Casein kinase I δ/ε phosphorylates topoisomerase IIα at serine-1106 and modulates DNA cleavage activity

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ABSTRACT

We previously reported that phosphorylation of topoisomerase (topo) IIα at serine-1106 (Ser-1106) regulates enzyme activity and sensitivity to topo II-targeted drugs. In this study we demonstrate that phosphorylation of Ser-1106, which is flanked by acidic amino acids, is regulated in vivo by casein kinase (CK) Iδ and/or CKIε, but not by CKII. The CKI inhibitors, CKI-7 and IC261, reduced Ser-1106 phosphorylation and decreased formation of etoposide-stabilized topo II–DNA cleavable complex. In contrast, the CKII inhibitor, 5,6-dichlorobenzimidazole riboside, did not affect etoposide-stabilized topo II–DNA cleavable complex formation. Since, IC261 specifically targets the Ca2+-regulated isoforms, CKIδ and CKIε, we examined the effect of down-regulating these enzymes on Ser-1106 phosphorylation. Down-regulation of these isoforms with targeted si-RNAs led to hypophosphorylation of the Ser-1106 containing peptide. However, si-RNA-mediated down-regulation of CKIIα and CKIIβ did not alter Ser-1106 phosphorylation. Furthermore, reduced phosphorylation of Ser-1106, observed in HRR25 (CKIδ/ε homologous gene)-deleted Saccharomyces cerevisiae cells transformed with human topo IIα, was enhanced following expression of human CKIε. Down-regulation of CKIδ and CKIε also led to reduced formation of etoposide stabilized topo II–DNA cleavable complex.

These results provide strong support for an essential role of CKIδ/ε in phosphorylating Ser-1106 in human topo IIα and in regulating enzyme function.

INTRODUCTION

Type II DNA topoisomerases, topoisomerase II (topo) α and β, regulate DNA topology by creating transient double stranded DNA breaks (1–3). Although, both enzymes exhibit significant sequence homology and catalyze redundant catalytic reactions, they are involved in different cellular functions. This difference may in part be due to differential regulation of these enzymes. Several different mechanisms have been shown to regulate topo II activity, including transcriptional, translational, as well as post-translational mechanisms. The major post-translational mechanisms that modulate topo II activity are phosphorylation, interaction with other proteins and proteasome-mediated degradation (1–3).

Both topo IIα and topo IIβ are phosphorylated at several sites, primarily in the divergent C-terminal region (4–8). Whereas, little is known about site-specific phosphorylation of topo IIβ, several in vitro and in vivo studies have identified specific phosphorylation sites in topo IIα. Within the C-terminal region of topo IIα phosphorylation of threonine-1342, serine(Ser)-1376, Ser-1469 and Ser-1524 catalyzed by casein kinase (CK) II (6,9–14), and of Ser-1212, Ser-1246, Ser-1353, Ser-1360 and Ser-1392 catalyzed by a proline directed kinase has been observed (15). Recently, it has been reported that Polo-like kinase 1 phosphorylates topo IIα at Ser-1337 and Ser-1524 (16).
In addition to the sites in the C-terminal region, phosphorylation of Ser-29 located in the ATP binding domain within the N-terminal region (17) and of Ser-1106 located within the catalytic core have also been reported (18). Whereas phosphorylation of Ser-29 is catalyzed by protein kinase C (17), the kinase responsible for phosphorylation of Ser-1106 has not yet been identified.

Since Ser-1106 is located in the catalytic domain of topo IIα and phosphorylation of this site enhances enzyme activity and sensitivity to topo II-targeted drugs in vivo (18), it is important to decipher the mechanism by which phosphorylation of Ser-1106 is regulated. The first step toward determining this mechanism would be to identify the kinase(s) that catalyzes phosphorylation at this site. Based on the acidic amino acid sequences that flank Ser-1106 at the amino- and carboxy-terminus, two potential kinases that could phosphorylate this site are CKI and CKII (19).

Although CKII has been recognized as a major kinase phosphorylating topo IIα, the role of CKI in phosphorylating topo IIα has not been explored. Unlike CKII, which consists of a tetramer of two catalytic subunits, α and β, and two regulatory β subunits (20–22), human CKI comprises of a superfamily of seven different isoforms that function as monomers (23,24). Structurally these isoforms, CKIα, β, γ1, γ2, γ3, δ and ε, are organized into three distinct regions – a short N-terminal region, a highly conserved kinase domain and a highly variable C-terminal domain, primarily involved in regulating enzyme function. The CKIα and CKIε isoforms are very similar in structure and exhibit 98% homology in the kinase domain and 50% homology in the C-terminal domain. Autophosphorylation of the C-terminal domain leads to inhibition of the enzyme, which can be relieved following dephosphorylation or proteolytic cleavage of this region, often via a Ca2+-dependent mechanism (25,26). Indeed, it has been suggested that dephosphorylation of CKIγ by the Ca2+/calmodulin-dependent phosphatase, calcineurin, enhances phosphorylation of DARP-32 by this isoform (27,28).

Our earlier studies demonstrating a Ca2+-dependent mechanism in regulating phosphorylation of Ser-1106 and in modulating sensitivity to topo II-targeted drugs (18) suggested that the kinase responsible for phosphorylating this site may be CKIδ and/or CKIε, rather than CKII. In this study we examined the role of CKIδ and CKII in phosphorylating Ser-1106 by attenuating the activity of these kinases with specific inhibitors or with targeted si-RNAs. Our results demonstrated that CKIδ, but not CKII, catalyzes the in vivo phosphorylation of Ser-1106 and regulates topo II–DNA cleavage activity.

MATERIALS AND METHODS

Reagents

CKI-7 was obtained from Seikagaku Kogyo, Tokyo. IC261 was kindly provided by ICOS Corp., Bothell, WA and 5,6-dichlorobenzimidazole riboside (DRB) was purchased from Calbiochem, La Jolla, CA, USA. Etoposide was purchased from Sigma-Aldrich, St Louis, MO, USA. Stock solutions of these compounds were made in dimethyl sulfoxide and stored at −20°C. The rabbit polyclonal antibody to topo IIα was a gift from Dr Ian Hickson, ICRF, Oxford, UK. Mouse monoclonal antibodies to CKIδ and CKIε were obtained from ICOS Corp., Bothell, WA (generous gift from Dr Anthony DiMaggio) and BD Biosciences, San Jose, CA, USA respectively. Goat polyclonal antibodies to CKIIα and CKIIγ were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Cell culture

HL-60 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM glutamate at 37°C in a humidified atmosphere of 5% CO2 and 95% air. HCT-116 cells, obtained from Dr Bert Vogelstein, Johns Hopkins University, Baltimore, MD, were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum and 2 mM glutamate at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cells were treated with CKI-7 (200 μM), IC261 (10 μM) or DRB (40 μM) for 3 h. When the combination of CKI or CKII inhibitor and etoposide was employed, cells were pretreated with CKI-7, IC261 or DRB for 3 h. Following this treatment, cells were washed, resuspended in inhibitor-free medium and incubated for an additional hour in etoposide.

Transfection with targeted si-RNAs

A 21-nucleotide duplex si-RNA (si-CKIδ/ε) with the sense sequence: 5’-CUGGGAGAAAGGCAACCdTdT-3' and antisense: 5’-GGUUGCCCUUCUUCCCCAGdTdT-3’, purchased from Qiagen, Valencia, CA, USA was used to target identical regions in CKIδ and CKIε (29). In addition the On-Target plus SMART pool si-RNA anti-CSNK1D human (si-CKIδ) and On-Target plus SMART pool si-RNA anti-CSNK1E human (si-CKIε) were purchased from Dharmacon, Lafayette, CO. For targeting CKIIγ RNA the siGENOM SMART pool CSNK2A1 (Dharmacon, Lafayette, CO) was employed. The CKIIγ si-RNA (sense: 5’-CAGUCUGAGGAGCCGCGAGdTdT-3’, antisense: 5’-CGGCUCUCAGACUdGdTdT-3’), previously described (30) was synthesized by MWG Biotech, Ebersberg, Germany. The control si-RNA (5’-GCUCAGAUCUAACGAGAdTdT-3’) was purchased from Dharmacon, Lafayette, CO. HCT-116 cells were incubated in serum-free McCoy’s medium for 6–10 h with the si-RNA (100 nM) in the presence of Lipofectamine 2000 (Invitrogen Life Technology, Carlsbad, CA) as described by the manufacturer. When the combination of si-CKIδ and si-CKIε was employed, the concentration of each si-RNA was reduced to 75 nM. Following the initial incubation, cells were washed and cultured in McCoy’s medium containing 10% fetal bovine serum and 2 mM l-glutamate for 24 h. At the end of the incubation period, cells were harvested for preparing cell lysates. When cells were transfected with si-CKIδ/ε about 50–70% of the cells that readily detached upon washing were used for preparing lysates for 2D-phosphopeptide maps of topo IIα, since both CKIδ and CKIε were maximally down-regulated in this population.
Transformation of *Saccharomyces cerevisiae* W303 cells

The wild-type (WT) *S. cerevisiae* W303 strain (ura3-1, trpl-1, leu2-3, 112, his3-11, 15 can1-100, ade2-1) and 7D, an *HRR25Δ* isolate isogenic to W303 (31) kindly provided by Dr. Anthony DeMaggio (ICOS Corp., Bothell, WA) were transformed with human topo IIΔ cDNA cloned in the pHT212 vector using the Yeastmaker lithium acetate transformation system (Clontech, Palo Alto, CA). Control transformations were carried out with the pHT212 plasmid (*LEU2*). Cells transformed with the pHT212 plasmid or pHT212 plasmid with the human topo IIΔ insert were selected on plates lacking leucine (18). The *HRR25Δ* isolate expressing human topo IIΔ was transformed with the human CKIΔ cDNA, (kindly provided by Dr. Jeff Kuret, Ohio State University, Columbus, OH), which was inserted in MluI and XbaI restriction sites of the modified pRS316 plasmid, YEpRS316. The YEpRS316 plasmid was constructed by restriction sites of the modified pRS316 plasmid, Columbus, OH), which was inserted in MluI and XbaI restriction sites of the modified pRS316 plasmid, YEpRS316. The YEplasmid plasmid was constructed by insertion of the Scal fragment from pYEpWOB6 which contains the 2μm origin. Control transformations were carried out with the YEpRS316 plasmid (*LEU2* and *URA3*). The transformed cells were selected on plates lacking leucine and uracil.

Metabolic labeling with [32P]-orthophosphoric acid

Log phase cultures of HL-60 or HCT-116 cells were incubated in phosphate-free RPMI-1640 supplemented with 10% dialyzed fetal bovine serum and 2 mM glutamine for 1 h at 37°C. Cells were then labeled with carrier-free [32P]-orthophosphoric acid (MP Biomedicals, Irvine, CA) for an additional 3 h. During the labeling period, the CKI or CKII inhibitor was added for experiments involving these treatments. Yeast cell lysates were labeled as previously described (18). Briefly, cells cultured overnight at 30°C with shaking (250 rpm) in synthetic dropout liquid medium lacking leucine were incubated in YPDA without phosphate medium, for 3 h with shaking, to a cell density corresponding to 0.6 units (A600). Following centrifugation, cells were resuspended into 20 ml of YPDA medium without phosphate containing 5 mCi of [32P]-orthophosphoric acid and incubated at 30°C for 1 h with shaking.

Preparation of cell lysates, immunoprecipitation and western blotting

Lysates of HL-60 and HCT-116 cells were prepared in radioimmunoprecipitation assay (RIPA) buffer as described earlier (18). Topo IIΔ protein immunoprecipitated from the cell lysate was subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane (18). The membrane was stained with Gelcode Blue staining reagent (Pierce Chemical Co., Rockford, IL) to visualize the topo IIΔ band, which was excised and processed for proteolysis with cyanogen bromide (CNBr) or trypsin (18). For determination of down-regulation of CKIΔ and CKIΔ, western blot analysis was carried out on cell lysates (20–40 μg) prepared from si-RNA transfected cells that were harvested just prior to labeling with [32P]-orthophosphoric acid (32). Cell lysate of *S. cerevisiae* cells were prepared in Y-PER lysis buffer after freezing the cell pellet in liquid nitrogen and human topo IIΔ present in the lysate was purified by Ni2+-nitrilotriacetic acid essentially as described earlier (18). Purified topo IIΔ was subjected to SDS–PAGE, transferred to nitrocellulose membrane and the stained topo IIΔ band was processed for phosphopeptide mapping.

Phosphopeptide mapping of 32P-labeled topo IIΔ

The 170 kDa 32P-labeled topo IIΔ band visualized by staining with Gelcode Blue staining reagent on the nitrocellulose membrane was excised and digested with trypsin essentially as described earlier (18). Following extensive washing of the membrane in water, topo IIΔ was proteolytically cleaved with 5 μg of trypsin-TPCK treated (Worthington Biochemical, Freehold, NJ) or with 2 μg of trypsin Gold® (Promega Inc., Madison, WI) in 1% ammonium bicarbonate, pH 8.3 at 37°C for 14–16 h. In initial experiments involving treatment with CKI inhibitors, proteolytic digestion with TPCK-treated trypsin consistently led to generation of two Ser-1106 containing peptides due to partial proteolysis (Figure 1B), whereas in subsequent experiments trypsinization with highly purified trypsin Gold® consistently

Figure 1. CKI inhibitors, CKI-7 and IC261, decrease phosphorylation of the topo IIΔ peptides containing Ser-1106. HL-60 cells were labeled with [32P] orthophosphoric acid for 3 h at 37°C in the absence or presence of CKI inhibitors, CKI-7 (200 μM) or IC261 (10 μM). Lysates of these cells were immunoprecipitated with topo IIΔ-specific antibodies. The immunoprecipitated protein was subjected to SDS–PAGE and transferred to nitrocellulose membrane. Two-thirds of the topo IIΔ band was cleaved with CNBr and one-third of the band was proteolyzed with trypsin. (A) CNBr fragments of topo IIΔ from cells treated in the absence or presence of CKI-7 or IC261 were separated in SDS–PAGE. (B) Phosphopeptide maps of tryptic digests of topo IIΔ from cells treated in the absence (Control) or presence of CKI-7 or IC261. The phosphochromatography buffer, n-butanol/pyridine/acetic acid/deionized water (5/3.3/1/4, v/v), was used for resolving peptides in the second dimension. Arrows indicate the position of Ser-1106 containing peptides.
led to generation of a single Ser-1106 containing peptide. The tryptic peptides released in the ammonium bicarbonate solution were transferred to fresh microcentrifuge tube and the membrane piece was washed with 100 μl of 20% acetonitrile. The pooled eluates were then concentrated by evaporation in a Savant Speed-Vac, washed three times with water and the peptides solubilized in pH 1.9 buffer (88% formic acid/glacial acetic acid/deionized water, 13:1:36, v/v). The peptides were then separated on thin layer cellulose plates by electrophoresis with pH 1.9 buffer in the horizontal dimension and chromatography in the vertical dimension (33). In initial experiments the phospho-chromatography buffer contained n-butanol/pyridine/acetic acid/deionized water (5/3.3/1/4, v/v). To improve migration of the Ser-1106-contained peptide an isobutyric acid buffer (isobutyric acid/n-butanol/pyridine/acetic acid/deionized water) (32.9/1/2.5/1.5/14.7, v/v), which resolves extremely hydrophilic phosphopeptides, was used.

Liquid chromatography–tandem mass spectrometry (LC–MS)

LC–MS was carried out on tryptic digests of stained topo IIα protein band excised from SDS–polyacrylamide gels. In-gel trypsin digestion was carried out as described earlier (34). Briefly, following washing/destaining in two aliquots of 50% ethanol/5% acetic acid (v/v), reduction with dithiothreitol and alkylation with iodoacetamide, the excised gel pieces were dried in a Speed-vac and incubated with 30 μl of 20 ng/μl trypsin in 50 mM ammonium bicarbonate on ice for 10 min. Any excess trypsin solution was then removed and 20 μl of 50 mM ammonium bicarbonate was added. Following overnight digestion at room temperature, the peptides were extracted from polyacrylamide pieces in two 30 μl aliquots of 50% acetonitrile/5% formic acid (v/v). These extracts were combined and evaporated to ~5 μl and then reconstituted in 1% acetic acid to a total volume of 25 μl for LC–MS analysis. The tryptic peptides in the extract (2 μl/injection) were separated by reversed-phase LC in a 10 cm × 50 μm (i.d.) Phenomenex Jupiter 10 μm C18 self-packed capillary column using a linear gradient of 2–70% acetonitrile containing 0.05 M acetic acid in 50 min at a constant flow rate of 0.2 μl/min. The effluent was analyzed using a Finnigan LCQ-Deca ion trap mass spectrometry system equipped with a Protean microelectrospray ion source (ThermoFisher, San Jose, CA) operated at 2.5 kV. Data interpretation was performed with the programs TurboSequest and Mascot. All matching spectra were verified by manual interpretation.

Selected reaction monitoring (SRM) mode was used to compare the extent of Ser-1106 phosphorylation in control scrambled si-RNA and si-CKIβ/ε treated HCT-116 cells. The SRM experiment consisted of a 5-scan event analysis in which one scan event was a standard MS scan and the other four were different SRM descriptors directed to various sets of control or Ser-1106 containing peptides, both unphosphorylated and phosphorylated. To verify peptide recovery from the digestion procedure and the mass spectrometry response, one ion of the trypsin autolysis peptide VATVSLPR at m/z 422 (+2) and one ion of the unmodified topo IIα native peptide EVTFVPGLYK at m/z 577 (+2) were monitored. These two descriptors served as controls. In addition, the ion transition m/z 484 → m/z 435 (+2) characteristic for phosphopeptide loss from phosphorylated Ser-29 contained in RLPs53VER peptide in topo IIα was monitored to confirm general phosphorylation of every sample. To determine the status of Ser-1106 phosphorylation, the peptide VPDEEENES1106DNEKETEK containing phosphorylated Ser-1106 as doubly charged (m/z 1116) or triply charged (m/z 744) ion and their corresponding unphosphorylated peptide ions were monitored.

DNA cleavable complex formation

The effect of etoposide on forming a stable topo II–DNA cleavable complex was determined by measuring the amount of precipitated protein–DNA complex and by evaluating depletion of topo IIα not complexed with DNA (band depletion). For measuring precipitated protein–DNA complex, cells were labeled for 24 h with 0.02–0.04 μCi/ml of [14C]-thymidine, specific activity 53 mCi/mmol (Amersham, Arlington Heights, IL). For measuring DNA cleavable complex in cells down-regulated for CKIβ plus CKIε, cells were treated for 6 h with scrambled si-RNA or si-CKIβ plus si-CKIε prior to labeling. Cells were then trypsinized, treated with etoposide for 1 h and the precipitated protein–DNA complex was assayed as previously described (35). For the band depletion experiment cells were treated similarly without the addition of [14C]-thymidine. Cell lysates were prepared in 2-fold concentrated LDS-sample buffer (Invitrogen Life Technology, Carlsbad, CA, USA). The lysates were incubated at 70 °C for 10 min, sonicated and centrifuged at 12 000 × g. An aliquot (10–15 μl) of the lysate was subjected to western blot analysis. The membranes were probed with antibodies to topo IIα and topo I (internal control), which does not form a stabilized DNA cleavable complex with the topo IIα-targeted drug, etoposide. Down-regulation of CKIβ and CKIε was also determined by western blotting with antibodies specific for CKIβ and CKIε.

RESULTS

Inhibitors of CKIβ and CKIε (CKI-7 and IC261) lead to hypophosphorylation of the CNBr and tryptic topo IIα peptides containing Ser-1106 and reduce formation of etoposide stabilized topo IIα–DNA cleavable complex

The presence of acidic amino-acid residues N-terminal to Ser-1106 and the Ca2+-dependency of phosphorylation of this site (18) suggested that phosphorylation of Ser-1106 may be regulated by protein kinase CKIβ and/or CKIε. Therefore we first examined whether two CKI inhibitors, CKI-7 and IC261, specific for CKIβ and CKIε (36), altered phosphorylation of Ser-1106 containing peptides. Treatment of HL-60 cells with CKI-7 or IC261 led to hypophosphorylation of the CNBr (peptide 34) and tryptic phosphopeptides that were previously shown to harbor Ser-1106 (Figures 1A and 1B, respectively). Comparison of the intensity of spots corresponding to the peptides containing Ser-1106 (normalized to two other peptides)
revealed that CKI-7 and IC-261 led to comparable decreases (20–40% and 25–30%, respectively) in phosphorylation of Ser-1106 in vivo. To confirm whether decreased phosphorylation observed in the presence of CKI-7 or IC261 compromises the functional activity of topo IIz, we examined the effect of CKI-7 and IC261 on formation of etoposide stabilized topo II–DNA cleavable complex. Pre-treatment of HL-60 cells with 200 μM CKI-7 or 10 μM IC261 for 3 h at 37 °C, 30 min prior to treatment with 5 μM etoposide for 1 h led to a significant (P < 0.05) decrease in the formation of etoposide-stabilized DNA cleavable complex (Figure 2). We also examined the effect of the CKII specific inhibitor, DRB, on formation of etoposide stabilized topo II–DNA cleavable complex. Pretreatment with 40 μM DRB did not alter etoposide stabilized topo II–DNA cleavable complex formation (Figure 2). These results suggest a role for CKIδ/ε in regulating the functional activity of topo IIz via phosphorylation at Ser-1106.

**Down-regulation of CKIδ and/or CKIε with targeted si-RNA decreases phosphorylation of the tryptic peptide containing Ser-1106**

To confirm the role of CKIδ and/or CKIε in phosphorylating Ser-1106 we down-regulated these two enzymes with three different sets of si-RNAs. These included si-CKIδ/ε—which targeted the nucleotide sequence (412–430) that is identical in the CKIδ and CKIε coding region; si-CKIδ—which is a smart pool targeted to the CKIδ isoyme; and si-CKIε—which is a smart pool targeted to the CKIε isoyme. The si-CKIδ and si-CKIε were used individually to down-regulate the specific isoyme or used in combination to simultaneously down-regulate both isoymes. Since, HL-60 cells are difficult to transfect we used the colon carcinoma cell line, HCT-116, for transfection of the si-RNAs. This cell line was chosen because it can be readily transfected and the phosphopeptide map of topo IIz in HCT-116 cells is similar to that in HL-60 cells (data not shown). Transfection of the three si-RNAs in HCT-116 cells led to significant down-regulation (~60–80%) of the targeted isoyme; si-CKIδ/ε and the combination of si-CKIδ and si-CKIε led to down-regulation of both CKIδ and CKIε, whereas si-CKIδ or si-CKIε when used individually, down-regulated only the targeted enzyme CKIδ or CKIε, respectively (Figures 3A, 5A and 8A). When both CKIδ and CKIε were down-regulated a slight increase in the G2 + M phase of the cell cycle was observed (data not shown).

The down-regulation of CKIδ and CKIε in HCT-116 cells transfected with si-CKIδ/ε resulted in significantly decreased (75%) phosphorylation of the tryptic Ser-1106 containing phosphopeptide, as compared to cells transfected with the control scrambled si-RNA (Figure 3B). This was not due to an increase in the G2 + M population observed in cells transfected with si-CKIδ/ε, since phosphorylation at Ser-1106 was not affected when cells were blocked in mitosis following treatment with nocodazole (Figure 3C). Comparison of phosphorylation of Ser-1106 in topo IIz present in cells transfected with scrambled si-RNA or si-CKIδ/ε by LC-MS (Figure 4) revealed findings that were similar to those obtained by 2D-phosphopeptide mapping. Although both phosphorylated and unphosphorylated Ser-1106 was detected in control cells transfected with scrambled si-RNA, only unphosphorylated Ser-1106 was detected in cells transfected with si-CKIδ/ε. Down-regulation of only one isoyme, CKIδ or CKIε (Figure 5A), also led to hypophosphorylation of the Ser-1106 containing peptide (Figures 5B and 5C, respectively), albeit to a lesser extent than that observed when both CKIδ and CKIε were down-regulated (Figure 3B). In cells treated with si-CKIδ, phosphorylation of Ser-1106 was 25–40% less than that observed in cells treated with scrambled si-RNA, whereas in cells treated with si-CKIε phosphorylation of Ser-1106 was 40–65% less than that observed in cells treated with scrambled si-RNA. Differential hypophosphorylation of Ser-1106 in si-CKIδ and si-CKIε transfected cells could be due to differences in the effectiveness of the two isoymes in phosphorylating this site or due to differential down-regulation of CKIδ (~60%) and CKIε (~80%).

To confirm that phosphorylation of Ser-1106 in vivo does not involve CKII we examined the effect of transfecting HCT-116 cells with si-RNAs to CKIIz and CKIIε’ on phosphorylation of Ser-1106. Results of this experiment revealed that transfectants, in which CKIIz and CKIIε’ were significantly down-regulated (Figure 6A), did not exhibit altered phosphorylation of Ser-1106 although phosphorylation of other tryptic peptides previously...
we compared phosphorylation of Ser-1106 in WT or HRR25Δ cells transformed with human topo IIα. As shown in Figure 7A, phosphorylation of the tryptic Ser-1106 peptide was significantly reduced in HRR25Δ cells. Transformation of the HRR25Δ cells expressing human topo IIα with human CKIε enhanced phosphorylation at Ser-1106 (Figure 7B). This finding provides further support for the role of CKIδ/ε, in particular the CKIε isozyme, in regulating phosphorylation at Ser-1106 in topo IIα.

Reduced phosphorylation of Ser-1106 in HCT-116 cells transfected with si-CKIδ plus si-CKIε leads to decreased formation of etoposide stabilized topo II–DNA cleavable complex

We previously demonstrated a functional role for Ser-1106 phosphorylation in topo IIα, based on the observation that mutation of Ser-1106 to alanine in human topo IIα led to decreased topo IIα function in vitro and in vivo in JN394 yeast cells transformed with human topo IIα (18). In this study we examined whether topo IIα function is affected when phosphorylation at Ser-1106 is altered by down-regulating the kinase(s), CKIδ and/or CKIε, involved in phosphorylating this residue. Down-regulation of CKIδ and CKIε by si-CKIδ plus si-CKIε (Figure 8A) led to a significant (P < 0.01) decrease in SDS–KCl precipitable 14C-thymidine labeled etoposide stabilized topo II–DNA cleavable complex (Figure 8B).

Since, formation of a stable topo IIα–DNA cleavable complex in the presence of etoposide leads to depletion of topo IIα not complexed with DNA, we also determined the amount of topo IIα in lysates of control or etoposide-treated HCT-116 cells that were transiently transfected with scrambled si-RNA or si-CKIδ plus si-CKIε. In cells transfected with si-CKIδ plus si-CKIε depletion of topo IIα following treatment with etoposide was less (~40–50%) than that observed in cells transfected with scrambled si-RNA (Figure 8C). This finding corroborates the previous data demonstrating decreased formation of the etoposide stabilized topo II–DNA cleavable complex in cells transfected with si-CKIδ plus si-CKIε. These results indicate that CKIδ and/or CKIε are involved in regulating topo IIα function via phosphorylation at Ser-1106.

DISCUSSION

In the present study we identify CKIδ and/or CKIε as upstream kinase(s) regulating in vivo phosphorylation of topo IIα at Ser-1106 and thereby modulating the DNA cleavage activity of the enzyme. The role of CKIδ and/or CKIε in phosphorylating Ser-1106 is based on several lines of experimental evidence. In vivo phosphorylation of the tryptic peptide that contains Ser-1106 is decreased when CKIδ and/or CKIε are inhibited by the CKI inhibitors, CKI-7 and IC261 (specific for CKIδ/ε), or when CKIδ and CKIε are down-regulated by targeted si-RNAs. Similarly, in human topo IIα expressing HRR25 (CKIδ and CKIε homologous gene) deleted S. cerevisiae cells, the tryptic peptide containing Ser-1106

Phosphorylation of Ser-1106 in human topo IIα expressed in HRR25Δ S. cerevisiae cells is reduced, but can be enhanced following transformation of the cells with human CKIε

Since human topo IIα expressed in S. cerevisiae cells is phosphorylated at Ser-1106 (18) and the S. cerevisiae gene, HRR25 is homologous to CKIδ and CKIε (37),

reported to be substrates for casein kinase II (6) was reduced (Figure 6B).
Figure 4. Peptides containing phosphorylated Ser-1106 are not detected by LC-MS analysis of topo IIα from si-CKIδ/ε transfected HCT-116 cells. HCT-116 cells were transfected with scrambled si-RNA or si-CKIδ/ε. Topo IIα protein present in these cells was purified by immunoprecipitation and SDS-PAGE. The stained topo IIα band in the gel was digested with trypsin and the peptides were analyzed by LC–MS as described in Materials and methods section. (A) Total ion chromatograms (upper panel) and trypsin autolysis marker at m/z 422 (lower panel) of topo IIα tryptic digests from cells transfected with scrambled si-RNA or si-CKIδ/ε. (B) SRM chromatograms for the doubly charged peptide ion containing phosphorylated Ser-1106 at m/z 1116 (upper panel) and CID spectrum of doubly charged peptide ion (lower panel) containing phosphorylated Ser-1106 in topo IIα, detected only in samples treated with scrambled si-RNA. (C) SRM chromatograms for the triply charged peptide ion containing phosphorylated Ser-1106 at m/z 744 (upper panel) and characteristic H$_3$PO$_4$ neutral loss for the m/z 744 (+3) phosphorylated peptide (middle panel). CID spectrum of triply charged peptide ion (lower panel) containing phosphorylated Ser-1106 in topo IIα was detected only in samples treated with scrambled si-RNA. Characteristic loss of H$_3$PO$_4$ is seen in both CID spectra. Detected b (N-terminal) and y (C-terminal) fragment ions are labeled in the spectra.
is also hypophosphorylated, and phosphorylation at this site can be enhanced following transformation of these cells with human CKIIε. The decrease in phosphorylation of the Ser-1106 tryptic peptide is indeed due to reduced phosphorylation at this site, since our earlier studies indicated that in vivo phosphorylation of this tryptic peptide was not observed when Ser-1106 in topo IIα was mutated to alanine (18). Furthermore, LC–MS analysis of phosphorylated Ser-1106 in topo IIα obtained from HCT-116 cells treated with scrambled si-RNA or si-CKIIε revealed the presence of phosphorylated Ser-1106 only in scrambled si-RNA treated cells.

The decrease in phosphorylation at Ser-1106 observed in cells exhibiting reduced kinase activity of CKIIε and CKIIα correlates with a decrease in topo IIα function. Inhibition of the CKI activity in cells treated with CKI inhibitors, CKI-7 or IC-261 leads to reduced formation of topo II–drug DNA complex in vivo. Similarly, when CKIε and CKIIα are down-regulated by targeted si-RNAs, formation of the etoposide stabilized topo II–DNA cleavable complex is reduced. These results indicate that the phosphorylation at Ser-1106 catalyzed by CKIIε and/or CKIε enhances the in vivo DNA cleavage activity of topo IIα.

Several different kinases, including CKII, protein kinase C, proline directed kinases e.g. cdc2 and Polo-like kinase 1 have been reported to phosphorylate topo IIα (6,9–17). However, this is the first report demonstrating CKI as a physiologically relevant kinase that modulates phosphorylation and activity of topo IIα. Our data demonstrating decreased phosphorylation of several peptides (excluding the Ser-1106 containing peptide) following down-regulation of CKIIα and CKIIαε with targeted si-RNAs provides evidence that CKII is also capable of

Figure 5. Down-regulation of CKIδ or CKIε with targeted si-RNAs in HCT-116 cells decreases phosphorylation of the topo IIα peptide containing Ser-1106. HCT-116 cells were transfected with scrambled si-RNA, si-CKIδ or si-CKIε as described in Materials and methods section. A small aliquot of the cells was lysed in RIPA buffer and subjected to western blot analysis to determine down-regulation of CKIδ and CKIε (A). The remaining cells were labeled with [32P] orthophosphoric acid for 3 h, lysed in RIPA buffer and the cell lysates were immunoprecipitated with topo IIα-specific antibodies. The immunoprecipitated topo IIα protein was subjected to SDS–PAGE and transferred to nitrocellulose membrane. The stained topo IIα band was excised, proteolyzed with trypsin and the labeled tryptic peptides analyzed by 2D-phosphopeptide mapping (B, C). The isobutyric acid buffer (isobutyric acid/n-butanol/pyridine/acetic acid/deionized water) (32.9/1.2.5/1.5/14.7, v/v) was used for resolving the peptides in the second dimension. The arrow indicates the position of Ser-1106 containing peptide.

Figure 6. Down-regulation of CKIα and αε with targeted si-RNAs does not alter phosphorylation of the topo IIα peptide containing Ser-1106. An aliquot of HCT-116 cells transfected with scrambled si-RNA or si-CKIα or si-CKIαε, as described in Materials and methods section, were lysed in RIPA buffer and subjected to western blot analysis to determine down-regulation of CKIα and CKIαε (A). The remaining cells were labeled with [32P] orthophosphoric acid for 3 h. Cell lysates prepared in RIPA buffer were immunoprecipitated with topo IIα-specific antibodies, subjected to SDS–PAGE and transferred to nitrocellulose membrane. The stained topo IIα band was excised, proteolyzed with trypsin and the labeled tryptic peptides analyzed by 2D-phosphopeptide mapping (B). The isobutyric acid buffer (isobutyric acid/n-butanol/pyridine/acetic acid/deionized water) (32.9/1.2.5/1.5/14.7, v/v) was used for resolving the peptides in the second dimension. The arrow indicates the position of Ser-1106 containing peptide.
phosphorylation of topo IIα in vivo. However, phosphorylation at CKII sites does not significantly affect sensitivity to topoII-targeted drugs, since treatment of HL-60 cells with the CKII inhibitor, DRB, did not significantly alter formation of topo II-drug–DNA complex in vivo. Since our study did not evaluate the effect of reduced phosphorylation at CKII sites on other functions of topo IIα, the functional role of phosphorylations at CKII sites remains unclear. It is possible that phosphorylation at CKII sites, most of which map to regions in the C-terminal domain, along with other phosphorylations within this region regulate accessibility of the catalytic site to its substrate. This mechanism would be analogous to that described for regulation of the activity of CKIδ and CKIε, wherein dephosphorylation of the C-terminal region activates the enzyme.

Our previous data demonstrating Ca2+-dependent phosphorylation of Ser-1106 (18), provides further support for the role of CKIδ and/or CKIε (Ca2+-regulatable enzymes), but not CKII, as physiologic kinases that modulate Ser-1106 phosphorylation. Although, the catalytic activity of CKIδ and CKIε does not require Ca2+2+, these enzymes can be regulated via Ca2+-dependent dephosphorylation or proteolysis (25,26). In neostriatal neurons, the metabotropic glutamate receptors activate CKIε by dephosphorylating the inhibitory C-terminal autophosphorylation sites by the Ca2+-dependent phosphatase, calcineurin (38). The activated CKIε then leads to phosphorylation of Ser-137 of DARPP-32. A scenario similar to that described in neostriatal muscles, could explain how phosphorylation of Ser-1106 is regulated by Ca2+2+ influx in neurons (27,39), could remove the inhibitory

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domain in CKI\(\alpha\) or CKI\(\epsilon\) and lead to activation of these kinases.

CKI isozymes are involved in regulating several different cellular processes (40). The CKI\(\alpha\) and CKI\(\epsilon\) isozymes have been shown to modulate the development process because of their role in the wnt signaling pathway. In addition, these isozymes play a role in circadian rhythm, cell division, apoptosis and neurodegenerative diseases. CKI\(\alpha\) and CKI\(\epsilon\) phosphorylate p53, tubulin and microtubule-associated proteins to regulate cell growth, chromosome segregation and stress response at the spindle apparatus and the mitotic centrosome. The identification of topo II\(\alpha\), which is also involved in regulating DNA replication and cell division, chromosome segregation and DNA repair, as another nuclear substrate of CKI\(\alpha\) and/or CKI\(\epsilon\) in this study, suggests that these CKI isozymes may be essential for regulating various aspects of DNA metabolism. In this regard, topo II\(\alpha\) could function as part of a protein complex that comprises of transcription factors, nuclear regulatory proteins and kinases (including CKI\(\alpha\) and/or CKI\(\epsilon\))/phosphatases that regulate phosphorylation/dephosphorylation of components of the complex. Thus, it would be important to determine whether CKI\(\alpha\) or CKI\(\epsilon\) is capable of associating with topo II\(\alpha\).

In summary our results demonstrate that CKI\(\alpha\) and/or CKI\(\epsilon\) are physiologically relevant kinase(s) that are involved in regulating site-specific phosphorylation at Ser-1106 and modulating the function of topo II\(\alpha\). Since Ser-1106 phosphorylation regulates sensitivity of cells to topo II-targeted drugs and expression of CKI\(\alpha\) and/or CKI\(\epsilon\) can be altered in cancer cells, one potential mechanism by which tumors develop resistance to topo II-targeted drugs could involve decrease in expression or activation of CKI\(\alpha\) and/or CKI\(\epsilon\). The correlation of Ser-1106 hypophosphorylation with etoposide resistance was not only observed in cell culture model systems but was also seen in blast cells isolated from patients with acute myelogenous leukemia. Comparison of Ser-1106 phosphorylation with etoposide induced apoptosis revealed that reduced phosphorylation at Ser-1106 was associated with decreased apoptosis (data not shown). Thus, it might be possible to identify sensitivity of tumors to topo II\(\alpha\)-targeting drugs by characterizing phosphorylation at Ser-1106 or by determining the expression level or activity of CKI\(\alpha\) and CKI\(\epsilon\) in tumor samples.

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