Cell Cycle Phase-specific Phosphorylation of Human Topoisomerase IIα

EVIDENCE OF A ROLE FOR PROTEIN KINASE C*

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Type II topoisomerases are essential for faithful cell division in all organisms. In human cells, the α isozyme of topoisomerase II has been implicated in catalyzing mitotic chromosome segregation via its action as a DNA unlinking enzyme. Here, we have shown that the enzymatic activity of topoisomerase IIα protein purified from HeLa cell nuclei was strongly enhanced following phosphorylation by protein kinase C. We have investigated the possibility that this kinase is involved in cell cycle phase-specific phosphorylation of topoisomerase IIα in HeLa cells. Two-dimensional tryptic phosphopeptide mapping revealed that topoisomerase IIα protein immunoprecipitated from metabolically labeled HeLa cells was differentially phosphorylated during the G2/M phases of the cell cycle. To identify sites of phosphorylation and the kinase(s) responsible for this modification, oligohistidine-tagged recombinant domains of topoisomerase IIα protein were overexpressed in Escherichia coli and purified by affinity chromatography. Phosphorylation of a short fragment of the N-terminal ATPase domain of topoisomerase IIα by protein kinase C in vitro generated two phosphopeptides that co-migrated with prominent G2/M phase-specific phosphopeptides from the HeLa cell-derived topoisomerase IIα protein. Site-directed mutagenesis studies indicated that phosphorylation of serine 29 generated both of these phosphopeptides. Our results implicate protein kinase C in the cell cycle phase-dependent modulation of topoisomerase IIα enzymatic activity in human cells.

In order for chromosomes to be faithfully transmitted from mother to daughter cells, DNA must be fully replicated and segregated evenly. Chromosome segregation can be affected only when all covalent DNA interlinks between replicated sister chromatids have been removed. The enzyme that catalyzes the disentanglement of replicated chromosomes via its ability to decatenate covalently interlinked duplex DNA molecules is DNA topoisomerase II, a highly conserved, homodimeric nuclear protein (see Wang (1985), Osheroff et al. (1991), Holm (1994), and Watt and Hickson (1994) for reviews). Evidence of a role for topoisomerase II in mitotic chromosome segregation has been derived largely from studies in lower eukaryotes.

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Yeast mutants defective in topoisomerase II activity fail to remove all chromosomal interlinks at mitosis and subsequently incur chromosomal breakage as cell division is attempted in the absence of proper segregation (DiNardo, et al., 1984; Holm, et al., 1985; Uemura and Yanagida, 1986; Uemura, et al., 1987; Holm, et al., 1989; Rose and Holm, 1993; Spell and Holm, 1994). This defective segregation leads to a rapid decline in cell viability (Goto and Wang, 1984; Uemura and Yanagida, 1984). Because yeast cells contain a single topoisomerase II gene, it has been possible to study topoisomerase II function using conditional lethal mutants defective in topoisomerase II function at the restrictive growth temperature. Similar studies in human cells have been hampered both by a lack of suitable mutants deficient in topoisomerase II activity and by the presence of two closely related topoisomerase II isozymes. The human isozymes are termed topoisomerase IIα (170-kDa form) and topoisomerase IIβ (180-kDa form) (Tsai-Pflugfelder et al., 1988; Drake et al., 1989; Chung et al., 1989; Jenkin et al., 1992; Austin et al., 1993) and are the products of distinct genes encoded on different chromosomes (Tsai-Pflugfelder et al., 1988; Tan et al., 1992; Jenkin et al., 1992).

Regulation of mitotic events in mammalian cells may require the action of a number of different protein kinases. The cyclin-dependent protein kinase, p34cdc2, is regarded as the master controller of mitotic events and phosphorylates a number of nuclear/nucleolar proteins, including histone H1 and nucleolin (reviewed by Norbury and Nurse (1992). Murray (1992), Nigg (1993), and Morgan (1995)). However, recent studies have implicated both mitogen-activated protein (MAP)1 kinase and protein kinase C (PKC) in the regulation of certain mitosis-specific functions. The role of MAP kinase has not been defined in detail, although this kinase is implicated in the generation of the mitosis-specific phosphorylated epitope recognized by the MPM-2 antibody (Kuang and Ashorn, 1993; Westendorf et al., 1994). This epitope is found in a number of nuclear proteins, including topoisomerase IIα and β (Taagepera et al., 1993). At least one mitotic role for PKC, triggering the depolymerization of the nuclear lamina, has been proposed (Goss et al., 1994).

Although little is known of the mechanisms by which the function of topoisomerase I is regulated in mammalian cells, a number of different protein kinases have been implicated in the modulation of topoisomerase II enzymatic activity. In general, dephosphorylation of eukaryotic topoisomerase II enzymes leads to loss of activity (Saito, et al., 1990; Cardenas and Gasser, 1993), whereas phosphorylation by casein kinase II or PKC causes a mild stimulation of activity (Ackerman et al., 1985; Rottman et al., 1987; Ackerman et al., 1988; Cardenas et al., 1992; Corbett et al., 1993a, 1993b). Regulation by casein kinase

1 The abbreviations used are: MAP, mitogen-activated protein; PKC, protein kinase C; TLC, thin layer chromatography.
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II is particularly noteworthy in that Saccharomyces cerevisiae or mouse topoisomerase II proteins that have been inactivated by dephosphorylation can be reactivated by this kinase (Saijo et al., 1990; Cardenas and Gasser, 1993).

Several previous studies have suggested that topoisomerase IIα protein from mammalian cells is phosphorylated in vivo on multiple sites (Saijo et al., 1990, 1992; Kroll and Rowe, 1991; Burden et al., 1993; Ganapathi et al., 1993; Kimura et al., 1994; Wells et al., 1994; Wells and Hickson, 1995). At least some of these sites of phosphorylation correspond to recognition sequences for casein kinase II (Wells et al., 1994). Moreover, topoisomerase IIα protein is hyperphosphorylated during the G2 and/or M phases of the cell cycle (Saijo et al., 1992; Burden et al., 1993; Wells and Hickson, 1995). However, although casein kinase II appears to phosphorylate yeast topoisomerase II protein in a cell cycle phase-specific manner (Cardenas et al., 1992), no evidence has been presented that this particular kinase is implicated in the M phase-specific hyperphosphorylation of topoisomerase II proteins from mammalian cells.

In this paper, we have studied the cell cycle phase-specific phosphorylation of human topoisomerase IIα protein. We have identified a serine residue in the N-terminal ATPase domain of topoisomerase IIα protein, which is modified specifically during the G2/M phases of the HeLa cell cycle. We have shown that this residue is a target for PKC stimulation of topoisomerase IIα protein purified from HeLa cell extract by phosphate-stimulated enzyme activity 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.

Purification of Human Topoisomerase IIα—Topoisomerase IIα protein is hyperphosphorylated during the G2 and/or M phases of the cell cycle (Saijo et al., 1992; Burden et al., 1993; Wells and Hickson, 1995). However, although casein kinase II appears to phosphorylate yeast topoisomerase II protein in a cell cycle phase-specific manner (Cardenas et al., 1992), no evidence has been presented that this particular kinase is implicated in the M phase-specific hyperphosphorylation of topoisomerase IIα proteins from mammalian cells.

In this paper, we have studied the cell cycle phase-specific phosphorylation of human topoisomerase IIα protein. We have identified a serine residue in the N-terminal ATPase domain of topoisomerase IIα protein, which is modified specifically during the G2/M phases of the HeLa cell cycle. We have shown that this residue is a target in vitro for PKC and that phosphorylation of topoisomerase IIα protein purified from HeLa cells by PKC strongly stimulates enzymatic activity in vitro.

RESULTS

Cell Synchronization—HeLa cells were synchronized at the start of S phase by the use of a double thymidine block. Exponentially growing cells were treated with 2 mM thymidine for 14 h and then released into thymidine-free medium. After 11 h, 2 mM thymidine was again applied for a 15-h period before release into thymidine-free medium.

Flow Cytometry—Cells were fixed for 30 min in ice-cold 70% ethanol/30% phosphate-buffered saline collected by centrifugation and were treated with RNase A (100 μg/ml final concentration) and propidium iodide (40 μg/ml) in phosphate-buffered saline for 30 min at 37 °C. Cell cycle distribution was then determined using a Beckton Dickinson FACScan and the data were analyzed using the Lysis II software.

Protein Gel Electrophoresis—Proteins were separated by discontinuous SDS-polyacrylamide gel system described by Laemmli (1970).
chromatography, and the topoisomerase IIα protein was purified to near homogeneity. This method of purification has been used previously to separate the α and β isozymes of human topoisomerase II (Drake et al., 1987; Strausfeld and Richter, 1989). The purified topoisomerase II preparation contained a predominant 170-kDa protein that was recognized by the CRB antiserum specific for topoisomerase IIα (at 1:200 dilution). The immunoreactive protein was detected with 125I-protein A. The position of the 170-kDa topoisomerase IIα protein is indicated by an arrow.

Regulation of Topoisomerase IIα Activity by PKC—The purified topoisomerase IIα protein was tested as a substrate for PKC in vitro. Fig. 2 shows that the purified topoisomerase IIα preparation was free from contaminating kinases and had no intrinsic autophosphorylation activity. However, the 170 kDa topoisomerase IIα protein was a substrate in vitro for phosphorylation by 3 isoforms of PKC. To study the effects of phosphorylation by PKC, the enzymatic activity of the topoisomerase IIα protein was assayed using supercoiled plasmid DNA as a substrate. Fig. 3 shows that the rate of plasmid relaxation catalyzed by the PKC-phosphorylated topoisomerase IIα protein was increased significantly over that catalyzed by the unmodified enzyme.

Topoisomerase IIα Protein Is Hyperphosphorylated During the G2/M Phases of the HeLa Cell Cycle—The α isozyme of topoisomerase IIα is a phosphoprotein in mammalian cells, and the level of its phosphorylation is regulated in a cell cycle phase-dependent manner. Studies with human and rodent cell lines have indicated that topoisomerase IIα protein is hyperphosphorylated during the G2/M phases of the mammalian cell cycle (Saijo et al., 1992; Kroll and Rowe, 1991; Burden et al., 1993; Wells et al., 1994; Burden and Sullivan, 1994; Kimura et al., 1994; Wells and Hickson, 1995). In order to identify the G2/M phase-specific sites of phosphorylation on human topoisomerase IIα protein, as well as the kinases responsible for this modification, HeLa cells were labeled with 32P orthophosphate, and the topoisomerase IIα protein was immunoprecipitated with the isozyme-specific CRB antiserum. The topoisomerase IIα protein was then digested with trypsin, and the resulting phosphopeptides were separated in two dimensions on thin layer chromatography (TLC) plates.

Fig. 4 shows a comparison of tryptic phosphopeptide maps for topoisomerase IIα protein derived either from an asynchronous culture of HeLa cells or from a culture synchronized via a double thymidine block and released into fresh thymidine-free medium for 8 h. Flow cytometric analysis revealed that the culture released from the cell cycle blockade contained 91% G2/M phase cells. A number of phosphopeptides that were either specific for or greatly enriched within the G2/M phase sample were evident (arrows in Fig. 4b). Among these phosphopeptides are several that we have shown previously to be dependent upon phosphorylation by a proline-directed kinase (identified by open arrowheads in Fig. 4) and represent phosphorylation of serine residues in the C-terminal regulatory domain of topoisomerase IIα protein (Wells and Hickson, 1995). However, two of the most prominent G2/M phase-specific phosphopeptides (indicated by arrows labeled A and B in Fig. 4b) have not been identified in previous studies. Thus, we sought to identify the kinase(s) responsible for phosphorylation of the serine or threonine residue(s) present in phosphopeptides A and B. To determine the identity of the phospho-acceptor residues, phosphopeptides A and B were excised from the TLC plate and subjected to phosphoamino acid analysis. This revealed, in each case, that serine was the sole phospho-acceptor.
II oligohistidine-tagged recombinant domains of topoisomerase II were expressed and purified from asynchronously growing HeLa cells (a) or a culture enriched for G2/M phase cells (b). Phosphopeptides were separated in the horizontal dimension by electrophoresis at pH 1.9 (anode on left) and in the vertical dimension by chromatography. The positions of the G2/M phase-specific phosphopeptides identified in previous studies (Wells and Hickson, 1995) are indicated by open arrow heads. The two G2/M phase-specific phosphopeptides studied in this paper are indicated by solid arrows and denoted A and B. The position of the origin (O) is indicated.

Fig. 5. The 18-kDa N-terminal domain of human topoisomerase IIα protein is a substrate in vitro for PKC. The E. coli-expressed 18-kDa fragment was electrophoresed on a 12% polyacrylamide gel and stained with Coomassie Blue (lane C). The recombinant protein was then incubated in the presence (lane 1) or absence (lane 2) of PKCβ1 and electrophoresed on a 12% gel, which was exposed to x-ray film after drying. Lane 3 shows the PKC preparation in the absence of the 18-kDa topoisomerase IIα fragment. The position of the topoisomerase IIα protein fragment is indicated by the arrows. The sizes (in kDa) of molecular mass standards run in parallel are shown on the right.

Fig. 6. The G2/M phase-specific phosphopeptides A and B are derived from the N-terminal 18-kDa fragment of topoisomerase IIα protein. a, two-dimensional tryptic phosphopeptide map of topoisomerase IIα protein extracted from G2/M phase-enriched HeLa cells. The positions of phosphopeptides A and B are indicated. b, a map of the 18-kDa N-terminal fragment phosphorylated in vitro by PKCβ1. The positions of three prominent phosphopeptides are shown. c, a 1:1 mix of the samples in a and b, indicating co-migration of phosphopeptides A and B from a with phosphopeptides 3 and 4 from b. d, a mix of the sample in a with phosphopeptide 3 extracted from a thin layer plate. Note the selective increase in intensity of phosphopeptides A and B from the in vivo sample due to co-migration with phosphopeptide 3.

whether the PKC-specific sites in the 18-kDa fragment and the G2/M phase-specific sites were identical, two approaches were undertaken. First, the entire in vitro and in vivo phosphopeptide samples were mixed, and the mixture was separated in two dimensions on TLC plates. Fig. 6 (a–c) shows that phosphopeptides 3 and 4 from the in vitro sample appeared to co-migrate with phosphopeptides A and B from the HeLa cell-derived
serine-29. Notetheabsenceofphosphopeptides3and4. The 18-kDa fragment containing a single amino acid substitution of alanine for serine-29.Notetheabsenceofphosphopeptides3and4. Twodimensional tryptic phosphopeptide map of the synthetic peptide co-migrates with phosphopeptide 3 from the recombinant N-terminal protein. This confirms that serine-29 is the target residue for PKC in vitro and is the only possible phospho-acceptor residue present in peptide A from the in vivo labeled topoisomerase IIα protein.

**DISCUSSION**

We have identified serine-29 as a site of phosphorylation of topoisomerase IIα protein from the human HeLa cell line and have shown that this residue is a substrate for PKC in vitro. Phosphorylation on serine-29 is increased greatly as HeLa cells traverse the G2/M phases of the cell division cycle. Further, we have shown that phosphorylation by PKC substantially increases the catalytic activity of purified topoisomerase IIα protein in vitro.

Many mitosis-specific events in mammalian cells are regulated by the action of protein kinases. The most extensively characterized of the mitosis-activating kinases is the p34\(^{\text{cdc2}}\) cyclin B complex. Phosphorylation of target proteins by p34\(^{\text{cdc2}}\) cyclin B initiates many of the hallmark events in mitosis, such as nuclear disassembly and chromatin condensation (reviewed by Norbury and Nurse, 1992; Murray, 1992; Nigg, 1993; Morgan, 1995). However, recent studies have indicated that kinases other than p34\(^{\text{cdc2}}\) cyclin B are intimately involved in the regulation of mitotic events. For example, MAP kinase appears to be at least one of the kinases responsible for the generation of the mitosis-specific phosphorylated epitope recognized by the MPM-2 antibody (Kuang and Asham, 1993; Westendorf et al., 1994). Moreover, although many isoforms of PKC are cell membrane-associated and unlikely, therefore, to be responsible for modulating nuclear events, certain PKC isoforms are clearly critical for cell cycle traverse and are strongly implicated in affecting an essential nuclear mitotic function (reviewed by Clemens et al. (1992)). Current evidence suggests that the translocation of the βII isosform from the cytoplasmic membrane to the nucleus and the subsequent phosphorylation of nuclear lamins at the G2/M phase transition are necessary for the depolymerization of the nuclear lamina (Goss et al., 1994). This was a role previously assigned to p34\(^{\text{cdc2}}\) cyclin B. Indeed, PKC is known to be required for the G2/M phase transition in at least some cell types, and the expression of the βII isoform has been shown to be essential for

![Phosphopeptide map](image-url)
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proliferation in a human leukaemic cell line (Usui et al., 1991; Levin et al., 1990; Murray et al., 1993). It is not unreasonable to assume, therefore, that PKCβII or another isoform of PKC that is located in the nucleus, such as PKCζ, is involved in the regulation of other nuclear factors that are required during mitosis. Our data are consistent with the proposal that activation of topoisomerase IIα during mitosis is mediated, at least in part, by PKC. However, we cannot rule out the possibility that kinases other than or in addition to PKC modify serine-29 of topoisomerase IIα in mitotic cells. Based upon the sequence context in which serine-29 lies and the data presented in this paper, it seems highly unlikely that any of the kinases previously implicated in modifying lower eukaryotic topoisomerase II proteins in vivo (casein kinase II and p34cdc2 kinase; Ackerman et al., 1988; Cardenas et al., 1992; Shiozaki and Yanagida, 1992), or the human topoisomerase IIα protein in vivo (casein kinase I and a proline-directed kinase; Wells et al., 1994; Wells and Hickson, 1995) are directly responsible for the modification of serine-29.

A number of previous studies have suggested that PKC may be involved in the regulation of topoisomerase IIα functions. The most extensively characterized system for an analysis of the effects of phosphorylation on topoisomerase IIα activity to date has been Drosophila. Osheroff and colleagues have shown that phosphorylation by PKC enhances the activity of Drosophila topoisomerase II approximately 2.5-fold and that this activation is mediated via an enhancement in the rate of ATP hydrolysis (Corbett et al., 1993a, 1993b). Moreover, topoisomerase IIα phosphorylated by PKC has an altered susceptibility to inhibition by certain antineoplastic agents that interfere with the catalytic cycle of topoisomerase II (DeVore et al., 1992). Similarly, Rottmann et al. (1987) showed that PKC phosphorylates topoisomerase IIα from the sponge, Geodia cydonium, in vivo and increases its activity around 2.5-fold in vitro. Our data on human topoisomerase IIα protein are in agreement with those obtained with the Geodia and Drosophila topoisomerase IIα proteins. Moreover, the location of the PKC target serine residue in the N-terminal ATPase domain of human topoisomerase IIα protein suggests an interesting possibility that this phosphorylation event is linked directly to alterations in ATP binding/hydrolysis. However, it should be noted that phosphorylation of Drosophila topoisomerase IIα by either casein kinase II or PKC (both of which apparently target the C-terminal domain) causes an increase in the rate of ATP hydrolysis (Ackerman et al., 1988; Corbett et al., 1992, 1993a, 1993b). Indeed, Ackerman et al. (1988) have provided evidence that casein kinase II is the predominant activity responsible for phosphorylating topoisomerase IIα in cultured Drosophila cells. Similarly, our previous data implicate casein kinase II as a key regulator of human topoisomerase IIα (Wells et al., 1994). The data presented here indicate both that the phosphorylation of serine-29 of human topoisomerase IIα is almost certainly not mediated by casein kinase II and that this strongly cell cycle-regulated modification would not necessarily have been detected in previous studies in which asynchronously growing cell cultures were employed.

There are a number of possible functions that phosphorylation of human topoisomerase IIα protein might perform. Considering the G2/M phase-specific nature of the modification of serine-29, it would seem likely that this modification is required to affect a mitosis-specific function. Our data indicate that phosphorylation by PKC can substantially increase the catalytic activity of purified human topoisomerase IIα protein. Indeed, the extent to which PKC can activate topoisomerase IIα in vitro may be a significant underestimate of the true effect of PKC on activity in vivo, because the purified enzyme was likely to be already in at least a partially phosphorylated state. It is possible that this activation may be linked to a requirement for a highly efficient catalytic activity throughout the short time period in the G2 and/or M phases in which chromosome segregation must be affected by topoisomerase II.

The data presented here indicate that phosphorylation of serine-29 gives rise to two tryptic phosphopeptides. We would suggest that this occurs via differential digestion of the protein by trypsin, which is known to occur at sites of adjacent lysine and arginine residues (Campbell et al., 1986). Indeed, serine-29 lies in a sequence that contains three consecutive target residues for trypsin (KKRLS<sup>30</sup>; in the one-letter amino acid code), which would appear to provide the opportunity for differential digestion to occur.

Few of the previous studies on topoisomerase IIα phosphorylation in lower or higher eukaryotic cells have analyzed the location of phospho-acceptor residues. In those studies where sites have been mapped definitively or predicted from the mobility of phosphopeptides on TLC plates, it is the C-terminal domain that has been implicated as the major target for kinases. In budding yeast topoisomerase II protein, the C-terminal domain is proposed to be a target for multiple phosphorylations by casein kinase II (Cardenas et al., 1992). Moreover, several of these C-terminal sites appear to be hyperphosphorylated at mitosis. Similarly, we have shown previously that human topoisomerase IIα protein is phosphorylated in vivo on two serine residues in the C-terminal domain by casein kinase II (Wells et al., 1994). The only previous data implicating the N-terminal ATPase domain of topoisomerase IIα as a target for phosphorylation have come from the work of Shiozaki and Yanagida (1992) on the fission yeast topoisomerase II enzyme. Their study indicated that phosphorylation was implicated in controlling nuclear localization. Clearly, therefore, PKC-mediated phosphorylation of the N-terminal domain of human topoisomerase IIα protein could regulate enzymatic activity and/or nuclear localization. Studies are in hand to address these possibilities.

We have examined the possibility that an N-terminal site for PKC-mediated phosphorylation is conserved in other eukaryotic topoisomerase II enzymes (see Caron and Wang (1994) for a review of sequence homologies). In human topoisomerase IIα, serine-29 lies in a sequence context that comprises predominantly basic amino acids. This sequence motif is conserved in all mammalian topoisomerase IIα enzymes. Moreover, this motif is also conserved in the β isoform of human topoisomerase II. Whether this indicates that topoisomerase IIβ protein is also a target for PKC in vivo will require further studies. Although accurate alignment of the human and lower eukaryotic topoisomerase II sequences is difficult due to general sequence divergence, there are potential target serine residues for PKC near to the N terminus of the yeast and Drosophila topoisomerase II proteins.

In summary, we have shown that PKC can modulate the enzymatic activity of human topoisomerase IIα and have identified a serine residue in the ATPase domain of the protein that is phosphorylated specifically during the G2 and/or M phases of the HeLa cell cycle. The challenge is now to identify the precise role of the cell cycle-regulated phosphorylations that occur on topoisomerase IIα protein and that appear to require the action of at least two distinct kinases (Wells and Hickson, 1995) and this work).

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