Casein Kinase II Stabilizes the Activity of Human Topoisomerase IIα in a Phosphorylation-independent Manner*

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Previous reports have indicated that topoisomerase II (topo II) co-purifies with and is a substrate for casein kinase II. We have carried out a detailed study of the effect that purified casein kinase II has on the activity of purified recombinant human topo IIα. Co-incubation of topo IIα and casein kinase II led to an apparent activation of the topo IIα; however, in experiments in which topo IIα was preincubated at 37 °C with or without native casein kinase II prior to assay for decatenation activity, it emerged that the kinase was exerting its “activating” function via a decrease in the rate of topo IIα enzyme inactivation during the incubation period. This stabilization of topo IIα by casein kinase II was ATP-independent and was observed in both mutated and truncated derivatives of topo IIα lacking the major casein kinase II phospho-acceptor sites, indicating the lack of a requirement for phosphorylation. Consistent with a nonenzymatic role for casein kinase II, stoichiometric quantities of kinase were required for topo IIα stabilization. These data indicate that casein kinase II plays a significant role in regulating human topo IIα protein action via stabilization against thermal inactivation.

DNA topo II is a ubiquitous and structurally conserved enzyme required for many different aspects of DNA metabolism (reviewed in Refs. 1 an 2). Studies in bacterial and lower eukaryotic species have revealed that topo II is required for cell viability due to an essential role in the segregation of newly replicated chromosomes during cell division (3–7). During replication, sister DNA molecules become knotted and catenated as a consequence of the difficulties inherent in the unwinding and copying of a double-stranded, helical nucleic acid structure (reviewed in Ref. 8). In eukaryotes, topo II catalyzes the disentanglement of sister chromatids and hence permits their faithful segregation during mitosis. Studies on yeast temperature-sensitive topo2 mutants have shown that both chromosome hypercondensation and sister chromatid segregation are dependent upon functional topo II, and that in the absence of topo II chromosomes break or nondisjoin as the mitotic spindle attempts to pull apart the still interlinked DNA molecules (3–8).

In mammalian cells, but not in yeasts, topo II exists as two closely related isoforms designated α (170-kDa form) and β (180-kDa form) (9–13). The respective roles of the two isoforms are not known currently, although several differences in their activities/regulation have been noted. For example, the α isoform is a strict proliferation marker in vitro and in vivo and is expressed at a high level in each cell cycle during the period just prior to cell division (14–17). This isoform is tightly associated with condensed chromatin during metaphase. In contrast, topo IIβ is ubiquitously expressed in human tissues and is apparently excluded from chromosomes around the time of mitotic chromosome condensation, although it is found in the nucleoplasm during interphase (14–18).

Topo II is a phosphoprotein in cells from both higher and lower eukaryotes (reviewed in Ref. 1). In those species that have been analyzed, the extent of this phosphorylation is modulated as cells traverse the different phases of the cell division cycle. In particular, topo IIα in mammalian cells is hyperphosphorylated during mitosis as a result of the actions of at least two different protein kinases (19–23). However, the precise functional role of these cell cycle-specific modifications is not clear at this stage.

The kinase most closely associated with the regulation of topo IIα is casein kinase II. Topo II from Drosophila, budding and fission yeast, as well as from mammalian cells, is a high affinity substrate for casein kinase II in vitro (24–29). Several studies have confirmed that this kinase also phosphorylates topo II in intact cells, primarily on sites located within the poorly conserved, noncatalytic C-terminal domain (25, 28–30). The fission yeast topo II is also phosphorylated on a site in the N-terminal ATPase domain by casein kinase II (28). To date, three sites have been identified within the C-terminal domain of human topo IIα that are phosphorylated by casein kinase II in vitro (29, 31). None of these sites is modified differentially during cell cycle transit, and only one, serine 1524 in humans, is highly conserved in all of the mammalian topo IIα enzymes. This residue is a major site of phosphorylation in several different human cell lines studied (29). Of the other two sites, serine 1376 is conserved in mouse and rat topo IIα, but its flanking sequence is not, while threonine 1342 is not conserved, being replaced by an aspartate in the rodent enzymes (32, 33).

In this study, we have investigated the role played by casein kinase II in regulating the activity of human topo IIα. We show that co-incubation of topo IIα with casein kinase II strongly influences the ability of topo IIα to decatenate kinetoplast DNA. We present evidence that casein kinase II is able to stabilize topo IIα against inactivation during incubation at 37 °C, and that this effect is not dependent upon phosphorylation of topo IIα.

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1 The abbreviations used are: topo, topoisomerase; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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Site-directed Mutagenesis—The topo IIα cDNA was mutated using the single-stranded oligonucleotide-directed mutagenesis method of Kunkel et al. (34), as described in Wells and Hickson (22). An AvrII/XhoI fragment containing the 3′ end of the topo IIα cDNA (12) was subcloned into pBlueScript and a single-stranded uracil-containing template generated using the Escherichia coli strain CJ236. For the mutation of serine 1524 to alanine (S1524A), this oligonucleotide 5′-TTCAAGGTCGTCCACAGCAGCCTTTT-3′ was used; for the mutation of serine 1524 to alanine (S1524A), this oligonucleotide 5′-ACCGTCTTTGGGCGTGTAAAGTA-3′ was used; for the truncation at alanine 1178 the oligonucleotide 5′-TTAAAACGCTCACAATTTCTCATA-3′ was used. The double phosphorylation site mutant was generated using the S1576A and S1524A oligonucleotides simultaneously. Double-stranded mutant cDNA transcript constructs were verified by dideoxy DNA sequencing. AvrI/XhoI fragments containing the mutated phospho-acceptor residues were then subcloned into pYEpWob6 previously digested with the same enzymes.

Overexpression and Purification of Human Topo IIα Enzymes—The expression construct pYEpWob6 (35) was the basis for the production of full-length and truncated human topo IIα proteins. Full-length, mutated, and truncated proteins were overexpressed in Streptomyces cerevisiae and lysates were prepared by freeze/thawing and shearing cells with glass beads (35). Soluble proteins were then loaded onto a hydroxyapatite (Utrigel) column, and the topo IIα protein was eluted using a 200–600 mM NaCl gradient. Peak fractions were pooled and loaded onto a 1 ml HitrapSP column (Pharmacia Biotech Inc.), and bound proteins were eluted using a 200 mM to 1 x NaCl gradient. Peak fractions containing ~95% pure topo IIα, as determined by Coomassie Blue staining of SDS-polyacrylamide gels, were adjusted to 50% glycerol and stored at ~70 °C.

Purification of Casein Kinase II from Rabbit Skeletal Muscle—All procedures were carried out at 4 °C. The tissue was homogenized in 2.5 volumes of 4 mM EDTA, 15 mM 2-mercaptoethanol, 0.1 mM phenylmethylthlykylfluoride, and 1 mM benzamidine at pH 7.0. Following centrifugation at 10,000 rpm for 10 min, the supernatant was collected and adjusted to pH 7.5 with 15 mM NH₄OH. Solid (NH₄)₂SO₄ was added slowly with stirring to 33% saturation. The precipitate was then resuspended in 20 volumes of buffer A (2 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂) supplemented with either 1 mM ATP or 110 μM [γ-32P]ATP (final specific activity >23 TBq/nmol). PKI, the peptide inhibitor of protein kinase A, and H-7 were obtained from Sigma.

Determination of the Stoichiometry of Phosphorylation—12 μg of wild-type topo IIα were incubated with 2.4 μg of casein kinase II and 110 μM [γ-32P]ATP in a total volume of 20 μl of kinase buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂) supplemented with either 1 mM ATP or 110 μM [γ-32P]ATP (final specific activity >23 TBq/nmol). PKI, the peptide inhibitor of protein kinase A, and H-7 were obtained from Sigma.

DNA Topo II Assays—DNA topo II activity was assayed by measuring the deacatenation of KDNA (Topogen). The standard mix for a 20-μl reaction consisted of 50 mM Tris-HCl, pH 7.9, 85 mM KCl, 10 mM MgCl₂, 0.5 mM EDTA, 1 mM ATP, 1 mM dithiothreitol, 20 μg/ml bovine serum albumin, and 240 ng of kDNA. The reaction mixture was incubated at 30 °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 loaded onto a 1% agarose gel in Tris borate EDTA buffer. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV illumination.

RESULTS

Co-purification of a Kinase with Topo IIα from HeLa Cell Nuclear Extracts—Our initial premise was that because casein kinase II phosphorylates human top IIα in vitro, it was very likely to play a role in the regulation of top IIα function. Moreover, a kinase with properties very similar to those of casein kinase II has been reported to co-purify with top IIα in extracts from Drosophila, mouse and yeast cells (26, 27, 36). To address whether this was also the case for human top IIα, we tested the highly purified top IIα preparations from HeLa cell nuclei that we have described previously (23) for kinase activity. Fig. 1 shows that a kinase capable of phosphorylating top IIα (as well as casein; data not shown) co-purifies with top IIα from HeLa nuclei. Next, we addressed whether this co-purifying kinase displayed two of the features diagnostic of casein kinase II; sensitivity to heparin and an ability to utilize GTP, as well as ATP, as a phosphate donor (reviewed in Ref. 37). As shown in Fig. 1, the co-purifying kinase was inhibited by heparin, but not by a specific peptide inhibitor of protein kinase A, or by H-7, the inhibitor of protein kinase C, cyclic nucleotide-dependent kinases, and myosin light chain kinase. Moreover, this kinase could use GTP as a phosphate donor (data not shown), suggesting that it was either casein kinase II or a very closely related enzyme. These data, together with those of previous studies, suggest that top IIα may be an important target for casein kinase II in human cells.

Purification of Recombinant Topo IIα Protein—Using this information, we set out to analyze the role of casein kinase II in regulating top IIα enzyme activity in vitro. However, using human nuclear extracts as a source of enzyme, it proved very difficult to separate top IIα from the co-purifying kinase. As a consequence, we adopted an alternative approach exploiting the recent description of an overexpression system for recombinant human top IIα using S. cerevisiae as a host (35). With this system, we prepared homogeneous human top IIα from yeast extracts (see Fig. 6 below) using a new purification protocol (see “Experimental Procedures”). In addition, we purified casein kinase II to apparent homogeneity from rabbit skeletal muscle...
muscle using an established protocol. We first confirmed that the recombinant topo IIα preparation lacked intrinsic kinase activity and that the topo IIα was a substrate in vitro for the purified casein kinase II (Fig. 2a). Analysis of this reaction showed that the stoichiometry of phosphorylation was approximately 2 moles of phosphate per mole of topo II monomer, even after extended incubation periods (Fig. 2b). Thus, we conclude that this yeast-based expression system provides a means to analyze the effects of casein kinase II on topo IIα activity without the compounding influence of a co-purifying kinase.

**Effect of Casein Kinase II on the Activity of Recombinant Topo IIα Protein**—We next tested whether the purified rabbit casein kinase II could influence the activity of the recombinant topo IIα protein. Fig. 3 shows that the purified human topo IIα displayed significantly higher activity in a decatenation assay following preincubation with casein kinase II than it did following an otherwise identical incubation in buffer containing either a control protein (BSA) or heat-inactivated casein kinase II. Thus, native casein kinase II is required for this activation of topo IIα. As a control, we confirmed that the casein kinase II preparation contained no contaminating topoisomerase activities (data not shown).

We performed a number of experiments to define the mech-
anism by which casein kinase II could influence the activity of topo IIα during the short period of preincubation at 37 °C prior to performing the decatenation assay at 30 °C. During the course of these studies, we found that the activity of the topo IIα enzyme declined markedly during such a preincubation period in the absence of casein kinase II, but was largely unaffected when casein kinase II was included in the incubation mixture (Fig. 4a). However, it should be noted that in neither case did the level of topo IIα activity rise above that of enzyme that had not been preincubated at 37 °C prior to performing the decatenation assay. Moreover, the decline in activity seen following a period of preincubation at 37 °C was not observed if the preincubation temperature was lowered to 25 °C before performing a decatenation assay (Fig. 4b).

Thus, the effect of casein kinase II was not to “activate” the topo IIα, but instead to maintain its activity at a relatively steady level during a period of incubation of 37 °C, presumably by preventing thermal inactivation. Hence, we will consider this to be stabilization rather than an activation of topo IIα by casein kinase II.

To address whether the effect of casein kinase II was to prevent inactivation of topo IIα during incubation at 37 °C, or to “renature” topo IIα that had already lost activity, we tested whether topo IIα inactivated by incubation at 37 °C in the absence of casein kinase II could be “reactivated” by subsequent addition of casein kinase II. Fig. 5 shows that, once inactivated, topo IIα could not be reactivated by casein kinase II, indicating that casein kinase II must be present during the preincubation period at 37 °C to exert its stabilizing effects.

**Fig. 4.** Changes in topo IIα decatenation activity following preincubation. Relative decatenation activity of recombinant topo IIα (0.3 μg per reaction) following a preincubation at 37 °C (panel a) or 25 °C (panel b) in the presence of 0.2 μg of casein kinase II (○) or in the presence of 0.6 μg BSA (□) for the times indicated on the x axis. After preincubation, samples were assayed for decatenation activity at 30 °C for 15 min. Data represent the mean of three independent experiments.

**Fig. 5.** Casein kinase II cannot restore activity to heat-inactivated topo IIα. Recombinant topo IIα (0.3 μg per reaction) was preincubated at 37 °C for the times indicated. At intervals, aliquots were removed and added to a decatenation reaction either in the presence of 0.6 μg of BSA (□) or in the presence of 0.2 μg casein kinase II (○), and the incubation was continued for a further 15 min at 30 °C. Data represent the percent DNA decatenated as a function of time of preincubation at 37 °C.

say. However, to exclude a role for phosphorylation by casein kinase II, we studied mutated and truncated derivatives of recombinant topo IIα. We have shown previously that casein kinase II phosphorylates topo IIα in vitro primarily on two sites in the C-terminal domain (29). These sites, serine 1376 and...
serine 1524, are also phosphorylated in vivo (29). In addition, Ishida et al. (31) have shown recently that threonine 1342 is phosphorylated in human cell lines. Recombinant topo IIα proteins containing mutation of serine 1376 to alanine (S1376A), serine 1524 to alanine (S1524A), or a double mutant topo IIα-SDM (lane 3), as indicated on the right. The positions and sizes of the molecular mass standards (lane MWM) are shown on the left in kDa. b, diagrammatic representation of the recombinant proteins used in the study.

To address whether casein kinase II was having a differential effect on the activity of the mutated/truncated versions of topo IIα in comparison with that of the wild-type enzyme, we analyzed the decatenation activity of these proteins following a 15-min preincubation period at 37 °C in the presence or absence of casein kinase II. As a preliminary step in this analysis, we determined the quantity of each of the topo II enzymes required to give an equivalent level of decatenation activity. Approximately equal levels of decatenation activity were exhibited by 0.34, 0.21, and 0.13 μg of the wild-type topo IIα, the topo IIα-SDM protein, and the truncated topo IIα-CTT protein, respectively. Following equalization of intrinsic decatenation activity in vitro by casein kinase II, two-dimensional tryptic phosphopeptide mapping was performed. Fig. 7a shows that digestion of wild-type topo IIα following phosphorylation by casein kinase II generated two predominant phosphopeptides, designated 1 and 2, which have been identified previously as being specific for serine 1524 and serine 1376, respectively (29). As expected, two-dimensional maps derived from the S1424A mutant protein lacked phosphopeptide 1 (Fig. 7d), and those from the S1376A protein lacked phosphopeptide 2 (Fig. 7c). Moreover, the topo IIα-SDM double mutant protein was only very poorly phosphorylated (0.13 mol of phosphate/mol of topo IIα monomer), and any specific radiolabel (i.e. that which migrated away from the origin) was present at a very low level (Fig. 7b), consistent with the notion that serine 1376 and serine 1524 are the major in vitro casein kinase II phosphorylation sites on topo IIα. In agreement with this, the truncated derivative of topo IIα (topo IIα-CTT) lacking the C-terminal domain, could not be phosphorylated by casein kinase II in vitro to an extent that permitted meaningful two-dimensional mapping analysis to be undertaken (stoichiometry of 0.02 mol of phosphate/mol of topo IIα monomer). Taken together, these data are also consistent with the results shown in Fig. 2 indicating a stoichiometry of phosphorylation of 2 mol of phosphate/mol of wild-type topo IIα monomer.
activity, the effect of casein kinase II on the activity of these three topo II preparations was compared. Fig. 8 shows that casein kinase II was able to stabilize the activity of all three of these proteins, indicating strongly that phosphorylation of residues of the C-terminal domain of topo II (or indeed elsewhere in the protein, since the reaction was ATP-independent and the topo II-CTT protein was not a substrate for casein kinase II in vitro) is not required for this stabilization of topoisomerase activity. It should be noted that we consistently observed a greater degree of resistance to inactivation by the truncated topo II-CTT enzyme than by any of the full length versions of this protein. Nevertheless, it is clear that all of the mutated/truncated topo II derivatives behave qualitatively in a very similar fashion to that of the wild-type topo IIα.

Since we were unable to identify any role for phosphorylation by casein kinase II in the stabilization phenomenon, we considered whether the kinase might be acting in a stoichiometric manner rather than enzymatically. Fig. 9 shows that detectable stabilization was observed only when the molar ratio of casein kinase II to topo II rose to at least 0.3 and that full stabilization required equimolar levels of kinase and topoisomerase.

**DISCUSSION**

We have shown that casein kinase II co-purifies with and strongly influences the activity of human topo IIα. This activation of topo IIα is apparently independent of any phosphorylation and does not require sequences present in the regulatory C-terminal domain of the enzyme. Although casein kinase II treatment increases the activity of topo IIα to a level above that of mock-treated enzyme, this does not represent true activation, in that the role of the kinase appears to be to stabilize topo IIα and hence prevent a decline in activity that normally occurs during a period of incubation at 37 °C. We would suggest that this effect on enzyme activity might be widespread among nuclear enzymes and therefore important for chromosome function, since casein kinase II has been found in association with a wide variety of other enzymes important for different aspects of DNA metabolism (see below). Of possible significance in this regard is the observation that that the β subunit of casein kinase II confers thermal stability upon casein kinase II subunits, in which the catalytic activity resides (38).

How might casein kinase II effect its role as an enzyme stabilizer? The most obvious mechanism is via direct association with topo IIα. Our data showing the co-purification of topo IIα with casein kinase II and the requirement for stoichiometric amounts of casein kinase II to effect stabilization of topo IIα enzyme activity, are consistent with this suggestion. Moreover, Bojanowski et al. (39) have shown that the β subunit of casein kinase II interacts with topo II from budding yeast. However, despite extensive efforts, utilizing a variety of different methodologies including immunoprecipitation and chemical cross-
linking, we have been unable to “trap” a stable complex containing pure human topo IIα with casein kinase II. This does not rule out the possibility that such an association occurs under certain conditions, or that the association is transient in nature. Casein kinase II is known to associate with a number of its other substrates, including topo I, p53, and protein phosphatase 2A (40–42). In addition, casein kinase II has been shown to stimulate topo I activity via a direct physical association (40).

Recently, Kimura et al. (43) reported that casein kinase II has no effect on the activity of mouse topo IIα. Further, they concluded that their previously reported results (27) showing a stimulation of topo IIα activity by casein kinase II could not be attributed to the kinase, but to the composition of the incubation buffer. Specifically, incubation in buffers containing low, but not high, concentrations of glycerol was stimulatory, and this effect was dependent upon topo IIα being present at a high concentration. Our results are partially in agreement with those of Kimura et al. (43) in that we could not identify a role for phosphorylation per se in the regulation of topo IIα activity by casein kinase II. However, our findings that native casein kinase II is a necessary component of the buffer, and that denatured casein kinase II is not active in this regard, are not consistent with their conclusions. Moreover, we have been unable to reproduce the stimulatory effects of manipulating the glycerol concentration alone, reported by Kimura et al. (43). The reason(s) for these discrepancies is not apparent at this stage.

We have shown that the C-terminal domain of topo IIα is not important for catalytic activity in vitro, and that phosphorylation of target residues within this domain is not required for the regulation of that activity by casein kinase II. It would be predicted, therefore, that a failure to phosphorylate these residues would not alter the known ability of the topo IIα CDNA to rescue temperature-sensitive yeast top2-4 mutants at the restrictive temperature (35, 44). Consistent with this prediction, we have shown that CDNAs carrying mutations of serine 1524 and/or serine 1376 will fully complement top2-4 strains. It is conceivable that the role of phosphorylation of these residues is normally not to regulate catalytic activity, but instead to influence subcellular or subnuclear localization in human cells, as has been shown for fission yeast topo II (28). Our data showing that residues 1179–1530 are dispensible for the catalytic activity of human topo IIα in vitro are consistent with previous analyses on truncated versions of the S. cerevisiae and Drosophila topo II proteins (45, 46). The possibility that phosphorylation of sites outside the C-terminal domain may influence the stability of the enzyme can be discounted, since the truncated protein, which was shown to incorporate only 0.02 mol of phosphate/mol of topo IIα monomer, is stabilized by casein kinase II in a fashion similar to that of the wild-type enzyme.

Our work and that of Kimura et al. (43) on mouse topo IIα have shown no direct effect on topo IIα activity of phosphorylation by casein kinase II. In addition, both studies stoichiometric quantities of kinase were necessary to give efficient phosphorylation. This is in contrast to reports of the effect of casein kinase II phosphorylation on topo II activity from lower eukaryotes. Topo II from Drosophila is a high affinity substrate for casein kinase II both in vitro and in vivo and is fully phosphorylated using substoichiometric quantities of kinase (24, 30). The phosphorylated enzyme has an enhanced rate of ATP hydrolysis and a 3-fold higher plasmid relaxation activity compared with that of the dephosphorylated enzyme, effects that are fully reversed by dephosphorylation of the enzyme (24, 30, 47). Similarly, S. cerevisiae topo II is readily phosphorylated by casein kinase II, resulting in a greater than 10-fold stimulation of decatenation activity (36). In Drosophila and yeast there is a single gene encoding topo II, whereas in mammalian cells there are two closely related, but genetically distinct, isoforms. Thus it is possible that higher eukaryotes have evolved a mechanism for differentially regulating the two isoforms.

Gasser and co-workers (36, 48) have reported that casein kinase II not only reactivates dephosphorylated yeast topo IIα, but also influences the extent of enzyme multimerization. Although it remains to be confirmed that higher order (greater than dimeric) forms of topo II have any functional significance in eukaryotic cells, it is formally possible that the regulatory phenomenon that we have observed reflects an ability of casein kinase II to prevent topo IIα from adopting a subunit composition that exhibits little or no catalytic activity. Further work is required to ascertain whether or not this suggestion is true.

The primary role of topo IIα in human cells is almost certainly to act during mitosis. It would appear that the level and activity of this enzyme is very tightly controlled by a combination of features acting at different levels. Thus, the mRNA is virtually absent from G0/G1 cells and only accumulates to high levels during late S phase (14–16, 49). The topo IIα protein peaks later, in G2/M, and is phosphorylated by at least two different kinases during mitosis (14, 22, 23). Indeed, it would appear that topo IIα activity is strongly activated only during a narrow time window when its decatenation function is required for mitotic chromosome condensation/ segregation. Casein kinase II might contribute to this tight regulation at (at least) two levels; either via modulation of intrinsic topo IIα enzyme stability, or via its known ability to modulate the action of other kinases/phosphatases required for mitosis, including protein phosphatase 2A and the universal mitotic controller, p34cdc2 (42) (reviewed in Ref. 37). It might be significant that both casein kinase II and topo IIα are among the proteins that are phosphorylated during mitosis, leading to the generation of the phosphoepitope recognized by the MPM-2 antibody (50, 51).

In summary, we have shown that the activity of topo IIα is maintained during an incubation at 37 °C by the action of casein kinase II in a manner that is independent of phosphorylation. The challenge is now to delineate the functional significance of this regulation in vivo and to address whether casein kinase II plays a widespread role in regulating the stability of other important nuclear enzymes.

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