultured at 30 °C in synthetic dropout liquid medium without uracil. The cells were collected by centrifugation and resuspended in YPDA medium. After adjusting the cell number to 2 × 10⁶ cells/ml, cells were treated for 24 h at 35 °C with VP-16 (0–200 μM) or m-AMSA (0.5–25 μM) at a final concentration of 2% Me₂SO. Following treatment, the control or treated cultures were diluted 1000-fold with sterile water and plated in triplicate usingYPD agar in 100 × 15-mm Petri dishes. After incubation for 3–4 days at 35 °C, the number of colonies on the control and treated drug plates were counted in an AlphaInnotech image analyzer (AlphaImager R544, Alpha Innotech Corp., San Leandro, CA).

Precipitation of Covalent Topo IIα-DNA Complex—Formation of covalent topo II-DNA complex by WT or S1106A-MT topo IIa was determined by the precipitation of 3′-end-labeled [32P]-pcDNA3 in a cell-free system as described by Zwelling et al. (32). Briefly, pcDNA3 was linearized with EcoRI and 3′-end-labeled with [32P]dATP. The 3′-end-labeled pcDNA3 was incubated with 10–200 μg WT or S1106A-MT topo IIa in the absence or presence of 1 μM ATP. To determine drug-stabilized DNA-cleavable complex formation in the presence of 1 μM ATP, 25 or 100 ng of WT or S1106A-MT topo IIa was incubated with 1–100 μM VP-16 for 30 min at 37 °C (10, 11, 32). The reaction was stopped by the addition of SDS, and the protein-DNA complex was precipitated by the addition of KCl (10, 11, 32). The precipitate was washed twice with 100 mM KCl and dissolved in water at 65 °C, and the solution was added to Ecolomos (ICN Pharmaceutical, Costa Mesa, CA). The level of radiolabel was then determined using a liquid scintillation counter.

RESULTS

CNBr Peptide 34 Is Hypophosphorylated in HL-60/S Cells Treated with BAPTA-AM and in HL-60/R Cells—Previously we have demonstrated the presence of two hypophosphorylated tryptic peptides in a derivative of HL-60 cells (HL-60/R) that are resistant to the topo II-targeting drug, VP-16 (10, 11). However, the location of the phosphorylation site(s) in topo IIa was not defined. To identify the region in topo IIα that contains these sites, we compared the one-dimensional maps of CNBr phosphopeptides generated from [32P]-labeled topo IIa immunoprecipitated from HL-60/S cells, HL-60/S cells treated with BAPTA-AM, and HL-60/R cells. Our results demonstrated that a CNBr phosphopeptide of an approximate molecular mass of 12 kDa was hypophosphorylated in HL-60/S cells treated with BAPTA-AM and HL-60/R cells as compared with HL-60/S cells (Fig. 1). Although the decrease in phosphoryla-
samples of tryptic digests of WT or S1106A-MT topo II (Tricine gels), transferred to PVDF membrane, and autoradiographed. The recombinant protein from these cells were metabolically labeled with \[^{32}P\]orthophosphoric acid. The recombinant protein was subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was stained with Coomassie Brilliant Blue R-250 (lanes 1 and 2) or immunoblotted with topo II\(\alpha\)-specific antibody (lanes 3 and 4).

Serine 1106 Is the Major Phosphorylation Site Present in CNBr Peptide 34—To determine the specific hypophosphorylation site in peptide 34, we performed LC-tandem mass spectrometry. This analysis led to the identification of a phosphorylated serine, Ser-1106, located in the catalytic domain of topo II\(\alpha\). The Ser-1106 phosphorylation site was detected initially in the 12-kDa CNBr fragment of topo II\(\alpha\) by data-dependent analysis. The CID spectrum of the peptide, VPDEDEENEEpSDNEKETEK, was detected. The detection of two phosphorylated peptides containing Ser-1106 (generated because of partial proteolysis) provides an explanation for the presence of two hypophosphorylated peptides in the two-dimensional tryptic phosphopeptide maps (Fig. 1B). The CID spectrum of these peptides is typical for a serine or threonine phosphopeptide, with a high abundance ion due to the loss of \(\text{H}_2\text{PO}_4^-\) and low abundance (but significant) sequence ions. Based on this spectrum, a highly specific, selected reaction-monitoring scheme was designed and used to detect this phosphopeptide in the more complex digest of intact topo II\(\alpha\).

**Hypophosphorylated Site in CNBr Peptide 34 and in Tryptic Peptides 2 and 3 Corresponds to Ser-1106**—Because Ser-1106 was identified as the major phosphorylation site in peptide 34, we determined the effect of mutation of this site to alanine on the phosphorylation of CNBr peptide 34 and tryptic peptides 2 and 3. Although the mammalian system was the basis of our findings, the facile expression of human topo II\(\alpha\) in yeast cells made this system an attractive model to carry out comparative studies of WT and S1106A-MT topo II\(\alpha\) protein. BJ201 yeast cells expressing WT or S1106A-MT topo II\(\alpha\) protein were metabolically labeled with \[^{32}P\](orthophosphoric acid. The recombinant protein from these cells was purified by Ni\(^{2+}\)-NTA column chromatography and subjected to SDS-PAGE. Following transfer to a nitrocellulose membrane, the stained topo II\(\alpha\) band was excised and digested with CNBr or trypsin. A, samples of CNBr digests of WT (lane 1) or S1106A-MT (lane 2) topo II\(\alpha\) were electrophoresed on SDS-polyacrylamide gels (10–20% Tris-Tricine gels), transferred to PVDF membrane, and autoradiographed. B, samples of tryptic digests of WT or S1106A-MT topo II\(\alpha\) protein were analyzed by two-dimensional phosphopeptide mapping and autoradiography.

**FIG. 3.** Differential phosphorylation of CNBr peptide 34 and tryptic peptides 2 and 3 generated from WT and SI106A-MT topo II\(\alpha\) protein expressed in BJ201 yeast cells. BJ201 yeast cells expressing WT or SI106A-MT-MTprotein were metabolically labeled with \[^{32}P\](orthophosphoric acid. The recombinant protein from these cells was purified by Ni\(^{2+}\)-NTA column chromatography and subjected to SDS-PAGE. Following transfer to a nitrocellulose membrane, the stained topo II\(\alpha\) band was excised and digested with CNBr or trypsin.

**FIG. 2.** Identification of Ser-1106 phosphorylation site by mass spectrometry. An in-gel tryptic digest of CNBr peptide 34 was analyzed using capillary column LC-tandem mass spectrometry. The data were analyzed using CID spectra to search NCBI nonredundant data base. The CID spectrum of the phosphopeptide containing Ser-1106 shows the facile loss of \(\text{H}_2\text{PO}_4^-\) to produce the doubly charged base peak in the spectrum (\(m/z\) 823.4). The peptide sequence and the site of phosphorylation (VPDEDEENEEpSDNEK) were subsequently established by interpretation of the low abundance fragment ions.

**FIG. 4.** Purification of WT and SI106A-MT topo II\(\alpha\) from BJ201 yeast cells. Extracts of yeast cells expressing WT or SI106A-MT topo II\(\alpha\) were purified by Ni\(^{2+}\)-NTA column chromatography. An aliquot (1.2 \(\mu\)g) of the purified preparation of WT (lanes 1 and 3) or SI106A-MT (lanes 2 and 4) topo II\(\alpha\) was subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was stained with Coomassie Brilliant Blue R-250 (lanes 1 and 2) or immunoblotted with topo II\(\alpha\)-specific antibody (lanes 3 and 4).
Site-specific Phosphorylation Regulates Topo IIα Function

Fig. 5. Comparison of the enzymatic activity of WT and S1106A-MT topo IIα protein. Varying concentrations (200 ng, lane 1; 100 ng, lane 2; 50 ng, lane 3; 25 ng, lane 4; 12.5 ng, lane 5; 6.25 ng, lane 6; 3.125 ng, lane 7) of WT or S1106A-MT topo IIα protein were incubated with kinetoplast DNA (kDNA). A, an aliquot of the reaction mixture was electrophoresed on a 1% agarose gel to separate the kDNA substrate from decatenated (minicircles) DNA, and the DNA bands were visualized by UV illumination of ethidium bromide-stained gels. The relative intensity of the bands was determined using an AlphaInnotech image analyzer. B, the percent decatenation, calculated as the ratio of the intensity of the decatenated band to the total intensity of the substrate band plus the decatenated band, was plotted versus concentration of WT (○) or S1106A-MT (○) topo IIα. C, time course of percent relative decatenation/μg of purified WT (○) or S1106A-MT (○) topo IIα.

S1106. It is interesting to note that the CNBr and tryptic phosphopeptide maps of human topo IIα from HL-60 and yeast cells (Figs. 1 and 3) are similar, although the relative intensities of individual phosphopeptides varies depending on the source of the topo IIα protein. The only major difference is the absence of a 4-kDa phosphopeptide in the CNBr maps and of phosphopeptide 1 in the tryptic maps of the recombinant protein (WT or S1106A-MT) expressed in yeast, as compared with native topo IIα expressed in HL-60 cells. This discrepancy is due to differential migration of the C-terminal CNBr or tryptic peptide, which is larger in the recombinant protein (due to the presence of additional amino acids transcribed from linker sequences) compared with the native protein. Indeed the C-terminal CNBr peptide has a molecular mass of 4 kDa and the C-terminal tryptic peptide phosphorylated at Ser-1524 corresponds to peptide 1 (18).

S1106A-MT Topo IIα Exhibits Reduced Enzymatic Activity and Formation of Protein-DNA Cleavable Complex—We next examined the functional role of Ser-1106 phosphorylation by comparing the catalytic activity and level of drug-stabilized, DNA-cleavable complex formation of recombinant WT or S1106A-MT topo IIα. For these studies the WT and S1106A-MT topo IIα proteins were expressed in the B201 yeast strain and purified by metal ion affinity chromatography using Ni²⁺-NTA columns. This procedure led to significant purification of topo IIα protein, which was present in equivalent amounts in the WT and S1106-MT protein preparation, as judged by Coomassie Brilliant Blue R-250 staining and Western blot analysis (Fig. 4). The catalytic activity of equivalent amounts of purified WT or S1106A-MT topo IIα protein was determined using 300 ng of kDNA as the substrate (31). As seen in Fig. 5, 4-fold more S1106A-MT topo IIα protein, as compared with WT topo IIα protein, was required to produce equivalent levels of decatenated minicircles from kDNA (Fig. 5, A and B). Comparison of the time course of relative decatenation activity by WT and S1106A-MT topo IIα revealed a 4-fold higher rate of reaction for WT, compared with S1106A-MT, topo IIα protein, based on the initial slope of the reaction curves (Fig. 5C). In these experiments (Fig. 5C), to observe measurable decatenation in the linear range of the slope, 50 ng of the mutant protein and 12.5 ng of the WT protein were required.

Because the S1106A-MT protein was enzymatically less active than the WT protein, we next determined whether these proteins also differed in their ability to form DNA-cleavable complex in the absence or presence of drugs. In the presence of 1 mM ATP, the level of topo II DNA-cleavable complex formation with the S1106A-MT topo IIα was 2-fold reduced compared with that of the WT protein (Fig. 6A). No difference was observed in the absence of ATP (Fig. 6A). In the presence of ATP
and the topo II-targeting drug, VP-16, a significant \((p = 0.01)\) 2–4-fold decrease in drug-stabilized, DNA-cleaveable complex formation was observed with the S1106A-MT compared with WT topo II protein (Fig. 6B). This difference was seen using two different concentrations of the topoII protein (25 and 100 ng as indicated above the panels in Fig. 6B).

S1106A-MT Topo II Protein Transformed in the JN394t2-4 Yeast Strain Is Resistant to the Cytotoxic Effects of the Topo II-targeting Drugs, VP-16 and m-AMSA—To confirm that hypophosphorylation of topoII at Ser-1106 confers a resistant phenotype in vivo, we tested drug sensitivity of yeast cells expressing WT or S1106A-MT topo II protein. Sensitivity to two topo II-targeting drugs, VP-16 and m-AMSA, was determined in the yeast strain JN394t2-4 transformed with the pYEpWob construct of WT or S1106A-MT human topo II protein. At 35 °C JN394t2-4 yeast cells do not grow unless the human recombinant topo II\(\alpha\) is expressed. The survival data in Table I demonstrate that JN394t2-4 cells transformed with S1106A-MT topo II are more resistant to the cytotoxic effects of different concentrations of VP-16 (up to 12-fold higher survival) and m-AMSA (up to 20-fold higher survival) than JN394t2-4 cells transformed with WT topo II\(\alpha\). This survival difference is not due to differential cellular expression of the WT and S1106A-MT protein, because extracts made from identical numbers of yeast cells transformed with the pYEpWob construct of human WT or S1106A-MT topo II\(\alpha\) express equivalent amounts of topo II\(\alpha\) protein (data not shown).

**DISCUSSION**

Post-translational modification of topo II\(\alpha\) by reversible phosphorylation is a key mechanism regulating its function. In this study, we identified a novel phosphorylation site, Ser-1106, in the catalytic domain of topo II\(\alpha\). Mutation of this site to alanine leads to a reduction in the catalytic activity of topo II\(\alpha\) and level of formation of enzyme-DNA-cleaveable complex. This results in decreased sensitivity to topo II-targeting drugs in vitro. To our knowledge this is the first report identifying a phosphorylation site in the catalytic domain of topo II\(\alpha\) that is capable of modulating its function.

Our previous observation demonstrating that specific sites in topo II\(\alpha\) protein were hypophosphorylated in cells resistant to topo II-targeting drugs (10, 11) provided the impetus for defining phosphorylation site(s) that were functionally relevant for topo II\(\alpha\) function, in particular regulation of sensitivity to topo II-targeting drugs. By using an integrated approach involving CNBr and tryptic phosphopeptide mapping of in vitro \(^{32}\)P-labeled topo II\(\alpha\), N-terminal Edman sequencing of CNBr peptides, and mass spectrometry, we were initially able to locate a region in the catalytic domain of the enzyme (amino acids 1041–1131) that harbored the hypophosphorylated site. Phosphorylation of this region could also be manipulated by altering intracellular Ca\(^{2+}\) transients using BAPTA-AM, which mimicked the resistant phenotype (10, 11). Mass spectrometric analysis of this region led to the identification of Ser-1106 as a major phosphorylation site. The functional significance of this site was established by comparing the activity of WT and Ser-1106-MT topo II\(\alpha\) protein in vitro and drug sensitivity of yeast cells transformed with these proteins in vivo.

Previous studies have identified several phosphorylation sites in topo II\(\alpha\), primarily in the C-terminal region. Consistent with the physiologic function of topo II\(\alpha\), phosphorylation of several sites is cell cycle phase-regulated (6–9). Although several kinases have been shown to phosphorylate topo II\(\alpha\), casein kinase II is the major kinase interacting with and phosphorylating several sites, including Ser-1342, Ser-1367, Ser-1469, and Ser-1524 in human topo II\(\alpha\) (14–21). In addition to casein kinase II, protein kinase C has been shown to phosphorylate Ser-29 (8), and a prolinc-directed kinase phosphorylates Ser-1212, Ser-1246, Ser-1353, Ser-1360, and Ser-1392 (9). Identification of most of these sites was based on in vitro studies employing purified protein kinases. However, in vivo phosphorylation of these sites was confirmed by matching tryptic phosphopeptides generated following phosphorylation in vitro and in vivo (8, 9). Despite these extensive studies, the functional significance of phosphorylation at these sites in vivo remains unclear. Although casein kinase II is capable of modulating the activity of mammalian topo II\(\alpha\), the mechanism by which this occurs is thought to involve stabilization of topo II\(\alpha\) but not phosphorylation per se (33, 34). Indeed, it has been shown that the C-terminal domain is not important for enzymatic activity, because deletion of this region or mutation of Ser-1376 and/or -1524 does not render the enzyme inactive (34). Rather, it has been proposed that phosphorylation at these sites may be important for subcellular localization of topo II\(\alpha\) (34). Furthermore, phosphorylation of Ser-29 does not affect the ATPase activity of topo II\(\alpha\), which is localized to the N-terminal region and required for the final religation step in the enzymatic reaction (35).

Resistance of tumor cells to topo II-targeting drugs have primarily focused on (a) overexpression of MDR1, which encodes P-glycoprotein resulting in enhanced drug efflux, and (b) point mutations or truncation in the topo II\(\alpha\) gene (5). Indeed, point mutations identified in model systems resistant to topo II-targeting drugs have been shown to confer drug resistance when tested in the JN394t2-4 yeast system (24). The functional

**Table I**

**Survival of JN394t2-4 yeast cells expressing WT or S1106A-MT topo II\(\alpha\) following treatment with VP-16 or m-AMSA for 24 h**

| Treatment | Survival (% control) | Ratio of S1106A-MT to WT |
|-----------|----------------------|-------------------------|
| VP-16     |                       |                         |
| 10 \(\mu M\) | 61.4 ± 7.8\(^a\) | 100.0 ± 12.9\(^b\) | 1.6 |
| 25 \(\mu M\) | 45.2 ± 7.8 | 92.0 ± 7.5 | 2.0 |
| 50 \(\mu M\) | 23.5 ± 5.2 | 69.2 ± 3.3 | 2.9 |
| 100 \(\mu M\) | 10.8 ± 3.5 | 49.5 ± 2.8 | 4.6 |
| 200 \(\mu M\) | 2.3 ± 1.1 | 28.2 ± 6.3 | 12.2 |
| m-AMSA    |                       |                         |
| 0.5 \(\mu M\) | 45.0 ± 13.1 | 100.0 ± 16.2 | 2.2 |
| 1.0 \(\mu M\) | 30.6 ± 4.9 | 95.4 ± 9.5 | 3.1 |
| 2.5 \(\mu M\) | 10.6 ± 5.2 | 60.8 ± 5.6 | 5.8 |
| 5.0 \(\mu M\) | 1.8 ± 0.5 | 38.4 ± 3.2 | 16.7 |
| 10 \(\mu M\) | 0.6 ± 0.2 | 12.6 ± 1.8 | 19.8 |

\(^a\) JN394t2-4 yeast cells expressing WT or S1106A-MT topo II\(\alpha\) were treated with the indicated concentrations of VP-16 or m-AMSA for 24 h.

\(^b\) Cell survival (%) ± S.E.

\(^c\) Survival of mutant cells was significantly higher than wild type cells, \(p = 0.05\).
role of site-specific phosphorylation on sensitivity of topo IIα to DNA-cleavable complex formation by topo II-targeting drugs in vitro or in vivo has not been addressed. In general, hypophosphorylated (10, 13) or hyperphosphorylated (12, 36) topo IIα has been correlated with drug insensitivity. Our results on resistance to topo II-targeting drugs in vitro and in vivo with S1106A-MT topo IIα provide evidence for a regulatory role of site-specific phosphorylation in sensitivity to topo II-targeting drugs. Thus, hypophosphorylation of topo IIα may be responsible for the lack of response of cancer patients to treatment with topo II-targeting drugs. Unlike S1106A-MT, the double mutant S1376A and S1524A topo IIα enzyme that is enzymatically active (34) is not resistant to the topo II-targeting drugs VP-16 or m-AMSA when tested in the JN394t2-4 yeast system (data not shown).

Ser-1106 is flanked by acidic amino acids, thereby fulfilling the consensus sequence requirement for both casein kinase I and casein kinase II (37). Thus these enzymes could serve as potential physiologic kinases regulating phosphorylation of Ser-1106. Although casein kinase II has been shown to phosphorylate several sites in the C-terminal region of topo IIα, the role of casein kinase I has not been evaluated, despite the presence of several casein kinase I consensus sites. Both casein kinase I and casein kinase II play an important role in regulating numerous cellular events. However, the presence of different isoforms of casein kinase I allows for a more diverse mechanism by which this enzyme can mediate signaling events. Of particular relevance to this study is the ability of isoforms of casein kinase I, casein kinase Iδ and casein kinase Iε (but not casein kinase II), to be activated by Ca2+-dependent dephosphorylation or proteolysis (38, 39). Indeed, it has been reported that Ca2+-dependent dephosphorylation of casein kinase Iε by calcineurin regulates phosphorylation and activation of DARP32 by metabotropic glutamate receptors in neostriatal neurons (40, 41). A mechanism similar to this could provide for an explanation for Ca2+-dependent phosphorylation of Ser-1106 by casein kinase Iδ or casein kinase Iε, which is supported by our preliminary data demonstrating decreased phosphorylation of CNBr and tryptic peptides containing Ser-1106 by two inhibitors of casein kinase I, CKI-7 and IC-261.2 However more

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Phosphorylation of Serine 1106 in the Catalytic Domain of Topoisomerase IIα Regulates Enzymatic Activity and Drug Sensitivity

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