Serine 1524 Is a Major Site of Phosphorylation on Human Topoisomerase IIα Protein in Vivo and Is a Substrate for Casein Kinase II in Vitro*

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Topoisomerase II protein is essential for cell proliferation and is known to exist as a phosphoprotein in cells from both lower and higher eukaryotic species. In this paper, we have investigated the phosphorylation of the α isozyme of human topoisomerase II. The topoisomerase IIα protein was phosphorylated predominantly on serine residues in the human tumor cell lines HeLa and NSCLC-3. Two-dimensional tryptic phosphopeptide mapping studies revealed several sites of phosphorylation in vivo, including a major site that was common to topoisomerase IIα protein from both HeLa and NSCLC-3 cells. To identify sites of phosphorylation, the regulatory C-terminal domain of human topoisomerase IIα protein was overexpressed in Escherichia coli as a hexahistidine-tagged fusion protein and purified by nickel chelate chromatography. Tryptic phosphopeptide mapping revealed that casein kinase II phosphorylated the C-terminal domain primarily on 2 serine residues in vitro, which were shown to be sites of modification in vivo. Site-directed mutagenesis studies identified these casein kinase II-specific phosphorylation sites as serine 1524 and serine 1376.

Many activities associated with DNA, including transcription, recombination, and replication, require alterations in DNA topology. DNA topoisomerases are enzymes that regulate chromosome structure through their ability to catalyze the interconversion of topological isomers of DNA. Depending upon their mechanism of action, topoisomerases are classified as either type I or type II; the former makes single strand cleavages in DNA, and the latter makes double strand cleavages (reviewed by Wang (1986), Hsieh (1990), Osheroff et al. (1991), and Watt and Hickson (1994)). Type II enzymes alone can segregate multiply intertwined daughter chromosomes during cell division in eukaryotic cells, and topoisomerase II protein has been shown to be essential for mitosis and meiosis in yeast (DiNardo et al., 1984; Holm et al., 1985; Uemura and Yanagida, 1986; Uemura et al., 1987; Rose et al., 1990; Rose and Holm, 1993).

The cell cycle phase specificity of topoisomerase II activity suggests that topoisomerase II activity might be temporarily regulated. Such regulation could involve post-translational modification of the topoisomerase II protein. Indeed, previous studies have indicated that topoisomerase II enzymes from a wide variety of eukaryotic sources exist as phosphoproteins (Sahyon et al., 1986; Rottman et al., 1987; Ackerman et al., 1988; Heck et al., 1989; Kroll and Rowe, 1991; Cardenas et al., 1992; Saijo et al., 1992; Shiozaki and Yanagida, 1992; Burden et al., 1993). For example, Drosophila topoisomerase II protein has been shown to be a substrate in vitro for casein kinase II, protein kinase C, and calmodulin-dependent protein kinase II, but not for protein kinase A (Ackerman et al., 1988; Sahyon et al., 1986). Topoisomerase II protein from budding yeast is also a substrate for casein kinase II in vitro (Cardenas et al., 1992). Moreover, in vivo studies have indicated that casein kinase II is likely to be an important regulator of topoisomerase II function in both Drosophila and budding yeast cells (Ackerman et al., 1988; Cardenas et al., 1992). In contrast to these results, work by Shiozaki and Yanagida (1992) has suggested that the activity of topoisomerase II protein from fission yeast may not be regulated in a similar manner by changes in phosphorylation status. Phosphorylation of topoisomerase II enzymes has also been demonstrated in cells from higher eukaryotes, including human and rodent cells, and appears to be cell cycle regulated (Heck et al., 1989; Saijo et al., 1992; Kroll and Rowe, 1991; Burden et al., 1993).

Interpretation of previous phosphorylation studies on topoisomerase II proteins from mammalian sources has been complicated by the recent observation that two closely related isozymes of this protein are expressed in mammalian cells but probably are not expressed in lower eukaryotes (Drake et al., 1989; Chung et al., 1989). The α isozyme, encoded by a gene on chromosome 17 in humans (Tsai-Pflugfelder et al., 1988), is a protein of 170 kDa, and the β isozyme, encoded by a gene on chromosome 3 (Tan et al., 1992; Jenkins et al., 1992), is 180 kDa. Despite strong sequence similarity in their catalytic domains, the α and β isozymes appear to have quite distinct biochemical and pharmacological properties (Drake et al., 1989) and may have independent functions in vivo.

Using an isozyme-specific antisera, we have specifically studied phosphorylation of the α isozyme of topoisomerase II protein in human cells. We show that this isozyme is phosphorylated at multiple sites in vivo and show that two of these sites, serine 1524 and serine 1376, are substrates for phosphorylation by casein kinase II in vitro.

MATERIALS AND METHODS

Cell Lines—HeLa S3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 3 mM l-glutamine,
and antibiotics in a humidified atmosphere containing 5% CO₂ at 37 °C. The non-small cell lung adenocarcinoma cell line, NSCLC-3, was grown in RPMI 1640 medium supplemented with 20% fetal bovine serum and 2% glutamine.

**Enzymes and Antibodies**—Recombinant human casein kinase II was produced in E. coli essentially as described by Kroll and Rowé (1991), except that cells were labeled for 14 h with 100 μCi/ml [³²P]orthophosphate (carrier free, Amerham) or 25 μCi/ml [³⁵S]methionine (>1,000 Ci/mmol, Amerham) in phosphate-free or methionine-free medium, respectively. The cell cycle phase distribution was not significantly altered during these periods of metabolic labeling. All buffers contained the following protease and phosphatase inhibitors: 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml soybean trypsin inhibitor, 1 mM benzamidine, 1 mM iodoacetamide, 50 μg/ml 1-chloro-3-0sylamido-7-amino-2-heptanone, 0.1 μM β-glycerophosphate, 0.1 μM p-nitrophenyl phosphate, 0.5 mM dithiothreitol, 100 μM sodium orthovanadate. The topoisomerase IIA protein was immunoprecipitated using the CRB antibody from the XhoI site of PET-14B (Novagen). cDNA fragments were amplified by the polymerase chain reaction (PCR) using the full-length topoisomerase IIA cDNA as a template (Jenkins et al., 1989). The 5′ and 3′ primers and incorporating XhoI recognition sites. The two different 5′ primers used had the sequences 5′-AGAGAGCTCAGGAGGCTGTGGAGCACAAGGA-3′ and 5′-AGAGAGCTCAGGAGGCTGTGGAGCACAAGGAAATGTGCGTTGCA-3′, and they were designed to encode C-terminal polypeptides commencing at Glu-1176 and Pro-1230, respectively. The identical 3′ primer included the natural stop codon of the topoisomerase IIA sequence. PCR was performed for 10 cycles with 1 μg of plasmid template under the following conditions: 94 °C for 0.5 min; 55 °C for 0.5 min; and 72 °C for 1 min. The initial cycle included a denaturation step for 3 min, and the five cycles had a denaturation step of 5 s.

The PCR products were separated on a 1% agarose gel, digested with XhoI, and cloned downstream of the initiation methionine in XhoI-digested PET-14B. This fused the C-terminal fragments of topoisomerase IIA protein to the PET-14B leader sequence, which encodes a hexa-histidine tag.

**Purification of Recombinant Proteins**—PET-14B derivatives containing the C-terminal domain fragments of the topoisomerase IIA protein were transformed into Escherichia coli BL21 (DE3), and transformants were grown to an OD₆₀ of 0.6 before addition of isopropryl-β-d-thiogalactopyranoside (0.4 mM) to induce expression from the T7 promoter in PET-14B. After 2 h of further growth, bacteria were harvested and lysed by sonication, and proteins were separated by nickel chelate chromatography as recommended by the supplier.

**Site-directed Mutagenesis**—Mutagenesis was performed by a PCR-based technique, essentially as described by Landt et al. (1990). This uses two sequential PCR reactions utilizing a single mutagenic primer and two vector-specific primers. Amplification was performed essentially as detailed above except that 5 and 10 cycles of amplification were used in the first and second rounds of PCR, respectively. Mutant forms of topoisomerase IIA C-terminal domain were cloned into PET-14B as described above. Control antibodies included those raised against the identical apparent molecular weight on SDS-polyacrylamide gel electrophoresis to that precipitated by the CRB antiserum. DNA Sequencing—Nucleotide sequences were performed on double-stranded plasmid templates using the dideoxy chain termination method and Sequenase enzyme (U S Biological Corp.).

**Phosphorylation Reactions**—The casein kinase II reaction buffer contained 60 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 50 mM NaCl, 25 μM KCl, 1 μM dihydrothreitol and 200 μM ATP. Where required, reactions contained 1–5 μC of [³²P]ATP (3,000 Ci/mmol, Amerham Corp.) and antibiotics in a humidified atmosphere containing 5% CO₂ at 37 °C.

**Metabolic Labeling, Nuclear Extraction, and Immunoprecipitation**—These procedures were carried out essentially as described by Kroll and Rowé (1991), except that cells were labeled for 14 h with 100 μCi/ml [³²P]orthophosphate (carrier free, Amerham) or 25 μCi/ml [³⁵S]methionine (>1,000 Ci/mmol, Amerham) in phosphate-free or methionine-free medium, respectively. The cell cycle phase distribution was not significantly altered during these periods of metabolic labeling. All buffers contained the following protease and phosphatase inhibitors. 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml soybean trypsin inhibitor, 1 mM benzamidine, 1 mM iodoacetamide, 50 μg/ml 1-chloro-3-0sylamido-7-amino-2-heptanone, 0.1 μM β-glycerophosphate, 0.1 μM p-nitrophenyl phosphate, 0.5 mM dithiothreitol, 100 μM sodium orthovanadate. The topoisomerase IIA protein was immunoprecipitated with the CRB antibody at a 1:40 dilution. This antibody specifically immunoprecipitates the α isoform of topoisomerase II.

**Phosphoamino Acid Analysis and Phosphopeptide Analysis**—The topoisomerase IIA protein immunoprecipitated by the CRB antibody from metabolically labeled cells was run on a 7.5% SDS-polyacrylamide gel. In parallel, the purified topoisomerase IIA C-terminal domains, which were phosphorylated in vitro by purified casein kinase II, were separated on 12% SDS-polyacrylamide gels. The SDS gels were dried down onto Whatman 3MM paper, and the positions of the radiolabeled topoisomerase IIA protein bands were detected by autoradiography. The appropriate region of the gel was excised in each case, and the protein was eluted as described by Morgan et al. (1989), except that the gel slice was rehydrated in 30% methanol for 15 min followed by soaking in 50 mM ammonium bicarbonate for 30 min. Two-dimensional electrophoresis on thin layer cellulose plates was as described by Cooper et al. (1983). Phosphopeptides were separated by electrophoresis at pH 1.9 or 4.72, followed by chromatography using phospho-chromatography buffer as described by Woodgett (1992).

**RESULTS**

**DNA Topoisomerase IIA is a Phosphoprotein in Human HeLa Cells with Serine as the Major Phosphate Acceptor Residue**—Nuclear protein extracts were prepared from HeLa cells grown in the presence of [³²P]orthophosphate or [³⁵S]methionine. Topoisomerase IIA protein was immunoprecipitated using the specific antiserum CRB and the precipitate analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The CRB antibody was raised to a peptide from the C-terminal domain of the predicted topoisomerase IIA protein sequence and, on Western blots of human cell nuclear extracts, recognizes a single protein of identical apparent molecular mass (170 kDa) to that of topoisomerase IIA protein (Smith and Makinson, 1989). The sequence of the peptide to which this antibody was raised is not conserved in the β isozyme of human topoisomerase II. Immunoprecipitation by this antiserum from HeLa cells grown in the presence of [³²P]orthophosphate revealed a single radiolabeled protein of 170 kDa with a mobility identical to that of a protein immunoprecipitated from control cells labeled with [³⁵S]methionine (Fig. 1A). We confirmed that this 170-kDa protein was the α isoform of topoisomerase II by carrying out additional control studies. First, three topoisomerase IIA-specific monoclonal antibodies, IF6, IF3, and 7E6 (Negri et al., 1992), immunoprecipitated a single protein of identical apparent molecular weight on SDS-polyacrylamide gel electrophoresis to that precipitated by the CRB antiserum. Second, the protein precipitated by the CRB antibody was recognized on Western blots by the IF6 antibody but not by a series of control antibodies including those raised against human topoisomerase IIB isoform (data not shown). Taken together, these data indicate that topoisomerase IIA is a phosphoprotein in human tumor cell lines, in agreement with the data of Kroll and Rowé (1991) and Ganapathi et al. (1993).

To identify the phosphorylated amino acid residues in the topoisomerase IIA protein, in vivo labeled protein from HeLa cells was purified from an SDS-polyacrylamide gel and subjected to partial acid hydrolysis. The released amino acids were then separated by two-dimensional electrophoresis. The majority of the ³²P-labeled material migrated with phosphoserine, but a small proportion (5–10%) of phosphothreonine was also evident (Fig. 1B). No phosphotyrosine was detected, even after long autoradiographic exposures. A similar result was obtained using topoisomerase IIA protein extracted from the NSCLC-3 adenocarcinoma cell line (data not shown).

**Phosphopeptide Mapping of in Vivo Labeled Topoisomerase IIA Protein**—The [³²P]-labeled topoisomerase IIA protein immunoprecipitated from HeLa cell extracts was digested with trypsin, and the resultant phosphopeptides were separated in two dimensions on thin layer cellulose plates by electrophoresis at pH 1.9 followed by chromatography. A representative tryptic phosphopeptide map of HeLa cell topoisomerase IIA protein is

\[ ^{N} J. \text{ Weh, C. M. Addison, A. M. Fry, R. Ganapathi, and I. D. Hickson, unpublished data.} \]
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Phosphorylation of the C-terminal Domain of Human Topoisomerase IIα Protein in Vivo—The recombinant C-terminal domain of topoisomerase IIα protein was tested as a substrate for a range of protein kinases in vitro. No significant autophosphorylation was seen in the absence of added kinase. Both the full C-terminal domain and the subdomain were substrates for casein kinase II (Fig. 3B), protein kinase C, protein kinase A, and P34cdc2 kinase (data not shown). Subsequent two-dimensional tryptic phosphopeptide mapping revealed multiple sites of phosphorylation for each of these kinases. However, only casein kinase II generated a phosphopeptide with a mobility closely matching that of the major in vivo phosphopeptide 1, and thus this kinase alone was studied further.

We next performed a comparison between the mobility of the tryptic phosphopeptides generated from the in vivo labeled topoisomerase IIα protein and those derived from casein kinase II phosphorylation of the C-terminal domain in vitro. The tryptic maps generated by casein kinase II phosphorylation of the C-terminal full domain and the subdomain were very similar, with the same two major phosphopeptides. These two high affinity sites must therefore lie in the C-terminal subdomain between Pro-1370 and Phe-1530. Fig. 4 shows data for the full C-terminal domain. Phosphoamino acid analysis of this sample revealed that phosphoserine represented approximately 90% of the total phosphorylated amino acids (data not shown).

To determine whether the two casein kinase II-specific phosphopeptides co-migrated with phosphopeptides generated by tryptic cleavage of in vivo labeled topoisomerase IIα protein, the in vitro and in vivo samples were mixed and then separated in two dimensions as before. Fig. 4 shows that the casein kinase II-specific phosphopeptide 1 clearly co-migrated with the in vivo phosphopeptide 1. The data in Fig. 4 also suggested that the second major casein kinase II-derived phosphopeptide (designated 2 in Fig. 4) co-migrated with a minor species from the in vivo sample. However, it was difficult to be definitive about this latter result because of the relative difference between the labeling efficiencies of these peptides in the in vitro and in vivo samples. To overcome this problem, we ran a two-dimensional map of the casein kinase II-phosphorylated C-terminal domain, excised phosphopeptide 2 from the thin-layer plate, then mixed the purified phosphopeptide with the complete in vivo sample, and separated the mixture in two dimensions. Relative to all other phosphopeptides in the in vivo sample (Fig. 5A), an approximately 2-fold increase in intensity of phosphopeptide 2 was apparent in the mix (Fig. 5B), indicating that co-migration had occurred. We also confirmed that the two casein kinase II-specific phosphopeptides co-migrated with phosphopeptides from the in vivo sample by performing two-dimensional separations with electrophoresis buffer at pH 4.72 instead of pH 1.9 (data not shown). Thus, we conclude that the two major sites in the C-terminal domain of topoisomerase IIα protein phosphorylated in vitro by casein kinase II are sites that are phosphorylated in human tumor cell lines.

Identification of Sites of Phosphorylation within Topoisomerase IIα Protein—To identify sites of phosphorylation, site-
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Directed mutagenesis of serine residues within the C-terminal domain of topoisomerase IIα protein was performed based on the known consensus for casein kinase II phosphorylation sites. Fig. 6 shows that a two-dimensional tryptic map generated from the purified C-terminal domain containing a single amino acid substitution replacing serine 1524 with alanine lacked phosphopeptide 1 (Fig. 6A), while an equivalent polypeptide with alanine replacing serine 1376 lacked phosphopeptide 2 (Fig. 6B). We considered the possibility that phosphorylation of serine 1376 could convert serine 1373 to a substrate for casein kinase II with the effect that phosphopeptide 2 could have represented phosphorylation of 2 serine residues on the same tryptic peptide. However, substitution of alanine for serine 1524 also affected the electrophoretic mobility of phosphopeptide 2 (data not shown). Thus, we conclude that a major in vivo site of phosphorylation in topoisomerase IIα protein is serine 1524, and a second site of phosphorylation is at serine 1376.

DISCUSSION

Post-translational modification of proteins by phosphorylation is one of the most important means by which enzymatic activity is regulated in human cells (Krebs and Beavo, 1979; Hunter, 1987). This modification can lead to inhibition or stimulation of activity and to altered affinity for a target protein or DNA. We have shown that the α isozyme of human topoisomerase II is a phosphoprotein in vivo and that a major site of phosphorylation is located at serine 1524. This site can be phosphorylated in vitro by casein kinase II. Our in vivo phosphoamino acid analysis revealed a small (5–10%) but consistent level of phosphothreonine, in contrast to the results of Kroll and Rowe (1991), who only modified serine was found. Further work is required to identify the site(s) of phosphorylation on threonine residues in human topoisomerase IIα protein.

Casein kinase II is a predominantly nuclear serine/threonine kinase with an essential role in cell proliferation in yeast (Krek et al., 1992; Padmanabha et al., 1990). Several nuclear targets for casein kinase II have been identified, including transcription and replication factors. For example, a requirement for
casein kinase II in the activation of human DNA ligase I has been reported (Prigent et al., 1992). Casein kinase II recognizes serine (or more rarely threonine) residues in a generally acidic context (Litchfield and Luscher, 1993) with the minimal consensus being SXXD/E. Moreover, multiple acidic residues adjacent to the target serine are frequently observed in strong casein kinase II recognition sites. The in vivo sites of phosphorylation identified in topoisomerase IIα protein lie in the sequences S1376DLEADD and EES1524DEDD and might therefore be expected to be high affinity recognition sites for casein kinase II. Serine 1524 is only 6 residues from the C terminus of the topoisomerase IIα protein, and it may be significant that other proteins, such as p53, are also phosphorylated at sites very close to their C terminus by casein kinase II (Meek et al., 1990).

We have shown that serine 1524 is a major site of phosphorylation in topoisomerase IIα protein in two different human tumor cell lines. It would seem likely, therefore, that modification of this residue is a common, if not universal, feature in exponentially growing human cell lines. Whether this modification is also seen in untransformed cells will require further study. The activity of purified human topoisomerase IIα protein is increased following phosphorylation by casein kinase II (Fry et al., 1992). The data presented here suggest that phosphorylation on serine 1524 might be important in the regulation of topoisomerase IIα enzymatic activity, since this site may be unique in being efficiently phosphorylated both in vivo and in vitro. To provide evidence to support this hypothesis, it will be necessary to purify wild-type topoisomerase IIα protein and to compare its activity with that of a mutant protein containing a serine 1524 to alanine substitution.

Our data do not necessarily imply that the other phosphopeptides seen with in vivo labeled topoisomerase IIα protein are generated by kinases other than casein kinase II. However, these phosphopeptides do not seem to be generated by casein kinase II phosphorylation of the C-terminal domain of topoisomerase IIα protein, or, if they are, they must represent sites in the recombinant C-terminal fragments not efficiently modified by casein kinase II in vitro.

Casein kinase II has been implicated in the control of topoisomerase II activity from several different eukaryotic species. The best characterized system is in Drosophila, where the single topoisomerase II enzyme is phosphorylated in vivo by casein kinase II (Ackerman et al., 1988), although specific sites of phosphorylation have not been reported. It is interesting to note, however, that the predicted Drosophila topoisomerase II protein sequence also contains consensus casein kinase II recognition sites very close to its C terminus. Not only is the activity of the Drosophila topoisomerase II enzyme regulated in vitro by casein kinase II, but also the susceptibility of the modified enzyme to inhibition by antineoplastic drugs is attenuated following phosphorylation by casein kinase II (Ackerman et al., 1985; DeVore et al., 1992). The presence of a casein kinase II-like enzyme has been reported to co-purify with topoisomerase II (presumed to be the α isozyme) from mouse FM3A cells. This kinase was able to reactivate dephosphorylated topoisomerase II and was suggested to play a key role in regulation of topoisomerase II activity in mouse cells (Saiko et al., 1990). A similar pattern has emerged from studies in budding yeast, where casein kinase II appears to co-purify with topoisomerase II and plays a central role in activation of dephosphorylated topoisomerase II protein (Cardenas et al., 1993; Bojanowski et al., 1993).
Based upon predicted mobilities of tryptic phosphopeptides during two-dimensional separation, it has been suggested that casein kinase II phosphorylates the C-terminal domain of the budding yeast enzyme to a quantitatively similar extent on at least 7 tryptic peptides representing up to 15 different sites of phosphorylation (Cardenas et al., 1992). While alignments of the C-terminal domains of the human and budding yeast topoisomerase II enzymes are complicated by the general sequence divergence within this domain, there are predicted casein kinase I1 sites in the budding yeast enzyme at similar sequence divergence within this domain, there are predicted casein kinase II sites in the budding yeast enzyme at similar locations to those mapped here for human topoisomerase IIa protein, although overall sequence similarity around these locations is weak. However, it seems unlikely that the C-terminal domain of the human enzyme is phosphorylated by casein kinase II on as many sites as that of the yeast enzyme, as we have only been able to detect two strong recognition sites in vitro. This may reflect altered patterns of topoisomerase II enzyme regulation by phosphorylation among different eukaryotic species. This is perhaps not too surprising bearing in mind that human cells express two topoisomerase II isozymes while yeast cells contain a single topoisomerase II protein.

In summary, we have identified two sites of phosphorylation in the topoisomerase IIa protein from cultured human cells and shown that these serine residues are substrates for phosphorylation in vitro by casein kinase II. We are now in a position to address whether phosphorylation of human topoisomerase IIa protein on specific sites by casein kinase II is an important regulator of its cellular functions.

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